Isolation And Characterisation Of Immunoglobulin Gene Superfamily Molecules From Lower Vertebrates (Fish)

by

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Molecular Medicine Research Group
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ABSTRACT

Acquired immunity in vertebrates is a major mechanism of the immune system, which enables the vertebrates to discriminate self and non-self. The most important feature of acquired immunity is the major histocompatibility complex (MHC) restricted-cellular immune response. This involves several important elements including MHC molecules and T cell antigen receptor (TCR) complex. MHC molecules are glycoproteins that recognise and present self and foreign peptides to T-cells via the TCR, causing the T cell to proliferate and secrete cytokines. There is increasing evidence that peripheral T cells and MHC restricted cellular immune responses occur in all vertebrates. However, the exact nature of the TCR and MHC in lower vertebrates has still to be established.

The aim of this thesis was firstly, to use the polymerase chain reaction (PCR) in conjunction with degenerative primers to the TCR β chain variable region (TCRBV) sequences of the horned shark to allow the amplification of the TCR-like genes from dogfish, Scylliorhinus canicula; secondly, to employ a similar strategy using degenerative primers to the banded houndshark MHC class-I α3 DNA sequence, to amplify MHC genes from S. canicula.

Using the degenerative primers, four clones from dogfish genomic DNA were obtained. These clones showed homology to TCR genes of other species. The amino acid sequence of clone 2C144 showed homology to the TCRBV region of several species. The highest similarities include 51.7% with the honed shark TCRBV, 45.1% with human TCRBV, 41% with monkey TCRBV, 40.7% with bovine TCRBV and 38.8 and 37.5% with chimpanzee and rat TCRBV, respectively. The clone 6C43 also showed homology to the TCRBV genes of several species (similarity is between 21.1% to 23.9%). Another two clones (6C53 and 6C54) were identified that showed high homology to the TCR δ chain variable region (TCRDV) of other vertebrates with 45.7% identity to the honed shark TCRDV region, 43.2% identity to mouse TCRDV, 32.2% identity to porcine TCRDV region, and 30.7% identity to human TCRDV. The highly conserved residues in the other vertebrates TCR are present in these four clones as well, such as WYRQ\textsuperscript{37} and YY(F)C\textsuperscript{92} motifs. Southern blotting analysis with the putative TCRBV (2C144) and TCRDV (6C53 and 6C54) suggested that polymorphism
existed between different fish. Northern blotting analysis with the probe 6C53 identified a transcript of approximately 2 kb in the spleen, lymphocytes and brain as well, with the probe 2C144 identified a transcript of approximately 2 kb in the lymphocytes of dogfish.

The sequences of nine clones obtained using cDNA as a template from dogfish together with degenerative primers showed high homology to the membrane-proximal domain of the MHC class II α chain in several species. These clones exhibited a high degree of homology to the nurse shark MHC class II α chain (74.1%), zebrafish MHC class II α chain (52.8%), mouse MHC class II I-A (49.6%), rat MHC class II α chain (49.9%), bovine MHC class II DYα chain and DQα chain (52.7% and 44.9%, respectively), human HLA- DP, -DQα and -DRα chain (44.4%) respectively. The cysteine residues of the membrane-proximal domain are conserved in the dogfish, suggesting that it may have a similar tertiary structure to mammalian MHC class II proteins. A highly conserved tryptophan residue at position 121 was found in the dogfish and an N-linked glycosylation site was present at position 133, whilst in higher mammals it is usually found at position 118. The Southern blotting analysis using probe DM9 showed that there may be more than four loci of MHC class IIA in dogfish.

These results suggest that the MHC class II, TCRB and TCRD genes are present in dogfish. The dogfish may have distinct T-cells, expressing either α/β or γ/δ heterodimers. Conserved key residues in both MHC class IIA, TCRB and TCRD suggest that these genes may encode functional MHC class II, TCRB and TCRD molecules. The MHC restricted cellular immune response may be present in dogfish. Dogfish TCRB, TCRD and MHC class II genes show high homology with several species including human, rat, bovine and other fish species, which implies that the immunoglobulin (Ig) superfamily has evolved from a common ancestor.
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AUTHOR'S DECLARATION

All of the studies presented in this thesis were performed by the author except for auto-sequencing, performed by Dr. Sian Ellard in the Royal Devon and Exeter Hospital.

This work has not been submitted for any other degree.

Signed: [Signature]

Date: 17/06/98
PUBLICATIONS AND CONFERENCES

B.M. Yang, J.E. Harris, M.L. Gilpin and A.G. Demaine. Isolation of major histocompatibility complex (MHC) class II genes from dogfish (Scyliorhinus canicula L.) (in preparation for submission)

B.M. Yang, J.E. Harris, M.L. Gilpin, M.L. Hibberd and A.G. Demaine. Isolation of T-cell receptor (TCR) genes from dogfish (Scyliorhinus canicula L.) (in preparation for submission)

13th European Immunology Conference in Amsterdam on 23-25th of June, 1997, presented the following abstract as a poster:

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<td>[α-^32P] dATP</td>
<td>alpha phosphate-32 dioxyadenosine</td>
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<td>[α-^35S] dATP</td>
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<td>A</td>
<td>Adenine or alpha (when prefixed by TCR only)</td>
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<tr>
<td>ABS</td>
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<td>2'-Deoxyribonucleotide 5'-triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-Deoxythymidine 5'-trisphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetra-acetic acid disodium salt</td>
</tr>
<tr>
<td>EMBL</td>
<td>European molecular biological library</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>G</td>
<td>Guanosine or gamma (when prefixed with TCR only)</td>
</tr>
<tr>
<td>[γ-32P] dCTP</td>
<td>gamma phosphate-32 dioxyadenosine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut associated lymphoid tissues</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>J</td>
<td>Joining region (gene segment of Ig superfamily)</td>
</tr>
<tr>
<td>kD</td>
<td>Kilo-dalton</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo-base</td>
</tr>
<tr>
<td>LMP</td>
<td>Low molecular mass protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoantibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>Mononey Murine Leukemia Virus reverse transcriptase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleotide</td>
</tr>
<tr>
<td>MT</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OligodT</td>
<td>Oligonucleotide ditriphosphate</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBD</td>
<td>Peptide binding site</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PNK</td>
<td>Polynucleotide kinase</td>
</tr>
<tr>
<td>PWM</td>
<td>Pokeweed mitogen</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activation gene</td>
</tr>
<tr>
<td>Rfp-Y</td>
<td>Restriction fragment pattern Y</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleotide</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Roswell Park Memorial Institute Medium 1640</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTase</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcript-polymerase chain reaction</td>
</tr>
<tr>
<td>sIg</td>
<td>Surface immunoglobulin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>T</td>
<td>Thymidine</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell antigen receptor</td>
</tr>
<tr>
<td>TCRA</td>
<td>T-cell antigen receptor α chain</td>
</tr>
<tr>
<td>TCRB</td>
<td>T-cell antigen receptor β chain</td>
</tr>
<tr>
<td>TCRD</td>
<td>T-cell antigen receptor γ chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCRG</td>
<td>T-cell antigen receptor δ chain</td>
</tr>
<tr>
<td>TEMED.</td>
<td>N,N,N',N'-Tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>T_{h} or Th</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>TL.</td>
<td>Thymus leukemia</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UT</td>
<td>Untranslated</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Variable region (gene segment of Ig superfamily)</td>
</tr>
<tr>
<td>X-gal.</td>
<td>5-Bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

1.1. General introduction

Immune responses in the more recently evolved endothermic (warm-blood) vertebrates (such as birds and mammals) are characterised by the participation of functionally distinct populations of lymphoid cells. These lymphocytes can be divided into B or T cells according to their surface markers and their functions. B cells are most readily identified by the presence of surface immunoglobulin (slg) and their ability to undergo clonal expansion and differentiation into antibody-secreting cells in response to the proper stimuli. T cells are also, clonally expandable lymphocytes but, in contrast to B cells, do not exhibit slg and are "educated" in the thymus during ontogeny to differentiate into specialised subpopulations capable of subsequently exhibiting either helper/inducer or cytotoxic functions. B cells are involved in humoral immunity and T cells participate in cell-mediated immunity relying on major histocompatibility complex (MHC)-peptide-based T cell recognition by the surface T cell antigen receptor (TCR). MHC and TCR together with antibodies all belong to the immunoglobulin (Ig) gene superfamily. They all have at least one Ig fold domain in their structures. The gene structures of TCR and antibodies are also very similar, and undergo somatic recombination during T and B cells development.

Recent phylogenetic studies have provided evidence that the TCR and MHC molecules emerged as early as the jawed fish (gnathan) stage (Hashimoto et al., 1990; Rast and Litman, 1994). The MHC-peptide-based T cell recognition system appears to have arisen at the emergence of the cartilaginous fish. Rast and Litman (1994) and Rast et al. (1995,1997) have reported that TCR α, β, γ, δ chains exist in the cartilaginous fish such as the horned shark (*Heterodontus francisci*) and the skate (*Raja eglanteria*). At the same time, Partula et al. (Partula et al., 1994,1995,1996) have identified TCR genes in a representative of the bony fish (teleost) the rainbow trout (*Oncorhynchus mykiss*) and Hordvik et al. (1996) also cloned TCR β-chain cDNAs from Atlantic salmon. Despite their considerable evolutionary distance (about 500 million years), the cartilaginous fish, teleost and mammalian TCR molecules remain structurally well
conserved, thus emphasising their fundamental importance in immunity. TCRs are disulphide-linked, membrane-bound heterodimers consisting of either α- and β- or, γ- and δ-chains. These heterodimers occur in conjunction with various accessory molecules to form signalling complexes. Although the exact function(s) of the TCR γδ system is uncertain, the function of the TCR αβ system is well characterised. TCR αβ recognises processed immunogenic peptides presented by MHC class I or class II molecules that are located on the surfaces of antigen presenting cells (APCs) (Germain, 1994).

The MHC together with the TCR molecules play a critical role in orchestrating the immune response at the molecular level (Townsend and Bodmer, 1989; Bjorkman and Parham, 1990; Germain and Margulies, 1993; Germain, 1994). The MHC molecules can be divided into two structurally and functionally distinct families of glycoproteins—the class I and class II molecules. The MHC class I molecules are encoded by the MHC class I region and are integral membrane proteins, which generally bind endogenously derived peptides from intracellular proteins. They have three domains (α1, α2, α3) with a fourth domain contributed by a noncovalently bound molecule called β2-microglobulin (β2-m). The major role of MHC class I molecules is to present peptides to the TCR of cluster differentiation (CD) 8+ T cells. The MHC class II molecule is a heterodimer with each protein, α and β chains containing two extracellular domains, that present extracellular peptides to the TCR of CD4+ T cells (Teh et al., 1988; Parnes, 1989). The MHC genes have been identified in many vertebrate groups except the jawless fish. A MHC gene was first reported in fish by Hashimoto et al. in 1990. Later, the genes encoding for the α chain of MHC class I and the β2-m, as well as the α and β chains of MHC class II molecules were isolated and sequenced from a number of different teleost species (reviewed in Dixon et al., 1995) and cartilaginous fish (Hashimoto et al., 1992; Kasahara et al., 1992, 1993; Bartl and Weisman, 1994a,b). APCs have also been shown to process and present antigens in fish (Vallejo et al., 1992).
1.2. Evolutionary position of fish

Fish are the oldest group of vertebrates (Pough et al., 1996). Jawless fish were the first vertebrates to appear in the fossil record. Their modern extant representatives are the hagfish and lampreys, which are the most primitive of all vertebrates. The earliest jawed fish diverged into three main lineages: one lineage being the cartilaginous fish and the representatives are the sharks, skates and rays; and two separate lineages of bony fish, one of the bony fish lineage is the Actinopterygii represented by most modern fish - teleosts form the majority; and the second lineage of bony fish is the Sarcopterygii, which have only a few modern representatives, such as the coelacanth and lungfish (Dipnoi), although these are the groups from which the tetrapods (amphibians, reptiles, birds and mammals) evolved (Fig. 1.1). Today, fish are the largest group of vertebrates with more than 20,000 species, the vast majority being teleosts such as the trout, salmon and carp. Interest in fish immunobiology is stimulated in part by their ancestry and location in evolution and also by a variety of commercial concerns regarding recent developments in their culture and hence the necessity to protect them against pathogens.

1.3. Immune mechanism in the dogfish

1.3.1. Lymphoid organs

The dogfish is a small ubiquitous shark of which there are many genetic varieties. Like other sharks, this fish has a well developed lymphoid system, such as the thymus, spleen, Leydig organ, epigonal and the gut-associated lymphomyeloid tissues (GALT) (Pulsford et al., 1982, 1984; Fänge and Pulsford, 1983; Hart et al., 1986; Lloyd-Evans, 1993a). The location of these organs is shown in Fig. 1.2 (Morrow, 1978; Fänge, 1982).

In Lloyd-Evans’ study (1993a), the structure and function of the developing immune system in embryonic and post-hatch dogfish were investigated. The results showed that a major feature of the developing immune system in S. canicula, is that the haemopoietic/lymphoid tissues develop in a certain order during the hatching stage. The first tissue to contain Ig⁺ cells is the liver at 2 months. The second one is the interstitial
Fig. 1.1. Evolution tree of vertebrates (modified from Pough et al., 1996).
Fig. 1.2. The lymphomyeloid organs of dogfish include the thymus, the spleen, the organ of Leydig and the Epigonal tissues.
kidney at 3 months. The third haemopoietic/lymphoid organs are the thymus, spleen, and Leydig organ. They appear at 4 months and the last tissues to differentiate are the epigonal and GALT. The haemopoietic/lymphoid nature of the kidney and thymus disappear at post-hatch whilst other lymphomyeloid tissues last throughout adult life.

The structure of dogfish spleen was investigated by Pulsford *et al.* in 1982. The results showed that small lymphocytes are one of the principal cell types in the spleen and surround the central macrophage to establish various types of cytoplasmic contact. Rosenthal *et al.* (1975) suggested that in such an association macrophages may present antigens to T-like cells.

The dogfish thymus structure was also studied by Pulsford *et al.* in 1984. The results showed that the thymus does not have a well defined cortical and medullary region, and that the tissue is composed mainly of a range of different sized lymphocytes located within a connective tissue capsule and reticular epithelial cell framework. Lymphoblasts, often in mitosis, are also present. The investigation suggests that the major function of the dogfish thymus appears to be the processing and export of small lymphocytes to reside in the blood, spleen, epigonal, Leydig organ and GALT.

At later stages of development (8 months and on), the cell density of the Leydig organ and epigonal tissues is less compact than at earlier stages, but Ig^+^ cells are found in both tissues throughout all stages (Lloyd-Evans, 1993a). The structural studies on lymphomyeloid tissues of dogfish (Fänge and Pulsford, 1983) showed that the cellular composition of the Leydig organ and epigonal tissues are remarkably similar both containing large numbers of developing granulocytes, blast cells, lymphocytes (forming nodule-like aggregations at some times), and plasma cells.

Ontogeny studies on the GALT in the dogfish (Hart *et al.*, 1986) have shown that the GALT develops with macrophage-like cells after the thymus and lymphoid-like tissue in kidney, but may come at the same time as the epigonal, Leydig and spleen. This suggests that as the developing embryo is exposed to antigens present in the seawater, the immune system develops in parallel and also indicates that the GALT in dogfish might be an important tissue in their immune system, because it contains small lymphocytes and eosinophilic staining granulocytes in later developmental stages. A
similar suggestion was made by Rombout and van den Berg (1989) and Rombout et al. (1989 a,b, 1993) in other species of fish. Their results showed that the second gut segment of carp was involved in the uptake and transport of antigens, antigen-presentation and antibody synthesis, suggesting that the GALT in carp takes part in the immune response in particularly local mucosal immunity.

In the study by Tomonaga et al. (1986), four species of sharks were investigated and the results showed that the GALT is present in these species and may represent a primitive form of Peyer's patches which are common in mammals. Finally, studies on the phagocytic cells and leukocytes in the peripheral blood of the dogfish (Parish 1981, Parish et al., 1986a, b, c) have shown that most of the principal mammalian leukocyte cell types are found in the dogfish blood.

All the above studies suggest that the dogfish lymphoid system is well developed. The main features include: there are plenty of mature and developing lymphocytes in the peripheral region of the thymus, and macrophages in the medullary region with very little Ig^+ cells in the peripheral region of the thymus. A high proportion of blast cells and plasma cells are located in the spleen. The cellular composition is very similar between the Leydig and the epigonal tissues, in which there are a large number of developing granulocytes, blast cells, lymphocytes and plasma cells, and less Ig^+ cells. The GALT is found in all later developmental stages of the dogfish. Most of the cells are small lymphocytes and Ig^+ cells in the spiral intestine. However, there is still no direct or definitive evidence to demonstrate the presence of distinct T and B cells subpopulation in extant elasmobranch fish. McKinney (1992a) hypothesised that shark T cells may be very similar to TCR γδ bearing mammalian T cells. The shark B cells may be the primitive equivalent of neonatal and newborn primary B cells. These hypotheses are shown in Fig. 1.3. and the suggestion of unusual features of shark lymphocyte function is summarised in Table 1.1.

1.3.2. Immune responses in the dogfish

Acute-graft-rejection have not been recognised in the cartilaginous fish although the chronic allogeneic reaction has been demonstrated in these species (Perey et al.,
Functional shark lymphocytes subsets are postulated. It is suggested that shark T and B cells are derived from a common precursor. Mature B lymphocytes may be similar to mammalian neonatal primary B cells, which synthesis multispecific, self-reactive IgM. Shark T lymphocytes may resemble mammalian TCR γδ cells, which respond to antigens in the context of self MHC (adapted from McKinney 1992a).
Table 1.1. Unusual features of shark lymphocyte function

<table>
<thead>
<tr>
<th>T cell</th>
<th>B cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of Hassall's corpuscles in non-thymic lymphoid tissue</td>
<td>Variable to absent anamnestic antibody responses</td>
</tr>
<tr>
<td>Thymic involution at ~ 3 weeks of age</td>
<td>Lack of affinity maturation</td>
</tr>
<tr>
<td>Lack of T cell help during antibody production</td>
<td>Plethora of natural antibodies</td>
</tr>
<tr>
<td>Chronic allograft rejection, &gt; 30 days</td>
<td>Multispecific antibodies</td>
</tr>
<tr>
<td></td>
<td>&quot;Autoreactive&quot; antibodies</td>
</tr>
<tr>
<td>Germline rearranged TCR genes</td>
<td>Germline rearranged Ig genes</td>
</tr>
</tbody>
</table>

Adapted from McKinney, 1992a.
1968; Borysenko and Hildemann, 1970; Hiledmann, 1970), whilst it has been shown that lymphocytes demonstrate proliferative responses to mitogens such as concanavalin A (Con A) and phytohaemagglutinin (PHA) which yield T cell like responses in these species (Lopez et al., 1974; Sigel et al., 1978; Pettey and McKinney, 1981). Therefore, MHC-restricted T cell immune responses may be present in the dogfish. This is also supported by the following observations. The dogfish has a complete set of lymphoid organs and TCR and MHC genes have already been isolated from several other shark species. The shark lymphocytes have been characterised by the presence or absence of slg, which is the slg<sup>-</sup> cells for B cells, whilst the slg<sup>+</sup> cells for the shark T cell-like subset, as well as perhaps other minor subsets of lymphocytes (McKinney, 1992a). Also previous work with the systemic immune response in dogfish (Morrow, 1978; Morrow et al., 1982) has shown that dogfish have the ability to produce specific antibody in response of a variety of soluble and particulate protein antigens, including some parasite antigens. Isolation and characterisation of the antibody molecule have shown it to be of the IgM class found in higher vertebrates (Lloyd-Evans, 1993b) and also that this is the only Ig class represented in the dogfish. The cellular functions in vitro were also investigated (Morrow, 1978) and it was found that dogfish leukocytes could be stimulated by a variety of mitogenic substances, such as ConA, pokeweed mitogen (PWM), PHA and lipopolysaccharides (LPS), but these results are insufficient to provide definitive evidence for lymphocyte heterogeneity. Evidence that the dogfish was capable of weak recognition of histocompatibility antigens was provided in vitro by mixed lymphocytes reaction (MLR) and in vivo by reaction to injected allogeneic leukocytes.

The local immune response in dogfish has also been investigated (Hart, 1987) in the dogfish. The results showed that specific antibodies were detected in the bile after sheep red blood cells and Vibrio were intubated into the gut by oral and anal routes or injected directly into the peritoneum.
1.4. Genetics of the TCR in non-mammalian vertebrates and comparison with the TCR in mammals

1.4.1. Isolation of the TCR

The polypeptide chain of the TCR was first identified on the surface of murine and human T cells using monoclonal antibodies (MAb) against cloned T-cell lines or hybridomas (Allison et al., 1982; Haskins et al., 1983; Kappler et al., 1983a,b; Kaye et al., 1983; Marrack et al., 1983a,b; Meuer et al., 1983a,b; Samelson et al., 1983; Staerz et al., 1984). These antibodies only recognised the T cells that were used as the immunogen. For example, Allison, et al. (1982) used a panel of hybridomas, which were constructed by fusion of P3x63Ag8 myeloma cells with spleen cells from a BALB/c mouse that had been immunised with a C57BL/Ka X-ray-induced lymphoma, C6XL. One of forty-three hybridomas secreted antibody (MAb 124-40) which reacted with the hybridoma cells but did not react with normal spleen cells. The antigen recognised by MAb 124-40 was isolated by radioimmunoprecipitation and found to be a glycoprotein composed of disulphide-bonded subunits of 39 kilo-dalton (kD) and 41kD. A cell surface component of similar structure, but not reactive with MAb 124-4, could be detected by two-dimensional electrophoresis in extracts of purified T cells, but not of B cells. These results suggested that the specific hybridoma antigen that was recognised by the MAb 124-40 might be a clonally expressed epitope carried by a T cell surface component.

Meuer et al. (1983a) used human MHC class I restricted T cells (CT8m, target specificity:HLA-A3) to immunise BALB/c mouse and the spleen cells were fused to mouse myeloma, NS-1. Two MAbs (anti-Ti1A and anti-Ti1B) against CT8m were produced. The antigens recognised by the anti-clonotypic antibodies were linked to the CD3 surface structure on the MHC class I specific CD8+ T-cell clone. This antibody identified a clonally unique antigen recognition structure (Ti1) comprised of a 49kD α-
chain and a 43kD β-chain, which is distinct from CD3 molecules and unrelated to the CD8 surface structure. To determine whether analogous receptor molecules could be found on other T cell clones of differing specificity, MAbs against a clonal structure (Ti2) on a MHC class II specific CD4+ T cells, were produced using the same method (Meuer et al., 1983b). The Ti2 structure on CD4 + was shown to be a disulphide-linked heterodimer like Ti1 on CT8M and was composed of a subunit of similar molecular weight. MAbs against Ti2 or Ti1 block antigen specific functions of the respective clone without showing any cross-reactivity. These findings suggested that each T cell uses an analogous Ti heterodimer for antigen specific function regardless of subset derivation or specificity.

Samelson et al. (1983) obtained MAb against the antigen receptor on a cloned mouse T-cell hybrid, which was a pigeon cytochrome c-specific and Ia molecule-restricted T cells. Two antibodies were isolated that specifically bound to the hybrid cells and the interleukin 2 (IL-2) release was inhibited from these hybrid cells. Lectin-induced IL-2 release was not affected by these antibodies. Immunoprecipitation and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of detergent lysates from surface-labelled hybrid cells revealed a heterodimeric structure, which was composed of two chains of 45-50kD and 40-44kD. Under reducing and nonreducing conditions the intermolecular disulphide bonds linking the chains were identified. It was suggested that this molecule is the antigen-specific receptor on the immunising T cell clone.

Haskins et al. (1983) used a similar method to that have mentioned above to identify the TCR proteins. A MAb (KJ1-26) against the T cell hybridoma DO-11.10 was produced from the B cell fusion of spleen cells from a (BALB.BxAKR) F1 mouse immunised with the T cell hybridoma DO-11.10, specific for cOVA/I-A4 and cross-
reacting with cOVA/I-A^b. This KJ1-26 completely inhibited the response of DO-11.10 to cOVA/I-A^d and also the production of IL-2. The molecule recognised by KJ1-26 has an apparent molecular weight determined by SDS-PAGE of 80-90kD under nonreducing conditions and 40-44kD after reduction. These results suggested that this molecule was closely involved in antigen/MHC recognition by T cells.

Kappler et al. (1983a) also identified the mouse TCR protein. The receptors for antigen plus a MHC gene product on a mouse T cell hybridoma specific for ovalbumin plus a MHC class II product were compared with those on another mouse T cell hybridoma, specific for a MHC class I product. In each case the putative receptor was identified by a clonotypic MAb. The two receptors had very similar size, being 70-85kD proteins. Under reducing conditions, they reduce to an acidic α-chain and a slightly basic β-chain of 40-43kD. The charge of both the acidic and basic polypeptides varied between the two receptors, being studied, showing that variable (V) amino acid sequences probably occurred in both chains (Kappler et al., 1983b).

In all these studies, the clonotypic MAbs precipitated a surface protein of ~80kD that separates into two chains of ~40kD on reduction (Allison et al., 1982; Staerz et al., 1984; Kappler et al., 1983a) or a ~45kD α-chain and a ~40kD β-chain (Samelson et al., 1983; Meuer et al., 1983a; Kappler et al., 1983b).

The identification of the TCR proteins using clonotypic antibodies now allowed the genes to be isolated. In 1984, Yanagi, et al. (1984) isolated four cDNA clones (YT30, YT35, YT53, and YT76) from a human T-cell line (MOLT-3). These four clones had similar restriction enzyme maps. The clone YT35 was used to probe the RNA from several T-cell lines, B-cell lines, normal bone marrow cells or bladder tumour cells from humans and a T cell line from mice. The results of Northern blotting and hybridisation showed no signal with RNA from B-cell lines, normal bone marrow cells or bladder
tumour cells. A single band was found in all T-cells with the greatest hybridisation in the thymus, followed by PHA-stimulated T lymphoblasts and a murine T-cell line. This suggests that these four cDNA clones are T cell specific. They used YT35 or YT76 to probe the total mRNA from MOLT-3 and the attached mRNA was isolated and transcribed in vitro using a rabbit reticulocyte lysate system. Two synthesised proteins were isolated, consisting of a 45kD and a 30kD proteins. The clone YT35 was sequenced and the deduced protein was found to have a high degree of similarity with Ig light-chain protein in mammals. Three important findings would be deduced from these studies: first, a human T cell-specific cDNA clone had been isolated and characterised; second, the putative protein sequence has been obtained; and third this protein contained two polypeptide chains and resembled human and murine Ig light chains.

Hedrick et al. (1984a) also isolated cDNA clones encoding T-cell specific membrane-associated proteins from mice and found a remarkable resemblance between the sequence of the amino acid of the clones and the Ig proteins of B cells. The membrane-bound polysomal RNA from an antigen specific, MHC restricted helper T cells (Th) hybridoma (M12, 2B4) was used to synthesise cDNA which was then subtracted with B cell mRNA (L10A). The subtracted cDNA was used as a probe to screen a selected cDNA library (TH-B) that had been constructed from a second T cell hybridoma (3.3T). They found 35 positive clones, 5 of these clones were reactive with B cell mRNA, and the remaining 30 fell into one of 10 distinct patterns of mRNA size and expression. One of these 10 different clones cross-hybridised strongly and gave a Northern blotting pattern superimposable on a rat Thy-1 cDNA clone, a classical T-cell membrane antigen not expressed on B cells. This clone was named as TM86 and used to screen a thymocyte cDNA library (Hedrick et al., 1984b). The clone TM86 and other three positive clones (86T1, 86T3, 86T5) from the thymus cDNA library were
sequenced. They showed homology to Ig with a putative protein structure similar to TCR polypeptide chains of mice. The gene was found to be rearranged in a variety of T cell lines by Southern blotting analysis.

Chien et al. (1984a) also isolated a putative TCR gene (p2B4.71) from a cDNA library constructed from the mRNA of the T11 cell hybridoma (2B4) by screening with probe 86T5 (Hedrick et al., 1984b). The Southern blotting results suggested that this gene was rearranged in T cells. Later, they (1984b) isolated another TCR gene by subtractive cDNA hybridisations, which was likely to code for the murine α chain of TCR.

Saito et al. (1984a) reported the complete primary structure of a heterodimeric TCR deduced from cDNA sequences. They used the subtraction-cloning method to isolate the TCR of cytotoxic T cells (CTL). The cDNA synthesised on the poly(A)+ RNA from CTL clone 2C (BALB.B) was subtracted twice with poly(A)+ RNA from a mouse B-cell lymphoma (A20-2J) and a library was constructed from the subtracted cDNA. They used two probes. The first was 2C cDNA prepared from the poly(A)+ RNA of membrane-bound polysomes by subtraction with poly(A)+ RNA from another B cell lymphoma (CH1). The second was the cDNA prepared from the total poly(A)+ RNA from A20-2J. They identified 10 sets of T-cell-specific clones according to the sizes of the corresponding mRNA present in 2C. Then they used representative cDNA clones of each set as hybridisation probes and compared EcoRI digested genomic DNA from 2C and BALB.B embryos by Southern blotting analysis, leading to the identification of two distinct classes of cDNA, one is the clone pHDS11 and the other is the pHDS4 and pHDS203. These clones were sequenced. The clone pHDS11 showed homology to the Ig chain as well as to the cDNA clones YT35 (Yanagi et al., 1984), TM86 (Hedrick et al., 1984b), p2B4.71 (Chien et al., 1984a). It was deduced that the clone pHDS11
encode the β-subunit of CTL 2C. Two other clones pHDS4/pHDS203 were considered to code for the α-subunit of the CTL receptor. A proposed TCR αβ structure was also described in this paper. Later, Saito et al. (1984b) identified another gene (pHDS58) that was also rearranged and expressed in clone 2C. This gene showed clonal diversity and encodes a polypeptide chain that contained Ig-like V and constant (C) domains and also carried N-glycosylation sites. Subsequently it was identified as the α-subunit of the TCR. Because there were some differences between the clone pHDS4/pHDS203 and pHDS58, the possibility of a second receptor was considered. Later, the pHDS4/pHDS203 clone was designated as the TCRG gene which actually encodes the γ chain of TCR γδ.

Sim et al., (1984) reported cDNA clones encoding the α-chain mRNA of the receptor of the human T-cell leukaemia line HPB-MLT. They used one cDNA clone (pGA-1) as a probe to hybridise different T cell and B cell lines, and found that the expression of α chain mRNA and rearrangement of a α chain V gene segment only occurred in T cells.

The suggestion that there may be a second TCR by Saito et al. (1984b) and the finding of the γ chain gene quickly led to a number of reports identifying the third gene family of the TCR now designed TCR γ gene (TCRG). These gene segments also undergo somatic rearrangement (Harday et al., 1985; Kranz et al., 1985a; Lefranc and Rabbitts, 1985; Murre et al., 1985; Rabbitts et al., 1985; Quertermous et al., 1986). The finding of a TCRG gene led to the identification of a novel subset of lymphocytes bearing a new TCR heterodimer, γδ (Brenner et al., 1986; Bank et al., 1986; Lanier et al., 1987). Later, Chien et al., (1987) identified and characterised the TCRD gene which encodes a δ chain of TCR γδ. They also found this gene to be located within the TCRA locus, both in mouse and man.

Brenner et al. (1986) used two framework MAbs (βF1 and WT31) which react
with most CD3 human peripheral blood lymphocytes (PBL) to immunoprecipitate the TCR αβ heterodimer from all human T cell lines examined that have αβ T-cell receptors and express the CD3 glycoprotein. They isolated βF1'WT'T3+ lymphocytes from immunodeficient patients, which were negative for TCR αβ. Chemical crosslinking experiments revealed that these lymphocytes expressed novel CD3-associated polypeptides, which were designated as TCR γ and δ chains.

At the same time, Bank et al. (1986) identified a functional CD3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. The immature T4'T8'T3+ cell line CII was isolated by treating the human thymocytes with anti-CD4 and anti-CD8 antibodies. The CD3 molecule on this cell line was immunoprecipitated with antibodies against CD3. Two additional bands of 44kD and 62kD were preprecipitated along with the CD3 complex. This data strongly suggested that a second set of TCR was present in human T cells.

Lanier et al. (1987) isolated a CD3'WT31' (TCR αβ') T cell line from normal peripheral blood and thymus which express no CD8 or CD4. They used anti-CD3 MAb to co-immunoprecipitate the CD3/TCR complex. The immune complex was analysed by SDS-PAGE and a disulfide-linked structure of ~90kD was co-immunoprecipitated with the CD3 complex. Further analysis showed that the structure contained two subunits of ~40kD and ~45kD and Northern blotting showed this cell line transcripts TCRG gene and also that the gene was rearranged.

1.4.2. TCR structure and function, distribution and gene organisation

1.4.2.1. TCR distribution and structure

It is well documented that the MHC class I and class II molecules together with the TCR are the principal molecules that influence self-nonself discrimination in
vertebrates (Blackman et al., 1988; Davis and Bjorkman, 1988; Flajnik, 1996; Kurosawa and Hashimoto, 1997) and play an important role in antigen recognition, signal transduction and lymphocyte proliferation. Most thymocyte and peripheral T cells express a TCR αβ, whilst the TCR γδ bearing cells are a small population which are found in the epithelium of the intestine (Jarry et al., 1990) and to a lesser extent in the skin (Brenner et al., 1988). T cells expressing the TCR γδ are found in virtually all organised lymphoid organs and diffusely distributed lymphoid tissues where they constitute a small subpopulation of T cells (mean, ~4%) (Groh et al., 1989). Occasionally, > 10-16% of CD3 cells express TCR γδ and around 1-10% in the peripheral T cell population in human and mouse express TCR γδ (Brenner et al., 1986).

Interestingly, TCR γδ cells are more numerous and localised in the sinusoids of the spleen compared to lower numbers in the periarteriolar white pulp and peripheral blood in human (Bucy et al., 1989; Falini et al., 1989), similar to the location found in the chicken spleen (Bucy et al., 1988). Approximately ~7% of T cells in the intraepithelium of the human intestine express TCR γδ, in contrast, TCR αβ cells were located in the lamina propria. The TCR γδ accounts for ~2% of the T cells in the thymus in human and are three times more numerous in the medulla than in the cortex (Falini et al., 1989). Another interesting point is that the TCR γδ cells are significantly more abundant in birds (Bucy et al., 1991) and the ruminant (Hein and Mackay, 1991). The repertoire of the TCR γδ in sheep is also extensive (Hein and Dudler, 1993). The distribution of TCR αβ and TCR γδ cells in amphibian and fish species has not been established.

The TCR is composed of a glycosylated polymorphic heterodimer of either αβ or γδ, which is associated with a nonpolymorphic membrane-bound complex of proteins collectively known as the CD3 complex (Clevers et al., 1988; Klausner et al., 1990) (Fig.1.4). The two polypeptide chains are linked by a cysteine bridge (disulphide-bonds)
Fig. 1.4. The outline structure of the TCR:CD3 complex. The TCR:CD3 complex contains eight polypeptide chains. Two are the disulfide-bonded α and β chains of the TCR. The other six chains, one γ and δ, two of ε and ζ chains, collectively called CD3, which will signal to the interior of the cell when antigen binding has occurred. The TCR and CD3 are co-ordinately expressed at the T cell surface (adapted from Janeway and Travers, 1996).
in the extracellular region close to the membrane of the T cell (Saito et al., 1984a). Each chain of the TCR has a hydrophobic leader sequence, 18-29 amino acids long, a 102-119 amino acid V region, a 87-113 amino acid C region, a small connecting peptide, a 20-24 amino acid transmembrane region, and a cytoplasmic region of approximately 5-10 amino acids. Each V and C region contains cysteine residues at positions consistent with the presence of a centrally located disulphide loop of 63-69 amino acids and a cysteine residue proximal to the transmembrane region that might be involved in the formation of an interchain disulphide bond. The hydrophobic transmembrane regions of the chains have an unusual feature: they contain positively charged amino acids that may play a role in interaction with components of the CD3 complex. Both α and β chains contain as many as three N-linked glycosylation sites which may be of complex or high mannose type (Allison and Lanier, 1987). The two domains in the external portion of each chain resemble the Ig V and C domains, respectively. Both chains have carbohydrate side chains attached to each domain. A short segment, analogous to an Ig hinge region, connects the Ig-like domains to the membrane and contains the cysteine that forms the interchain disulphide bond.

Novotny et al. (1986) compared the extracellular segments of the TCR α, β, γ polypeptide chains and N-terminal segment of CD8+ cells. The comparative studies showed that these four T-cell polypeptide chains are organised into Ig-like domains consisting of multistranded, antiparallel β-sheet bilayers. The structure of a C scaffold for antibody-binding sites are conserved in the TCR chains (Fig.1.5).

Chothia et al. (1988) analysed about 200 complete or partial sequences of V domains of TCR α and β chains and compared these with Ig of known structure. They found 40 sites that were crucial to the conserved structure of the V domains of Ig were also present in the TCR α and β chains (same or very similar residues). They also found
Fig. 1.5. Three-dimensional structure of a TCR αβ is from 2C cell line (Garcia et al., 1996). Backbone ribbon of the 2C TCR shows that the α chain is (residues 1-213) in pink and the β chain is (residues 3-247) in blue. Both α and β chains are β strands indicated by arrows and fold into Ig domains labeled according to the standard convention used for Ig folds. The disulfide bonds are shown in yellow balls, which are present within each domain and interchain disulfide. The CDRs are numbered from 1 to 4 for each chain.
that the TCR α and β dimer has a framework structure very close to that of the Ig. This structure contains three hypervariable regions or so called complementarity-determining regions (CDRs) in the V domain. These hypervariable loops are clustered together to form the antigen/MHC binding site. The first and the second loops have limited sequence variability. This suggests that these regions are involved in the recognition of the MHC proteins, whilst the third loop known as CDR3 will recognise the antigen presented by MHC molecules. The Va and Vβ CDR3s straddle the central region of the peptide within the α1 and α2 helics (Fig. 1.6).

The crystal structure of the α and β chain of the TCR (Bentley et al., 1995; Bentley and Mariuzza, 1996; Fields et al., 1994, 1995; Garcia et al., 1996) shows structural homology of the extracellular portion of the α and β chain of the murine TCR to Ig. The structure of the first and second loops suggests that they adopt a more restricted site of conformation in TCR β chains than those found in Ig. The third loop had certain structural characteristics in common with those of Ig heavy chain V domains. The V and C domains were in contact and this may restrict the flexibility of the β chain. Later, the crystallisation of the TCR δ chain V domain of a human TCR γδ was reported (Lebedeva et al., 1996). Comparative sequence analyses of the human CDR3 region of TCR α and β chains (Moss and Bell, 1995, 1996) shows that the length of the CDR3 region in both TCRA and TCRB sequences is found to be relatively conserved and very similar. The pattern of amino acid usage in the CDR3 region is different between these two sequences. There is a predominance of charged and polar residues in the TCRA transcript, whereas TCRB has a greater concentration of glycine residues. These findings suggested that the TCRA and TCRB genes may utilise different mechanisms to generate diversity in the CDR3 region due to a lack of diversity (D) segment in TCRA.
Fig. 1.6. The TCR and MHC-peptide complex interaction. The 2C-H-2K\textsuperscript{b}-peptide complex are shown in backbone tube. The MHC-peptide complex is below with the octamer peptide P1-P8 which locates between TCR and MHC and is shown as a large tube in yellow. The TCR is above within the CDR 1 and CDR 2 of \( \alpha \) chain in pink, hyper variable region 4 is in white. CDR 1 and CDR 2 of \( \beta \) chains in blue, hepervariable region 4 in orange. The CDR3s are coloured in yellow (adapted from Garcia et al., 1996).
1.4.2.2. TCR gene organisation

Four different loci—TCRA, TCRB, TCRG and TCRD encode the polypeptide chains for the TCR. The arrangement of the gene segments of the genes that encode for the antigen/MHC-binding portion of the TCR have similar features to Ig with separate V, diversity (D) for TCRB and TCRD, joining (J) and C gene segments.

