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Currently little is known about the mechanisms and locations of lymphocyte development in teleosts. In this study several aspects of the underlying factors which govern B lymphocyte development in trout were investigated which included: the isolation and characterization of immunoglobulin heavy chain (IgH) genes, the recombination activating genes 1 and 2 (RAG1 and RAG2) and the use of cellular markers to identify tissues harboring precursor B-cells.

Immunoglobulin heavy chains are part of the structural components which make up antibody molecules produced by B-cells. We isolated various full-length IgH cDNA clones, some of which contained the secreted while others contained the membrane bound form of IgH. Upon characterization of the membrane bound forms, typical features common to all IgH cDNAs were found including a

leader peptide, a variable region and constant domain containing transmembrane (TM) segments as well. Further sequence analysis of this region revealed that the TM domains were spliced directly to the CH3 domains which results in the loss of the entire CH4 region. Our results support previous observations of unusual splicing events in fish IgH genes.

RAG1 and -2 in mammals have been shown to be essential for carrying out V (D) J recombination of lymphocyte receptors and are found to be expressed within primary lymphoid tissues and precursor lymphocytes. We isolated the RAG locus from a rainbow trout genomic library and characterized their conservation and expression. Overall the complete amino acid sequences of RAG1 and RAG2 displayed 78% and 75% similarity when compared to RAG genes from higher vertebrates thus demonstrating the highly conserved nature of these genes. Tissue specific expression of both genes was primarily associated with the thymus and pronephros in both juvenile and adult trout. Based upon these observation we conclude that the thymus and pronephros likely serve as the tissue sites for V (D) J recombination in trout and are thus primary lymphoid organs.

Finally we addressed the question as to where B-cell lymphopoiesis occurs in trout. Our results using both immunofluorescence and confocal microscopy putatively demonstrate that the thymus harbors precursor B-cells and thus alludes to a dual function for both B and T-cell development in trout.

B-Cell Development in Rainbow Trout: a Molecular/ Cellular Based Approach

by

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I understand that my thesis will become part of the permanent collection at Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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John D. Hansen, author

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I would like to dedicate this thesis to my parents without their influence and guidance, this would not be possible. Because of their unhesitating support over the years I hope that I can fully express my sincere gratitude in some way. ***THEY'RE GREAT!*** I would also like to take the time to tell Brenda, my best friend and the woman for whom I love more than all of science, that I am indebted for her patience and support over this long haul. It is through her that I have really learned to appreciate life itself and not to let work take precedent over the really important things.

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I would also like to acknowledge and in some way dedicate a portion of this thesis to all of the trout and salmon who have given their lives to science. Hopefully some day we will be able to return this favor in the way of disease prevention and teaching humankind better management practices for they are truly magnificent and their loss would be unforgivable.

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CONTRIBUTION OF AUTHORS

Dr. Stephen L. Kaattari and Dr. Jo-Ann C. Leong were major advisors on all manuscripts. Dr. Jo-Ann C. Leong appeared on only one publication, but was an excellent editorial source for all publications. Dr. Carol H. Kim was responsible for conducting the majority of the immunofluorescence and confocal analysis in chapter 4.

Table of Contents

Table of Contents (cont)

Table of Contents (cont)

List of Figures

B-Cell Development in Rainbow Trout: a Molecular/Cellular Based Approach

CHAPTER 1

Introduction and Literature Review

Introduction

The characterization of genes involved in the immune response of salmonids is of interest from a phylogenetic point of view as well providing insights into the mechanisms controlling the overall development of the immune system in fish. The genes encoding the immunoglobulin heavy and light chains are by far the most heavily investigated in lower vertebrate taxa (Wilson and Warr 1992). Relatively little is known about the ontological sites and mechanisms involved in the generation of the primary immunoglobulin and antigen receptor repertoires of B and T-cells in fish.

In mammals, the progenitor cells of the lymphocyte lineages (B and T cells) originate within the bone marrow. T-cell progenitors migrate to the thymus where they mature, whereas the B-cell progenitors remain in the bone marrow microenvironment during development. In these tissues, site specific recombination of variable region gene segments leads to the genesis of the primary immune repertoire. Recently two genes, the recombination activating genes 1 and 2 (RAG1 and RAG2) have been demonstrated to be essential for the somatic recombination of lymphocyte receptor variable region genes

(Schatz et al 1989, Carlson et al 1991). In teleost fish, early histological analysis indicated that the thymus may be the primary source of lymphocytes (Grace and Manning 1980, Josefsson and Tatner 1993). Indeed the thymus is the first lymphoid tissue to develop and to display lymphocytes during the ontogeny of fish, but others contend that the pronephros is the bone marrow equivalent in fish based upon observations of hematopoietic foci and functional analysis (Razquin et al 1990, Irwin and Kaattari 1986). Therefore it has yet to be clearly shown where lymphopoiesis occurs in teleost.

This thesis deals with the isolation and characterization of genes involved in the generation of the immune repertoire in trout. The first manuscript, Chapter 2, consists of the isolation cDNAs coding for membrane bound forms of immunoglobulin heavy chains in trout and discusses relevant features found from sequence analysis. Chapter 3 describes the isolation of RAG1 from a rainbow trout genomic library using a degenerate PCR-based approach and defines the tissues and lymphocyte phenotypes which specifically express RAG1. This work provides an initial step in the determination of the tissues most likely associated with V (D) J recombination. Finally in Chapter 4, I addressed several features involved in the development of the trout immune system. This analysis provided a more sensitive approach in identifying the primary lymphoid tissues in trout using both molecular and cellular based approaches.

Literature Review

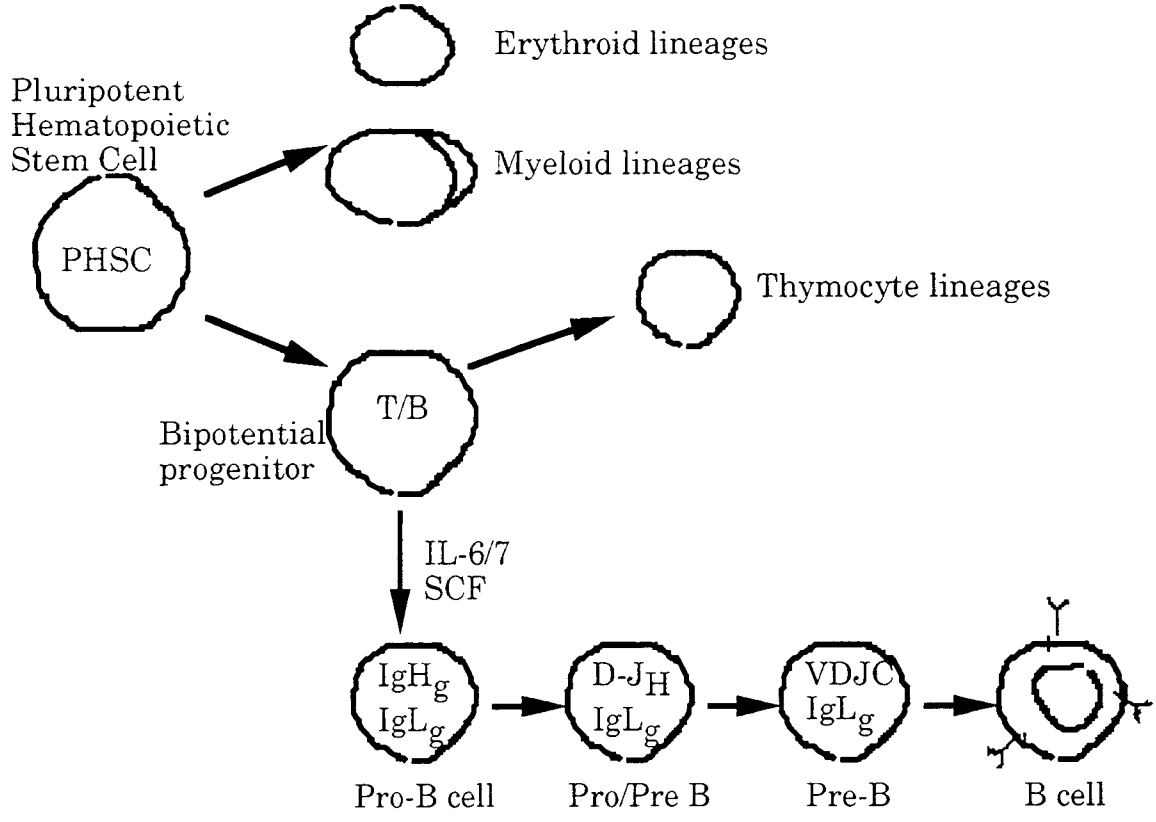
B cell development

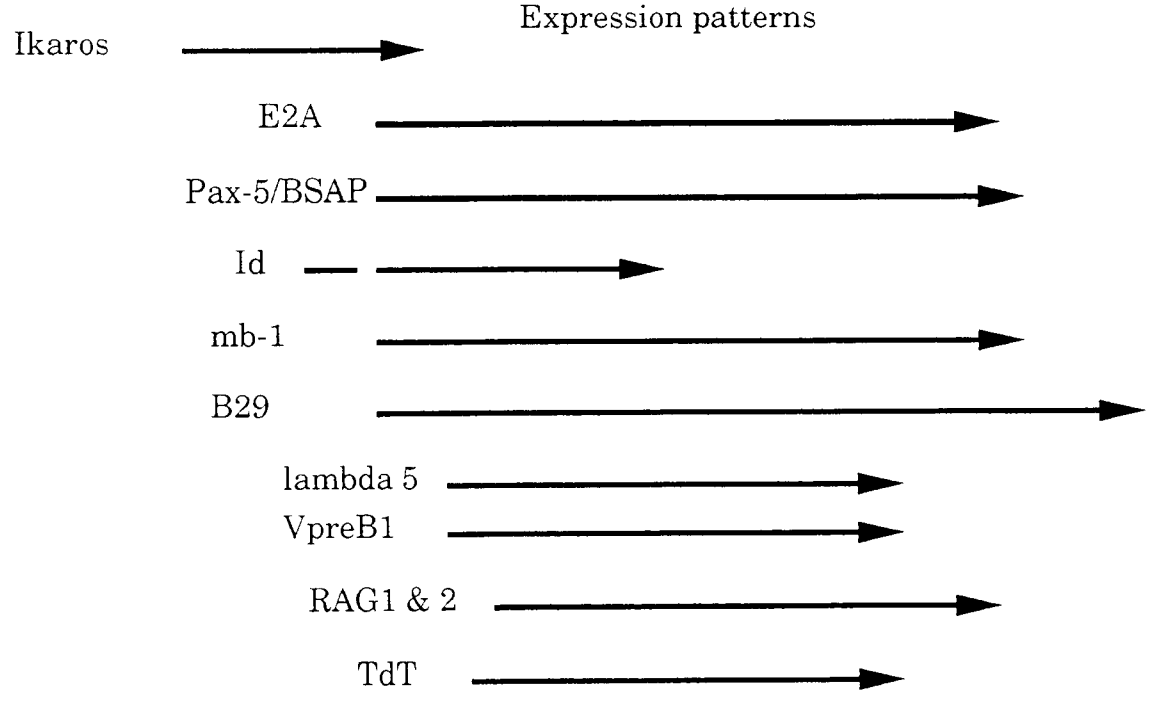
The generation of mature B and T lymphocytes in mammals is a complex process that occurs in the bone marrow and thymus respectively. Aside from maturing in different tissues, both are common descendants of pluripotential hematopoietic stem cells, which also form the myeloid and erythroid lineages. The differentiation of B lymphocytes from bipotential progenitors (B/T) proceeds through multiple steps that are under the control of coordinately expressed lineage-specific genes. The overall process can be thought of as two major events during B cell differentiation: the recombination and expression of immunoglobulin genes. A cascade of developmental factors (Fig 1.1) control the productive and sequential rearrangement of immunoglobulin genes such that the heavy chain loci are rearranged first, expressed, then the light chain loci are similarly rearranged and expressed (Blackwell and Alt 1988).

Pluripotential hematopoietic stem cells in the bone marrow bind to stromal cells where they receive initial signals in the form of stem cell factor and interleukins-6 and 7 to induce the differentiation into B-cell progenitors (Palacios and Samaridis 1992, Faust et. al 1993, Dong and Wortis 1994). The establishment of various stem, progenitor and pre-B cell lines has proved invaluable in the process of determining the development of B lymphocytes. It appears that binding of the stem cell surface molecule CD44 to hyaluronic acid on bone marrow stromal cells

Figure 1.1 B-cell development. Schematic representation of transcription factor expression and the appearance of stage specific proteins during the ontogeny of a B-cell. At the progenitor B-cell (pro-B) stage, the immunoglobulin variable region genes of both the heavy and light chains are in the germline configuration (IgH_g and IgL_g). At the final stage designated "B-cell", all Ig genes are fully rearranged, expressed and associated with mb-1 (Ig-alpha) and B29 (Ig-beta) to form the complete B-cell receptor. Placement of lineage specific markers is based upon Palacios and Samaridis 1992, Ikuta et al 1992, Li et al 1993 and Bain et al 1994.

Figure 1.1


Erythroid lineages
Pluripotent
Hematopoietic
Stem Cell
Myeloid lineages
PHSC
Thymocyte lineages
T/B
Bipotential
progenitor
IL-6/7
SCF
IgHg
IgLg
D-JH
IgLg
VDJC
IgLg
Pro-B cell
Pro/Pre B
Pre-B
B cell


Expression patterns
Ikaros
E2A
Pax-5/BSAP
Id
mb-1
B29
lambda 5
VpreB1
RAG1 & 2
TdT

induces upregulation of the receptors for stem cell factor (c-Kit) and interleukins 6r and 7r. This binding also induces stromal cells to express and secrete interleukins 6 and 7 which induce progenitor proliferation. At about this point in the early life of the B cell progenitor a virtual cascade of transcription factors are synthesized which will directly and indirectly influence the fate of the developing B-cell.

Transcription factors involved in B cell ontogeny

It appears that a group of transcription factors control the development of progenitor B-cells through the mature B cell form. In a progenitor B-cell all of the immunoglobulin genes are in the germline configuration. Two camps seem to dominate the thoughts regarding how lymphocytes descend from stem cells: one believes that B-cell, T-cell, monocyte and erythrocyte lineages are directly derived from one stem cell while others contend that an intermediate bipotential precursor is involved (Ikuta et al 1992). Regardless of which is correct, researchers have been successful in resolving some of the various stages in B-cell development.

Recently, a putative master switch in the process of committing stem cells to the lymphocyte lineages has been characterized. The gene *Ikaros* which codes for four alternatively spliced zinc finger DNA binding proteins is thought to be the master switch (Georgopoulus et al 1992, Hahm et al 1994). Some of the different isoforms produced by the *Ikaros* gene have binding affinities for the *TdT*, *lambda 5* and *VpreB* promoters which are utilized in later stages of B cell development. In mice homozygous for a disrupted/dysfunctional *Ikaros* gene there is a

complete failure to generate B and T cell progenitors, while the erythroid and myeloid lineages appear to develop normally (Georgopoulus et al 1994).

Once committed to the B and T-cell pathways there are several other transcription factors that dictate B cell development including the products of the E2A, Pax5 and Id genes. The E2A gene encodes two basic helix-loop-helix DNA binding proteins, E12 and E47, which are produced by differential mRNA splicing. These transcription factors bind to the enhancer regions of the immunoglobulin heavy and light chains (Murre et al 1991, Bain et al 1993) and are thought to induce their subsequent transcription. Bain et al (1993) demonstrated that although the E2A products are produced in a variety of cell types, DNA binding activity was only associated with nuclear extracts from pre-B cells. It has also been shown that expression of cDNA constructs containing E2A transfected into precursor T-cells resulted in the expression of germline immunoglobulin heavy chain genes followed by induction of V-D-J recombination at the immunoglobulin heavy chain loci (Schlissel et al 1991). Using knockout technology the EA2 genes have been shown to be required for the differentiation of progenitor B cell to the precursor B-cell stage. In these mice there is a failure of the pre-B cells to rearrange their immunoglobulin loci and as well as displaying significantly lower levels products related to B-cell differentiation (lambda 5, VpreB1) (Zhuang et al 1994).

EA2 gene products appear to be under the influence of the Id gene products, Id-1 and -2, via post-translational control. The Id gene which codes for a group helix-loop-helix proteins is heavily expressed

during the early progenitor stages and is then down regulated during B cell differentiation (Sun et al 1991, Wilson et al 1991). It appears that the products of Id can inhibit the action of the Ig heavy chain enhancers (Wilson et al 1991) as well as forming inactive heterodimers with E12 and E47 though their helix-loop-helix motif (Sun et al 1991, Riechmann et al 1994). Taking this into account along with the expression patterns of both the EA2 and Id genes, Sun (1994) tested whether the Id gene products are responsible for inactivating EA2 proteins at the pro-B stage and then, upon downregulation, allow the E12/E47 products to become active during precursor stages. In transgenic mice that overexpress the Id gene, precursor B-cells failed to develop. In addition it was found that there were significantly lower levels of RAG1, RAG2 and lambda 5 expression. These results indicate that the Id products inactivate E12/E47 during early stages of development and upon down regulation of Id, E12/E47 are free to bind to their target sites to allow for the transition to the precursor B stage.

Another B-cell differentiation factor is produced by the Pax-5 gene, a member of the paired box family of DNA binding proteins. The Pax-5 gene codes for a product known as the B-cell specific activating protein, BSAP, which is found in all early stages of B-cell development (Adams et al 1992). Potential targets for BSAP include the VpreB, lambda 5 promoters and several regulatory regions found within the Ig loci (Okabe 1992, Liao et al 1992). BSAP has also been implicated in the proliferation of B cells and the switch recombination events which lead to the production of the different immunoglobulin isotypes found in plasma cells (Max et al 1995). Mice with a homozygous disruption of Pax-5 have B cells arrested in the early precursor stages of development

(Urbanek et al 1994). Thus, BSAP is another essential transcription factor involved in the overall ontogeny of B-cells.

Genes involved in Ig receptor generation

As previously mentioned , the transcription factors produced by the Ikaros, EA2, Id and Pax-5 genes have been found to be important regulators of early events in B cell development. These events include the up and down regulation of genes involved in the generation of B cell antigen receptors. This regulation involves the products of three sets of genes expressed during the early stages of B cell development. The first set includes the genes essential for the recombinatorial events which generate mature immunoglobulin heavy and light chain genes (RAG1, RAG2 and TdT). The second set includes the genes that encode the surrogate light chain proteins, lambda 5 and VpreB. Finally is the set responsible for the assembly and function of the membrane bound antigen receptor (Mb1 and B29).

The immunoglobulin heavy and light chains are encoded by multiple germline gene elements (variable, diversity and joining) which are somatically recombined by a site specific recombinatorial process in developing B lymphocytes (Schatz et al 1992). The recombination activating genes, RAG1 and 2, have been shown to be essential for this rearrangement (Schatz et al 1989, Carlson 1991). Transfection of the RAG genes into non-lymphoid cells can induce V (D) J recombination and were cloned based upon this capability (Schatz 1989). The expression patterns of RAG1 and 2 are unique to the sites and cell types that undergo V (D) J recombination. The exact role of the gene products

of RAG1 and 2 is not precisely known, but several current studies have indicated that they are actual components of the V(D)J recombination machinery. Their obligate role in the recombinatorial process has been demonstrated in mice made deficient in either RAG1 and RAG2 (Mombaerts et al 1992, Shinkai et al 1992). In these studies, knockouts for RAG1 or 2 resulted in mice lacking mature B and T cells. This phenotype can be rescued by the introduction of immunoglobulin or T-cell receptor transgenes into RAG deficient mice (Spanopoulou et al 1994, Shinikai et al 1993, Mombaerts et al 1992). On a similar note, expression of the RAG genes coincides with variable region recombination in RAG inducible B-cell lines (Oltz et al 1993). Some believed that the RAG genes code for yet another set of transcription factors involved in lymphocyte development or that RAG1 was a type of topoisomerase, but mutagenic analysis disfavors these hypotheses (Silver et al 1993).

Terminal deoxynucleotidyl transferase, TdT, is believed to be enzyme directly involved in the base addition events (N insertions) observed during the recombination of antigen receptors (Kallenbach et al 1992). The base addition events contribute to the overall diversity observed in antigen binding sites. Disruption of the TdT gene in mice results in a total lack of N-insertions in the immunoglobulin heavy chains (Gilfillan et al 1993), thus limiting the potential diversity of the primary Ig repertoire. The expression patterns for TdT corresponds with that of the RAG genes, but its activity ceases at the time of light chain rearrangement. This fact explains the lack of N-insertions in the variable regions of light chains.

The surrogate light chains, lambda 5 and VpreB1, consist of two noncovalently associated proteins which form a light chain-like structure during the early precursor stages and are essential for the proper development of B cells (Melchers et al 1994). They are expressed in all early stages, but are shut off following the rearrangement of a function light chain. In the late progenitor stages they are found on the surface of the B cell complexed with gp130 and gp 35-65 (surrogate heavy chain). This suggests that the surrogate light chain may act as a receptor prior to the appearance of heavy chain molecules. Lambda 5 knockout mice have provided evidence that surrogate light chain expression is essential for B cells to proceed past this point (Kitamura et al 1992, Ehlich et at 1993). In the subsequent developmental stage, the surrogate light chains are found associated with a truncated form of immunoglobulin heavy chain. It is believed that this complex serves to screen for proper reading frame formation during heavy chain rearrangements. Finally the surrogate light chains are found on the surface complexed with functional heavy chain. This complex is thought to stimulate clonal expansion due to proper association with the heavy chain, mediate allelic exclusion at the heavy chain loci, and to signal for the activation of light chain rearrangement (Kitamura and Rajewsky 1992, Melchers et al 1993). Once a functional light chain is produced, it displaces the surrogate light chains which are thought to halt further light chain rearrangement.

The genes responsible for forming the complete membrane bound B-cell antigen receptor include the products of the mb-1 and the B29 genes. These genes encode the immunoglobulin-alpha and -beta chain respectively (Sakaguchi et al 1988, Hermanson et al 1988). The two

transmembrane glycoproteins together are required for proper localization of membrane immunoglobulin on the surface of pre-B cells and mediate signal transduction of engaged membrane bound immunoglobulin antigen receptors (Matsuuchi et al 1992) in a way analogous to CD3 in T-cell receptors. These genes are expressed during late progenitor stages and in all stages prior to the differentiation of B-cells into plasma cells. Northern blot analysis has indicated that mb-1 expression precedes DH-JH rearrangement in late progenitor B cells and thus marks one of the earliest events in B cell differentiation (Palacios and Samaridis 1993). Besides governing the proper localization of mIg and inducing proliferation, the alpha and beta chains are involved in antigen internalization and processing (Patel and Neuberger 1993). Additionally, a recent paper has indicated that the beta chain is essential for allelic exclusion of immunoglobulin heavy chains (Patavasiliou et a 1995).

Generation of lymphocyte receptor diversity

The specificity of the humoral immune response and its ability to adapt to new antigens is largely due to the plasticity of the antibody response which is produced by B-cells (Honjo and Habu 1985). It has been clearly shown that fish possess B-cells with of the same general characteristics as that found in mammalian B-cells (rev Kaattari 1992). B-cells in general can be defined as those lymphocytes which express membrane bound immunoglobulin and can secrete immunoglobulins in response to antigenic stimulation with specificity for these antigenic stimuli (McKinney et al. 1977, Marchalonis 1982, Sima and Vetvicka

1990). The activated B-cell (antigenically stimulated) undergoes a rapid proliferative response to generate a pool of B-cell clones. These clones actively secrete antibodies (plasma cells) while other clones act as a memory pool for future encounters with the same antigenic stimuli.

Thus far, it appears that immunoglobulin production is restricted to the jawed vertebrates (gnathostomata). Molecules resembling immunoglobulins have been detected in the sera of hagfish (Varner et al 1991), but upon actual cloning and sequencing the cDNAs were found to encode components of the complement system (Ishiguro et al 1992). Therefore further studies need to be undertaken to determine if the agnathans (hagfish and lampreys) actually have true B-cells capable of producing antibodies.

In the lymphoid system of vertebrates, immunoglobulins (antibodies) serve as the effector molecule for B-cells. They can be found on the surface of B-cells as well as in the circulation. Immunoglobulins can exert their effector roles in a variety of ways: via neutralization of toxin or viruses, as adhesins, as opsonins for phagocytes, through complement fixation, roles in ADCC or as agglutinins. Over the last two decades a wealth of information has been obtained in regard to fish immunoglobulins and the genes that encode them. Fish immunoglobulins, like mammalian immunoglobulins are composed of a basic monomeric structure containing 2 heavy chains (~70 kilodaltons) and 2 light chains (~22 kilodaltons) (Wilson and Warr 1992). In mammals, B-cells have the ability to generate five major classes (isotypes) of antibodies, which differ functionally (effector role), physically (isoelectric point, molecular weight) and antigenically (allo-, idio- and isotypic differences). Regardless of the immunoglobulin

isotypes which are characterized by distinct constant domains, antibodies produced by each B cell clone are uniquely specific for distinct antigenic determinants. One can think of the antibody/ antigen interaction as a lock and key type of event, where the lock is the antibody's antigen binding site and the key is the antigen. The combining site, or antigenic cleft is composed of the association of the variable regions of the heavy and light chain polypeptides. Variable regions are composed of three complementary determining domains (CDRs) and four framework regions. CDRs form the antigenic cleft, while the framework regions are responsible for overall stability of the antigen combining sites (Tonegawa 1983).

It seems that practically any foreign substance is capable of eliciting an antibody response. Even more so, the response itself to a simple antigen can be quite diverse, comprising antibodies with differing degrees of affinity and fine specificity. This massive collection of antibodies which is capable of binding practically any type of antigen, is known as the B-cell immune repertoire and is believed to be composed of at least 10^{11} different antibody molecules (Berek and Milstein 1988). Prior to the ability to biochemically examine immunoglobulins in fine detail there were two main theories as to the generation of such large repertoires of antibodies. The germline theorists proposed that for every different immunoglobulin, there is a separate germline gene and that an individual acquires this repertoire simply by inheritance. On the other side were the somatic theorists who conjectured that diversity was accomplished by mutation of one of a limited number of inherited genes within each B-cell. It turned out that both camps were correct to a certain degree. The immunoglobulin repertoire is generated from a

large, but limited number of antibody genes by somatic rearrangement and the repertoire is further diversified by somatic hypermutational events within the CDRs of antibody molecules (Alt et al 1986, Schatz et al 1992).

