VH Gene Usage in VHa2 Allotype-Producing B Cells from Mutant Alicia Rabbits

Anusorn Boonthum
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Recommended Citation
https://ecommons.luc.edu/luc_diss/3426

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1998 Anusorn Boonthum
LOYOLA UNIVERSITY CHICAGO

VH GENE USAGE IN
VHa2 ALLOTYPE-PRODUCING B CELLS
FROM MUTANT ALICIA RABBITS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

BY
ANUSORN BOONTHUM

CHICAGO, ILLINOIS
JANUARY, 1998
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my mentor, Dr. Katherine L. Knight, for her guidance throughout my graduate education. Particularly, I am grateful for her instilling in me a scientific mind. In addition, I wish to acknowledge the generous contribution of time and constructive criticisms by my committee members; Dr. Charles F. Lange, Dr. Manuel O. Diaz, Dr. Hans-Martin Jäck and Dr. John Nawrocki.

I would also like to thank Helga Spieker-Polet and Pi-Chen Yam for their help in the generating of hybridomas, Shi-Kang Zhai for DNA sequencing, and Raouchania Purnyn for assisting the recombination experiments. I would like to extend my thanks to Candance Winstead, Rob Barrington and Dennis Lanning for editing this manuscript and Chainarong Tunyaplin for illustration. Special thanks go to Mae Kingzette and her family for giving me a home. Additionally, I thank all the members of Dr. Knight’s laboratory and the departmental staff for their help and friendship during my time at Loyola.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

LIST OF TABLES .................................................................................................................... vii

LIST OF FIGURES ................................................................................................................ viii

ABSTRACT ............................................................................................................................. xi

INTRODUCTION ...................................................................................................................... 1

Chapter

I. LITERATURE REVIEW ........................................................................................................ 3

   Immunoglobulin Structure and Rabbit Allotypes ............................................................. 4

   Immunoglobulin Gene Rearrangement ............................................................................ 7

   V\textsubscript{H} Gene Usage in Mouse and Human ......................................................... 13

   Rabbit V\textsubscript{H} Genes and Repertoire Formation ................................................ 20

II. MATERIALS AND METHODS ....................................................................................... 38

   Animals ............................................................................................................................ 38

   Probes ............................................................................................................................. 38

   Cell Culture Media ......................................................................................................... 39

   Generation of Alicia Hybridomas ............................................................................... 40

   Detection of V\textsubscript{H}a2 Ig Secreting Hybridomas ................................................ 42

   Preparation of Genomic DNA ....................................................................................... 43

   Southern Blot Analysis ................................................................................................... 44
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Number of hybridomas generated from Alicia rabbits.</td>
<td>61</td>
</tr>
<tr>
<td>2.</td>
<td>Southern blot analysis of DNA from a2-secreting and a2-negative secreting Alicia hybridomas hybridized with probe A and JH probe.</td>
<td>69</td>
</tr>
<tr>
<td>3.</td>
<td>Recombination frequencies of recombination substrates containing 3' V_H1 or IL-10 as negative control.</td>
<td>123</td>
</tr>
<tr>
<td>4.</td>
<td>Recombination frequencies of 3' V_H1 subclones, fragment 2, 3 and 4.</td>
<td>124</td>
</tr>
<tr>
<td>5.</td>
<td>Analysis of recombination substrates by hybridization with transcription terminator probe.</td>
<td>129</td>
</tr>
<tr>
<td>6.</td>
<td>Analysis of recombination substrate containing 2-kb Hind III DNA region 3' of V_H1 by chloramphenicol selection and hybridization with transcription terminator probe.</td>
<td>130</td>
</tr>
<tr>
<td>7.</td>
<td>Analysis of recombination substrate containing 400 bp Pst/Hind III DNA region 3' of V_H1 (fragment 1) by hybridization with transcription terminator probe.</td>
<td>131</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Comparison of the nucleotide sequences of $V_{Ha}$ genes, $V_{Hx}$, $V_{Hy}$ and $V_{Hz}$, with that of $V_{HJ}$ from a2 allotype rabbits.</td>
<td>28</td>
</tr>
<tr>
<td>2.</td>
<td>The 10 kb deletion of DNA at the 3' end of $V_{H}$ locus of Alicia rabbits.</td>
<td>34</td>
</tr>
<tr>
<td>3.</td>
<td>Primers for PCR of VDJ genes and their promoters.</td>
<td>51</td>
</tr>
<tr>
<td>4.</td>
<td>Recombination substrates.</td>
<td>53</td>
</tr>
<tr>
<td>5.</td>
<td><em>In vitro</em> recombination assay.</td>
<td>55</td>
</tr>
<tr>
<td>6.</td>
<td>Analysis for the status of hybridomas from mutant Alicia rabbit.</td>
<td>63</td>
</tr>
<tr>
<td>7.</td>
<td>Southern analysis of <em>BamHI</em>-digested DNA from 14 Alicia hybridomas.</td>
<td>66</td>
</tr>
<tr>
<td>8.</td>
<td>Southern analysis of hybridoma 1E6 genomic DNA digested with <em>EcoRI</em> using a rabbit $J_{H}$ probe.</td>
<td>71</td>
</tr>
<tr>
<td>9.</td>
<td>Southern analysis of 1E6 genomic DNA digested with <em>BamHI</em> using a rabbit $J_{H}$ probe.</td>
<td>74</td>
</tr>
<tr>
<td>10.</td>
<td>Restriction map of 4.7 kb <em>BamHI</em> genomic DNA fragment that contains the VDJ gene from hybridoma 1E6.</td>
<td>76</td>
</tr>
<tr>
<td>11.</td>
<td>Comparison of translated amino acid sequences of 1E6 V region with the partial amino acid sequence of pool a2 Ig.</td>
<td>79</td>
</tr>
<tr>
<td>12.</td>
<td>Southern analysis of hybridoma 8E1 genomic DNA digested with <em>EcoRI</em> using a rabbit $J_{H}$ probe.</td>
<td>82</td>
</tr>
<tr>
<td>13.</td>
<td>Southern Analysis of 8E1 genomic DNA digested with <em>BamHI</em> using a rabbit $J_{H}$ probe.</td>
<td>84</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Restriction map of genomic VDJ gene from hybridoma 8E1</td>
<td>86</td>
</tr>
<tr>
<td>15.</td>
<td>Comparison of nucleotide sequence of hybridoma 8E1 VDJ gene and its deduced amino acid sequence with those of germline V_H4</td>
<td>88</td>
</tr>
<tr>
<td>16.</td>
<td>Comparison of genomic VDJ gene from hybridoma 8E1 with VDJ gene of hybridoma 1E6</td>
<td>90</td>
</tr>
<tr>
<td>17.</td>
<td>Comparison of genomic VDJ gene from hybridoma 8E1 with germline V_H4 gene segment</td>
<td>93</td>
</tr>
<tr>
<td>18.</td>
<td>Nucleotide sequences of the promoter regions of VDJ genes from 17 hybridomas compared with the promoter regions of known germline V_H genes from the ali haplotype</td>
<td>96</td>
</tr>
<tr>
<td>19.</td>
<td>Comparison of nucleotide sequence of hybridoma 1B3 VDJ gene and its promoter with nucleotide sequences of germline V_H4 and V_H9</td>
<td>98</td>
</tr>
<tr>
<td>20.</td>
<td>Comparison of nucleotide sequence of hybridoma 1F7 VDJ gene and its promoter with nucleotide sequences of germline V_H4 and V_H9</td>
<td>100</td>
</tr>
<tr>
<td>21.</td>
<td>Comparison of nucleotide sequence of hybridoma 4A4 VDJ gene and its promoter with nucleotide sequences of germline V_H4 and V_H9</td>
<td>102</td>
</tr>
<tr>
<td>22.</td>
<td>Comparison of nucleotide sequence of hybridoma 6A1 VDJ gene and its promoter with nucleotide sequences of germline V_H4 and V_H9</td>
<td>104</td>
</tr>
<tr>
<td>23.</td>
<td>Comparison of nucleotide sequence of hybridoma 8E1 VDJ gene and its promoter with nucleotide sequences of germline V_H4 and V_H9</td>
<td>106</td>
</tr>
<tr>
<td>24.</td>
<td>Comparison of nucleotide sequence of hybridoma 10A2 VDJ gene and its promoter with nucleotide sequences of germline V_H4 and V_H9</td>
<td>108</td>
</tr>
<tr>
<td>25.</td>
<td>Comparison of nucleotide sequence and its deduced amino acid sequence of hybridoma 1C3 VDJ gene with nucleotide sequence of germline V_H4 and its promoter</td>
<td>110</td>
</tr>
<tr>
<td>26.</td>
<td>Comparison of nucleotide sequence and its deduced amino acid sequence of hybridoma 4C4 VDJ gene with nucleotide sequence of germline V_H4 and its promoter</td>
<td>112</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>27.</td>
<td>Somatic gene conversion in FR1 of 6 $V_{H}\alpha$-utilizing hybridomas.</td>
<td>114</td>
</tr>
<tr>
<td>28.</td>
<td>Recombination enhancer hypothesis</td>
<td>117</td>
</tr>
<tr>
<td>29.</td>
<td>Recombination substrates containing the 3' $V_{H}$/ DNA fragments</td>
<td>119</td>
</tr>
<tr>
<td>30.</td>
<td>An example of in vitro recombination assay detected by differential hybridization</td>
<td>126</td>
</tr>
<tr>
<td>31.</td>
<td>Model for the appearance of $V_{H}\alpha_2$ Ig molecules in adult Alicia rabbits</td>
<td>143</td>
</tr>
<tr>
<td>32.</td>
<td>Alicia rabbits use at least five $V_{H}$ genes to encode $V_{H}\alpha_2$ Ig molecules</td>
<td>160</td>
</tr>
<tr>
<td>33.</td>
<td>Comparison of nucleotide sequence of a cDNA encoding RA-CD40L with that of human and mouse CD40 ligand</td>
<td>164</td>
</tr>
<tr>
<td>34.</td>
<td>Comparison of nucleotide sequences of the first exon of genomic RA-IL-10 clone, human and mouse IL-10</td>
<td>168</td>
</tr>
</tbody>
</table>
ABSTRACT

Rabbit B cells preferentially use only one $V_H$ gene, $V_{H1}$, in their VDJ gene rearrangements. Three allelic $V_{H1}$ genes, $V_{H1-a1}$, $V_{H1-a2}$ and $V_{H1-a3}$ encode $V_H$ allotypes, designated $V_{Ha1}$, $V_{Ha2}$ and $V_{Ha3}$ allotype, respectively. Because the $V_{Ha2}$ allotype-encoding gene, $V_{H1-a2}$, is frequently used in normal a2 allotype rabbits, most of their serum immunoglobulin (Ig) molecules bear $V_{Ha2}$ allotype ($V_{Ha2}$ Ig). However, in mutant Alicia rabbits, the preferentially utilized $V_{H1-a2}$ gene, is deleted. As a result, young Alicia rabbits produce predominantly Ig molecules that do not have $V_{Ha2}$ allotypic specificity. However, $V_{Ha2}$ Ig appear in the serum as Alicia rabbits age. What $V_H$ gene(s) then are used to encode these $V_{Ha2}$ Ig molecules? Because rabbit VDJ genes are diversified by a somatic gene conversion-like process, one possibility is that $V_{Ha2}$ Ig in aged Alicia rabbits are derived from somatic gene conversion. Another possibility is that the $V_{Ha2}$ Ig molecules are derived from $V_{Ha2}$-encoding genes other than $V_{H1}$.

To address these questions, I generated $V_{Ha2}$ Ig-producing hybridomas from aged Alicia rabbits. A total of 17 hybridomas were examined for $V_H$ gene usage by PCR-amplification for the VDJ genes and their promoter regions, whose nucleotide sequences
differ among various $V_H$ genes. Nucleotide sequence analysis of VDJ genes and their promoters revealed that a total of five $V_H$ genes were used to encode $V_{Ha2}$ Ig molecules; nine of them used $V_{H4}$. The other four $V_H$ genes are novel, as yet unidentified germline $V_H$ genes. Analysis of diversified $V_{H4}$ genes showed that the appearance of $V_{Ha2}$ allotype-associated amino acids was due to somatic gene conversion events using the $V_{Ha2}$-encoding pseudogene, $V_H9$, as a donor. Thus, one mechanism used for generating $V_{Ha2}$ Ig in aged Alicia rabbits is somatic gene conversion.

The rearrangement of multiple germline $V_H$ genes in B cells of Alicia rabbits contrasts with the rearrangement of a single $V_H$ gene, $V_{H1}$, in most B cells of normal rabbits. This observation led to the hypothesis that the preferential utilization of $V_{H1}$ in normal rabbits is due to a nearby $cis$-acting element, designated a recombination enhancer. Deletion of such an enhancer along with $V_{H1}$ in the 10 kb of DNA deletion in Alicia rabbits then may allow rearrangement of other $V_H$ genes, as observed in Alicia rabbits. Hence, I searched for such an enhancer by performing an $in vitro$ recombination assay using recombination substrates containing various DNA regions, $3'$ of $V_{H1}$. In this analysis, no difference in recombination frequencies between constructs containing $3'$ $V_{H1}$ DNA and constructs containing the control DNA was observed.

In summary, I showed conclusively that $V_{Ha2}$ Ig molecules are generated in Alicia
rabbits, at least in part, from somatic gene conversion events. In addition, I found that, unlike normal rabbits that utilize predominantly one $V_H$ gene, Alicia rabbits use several $V_H$ genes in their VDJ gene rearrangements. Further studies of Alicia rabbits will likely provide an important insight into the molecular basis for preferential utilization of $V_H$ genes in rabbits and other species.
INTRODUCTION

One of the essential factors that counteract microbial invaders and harmful substances is antibody, a protein capable of binding foreign antigens. To be able to encounter a wide variety of pathogens, the organisms must have the ability to generate a large number of different antigen-binding specificities. In mice and humans, combinatorial joining of V(D)J gene segments is a major mechanism for generating a diverse antibody repertoire (reviewed in Tonegawa, 1983; Alt et al., 1987; Rajewsky, 1996). In contrast, rabbits use predominantly one $V_H$ gene, $V_{HJ}$, in their VDJ gene rearrangements in spite of the fact that they have hundreds of $V_H$ genes, half of which appear to be functional (Becker et al., 1990; Knight and Becker, 1990; Friedman et al., 1994; Raman et al., 1994; Tunyaplin and Knight, 1995). The preferential utilization of one $V_H$ gene limits the extent to which combinatorial joining can contribute to the generation of a diverse antibody repertoire. To create antibody diversity, rabbit VDJ genes undergo, early in life, massive alteration in their nucleotide sequences by the process of somatic gene conversion in which upstream $V_H$ genes serve as donors (Becker and Knight., 1990; Raman et al., 1994; Crane et al., 1996). Remarkably, diversification of rabbit VDJ genes represents the first example of somatic gene conversion in mammals.