1.4.2.2.1. TCRA genes

The TCRA locus is located on the long arm of chromosome 14 (14q11-12) in human (Croce et al., 1985; Caccia et al., 1985; Collins et al., 1985; Rabbitts et al., 1985) and on chromosome 14 of the mouse (Kranz et al., 1985b; Dembic et al., 1985). The human TCRA locus consists of 70-80 V segments, each containing an exon encoding a V region preceded by an exon encoding the leader sequence that targets the protein to the endoplasmic reticulum for transport to the cell surface. A cluster of approximately 60 J segments is located a considerable distance from the V segments. The J segments are followed by a single C-domain segment, which contains four exons for the C and hinge domains and a single exon encoding transmembrane and cytoplasmic regions. The TCRA locus is interrupted between the J and V segments by another TCR locus—the TCRD locus (Chien et al., 1987) (Fig. 1.7).

1.4.2.2.2. TCRB genes

The TCRB locus spans about 600 kb on chromosome 7q32-35 in humans (Caccia et al., 1984; Barker et al., 1984; Isobe et al., 1985; Morton et al., 1985) and on chromosome 6 of the mouse (Caccia et al., 1984). The TCRB locus (Fig. 1.8) in human has a cluster of about 50 different functional V segments followed by two separate clusters each containing a single D segment, together with six or seven J segments and a
Fig. 1.7. The organisation of the human TCRA gene, there are about 70-80 variable, 60 joining and a constant gene segments (adapted from Janeway and Travers, 1996).

Fig. 1.8. The organisation of the human TCRB gene, there are about 50 variable, a diversity, 6-7 joining and a constant gene segments (adapted from Janeway and Travers, 1996).
single C segment.

1.4.2.2.3. TCRG genes

The human TCRG locus resides on the short arm of chromosome 7 (7p15) (Murre et al., 1985; Rabbitts et al., 1985) and the mouse TCRG is on chromosome 13 (Kranz et al., 1985b). These genes are organised in a similar manner to those of the TCRB genes (Strauss et al., 1987). Two neighbouring TCRGJ, TCRGC gene clusters are flanked on their 5' sides by an array of TCRGV genes. Approximately 14 V segments have been identified (Forster et al., 1987; Huck et al., 1988), although a number of pseudogenes are also present (Strauss et al., 1987). The five J segments identified in the TCRG locus can be divided into two groups comprising TCRGJ1-3 located upstream of TCRGC1, and TCRGJ4-5 located upstream of TCRGC2 (Quertermous et al., 1986). The two TCRGC segments are structurally similar to the TCRAC and TCRBC genes, but have three exons, which encode the C domain, the connection peptide, transmembrane and intracytoplasmic portions, respectively (Fig. 1.9).

1.4.2.2.4. TCRD genes

The human TCRD locus occupies the space between the TCRAV and TCRAJ gene segments (Chien et al., 1987; Griesser et al., 1988). There are three TCRDD segments (Loh et al., 1988) and three TCRDJ segments, and a single constant segment. There are at least six TCRDV segments interspersed among the TCRAV gene segments (Takahara et al., 1989) (Fig. 1.10).

The multiple V, D, and J segments in TCRA, TCRB, TCRG and TCRD loci undergo somatic recombination during T cell development to produce functional VDJ encoding for TCRB and TCRD chains or VJ gene segments for TCRA and TCRG
Fig. 1.9. The organisation of the TCRG genes in human. More than 14 V segments and 5 J sections and 2 C segments are constituted the TCRG genes (adapted from Janeway and Travers, 1996).

Fig. 1.10. The organisation of the TCRD genes in human. There are TCRDD segments and three TCRDJ segments, a single constant segments. There are at least six TCRDV (adapted from Janeway and Travers, 1996).
chains. This process is similar in many ways to that which occurs during Ig gene rearrangement in B cells. Rearrangements of two different sets of receptor genes distinguishes two T cell lineages, one expressing αβ and another expressing γδ. The gene recombination between V and C genes generates deletions and rearrangements and involves the same mechanism, the 12-23 rule (Lewin, 1994). A consensus sequence is found at the boundaries of all germ-line segments that participate in joining reactions. Each consensus sequence consists of a heptamer separated by either 12 or 23 base pair (bp) from a nonamer (Fig. 1.11). The rule that governs the joining reaction is that a consensus sequence with one type of spacing can be joined only to a consensus sequence with the other type of spacing. Since the consensus sequences at V and J segments can lie in either order, the different spacing does not impart any directional information, this prevents one V segment from recombining with another, or one J segment from recombining with another. The genes involved in the recombination are recombinase genes (RAG) (Lin and Desiderio, 1995; Thompson, 1995). During development of the T cell, the TCR α and β chain genes are rearranged by joining discrete segments. For the α chain, a V gene segment rearranges to a J gene segment to create a functional exon. The VJ exon is transcribed and spliced to C and generates the mRNA which then is translated to yield the TCR α-chain protein. For the β chains, like the Ig heavy chains, the V domain is encoded by three gene segments, V, D, and J. These gene segments are rearranged and generate a functional exon. This exon is transcribed and spliced to join VDJ to C and the resulting mRNA is translated to yield the TCR β-chain protein. The α and β chains pair to yield the TCR αβ heterodimer soon after their biosynthesis (Fig.1.12). The TCRG and TCRD genes recombined in the same way to generate a functional TCR γδ heterodimer.
Fig. 1.11. (A) Recombining sites contain consensus sequences (heptamer and nonamer) which are present in inverted orientation at each pair. There is a 12 bp spacing at one member of the pair between the two consensus sequences. The other has 23 bp spacing. (B) The consensus sequences are breakaged and reunined to generate TCR genes (adapted from Lewin, 1994).
Fig. 1.12. The rearrangement and expression of TCR α- and β-chain genes. The TCRA and TCRB genes contain discrete segments V(D)JC, which are joined by somatic recombination during development of the T cell. A TCRAV gene segment rearranges to a TCRAJ gene segment to create a functional exon. The TCRAVJ exon is transcribed and spliced to TCRAC. The mature mRNA is then translated to generate the TCR α-chain protein. For the β chain, the procedure is the same as that in α chain (adapted from Janeway and Travers, 1996).
1.4.3. TCR function

1.4.3.1. TCR αβ function

T cells co-ordinate the immune response through their autonomous programme of differentiation that is induced following antigenic stimulation. The first step in this process is the effective contact between the MHC/antigen complex and the TCR αβ. The TCR αβ recognises antigen held within the groove of MHC molecules, which are anchored in the membrane of the APCs. During the contact, TCR αβ acts as a multi-component signalling device between cells (Janeway, 1992). The polypeptide chains of the TCR are associated with the CD3 complex, which play an essential role in the signalling and is often considered to be a part of the TCR. The CD3 complex acts as the messenger from extracellular to intracellular events.

TCR αβ bearing T cells can be divided into two main subsets according to their surface marker. The first expresses the CD8 cell surface marker and the second expresses the CD4 surface marker. The CD4+ cells contain two functional classes: Th1 cells (inflammatory cells) and Th2 cells (anti-inflammatory cells). Generally, CD8+ TCR αβ cells recognise the antigens within cells, which have been processed and then presented by MHC class I bearing cells. The main function of the CD8+ cells (cytotoxic T cells) is to kill infected cells. CD4+ TCR αβ cells recognise the peptides that are processed and presented by MHC class II bearing cells. Th1 cells activate macrophages to kill the bacteria they harbour, and Th2 cells activate B cells to synthesis and secrete antibodies (Parnes, 1989; Janeway, 1992) (Fig. 1.13).

1.4.3.2. TCR γδ function

The function of TCR γδ T cells has still to be elucidated. The majority of TCR γδ cells lack CD4 and CD8 (Brenner et al., 1986; Lanier et al., 1987; Groh, et al., 1989).
TCR \( \alpha \beta \) bearing T-cells express surface markers either CD4 or CD8. The CD4 and CD8 molecules are important in antigen recognition by T cells and also these two lymphocyte subsets have different functions in immune system. CD8 molecules bind to a site on the \( \alpha 3 \) domain of class I MHC molecules, whereas CD4 binds to a site between the \( \alpha 1 \) and \( \alpha 2 \) domains of a class II molecules. And CD8 lymphocytes recognise the peptide antigens presented by MHC class I bearing cells and kill the infected cells, whilst CD4 lymphocytes recognise the peptides presented by MHC class II bearing cells, which normally are professional antigen presenting cells and activate macrophages to kill the pathogens or activate B cells to produce antibodies (MHC function will be discussed in section 1.5.2.3).
But ~1-4% and ~10-70% TCR γδ cells of peripheral blood TCR γδ cells expressed CD4 and CD8, respectively (Groh et al., 1989) and CD8 is expressed on approximately 50% of TCR γδ T cells in splenic sinusoids and 30% in peripheral blood (Bucy et al., 1989). Only a tiny fraction of peripheral TCR γδ T cells express CD4 (Groh et al., 1989; Bucy et al., 1989).

The TCR γδ and TCR αβ T cells probably have a similar function in the immune system (Haas et al., 1993; Bluestone et al., 1991, 1995). TCR γδ T cell clones have the ability to produce a variety of biologically active cytokines and cytotoxic function. Cron et al. (1989) found that the activated TCR γδ cells which produce significant levels of IL2, interleukin-3 (IL3), interferon-γ (IFN-γ), and tumour necrosis factor (TNF) when stimulated with immobilised anti CD3-ε MAb in vitro and lysed syngeneic Fe receptor (FcR)-bearing targets with the presence of anti CD3-ε MAb or lysed the natural killer (NK)-sensitive line without anti CD3-ε MAb.

Patel et al. (1989) obtained CD3⁺CD4⁻CD8⁻ TCR γδ cells from healthy adult peripheral blood by depleting CD4⁺ and CD8⁺ T cells. Twelve CD3⁺CD4⁻CD8⁻ T cell clones were identified and stimulated with immobilised MAb to CD3. All of these clones secreted IL-2, IFN-γ, and lymphotixin/TNF-like factors and also found to have an unrestricted MHC cytotoxic function. In Spits and co-worker's study (1990), the TCR γδ cells were found to secrete IL-2, INF-γ and granulocyte/macrophage colony-stimulating factor (GM-CSF) and could lyse target cells. The lysis of target cells by TCR γδ cells was also demonstrated by Borst and co-workers (1987). The cloned CD3⁺CD4⁺ CD8⁺WT31⁺ TCR γδ cells lysed a variety of tumour cells without MHC-restriction.

In Patel and co-worker’s study (1989), similar helper T cell function was found in the TCR γδ cells. Nine of the twelve TCR γδ cell clones supported the B cell differentiation after stimulation with anti-CD3, and this function had a positive
correlation with the amount of IFN-γ secreted. It has also been shown that a TCR γδ cell line can induce Ig secretion in B cell lines (Sperling et al., 1989).

Another potential function was suggested by Janeway et al. (1988), which was the immunological surveillance of epithelia to detect and destroy infected or transformed epithelial cells.

The TCR γδ cells have some function in infectious disease. The TCR γδ cells have been shown to accumulate in the draining lymph nodes of mice immunised with Mycobacterium tuberculosis (MT) (Janis et al., 1989). The role of TCR γδ cells in the primary response to MT in athymic mice B10.A was studied by immunising these mice with MT and collecting the draining cells into axially and popliteal lymph nodes. The CD3⁺CD4⁻CD8⁻Vβ8⁻ T cells were found to have increased substantially compared to CD3⁺CD4⁺ or CD8⁺ T cells. The total number of CD3⁺CD4⁺CD8⁻Vβ8⁻ T cells increased 20-~25-fold after priming. These T cells expressed the IL-2 receptor, responded to solubilised MT antigen and produced IL-2. Similar activation was obtained from the TCR γδ cells of pneumonia mice infected with influenza A virus (Carding et al., 1990; Eichelberger et al., 1991).

With the exception of these related functions with TCR αβ cells, the TCR γδ cells also have some strikingly different characteristics, in particular the specificity of recognition of TCR γδ cells (Chien et al., 1996). That the TCR γδ cells respond to antigens without requiring MHC molecules and could not be inhibited by antibodies against the classical MHC proteins has been demonstrated by several investigations (Brenner, et al., 1987; Borst et al., 1987; Janis et al., 1989; Patel et al., 1989; Kabelitz et al., 1990a, Schild et al., 1994).

The TCR γδ bearing cells possess non-MHC-restricted cytotoxic activity (Brenner et al., 1987). T cell lines generated from adult peripheral blood that express
TCR γδ have spontaneous effector cytotoxic capability and this function was not inhibited by a variety of MAbs directed against monomorphic MHC class I (W6/32, anti-HLA-A, B, C, 4E and 131) or class II (LB3.1 and anti-Leu10) determinants. This suggests that these TCR γδ cells are non-MHC class I or class II restricted CTL.

In order to determine the activation of TCR γδ cells in vivo in direct response to MT, purified TCR γδ cells were incubated in vitro with a MAb to a class II MHC protein (Janis et al., 1989). The results showed that the TCR γδ cells activation was not at all blocked and indicated that MT-reactive TCR γδ cells do not require MHC class II molecules during antigen recognition.

In Kebelitz and co-worker’s studies (1990a), frequency and specificity of TCR γδ cells activated by allogeneic and autologous stimulator cells were investigated. They cultured TCR γδ cells with allogeneic and autologous peripheral blood stimulator cells under limiting dilution culture conditions. The majority of TCR γδ cells proliferated at the same level to allogeneic and autologous peripheral blood stimulator cells. This indicates that the TCR γδ cells do not discriminate allogeneic target cells from the autologous and also suggests that these T cells do not recognise classical allelic MHC class I or class II antigens.

Schild et al. (1994) used two TCR γδ cell clones, LBK5 (specific for the class II, IEb) and G8 (specific for the non-classical MHC class I, thymus leukaemia (TL)10b) to investigate the recognition properties of these clones. The results showed that these clones had been activated without MHC class I and II to process the antigens.

In contrast, some investigations showed that the TCR γδ cells do recognise antigens in conjunction with the requirement of MHC class I or class II molecules. These were demonstrated by several experiments in mice (Matis et al., 1989; Rellahan et al., 1991) and in humans (Ciccone et al., 1989; Jitsukawa et al., 1988; Bosnes et al., 1990;
Spits et al., 1990; Vandekerckhove et al., 1990).

In Matis et al. (1989) and Rellahan et al. (1991)'s work, they both developed an alloreactive murine TCR γδ T cell line, LBK5 (I-E^k.h.s) or LKD1 (I-A^d). Inhibition of lysis of LKD1 by the I-A^d-specific MAb MK.D6 and inhibition of lysis of LBK5 by the I-E-specific MAb were observed. These results suggested that some TCR γδ might recognise MHC antigens.

In Spits's group (1990), two TCR γδ cell clones were generated from donor peripheral lymphocytes. These two clones (ES-204 and ES443) were specific for HLA-A2 and the cytolysis activity of both clones was inhibited by anti-HLA-A2 MAb (CR-11-351).

Vandekerckhove et al. (1990) studied the CTL repertoire of a long term surviving patient who had an HLA mismatch kidney allograft. Two donor-specific TCR γδ CTL clones were obtained from this patient that recognised both donor's class I and class II antigens. This reaction could be inhibited by specific antibodies.

Dual recognition of a human TCR γδ cell clone was also found using tetanus toxin and mycobacteria as the antigens. This clone could recognise the tetanus toxin peptide presented by HLA-DRw53 and the mycobacteria without MHC-restriction (Holoshitz et al., 1992).

Moreover, several studies have shown that the TCR γδ cells may recognise the antigens presented by MHC-like proteins such as TL region-encoded proteins (Houlden et al., 1989; Tonegawa et al., 1989; Vidovic et al., 1989; Ito et al., 1990), heat shock protein (Hsp) and MT (Holoshitz et al., 1989; O'Brien et al., 1989; Kabelitz et al., 1990b), and may be stimulated by nonpeptidic mycobacterial ligands or nopeptide antigens (Constant et al., 1994, Tanaka et al., 1995).

Houlden and his co-workers (1989) isolated a clone TCR γδG8 CTL, which
recognised a novel TL-encoded protein in mouse. Tonegawa et al. (1989) also developed a number of TCR γδ T cell hybridomas from fetal and adult mouse thymocytes and screened them for specificity using a growth-inhibition assay. They identified one TCR γδ hybridoma (KN6), whose growth was inhibited by syngeneic (C57BL/6) but not allogeneic mice spleen cells (BALA/c, CBA/J, and AKR/J). Further analyses show that the KN6 ligand was located in or distal to the TL region and sequencing results showed that the gene for the ligand is a novel MHC class I gene (27h) in strain C57BL/6 and belong to TL region (Ito et al., 1990). This indicates that TCR γδ cells could recognise a self-histocompatibility complex TL region product.

Vidovic and co-workers (1989) demonstrated that a TCR γδ clone DGT3 (CD4−CD8−) that recognised synthetic copolymer poly (Glu50Tyr50) in conjunction with a Qal cell surface product. Their results suggest that the TCR γδ cells recognition of foreign antigen was associated with self-Qa product.

Holoshitz et al. (1989) have isolated TCR γδ clones from the synovial fluid of a rheumatoid arthritis patient. These clones respond to MT antigen specifically. Further analysis showed that one of these clones responded to a purified preparation of the Hsp65 from MT. In contrast, O'Brien and co-workers studies (1989) showed that the TCR γδ cells spontaneously produced IL-2 and were reactive to purified protein derivation from MT, but gave a weak response to Hsp65. Kabelitz et al. (1990b) showed that the TCR γδ cells specially recognise killed MT, but not Hsp65 and a purified protein derivative. This indicates that the antigenic components of MT are highly stimulatory for human TCR γδ cells rather than HSP65.

Nonpeptidic antigens can stimulate TCR γδ cells (Constant et al., 1994). In this study, four ligands from MT strain H37Rv were isolated. These ligands stimulate human TCR γδ cells to proliferate. The structural analysis showed that one of these ligands is a
5' triphosphorylated thymidine-containing compound and indicates that some TCR γδ cells recognise non-peptide ligands. Similar results were obtained by Tanaka et al. (1995). They isolated natural antigens from *Mycobacterium*, which were recognised by TCR γδ cells (Vγ2Vδ2). These compounds are isopentenyl pyrophosphate and related prenyl pyrophosphate derivatives. This provides evidence that human TCR γδ cells can recognise naturally occurring small non-peptidic antigens.

The difference in the recognition of antigen-MHC complexes by TCR γδ and TCR αβ may be a function of CDR3 length (Rock et al., 1994). The length of the CDR3 in Ig light chains tend to be short and of a conserved length, whilst those of Ig heavy chain are longer with a wide range of lengths. This may reflect the fact that Igs recognise a variety of different antigenic surfaces, from small molecules to large pathogens. For the TCR αβ chains, the CDR3 length distributions are about equal in length and the conserved. This may provide the requirement for α and β chains of the TCR to contact both the MHC and bound peptide. But in the TCR γδ, the γ chain CDR3 loops are short with a relatively fixed length, and the δ chain CDR3 loops are longer with flexible length distribution. Therefore, the CDR3 lengths in the γδ TCR are more similar to Ig light and heavy chains than to TCR αβ. This indicates that the TCR γδ cells have an Ig-like capacity to recognise different antigens directly.

1.4.4. Current status of TCR genetics research in lower vertebrates

It has been generally accepted that the capacity to reject allografts is widely spread throughout the Animal Kingdom (Cooper et al., 1992, Manning and Nakanishi, 1996), but the acquired immune system involves the full range of T and B-cell with recognition of non-self by cellular receptors (TCR and sIg) to eliminate the non-self antigens of the organism. Although the MHC and TCR genes have only been isolated
from jawed fish, some immune-like phenomena has been reported in invertebrates (Sun et al., 1990) and also lymphocyte-like cells have been found in earthworms (Beck and Habicht, 1996). So the phylogenetic origins of T-cell immunity and the TCR genes, have generated much interest and the nature of the development and evolution of MHC and TCR from lower vertebrate to higher vertebrates has become an important area of investigation (Marchalonis and Schluter, 1990). To date, TCR α, β, γ and δ genes have been isolated from all vertebrates except jawless fish. The isolated non-mammalian TCR genes are shown in Table 1.2.

1.4.4.1. TCR in Fish species

Fish span an enormous range on the evolutionary tree. There are three major groups of extant fish (Pough et al., 1996). They are the jawless fish (the Agnatha), such as the lampreys and hagfish; the cartilaginous fish (the Elasmobranchs), including the sharks, skates and rays; the bony fish (the Teleosts) including the salmon, trout, cyprinids some lungfish and one lobe-finned fish.

Because such large groups display a broad adaptive radiation, the immune response is likely to show large differences (McCumber et al., 1982; Kaastrup et al., 1988; Miller et al., 1985,1986). To understand the evolution of the MHC protein-based immune recognition system, it is important to isolate and characterise contemporary representatives of gene elements encoding for MHC and TCR molecules in more primitive species. However, until 1994, TCR-like genes had not been reported in fish. The first report by Rast and Litman (1994) involved a polymerase chain reaction (PCR) approach, which was used to amplify TCR-like products from the genomic DNA of the horned shark (Heterodontus francisci). Consequently, one of these products was used as
Table 1.2. Current status of TCR genes in non-mammals

<table>
<thead>
<tr>
<th>Species</th>
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<th>References</th>
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<tr>
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<tr>
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<td>Fellah et al., 1993</td>
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<tr>
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<td>Fellah et al., 1994</td>
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<td>Hawke et al., 1996</td>
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<tr>
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<td>TCRA,B,G and D</td>
<td>Rast et al., 1997</td>
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a probe to screen a spleen cDNA library. Several clones were obtained. A full-length cDNA clone HF6, was found to be highly related at the nucleotide sequence and predicted peptide levels to higher vertebrate TCR \( \beta \)-chain genes. A total of 31 positions were fully conserved, while an additional 22 residues were shared by the shark sequence and TCR \( \beta \)-chain genes from at least three of five other species (axolotl, chicken, mouse, rabbit, and human). A total of 60 residues in this clone are shared with at least two of these five sequences. A functionally important lysine residue is present at position 269, which interacts with CD3 in mammals (Alcover et al., 1990). Southern blotting analysis of Clamped Homogeneous Electrical Field (CHEF)-separated shark genomic DNA showed that TCRBC and TCRBV regions are linked together. A genomic DNA library was also screened with the probes (clone HF6 C and V regions). Subsequently, the positive clones were hybridised with a TCRJ probe. The results showed that 191 TCRV\(^{+}\) and 103 TCRC\(^{+}\) clones were identified without cross-reaction between probes V and C. Twelve TCRV\(^{+}\) clones were mapped with restriction endonucleases and each was unique. Eight among them had two TCRV segments and eleven were positive with probe TCRJ. Also 12 TCRC clones were analysed in the same way. Ten contained a single unique TCRC sequence and five hybridised with the probe TCRJ. These results indicate that a large number of TCRC segments are present in the shark TCR germline and linked with V and J segments and suggest that the organisation of the shark TCR genes may be similar to that of the Ig in this species, and are possibly present at multiple loci on different chromosomes (Litman et al., 1993a,b). Each cluster contains possibly two V, one D, at least four J and one C segment. Typical recombination signal sequences 3'-V CACAGTG-23 bp spacer-TCAAAAACA; 5'-J GATTTTGTG-12 bp spacer-CAGTGTG are present in the representative genomic V and J segments. This provides strong evidence that fish TCR genes undergo somatic rearrangement during T cell
development. The presence of TCR-like genes at such an early stage of vertebrate evolution also provides direct evidence that an integral component of T-cell recognition immunity system is present in these phylogenetically primitive vertebrates.

Subsequently, Hawke *et al.* (1996) reported that the TCR genes of the horned shark are more related to human TCRB genes. They exhibited considerable V family multiplicity and might utilise combinatorial mechanisms in the generation of immunological diversity. They compared 55 spleen cDNA clones encoding TCRB genes from the horned shark; 7 diverse TCRBV families are defined according to overall sequence identity. Also, at least 18 putative TCRBJ sequence types are recognised and a consensus diversity element was identified that is related to mammalian TCRBD gene segments. The 7 TCRBV families were named as TCRBV1-7, one of them- sequence Hf89 (shark TCRBV4 family) has the highest sequence identity (54% at nucleotide and 45% at amino acid level) with human TCRVB 20. Rast and co-workers (1995) also identified two TCRD-like genes in horned shark and TCRA gene in pufferfish. These results suggest that the distance possibility exists that the α/δ divergence took place after the divergence of the cartilaginous fishes and leading to the mammals.

At the same time, Partula *et al.* (1994,1995,1996) identified TCRA and TCRB genes in a bony fish- the rainbow trout (*Oncorhynchus mykiss*). Eight TCRAV segments were identified and classified into six different families. Thirty-two different TCRAJ gene segments were identified. They found CDR3 was well conserved between trout and mammals, suggesting that this region has been under continuous selective pressure during jawed vertebrate evolution. Three TCRBV segments were obtained that belonged to different TCRBV families named respectively as TCRBV1, TCRBV2, and TCRBV3. A limited number of amino acid residues are conserved among the three TCRBV segments, including Gln-6, Pro-8, Cys-23, Try-34, and Cys-92. Residues Asp-86 and
Ser-87 are conserved in human and mouse subgroup I TCRBV and these sequences are also found in the trout TCRBV1, where Asp-86 forms a salt bridge with Lys-64 residue (Schiffer et al., 1986). TCRBV also has significant similarity with the human TCRBV 20 family. The 10 trout TCRBJ segments were completely conserved among all the TCRBJ sequences as in other vertebrates. The invariant mammalian TCRBJ residues were well conserved. 66 TCRBV-TCRBJ junction sequences were also compared, which led to the identification of a TCRBD-like sequence (GGACAGGG). This TCRBD-like sequence is similar to the chicken and mammalian TCRBD, but is shorter than that in the latter species. Nine cDNA clones were obtained from rainbow trout. The trout TCRBC transmembrane region is the same size as those in other species and several important residues are well conserved including the Lys271, which is supposed to interact with the CD3δ element of the CD3 complex in mammals. Southern blotting analyses with a TCRBC probe indicate that the trout could have at least three TCRBC isotypes. The trout TCRBC most closely resembles the chicken and the axolotl TCRBC sequences when comparing them with known sequences, but is shorter comparatively than mammalian TCRBCs. Despite their considerable evolutionary distance (about 400 million years), the TCRB chains of teleost and tetrapods remain structurally well conserved, thus emphasising their prime importance in immunity. The cDNA analysis of the rainbow trout TCRB suggests that several TCRBV segments, and at least one TCRBD, ten TCRBJ and one TCRBC region rearrange and these may be clustered at the genomic level. The three TCRBC segments hybridising with a TCRBC probes suggested that there might be more than one genomic TCRB cluster similar to the arrangement found in the shark (Rast and Litman, 1994).

Hordivik et al., (1996) have reported the cloning of TCRB from Altantic salmon cDNAs. They used reverse transcriptase PCR (RT-PCR), isolated and characterised
twenty-five distinct cDNA fragments. Key residues such as two conserved cysteines which make the intrachain disulphide bridge, and one conserved lysine in the transmembrane part which is an important residue interacting with the CD3 complex (Manolios et al., 1994) were also found in this fish species. An octamer sequence (GGACAGGGG) was found, which is very similar to amphibian, avian, and mammalian TCRBD sequence in the VDJ junction from salmon as well as in trout (Partula et al., 1995). Generally, the hinge region cysteine residue is involved in covalent bonding between the α and β chains in mammalian TCRs and is present in lower vertebrates such as horned shark (Rast and Litman, 1994) but appears to be absent in both salmon and trout TCR β chains. They suggest that the disulphide interchain bonding between α and β chains are absent in the hinge region in this species, which may be similar to the situation with the Ig heavy chain membrane form (Hordvik et al., 1992) and that they may have alternate patterns of TCRB/TCRA interchain bonding.

Recently, Rast et al. (1997) have identified and characterised TCR α, β, γ and δ genes in clearnose skate (R. eglanteria). These cDNA sequences show extensive variable region diversity; also the putative diversity segments were identified with varying degrees of junctional diversity. Each of TCRA, TCRB, TCRG and TCRD has several families.

All of the above results indicate that both TCR αβ and TCR γδ genes emerged at an early level of vertebrate phylogeny and that the three major known classes of rearranging antigen receptors are present in the common ancestor of the present-day jawed vertebrates.

An attempt to amplify the TCR genes from jawless fish has never been successful. This may indicate that the TCR emerged after the jawless fish stage (Rast et al., 1995).
1.4.4.2. TCR in Amphibian species

The most striking feature of amphibians is the metamorphosis between the juvenile and adult stages. This metamorphosis can involve virtually complete remodelling of the anatomy accompanying changes in size, physiology and behaviour to facilitate life in a new environment (Fox, 1981). The immune system also undergoes enormous changes and has its origins in the two different lifestyles of the amphibians (Du Pasquier, 1989; Turpen and Smith, 1989). Now, several reports (Fellah et al., 1993, 1994; Rast et al., 1995) have been published identifying TCR genes in amphibian.

Fellah et al., (1993) reported the isolation of TCR genes from the cDNA of an amphibian species, the Mexican axolotl (Ambystoma mexicanum). They identified several sequences highly homologous to both the avian and mammalian TCR β chain genes. For example, axolotl TCRBV22 segment is similar to the mouse TCRBV14 family and corresponding human TCRBV18 family. The axolotl TCRBJ segments from two different clones are also conserved. The extracellular regions of the two axolotl β chain C domain isotypes TCRBC1 and TCRBC2 show a high degree of homology. Among the first 100 codes at the 5' end, 88 amino acids are completely conserved and 2 amino acids present synonymous substitutions, but 3'UT and transmembrane sequences of TCRBC1 and TCRBC2 are highly divergent. Thus suggesting that this structure has been preserved from an early tetrapod stage of evolution. In addition the lysine residue at positions 271 that are thought to interact with CD3 is present. The axolotl TCRBV is very similar to the mammalian TCRBV and most of the characteristic residues including the invariant amino acid Gln-6, Pro-8, Trp-34, Tyr-35 are conserved. The results show that the axolotl TCRBC is most closely related to the chicken TCRBC and various mammals TCRBC1/TCRBC2 chains, but shorter than that of mammalian species.
Extensive nucleotide sequence homology was present between TCRBC1 and TCRBC2 genes of the axolotl. The TCRBC sequences are similar to chicken TCRBC.

Fellah and co-worker (1994) also reported the isolation of thirty-six rearranged TCRBV genes and eleven different TCRBV segments were identified. These eleven TCRBVs could be classified into 9 families on the basis of a minimum of 75% nucleotide identity. At the amino acid level, all of the 36 sequences have mammalian and avian TCRBV features, including the conserved residues at position Cys23, Trp34, Arg69, Try90, and Cys92. The database search found that some of the TCRBV families consistently matched the human TCRBV13 family and its homologue mouse TCRBV8. Most of the axolotl TCRBV families have fewer than 35% identical nucleotides and the less related families have no more that 23.2% identity. Therefore, a genetic distance exists between the axolotl TCRBV families than between the different TCRBV families of any mammalian species. This suggests that the TCRBV gene repertoire in the axolotl is probably larger than the current estimation.

Rast et al. (1995) reported the identification and characterisation of TCRA-like gene in the African clawed frog (*Xenopus laevis*). One sequence was identified that exhibits similarity to the rat Ig light chain and TCRAV. Therefore, a similar gene structure between Ig light chain and TCRA and the two sets of TCRs may be present in amphibian.

1.4.4.3. TCR in avian species

The chicken TCR was the first to be identified from non-mammalian species (Tjoelker et al., 1990). Sequence analysis of cDNAs derived from the chicken TCRB locus suggests that at least two TCRBV families, one TCRBD region, three TCRBJ elements and a single TCRBC region are present. The TCRB gene segments appear to
possess conserved cysteine residues at positions 23 and 92 that are involved in the formation of intrachain disulphide bond (Saito et al., 1984a). Conserved tyrosine, serine, aspartic acid and phenylalanine residues are also present at position 65, 86, 87 and 90. Some important residues including the cysteines at positions, 147 and 212, that are involved in the formation of an intrachain disulphide bond, are present in the TCRBC region. A cysteine at position 247, which may form a disulphide bond with a corresponding cysteine on the TCR α chain has been conserved in the chicken sequence. These suggest that the important structural features of the TCRB polypeptide are conserved between avian and mammalian species and also suggest that these TCR genes in chicken may have a similar structure to mammalian TCR genes.

McCormack et al. (1991) analysed chicken TCRB diversity. They found that most rearranged chicken TCRB genes have at least one glycine in the TCRD segment. This confirmed the hypothesis that the germ-line TCRBD nucleotide sequences of chicken, mice, rabbits, and humans have been highly conserved and all three possible reading frames encode a glycine residue. As we know, glycine in this region is the striking feature of the CDR3 and is important in recognising the antigen/MHC complex. Almost all-functional TCRBVDJ junctions encode a glycine. Therefore TCRBD-encoded glycine in the chicken TCR β chain may be important for the antigen-binding CDR3 loop in the structure and function of TCR αβ.

The genes encoding the TCR α chain in the chicken have been identified by Göbel et al. (1994). Southern blotting analysis using TCRAV and TCRAC as probes to hybridise chicken genomic DNA showed that the chicken genome contains a large TCRAV family and many TCRAJ segments, but appears to be composed of a single TCRAC gene in the chicken TCRA locus.

In chicken, a large subpopulation of avian T cells expressing a homologue of the
mammalian TCR γδ were reported by Sowder et al. (1988). They used an antibody against TCR γδ from a hybridoma that identified a CD3-associated heterodimer that resembles the mammalian TCR γδ. They found that the antibody reacted by indirect immunofluorescence with a subpopulation of Ig-negative lymphocytes. In tissues from adult chicken, this antibody reacted with ~10% thymocytes, ~15% blood mononuclear cells, ~25% spleen cells, <1% with bursal cells and bone marrow. Lahti et al. (1988) also found that ~70% of TCR γδ+ cells in spleen express CD8. They suggested that CD8+/TCR γδ+ cells may possibly recognise MHC class I molecules. The avian TCR γδ cells were located in the splenic sinusoids and the intestinal epithelium (Bucy et al., 1988, 1991). Two clones (Gd186 and Gd187) from a chicken genomic DNA were isolated and identified as the TCRG genes of the chicken (Rast et al., 1995). The chicken spleen cDNA library was screened using these two clones as probes. Four positively hybridised clones were purified and sequenced. One of them named as Gd186cDNA showed a higher alignment score with TCRG of various species including human and mouse. The transmembrane regions of the chicken Gd186cDNA, mouse, and human genes were identical at 9 out of 25 positions, including a conserved Lys312 which is thought to interact with CD3y in mammals (Alcover et al., 1990).

In the early studies in chicken, another TCR was identified and designated TCR3 (Lahti et al., 1988; Chen et al., 1989). These TCR3 cells are CD3+/TCR αβ+/TCR γδ+. TCR3 accounted for ~5% of peripheral blood lymphocytes in one-month-old chickens, and ~16% in peripheral blood lymphocytes in six-month-old chickens; ~88% TCR3 cells express CD4, and ~12% TCR3 express CD8. At least one of the two chains in the TCR3 complex is physically distinct from those of the TCR αβ and TCR γδ isotypes. Later, Char and co-workers (1990) produced a MAb that identified this TCR3 molecule. The results showed that TCR3 cells are CD8+ and CD4+ T cells and the pattern of
histological localisation of TCR3 cells in the peripheral blood lymphocytes is similar to the TCR αβ subset. They concluded that TCR αβ and TCR3 in the chicken might present αβ subfamilies of TCR that are sequentially expressed on developmentally discrete sublines of T cells. Lahti and co-worker (1991) also suggested that chickens have two genomic TCRBV gene families and that expression of the TCR αβ and TCR3 epitopes correlates with the expression of these two V gene families. Thus, the TCR αβ and TCR3 cells appear to be subclasses of TCR αβ cells with different specificities and this data suggests that the TCR αβ cells can be divided into two distinct and evolutionarily conserved lineages based on TCRBV gene segment usage. Cooper et al. (1991) concluded that TCR αβ and TCR3 are two sublineages of avian T cells that have been defined as TCR αβ cells, each of which uses a different TCRBV gene family. They propose that these two separate sublineages of TCR αβ cells may recognise the same MHC class II alloantigens and initiate a graft-versus-host reaction.

1.4.4.4. Comparison with mammalian species

1.4.4.4.1. TCR genes structure

With the exception of the jawless fish, the TCRA, TCRB, TCRG and TCRD genes have been isolated and characterised from a number of lower vertebrates (Tjoelker et al., 1990; McCormack et al., 1991; Fellah et al., 1993, 1994; Göbel et al., 1994; Rast and Litman, 1994; Rast et al., 1995, 1997; Partula et al., 1994, 1995, 1996). Although the function and gene arrangement in lower vertebrates need to be fully characterised, the existing data suggests that: the TCR genes in lower vertebrates exist in separate V, D, J segments, and may undergo somatic rearrangements during T cell development in a similar manner to mammalian TCRs and most of the TCR sequences isolated from fish contain recombining signal sequences. In the human, the V gene is composed of two
exons and one intron. The first exon (approximately 250 bp) encodes a hydrophobic leader sequence, and the second exon (approximately 250-350 bp) codes for the remainder of the V region. There are two TCRBC genes, each having four exons. The first and second exons encode the majority of the extracellular constant domain. The third is for a hydrophobic putative transmembrane region, which contains a highly conserved lysine residue about midway along the sequence. The fourth codes for the cytoplasmic domain as well as 3'UT sequence. Because the data is limited in lower vertebrates, the gene organisation in these species has not yet been identified. But the limited data suggests that the genes are organised in multiclusters in the germline of elasmobranch fish. This is similar to the organisation of the Ig heavy chain genes, where the organisation of shark and skate Ig genes is also as a multicluster i.e., repeats of V-D1-D2-J-C (Warr, 1995). But the organisation of the shark TCRB genes may be slightly different to the shark Ig gene. Each cluster may contain two V, one D, at least four J and one C segment. Combinatorial diversity in the gene rearrangements may also be present in the cartilaginous fish (Rast and Litman, 1994, Hawke et al., 1996). Although the TCRA, TCRB, TCRG and TCRD genes have all been identified in the lower vertebrates, the chromosomal location is still unknown. Chromosomal location of TCR genes within the fish group is likely to be complex, as they are primitive vertebrates, and each fish species has very different number of chromosomes (Klinkhardt et al., 1995).

1.4.4.2. Protein structure of TCRs

In mammals, the TCR protein contains a leader, a V region, a J region, a C region, a connecting peptide, a transmembrane region and a cytoplasmic region. In mouse, the V domain of the α and β chains is about 110 amino acids long. Positively charged amino acids may play a role in the interaction of the α/β heterodimer with other
membrane protein containing negatively charged amino acids in their transmembrane protein. The positively-charged lysine side chain may be important in interactions with the CD3 polypeptides (John et al., 1989; Alcover et al., 1990; Manolios et al., 1990, 1994), four of which are known to have a conserved negative charge (Asp or Glu) in their putative transmembrane regions (Van den Elsen et al., 1984; Gold et al., 1986; Weissman et al., 1988). Many conserved amino acids in Vα and Vβ chains are important for protein structure and receptor function. Two cysteines separated by 63-69 residues provide intrachain disulphide linkages. Recent data in lower vertebrates shows that functional residues are conserved including lysine in the transmembrane region and residues at positions Cys23, Try34, Arg69, Tyr90 and Cys92. The conserved residues in the TCRs of lower vertebrates may be involved in maintaining the same three-dimensional structures as these are found in mammals suggesting that the recognition of antigen by the T cells also utilise similar mechanisms in lower vertebrates.