Immunoglobulin polypeptides (heavy and light chains) consist of separate structural domains: a variable region which forms the antigen combining site, and the constant domain which is responsible for effector function. The gene segments which encode for the heavy (V D J) and light (V J) chain variable regions are encoded by multiple germline gene segments which are somatically recombined in an orderly fashion during the development of B-cells in the bone marrow as depicted in figure 1.2 (Tonegawa 1983, Alt et al 1986, Schatz 1992). The heavy chain variable region is generated by the joining of three gene segments; V_H, D_H and J_H all of which belong to multigene families based upon DNA sequence similarity analysis (Seidman et al 1978). Recombination begins with the joining of D_H to J_H which is then recombined with up to one of 1000 V_H gene segments (Sakano et al 1980, Kurosawa and Tonegawa 1982, Pascual and Capra 1991) and is believed to be mediated by the products of the recombination activating genes 1 and 2 (Schatz et al 1992). Considering that in mice there are more than 20 D_H gene segments, ~6 J_H segments and possibly more than 1000 V_H gene segments it is not hard to imagine the generation of 10^{5-6} different heavy chains through random joining of these gene segments (Bangs et al 1991). Light chain diversity is generated by the joining of 2 gene segments V_L and J_L which are also members of multigene families (Brack and Tonegawa 1977). Thus the random association of

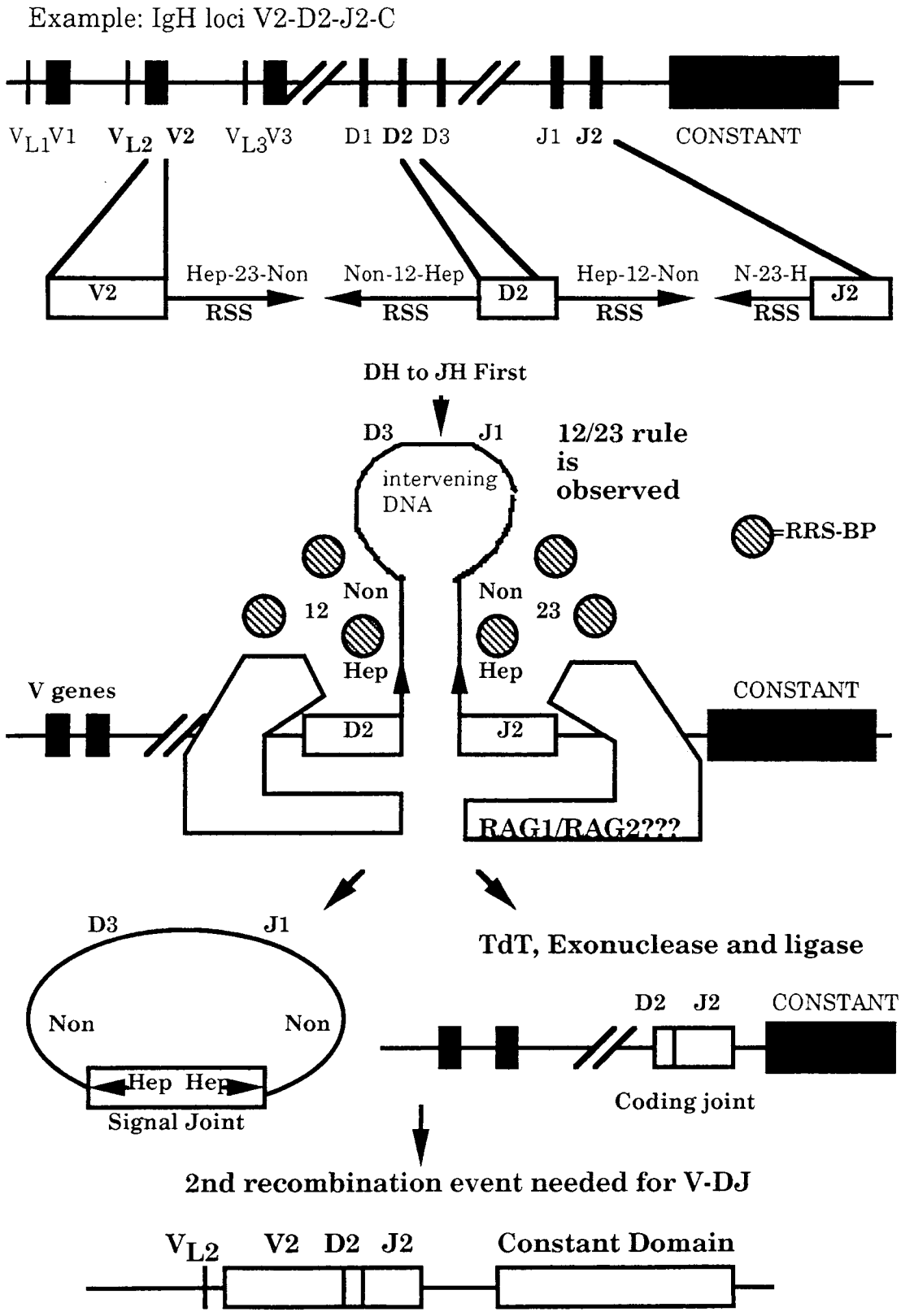
recombined heavy and light chains generates a considerable amount of antibody diversity in itself.

Recombination of the various variable gene segments is mediated in part by highly conserved sequence motifs which flank one or both sides of the gene segments involved in the generation of antibody diversity (Sakono et al 1979, Sakano et al 1980, Hesse 1989). The motifs, termed recombination signal sequences (RSSs), consist of a highly conserved dyad-symmetric heptamer sequence which is separated from an A/T rich nonomer sequence by either a 12 or 23 bp non-conserved spacer. All gene segments involved in variable region recombination have one type of configuration (12 or 23 by spacer) and the joining partner has the opposite type (12/23 rule). The non-conserved spacer corresponds roughly to 1 or 2 helical twists such that the heptamer and nonamer are positioned on the same side of the helix permiting recombination. The joining of two regions such as D_H to J_H involves recognition of RSSs, double stranded cuts at the heptamer segment border followed by re-ligation of the two coding sequences forming a coding joint (Roth et al 1992). Pairing of the RSSs between two segments to be joined results in the looping out of intervening DNA which is subsequently lost when recombination occurs at the ends of the heptameric sequences. This pairing process is believed to be mediated in part by a set of proteins which specifically recognizes the signals and brings them together prior to recombination. Such proteins would also need to discriminate spacer lengths in order to comply with the 12/23 spacer rule. Using radiolabeled RSSs as probes, several investigators have isolated cDNA clones which when expressed were shown to specifically recognize and bind to the recombination signal sequences

Figure 1.2 Basic steps involved in the somatic recombination events leading to the generation of the primary immunoglobulin repertoire. Shown is the proposed process for the rearrangement of the variable region gene segments variable (V), diversity (D) and joinging (J) for the Ig heavy chain loci. The leader peptide is designated as V_L. A similar process is utilized for the generation of the light chain variable region, the difference being the lack of diversity gene segments. Recombination signal sequences (RSS) which are composed of a highly conserved heptamer (Hep), a 12 or 23 bp spacer and a conserved nonamer sequence are found flanking each of the germ-line variable region gene segments and are brought together by a process which has yet to be fully defined (Tonegawa 1983, Hesse et al 1989). Studies indicate a set of proteins known as RSS binding proteins which may be partly responsible for holding gene segments in place (Matsunami et al 1989). The recombination activating proteins -1 and -2 are also believed to mediate the joining of gene segments (Schatz et al 1989, Carlson et al 1991). Shown are the steps for the generation of a unique heavy chain, a similar single recombination event would be utilized to join the light chain variable region. Junctional diversity is created prior to the actual joining of the two gene segments in question and is accomplished by base trimming via an exonuclease and base addition thought to be due to terminal deoxynucleotidyl transferase (TdT) (Landau et al 1987, Kallenbach et al 1992, Heinrich et al 1984). The intervening genes and recombination signal sequences are looped out and lost as a deletional circle during the joining process. The signal joint containing the recombination signal sequences and the coding joint are most likely joined by DNA ligase.

Figure1.2

Generation of immunoglobulin diversity



(Matsunami et al 1989,Shirakata et al 1991, Amakawa et al 1993). Upon sequence analysis, these clones displayed a high degree of similarity to the non-histone chromosomal protein HMG-1. A small region within the clones (40 amino acids) also suggests some similarity to the resolvase and integrase families which are involved in genome rearrangements. As stated before, recombination occurs between segments with different spacer lengths, ensuring that specific gene segments are joined in the proper order. For example, DH gene segments in mice are flanked on both sides by RSSs with 12 bp spacers and VH and JH are flanked 3' and 5' respectively by RSSs containing 23 bp spacers (Kurosawa et al 1981). Therefore joining of V_H to J_H in the assembly of the immunoglobulin heavy chain variable region is not possible due to the 12/23 spacer rule. A similar process is used for assembly of the light chain, the main difference being that the light chain is encoded by 2 gene segments, VH and JH, which are also governed by the 12/23 spacer rule.

Even though recombinatorial events can lead to a wide diversity of antigen receptors, the process is not flawless thus resulting in some amount of junctional diversity. When the double stranded cuts occur at the two heptameric sequences (Roth et al 1992,1993), coding joint imprecision may occur as the result of base loss and/or addition prior to ligation of the two gene segments (coding joint). The joining events between variable region gene segments can be imprecise in two aspects: bases can be trimmed via exonucleases and bases can be added between the joining junctions (Alt and Baltimore 1986, Landau et al 1987, Lieber et al 1988, Kallenbach et al 1992) further adding to diversity. There are two type of base additions seen in variable region assembly, one is

templated and the other random. P insertions, which occur on both heavy and light chains, are formed by a filling in process mediated by asymmetric cleavage of a hairpin intermediate during the recombination process resulting in palindromic insertions. N-insertions which occur only on the heavy chain are not encoded by the gene segments, but are simply inserted in a random fashion by the enzyme terminal deoxynucleotidyl transferase.

The joining of V-D-J segments produces the third CDR region, thus base trimming and addition events prior to joint ligation greatly enhances binding site diversity in antibody molecules (Tonegawa 1983). Occasionally, when a cell undergoes a null (nonproductive) V (D) J rearrangement due to a frameshift in the coding joint, the rearranged heavy chain can be rescued. A cryptic recombination signal has been found in mice which has been shown to allow replacement of a VH region with a different VH segment which then may be ligated in the proper reading frame resulting in a now functional rearrangement (Reth et al 1986)

The final stage of antibody diversity is generated through a process known as somatic hypermutation which is dependent upon antigen stimulation and T-cell help (Heinrich et al 1984, Manser 1987). In mammals somatic hypermutation is a process leading to point mutations in the complementary determining regions of immunoglobulins. These somatic mutants occur within germinal centers (Berek et al 1991, Kallberg et al 1993) which are the sites of intense B-cell clonal expansion and maturation (Kroese et al 1987, Jacob et al 1991), differentiation into memory and plasma B-cells (Coico et al 1983, Tew et al 1992), and cell death during an antibody response (Liu et

al 1989). A germinal center is developed by 2-3 antigen stimulated B-cells (centroblasts) which colonize a follicle in a secondary lymphoid tissue (spleen or lymph node).

Within the germinal centers, follicular dendritic cells take up antigen and hold it in the form of a non-processed immune complex (native) for considerable periods of time (months to years). Centroblasts possessing membrane bound antibodies with intermediate to high affinity for the antigen bind and are stimulated to undergo rapid proliferation. During this rapid proliferative response, the machinery responsible for V-region hyper-mutational events is initiated resulting in a wider repertoire from which higher affinity B-cells (antibodies) are selected for stimulation by antigen (affinity maturation). Centroblasts with lower affinity antibodies are not able to remain bound to the antigen and are negatively selected via apoptosis, where as centrocytes with higher affinity remain bound which correlates with the expression of bcl-2, an inhibitor of apoptosis (Berek 1992, Green and Scott 1994). Centroblasts undergoing these proliferative responses down regulate their overall surface antibody expression ten-fold, thus only those cells with high affinity can remain bound to the antigen complex. Thus we know where affinity maturation occurs, and some of the cell types involved, but little is still know about the machinery involved in the generation of mutants. Recently investigators have found ways of dissecting this problem by using methods for isolating and analyzing single cells during a response within the germinal centers (Kuppers et al 1993, Pascual et al 1994)Through this type of work it may be possible to isolate the enzymes responsible for the observed mutational events.

Affinity maturation thus can be thought of as a true form of natural selection. First via somatic hypermutation, variability is generated in the antigen-binding regions resulting in higher and lower affinity antibodies which is then followed by selection of those B-cells possessing antibodies with the highest affinity for the antigen in question. These selected B-cells then further differentiate into short lived antibody secreting plasma cells required for the primary response or may form a pool of high affinity memory B-cells.

Immunoglobulin gene families

Within the last ten years a great deal of knowledge has be gained in the field of vertebrate immunogenetics. One of the most intensely investigated areas concerns the genes encoding the heavy and light chains of immunoglobulins. It has long been believed that mammals contain hundreds if not thousands of V_H gene segments which compose a portion of the actual variable region (antigen binding site) in antibodies. Though this may be true for rodents, it does not appear to hold true for humans. Recently the entire V_H, D_H and J_H gene families in humans have been mapped and characterized. The V_H genes in humans fall into 7 distinct families (Matsuda et al 1993) and approximately 14 in mice (Tutter et al 1991). Investigations have discovered that the actual functional repertoire in humans is only composed of ~51 V_H, 30 D_H and 6 J_H gene segments and that the locus spans a distance of ~1.1 mbp (Cook et al 1994). Although hundreds to thousands of variable region gene segments have been identified, the vast majority have proved to be either pseudogenes or lacking in the

proper flanking sequences which govern rearrangement. Thus some may view the human repertoire as being restricted which has been widely used to describe the immune response in the lower vertebrates (Du Pasquier 1982). This limited repertoire of variable region genes only serves to stress the importance of junctional diversity, random association of heavy and light chains and the role of somatic hypermutation in the generation of a large functional repertoire of antibodies.

Immunoglobulins are found in all classes of vertebrates, the exception being the Agnathans which has yet to be fully investigated. In mammals there are five classes of heavy chains and two of light chains based upon amino acid analyses and functional criteria. Antibodies also have the potential to be found as secreted or membrane bound forms (Ig receptor), the difference being due to the hydrophobicity or philicity of their respective carboxyl tails which are generated by alternative mRNA splicing events. Fish in general possess a high molecular weight antibody most similar to the mammalian IgM molecule. In carp one report has described differences in antibodies found in the serum and mucus, but have not been defined functionally (Rombout et al 1993) In elasmobranchs (sharks and rays) and Dipnoi (lungfish) immunoglobulins are composed of a pentameric structure while a tetrameric form is found in osteichthyan fish (bony fish) (Wilson and Warr 1992). These multimeric forms seems to be the predominant species of immunoglobulins, but low molecular weight forms (monomers) have been reported in one teleost (Lobb and Clem 1981) and in the elasmobranchs (Kobayashi et al 1984, Kobayashi and Tomenaga 1988). The antibodies produced by fish are considerably less diverse and

lower in intrinsic affinity compared to mammalian immunoglobulins, but are comparable though in overall avidity.

The heavy chain loci in fish:

The basic monomeric immunoglobulin structure is composed of two heavy and two light chains connected via disulfide linkages. The heavy chain molecules are formed by the fusion of variable, diversity and joining gene segments by somatic recombination and the addition of constant domain gene segments (3-4) by mRNA splicing. Thus the heavy chain locus is composed of V_H, D_H, J_H gene families and C (constant) gene segments. The vertebrate organizational pattern for the gene segments in the heavy chain loci is quite diverse (figure 1.3). The mammalian and anuran amphibian loci is commonly referred to as the translocon type of pattern, where variable region gene families occur in the order of V_H, D_H, J_H followed by one group of constant region genes (Early et al 1980a, Schwager et al 1988). It has now been shown via Southern blotting, gene titration and sequence analysis of genomic clones that the teleost heavy chain locus is of the mammalian type (Ghaffari and Lobb 1989, Amemiya and Litman 1990, Matsunaga et al 1990, Lee et al 1993). Recently, Southern blot analysis has revealed that the Holstean fish are of the mammalian type as well (Wilson et al 1995a).

Another commonality found in the IgH loci of bony fish and mammals, is the finding of a large assortment of VH gene families which have been extensively characterized in the channel catfish and rainbow trout using Southern blot and PCR (Ghaffari and Lobb 1991, Roman and Charlemange 1994, Anderson and Matsunaga 1995). The

catfish seem to possess ~6-7 V_H gene families while the trout appears to be more heterogeneous having as many as 12 families. These various gene families may play specific roles against particular pathogens or could be expressed differentially during ontogeny to suit the needs of developing fish. IgV genes are categorized into a small number of separate IgV families based upon overall nucleotide similarity, where 80% similarity is used as the standard (Brodeur and Riblet 1984). Using nucleotide sequence analysis of the constant regions from immunoglobulin heavy chain cDNA clones, isotypic and allotypic differences have also been described in teleosts, whether these differences equate to functional differences has yet to be determined (Ghaffari and Lobb 1989, Hordvik et al 1992, Hansen et al 1994). A cDNA clone believed to encode the second class of immunoglobulin seen in the skate *Raja kenojei* has also been characterized (Harding et al 1990).

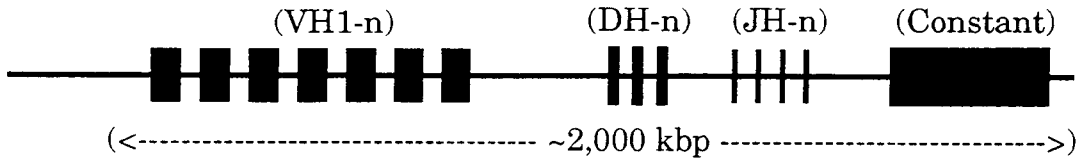
In avian lineages, a quite different theme for their organizational pattern exists, which consists of a single functional VH and JH gene that undergo gene conversion events with upstream pseudogenes to generate diversity post-recombination (Reynaud et al 1989). The pattern found in elasmobranchs is quite different in that multiples (possibly hundreds) of prefused and nonjoined VDJC clusters are found in the IgH locus (Litman et al 1985, Kokubu et al 1988, Harding et al 1990). The IgH locus of the coelocanth, *Latimeria chalumnae*, has also been partially characterized. In this fish the VH and DH genes have been found to be separated by only two to three hundred base pairs and it thus appears that the coelacanth IgH arrangement is most likely a transitional state between the elasmobranch and teleost IgH and

Figure 1.3. Schematic representation of the IgH loci in vertebrates. Mammalian, amphibian, teleostean and holostean loci are based on the work of Early et al 1980, Kawakami 1980, Schwager et al 1988, Du Pasquier et al 1989, Ghaffari and Lobb 1989, Amemiya and Litman 1990, Lee et al 1993 and Wilson et al 1995a,b. Partial characterization of coelacanth heavy chain loci is the work of Amemiya et al 1993. Elasmobranch arrangement is based on studies conducted by Litman et al 1985, Kokubu et al 1988 and Harding et al 1990. Finally the avian organizational pattern is from Reynaud et al 1989. Figure modified from Wilson and Warr 1992.

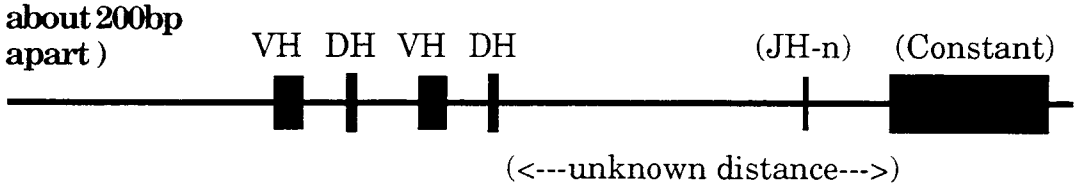
Figure 1.3

Heavy chain loci of vertebrates

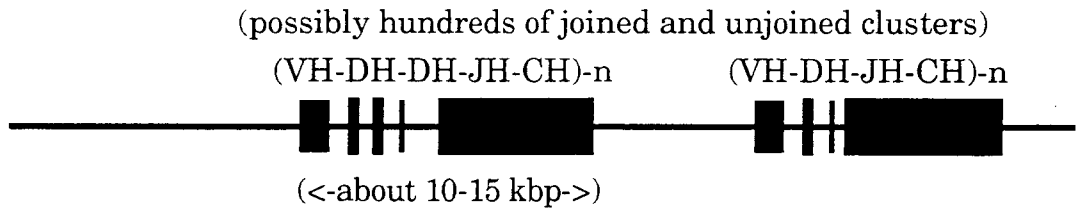
Mammals, Amphibians, Teleosts and Holosteans



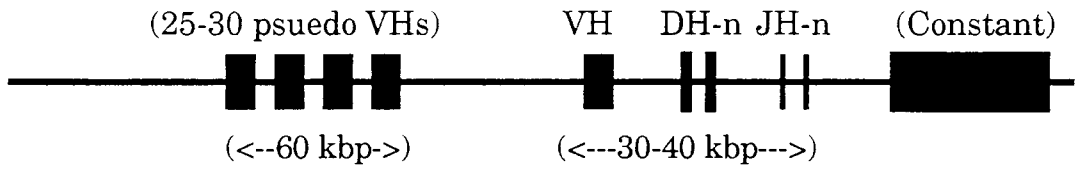
Coelacanth (VH-DH about 200bp apart)



Elasmobranchs (sharks and rays)



Avian species



therefore represents a third type of IgH gene organization for fish (Amemiya et al 1993).

cDNA clones encoding both the secreted and membrane bound forms of IgH have been isolated from a variety of fish. It appears that teleosts utilize an unusual splicing event compared to other vertebrates to yield their membrane bound forms of IgH. In mammals and elasmobranchs a cryptic donor site is found at the end of the fourth domain of the constant region which is utilized to splice to hydrophobic TM gene segments (Early et al 1980, Kokubu et al 1988). In teleosts, the typical 3' splice site of the C3 domain is spliced to the TM genes, leading to the deletion of the entire C4 domain (Bengten et al 1991,Warr et al 1992, Anderson and Matsunaga 1993, Hansen et al 1994) The Holostean fish, bowfin and gar, which appear in the fossil record sometime between the elasmobranch and teleosts, appear to use both the mammalian and teleost type of splicing events to yield 2 types of membrane bound IgH, both of which appear to be functional (Wilson et al 1995b).

The light chain locus

The immunoglobulin light chain organizational pattern in fish has recently been established. All vertebrate light chains are composed of VH, JH and a single constant domain. As previously discussed , the IgH gene organization in vertebrates has been extensively investigated to a much greater extent compared to the light chain loci. The light chain gene organization has only recently been characterized for the lower vertebrates. In mammals and amphibians, clusters (100s- 1,000s) of V_H

gene segments precede a few J_H gene segments followed by the C segments (Zachau et al 1989, Schwager 1991). Thus the pattern for IgH and IgL loci are similar to one another in mammals and amphibians. Avian species have a single functional V_H, J_H and C gene and utilize gene conversion events from a pool of upstream V_H pseudogenes in the same fashion as they do for IgH chains (Reynaud et al 1987, McCormack and Thompson 1990).

In the elasmobranchs, the multicluster type of gene organization has been found in the horned, sandbar and nurse shark (Shamblot et al 1989, Hohman et al 1992, Greenberg et al 1993) and has recently been extended to the spotted ratfish and little skate which are also members of the Chondrichthyes class (Rast et al 1994). In these species the light chain gene components for the variable region may be fully joined or unjoined in the multiclusters. The question that remained, is whether the teleosts would hold to the mammalian type of IgL organization as they do for the IgH loci. This question was answered by Daggfeldt and coworkers (1993) in their study of the light chain gene organization in Atlantic cod and rainbow trout. To do this polyclonal antisera was generated against the cod light chain and used to screen cDNA expression libraries. To the surprise of most comparative immunologists, they found that the teleost IgL loci is of the multicluster type of arrangement typical of the elasmobranchs, which may lead to limited diversity.

In mammals there are two classes (isotypes) of light chains, designated as lambda and kappa, each of which is encoded by separate loci (Zachau et al 1989, Selsing et al 1989). It appears that the lambda designation may not be fully realistic for Hayzer (1990) has documented

that the lambda loci may contain several functional C region gene segments. Whether these "other" C segments are utilized has not been clearly shown. The amphibian organization which is represented by Xenopus has two types of light chains known as pi and sigma (Schwager et al 1991). In sigma, it appears that a recent gene duplication event has occurred. Expression and sequence analysis indicates that two types of sigma are made, each with their own set of variable region genes. Using mAbs Hsu et al (1992) demonstrated that three types of light chains (u,x and v) are produced *in vivo* based upon western blot and 2-D gel electrophoresis analysis which correlates with the above findings. These authors suggest that although the repertoire in amphibians is similar on a genetic basis, compared to mammals, it may be restricted possibly due to a preferential pairing of heavy and light chains.

Lobb et al (1984) using mAbs has also found two types of light chain isotypes, known as F and G, in the channel catfish. Immuno-precipitation analysis of these two isotypes demonstrated a preferential usage of the G light chain during the early response and the F isotype during the later response. In rainbow trout studies have suggested that at least three types of light chain isotypes exist based upon mAb analysis of lymphocyte cell populations (Sanchez and Dominguez 1991, Sanchez et al 1995). Upon LPS induction, the two trout light chain reagents were shown to account for ~55-70% of total Ig in *in vitro* cultures and ~30% of total Ig in sera whereas a heavy chain reagent, mAb 1-14 (Deluca et al 1983), reacted with ~95% of total trout Ig. Based upon genomic and cDNA analysis there could be a wide variety of potential light chain isotypes in all teleosts. Although it doesn't appear that trout possess additional heavy chain isotypes (Lee et al 1993, Anderson and

Matsunaga 1993), IgH isotype heterogeneity has been reported in the channel catfish (Lobb and Clem 1982, Lobb and Olsen 1988) and Atlantic salmon (Hordvik et al 1992) based upon surface determinants, cDNA sequence and chromatographic analysis. The elasmobranch light chains have yet to be fully analyzed on a cellular basis, but sequence data predicts that a variety of light chain isotypes are possible.

Is the repertoire and maturation process restricted?

As mentioned earlier, most comparative immunologists will agree that the immune response in lower vertebrates is restricted in comparison with the mammals. The main question then, is what causes this restriction in antibody heterogeneity. Many believe that the observed restriction is due to either the lack of: somatic hypermutation, the failure to select mutants, limited junctional diversity, preferential pairing of heavy and light chains or possibly inadequate numbers of germline variable region gene segments.

In the amphibian *Xenopus*, Wilson et al (1992) set out to determine if somatic mutational events occurred during an immune response to DNP-KLH. In mice somatic hypermutation rates have been estimated to be between 10^{-3} to 10^{-5} per base pair per cell mitosis (Wabl et al 1985, Rajewsky et al 1988) resulting in 10^3 to 10^4 fold increase in the overall affinity of antibodies upon immunization. In the *Xenopus* study it was found that the mutational rate was about 7 times lower than that seen in mice resulting in a slight rise (~5 fold) in antibody affinity. The authors suggest that the mutational machinery is present and does indeed act upon functionally rearranged variable region genes, but due to the lack

of germinal centers, mutants are not properly selected. Investigations have shown that *Xenopus* does have a vast array of variable region genes, therefore restriction in this species is probably not due to a lack in the germline gene segments. Junctional diversity was then analyzed in *Xenopus* by characterizing the role of terminal deoxynucleotidyl transferase, TdT (Lee and Hsu 1994). Expression analysis demonstrated that TdT is expressed in adults, but not in tadpoles. This correlates with previous findings that showed a lack of N region insertions in tadpoles, but not in adults which display a more heterogeneous antibody repertoire (Hsu and Du Pasquier 1992).