The fact that preferential utilization of a single $V_H$ gene and somatic gene conversion occur in rabbits may imply that they are unique mechanisms for generating
the antibody repertoire in rabbits. However, both preferential $V_H$ gene usage and somatic gene conversion have been observed in other species. Because these two phenomena are prominent in rabbits, it opens a window of opportunity to investigate how these processes are operated and regulated at the cellular as well as molecular level.

In this study, the antibody repertoire of the mutant Alicia rabbits, lacking the preferred $V_H^I$ gene, was analyzed at the level of $V_H$ gene usage. Surprisingly, several $V_H$ genes were found to be used in VDJ gene rearrangements of Alicia rabbits. This finding is in sharp contrast to $V_H$ gene usage in normal rabbits and led to an attempt to explore the molecular mechanism underlying the preferential utilization of $V_H^I$ gene segment. In addition, upon analyzing Alicia VDJ genes, I discovered that the somatic gene conversion process can contribute to the generation of $V_H^{a2}$ allotypic determinants. This finding provides an explanation for the appearance of $V_H^{a2}$ Ig molecules in adult Alicia rabbits that genetically do not have the frequently used $V_H^{a2}$-encoding gene, $V_H^I$. Further studies of Alicia rabbits will likely provide greater insight into the molecular basis for preferential $V_H$ gene usage as well as into the molecular and cellular mechanisms that shape the antibody repertoire.
CHAPTER I

LITERATURE REVIEW

More than a century ago, Behring and his colleagues, Kitasato and Wernicke, at Koch's Institute discovered antibody by documenting that the serum of individuals recovered from tetanus and diphtheria infections could neutralize tetanus and diphtheria toxins, respectively and confer immunity to normal animals (reviewed in Paul, 1993). This discovery opened the development of immunotherapy against illness and death caused by many infectious diseases. Since then, antibodies or immunoglobulins (Ig) have been studied extensively. The first breakthrough came from the laboratories of Porter and Edelman who studied the structure of IgG antibodies in rabbit and human, respectively. Together, they demonstrated that IgG consists of four polypeptide chains, two heavy chains and two light chains (Fleischman et al., 1962, Edelman et al., 1969). Another major breakthrough came from the study in 1975 by Köhler and Milstein who developed a method to prepare antibodies of the desired specificities, the hybridoma technology (Köhler and Milstein, 1975). Their technique has allowed scientists to produce monoclonal antibodies specific for a single antigenic determinant of any desired immunogen. Perhaps the next remarkable breakthrough was made by Tonegawa and Hozumi of Basel Institute for Immunology in 1976 (Hozumi and Tonegawa, 1976). By
using molecular techniques, they showed conclusively that Ig genes are inherited as gene segments. These gene segments are recombined somatically to form a complete Ig gene during the development of an antibody-producing cell. The power of recombinant DNA technology led to the rapid progress in our understanding of the organization, structure and expression of Ig genes. These findings paved the way for engineering antibodies in vitro. Thus, whereas Behring began the process of passive immunization, in the last 100 years immunologists have investigated the immune system extensively to the point now that human monoclonal antibodies of desired specificities can be developed through molecular technology for immunotherapy.

The discovery of Ig gene rearrangement has led to the enormous interest in the studies on Ig variable region genes of heavy chain (V_H) and light chain (V_L). The following are the questions that have been investigated extensively during the past two decades. How many V_H and V_L genes are there in the genome? Is the V gene repertoire randomly expressed or is there a genetic program for the order of V gene expression? What factors influence the rearrangement and the expression of Ig genes? Some of these findings will be summarized in this chapter. Particularly, I will review the studies of Ig structure and immunogenetics relevant for study of V_H gene usage in Alicia rabbits.

**Immunoglobulin Structure and Rabbit Allotypes**

An antibody or Ig monomer is composed of two pairs of polypeptide chains: each pair comprising of a heavy chain (H) and a light chain (L) (reviewed in Potter, 1973,
Kabat et al., 1991). All four chains are held together by disulfide bonds. Each Ig chain consists of a variable region and a constant region. This structural information was obtained from two major discoveries. The first was the findings by Porter and by Edelman that Ig molecules could be dissociated into smaller subunits. Edelman treated γ-globulin with reducing agents and found that it separated into two kinds of polypeptide chains, H and L chains (Edelman, 1959, 1969). Porter showed that digestion of rabbit IgG with the enzyme papain cleaved the Ig molecules into two antigen-binding fragments (Fab) and one crystallizable fragment (Fc) (Porter, 1959). The Fab fragment consists of a L chain associated with the amino-terminal half of an H chain. The Fc part is made up of the carboxy-terminal halves of the two H chains. The second major discovery about Ig structure was obtained from homogeneous antibody populations, human myeloma proteins and mouse plasmacytomas (Hilschman and Craig, 1969). The availability of monoclonal antibodies and the ability to separate H and L chains allowed investigators to directly sequence H or L chain (reviewed in Kabat et al., 1991). Analyses of primary amino acid sequences of H and L chains revealed that the sequence of the amino-terminal region of each polypeptide chain was more variable than that of the carboxy-terminal region. On the basis of these findings the variable (V) and constant (C) domains were identified. However, the variability in amino acid sequences was not distributed evenly throughout the V region. When Kabat and Wu analyzed amino acid sequences of several H and L chains, they found that the variability in sequences of both chains was localized in three hypervariable regions designated complementarity determining regions (CDR) surrounded by the relatively invariable framework regions (FR).
The structural information obtained from protein chemistry studies is supported by electron microscopy and X-ray crystallography (reviewed in Kabat et al., 1991). These physical methods confirm the Ig structure previously predicted by Porter and Edelman. That is, the IgG molecule is Y shaped in which each arm of the Y represents Fab and the stem of the Y represents Fc (reviewed in Alzari et al., 1988). Furthermore, crystallographic analyses of Fab fragments ratify the Kabat notion that the CDR makes contact with antigens.

Polymorphic forms of Ig chains are called allotypes. Ig allotypes have been described in many species including mouse and human, however, this review will discuss only allotypes of rabbit Ig molecules. Rabbit allotypes were first identified by Oudin in 1956, two years before the structure of Ig was published (Oudin, 1956). Notably, rabbit Ig present the only case in which allotypic determinants are found in both \( V_H \) and \( C_H \) regions (reviewed in Kindt, 1975, Kindt and Capra, 1984, Knight, 1992). Allotype a, w, x and y are associated with determinants in the \( V_H \) regions while allotype d or e, f or g and ms or n are found in the \( C_H \) regions of \( \gamma, \alpha \) and \( \mu \) heavy chains, respectively.

The \( V_{H_\alpha} \) allotypes, a1, a2, or a3, are present in the \( V_H \) regions of 70 to 90 % of serum Ig molecules isolated from normal rabbits (Dray et al., 1963 a and b). These molecules are designated a-positive molecules (\( V_{H_\alpha}^+ \)). Amino acid sequence analysis of IgH molecules from each of the \( V_{H_\alpha} \) allotypes showed that the \( V_{H_\alpha} \) specificities correlate with particular amino acids at several positions distributed within the FR1 and FR3
(Mage et al, 1984). The 10-30% of normal Ig molecules that do not have the $V_H a$ allotype are collectively classified as a-negative ($V_H a^-$) (Kim and Dray, 1973). Conversely, $V_H a^-$ molecules are predominantly present in serum of $V_H a$ allotype-suppressed rabbits in which the animals are exposed perinatally to anti-a allotype antiserum (David and Todd, 1969, Vice et al, 1970, Kim and Dray, 1973, Short et al, 1991). Another major source of $V_H a^-$ Ig molecules is serum Ig of young Alicia rabbits, the mutant a2 rabbits (Kelus and Weiss, 1986). These mutant rabbits are genotypically $a^2/a^2$, however phenotypically they produce very low levels of $V_H a^2$ Ig molecules. The molecular basis for the genetic defect in these mutant rabbits will be described in the section regarding Alicia rabbits. While three $V_H a$ allotypes are characterized, four allotypic specificities, $x32$, $y33$, $w34$ and $w35$ have been identified on some of the $V_H a^-$ molecules (Kim and Dray, 1973; Roux, 1981). However, protein sequence analysis of these molecules failed to characterize amino acid residues associated with the $V_H a^-$ Ig.

**Immunoglobulin Gene Rearrangement**

Studies on rabbit allotypes provided the first clue that $V_H$ and $C_H$ regions of IgH chains are encoded by separate genes (Todd, 1963; Feinstein, 1963; Kindt and Todd, 1969). By using specific antisera, Todd identified $V_H a$ allotypes on both IgG and IgM molecules (Todd phenomenon). The similar finding for IgG and IgA was reported by Feinstein and the presence of $V_H a$ allotype on IgE was found by Kindt and Todd. These findings led immunologists to suggest that the $V_H$ and the $C_H$ regions are encoded by
separate genes and that a single $V_H$ gene can interact with a different $C_H$ gene. Additionally, these data provided experimental evidence to support Dreyer and Bennett's revolutionary idea that two genes encode one polypeptide chain (Dreyer and Bennett, 1965). However, this was not generally accepted outside the immunology community, until 1976. In that year, Tonegawa and his associate, Hozumi, showed at the molecular genetic level that the $V$ and $C$ regions of the mouse $\kappa$ light chain are encoded by genes separated in embryonic cells (Hozumi and Tonegawa, 1976). These genes are located far apart in the germline and are joined together during B cell development by a process called Ig gene rearrangement (reviewed in Tonegawa, 1983).

Since then, rapid progress has been made in order to understand the molecular mechanism of Ig gene rearrangement. A major finding was that the $V$ regions of both IgH and IgL chains are also encoded by multiple gene segments. The $V_H$ region is encoded by three gene segments, $V$ (variable), $D$ (diversity) and $J$ (joining) and the $V_L$ region is encoded by $V$ and $J$ gene segments (Early et al., 1980; Seidman et al., 1979). In this section, I will review the current view of V(D)J gene rearrangement.

Gene rearrangement to generate antigen receptor genes is a process common to both B and T cells, however Ig gene rearrangements occur only in B cells whereas the rearrangements of T-cell receptor genes occur only in T cells (reviewed in Lewis, 1994a). Because the objective of this dissertation project is to examine $V_H$ genes used in V(D)J gene rearrangements occurring in B cells, this review will focus on IgH gene
rearrangement. The rearrangement begins with the joining of one D and one J\textsubscript{H} gene segment during the development of pro-B cells (Alt et al., 1984; Hardy et al., 1991). Following the DJ gene rearrangement, one of the many V\textsubscript{H} gene segments is recombined with the DJ genes to form a complete IgH variable region gene at the late pro-B cell stage.

The process of V(D)J rearrangement is initiated by the recognition of RSS (Recombination Signal Sequence) by the V(D)J recombination machinery (reviewed in Lewis, 1994a). These DNA elements are located in the intronic region 3' of V\textsubscript{H} gene segments, on both sides of D genes and 5' of J\textsubscript{H} gene segments. Nucleotide sequence analysis of several germline V, D and J gene segments revealed that an RSS consists of two conserved DNA motifs, a heptamer and a nanomer, separated by non-conserved nucleotides of 12 base pairs or 23 base pairs in length (Early et al., 1980; Sakano et al., 1981). Thus, two types of RSS are classified on the basis of the difference in spacer length, 12 and 23 base pairs, that are equivalent to 1 and 2 turns of DNA helix, respectively. The finding of two types of RSS adjacent to gene segments capable of undergoing recombination led Early to propose that V(D)J recombination could occur between the gene segment with a 12-base pair spacer and the gene segment with a 23-base pair spacer (the "12/23 rule"). In fact, studies of V(D)J recombination substrates, either chromosomally integrated or extrachromosomal, supported this hypothesis (Akira et al., 1987; Hesse et al., 1989).
Recent studies showed that the RSS is recognized by a lymphoid-specific recombinase, composed of RAG-1 and RAG-2 (for “recombination Activating Gene” products) (McBlane et al., 1995; Eastman et al., 1996; Ramsden et al., 1996; Spanopoulou et al., 1996; van Gent et al., 1996). These studies of the initial step of V(D)J recombination relied upon previous knowledge of RAG-1 and RAG-2, as well as the availability of a cell-free system (Schatz et al., 1989; Oettinger et al., 1990; Sadofsky et al., 1995; van Gent et al., 1995). By serially transfecting genomic DNA into recombination incompetent cells and assaying for DNA capable of inducing V(D)J recombination activity, Schatz et al were able to clone the genomic RAG-1 (Schatz et al., 1989). Then they used a RAG-1 genomic probe to isolate RAG-1 cDNA. Surprisingly, four independent RAG-1 cDNA clones functioned very poorly in a chromosomally integrated recombination assay. One explanation for this observation was that there might be another gene which resided in the genomic clone and was required for V(D)J recombination. Indeed, Oettinger et al identified RAG-2 from this genomic clone (Oettinger et al., 1990). The indispensability of these two proteins in V(D)J recombination was clearly shown by gene targeting experiments. In RAG-1 or RAG-2 deficient mice, lymphocyte development is arrested prior to the developmental stage when the recombination normally occurs (Mombaerts et al., 1992; Shinkai et al., 1992). Taken together, these studies demonstrated that both RAG proteins are absolutely required for V(D)J recombination and lymphocyte development.

The development of a cell-free system capable of supporting the initial step of
V(D)J recombination made possible the elucidation of the biochemical mechanism of V(D)J recombination. This system was developed by Gellert’s group at NIH using either nuclear extracts from recombinase expressing cells, 103/BCL-2 pre-B cell lines, or recombinant RAG-1 and RAG-2 proteins (McBlane et al., 1995; van Gent et al., 1995). They showed that RAG-1 and RAG-2 proteins are able to cleave oligonucleotide substrates containing a single RSS when Mn\(^{2+}\) is presence as divalent cation (McBlane et al., 1995; Ramsden et al., 1996; van Gent et al., 1996). The cleavage products were blunt, 5’ phosphorylated signal ends and hairpin coding ends as detected in vivo. With Mg\(^{2+}\) as divalent cation, efficient cleavage requires the presence of a 12-base pair and a 23-base pair RSS, as observed in many previous studies (Eastman et al., 1996; Ramsden et al., 1996; van Gent et al., 1996). These data suggest that RAG-1 and RAG-2 are necessary and sufficient for RSS-directed cleavage of recombination substrates.