1.4.4.4.3. TCR families in different species

Yoshikai et al. (1986) found that the subfamilies TCRVA1, TCRVA2 and TCRVA3 of mammals could be classified into four subgroups by amino acid residues at position 61 and 62 according to Schiffer and co-worker's suggestion (1992). Sequences with Gly 61, Phe 62 were classified as group I, Arg 61, Phe 62, subgroup II, and Arg 61, Leu 62 subgroup III as in TCRVA1, TCRVA2 and TCRVA3 respectively. Other sequences with different amino acid pairs at position 61 and 62 and incomplete sequences are collected into a miscellaneous subgroup. Schiffer et al., (1986) have demonstrated that the TCRB chains of the TCR can be divided into three subgroups according to conserved residues at certain positions. These were distinguished by Subgroup I having an invariant Phe at position 65, and a salt bridge between a positively

77
charged residue at position 64, and Asp at position 86. Subgroup II has an invariant Tyr at position 65 and Gly at position 63 and cannot form a salt bridge. They are classified as Subgroup III, where there is an invariant Tyr at position 65, but can have a reverse salt bridge formed between Asp 64 and Arg 86. The γ and δ chains of the TCR have not demonstrated subgroups as yet. Following regulation, some trout TCRBV segments are classified into subgroup II VB sequences (Partula et al., 1995).

Traditionally, 75% identity at the nucleotide level has been used to delineate subfamilies (Tyonaga and Mak, 1987; Wilson et al., 1988). The human TCRAV gene segments can be grouped into 32 subfamilies (Arden et al., 1995a) (Table 1.3). Among these, 26 subfamilies contain only a single member. The TCRBV subfamily has 34 members and some of these families consist of multi-membered groups (Table 1.4). TCRBV5, 6, 8 and 13 are the largest. Eighteen TCRGV gene segments can be divided into six distinct subfamilies (Table 1.5). There are also 5 different TCRDV subfamilies in human (Table 1.6), most of which are single-member subfamilies. The mouse TCR families have also been identified (Arden et al., 1995b) (Table 1.3-Table1.6). To date, many subfamilies of TCR have been identified in non-mammalian TCR. The chicken TCRBV family at least has 2 TCRBV, 3 TCRBJ and a single TCRBD and TCRBC subfamilies (Tjoelker et al., 1990). The amphibian TCRBV also can be classified into 9 subfamilies (Fellah et al., 1994). There are at least 7 TCRBV subfamilies and 18 putative TCRBJ sequences, a TCRBD in the horned shark (Hawke et al., 1996). Rast et al., (1997) have investigated TCR genes from another cartilaginous fish, the skate. A number of families are present in each of the TCRA, TCRB, TCRG and TCRD genes in this species. The results are shown in Table 1.7. Partula et al., (1994,1995,1996) showed that the trout TCRA genes have 6 TCRAV, 32 TCRAJ and 1 TCRAC, and the genes for the TCRB chains have 3 TCRBV, 10 TCRBJ and 1 TCRBD and 3 TCRBC,
Table 1.3. The TCRAV gene families in mammals

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The data are derived from Arden et al., 1995a,b.
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The data are derived from Arden et al., 1995a,b.
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The data are derived from Arden et al., 1995a,b.
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The data are derived from Rast et al., 1997.
respectively. The presence of a large number of TCR families in lower vertebrates suggests that this diversity is required for the large amounts of pathogens in their environment.

1.5. MHC genetics in non-mammalian and compared with mammalian species

The immune system is able to deal with a wide variety of immunological conditions via a complex series of interactions. It is now clear that many of the proteins in vertebrates that are involved in the immune response are coded for genes that are located in the MHC (Campbell and Trowsdale, 1993; Trowsdale, 1993, 1995). The MHC plays a fundamental role in controlling the immune system. These MHC genes can be divided into three groups: designated class I, class II and class III (Hood et al., 1983; Klein, 1986; Campbell and Trowsdale, 1993; Trowsdale, 1995). The molecules encoded by MHC class I and class II regions are mostly glycoproteins that are involved in the recognition, processing and presentation of self and foreign peptides to T cells (Bjorkman and Parham, 1990; Trowsdale, 1993). The MHC class I genes code for the classical HLA-A, B, and C and the non-classical HLA-E, F, G, and H in human and the H-2K, H-2D and H-2L antigens in the mouse. The class II region encodes the HLA-DR, DQ and DP molecules in human and I-A and I-E in the mouse. The class II also contains the genes encoding for the lower molecular polypeptides (LMP) and transporter associated with antigen processing (TAP) that are involved in antigen processing and presenting. The MHC class III region encompasses a range of different genes, tightly clustered, some of which may be involved in the immune system such as complement components C2, C4 and factor B (Bf) (Woods et al., 1982, 1984; Campbell and Porter, 1983; Belt et al., 1984; Carroll et al., 1984), and tumour necrosis factor (TNF) genes
Still there are some genes which may be related to immunity in this region, such as HLA-B-associated transcripts (BATs) (Spies et al., 1989a,b), Hsp70 1H and Hsp70 2 (Sargent et al., 1989).

1.5.1. History of MHC

What is the MHC? Klein (1986) gave his own definition: the MHC is a group of genes coding for molecules that provide the context for the recognition of foreign antigens by T cells. The MHC (H2) was discovered by Gorer in the mouse in 1936 and Briles et al. (1950) described the MHC in the chicken known as B complex. The human leukocyte antigen (HLA) system was discovered by Dausset in 1958. Doherty and Zinkernagel showed conclusively that the function of the MHC molecules is the discrimination of self from non-self by the immune system (Doherty and Zinkernagel, 1974; Zinkernagel and Doherty, 1974a,b).

The first mammalian MHC gene was isolated in the man by Ploegh et al. in 1980. A few years later, the first non-mammalian MHC gene was identified in the chicken by Bourlet et al. (1988). Most of the immunologic phenomena linked to the murine H-2 and human MHC complexes are also associated with the chicken B complex, e.g. resistance to neoplastic disease (Pazderka et al., 1975; Collins et al., 1977), control of T cell-B cell interactions and susceptibility to autoimmune diseases (Bacon and Rose, 1979).

In mouse and man, the MHC class I and II antigens have been extensively studied by serological, cellular and biochemical techniques, and the corresponding class I and II genes have been isolated and characterised in detail. The structure of the MHC shows much more variety in lower vertebrates than in mammals. DNA clones for MHC class I and II molecules have been isolated from birds (Bourlet et al., 1988; Guillemot et al., 1985).
amphibians (*Xenopus laevis*) (Flajnik *et al.*, 1984; Flajnik and Du Pasquier, 1990), reptile (Grossberger and Parham, 1992), teleost fish (carp: Hamashimoto *et al.*, 1990), and cartilaginous fish (shark: Hashimoto *et al.*, 1992; Kasahara *et al.*, 1992). Up to now, MHC class I and class II genes have been found in all vertebrates except jawless fish. Preliminary results suggest that the MHC might exist in hagfish and lamprey (Kandil *et al.*, 1996), suggesting that the MHC may appear as early as the tunicate stage or even earlier.

1.5.2. Gene organisation, distribution, structure, and function of MHC class I and class II molecules

1.5.2.1. Gene organisation

The MHC consists of 3 regions designated class I, II, and III (Flavell *et al.*, 1986; Klein, 1986; Trowsdale, 1993) (Fig. 1.14) and is located on human chromosome 6 and on mouse chromosome 17. In mouse, the class I region contains class I-encoding sequences with the exception of the H2K gene, which is located on the proximal flank of the class II region. The class I genes are interspersed with genes of a variety of functions and expression patterns. The class II region is more homogeneous and contains pairs of genes encoding the class II products. In addition, there are two sets of genes involved in antigen processing and presenting: the TAP and LMP proteins are responsible for producing the transporting peptides for class I molecules (Beck *et al.*, 1992; Monaco, 1992). There are also genes called DM (RING6 and RING7) in human and M in the mouse which are involved in loading peptides onto class II molecules (Cho *et al.*, 1991; Kelly *et al.*, 1991; Fling *et al.*, 1994; Morris *et al.*, 1994). The canonical MHC arrangement seems to have been broadly conserved throughout mammals. Among the mammalian population, any single species may contain many loci encoding each type of MHC proteins (class Iα, class IIα and class IIβ) and may have up to 100 alleles for each
Fig. 1.14. The organisation of the MHC class I, class II and class III regions of the human MHC. Genetic distances are approximately in kb (adapted from Janeway and Travers, 1996).
locus (Klein, 1986).

The typical mammalian MHC gene in the class I region codes for integral membrane proteins (Bjorkman and Parham, 1990). The first exon contains the 5'UT sequence in addition to the signal sequence. Then there are three separate exons for each of the external domains, α1, α2, and α3. The fifth exon codes for the transmembrane region, then the last two or three exons are used for the cytoplasmic domain and the 3'UT region.

The MHC II genes consist of six exons separated by five introns (Carson and Trowsdale, 1986). Exon 1 codes for the 5'UT region, the leader peptide of the immature protein, and a few N-terminal amino acid residues of the mature protein. Exons 2 and 3 encode most of the extracellular portion of the protein, the α1 or β1, α2 or β2 and domains, respectively. Exon 4 encodes the connecting peptide, the transmembrane region, and part of the cytoplasmic domain. Exon 5 codes for most of the cytoplasmic domain, and exon 6 for the rest of the cytoplasmic domain and the 3'UT region.

1.5. 2.2. The structure of the MHC molecules

The MHC encodes molecules, which are members of the Ig gene superfamily (Williams and Barclay, 1988). These proteins generally contain an extracellular region which in MHC class I molecule is approximately 280 amino acids and occupy position 1 to 283; a transmembrane peptide of 23 amino acids and located at position 284 to 306. The rest is a cytoplasmic peptide, which is 25-32 amino acids and located at position 307 to 338 or 348.

The MHC class I molecule is a heterodimer of a membrane-spanning α chain (43kD), non-covalently associated with β2-m (12kD), which does not span the membrane. The α chain folds into three domains, α1, α2, α3. The α3 domain and β2-m show similarities in amino-acid sequence to Ig C domains and have a similar folded
structure, while the α1 and α2 domains fold together into a single structure consisting of two segmented α helices lying on a sheet of eight anti-parallel β-strands. The folding of the α1 and α2 domains create a long cleft or groove, where peptide antigens bind to the MHC molecules (Fig. 1.15). They form complexes with peptides derived from intracellular degradation of self and non-self molecules. The α3 domain contains a site, which interacts with the CD8 molecule (Salter et al., 1990).

The MHC class II molecules consist of two transmembrane glycoprotein chains, α (34kD) and β (29kD). Each chain has two domains, and the two chains together form a compact four-domain structure similar to that of the class I molecule. The α1 and β1 domains form the peptide-binding cleft (Brown et al., 1988). The α2 and β2 domains of the MHC, like the α3 and β2-m domains of the MHC class I molecule, have amino acid sequence and structural similarities to Ig C domains; both of them span the membrane (Fig. 1.16). The MHC class IIβ2 domain also contains a site for binding CD4 molecules (Cammarota et al., 1992; König et al., 1992).

There are some important residues in the MHC proteins that are required to form a groove for antigen binding and recognition by the TCR. These residues include Tyr-7, Tyr-59, Arg-62, Glu-63 and Tyr or Arg-84 in the α1 domain and Thr-143, Lys-146, Trp-147, Try-159, Try-171 in the α2 domain in murine H-2Kb (Fig. 1.17). Crystallographic analysis of human and mouse MHC class I molecules have identified the conserved residues that interact with the main chain atoms of the peptide N- and C-termini of peptide (Bjorkman et al., 1987a,b; Madden et al., 1991; Saper et al., 1991; Fremont et al., 1992; Matsumura et al., 1992). In MHC class II molecules, five residues (β-Asn-82, α-Asn-62, α-Asn-69, β-Trp-61, α-Arg-76) have been suggested as interacting with the extended main chain atoms of the bound peptide and contribute to the peptide side chain affinity to the binding interaction (Fig. 1.18) (Brown et al., 1993).
Fig. 1.15. The structure of a MHC class I molecule. (a) A computer graphics representation of a human MHC class I molecule, HLA-A2 has been cleaved from the cell surface by the enzyme papain (one can not see the transmembrane region and the short stretch of peptide). (b) A ribbon diagram of the structure. The $\alpha$ chain folds into three domains, $\alpha_1$, $\alpha_2$, and $\alpha_3$. The $\alpha_3$ domain and $\beta_2$-m show similarities to Ig C domains and have a similar folded structure. (c and d) The $\alpha_1$ and $\alpha_2$ domains fold together into a single structure consisting of two segmented $\alpha$ helices lying on a sheet of eight antiparallel $\beta$-strands. The folding of the $\alpha_1$ and $\alpha_2$ domains creates a long cleft or groove, which is the site at which peptide antigens bind to the MHC molecules. The $\alpha_3$ domain and $\beta_2$-m connect with non-covalent bond. Only $\alpha$ chain span the cell membrane (adapted from Janeway and Travers, 1996).
Fig. 1.16. The MHC class II molecules structure. (a) A computer graphics representation of a human MHC class II molecule, HLA-DR1. (b, c and d) The MHC class II molecule is composed of two transmembrane glycoprotein chains $\alpha$ and $\beta$. Each chain contains two domains, $\alpha_1$ and $\alpha_2$ or $\beta_1$ and $\beta_2$. The ribbon diagram shows that the $\alpha_2$ and $\beta_2$ domains have structure similarities to Ig constant domains. The $\alpha_1$ and $\beta_1$ folds to form the peptide-binding cleft. Both chains span the membrane (adapted from Janeway and Travers, 1996).
Fig. 1.17A and B. The conserved residues that interact with the main chain atoms of the N- and C- termini of peptide are shown in red (highly conserved) and green (less conserved). At the N- and C-termini of the peptide backbone hydrogen bonds (dotted lines) are formed (adapted from Matsumura et al., 1992).
Fig. 1.18. The distribution of conserved residues in the class II peptide-binding site. Three conserved asparagine residues, 62 and 69 in the α1 helical region and 82 in the β1 helical region. Conserved tryptophan β 61 and arginine α 76 locate in the side chain. A special arrangement of hydrogen bonds from conserved class II HLA residues to the peptide main chain (yellow) (adapted from Stern et al., 1994).
The structural characteristics of these peptides, which bind to MHC molecules, have also been defined. It is now known that the majority of peptides that bind to MHC Class I are 8-10 residues long, with allotype-specific binding motifs containing up to three anchor positions. This corresponds to the presence of six pockets and the close-ended structure of the MHC class I peptide-binding groove. In contrast, associated with MHC class II are 10-34 residues in length and are commonly presented in nested sets with extensions or truncations at the N- or C-terminal ends. Binding motifs for MHC class II appear to contain up to four anchor positions, with more loosely defined amino acid preferences. The binding peptides differ in length between the class I and class II molecules and may account for small structural differences between the two binding sites and the positioning of key residues that mark hydrogen bonds to the bound peptides (Fig. 1.19).

1.5.2.3. The distribution and function of MHC class I and class II molecules

The class I molecules are mainly on somatic cells. The highest class I antigen expression can be detected on almost all lymphoid and haematopoietic cells. Medium or very low expression of these antigens occurs on muscle cells, myocardium, skeletal tissue, hepatocytes and on various endocrine glandular cells. Almost no class I antigen expression can be detected on nervous, sperm, trophoblast and pancreatic acinar cells (Daar et al., 1984). The MHC class I cells present endocellular pathogen antigens to CD8 cells. The cells expressing class II molecule are mainly involved in presenting exogenous antigen to CD4 T cells (Fig. 1.13). These cells include keratinocytes, dendritic monocytes, blood monocytes, Kupffer cells, astrocytes, undifferentiated myeloid cells, B cells, activated T cells and endothelial cells (David-Watine et al., 1990; Kao and Riley, 1993).
Fig. 1.19. MHC molecules bind peptides tightly within the cleft in crystal structures. (a) The peptide is bound in an elongated conformation with both ends tightly bound at either end of the cleft in the case of MHC class I molecules. For MHC class II molecules (b), the peptide is also bound in an elongated conformation but the ends of the peptide extends beyond the cleft. (c and d) TCR recognises the upper surface of MHC class I or class II molecules. The residues of the MHC molecule are shown in white and the peptide is in red (adapted from Janeway and Travers, 1996).
1.5.2.4. β2-m

β2-m was isolated from a patient's urine by Bergård and Bearn in 1968. The complete and partial protein sequences were performed by Peterson et al. and Smithies and Poulak in 1972, respectively. β2-m is a member of the Ig gene superfamily. It is 99 amino acids long, including two cysteine residues, which form the disulphide bond. The chromosome location is 2 in mouse and 5 in human, respectively. β2-m assembles with MHC class I molecules to stabilise the structure.

1.5.3. MHC in fish species

In order to understand the evolutionary origin and function of the MHC in lower vertebrates, fish have been used as an example of a primitive vertebrate. The MHC function in lower vertebrates has been demonstrated from studies of acute or chronic allograft rejection (Hildemann, 1970; Rijkers et al., 1977; Botham et al., 1980; Kaasstrup et al., 1989; reviewed by Manning and Nakanishi, 1996), MLR (Caspi and Avtalion, 1984; Miller et al., 1986; Kaasstrup et al., 1988) and in vitro antibody responses (Clem et al., 1985; Miller et al., 1985). All the above give strong support to the existence of MHC molecules in teleost fish. However, attempts to isolate MHC proteins have not always been successful. The first MHC genes to be isolated in fish were reported by Hashimoto et al. in 1990. Later, the genes encoding the α chain of the MHC class I and the β2-m, as well as the α and β chains of MHC class II molecules were isolated and sequenced from a number of different teleost and jawed fish (Hashimoto et al., 1992, Kasahara et al., 1992, 1993; reviewed by Dixon et al., 1995). The current status of the isolation of MHC genes in fish is summarised in Table 1.8. Most of these MHC genes were isolated using PCR with two primers which were designed from the two highly conserved amino acid sequence blocks (identified by Hashimoto et al. in
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This table is based on the Manning and Nakanishi's review (1996).
surrounding two cysteine residues in the second domain of MHC class II as well as the third domain of class I α chains of humans, mice, and chickens. The putative MHC sequences were used as probes to screen the cDNA library to obtain full-length sequences and to screen genomic DNA to characterise the gene organisation of the MHC genes. These data show that the MHC class I and II gene structures in both teleost and cartilaginous fish are very similar to that in mammals and also the predicted proteins contain evolutionarily conserved residues or regions.

The identification of a shark sequence resembling the MHC class I α 3 domain was performed by Hashimoto et al. (1992). A clone named DI was sequenced, which showed similarity to MHC class Iα3 of other species. Using DI as a probe to screen a shark genomic library a complete putative exon, λDS-1 was identified. Several conserved amino acids including Gln-46, Glu47, Ile-53, and Pro-55 present in this sequence. These conserved residues are considered to be important for the interaction of the T-cell co-receptor CD8 with the MHC class I α3 domain (Salter et al., 1990). The MHC class IIA (Kasahara et al., 1992), and class IIB genes (Bartl and Weissman, 1994b) from the nurse shark were also isolated and characterised. Two cDNA clones pSaα5-1 and pSaαB-1 were identified as MHC class IIA genes and also two genomic clones were identified as MHC class IIB. The two MHC class IIA genes encode three amino acid substitutions in the α1 domain; two of them were located at positions that are thought to interact with processed peptides. These two clones may encode putative allelic products. The deduced MHC class II α chains show highly structural similarity to their mammalian counterparts. The two class IIB gene clones 8 and 11 from the nurse shark encode proteins, which differ by 13 amino acids located in the putative antigen-binding cleft. The sequences of these genes and the predicted proteins are very similar to mammalian class IIB genes. This suggests that the shark MHC molecules might have a similar tertiary
structure to their mammalian counterparts. The polymorphism of the nurse shark MHC class IIA genes was reported by Kasahara et al. (1993). They isolated two MHC class IIA loci from 12 nurse sharks and named these loci as Gici-DAA and Gici-DBA. Gici-DAA has six and Gici-DBA has five alleles. These individual alleles usually differ by multiple nucleotides. Most of the nucleotide substitutions were located at the putative antigen-binding sites. Non-synonymous substitutions (amino acid changed) occurred more frequently than synonymous substitutions (no-amino acid changed). The polymorphism pattern is essentially similar between the nurse shark and mammalian MHC genes. As expected, the most polymorphic residues are clustered at the putative antigen-binding sites. These results suggest that the MHC class II genes in the nurse shark are functional. The polymorphic pattern in the nurse shark MHC class IIA gene has three notable features. First, the Gici-DAA loci are present in all individuals. Second, all sharks with Gici-DBA loci are heterozygous at the Gici-DBA locus. Finally, it seems these two MHC class IIA loci are not strongly linked, because the Gici-DBA alleles are not associated with specific Gici-DAA alleles.

The MHC genes in teleost fish have been studied in more detail compared to that of cartilaginous fish. These genes have been identified in quite a few species (see Table 1.8). β2-m has also been isolated and characterised in several teleost fish species (Dixon et al., 1993; Ono et al., 1993b). The MHC genes in teleost fish have the following major features: in the rainbow trout, a complete coding sequence MHC II β chain was obtained by Glamann (1995). A single B gene encodes a 247 amino acid long polypeptide, which is similar in size to mammalian, avian, amphibian, and other teleost β chains. The amino acid sequence encoded by this class II B gene has approximately 30% homology to mammalian, amphibian, and avian class II β chains. Most conserved residues are located in the Ig-like domain and in the transmembrane region. The most conserved motif in this
sequence is VGYT (residues 49-52), which is also present in other fish. According to the crystal structure of HLA-DR molecules (Brown et al., 1993), these residues contribute to the dimeric interface. A conserved segment RFNS (residues 39-42) that is involved in the cell to cell interaction with the T cell subset molecule CD4 is also present (Auffray and Novotny, 1986). The isolation of multiple β-chains encoding clones from individual fish gave rise to no more than two variants and restriction fragment length polymorphism (RFLP) analysis found only four bands using a β-chain probe. These suggest that there may be no more than one MHC class II β chain-encoding locus transcribed in the rainbow trout. The RFLP analysis in another study (Juul-Medsen et al., 1992) also showed that the rainbow trout contains very few MHC class IIB genes.

Rodrigues et al. (1995) studied the MHC class IIB transcripts in the tissue of the common carp. The results show that the highest level of the class IIB transcription is found in the thymus, followed by whole blood, head kidney, spleen and the second segment of the gut. No class II transcripts were detected in skeletal muscle.

Sültmann et al., (1993) and Ono et al. (1992) investigated zebrafish MHC class II α and β chain-encoding genes, respectively. The phylogenetic analysis in both studies suggests that the zebrafish class II genes appear to be derived from a different ancestor than the various class II gene families of other vertebrates, including mammalian and shark species MHC class II genes, because the zebrafish MHC class II polypeptides form a distinct branch which is not directly associated to any other human MHC class II gene families in the phylogenetic tree analysis.

Unlike the MHC genes in cartilaginous fish, the intron-exon structure of the teleost MHC gene, especially MHC class IIB genes is now becoming clear (Ono et al., 1992, 1993a,c; Sültmann et al., 1994). The teleost MHC gene sequences show conserved features at both the nucleotide and protein level when compared to higher
Vertebrates.

With respect to the MHC class IA genes the three putative exons for encoding MHC class I α1, α2, and α3 domains were identified in carp (Hashimoto et al., 1990). Between these exons, two introns of 179 bp and 439 bp were found. The intron-exon appears to correspond well to that found in the exon 2 to exon 4 region of higher vertebrate class I genes.

Hashimoto and co-workers (1990) identified two exons for the MHC class IIB gene. These two exons encode for the MHC class II β1 and β2 domains, respectively. There is a 189 bp intron between them. Ono and co-workers (1993a) identified four exons in carp. Further studies in gene structure of several fish species showed that MHC class IIB gene generally have 5-6 exons and 4-5 introns. Zebrafish class IIB genes (Ono et al., 1992; Sültmann et al., 1994) contain 5 exons. Exon 1 contains the 5’UT region and the region coding for the leader peptide. Exon 2 and 3 code for the β1 and β2 domains, respectively. Exon 4 encodes the connecting peptide, the transmembrane region, and part of the cytoplasmic tail. Exon 5 encodes the remainder of the cytoplasmic tail and the 3’UT region containing the polyadenylation signal AATAAA. The exon-intron organisation of the zebrafish class II genes is similar to that of the mammalian genes, but the introns are characteristically short, ranging in length from 74 to 362 bp. In cichlid fish, the MHC class IIB gene consist of six exons, exon 1 to exon 6, encoding the leader peptide (exon1), β1 domain (exon1 and exon 2), β2 domain (exon3 and 4), connecting peptide and transmembrane region (exon 5), cytoplasmic domain (exon 5 and 6), and the 3’UT region (exon 6). The exons are separated by relatively longer introns than that in the zebrafish, the length of the longest intron being 1.3 kb (intron 2). With the exception of intron 2, other introns are still shorter compared to the mammals. These introns are from 68 to 408 bp. The possible exon-intron relation of MHC gene in
teleost fish is shown in Fig. 1.20. The data were mainly obtained from carp and zebrafish MHC class I and class II genes. The MHC class IIA gene organisation was also identified by Sultmann and co-workers (1993). The gene consists of four exons interrupted by very short introns (<200 bp) and is similar to that of the mammalian class IIA genes.

1.5.4. MHC in amphibian and reptile species

MLR, skin graft rejection and some red cell antigens have been studied in the *Xenopus laevis* (Du Pasquier *et al.*, 1975). They used a sibship of 56 individuals whose parents were heterozygous. Therefore, 4 different haplotypes (a, b, c, and d) are present in the MLR reactive genetic region. The 56 individuals have been typed for the MLR region and classified into 4 groups (a/c, a/d, b/c, and b/d). Skin graft exchange was performed between different groups. It has been shown that graft rejection takes longer time in identical sibs than in unidentical sibs. When graft experiments were compared between different sibs, the rejection reaction took longer if the sibs shared a MLR haplotype compared to sibs with no sharing of MLR haplotypes. The results suggest that the homology of MHC is present in amphibians. The MHC has been well defined at both the biochemical and functional levels in *Xenopus laevis* (Flajnik and Du Pasquier, 1990). The MHC class II molecules were identified and characterised using a rabbit antibody against human class II β-chain serum and immunoprecipitates (Kaufman *et al.*, 1985a). MHC polymorphism has been detected by two-dimensional gel electrophoresis (Kaufman *et al.*, 1985b).

The MHC in *Xenopus* is called the XLA complex. The MHC class I and class II loci of the XLA complex have been identified (Flajnik *et al.*, 1993; Sato *et al.*, 1993). XLA antigens induce humoral responses, skin-graft rejection and MLR similar to
Fig. 1.20. The possible teleost MHC class IIB gene organisation (Ono et al., 1993d).
mammals. The number of XLA loci and their chromosomal arrangements are not known, but a similar organisation to the MHC of mammals has been suggested including three regions MHC I, MHC II and MHC III. Southern blotting analysis has shown that Bf is linked to the MHC class I and class II genes (Kato et al., 1994), and Hsp70 genes are also linked to the *Xenopus* MHC (Salter-cid et al., 1994). LMP-7 genes were isolated from *Xenopus* and are linked more closely to MHC class II than to class I or class III genes (Namikawa et al., 1995). Recently, Nonaka, et al. (1997) investigated the MHC organisation in *Xenopus* and the results suggest that a primordial organisation of MHC gene clusters exist in the *Xenopus* MHC loci. That is the single copy *Xenopus* class I gene is located between the class II and class III regions and is closely linked with the processing and transport genes; this is in contrast to mammalian MHC gene loci.

In Shum and co-workers’ (1993) experiments, *X.laevis* genomic DNA was digested with eight different restriction enzymes, only one band was found to hybridise to the toad α3-probe irrespective of the restriction enzyme. This suggests that there may be only one class I locus present in the MHC of *X. laevis*. Another interesting finding is that although *Xenopus* specialises in polyploidization, and there exist species with different numbers of chromosomes ranging from 20 (2n) to 108 (12n) chromosomes (Kobel and Du Pasquier, 1986), only two MHC haplotypes are expressed with exception in *X.rumenzoriensis*, (12n) which can express three or more MHC haplotypes (Du Pasquier et al., 1977). This suggests that the maintenance of diploidization is mediated by the deletion of MHC genes.

Another feature is the distribution of MHC class I and II molecules in *X. laevis*. As a result of metamorphosis, the immune system undergoes a number of changes (Flajnik et al., 1987; Du Pasquier et al., 1989). One of these changes is the pattern of expression of MHC molecules. Using immunoprecipitation with alloantisera or...
xenoantisera or radiolabelled spleen and thymus lysates or by MLR analysis, the same class II molecules are found to be expressed throughout ontogeny although with a different tissue distribution. In the tadpole, class II antigens are expressed on B cells and macrophages, whereas in the adult, they are expressed on some thymic lymphocytes and virtually all peripheral B and T cells (Du Pasquier and Flajnik, 1990; Flajnik et al., 1990). In contrast, the MHC class I is not detected in tadpoles, but appears on all tissues at the climax of metamorphosis (Flajnik et al., 1986).

Rollins-Smith and Blair (1990) found that the expression of MHC class II antigens on adult T cells in *Xenopus* is metamorphosis-dependent. Young postmetamorphic toads show expression of class II MHC antigens on a high proportion of thymocytes and most peripheral T and B cells. If the larvae’s metamorphosis was inhibited by treatment with sodium perchlorate, these larvae have few class II+ thymic lymphocytes, and the proportion of class II+ splenic lymphocytes is approximately equal to that of IgM+ B cells, whilst in the control animal the class II antigens are virtually absent from thymic lymphocytes and peripheral T cells and could be found in increasing numbers in both populations after metamorphosis. Thus, perchlorate-treated animals retained the larval pattern of class II expression suggesting that the emergence of class II+ T cells is dependent on metamorphosis. In Flajnik’s review (1996), it is proposed that the immunocompetent tadpole expresses only enough of its available adaptive immune system to allow for protection against pathogens. The full array of defence is not developed in order to avoid autoimmunity at metamorphosis; i.e. the repertoire and expression of only one MHC molecule in tadpole life lessens the chance that clones responsive to adult autoantigens will be activated and then destroy the organism at metamorphosis (Lee and Hsu, 1994; Salter-Cid and Flajnik, 1995).

Exon-intron organisation of *Xenopus* MHC class II β-chain genes has been
identified (Kobari et al., 1995). Three class II β chain genes are designated Xela-DAB, Xela-DBB, and Xela-DCB. The Xela-DAB gene has been analysed in detail and is made up of at least six exons, with an exon-intron organisation similar to that of a typical mammalian class II β chain gene.

The MHC class I, class II and non-classical MHC I genes have been isolated and sequenced (Flajnik et al., 1991a, 1993; Sato et al., 1993; Shum et al., 1993; Sammut et al., 1997). All results show that the MHC class I and MHC class II genes and predicted proteins in amphibians are similar to higher vertebrates.

Reptilian class I MHC genes have been isolated from snakes and lizards (Grossberger and Parham, 1992). Five different MHC class I cDNA sequences from lizard have been found, which suggests that the lizard may have at least three loci. Like the MHC class I in the mammals, much of the polymorphism in the sequences is at, or near residues, which could come into contact with antigen or the TCR (Bjorkman et al., 1987b; Bjorkman and Parham, 1990).

Radtkey et al. (1996) investigated variation and evolution of MHC class I in sexual and parthenogenetic geckos (reptile) and found that the variability in the sexual species is similar to mammalian- class I genes and are highly variable in outbreeding sexual populations. Asexual lineages with little or no class I diversification over thousands of generations have persisted to this day. On the other hand, the high level of heterozygosity in the parthenogenetic species (a consequence of their hybrid origin) may provide clonal lineages with adequate antigen presenting diversity to survive and compete with sexual relatives.
1.5.5. MHC in avian species

The MHC in chicken was studied in the early 1920's, and is the second MHC complex to be discovered after the mouse H-2 complex, even before the discovery of the HLA complex (the earlier event reviewed by Pazderka et al., 1975). The chicken B complex was first described as a genetic locus controlling blood group antigens in 1948 (Briles and McGibbon, 1948; Briles et al., 1948, 1950). Like H-2, the B region proved to be complex and related to the skin graft rejection following serological and histogenetic testing in 1961 by Schierman and Nordskog. The chicken MHC contains three classes of loci, B-F, B-L and B-G (Crone and Simonsen, 1987). The investigations of the biochemical structure, functional attributions and tissue distributions of B-F and B-L showed that B-F and B-L in chicken are equivalents of mammalian MHC class I and class II respectively, but a mammalian counterpart of B-G antigen (erythrocyte-specific class IV alloantigens) has not been identified. In Kaufman and co-worker's studies (1991), the B-G genes, B-G antigens and tissue distribution were investigated. The B-G molecules have at least three different classes: these expressed on erythrocytes alone, lymphocytes alone and erythrocytes and thrombocytes. They suggested that B-G molecules might have a function in the development of some B cells in the chicken. B-G antigens have many alleles like B-F and B-L antigens (Guillemot et al., 1989), and size polymorphism also was observed (Kaufman et al., 1990). They indicate that the size polymorphism of chicken B-G antigens is caused by length variation in the cytoplasmic heptad repeat region. B-G antigens seem related to certain immunological phenomena in chicken including the "adjuvant effect", the "preferential response" and "natural antibody reaction" which were discovered during the generation of alloantisera and monoclonal antibodies to chicken red blood cell surface molecules (Kaufman and Salomonsen, 1992).
Except with BG in chicken MHC, B complex displays several other distinctive features (Guillemot et al., 1988, 1989). The map of the B complex showed that the chicken MHC class I (B-F) and class II (B-L) genes are closely linked to each other, B-F and B-Lβ genes are very much smaller and occupy a short distance. The results also showed that some non-MHC I and MHC II genes were found within the B complex. These genes are not homologous to mammalian MHC III genes. The five B-Lβ and six B-F genes were identified by Southern blotting using corresponding probes. These five different chicken B-Lβ genes may belong to two families. A recent report indicates that two class I and two class II chicken MHC-like genes map to the chicken restriction fragment pattern Y (Rfp-Y) system outside of the B complex (Miller et al., 1994). This system is structurally distinct from their counterparts in the B system and perhaps it is representative of a gene coding for a recognition molecule of another kind. This system may stand for an independent MHC-like locus (Briles et al., 1993).

Kroemer et al., (1990) reported the localisation of 18 genes within 320 kb of DNA cloned from the B complex of the inbred CB line. They also found that the B-L and B-F genes were closely associated with each other. At least five B-Lβ and six B-Fα genes were found. Whether the polymorphic B-G antigens have functions similar those of mammalian MHC determinants or whether they merely represent an avian blood group system coincidently linked to the MHC has still to be determined. Although the chicken B region is without a class III region homologous to that found between the class I and II regions in the H2 and HLA complex, several non-class-I and II genes are interspersed with B-F and B-Lβ genes. This may explain their presence in the MHC region just like MHC class III region in mammals is coincidental and only class I and class II genes should be placed in the MHC in stricto sensu (Klein, 1986).

Like mammals, the chicken MHC class I contains three domains α1, α2, α3
which are encoded by separate exons. For MHC class II the overall intron-exon structure has been conserved dividing the B-Lβ gene into a signal peptide, β1 (amino acid 6-94), β2 (96-188), transmembrane and cytoplasmic exons (Bourlet et al., 1988; Guillemot et al., 1986, 1988, 1989; Zoorob et al., 1990). The four introns were found to be very short (86-108 nucleotides) when compared to their homologues in human and murine class II β chain genes (400-3000 bp).

In conclusion, the chicken B locus appears to be very different from its human and murine equivalents in the following respect: class I and class II loci are closely linked containing intermingled B-F and B-Lβ together with non-class-I, non-class II genes, several of which are unrelated to mammalian class III genes. There is no evidence for a separate class III region homologous to that found between the class I and II region in the H-2 and HLA complexes. The telemetric boundary of the B complex is placed in the immediate vicinity of the nucleolus organizing region. The overall size of the MHC is probably reduced compared to mammals.

1.5.6. Comparison MHC of lower vertebrates with mammalian

The MHC gene location in chromosomes has been identified in some species. The MHC maps for lower vertebrates species, including those of bird, fish, and amphibian show many similarities to the mammalian MHC (Kasahara et al., 1995; Dixon et al., 1995). For example, there are at least two regions the class I and II; the genes exist as clusters; a similar pattern of MHC polymorphism is present; the molecular structure is very similar, e.g. MHC class I molecules contain three domains and MHC class II molecules contain four domains. In addition, some residues (Tyr-7, Tyr-59, Tyr or Arg-84, Thr-143, lys-146, Trp-147, Try-159, Try-171) in MHC class I molecules are perfectly conserved in human, chickens, amphibians, and fish. This suggests that those
molecular mechanisms by which class I molecules bind peptides are basically the same in
diverse animals ranging from the bony fish to humans. Also, the conserved residues (β-
Asn-82, α-Asn-62, α-Asn-69, β-Trp-61, α-Arg-76) are present in MHC class
II molecules in all vertebrates. Although Arg-76 is not present in the non-mammalian
class II α-chains, all other residues appear to be present in other species. This suggests
that the mammalian and non-mammalian class II molecules presumably bind peptides in a
similar manner. Some of these residues are highly conserved from the lower vertebrates
to higher vertebrates.

There are however some distinct differences between all vertebrates. This
difference may provide some insight into the evolution of the MHC (Klein et al., 1983).
They may also help to explain the origin and maintenance of the clustering of MHC
genes. From Trowsdale's review (1995), one of the differences between MHC genes of
mammalian and non-mammalian species is the intron size. The more primitive the
vertebrate, the shorter the intron (Fig. 1.21). Some N-linked glycosylation sites are in
different positions, generally, those in mammals and amphibian are in the same positions,
and fish have different N-linked glycosylation sites in both class I and class II molecules.