Somatic mutation events have also been observed in the horned shark, *Heterodontus*, using a PCR-based approach to analyze the hyper-variable regions of expressed immunoglobulins. cDNAs of the variable regions were found to have minor, but definite signs of somatic mutation (Hinds-Frey et al 1993). Whether these mutations result in higher affinity antibodies was not investigated. It is reasonable to assume that the mutations would result in antibodies possessing higher and lower degrees of affinity. Immunologic memory has been demonstrated in teleosts (Lamers et al 1985, Arkoosh and Kaattari 1991), but affinity maturation greater than 10-fold has yet to be observed. Using an ELISA based approach, slight maturation in overall affinity (~4 fold) has been observed in trout (Shapiro and Kaattari 1994). One aspect that needs to be determined in teleosts is the observation of somatic mutants as observed in the amphibians and elasmobranchs. In one study, a histological investigation during the secondary immune response in carp reported the formation of what could be interpreted as a primitive germinal center in fish (Imagawa et al 1991), but true germinal centers

do not occur in the lower vertebrates. Similarly the process of class switching which is a hallmark of the secondary response in mammals, is not observed in teleost fish.

Further investigations are needed to answer the questions regarding the limited diversity and poor affinity maturation seen in the lower vertebrates. Looking at the germline elements it does not appear that the lower vertebrates are limited as far their potential of generating unique antigen binding sites by recombinatorial processes is concerned. Some will argue that the multicluster type of arrangement seen in the IgH loci in elasmobranchs and IgL loci of elasmobranchs and teleosts would be expected to limit the overall potential diversity of antibodies. Seeing that some clusters are in the nonjoined configuration the possibility to rearrange with other clusters due to the presence of recombinatorial signal sequences still exists. The consensus is that the low degree of affinity maturation must be due to the lack of germinal centers which are required for the proper selection of high affinity antibodies as seen in mammals. Another possibility for the overall lack of diversity and affinity maturation may be due to regulatory mechanisms or environmental factors.

Since the germ line Ig elements have been characterized in fish, investigators are now trying to elucidate the factors that control transcription of immunoglobulin loci. Recently the first immunoglobulin enhancer has been cloned and characterized from a teleost fish. Magor et al (1994) found that in channel catfish that the IgH enhancer was functional in both catfish and murine cell lines, thus demonstrating conservation of function. The position of the enhancer was not conserved, for it was found to be near the TM2 gene segments in

catfish where as in mammals it is located in the D gene segment area. These authors are currently attempting to identify other regulatory elements involved in immunoglobulin gene transcription in catfish which will no doubt be aided by the recent establishment of B cell lines in this teleost (Miller et al 1994). Similar findings have been reported in rainbow trout where reporter constructs containing murine immunoglobulin promoter/enhancer coupled to chloramphenicol transferase were used in the creation of transgenic trout. Expression of the murine promoter/CAT construct was observed only within B lymphocytes in the respective transgenic trout, whereas trout possessing the CMV/CAT contruct had expression in a variety of cell and tissue types (Michard-Vanh'ee et al 1994). These studies demonstrate that regulatory mechanisms involved in immunoglobulin transcription have been conserved over the last 150 million years.

Other molecules of the immunoglobulin superfamily found in fish

The immunoglobulin supergene family includes genes encoding immunoglobulins, T-cell receptors, components of the complement system, the major histocompatibility complex (MHC) plus a variety of other receptor-related molecules. The previous section was devoted to immunoglobulin genes due to the wealth of knowledge about them. Relatively little is known of T-cell receptor (TCR) molecules in the lower vertebrates even though immune responses typically mediated by these molecules have been cited in variety of investigations. Recently cDNAs coding for TCR-beta subunits have been isolated from amphibian, teleost (trout) and sharks using polymerase chain reaction technology (Fellah

et al 1993, Partula et al 1994, Rast and Litman 1994). All of these clones were shown to display conserved regions typically found in mammalian TCRs. In amphibians northern blot analysis demonstrated expression in the thymus and it was later determined that the beta subunits are organized in clusters of nine families similar to that found in mammals (Fellah et al 1994). Thymic RNA was the source of the trout clones suggesting that T-cells may develop within the thymus of teleosts. Shark TCR clones also proved to be of the multicluster type of gene arrangement as are their immunoglobulin genes.

The fish major histocompatibility complex genes have been characterized. The human complex spans some 2 mbp and encodes both class I and II plus other genes involved in antigen presentation (Campbell and Trowsdale 1993). The organizational pattern has yet to be defined in fish. cDNAs coding for class I, II and III (B_2-microglobulin) MHC molecules have been cloned in fish (Dixon et al 1995). The information drawn from these studies will no doubt aid in defining the mechanisms utilized by fish for antigen presentation and processing.

Recently a novel type of antigen receptor has been cloned and characterized in fish. The molecule designated NAR (nurse shark antigen receptor) was cloned using 3' RACE technology (Greenberg et al 1995) and based upon sequence and organizational analysis can be considered to be a new member of the immunoglobulin supergene family. NAR found as a dimer in sera, contains five constant regions and one variable region and appears to undergo somatic diversification.

Lymphocyte origins

In vertebrates, the cells of the lymphoid system originate, develop and reside in specialized organs and environments. Over the course of two decades it has been demonstrated that the sites for the primary production of mammalian lymphoid, myeloid, and erythroid precursors include the yolk sac, liver, bone marrow and spleen during fetal development and the primarily the bone marrow in adults (Johnson and Moore 1975, Owen et al 1977, Kincade 1981, Whitlock et al 1985, Ikuta et al 1992, Schatz et al 1992). These precursor cells differentiate in the bone marrow to form pre-B and pre-T cells (LePault 1983). The progenitors for T-cells migrate to the thymus where they differentiate into T-cell subsets (Owen 1972), while the pre-B cells remain in the marrow to undergo B-cell differentiation (LePault 1983, Ikuta et al 1992).

Precursor B lymphocytes, pre B-cells, which express cytoplasmic Ig heavy chain, but lack surface Ig, have been detected in the livers of 11 day old mice and 7 week old human fetuses prior to the appearance of surface IgM positive B-cells (Raff et al 1976, Gathings 1977, Andrew and Owen 1978, Velardi and Cooper 1984). More recently investigators were able to isolate progenitor cells from murine yolk sac and embryos at somite stage 10 which corresponds to day-8 of gestation. These cells were capable of differentiating into both B and T cells and that the progenitors were diverse with respect to allotype and VH gene usage (Cumano et al 1993, Palacios and Imhof 1993, Godin et al 1995). Combined, these studies and others lay the foundation for the contention that these cells are the precursors of functionally mature B lymphocytes which can be found in various proportions in all lymphoid tissues.

Lymphocyte ontogeny in fish

Investigations dealing with the immune system of fish is of phylogenetic interest mainly because fish are the first group of animals displaying the basic characteristics of higher vertebrate immune systems such as that found in mammals (Faisel and Hetrick 1992). These include the possession of well defined lymphoid organs and tissues, lymphocytes, and B/T cell cooperation. Using serological markers or functional assays, B cells have been detected in all fish immune organs. These include the thymus, spleen, kidney and gut (Ellis 1977, Fange 1982, Faisel and Hetrick 1992). Agnathans (hagfish and lampreys) lack well defined lymphoid tissue and lymphocytes (Riviere et al 1975, Page and Rowley 1982). Hagfish are devoid of thymus and spleen and it appears that lymphocytes are produced either in the head kidney or the gut. Lampreys also lack a well defined thymus, but appear to have a primitive spleen and tissues which may be the precursor of a bone marrow environment (Takeda, 1981, Zapata 1983). All jawed vertebrates, gnathostomata, have a well developed thymus, spleen, and gut associated lymphoid tissue. In cartilaginous fish, ontological studies have shown that the liver is the first organ to contain immunoglobulin positive cells at 2 months post hatch, followed by the kidney, thymus and later the spleen (Lloyd-Evans 1993). It has also been suggested that the spleen of the skate may be the site for B-cell development in this animal based upon the observation of double-isotype producing lymphocytes, but further studies need to be conducted to validate this point (Kobayashi et al 1985).

Mammalian B-cells are known to originate from pluripotent hematopoietic stem cells in the bone marrow, but the exact ontological locations of B-cell development has yet to be fully defined in teleosts. The anterior kidney, spleen and peripheral blood contain the vast majority of mature B-lymphocytes in fish, with a small population being found in the thymus. Most investigators agree that the anterior kidney and trunk kidney are the major sites of erythrocyte, monocyte and B-lymphocyte development (Ellis 1977, Zapata 1979, Bothman and Manning 1981) and are generally referred to as the bone marrow equivalent in teleost fish. The spleen is also considered a hematopoietic organ in teleost fish, but its role is thought to be strictly limited to erythropoiesis (Rowley et al 1988). The fetal and adult liver is not thought to play a role in hematopoiesis in teleost fish (Zapata and Cooper 1990)

During lymphocyte ontogeny in fish, the thymus is the first tissue to display lymphocytes prior to their appearance in the kidney, spleen and blood (Ellis 1977, Grace and Manning 1980, Secombes et al 1983, Razquin et al 1990, Josefsson and Tatner 1993). The thymus in fish is also the first defined lymphoid tissue to develop during ontogeny and as in higher vertebrates it is composed of lymphocytes and lymphoblasts contained within a network of reticular epithelial cells (Tatner and Manning 1983, Chilmonczyk 1985). The thymus in fish originates as a thickening from the pharyngeal epithelium, but instead of budding off as in higher vertebrates, it remains in direct contact with the pharynx (Chilmonczyk 1992). Thymic infiltration by lymphocytes has been detected 22 days pre-hatch in Atlantic salmon and 5 days pre-hatch in rainbow trout (Ellis 1977, Grace and Manning 1980). Using tritiated thymocytes, Tatner (1985) demonstrated that these *in-situ* labeled

thymocytes could be traced to the kidney and spleen after intrathymic injection. This observation demonstrates that thymocytes in fish as in mammals are exported from the thymus to peripheral organs and blood.

In rainbow trout, thymocytes are separated from the external environment by a single layer of epithilial cells (Tatner and Manning 1982). These authors postulate that this may lead to direct stimulation of thymocytes early in the ontogeny of fish which may be related to the early appearance of cellular immunity. The origins of thymocyte precursors has yet to be fully defined. One possibility is that the origin of the first thymocyte progenitor is external, such as being derived from the yolk sac or kidney and these stem cells are maintained throughout life within the thymic environment. It is not absolutely clear whether lymphocyte precursors originate from organs other than the thymus, but histological and functional studies indicate that the thymus is most likely the primary lymphoid tissue for T-cell development in fish (Chilmonczyk 1992). In support of the putative role of the thymus for T-cell development in fish is the finding that thymectomy of young trout resulted in decreased numbers of lymphocytes in the spleen, but not in the kidney (Manning et al 1982 a,b). The use of serological markers to define lymphoid populations will certainly resolve this question.

The thymus may be the first organ to contain lymphocytes, but the anterior kidney is the first organ during development to contain hematopoietic foci (Ellis 1977, Grace and Manning 1980, Bothman and Manning 1981, Razquin 1990, Josefsson and Tatner 1990). In Atlantic salmon, Ellis (1977) noted that hematopoietic foci were present in the kidney prior to the appearance of lymphocytes in the thymus. Most

investigators believe that the anterior kidney is the site of B-cell differentiation (Kaattari and Irwin 1985, Irwin and Kaattari 1986) and serves as the source of progenitors for thymocytes as well. Castillo et al (1993) reported the appearance of lymphocytes displaying cytoplasmic heavy chain at day 12 pre-hatch in trout and that surface Ig postitive ($sIgM^{+}$) cells were observed 4 days later. These authors designated the cells observed at day 12 as "pre-B" cells, this may be the case, but for a true definition of pre-B cells, the absence of cytoplasmic light chain needs to be demonstrated. Razquin and coworkers (1990) demonstrated that the anterior kidney is the first site during trout ontogeny to bear sIg^{+} cells at about day 4-5 post hatch. Approximately 3 weeks later sIg^{+} positive cells could be found in the spleen and thymus. This correlates with the findings of Secombes et al (1983) who showed that sIg^{+} cells were first detected within the kidney in carp as well. As mentioned earlier the first sIg^{+} cells were detected before the kidney becomes lymphopoietic (Castillo et al 1993), therefore progenitors of B cells most likely have origins in other tissues during embryonic development and then colonize the kidney later during ontogeny. Histoenzymatic and immuno-histochemical analysis of rainbow trout kidney (Castillo et al 1987, Razquin 1990) reveal that it possesses a framework of reticular cells and phagocytes which resemble the stromal microenvironment of mammalian bone marrow. Therefore the kidney likely serves as the primary organ for B-cell development as well as being the source of stem cells in developing fish.

Affinity analysis has also been used in mammalian models to help characterize the development of B cell populations in proposed lymphopoietic organs (Goidl and Siskind 1974). In these studies organs

displaying repertoires of homogeneous B cells with low affinity were considered to be primary organs for B cell development whereas organs with more heterogeneous populations of B cells were designated as secondary lymphoid organs. Using plaque forming analysis in immunized coho salmon it was determined that the anterior kidney possessed restricted B-cell populations with low affinity while the spleen and posterior kidney contained a more heterogeneous population of B cells with a higher average affinity these clones (Kaattari and Irwin 1985, Irwin and Kaattari 1986). The bone marrow in mammals has also been shown to contain a population of cells which are capable of regulating immunologic processes (Mortari et al 1986). Using irradiated and non-irradiated pronephros cells, DeKoning (1992) demonstrated that the trout kidney possess cells capable of regulating the mitogenesis of splenic and PBLs stimulated with lipopolysaccharide. These results provide further support for the notion that the kidney of fish has functions similar to that found in the bone marrow of mammals.

With the advent of molecular biology investigators have the ability to take a different approach than the traditional use of serological and functional markers to define the organs involved in lymphocyte development. In the Atlantic cod, northern blot analysis determined that the major expression of the membrane-bound and secreted forms of IgH were found in the head kidney when compared to the liver, ovaries and spleen (Bengten et al 1990). The expression in the liver is somewhat surprising seeing that this organ is relatively devoid of lymphocytes. A plausible explanation could be that their liver preparation was contaminated via peripheral blood lymphocytes or possibly that the liver

serves some rudimentary function in lymphopoiesis. A similar study was conducted in Atlantic cod and rainbow trout using Ig light chain cDNA probes (Daggfeldt et al 1993). In this study light chain expression was noted in both the kidney and spleen in northern blots. Of interest was the finding that the constant domain of IgL was transcribed prior to rearrangement and regularly spliced to the J segment. The presence of these nonrearranged transcripts and multiple forms of IgL transcripts in general could be interpreted as identifying these organs as sites for B cell lymphopoiesis (Daggfeldt et al 1993). Reports investigating mammalian pre-B cells have shown similar findings and it is believed to be necessary for the induction of rearrangement (Nelson 1985, Schlissel et al 1989, Schlissel and Morrow 1994). Thus these findings suggest that the head kidney as well as the spleen in teleost fish may house populations of putative pre-B cells. Bernstein and co-workers (1994) proposed that the thymus and spleen are putative sites for lymphocyte development in sharks based upon RT-PCR expression analysis of shark RAG-1. These were the only organs investigated and negative controls were inappropriate, thus any conlusion drawn from this study are purely speculative and await confirmation.

Basic types of teleost leukocytes

The aspects governing the immune response in virtually all vertebrates is due to the activation or induction of immune cell types. The following sections will cover the basic 5 types of leukocytes seen in fish as follows: 1. monocytes/macrophages 2. granulocytes 3. T lineage lymphocytes, 4. Natural Killer/ non-specific cytotoxic cells and 5. B

lineage lymphocytes (brief coverage). The function of these cells in the immune response in vertebrate taxa including fish can be categorized into the nonspecific (innate), adaptive, or specific immunity (Golub and Green, 1991).

Specific immune interactions display four main features: self/non-self recognition, inducibility via antigen, antigen specificity and a display of a memory component. Whereas nonspecific immune reactions generally deal with only two of these components, that is being inducible by antigen and the ability to distinguish self and non-self. The cells of the myeloid lineage, monocytes/macrophages/granulocytes are generally thought of as components of the innate and nonspecific immunity, whereas both the monocytic and lymphocytic lineages (B and T-cells) are thought of as the main mediators of the specific immune response. It will be discussed though how the monocyte lineage is involved in both the nonspecific immune response as phagocytes and the specific immune response as antigen processing and presenting cells. The latter of which is a key component for T-dependent immune response which leads to an enhanced production of antibodies via interactions of all three cell types.

Monocytes and macrophages

As in mammals, one refers to circulating mononuclear cells as monocytes, while macrophages are either wandering or fixed within tissues. A complete cytochemical analysis of these cells types in fish can be found in the review by Rowley et al 1988. There are basically two main types of macrophages in fish that have been characterized: 1.

melanomacrophages which contain melanin and other pigments such as lipofuscin and haemosiderin (Argius, 1985), 2. monocytes and macrophages which seem to differ both in size and lysosome quantities. By far the melanomacrophages are the most numerous in fish.

Fish monocytes and macrophages possess the same basic functions as seen in mammalian species, such as phagocytosis of antigen-antibody complexes (reviewed by Secombes and Fletcher, 1992), antigen processing and presentation (Reviewed by Vallejo et al. 1992), production of cytokine-like molecules (Secombes, 1991) and potential immunomodulating factors such as leukotrienes (Secombes and Fletcher, 1992). In general the monocytes and macrophages are the key players in the overall specific vertebrate immune response. Through their actions, they induce and modulate both B and T-cells in almost all aspects of the specific immune response.

Various receptor types have been demonstrated on fish macrophages which are thought to enhance phagocytosis and killing abilities. Studies using *Onchorynchus rhodurus* have shown lectin-like molecules to be present on the surface of fish macrophages. Investigators have demonstrated opsinization of complement in rainbow trout (Honda et al.,1985 , Michel et al., 1990) which is indicative that fish macrophages possess complement receptors. Griffin (1983) has shown that macrophages also possess a Fc receptor (FcR) in anti-*Yersinia ruckeri* immune responses in rainbow trout. In a more phylogenetically distant species, it was shown that the nurse sharks, *Ginglymostoma cirratum*, most likely have Fc receptors as well on splenic macrophages as demonstrated by an erythrocyte/antibody rosette assay (Haynes et al 1988). It has yet to be determined if the cyclostomes

(hagfish, lampreys) possess these basic receptors as well. Most likely it will be found that cyclostomes possess complement receptors since it has been shown that lampreys have genes which encode portions of the complement system.

It is generally accepted that monocyte's and macrophage's key role in the specific immune response is that of antigen processing and presentation. Through this function they are able to stimulate the cellular arm (T-cells) of the immune system via presentation of foreign peptides in association with MHC class II molecules to antigen receptors on T-helper cells. This is a crucial event for B and T cell cooperation during the immune response. Antigen processing and presentation has been clearly shown in the carp (Rombout and Van de Berg, 1989) and channel catfish (Vallejo et al. 1990., Vallejo et a. 1991a). In the carp studies, it was shown that antigenic deteminants of ferritin or*Vibrio anguillarum* were processed and presented on the surface of gut melanomacrophages after anal intubation. This presentation resulted in cellular proliferation. More extensive studies on this topic have been performed in the channel catfish by *in vitro* antigen pulsing of *in vivo* primed antigen presenting cells (APCs). A key feature of this and subsequent studies involving the catfish immune response was the generation of spontaneous (~30-40%) long term leukocytes lines from non-stimulated PBLs (Vallejo et al. 1991b). In this study, macrophage like cell lines were pulsed *in vivo* with antigen, fixed in paraformaldehyde and added to autologous PBLs *in vitro*. Both antigen specific proliferation and enhanced antibody production to T-dependent (TD) antigens were measured. In these studies PBLs that were fixed prior to antigen pulsing, displayed no marked proliferation or antibody

production when co-cultured with autologous PBLs. The same situation occured when the investigators used allogeneic PBLs as responders. The results of the channel catfish studies indicate that a MHC type of molecule regulates presentation of processed peptides and that the response in teleost is restricted (Vallejo et al. 1991c) as is seen in higher vertebrate species.

As mentioned earlier, cytokine like production has been suggested in teleost species. Most investigators have focussed on an interleukin-1 (IL-1) like factor produced by fish macrophages. IL-1 is known to be a pleotrophic molecule with many attributes, one being the essential co-stimulatory signal for T-cell activation and proliferation (Weaver and Unanue 1990). IL-1 appears to be a very conserved molecule which evolved early in the phylogeny of the vertebrates for IL-1-like molecules and associated activities have been reported in a number of teleost species including carp (Verburg-van Kemenade et al 1995), channel catfish (Clem et al., 1991) and rainbow trout (Ortega 1993). Thus far the catfish and rainbow trout models have shown the greatest similarity to mammalian IL-1 molecules. Clem and cowokers demonstrated that LPS stimulated catfish monocytes produced supernatants which possessed an activity which could replace monocytes in *in vitro* assays. It was also shown that the factors (cytokines) within the stimulated supernatants were able to induce proliferation of murine cortical thymocytes *in vitro* and that this activity could be reduced if mammalian anti-IL-1 polyclonal sera was included. In the studies involving rainbow trout, Ortega demonstrated biological activity in plaque forming assays using supernatants derived from LPS or antigen stimulated adherent macrophages. The "factor" was later determined to have a molecular

weight of approximately 20 kda, similar to that of mammalian IL-1. Thus it is clear that fish leukocytes are capable of secreting factors which can potentially activate and regulate other leukocyte populations.

Granulocytes

Fish granuloctyes have been characterized primarily based upon giemsa staining, ultrastructure, resemblence to mammalian granulocytes, enzymatic activity (i.e. peroxidase) or via Sudan black staining (Ainsworth, 1992., Bielek, 1981 and Ellsaesser et al. 1985). To date, two comprehensive reviews may be found on teleost granulocytes (Ainsworth 1992 and Hine 1992). Catfish granulocytes have been characterized using mAbs specific for neutrophils (Bly et al. 1990). Neutrophils appear to be the only class of granulocytes found within catfish (Ellsaesser et al. 1985), but other classes such as eosinophils and basophils have been distinguished in carp (Temmink and Bayne 1987). More recently, Cross and Matthews (1991) have described yet a fourth class of granuloctyes in carp, further demonstrating the parallel of immune cell types found within all vertebrates. This fourth type, an eosinophilic granular cell, is present within the skin, but can not be found in the peripheral blood system. This resembles a primitive cell type found within salmonids which is believed to be a mast cell (Vallejo and Ellis 1989; Powell et al. 1990).

As in mammalian species, teleost granuloctyes are involved in the inflammatory response (Hine, 1992) and have been shown to demonstrate chemotactic and phagoctytic activities (Ainsworth 1992). Studies in both plaice and carp clearly show that fish granuloctyes were

efficient in phagocytizing complement opsonized particles, but antibody opsonized particles revealed little effect (Nash et al. 1987; Matsuyama et al., 1992). From these experiments one could conclude that fish granulocytes possess receptors for complement, but not for antibodies (no Fc-like recptors) which differs from that seen in mammalian granulocytes (both receptor types). Carp neutrophils have also been shown to secrete a factor similar to interleukin-1 (Verburg-van Kemenade et al 1995). All of these studies indicate similarities and subtle differences of fish granulocytes to their mammalian counterparts.

Thymus derived lymphocytes

Thymus derived lymphocytes, or T-cells, are a set of lymphyocytes usually defined by the presence of cell surface receptors (TCR) which interact with MHC class I and II molecules and differentiate self from non-self. Their involvement in the immune response can be simplified to two functions, 1) detection of non-self antigens on autologous cells via contact of TCR with MHC presented antigens and 2) enhancement of antibody production by B-cells via T-cell help in the form of secreted lymphokines. Numerous reports in fish have described morphological, functional and mitogenic responses of fish lymphocytes which are reminscent of mammalian T-cells. Ellis was the first to give a complete morphological description of the cell types found within fish. He posed that fish lymphocytes are morphologically similar to their preposed mammalian counterparts (Ellis 1977b). They are generally small in size (3-10 microns) and have a high nuclear to cytoplasm ratio. The

lymphocytes found within teleosts are comprised of a rather heterogenous mixture thought to be similar to mammalian leukocytes.

In mammals, a standard test for lymphoctyes is their proliferative response to the B cell mitogens pokeweed mitogen (PWM) and lipopolysacchride (LPS) and to the T-cell mitogens, phytohemagglutinin and concanavalin A) (ConA) (Harwell et al 1976). In this assay, lymphocytes are stimulated *in vitro* with known B or T-cell mitogens and their proliferative response is measured via 3-H thymidine uptake. Using this basic method investigators have been able to clearly distinguish that fish lymphocytes are composed of a heterogenous mixture (Etlinger et al 1976, Cuchens and Clem 1977, Warr and Simon 1983, Caspi et al 1984). Etlinger's study showed how the mitogenic response is tissue specific as well, indicating that specific fish tissues most likely harbor distinct lymphoid populations. Development of mAbs specific for lymphocyte surface determinants allowed investigators to partition fish lymphocytes into sIg- (T-cells, macrophages and pre-B cells) and sIg^+ (B-cells) fractions (DeLuca et al 1983, Sizemore et al. 1984, Ainsworth 1990). These studies revealed that the sIg^- fractions in the presence of adherent cells responded well to the classical T-cell mitogens, but not to B-cell mitogens. According to Ellsaesser and coworkers (1988), the phenotypic class of lymphocytes within the thymus were of the sIg- nature, indicating that the thymus harbors a large population of potential fish T-cells. In similar studies, mAbs generated against catfish sIg- lymphocytes demonstrated that this pool of lymphocytes could act as helper cells in antibody production assays in response to T-dependent antigens (Miller 1987). This reagent however reacts with sIg- thymocytes, thrombocytes, neutrophils and

brain cells, therefore they can not be considered to be T-cell specific, but simply specific for sIg- lymphocyte phenotypes. Another aspect typical of T-cells is their role in the mixed lymphocyte reaction (MLR). A number of reports have described fish lymphocytes (T-cells) acting as both responders and stimulators in MLR assays (Ellis 1977a, Caspi and Avtalion 1984, Miller et al. 1986, Kaattari and Holland 1990). In these assays, when lymphocytes from two unrelated individuals are mixed together in culture, proliferation indicates the ability to distinguish allogenic differences in the major histocompatiblity antigens (MHC) present on the cell types.

Some of the earliest methods used to show that fish could discern self from non-self came from experiments which examined both allograft rejection ability (Bothman and Manning 1981) and delayed type hypersensitivity reactions (Bartos and Sommer 1981., Pauley and Heartwell 1983., Stevenson and Raymond 1990). So far the ability to reject allografts has been shown to be present in the most primitive species of fish including elasmobranchs and agnathans. Rejection is typically slow in these more phylogenetically distant groups, whereas teleosts display a fairly rapid response. In 1 year old carp, first-set rejection of scale implants occurs around day 14-16 and second-set grafts start to be rejected as early as days 6-7 indicating the presence of a memory component in the cellular arm of the immune system of teleosts. In this study autografts displayed no signs of rejection. Further studies showed that the cellular response develops quite early in the ontogeny of carp. In these studies, sixteen day old fry were capable of rejecting allografts and to show a more rapid response when second set grafts were implanted, thus showing that memory is present in

young fish as well (Manning et al 1982a, b). Other tests have demonstrated that fish lymphocytes display delayed type hypersensitivity reactions when antigen is injected into antigen primed fish. When killed mycobacteria was injected into primed fish, inflammation resulting from an accumulation of leukocytes could be seen within a few days at the site of injection. Therefore these studies dealing with graft rejection and DTH clearly prove cellular immunity in fish.