Subsequent to cleavage by two lymphoid-specific RAG proteins, several ubiquitous DNA repair proteins are required to join broken DNA and form coding joint and signal joint (reviewed in Bogue and Roth, 1996; Chu, 1996). One of these proteins is DNA-dependent protein kinase (DNA-PK), a serine/threonine protein kinase whose activity is activated through the interaction with DNA (reviewed in Anderson and Carter, 1996). It is made up of a catalytic subunit, DNA-PK\(_{CS}\) (the product of murine scid gene), and its DNA binding subunits, Ku70 and Ku80. The indispensable function of DNA-PK\(_{CS}\) and Ku70/80 in V(D)J recombination is evident as the lack of antigen receptors in scid mice and mice deficient for the Ku80 gene, respectively (Bosma et al., 1983;
Nussenzweig et al., 1996; Zhu et al., 1996). Several studies showed that Ku binds to altered DNA structures such as DNA nicks, DNA broken ends and hairpins (Mimori et al., 1986; Blier et al., 1993; Morozov et al., 1994). Because mutations in Ku70/80 impair both signal and coding joint formation, it is possible that after RAG-1 and RAG-2 cleave DNA at RSS, Ku binds to both the blunt signal ends and hairpin coding ends (Pergola et al., 1993; Taccioli et al., 1993). When Ku binds to its DNA substrates, it recruits DNA-PKcs and the interaction of DNA-PKcs with Ku-DNA complex results in activation of the kinase activity (Gottlieb and Jackson, 1993). DNA-PKcs, a 460 KDa polypeptide, has been shown to phosphorylate several DNA-binding proteins in vitro, including its DNA binding subunit, the Ku autoantigen (reviewed in Anderson, 1996). Mutation in DNA-PKcs (scid) leads to the accummulation of hairpin coding ends, suggesting that DNA-PKcs plays an important role in opening of hairpin (Roth et al., 1992). However, artificial hairpins introduced into scid cell lines are opened normally (Lewis, 1994b). At the present time, the mechanism of hairpin resolution is not clearly defined. Nevertheless, it is generally accepted that coding end hairpins must be resolved prior to joining. Analysis of engineered hairpins revealed that asymmetric cleavage of hairpin can contribute to P (Palindromic)-nucleotide addition at the coding junctions (Lewis, 1994b). Some coding junctions have extra nucleotides that are added by the enzyme Terminal Deoxynucleotidyl Transferase (TdT) (Alt and Baltimore, 1982). Thus, once hairpins are opened, the coding junctions can be accessible to exonuclease enzyme and can be further modified by either P-nucleotide addition or N-region addition resulting in junctional diversity.
**V_H Gene Usage in Mouse and Human**

In mammals, the genes encoding the V region of the Ig H chain are assembled during B cell development by rearrangement of V_H, D_H and J_H gene segments (reviewed in Tonegawa, 1983; Alt et al, 1987). The combinatorial joining of these gene segments is a principal mechanism for generating the primary IgH repertoire in mouse and human (reviewed in Alt et al, 1987). During the past two decades, the VDJ repertoire of B lineage cells has been extensively investigated, especially focusing on the utilization of V_H genes. This is due to the fact that V_H genes encode the majority of the V_H domain and there are hundreds of V_H genes available in the genome. Furthermore, there is a preference for using certain V_H genes not only in normal physiological conditions but also in pathogenic states, such as in B-cell malignancies, autoimmune diseases and some types of microbial infections. Most importantly, knowledge of V_H gene usage is essential for analyzing the development of antibody repertoire and for investigating the molecular and cellular mechanisms involved in shaping the B cell repertoire. In this section, the pattern of V_H gene utilization in mouse and human will be discussed in the context of IgH repertoire development as well as the mechanisms shaping the repertoire.

**V_H Gene Utilization in Murine B-Lineage Cells**

Initially, the V_H gene repertoire in the mouse was studied at the level of V_H gene families. All known mouse V_H gene segments are grouped into 15 families on the basis of their DNA homology (Brodeur and Riblet, 1984; Winter et al., 1985; Rathburn et al.,
1987; Köfler, 1988; Reininger et al., 1988; Tutter et al., 1991; Köfler et al., 1992; Mainville et al., 1996). Although the physical map of the \( V_H \) locus is still incomplete, the organization of the \( V_H \) gene family in the IgH chromosomal region appears to be conserved among the mouse strains (Mainville et al., 1996). The \( V_H 7183 \), consisting of 15 members, is the 3' most \( V_H \) gene family in BALB/c mice (Carlsson et al., 1992; Atkinson and Wu, 1993). The \( V_H J558 \) family, located at the 5' end of the IgH locus, is estimated to have 60 to 100 members (Brodeur and Riblet, 1984).

During fetal development, the \( V_H \) gene family located at the 3'end of the \( V_H \) locus is preferentially used in VDJ gene rearrangements (reviewed in Alt et al., 1987; Perlmutter, 1987; Wu et al., 1990). The first evidence came from the study in pre-B cell lines by Yancopoulos and colleagues (Yancopoulos et al., 1984). They examined VDJ gene rearrangements in A-MuLV-transformed cell lines derived from fetal liver of BALB/c mice by Southern as well as Northern blot analysis and found that 11 of 12 VDJ gene rearrangements used the 3' most \( V_H \) gene family, \( V_H 7183 \). Similar results were reported by Perlmutter et al. (1985) who performed Northern analysis of nine fetal liver hybridomas and found that seven pre-B hybridomas expressed members of the \( V_H 7183 \) gene family. Further, Alt's group determined \( V_H \) gene expression in normal lymphoid tissues and showed that \( V_H \) gene usage in fetal liver also displayed a bias toward the 3' \( V_H \) gene family (Yancopoulos et al., 1988; Malynn et al., 1990). In theory, preferential \( V_H \) gene usage could be due to preferential \( V_H \) gene rearrangement or to receptor-
mediated selection of B cells that express a particular \( V_H \) gene or both. The fact that A-MuLV transformed cells isolated from fetal liver do not express surface Ig and that their IgH loci which initially contain the rearranged DJ genes on both alleles undergo V to DJ joining during multiplication in culture, the preferential usage of the 3’ \( V_H \) gene family early in ontogeny of the mouse is likely due to preferential rearrangement.

Subsequently, several investigators reported that not only \( V_H 7183 \) but \( V_H Q52 \) family members are also frequently used in pre-B cells during fetal life (Lawler et al., 1987; Osman et al., 1988). These two family members are interspersed at the 3’ end of the \( V_H \) locus in several mouse strains and are generally referred to as 3’ \( V_H \) gene families (Brodeur et al., 1988). Further analysis of \( V_H \) gene usage in A-MuLV transformed pre-B cell line derived from NIH/Swiss mice revealed that \( V_H Q52 \), the 3’most \( V_H \) gene family in this mouse strain, are predominantly used (Reth et al., 1986). This finding together with previous observations in BALB/c mice led Alt and colleagues to propose the position-dependent hypothesis, which predicts that the chromosomal position of \( V_H \) gene, in close proximity to \( D_H \) gene segments, affects the frequency of VDJ gene rearrangements (Alt et al., 1987). However, there is considerable evidence that proximity alone cannot be responsible for the nonrandom utilization of \( V_H \) genes (Riley et al., 1986; Gauss and Lieber., 1992; Atkinson et al., 1993). Homology-directed recombination and RSS may also play a role in the preferential usage of the 3’ \( V_H \) gene families during fetal life of the mouse (Chukwuocha and Feeney, 1993; Connor et al., 1995).
From what we know now, it seems likely that fetal repertoire is limited, in part, by the restricted usage of particular $V_H$ genes. After birth, a more diverse repertoire gradually replaces the repertoire used during fetal development (Perlmutter et al., 1985; Malynn et al., 1990). Analysis of hybridomas and B cell colonies derived from LPS-activated spleen cells of adult mice showed that all examined $V_H$ families are expressed and their expression correlates with the size of the family rather than the chromosomal position (Dildrop et al., 1985; Wu and Paige, 1986; Schulze and Kelsoe, 1987). In accord with studies at the monoclonal level, quantitative Northern analysis of polyclonal splenic B cells in adult mice demonstrated that $V_H$ family usage is randomized corresponding to the complexity of $V_H$ families in the genome (Yancopoulos et al., 1988; Malynn et al., 1990).

At present, we do not yet understand the possible reason nor the mechanism underlying the difference in patterns of $V_H$ gene usage between fetal and adult life. One explanation is that the rearrangement of $V_H$ gene segments is developmentally regulated (Perlmutter, 1987). This is supported by the observations that the ability to respond to specific antigens develops in a programmable fashion. For instance, the fetus can respond to dinitrophenyl (DNP) haptens by day 14 of fetal life while the response to dextran antigen is not achieved until after the first five days of life (Klinman and Press, 1975; Stohrer and Kearney, 1984; Riley et al., 1986). Anti-DNP antibody is encoded by $V_H7183$, the preferentially rearranged $V_H$ gene family in fetal pre-B cells, while anti-
dextran antibody is encoded by the frequently used $V_H$ gene in adult B-lymphoid cells, $V_H$J558. Therefore, it seems that there is a correlation between the ability to respond to particular antigens and the timing of $V_H$ gene expression. This correlation may reflect programmed rearrangement of $V_H$ genes. However, the idea of a developmentally programmed rearrangement cannot explain the observation that A-MuLV pre-B cell lines derived from adult bone marrow still preferentially rearrange the fetal version of the $V_H$ gene, $V_H$7183. The second possibility is that the change in pattern of $V_H$ gene usage during ontogeny is due to the difference in B cell population in fetal and adult tissues. Essentially all B cells in fetal lymphoid tissues are CD5+ B cells whereas most of the B cells in adult spleen are CD5- B cells (reviewed in Förster et al., 1991; Kantor and Herzenberg, 1993; Hardy, 1994). Studies on the $V_H$ gene repertoire of CD5- B cells revealed that a restricted set of $V_H$ gene segments is predominantly used in their VDJ gene rearrangements. However, the 3' $V_H$ gene families typically expressed during fetal life are not preferentially used in CD5+ B cells (Andrade et al., 1989; Freitas et al., 1990; Andrade et al., 1991). The third possibility is that $V_H$ gene replacement may interfere with biased usage of the 3' $V_H$ gene families (Alt, 1986). Although $V_H$ gene replacement has been shown to play an important role in receptor editing, the relevance of this mechanism regarding the random use of $V_H$ genes in adult mice remains to be determined (Chen et al., 1995). Lastly, $V_H$ gene expression may reflect the restricted usage of individual $V_H$ genes in a given family and the selected $V_H$ genes may be distributed over the $V_H$ locus resulting in the randomization pattern. To date, the mechanism for the
randomization of \( V_H \) gene families later in life is still obscure and remains a major challenge for investigator.

One factor that should be considered in the analysis of \( V_H \) gene family usage is strain-dependent variation. This notion is first described by Wu and Paige who examined \( V_H \) gene usage by RNA colony blot assay (Wu and Paige, 1986). In this method, RNA transcripts of B cell clones growing in semi-solid agar were blotted on membranes and hybridized with \( V_H \) family and \( C\mu \) probes. They found that BALB/c mice frequently use the \( V_H7183 \) family whereas the C57BL/6 mice preferentially use \( V_HJ558 \) family. By using in situ hybridization, Jeong et al (1988) analyzed the \( V_H \) repertoire in LPS-activated B cells from six mouse strains and also found that \( V_H \) gene family utilization is strain-dependent (Jeong et al., 1988). These findings were later confirmed by studies from several laboratories including Alt's laboratory (Yancopoulos et al., 1988; Osman et al., 1988; Sheehan and Brodeur, 1989; Freitas et al., 1989; Viale et al., 1992). One possible explanation for strain-dependent \( V_H \) gene usage is that \( V_H \) gene utilization is determined by a locus outside the \( V_H \) chromosomal region (Wu and Paige, 1988; Viale et al., 1992). At present, the nature of this locus has not been characterized. Because the number of germline \( V_H \) genes and the copy number of functional \( V_H \) genes are different among the mouse strains, these factors may also influence the observed frequency of \( V_H \) gene usage.
**V<sub>H</sub> Gene Utilization in Human B-Lineage Cells**

The human germline V<sub>H</sub> repertoire consists of approximately 50 functional V<sub>H</sub> gene segments which are members of seven V<sub>H</sub> gene families (Matsuda et al., 1993; Cook et al., 1994). Each family contains a different number of V<sub>H</sub> gene segments, ranging from one for V<sub>H</sub>6 to more than 50 for V<sub>H</sub>3, of which 22 are functional genes. In contrast to the mouse, the human V<sub>H</sub> family members are highly interspersed among each other (reviewed in Pascual and Capra, 1991). Recently, the human V<sub>H</sub> locus has been completely mapped and almost all V<sub>H</sub> gene segments have been sequenced (Matsuda et al., 1993; Cook et al., 1994). This information led investigators to rename all previously known V<sub>H</sub> gene segments by the family number and the order from the 3' end of the V<sub>H</sub> locus (reviewed in Honjo and Matsuda, 1995). For example, V6-1 indicates that this gene segment is the member of V<sub>H</sub>6 family and is the most D proximal V<sub>H</sub> gene.

Furthermore, restriction fragment length polymorphism (RFLP) and DNA sequencing have demonstrated that many human V<sub>H</sub> genes are polymorphic. This polymorphism is the result of gene duplication and deletion events which may be small, involving a single V<sub>H</sub> gene segment, or may be extensive, involving multiple V<sub>H</sub> genes. Consequently, the number of V<sub>H</sub> gene segments in each haplotype is different and this variation in germline composition may influence V<sub>H</sub> gene utilization in humans.

Early in human ontogeny, as in the mouse, there is a bias in utilization of certain V<sub>H</sub> genes. Cuisinier et al examined the human V<sub>H</sub> repertoire at seven weeks of gestation
in which B-lymphopoiesis just begins and only pre-B cells can be detected in fetal liver (Gathings, 1977; Cuisinier et al., 1989). By using an RNA dot-blot hybridization technique, they found that only \( V_{H}5 \) and \( V_{H}6 \) families were expressed in fetal liver (Cuisinier et al., 1989). By eight weeks of gestation, all of the \( V_{H} \) families were expressed in fetal liver as determined by anchored-PCR and nucleotide sequence analysis (Cuisinier et al., 1993). In particular, the \( V_{H}3 \) family which contains more than 20 functional \( V_{H} \) gene segments distributed across the entire \( V_{H} \) locus was utilized most frequently.

The initial observation that chromosomal position of a \( V_{H} \) gene determines \( V_{H} \) gene utilization in mouse embryos led researchers to ask whether a similar phenomenon occurs in humans. Pascual et al analyzed fetal VDJ genes and found that two members of the \( V_{H}5 \) family, one located 300 kb and the other 800 kb from the D locus were used at the same frequency (Pascual et al., 1993). This finding suggests that \( V_{H} \) gene utilization in human does not exclusively correlate with the position of \( V_{H} \) gene segments within the \( V_{H} \) locus as observed in early ontogeny of the mouse.