Kasahara et al. (1995) analysed some representative MHC class I and class II
molecules and suggested that the MHC molecules of lower vertebrates also play a critical
role in the positive and negative selection of T cells, and that many of the parameters
involved in thymic selection do not differ fundamentally between mammalian and non-
mammalian species. At present, the MHC gene map, gene locus and structures have not
been described in detail in any fish species.
Fig. 1.21. Comparison of MHC class II A (A) and class II B (B) chain gene organisation in different vertebrate species (adapted from Trowsdale, 1995).
1.6. Ig gene superfamily

1.6.1. Ig gene superfamily components

The Ig superfamily contains many members. The idea originates from the time when Ig chains were first sequenced and sequence similarities were found within C regions of heavy and light chains. Ig chains probably evolved from a primordial ancestral gene coding for approximately 100 amino acids. The domains within the Ig chains all contain a characteristic intrachain disulphide bond and it has been proposed that each of the domain is an independent structural unit (Edelman, 1970). Later, the domain hypothesis was firmly established when the structures of V and C domains were determined to reveal a common fold (reviewed by Williams and Barclay, 1988). This common fold was called the Ig fold, which contains a globular domain structure of approximately 110 amino acids in length, which form a sandwich of two β-sheets of three or four β-strands. These β-strands are held together by a conserved intradomain disulphide bond which stabilises the structure (Fig. 1.22a). Thus, the Ig gene superfamily means a gene family whose members all possess one or more Ig folds (Peterson et al., 1972, Leahy et al., 1992). The Ig superfamily are mainly cell surface molecules, which include Ig, slg, TCR, MHC, β2-m associated antigens, T cell adhesion molecules, T subset antigens, brain/lymphoid antigens, Ig receptors, neural molecules, tumour antigens, growth factor receptors and also a number of non-cell surface molecules (Williams and Barclay, 1988; Marchalonis et al., 1994). The Ig-related molecules commonly form dimers (Fig. 1.22b) and stable dimers are often disulphide-linked, except the MHC antigens and β2-m associated antigens. The carbohydrate structures are the dominant features in some Ig-superfamily molecules and the N-linked structure varies in type and contents. Great diversity exists within transmembrane sequences and cytoplasmic domains of Ig-related molecules.
Fig. 1.22. A model of Ig fold. It is a globular domain structure, which is approximately 110 amino acids in length and fold into two \(\beta\)-pleated sheets of three or four \(\beta\)-strands. These two \(\beta\) sheets are held together by an intradomain disulphide bond. (b) Both TCR and sIg contains Ig domains and belong to Ig superfamily (Davies, 1997).
1.6.2. Evolution of the Ig gene superfamily (MHC and TCR)

It is clear from the contemporary data that all modern vertebrates possess the hallmarks of an adaptive immune system. Ig, TCR, MHC class I and II molecules and other receptors involved in the immune systems are all members of the Ig superfamily and have not been found in invertebrates (Schluter et al., 1994). Otherwise, all vertebrates from shark to man possess Ig that is essentially the same (Hohman et al., 1992). This suggests that the Ig system not only has common ancestral molecules, but also that the common ancestral molecule was in existence at the time of the divergence of vertebrates and invertebrates. Thus, the rearrangement of the Ig system seems to have rapidly arisen at the time of the emergence of the chordates from the protordates (Marchalonis and Schluter, 1990).

1.6.2.1. TCR and Ig co-evolution?

Edelman (1987) suggested that Ig genes may come from utilisation of an unrelated molecule containing an Ig domain(s), such as a neural adhesion like molecule or the Ig system may have arisen independently and the similarity of the Ig domain is the result of convergent evolution imposed by the functional properties of the Ig fold. Schluter et al. (1994) used two specific antibodies (shark IgM heavy chain and TCRBJ) to probe conserved antigenic motifs in the invertebrates. They identified and characterised Ig-related molecules in tunicates. These molecules reacted with antibodies raised to shark IgM heavy chain and also reacted with Ig of shark, amphibian, bird, rodent, and human origin, and also show peptide sequence homologies. Therefore, Ig-related molecules appear to be present in tunicates and mammals. Other studies have isolated hemolin from invertebrate’s blood (Sun et al., 1990). Hemolin is a member of
the Ig superfamily and binds to microbial surfaces and participates in their removal (Beck and Habicht, 1996). This indicates that the antibody-based immune responses thought to be previously restricted to vertebrates may have some origins in invertebrates.

What is the successive order of appearance in evolution of the families of antigen receptors that are expressed by B and T cells? Litman and Rast (1996) determined that an order of emergence could be inferred, e.g., a TCR αβ-like system emerged first and gave rise (through duplication and divergency) to an Ig-like system because shark have a TCR αβ-like system and are more closely related to mammals than other receptors. Also, phylogenetically, the T-cell recognition system is thought to be much older than the Ig system, perhaps appearing as early as the tunicates (Scofield et al., 1982), whereas Ig gene products seem to have first appeared in the lower fishes (Manning and Turner, 1976). In ontogeny, the TCR γδ appears earlier than TCR αβ bearing cells (Pardoll et al., 1987). There is plenty of data to indicate that both Ig and TCR derived from a common ancestry and structural constraints in that they share a similar primary structure, genomic origins and similar mechanisms of V-J or V-D-J rearrangement (Davis and Patten, 1987). It is postulated that, the gene for this CD8/TCR/Ig ancestor duplicated and diverged to give a gene leading to CD8 production and another gene leading to the TCR/Ig. The TCR/Ig gene was invaded by a transposon to create the split V region of the ancestral TCR, which later gave rise to antibody genes (Davis and Bjorkman, 1988).

Litman and Rast (1996) studied the origin and structure of Ig and TCR genes in the most phylogenetically distant jawed vertebrates and compared them to mammals. They suggest that phylogenetically, the ancient gene structures have true vertical relationships with the antigen receptor gene loci, which have been found in higher vertebrate species. TCR and sIg have very similar structures and sequences. This provides evidence that these genes have evolved from a common ancestor. This is also
supported by the fact that the same recombinases is used which rearrange TCR and Ig gene segments.

The basic organisation of introns and exons in Ig V gene segments has been well conserved during the evolution of vertebrates. All higher vertebrates have several V gene segments in their Ig loci. This almost certainly arose by gene duplication. Heavy chain genes contain D and J gene segments whereas light chain genes have J gene segments. The overall origination of these segments has evolved from at least three different lines and has resulted in three distinctive patterns. They may come from one ancestral gene which undertook gene duplication, capture of transposon, and finally developed into three Ig gene patterns. In modern elasmobranchs (shark and rays), the gene segments are organised into clusters separated by long spaces (VD1D2JCn-VD1D2JCn-1-VD1D2JCn-2...VDJC1). For example, the shark (Heterodontus francisci) has hundreds of these clusters, which occupy a significant proportion of its genome (Litman, 1996). Mammals have a different pattern where the gene segments are clustered (VVV......DDD......JJJ......C.). Birds have evolved a third distinct pattern. There is a single functional V\_H gene but several V pseudogenes. (Fig. 1.23).

It is proposed that the TCR γδ dimer may represent the more primitive T-cell system (Janeway et al., 1988), and this is consistent with the fact that the TCR αβ and γδ genes have close structural homology (Saito et al., 1984a,b), indicating a common evolutionary origin. Matsunaga and Dahl (1989) proposed that the TCR γδ dimer employs a very limited V gene repertoire. During T-cell ontogeny, the ancestral Cδ -line gene may diverge to form the TCR Cα and Cδ gene by duplication, then during T-cell development, T\_H cell developed from TCR γδ to TCR αβ, MHC class I to class II and primordial CD4/8 to CD4 and CD8.
Fig. 1.23. There are three main patterns of Ig gene organisations found in vertebrates elasmobranchs (shark and rays), birds and mammals. Their possible evolutionary relationships are indicated (Davies, 1997).
1.6.2.2. Class evolution within the MHC

Because the membrane-distal domains of MHC molecules have the following similar features: approximately the same size as Ig domains, the intradomain disulphide bond (in α2 of class I and β1 of class II), Ig-like domains formed by cysteine residues, some investigators believe that these domains are certainly members of the Ig superfamily (Orr et al., 1979), and may be derived from Ig-like domains (Fig.1.24). Other investigators suggest that the peptide-binding domains (PBD) could have been derived from another protein family, which has not been identified. Flajnik and co-worker's (1991b) hypothesise that a class I-like protein is the most likely ancestor of all MHC molecules and that MHC proteins were assembled from different gene families with class I molecules emerging before class II. They also propose that the peptide-binding structure existed before the emergence of the MHC.

Hughes and Nei (1993) constructed a phylogenetic tree of C-domains from MHC and related molecules (Hsp70). The results showed that the MHC PBD is more similar to Ig V domain than to Hsp70 domain in alignments. They hypothesised that the ancestral MHC molecule had a class II-like structure, then gave rise to MHC class II first before MHC class I.

Kaufman (1990) also suggested that it seems most likely that a non-polymorphic class II molecule with a non-immunological function was adopted as an allore cognition system possibly in colonial invertebrates, before the creation of class I molecules in evolution while arose by exon shuffling. Thus these nonpolymorphic class I molecules would be due to later evolutionary events.

However, Kasahara et al. in their review (1995) suggested that the PBD of the MHC molecule might have been derived from that of the evolutionarily more ancient Hsp70 molecules because the PBDs of MHC molecules and Hsp70 share a number of
Fig. 1.24. Modern MHC molecules and antigen receptors may have co-evolved from a primitive allore cognition phenomenon. (A) Allore cognition might have been mediated by two Ig superfamily members, which are the promodial MHC molecule and the receptor on the surface of an effector immunocyte. (B) Diversity, duplication and rearrangements may have generated TCR and slg and increased the spectrum of allogenic self molecules they could recognise, and also the promodial MHC molecule evolved into MHC class I and class II molecules (Davies, 1997).
similarities. Davies (1997) introduced the suggestion that the domains that form the peptide-binding cleft are unlike Ig folds and it is possible that they have been acquired by exon shuffling from a different gene. A recent report (Kasahara et al., 1997) hypothesised that chromosomal duplication created the MHC class I first in the adaptive immune system.

MHC II genes are extremely polymorphic, with many alleles differing in > 10% of their nucleotide sequences. The mechanisms responsible for the generation of this diversity are controversial. She et al. (1991) analysed the diversification pattern in the MHC class IIA β gene by sequencing exon 2, 3 and a segment of intron 2 in alleles derived from 14 species of mice and rats. They concluded that most class II genes encoding the β subunit undergo genetic exchanges between different alleles through recombination and possibly some exchanges among different loci through gene conversion. Most polymorphisms at the MHC loci are ancestral and different in various genes. The extensive diversity of the MHC is a result of both mutational accumulations over evolutionary periods and recombination or gene conversion. The convergent evolution, gene duplication and deletion and exon shuffling have played a role in MHC diversification. Selection for antigen-binding site diversity is very important in maintaining MHC polymorphism.

In Salter-Cid and Flajnik’s review (1995), two hypotheses of polymorphism generation were introduced. One is the “trans-species hypothesis”. This suggests that much of the divergence among MHC alleles in recently evolved species is caused by inheritance of sets of divergent alleles, not by hypermutational diversification occurring after the origin of a given species. This means there is no relationship between speciation and generation of new MHC alleles. Another hypothesis suggests that MHC polymorphism is due to large-scale recombination and this generates new alleles.
According to this hypothesis, the exchange of DNA segments from one MHC gene to another is due to genetic mechanisms such as gene conversion.

1.7. The aim of this project

In order to contribute to the understanding of the role and evolution of the MHC and TCR in primitive immune recognition systems, a class of lower vertebrates, dogfish (cartilaginous) has been chosen for the isolation and characterisation of MHC and TCR genes. It is interesting that this fish has a thymus, but does not show acute graft rejection (Borysenko et al., 1970). The MHC class I and class II genes and TCR genes have been identified from several shark species (Hashimoto et al., 1992; Kahahara et al., 1992, 1993; Bartl and Weissman, 1994a, 1994b; Rast et al., 1994, 1995, 1997; Hawke et al., 1996). The dogfish (Scyliorhinus canicula L.) is an archetypal representative of a cartilaginous fish, which has been extensively studied (Morrow, 1978; Parish, 1981; Hart, 1987) and lends itself to studies on its immune mechanisms. It is a manageable species, which takes well to captivity and is of adequate size to investigate all elements of the immune system. The primary aim of this study is therefore:

1. To isolate and characterise the TCR and MHC genes from the dogfish by using PCR, cloning, sequencing, Southern and Northern blotting techniques.

2. To investigate any conserved residues, which may be critical in understanding the evolution of immunity and gene loci.
CHAPTER 2. MATERIALS AND METHODS

2.1. Fish

Dogfish (*Scyliothinus canicula* L.), rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio* L.) were obtained from stocks of fish routinely held in the aquarium department at the University of Plymouth. Dogfish were mature and about 60cm long. Trout and carp were about 20-25cm long and 1 year old. Both sexes were used.

2.2. Isolation of lymphocytes from fish blood

Peripheral blood from dogfish, trout and carp was collected from the caudal vein (Based on Rowley’s method 1990) of the fish into 5ml syringes, previously flushed with the anticoagulant, 5% ethylenediaminetetra-acetic acid disodium salt (EDTA), pH 8.0 and transferred to 15ml Falcon tubes (Becton Dickinson and Company, USA). Each blood sample was diluted 1:4 with Roswell Park Memorial Institute Medium 1640 (RPMI 1640) without glutamine (Gibco, Life Technologies, UK), and an equal volume of Histopaque (Sigma) or Lymphocytes Separation Medium (ICN, UK) was carefully added to the bottom of the diluted blood using a 10ml syringe with a long filling tube. Blood cells were separated by density gradient at 1,800 rotations per minute (rpm) in a MSE Mistral 100 Centrifuge (UK) for 15 min. The lymphocytes were removed from the interface, transferred to a 15ml Falcon tube and washed twice for 10 min. in RPMI 1640 medium and recovered by centrifugation at 1,700 rpm. Finally, cells were re-suspended in a 5ml volume of RPMI 1640 medium. Their viability was assessed by mixing an equal volume of the cell suspension with 0.2% trypan blue and 3mM sodium azide and observing them in a haemocytometer chamber under a microscope (Kyowa,
Tokyo). Viable cells appeared brightly refringent whilst dead cells stained blue as membranes of viable cells exclude the trypan blue which passively diffuses into dead cells (Dealtry, 1992). Cells for the lymphocyte proliferation assay were used immediately (Section 2.3). Cells for storage were divided into aliquots of $5 \times 10^6$ cells and placed in 2ml sterile cryogenetic vials (Corning, New York) and centrifuged at 1,700 rpm for 10 min. The supernatant was discarded and the pellet was snap frozen in liquid nitrogen for 5 min. before transferring to -80°C for storage prior to extraction of RNA.

2.3. Lymphocyte proliferation assay

Cells were centrifuged at 1,700 rpm for 10 min. and re-suspended to a volume which gave $5 \times 10^6$ cells/ml densities with complete RPMI 1640 medium containing 10% fetal calf serum (FCS), 0.05μg/ml gentamicin sulphate and 20mM L-glutamine. A 100μl aliquot of cell suspension was added to each well of a 96 well flat-bottomed cell culture plate (Flow, ICN Biomedicals Ltd. UK). Various mitogens (Sigma) were added to the complete medium in a 100μl volume resulting a final concentration of Con A at 10 μg/ml, 25μg/ml, or 50μg/ml, LPS at 50μg/ml, 100μg/ml, or 250μg/ml, or PHA at 2.5μg/ml, 5μg/ml, or 10μg/ml, respectively. Urea and NaCl were added in culture medium at a final concentration of 2.16% and 0.2M respectively for dogfish lymphocytes (Grogan and Lund, 1990; McKinney, 1992b). Control wells received medium only. Assays were performed in triplicate. Cells were incubated at 18°C. in a 10% CO₂ atmosphere for three days. The CO₂ atmosphere was obtained by either placing a 10% volume of CO₂ in a sealed box with the plate or incubating the plate in a box containing an Alka Seltzer tablet (Sigma). After three days incubation, $³H-$
thymidine (Sigma) 1μCi. in 25μl complete medium was added to each well. Cells were harvested 24 hours later using a Skatron semi-automated cell harvester (Norway) onto Beckman (USA) filters impregnated with Xtalscint. The specific activity was measured on the ³²C channel of a Philips β emission counter. The results are expressed as stimulation indices (SI) calculated from the mean counts per minute (cpm) of triplicate control cultures and the mean cpm of triplicate stimulated cultures according to the following formula:

\[
SI = \frac{\text{Mean cpm of stimulated cultures}}{\text{Mean cpm of nonstimulated control cultures}}
\]

In order to increase the ratio of T-like lymphocytes used for making cDNA, fish lymphocytes were cultured in the above conditions with the mitogen Con A or PHA in a 1 ml volume in a 24 well flat bottomed cell culture plate (Cell Wells, Corning, New York). After three days culture, the viability of the cells was assessed by taking 50μl of culture. The remaining cells were collected by centrifugation at 1700 rpm for 10 min. and re-suspended at a density of 5x10⁶ per ml. Cells were snap frozen in liquid nitrogen for 5 min. and stored at -80° freezer prior to extracting RNA.

2.4. Isolation of cells from dogfish spleen, kidney, liver, heart, testes, Leydig tissues, epigonal tissues and brain

Dogfish were killed by an overdose of anaesthetic MS222 (Tricaine methaesulfonate), 500mg in 1 L of double distilled water (ddH₂O), followed by a blow to the head (Tatner, 1990). Once the dogfish expired, it was placed on ice and bled by
severing the caudal peduncle. The fish was laid on its dorsal side and its integument was sprayed with 70% ethanol. A scalpel blade immersed in 70% ethanol was used to cut the dogfish starting from the anterior head to a point just prior to the anterior of the vent. The spleen, kidney, liver, heart, testes, Leydig tissues, epigonal tissues and brain were then removed. All organs were placed in petri dishes kept on ice prior to being transferred to a sterile plastic bag where the tissue was subsequently minced by rolling a bottle over the bag. Organ samples were immediately snap frozen in liquid nitrogen for 5-10 min. prior to storage at -80°C before extracting the RNA.

2.5. Extraction of RNA from lymphocytes and tissue

All RNA and cDNA preparations were performed using 0.1% diethylpyrocarbonate (DEPC) (Sigma, UK) treated and autoclaved centrifuge tubes, pipette tips, glassware and solutions to remove RNase. Two methods were used for extracting RNA. During each procedure, gloves were wore and changed often.

2.5.1. Extraction of total RNA using RNAzol B

RNAzol B (Biogenetics, UK) contains guanidium isothiocyanate and phenol. RNAzol B was added to frozen lymphocytes in the original container at a ratio of 200μl of RNAzol B to 10^6 cells and mixed by pipetting until the cell pellet was completely homogenised and then transferred to a 1.5ml microfuge tube. Chloroform at a ratio of 1:10 of chloroform:RNAzol B was added, and after vortexing the contents for 15 seconds the tube was placed on ice for 15 min. Following centrifugation at 13,000 rpm in a Biofuge 13 centrifuge (Heraeus, Germany) for 15 min. at 4°C, the upper layer was carefully transferred to a new microfuge tube. An equal volume of isopropanol (BDH, UK) was added and following vortexing, the tube was placed on ice for a further
15 min., and thereafter centrifuged at 13,000 rpm for 15 min. at 4°C. Finally, the pellet was washed with 1 ml of 70% cold ethanol, air-dried, and re-suspended in 25 μl of DEPC treated ddH₂O.

RNA was extracted from spleen, kidney, liver, heart, testes, Leydig tissues, epigonal tissues and brain samples by using the same procedure.

2.5.2. Extraction of total RNA using the RNeasy Kit (QIAGEN, Germany)

A 350 μl volume of Lysis Buffer RLT (containing guanidium isothiocyanate and β-mercaptoethanol) was directly added to 5 x 10⁶ frozen lymphocytes mixed by shearing quickly through a needle of a syringe within 1 min. The resulting lysate was centrifuged at 13,000 rpm for 3 min. in a microfuge. A 350 μl volume of 70% ethanol was added to the tube, and mixed by pipetting. Next, the mixture was applied to a RNeasy spin column held in a collection tube and centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and a 700 μl volume of Wash Buffer RW1 (QIAGEN, Germany) was added to the column. Following centrifugation the flow-through was again discarded. The spin column was washed with 2 x 500 μl volume of Wash Buffer RPE (containing 80% ethanol) and centrifuged as above. The column was then spun for 2 min. to evaporate any residual ethanol. Finally, the RNA was eluted with a 30-50 μl volume of DEPC treated ddH₂O into a fresh collection tube.

The optical density (OD) value of each sample was measured by a scanning wavelength between 240 nm and 280 nm in a spectrophotometer (CECIL 5000 Striss, England) to assess both the quality and quantity of the RNA. The OD₂₄₀:OD₂₈₀ ratio was usually above 1.75, which indicated freedom from protein contamination. The concentration in μg/μl was calculated by the following formula: OD₂₆₀ x 40 x dilution factor /1,000. The quality was also assessed by electrophoresis through a 1% agarose
gel containing 0.01% ethidium bromide (Sharp et al., 1973). Ethidium bromide contains a planar group that intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases cause dye to bind to DNA and display an increased fluorescent yield compared to the fluorescence of the unbound dye in free solution. Ultraviolet (UV) radiation at 254nm is absorbed by the DNA and transmitted to the dye; radiation at 302nm and 366nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590nm in the red-orange region of the visible spectrum. Because the fluorescent yield of ethidium bromide:DNA complexes is much greater than that of unbound dye, small amounts of DNA can be detected in the presence of free ethidium bromide in the gel. Ethidium bromide can be used to detect both single- and double stranded nucleic acids (both DNA and RNA). Generally, RNA gives a 28S band of 6333 bases and an 18S band in 2366 bases under UV radiation (UVP, USA) at 590nm. Then RNA was stored at -80°C until used.

2.6. Synthesis of first strand cDNA from mRNA

2.6.1. Synthesis of first strand cDNA for PCR and cloning

A 4μl volume of oligo-dT (0.5μg/μl) was added to 20μg of total RNA in a microfuge tube which was pre-heated at 60°C for 2 min., mixed briefly, spun down, incubated at 55°C for 3 min., then placed on ice for 3 min. A 0.55μl aliquot of RNasin (33w/μl) (Promega, USA) was added, the tube contents mixed, spun down, and the following was added: 1μl of four 2'-deoxyribonucleotide 5'-triphosphates mixture (dNTPs) (25mM), 4μl 0.1M dithiothreitol (DTT), 8μl 5 x reverse transcriptase (RTase) buffer, 0.75μl Moloney Murine Leukemia Virus (M-MLV) RTase (200u/μl) (Gibco Life Technologies, UK). The final volume was made up to 40μl by adding an
appropriate amount of DEPC treated ddH₂O, the contents mixed, the tube spun down and incubated at 37°C for 45 min. The first strand cDNA was extracted with phenol/chloroform and precipitated with 1 volume of 4M sodium acetate pH 5.2 and 4 volumes of absolute ethanol, washed with 95% ethanol and 100% ethanol, dissolved in 10-20μl ddH₂O, and stored at -20°C until used for PCR and cloning.

2.6.2. Synthesis of first strand cDNA for construction of cDNA library

A 4μl aliquot of Xba I primer/adaptor (1μg/μl) was added to 20μg of RNA in a microfuge tube which was pre-heated at 60°C for 2 min. The contents were mixed, spun down, incubated at 55°C for 3 min., and the tube was placed on ice for 3 min. A 0.55μl aliquot of Rnasin (33u/μl) (Promega, USA) was added, mixed, spun down, and the following was added: 1μl of dNTPs (25mM), 4μl of 0.1M DTT, 8μl of 5 x RTase buffer, 0.75μl of M-MLV RTase (200u/μl) (Gibco Life Technologies, UK), 1μl of [α-³²P]-dCTP (3000Ci/mmol) (Amersham Life Science, England). The final volume was made up to 40μl by adding an appropriate amount of ddH₂O. The contents of the tube were mixed, spun down and incubated at 37°C for 45 min. Thereafter, the first strand of cDNA was extracted with phenol/chloroform and precipitated with 1 volume of 4M sodium acetate pH 5.2 and 4 volumes of absolute ethanol, washed with 95% ethanol and 100 % ethanol, and dissolved in 100μl of DEPC treated ddH₂O. The precipitation procedure was repeated once and the first strand cDNA was finally dissolved in 115μl of DEPC treated ddH₂O. The concentration of cDNA was estimated by determining the incorporation of [α-³²P]-dCTP in a multi-purpose scintillation counter LS6500 (Beckman, USA).
2.7. Construction of cDNA library

2.7.1. Synthesis of second strand cDNA

To the suspension of the first strand of cDNA (115μl), the following was added: 1.5μl of 2M Tris (pH 7.5), 3.0μl of 0.1M DTT, 1.0μl of 1M MgCl₂, 1.5μl of 1M ammonium sulphate, 7.5μl of 2M KCl (DEPC treated, autoclaved), 7.5μl of 1mg/ml bovine serum albumin (BSA), 4.0μl of 25mM dNTPs, 2.0μl of RNase H (Gibco Life Technologies, UK) and 7.0μl of E.coli DNA polymerase I (Biolabs, UK). After mixing, the contents of the tube were incubated at 14°C for 60 min. and at room temperature (RT) for a further hour. Double stranded cDNA was extracted using the same method as for recovering first strand cDNA, and analysed by counting the cpm in a multi-purpose scintillation counter LS6500 (Beckman, USA) as well as by electrophoresis through a 1% agarose gel. The quantity of DNA was be determined by the following formula: OD₂₆₀ x 45 x dilution factor/1,000. The majority of cDNA appears as a smear above 1 kb in the 1% agarose gel.

2.7.2. Blunt-ending the DNA

The cDNA was resuspended in 10μl of ddH₂O, and the following was added: 10μl of Klenow fragment (1,000u/ml) (Pharmacia, UK), 0.62μl of 2 M Tris-HCl (pH 7.5), 0.25μl of 1M MgCl₂, 0.25μl of 0.1 M DTT, 0.63μl of 2mg/ml BSA, 0.25μl of 25 mM dNTPs, and made up to a final volume of 25μl with ddH₂O. The tube was incubated in a water bath at 37°C for 30 min. The cDNA was precipitated as above, and resuspended in 19μl of ddH₂O.
2.7.3. Ligation of adaptor to cDNA

A 1µl aliquot of Eco R I adaptor (10pmol/µl) (Promega, USA) and 19µl of cDNA was added to the Ready-To-Go T4 DNA ligase tube (Pharmacia, UK), which contained reaction buffer, 0.1 mM ATP, and 6 units of T4 DNA ligase. The reaction was incubated at RT for 3-5 min., mixed gently by pipetting up and down, spun briefly to collect the contents at the bottom, then incubated at 16°C for 30-45 min. The ligation reaction was stopped by heating at 70°C for 10 min.

2.7.4. Phosphorylation reaction

A 25µl aliquot of ddH₂O was added to the Ready-To-Go T4 Polynucleotide Kinase (PNK) (Pharmacia, UK), which contained reaction buffer, 10 units of T4 PNK, 0.2µM of ATP and incubated for 2-5 min. at RT. The DNA solution, containing 5-10pmol of 5' DNA ends, was heated at 65°C for 5 min., cooled to RT and transferred to the Kinase Ready-To-Go tube. The total volume was adjusted to 50µl with ddH₂O, the tube was then incubated in a water bath at 37°C for 30 min., and the reaction stopped by adding 5µl of 250mM EDTA. The DNA was concentrated to 17µl by precipitation.

2.7.5. Digestion with Xba I

A 1µl aliquot of restriction endonuclease Xba I (15u/µl) (Promega, USA), and 2µl of buffer H was added to the tube containing 17µl of DNA, mixed and incubated at 37°C for 2-4 h. The DNA concentration was adjusted to 10-20ng/µl by precipitation.

2.7.6. Ligation of Lambda GEM-2 and cDNA insert

Before ligation, the quantity and quality were assessed by spectrophotometry
(240nm to 280nm) and by electrophoresis through a 1% agarose gel.

A 2μl aliquot of lambda vector DNA (0.5μg/μl, 0.018pmol) (Promega, USA), 4μl of cDNA, 1μl of 10 x T4 ligase buffer, and 3μl of T4 DNA ligase (3u/μl) were mixed in a microfuge tube and incubated at RT for 3 h.

2.7.7. Preparation of bacterial culture

The day before packaging was to be performed, a single colony of LE392 bacteria (Promega, USA) picked from a freshly streaked LB plate, was used to inoculate 3ml of LB medium supplemented with 0.2% maltose and 10mM MgSO4. The tube was shaken at 37°C until the OD value of the culture reached 0.6-0.8 (5-8 h) and the bacteria was stored at 4°C for up to 24 h.

2.7.8. Packaging and titration of ligated DNA

As soon as the packaging extract (50μl) (Promega, USA) was thawed on ice, 10μl of the ligation reaction was added to the extract, mixed by gently tapping the bottom of the tube several times, and incubated at 22°C for 3 h. To the packaging mixture (60μl), 445μl of phage buffer (20mM Tris-HCl, pH 7.4, 100mM NaCl, 10mM MgSO4) and 25μl of chloroform were added, mixed by inversion and the chloroform was allowed to settle to the bottom of the tube.

A dilution series of the packaging mix from 1:10 to 1:100,000 was made in phage buffer. A 100μl volume of each dilution (in duplicate) was added separately to 100μl of the prepared bacteria and incubated at 37°C for 30 min. to allow phage absorption. A 3ml volume of molten TB top agar (45°C) was added, mixed by quickly inverting, and then poured immediately onto LB plates pre-warmed to 37°C. When the top agar set the plates were inverted and incubated at 37°C overnight.
2.7.9. Isolation of bacteriophages λ and detection of recombinant insertion

2.7.9.1. Isolation of bacteriophages λ (Sambrook, et al., 1989)

Picking bacteriophage λ plaques from plates

A Pasteur pipette was used to suck the bacteriophage λ plaque from the plate which was then placed into 1ml of SM buffer (0.01% gelatin, 50mM Tris-HCl, pH 7.5, 8mM MgSO4) containing one drop of chloroform. This solution was left at RT for 1-2 h. to allow the bacteriophage particles to diffuse out of the agar and was stored at 4°C until needed.

Plate lysate

Bacteria LE392 was freshly cultured in a LB plate at 37°C overnight and a single colony was transferred to 5ml of LB medium supplemented with 0.2% maltose and 10mM MgSO4, and incubated at 37°C overnight with shaking. Next, the culture was centrifuged at 4,000xg for 10 min. The supernatant was discarded and the bacteria was resuspended in 10ml of the same medium as above and stored at 4°C up to 1 week before use.

The stored bacteriophages (100μl) were mixed with 100 μl of the bacteria-suspension, and the mix was incubated at 37°C for 20 min. Next, 2.5 ml of melted TB top agarose was added to the mix, poured onto LB agar plates pre-warmed to 37°C, and left to set for 30 min. The plates were then inverted, incubated at 37°C for 9-15h. until the plaques became confluent. Each plate was overlaid with 3ml of λ diluent, and the bacteriophage particle was allowed to elute over a 1-2h. period at RT with constant gentle shaking. The top agarose was scraped with a spatula and transferred to a 50ml centrifuge tube. The tube was incubated at RT for 30 min. with shaking, then
centrifuged at 8,000-10,000xg for 10 min. at 4°C. The supernatant was carefully withdrawn, transferred to a fresh tube to remove the bacteria debris, 0.3 %(v/v) chloroform was added to the resulting mixture and stored at 4°C for up to 6 mouths.

Rapid analysis of bacteriophage λ

The bacteriophage λ solution (3ml) was mixed with 1μl each of RNase A (1mg/ml) and DNase I (1mg/ml), and incubated at 37°C for 15 min. An equal volume of a solution containing 20% w/v polyethylene glycol (PEG 8000) and 2M NaCl in λ diluent (10mM Tris.HCl, pH 7.5, 10mM Mg++) was added to the bacteriophage λ solution, and incubated at 0°C for 1 h. The mixture was centrifuged at 10,000xg for 10 min. at 4°C. The bacteriophages were resuspended in 0.5ml of TE buffer (pH 8.0), mixed and a 5ml volume of 10% SDS was added. The mix was then incubated at 68°C for 5 min. A 10μl volume of 5M NaCl was then added with an equal volume of phenol/chloroform, mixed and centrifuged at 12,000 rpm for 15 min. Next, bacteriophage DNA was extracted once with chloroform alone. The aqueous phage was transferred to a fresh tube, precipitated with an equal volume of isopropanol, recovered by centrifugation at 12,000 rpm for 15 min at 4°C in Biofuge 13 centrifuge (Heraeus, Germany), washed with 70% ethanol, dried and resuspended in 50 μl of TE buffer (pH 8.0).

2.7.9.2. Detection of recombinant DNA

The bacteriophage DNA (10μg) was digested with 0.5μl of restriction enzyme Bam HI (40u/μl) (Boehringer Mannheim, UK) at 37°C for 2-4h. to release the inserted DNA. The resulting DNA was analysed by electrophoresis through a 1% agarose gel and photographed on an UV transilluminator (UVP, USA).
2.8. Extraction of fish genomic DNA

Dogfish DNA was extracted by using a Nucleon II kit (Scotlab, UK). The following procedure was employed: a 40ml volume of Nucleon A (10mM Tris-HCl, 320mM sucrose, 5mM MgCl₂, 1% Triton X-100, pH 8.0) was added to 1ml of blood, the sample was shaken for 4 min. and centrifuged at 1,300xg for 4 min. at RT. The supernatant containing the lysed cells was discarded and 2ml of Nucleon B (400mM Tris-HCl pH 8.0, 60mM EDTA, 150mM NaCl, 1%SDS) was added to resuspend the pellet, and incubated for 30 min. at 37°C to ensure that the nuclear membranes were disrupted and the mixture cleared. Next, the suspension was transferred to a 15ml Falcon tube, 500μl of 5M sodium perchlorate was added to deproteinise the DNA solution and the mixture was gently shaken at RT for 15 min., followed by a second incubation at 65°C for 25 min. A 2ml volume of chloroform (-20°C) was added and the tube was shaken at RT for 10 min., followed by centrifugation at 800xg for 1 min. A 300μl volume of resuspended Nucleon silica was added and the mixture was centrifuged at 1400 xg for 3 min. at RT. Without disturbing the Nucleon silica suspension layer, the aqueous phase containing the DNA was carefully transferred to a new tube. The solution was respun at 1300xg to pellet any residual Nucleon silica and the supernatant was carefully decanted into a new tube. The DNA was precipitated by placing the tube at -20°C for 2-3 h. followed by the addition of 2 volume of cold 100% ethanol (-20°C) and gentle inversion. The precipitated DNA was spooled out with a glass pipette with a sealed tip and washed in 70% ethanol. Any excess ethanol was drained off and the DNA was dissolved in 500μl of ddH₂O. Absorbency of the DNA between 260nm and 280nm was measured, its concentration was calculated (formula: μg/μl=OD₂₆₀ x 50 x dilution factor/1,000) and the quality was analysed by electrophoresis through a 0.3% agarose gel poured onto a 1% agarose gel support. The
DNA was stored at -20°C until required.

2.9. Design of primers and amplification of fish TCR-like and MHC-like genes

2.9.1. Design of primers

Several sets of primers or degenerate primers were prepared using the known fish TCRB gene sequences (Table 2.1) and fish MHC class I and class II gene sequences (Table 2.2). These primers were synthesised using a commercial Pharmacia Gene Assembler and were of 18 to 30-mers in length with a random base distribution and a guanosine and cytidine (G+C) ratio of approximately 50%. Primers were checked using DNASTAR software (Lasergene, USA) to avoid the possible "primer-dimers" or "hairpins". For the template of recombinant plasmid with cDNA insertion (non-directional), the M13 sequencing primers (M13-40 forward, M13-17 reverse) (Promega, USA) were used simultaneously with primers TCRF7.1, TCRF7.2 and TCRR7 (the three primers were designed from known dogfish TCRDV-like gene, clone 6C53 in chapter 3) and used in PCR for amplification of TCRD genes (Table 2.3).

2.9.2. Amplification of fish TCR-like and MHC-like genes

The PCR mixture contained either 100-500ng genomic DNA or 100-300ng cDNA, or 100-300ng plasmid with cDNA insert template with 10mM Tris-HCl, pH 9.0, 1.5-4.5mM MgCl₂, 50mM KCl, 0.1% Triton X-100, 0.01% gelatin, 0.2mM, primers at 100pMol., and 1.6-2.2 units of *Taq* polymerase (HT Biotechnology Ltd, England) made up to a 50µl final volume by adding ddH₂O. The thermal cycling protocol was showed in Chapters 3 and 4. When the primers were initially used, four control tubes were used. These included one without template, one without 5'-primer, one without 3'-primer, and one without both primers. When the PCR was finished, the PCR products were treated as described in the following sections.
Table 2.1. Primer sequences for amplification of fish TCR β chain genes in PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Region</th>
<th>expected size (bp)</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRF1</td>
<td>5'-ATCTACTGGTATCGCCAGCAGAG</td>
<td>Variable</td>
<td>~190</td>
<td>Rast and Litamn. 1994</td>
</tr>
<tr>
<td>TCRR1</td>
<td>5'-CTAGCAGCAGCAGTAATACACCGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF2</td>
<td>5'-GCAGAGGGGAAAACGGTGAAAA</td>
<td>Variable+Costant</td>
<td>~420</td>
<td>Rast and Litamn. 1994</td>
</tr>
<tr>
<td>TCRR2</td>
<td>5'-AAATGCCTGACGGCGAGCAGACCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF3</td>
<td>5'-GGAGATGGGACCAAACTGGTTG</td>
<td>Constant</td>
<td>~471</td>
<td>Rast and Litamn. 1994</td>
</tr>
<tr>
<td>TCRR3</td>
<td>5'-GCAGATGAGGATCAGGTATGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF4</td>
<td>5'-CCAGGAGAAGGAGAATGAGAGG</td>
<td>Variable</td>
<td>~240</td>
<td>Partula et al., 1994</td>
</tr>
<tr>
<td>TCRR4</td>
<td>5'-GGGTGAGTTGGTGAAGCGGTCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF5</td>
<td>5'-GCAGGTTAGCTTTTCATTAC</td>
<td>for sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF6</td>
<td>5'-CCGAATTCCTGGTAYCRNCA</td>
<td>Variable</td>
<td>~190</td>
<td>Rast and Litamn. 1994</td>
</tr>
<tr>
<td>TCRR6.1</td>
<td>5'-CGGATCCGCRGARTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRR6.2</td>
<td>5'-CGGATCCGCRGARAARTA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key to symbols: D=G+A+T, N=A+T+C+G, Q=5'-Biotin, R=A+G, Y=C+T.
Table 2.2. Primer Sequences for amplification of MHC class I and II genes from fish in PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Region</th>
<th>Expected size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCF1</td>
<td>5'-CAACAGCACTGTGGGGAAGTTTG</td>
<td>MHC class II β</td>
<td>-360</td>
<td>Hordvik et al., 1993</td>
</tr>
<tr>
<td>MHCR1</td>
<td>5'-GAGTGGATCTGGTAGTACCAGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tu803</td>
<td>5'-CACWSCYKGAROKMCTWYTAC</td>
<td>MHC class I α1 and α2</td>
<td>-360</td>
<td>Takeuchi et al., 1995</td>
</tr>
<tr>
<td>Tu672</td>
<td>5'-GCDATRAARTCCTYKCCRTCTRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHCF2</td>
<td>5'-CTGTYAKGTGAMWGRWTCTAYC</td>
<td>MHC class I α3</td>
<td>-90</td>
<td>Hashimoto et al., 1992</td>
</tr>
<tr>
<td>MHCR2</td>
<td>5'-GMKRCTGSKGTGCTCCACKTGRC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHCF3</td>
<td>5'-GAYGGWGANGAAGNTKTWCYANYRGAYYTTC</td>
<td>MHC class II α</td>
<td>-550</td>
<td>See Section 4.3. in Chapter 4.</td>
</tr>
<tr>
<td>MHCR3</td>
<td>5'-GTGCTCCACGTGCGAGGARTACATG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Primer sequences for amplification of whole TCRD genes from plasmids with dogfish cDNA insertions in PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRF7.1</td>
<td>5'-TCTTTGGGACGTTCAG V+C or V</td>
<td></td>
</tr>
<tr>
<td>M13 -40 forward</td>
<td>5'-GTTTTCCCAGTCACGAC</td>
<td>V+C or V</td>
</tr>
<tr>
<td>TCRF7.2</td>
<td>5'-GAAAGTGTCGAAAAGGAGCTG V+C or V</td>
<td></td>
</tr>
<tr>
<td>M13 -40 forward</td>
<td>5'-GTTTTCCCAGTCACGAC</td>
<td>V+C or V</td>
</tr>
<tr>
<td>TCRR7</td>
<td>5'-CTTTCCAGGGACGGTGCTCTTC V+C or V</td>
<td></td>
</tr>
<tr>
<td>M13 -40 forward</td>
<td>5'-GTTTTCCCAGTCACGAC</td>
<td>V+C or V</td>
</tr>
<tr>
<td>TCRF7.1</td>
<td>5'-TCTTTGGGACGTTCAG V+C or V</td>
<td></td>
</tr>
<tr>
<td>M13 -17 reverse</td>
<td>5'-CAGGAAACAGCTATGAC</td>
<td></td>
</tr>
<tr>
<td>TCRF7.2</td>
<td>5'-GAAAGTGTCGAAAAGGAGCTG V+C or V</td>
<td></td>
</tr>
<tr>
<td>M13 -17 reverse</td>
<td>5'-CAGGAAACAGCTATGAC</td>
<td></td>
</tr>
<tr>
<td>TCRR7.2</td>
<td>5'-CTTTCCAGGGACGGTGCTCTTC V+C or V</td>
<td></td>
</tr>
<tr>
<td>M13 -17 reverse</td>
<td>5'-CAGGAAACAGCTATGAC</td>
<td></td>
</tr>
</tbody>
</table>

Note: V=Variable region, C=Constant region.
2.10. Separation and purification of PCR fragments

An 8μl aliquot of the PCR mixture was mixed with 2μl of DNA loading buffer and the PCR products were separated by electrophoresis (Boehringer Mannheim, Germany) in a 1% agarose gel made in 0.5 x TBE buffer run at 100-150 volts for 1-2 h. Gels were photographed under UV illuminator (UVP, USA). When the expected size products were found, a further sample of PCR mixture was run through another 1% gel using either low-melting-point agarose gel or normal agarose in 1 x TAE or 0.5 x TBE buffer respectively. Then expected size DNA bands were excised using a clean, sharp scalpel blade, and recovered using one of the following procedures.