Cytokine-like factors similar to those secreted by stimulated mammalian T-cells have also been described in fish. Using supernatants from ConA and phorbol myristate acetate stimulated trout PBLs, macrophages displayed enhanced killing capabilities and increased respiratory burst activity (Graham and Secombes 1988a). A futher investigation using panned cells, showed that sIg^- pools and not sIg^+ secrete this interferon-like factor, indicating that T-cells and or accessory cells are needed for this type of activation (Graham and Secombes 1988b). Tamai and coworkers (1993) have recently cloned and expressed a putative cDNA thought to encode flatfish interferon-gamma. The amino acid sequence showed an average of 23% similarity to mammalian interferon-gamma and the expressed recombinant protein displayed antiviral activity in flatfish cells lines infected with a flatfish rhabdovirus. Upon close inspection of this analysis, whether or not this cDNA actually ecodes an interferon-like molecule will have to await future investigations.

IL-2 which is produced by IL-1 (secreted from macrophages) induced helper T-cells, is one of the most intensively studied monokines in mammalian immunology shown to directly stimulate the proliferation of T-cells displaying an IL-2 receptor. Supernatants from

PHA-stimulated carp lymphocytes were shown to induce the proliferation of lymphoblasts in way similar to that observed when murine IL-2 was added (Caspi and Avtalion 1984). Further investigation in carp showed a similar response, but in this the study supernatants were derived from MLR cultures (Grondel et al 1984). To date, the only molecular data regarding fish IL-2 comes from PCR amplification of flatfish IL-2 (Tamai et al, 1992). In this study, a cDNA putatively encoding flatfish IL-2 was isolated and expressed within transfected COS cells resulting in the production of a 14 Kda protein. A region of the amino acid the sequence displayed 42% similarity to mammalian IL-2. The authors have yet to demonstrate that the synthesized protein has biological activity on fish leukocytes and, until this has been accomplished, its role in the fish immune response is still tentative.

Basically these studies show that fish leukocytes are capable of producing and responding to"factors" which resemble those seen in mammalian T-cell analyses. Various laboratories are currently attempting to isolate and clone these genes which encode fish lymphokines. Until these factors have been isolated, their regulatory role in the overall immune response in fish will depend upon the use of supernatants from stimulated leukocytes.

Non-specific cytotoxic cells/ natural killer cells

Natural killer cells (NK) are a class of lymphocytes with the ability to lyse and thus kill cells displaying foreign antigenic determinants without the need of prior activation, thus acting as a type of natural immune surveillance (Trinchieri 1989). Their main purpose is to

identify and kill virally infected, cancerous cells and intracellular parasites. NK cells are generally the earliest component of the host immune response to viral infections. In mammals, when NK cells are stimulated by IL-2 they are known as lymphokine-activated killer cells (LAK) and their associated killing activity is increased as is their ability to lyse a larger spectrum of targets.

In teleosts the cellular homologue of NK cells are a class of cells known as non-specific cytotoxic cells (NCC). Using channel catfish, Graves and co-workers demonstrated that NCCs isolated from the pro-nephos demonstrated a rapid killing capability of human tumor cell lines. In this study, over 90% of the target cells were lysed within a 90 minute time peroid (Graves et al., 1984). Greenlee el al (1991) later showed that the killing resulted from a direct cell to cell contact by necrotic and apoptotic mechanisms of agranular NCCs as shown by chromium release assays and DNA fragmentation of target cells. The exact mechanism by which NCCs lyse target cells is unknown and must be somewhat different from NK cells due to the lack of cytoplasmic granules. Also the overall kinetics of killing, general morphology and target cell specificity are different for NCC and NK cells (Evans and Jaso-Friedmann 1992).

Various mAbs have been generated against teleost NCCs that cross react with a conserved determinant (receptor) on NK cells (Evans et al 1988, Harris et al. 1991, Harris et al 1993). Using these anti-NCC reagents, investigators have shown that the mAbs most likely bind to the actual receptor on these cell types as shown by an inhibition of cytotoxicity against a variety of transformed cell lines. However, these reagents failed to diminish the ability of NCC or NK cells in their role in

antibody-dependent cell mediated cytotoxicity, thus lending further support that the mAb is an anti-NCC receptor reagent.

As discussed earlier, when NK cells are activated (LAK) they display an enhanced killing ability. Recently a factor termed natural killer enhancing factor (NKEF) has been cloned and characterized in mammals which significantly augments NK cytotoxicity (Shau et al 1994). NKEF was shown to be related to a class of molecules produced in organisms undergoing oxidative stress, therefore the factor may likely play a dual role in NK cells by increasing cytotoxicity and as a protective mechanism to deal with oxidative stress. Using a degenerate primer approach, a full-length cDNA for rainbow trout NKEF has been cloned and sequenced (Mourich et al 1995). This trout clone displays 70% and 84% similarity at the nucleotide and amino acid level to the human clone of NKEF, thus showing a high degree of sequence conservation over the course of vertebrate evolution. Studies are currently underway using recombinantly produced trout NKEF to determine its role in enhancing NCC cytotoxicity of virally infected and transformed trout cell lines and thus potentially demonstrating a conserved function.

Fish B -Cells

The specificity of the humoral immune response and its ability to adapt to new pathogens/antigens is largely due to antibodies which are produced by B-cells (Honjo 1985). Investigations have shown that fish possess B-cells with many of the same general characteristics as found in mammalian B-cells (Kaattari 1992). B-cells can be defined as those lymphocytes which express membrane bound immunoglobulin

(receptor) and in response to antigenic or mitogenic stimulation can secrete immunoglobulins (effector) with specificity to the antigenic stimuli (McKinney et al 1977, Marchalonis 1982). These activated B-cells undergo a rapid proliferative response to generate a pool of memory and plasma B cells. The plasma cells are capable of secreting large quantities of antibodies while the memory pool serves as a "watch-dog" for future encounters with the same antigenic stimuli. This memory pool is largely responsible for the secondary immune response. Upon secondary encounter of the same antigen, the relatively quiescent pool of memory cells are induced to quickly produce large quantities of antigen specific antibodies. One report contends that B cells found in the primary and secondary immune response are actually formed from separate precursor subpopulation (Linton et al 1989). In mammals, a class switching event is seen in the transition from the primary to secondary response. Class switching events during an immune response nor true affinity maturation has yet to be demonstrated in fish.

As discussed earlier, a classical measure for discerning lymphocytes is their reactivity to specific mitogens. Early evidence for distinct populations of lymphocytes (B and T-cells) were derived from *in vitro* mitogenic characterization of various fish organs. The B-cell mitogens, LPS and PWM, have been used in several studies to demonstrate that fish B-cells react in a similar way as compared to mammalian B-cells. Generally speaking when exposed to B-cell mitogens, fish B-cells undergo a proliferative response and increase the production of polyclonal antibodies (Etlinger et al. 1977, Kaattari and Irwin 1985, Kaattari and Yui 1987). Kaattari and Irwin demonstrated that lymphocytes from the pronephros (head kidney) responded well to

O-extract preparations of *Vibrio anguilarium* in plaque forming assays. Similar studies using panned sIg^+ lymphocytes from carp demonstrated that upon stimulation with LPS, a marked proliferative response was seen compared to sIg- lymphocytes [(T-cells) (Koumans van-Diepen et al. 1984)].

The hemolytic plaque assay has been used in fish immunology to define antibody producing cells (plasma cells). Briefly, this assay is composed of coupling a specific antigen to sheep red blood cells and adding antigenically primed peripheral blood leukocytes in the presence of comlement. If B-cells specific for the antigen in question are present they will produce antibodies which bind to the antigen followed by complement which will lyse the target red blood cells. The amount of plaques formed is indicative of the quantitiy of antibody producing B-cells present and thus can serve as a measure of memory as well. Using this technique pioneering studies in teleost immunology were able to discern that antibody producing cells could be found in the spleen, peripheral blood and pronephros in trout and perch (Chiller et al 1969a, Chiller et al 1969b, Pontius and Ambroisius 1971). In teleosts memory induction does occur, but only by 10-30 fold over the primary response, as compared to mammals which exhibit roughly 100 fold or more improvement. In trout *in vivo* and *in vitro* analysis of antibody producing cells demonstrated that memory occurs, but not to the degree found in mammals. The authors indicated though that memory in fish may be attributed to a simple exansion of the B-cell precursor pool due to polyclonal activation based upon limiting dilution analysis of splenic responders (Arkoosh and Kaattari 1991). Another explantion may be related to findings by Gray and coworkers. In these studies it was found

that low levels of antigen must remain present to maintain lymphocyte memory to the said antigen in murine models (Gray and Skarvall 1989, Gray and Matzinger 1991). Most memory studies in fish are based upon injection of the antigen along with a suitable adjuvant and then some time later a second injection is given and the response is measured. Few studies have documented memory spanning years which would be an essential quality in the design of efficacious vaccines against fish pathogens. Some qualities that are found in both mammalian and teleost secondary immune responses include a heightened antibody titer, faster response and increased sensitivity to the antigen in question (Kaattari 1992).

Chapter 2

Complete Nucleotide Sequence of a Rainbow Trout cDNA Encoding a Membrane-Bound Form of Immunoglobulin Heavy Chain

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Introduction

The molecular characterization of genes involved in the humoral and cellular immune response in fish is important in the understanding of immune development in vertebrate species. There have been several reports dealing with immunoglobulin heavy chain (IgH) genes from primitive taxa ranging from elasmobranchs (Kokubu et al., 1988) to Atlantic salmon (Hordvik et al., 1992). Recently, there have been two reports dealing with cDNAs encoding for complete secretory IgHs along with partial clones of membrane-bound forms of IgH in trout (Lee et al., 1993 and Andersson and Matsunaga 1993). Polymerase chain reaction amplification (PCR) with degenerative primers were used to derive a rainbow trout VH probe that, in turn was used to screen a peripheral blood leukocyte (PBL) cDNA library from a single rainbow trout (*Onchorynchus mykiss*) , Shasta strain. Several full length secretory and membrane-bound forms of trout IgHs were isolated. Here we briefly describe the complete nucleotide sequence from one of the membrane-bound forms of trout IgH, clone RBTIGTM2.

Materials and Methods

An alignment of amino acid and nucleotide sequences of several vertebrate VH genes were used as the basis for designing degenerative oligonucleotide primers which were as follows: Sense FR1=5'-CCISAIMGNCCTGYAMAGCCTCYGGITT-3' and antisense FR3=5'-CCGGATCCGCACAITAATAAVNYGCNGTGTCYTC-3'. The FR3 primer incorporates the fact that most VH genes end in the amino acid motif of Y-Y-C-A-R. PCR was conducted using genomic trout DNA as the template. The resulting amplified product was cloned using a TA cloning kit (Invitrogen) and then sequenced for verification of identity. The VH gene clone was then radiolabeled using random hexamers (BRL) and used to screen a trout PBL cDNA library which had been constructed in a Uni-ZAPXRII vector system as per manufacturer's instructions (Stratagene). The library was screened under high stringency conditions and several putative clones were isolated and characterized. Sequencing of the VH clone and full length IgH clones were accomplished using both manual and automated sequencing in both directions. Manual sequencing was performed using the Sequenase 2.0 system (USB) and automated sequencing was conducted using an ABI 373A sequencer located at the OSU Center for Gene Research and Biotechnology Central Services Facility. All sequence analysis were conducted using the Genetics Computer Group Sequence Analysis Software Package Version 7.2-UNIX.

Results and Discussion

Clone RBTIGTM2 encodes 1703 nucleotides, including an 18 amino acid leader peptide, VH region, CH1-3 domains and a TM domain followed by 3' UT sequences including a putative polyadenylation site (Fig 1). The nucleotide sequence of the RBT VH region was most similar to ladyfish (Amemiya and Litman 1990; ~81% identity). The CH1-3 domains were most similar to trout (Lee et al., 1993; ~98% identity) and to Atlantic salmon (Hordvik et al., 1992; ~95% identity). One notable difference was the addition of an extra codon (GCA) between the CH2-CH3 boundary which was not seen in other published reports of trout IgH cDNAs. The TM domain was completely conserved when compared to the other trout TM sequences. This clone adds further support to the unusual splicing patterns seen in other teleosts including Atlantic salmon (Hordvik et al., 1992), Atlantic cod (Bengten et al., 1991) and catfish (Wilson et al., 1990), where the entire CH4 domain was missing in the membrane bound forms of IgH. A second full length membrane bound IgH was sequenced and compared to RBTIGTM2. The second clone possessed a limited number of nucleotide differences in the constant domains (Fig 1) which resulted in 5 amino acid substitutions. Although this clone could conceivably be representative of another isotype, the limited amino acid variance suggests an allotypic variant of RBTIGTM2.

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Fig. 2.1. Nucleotide and predicted amino acid sequence of a complete rainbow trout membrane-bound form of IgH from clone RBTIGTM2. The assignment of domains and segment boundaries is based on comparisons with previously published trout IgH genes (Lee et al., 1993). Cysteinyl residues assumed to form intradomain disulfide bridges are in parentheses. Putative glycosylation sites are boxed and the proposed polyadenylation site is underlined. The additional codon not seen in other trout IgHs is shown in brackets. Allelic differences are shown below the primary amino acid translation. This sequence along with the other membrane-bound IgH have been submitted to the GenBank/EMBL databases under the accession numbers UO4616 (RBTIGTM1) and UO4615 (RBTIGTM2).

FIG. 2.1

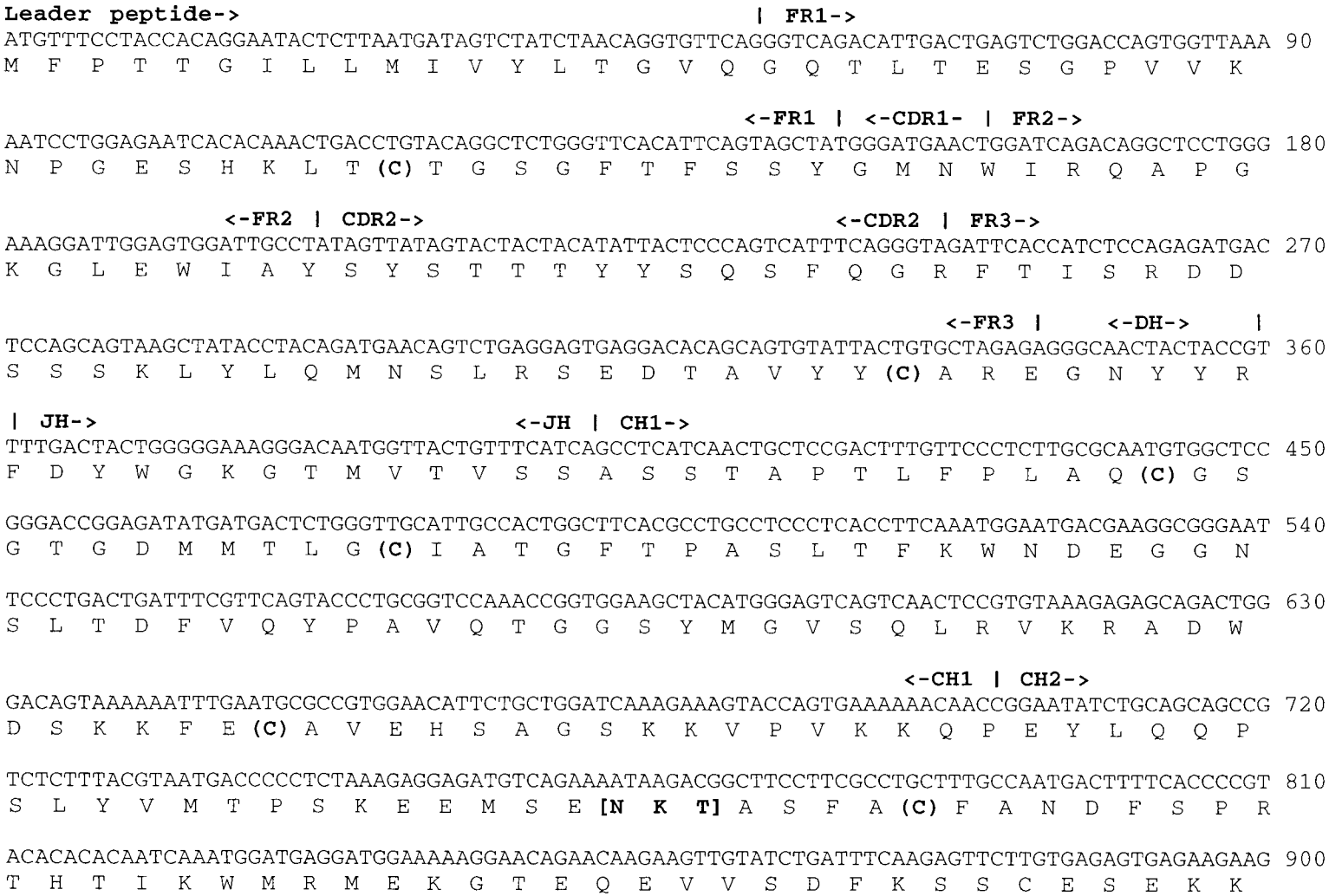
Leader peptide-> | FR1->
ATGTTTCCTACCACAGGAATACTCTTAATGATAGTCTATCTAACAGGTGTTCAGGGTCAGACATTGACTGAGTCTGGACCAGTGGTTAAA 90
M F P T T G I L L M I V Y L T G V Q G Q T L T E S G P V V K
<-FR1 | <-CDR1- | FR2->
AATCCTGGAGAATCACACAAACTGACCTGTACAGGCTCTGGGTTCACATTCAGTAGCTATGGGATGAACTGGATCAGACAGGCTCCTGGG 180
N P G E S H K L T (C) T G S G F T F S S Y G M N W I R Q A P G
<-FR2 | CDR2-> <-CDR2 | FR3->
AAAGGATTGGAGTGGATTGCCTATAGTTATAGTACTACTACATATTACTCCCAGTCATTTCAGGGTAGATTCACCATCTCCAGAGATGAC 270
K G L E W I A Y S Y S T T T Y Y S Q S F Q G R F T I S R D D
<-FR3 | <-DH-> |
TCCAGCAGTAAGCTATACCTACAGATGAACAGTCTGAGGAGTGAGGACACAGCAGTGTATTACTGTGCTAGAGAGGGCAACTACTACCGT 360
S S S K L Y L Q M N S L R S E D T A V Y Y (C) A R E G N Y Y R
| JH-> <-JH | CH1->
TTTGACTACTGGGGGAAAGGGACAATGGTTACTGTTTCATCAGCCTCATCAACTGCTCCGACTTTGTTCCCTCTTGCGCAATGTGGCTCC 450
F D Y W G K G T M V T V S S A S S T A P T L F P L A Q (C) G S
GGGACCGGAGATATGATGACTCTGGGTTGCATTGCCACTGGCTTCACGCCTGCCTCCCTCACCTTCAAATGGAATGACGAAGGCGGGAAT 540
G T G D M M T L G (C) I A T G F T P A S L T F K W N D E G G N
TCCCTGACTGATTTCGTTCAGTACCCTGCGGTCCAAACCGGTGGAAGCTACATGGGAGTCAGTCAACTCCGTGTAAAGAGAGCAGACTGG 630
S L T D F V Q Y P A V Q T G G S Y M G V S Q L R V K R A D W
<-CH1 | CH2->
GACAGTAAAAAATTTGAATGCGCCGTGGAACATTCTGCTGGATCAAAGAAAGTACCAGTGAAAAAACAACCGGAATATCTGCAGCAGCCG 720
D S K K F E (C) A V E H S A G S K K V P V K K Q P E Y L Q Q P
TCTCTTTACGTAATGACCCCCTCTAAAGAGGAGATGTCAGAAAATAAGACGGCTTCCTTCGCCTGCTTTGCCAATGACTTTTCACCCCGT 810
S L Y V M T P S K E E M S E [N K T] A S F A (C) F A N D F S P R
ACACACACAATCAAATGGATGAGGATGGAAAAAGGAACAGAACAAGAAGTTGTATCTGATTTCAAGAGTTCTTGTGAGAGTGAGAAGAAG 900
T H T I K W M R M E K G T E Q E V V S D F K S S C E S E K K

FIG. 2.1 Continued

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AGTGAGACAACTCTGTACAGCACAACCAGCTATCTCAGAGTCAATGAGAGTGAGTGGAAGAGTGAAGAAGTAACATTCACTTGCGTGTTT 990
S  E  T  T  L  Y  S  T  T  S  Y  L  R  V [N  E  S] E  W  K  S  E  E  V  T  F  T (C) V  F

                                                  <-CH2 | CH3->
GAGAACAAAGCTGGAAATGTGAGGAGAACTGTGGGCTACACTTCATCAGATGCAGGTCCAGTCCATGGACATTCAGTAGTCATTACGATC 1080
E  N  K  A  G  N  V  R  R  T  V  G  Y  T  S  S  D {A} G  P  V  H  G  H  S  V  V  I  T  I

ATCGAGCCGTCTCTTGAGGATATGCTTATGAACAAAAAAGCGCAGCTTGTGTGTGATGTCAATGAACTAGTTCCTGGCTTCCTGAGCGTC 1170
I  E  P  S  L  E  D  M  L  M  N  K  K  A  Q  L  V (C) D  V  N  E  L  V  P  G  F  L  S  V
                                       E                 I                    M

AAATGGGAAAATGACAATGGAAAGACCTTAACCAGCCGAAAGGGTGTCACTGACAAAATTGCCATACTTGACATCACTTATGAGGACTGG 1260
K  W  E  N  D  N  G  K  T  L  T  S  R  K  G  V  T  D  K  I  A  I  L  D  I  T  Y  E  D  W

                                                                                <-CH3 | TMD->
AGCAATGGGACAGTATTCTACTGCGCTGTAGATCACATGGAAAACCTGGGGGGACTTGGTAAAGAAAGCCTACAAGAGGGAGACCGATTGT 1350
S [N  G  T] V  F  Y (C) A  V  D  H  M  E  N  L  G  D  L  V  K  K  A  Y  K  R  E  T  D  C
                                       L                 G
CTCGTGTTGACTGACTGTCCATGCAGCAACACCATGGAAACCGACAGGGACAGCATGGGAAAAACAGCCTTCACCTTCATCATACTCTTC 1440
L  V  L  T  D  C  P  C  S  N  T  M  E  T  D  R  D  S  M  G  K  T  A  F  T  F  I  I  L  F

CTCATAACTCTGCTGTATGGCGTTGGAGCAACTGCCATCAAGGTGAAATGAAGAAACTGAGTTTGAAGTACTTGAAGAAGATATTACTTT 1530
L  I  T  L  L  Y  G  V  G  A  T  A  I  K  V  K  STOP signal        3' untranslated region

TCATACTTACAATACAGTATATGAATGGATACAATACATGTAACTGTTCTCCTGCTTGTCTTTATTGTTGATTTTGTTTGAATAGTTCAA 1620

CTGTAGAGGTTAGATTTGTAAATATTTCATTGTGCCTTTTTAAACTATTAAGATAATAAAGTGTAACTGAAGTGAAAAAAAAA 1703
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CHAPTER 3

The Recombination Activating Gene 1 (*RAG1*) of Rainbow Trout (*Oncorhynchus mykiss*): Cloning, Expression and Phylogenetic Analysis.

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Abstract

The characterization of genes involved in the generation of the immune repertoire is an active area of research in lower vertebrate taxa. The recombination activating genes have been shown to be essential for *V (D) J* recombination of T cell antigen receptor (*TCR*) and immunoglobulin (*Ig*) genes, leading to the generation of the primary repertoire. As *RAG1* is critical to the differentiation of pre-B and -T cells, its expression within an associated primary lymphoid organ can serve as a developmental marker. To examine the ontogeny of lymphocytes in *Oncorhynchus mykiss*, we cloned *RAG1* from trout and examined its tissue and lymphocyte specific expression. The polymerase chain reaction, coupled with degenerate oligonucleotide primers was used to amplify a homologous probe (633 bp) from rainbow trout genomic DNA, which in turn was used to isolate a lambda genomic clone. Sequence analysis of this genomic clone confirmed the *RAG1* nature of this gene (3,888 bp) and revealed an internal intron of 666 bp. When compared to other previously reported *RAG1* sequences, the predicted amino acid translation (1073 aa) displayed a minimum of 78% similarity for the complete sequence and 89% similarity in the conserved region (aa 417-1042). Using northern blot analysis, expression of *RAG1* was found to be limited to surface immunoglobulin negative (sIg^-) lymphocytes within the thymus. This data forms the basis for a proposal that the thymus of teleost species plays an essential developmental role in lymphopoiesis and thus, can be regarded as a primary lymphoid organ.

Introduction

In mammals, the primary immune repertoire is generated somatically by site specific recombination events that occur in pre-B and -T lymphocytes (Schatz et al. 1992). *V (D) J* recombination of antigen receptor and immunoglobulin germline gene segments is thought to be facilitated by recombinational signal sequences (RSS) that are found within the noncoding sequences flanking the variable region gene segments (Sakano et al. 1980; Hesse et al. 1989). These flanking sequences consist of a highly conserved palindromic heptamer which is separated from an A/T rich nonamer by either a 12 or 23 bp conserved spacer sequence. The high degree of sequence conservation of these RSSs lend support to the notion that a common *V (D) J* recombinase has also been conserved over the course of vertebrate evolution (Tonegawa 1983; Litman et al. 1985; Yancopoulos et al. 1986; Hesse et al. 1989; Schatz et al. 1992). Besides random association of variable region gene segments, diversity of lymphocyte receptors is further enhanced by such events as imprecise joining of gene segments due to P and N insertions, base trimming and somatic hypermutation (Landau et al. 1987; Kallenbach et al. 1992; Heinrich et al. 1984).

The recombination activating genes 1 and 2, have been shown to be essential for the recombinatorial process leading to the genesis of *TCR* and *Ig* repertoires (Schatz et al. 1989; Carlson et al. 1991). These genes were initially isolated by their cosegregation with *V (D) J* recombination activity on artificial substrates when transfected into NIH 3T3 fibroblasts (Schatz and Baltimore 1988, Schatz et al. 1989).

V (D) J recombination has been shown to occur within the primary lymphoid organs of mammals, bone marrow and thymus, and thus can serve as a developmental marker for primary lymphoid organs. Studies utilizing targeted germline gene disruption of *RAG1* and *RAG2* have shown them to be critical in activating or carrying out *V (D) J* recombination (Mombaerts et al. 1992; Shinkai et al 1992). In these studies, mice that were made deficient in *RAG1* or *RAG2* were not capable of producing mature B or T lymphocytes, resembling the SCID mouse scenario (Bosma et al. 1983; Schuler et al. 1986). It was later determined that the SCID mutation resided on a different chromosome than the *RAG* genes, but the possibility remained that recombination may be impaired in this disease state (Schatz et al. 1992).

The carboxyl-terminus of *RAG1* shares some sequence similarity to the yeast protein *HRP1* (Wang et al. 1990) which, in turn, has sequence similarity to yeast topoisomerase 1 (Aguilera et al.1990). Recently, it has been shown that the *RAG1* C-terminal end alone was capable of carrying out *V (D) J* recombination in transfected cells carrying variable region gene segments on plasmids (Silver et al. 1993). The relationship between *RAG1* and topoisomerase-1 was further investigated with mutation of a specific tyrosine residue in the C-terminus of murine *RAG1* (Y998). This mutation which was created because it corresponded to the proposed active site of yeast *HRP1* (Y532), was still able to effect *V (D) J* recombination activity. Thus, the actual active site of *RAG1* has not been determined and any relationship between *RAG1* and *HRP1* is speculative at this time.