**Rabbit \( V_{H} \) Genes and Repertoire Formation**

Similar to mouse and human, during B cell development, IgH variable region genes of rabbit are assembled by rearrangement of three non-contiguous gene segments, \( V_{H} \), D and \( J_{H} \). While combinatorial joining of these gene segments generates a large
primary VDJ repertoire in mouse and human, this process does not contribute as much to the IgH repertoire in rabbit. As will be described below, this is due to the fact that rabbit B cells use only a few \( V_H \) genes in their VDJ gene rearrangements. To gain insight into IgH repertoire formation in rabbits, we need to know which \( V_H \) genes are used in VDJ gene rearrangements; how they are organized on the IgH locus; whether chromosomal position affects the utilization of \( V_H \) genes and most importantly how VDJ genes are diversified. The following review will present the current information regarding genomic organization and utilization of rabbit \( V_H \) genes as well as the somatic diversification of the rearranged VDJ genes. Additionally, the historical viewpoint on the investigations of \( V_H \) gene usage will be included. One of the discoveries that significantly contributed to the clarification of the allelic inheritance of \( V_H ^a \) allotype is the study by Knight and Becker in 1990 on a spontaneously arising mutant rabbit, Alicia. Because the objective of this dissertation project is to identify \( V_H \) genes used by Alicia rabbits to produce Ig molecules carrying the \( V_H ^a \) allotypic specificity, the last section of this review will be devoted to the Alicia rabbit.

**\( V_H \) Gene Organization**

The rabbit \( V_H \) locus resides on chromosome 16 and, as in mouse and human, contains multiple \( V_H \), D and \( J_H \) gene segments (Medrano and Dutrillaux, 1984; Becker et al., 1989). On the basis of DNA cloning and restriction enzyme analysis, Becker et al reported that the \( V_H \) and \( J_H \) gene segments were separated by 63 kb, a distance in
according with that reported in the human V<sub>H</sub> locus (Schroeder et al., 1988; Becker et al., 1989). The J<sub>H</sub> chromosomal region, located 8 kb upstream of the C<sub>µ</sub> gene, contains six J<sub>H</sub> gene segments, of which five, J<sub>H</sub> 2 to J<sub>H</sub> 6, are functional (Knight et al., 1985; Becker et al., 1989). At present, eleven D gene segments have been reported between the V<sub>H</sub> and J<sub>H</sub> gene segments (Becker et al., 1990; Friedman et al., 1994). To determine the number of germline V<sub>H</sub> gene segments, Gallarda et al. (1985) performed quantitative genomic blot analysis using a pan-V<sub>H</sub> probe and estimated that the germline contained at least 100 V<sub>H</sub> genes (Gallarda et al., 1985). This finding is consistent with that of Currier et al. who determined the number of V<sub>H</sub> genes from V<sub>H</sub>-containing cosmid clones (Currier et al., 1988). Within the 765 kb of genomic DNA represented in these clones, the authors estimated that approximately 100 V<sub>H</sub> gene segments were present, each separated by, on average, 5 kb. Because the entire V<sub>H</sub> chromosomal region was not represented in these cosmid clones, it is likely that the rabbit genome contains more than 100 V<sub>H</sub> gene segments. Of approximately 50 germline V<sub>H</sub> genes examined, nearly one half of them appear to be functional genes (Bernstein et al., 1985; McCormack et al., 1985; Gallarda et al., 1985; Currier et al., 1988; Knight and Becker, 1990; Raman et al., 1994). Notably, the 3' most V<sub>H</sub> gene, V<sub>H</sub>1, is reported to be a functional gene in all three allotype rabbits (Knight and Becker, 1990).

According to nucleotide sequence comparisons, all rabbit V<sub>H</sub> genes studied are more than 80% similar to each other, and in FR2 they are greater than 90% identical
(Gallarda et al., 1985; Currier et al., 1988; Knight and Becker, 1990; Short et al., 1991; Roux et al., 1991; Raman et al., 1994; Friedman et al., 1994). Thus all rabbit V\textsubscript{H} genes are members of a single gene family. This is in contrast to human and mouse V\textsubscript{H} genes which have been grouped into 7 and 15 gene families, respectively (Köfler et al., 1992; Cook et al., 1994; Mainville et al., 1996). On the basis of both the similarity of amino acid sequences and cross-hybridization of V\textsubscript{H} family probes, the rabbit V\textsubscript{H} gene family is thought to be the homologue of the human V\textsubscript{H}3 family and of the mouse S107 and 7183 families (Gallarda et al., 1985; Tutter and Riblet, 1989).

**V\textsubscript{H} Gene Usage**

One intriguing feature of rabbits is that throughout their ontogeny, only a few V\textsubscript{H} genes are used in VDJ gene rearrangements in spite of the fact that there are hundreds of V\textsubscript{H} genes available in the genome. In mouse and human, the restricted utilization of V\textsubscript{H} genes is observed only in fetal and neonatal lives. The other unique feature of rabbits is that the V region of most IgH molecules have V\textsubscript{H}a allotypic markers a1 a2 or a3 and these molecules are designated V\textsubscript{H}a-positive (V\textsubscript{H}a\textsuperscript{+}). The serum Ig molecules that do not have the V\textsubscript{H}a allotype are classified as V\textsubscript{H}a-negative (V\textsubscript{H}a\textsuperscript{-}). Here, I will review the observations suggesting that only a restricted number of V\textsubscript{H} genes are used to encode V\textsubscript{H}a\textsuperscript{+} and V\textsubscript{H}a\textsuperscript{-} Ig molecules. Particularly, I will present the evidence indicating that one V\textsubscript{H} gene segment, V\textsubscript{H}a, is preferentially used in VDJ gene rearrangement of normal rabbits.
Preferential $V_H$ Gene Usage and The Allelic Inheritance of $V_{Ha}$ Allotype

It is noteworthy to mention that the knowledge of $V_H$ gene usage in rabbits was initially obtained from studies performed to explain the allelic inheritance of the $V_{Ha}$ allotype. The $V_{Ha}$ allotypic specificities $a1$, $a2$ and $a3$ are present on approximately 80% of serum IgH molecules and they are encoded by allelic genes $a1$, $a2$ and $a3$, respectively (Dray et al., 1963 a and b). Because the majority of serum Ig molecules bear the $V_{Ha}$ allotype, it was assumed that most germline $V_H$ genes encode the $V_{Ha}$ allotypes. The $V_{Ha}$ allotypes are inherited as autosomal co-dominant alleles and it was this allelic inheritance that had puzzled immunologists for over 30 years (reviewed in Knight 1992). The problem was that if the germline contained hundreds of $V_H$ genes that could encode the $V_{Ha}$ allotype and if each of the $V_H$ genes was used in VDJ gene rearrangements, how could these $V_{Ha}$ allotypes be inherited in such an allelic manner. In other words, why weren't the allotype-encoding $V_H$ genes shuffled during meiotic recombination such that they would no longer be inherited in an allelic fashion?

To explain the allelic inheritance phenomenon of $V_{Ha}$ allotype, Knight and Becker cloned and sequenced the D-proximal $V_H$ genes from rabbits of three allotypes (Knight and Becker, 1990). By analyzing nucleotide sequences of germline $V_H$ genes, they found that the 3' most $V_H$ gene, $V_{Hl}$, from $a1$, $a2$ and $a3$ allotype rabbits encoded prototypic $V_{Ha}$ allotype $a1$, $a2$ and $a3$ respectively. The next step was to investigate whether these genes are used in VDJ genes encoding $V_{Ha}^+$ Ig molecules. To do this, they
examined VDJ gene rearrangements of leukemic B cell lines derived from tumors in transgenic rabbits carrying the Eμ-myc transgene (Knight et al., 1988; Becker et al., 1990). These leukemic B cell lines were the only available sources of clonal B cell populations at that time. By comparing the nucleotide sequences of the V regions of the rearranged VDJ genes to sequences of germline VH genes of the same allotype, they found that seven of eight genes have nucleotide sequences that matched with VH\textsubscript{J} sequence. Moreover, analysis of regions immediately upstream of these seven VDJ genes showed that they were identical to the same region upstream of germline VH\textsubscript{J}. These data indicated that most rabbit B cells used VH\textsubscript{J} in their VDJ gene rearrangements. Furthermore, the evidence for the preferential utilization of the VH\textsubscript{a} allotype-encoding VH\textsubscript{J} was strongly supported by the findings obtained from the studies in mutant Alicia rabbits, having a defect in VH\textsubscript{a} allotype expression. As will be described in the section of Alicia rabbits, Knight and Becker discovered that the defect in the expression of VH\textsubscript{a} allotype in Alicia rabbits was due to a deletion in VH\textsubscript{J}. Because 80% of Ig molecules in normal rabbits carried the VH\textsubscript{a} allotype and essentially serum Ig in young Alicia rabbits, lacking VH\textsubscript{J}, did not have VH\textsubscript{a} allotypic specificities, Knight and Becker concluded that the allelic VH\textsubscript{J} encoded the IgH chain with VH\textsubscript{a} allotypic determinants and that VH\textsubscript{J} was preferentially used in VDJ gene rearrangements. Therefore, it has become clear that the allelic inheritance of the VH\textsubscript{a} allotype is due to the preferential utilization of the VH\textsubscript{a} allotype-encoding VH\textsubscript{J} in VDJ gene rearrangements. In retrospect, the reason that the VH\textsubscript{a} allotype issue was previously difficult to understand is because the assumption that
the germline had hundreds of $V_{H\alpha}$ allotype-encoding genes and that each of these $V_{H}$ genes were equally used in VDJ gene rearrangements.

**$V_{H}$ Gene Usage in $V_{H\alpha}^{+}$ Ig Molecules**

Eighty percent of serum Ig molecules are $V_{H\alpha}$-positive ($V_{H\alpha}^{+}$) and are encoded by the $V_{H\alpha}^{+}$ gene, $V_{Hl}$. As mentioned above, analysis of $V_{H}$ gene usage in leukemic B cell lines demonstrated that B cells expressing $V_{H\alpha}^{+}$ used $V_{Hl}$ in their VDJ gene rearrangements (Knight et al., 1988; Becker et al., 1990). Studies of a mutant rabbit, Alicia, supported this finding (see more detail in section of mutant Alicia rabbit). Another line of evidence that $V_{Hl}$ is used to encode $V_{H\alpha}^{+}$ Ig molecules was provided by Raman et al, who showed that in eight of nine $V_{H\alpha}^{+}$-encoding VDJ genes from rabbit x mouse heterohybridomas, $V_{Hl}$ was used (Raman et al., 1994).

The Predominance of $V_{H\alpha}^{+}$ Ig reflects the preferential utilization of $V_{Hl}$

The findings that most $V_{H\alpha}^{+}$ Ig molecules were encoded by $V_{Hl}$ and that these $V_{H\alpha}^{+}$ Ig represented 80% of total Ig molecules led Knight and Becker to propose that $V_{Hl}$ was preferentially used in VDJ gene rearrangements of rabbit B cells (Knight and Becker, 1990). This idea was later supported by the studies on $V_{H}$ gene usage in fetal and neonatal rabbits. By analyzing fetal VDJ genes, Tunyaplin and Knight found that all of 23 productive VDJ genes used $V_{Hl}$ (Tunyaplin and Knight, 1995). Moreover, Friedman et al showed that $V_{Hl}$ was used in approximately 80% of VDJ genes found in bone
marrow and spleen of neonatal rabbits (Friedman et al., 1994). Also, as mentioned earlier, most rabbit x mouse heterohybridomas derived from adult rabbits utilized $V_\text{H}l$ (Raman et al., 1994). Taken together, it appears that $V_\text{H}l$ is preferentially used in rabbit B cells early in life and this preferential usage of $V_\text{H}l$ continues throughout adult life. Such restricted use of one $V_\text{H}$ gene is striking, especially since nearly 50% of the germline $V_\text{H}$ genes appear functional. Although the preferential utilization of $V_\text{H}$ genes has also been reported in the mouse and human, it appears only in their fetal and neonatal lives.

$V_\text{H}$ Gene Usage in $V_\text{H}a^-$ Ig molecules

Ten to twenty percent of serum Ig molecules in normal rabbits that do not have the $V_\text{H}a$ allotype are collectively classified as $V_\text{H}a^-$ and are encoded by $V_\text{H}a^-$ genes (Kim and Dray, 1973; Short et al., 1991). In normal a2 allotype rabbits, three different $V_\text{H}a^-$ genes, $V_\text{H}x$, $V_\text{H}y$ and $V_\text{H}z$ are used to encode these $V_\text{H}a^-$ molecules (Short et al., 1991; Friedman et al., 1994). $V_\text{H}x$ and $V_\text{H}y$, are found to be predominantly used in young Alicia rabbits that have $V_\text{H}l$ deleted from the genome. The other $V_\text{H}a^-$ gene, $V_\text{H}x^-$, was found in VDJ gene rearrangements of neonatal rabbits (Friedman et al., 1994). Comparison of the deduced amino acid sequence of $V_\text{H}y$ with that of the $V_\text{H}a^+$ encoding gene, $V_\text{H}l$, revealed an insertion of four amino acids at position 10 in FR1 (Figure 1) (Short et al, 1991). The deduced amino acid of the potential $V_\text{H}x$ gene showed an insertion of glutamic acid (codon GAG) at position 2. These unique characteristics of the $V_\text{H}a^-$ genes will be useful
Figure 1: Comparison of the nucleotide sequences of $V_{H\alpha^{-}}$ genes, $V_{H\alpha}$, $V_{H\beta}$ and $V_{H\delta}$, with that of $V_{H\lambda}$ from a2 allotype rabbits. The FR and CDR are according to Kabat et al., 1991. Dots indicate nucleotide identity; a slash indicates nucleotide deletion.
<table>
<thead>
<tr>
<th>FR1</th>
<th>CAG // / TCG GTG AAG GAG TCC GAG GGA GGT // / / / / / / / / CTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>GAG CA. C. . . . . . . . G. . . . C /// / /// / /// / /// . . G</td>
</tr>
<tr>
<td>VHX</td>
<td>/// CA. C. . G. C. . . GA ... A GCC GGA GGA GGC ... G</td>
</tr>
<tr>
<td>VHY</td>
<td>/// / CA. C. . G. C. . . GA ... C /// / /// / /// . . G</td>
</tr>
<tr>
<td>VHZ</td>
<td>F K P T D T L T L T C T V S G</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CDR1</th>
<th>TTC AAG CCA ACG GAT ACC CTG ACA CTC ACC TGC ACA GTC TCT GGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHX</td>
<td>. . G. G. G ........</td>
</tr>
<tr>
<td>VHY</td>
<td>. . T GG. . GA T. . . GA . . TG. . A. C. .........</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CDR2</th>
<th>P G N G L E W I G A I G S</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>CCA GGG AAC GGG CTG GAA TGG ATC GGA GCC ATT // / /// / GGT AGT</td>
</tr>
<tr>
<td>VHY</td>
<td>. . T C TGG . . T. ........</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FR2</th>
<th>P G N G L E W I G A I G S</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>AGT GGT AGC GCA TAC TAC GCG AGC TGG GCG AAA AGC CGA TCC ACC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FR3</th>
<th>AGT CTG ACA GCC GCG GAC ACC ACC ACC CTG AAC ACG GTG ACT CTG AAA ATG ACC</th>
</tr>
</thead>
</table>

| S L T A A D T A T Y F C R A | I T R N T N L N T V T L K M T |

<table>
<thead>
<tr>
<th>VH1</th>
<th>AGT CTG ACA GCC GCG GAC ACC ACC ACC CTG AAC ACG GTG ACT CTG AAA ATG ACC</th>
</tr>
</thead>
</table>
for identifying the $V_H$ genes utilized in the VDJ rearrangements that encode $V_{H^a}$ molecules.