Traditional method

The excised bands were placed into 0.5ml-microfuge tubes pierced with a needle, and containing a bed of glass wool. Each 0.5ml tube was then placed in a 1.5ml microfuge tube, and spun at 10,000 rpm for 10 min. The DNA was precipitated by adding a 1/10th volume of 3M sodium acetate, pH 5.2 and 2-3 volumes of 100% ethanol. After mixing the tube was placed at -70°C for several h., then centrifuged at 13,000 rpm for 15 min., the pellet washed with 70% ethanol and air-dried. Finally, the pellet was dissolved in a 10-20μl volume of ddH₂O.

Wizard PCR Preps DNA Purification System (Promega, USA)

A 300μl volume of cut gel (low melting-point-agarose in 1 x TAE buffer) was transferred to a 1.5ml microfuge tube and incubated at 70°C until the agarose was completely melted. Next, 1ml of resin was added and mixed. The mixture was loaded into a Wizard Mini-column and forced through using a syringe and a 2ml volume of 80% isopropanol was added to wash the column. The column was transferred to a 1.5ml microfuge tube and centrifuged at 12,000xg for 20 seconds to dry the resin prior to
being transferred to a new 1.5ml microfuge tube. A 50μl volume of ddH₂O was applied to the column, incubated for 1 min. and then centrifuged at 12,000xg for 20 seconds to elute the bound DNA fragment.

_Qwik-Precip_

After the cut gel was centrifuged at 10,000 rpm for 10 min. as in traditional method, the DNA was collected in a 1.5ml microfuge tube, and a 0.1 volume of 5M NaCl, plus 1-2μl of "Qwik Precip" (Advance Genetic Technologies Corp., USA) were added into the tube together with 2-3 volumes of cold 100% ethanol. The sample was briefly mixed and centrifuged for 2-3 min. at 13,000 rpm at RT. The sample was then mixed vigorously and centrifuged again for another 30 seconds. The supernatant was removed and the tube was rinsed with 70% ethanol and air-dried. The precipitated DNA was then resuspended in 50μl of ddH₂O.

_Dynabeads_

First, the Dynabeads M-280 Streptavidin (magnetic beads coated with streptavidin) (Dynal, UK) was resuspended by gently shaking the vial to obtain a homogeneous suspension. A 100μl volume of the Dynabeads was transferred to a 1.5ml microfuge tube and placed in the Dynal Magnetic Particle Concentrator for Microtubes of Eppendorf Type (MPC-E) (Dynal, UK). The supernatant was removed by pipette, and the beads were washed twice with 100μl of PBS, pH 7.4 + 0.1% BSA to remove NaN₃, and re-suspended in 100μl of PBS, pH 7.4+0.1% BSA. Secondly, a 20μl (200μg) volume of the pre-washed beads was transferred to a 1.5ml microfuge tube, and placed in the Dynal MPC-E (Dynal, UK). The supernatant was removed whilst the tube was kept by the magnet. The beads were re-suspended in 20μl of 2 x binding and
washing (B+W) buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA, 2.0mM NaCl), mixed gently and the supernatant was removed with a pipette while the tube was kept in the magnet. The beads were resuspended in 40μl of 2 x B+W buffer and added to a 40μl volume of PCR product, containing one primer labelled with biotin. The tube's contents were mixed and incubated at RT for 15 min, while keeping the mixture suspended by gentle rotation of the tube. The tube was placed in the MPC-E, and the supernatant was removed by pipette, followed by another wash with 40μl of 2 x B+W buffer, and removal of the supernatant in the MPC-E. The beads were then resuspended in 8μl of a freshly prepared 0.1M NaOH solution, and incubated at RT for 10 min. The tube was placed in the MPC-E to collect the beads and the NaOH supernatant was transferred (containing the non-biotinylated strand) to a fresh tube. The beads were washed once with 50μl of 0.1M NaOH, once with 40μl of B+W buffer, and once with 50μl of TE buffer. The supernatant was removed and the beads were resuspended in 18μl of ddH₂O.

Purified PCR products were either reamplified, cloned or directly sequenced.

2.11. Preparation of fresh or frozen competent JM109 E. coli

The following procedure was used (Hanahan, 1983). The commercial bacteria strain E. coli JM109 (Promega, UK) was plated directly from a frozen stock onto the surface of a LB agar plate using a sterile loop. The plate was incubated overnight at 37°C. The following day, one well-isolated clone (1-2cm in diameter) was transferred to a 50ml Falcon polypropylene tube containing 10ml of LB medium and incubated overnight at 37°C with shaking at 250 rpm. Then, 50-100μl of the culture was transferred to a 1L flask containing 100ml of LB culture medium, and grown at 37°C
with shaking at 250 rpm. The growth of the culture was monitored by measuring OD₆₀₀ every 20-30 min. until the OD₆₀₀ reached 0.5-0.7.

The bacteria was transferred to a sterile, ice-cold 50ml Falcon polypropylene tube and cooled to 0°C on ice for 10 min. The bacteria was recovered by centrifugation at 4000 rpm for 10 min. at 4°C in a Sorvall RC 3B Plus centrifuge (USA). The supernatant was decanted and the tube was inverted for 1 min. to allow the last traces of media to drain away. The bacteria was resuspended by gentle vortexing in 20ml of ice-cold transformation buffer FSB, stored on ice for 10 min., and then recovered by centrifugation at 4000 rpm for 10 min. at 4°C. The buffer was decanted, and the tube was inverted for 1 min. After the bacteria was resuspended by gentle vortexing in 4ml of ice-cold transformation buffer FSB, a 140μl volume of dimethyl sulfoxide (DMSO) was gently mixed with the bacteria, and the tube was kept on ice for 15 min. An additional 140μl volume of DMSO was added, gently mixed and the tube was returned to the ice. The bacteria was immediately dispensed in 200μl aliquots into chilled, sterilised microfuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until use.

2.12. Cloning

Blunting/Kinasing Reaction

A 1-16μl volume of PCR product or cDNA (20-1000ng, depending on the DNA size), was added to a tube containing 1μl of Klenow Fragment, 2μl of 10 x blunting/kinasing buffer, 1μl of PNK, and ddH₂O to a final 20μl volume, gently mixed and incubated at 37°C for 30 min. Next, 20μl of phenol/chloroform was added, mixed and the tube was centrifuged at 13,000 rpm (Heraeus-Biofuge 13, Germany) for 1 min. The upper layer was collected and purified using a MicroSpin Column (Pharmacia,
UK).

**Ligation of DNA into pUC18 plasmid**

The recovered 2-7µl of purified sample were mixed with the following: 1 µl (50ng) of blunt-ended, Sma I digested and dephosphorylated linear plasmid pUC18 (Sma I/BAP), 10µl of T4 DNA ligase 2 x ligation buffer, 1µl of 0.1M DTT solution, 1µl of T4 DNA ligase, and ddH2O to 20µl. The solution was incubated at 16°C for 1-2 h. Alternatively, Promega T4 DNA ligase was also used. The recovered 2-5µl of purified sample were mixed with the following: 1µl (50ng) of blunt-ended, Sma I/BAP pUC18, 1µl of 10 x ligation buffer, 3µl of T4 DNA ligase (3µl/µl) (Promega,UK), and ddH2O to 10µl. The solution was incubated at RT for 3 h., or at 4°C for 16-18 h.

**Transformation of JM109 competent cells and screening for recombinants**

The frozen competent cells were removed from -80°C storage and placed on ice for 5 min. The tubes were gently mixed and 100µl of competent cells per transformation was placed in a 0.5 ml sterile pre-chilled PCR tube (on ice). Then, 10µl (1-50ng) of the recombinant plasmid were added to the cells and placed on ice for 10 min. The cells were heat shocked for 45-50 seconds in a water bath at 42°C, and the tube was immediately placed on ice for 2 min. Next, 400µl of S.O.C. medium (RT) were added and the tube was incubated at 37°C for 60 min. with shaking at approximately 225 rpm (Grant, Cambridge, England). Several aliquots of the transformation mixture (100-200µl) were then spread onto LB antibiotic plates (final concentration of ampicillin, 50µg/ml) with a top gel containing 40µl of 2% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and 40µl of 100mM Isopropyl β-D-thiogalactopyranoside (IPTG). The plates were kept on the bench for 30 min., before inverting and incubating
at 37°C overnight. Blue/white colour screening of colonies for recombinants was used.
The next day, white clones were transferred to new LB antibiotic plates and incubated at
37°C overnight. The recombinant clones were picked separately and grown in a 10ml
volume of LB medium containing ampicillin (100μg/ml) in 50ml Falcon tubes at 37°C in
a shaking water bath overnight. The plasmid was characterised by using techniques
described below.

2.13. Extraction of plasmid and detection of recombinant insertion

2.13.1. Extraction of plasmid

Two methods were used.

*The method was based on that of Birnboim and Doly (1979)*

A 1.5ml volume of overnight culture of plasmid-containing bacteria was removed
from the Falcon tube, placed into a 1.5ml microfuge tube and spun for 30 seconds. The
supernatant was discarded and the pellet re-suspended in 100μl of Solution I (50mM
glucose, 25mM Tris, pH 8.0, 10mM EDTA, pH 8.0). Next, 200μl of freshly made
Solution II (0.2N NaOH, 1%SDS) were added and the tube was kept on ice for 5 min.
A 150μl volume of Solution III (5M sodium acetate, pH 4.6) was added, the solution
was then mixed and the tube kept on ice for a further 5 min., after that it was centrifuged
at 13,000 rpm for 5 min. The supernatant was transferred to a new microfuge tube and
1ml of cold 100% ethanol (-20°C) was added, mixed, and kept on ice for 10 min. After
centrifugation for 3 min., the precipitated plasmid DNA pellet was re-suspended in 100μl
of Solution IV (50mM Tris-HCl, 100mM sodium acetate, pH 4.6) and reprecipitated by
adding 250μl of 100% cold ethanol. The precipitate was washed with 70% ethanol, air-
dried and resuspended in 15μl of ddH₂O.
Quantum Prep Plasmid Miniprep Kit (Bio-Rad)

A 1.5ml volume of an overnight culture of plasmid-containing bacteria was transferred to a tube and centrifuged at 13,000 rpm for 30 seconds. The supernatant was removed and 200µl of Cell Resuspension Solution (Quantum Prep Plasmid Miniprep Kit) were added to the pellet and mixed. A 250µl volume of Cell Lysis Solution (Quantum Prep Plasmid Miniprep Kit) was added and mixed by inverting the capped tube 10 times. Following addition of 250 µl of Neutralisation Solution (Quantum Prep Plasmid Miniprep Kit), the tube contents were mixed as above, and the tube was centrifuged at 13,000 rpm for 5 min. A white pellet formed along the inside or at the bottom of the tube. A 200µl volume of the well mixed Quantum Prep matrix was added directly to the white pellet, mixed gently by pipetting up and down twice, immediately transferred to a Spin Filter inserted in a 1.5ml microfuge tube and centrifuged at 13,000 rpm for 30 seconds. The eluant was discarded and the Spin Filter inserted in a 1.5ml microfuge tube and centrifuges at 13,000 rpm for 30 seconds. The eluant was discarded and the Span Filter was washed with 500µl of Wash Solution (containing 95% ethanol) twice by centrifuging at 13,000 rpm for 30 seconds and 2 min., respectively, to remove residual traces of ethanol. Next, the Span Filter was transferred to a fresh 1.5ml microfuge tube, 50-100µl of ddH2O were added and centrifugation at 13,000 rpm for 30 seconds was used to elute the DNA. The plasmid DNA was stored at -20°C until required.

2.13.2. Detection of the recombinants

The plasmid (pUC18 recombinants) containing PCR product and cDNA fragments inserts produced from both methods (Section 2.13.1) were digested by restriction enzymes EcoR I and Pst I (1µ/µg DNA) (Boehringer Mannheim, Germany).
at 37°C for 2-4 h. to release the inserted DNA. The presence and size of the insertions were determined by electrophoresis of the digested DNA through a 1% agarose gel at 100 volts for 1 h. The gel was visualised with UV illumination and photographed using an image analyser. The plasmid carrying cDNA fragments were used as templates for further PCR.

2.14. Bacterial culture for extraction and purification of the plasmid for sequencing

2.14.1. Bacterial culture

Clones were streaked onto freshly made LB agar plates containing ampicillin (100µg/ml), and incubated at 37°C overnight. The following day, a single isolated colony was picked and transferred into LB broth medium containing ampicillin (100µg/ml); either a 400ml volume in a sterilised 2L flask (large-scale) or into a 30 ml volume in a sterilised 100ml flask (small-scale). The cultures were incubated at 37°C overnight with shaking and then transferred to 50ml or 500ml Oak Ridge centrifuge tubes (Nalgene, Nalga Company, USA) and the bacteria was pelleted by centrifugation at 6,000 rpm for 20 min. at 4°C. The supernatant was discarded and DNA was extracted from the pellet as follows.

2.14.2. Extraction and purification of the plasmid for sequencing

2.14.2.1. Small scale

For small scale preparation of plasmid DNA, the QIAGEN Plasmid Kit (Qiagen Ltd. Germany) were used. The bacterial pellet was suspended in 4ml of buffer P1(50mM Tris-HCl, pH 8.0, 10mM EDTA, 100µg/ml RNase A), then 4ml of buffer P2 (200mM NaOH, 1%SDS) was added to lyse the cells followed by chilled buffer P3
(3.0M potassium acetate, pH 5.5). The tubes were incubated on ice for 15 min., and centrifuged at > 20,000g (MSE, Europa 24M, UK) at 4°C for 30 min. QIAGEN-tip-100 (column) was equilibrated with 4ml of buffer QBT [750mM NaCl, 50mM 3-N(Morpholino)propanesulfonic acid (MOPS), pH 7.0, 15% ethanol, 0.15% Triton X-100], and the column was then allowed to empty by gravity flow. The supernatant from the centrifugation step was loaded into the QIAGEN-tip. The QIAGEN-tip was then washed with 2 x 10ml of buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% ethanol), and the DNA was eluted with 5ml of buffer QF (1.25M NaCl, 50mM Tris-HCl, pH 8.5, 15% ethanol). After precipitation with a 0.7 volume of isopropanol, the DNA was washed with 2ml of 70% ethanol, air-dried for 5 min. and dissolved in 20-50μl of ddH₂O.

2.14.2.2. Large scale

For large-scale preparation of plasmid DNA, equilibrium centrifugation in Cesium Chloride (CsCl)-ethidium bromide gradients was used (Sambrook et al., 1989). The bacteria was harvested by centrifuging the culture at 6000 rpm (MSE, Europa 24 M, UK) at 4°C for 20 min. The pellet was resuspended in 10ml of Solution I (50mM glucose, 25mM Tris-HCl, 10mM EDTA). Next, 20ml of Solution II (1%SDS, 0.2N NaOH) was added and after mixing, kept on ice for 5 min. Then 15 ml of Solution III (3M sodium acetate, pH 4.8) was added, the solution was incubation on ice for 10 min. followed by centrifugation at 7,000 rpm for 20 min. at 4°C. After carefully retaining the supernatant and allowing it to warm to RT, a 0.6 volume of isopropanol was added and the mixture was kept at RT for 15-30 min. After centrifugation at 15°C (9000 rpm for 15 min.), the DNA pellet was washed with 1ml of 70% ethanol, air-dried for 10 min., and dissolved in 2.5ml of TE buffer (pH 8.0). Next, 2.50g of CsCl was added to the DNA solution followed by 0.25ml of ethidium bromide solution (10mg/ml), and after
mixing, the preparation was centrifuged at 13,000 rpm for 3 min. The supernatant was transferred to a 13x23mm Quick-Seal centrifuge tube (Beckman, USA) which was sealed by heating, and centrifuged at 80,000 rpm in a TLA-100.3 fixed angle rotor (Beckman TL-100 Ultracentrifuge, USA) for 24 h. at 20°C. The superhelical plasmid DNA from CsCl gradients was collected using a needle and syringe observed under UV light and the DNA sample was transferred to a plastic Bijoux bottle. An equal volume of isopropanol saturated with CsCl and ddH₂O was added, mixed, and left on the bench for several min. Next, the upper layer (pink-colour) was taken off, and an equal volume of isopropanol was again added. This procedure was repeated until the solution became clear. The DNA was precipitated with the addition of a 1/10 volume of 3M sodium acetate and 3 volumes of cold 100% ethanol, washed with 70% ethanol, air-dried, resuspended in 100μl of ddH₂O. The quantity of DNA was determined using spectrophotometry and its quality was assessed by electrophoresis through a 1% agarose gel. The DNA was stored at -20°C until required.

2.15. Bacterial culture storage and recovery

2.15.1. Short term storage

The colony containing recombinant plasmid pUC18 was scraped from the original plate and inoculated to a fresh LB plate with antibiotics, and incubated at 37°C overnight. The next day, a well-isolated single colony was picked with a sterile inoculating loop and streaked onto a fresh LB plate with ampicillin (100μg/ml) and incubated at 37°C overnight. The plate was sealed with parafilm and stored at 4°C for 2-3 months.
2.15.2. Long term storage

The colony containing recombinant plasmid pUC18 was scraped from the original plate and inoculated to a fresh LB plate with antibiotics, and incubated at 37°C overnight. The next day, a well-isolated single colony was picked with a sterile inoculating loop and grown in 10ml of LB culture medium in a 50ml flask with shaking at 37°C overnight. Afterwards, sterile glycerol was added to the bacterial culture at a ratio of 0.85ml of bacterial culture to 0.15ml of glycerol, mixed completely, transferred to 1.8ml sterile cryogenic vials (Corning, New York) in 1ml aliquots, snap frozen in liquid nitrogen, then the tubes were stored at -70°C for long term storage (up to 2 years) (Sambrook et al., 1989).

2.15.3. Recovery of long term storage bacteria

Immediately after remove from -70°C, the bacteria was scraped from the frozen surface of the storage culture with a sterile inoculating loop, and immediately streaked onto the surface of a LB agar plate containing antibiotics and incubated at 37°C overnight. The remaining frozen culture was returned to storage at -70°C.

2.16. DNA base sequencing

Five different methods were used for the sequencing of recombinant plasmid DNA or, direct sequencing of PCR products (based on the chain-termination DNA sequencing method, Sanger et al., 1977).

2.16.1. Sequencing PCR products by 5'-End-labelled Primer (Promega, USA)

Radiolabelling of oligonucleotide primers

Approximately 10pmol of PCR primer (Sense or Antisense), 2µl of 10 x T4
PNK buffer and 10pmol of $[\gamma^{32}\text{P}]$ dATP (Amersham Life Science, UK) were mixed in a microfuge tube, and then 2μl of T4 PNK (5-10u/μl) (Promega, USA) were added. The final volume was made up to 10μl by adding ddH$_2$O. The solution was incubated at 37°C for 10-30 min., then heated to 100°C for 2 min. to inactive the T4 PNK, and stored at -20°C up to one month before use.

**Extension/termination reaction**

The 0.5ml PCR tubes were prepared and labelled with G, A, T, C (standing for the Guanosine, Adenine, Thymidine and Cytidine) for each set, then 2μl of 2',3'-dideoxyguanosine 5'-triphosphates (ddGTP), 2',3'-dideoxyadenosine 5'-triphosphates (ddATP), 2',3'-dideoxythymidine 5'-triphosphates (ddTTP) or 2',3'-dideoxycytidine 5'-triphosphates (ddCTP) was added into the tubes labelled with G, A, T and C, respectively and kept on ice or 4°C. Then, the following were mixed in a fresh tube: 4-40pmol of template DNA, 5μl of 5 x sequencing buffer, 1.5μl of labelled primer, and ddH$_2$O was added to a final volume of 16μl. Next, 1μl of Taq DNA Polymerase (5u/μl) (Promega, USA) was added to the mixture and this was distributed in 4μl aliquots to each set containing one of the four 2',3'-dideoxyribonucleotide 5'-triphosphates (ddNTPs). The reactions were performed in a thermal cycler using the programme appropriate for each PCR using the same template and primers as before. After each reaction, 3μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, and 0.05% Xylene cyanol FF) were added and this was stored at -20°C (if necessary) before loading onto the sequencing gel.
2.16.2. Direct sequencing of PCR products using the Sequenase PCR Product Sequencing Kit (Amersham Life Science, UK)

Enzymatic pre-treatment of PCR product

The PCR product was treated with Exonuclease I and Shrimp Alkaline Phosphatase. The Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase removes the remaining dNTPs from the PCR mixture which would interfere with the labelling step of the sequencing process. A 1 μl aliquot of the Exonuclease I (10.0u/μl) and 1 μl of the Shrimp Alkaline Phosphatase (2.0u/μl) were added to a maximum of 5 μl of PCR amplification mixture in a 0.5ml PCR tube, creating a total volume of 7 μl. The solution was mixed, placed in a thermal cycler, incubated at 37°C for 15 min., and then heated to 80°C for 15 min. to inactive the enzymes.

Annealing reaction

The treated PCR product (1-9 μl, 0.5pmol) was transferred to a new 0.5 ml fresh PCR tube, 1 μl of primer (5-10pmol/μl) was added and ddH2O was added to adjust the total volume to 10 μl. The DNA was denatured by heating for 2-3 min. at 100°C in a thermal cycler, then quickly cooled by placing the vial directly in an ice-water bath for 5 min. The reaction solution was centrifuged briefly and kept on ice before use.

Labelling reaction

To the ice-cold annealed DNA mixture (10 μl), the following was added: 2 μl of 5 x reaction buffer (200mM Tris-HCl, pH 7.5, 100mM MgCl2, 250mM NaCl), 1 μl of
0.1M DTT, 2µl of 1:5 diluted labelling mixture (7.5µM dGTP, 7.5µM dCTP, 7.5µM dCTP), 0.5µl of [α-32P] or [α-35S]dATP (10µCi/µl), and 2µl of Sequenase version 2.0 T7 DNA Polymerase (1.6u/µl with 2u/µl inorganic pyrophosphatase in 20mM Tris- HCl, pH 7.5, 2mM DTT, 0.1mM EDTA, and 50% glycerol) for a total volume of 17.5µl. The mixture was thoroughly mixed and incubated for 2-5 min. at RT or up to 15 min. on ice.

**Termination reaction**

Termination tubes were labelled G, A, T, and C, and filled respectively with each termination mixture, i.e.; ddG (80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 50mM NaCl), ddA (80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 50mM NaCl), ddT (80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 50mM NaCl), and ddC (80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 50mM NaCl). The tubes were warmed to 37°C at least 1 minute before the termination reaction was started. When the labelling incubation was completed, 3.5µl of labelling mixture were transferred to each of the tubes labelled G, A, T and C. After mixing, the tubes were returned to 37°C for a further 5-10 min. incubation. When the termination reactions were finished, 4µl of stop solution were added to each of the tubes, mixed thoroughly and stored on ice ready to load onto the sequencing gel. Samples labelled with 35S could be stored at -20°C for 1 week before use, but samples labelled with 32P were used the same day.
2.16.3. Sequencing of PCR products with biotin-labelled primer, after treatment with Dynalbeads M-280

**Annealing and labelling reaction**

To 9µl of PCR product (from section 2.10), 1µl of primer (TCRF5) was added, heated at 100°C for 3 min., then immediately cooled on ice. Then 2µl of 5 x reaction buffer, 1µl of 0.1M DTT, 2µl of 1:5 diluted labelling mixture, 1µl of [α-32P] dATP, and 2µl of T7 DNA Polymerase (Amersham Life Science, UK) were added to the denatured DNA, mixed and left on ice for 15 min.

**Termination reaction**

This reaction was performed as described in section 2.16.2.

When the termination reactions were finished, 4µl of stop solution were added to each termination reaction tube, and mixed thoroughly and heated at 72°C for 2 min. The beads were then collected by placing the tube in the MPC-E and the supernatant (containing single stranded DNA termination products) was removed and transferred into new tubes ready for loading onto the sequencing gel.

2.16.4. Sequenase Sequencing Kit Version-II (Amersham Life Science, UK)

**Denaturing reaction**

Purified plasmid with insertions was used. Approximately 3-5µg of plasmid DNA were denatured by adding a 0.1 volume of 2M NaOH and 2mM EDTA and incubating at 37°C for 30 min. The mixture was neutralised by the addition of a 0.1 volume of 3M sodium acetate (pH 4.5-5.5). The DNA was precipitated with 2-4 volumes of 100% ethanol at -70°C for 15 min. and recovered by centrifugation at
13,000 rpm for 15 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 7μl of ddH₂O prior to performing the next step.

Annealing reaction

To the 7μl of denatured plasmid DNA, 1μl of M13 primer (either M13-40 forward, or M13-17 reverse) and 2μl of 5 x reaction buffer were added. The mixture was incubated at 65°C for 2 min., then kept in a small beaker of water initially at 65°C, but allowed to cool slowly over a period of 30 min. on the bench to a temperature of approximately 35°C.

Labelling reaction

The labelling reaction was performed by adding 1μl of 0.1M DTT, 2μl of 1.5mM labelling mixture, 0.5 μl of [α-³²S] dATP or 0.5 μl of [α-³²P] dATP, and 2μl of Sequenase Version 2.0 T7 Polymerase to the ice-cold annealed DNA mixture (10μl), and incubating for 15 min. on ice.

Termination reaction

This reaction was performed as described in section 2.16.2.

When the termination reactions were finished, 4μl of stop solution were added to each of the tubes, mixed thoroughly and stored on ice ready to load onto the sequencing gel. Samples labelled with ³⁵S could be stored at -20°C for 1 week before use, but samples labelled with ³²P were used the same day.
2.16.5. Cycle sequencing using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer, USA)

For each reaction, 250ng of double stranded recombinant plasmid (pUC18), 3.2pmol of sequencing primers (M13-17 reverse or M13-40 forward), and 8μl of reaction mixture [A-Dye terminator, G-Dye terminator, T-Dye terminator, C-Dye terminator, dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0), MgCl₂, thermal stable pyrophosphatase, and AmpliTaq DNA Polymerase, FS] were mixed together and made up to 20μl with ddH₂O. The sequencing reactions were performed in a thermal cycler (40 wells, Techne, USA) as follows: one cycle of 5 min. at 96°C; and 25 cycles of 95°C for 30 seconds, 45°C for 15 seconds, and 72°C for 4 min. The PCR products were purified using the ethanol precipitation protocol. To each tube, 2μl of 3M sodium acetate, pH 4.6, and 50μl of 95% ethanol were added, mixed, and placed on ice for 30 min., or placed at -80°C for 15 min. The mixture was centrifuged at 13,000 rpm for 15-30 min. at RT, and the supernatant was carefully removed. The DNA pellet was rinsed with 250μl of 70% ethanol and after spinning, the supernatant was discarded and the pellet dried at 55°C in a thermal cycler. Next, the pellet was resuspended in 6μl of loading buffer [deionized formamide and 25mM EDTA (pH 8.0) containing 50mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran]. Samples were sent to the Royal Devon and Exeter Hospital for sequencing in their ABI 373 (34 wells) DNA Sequencer (Perkin Elmer, USA).

2.17. Preparation and electrophoresis of manual sequencing gels

Glycerol tolerant gel buffer was used in electrophoresis. The buffer was made in 20 times concentration by adding Tris-base (216g), Taurine (72g), and EDTA (4g) to
ddH$_2$O to a final volume of 1,000ml and autoclaved.

The Bio-Rad Sequence Gen II electrophoretic system (Bio-Rad, USA) was used employing 21x40cm glass plates with 0.25cm spacers and with a 24 space sharktooth comb. Before use, the glass plates and spacers were washed in warm detergent solution, rinsed thoroughly in tap water, followed by ddH$_2$O, and finally rinsed with 70% ethanol and set aside to dry in air. The inner surface of the backglass plate was siliconised by treatment with a small amount of Repelcote (2% solution of dimethyldichlorosilane in octamethylocyclotetra-siloxane) (BDH,UK). Excess Repelcote was wiped off with a piece of paper towel, and the plate was dried in air (This prevents the gel from sticking tightly to both plates and reduces the possibility that the gel would tear when it was removed from the plates after electrophoresis). Some Vaseline was wiped along the sides of the back plate, and then two spacers were put over the Vaseline. The front plate was laid on top of the back plate and the two plates were clamped together. Next, the plates were positioned in the casting tray and the whole apparatus was stood at an angle of approximately 45°.

Denaturing gels were made by either of the following ways.

*Flowgel ready-made liquid (National Diagnostics, USA)*

A 6-8% sequencing denature gel was made by adding 7.5ml of 20 x glycerol tolerant gel buffer, 7.5ml of ddH$_2$O, 99ml (6% gel) or 87ml (8% gel) of Ultra Pure Sequagel concentrate solution (237.5g acrylamide, 12.5g methylene bisacrylamide and 500g urea) and 36ml (6% gel) or 58ml (8% gel) of Ultra Pure Sequagel diluent (500g 8.3M urea).

*Bio-Rad 19:1 acrylamide/bisacrylamide liquid*

The urea (63g) was dissolved in 40ml of ddH$_2$O, then 7.5ml of 20 x glycerol
tolerant gel buffer and 30ml of 40% acrylamide/bis mix (19:1) were added and the final volume adjusted with ddH₂O to 150ml, mixed and degassed under vacuum.

Once the gel mix was prepared as above (either Flowgel or Bio-Rad), 50ml were transferred into a 100ml flask, and 0.8ml of 10% ammonium persulfate (APS) (Sigma) and 40μl of N,N,N',N'-Tetramethylenediamine (TEMED) (National Diagnostics, USA) were added, mixed and, immediately poured into the casting tray to seal the bottom of plates to prevent leaking. Following polymerisation, 1ml of 10% APS and 50μl of TEMED were added to the remaining gel mix, which was loaded immediately using a 50ml syringe with a filling tube and the solution was pushed into the gel forming apparatus between the two plates. During the procedure air bubbles were carefully removed by gentle tapping of the glass. When the gel level reached the top, the flat side of the comb was inserted halfway into the space between the plates. When the gel was set, the casting tray was removed, and the apparatus was placed vertically into the lower buffer tank and half-filled with 1x glycerol tolerant buffer. Next, the apparatus was connected to a constant power supply (ECPS, 3000/150, Pharmacia), and the gel was subjected to 1,600 volts for about 30min. until the temperature of the gel reached 50-55°C. Afterwards the gel was disconnected from the power, the comb was removed and re-inserted with sharktooth side to generate wells. The sequencing samples (2.5-3μl) were pre-heated at 75-80°C for 2 min. before loading into the wells in the order G, A, T and C. The gel was electrophoresised for 1.5 to 4 h. After electrophoresis, the gel was soaked in 5% acetic acid and 15% methanol for 5-15 min. to remove the urea. The gel was dried at 75°C for 2-4 h. in a vacuum machine (HETO Lab Equipment, Denmark) and placed next to film (Kodak, USA) for autoradiography in a Cronex cassette at -80°C for 4-36 h. depending on the intensity of the radiolabelling. After developing the film the sequences were read on a light box.
2.18. Sequence analysis

All of the sequences were analysed using the DNASTAR software package (LASERGENE, USA) and compared with EMBL, GenBank and Prito Swiss Protein Databases. The sequences of interest were analysed further for alignment and a phylogenetic tree was constructed.

2.19. Southern blotting and hybridisation

2.19.1. Restriction enzyme digestion of fish Genomic DNA

Dogfish genomic DNA (5-10μg) was digested in 1.5ml microfuge tubes using 10-20 units of restriction enzymes, EcoR I, Hind III, or Bgl II (Boehringer Mannheim, Germany). Reactions were performed in a total volume of 30μl, using restriction enzyme buffer, which was supplied with the enzyme by the manufacturers, and ddH2O to make up the final volume. Digests were incubated at 37°C for 16 h. The efficiency of digestion was checked by running a 3μl aliquot of each digest together with 1.5μl of loading/track dye and 7μl of ddH2O in a 0.6% horizontal slab/agarose gel for 1 h. If the digestion was incomplete, 5-10 more units of enzyme were added in 3μl of 1 x buffer and incubated for a further 4 h. or up to 3 days. The enzyme volume in the reaction was always kept below 10% to prevent non-specific cleavage of DNA due to high concentration of glycerol, present in the enzyme preparations.

2.19.2. Separation of DNA fragments by agarose gel electrophoresis

DNA fragments were separated by electrophoresis in 0.6% horizontal slab agarose gels; 2.1g of agarose were added to 350ml of 0.5 x TBE buffer in a flask or glass bottle and heated in a microwave oven (800 watts) for 2-3 min. to dissolve the
agarose. The solution was cooled to 60-65°C and 35μl of ethidium bromide (10mg/ml) were added and mixed by swirling. The agarose solution was poured into a 20x24 cm tray and a 20 or 28 toothcomb was used to generate sample wells. After the gel was set, it was placed at 4°C to harden the agarose. Next, the gel was submerged in 0.5 x TBE buffer in the electrophoresis tank and the comb was carefully removed. The digested DNA were mixed with a 1/10 volume of sample loading buffer and loaded onto the gel. A lambda DNA Hind III digest, or 1kb DNA ladder was run in a separate lane to provide molecular weight markers. The gel was run for 16-20 h. at a constant voltage of 70. The migration of the DNA was checked by visualising the separation of the lambda markers and 1kb DNA ladders under UV light, and a photograph was taken. Next, the gel was soaked in denaturation/transfer solution (0.4N NaOH, 1M NaCl) for 15 min. to denature the DNA with shaking. This was repeated once and the gel was ready for transfer of DNA.

2.19.3. Setting up a capillary blot

The method was based on Southern blotting (Southern, 1975). First, the blotting station was assembled: 2 sheets of 3 MM Whatman chromatography paper were placed over a 30x20cm plastic support with the edges of the paper hanging down into a plastic container containing 0.4N NaOH (denaturing buffer) to act as a wick. The 3MM paper was wetted with 0.4N NaOH and any air bubbles were smoothed out with a glass rod. Then, the gel was removed from the electrophoresis tank, trimmed to remove unused areas, the top right hand corner of the gel was cut and this allowed the gel to be orientated during the procedure. The gel was inverted and placed on the support, centred on the wet 3MM papers and any air bubbles between the gel and the paper were removed as before. The blotting station was covered with Saranwrap, leaving the area over the gel free, this served as a barrier to prevent liquid from flowing directly from
the reservoir of 0.4N NaOH to the paper towels placed on top of the gel, causing a short
cut circuit in the system. The dimensions of the gel were used as a template to cut the
Hybond Nylon N' membrane (Amersham Life Science, UK) and the membrane was
then placed on top of the gel and smoothed out. Two sheets of Whatman 3MM paper
pre-moistened with 0.4N NaOH were placed on top of the nylon membrane and
smoothed out as before. Two parallel stacks of paper towels were placed on top of the
sheets of 3MM Whatman paper, and a glass plate and 500g weight were placed on top
of the paper towels. The gel was blotted overnight. Next day, all paper towels were
removed and the nylon membrane was washed for 10 min. in 3xSSC at RT and the gel
was discarded. After being labelled and washed, the nylon membrane was placed
between 2 sheets of 3MM Whatman paper and blotted gently, the membrane was then
wrapped in Saranwrap and stored at 4°C until probed.

2.19.4. Isolation of probe DNA from plasmid

About 10μg of recombinant plasmid DNA were digested with restriction
enzyme EcoR I and Pst I (10-20u) in a 10μl volume in 1.5ml microfuge tubes held at
37°C for 2-4 h. After digestion, the samples were loaded onto a 1% agarose gel or 1%
low melting-point agarose gel. Electrophoresis was performed in a mini-gel tank in
0.5% TBE buffer or 1 x TAE buffer with 10μl of ethidium bromide (10mg/ml). The gel
was run at 75 volts for 1-2 h. The identified DNA was cut out of the gel using a sharp
scalpel whilst visualised under UV. Then the DNA was purified by one of the methods
in section 2.10.

2.19.5. Radiolabelling DNA probes (Random primer method)

The probe was labelled with [α-32P] dCTP, using a random primer DNA
Labelling Kit (Boehringer, UK). Approximately 25ng of DNA were denatured by heating at 100°C for 10 min. and cooling on ice. Next, 1μl of dATP (0.5mM/l), 1μl of dTTP (0.5mM/l), 1μl of dGTP (0.5mM/l), 2μl of reaction mixture, and 5μl of [α-32P]dCTP (3,000Ci/mmol) were added and mixed. Then 1μl of Klenow fragment (2u/μl) was added and the tube was incubated at 37°C for 30 min. The labelled probe was then purified as follows.


Labelled DNA was separated from unincorporated [α-32P]dCTP by column chromatography through a Sephadex G-50 column (Sambrook et al., 1989). The Sephadex G-50 was prepared by slowly adding 30g of Sephadex G-50 to 250ml TE buffer (pH 7.6) in a 500ml bottle. The bottle was left to stand at RT overnight or heated to 65°C for 1-2 h., then cooled to RT. The supernatant was decanted and replaced with an equal volume of TE buffer (pH 7.6) to equilibrate the resin.

The Sephadex G-50 column was prepared in a disposable glass-silicated Pasteur pipette plugged with a small amount of sterile glass wool. A long sterile bio-loop was used to push the wool to the bottom of the glass pipette.

The column was filled with a slurry of Sephadex G-50. Care was taken to avoid producing air bubbles and to ensure that the Sephadex G-50 was well mixed and not separated into resin and TE buffer. The Sephadex G-50 was continually added until it packed to a level 1 cm below the top of the column.

The labelled DNA sample was mixed with 180μl of TE buffer (pH 7.4) and loaded onto the top of the column. As soon as the DNA sample had entered the Sephadex G-50 a further 180μl of TE buffer was added to wash the DNA sample through the column. The sample was repeatedly washed through the column with a
series of additional 180μl aliquots of TE buffer. Fractions of the eluted liquid from the column were immediately collected in 1.5 ml plastic tubes. A total of 12 tubes were collected with approximately 180μl in each.