The *RAG* genes have been isolated from vertebrate species including human, mouse, chicken and more recently from rabbit and

Xenopus (Schatz et al. 1989; Oettinger et al. 1990; Carlson et al. 1991; Fuschiotti et al. 1993; Greenhalgh et al. 1993). In mammals coexpression of *RAG1* and *RAG2* was found within the lymphopoietic organs of the thymus, bone marrow and fetal liver (Schatz et al. 1992). In rabbits, expression of *RAG1* and *RAG2* was seen within the thymus. It appears that the rabbit *RAG2* gene may undergo alternative splicing events yielding a large 4.4 kilobase (kb) and a smaller 2.2 kb mRNA species. Using northern blot analysis, the major site of *RAG1* and *2* expression in the amphibian *Xenopus* was seen within the thymus, with slight expression of *RAG2* detected in the ovaries. The more sensitive assay, reverse transcription-PCR (RT-PCR), found that *RAG1* and *RAG2* were expressed within the thymus, liver, and spleen in juvenile frogs, and within the thymus and bone marrow of adults. In avian species, *RAG1* and *RAG2* were expressed within the thymus, but expression of *RAG2* in the absence of *RAG1* occurred in the bursa of Fabricius, the site of gene conversion of *Ig* repertoires. It was later shown that *RAG2* was not essential for further gene conversion events, but it is possible that *RAG2* may be required for initiating gene conversion events (Takeda et al. 1992).

Rainbow trout and other fish possess immune systems with many of the same features and functions found in mammals including antibodies, *V (D) J* recombinatorial mechanisms, B/T cell cooperation, and distinct lymphoid tissue and organs (Reviewed in Faisal and Hetrick 1992). The genomic organization of the immunoglobulin heavy and light chain loci has recently been characterized in teleosts (Amemiya and Litman 1990; Wilson et al. 1990; Daggfeldt et al. 1993). The heavy chain organization is similar to that found in mammals

(Earley et al 1980), but the light chain repertoire is more elasmobranch-like in that a multiple *V-(D)-J-C* cluster type of arrangement is found (Litman et al. 1985; Kokubu et al. 1988; Hohman et al. 1992; Rast et al. 1994). In amphibians, both the heavy (Schwager et al. 1988; Du Pasquier et al. 1989) and light chain loci (Schwager et al. 1989) are comparable to mammalian organization (Reviewed by Zachau et al. 1989). The cDNAs encoding *TCR* subunits have been recently isolated from amphibian, teleost, and elasmobranch species and their isolation provides the initial characterization of these T-cell receptor genes (Fellah et al. 1993; Partula et al. 1994; Rast and Litman 1994).

Early histological analysis indicated that the thymus may be the primary source of lymphocytes in teleosts (Grace and Manning 1980; Tatner 1985; Josefsson and Tatner 1993), but it also been conjectured that the pronephros (anterior kidney) could be the bone marrow equivalent (Ellis 1977; Razquin et al. 1990). To date, very little is known about the actual sites and mechanisms involved in teleost lymphopoiesis. To determine the likely location of *V (D) J* recombination, and thus the primary lymphopoietic tissue(s) in trout, we cloned *RAG1* and examined its expression patterns during ontogeny.

Animals

Rainbow trout (*Oncorhynchus mykiss*, shasta strain) were obtained from the Marine Freshwater Biomedical Sciences Center at Oregon State University, and maintained at the Salmon Disease Laboratory in Corvallis, Oregon. This facility receives pathogen-free water at a constant temperature of 12° C. All fish were fed Oregon Moist Pellet commercial salmon food daily. Tissue samples were obtained from trout sacrificed by anesthetic overdose in benzocaine (ethyl p-aminobenzoate, Sigma, St. Louis, MO) (Kaattari and Irwin 1985). Tissues were used fresh or frozen immediately with liquid nitrogen and stored at -85° C until needed.

Rainbow trout RAG1 probe

The polymerase chain reaction (PCR) was executed using degenerate primers based upon an alignment of the highly conserved 3' region of *RAG1* from all previously cloned *RAG1* genes. This procedure amplified a homologous probe (633 bp) from rainbow trout genomic DNA which in turn was used for northern blot analyses and to screen a rainbow trout genomic library. All primers used for PCR amplification and sequencing were synthesized at the Oregon State Center for Gene Research and Biotechnology Central Services facility. The *RAG1* degenerate primers were as follows: Sense *RAG1*L1= 5'- CAYTGYGA YATHGGIAAYGC-3' (aa 830-836) and antisense *RAG1*R1= 5'-RTGNG CRTTCATRAAYTTYTG-3' (aa 1035-1041). Numbers in parentheses

refer to primer positions relative to the coding region of rainbow trout *RAG1*. Primers were purified using Oligoclean (BMB, Indianapolis, IN). Rainbow trout genomic DNA was isolated by standard methods (Strauss 1989) and used as the template in a hot-start PCR. Genomic DNA (500 ng) was added to a 1x PCR cocktail (98 ul) containing 50 mM KCl, 10 mM Tris-HCl, pH9.0, .01% gelatin, 2.5 mM $MgCl_2$, 200 uM dNTPs, 0.5 U Perfect Match (Stratagene, LaJolla, CA) and 250 pmoles of each primer. PCR samples were then overlaid with mineral oil and heated to 95°C for 10 min, cooled to 80°C at which time 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) was added and then the profile of 95° C for 45 sec, 54° C for 1 min and 72° C for 1.5 min for 35 cycles followed by an additional extension time of 7 min at 72° C was used for amplification. 5 ul of the reaction was electrophoresed through a 1.5 % agarose gel and a product (633 bp) of the expected size was observed. The product was subsequently cloned using the TA cloning kit from Invitrogen as per manufacturer's specifications (SanDiego, CA) and sequenced for verification of identity. The rainbow trout *RAG1* PCR clone, pTARAG1, was digested with *Eco* R1, purified (Geneclean, Bio 101, LaJolla, CA) and used as a homologous probe for northern blot analysis and library screening. The probe was randomly labeled with [32-P] dCTP according to the protocol of Feinburg and Vogelstein (1983) using a commercial kit (BRL, Gaithersburg, MD) to a specific activity of 1.5 X 10^9 cpm/ug. Non-incorporated nucleotides were removed using G-50 Quick-Spin columns (BMB) prior to use in hybridizations. All hybridizations and washings for library screening, Southern and northern analyses took place in a Techne Hybridiser, HB-1D (Techne, Inc, Princeton, NJ).

Library screening and restriction analysis

A rainbow trout genomic library (a kind gift from Dr. Thomas Chen, Center of Marine Biotechnology, University of Maryland, Baltimore, MD) was used for isolating genomic clones encoding the trout *RAG* locus. The library was constructed into the *Bam* HI site of lambda Dash-2 (Stratagene) after a partial *Sau* 3a digestion of trout testis DNA. The amplified library was titered, plated and plaque-lifted onto BA-85 supported nitrocellulose (Schleicher & Schuell, Keene, NH) using the manufacturer's suggested conditions. Approximately 1 X 10^6 PFUs were screened in duplicate with the randomly labeled PCR generated *RAG1* probe. Filters were prehybridized for 4 h at 68° C in 5x SSC, 5x Denhardt's solution and 0.5% SDS (w/v) and then hybridized for 18 h under the same conditions with the addition of radiolabeled probe. Duplicate filters were washed at low stringency (42° C, 2x SSC/0.5% SDS) and finally at high stringency (65° C, 0.2x SSC/ 0.2% SDS). Autoradiography was then conducted using Kodak XAR-5 film and one intensifying screen for 18 h at -20° C. Upon tertiary screening, positive plaques were confirmed via PCR using trout specific primers derived from sequencing the PCR amplified trout *RAG1* probe. Upon this secondary confirmation, positive plaques were selected and used for small scale phage preparations and restriction endonuclease analysis. Phage DNA was digested with restriction endonucleases (Promega), separated by agarose gel electrophoresis, transferred to Nytran (S & S) using 10x SSC and UV fixed. The blot was then hybridized and washed as described for library screening. The majority of trout *RAG1* was

localized to a 6.6 kbp *Eco* RI fragment which was cloned (pRBTRAG1) into pGEM-3Z for sequencing.

Cloning the 5' end of trout RAG1

5' RACE technology based upon minor modifications of the methods of Frohman et al (1988) and Renu et al (1992) were used to amplify and clone the 5' end of trout RAG-1. Briefly, 500 ng of total thymic RNA was reverse transcribed using 100 pmoles antisense anchor primer (5'-CCA-GGA-AGC-CCT-TAG-CTG-3', bp 1079-1096) and 200 U M-MLV reverse transcriptase (Promega) for 60 min at 42° C and then at 52° C for 30 min. After degradation of the RNA template with 0.5 U of RNase H (Promega), excess primers and dNTPs were removed using a Microcon-30 spin filter (Amicon, Inc, Beverly, MA). The subsequent retentate was concentrated and the first strand cDNA was tailed with dATP using 12 U TdT (Promega). Following the addition of the poly A tail, the products were amplified using 15 pmoles dT-adapter primer (5'-GAC-TCG-AGT-CGA-CAT-CGA-T_{17}) and 25 pmoles anchor primer using the following profile: 50° C for 5 min, 72° C for 45 min followed by 5 cycles of PCR, 95° C for 45 sec, 50° C for 1 min and 72° C for 2 min. The final cycle was extended for 5 min at 72° C. The reaction products were again filtered to remove dNTPs and primers and an aliquot was then used in a second amplification reaction using 25 pmoles each of nested gene specific primer (5'-CAG-CAG-TCT-GGC-ACT-AAG-3', bp 871-888) and adapter primer (5'-GAC-TCG-AGT-CGA-CAT-CG-3') for 30 cycles at 95° C for 45 sec, 55° C for 1 min, 72° C for 2 min followed by an extension time of 10 min at 72° C. The product was gel purified and cloned using the TA

cloning kit. This cDNA clone was partially sequenced and a sense primer (5'-CCG-TTG-CTG-ACA-CTA-TGG-3', bp -14 to + 4) was constructed . The sense primer, along with the nested antisense primer used in the 5' RACE RT-PCR amplification, were used to amplify the 5' end of trout *RAG1* from genomic DNA. Reaction conditions were similar to that used to generate the trout *RAG1* PCR probe, except 25 pmoles of each primer was used. Products were purified, cloned (TA cloning kit) and sequenced in both directions from two independent PCR amplifications.

RNA isolation and northern analysis

Total RNA was isolated from specified tissues by homogenization in 4M guanidine isothiocyanate buffer followed by organic solvent extraction and finally precipitated using isopropanol (Chomczyncki and Sacchi 1987). RNA concentrations and relative purity were checked by UV spectroscopy (A260/280) prior to loading. Equivalent amounts of RNA were loaded onto a 1.2%/6.6% agarose/formaldehyde gel, electrophoresed, transferred to Nytran using 10X SSPE and UV fixed. Prior to hybridization, the blot was stained with Methylene Blue (MRC, Inc., Cincinnati, OH) for a qualitative assessment of integrity. The blot was then scanned by laser densitometry (Biomed Instruments, Inc., model #SL-DNA, Fullerton, CA) to establish that equivalent amounts of RNA had been transferred to the membrane based upon ribosomal RNA staining. Methylene blue was removed during prehybridization (50% formamide, 5X SSPE, 0.5% SDS and 5X Denhardt's soln at 42° C for 2 h) and the blot was hybridized overnight using the radiolabeled PCR

generated probe under the same conditions as described for prehybridization. The blots were then washed as previously described for library screening and exposed to Kodak X-OMAT AR film for 48 h at -80° C. The molecular weight of trout *RAG1* message was calculated by comparison to rRNA bands and to RNA molecular weight markers (Promega).

Cell partitioning into sIg^- & sIg^+ populations

Single cell suspensions from the tissue samples were prepared using a Cellector tissue sieve (VWR Scientific, Seattle, WA). Cells were washed twice in cold PBS (pH 7.6) and underlaid with an equivalent amount of Histopaque-ficoll 1077 (Sigma). After centrifugation, the interface layer of cells was collected, washed twice, and viability determined by trypan blue exclusion. Only those cells displaying at least 95% viability were used in subsequent panning assays. For panning, monoclonal antibody (mAb) 1-14 (mouse anti-trout Ig heavy chain, DeLuca et al. 1983) was conjugated to tosylactivated M-450 Dynabeads (Dynal, Inc, Lake Success, NY). Cells were brought to a concentration of 5×10^7 cells/ml in RPMI/2% FCS and approximately 1.5×10^8 mAb 1-14/M450 beads were then added per ml of cells. After incubation on a rotator at 4° C, positively selected cells (sIg^+) were collected using a magnetic particle concentrator (Dynal), washed twice in PBS and enumerated. Supernatants were panned again to ensure depletion of sIg^+ cells and sIg^- cells were then recovered from the final supernatant by centrifugation, washed twice and enumerated. Cell partitioning into sIg^- and sIg^+ populations was confirmed by fluorescence microscopy

using mAb 1-14 and goat anti-mouse IgG conjugated with Texas Red® (Molecular Probes, Eugene, OR). RNA was isolated from equivalent numbers of sIg^- and sIg^+ cells. RNA isolation and northern blot analysis were as previously described.

Sequencing

Sequencing of the trout *RAG1* clones was accomplished using both manual and automated DNA sequencing methods based upon dideoxy chain termination chemistry (Sanger et al. 1977). Manual sequencing was performed using the Sequenase 2.0 system (USB, Cleveland, OH) and automated sequencing was carried out using an ABI 373A sequencer located at the Center for Gene Research and Biotechnology on the Oregon State campus. Sequences were determined in both directions using specific or universal primers.

Computer programs

All amino acid sequence comparisons were conducted using the best fit function in the Genetics Computer Group (GCG) Analysis Software Package Version 7.2-UNIX (Devereux et al. 1984). Positions with insertions/deletions were omitted from these comparisons. Sequence alignments were performed using the Clustal function (Higgins et al. 1992) along with manual corrections in the Genetic Data Environment (GDE version 2.2, beta release by Steve Smith) software package. Trees were constructed using the DeSoete algorithm (DeSoete 1983) and branching positions were verified by bootstrap parsimony (Felsenstein 1989), a component of Phylip (version 3.5 release by Joseph Felsenstein)

Isolation and characterization of RAG1 from rainbow trout

A portion of trout *RAG1* was amplified from rainbow trout genomic DNA using degenerate oligonucleotide primers. These primers were based upon a highly conserved block of amino acid residues found in the 3' region of the gene as a result of a sequence alignment of all known *RAG1* genes (unpublished data). The product, 633 base pairs (bp), was cloned and sequenced to determine its identity as being derived from trout *RAG1*. This PCR generated probe was then employed to screen a rainbow trout genomic library under highly stringent conditions. Upon tertiary screening, positive plaques were confirmed as clones of *RAG1* via PCR with trout specific primers. These positive plaques were purified and characterized via restriction endonuclease analysis. Phage DNA from four plaques were digested with a battery of restriction enzymes, blotted and hybridized with the trout *RAG1* probe. A 6.6 kilobase pair (kbp) *Eco* R1 fragment hybridized with the trout *RAG1* probe and this fragment was subsequently isolated, subcloned and sequenced.

Upon sequence analysis of this genomic clone it was found that the clone was truncated at the 5' end. The remaining 5' end of trout *RAG1* was amplified and cloned from thymic total RNA using 5' RACE RT-PCR technology. This cDNA clone was sequenced and used to construct a sense primer to amplify the 5' end of *RAG1* from rainbow trout genomic DNA. The sequence of rainbow trout *RAG1* along with the locations of a putative nuclear localization signal (NLS) (Dingwall

and Laskey 1991) and a zinc finger motif (Brunk et al. 1991; Freemont et al. 1991) are depicted in Figure 3.1 a and b. Sequence analysis of this genomic clone revealed an internal intron of 666 bp.

Although PCR clones from zebrafish (595 bp) and the nurse shark (318 bp) have recently been reported (Greenhalgh and Steiner 1995), to our knowledge, this is the first report of a complete teleost *RAG* gene and of an intron existing within a *RAG* gene. Sequences analysis of the trout *RAG1* gene and its predicted amino acid translation were compared to the other full length clones of *RAG1* (human, mouse, chicken, *Xenopus* and rabbit). The predicted amino acid translation for trout *RAG1* was compared to that of the other *RAG1* genes and found to have a minimum of 78% and 89% similarity for the complete and conserved regions (aa 417-1042). This analysis revealed that trout *RAG1* was most similar to chicken *RAG1* at the protein level for the complete and conserved regions with 81% and 92% respectively. The presence of the cysteine motif, putatively a zinc-finger, supports the notion that *RAG1* interacts in some way with chromosomal DNA to orchestrate *V (D) J* recombination. Our findings of *RAG1* in trout lend further support to the concept of a specific recombinase complex or recombination activating factor(s) being conserved throughout vertebrate evolution.

Tissue and lymphocyte-specific expression of RAG1

Using northern blot analysis, we then examined the tissue specific expression of *RAG1* in lymphoid organs of trout. As Figure 3.2 demonstrates, a strong signal (5.8 kb) was detected in both 3 month

(juvenile) and adult (> 2 years) fish using the trout specific *RAG1* probe. This hybridization for *RAG1* specific RNA was only found in thymic tissue. The signal strength for the thymic RNA was the same intensity for all time points assayed, including 2, 6 and 12 month old trout (unpublished data). The lower molecular weight species corresponding to 3.5 kb was not detected when poly A^+ mRNA was used in the northern blot. No signal was observed in the other tissues even with prolonged exposure (5 days). The trout *RAG1* message (5.8 kb) is smaller in size to *RAG1* mRNAs (6.6 kb) in other vertebrates. The size of the message suggests that the trout *RAG1* gene has a large 3' noncoding region similar to that found in human and murine *RAG1* mRNAs. To determine whether this was the case, a cDNA library was constructed and screened with the trout *RAG1* probe. A putative poly adenylation signal and poly A tract was found approximately 2.6 kbp downstream of the termination codon within the cDNA clone (unpublished data).

The source of the lymphocytes specifically expressing *RAG1* was more precisely delineated by an initial partitioning of lymphocytes from various tissues into sIg^- and sIg^+ pools. This was accomplished by conjugating mAb 1-14, mouse anti-trout Ig heavy chain, to magnetic beads which were used in a double panning assay to select the various lymphocyte phenotypes. RNA from equivalent amounts of sIg^- and sIg^+ cells were used in the northern blot, for hybridization to the trout *RAG1* probe. As can be seen in Figure 3.3, only sIg^- cells from the thymus displayed a strong hybridization signal at 5.8 kb. A faint signal (60 fold less by densitometry scanning, data not shown) was also observed in sIg^+ cells from the thymus. This latter expression was most likely due to the transient expression of *RAG1* in immature lymphocytes which

have recently emerged within the thymus (Ma et al. 1992; Petrie et al. 1993).

Since expression of *RAG1* is limited to precursor lymphocytes and primary lymphoid organs in mammals, the presence of *RAG1* mRNA is characteristic of a primary lymphoid organ where *V (D) J* recombination occurs. The data presented here demonstrates that expression of *RAG1* in trout is limited to sIg^- lymphocytes in the thymus, and suggests that the thymus is most likely a primary lymphoid organ. Since teleost thymic cells also express T cell receptor mRNAs (Partula et al. 1994) and *RAG1*, the recombination events leading to diversity of T cell receptors more than likely occurs in the thymus. *RAG1* transcripts have also been detected by the more sensitive RT-PCR assay, in the kidney tissue of juvenile and adult trout (unpublished data). Thus, *V (D) J* recombination may also occur within the kidney, but at levels much lower than that seen in the thymus. These data all indicated that the thymus and possibly the kidney, serve as the primary locations of lymphocyte development in teleosts.

Phylogenetic analysis

Due to the high degree of conservation of *RAG1* and the hypothesis that it serves a similar role in all organisms carrying out *V (D) J* recombination, we decided to conduct a phylogenetic comparison of *RAG1* genes, also known as a gene tree (Fig 3.4). To do this, a sequence alignment was performed using the Clustal alignment program (Higgins et al. 1992) and trees were constructed using the DeSoete algorithm (DeSoete 1983). Bootstrap parsimony (Felsenstein 1989)

analysis of 1,000 replications was performed to confirm branching positions. Trees based upon the complete and highly conserved region of *RAG1* (aa 412-1042) were also compared with no variation in the branching orders seen, therefore we chose to use the tree based upon the complete coding region of *RAG1*. As mentioned earlier, the conserved region was found to be sufficient in carrying out *V (D) J* recombination in vitro (Silver et al. 1994). The branching order among these genes matched the known evolutionary pattern for the organisms from which they came. Starting with trout *RAG1*, the next divergence is a lineage leading to amphibians and birds, which is consistent with the known evolution of these groups, and finally mouse, human and rabbit genes diverged as a relatively shallow cluster. The position of the mouse *RAG1* gene supports the general hypothesis that the order Rodentia diverged sometime prior to the divergence of lagomorphs, primates and the other mammalian orders (Li et al. 1990). Overall, the disposition of taxa within the tree was consistent with paleontological evidence on the evolution of these organisms (Pough et al. 1989).

In other words, the *RAG1* gene may server as a molecular clock. Typically, genes which make good molecular clocks are genes with highly conserved functions which have not been transferred laterally in evolution (Li and Graur 1991). For the *RAG1* gene, this conclusion is supported by the observations that *RAG1* amino acid sequences are at least 78% and 89% similar in the complete and conserved regions of *RAG1*. Sequence alignment of all cloned *RAG1* genes shows that relatively few insertions and deletions occur within the conserved region and that most nucleotide substitutions are found in the 3rd codon position, so they are essentially synonymous substitutions . Other

differences found within this region were composed mainly of conserved or similar amino acid substitutions.

In summary

We were able to clone *RAG1* from rainbow trout using a degenerative PCR-based approach and to show that expression of this gene was limited to sIg^- thymocytes within our northern blot analyses. *RAG1* expression analysis indicates that the thymus and possibly the kidney serve as the major sites for *V (D) J* recombination of lymphocytes in teleosts. Also due to the high conservation of this gene and its proposed function in all vertebrates, we suggest that *RAG1* may serves as a molecular clock for evolutionary analyses of various taxa that carry out *V (D) J* recombination. We are currently investigating the expression of *RAG1* during earlier stages of trout ontogeny and characterizing the role of a newly discovered trout *RAG2* in lymphocyte development (unpublished work, Hansen et al.).

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A.

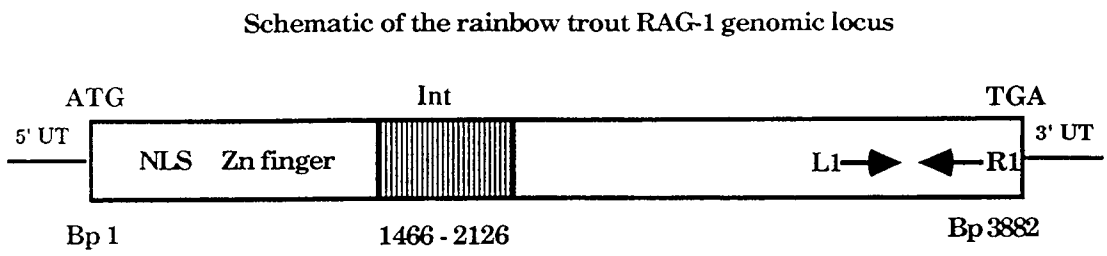

Fig. 3.1. **a**, Schematic representation of the rainbow trout *RAG1* genomic clone. The location of the intron (bp 1466-2131) is labeled as Int. The initiation codon site at +1 was chosen based upon matching the consensus initiation site for eukaryotic translation (Kozak 1986). Locations of degenerate primers initially used to generate the *RAG1* probe are indicated as L1 (RAGL1) and R1 (RAGR1). Also depicted in this schematic are the relative locations of a possible nuclear localization signal (Dingwall and Laskey 1991) and zinc finger-like motif, C3HC4 type (Brunk et al. 1991; Freemont et al. 1991). The location of a putative poly-adenylation signal would be located approximately 2.6 kbp downstream from the TGA stop codon in the 3' untranslated region (unpublished data). **b**, The nucleotide sequence for the coding region of rainbow trout *RAG1* and its predicted amino acid translation is shown. The location of a stretch of amino acid residues possibly representing a NLS are underlined and the conserved residues involved in forming a potential zinc finger motif are indicated by boxes (residues 310-348). The nucleotide and predicted amino acid sequence for trout *RAG1* have been deposited with GenBank under accession # U15663.