**Somatic Diversification of VDJ Genes**

Collectively, the data of $V_H$ gene usage in $V_{H^a^+}$ and $V_{H^a^-}$ Ig molecules indicate that nearly all of the VDJ antibody repertoire derives from a small number of germline $V_H$ genes. Because only a few $V_H$ genes are used in VDJ gene rearrangements, combinatorial joining of $V_H$, D and $J_H$ gene segments is limited and does not contribute extensively to the generation of antibody diversity. How then is antibody diversity generated in rabbit B cells? Similar to mouse and human, antibody diversity in rabbit is generated by junctional diversity, N-region addition and combinatorial association of H and L chains. In contrast, much of the IgH diversity in rabbit is generated by a somatic gene conversion-like process (Becker and Knight, 1990). This finding was first reported by Becker and Knight who compared nucleotide sequences of VDJ genes that utilized $V_H/l$ with the sequence of the germline $V_H/l$ and found clusters of nucleotide changes in $V_H$ regions of VDJ genes. The mutation tracts often had either codon insertions or deletions that are characteristic of gene conversion processes. Most importantly, they showed that the nucleotide sequences of the diversified regions were identical to the sequences of $V_H$ genes upstream of $V_H/l$. Their data demonstrated conclusively that these VDJ genes had undergone diversification through a somatic gene conversion-like mechanism that used the upstream $V_H$ genes as donors.
Somatic gene conversion was first described as a mechanism for the generation of antibody diversity in chicken $V_H$ and $V_{\lambda}$ genes (Reynaud et al., 1987; Reynaud et al., 1989). Among mammals, rabbit was the first species in which somatic gene conversion was shown to play a major role in the diversification of VDJ genes. Evidence for somatic gene conversion of Ig genes in mouse and human has been limited; however, Xu and Selsing recently showed that a VDJ transgene had diversified using the upstream transgene construct as donor (Xu and Selsing, 1994). Most recently, somatic gene conversion was shown to play an important role in the diversification of bovine $V_{\lambda}$ genes (Parng et al., 1996). All of these experiments suggest that somatic gene conversion-like events occur in many species, but that, whereas it may contribute a major part of the antibody diversity in species such as rabbit, bovine and chicken, it may be a minor mechanism for developing antibody diversity, in species such as mouse.

During an immune response against foreign antigens, human and mouse VDJ genes undergo somatic hypermutation in germinal centers of peripheral lymphoid tissues (Berek et al., 1991; Jacob et al., 1991; Jacob and Kelsoe, 1992; Kuppers et al., 1993; Pascual et al., 1994). In contrast, chicken $V_H$ and $V_L$ genes diversify in the bursa of Fabricius by somatic gene conversion (Reynaud et al. 1987 Reynaud et al., 1989; Thompson and Neiman, 1987). The somatic gene conversion occurs before hatching, prior to exposure to exogenous antigens. Rabbit VDJ genes also diversify by a gene conversion-like process, but unlike chicken this does not occur during fetal development. At birth, the VDJ genes are undiversified but by 6 to 8 weeks of age essentially all VDJ
genes had undergone extensive somatic diversification (Friedman et al., 1994; Crane et al., 1996). Because gut-associated-lymphoid-tissues (GALT) develop in rabbit beginning at 1 to 2 weeks of age and because GALT have histologic characteristics similar to that of the chicken bursa (Archer et al., 1963, Cooper et al., 1966), Knight and Crane proposed that rabbit B cells undergo somatic diversification in GALT (Knight and Crane, 1994). In support of this idea, Weinstein et al (1994) recently showed that VDJ genes diversified, apparently by gene conversion, in germinal centers of the appendix of six week-old rabbits. It will be important to investigate whether the gene conversion-like process is antigen driven or developmentally regulated.

**Mutant Alicia Rabbits**

The mutant rabbit, Alicia, was identified by Kelus and Weiss at the Basel Institute for Immunology (Kelus and Weiss, 1986). By allotyping serum Ig, they found that an a<sup>1</sup>/a<sup>2</sup> heterozygous rabbit had become spontaneously defective in expression of V<sub>H</sub>a<sub>2</sub> allotype and from this first Alicia heterozygote, they were able to generate Alicia homozygotes. This Alicia rabbit is genotypically a<sup>2</sup>/a<sup>2</sup>, however, phenotypically, it expressed exceedingly low levels of the V<sub>H</sub>a<sub>2</sub> allotype.

**Contribution of Ali Mutation to the Discovery of the Preferential Usage of V<sub>H</sub>1**

By the time that the Alicia mutation (ali) was discovered, it had been widely assumed that the germline of rabbits contained hundreds of V<sub>H</sub>a allotype-encoding genes and that each of these genes was expressible. It, therefore, was difficult to envision what
kind of mutation could dramatically affect the $V_{H}a$ allotype expression. Also, at that time, the explanation for the allelic inheritance of $V_{H}a$ allotype was still elusive. As described earlier, Knight and Becker (1990) had attempted to solve the problem of allelic inheritance of $V_{H}a$ allotype by showing that most leukemic B cell lines preferentially used the $V_{H}a$ allotype-encoding gene, $V_{H}/$. $V_{H}/$ was located at the 3' end of the $V_{H}$ chromosomal region. Because Alicia rabbits had a defect in $V_{H}a$ allotype expression, they hypothesized that the mutation in Alicia rabbits might be associated with $V_{H}/$. To test this hypothesis, they cloned the 3' end of the $V_{H}$ chromosomal region from normal and Alicia rabbits and then performed restriction enzyme analysis. By comparing the restriction maps of Alicia cosmid clones to that of normal rabbits (Figure 2), they found a 10 kb deletion in the $V_{H}$ locus including the prototypic $V_{H}a2$-encoding gene, $V_{H}/$ (Knight and Becker, 1990). In addition, using transverse alternating field electrophoresis and Southern analysis, Allegrucci and co-workers detected a deletion of 10-15 kb DNA near the 3' end of the $V_{H}$ chromosomal region (Allegrucci et al, 1990; Allegrucci et al, 1991). Taken together, these experiments clearly demonstrated that the ali mutation results from the deletion of the nominal $V_{H}a$ allotype encoding $V_{H}/$ gene. Generally speaking, the mutant Alicia rabbit represents a $V_{H}/$ knock-out rabbit.

The finding that mutant Alicia rabbit has a deletion encompassing $V_{H}/$ supported the idea that $V_{H}/$ is preferentially used in VDJ gene rearrangements of rabbit B cells. The loss of the expression of $V_{H}a2$ allotype correlates with the loss of $V_{H}/$, indicating that
Figure 2: The 10 kb deletion of DNA at the 3’ end of $V_H$ locus of Alicia rabbits.
most $V_H a_2$ Ig molecules are derived from $V_H l$. Because most Ig molecules in normal rabbits have markers designating them as $V_H a$ allotype a1, a2 or a3, Knight and Becker suggested that $V_H l$ is utilized in most rabbit B cells (Knight and Becker, 1990). As mentioned above, the data from studies of $V_H$ gene usage in B cells from fetus, newborn and adult rabbits conclusively confirm this notion (Raman et al., 1994; Friedman et al., 1994; Tunyaplin and Knight, 1995).

**$V_H$ Gene Usage in Alicia Rabbits**

$V_H$ gene usage in Alicia rabbits was firstly reported by DiPietro and colleagues who analyzed VDJ gene repertoire of 8-week-old Alicia rabbit (DiPietro et al., 1990). They determined nucleotide sequences of VDJ genes cloned from a splenic cDNA library and found that 6 of 7 cDNA clones used $V_H y$, a $V_H a^-$ gene, in their VDJ gene rearrangements. Their data explained the presence of $V_H a^-$ Ig molecules in most serum Ig of young Alicia rabbits described by Kelus and Weiss (1986). Further, the frequent use of $V_H a^-$ genes in young Alicia rabbits was confirmed by Chen et al who PCR-amplified VDJ genes from splenic RNA of 2-to 8-week-old Alicia rabbits (Chen et al, 1993). By screening cDNA clones with specific oligomer probes, they found that 158 of 159 cDNA clones isolated from 2-week old Alicia rabbit appeared to be $V_H a^-$-encoding VDJ genes, $V_H x$ and $V_H y$. At 2 weeks of age, these two genes were used at approximately the same frequency. But by 8 weeks of age, all $V_H a^-$-encoding VDJ genes were $V_H y$, as reported by DiPietro et al. The germline counterpart of the $V_H y$-encoding VDJ gene was cloned and
found to be located more than 50 kb upstream of $V_{HJ}$ (Knight, unpublished data). These findings suggest that in rabbits lacking the 3’ most $V_H$ gene, $V_{HJ}$, the next 3’ functional $V_H$ gene, $V_{HJ}'$, is not frequently used in VDJ gene rearrangements. Therefore, the preferential usage of $V_{HJ}$ in normal rabbits is not simply due to the close proximity of $V_{HJ}$ to D and $J_H$ gene segments.

Because normal rabbits preferentially use the $V_Ha$ allotype-encoding gene, $V_{HJ}$, in their VDJ gene rearrangements, 80% of their serum Ig molecules are $V_Ha^+$. In contrast, most Ig molecules in young Alicia rabbits are $V_Ha^-$ and are encoded by $V_Ha^-$ genes. However, $V_Ha2$ Ig appear in the blood circulation as Alicia rabbits age. What is the origin of these $V_Ha2$ Ig molecules in light of the fact that the preferentially utilized $V_Ha2$-encoding gene, $V_{HJ}$, is deleted? What $V_H$ gene(s) are used to encode these $V_Ha2$ Ig molecules? Because somatic gene conversion has been shown to be a major mechanism to generate antibody diversity in normal rabbits (Becker and Knight, 1990), is it possible that the $V_Ha2$ Ig in aged Alicia rabbits are derived from the rearranged $V_Ha^-$ genes that have been gene converted by a non-$V_H1$, $V_Ha2$-encoding gene? Alternatively, is it possible that the $V_Ha2$ Ig molecules are derived from, as yet unidentified, $V_Ha2$-encoding genes other than $V_{HJ}$. These are the questions that I have focused in this dissertation project.
CHAPTER II

MATERIALS AND METHODS

Animals

Two mutant Alicia rabbits (ali/ali) used in this study, number 20297 and 20304, were two years and 10 months old and were the gift of Dr. Andrew Kelus (Basel Institute of Immunology, Basel, Switzerland). Alicia rabbit number 20304 was used in fusions 1 and 2 while number 20297 was used in fusions 3. Alicia rabbits as well as colony rabbits of known V_{H}a allotypes were maintained at the animal facility of Loyola University Chicago Medical Center.

Probes

V_{H} probe

A pan-V_{H} probe used in the present study is the p181 V_{H} probe derived from codons 7 to 66 of a germline V_{H} gene segment isolated from an a3 allotype rabbit (Gallarda et al., 1985). This probe was used to detect the germline V_{H} gene segments and the rearranged VDJ genes of Alicia rabbits.
**J_H probe**

The coding regions of the five J_H gene segments are nearly identical. Therefore, a single probe containing any J_H gene segment can detect all germline J_H genes as well as rearranged J_H genes (Becker et al., 1989). In this study, pJ5 (1.2 kb AccI/BamHI of germline DNA fragment containing J_H4, J_H5 and J_H6) was used in genomic blot analysis and in the isolation of Alicia VDJ genes from size-selected genomic libraries.

**Cell Culture Media**

RPMI medium supplemented with 10% fetal calf serum (FCS) and 1 mg/ml G418 (GIBCO/BRL purity 705 µg/mg) was used to culture the mouse pre B cell line, 103/BCL-2. The components of RPMI medium are as follows: RPMI-1640, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 mM HEPES buffer, 5 mM 2-mercaptopethanol and 0.2% sodium bicarbonate. In addition, the following anti-microbial agents were included in the medium: 50 unit/ml Penicillin, 50 µg/ml Streptomycin, 30 µg/ml Gentamicin and 0.5 µg/ml Fungizone. Most ingredients were purchased from GIBCO/BRL.

Rabbit splenocytes cultured in the “CD40 system” and Alicia hybridomas were grown in modified RPMI supplemented with 15% normal rabbit serum (NRS) and FCS, respectively. Modified RPMI medium consists of the basic ingredients as RPMI medium (500ml), plus the following mixture solution: 5 ml of 100x vitamin, 10 ml of 50x MEM-essential amino acid and 5 ml of 100x MEM-non essential amino acid supplements. These three supplements were also obtained from GIBCO/BRL.
Generation of Alicia Hybridomas

Cell Fusion

Alicia hybridomas were generated by fusing spleen cells of Alicia rabbits with the rabbit hybridoma fusion partner, 240E1-1-2, developed by Spieker-Polet et al (1995). Prior to fusion, Alicia rabbits were immunized by injection of heat-killed Bacteroides in complete Freund's adjuvant subcutaneously, intramuscularly and intraperitoneally. Three weeks later, the animals were boosted twice with the same antigen but in incomplete Freund's adjuvant. Four days after the last immunization, fusions were performed using the procedure as described by Spieker-Polet et al (1995). In one experiment, Alicia splenocytes were activated in vitro before the fusion by co-culturing of spleen cells with CD40 ligand-expressing CHO cells (Spriggs et al., 1992). According to Spieker-Polet's method, Alicia spleen cells were fused with the rabbit fusion partner at a ratio of 2:1 in the presence of 50% PEG 4000 (EM Science, cherry Hill, NJ 08304) at 37°C.

The fusion was performed by, first, mixing Alicia spleen cells and the rabbit fusion partner at a ratio of 2:1 in serum-free RPMI medium. The cell mixture was then spun down and the supernate was completely discarded. Subsequently, the fusion was conducted at 37°C by placing the tube containing the mixed-cell pellet in a beaker containing warm water. One ml of sterile prewarmed 50% PEG was added drop-by-drop with continuous mixing over one minute and the cell mixture was further gently shaken for an additional minute. Then, one ml of prewarmed serum-free RPMI medium was added drop-by-drop with continuous mixing over one minute; followed by slowly adding
five ml of serum-free RPMI medium over a period of one minute. Finally, 34 ml of the medium was slowly added over the last one minute. Following the fusion step, the cell mixture was spun down and the cell pellet was then resuspended in prewarmed modified RPMI supplemented with 15% FCS at a concentration of $4 \times 10^5$ cells/ml. Then, the cells were distributed into 48-well flat-bottom plate at 0.5 ml of cell suspension per well. After 72 hours, 0.5 ml modified RPMI containing 15% FCS and 2X of hypoxanthine/aminopterine/thymidine (HAT) was added to each well. The medium was changed with 1X HAT containing medium every 5-6 days. The wells were screened visually for the development of hybridoma clones and the culture supernates were tested for the presence of Ig molecules by ELISA (Enzyme-linked immunoassay).