The specific activity was determined using a multiple purpose scintillation counter. The whole of the collected sample was counted. Collecting the unincorporated nucleotides allows the labelling efficiency to be calculated. Incorporation was routinely 70%, with specific activities of 10^5 cpm/μg. The nucleotides are smaller molecules than DNA molecules and therefore pass through the holes in the Sephadex G-50 beads and through the column at a much slower rate than the DNA molecules that pass between the beads and through the column at a faster rate. Because of this, the leading peak of radioactivity consists of nucleotides incorporated into DNA, and the tailing peak consists of unincorporated [α-32P] dCTP. The radioactive fractions in the leading peak were pooled and stored at -20°C and used within three days.

2.19.7. Hybridisation of radio-labelled probes to immobilised nucleic acids

Hybridisations were performed in a sealed glass tube within a purpose built hybridisation oven (Techne, USA). According to the size of the membrane used, two different sizes of cylindrical siliconised borosilicate glass tubes were used. One was 20cm in length and 3cm in diameter, another was 20cm in length and 8cm in diameter, both were sealable with screw caps. Two methods were employed in the hybridisation of radiolabelled probes to immobilised DNA and RNA.

2.19.7.1. Hybridisation with home-made-hybridisation buffer (Sambrook et al., 1989)

Before exposure to the radioactive probe, the membranes were pre-heated at 65°C for 1-4 h. in 12ml (for 20x8cm tube) or 6ml (for 20x3cm tube) of hybridisation
buffer [6 x SSC, 0.5% SDS, 5 x Denhardt's solution (50x=5g Ficoll, 5g polyvinylpyrrolidone, 5g bovine serum albumin. in 500ml of ddH₂O, Denhardt, 1966), 5% Dextran sulphate and 0.2mg/ml sonicated salmon sperm DNA]. The labelled probe > (5x10⁶) cpm was denatured for 5 min. at 100°C, placed on ice for 10 min., added to the hybridisation tube, and incubated overnight. The next day, the membrane was washed with 60ml of preheated (65°C) 2 x SSC, 0.3% SDS for 10 min., changed to 1 x SSC, 0.3% SDS twice for 10 min.

2.19.7.2. Hybridisation with commercial Rapid-hyb buffer (Amersham Life Science, UK)

Rapid-hyb buffer contains chemical blocking agents. This removes the requirements for heterologous DNA to prevent non-specific binding of the probe to the membrane. A 10ml (for 20x8cm tube) or 5ml (for 20x3cm tube) aliquot of the rapid hybridisation buffer was pre-warmed to 65°C in a hybridisation oven, and then the filter was immersed completely in the buffer at 65°C for at least 15 min. with rotation. The 32P random primer labelled DNA probe was boiled for 5 min. and placed on ice for 5 min. Next, the probe was added to the hybridisation tube at a concentration of >5x10⁶ cpm per filter and hybridisation was carried out at 65°C for 2-4 h. with continuous rotation. Post hybridisation washings were carried out to remove non-specifically bound probe. The membrane was washed with 50ml of the washing solution 2 x SSC, 0.1% SDS at RT for 20 min. Then the membrane was washed twice with 1 x SSC, 0.1% SDS at 65°C for 15 min. for each time.

Finally, excess liquid was removed by blotting the membranes between two sheets of Whatman 3MM paper. The damp membrane was wrapped in Saranwrap and placed in Cronex cassettes between Cronex lightening plus intensifying screens. In a dark room the membrane was covered with a sheet of Kodak XAR5 X-ray film,
(Kodak, USA) and the cassette was firmly closed and stored at -80°C for 7 days. After developing, the film was analysed on a light box.

2.20. Northern blotting and hybridisation

2.20.1. Separation of total RNA by agarose gel electrophoresis

Approximately 5µg of RNA in a volume of 6 µl were mixed with 12.5µl of formamide (deionized by mixing 1.0g of bed resin with 10ml of formamide and stirring for 10-20 min., the mixture was filtered through filter paper, and aliquoted in 1ml amounts and stored at -20°C), 2.5µl of 10 x MOPS buffer, and 4µl of 37% (v/v solution) formaldehyde. The mixture was incubated at 65°C for 5 min., placed on ice for 2 min., and 2.5µl of Loading buffer [50% (v/v) glycerol containing 0.1mg/ml bromophenol blue] was added. A RNA Molecular Weight Marker II (Boehringer Mannheim, Germany) 5µl of a 1mg/1ml stock treated as above, was used for standardisation with the migration of the RNA samples.

A 1-1.5% agarose gel was prepared as follows: 1-1.5g of agarose, 10ml of 10 x MOPS buffer, 73ml of ddH₂O were placed into a flask or glass bottle, heated in a microwave oven (800 watts) for 2-3 min. to allow the agarose to dissolve and then cooled to 50°C. Next, 17ml of 37% formaldehyde was added, mixed and the gel solution poured into a 11x14cm tray with a 14-tooth comb in a fume hood. After the gel was set, the tray was immersed in a electrophoresis tank containing 1 x MOPS buffer and the comb was carefully removed. RNA samples and the RNA marker (7.4/5.3/2.8/1.9/1.6kb) were loaded into the wells. The gel was run at 100 volts for 3-4 h. until the tracking dye had migrated approximately 8 cm. When the electrophoresis was complete, the RNA marker lane was cut off and stained with 5µg/ml ethidium
bromide in 1xMOPS buffer for 5 min. in the dark with gentle shaking, then destained in ddH₂O for 2 h. in the dark with gentle shaking. A ruler was placed alongside the gel and a photograph was taken under UV light.

2.20.2. Northern blotting

A blotting station was assembled as for Southern blotting (Section 2.19.3) except the transfer buffer used was 20 x SSC or 20 x SSPE. After blotting, the nylon membrane was removed and washed for 10 min. in 3 x SSC at RT and the gel was discarded. After washing, the nylon membrane was baked in an oven (Techne, USA) at 80°C for 2 h. to fix the RNA. Finally, the membrane was placed between 2 sheets of 3MM Whatman paper, wrapped in Saranwrap and stored at 4°C until probed.

2.20.3. Hybridisation of radio-labelled probes to immobilised nucleic acids

The probe used in Northern hybridisation was isolated from recombinant plasmid (Section 2.19.4), radiolabelled (Section 2.19.5) and separated (Section 2.19.6) by the same method given in Southern hybridisation. The Northern hybridisation was also performed in the same way as the Southern hybridisation (Section 2.19.7).
CHAPTER 3. ISOLATION AND CHARACTERISATION OF TCR GENES FROM FISH

3.1. Amplification of TCRBV region from dogfish, trout and carp

A set of primers was designed from the published TCRB gene HF6 sequence (Fig. 3.1) of the horned shark (Rast and Litman, 1994) and flanked highly conserved TCR and Ig light chain motifs WYRQ and YYCA. These primers were named as TCRFl (sense) and TCRRI (antisense). The sequences were as follows:

TCRF1 5' - ATGTACTGGTATCGCCAGCAGAG;
TCRR1 5' - CTAGCAGCACAGTAAATACACCGC.

The expected PCR products were approximately 190 bp. To determine the optimum conditions for PCR, a Mg++ titration was performed (1.5mM to 4mM MgCl₂/per reaction) whilst the annealing temperature was adjusted in a stepwise manner (from 40°C to 55°C). Finally, PCR was performed at 94°C for 1 min.; at 45°C for 2 min.; at 72°C for 2 min. for 35 cycles (the same PCR conditions were used in following sections unless stated otherwise). Multiple PCR products were obtained from dogfish, trout and carp genomic DNA and cDNA prepared from lymphocytes. The results are shown in Fig.3.2. PCR products ranged from 200 to 1,000 bp in size. The amplified PCR products from trout cDNA are in lanes 3 and 4, where the Mg⁺⁺ concentration was 2mM and 3mM respectively. A weak band of approximately 200 bp and a second band of 600 bp were obtained. The 600 bp band is stronger at 3mM Mg⁺⁺ concentration compared to 2mM Mg⁺⁺ concentration. There was a weak PCR product at ~250 bp from carp cDNA. Two bands of approximately 600 and 1000 bp were obtained from dogfish cDNA shown in lanes 6 and 7. A doublet in the region of 400 bp was obtained following amplification of trout genomic DNA (lanes 8, 9 and 10). Finally, the last four lanes were PCR...
Fig. 3.1. Nucleotide and predicted amino acid (numbered 1-239) sequence of horned shark TCRB chain from cDNA clone HF6 (Rast and Litman, 1994). Regions of the nucleotide sequence that correspond to the primers TCRF1and TCRR1 are in red, primers TCRF2 and TCRR2 are in green and primers TCRF3 and TCRR3 are in blue, respectively.

Leader→ Variable→
M F L H S P V Q E R S R Y P V G N R L T V A E
ATCTCACAATTGCTTGGAAAAACTTACAGGCTCTTACAGC AAAGTTTGACCTGA

168
Fig. 3.2. Multiple PCR products were obtained from dogfish, trout and carp genomic DNA and cDNA prepared from lymphocytes. The primers used in the PCR were TCRF1 and TCRR1. The Mg$^{++}$ concentration is shown at the top of each lane. The 123 DNA ladder was used in lane 1. Control sample (without template) was in lane 2. The PCR products from trout cDNA was in lanes 3 and 4. Lane 5 was loaded by the PCR products from carp cDNA. The PCR products from dogfish cDNA were in lanes 6 and 7. Lanes 8-10 were PCR products from trout genomic DNA. Lanes 11-14 were the PCR products from dogfish genomic DNA.
products derived from dogfish genomic DNA. A Mg$$^{+}$$ concentration of 1.5mM gave rise to a single band of approximately 190 bp. However, when the Mg$$^{+}$$ concentration was increased from 2mM to 4mM three bands of approximately 190, 250 and 600 bp were obtained (last three lanes). The appropriate bands (~190 bp) from lanes 11 to 14 were excised from agarose gel and purified. Products were reamplified using 1.5mM Mg$$^{+}$$, annealing temperature at 48°C for 2 min. A very strong single band was obtained (Fig. 3.3) and sequencing was performed using the 5'-end-labelled primer protocol. It was only possible to obtain sequences of approximately 100 bp. Two sequences are shown in Fig. 3.4. These two sequences were derived from the same PCR template with different sequencing reactions. There are sequence similarities between them. Both primers and open reading frame (ORF) were absent in these sequences. These sequences were compared to the GenBank and EMBL databases, and there was no homology with any members of the Ig gene superfamily. Therefore, a second set of primers was designed using the horned shark TCRBV gene sequence then used for amplification of the TCRBV region. The primers were named TCRF2 and TCRR2

$$5'$$ (sense)-GCAGAGGGGAACGCTGGAA; 
$$5'$$-(antisense)-AAGTCGCTGACGAGGCAGACCA (Fig. 3.1).

The expected PCR products were approximately 420 bp (excluding the intron). Two bands of approximately 400 bp were obtained from dogfish genomic DNA and approximately 500 to 600 bp were obtained from trout genomic DNA (Fig. 3.5). When Mg$$^{+}$$ concentration was at 4mM, no PCR product was obtained from either dogfish or trout genomic DNA. It was unknown if the amplified fragments contain an intron sequence, therefore, all four bands were purified from 1% agarose gel to provide templates for re-PCR and further sequencing. Despite many attempts, it proved impossible to re-PCR any of the fragments. It was, therefore, decided to repeat the PCR using genomic DNA as the template and the subsequent small amount of purified DNA
Fig. 3.3. The single PCR product was obtained from the template which (~190) was removed from 1% agarose gel and purified. The primers used in PCR were TCRF1 and TCRR1. A 123 DNA ladder was used in lane 1. Lane 2 was control sample (without DNA). Lanes 3-5 were PCR products in the same condition.
Fig. 3.4. Nucleotide sequences of the PCR product (Fig. 3.3) using 5'-end-labelled primer protocol. Two examples are showed in follows. Sequence 1 is in black and 99 bp long, while sequence 2 is in red and 117 bp long.
Fig. 3.5. Multiple PCR products were obtained from dogfish (lanes 2-8), trout (lanes 9-14) genomic DNA. The primers used in PCR were TCRF2 and TCRR2. A 123 DNA ladder was used in lane 1. Lane 2 was control sample. The Mg$^{++}$ concentration was indicated at the top of each lane.
for direct sequencing. Any resulting sequence would then be used to design additional primers specific for that particular species. The DNA sequencing of the template gave unreliable results and it was therefore not possible to continue with this strategy. Attempts were made to amplify the TCR genes using the combined primers TCRF1 and TCRR2 or TCRF2 and TCRR1 with expected products of approximately 370 and 250 bp respectively (excluding the intron) (Table 3.1). Several bands from 200 to 600 bp were obtained from dogfish and trout genomic DNA using these primers (Fig. 3.6). A single re-PCR product of approximately 250 bp was obtained from trout genomic DNA with primers TCRF2 and TCRR1 (Fig. 3.7) and sequenced. Again, the sequencing results were inconsistent.

The likely reason for the inconsistency of the sequencing results is the heterogeneity of the PCR products because they were obtained from the TCRV regions. Amplification of the conserved TCRC region might be more successful than the TCRV region. Therefore, a third set of primers for the TCRBC region was used as follows.

3.2. Amplification of TCRBC region from dogfish, trout and carp

Two primers TCRF3 (sense) and TCRR3 (antisense) were designed from the known horned shark TCRB sequence (Rast and Litman, 1994). The primer positions are shown in Fig 3.1. The primer sequences were

TCRF3 5'-GGAGATGGGACCAACTGGTTG;
TCRR3 5'-GCAGATGAGGATCAGGTATGTC.

The expected PCR products were approximately 450 bp (excluding the intron). PCR reactions were performed using cDNA prepared from dogfish, trout and carp lymphocytes as template in order to circumvent problems due to intron incorporation. The multiple sized bands ranging from 130 to 500 bp were obtained from the dogfish, trout and carp cDNA (Fig. 3.8). A band corresponding to 500 bp from dogfish was
Table 3.1. The combination of primers TCRF1, TCRF1, TCRF2 and TCRR2 was used in PCR to generate TCRBV genes from dogfish

<table>
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<th>Primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRF1+TCRR2</td>
<td>~370</td>
</tr>
<tr>
<td>TCRF2+TCRR1</td>
<td>~250</td>
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</table>
Fig. 3.6. Multiple PCR products were obtained from dogfish and trout genomic DNA. The primers used in PCR were TCRF2 and TCRR2 or TCRF2 and TCRR1 or TCRF1 and TCRR2. The 123 ladder DNA marker was used. T is for trout genomic DNA, D is for dogfish genomic DNA. Mg$^{2+}$ (mM) titration is from 1.5mM, 2mM, 2.5mM and 3mM for each group from the right to the left wells. Each group included four wells.
Fig. 3.7. A single band of approximately 250 bp was obtained when re-PCR was performed. The template was PCR product from trout genomic DNA. The primers were TCRF2 and TCRR1. The Mg$^{+}$ concentration was 1.5mM (lane 3) and 2mM (lane 4) respectively. Lane 1 was the 123 DNA ladder and lane 2 was control sample.
Fig. 3.8. Multiple PCR products were obtained from carp (lanes 2-5), dogfish (lanes 6-8) and trout (lanes 9-11) cDNA. The primers used in PCR were TCRF3 and TCRR3. The 123 DNA ladder was used in lane 1. The Mg$^{++}$ concentration was 1.5 to 3mM indicated at the top of each lane.
excised from agarose gel and purified. Re-amplification was performed but despite many attempts, it was difficult to obtain a clear and reproducible single band. It was therefore decided to generate a PCR product from cDNA of dogfish again and the PCR product was separated on a 1% agarose gel. The sequencing was performed using the Sequenase PCR Product Sequencing Kit or 5'-end-labelled primer. Unfortunately, sequencing results were still inconsistent.

3.3. Amplification of TCRBV region from dogfish using biotin-labelled primer

The principal reason for the inability to generate sequence from the PCR products was probably the quality and heterogeneity of the sequencing templates. Therefore a set of primers labelled with biotin was constructed from a known trout TCRB sequence (Partula et al., 1994) (Fig. 3.9) and used for amplification of TCRBV region from dogfish and trout genomic DNA. An internal primer was prepared for sequencing. The primer sequences were as followings:

TCRF4 5'(sense)-CCAGGAGAAGGAA TGAAGCAGG;

TCRR4 5'(antisense)-*QGGTGAGTTTGGTGCCGGCTCCA;

TCRF5 5'(sense) -GCAGGTA@CTTTCATTAC for sequencing.

The expected PCR products were approximately 230 bp (excluding the intron). A single band was obtained from dogfish genomic DNA approximately 300 bp (Fig. 3.10). A PCR product at approximately 150 bp was obtained from trout genomic DNA. The PCR products were purified by magnetic beads coated with streptavidin, and sequencing was performed using either the Sequenase PCR Product Sequencing Kit or 5'-end-labelled primer. The primer TCRF5 was used in the sequencing reaction. These sequences were also inconsistent.

*Q=Biotin
Fig. 3.9. Nucleotide sequence of trout TCRB chain variable region from cDNA clone TCRBV1 (Partula et al., 1994). Regions of nucleotide sequence corresponding to the primers TCRF4 and TCRR4 are in red, primer TCRF5 is underlined.

TCRBV1
TACAGACAGCTTCCAGGAGAAGGAATGACAGGGTAGCTTTCATATTACTCCAGTTCTCA
ACCTGACTATTCAGGGGAATTCAGTAAAGACAAATACCCAACTGTTAAGGCTGTAGCTG
AGAGTGGGATCTTTTCACAGTGAAGAGTTGGAGACAGGAGACAGTGGAATGTACTTCTGT
TCRB
GCTGTTGAGTGAATTGGACAGGGGAGTGACCTGAGCCATTTTTGGAGCCGGCACAAACCTC
ACCTTTTGGAT
Fig. 3.10. A single band of approximately 300 bp was obtained from dogfish genomic DNA (lanes 7-10) when the primers TCRF4 and TCRR4 were used. A weak band of approximately 150 bp was obtained (lanes 4-6) from trout genomic DNA. The Mg\textsuperscript{2+} concentration is indicated at the top of each lane.
3.4. Amplification of TCRBV region from dogfish using degenerative primers

A major problem of all the above strategies was the difficulty in generating reproducible PCR products for sequencing. Therefore, a different strategy was devised in an attempt to isolate and characterise the TCRBV gene from the dogfish. Degenerative primers were prepared that utilised the highly conserved TCR and Ig light-chain framework region 2 (FR2) WYRQ and YY(F)CA in FR3. The related motifs complementary to these conserved FR2 and FR3

5'-(sense) CCGAATTCTGGTA(TC)C(GA)*NCA(TCRF6);
5'-(antisense) CGGATCCG(GA)CA(GA)TA(GA)TA(TCRR6.1);
5'-(antisense) CGGATCCG(GA)CA(GA)AA(GA)TA(TCRR6.2)

were prepared (Rast and Litman, 1994), respectively. The expected products were approximately 190 bp. The PCR was performed with annealing temperature at 45°C-48°C, Mg²⁺ concentration at 1.5mM-2.5mM. The PCR products were separated by 1% agarose gel electrophoresis and multiple bands were obtained of approximately 130 to 900 bp from genomic DNA (Fig. 3.11). Three bands of 150, 190-200 and 260 bp were removed from 1% agarose gel and purified (Fig. 3.12). Due to difficulties experienced in direct sequencing from PCR products or even from highly purified PCR products, these products were cloned in order to obtain a single and purified PCR fragment for sequencing. The previous PCR products in sections 3.1. and 3.2. were cloned and sequenced as well.

*N=G, A, T and C
Fig. 3.11. Multiple PCR products were obtained from dogfish genomic DNA when the primers TCRF6 and TCRR6.1 or TCRR6.2 were used. The 123 DNA ladder was used in lane 1. Lanes 2-5 were PCR products with primers TCRF6 and TCRR6.1 and lanes 6-9 were PCR products with primers TCRF6 and TCRR6.2. The Mg\(^{2+}\) concentration is indicated at the top of each lane.
Fig. 3.12. The approximately 190-200 bp PCR fragments were removed from 1% agarose gel in Fig.3.11, and purified by salt-ethanol precipitation. Lane 2 was PCR product with primers TCRF6 and TCRR6. Lanes 6-7 were PCR products with primers TCRF6 and TCRR6. A PCR marker was used in lane 1.
3.5. Characterisation of cloning PCR fragments

3.5.1. PCR fragments from dogfish genomic DNA with primers TCRF6, TCRR6.1 and TCRR6.2

Three main bands were purified (section 3.4) (Fig. 3.12) and cloned. A total of 66 clones with inserts were obtained (Fig. 3.13). A total of 32 clones were sequenced and full-length sequences were obtained in 25 clones (Table. 3.2). All of these 25 clones were sequenced in both directions to confirm the sequence as well as two separate methods (1) Sequenase Sequencing Version II; (2) Cycle sequencing using ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase FS. These 25 sequences were compared with the GenBank and EMBL database (release 100) and Swiss-Prot databases (release 100) using the DNASTAR software package (LASERGENE, USA). 4 of the 25 clones were related to the known TCR sequences in the database. Two clones 2C144 and 6C43 are 191 and 194 bp in length, respectively. The sequencing results of 2C144 are shown in Fig. 3.14 (Sequenced by Cycle sequencing using ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase FS) and Fig. 3.15 (sequenced by Sequenase Sequencing Version II). The nucleotide sequences and predicted amino acid sequences of the clone 2C144 and clone 6C43 are shown in Fig. 3.16A and B. The primers TCRF6 and TCRR6.1 were found in sequence 2C144 and the primers TCRF6 and TCRR6.2 were found in sequence 6C43. The similarity of nucleotide sequences between 2C144 and 6C43 are less than 75% and the predicted amino acid sequence of 2C144 differs substantially from 6C43 (Fig. 3.16C and D).

A computer search showed that these clones had homology to the TCRBV region of various species. The numerical positions of these two sequences according to the report of Kabat et al. (1991) corresponded to the amino acid segment between 32 and 95 of the TCRBV of mammals. A computer analysis showed that the highest
Fig. 3.13. Plasmid pUC18 were isolated from JM109 and digested with restriction enzyme Eco R I and Pst I at 37°C for 2 h. Then 2μl tracing dye was added and the mixture was loaded onto 1% agarose gel. The inserted PCR fragments were separated from pUC 18 (2887 bp). The 1 kb DNA ladder and 123 DNA ladder were used to determine the size of the inserts.
Table 3.2. Bi-directional sequencing of cloned PCR products from dogfish

<table>
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<th>Name</th>
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<th>Primers</th>
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*S for Sequenase Sequencing Version II
*C for Cycle Sequencing by ABI PRISM Dye termination Cycle Sequencing Ready Reaction Kit with Polymerase FS.
Fig. 3.14. The sequencing result of the clone 2C144 which was sequenced by cycle sequencing using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS and the sequence was read by the ABI PRISM 377 DNA Sequencer. Sequencing primer was M13-universal reverse.
Fig. 3.15. The sequencing result of the clone 2C144, which was sequenced using Sequenase Sequencing Version II and the nucleotide order was G, A, T, C.
Fig. 3.16. The nucleotide sequences and predicted amino acid sequences of the clone (A) 2Cl44 and clone (B) 6C43. The primer positions were underlined. (C) and (D) Alignment of dogfish TCR β chain V region sequences of clone 2Cl44 and 6C43. These two sequence identities had less than 75% homology. Gap "-" was introduced to obtain maximum match and identical nucleotides were indicated by "+".
alignment of the predicted amino acid sequence of clone 2C144 was with the horned shark TCRBV region at 46.1-51.7%, human TCRBV13 and TCRBV12 at 45.1%, monkey TCRBV at 38-41%, bovine TCRBV22 at 40.7%, chimpanzee TCRBV at 38.8%, and rat TCRBV8 at 37.5%, respectively (Table. 3.3). The alignment with other species is shown in Fig. 3.17. There are 12 positions that are fully conserved, whilst an additional 20 positions are shared between the dogfish and TCRBV genes from at least one of the six other species. As expected, tryptophan 34, tyrosine 35, arginine 36 are all conserved. A cysteine residue was found in position 92 whilst a phenylalanine and arginine residue was present at positions 65, and 69, respectively. The clone 6C43 also showed similarity with the TCRBV gene of other species including rabbit and mouse (Table. 3.4) and contained conserved amino acid sequence motifs, including WY35Q37 and YY(F)C92, as well as R69.

Another two clones designed as 6C53, 6C45 are 194bp in length. The sequencing results of 6C53 are shown in Fig. 3.18 (sequenced by Cycle sequencing using ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit with AmpliTag DNA Polymorase FS) and Fig. 3.19 (sequenced by Sequenase Sequencing Version II). The nucleotide and predicted amino acid sequences of clone 6C53 and clone 6C45 are shown in Fig. 3.20. The primers TCRF6 and TCRR6.2 were found in both 6C53 and 6C45. These two clones were identical at the amino acid level with the exception of a single residue at position 38, where threonine was present in clone 6C53 and serine in clone 6C45. A search of the database showed that these two amino acid sequences shared homology to the TCRDV genes of a number of species. The greatest homology was with the horned shark (45.3%) (Table. 3.5). The alignment with other species is shown in Fig. 3.21. In this alignment, there are 12 positions that are fully conserved and an additional 22 residues that are shared between the dogfish sequence and TCRDV genes from at least one of the four other species. The predicted amino
<table>
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<th>Score (%)</th>
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*All sequences are from GenBank and EMBL in DNASTAR software package (LASERGENE, USA).
Fig. 3.17. Alignment of predicted peptide sequence, corresponding to V region of the dogfish 2C144 homologs, with the part of V region s of six other vertebrate of TCRBV chain genes from the horned shark (HFU09934), monkey (MMU04564), rat (RNTCRBV82), bovine (BOVBTB22), chimpanzee (PTU04555), human (HSU66059). GenBank identifiers for the specific sequence shown are in brackets. Boxes indicate residues that match dogfish exactly. Gap"-" was introduced to maximise the match.
Table 3.4. The computer search results of clone 6C43 from Swiss & PIR & Translated release 100

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* All sequences are from GenBank and EMBL in DNASTAR software package (LASERGENE, USA).
Fig. 3.18. The sequencing result of the clone 6C53 which was sequenced by cycle sequencing using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS and the sequence was read by the ABI PRISM 377 DNA Sequencer. Sequencing primer was M13-Forward-40 primer.
Fig. 3.19. The sequencing result of the clone 6C53, which was sequenced using Sequenase Sequencing Version II and the loading order was G, A, T and C.
Fig. 3.20. The nucleotide sequences and predicted amino acid sequences of the clone 6C53 and clone 6C45. The nucleotide differences between 6C53 and 6C45 are indicated by "*"; the amino acid differs in position 38. The primer positions are underlined.

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6C53  CCGAATTCTGCTAGCCAGTACCCACCGGTTCCATGCCCATTTCTGTGCTGACAGAAAC
6C45  CCGAATTCTGCTAGCCAGTACCCACCGGTTCCATGCCCATTTCTGTGCTGACAGAAAC

*  *  *

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6C53  AGATACAGCGAGAACGCAGGAAAATCTTTGGGACCGTTTTTCAGCGGAGTTTGATCAT
6C45  AGATACAGCGAGAACGCAGGAAAATCTTTGGGACCGTTTTTCAGCGGAGTTTGATCAT

*  *

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</table>

6C53  GTGAAGAGCCAGCGAGCGGTTCCCGGCTGCCGCTGACAGACTCCGCGGTGTAT
6C45  GTGAAGAGCCAGCGAGCGGTTCCCGGCTGCCGCTGACAGACTCCGCGGTGTAT

*  *

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<th>A</th>
<th>D</th>
<th>P</th>
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</thead>
</table>

6C53  TTCTGCGCGATCCG
6C43  TTCTGCGCGATCCG
Table 3.5. The computer search results of clone 6C53 and 6C45 from Swiss & PIR & Translated release 100

<table>
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<tr>
<th>Species</th>
<th>Score (%)</th>
<th>TCR Region</th>
<th>Entry Number</th>
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</thead>
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<tr>
<td>Horned shark</td>
<td>45.7</td>
<td>TCRDV</td>
<td>HFU22671</td>
</tr>
<tr>
<td>Mouse</td>
<td>43.2</td>
<td>TCRDV</td>
<td>C30583</td>
</tr>
<tr>
<td>Mouse</td>
<td>42.2</td>
<td>TCRDV(VD1D2J1)</td>
<td>MUSTCDXG</td>
</tr>
<tr>
<td>Pig</td>
<td>32.2</td>
<td>TCRDV(VD5.2)</td>
<td>PIGRTCRD25</td>
</tr>
<tr>
<td>Human</td>
<td>30.7</td>
<td>TCRDV3</td>
<td>HUMTCRDBBF</td>
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<tr>
<td>Rabbit</td>
<td>27.4</td>
<td>TCRDV5</td>
<td>RABTCRDC</td>
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<tr>
<td>Horned shark</td>
<td>26.1</td>
<td>TCRDV</td>
<td>HFU22673</td>
</tr>
</tbody>
</table>

a: All sequences are from GenBank and EMBL in DNASTAR software package (LASERGENE, USA).
Fig. 3.21. Alignment of predicted peptide sequence, corresponding to TCRDV region of the dogfish 6C53 and 6C45 homologs, with TCRDV chain genes of four other vertebrates from the horned shark (HFU22671), mouse (C30583), pig (PIGRTCR), human (HSTRDWM). Genbank identifiers for the specific sequence shown are in brackets. Boxes indicate residues that match dogfish exactly. Gap"-" is introduced to maximise the match.
acid sequence of the clone 6C53 suggests that it is part of a TCRDV region, and corresponds to positions 32 to 95 (Kabat et al., 1991). Conserved phenylalanine and asparagine residues were found at positions 64 and 86, respectively. These two residues would form a salt bridge. Also, the highly conserved residue tryptophan 34, tyrosine 35, arginine 36, tyrosine 90, cysteine 92 are present in these clones.

3.5.2. PCR fragment from trout with primers TCRF2 and TCRRI

A purified band approximately 250 bp (section 3.1) was cloned into plasmid pUC18 and four clones were obtained with inserts of similar size (Fig. 3.22) and named as C3, C5, C7 and C8. The double-stranded plasmid templates of these 4 clones were sequenced using Sequenase Sequencing Version II. Sequences of clone C3 and C7 are shown in Fig. 3.23. These sequences were compared with GenBank and EMBL. None of them belonged to the Ig supergene family although they exhibited short ORFs.

3.5.3. PCR fragments from dogfish cDNA with primers TCRF3 and TCRR3

Two PCR products using primers TCRF3 and TCRR3 from dogfish lymphocyte cDNA (section 3.2) were cloned into pUC18. 37 clones were obtained with inserts, and two of these clones were fully sequenced. 11C151 and DT34 were 395 and 320 bp respectively (Fig. 3.24). The PCR primers were present in clone DT34, not in 11C151. A computer search showed that they did not belong to the Ig gene superfamily although ORFs were found in some regions of these two sequences. The amino acid sequences translated from these two ORFs showed no definite homology to one certain protein in the database.

3.6. Southern and Northern blotting analysis using probes 2C144 and 6C53

Southern blotting analysis using 2C144 as a probe (Fig. 3.25) showed that 2
Fig. 3.22. Plasmid pUC18 were isolated from JM109 and digested with restriction enzyme *Eco R I* and *Pst I* at 37°C for 2 h. Then 2μl tracing dye was added and the mixture was loaded onto 1% agarose gel. The inserted PCR fragments were separated from plasmid pUC 18 (2887 bp). The 1 kb DNA ladder was used in both side lanes. The samples 3, 5, 7 and 8 had inserts.
Fig. 3.23. Nucleotide sequences of clones C3 (210bp) and C7 (175bp).

Clone C3
GTCTCCCCTTTTGCCACCTTTTGATGCTATATCTCCTGCACCCCCGTGACTACTT
TGATGCTATGCTCTTAGCTGCACCCCCGTGACTGCTTTGATACATATATTCGGGCTG
CACCCCTGTTGGACACCTTTTGTGCTATATCTCCTGCTGACCCCCGTTAACTACTTTG
ATGACTATGTCTCTAGACGACACAGTACAGCC

Clone C7
GAAGTTCAATCTCATGGTTCTCTGCACGGGCTCATATTTCTGTGCGTGTTAGGGGAACAT
TGTTAATGACTATATGCGATGAGCAATGGTGAAATTCCCCTTTAACTTAGTCCAG
ATGAGTCCCTGGGACGTCCTTTATCCACCACAGGATCGTCGTGTCATTATGTGGCG
Fig. 3.24. Nucleotide sequences of clone 11C151 and DT34. The primers TCRF3 and TCRR3 are underlined.

11C151

TGCTATATGAGACCTTATCGAAAGCCTTCTGAAAGCCTAAATCCACTGGTTCTCCCTGG
TCAACTCTACTAGTTGAAATGAAAATCACTTAGTGTCACAAGTAGGGCTTCAAAATGAGTT
TACTGTGAAAGCAGAGCTAGTCGAATCCGCTGTTCTAAATGGGAAATGAAAATGAAAATCCAC
TCTACATAGTTGAAATGAAAATGAAAATGAAAATGAAAATGAAAATGAAAATGAAAATGAAAATG

DT34

GGAGATGGGACCAAACTGGTTGAAATTGTGCAGCGAGGTGTTATTACTATGAGTGTGGGGG
GATTACAAACTCCTCATCATATCTAGGTTTCTAATCCCTTATGGAAGGCTCTAACCCTTATTG
AGAAGACTCTG...GAGCCACCCCTATCAACACTCTCTTGCAGGAGATCGAGCAATACATT
GGATTTGGATTGATGATGTCTTTATTGTCACATGTACGGGATATGGGAAAGGAT
TTTCTCGAGCAACAGCTCAACAGACATCTGTATCCCTCATCTGC
Fig. 3.25. Clone 2C144 and 6C53 were digested with *EcoR* I and *Pst* I at 37°C for 2 to 4 h. The samples were loaded onto a 1% agarose gel. The plasmid inserts were purified and used as the TCRBV (2C144) and TCRDV (6C53) probes, respectively.
bands were present in all four fish digested with \textit{EcoR I} (size: 1 and 1.2 kb). There was also variability in the size of the fragment between individual fish when dogfish genomic DNA was digested with \textit{EcoR I}, \textit{Bgl II} and \textit{Hind III}. At least 3 bands present, depending upon the restriction endonuclease employed (Fig. 3.26).

The results of the Southern blotting using the 6C53 as a probe (see Fig. 3.25) in conjunction with the endonuclease \textit{EcoR I} identified five bands at 1, 2, 2.4, 2.5 and 3 kb in all four fish, also some fish had additional bands of 0.7, 0.9, 1.3 and 1.5 kb (Fig.3.27).

Northern blot and hybridisation with the probe 6C53 (TCRDV) identified a transcript of approximately 2kb that was present in the spleen and lymphocyte as well as brain, but absent from the liver and kidney (Fig. 3.28). Northern blot and hybridisation with the probe 2C144 (TCRBV) also identified a transcript of approximately 2kb in lymphocytes although not in brain (Fig. 3.29).

3.7. Amplification of whole TCRD gene from dogfish cDNA mixture

To obtain the full-length sequences of the TCR genes of \( \delta \) chain, the double stranded cDNA-RNA prepared from dogfish lymphocytes and synthesed using oligdT, was ligated into pUC18. For amplifying TCRD sequences from plasmid with cDNA inserts, three primers were designed based on the TCRDV-like gene 6C53. The primer sequences were determined by Primerselect Program of DNASTAR (LASERGENE, USA) and were as follows:

\[
\begin{align*}
5'(\text{sense})-\text{TCTTTGGGGGACC} & \text{GGTTTTCAG} \quad (\text{TCRF7.1}); \\
5'(\text{sense})-\text{GAAAGTGTGCA} & \text{AAACGGAGCT} \quad (\text{TCRF7.2}); \\
5'(\text{antisense})-\text{CTTTCAGGGGGACG} & \text{GTGCTTTC} \quad (\text{TCRR7}) \quad (\text{Fig. 3.30}).
\end{align*}
\]

These primers combined with the sequencing primers M13-40-forward (GTTTCCCAGTCACGAC) and M13 Universal reverse (CAGGAAAACAGCTATGAC)
Fig. 3.26. Southern blot of dogfish whole blood genomic DNA from dogfish No.1, No. 2, No. 6, and No.7 digested with EcoRI (E), BglII (B) and HindIII (H) and hybridised to $^{32}$P-labelled TCRBV probe 2C144.
Fig. 3.27. Southern blot of dogfish whole blood genomic DNA from dogfish No.3, No.4, No.6, and No.8 digested with EcoR I and hybridised to 32P-labelled TCRDV probe 6C53.
Fig. 3.28. Northern blot of dogfish brain (B), Epiganal tissues (E), kidney (K), Liver, lymphocytes (L), skeletal muscle (M), red cells (R) and spleen (S) and hybridised to $^{32}\text{P}$-labelled TCRBV probe 6C53.

[Image of the Northern blot with labels B, M, E, K, Liver, S, L, R and a 2kb marker on the right]
Fig. 3.29. Northern blot of dogfish brain (B), kidney (K), Liver, lymphocytes (L) and spleen (S) and hybridised to $^{32}$P-labelled TCRDV probe 2C144.
Fig. 3.30. The nucleotide sequence of clone 6C53 and the position of primers TCRF7.1 and TCRF7.2 are in red and blue, respectively. Primer TCRF7 is underlined.

CCGAATTCTGGTACCGCAGTATCCACCGGGTCCATTTGAAAAACAGATACAGCAG
GAACGCAGCAGAAATCTTTGGGGGACCGTTTTTACATCAGGAAAGGCAACCGGCTCCCCCTG
AAAGTGTCGAAAACGGAGCTGACAGACTCCCGGGTGTATTTCTCGCGGGATCC
were used to allow amplification of whole TCRD genes from the cDNA inserts, which could be ligated into the plasmid in 2 possible directions (Fig. 3.31). When the primers were used in combination in Table 3.6, the corresponding size of the PCR products would be obtained, which ranged from 600 to 650 bp for TCRD V region and 900 to 1000 bp for TCRD V+C region. Multiple PCR products were obtained which varied in size from 200 to 1000 bp (Fig. 3.32). The bands of approximately 600 bp and 1000 bp were purified from agarose gel and ligated into pUC18 and transformed to bacterial JM109 cells. A total 28 from 59 clones that were obtained were sequenced by ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS. The sequences with longer ORFs (above 30 amino acids long) are shown in Fig. 3.33. None of these clones obtained had a full-length sequence or belonged to the Ig gene superfamily. The results are described in Table 3.7.

The strategies for isolating TCR genes from dogfish are represented in Fig. 3.34. The PCR results with different sets of primers are shown in the Table 3.8. With the exception of the sequences from the PCR products performed with primers TCRF6, TCRR6.1 and TCRR6.2, all other sequences did not proved to be the Ig gene superfamily.
Fig. 3.31. The diagram of plasmid with dogfish cDNA insertion and the position of primers-binding and the possible products of PCR.
Table 3.6. The primers used for amplification of whole TCRD gene from dogfish cDNA mixture

<table>
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<tr>
<th>Name</th>
<th>Expected Region</th>
<th>Expected Size (bp)</th>
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<tr>
<td>TCRF7.1</td>
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<tr>
<td>M13-forward-40</td>
<td>V+C</td>
<td>~1000</td>
</tr>
<tr>
<td>TCRF7.2</td>
<td></td>
<td></td>
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<tr>
<td>M13-forward-40</td>
<td>V+C</td>
<td>~1000</td>
</tr>
<tr>
<td>TCRF7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13-Universal reverse</td>
<td>V or V+C</td>
<td>~600 or 1000</td>
</tr>
<tr>
<td>TCRF7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13-Universal reverse</td>
<td>V or V+C</td>
<td>~600 or 1000</td>
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<tr>
<td>TCRR7</td>
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<tr>
<td>M13-forward-40</td>
<td>V or V+C</td>
<td>~600 or 1000</td>
</tr>
<tr>
<td>TCRR7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13-Universal reverse</td>
<td>V</td>
<td>~600</td>
</tr>
</tbody>
</table>

V: Variable region
C: Constant region
Fig. 3.32. Multiple PCR products were obtained from cDNA of dogfish lymphocytes. The primers used in PCR were TCRF7.1 and M13-40 forward (lane 1); TCRF7.1 and M13-universal reverse (lane 2); TCRF7.2 and M13-40 forward (lane 3); TCRF7.2 and M13-universal reverse (lane 4); TCRR7 and M13-40 forward (lane 5); TCRR7 and M13-universal reverse (lane 6).
Fig. 3.33. Nucleotide sequences of clone c1c3, c8c7, c8c8, c14c1, c20c9, c22c17, c22c18, c22c19, c24c5, c24c19 and c24c23. The open reading frames are in red.