B. TROUT *RAG1*

```
ATGGAGGAGACATATGCCCCCCGGTGCTCCATGCCGGCCGAGCTCCATCATCCCTACTCCAAGTTCTCAGACTGGAAGTTCAAGCTGTTCCGGGTCAGGTCCATGGAGAGGGCCCCACTG-120
M  E  E  T  Y  A  P  R  C  S  M  P  A  E  L  H  H  P  Y  S  K  F  S  D  W  K  F  K  L  F  R  V  R  S  M  E  R  A  P  L  -40

CCCGGGGAGATGCAGCTAGAGAGAGGGGCCTTGTCTGGTGTTGTGGCCTCTGCACCCCTGGGGGAAACTGTGGGGGATGTGGTGGGTCTCCCAGGGAGTGTGATGAAGCTTTGGCTGGGG-240
P  G  E  M  Q  L  E  R  G  A  L  S  G  V  V  A  S  A  P  L  G  E  T  V  G  D  V  V  G  L  P  G  S  V  M  K  L  W  L  G  -80

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GTGGACGTGGCGGGGGACATGGAGGTCGTCCATCCACCATTCTTCTGCCAGCGCTGCTGGACATTGGCCATGCGAGGAGGGGGCTTCTGCAGCTTCTCCAGGACCCATGTCCCTGGGTGG-600
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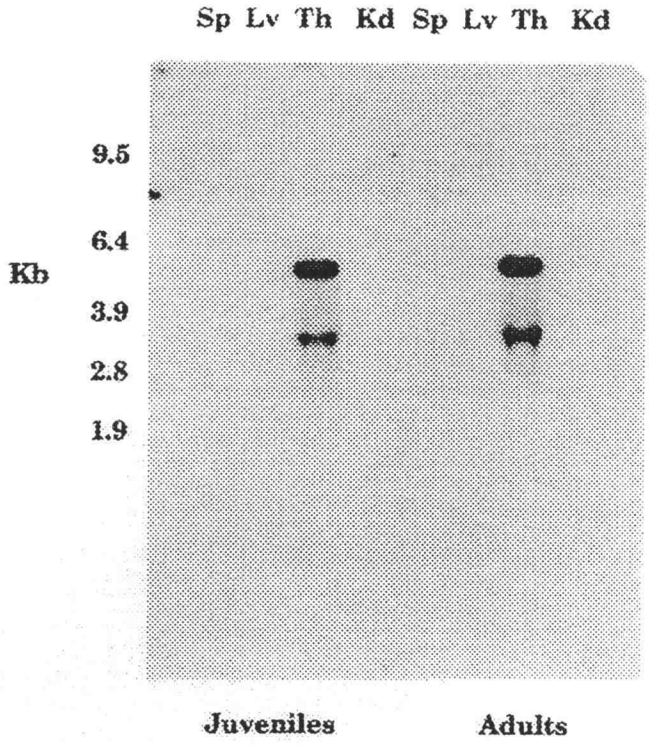

Fig. 3.2. Tissue specific expression of *RAG1* in rainbow trout. Northern blot analysis of total RNA (8ug) from spleen (Sp), thymus (Th), liver (Lv) and anterior kidney (Kd) from juvenile (3 months) and adult trout (30 months) using a trout specific *RAG1* probe. *RAG1* expression (5.8 kb message) is only detected in the thymus at similar intensity levels. No signal was seen in other tissues even with prolonged exposure (5 days). The lower molecular weight species, 3.5 kb, is not seen when poly A^+ mRNA was used. Equivalent amounts of total RNA were loaded and transferred as verified by methylene blue staining. The molecular weight of trout *RAG1* message was calculated by comparison to rRNA bands and to RNA molecular weight markers.

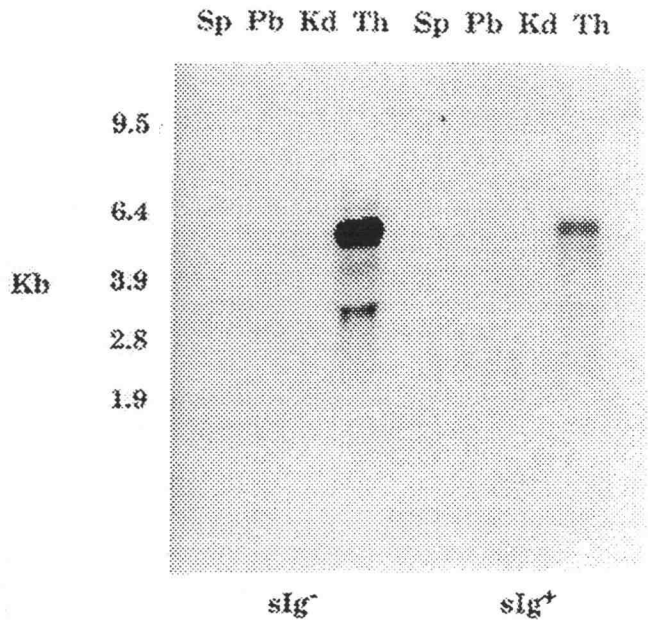

Fig. 3.3 Immunoglobulin phenotypes of lymphocytes expressing *RAG1* in rainbow trout. Northern blot analysis of total RNA (5ug) from sIg^+ and sIg^- lymphocyte populations from the spleen (Sp), peripheral blood (Pb), anterior kidney (Kd) and thymus (Th) from 3 month old trout was hybridized with the trout specific *RAG1* probe. Cells were isolated using a double panning assay which incorporated mAb 1-14, a mouse anti-trout Ig heavy chain reagent (DeLuca et al. 1983). A strong signal for *RAG1* expression was seen in the sIg^- cells from the thymus. A weak signal was detected in the sIg^+ cells from the thymus which was most likely due to the transient expression of *RAG1* in immature lymphocytes (Ma et al. 1992; Petrie et al. 1993). Relative % of sIg^+ cells/tissue were as follows; spleen ~40%, PBLs ~65%, kidney ~55% and thymus ~3% as determined by immunofluorescence. RNA was isolated from equivalent numbers of sIg^- and sIg^+ cells and equivalent amounts of RNA were loaded and transferred as verified by methylene blue staining.

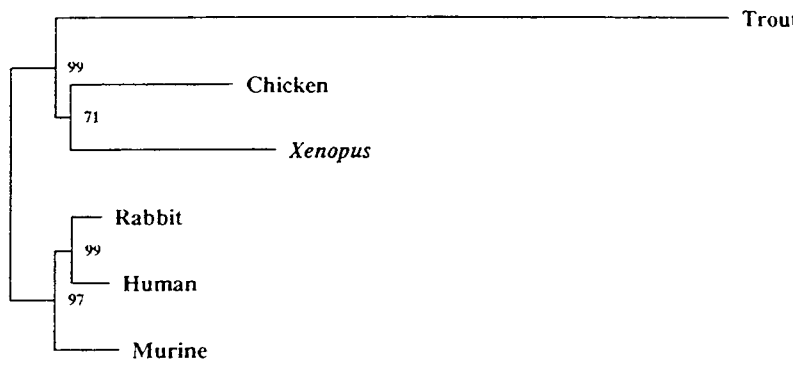

Fig. 3.4. Unrooted phylogenetic tree of the *RAG1* complete coding region amino acid sequences. An amino acid sequence alignment was conducted using the Clustal alignment function (Higgins et al. 1992) in GDE and a tree was then constructed using the DeSoete algorithm (DeSoete 1983) in GDE. Sequence positions having insertions and or deletions were omitted from the analysis. Trees based upon the complete and conserved regions of *RAG1* gives essentially the same branching order. Branching positions were verified by bootstrap parsimony (Felsenstein 1989) analysis of 1,000 replications. Numbers at the forks represent %s from the 1,000 replicants. *RAG1* sequence data for the chicken (M58530), *Xenopus* (L19324), human (M29474), mouse (M29475) and rabbit (M77666) were obtained from GenBank using the fetch command in GCG (Devereux et al. 1984). Accession numbers are indicated in parentheses.

CHAPTER 4

Thymic Involvement in Raibow Trout B-cell Lymphopoiesis: Characterization of Ontological Markers Associated with B-cell Development

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Abstract

The recombination activating gene 2 (*RAG2*) of trout has been cloned and characterized. Using degenerate primers based upon an alignment of amino acid residues from previously cloned *RAG2* genes, a portion of trout *RAG2* (1,140 bp) was amplified from genomic DNA. The identity of the fragment was confirmed and then used as a probe to isolate restriction fragments corresponding to *RAG2* from *RAG1* positive phage DNA. The trout *RAG2* gene (1601 bp) displays an average of 60% and 75% similarity at the nucleotide and amino acid level when compared to clones from other species and was found to contain an acidic region in the carboxyl terminal end. Based upon sequence and restriction analysis, *RAG1* and *-2* in trout as in all species studied to date are convergently transcribed and found to be tightly linked (2.8 kb apart). Northern blot analysis of one year old trout demonstrated strong expression of *RAG2* in the thymus, with a much weaker signal being detected in the pronephros. Using the more sensitive assay, RT-PCR, the highest expression of both *RAG1* and *-2* were detected in the thymus and pronephros, with fainter signals being observed in the spleen, mesonephros and liver. Expression of both genes was observed in embryos beginning at day 10 post fertilization. Finally, putative pre-B cells were detected in the thymus using both immunofluorescence and confocal microscopy.

Introduction

In mammals, the cells of the lymphoid lineages originate from pluripotent hematopoietic stem cells located within the bone marrow. Progenitors of the T-cell lineages migrate to the thymus to mature, whereas progenitors of the B-cell lineages develop within the bone marrow microenvironment. The primary Ig and antigen receptor repertoires for B and T-cells are generated by site-specific recombination events of variable region gene segments, termed V (D) J recombination (Schatz et al 1992) within the primary lymphoid organs. This process is believed to be facilitated in part by evolutionary conserved sequence motifs found flanking the germline variable region genes (Schatz et al 1992). The motifs, termed recombination signal sequences (RSSs), consist of a highly conserved dyad-symmetric heptamer sequence which is separated from an A/T rich nonomer sequence by either a 12 or 23 bp non-conserved spacer (Sakano et al 1980, Hesse et al 1989). The length of the spacer is believed to govern the sequential joining of the gene segments. This pairing process is believed to be mediated by a set of proteins which would specifically recognize the RSSs and bring them into close proximity prior to recombination. Using radiolabeled RSSs as probes, investigators have isolated cDNA clones which when expressed were shown to specifically recognize and bind to the RSSs (Matsunami et al 1989, Shirakata et al 1991). It has yet to be demonstrated if these genes are critical for V (D) J recombination to occur, but the RSS motifs are required for the joining of two gene segments. Prior to the actual formation of the coding joint, further events can occur to enhance diversity including base trimming as well as templated (P) and non-

templated (N) insertions (Landau et al 1987, Kallenbach et al 1992). Following surface Ig expression, the antibody repertoire may be further diversified by somatic hypermutation, an antigen-dependent response occurring within the germinal centers (Berek 1991). The overall process and machinery involved in V (D) J recombination is poorly understood, but recently two genes known as the recombination activating genes 1 and -2 have been shown to be essential for recombinational activity (Schatz et al 1989, Carlson et al 1991).

Transfection of the RAG locus into fibroblasts results in V (D) J recombination of artificial substrates which was the basis for the isolation of these two genes (Schatz et al 1989). Expression of the RAG genes coincides with the ontological locations and cell types known to undergo V (D) J recombination. The exact role of RAG1 and RAG2 are not precisely known, but studies involving knockouts of either of these genes have demonstrated that they are critical for the proper development of mature B and T lymphocytes (Mombaerts et al 1992, Shinikai et al 1992). This phenotype can be circumvented by the introduction of Ig and TCR transgenes into mice disrupted for the RAG genes (Spanopoulou et al 1994, Shinikai et al 1993). Similarily, expression of the RAG genes coincides with variable region recombination in RAG inducible B-cell lines (Oltz et al 1993). Mutagenic analysis of RAG1 and RAG2 have demonstrated that the C-terminal end of RAG1 and the first three quarters of RAG2 are all that is needed to induce V (D) J recombination of artificial substrates in transfected cells (Silver et al 1993, Cuamo et al 1994). Although the exact role of the RAG genes is unknown these studies elude that RAG1 and RAG2 are most

likely actual components of the V (D) J recombination machinery and not merely transcription factors.

The overall genomic organization for both the heavy and light chain immunoglobulin loci have been well characterized in virtually all vertebrates. In mammals and amphibians the heavy chain loci is arranged such that VH, DH and JH gene segments are recombined to form the variable region which is then spliced to constant region gene segments (Early et al 1980, Schwager 1988, Du Pasquier 1989). In chickens and rabbits, the immunoglobulin heavy and light chain primary repertoires are generated by the recombination of a single functional V gene to the other variable region gene segments which are further diversified by somatic gene conversion events (Reynuad et al 1989, McCormack and Thompson 1990, Knight and Crane 1994). The elasmobranch heavy and light chain loci are commonly referred to as the "multicluster type" of arrangement where tandem arrays of joined and nonjoined V(D)JC clusters can be found (Kokubu et al 1988, Rast et al 1994). In teleosts the heavy chain loci is of the mammalian type of organization (Wilson et al 1990, Bengten et al 1993) and the light chains have been shown to posses the multicluster pattern as found in the elasmobranchs (Daggfeldt et al 1994). The IgH loci of the coelocanth *Latimeria chalumnae* has also been partially characterized and was found to consist of clusters of VH and DH gene segments which were two to three hundred base pairs apart (Amemiya et al 1993) and thus may represent a transitional state between the elasmobranchs and teleosts. More recently Wilson and coworkers (1995) have extended the mammalian IgH organizational pattern to the Holostean fish, bowfin and gar.

In mice, coexpression of the RAG genes are found within the bone marrow and thymus, the ontological sites of B and T-cell lymphopoiesis. In Xenopus, expression of RAG1 and RAG2 was found in the spleen, thymus and liver of juvenile frogs. The spleen is believed to be the site for B-cell development. In chickens, expression of RAG1 and RAG2 were found in the thymus (organ for T-cell lymphopoiesis), with sole expression of RAG2 within the bursa of Fabricius, the site of further diversification of Ig repertoires by gene conversion events. Later Takeda and coworkers (1992) demonstrated that RAG2 expression was not essential for gene conversion events, but that RAG2 may be involved in initiating the process. Bernstein and coworkers (1994) using RT-PCR demonstrated expression of RAG1 in the thymus and spleen in sharks, the putative locations for lymphopoiesis in elsamobranchs. In teleosts the Ig loci have been characterized, but the ontological locations of lymphopoiesis are not clear. Some believe that the thymus may serve as the primary source of lymphocytes (Grace and Manning 1980, Josefsson and Tatner 1993) whereas others believe that the pronephros (anterior kidney) may be the bone marrow equivalent in teleosts (Ellis 1977, Irwin and Kaattari 1986, Razquin et al 1990.) The thymus, based upon morphological and histological analyses is considered to be the organ for T-cell lymphopoiesis (Chilmonczyk et al 1992). Previously we reported the isolation and characterization of RAG1 from rainbow trout. In that study expression of RAG1 was found to be limited to sIg^- lymphocytes within the thymus using northern blot analysis (Hansen et al 1995). Here we describe the isolation and characterization of RAG2 from trout and extend our analysis of lymphopoiesis in this teleost using a molecular and cellular based approach.

Animals

Rainbow trout (*Oncorhynchus mykiss*, shasta strain) were obtained from the Marine Freshwater Biomedical Sciences Center at Oregon State University, and maintained at the Salmon Disease Laboratory in Corvallis, Oregon. This facility receives pathogen-free water at a constant temperature of 12° C. All fish were fed Oregon Moist Pellet commercial salmon food daily. Embryos were generated from the eggs of a single female which were fertilized with the milt from a single male. Embryos and tissue samples were obtained from trout killed by anesthetic overdose in benzocaine (ethyl p-aminobenzoate, Sigma, St. Louis, MO) (Kaattari and Irwin 1985) and bled via the caudal vein prior to dissection to rid the organs of erythrocytes. Tissues were used fresh or frozen immediately with liquid nitrogen and stored at -85° C until needed.

Trout RAG2 probe

PCR was conducted using degenerate primer sets based upon an amino acid alignment of all cloned RAG2 genes. The proceedure was used to amplify a portion of RAG2,1,142 bp , from rainbow trout genomic DNA. All primers used for PCR amplification and sequencing were synthesized at the Oregon State Center for Gene Research and Biotechnology Central Services facility. The RAG2 degenerate primer set was as follows: sense RAG2L1 5'-TTY-GGI-CAR-AAR-GGI-TGG-3' (bp 91-108) and antisense RAG2R1 5'-TCC-TCR-TCR-TCY-TCR-TA-3'

(bp 1213-1232). Numbers in parentheses refer to primer position relative to the complete RAG1 sequence. All primers were desalted using Oligoclean (BMB, Indianapolis, IN). Rainbow trout DNA was isolated using the procedure of Strauss (1989) which was subsequently used in PCR. Genomic DNA (250ng) was added to a 1 x PCR cocktail containing 50mM KCl, 10mM Tris-HCl, pH 9.0, .01% gelatin, 3mM $MgCl_2$, 200 mM dNTPs, and 150 pmoles of each primer. PCR samples were then overlaid with mineral oil, heated for 10 minutes at 95 °C, cooled to 80 °C at which time 2.5 units of Taq polymerase (Promega) and 0.2 units of Perfect Match (Stratagene) were added and the profile of 94 °C for 45 s, 56 °C for 1min, and 72 °C for 45 s for a total of 30 cycles followed by an additional extension time of 5 min at 72 °C was used for amplification. One tenth of the reaction was electrophoresed through a 2% agarose gel and a product of expected size (~1,142) was observed. The amplified fragment was cloned into pCRII (Invitrogen) and sequenced for verification of identity.

The PCR clone, pTARAG2, was digested with Eco R1 and the insert was gel purified (Geneclean:Bio 101) and used as a homologous probe in Southern and northern blots. The fragment was randomly primed with [32] dCTP (Amersham) using a commercial kit (BRL) to a specific activity of 1 x 10^9 cpm/ug. The probe was then cleared of non-incorporated nucleotides using G-50 Quick-Spin columns (BMB) prior to hybridization. All hybridization and washings for Southern and northen blot analysis took place in a Techne Hybridiser, HB-1D (Techne)

Genomic cloning of RAG2 and restriction analysis of the RAG locus

Previously we described the isolation of RAG1 from a rainbow trout lambda genomic library (Hansen et al 1995a). In all species investigated to date, the RAG genes have been shown to be tightly linked (Schatz et al 1992). Using this knowledge we analysed RAG1 positive lambda clones for the presence of trout RAG2. Phage DNA from selected clones were digested with Eco R1, Bgl II and Hind III restriction endonucleases (Promega), separated on a 0.8 % agarose gel, stained with EtBr, transfered to Nytran (S & S) using 10 x SSC and UV fixed. The blot was then probed under high stringency (65 °C 5x SSC,5x Denhardts soln and 0.5% SDS) conditions overnight with the radiolabeled RAG2 probe, washed (65 °C 0.2x SSC/0.2% SDS) and exposed to Hyperfilm (Amersham). The majority of RAG2 was localized to a 4.8 kbp Bgl-II restriction fragment which was cloned into pGEM-3Z for sequencing. The blot was then stripped and reprobed under the same conditions with a radiolabeled probe corresponding to the 5' end of RAG1 (Hansen et al 1995) to determine its map position.

Amplification and cloning of the 5' end of trout RAG2

We have previously described minor modifications of the 5'RACE methods of Frohman and co-workers (1989) and Renu and co-workers (1992) were used to amplify and clone trout RAG1 (Hansen et al 1995a). The same methodology was employed to amplify and clone the 5' end of trout RAG2. Briefly, first strand cDNA was synsthesized using 10 ng of thymic poly A^+ mRNA, M-MLV reverse transcriptase (Promega) and an antisense anchor primer (R2RACE1 5'-TGG-AGG-AGA-TCT-CGT-

TGT-3', bp 296-313). The mRNA template was degraded with Rnase H and dA tailed using TdT (Promega) and dATP. Second strand synthesis was accomplished using Taq polymerase and a dT_n-adapter primer by PCR. Reaction products were filtered (Microcon-30 spin filter, Amicon) and used in a second PCR amplification with a nested gene specific primer (R2RACE2 5'-GAG-ATG-GGT-CGC-AGT-TTG-3', bp 165-182) and the adapter primer. An aliquot from this nested PCR was then diluted 1:100 with TE and 1 ul of this dilution was used in a second nested PCR amplification (R2RACE3 5'-GAA-GAC-CCC-AGT-GGG-ACA-3', bp 121-138). The product (~220 bp) was cloned and sequenced to construct a sense primer (5'-CCT-GCG-GTA-GAT-GTC-3', bp -10 to +5) for the amplification of the 5' end from trout genomic DNA. Products were gel purified, cloned (pCRII) and sequenced from two independent PCR amplifications.

RNA preparation and northern analysis

Total RNA was extracted from specified tissues and embryos using the Trizol (MRC,inc) reagent according to manufacture's specifications. Poly A+ mRNA was further isolated from total RNA using Oligotex-dT (Qiagen). RNA samples (15 ug total and 5 ug Poly A) were fractionated on a 1.2%agarose-6.6% formaldehyde-1x MOPS gel, transefered using 10X SSPE and UV fixed. Prior to hybridization, blots were stained with Methylene blue (MRC) for a qualitative assessment of integrity. The blots were then photographed (Polaroid film 667) and positives were scanned by densitometry (Image QuaNT v4.1b, Molecular Dynamics) to establish equivalency of loading based upon rRNA bands. Methylene

blue was removed during prehybridization (50% formamide, 5x SSPE, 3X Denhardts soln and 0.5% SDS at 42 °C) and the blots were probed overnight using the same conditions as with prehybridizations with the addition of the trout RAG2 radiolabeled probe. Blots were washed under high (RAG2- 0.2x SSPE/0.2% SDS at 65 °C) or moderate stringency (xTdT-1X SSPE/0.2% SDS at 55 °C) and exposed to Hyperfilm (Amersham) for periods ranging from 2-7 days at -80 °C. The molecular weight for RAG2 was calculated by comparison with RNA molecular weight standards (Promega) and to rRNA bands.

RT-PCR expression analysis of trout RAG1 and -2

The method used was based upon modifications from Greenhalgh et al (1993). Total RNA from specified tissues were incubated with RQ1 RNase free DNase (Promega) at 0.667 units per ug RNA in 1x digestion buffer (50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine and 10 mM NaCl) for 20 minutes at 37 °C. The reactions were extracted 2 times with an equal volume of DEPC ddH_20 saturated phenol/chloroform, 1 time with chloroform and finally precipitated with 3M Na-acetate and ethanol. RNA samples were quantitated by UV spectroscopy (A260) and aliquots were gel electrophoresed and stained with EtBr for an integrity check of the RNA. One ug of RNA was incubated with 100 ng random primers (Promega) in 10 ul DEPC H_2O at 65 °C for 3 minutes and placed on ice. The reactions were then reverse transcribed using 200 U M-MLV reverse transcriptase (RNase H minus, Promega) at 37 °C for 60 minutes in a total of 20 ul 1x RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 mM dNTPs and 5 U RNasin

(Promega). Following RT, samples were incubated for an additional 5 min at 100°C and stored at -80 °C. Duplicate sets of samples were used, one contained reverse transcritase and the other did not. Portions of the RT reactions ranging from 1/200 to 1/5 of the total were added to 50 ul PCR reaction mixtures (supplemented with 2 uCi of [32]-P dCTP) containing 25 pmoles each of the RTRAG1 (sense 5'-GGA-GAA-GAT-GAG-GCG-ATC-ACC-A-3', bp 2002-2023 and antisense 5'-TTG-GCA-GAG-ACG-CCT-TTG-ACT-3', bp 2429-2450) and RTRAG2 (sense 5'-GTG-GCT-GCA-ACC-GCA-AAG-T-3', bp 341-359 and antisense 5'-CTC-TTG-CCA-AAG-CTA-GGT-GGA-A-3', bp 616-637) primers and overlaid with 2 drops of mineral oil. The reactions were heated to 80 °C at which time 1.5 U of Taq DNA polymerase was added. PCR amplification for both RAG1 and RAG2 were conducted using the following profile: 94 °C 45 sec, 57 °C 1 min and 72 °C 45 sec for 24 cycles followed by a final extension time of 5 min at 72 °C. One tenth of the reaction products were fractionated on 6% polyacrlamide gels, stained with EtBr for visualization of PCR molecular weight markers (Promega) and dried. The dried gels were then exposed to Hyperfilm for detection of amplified products.

Immunofluorescence and confocal microscopy

Methods: Spleen , peripheral blood , kidney and thymus lymphocytes were isolated by ficoll gradient centrifugation. Cells were fixed, permeabilized with Triton-X 100, and allowed to adhere to polylysine coated coverslips. Live cells were used as controls. The samples were blocked with 1% BSA, 0.1% Tween-20 in PBS (pH 7.6) for 30 minutes and

then incubated with a 1:100 dilution (~50 ug/ml) of mAb 1-14 (mouse anti trout Ig heavy chain; DeLuca et al 1983) in PBS/1% BSA for one hour. The cells were washed and subsequently incubated with goat anti-mouse IgG conjugated with Texas Red® (5 ug/ml, Molecular Probes) for one hour. After washing the samples, fluorescein conjugated Concanavalin A (10 ug/ml, Molecular Probes) was added for 5 minutes. The cells were washed and then subjected to a dehydration series of 50%, 75%, and 100% ethanol and mounted in Cytoseal (Stephens Sci). Cells were analyzed by fluorescence microscopy using a Nikon Labophot II equipped with a CF E Plan Achromat 100X 1.25 N.A. objective lens and a multiband filter set, DAPI/fluorescein/Texas Red® (Omega® Optical). Photomicrograph images were recorded with Ektachrome 400 ASA daylight film (Kodak). Cell preparations for confocal microscopy were the same as that used in immunofluorescence. Cells were visualized using a Zeiss LSM-410 confocal microscope.

Sequencing

Sequencing of trout RAG2 clones was attained using both manual and automated DNA sequencing methods based upon dideoxy chain termination chemistry. Manual sequencing was performed using the Sequenase 2.0 system (USB) and automated sequencing was conducted using an ABI 373A sequencer located at the Center for Gene Research and Biotechnology on the Oregon State campus. Sequences were determined in both directions using RAG2 gene specific or universal primers.

Computer analysis

Amino acid and nucleotide sequence comparisons were conducted using the BEST FIT function in GCG V7.2 unix (Deveroux et al 1984). Positions with insertion/deletions were omitted from these comparison. Sequence alignments were performed using PILEUP found in GCG along with minor manual corrections. Phylogenetic trees were constructed using the DeSoete algorithm (DeSoete et al 1983) and banching positions were confirmed by bootsrap parsimony (Felsenstein 1989), found in Phylip V3.5. Databases searches were conducted using BLAST 1.4.8MP unix (Altschul et al 1990). Primers utilized for sequencing and PCR amplifications were chosen using Right Primer v 1.01 (Biodisk) and densitometric analyses were accomplished using Image QuaNT V4.1b from Molecular Dynamics.

Results

RAG2 cloning and sequence analysis

Previously we described the isolation of a lambda phage genomic clone encoding trout RAG1 using a degenerate PCR based approach (Hansen et al 1995a). We employed the same basic technique in this report to isolate genomic clones of trout RAG2. Reports dealing with the isolation of the RAG locus indicated that in all species studied to date, RAG1 and RAG2 are tightly linked (~6-8 kbp apart). Initially we used probes derived from *Xenopus* and murine RAG2 to identify restriction fragments containing RAG2 from RAG1 positive lambda phage DNA under low stringency conditions. A similar approach was successful in the isolation of RAG2 from Xenopus and rabbit using murine and chicken RAG2 probes (Greenhalgh et al 1993, Fuschiotti et al 1993), but this approach failed to identify trout RAG2 clones. Therefore we generated a set of degenerate primers, based upon an amino acid alignment of all cloned RAG2 genes, to amplify a homologous probe for RAG2 from trout genomic DNA by PCR. An expected product of 1,142 bp was observed upon amplification. The product was then cloned and sequenced to validate its identity as being derived from trout RAG2. A BLAST search was conducted on the amplified sequence and was shown to be most similar to RAG2 sequences from other species.

Restriction digests of RAG1 positive phage DNA were electrophoresed, blotted and hybridized with the trout RAG2 PCR generated probe. Upon autoradiography, a 4.8 kbp *Bgl* II fragment was shown to hybridize with the trout *RAG2* probe. This fragment was gel

purified, subcloned into pGem-3Z and sequenced. Sequence analysis revealed that the *Bgl* II clone contained the majority of trout *RAG2* (amino acids 107-533), the 3' UT region for both *RAG* genes along with the carboxyl-terminal end of RAG1 (aa 841-1,072). Based upon the orientation and distance between the genes it was determined that, as in other species, trout *RAG1* and *RAG2* are convergently transcribed. The distance between the two genes was only ~2.8 kbp apart, demonstrating that the trout RAG genes are tightly linked (Figure 4.1a). Southern blot analysis was also performed on trout genomic DNA and it appears that an additional locus may exist for the RAG genes (data not shown).

As mentioned above the genomic clones for RAG2 were found to be truncated at the 5' end. Using the technique of 5' RACE, thymic mRNA was used as a template to amplify and clone cDNAs coding for the 5' end of RAG2. The cDNAs were sequenced and used to compose a sense primer for the amplification of the 5' end from trout genomic DNA. Three independent clones were sequenced. The sequence of trout RAG2 along with its predicted amino acid translation are shown in figure 4.1b. The initiation codon was based upon an alignment of all cloned RAG2 genes (figure 4.2), which predicts that trout RAG2 would consist of 533 amino acids. An acidic region is found near the carboxyl terminal end which is characteristic of all cloned RAG2 genes thus far.

The nucleotide and amino acid sequence of trout RAG2 was then compared to the other published clones for RAG2. Trout RAG2 averaged 60% and 75% similarity at the nucleic and amino acid level when compared to the other clones of RAG2. Based upon the alignment found in figure 4.2, a phylogenetic gene tree (figure 4.3) was constructed using the DeSoete treetool to further illustrate the conserved nature of RAG2.

Bootstrap parsimony analysis of 200 replicants was then used to support the branching positions. The overall branching order for the taxa within the tree, is consistent with the paleontological records for these organisms (Pough et al 1989).

Expression analysis of trout RAG1 and RAG2

The tissue specific expression pattern of RAG2 was initially investigated using northern blot analysis. Figure 4.4 shows the results of total RNA samples prepared from a 1-year old trout which were blotted and hybridized with the trout RAG2 probe. In the thymus, a strong signal corresponding to a 2.2 kb transcript was observed along with a much weaker signal at ~3.8 kb. The size of the 2.2 kb message is consistent to other reported RAG2 transcripts. The transcript detected at ~3.8 kb could possibly be due to an alternative splicing event or may be the result of incomplete processing of RAG2 hnRNA. A faint signal at 2.2 kb is also observed in the pronephros sample. The pronephros signal was found to be 510x less abundant in comparison to the thymic signal as determined by densitometry. No signal was detected in the other tissues. Similar patterns for RAG2 expression were observed using juvenile trout (data not shown).

Using the more sensitive expression assay, RT-PCR, tissue samples from 1-year old trout were analyzed for the presence of both RAG1 and RAG2 transcripts. Total RNA was first treated with RNase-free DNase to eliminate the possibility of amplification resulting from minute quantities of genomic DNA or frequently used plasmids containing RAG1 or RAG2. The samples were then organically

extracted, precipitated and quantified. An aliquot of each sample was also gel electrophoresed and stained with EtBr for a qualitative assessment of integrity. RNA samples were reverse transcribed using random primers (hexamers) and for all analyses RNA samples without reverse transcriptase were used as negative control. Duplicate sets of samples with and without reverse transcriptase treatment were then used in a semi-quantitative PCR assay with RAG1 and RAG2 gene specific primers. The reactions were amplified in the presence of [32]-P dCTP for detection of products by autoradiography. Signal intensity correlated with the volume of reverse-transcribed products that were added to the PCR reaction.

The results depicted in figure 4.5 a (RAG1) and b (RAG2) reveal that the RAG transcripts can be found in a variety of tissues using this sensitive method. All samples used in the PCR reactions contained ~1/50 and ~1/100 of the RNA used in northern blots for RAG1 (Hansen et al 1995a) and RAG2 respectively, with the exception of the pronephros (~1/150 for RAG1, ~1/300 for RAG2) and thymus (~1/1500 RAG1, ~1/3000 for RAG2) samples. Strong signals corresponding to the expected sizes of the amplified portions of the RAG1 and RAG2 (448 and 296 bp) were observed in the thymus and pronephros, followed by much fainter signals in the spleen, mesonephros and liver. Taking the dilutions into perspective, the signals seen in the thymus and pronephros are quite considerable. Amplified products were not observed in the samples corresponding to the upper and lower gastro-intestinal tract or brain. All RNA samples which did not receive reverse transcriptase treatment were negative (data not shown).

We then directed our attention to the point in ontogeny when embryos begin to express the RAG genes. To do this, eggs were collected at various points post fertilization, embryos were isolated and RNA from three individuals was harvested per time point. Using the RT-PCR assay as described above, we analyzed RNA from day 7,10, 13 and 18 post fertilization. Faint transcripts for both genes could be detected beginning on the tenth day post fertilization as shown in figure 4.6. This is consistent with the observations by Castillo and coworkers (1993) of the first cIg^+ cells in developing trout embryos. The relative levels are similar for both genes, with the expression of RAG1 being slightly more intense than that of RAG2 at all time points.

Immunofluorescence and confocal analysis of putative pre-B cells

The expression patterns observed for the RAG genes indicates that the thymus and pronephros are most likely the sites of lymphopoiesis in trout. To determine the location of developing pre-B cells in trout we analyzed tissue preparations using both immunofluorescence and confocal imaging. Cytoplasmic expression of immunoglobulin heavy chain in the absence of surface expression of Ig is a hallmark of pre-B cells. Using a mouse anti-trout heavy chain reagent coupled to Texas Red, mAb1-14 (DeLuca et al 1983), we assayed thymic, peripheral blood, pronephros and splenic cell preparations for the presence of putative pre-B cells. ConA labeled FITC was used to demonstrate lymphocyte surfaces. Nonpermeabolized cells (controls) displayed only ConA FITC staining alone (T-cells and monocytes) or together with surface Texas Red signal (mature B-cells) in all tissues examined (data not shown).

No red cytoplasmic signal was observed in nonpermeabilized cells. Both intracellular and extracellular, or exclusively extracellular, Ig-Texas Red signals were detected in the fixed and permeabilized cells of the peripheral blood, pronephros and spleen; however, no cells were stained red exclusively inside the cell (data not shown). In the fixed and permeabilized cells of the thymus (figure 4.7a), cells exclusively expressing cytoplasmic Ig were found without evidence of surface staining for Ig. We extended this observation using laser confocal microscopy to verify that true cytoplasmic expression of IgH in the absence of a surface signal was being observed in the thymic preparations. Figure 4.7b clearly demonstrates that this is indeed the case and implies that the thymus in trout harbors cells which may be the precursors of mature B-cells.

Discussion

This study addressed several questions regarding lymphopoiesis in rainbow trout including: the isolation and characterization of a RAG2 homologue, the expression patterns of both RAG1 and RAG2 in tissue samples, the point in time at which embryos initiate V (D) J recombination as measured by expression of the RAG genes and the location of precursor B-cells in trout tissue.

Using a degenerate PCR based approach, we were able to amplify a portion of trout RAG2 from genomic DNA. Primers were selected based upon the high degree of amino acid conservation of RAG2 amino acid sequences. The amplified product was verified as being derived from trout RAG1 and used as a probe to identify restriction fragments from RAG1 positive phage DNA. The majority of RAG2 was isolated was found in a 4.8 kbp Bgl II restriction fragment. The remaining 5' end was obtained by PCR amplification of genomic DNA.

Our results demonstrate that the RAG locus in teleosts is similar to that found in all species thus far examined (fig 4.1a). The RAG genes are tightly linked and are found to be convergently transcribed (Schatz et al 1992). In all species investigated prior to trout, the genes were found to be ~6-9 kbp apart, but in our analysis the distance between the respective 3' ends was less than 3 kbp apart. Due to the close proximity of the genes, a post-transcriptional method of regulation may be possible via anitsense transcripts of either RAG1 or RAG2. We have yet to completely sequence the 3' UT region, but anticipate finding poly-adenylation signals for both genes in this area. Previously investigators had postulated that the origins of the RAG genes might be from the

recombinatorial machinery of a virus or fungus that subsequently introduced the locus into the genome of vertebrates which in turn evolved into the central mechanism mediating V (D) J recombination (Oettinger et al 1990). This is still pure speculation, but it will be interesting to see what the organization of the RAG locus will be in the elasmobranchs and if this will shed some light on the subject.

The trout RAG2 open reading frame of 1601 bp coding for 533 amino acids, is similar in size to that found in all RAG2 genes. The overall conservation of RAG2 can be observed in the alignment of the amino acid sequences from vertebrates (fig 4.2). Contrary to what is seen in alignments of RAG1, conserved substitutions in RAG2 are evenly distributed throughout the entire sequence. Taking this into consideration along with the observation that cysteine and proline residues are absolutely conserved within the alignment, it appears that the overall structure of RAG2 has been maintained during evolution. The alignment also dipicts absolute conservation of serine and a threonine residues believed to be potential regulators of RAG2 activity via phosphorylation events (Lin and Desiderio 1995). Thus it appears that essential regulatory and conformation residues have been conserved during evolution.

The predicted amino acid sequence was 53% identical and 75% similar overall in comparison to the other RAG2 sequences. As found in all other RAG2 amino acid sequences a stretch of acidic residues can be observed in trout as well from amino acids 367-417. A BLASTP search of this region resulted in matches that contained acidic regions such as *Drosophila* Troponin-T, UBF1 and papillomavirus early protein E7. Based upon the conserved nature of RAG2 a phylogenetic gene tree was

conducted using the complete coding region of all cloned RAG2 genes. The tree (fig 4.3) generated in this study is quite similar to that of RAG1 (Hansen et al 1995). Overall both analyses demonstrate that the branching positions of the taxa within the trees are consistent with the known evolution of these organisms. Therefore the RAG genes have the potential of being utilized as molecular clocks for future phylogenetic comparisons within species that undergo V (D) J recombination.

Tissue specific expression of the RAG genes has been shown to occur within tissues and cell types known to undergo V (D) J recombination of Ig and TCR molecules. Therefore expression of the RAG genes coincides with the location of hematopoietic and primary lymphoid tissues as well as precursor B and T lymphocytes. In both juvenile and adult trout a high degree of mRNA expression of both RAG1 (Hansen et al 1995) and RAG2 were found in the thymus using northern blot analysis (fig 4.4). Within the RAG2 northern blots an additional band at approximately 3.2 kb was noted. This signal most likely represents incomplete processing of hnRNA or alternative splicing events. Analysis of RAG2 expression in other vertebrates also noted the appearance of additional transcript sizes. An extremely faint band corresponding to the pronephros sample was detected when hybridized with the RAG2 probe. Based upon these initial northern blot analyses, it appears that the thymus and possibly the pronephros serve as primary lymphoid organs in trout.

For a more sensitive analysis of RAG expression, we employed a RT-PCR assay to assess relative expression levels of these genes in a variety of tissue sources (fig 4.5). Expression of both genes was noted within the thymus, spleen, pronephros, as well as faint signals within

the mesonephros and liver. No expression of either gene was found in the samples derived from the brain or upper and lower gastrointestinal tract. The signal intensities of both genes was greatest within the thymus and pronephros which further suggests that these organs serve as the locations for V (D) J recombination of lymphocyte receptors in trout.

We also determined the time at which embryos begin to express the RAG genes (fig 4.6). In our analysis expression could be detected at day 10 post fertilization and was found at all time points past the initial detection. Previously, Castillo and coworkers (1993) investigated the ontological appearance of proposed pre-B cells in trout. In their immunofluorescence study, pre-B cells as determined by cytoplasmic expression of IgH could be observed at day 14 post fertilization followed by surface expression at day 18 post fertilization. Our results are therefore consistent with the expression of the RAG genes prior to the actual recombination of IgH as observed in mammals.

During the development of trout the thymus is the first tissue to display lymphocytes prior to their appearance in the kidney and spleen (Ellis 1977, Secombes 1983, Razquin 1990). The thymus in fish is also the first lymphoid tissue to develop during ontogeny and, as in higher vertebrates, is composed of a lymphocytes and lymphoblasts contained within a reticular network of epithelial cells. Thymic infiltration by lymphocytes has been detected as early 22 days pre-hatch in Atlantic salmon and 5 days pre-hatch in rainbow trout (Ellis 1977, Grace and Manning 1980). Most investigators will agree though that the pronephros is the site of hematopoiesis and is generally referred to as

the bone marrow equivalent in teleost fish (Ellis 1977, Razquin et al 1990). In their study dealing with immunoglobulin light chains in fish, Daggfeldt and coworkers (1993) noted heavy expression of nonrearranged and multiple forms of IgL transcripts in the pronephros and spleen which was suggested to be indicative of the presence of pre-B cells. Previously we noticed expression of RAG1 in sIg^+ lymphocytes from the thymus. It is quite possible that this expression was due to newly emergent immature B-cells which were transiently expressing *RAG1* or pre-B cells with IgH associated with surrogate light chain. We therefore decided to analyze various tissues for the presence of cells expressing cytoplasmic IgH in the absence of surface expression. Using immunofluorescent analysis we found what could be perceived as pre-B cells only within thymic cell preparations. In support of these findings, cytoplasmic only expression was also observed in thymocytes using confocal microscopy as well. Clearly these results suggest that the thymus in trout may contain pre-B cells, but absolute confirmation will have to await analysis using both IgH (Warrs1-14) and IgL (not available yet) serological reagents.

In summary, our investigation has shown several aspects associated with lymphopoiesis in trout. Firstly, our characterization of the RAG locus has demonstrated that the RAG genes are highly conserved in sequence as well as in organization. We then examined the tissue specific expression patterns of both genes which indicated that V (D) J recombination most likely occurs within the thymus and pronephros in trout. Next it was shown that expression of the RAG genes correlated with the first onset of cIgH in embryos. Finally

we propose that pre-B cells may be located within the thymus which suggests that the thymus in trout may play a dual role in the development of B and T lymphocytes. We are currently investigating other markers such as TdT which are associated with the development of precursor B-lymphocytes in trout.

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A.

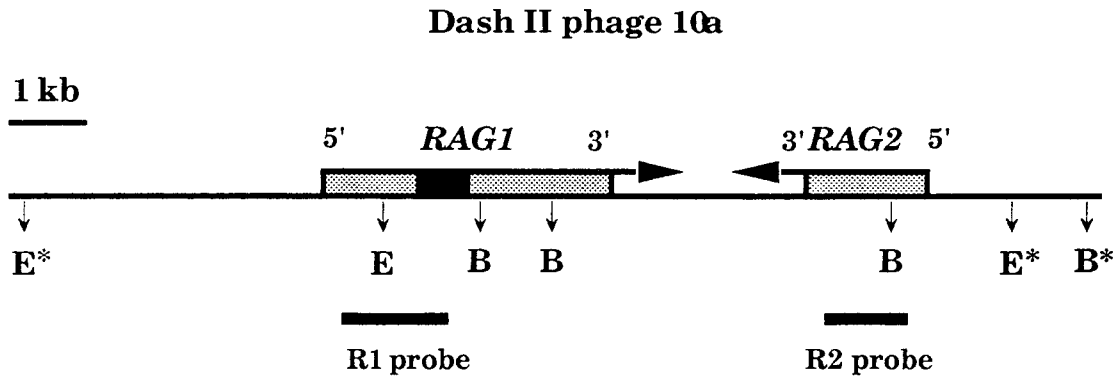

Figure 4.1 (A) Schematic representation of the *RAG* locus in rainbow trout. The dotted boxes represent the coding regions of *RAG1* and *RAG2* with arrows indicating the direction of transcription. The black box indicates the *RAG1* intron. The *Eco* R1 and *Bgl* II restriction sites located within the phage polylinker region are indicated by asterisks. Restriction sites shown are E= *Eco* RI and B= *Bgl* II. The approximate location of the *RAG1* and *RAG2* probes are also shown. **(B)** The complete nucleotide sequence of rainbow trout *RAG2* and its predicted amino acid translation are shown. The acidic stretch is underlined. This sequence has been deposited with Genbank under accession #U31670.

Fig 4.1B

Rainbow trout RAG2