**Cloning of Alicia Hybridomas**

The $V_{\text{H}}a2$-Ig secreting hybridomas were subcloned by limiting dilution using the rabbit fusion partner, HAT-sensitive cells, as feeder cells and HAT as a selective agent. In general, Alicia hybridoma cells were mixed with feeder cells and the mixed-cells were distributed into 48-well microplates such that the cell density of hybridoma and feeder cells was $0.5 \times 10^4$ and $5 \times 10^4$ cells per well, respectively. Following two days in culture, the complete medium supplemented with 2X HAT was added and the medium was changed every 5-6 days. Clones usually were observed after 2-3 weeks. In this study, the clones were generally presented in less than 30% of the wells. If monoclonal hybridomas were obtained, their culture media were then re-tested for the presence of
V_{\text{H}}a2 Ig molecules by ELISA. Once V_{\text{H}}a2 Ig-secreting hybridomas were detected, their cells were expanded for preparing genomic DNA.

**Detection of V_{\text{H}} a2 Ig Secreting Hybridomas**

The production of V_{\text{H}}a2 Ig molecules from Alicia hybridomas was determined by ELISA. Flat-bottomed 96 well microtiter plates (Becton Dickinson) were coated with 100 µl of 1 ug/ml affinity purified anti-V_{\text{H}}a2 allotype antibody in carbonate buffer pH 9.6. After overnight incubation at room temperature (RT), wells were washed four times with Phosphate Buffer Saline (PBS) pH7.2 which contained 0.05% Tween 20. Hybridoma culture supernates (100 µl per well) were then added and incubated at RT for 2 hours. Culture supernates of the fusion partner and transfectomas producing V_{\text{H}}a1 and V_{\text{H}}a3 Ig were used as negative control. Culture supernate of transfectomas expressing V_{\text{H}}a2 Ig and sera of known a2 allotype rabbits were also included as positive control. To one well, PBS-Tween alone was added as a blank. The plates were incubated and washed again. Following, biotinylated anti-a2 antibody at concentration 1 ug/ml in PBS were transferred into the wells (100 µl per well) and incubated for 2 hours at RT. After a further washing step, avidin-peroxidase complex (Vectastain, Vector laboratories Inc.) was added. After 30 min, the wells were washed and developed with ABTS substrate (0.5 ml) in citrate buffer pH4.2 (25 ml) and hydrogen peroxide (12.5 µl). The reaction was read at OD 405 nm and the results were reported as positive reaction if the absorbance of hybridoma was higher than the negative control.
Preparation of Genomic DNA

Genomic DNA was prepared from V_{H}lg-secreting hybridomas, the rabbit fusion partner and Alicia kidney tissue (Blin and Stafford, 1976).

Isolation of Nuclei

The isolation of genomic DNA from tissues requires a step of homogenization. First, 0.2-0.4 gm of frozen tissue was placed in a polypropylene tube containing 5 ml of sterile isotonic saline solution in the presence of 10 mM Tris-HCl pH 7.4 and 1 mM EDTA. The tissue was then homogenized with a Polytron until no pieces of tissue were visible. After that the homogenated tissue was centrifuged at 250 g for 5 minutes. For Alicia hybridomas and the rabbit fusion partner, approximately 1×10^7 cells were spun out of the serum-containing medium. The cell pellet was then resuspended in 10 ml of cold nuclei lysis buffer containing 0.32 M Sucrose, 0.01 M Tris-HCl pH 7.6, 5 mM MgCl₂ and 1% triton X-100. After the cells were lysed, the nuclei were collected by centrifugation at 1,500g for 15 minutes.

Isolation of Genomic DNA

Nuclei were resuspended in 300 µl of digestion buffer consisting of 0.024 M EDTA, 0.15 M NaCl, 0.04 M Tris-HCl pH 7.4. Then, each nuclei preparation was transferred to a microtube and mixed to obtain homogenous nuclei suspension. After that, proteinase K and SDS were added to a final concentration of 200 µg/ml and 0.05%,
respectively. This step of proteinase K digestion was carried out at 37°C for 18-24 hours. Subsequently, the sample was treated with RNase A at a final concentration of 75 µg/ml at 37°C for at least 4 hours. The next step was to purify genomic DNA by using phenol/choroform/isoamyl alcohol (25:24:1) (Sevag, 1935). This was accomplished by extracting the DNA samples twice with equal volume of phenol, then, twice with phenol/choroform/isoamyl alcohol and, lastly, twice with chloroform and isoamyl alcohol (24:1). The aqueous phase was collected and dialyzed against 4 liters of 10 mM Tris pH 7.5, 1 mM EDTA (TE) over two days with at least five changes of TE.

**Southern Blot Analysis**

Southern blot analysis was conducted as previously described by Southern (1975). Briefly, genomic DNA prepared from Alicia hybridomas, the rabbit fusion partner and Alicia liver and kidney tissues were restricted with *EcoRI* or *BamHI* at 37°C for 4-24 hrs. Once genomic DNA samples were completely digested (as all DNA ran out off the wells after electrophoresis on minigel), the DNA samples were fractionated by agarose gel electrophoresis. Generally, 1% agarose gel in E buffer (0.05 M Tris, 0.02 M NaOAc, 0.02 M NaCl, 0.002 M EDTA, pH was adjusted to 7.5 to 7.6 with 21 ml glacial acetic acid) was used throughout this study. The DNA samples were run along with a *HindIII*-digested λ DNA as molecular weight standard. After running the gel at 1 V/cm overnight, the gel was stained with ethidium bromide to visualize the DNA under UV light. Then, the gel was soaked in denaturing buffer (1.5 M NaCL, 0.5 M NaOH) for approximately 30 minutes and, then, followed by soaking in neutralization buffer (1.5 M
NaCl, 0.5 M Tris-HCl, pH 7.0) for 30 minutes. Subsequently, the DNA fragments in agarose gel were blotted overnight onto nitrocellulose membrane by capillary transfer method using 20x SSC as transfer buffer. To immobilize the DNA to the nitrocellulose membrane, the genomic blot was baked at 80°C for 1-2 hours.

To analyze the status of Alicia IgH gene rearrangements, the genomic blot was hybridized with a rabbit J\text{H} probe, pJ5. The probe was labeled with $^{32}$P-dCTP by the random primer method. Prior to hybridization, the DNA blot was prehybridized in genomic hybridization solution (40% deionized formamide, 4X SSC, 6 mM Tris-HCl pH 7.5, 0.8X Denhardt's solution and 10 mg/ml of sheared salmon-sperm DNA) at 42°C. After prehybridization for six hours, the probe with a specific activity of at least $1 \times 10^8$ cpm/µg was added and the hybridization was carried out at 42°C overnight. Then, the blot was washed in 1X blot wash solution (1X SSC, 0.1% SDS, 0.1% Sodium pyrophosphate) at 68°C and exposed to x-ray film in a cassette with intensifying screen at -80°C.

**Cloning of VDJ Gene from a Size-Selected Library**

Genomic Alicia VDJ fragments were isolated from size-selected genomic libraries of two $V_{\text{H}a2}$ Ig-producing hybridomas, 1E6 and 8E1. According to Southern blot analysis, the VDJ genes of 1E6 and 8E1 clones were found on approximately 5 and 6 kb $Bam$HI fragments, respectively. Therefore, their genomic DNA were restricted with $Bam$HI and the restricted DNA were electrophoresed in 1% low-melting point agarose
gel. The agarose fractions containing 1E6 and 8E1 VDJ genes were cut out and the genomic DNA fragments were isolated by digesting the agarose gels with agarase enzyme (New England Biolabs). First, the agarose gels were melted at 65°C in 50 mM Bis Tris-HCl (pH 6.5) and 10 mM EDTA for 10 min then were cool to 40°C. Subsequently, β-Agarase I (1 unit per 200 µl) was added and incubated for 1 hr at 40°C. After spinning down undigested agarose, DNA in the supernates were precipitated by ethanol. To construct the genomic libraries, the purified DNA fragments were cloned into BamHI cut pGEM-3 plasmid vector (Promega). The vector was prepared by digestion of 20 µg of pGEM-3 plasmid DNA with BamHI and dephosphorylation with calf intestine phosphatase. Similar to the preparation of insert DNA, the vector DNA was gel-purified and isolated from the gel by agarase digestion.

The next step was to ligate genomic DNA fragments to vector. To optimize the ligation reaction, the ratio of insert DNA fragments to vector was varied. A small scale ligation reaction was set up and the efficiency of ligation was tested by electrophoresis of the ligation mix with and without insert fragments on agarose gel. Also, a ligation mix in the absence of T4 DNA ligase enzyme was run on the gel as control. If the recombinant formation was achieved, the appearance of DNA with expected size of recombinant molecules would be observed and the intensity of vector and insert bands in the tube with ligase would be less than those in the tube without ligase. In addition, the efficiency of recombinant formation was determined by transformation of the ligation mix into E. coli and assay for the number of E. coli containing the insert fragments, a large-scale ligation
reaction would then be set up and transformed into ElectroMax DH10B cells (GIBCO BRL). The libraries were plated on medium containing ampicillin at the cell density 15,000 cells per plate. Following incubation, the bacterial colonies were transferred onto nitrocellulose membranes. After that, the processes of denaturation, neutralization and fixing the DNA on the membranes were performed.

The libraries were first screened with pJ5 probe. The J<sub>H</sub> -hybridizing clones were rescreened with a pan V<sub>H</sub> probe, p181. The clones that hybridized with both probes were colony-purified and grown up in 500 ml of L broth containing ampicillin as a selective agent. Plasmid DNA containing a VDJ gene was then prepared from the bacterial culture by triton-lysozyme lysis and centrifugation in CsCl/EtBr gradients.

**Large-Scale Preparation of Plasmid DNA**

Plasmid DNA used in this study was prepared by Triton-lysozyme lysis and centrifugation in CsCl/EtBr gradients. This was performed by, first, growing *E. coli* harbouring the needed plasmid in 500 ml of L broth containing appropriate antibiotic. After culture overnight at 37°C with shaking, the bacterial cells were harvested by centrifugation at 5000 RPM in Sorvall centrifuge for 15 min at 4°C. The bacterial pellet was then resuspended in 15 ml of Sucrose buffer (15% Sucrose, 0.05 M Tris, 0.05 M EDTA) and transferred into 50 ml Sorval tube. Subsequently, 5 ml of 10 mg/ml freshly prepared lysozyme in sucrose buffer was added and the mixture was incubated on ice for
at least 30 minutes. The next step was to the lyse the bacterial cell wall by adding 12 ml of Triton X-100 buffer (0.1% Triton X-100, 0.05 M Tris pH 8.0, 0.05 M EDTA). After incubation at 37°C for 30 minutes, the plasmid DNA was isolated from bacterial cell debris by centrifugation at 15,000 RPM for one hour. The supernatant was then collected into a 50 ml Falcon tube and a half volume of PEG solution (30% PEG, 1.5 M NaCl) was added to precipitate plasmid DNA. This step required incubation on ice for at least 30 minutes, followed by centrifugation at 2500 RPM for 15 minutes. After getting rid of residual PEG solution, the pellet was resuspended in CsCl solution (221.6 g of CsCl in 210 ml TE and 0.8 ml of 10 mg/ml EtBr) and transferred into a Quick-seal centrifuge tube (Beckman) for ultracentrifugation. The tube was then sealed and centrifuged for at least 18 hours at 55,000 RPM. After that the plasmid DNA band was collected by using a needle and syringe under long wavelength UV lamp. Then the ethidium bromide was removed by extracting the plasmid DNA sample with an equal volume of isopropanol saturated with CsCl. To remove CsCl, the plasmid DNA was dialysed against 4 liters of TE with at least three changes. The DNA was then quantitated by measuring OD at 260 nm. In general, this method yields approximately 1 mg of plasmid.

**PCR Amplification and Cloning of VDJ Genes**

Alicia VDJ genes and their promoters were PCR-amplified from genomic DNA of VHα2 Ig-secreting hybridomas. The upstream primer, 5'-TCGAATTCTCTGAATCAT-ATCACAGCCAT-3', was derived from the promoter region. 216 to 236 bp 5' of the
ATG start codon (Figure 3) (Tunyaplin and Knight, 1995). This oligomer is able to
prime the promoter region of most known V_H genes, but cannot prime the promoter
region of the VDJ gene of the rabbit fusion partner. The downstream primer, 5’-
GGTAAGCTTCACCTGA(G/A)GAGACGGTGACCAGGG-T-3’, was derived from the
conserved region of rabbit J_H genes (Becker et al., 1989). The PCR reaction contained
0.5 µg to 1 µg of genomic DNA template; 0.2 µM of each PCR primer; 50 µM mixture of
dATP, dCTP, dGTP and dTTP; 2.5 units of Taq polymerase and 1x PCR buffer in the
final volume of 50 µl. The amplification was performed by hot start method for 30
cycles. Each cycle consists of the denaturation step at 94°C for 45 seconds, the annealing
step at 60°C for 45 seconds and the extension step at 72°C for 45 seconds. The PCR
products were analyzed on nondenaturing polyacrylamide gels.

If the products with expected size, approximately 700 bp, were detected, they,
then, were restricted with EcoRI and HindIII, the restriction enzyme sites at the end of
promoter and J_H PCR primers, respectively. Subsequently, they were cloned into M13
mp18/19 and the recombinant M13 phage were screened for the VDJ genes by
hybridization with a pan V_H probe, p181. The hybridization was performed at 68°C
overnight in 3x hybridizing solution (3x SSC, 5x Denhardt’s, 0.1% SDS and 100 µg/ml
Salmon sperm DNA). After washing in 1x blot wash at 68°C, the autoradiography was
performed. Once the V_H-hybridizing clones were obtained, their nucleotide sequences
were determined.
Figure 3: Primers for PCR of VDJ genes and their promoters. The upstream primer is derived from the conserved promoter region, -216 to -236 bp 5' of the ATG start codon. The downstream primer is derived from the conserved region of rabbit $J_{H}$ genes.
Primers for PCR of VDJ Genes and Their Promoters
In vitro Recombination Assay

The recombination substrate, pWTSJD, used in the present study is shown in Figure 4. This extrachromosomal vector was kindly provided by Dr. Susanna Lewis. The assay was performed under supervision of Dr. Gillian Wu (University of Toronto) and the procedure is summarized in Figure 5A and 5B.