C1c3
TTTCTTCTGGNGGTGCTCTGCTTTNGNTTNGTATCCCGGCTTCCAAATACCTCTCGGCCTCANTACAATTTTT
TTTGAGANCCGCTAANNTGGCCCTTTATGATCAGGTGCATCGACCTGACTMRRGACCTGATCTNGCATGCTG
ACCCTGCTGACCCCGGCTTCTCTTCAACATCCTCTTTCTGGATGCNGATTTNGATCNGGATCTANGCTATG
TGATCTCAGCATTACTCTGCTTGAATNNAACGATGAGGCGACACCCGTGCTCTGTATATGCCGA

C8c7
CTCTCTCTTNGCTCAAGCTACATGGGATTCCGGAGAACGCGTGTCCCAGAACTTTAATGTCTTGTCC
CTAACTACCTTCATAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGGACTGG
TATCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA

C8c8
GCTCACGAAAACCCCGCTGAACCCCAAGCCAACCGGAAGAAAATAGACACAGATATATGTCGAGACCT
TCACCTACCTTCATAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGGACTGG
TATCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA

C14c1
TTNTTTTTTNNNNNGCGGCGCCTTTTTTNNAAACNGNCTGGCGGTNNGCCTACGGCTACCCGCTACGCTG
TTCCCTCCATCCCTCATTCTCTCCTCTCCTGATGATGCTCTGACATCAGTGCTG
GTCACCAAGCACAATTTCTCTGCTAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGG
ACTCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA

C20c9
TTTTTTTTTTTNNNNGGGCCCTTTTTTNNAAACNGNCTGGCGGTNNGCCTACGGCTACCCGCTACGCTG
TTCCCTCCATCCCTCATTCTCTCCTCTCCTGATGATGCTCTGACATCAGTGCTG
GTCACCAAGCACAATTTCTCTGCTAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGG
ACTCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA

C22c17
TTNTTTTTTTTTTTNNNNNNNGCGGCGCCTTTTTTNNAAACNGNCTGGCGGTNNGCCTACGGCTACCCGCTACGCTG
TTCCCTCCATCCCTCATTCTCTCCTCTCCTGATGATGCTCTGACATCAGTGCTG
GTCACCAAGCACAATTTCTCTGCTAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGG
ACTCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA

C22c18
ANCAAAANATCTTGTGGGGGATCTGGATTTTGCTGCTAGAAAAGAGACNGCAGCCAGATGCTTGGACNCAA
TGAGTGAGCTCCTCGGTGCTGGATGTGCGCTTCTCCCTCGGGGAAACTCTCGGTGCTGGATGCTCTGGC
NTACTGTAACAGCAGCGNGTGGATCNAACACACCGAATAATCTCAGACNCAAAACATCATTCTCTTCTATAT
ACAAAAANAAACNGCACAATGACTTTGCTTAAAANATGATTCAGNATCNGCCTCNATTATATTNCTCN
CTACTAGTNTGGGTGCGANGNHTACTNAACNTCTCGGCTTTATGCTTTAGATTAACAGTTTATNCCCGA
CNACTTTTATNTTTGTGGAAACATGCACCTTCGCTCTTGGCTGCTGCGGCGGCGGAGATGAGGCGTAN

C22c19
TTTTTTNTTTNNNCCGGCCCTTTTTTNNAAACNGNCTGGCGGTNNGCCTACGGCTACCCGCTACGCTG
TTCCCTCCATCCCTCATTCTCTCCTCTCCTGATGATGCTCTGACATCAGTGCTG
GTCACCAAGCACAATTTCTCTGCTAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGG
ACTCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA

C24c5
TTNTNNNNNNNNCCGGCCCTTTTTTNNAAACNGNCTGGCGGTNNGCCTACGGCTACCCGCTACGCTG
TTCCCTCCATCCCTCATTCTCTCCTCTCCTGATGATGCTCTGACATCAGTGCTG
GTCACCAAGCACAATTTCTCTGCTAGATGCTCTCCATCCAN6CTGTGATGCTCCCTCAATGCTCTATGGAGG
ACTCGTGAATGGAAGCTGCTGGATCTGGTCTCACCACACACTGTCGACCCACTGAGATGTTAATGCCCTAC
CTAATGTTCTGCTGATATGCCGA
Fig. 3.33. (continues)

C24c9
TTTCTTTNTTCTTCTCCTCTTNTNNTTNTTCCTTTNTNCNNCCNCNNCGNNTNTNTCTCNNT
TTNTNCTTNTNNCCNNNCNGTTTANTNCCTCCTCCTCGAGTCCTTCTGTGGATATGATGGAGCTACT
TGACCCCTCACACTAAATAGAGAAATGATATTATTACCTAACGAGCAACACTCGGCGCTTTAGAACATATC
AACACTTTGGAAACACACTCTGCACTCGCAAACATTAGTCACAGGAGTGCTCAGATTCAATCCACCAATGGC
AAAGGAGGCGACACTCTGAATATGAAACCAATCTCCAGGTCATCGATGCAGGATTTCCTGAGCGTGCTTT
TTTGGAGCTGGATCAGATTCAAAACGATTTTGCTGTAAGTGAAACACAGGAAAGCTGTCAAGT
CACTGCGGCAAACAGATTGGCGCTCGCCGANGGCGAGGGCCACTACCTGCACCTTCTGGTGGAGGAATA
NCCTGCACTGCGCA

C24c23
ATTTNNNNNTNNCCNNTCNTGGGAGGTGCCTTTCTTGGAGATGTTCTCTCAGGCGAGTGATTATGGCT
CAGCTGCCGTCAGCTGCCATTCTCTCCTCACCAGAANNTGCGAGGGACGCTCCCNCCCTCCGAGGC
CAAACCTTCTGGGGCGCACTGAGCTAGCAGCGCTTTCTCCTGGGTGTTCTCGAGCTTACATCAGGAAAATGTTTTGAC
ATTGACCCAGGCTCCAAAAGGACACAGCTCAGGAATCTCGCATGAGTACCTGAGATTTTGCTTATA
TTTACAGGTGCTGGCCTTTCTGCTTCTGCTGAACTTCTCTGGTGCATGTGTTTATCAAATGGTTGATATGTTCTAAGGCGCTAGTTAAGGCTGAGTGAAGAG
TGCTTTTATCAAGAAGCTATTGTATCTTAAAGGCGCGTAAAGGCTGAAGCTTAAGGCAATAATTATCCTCCTCC
TAAAGGCTGTTAGGCTAGCTGAGCAGCGCTCCATCNTRATATNCAGATCNAANAACACCGCTCCCTGAAAA
Table 3.7. The sequencing results of clones of PCR products from dogfish cDNA mixture with primers in Table 3.6.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
<th>Expected Size (bp)</th>
<th>ORFs (bp)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1C3</td>
<td>M13R+TCRF7.2 V or V+C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>276</td>
<td>138</td>
<td>No&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C6C1</td>
<td>M13R+TCRR7 V</td>
<td>232</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C8C7</td>
<td>M13F+TCRF7.1 V+C</td>
<td>278</td>
<td>-</td>
<td></td>
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<tr>
<td>C8C8</td>
<td>M13F+TCRF7.1 V+C</td>
<td>234</td>
<td>177</td>
<td>Actin</td>
</tr>
<tr>
<td>C14C1</td>
<td>M13F+TCRR7 V+C</td>
<td>263</td>
<td>101</td>
<td>No</td>
</tr>
<tr>
<td>C20C6</td>
<td>M13R+TCRF7.1 V or V+C</td>
<td>too short</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C20C9</td>
<td>M13R+TCRF7.1 V or V+C</td>
<td>230</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C20C13</td>
<td>M13R+TCRF7.1 V or V+C</td>
<td>Too short</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C22C14</td>
<td>M13R+TCRR7 V</td>
<td>too short</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C22C15</td>
<td>M13R+TCRR7 V</td>
<td>too short</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C22C17</td>
<td>M13R+TCRR7 V</td>
<td>242</td>
<td>102</td>
<td>No</td>
</tr>
<tr>
<td>C22C18</td>
<td>M13R+TCRR7 V</td>
<td>395</td>
<td>183</td>
<td>No</td>
</tr>
<tr>
<td>C22C19</td>
<td>M13R+TCRR7 V</td>
<td>339</td>
<td>96</td>
<td>No</td>
</tr>
<tr>
<td>C22C20</td>
<td>M13R+TCRR7 V</td>
<td>211</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C5</td>
<td>M13F+TCRR7 V or V+C</td>
<td>422</td>
<td>390</td>
<td>Ras inhibitor</td>
</tr>
<tr>
<td>C24C9</td>
<td>M13F+TCRR7 V or V+C</td>
<td>504</td>
<td>204</td>
<td>Ras inhibitor</td>
</tr>
<tr>
<td>C24C11</td>
<td>M13F+TCRR7 V or V+C</td>
<td>too short</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C12</td>
<td>M13F+TCRR7 V or V+C</td>
<td>too short</td>
<td>-</td>
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</tr>
<tr>
<td>C24C14</td>
<td>M13F+TCRR7 V or V+C</td>
<td>144</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C15</td>
<td>M13F+TCRR7 V or V+C</td>
<td>too short</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C19</td>
<td>M13F+TCRR7 V or V+C</td>
<td>207</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C20</td>
<td>M13F+TCRR7 V or V+C</td>
<td>79</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C22</td>
<td>M13F+TCRR7 V or V+C</td>
<td>86</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C24C23</td>
<td>M13F+TCRR7 V or V+C</td>
<td>486</td>
<td>153</td>
<td>No</td>
</tr>
<tr>
<td>C24C25</td>
<td>M13F+TCRR7 V or V+C</td>
<td>too short</td>
<td>-</td>
<td></td>
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<tr>
<td>C24C26</td>
<td>M13F+TCRR7 V or V+C</td>
<td>too short</td>
<td>-</td>
<td></td>
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<tr>
<td>C24C27</td>
<td>M13F+TCRR7 V or V+C</td>
<td>131</td>
<td>-</td>
<td></td>
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<tr>
<td>C24C29</td>
<td>M13F+TCRR7 V or V+C</td>
<td>Too short</td>
<td>-</td>
<td></td>
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</tbody>
</table>

<sup>a</sup>: V-Variable region  
<sup>b</sup>: C-Constant region  
<sup>c</sup>: No- means no definite homologous to any sequences in database
Fig. 3.34. Flowchart of the strategies for the isolation of dogfish TCR genes in this project.

Genomic DNA extracted from dogfish whole blood

- PCR with primers TCRF1 and TCRR1 (For V region of TCR) → -190bp PCR products were obtained → Directly sequencing (not Ig gene superfamily)

- PCR with primers TCRF2 and TCRR2 (For V region of TCR) → -420bp PCR products were obtained → Directly sequencing (inconsistent)

- PCR with primers TCRF3 and TCRR3 (For C region of TCR) → -480bp PCR products were obtained → Directly sequencing (not Ig gene superfamily)

- PCR with primers TCRF4 and TCRR4 (For V region of TCR, TCRR4 was biotin-labelled) → -300bp PCR products were obtained → Directly sequencing (inconsistent)

- PCR with degenerative primers TCRF6, TCRR6.1 → -190bp PCR products were obtained → Inserting the PCR fragments into plasmid pUC 18 and TCRR6.2 (For V region of TCR)

- Compared with GenBank and EMBL database → Sequencing the insertions

- Identified 2 TCRBV genes and 2 TCRDV genes → Suggesting several TCRV segments in dogfish germline

- Northern blot
  - TCRBV
    - Expressing in lymphocytes
  - TCRDV
    - Expressing in the spleen, lymphocytes and brain

- Southern blot
Table 3.8. Summary of the results of isolation TCR genes from dogfish using different sets of primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Expected Size (Excluding Intron) (bp)</th>
<th>Expected Genes</th>
<th>Sequences Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRF1</td>
<td>~ 190</td>
<td>TCRBV</td>
<td></td>
</tr>
<tr>
<td>TCRR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF2</td>
<td>~ 420</td>
<td>TCRBV</td>
<td></td>
</tr>
<tr>
<td>TCRR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF3</td>
<td>~ 470</td>
<td>TCRBV</td>
<td></td>
</tr>
<tr>
<td>TCRR3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF4</td>
<td>~ 234</td>
<td>TCRBV</td>
<td></td>
</tr>
<tr>
<td>TCRR4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF6</td>
<td>~ 190</td>
<td>TCRBV</td>
<td></td>
</tr>
<tr>
<td>TCRR6.1</td>
<td></td>
<td></td>
<td>TCRBV or TCRDV</td>
</tr>
<tr>
<td>TCRR6.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF7.1</td>
<td>~ 1000</td>
<td>TCRDV+C</td>
<td>Actin</td>
</tr>
<tr>
<td>M13-forward-40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF7.2</td>
<td>~ 1000</td>
<td>TCRDV</td>
<td></td>
</tr>
<tr>
<td>M13-forward-40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF7.1</td>
<td>~ 600 or 1000</td>
<td>TCRDV or V+C</td>
<td></td>
</tr>
<tr>
<td>M13-Universal reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRF7.2</td>
<td>~ 600 or 1000</td>
<td>TCRDV or V+C</td>
<td></td>
</tr>
<tr>
<td>M13-Universal reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRR7</td>
<td>~ 600 or 1000</td>
<td>TCRDV or V+C</td>
<td>Ras inhibitor</td>
</tr>
<tr>
<td>M13-forward-40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCRR7</td>
<td>~ 600</td>
<td>TCRDV</td>
<td></td>
</tr>
<tr>
<td>M13-Universal reverse</td>
<td></td>
<td></td>
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</tbody>
</table>

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3.8. Discussion

The initial results using primers TCRF1 and TCRR1 demonstrated that although a single PCR product in expected size was obtained and purified, it probably contained several products. This was supported by sequences, which were shown in Fig. 3.4. It was therefore extremely difficult to employ the direct sequencing technique for these products.

Although TCRF1 and TCRR1 were designed from phylogenetically well conserved regions containing the peptide motif WYRQ and YY(F)CA, the nucleotide sequences for these conserved regions varied considerably from species to species; even from family to family in the same class. For instance, the nucleotide sequences for the region WYRQ was TGGTATCGCCAG in horned shark TCRB gene (Rast and Litman, 1994); TGGTATAGACAA in axolotl TCRB gene (Fellah, et al., 1994). Therefore, degenerative primers would be needed.

The primers TCRF2, TCRR2, TCRF3, TCRR3, TCRF4, TCRR4 were used in the PCR and were based upon the known fish TCRB sequences. These are not particularly conserved and therefore, amplification products may contain unrelated sequences. The primers TCRF7.1, TCRF7.2, and TCRR7 were based on the known dogfish TCRDV-like (clone 6C53) genes, but it still proved difficult to amplify the TCR sequences. After the PCR fragments were cloned and sequenced, it was evident that none of them were related to the Ig gene superfamily.

Several studies (Johnson, 1987; Davis and Bjorkman, 1988; Shamblott and Litman, 1989a,b; Rast et al., 1994) have shown that the predicted peptide sequences derived from vertebrate Ig light-chain, TCR, and CD8 genes have two four-amino acid stretches in FR2 (WYRQ) and FR3 [Y(Y/F)CA] and these exhibit little phylogenetic variation between diverse species. Rast and Litman (1994) used specific degenerative
primers that were complementary to these two stretches to successfully amplify TCRBV region genes from the genomic DNA of the horned shark. Later, Rast (1995, 1997) also used these degenerative primers amplifying the TCRA gene from pufferfish as well as the TCRD gene from the horned shark and TCRA, B, G and D genes from the skate. These primers proved to be very efficient in the amplification of TCR genes from the genomic DNA of the lower vertebrates. When these primers were employed in our studies, PCR products were of a constant size.

From previous experiments, direct sequencing from PCR products was not successful. This could have been due to a number of factors. It is likely that templates were heterogenous and sequencing of multiply template gave diffuse bands, and a loss of resolution. Cloning the products was quite important to obtain good sequencing data. Almost identical strategies were adopted for the molecular cloning of shark (Rast and Litman, 1994) and trout (Partula et al., 1994) TCRB and TCRA genes. They all used conserved degenerative primers and searched for phylogenically conserved genes by amplifying the genomic DNA. This has the advantage of targeting all possible variants of a multigenic family, including those genes that are rarely or never (pseudogenes) transcribed. This strategy has been well suited to detecting TCR genes in dogfish as well and may allow the detection of other genes that are important in immune system.

A comparison of the two sequencing methods using either Sequenase Sequencing Kit and Manual reading on a light box; or, sequencing with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS with the sequencing read by the ABI PRISM 377 DNA sequencer showed that the latter sometimes gave unsatisfactory data. The optimum is to combine these two methods for obtaining the most reliable sequencing results.
Amplification of TCRD gene sequences from dogfish lymphocyte cDNA, cloning into the plasmid pUC18 and then the PCR fragments were subsequently subcloned into the same plasmid pUC18. This resulted in reading difficulty because of non-directional cDNA insertion. The best method is to subclone into a different plasmid or by using directional cDNA insertion.

The results present here provide evidence of the extensive phylogeny of the TCR system. Several comparisons of TCR sequence derived from dogfish sequences with shark, rat, bovine, monkey, chimpanzee, and human, TCRBV (2C144 and 6C43) and TCRDV (6C53 and 6C45) sequences showed that certain motifs are conserved. This suggests that these amino acid residues are critical for the secondary and tertiary structure of TCR-β and TCR-δ chain molecules in dogfish.

The predicted amino acid sequence of 6C43 differs substantially from 2C144, suggesting that these clones may belong to different TCRBV families. In the study of Hawke et al., (1996), they identified at least 7 TCRBV families in the horned shark. Our results suggest that dogfish may also have a number of TCRBV families. The analysis of the sequences suggests that TCRB of dogfish are similar to human TCRBV13 or TCRBV12 families.

The Southern blotting analysis suggests that there are polymorphic as well as allelic differences between dogfish. Rast and Litman’s (1994) work suggested that TCRBV loci are multi-clustered in the horned shark, with each cluster containing several TCRBV and TCRBJ segments and a single TCRBC segment. Analysis of the rainbow trout TCRB sequences obtained from cDNA suggests that several TCRBV segments, at least one TCRBD, 10 TCRBJ, and a TCRBC region rearrange preferentially and these must be clustered at the genomic level. This was supported by the results obtained using a TCRBC probe which suggested that there may be more than one genomic TCRB
cluster in the trout (Partula et al., 1995). Further studies are now required to confirm whether the TCRB loci are clustered in the dogfish.

Clone 6C53 and 6C45 are almost identical. Only a amino acid difference between them may be caused by PCR or point mutation. These two clones showed homology to TCRDV in a number of species and indicate that the genes coding for the TCR αβ and γδ are present in the jawed fish-dogfish. These results are consistent with Rast and co-workers’ (1995, 1997) findings.

The results of the Southern blotting using the 6C53 as a probe in conjunction with the endonuclease EcoRI showed that five bands were present in all four fish. Certain fish display extra bands suggesting that there may be variability in the number of copies of the gene or RFLP. The Northern blotting suggests that TCRD is expressed in dogfish spleen and lymphocytes. TCR γδ cells may form in a large proportion in spleen T cells in the dogfish. TCR γδ cells have been found in all of vertebrates except jawless fish. Haas et al. (1993) suggested they may perform some unique function(s), because these T cells are predominant in some situations where TCR αβ cells are not active and thus may make a different contribution to the immune system compared to TCR αβ cells. It is interesting that TCRDV, not TCRBV was expressed in the dogfish brain. This may support the option that the meninges of Elasmobranch is a lymphohaemopoietic tissue (Chiba et al., 1988) and also suggests the TCR αβ cells and TCR γδ cells may have different tissue distribution. TCRBV 2C144 was also expressed in lymphocytes. The results suggest that these two genes may encode functional TCR proteins in dogfish.

Rast et al. (1995) showed the unequivocal assignment of putative TCRV segments as a TCR component and that it remains largely dependent on the identification of a full length copy of cDNA sequence. However, isolation of a corresponding cDNA
can be problematic. They could not recover the corresponding TCRs despite using several cDNA libraries and using a large number of PCR fragments from zebrafish as probes because the levels of representation of the sequences was less than 1 in $5 \times 10^5$ within the libraries. We identified two TCR fragments (6C53 and 6C45) from dogfish but have failed to recover the corresponding TCRs from the cDNA mixture. This may be the reason why it proved extremely difficult to amplify TCRs from dogfish cDNA despite obtaining the germline sequence. Attempts were made to circumvent this problem by stimulating the lymphocytes with mitogen such as Con A and PHA, and using these cells to prepare cDNA. However, using RNA from stimulated lymphocytes did not make any difference.

The alignments of the predicted peptide sequences, corresponding to dogfish 2C144, 6C53 or 6C45, invariably showed that the highest alignment score is with the horned shark TCRBD or TCRDV sequences. Rast et al. (1995) pointed out that the assignment of genomic amplification products such as Ig/TCR-like V segments is based only in part on the alignment with family-specific residues. Genuine products typically can be identified both by the presence of consensus residues and through database searches, which tend to show matches exceeding 30% overall identity for authentic products. Despite clone 6C43 showing matches of less than 30% through database searches, several conserved consensus residues were present, suggesting it is the TCRBV gene sequence.

In conclusion, these results suggest that the dogfish may have two sets of T-cells that express either TCR $\alpha\beta$ or TCR $\gamma\delta$. Further work is now required to elucidate the exact nature of these TCRs. The data presented in this thesis strongly suggest that all of the major antigen receptor genes that were first identified in mammals (Ig heavy and light chains, TCR$\alpha$, TCR$\beta$, TCR$\gamma$, and TCR$\delta$) were also present at the divergence of
the bony from the cartilaginous fish. Although the TCR structure appears similar in these lower vertebrate groups, the immune system of cartilaginous fish differs radically from that of mammals in many historical, molecular, and functional aspects. With the appearance and publication of new sequences in fish, it will soon be possible to characterise the lymphocytes both at the molecular and cellular level. It may be possible to identify the most divergent living taxonomic group to possess a mammalian-type adaptive immune system.
CHAPTER 4. ISOLATION OF MHC GENES FROM FISH

4.1. Amplification of MHC class II β chain from dogfish, trout and carp

The primers for amplification of MHC class II β chain were designed from the published MHC class IIB chain of Atlantic salmon sequence clone c144 (Hordvik et al., 1993). The sense primer (MHCF1) was located in the β1 region for codons 36 to 43 of the MHC class IIB chain and antisense primer (MHCR1) was located in the β2 region for codons 148 to 154 (Fig. 4.1). The sequences of these two primers were as follows:

MHCF1 5'-CAACACGACTGTGGGGAAGTTTG;
MHCR1 5'-GAGTGGATCTGGTAGTACCAGTC.

The expected PCR product was approximately 360 bp (not including the intron). When PCR was performed with a hot start at 95°C for 10 min, then at 95°C for 1 min, at 50°C for 2 min; 72°C for 2 min for 35 cycles with an extension at 72°C for 5 min, a single band of 360 bp was amplified from trout and carp cDNA prepared from lymphocytes (Fig. 4.2). To obtain products from cDNA prepared from lymphocytes of dogfish, the annealing temperature was changed to 40°C, 45°C, a titration of Mg++ concentration was also performed in the range of 1.5 to 4mM, and multiple products of approximately 250, 360 and 500 bp were obtained from trout and dogfish genomic DNA, again a single bind at ~360 bp from trout and carp cDNA, but still not from cDNA of dogfish (Fig. 4.3).

4.2. Amplification of MHC class I genes

4.2.1. Using primers Tu672 and Tu803

The primers Tu672 (antisense) and Tu803 (sense) were used (Takeuchi et al., 1995) for the amplification of MHC class I genes from dogfish. The antisense primer Tu672, which was specific for codons 28 to 35 of the exon 3 class I genes and the primer
Fig. 4.1. The sequences of β1 and β2 regions of Atlantic salmon MHC class II β chain sequence (c144) (Hordvik et al., 1993). The two primers were designed from it and used to amplify trout, carp and dogfish MHC class II β chain genes. The primer positions were underlined.

→Leader peptide

V A V V P L L S Q Q R N M S M S I F C V
CCGTTGGCTGTCGTTCCGTCGTCGACGAGGAAACATGCGGATATCTCTCTGCGTT

→β1

S L T L V L S I F S G T D G Y F Y H M T
TCCCTGACCCTGGTTTTGCTCCATTTCTGGAAACAGATTGAATATTTATCATATGATGACA
QCRRYSSSKDLQGIELITSYVFNCAGTGCAGATACCTCCTCAAAAGGCCATGCTAGGTGATTACCCGTGATGTTTCAAT
QAENIRFNSSTVGFKYGTEHGCAGGCAAAATATCAATTCAACAGCAGTCTGGGAAAGTTATGATACCTGACGATGGA
VKNAEAWNKGPELAGELGVLEGTGAGAAAATGCAAGCCTGGAAACAAAGGTCTCGTGCTGGAGAGCTAGGGTGCTGGAG

→β2

R Y C K F N A P I D Y S A I L D K T V E P
CGTACTGTAAGTTTAAAGCTCTTATCGACTACAGGCCCATATCGACGACGACGTTGACGCCCC
H V R L S S V A P P S G R H P A M L M C S
CATGTCAGACGCTGCTAGTGCCTGGCCCCAGTCGCGACGACGACGCTGTGATGCTGACGC
A Y D F F P K P I R V T W L R D G R E V K
GCCTACGACGCTCTTCTCCCAAAACATCAGAGTGGTGGTGGGAGGCAAGAGGCAGTTGAGGTGAAG
SDVTSTEELANGDWWYQIHSHTCCTGATGTAACCTCCACTGAGGACGCTGCTGACGCTACCACTGGCAAGAGATAGTTGAGCTGGAG
LEYTPRSGEKISKCMVHEHISLTLTGAGGAAATACACCCCAAGCTTGAGAAGAGATCTCTCGCATGCGGAGGCACATCGCGCTACT
EMPVMYWH
GAGCCCATGGTGATCTCAGGG
Fig. 4.2. Characterisation of PCR products amplified from dogfish (lane1), trout (lane2) and carp (lane 3) cDNA prepared from lymphocytes, respectively. A single product of ~360 bp is indicated by an arrow. Lanes 4 and 5 were trout genomic DNA and lanes 6 and 7 were dogfish genomic DNA. There was no definite band in the lanes 4, 5, 6, and 7 and appears as smear. Lanes 8 and 9 were control and a 123 DNA ladder, respectively.
Fig. 4.3. Characterisation of PCR products amplified from trout (lane 1) and dogfish (lane 2) genomic DNA. Multiple products ~120, 250, 360 and 600 bp are indicated by arrows. The PCR was performed in 94°C for 1 min., 45°C for 1 min., 72°C for 1 min. and for 35 cycles. Lanes 3 and 4 were PCR products from carp and trout cDNA prepared from lymphocytes, respectively. Lanes 5 to 9 were dogfish cDNA at different Mg++ concentration indicated at the top. Lanes 10 and 11 were control (without DNA) and a 123 DNA ladder, respectively.
Tu803, which encompasses exon 2 codons 3 to 9 of the putative zebrafish MHC class I gene (Fig. 4.4). The expected product was approximately 360 bp from cDNA and 1030 bp from genomic DNA according to zebrafish MHC class I gene organisation (Takeuchi et al., 1995). These two primers were used in RT-PCR on dogfish cDNA and a doublet in the range of 500 bp was produced. The reaction was sensitive to Mg\textsuperscript{2+} concentration (Fig. 4.5). However, it was difficult to obtain clear and reproducible PCR products and a new set of primers was designed as follows.

4.2.2. Using primers MHCF2 and MHCR2

Two degenerative primers (MHCF2, MHCR2) were designed that were based on the amino acid positions corresponding to the highly conserved regions surrounding cysteine residues in the MHC membrane-proximal domains, and the banded houndshark MHC class I α3 domain sequences (clone DI and λDS-1) (Fig. 4.6) (Hashimoto et al., 1992). The primer sequences were

\[
\text{5'}(\text{sense})-\text{CTGT(C/T)A(G/T)GTA(A/C)(A/T)G(A/G)(A/T)TTCTA(C/T)}
\]

for codons 23 to 30 of MHC class I α3 domain;

\[
\text{5'}(\text{antisense})-\text{G(N/C)(N/G)CTG(G/C)(G/T)GTGCTCCAC(G/T)TG(A/G)C}
\]

for codons 80 to 87 of MHC class I α3 domain.

Unspecified multiple bands were produced (Fig. 4.7) using dogfish and trout genomic DNA as the template and an annealing temperature from 40°C to 55°C and Mg\textsuperscript{2+} concentration from 1.5 mM to 4 mM with a hot start at 95°C for 10 min. A single band of approximately 500 bp was consistently obtained from trout genomic DNA (Fig. 4.8) when the annealing temperature rose to 60°C. This was cloned and sequenced but was unrelated to any known MHC sequences. A single band of approximately 190 bp was also obtained using cDNA prepared from lymphocytes of a single dogfish (Fig. 4.9) and this
Fig. 4.4. The part of sequence of MHC class I gene of zebrafish. The corresponding positions of the degenerative primers Tu672 and Tu803 are underlined.

Exon 2
GGTGCAACCACAGCTGGAAAGCCTATTACACTGGAAACTGGGTTGACTGAGTTCCAGAGTTTGCTGGCAGCTTAATCCTAGATGATCAGTTGATGGGGTACTTCGACTCCAGAGTTTGCTGAGATGGATGATGATGGCAACAAACAAGTACATTGGCAGATTGAGGGAGTGCTACTGGAAATTTCAAGA

Exon 3
ACAACATTACAGTTATGATGAGCGCTTTCAACCAAACAAAGATGTTCCACACCTTCAGGTCATGTACGGCTGTGAGATGGATGATGATGGCAACAAACAAGTACATTGGCAGATTGGCTATCAGATGGAGAGGACTTCATCAGTTGGATAAA
Fig. 4.5. Characterisation of RT-PCR products amplified from dogfish cDNA prepared from lymphocytes. A doublet in the range of ~500 bp was indicated by arrows and Mg$^{++}$ concentration is at the top of each lane. Lane 6 was the PCR control without cDNA, and lane 7 was a PCR marker.
Fig. 4.6. The sequences of clones DI and λDS-1 (banded houndshark MHC class I α3 domains, Hashimoto et al., 1992). These two sequences are identical except for a single nucleotide difference indicated by red besides the primer positions. The degenerative primers MHCF2 and MHCR2 were derived from these two sequences. The primer positions in DI and λDS-1 are underlined.

DI

\[
\begin{align*}
CYVTDFYPSGINATWLH & \\
TGTTAGGTGACAGATTTCTACCCAAAGTGCCATCAACGCAACCTGGGTGAC & \\
TGTCATGTGAATTGCATCTCAAAGTGCCATCAACGCAACCTGGGTGAC & \\
CHVNGFYPSGINATWLH & \\
\end{align*}
\]

λDS-1

\[
\begin{align*}
NGTVQQEVLSRILPN & \\
AACGGTGACGGCACCCTTCCCCAGGACACCCTGGGTGACGATCACCCTGGGTGAC & \\
AACGGTGACGGCACCCTTCCCCAGGACACCCTGGGTGACGATCACCCTGGGTGAC & \\
NGTVQQEVLSRILPN & \\
\end{align*}
\]

DI

\[
\begin{align*}
TDGFQTTLQISVTTPQ & \\
ACAGACGGGACCTTCCCCAGGACACCCTGGGTGACGATCACCCTGGGTGAC & \\
ACAGACGGGACCTTCCCCAGGACACCCTGGGTGACGATCACCCTGGGTGAC & \\
TDGFQTTLQISVTTPQ & \\
\end{align*}
\]

λDS-1

\[
\begin{align*}
RDYTCTCPVEDSS & \\
AGAGACCTACCATCCTGGTAGGACACCCAC & \\
AGAGACCTACCATCCTGGTAGGACACCCAC & \\
RDYTCTCPVEDSS & \\
\end{align*}
\]
Fig. 4.7. Unspecific multiple PCR products were obtained from dogfish and trout genomic DNA. Mg++ concentration is indicated at the top of each lane. Lanes 1 to 4 were PCR products from dogfish genomic DNA and lanes 5 to 8 were PCR products from trout genomic DNA. Lane 9 was a control sample (without DNA) and lane 10 was a 123 DNA ladder.
Fig. 4.8. A single PCR product at ~500 bp from trout genomic DNA was consistently amplified using the primer MHCF2 and MHCR2 for MHC class I α 3 domain. These four lanes were in the same PCR conditions as in Fig. 4.7 with exception that Mg\(^{2+}\) concentration was 1.5mM.
Fig. 4.9. A single band of approximately 190 bp was amplified from cDNA prepared from dogfish lymphocytes and is indicated by an arrow. Lanes 3-7 were PCR samples from dogfish with different Mg\(^{++}\) concentration; lane 2 was control sample without template; lane 1 was 1 kb DNA ladder. These two bands on the bottom were dimerisation-primers. The PCR was performed in 94\(^{0}\)C for 1 min., 45\(^{0}\)C for 1 min., 72\(^{0}\)C for 1 min. and for 35 cycles with a extension at 72\(^{0}\)C for 5 min.
was subsequently cloned into pUC18. Twenty-seven clones with insertions were isolated. Eleven of these recombinant clones were characterised by sequencing the inserts (Fig. 4.10). The nucleotide sequences of nine of these clones (designated DM1, DM2, DM9, DM13, DM17, DM20, DM24, DM28, and DM29) were homologous to MHC class IIA genes of a number of species. Four of these clones (DM2, DM9, DM20, and DM29) were exactly 193 bp in length, three (DM24, DM28, and DM13) were 188, 166 and 149 bp respectively and the remaining two (DM1 and DM17) were 271 and 239 bp respectively. The first 193 bp of both DM1 and DM17 were homologous to the other seven clones. None of the nine clones were identical. These nine clones shared >97% nucleotide sequence homologous with only 21 nucleotide changes between them (Fig.4.11). DM9 and DM29 were the most diverse from the consensus sequence (DM2) with 8 differences at positions 12, 13, 15, 22, 106, 186, 190, 192 and 13, 35, 36, 98, 106, 166, 185, 190, respectively. Comparative nucleotide sequence analysis showed that these dogfish clones were homologous to the nurse shark MHC class IIA chain (68.4%), the banded houndshark MHC class I α3 domain-like gene (28.8%), human HLA-DQB (27.7%), rat MHC class II β1 chain (25.5%), and bovine MHC class II β chain (24.4%) (Table 4.1).

The putative amino acid sequences of these nine clones are shown in Figure 4.12. There are 9 amino acid residue differences between the clones at positions 4, 5, 7, 10, 12, 32, 33, 62 and 64. Five of the nine clones had amino acid substitutions at positions 4 and 62 with 3 varied amino acids. Some of the nucleotide substitutions were silent, for instance, the C → T substitution at nucleotide position 73, which encodes an aspartic acid at amino acid position 24. A silent substitution was also present at nucleotide position 76 (C → A substitution), 106 (A → G substitution), 151, 166 and 190 which were all C → T substitutions respectively. The amino acids are not substituted, as they still encode for glycine at position 25, leucine at position 35, serine at positions 50 and 63 and tyrosine at
Fig. 4.10. The nucleotide sequences of selected clones from PCR products obtained from dogfish cDNA. The sequencing was performed using Sequenase Sequencing Version II and labelled by $[^{35}\text{S}]-\text{dATP}$. The loading order was G, A, T and C. The M13-Universal reverse sequencing primer was used in sequencing reactions.

DM9  DM13  DM28

← Start point
Fig. 4.11. The nucleotide sequences of cDNA clones DM2, DM9, DM20, DM29, DM24, DM28, DM13, DM1 and DM17 from dogfish. The boxes indicate residues that differ from DM2.
Table 4.1. The score of the nucleotide sequences of dogfish clones with the homologous to MHC class IIA2 genes of different species in a computer search from GenBank and EMBL database release 100.

<table>
<thead>
<tr>
<th>Species</th>
<th>The highest homologous Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nurse shark</td>
<td>68.4% MHC class IIA</td>
</tr>
<tr>
<td>Banded shark</td>
<td>28.8% MHC class IA3</td>
</tr>
<tr>
<td>Mole rat</td>
<td>35.4% MHC class IIB1</td>
</tr>
<tr>
<td>Human</td>
<td>27.7% HLA-DQB</td>
</tr>
<tr>
<td>Bovine</td>
<td>24.4% MHC class IIB</td>
</tr>
</tbody>
</table>

The MHC class I and II genes from various species are derived from GenBank and EMBL database. The entries for these sequences are GINMHAA (nurse shark), TRKAMHC3A (banded shark), SEHMHPBO3 (mole rat), HUMMHDRDQ (human), BTU69097 (bovine),
Fig. 4.12. The predicted peptide sequences of cDNA clones DM1, DM2, DM9, DM13, DM17, DM20, DM24, DM28 and DM29 from dogfish. The boxes indicate residues that differ from DM2.
A search of the databases using the predicted amino acid sequences showed that the highest peptide alignment score is with the MHC class IIA chain of a number of species (majority was over 40%). The most striking homology was with the membrane proximal region of the MHC class IIA chain of the nurse shark (62.2-74.1% sequence identity), followed by zebrafish (48.4-52.8%), mouse (36.7-49.6%), rat (36-49.9%), bovine class II DQ A chain (45.5%) and human class II DP and DQ A chain (44.4%) (Table 4.3). According to the Kabat nomenclature (Kabat et al., 1991) the amino acid sequences corresponded to the MHC class IIA2 from position 107 to 170. Eleven amino acid residues were conserved between higher vertebrates and the dogfish. The conserved residues include cysteines at position 107 and 163 and a tryptophan at position 121. A putative N-linked glycosylation site occurs at position 118 in most vertebrates, however this was absent in the dogfish as well as the nurse shark although present in the zebrafish, but a potential N-linked glycosylation site was found at position 133 in both the nurse shark and dogfish (Fig. 4.13).