```
                                                                                                                    90
ATGTCTCTGCAACCACTGACTGCAGTGAACTGTGGCAGCCTCCTGCAGCCTGGCTGCTCCCTGTTACAGCTGGATGGTGACATATTTCTG
M  S  L  Q  P  L  T  A  V  N  C  G  S  L  L  Q  P  G  C  S  L  L  Q  L  D  G  D  I  F  L
                                                                                                                   180
TTTGGGCAGAAGGGGTGGCCCAGGCGCTCCTGTCCCACTGGGGTCTTCGGGGTACGCCTAAAGCATGGGGAGCTCAAACTGCGACCCATC
F  G  Q  K  G  W  P  R  R  S  C  P  T  G  V  F  G  V  R  L  K  H  G  E  L  K  L  R  P  I
                                                                                                                   270
TCCTCCTCAAATGACTCCTGCTATCTTCCCCCTCTGCGTTGTCCCGCCTTGACCCGCCTTGAGCCCCATGATGGACACCCAGAGGGCTAC
S  S  S  N  D  S  C  Y  L  P  P  L  R  C  P  A  L  T  R  L  E  P  H  D  G  H  P  E  G  Y
                                                                                                                   360
CTCATCCATGGAGGGCGAACCCCAAACAACGAGATCTCCTCCAGCCTCTACCTGCTGACTCTGGACAGCCGTGGCTGCAACCGCAAAGTG
L  I  H  G  G  R  T  P  N  N  E  I  S  S  S  L  Y  L  L  T  L  D  S  R  G  C  N  R  K  V
                                                                                                                   450
ACCCTGCGCTGTCAGGAGAGAGAGTTGGTGGGAGAGCAGCCAGGGCCCCGATACGGCCACACACTTAGCATGGTGCAGAGTCTGGGCAAG
T  L  R  C  Q  E  R  E  L  V  G  E  Q  P  G  P  R  Y  G  H  T  L  S  M  V  Q  S  L  G  K
                                                                                                                   540
CGGGCCTGCGTTGTGTTTGGGGGCAGATCCTACATGCCGGCCGGGGAGCGGACAACAGAGAACTGGAACAGCGTTGTGGACTGCCCTCCT
R  A  C  V  V  F  G  G  R  S  Y  M  P  A  G  E  R  T  T  E  N  W  N  S  V  V  D  C  P  P
                                                                                                                   630
CAGGTGTTCATCATCGACTTAGAATTTGGCTGCTGCTCTGCCCACACCTTACCTGAGCTCACTGACGGCCAGTCCTTCCACCTAGCTTTG
Q  V  F  I  I  D  L  E  F  G  C  C  S  A  H  T  L  P  E  L  T  D  G  Q  S  F  H  L  A  L
                                                                                                                   720
GCAAGAGACGACTATGTCTACTTCCTTGGGGGGCAAAGTCTGTCGCTTGATTTTCGCCCCCCTCGGGTATATAGCCTGAGGGATGGGGTT
A  R  D  D  Y  V  Y  F  L  G  G  Q  S  L  S  L  D  F  R  P  P  R  V  Y  S  L  R  D  G  V
                                                                                                                   810
CCTGAAGGGAAGCCCGCTGTTTCCTGTAGCACCTGGACACCGTCCATGTCCATCTCCAGTGCTATTGCCACCCGTGTGGGGCCCTCCCAT
P  E  G  K  P  A  V  S  C  S  T  W  T  P  S  M  S  I  S  S  A  I  A  T  R  V  G  P  S  H
                                                                                                                   900
GAGTTCATTATCCTGGGTGGGTATCAGTTAGAGACCCAAAAGAGGATGGAGTGCAGCAGTGTGGTGCTGGATGACTCTGGGATAAACATC
E  F  I  I  L  G  G  Y  Q  L  E  T  Q  K  R  M  E  C  S  S  V  V  L  D  D  S  G  I  N  I
                                                                                                                   990
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Fig 4.1B continued

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GAGCCCAGAGAGGCCCCTGAGTGGACAGGGGAAATCAAACACAACCACACCTGGTTCGGAGGCAGCATGGGTGGAGGGAGCGCTCTGATT
E  P  R  E  A  P  E  W  T  G  E  I  K  H  N  H  T  W  F  G  G  S  M  G  G  G  S  A  L  I
                                                                                        1080
GGGATCCCCTCTGAGGGCAGGCAAGCCACGCCCGAAGCACATTACTTCTACCAGGTGTGCTTCCAAAAGGAGGGGGAAGGAAAGGGTGAA
G  I  P  S  E  G  R  Q  A  T  P  E  A  H  Y  F  Y  Q  V  C  F  Q  K  E  G  E  G  K  G  E
                                                                                        1170
GATGGGAACCAGGTCTGCAGCCAGGAGTCCACAGACTTTGAGGACTCCGCCCCTCTGGAGGACTCTGAGGAGCTGTACTTCGGCCGCGAG
D  G  N  Q  V  C  S  Q  E  S  T  D  F  E  D  S  A  P  L  E  D  S  E  E  L  Y  F  G  R  E
                                                                                        1260
CCCCATGAGCTGGAGGACAGCAGCGAGGGAGAGGGGGATACGTACAATGAAGAGGATGAGGAGGATGAGAGCCAGACAGGCTATTGGGTC
P  H  E  L  E  D  S  S  E  G  E  G  D  T  Y  N  E  E  D  E  E  D  E  S  Q  T  G  Y  W  V
                                                                                        1350
AAATGTTGCCTGGGCTGCCAGGTGGACCCCAACACTTGGGAGCCCTACTACTCCACAGAACTGCTGCGGCCAGCCATGATCTACTGCTCC
K  C  C  L  G  C  Q  V  D  P  N  T  W  E  P  Y  Y  S  T  E  L  L  R  P  A  M  I  Y  C  S
                                                                                        1440
AAAGGGGAGGGAGGCCACTGGGTCCATGCCCAGTGTATGGAGCTGACTGAGGGCCTGCTGGTGAGGCTCTCGCAGGGAAACGGCAAGTAC
K  G  E  G  G  H  W  V  H  A  Q  C  M  E  L  T  E  G  L  L  V  R  L  S  Q  G  N  G  K  Y
                                                                                        1530
TTCTGCCTGGACCACGGGGGACTGCCCCGCCAGGAGATGACCCCGCCACGTCAGGTGCTGTCCCTGAAGAGGAGCCCCATGAAACCCCAG
F  C  L  D  H  G  G  L  P  R  Q  E  M  T  P  P  R  Q  V  L  S  L  K  R  S  P  M  K  P  Q
                                                                                        1602
CACAGGAAGGGCCCAATGATGCGAAGGATGACACCCGCCAAGAAGCGCTTCTTCAGGAGGCTGTTTGAGTAA
H  R  K  G  P  M  M  R  R  M  T  P  A  K  K  R  F  F  R  R  L  F  E  STOP
```

Fig 4.2

Alignment of *RAG2*

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Trout-R2      MSLQPLTAVN CGSLLQPGCS LLQLDGDIFL FGQKGWPRRS CPTGVFGVRL KHGELKLRPI SSSNDSCYLP PLRCPALTRL 80
Rabbit-R2     ....MI.VR. NTA.I...F. .MNF..Q..F .......K.. ......HFDI .QNH...K.A VF.K...... ...Y..TCTF
Xenopus-R2    .T.RIV.PGS NT..I...F. ..HFSSHV.Y L......K.. ......LLD. .NND.....A TFT....... ...H..VCSF
Chicken-R2    ....MVS..S NS......S. ..NF..HV.F .......K.. ......FLDI .QN...MK.A AF.R...... ...Y..ICT.
Mouse-R2      ....MV.VGH NIA.I...F. .MNF..QV.F .......K.. ......HFDI .QNH...K.A IF.K...... ...Y..TCSY
Human-R2      ....MV.VS. NIA.I...F. .MNF..QV.F .......K.. ......HLDV ..NHV..K.T IF.K...... ...Y..TCTF

Trout-R2      E-PHDGHPEG YLIHGGRTPN NEISSSLYLL TLDSRGCNRK VTLRCQEREL VGEQPGPRYG HTLSMVQSLG KRACVVFGGR 160
Rabbit-R2     QGSSESEKQQ .I....K... ..L.DKI.VM SVVCKN-.K. ..F..T..D. ..DV.EA... .S.DV.Y.R. .SMG.L....
Xenopus-R2    SASQG.EITQ ......K... ....HK..IM .MAFPV-.KR FS.C.S.KD. A.DV.EA... .SMNV.F.R. .N.V.M....
Chicken-R2    RGNGESDKHQ .I....K... .DL.DKI.IM SMVNKT-TK. T.FQ.I.KD. G.DV.EA... ..INV.H.R. .SMI.I....
Mouse-R2      KGSI.SDKHQ .I....K... ..L.DKI.IM SVACKN-.K. ..F..T.KD. ..DV.E.... .SIDV.Y.R. .SMG.L....
Human-R2      KGSLESEKHQ .I....K... ..V.DKI.VM SIVCKN-.K. ..F..T.KD. ..DV.EA... .SINV.Y.R. .SMG.L....

Trout-R2      SYMPAGERTT ENWNSVVDCP PQVFIIDLEF GCCSAHTLPE LTDGQSFHLA LARDDYVYFL GGQSLSLDFR PPRVYSLRDG 240
Rabbit-R2     ....SNQ... .K....A..L .H..LV.F.. ..ATSYI... .Q..L...VS I..N.T..I. ..H..ANNI. .ANL.RI.VD
Xenopus-R2    ....LNQ... ....N.I..E .L.YL...Q. ..STSFN.R. .Q..L...VS ...N.T..IF ..H..GNN.. ..N..KIKVD
Chicken-R2    ..I.LAQ... .K.......L .S..LV.F.. ...TSYI... .Q..L...VS V....TI.I. ..H..QNNT. ..SL.K.KVD
Mouse-R2      ....STQ... .K....A..L .H..L..F.. ..ATSYI... .Q..L...VS I..N.T..I. ..H..ASNI. .ANL.RI.VD
Human-R2      ....STH... .K....A..L .C..LV.F.. ..ATSYI... .Q..L...VS I.KN.TI.I. ..H..ANNI. .ANL.RI.VD

Trout-R2      VPEGKPAVSC STWTPSMSIS SAIATRVGPS HEFIILGGYQ LETQKRMECS SVVLDDSGIN IEPREAPEWT GEIKHNHTWF 320
Rabbit-R2     L.L.S..IN. TVLPGGI.V. ...L.QTNND -..V.V.... ..N....V.N I.S.E.NK.E .QEM.T.D.. PD...SKI..
Xenopus-R2    L.L.S..... TVINSKI.F. .S.V.QTS.D -..V.V...E SDS...LI.N G.F...ET.D .QEI.T.D.. .....SK...
Chicken-R2    L.L.S.C.T. .ILPGGI.V. .G.V.QT.DT -..VLV.... SDN....I.N TI..E.NK.E .VE.VS.D.. PD...CRM..
Mouse-R2      L.L.T...N. TVLPGGI.V. ...L.QTNND -..V.V.... ..N....V.. L.S.G.NT.E .SEM.T.D.. SD...SKI..
Human-R2      L.L.S...N. TVLPGGI.V. ...L.QTNND -..V.V.... ..N....I.N IIS.E.NK.E .REM.T.D.. PD...SKI..
```

Fig 4.2 continued

```
Trout-R2     GGSMGGGSAL IGIPSEGRQA TPE-AHYFYQ V-CFQKEGEG KGEDGNQVCS QEST-DFEDS APLEDSEELY FGREPHELED 399
Rabbit-R2    .SN..N..VF L...GDNK.I VS...F...M LK.--T.DDV HEDQRTFTN. .T..E.PG.. T.F.....FC .SA.ANSFDG
Xenopus-R2   .AD..K.AV. F...VDNKHQ STDCSFF..V LNFGDNDPAL Q------T.. .G..EEQ... M.......FT .N.DGNIFDE
Chicken-R2   .CD..K..V. L...GANK.L ISD..N...I LR.NKA.EDE EE.LTA.T.. .A..E.QG.. T.F.....FS .SA.ASSFDV
Mouse-R2     .SN..N.TIF L...GDNK.. MS...F...T LR.--S.EDL SEDQKIVSN. .T..E.PG.. T.F.....FC .SA.ATSFDG
Human-R2     .SN..N.TVF L...GDNK.V VS..GF...M LK.--A.DDT NE.QTTFTN. .T..E.PG.. T.F.....FC .SA.ANSFDG

Trout-R2     SSEGEGDTYN EEDEEDESQT GYWVKCCLGC QVDPNTWEPY YSTELLRPAM IYCSKGEGGH WVHAQCMELT EGLLVRLSQG 479
Rabbit-R2    --DD.F.... .D..D...E. ...IT..PT. D..I...V.F .....NK... ....H.D.-. ....K..D.A .RT.IH..E.
Xenopus-R2   --D-------. .D..D...V. ...I...PD. DM.R.....F .....NK.S. .F...DG.-. ...S...D.S .TM.KY...N
Chicken-R2   --DD-I.... .D......E. ...II..AS. NI.I...V.F .....NK... .L..S.S.-. .......D.S .SM.LQ..EA
Mouse-R2     --DD.F.... .D..D...V. ...IT..PT. D..I...V.F .....NK... ....H.D.-. .....S.D.E .RT.IH..E.
Human-R2     --DD.F.... .D......E. ...IT..PT. D..I...V.F .....NK... ....H.D.-. .......D.A .RT.IH..A.

Trout-R2     NGKYFCLDHG GLPRQEM-TP PRQVLSLKRS PMKPQHRKGP MMRRMTPAKK RFFRRLFE* 536
Rabbit-R2    SN..Y.NE.V EIA.A-LQ.. -KRTIP.RKP ...SL.K..S G-KIL..... S.L....D.
Xenopus-R2   .I....NE.V EVA.G-VQ.. -EKTPPV.KT SL.SVRKRTT I-N.LSAV.. S.L......
Chicken-R2   .V....NE.V H.NKG-LQ.. -KKAVH..KQ ...RL.K.KT .-KLT..V.. S.L......
Mouse-R2     SN..Y.NE.V QIA.A-LQ.. -KRNPP.QKP ...SL.K..S G-KVL..... S.L....D.
Human-R2     SN..Y.NE.V EIA.A-LH.. -QR..P..KP ...SLRK..S G-KIL..... S.L....D.
```

Figure 4.2 Comparison of the predicted amino acid sequences from trout, rabbit, *Xenopus*, chicken, mouse and human *RAG2*. The region essential for RAG2 associated V (D) J recombination activity is underlined. Serine and threonine residues believed to be involved in modulating the RAG2 protein by phosphorylation are in bold.