Transfection

The recombination plasmids were transfected into the 103/BCL-2 cell line by using DEAE-Dextran (Hesse et al., 1987; Lieber et al., 1987; Conner et al., 1995). This cell line, a gift of Dr. Naomi Rosenberg, was derived from pre-B cells transformed by a temperature-sensitive (ts) mutant of the Abelson virus and transfected with human BCL-2 gene (Chen et al., 1994). The cells were cultured in RPMI-supplemented with 10% FCS and 1 mg/ml G418 at the permissive temperature (34°C), at a density of approximately 1 x 10^6 cells/ml. At the day of transfection, the cells were aliquoted into 1-2 x 10^7 cells/transfection and washed with phosphate buffered saline solution (PBS). Then, cell pellets were resuspended in transfection mix containing 250 mg/ml freshly prepared DEAE-Dextran solution, 50 mM Tris-HCl pH 7.4, 1 mg of plasmid DNA in serum free RPMI medium. After incubation at 37°C for 10 minutes, the transfected cells were washed with PBS and cultured at 34°C in complete RPMI medium. Following 18 hours, the cells were maintained at 39°C for 18-24 hours to induce recombinase activity.
Figure 4: Recombination substrates. Four DNA fragments derived from the region 3’ of $V_{Hl}$-a2 gene were cloned into pWTSJD plasmid at HindIII site located between ptac and 12 base pair spacer of recombination signal sequence (RSS12).
Recombination Substrate

[Diagram of recombinant DNA with specific elements labeled: Col E1 ori, Amp r, Ptac, RSS12, RSS23, Transcription terminator, Cloning site, pWTSJD, Hind III, CAT, Polyoma Early]
Figure 5: *In vitro* recombination assay. A.: Analysis of recombination by chloramphenicol selection. B: Analysis of recombination by differential hybridization.
In vitro Recombination Assay

Recombination substrate
DNA: Amp$^r$

Transfection

103/BCL-2 pre-B cell line

34°C 18-24 hr
39.5°C 18-24 hr

Recover Plasmid DNA

E. coli DNA transformation
Growth selection

• • •

Amp$^r$ Cam$^r$

Amp

Amp + Cam
In vitro Recombination Assay

Recombination substrate DNA: Amp$^r$

Transfection

103/BCL-2 pre-B cell line

34°C 18-24 hr
39.5°C 18-24 hr

Recombinant plasmid:
Amp$^r$ Cam$^r$

Recover Plasmid DNA

E. coli DNA transformation Growth selection

pick onto grid

membrane transfer

hybridize with terminator

= non-recombinant
Harvesting the Plasmid DNA

The recombination plasmids were recovered from the 103/BCL-2 pre-B cells by alkaline lysis method. To accomplish this, the 103/BCL-2 cells were collected first by centrifugation at 800g for 10 minutes at 4°C. The cell pellets were then washed with cold PBS and spun down again. The pellet from each transfection was resuspended in 200 µl of buffer containing 50 mM glucose, 25 mM Tris-HCL pH 8.0 and 10 mM EDTA and transferred to a microcentrifuge tube. Then the transfected cells were lysed by adding 400 µl of freshly prepared alkaline solution consisting of 0.2 N NaOH and 0.6% SDS. After gentle mix by repeated inversion, 300 µl of 7.5 M ammonium acetate was added and the tube was kept on ice for 10 minutes. To isolate plasmid DNA from cell debris, the tube was centrifuged at 12,500 rpm for 15 minutes. The plasmid DNA was further purified by phenol-chloroform extraction as described earlier in genomic DNA preparation. After that, the sample was transferred to a microcentrifuge tube and 0.8 volume of isopropanol was added. The DNA was then precipitated by centrifugation at 12,500 rpm for 15 minutes at 4°C. Following, the DNA pellet was washed with cold 70% ethanol and centrifuged again. After air dry, the DNA pellet was resuspended in 30 µl sterile water.

Detection of Recombined Plasmids

To obtain a more reliable measurement of recombination frequencies (R), DNA molecules adhering to the outside of the pre-B cells without replication inside the cells
were first eliminated prior to transformation into bacteria (Lieber et al., 1987). This was achieved by digestion of the harvested DNA with restriction enzyme *DpnI*, an enzyme that cleaves DNA molecules with a bacterial dam methylation. After ethanol precipitation, the DNA was transformed into a recombination deficiency strain of *E. coli*, SURE strain (Strategene), by electroporation.

Analysis of recombination was performed by means of either chloramphenicol selection (Figure 5A) or differential hybridization (Figure 5B). For chloramphenicol selection, the transformants were cultured on agar plates containing ampicillin (Amp) and on plates containing Amp plus chloramphenicol (Cam). The frequency of recombination (R) was calculated by dividing the number of colonies on Amp plus Cam with the number of colonies on Amp alone, then multiplying by 100. For differential hybridization, the transformants were grown first on Amp plates and then picked on grids. Subsequently, the bacterial colonies on grids were transferred onto nitrocellulose membranes and hybridized with a probe derived from the prokaryotic transcriptional terminator that is deleted when rearrangement has occurred (see Figure 4 and 5B). Then, the terminator probe was stripped off and the blots were re-hybridized with the recombination vector or the tested DNA fragment. The R value was determined as the ratio of the number of colonies that did not hybridize with the terminator probe and the number of colonies that hybridized with the vector or the tested DNA fragment.
CHAPTER III

RESULTS

The goal of my dissertation project is to examine the molecular mechanism for the appearance of \( V_{\text{H}a2} \) Ig molecules in aged Alicia rabbits. To accomplish this, I generated \( V_{\text{H}a2} \) Ig-producing hybridomas from adult Alicia rabbits and then identified the \( V_{\text{H}} \) gene segments used in VDJ gene rearrangements of the \( V_{\text{H}a2} \) Ig-producing clones. First, spleen cells from three-year-old Alicia rabbits were fused with a rabbit fusion partner, 240E1-1-2, to generate stable rabbit x rabbit hybridomas and \( V_{\text{H}a2} \) Ig-producers were detected by ELISA. The hybridomas obtained from fusions 1 and 2 were derived from the same rabbit (see materials and methods). While the first fusion was performed by using standard hybridoma technique as described by Spieker-Polet et al.(1995), the second fusion was modified by activating spleen cells with CD40 ligand-expressing CHO cells prior to the fusion (Spieker-Polet et al., 1995, Spriggs et al., 1992). The hybridomas obtained from the third fusion were derived from Alicia rabbit number 20297 that died from an unknown cause approximately two hours before the fusion. From a total of 52 Alicia hybridomas, 37 clones secreted \( V_{\text{H}a2} \) Ig molecules and 34 of these could be IgG isotype as determined by ELISA (Table 1). The possible explanation for high frequency
Table 1  Number of hybridomas generated from Alicia rabbits.

<table>
<thead>
<tr>
<th>Fusion</th>
<th>IgG</th>
<th></th>
<th>non-IgG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a&lt;sup&gt;+&lt;/sup&gt;</td>
<td>a&lt;sup&gt;-&lt;/sup&gt;</td>
<td>a&lt;sup&gt;+&lt;/sup&gt;</td>
<td>a&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
of $V_{\text{H}a2}$ Ig-producing hybridomas will be further discussed in discussion. Because these hybridomas were derived from hyperimmunized rabbits, most of them produced IgG isotype. However, this also could be due to anti-rabbit light chain activity in goat anti-rabbit $\gamma$ chain antibody used in this study. Nevertheless, it suggested that some hybridomas derived from old Alicia rabbits produced $V_{\text{H}a}$-negative ($V_{\text{H}a^-}$) Ig molecules, the major Ig in young Alicia rabbits. These hybridomas will be useful for analysis of $V_{\text{H}}$ gene usage in $V_{\text{H}a^-}$ Ig molecules. In this dissertation project, only $V_{\text{H}a2}$ Ig-producing hybridomas were selected for analysis of $V_{\text{H}}$ gene used to encode $V_{\text{H}a2}$ Ig molecules. I describe below the results of analyses of these hybridomas including Southern blot analysis, cloning of Alicia VDJ genes and identification of utilized $V_{\text{H}}$ genes by analysis of promoter regions.

**Southern Analysis of Hybridomas**

**Analysis of $J_{\text{H}}$-Hybridizing Fragments**

To examine VDJ gene rearrangements in hybridomas derived from Alicia rabbits, Southern blot analysis was conducted. Genomic DNA prepared from 13 hybridomas, from the fusion partner and from Alicia kidney tissue were restricted and Southern blots of restriction-digested DNA were hybridized with a rabbit $J_{\text{H}}$ probe, pJ5. As many as five $EcoRI$ restricted fragments hybridized with the $J_{\text{H}}$ probe (Figure 6). Two of these, 4.1 and 13 kb fragments, represent the VDJ gene and germline $J_{\text{H}}$ of the fusion partner, respectively (lane 2). The 0.7 and 12 kb fragments represent germline $J_{\text{H}}$ of the Alicia B
Figure 6: Analysis for the status of hybridomas from mutant Alicia rabbit.

Top. Partial restriction map of $J_H$ loci of the fusion partner and ali. Solid boxes denote the $J_H$ gene segments. $\varnothing = \text{BamHI}$; $\mathcal{T} = \text{AccI}$; $\uparrow = \text{EcoRI}$. Solid lines represent confirmed restriction site by cloning and sequencing. Dotted lines represent presumed restriction sites based on analogy to the a1 haplotype; $J_H$ probe hybridized with a 0.7 Kb $\text{EcoRI}$ fragment on genomic blots of the a1 and ali haplotypes as well as the fusion partner.

Bottom. Southern analysis of $\text{EcoRI}$-digested DNA from 13 Alicia hybridomas. Genomic DNA prepared from the hybridomas were digested with $\text{EcoRI}$ and the blot was probed with the rabbit $J_H$ probe. The numbers and arrows refer to sizes of restricted DNA fragments in kb. GL indicates germline $J_H$ fragments, while 0.7 kb and 12 kb represent germline $J_H$ of the fused Alicia B cells and 13 kb represents germline $J_H$ of the fusion partner. The 4.1 kb represents the VDJ gene of the fusion partner. Alicia kidney and the rabbit fusion partner, 240E1-1-2, are shown as control.
cells (lane 1). The remaining fragments represent the rearranged JH genes of the fused Alicia B cells. These fragments, containing rearranged VDJ or DJ genes, ranged in size from 0.9 kb to 8 kb. For example, clone 1E6 (lane 13) has germline JH on the unexpressed alicia allele (0.7 kb and 12 kb), germline JH of the fusion partner (0.7 kb and 13 kb), VDJ of the fusion partner (4.1 kb) plus a 2.2 kb of JH-hybridizing fragment which must represent the VDJ gene of the fused Alicia B cell. From analyzing of all 13 clones, it appears that the VDJ genes of the ali haplotype may reside on DNA fragments of several different sizes. If so, this would suggest that several different VH genes were used in the VDJ gene rearrangements.

The status of JH gene rearrangement of several hybridomas was also analyzed by Southern blot analysis of BamHI restricted genomic DNA. The results of analysis of 14 hybridomas is shown in Figure 7. The 1.7 and 2 kb BamHI fragments represent germline JH genes of the fused Alicia B cells and the fusion partner, respectively. The 1.7 kb BamHI fragment also represents the VH\textsubscript{Hy}-utilizing VDJ gene of the fusion partner (see materials and methods). Because VH\textsubscript{Hy} has a BamHI site located in its FR1, the size of the restricted fragment containing fusion partner VDJ gene is smaller than the size of the DNA fragment containing the germline JH gene. Similar to Southern blot analysis of the hybridomas with EcoRI digestion, Southern analysis with BamHI digestion also showed several different sizes of JH-hybridizing fragments, suggesting that several VH genes may have been used in VDJ gene rearrangements.
Figure 7: Southern analysis of BamHI-digested DNA from 14 Alicia hybridomas. Genomic DNA prepared from the hybridomas were digested with BamHI and the blot was probed with the rabbit J_H probe. The size of standard molecular weight DNA, HindIII digested λ-phage DNA, is shown. GL refers to germline J_H fragments, 2.0 kb DNA fragment representing germline J_H of the fusion partner and the 1.5 kb fragment representing germline J_H of the fused Alicia B cells. The 1.5 BamHI fragment also represents the VDJ gene of the fusion partner (see text). Partial restriction map of J_H loci of the fusion partner and the ali is shown on the top (see Figure 6).
Rearrangement on the Unexpressed Allele

Previous study of IgH gene rearrangement on the unexpressed allele by Tunyaplin and Knight (1995) showed that approximately 50% of B cells have the unexpressed allele in germline configuration, 40% have DJ and 10% have VDJ gene rearrangements. I therefore analyzed 10 of the 52 total hybridomas for the configuration of V, (D) and J genes on the unexpressed allele. I performed Southern blot analysis with the J_{H} probe and probe A, a probe derived from the region 5' of all known germline D_{H} gene segments (Knight and Becker, 1990). If no rearrangement occurs, both probes A and J_{H} will hybridize with the germline sized fragments. If the unexpressed allele has a DJ gene rearrangement, only probe A will hybridize to a germline sized fragment and the J_{H} probe will hybridize to a non-germline sized fragment. If the unexpressed allele has a VDJ gene rearrangement, no probe A-hybridizing fragment will appear and the J_{H} probe will hybridize to a non-germline sized fragment. As summarized in Table 2, 2 of the 10 hybridomas appeared to have IgH in germline configuration on the unexpressed allele. 5 hybridomas presumably had a DJ gene rearrangement and 3 hybridomas appeared to have VDJ genes on the unexpressed allele. These data suggest that VDJ genes in Alicia rabbits are present on the unexpressed allele in a higher frequency than previously found in normal rabbits. The molecular basis for this difference is not understood.

Isolation and Identification of VDJ Genes Cloned from Genomic Libraries

The germline V_{H} gene used in VDJ gene rearrangements can be determined by directly comparing the nucleotide sequence of the VDJ gene with that of the germline V_{H}
Table 2  Southern blot analysis of DNA from a2-secreting and a2-negative secreting Alicia hybridomas hybridized with probe A and J_H probe.

<table>
<thead>
<tr>
<th>Hybridoma (allotype)</th>
<th>Germline probe A (^1)</th>
<th>Germline J_H</th>
<th>Status of the unexpressed allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>8E1 (a2)</td>
<td>+(^2)</td>
<td>+</td>
<td>Germline</td>
</tr>
<tr>
<td>10C2 (a2)</td>
<td>+</td>
<td>+</td>
<td>Germline</td>
</tr>
<tr>
<td>2B1 (a-)</td>
<td>-</td>
<td>-</td>
<td>VDJ</td>
</tr>
<tr>
<td>8A1 (a+/-)</td>
<td>-</td>
<td>-</td>
<td>VDJ</td>
</tr>
<tr>
<td>9F2 (a2)</td>
<td>-</td>
<td>-</td>
<td>VDJ</td>
</tr>
<tr>
<td>4A5 (a-)</td>
<td>+</td>
<td>-</td>
<td>DJ</td>
</tr>
<tr>
<td>4C7 (a-)</td>
<td>+</td>
<td>-</td>
<td>DJ</td>
</tr>
<tr>
<td>7D3 (a+/-)</td>
<td>+</td>
<td>-</td>
<td>DJ</td>
</tr>
<tr>
<td>9A6 (a2)</td>
<td>+</td>
<td>-</td>
<td>DJ</td>
</tr>
<tr>
<td>11F4 (a2)</td>
<td>+</td>
<td>-</td>
<td>DJ</td>
</tr>
<tr>
<td>6 additional hybridomas</td>
<td>ND</td>
<td>+</td>
<td>Germline or VD</td>
</tr>
<tr>
<td>15 additional hybridomas</td>
<td>ND</td>
<td>-</td>
<td>DJ or VDJ</td>
</tr>
</tbody>
</table>

\(^1\) Probe from 3' of \(V_H\) that identified DNA that would be deleted during VDJ gene rearrangement but not during DJ gene rearrangement.