The DM9 (Fig. 4.14) was used as a probe to investigate the number of MHC class II genes with the restriction endonucleases Hind III, Bgl II or Bgl II/Hind III in 7 fish (Fig. 4.15). The results showed that all seven fish had a 9 kb Hind III fragment, six fish had a 2.5 kb fragment, five fish had a 3.5 kb fragment, and three fish had a 3 kb fragment. There were also a 2.25 kb fragment in two fish and a 5 kb fragment in a single fish (Fig. 4.16). Using the restriction endonuclease Bgl II, six fish had a 2.8 kb fragment whilst three fish had fragments of 1, 2.5 and 5 kb. There were a 4, 6 and 10 kb of fragments in one or two fish, respectively (Fig. 4.17). The DNA of fish digested with both Bgl II/Hind III, yielded fragments of 2.3 kb in all fish, together with fragments of 2.8 and 3.5 kb in four fish. There were fragments at 1, 4.25, 5 and 8 kb in one to three fish (Fig. 4.18).
Table 4.2. The silent substitution of nucleotides in clone DMI, DM2, DM9, DM13, DM17, DM20, DM24, DM28 and DM29

<table>
<thead>
<tr>
<th>Nucleotide Position</th>
<th>Substitution</th>
<th>Amino Acid Position</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>C→T</td>
<td>24</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>76</td>
<td>C→A</td>
<td>25</td>
<td>glycine</td>
</tr>
<tr>
<td>106</td>
<td>A→G</td>
<td>35</td>
<td>leucine</td>
</tr>
<tr>
<td>151</td>
<td>C→T</td>
<td>50</td>
<td>serine</td>
</tr>
<tr>
<td>166</td>
<td>C→T</td>
<td>55</td>
<td>tyrosine</td>
</tr>
<tr>
<td>190</td>
<td>C→T</td>
<td>63</td>
<td>serine</td>
</tr>
<tr>
<td>Species</td>
<td>Amino Acid Sequence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogfish DM2</td>
<td>CQVNGFYPYPRSMK1KKKINEDLTGDVNITEYYKLDYSYQRFTYKFTPSPGDMYSCHVEHRSI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nurse shark IIA</td>
<td>CFADGFYPPHITMWNRENPMTDGDQNFYIKEDFTFRRSYSLSVIPSPGDMYSCHVEHSSL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish IIA</td>
<td>CHVTGGPPVNYVRNVTNNDIVTEISFSQYRNDSGDTFNMSALKFTPAEQDSYCTNVHRSI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IIA</td>
<td>CIVDDIFPPVINVTWLRNSQPTIKGVAQTSFYSQPNHRKFHYLTFVPSAEVDVYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat IIA</td>
<td>CMVEDIFPPMNINITWLRNSQPTIKGVITQTSFYSQPNHRKFHYLTFVPITAEDVYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine IIA</td>
<td>CQVDNIFPPVINITYFYNGHFVAEIGIETTFYPSDKHLSFLKFSYLTFPLFTSDFVYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLADPA IIA</td>
<td>CHIDKFFPVVLNVTNLNGELVTEGVAESLFLPRTDYSFHKFHYLTFVPSAEVDVYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLADRA IIA</td>
<td>CFIDKFTPVPVNNLTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPSTEVDVYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLADQA IIA</td>
<td>CLVDNIFPPVINITWLSNGHSTVETGVESTSFLSKDSFHFKISLYLTLFPLPSADEAYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLADZA IIA</td>
<td>CIVDNIFPPVINITWLRNGQTVTEGVAQTSFYSQPDHLFRKFHYLPFLPSEAEVDVYDCRVEHGL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig 4.13. The comparison of the membrane-proximal domain of amino acid sequences of dogfish MHC class II A chain (DM2) and representative MHC proteins of other species. Sequence alignment was performed by the clustal method to maximize sequence similarity. Boxes indicate completely conserved residues in all sequences. The potential N-linked glycosylation sites are highlighted in the shaded boxes. GenBank identifies for the specific sequences shown as follows: shark class II A (A46185), zebrafish class II A chain (L19450), mouse MHC class II A chain (M21931), rat class II A chain (S80420s3), bovine class II A chain (B37206), HLA DPA (HA2P-human), HLADRA(P01903), HLADRA(A37044), HLADZA(HA2Z).
Fig. 4.14. Clone DM9 was digested with enzyme \textit{Eco R I} and \textit{Pst I} at 37\(^\circ\)C for 2 to 4 h. The samples were loaded onto a 3% agarose gel. The plasmid inserts were purified and used as the MHC class IIA2 probe.
Fig. 4.15. Seven dogfish genomic DNAs (5-10µg) were digested with *Hind* III at 37°C for three days. Then, the DNA samples were loaded onto a 0.6% agarose gel and the electrophoresis was performed at a constant voltage of 70 overnight. After electrophoresis, the migrations of the DNAs were measured by visualising the separation of the lambda markers and 1 kb DNA ladders under UV light with a rule. A photograph was taken.
Fig. 4.16. Southern blot showing genomic DNA from dogfish No.1, No2, No4, No5, No6, No7, and No.8, digested with Hind III and hybridised to $^{32}$P-labelled MHC class IIA2 chain probe DM9.
Fig. 4.17. Southern blot showing genomic DNA from dogfish No.1, No2, No4, No5, No6, No7, and No.8, digested with $Bgl$ II and hybridised to $^{32}P$-labelled MHC class IIA2 chain probe DM9.
Fig. 4.18. Southern blot showing genomic DNA from dogfish No.1, No2, No4, No5, No6, No7, and No.8 digested with Hind III/Bgl II and hybridised to $^{32}$P-labelled MHC class IIA2 chain probe DM9.
There are no *Bgl* II and *Hind* III restriction sites in DM9. A restriction map was constructed according to the Southern blotting analysis results. There may be four MHC class IIA loci in fish No. 2 and fish No. 4 (Fig. 4.19). It was difficult to deduce the restriction maps in fish No. 1, 5, 6, 7 and 8.

A phylogenetic tree was constructed to predict the divergence between sequence pairs. The membrane-proximal domain is not a binding site for peptides, hence this domain could be expected to evolve at a constant rate and reflect the evolutionary history of the MHC. The membrane-proximal domains from different species were chosen to construct the phylogenetic tree. The phylogenetic tree showed the relationship between the dogfish class IIA chain (clone DM9) and to the class IIA chains of a number of species including man, rat, mouse, bovine, zebrafish and the nurse shark. The results showed that the dogfish DM9 sequence was more closely related to the nurse shark class IIA chain and was more distantly related to the class II sequences of other species with the exception of the teleost zebrafish (Fig. 4.20).

4.3. Amplification of MHC class II α chain from dogfish genomic DNA

Based on the alignment of the MHC class IIA sequence with other species, another set of primers was designed. The sense primer (MHCF3) corresponding to codons 27 to 36 of the MHC class IIA-[5' GA(C/T)GG(A/T)GANGAGNT(G/A/T)C(C/T)AC(T)N(T)(A/G)GA], was derived from the conserved region of several species including nurse shark (Kasahara et al., 1992), zebrafish (Sültmann et al., 1993), mouse (Bishop et al., 1988), human DR (Lee et al., 1982), human DQ (Jonsson. et al., 1989). The antisense primer (MHCR3) corresponding to codons 160 to 167 of MHC class IIA-[5' GTGCTCCACGTGGCAGGA(A/G)TACATG], was designed from the conserved region of known dogfish MHC class II α2 chain from the nine clones DM2 to
Fig. 4.19. Possible MHC class IIA gene loci in dogfish. H and B are restriction enzyme *Hind* III and *Bgl* II, respectively. The possible locus is labelled by the number 1, 2, 3 and 4.

Fish No. 2.

Fish No. 4.
Fig. 4.20. Phylogenetic tree showing relationship of the dogfish class IIA chain (DM9) to representative MHC II α2 chain of other species. The source of sequences is as described in the legend to Fig. 4.13.
DM29. A product of approximately 550 bp including the intron should be obtained based on the zebrafish MHC class II α chain gene organisation (Sültmann et al., 1993). PCR was performed from genomic DNAs of 8 individual dogfish and these two primers produced three bands of approximately 500-750 bp from all 8 dogfish genomic DNA (Fig. 4.21). The 550 bp fragments from two fish were cloned, and four recombinant clones were characterised. Three clones C1-5, C1-11 and C1-39 were from the first dogfish, other one C2-20 was from the second dogfish. The plasmids were isolated and the recombinants were partially sequenced. 486 bp of C1-5 was sequenced in length, 153 bp of C1-39 and C2-20 were sequenced, the C1-11 was only sequenced last 133 bp. The antisense primer MHCR3 was found in all four sequences. These four sequences were identical in the last 153 bp except for C1-11 (Fig. 4.22). Although there were ORFs, they showed no homology to MHC class IIA genes. The strategies of amplification and characterisation of dogfish MHC class II genes are depicted in Fig. 4.23.
Fig. 4.21. Characterisation of PCR products amplified from 8 dogfish genomic DNAs using primers MHCF3 and MHCR3 for amplification MHC class IIA chain genes. Approximately 500-750bp were amplified from all fish. Lane 1 was fish 8, lane 2 was fish 7, lane 3 was fish 6, lane 4 was fish 5, lanes 5-6 were fish 4, lane 7-8 are fish 3, lanes 9-10 were fish 2, lanes 11-12 were fish 1, and lane 13 was 1 kb DNA ladder.
Fig. 4.22. The nucleotide sequences of clones C1-5, C1-11, C1-39 and C2-20. 493 bp of clone C1-5 was sequenced and 153 bp of clone C1-39 and C2-20 were sequenced. The clone C1-11 was sequenced 133 bp. The last 153 bp of clone C1-5 are identical to all other three clones. The antisense primer MHCR3 is underlined.

1-5 AGCAGCAACTAATTCCAAAACTCCCATCAGTCTTCATGGGCTTTCAGGGAGA
1-5 TTCGGAAAAATGTTACCAATACGTAAACCTTCTGTAAGGGGACGCATTCTCGC
1-5 TCAGTACTGCAAAGGCTATGGAAGAGCAGATCTGTATGTGGATCGTTATAT
1-5 CTCGAAACCCACATCCCTGCAATTTGATCCACTGAGACATACCTGATT
1-5 ACCCAACCTCCAAATCCCTGCTCCCCATATATATTTTTAGATGTTTTTTCTTGT
1-5 TGCGCCTTGTATATTACAGGTGTAACAAAGATGAAGAAAAAAGGAGAT
1-5 GATAAA...

1-5 ...ACCAAGATTAATCAAGGAAGCAAGCAAGTGAAGTTAGAATCATTTTGTCAT
1-11 ...ACCAAGATTAATCAAGGAAGCAAGCAAGTGAAGTTAGAATCATTTTGTCAT
1-39 ...ACCAAGATTAATCAAGGAAGCAAGCAAGTGAAGTTAGAATCATTTTGTCAT
2-20 ...ACCAAGATTAATCAAGGAAGCAAGCAAGTGAAGTTAGAATCATTTTGTCAT

1-5 TGAAAAATCAAGGTGTTGGGGAAATAAAGCTGCGGATGTATATATACATAG
1-11 TGAAAAATCAAGGTGTTGGGGAAATAAAGCTGCGGATGTATATATACATAG
1-39 TGAAAAATCAAGGTGTTGGGGAAATAAAGCTGCGGATGTATATATACATAG
2-20 TGAAAAATCAAGGTGTTGGGGAAATAAAGCTGCGGATGTATATATACATAG

1-5 AGGCACCTGAAAGAGATTTAATTATTTGCTATGATCTCTCTGCACGTGGAGC
1-11 AGGCACCTGAAAGAGATTTAATTATTTGCTATGATCTCTCTGCACGTGGAGC
1-39 AGGCACCTGAAAGAGATTTAATTATTTGCTATGATCTCTCTGCACGTGGAGC
2-20 AGGCACCTGAAAGAGATTTAATTATTTGCTATGATCTCTCTGCACGTGGAGC
cDNA synthesised from dogfish mRNA extracted from lymphocytes

\[ \text{PCR with primers MHCF1 and MHCR1} \rightarrow \text{No PCR product was obtained} \] (For MHC class II B gene)

\[ \text{PCR with primers Tu672 and Tu803} \rightarrow \text{A doublet in the range of ~ 500bp was produced and not reproducible} \] (For MHC class I A genes)

\[ \text{PCR with primers MHC F2 and MHCR2} \rightarrow \text{190bp PCR products were obtained} \rightarrow \text{Inserting the PCR fragments into plasmid pUC 18} \] (For MHC class I A genes)

Compared with GenBank and EMBL database

Sequencing the insertions

Identified 9 MHC class II A2 genes

The 7 dogfish genomic DNA was digested with several restriction endonuclease and hybridised with probe (DM9)

Dogfish may have four MHC class IIA loci
4.4. Discussion

The primers MHCF1, MHCR1, Tu672 and Tu803 were all designed from the known zebrafish and Atlantic salmon MHC genes. These primers produced either no band or multiple bands from dogfish. A single band of the expected sized was produced from trout and carp cDNA and suggested that zebrafish and Atlantic salmon MHC genes were distinct from the dogfish, but more related to the trout and carp.

In the first instance the primers MHCF2 and MHCR2 were used for amplification of MHC class I α3 chain from fish, but in fact the MHC class II α2 chain was amplified. This was not surprising because the two primers were designed from the two highly conserved amino acid motifs around two cysteine residues in the membrane-proximal domain, that is the second domain of MHC class II and the third domain of MHC class I (Hashimoto et al., 1990, 1992; Kasahara et al., 1992; Bartl and Weissman, 1994b). The conserved cysteine residues, which are involved in an intradomain disulphide bridge, are present at positions 107 and 163 in clones DM1, DM2, DM9, DM17, DM20, DM24 and DM29 of the dogfish together with a conserved tryptophan residue at position 121. This strongly suggests that these sequences are members of the Ig superfamily gene. The N-linked glycosylation site occurs at position 118 in both nurse shark and dogfish MHC class IIA and this suggests that the most primitive vertebrates may have a similar structure of MHC molecules.

The additional sequences present in clones DM1 and DM17 may be a PCR artifact. In this reaction, some PCR fragments have been ligated together before inserting into the vector. Clone T17 of trout was 415 bp. Normally there is no intron within this gene, therefore this longer sequence cannot code for one domain of Ig.

The Southern blot and hybridisation results suggest that either the number of MHC class IIA genes may vary between individual dogfish, or, that RFLP occurs. The
non-identical nine clones show that polymorphism and multi-loci are present in dogfish MHC class II genes. This is supported by the Southern blot and hybridisation with DM9 probe. According to the restriction map, it seems that the distances between MHC class II loci are very short. This may be due to the short introns in gene loci. At present, the number of the MHC loci in fish is not mapped yet, although several reports suggest that the jawed fish at least have two MHC class IIA loci (Kasahara et al., 1993).

The phylogenetic tree showed that zebrafish MHC class II derived from a separate branch of the MHC class II compared to other species. This also suggests that zebrafish MHC class II genes are derived from a different ancestor than the various class II gene families of other vertebrates and the cloned shark class II A gene (Ono et al., 1992; Sültmann et al., 1993).
5.1. General discussion

A major difference in the immune system between vertebrates and invertebrates is in ability to develop acquired immunity. Invertebrates tend to use 'innate' immunity to defend against non-self antigens and organisms, whilst vertebrates utilise 'acquired' immunity for protection of the body against non-self antigens and organisms. The most outstanding features of the acquired immune system are processing and presentation of foreign peptides by MHC molecules, the recognition of this complex by TCR, and the ability of B-cells to bind with foreign antigens and secrete antibodies. With the exception of the jawless fish, TCR, MHC class I and class II genes have been isolated and characterised in all vertebrate species. Dogfish is one of the shark species and to date, there has been no report of identification of TCR and MHC genes in this species. This thesis describes the isolation of MHC and TCR genes from the most primitive vertebrate dogfish (*Scyliothinus canicula* L.) and provides data to support the notion that MHC and TCR genes are present in dogfish. Although the physical structure of Ig, TCR and MHC molecules in primitive vertebrates have still to be elucidated, their identification at such an early stage of vertebrate phylogeny provide evidence that TCR recognition systems operate in these vertebrates.

Two TCRBV (2C144, 6C43) and two TCRDV (6C53, 6C45) genes were isolated and their characterisations show that they have strong homology to TCRBV and TCRDV genes of various species including human, chimpanzee, monkey, mouse, rat, bovine, pig, rabbit, and horned shark.

The predicted amino acid sequences of dogfish TCRBV and TCRDV show that they
contain certain key amino acid residues that are extensively found in other species. These include the TCRV motifs W\textsuperscript{34}Y\textsuperscript{35}Q\textsuperscript{37} and YY(F)C\textsuperscript{92}. Cysteine at position 92 is also present in the dogfish and this probably forms an intradisulfide bond with another cysteine residue at position 23. These are critical residues and are found in all sequences of the Ig superfamily. These conserved residues suggest that the dogfish TCR is likely to have the same primary, secondary and three-dimensional structure as TCRs found in higher vertebrate species. It also suggests that the TCR in the dogfish may undertake the recognition of MHC-peptide complexes presented by antigen presenting cells in a similar manner to that found in other species.

The majority of fish Ig genomic genes are arranged as multiclusters with the exception of teleost Ig light chain genes. Recombinase genes have been isolated from the shark and zebrafish (Bernstein et al., 1994,1996; Greenhalgh and Steiner, 1995) and the typical recombination signal sequences have also been identified in the horned shark (Rast and Litman, 1994). This indicates that the Ig genes of these primitive vertebrates undergo somatic gene rearrangement. Rast and Litman (1994) suggested that the shark TCR genes are multiclustered. In the shark, the TCRB loci consists of a multicluster that includes several V segments, a possible D segment, many J segments and a single C segment. Partula and his co-workers' (1994) showed that in the rainbow trout there is at least one TCR gene cluster in the germline. Southern blot analysis using 2C144 (TCRBV gene) as a probe showed that up to 3 bands were present dependent upon the restriction endonuclease enzyme employed (\textit{EcoR} I, \textit{Bgl} II and \textit{Hind} III). There was variability in the size of the fragment between individual fish. This suggested that the TCRBV families have either a number of members or RFLPs, or both. The clone 6C45 and 2C144 seem to be different
TCRBV families, which indicates that dogfish also have several TCRBV gene families. It remains to be seen if the TCRB loci are clustered in the dogfish. But the results do suggest that there are several TCRBV families in the dogfish. The results of the Southern blot using the 6C53 as a probe in conjunction with the endonuclease EcoRI identified five bands in all four fish, although some fish display extra bands suggesting that there may be variability in the number of copies of the gene or RFLP. Hawke et al. (1996) reported cDNA clones representing 7 TCRVB families from shark and analysed VDJ junctional diversity and variation in the TCRCB regions and indicated that a remarkably high degree of complexity in TCR genes probably existed in the common ancestor of all living jawed vertebrates. Dogfish was expected to be the same as shark in TCR gene organisation. To confirm this, further work is required to isolate entire TCR gene sequences.

Northern blot and hybridisation identified a transcript of approximately 2 kb which was present in the spleen, lymphocytes and brain as well, and absent from the liver and kidney of dogfish for probe 6C53 and a transcript of approximately 2 kb which was present in lymphocytes of dogfish for probe 2C144. These results indicate that dogfish may have two sets of T-cells that express either TCR αβ or TCR γδ and also suggest that TCR αβ and TCR γδ cells may be distributed differently between tissues.

The MHC class II genes coding for class IIA2 chain were isolated and characterised from cDNA prepared from dogfish lymphocytes. These genes share homology to MHC genes found in several species including the nurse shark, zebrafish, mouse, rat, bovine and human. The closest relationship was with the third exon of the nurse shark MHC class IIA genes (>60%). It is therefore likely that dogfish express and utilise MHC molecules in the recognition and presentation of peptides.
Analysis of the nine sequences from the cDNA of a single dogfish showed that these were closely matched with only a few of substitutions. This suggests that there may be several MHC class II gene loci in the dogfish which is similar to the results obtained in other fish species (Ono et al., 1992; Stet et al., 1993; Sультmann et al., 1993). This may be caused by chromosomal and/or gene duplication or mutations occurring during duplication. Otherwise, the dogfish MHC class II genes are located in several chromosomal regions analogous to the organisation of the Ig genes in shark species. A single MHC allele can bind many peptides (Rammensee et al., 1993). Therefore, the acquisition of a large number of functional MHC molecules with distinct peptide-binding capabilities may appear advantageous to the bodies. Although data on the MHC class II α1 domain sequences is unavailable it can be proposed that these nine sequences may represent different gene loci in MHC class IIA genes while may enable a number of different pathogenic-peptides in the environment to be recognised. There are several key residues conserved in the dogfish MHC class II α2 domains. Two cysteine residues are present in all nine clones at position 107 and 163 and form an intradomain-disulfide bond, which is an important structure for stabilising MHC molecules. Other conserved residues such as tryptophan strongly suggest that these genes are members of the Ig gene superfamily. A putative N-linked glycosylation site is present in these nine sequences at position 133 as in the nurse shark, but different from other species including zebrafish, mouse, rat, bovine, and human. This suggests that the cartilaginous fish may have a slightly different structure from other species.

A lot of data has showed that shark Ig light-chain genes exhibit greater nucleotide sequence identity with certain mammalian TCR genes than with higher vertebrate Ig light-chain genes (Shamblott and Litman, 1989a; Rast et al., 1994). Comparison of the predicted
peptide sequences of these light-chain, higher vertebrate Ig light-chain, TCR, and CD8 genes showed that two four-amino acid stretches in FR2 of both TCR and Ig light-chain (WYRQ) and FR3 [Y(Y/F)CA] are highly conserved and exhibit little phylogenetic variation (Johnson, 1987; Davis and Bjorkman, 1988). These motifs are found in nearly all TCR isotypes and Ig light-chain proteins. Rast’s group (1994, 1995, 1997) designed one 5’ degenerated primer complementary to the WYRQ position and two degenerated 3’ primers complementary to the Y(Y/F)CA position and successfully amplified and isolated TCRA, TCRB, TCRG and TCRD gene segments from horned shark, skate, frog, and pufferfish. Sequentially, they screened a cDNA library and obtained full length cDNA sequences of TCRB from horned shark. Partula’s group (1994, 1995, 1996) designed similar primers and amplified the TCRBV genes from rainbow trout. These results show that the use of primers against these motifs is a very efficient method of amplifying and isolating TCR genes from non-mammalian species. In this project, short, minimally degenerate PCR primers were employed and these identified two TCRBV and two TCRDV gene segments from dogfish genomic DNA. These conserved motifs (WYRQ) and [Y(Y/F)CA] sequences are present in these four sequences. Rast et al. (1995) pointed out that the use of genomic DNA as the PCR template for identification of TCR homology was advantageous as there was no need to take account of tissue and developmental stage-specific expression of the gene.

Hashimoto and co-workers (1990) identified the two conserved residue blocks that flank two cysteines in the second domain of MHC class II and α3 domain of MHC class I. Most researchers have used degenerative primers designed from these two blocks and have successfully isolated MHC class I and class II genes from genomic DNA and cDNA of several fish species. This strategy was also employed in this project. Two primers that flank
these two cysteine residues were designed that correspond to the shark MHC class I α3 domain and were used to investigate the MHC class I genes in dogfish. In practice, however, these primers amplified a product of the MHC class II genes corresponding to the α2 domain from dogfish lymphocyte cDNA. This indicates that this conserved region is common to the MHC class I α3 and MHC class II α2 domain and also provides some evidence that the MHC class I and class II genes evolved from the same ancestral genes.

Rast, Litman and Hashimoto groups have pioneered the isolation and characterisation of TCR and MHC genes in fish. The design of primers is critical for the successful amplification of genes across different species. From this project, it was apparent that the primer was critical for successful amplification of the relevant gene.

DNA recombination is a technique that allows large quantities of cloned target DNA to be obtained in highly purified forms. The selection of the vector is an important factor in cloning. This project used plasmid pUC 18, which is a high copy number plasmid with multi-cloning sites and allows the easy identification of recombinants by the colour of the colonies. At the onset of this project, cloning of PCR products was not used and this resulted in the poor quality of the sequencing. Subsequent cloning of the products improved the sequencing results dramatically.

Construction of a dogfish cDNA library is an important step for isolating whole length target DNA sequences, investigating the subfamilies of Ig superfamily, and identifying conserved functional residues. Many previous studies on the isolation of the TCR and MHC genes from lower vertebrates have applied strategies to obtain and characterise the Ig superfamily genes. It was not feasible to construct a dogfish lymphocyte cDNA library in this project, the reasons for this are many and include the quality of the RNA from dogfish
and synthesis of double strand cDNA.
In conclusions, several novel observations are presented in this thesis.

1. MHC class IIA genes, TCRB and TCRD genes are present in dogfish.

2. Dogfish may have two sets of lymphocytes-B and T cells.

3. Dogfish may have two subpopulations of TCR bearing T cells, one is TCR αβ cells and another is TCR γδ cells.

4. Dogfish TCRBV and TCRDV are expressed in lymphocytes and spleen cells suggesting dogfish TCR genes may encode functional TCR proteins.

5. The MHC class II genes are present in dogfish and may encode functional MHC class II molecules.

6. The conserved key residues in both TCR and MHC class II genes of dogfish suggest that the lower vertebrates have similar primary, secondary, and three-dimensional structures to those of the higher vertebrates.

7. Dogfish TCRB, TCRD and MHC class II genes show high homology with several species including human, mouse, rat, bovine and other fish species. As dogfish is the most primitive class of vertebrates from which the MHC genes and TCR genes have been isolated, it implies that the Ig superfamily has evolved from a common ancestor.
5.2. Further work

The analysis of full length sequences is important for investigating the evolutionary origin and gene organisation of the Ig gene superfamily. In order to obtain the full length TCR and MHC genes, it would be beneficial to construct a cDNA library from dogfish lymphocytes or spleen cells.

Dogfish TCRBV region (2C144, 6C43), TCRDV region (6C53 and 6C45), and MHC class II α2 region (DM2, DM9) would be used as probes to screen the cDNA library. Positive clones would be isolated, sequenced and compared with the GenBank and EMBL databases to find any similarity with known TCR or MHC genes.

If it was possible to obtain the TCR genes, these could be used to perform chromosomal mapping on large scale with pulse field gel electrophoresis. A dogfish genomic DNA library could be constructed and screened by TCRV, TCRJ and TCRC. It would then be possible to calculate the number of TCRV, TCRJ, TCRD and TCRC segments in the dogfish germline. This would subsequently allow the genomic organisation of the TCR loci to be elucidated.

The α1 and α2 domains of MHC class II would be amplified in a population of dogfish to investigate polymorphism and gene loci. The variability plots of residues (Wu and Kabat, 1970) would be made from sequences of the MHC class II α1. We could identify if the functional residues in PBDs are conserved in the dogfish.

From both the TCR and MHC genes, the predicted protein sequences could be obtained. These TCR and MHC molecules could be compared with other species to measure the degree of conservation of critical amino acid residues and their influence upon the 3 dimensional structure of both the TCR and MHC proteins. This would allow their
relationship to be studied.

The MHC class I, class IIB, TCRA and TCRG genes would be isolated in dogfish using the same strategies: PCR, cloning and sequencing. We would like to investigate if TCRD and TCRA loci are organised similarly in fish as in mammalian species, and if the MHC I and MHC II genes are closely linked together.

Once the protein sequence is known, it may be possible to raise MAbs that could be used to investigate the expression of these genes in the blood and tissue of dogfish. Amplification of T and B cell specific markers such as CD3 and CD5 would help enormously in the quest to determine immunological mechanisms in this species.

CD4 and CD8 act as co-receptor with the TCR complex during MHC/peptide-TCR recognition. Identifying CD4 and CD8 would help to prove that the dogfish use a similar MHC/peptide-TCR recognition system to higher vertebrates.

Finally, it would be interesting to characterise TCR expression in the embryonic and newborn dogfish, in comparison to adult fish and to measure the distribution of TCR αβ and TCR γδ at different stage of fish development. Which TCR is expressed first, TCR αβ or TCR γδ and are these distinct lineages?
Appendix

Part A. Reagents for cloning

1) Ampicillin (stock solution) 50 mg/ml

A 1g amount of ampicillin was dissolved in 20ml ddH₂O and stored at -20°C.

2) 5-bromo-4-chloro-3-indolyi-β-D-galactoside (X-gal)

A 20mg/ml concentration of 5-bromo-4-chloro-3-indolyi-β-D-galactoside was made by dissolving 100mg of 5-bromo-4-chloro-3-indolyi-β-D-galactoside in 5ml of dimethyl-formamide. The tube containing the solution was wrapped in aluminium foil and stored at -20°C.

3) Dimethyl sulfoxide (DMSO)

High quality DMSO was purchased (Sigma, UK). To avoid oxidation products of DMSO, which is an inhibitor of transformation, the DMSO was divided into 1ml aliquots in sterile tubes and stored at -70°C. Each aliquot was used only once and discarded.

4) FSB

<table>
<thead>
<tr>
<th>Reagent</th>
<th>amount required/L</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M potassium acetate (pH 7.5)</td>
<td>10mL</td>
<td>10mM</td>
</tr>
<tr>
<td>MnCl₂4H₂O</td>
<td>8.91g</td>
<td>45mM</td>
</tr>
<tr>
<td>CaCl₂2H₂O</td>
<td>1.47g</td>
<td>10mM</td>
</tr>
<tr>
<td>KCl</td>
<td>7.46g</td>
<td>100mM</td>
</tr>
<tr>
<td>Hexaminecobalt</td>
<td>0.80g</td>
<td>3mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100mL</td>
<td>10%</td>
</tr>
</tbody>
</table>
Potassium acetate 1M was prepared by dissolving 9.82g of potassium acetate in 90ml of ddH₂O. The pH of the solution was then adjusted to 7.5 with 2M acetic acid, and ddH₂O was then added to bring the final volume to 100ml. The solution was divided into 10ml aliquots and stored at -20°C.

FSB was prepared by mixing all of the components in the above table with 800ml of ddH₂O. After the components were dissolved, the pH of the solution was adjusted to 6.4 with 0.1N HCl. The volume was adjusted to 1 L with ddH₂O. The solution was sterilised by filtration through a disposable Whatman filter (0.25-micro pore size), dispensed into 40ml aliquots in tissue-culture flasks and stored at +4°C.

5) Isopropylthio-β-D-galactoside (IPTG)

A 2g amount of IPTG was dissolved in 8ml of ddH₂O. The final volume was adjusted to 10ml, sterilised by filtration through a 0.22-micron pore filter, and stored at -20°C.

6) LB Medium

Bacto-tryptone (Difco) 10g
Bacto-yeast extract 5g
NaCl 5g

ddH₂O was added to 1 L, the pH was adjusted to 7.5 with 10N NaOH, then autoclaved.

7) LB medium with 0.2% maltose and 10mM MgSO₄

1ml of 1M MgSO₄ was added to cooled autoclaved LB medium. 1ml of 20%
maltose was added to LB medium immediately before use.

8) Phage buffer

1M Tris-HCl (pH 7.4), 1M NaCl and 1M MgSO₄ were mixed and ddH₂O was added to make that the final solution contained 20mM Tris-HCl, 100mM NaCl and 10mM MgSO₄ in 100ml volume.

9) SM buffer

This buffer was used for storage and dilution of bacteriophage stocks.

- gelatin 0.01%
- Tris-HCl (pH7.5) 50mM
- MgSO₄ 8mM

100ml of the buffer were sterilised by autoclaving. After the solution had cooled, 50ml aliquots was dispensed into sterile containers, and stored at RT.

10) S.O.C medium

- Bacto-tryptone (Difco) 20g
- Bacto-yeast extract 5g
- NaCl 0.5g

The above were dissolved in 950ml of ddH₂O. A 10ml volume of a 250mM solution of KCl was added and the pH was adjusted to 7.0 with 5 N NaOH and autoclaved. Before use, 5ml of a sterilised solution of 2M MgCl₂ (autoclaved) and 20ml of a sterile 1M solution of glucose (sterilised by filtration) were added, and stored at 20°C.
11) Bacterial strain JM109, LE392 (Promega, USA)

12) LB plates

A 15g amount of Bacto-agar was added to 1 L of LB medium without (bactiophage transformation) or with ampicillin (plasmid transformation), autoclaved, and 25 ml was poured into 90mm petri dishes, and stored at 4°C up to one month.

13) TB top agar (100ml)

Bacto-tryptone (Difco) 
NaCl
agar 

1g
0.5g 
0.8g

After autoclaving and cooling, 1ml of 1M MgSO₄ was added.

14) TB top agarose (100ml)

Bacto-trypton (Difco) 
NaCl
agarose 

1g
0.5g 
0.6g

After autoclaving and cooling, 1ml of 1M MgSO₄ was added.

Part B. Reagents for DNA and RNA work

1) Caesium Chloride 10mg/ml

A 2.50g amount of Caesium Chloride was dissolved in 2.5 ml of TE buffer (pH 8.0).
2) Denhardt's solution

50X:

Ficoll 5g
Polyvinylpyroliodone 5g
bovine serum albumin 5g

The above components were mixed in 500ml of ddH₂O, filtered, dispensed in 20ml aliquot, and stored at -20°C.

3) DEPC treated ddH₂O (0.1%) A 500ml-glass bottle was backed and autoclaved overnight, then 500ml of ddH₂O and 0.5ml of diethylpyrocarbonate (DEPC) were added to the bottle and mixed. The bottle was kept on the bench for several h., then autoclaved.

4) Deionised formamide

A 10ml volume of formamide and 1g of mixed bed resin were stirred for 15-20 min. The mixture was filtered through filter paper, then aliquoted into 1ml volume and stored at -20°C.

5) EDTA 0.5M (pH 8.0)

A 186.1g amount of EDTA was added to 800ml of ddH₂O and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with 10N NaOH, and autoclaved.

6) Ethidium Bromide 10mg/ml

A 1g amount of Ethidium Bromide was added to 100 ml of ddH₂O, stirred on a
magnetic stirrer for several h. The solution was covered in aluminium foil and stored at RT.

7) Gel loading buffer

(1) Type II

- Bromo phenol blue 0.25%
- Xylene cyanol FF 0.25%
- Ficoll (Type 400; Pharmacia) in water 15%

(2) Type IV

- Bromo phenol blue 0.25%
- Glycerol in water 30%

(3) Loading buffer for ABI 373

- Blue dextran 3g
- EDTA (N₂) 1.86g
- ddH₂O 100ml

Then 5 ml of demonised formamide and 1 ml of dextran blue/EDTA were mixed before use.

8) Glycerol Tolerant buffer

20 x:

- Tris-base 216g
- Taurine 72g
- Na₂EDTA·2H₂O 4g

The above were dissolved in 1 L of ddH₂O, then autoclaved.
9) Hybridisation buffer

A 10g amount of dextran sulphate was added to 100ml of ddH₂O, heated on a stirrer until dissolved, then 20ml of 50 x Denhardt's solution was added. When the solution was cooled, 10ml of 10% SDS, 60ml of 20 x SSC, and 5ml of boiled and sonicated salmon sperm DNA (10mg/ml) were added, mixed, and stored at -20° C.

10) 10 x MOPS

A 41.86g amount of 3-[N-Morpholino] propane-sulphonic acid was dissolved in 800ml ddH₂O, then 100ml 5M of Na Acetate, pH7.0, and 0.5M of Na, EDTA, pH 8.0, were added, mixed and made up to a final volume of 1 L with ddH₂O, and autoclaved.

11) Phenol:chloroform

An equal volume of equilibrated phenol and chloroform were mixed, left to sit for 2-4h until the mixture became clear. The solution was covered with foil and stored at 4°C for up to one month.

12) Solution I

| Glucose | 50mM |
| Tris-Cl (pH 8.0) | 25mM |
| EDTA (pH 8.0) | 10mM |

13) Solution II

| 0.2N NaOH (freshly diluted from a 10N stock) |
| 1% SDS |
14) Solution III (pH 4.6)

- 5M potassium acetate 60ml
- Glacial acetic acid 11.5ml
- ddH₂O 28.5ml

OR:

3M Na Acetate (pH 4.8).

15) Solution IV

- Tris-HCl 50mM (pH 8.0)
- Na Acetate 100mM

16) 20 x SSC

A 175.3g amount of NaCl and 88.2g of sodium citrate were dissolved in 800ml of ddH₂O. The pH was adjusted to 7.0 with a few drops of a 10N NaOH. The final volume was made up to 1 L by adding ddH₂O, then autoclaved.

17) 20 x SSPE

A 175.3g amount of NaCl, 27.6g of NaH₂PO₄·H₂O and 7.4g of EDTA were dissolved in 800ml of ddH₂O. The pH of the solution was adjusted to 7.4 with 10N NaOH and the volume was adjusted to 1 L with ddH₂O, and sterilised by autoclaving.

18) Stop solution (for sequencing reaction)

- formamide 95%
- EDTA 20mM
bromophenol blue 0.05%
xylene cyanol FF 0.05%

19) Tris-acetate (TAE) buffer
50 x:
   Tris-base 242g
   Glacial acetic acid 57.1ml
   0.5 M EDTA (pH 8.0) 100ml

20) Tris-borate (TBE) buffer
5 x:
   Tris base 54g
   Boric acid 27.5g
   0.5 M EDTA (pH 8.0) 20ml

21) Tris-EDTA (TE) buffer
pH 7.4
   Tris.Cl (pH 7.4) 10mM
   EDTA (pH 8.0) 1mM

pH 8.0
   Tris.Cl (pH 8.0) 10mM
   EDTA (pH 8.0) 1mM
Part C. Reagents for lymphocyte proliferation

1) Complete Medium

RPMI with 10% FCS, 0.05ng/ml glutamacine, 20mM glutamine.

2) For dogfish lymphocyte culture

2.16% Urea and 0.2M NaCl were added to Complete Medium.

3) Mitogen PHA, LPS, and ConA

Concanavalin A (ConA) and phytohaemagglutinin (PHA) (Sigma, UK) were dissolved in RPMI at a concentration of 1 mg/ml and filter sterilised (Whatman, USA). Lipopolysaccharides (LPS) (Sigma, UK) was dissolved in PRMI at a concentration of 10mg/ml and pasteurised by incubation in a 70°C water bath for 1 h. All solutions were stored at -20°C.
LIST OF MAIN SUPPLIERS

Amersham Life Science
Amersham International plc.
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA, UK
Tel: 01494-544000
Fax: 01494-542929

BDH
Merck Ltd.
Hunter Boulevard
Magna Park, Lutterworth
LE17 4XN
Tel: 0800-223344
Fax: 01455-558586

BioLabs
New England BioLabs
32 Tozer Road
Beverly, MA 01915
USA
Tel: 01508-927-5054
Fax: 01508-927-5054

Bio-Rad
Bio-Rad Laboratories Ltd.
Bio-Rad House,
Mayland Avenue
Hemel Hempstead
Herts HP2 7TD
Tel: 01442-232552
Fax: 01442-259118

Boehringer Mannheim
Boehringer Mannheim
(Diagnostic and Biochemicals) Ltd.
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East Sussex BN7 1LG
Tel: 0800-521578
Fax: 0800181087
Dynal
Dynal (UK) Ltd
Station House,
26 Grove Street
New Ferry,
Wirral, L62 5AZ UK
Tel: 0151-6446555
Fax: 0151-645-2094

Flowgen
Flowgen Instruments Ltd.
Lynn Lane, Shenstone,
Lichfield,
Staffs WS14 0EE
Tel: 01543-483054
Fax: 01543 483055

HT
HT Biotechnology Ltd
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Cambridge CB5 8QD
England
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Fax: 01223-412583

ICN
ICN Biomedical Ltd.
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Fax: 0800-614735

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Fax: 0141-814 6317
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Perkin Elmer Applied BioScience
Kelvin Close
Birthwood Science Park North
Warrington
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Tel: 01925-825650
Fax: 01925-282502

Pharmacia Biotech
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Promega
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Tel: 0800-378994
Fax: 0800181037

QIAGEN
QIAGEN Ltd.
Boundary Court
Gatwick Road
Crawley, West Sussex
RH10 2AX
Tel: 01293-422900
Fax: 01293-422922

Sigma
Sigma-Aldrich Company Ltd.
Fancy Road,
Poole,
Dorset,
BH12 4QH, England
Tel: 01202-733114
Fax: 01202-715460
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