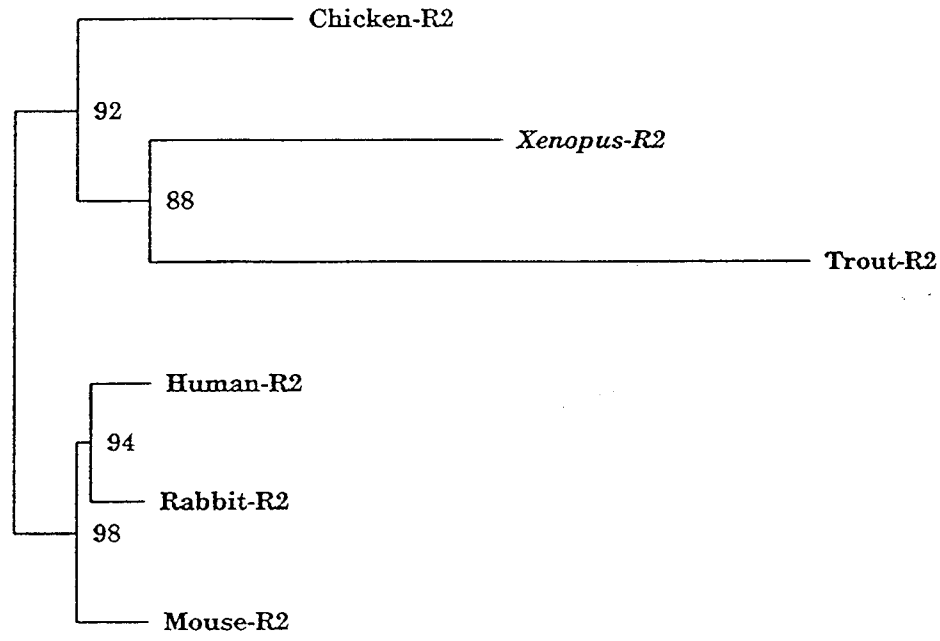


Figure 4.3 Unrooted phylogenetic tree of the *RAG2* complete amino acid sequences. An amino acid alignment was conducted using the Clustal alignment function found in GDE along with manual corrections and a tree was then constructed using the DeSoete algorithm in GDE. Positions have insertions/deletions were masked and branching positions were confirmed by bootstrap parsimony analysis of 200 replicants. Numbers at the forks represent %s obtained from the bootstrap analysis.

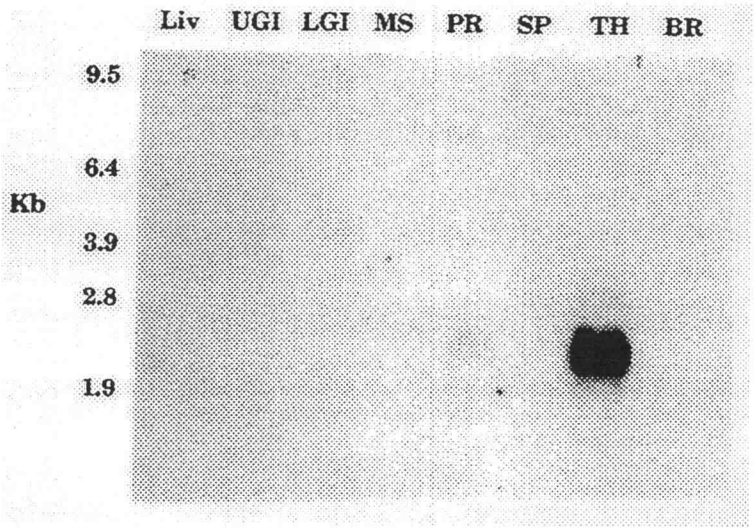


Figure 4.4 Northern analysis of RNA from various tissues of a one year-old trout. Total RNA was extracted from liver (Liv), upper GI (UGI), lower GI (LGI), mesonephros (MS), pronephros (PR), spleen (SP), thymus (TH) and brain (BR) and 15 ug was electrophoresed, blotted and hybridized with a trout-specific RAG2 probe (3 day exposure). *RAG2* expression (2.2 kb) is observed in the thymus with a much weaker signal (510x less as determined by densitometry) being detected in the pronephros. The additional band at approximately 3.2 kb in the thymus is most like due to incomplete processing of hnRNA or a splice variant. Equivalent amounts of total RNA were loaded and transferred as verified by methylene blue staining. The molecular weight markers are indicated.

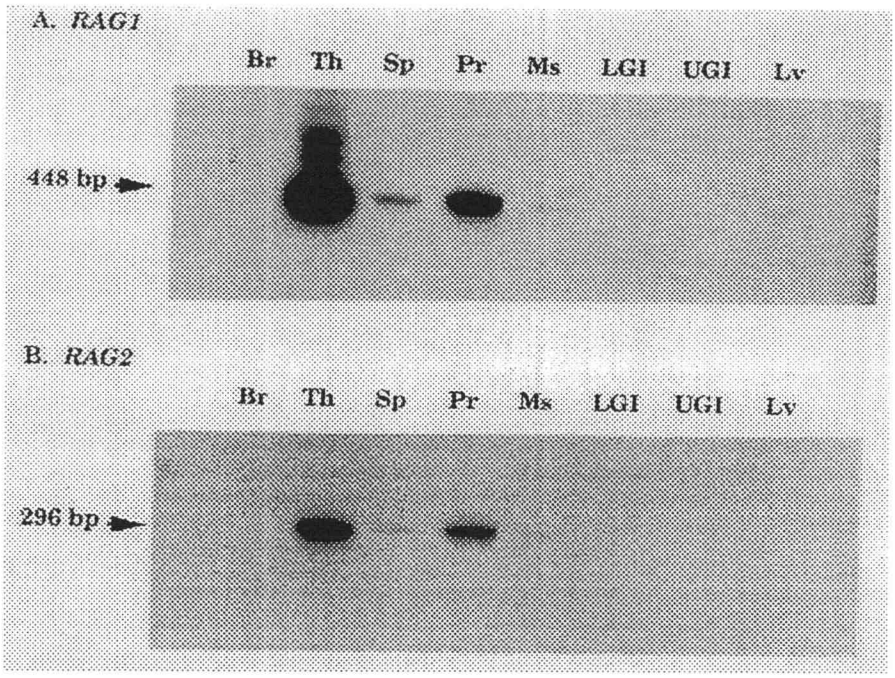


Figure 4.5 RT-PCR expression analysis of *RAG1* (**A**) and *RAG2* (**B**) from various tissues of a one year-old trout. Total RNA was extracted from the specified tissues, treated with RNase-free DNase, extracted, precipitated and quantified. Equivalent amounts of RNA were then incubated with random hexamers with or without reverse transcriptase. cDNA aliquots were then amplified with *RAG1* or *RAG2* gene specific primers by PCR in the presence of ^{32}P-dCTP. The amount of cDNA used for the thymus and pronephros amplifications were 1/20 and 1/3 in comparison to that used for the other samples. One tenth of the amplified samples were electrophoresed in a 6 % polyacrylamide gel, dried and exposed overnight. PCR product sizes were verified by molecular weight standards and are indicated by arrows for *RAG1* and *RAG2*. All samples which did not receive reverse transcriptase treatment were negative (data not shown).

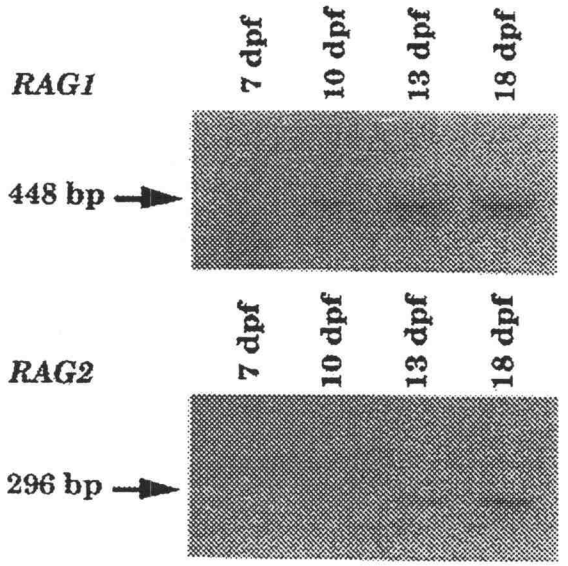


Figure 4.6 RT-PCR analysis of trout embryos expressing *RAG1* and *RAG2*. Total RNA was extracted from 3 embryos at days 7, 10, 13 and 18 post fertilization. RNA was then treated with RNase-free DNase, extracted, precipitated and quantified. Equivalent amounts of RNA were then incubated with random hexamers with or without reverse transcriptase. cDNA aliquots (1/4 of RT reaction) were then amplified with *RAG1* or *RAG2* gene specific primers by PCR in the presence of ^{32}P-dCTP. One tenth of the amplified products were electrophoresed in a 6% polyacrylamide gel, dried and exposed to film for 3 days. Expression of both genes is observed beginning on day 10 post fertilization. All samples not treated with reverse transcriptase were negative (data not shown).

a

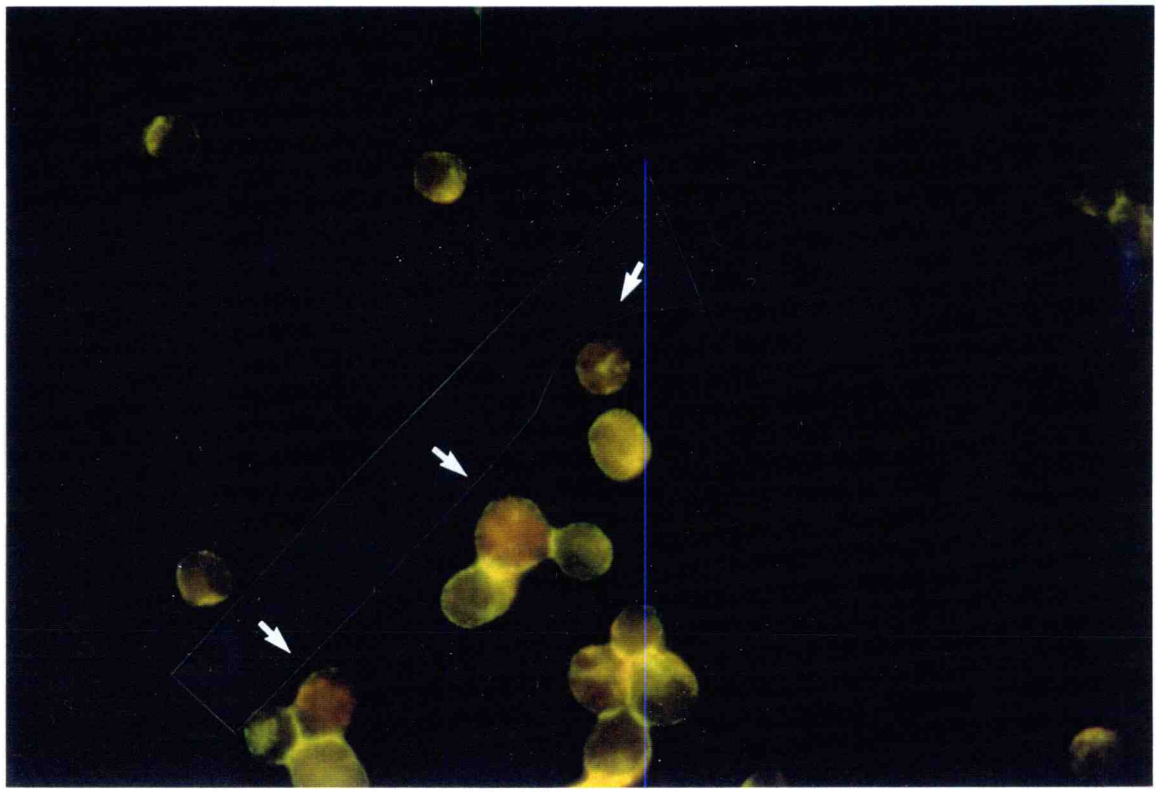
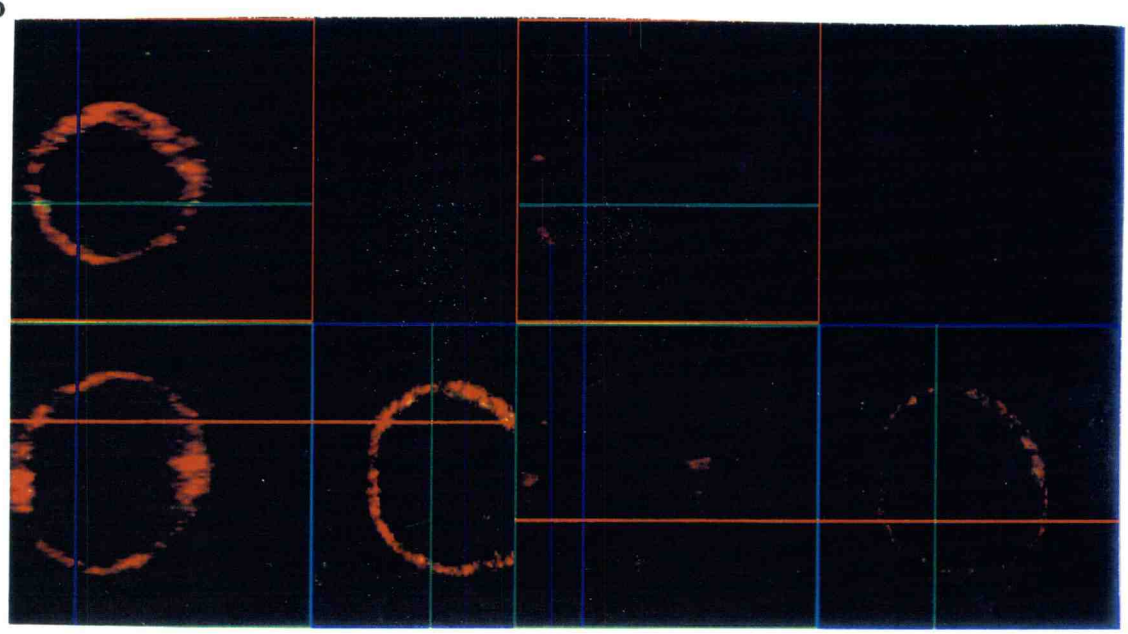
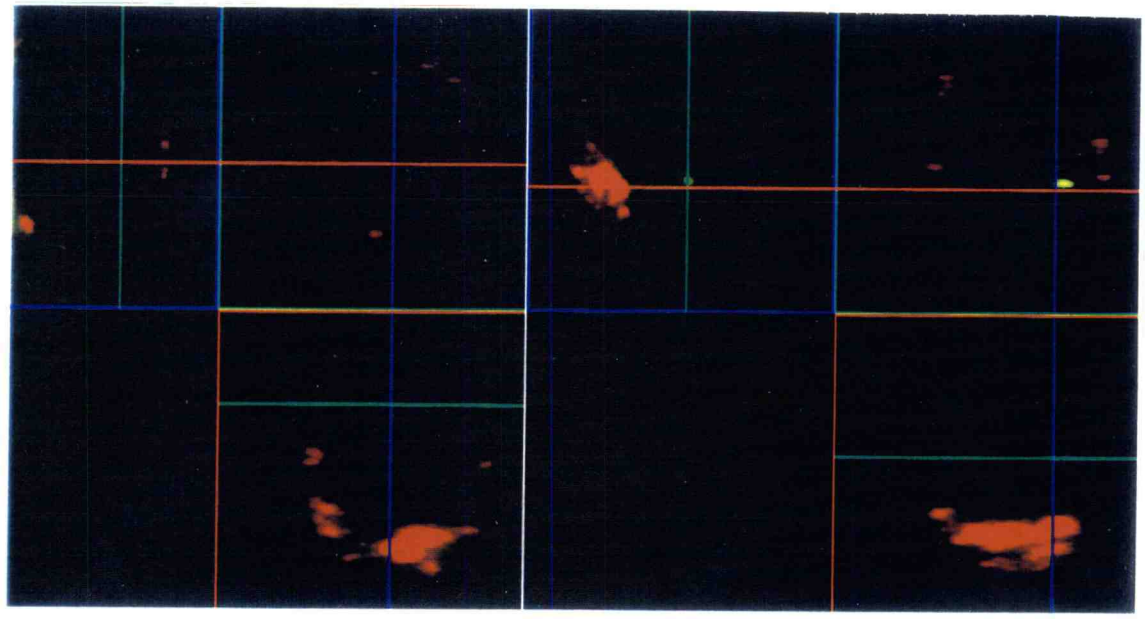


FIG 4.7 **a.** Fluorescence microscopy reveals putative pre-B cells within the thymus of trout. Fixed and permeabilized cells from the spleen, peripheral blood and kidney showed ConA-fluorescein staining alone, with surface alone or a combination of ConA-fluorescein/ extracellular Ig Texas Red®/ intracellular Ig Texas Red® (data not shown). No cells from these sources were positive only for cytoplasmic red staining. Fixed and permeabilized cells from the thymus displayed a number of cells that were positive for cytoplasmic Ig expression with the absence of a surface signal. Arrows indicate putative pre-B cells. **b.** Confocal microscopy of fixed and fixed/permeabilized cells from the thymus.

b



FIXED



FIXED AND PERMEABILIZED

CHAPTER 5

Concluding Remarks

The overall thrust of this thesis was to develop markers which assess the ontological locations of lymphopoiesis in trout. In mammals research in this area has been conducted for many years now with a wide variety of mAbs and molecular probes being available, but relatively little work in this area has been conducted in teleosts. The traditional method of using serological markers such as mAbs has been somewhat hampered in studies involving fish. Reagents are available to identify a few cell types within trout, but these are mainly limited to those cells expressing surface Ig. As far as the Ig heavy and light chain loci are concerned, great strides have been made in characterizing the relative organizational pattern for these loci (Wilson and Warr 1992). Few studies however looked at the expression of these receptor genes. It appears that expression is associated with tissues normally found harbor populations of mature B-cells. Recently the cDNAs encoding MHC class I and II molecules as well as clones for the beta chain of T-cell receptor have been isolated and partially characterized in fish (Dixon et al 1995, Partula et al 1994). These clones provide a great opportunity for those interested in characterizing the development of the immune system in trout and other teleosts. They may be used outright as nucleic acid probes for expression analyses or moreover for providing a source of recombinately expressed proteins from which mAbs can be generated. With the advent of the polymerase chain reaction coupled

with degenerate primers, a wide variety of immunologically relevant genes will most certainly be found.

In Chapter 2 it was demonstrated by using an amino acid alignment of higher vertebrate variable region genes that a trout specific probe could be amplified from genomic DNA using a degenerate primer set in a PCR assay. What was not discussed was that this primer set also had the ability to amplify variable region genes from a wide variety of vertebrate species which may be useful for future phylogenetic comparisons. Using the amplified probe, cDNAs from a peripheral blood lymphocyte library were isolated and found to encode full-length secreted and membrane bound forms of IgH. Approximately 20-25 different full-length IgH cDNAs were isolated based upon restriction and partial sequence analysis of the inserts. Two of these clones, were sequenced and found to encode membrane bound forms of IgH as expected which showed strong similarity to other IgH clones from vertebrates.

In catfish and Atlantic salmon, a quite different theme was found for the generation of mIgH as compared to that found in mammals and sharks. In these teleost species, an unusual splicing event occurs which leads to the complete loss of the CH4 domain. Previously investigators had reported partial cDNAs coding for trout mIgH, we on the other hand possessed complete clones which helped to validate that the splicing events found in the catfish and Atlantic salmon were typical of teleosts. Our mIgH cDNA clones also hinted at the possibility that they may contain isotypic differences, but it was concluded that the limited amino acid variability found most likely represented allotypic differences.

Our next goal in Chapter 3 was to identify a molecular marker associated with early events in the development of trout lymphocytes. Based upon the presence of recombination signal sequence motifs on all genomic variable region clones from mammals to fish, we asked the question if a common recombinase has also been conserved over course of evolution to perform V (D) J recombination of lymphocyte receptors. In mammals using a transfection based assay, genomic clones containing the recombination activating genes (RAG1 and RAG2) were isolated which proved to be essential for V (D) J recombination of lymphocyte receptors. We initially attempted to isolate trout RAG1 using heterologous probes, but with little success. We therefore constructed an amino acid alignment of all cloned RAG1 genes. The alignment displayed a highly conserved region in the carboxyl terminal end of RAG1 which would eventually be the basis for our primer selection.

Our initial PCR amplifications of RAG1 were not too encouraging as products of all sizes were observed. Success came upon the use of Hot-start PCR which basically entails fully denaturing the template for a period time prior to the addition of Taq polymerase in the PCR assay. Once we had concluded that the product of this reaction was derived from trout, it was used to isolate RAG1 from a lambda genomic library. The clone was sequenced and was shown to be most similar to the other RAG1 sequences upon a BLAST search. An interesting finding within the sequence analysis was the identification of an intron within RAG1. To this point, all RAG1 genes were shown to consist of a single exon, possibly during the course of evolution the intron was lost. Further analysis of other teleost and elasmobranch RAG1 genomic clones will

help to resolve if the intron is only found within teleosts and then possible lost at some later point in evolution. It should also be noted however, that southern blot analysis of genomic DNA indicated the presence of possibly another locus for the RAG genes which was not pursued. RAG1 in this second locus may be devoid of the intron.

Since our interest relied mainly on the use of RAG1 as a molecular marker of lymphopoiesis in trout, we used it as a probe to assess its tissue specific expression in juvenile and adult trout. In mammals expression of the RAG genes is limited to the primary lymphoid organs, the thymus and bone marrow. In our northern blot analyses using both total and poly A^+ RNA, expression was found to be limited to the thymus in both juvenile and adult trout at relatively the same levels. This was surprising for we anticipated expression to be found in the thymus as well as the pronephros, which is believed to be the primary organ for B-cell development. Using a double panning assay, we more precisely investigated the expression of RAG1 in sIg^- and sIg^+ cells from various lymphoid sources. Again the only cell type displaying RAG1 expression were those from the thymus. The sIg^+ expression in the thymocyte preparation was a bit surprising, but we attribute it to transient expression of RAG1 in immature cells which had recently undergone V (D) J recombination (possibly immature B-cells). Another possibility is that the sIg^+ cells found expressing RAG1 are early pre-B cells displaying expression of surface heavy chain and surrogate light chain. These findings confirmed our initial beliefs that the thymus can be regarded as a primary lymphoid tissue in trout. This is also supported by the findings of T-cell receptor mRNA transcripts in the thymus of trout (Partula et al 1994).

In Chapter 4 it was demonstrated that the RAG locus in teleosts is similar to that found in all other species thus far examined. Both genes are tightly linked and were found to be convergently transcribed based upon sequence analysis. An interesting finding was that the common 3' untranslated region in trout was much smaller in comparison to that of all other species. It may be that the close proximity of the respective 3' ends leads to regulation of the respective transcripts via an antisense mechanism. Taking the size of the entire locus (~8.2 kbp) into consideration, it not beyond the imagination that a viral vector may have introduced the RAG genes into the vertebrate genome. As of yet the 5' flanking regions of the RAG genes has not been characterized. It will be extremely intriguing if sequence motifs resembling direct repeats or long terminal repeats are found which would support the viral hypothesis for the origin of the RAG genes in vertebrates. Using PCR many investigators have recently tried to amplify portions of RAG1 from more distant species such as the lamprey or even protochordates without luck. Sequence analysis of the amplified products displayed no signs of RAG1. Therefore the process of V (D) J recombination may have been initiated by the introduction of the RAG genes into the direct predecessors of fish.

Keeping with the general theme of this thesis we extended our initial expression analyses of the RAG genes to include RAG2. In northern blots, expression of RAG1 was found in the thymus with an extremely faint signal observed in the pronephros. The expression of RAG2 and not RAG1 as noted by the northern blots is reminiscent of the situation in chickens. It has been postulated that RAG2 in avian species is involved in gene conversion events which further diversify the Ig

repertoires. Interestingly a variety of pseudo-variable region genes have been noted in teleost. Whether these are relevant findings will have to await future studies. Using a more sensitive expression assay (RT-PCR) we found transcripts of the appropriate sizes for the RAG genes in a variety of tissue sources. The heaviest levels of expression were found in the thymus and pronephros. Weaker signals were also noted in other tissue sources which may be due to immature cells which were recently exported to these tissues or that these tissues play some sort of rudimentary role in lymphopoiesis. Therefore based upon the overall expression patterns of the RAG genes, we conclude that the thymus and pronephros are most likely the sites of V (D) J recombination in trout and thus can be considered to be primary lymphoid organs.

Our final analysis of lymphopoiesis in trout consisted of a cellular based approach to identify tissues harboring precursor B-cells. By strict mammalian definition, an early precursor B lymphocyte is that cell which expresses cytoplasmic IgH in the absence of surface expression, but has yet to rearrange its IgL germline genes. In mammals plasma cells are known to internalize surface Ig, but in fish it has been shown that Ig is still present on plasma cells. Our data using both confocal and immunofluorescence microscopy is consistent with our hypothesis that the thymus is the location for lymphopoiesis of both B and T lymphocytes. Confirmation of this hypothesis will have to await the use of mAbs specific for all light chain isotypes, but due to the organizational pattern of the light chain loci it may be some time before these reagents are available. Therefore the data presented in this thesis lays the foundation for future studies looking at the overall development of the

immune system in fish as well as providing insight for studies dealing with the evolution of the immune systems in higher vertebrates.

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