\(^2\) + refer to hybridization; - refer to no observable hybridization.
genes, until the rabbit is 5 to 6 weeks of age. After 6 weeks of age, the VDJ genes, even those that encode the $V_H$ region of IgM from peripheral blood B cells, are extensively diversified by somatic gene conversion and somatic hypermutation (Short et al., 1991; Crane et al., 1995). Consequently, it is difficult to identify unequivocally the germline $V_H$ gene used in diversified VDJ gene rearrangement from rabbits older than 6 weeks by analyzing only the nucleotide sequence of the VDJ gene. To definitively identify the utilized germline $V_H$ gene, the region upstream of the rearranged VDJ gene should be cloned and compared with the region upstream of the candidate germline $V_H$ gene. This means that the utilized VDJ gene must be cloned as a genomic DNA fragment with the 5' region included.

One approach for cloning genomic VDJ genes and their upstream regions from $V_{H\alpha 2}$ Ig-producing hybridomas was to construct size-selected genomic libraries. Genomic blot analysis of Alicia hybridoma DNA hybridized with the rabbit $J_H$ probe showed that hybridomas 1E6 and 8E1 had only one rearranged VDJ gene on the ali chromosomes (Figure 6, 8, 12). Therefore, size-selected libraries were constructed from genomic DNA of these two hybridomas.

**Cloning of Genomic VDJ Gene from 1E6 Hybridoma**

The size of genomic DNA fragment containing the Alicia VDJ gene of hybridoma 1E6 was determined as above by Southern blot analysis. Basically, the genomic DNA prepared from the hybridoma, Alicia kidney and the rabbit fusion partner were restricted
Figure 8: Southern analysis of hybridoma 1E6 genomic DNA digested with EcoRI using a rabbit $J_H$ probe.
Germline Fusion Partner (13 kb)
Germline Ali (12 kb)
VDJ of Fusion Partner (4.1 kb)
VDJ of 1E6 (2.2 kb)
Germline Ali (0.7 kb)
with EcoRI as well as with BamHI and Southern blots were hybridized with the rabbit JH probe, pJ5. The JH-hybridizing bands were found on 0.7, 2.2, 4.1, 12 and 13 kb EcoRI fragments (Figure 8). By comparing the JH-hybridizing pattern of hybridoma 1E6 with those of the Alicia germline DNA and the rabbit fusion partner, I concluded that the 2.2 kb EcoRI fragment represented 1E6 VDJ gene. By analogy to analysis of EcoRI digestion, Southern blot analysis of BamHI digested DNA revealed that the VDJ gene of 1E6 hybridoma resided on 5 kb BamHI fragment (Figure 9). Because the entire germline JH locus was localized on a 2 kb BamHI fragment, the 5 kb BamHI fragment could have a VDJ gene with at least 3 kb of the upstream region. Thus, this 5’ region would be useful for identification of the utilized VH gene.

Hence, the 1E6 genomic library was constructed by cloning the DNA fragments of approximately 4.5 - 5.5 kb BamHI fragments into pGEM-3 plasmid vector. The library was first screened with the pJ5 probe. From a total of 400,000 colonies, ten clones hybridized with the pJ5 probe and one of these hybridized to pan VH probe. This clone was colony-purified and grown for plasmid DNA preparation. The presence of a genomic VDJ gene was verified by restriction enzyme digestion and Southern blots that were hybridized with VH and JH probes. As shown in the partial restriction map of 1E6 genomic clone, the VDJ gene was found on a 500 bp PstI/BstEII fragment (Figure 10).

To determine whether the 1E6 VDJ gene encodes VHα2 allotype, the 500 bp PstI/BstEII fragment was cloned into the Pst I/Sma I restriction sites of M13 mp18/19 and its
Figure 9: Southern Analysis of 1E6 genomic DNA digested with *BamHI* using a rabbit $J_H$ probe.
Alicia Kidney

Fusion Partner

Hybridoma 1E6

→ VDJ gene of 1E6 (5 kb)

→ Germline Fusion Partner (2 kb)

→ Germline Ali and VDJ of Fusion Partner (1.7 kb)
Figure 10: Restriction map of the 4.7 kb *BamHI* genomic DNA fragment that contains the productive VDJ gene from hybridoma 1E6.
Restriction Map of
Genomic VDJ Gene from Hybridoma 1E6
nucleotide sequence was determined by Sanger dideoxy sequencing in both orientations. The nucleotide sequence and the deduced amino acid sequence of the 1E6 V region is shown in Figure 11. Comparison of the deduced amino acid sequence of the V_H region of the 1E6 VDJ gene with the partial amino acid sequence of the pool serum a2 Ig molecules demonstrated that 1E6 encoded 10 of 11 known V_Ha2-allotype-associated residues. Therefore conclude that the 1E6 VDJ gene encodes V_Ha2 Ig molecules (Figure 11).

To determine the V_H gene utilized in the VDJ gene rearrangement of 1E6, the restriction map of the region 5' of the 1E6 VDJ gene was compared with those of all known germline V_H genes previously cloned from cosmid and phage libraries (Gallarda et al., 1985, Currier et al., 1988, Knight and Becker, 1990). The map of the region upstream of the 1E6 VDJ gene did not match with the upstream region of any previously identified germline V_H genes. Therefore, I prepared a 100 bp BamHI/XbaI fragment as a probe, from the region 5' of the cloned 1E6 VDJ gene and used it to screen previously unmapped cosmid and phage clones containing germline V_H genes of either normal a2 or mutant Alicia rabbits. Two V_H containing phage clones, phage 4-1 and 4-2, obtained from an EMBL4 phage library of genomic DNA from an a2 allotype suppressed rabbit hybridized with this probe (Short et al., 1991). These clones were further restriction mapped and compared with the restriction map of the region 5' of the 1E6 VDJ gene. However, their restriction maps did not match with the upstream region of the 1E6 clone. This means that the Alicia B cell from which hybridomas 1E6 was derived used an as yet unidentified V_H gene in its VDJ gene rearrangement.
Figure 11: Comparison of translated amino acid sequences of 1E6 V region with the partial amino acid sequence of pool a2 Ig. $V_H^{a2}$ allotype-associated residues are boxed (reviewed in Mage et al., 1984).
FR1

Ali 1E6  CAGTCAGTGAGAGCTGAGGGAGGCTCTTGAGCAGCTGAGTACCTGAGCACCTACCC
          Q S V K E S E G G L F K P A D T L L L L
Pool a2  S V K E S E G G L F K P T D T L L L L

CDR1

Ali 1E6  TGCACAGCTCTGGATTTACCACAGTAGCTATGGAAGTGGAGCTGGTGCCGGCTCCA
          C T A S G F T I S S Y G V S W V R Q A P
Pool a2  C T A S G F S S Y G S W V R

FR2

Ali 1E6  GGGAGGGGGCAATCGAGCCATCGAGGCAGCCATATAGTAAATGTAGAACATACGCGACC
          G K G P E W I G A I D I N G R T Y Y A T

CDR2

Ali 1E6  GGGAGGGGGCAATCGAGCCATCGAGGCAGCCATATAGTAAATGTAGAACATACGCGACC
          G K G P E W I G A I D I N G R T Y Y A T

FR3

Ali 1E6  TGGGCAAGAGCCGGCCACCACCATAGAATGTCAACAGAAGCAACGAGTCTGAGA
          W A K S R A T I T R N V N E N T E N T V T L R
Pool a2  S S I T I R N E

Ali 1E6  GTGACAGTCTGACAGCCGGCCACGGTCTGAGA
          V T S L T A A D T A T Y F C A R
Pool a2  T S L T A A D T A T Y F C A R
Cloning of Genomic VDJ Gene from 8E1 Hybridoma

According to Southern blot analysis with the J<sub>H</sub> probe, the productive VDJ gene of hybridoma 8E1 was found on 2.6 kb EcoRI and 6 kb BamHI fragments, respectively (Figure 12 and 13). Thus, the 8E1 genomic library was constructed by cloning approximately 5.5 - 6.5 kb BamHI fragments into pGEM-3 plasmid vector and transforming these DNA into electrocompetent DH10B cells. By screening approximately 400,000 colonies, six clones hybridized with the pJ5 probe. After colony-purification and re-hybridization with pan V<sub>H</sub> and J<sub>H</sub> probes, a clone that hybridized with both probes was chosen for analysis of the rearranged VDJ gene.

By restriction enzyme analysis and Southern blot hybridization of V<sub>H</sub> and J<sub>H</sub> probes, the 8E1 VDJ gene was also found on a 500 bp PstI/BstEII fragment (Figure 14). This fragment was then cloned into the PstI/BstEII sites of the single-stranded sequencing vectors, M13 mp18 and M13 mp19. The nucleotide sequences were analyzed in both orientations. Similar to the 1E6 clone, the deduced amino acid sequence of the 8E1 VDJ gene showed that this rearranged V<sub>H</sub> gene encodes 9 of the 11 V<sub>H</sub>a2-allotype-associated amino acids (Figure 15). Thus 8E1 also encodes V<sub>H</sub>a2 Ig molecules.

The 8E1 genomic clone was restriction-mapped and the region 5' of the 8E1 VDJ gene was compared with that of the 1E6 VDJ gene. As expected from their different sized J<sub>H</sub>-hybridizing fragments, the restriction maps of the region upstream of these two VDJ genes were different from each other (Figure 16). This result showed conclusively
Figure 12: Southern analysis of hybridoma 8E1 genomic DNA digested with EcoRI using a rabbit $J_H$ probe.
Germline Fusion Partner (13 kb)
Germline Ali (12 kb)
VDJ of Fusion Partner (4.1 kb)
VDJ of 8E1 (2.6 kb)
Figure 13: Southern Analysis of 8E1 genomic DNA digested with *Bam*HI using a rabbit $J_H$ probe.
Germline Fusion Partner (2 kb)

Germline Ali and VDJ of Fusion Partner (1.7 kb)

VDJ of 8E1 (6 kb)
Figure 14: Restriction map of genomic VDJ gene from hybridoma 8E1.
Restriction Map of
Genomic VDJ Gene from Hybridoma 8E1
Figure 15: Comparison of nucleotide sequence of hybridoma 8E1 VDJ gene and its deduced amino acid sequence with those of germline $V_{H4}$. Dots indicate identity, $V_{Ha2}$ allotype-associated residues are boxed (reviewed in Mage et al., 1984).
Figure 16: Comparison of genomic VDJ gene from hybridoma 8E1 with VDJ gene of hybridoma 1E6.
Comparison of Hybridoma 8E1 with 1E6

1E6

5'  VDJ  3'

8E1

5'  VDJ  3'

$V_H^5$

↑ Xbal  ↓ EcoRI  ● HindIII  T Hpal
that the hybridomas 1E6 and 8E1 used different $V_H$ genes in their VDJ gene rearrangements.

When the restriction map of the region 5' of the 8E1 VDJ gene was compared with those of known germline $V_H$ genes, it was identical to the region 5' of germline $V_{H4}$ gene segment (Figure 17). Moreover, the nucleotide sequence of the $V_H$ gene immediately upstream of the 8E1 VDJ matched the sequence of germline $V_{H5}$, the $V_H$ gene segment upstream of germline $V_{H4}$. On the basis of these analyses, I concluded that hybridoma 8E1 used $V_{H4}$ in its VDJ gene rearrangement.

**Identification of Utilized $V_H$ Genes by Analysis of Promoter Regions**

As mentioned earlier, Southern blot analysis of Alicia hybridomas demonstrated that, unlike normal rabbits, Alicia rabbits appear to use several $V_H$ genes in their VDJ gene rearrangements. To establish this and to more accurately determine how many $V_H$ genes are used by Alicia B cells, the $V_H$ gene segments used in VDJ gene rearrangements must be unequivocally identified. Because germline $V_H$ genes can be identified on the basis of the nucleotide sequences of the promoter regions and the promoter regions are not altered by the somatic diversification process, I identified the $V_H$ genes used in Alicia hybridomas by analyzing nucleotide sequences of the promoter regions.

The promoters and VDJ regions were PCR-amplified using a 5' primer derived from 216 to 236 bp upstream of the ATG start codon and a 3' $J_H$ primer derived from
Figure 17: Comparison of the genomic VDJ gene from hybridoma 8E1 with germline $V_{\gamma}$ gene segment.
Comparison of Hybridoma 8E1 with Germline $V_H^4$ alig

Germline $V_H^4$-V_H$^5$ alig

8E1

 Marker: EcoR I, HindIII, Hpal
conserved region of all rabbit J_H gene segments. The nucleotide sequences were determined and compared to each other and to the promoter regions of germline Alicia V_H gene segments. Nucleotide sequence analysis of promoter regions from 17 V_Ha2 Ig-secreting hybridomas showed that at least five different V_H genes were used in VDJ gene rearrangements. Nine of the hybridomas used the V_Ha7-ali gene, the other eight hybridomas used four different, as yet unidentified V_H genes (Figure 18). One of these four unidentified V_H genes was used frequently, in 5 of the 17 total hybridomas.

Since the nucleotide sequence of germline V_Ha7 is known, I determined whether V_Ha2 Ig molecules in Alicia are generated by a somatic gene conversion process. The nucleotide sequences of the rearranged V_Ha7 clones were compared to the sequence of germline V_Ha7 and the diversified regions were then compared to all known germline V_H genes (Figure 19-26). I found clusters of nucleotide changes in FR1 that potentially resulted from a somatic gene conversion event in which the V_Ha2-encoding pseudogene, V_H9, served as a donor (Figure 19-24, 27). These converted sequences encoded V_Ha2 allotype-associated amino acids, indicating that V_Ha2 Ig molecules in aged Alicia rabbits are derived, at least in part, from somatic gene conversion of rearranged V_Ha7 genes.

**Search for Recombination Enhancer**

In striking contrast to the rearrangement of multiple V_H genes in B cells of Alicia rabbits, most B cells in normal rabbits use one V_H gene, V_H1, in their VDJ gene
Figure 18: Nucleotide sequences of the promoter regions of VDJ genes from 17 hybridomas compared with the promoter regions of known germline V<sub>H</sub> genes from the alig haplotype. Dots represent identity to the germline V<sub>H</sub>A sequence. Numbers above the V<sub>H</sub>A sequence indicate the nucleotide position relative to the ATG start codon. The sequences start with nucleotide position -215 and end with -56 relative to the position of the translated start codon ATG.
The table presents information on various germlines and hybridomas, along with their corresponding sequences in the Germline VH4, VH6, VH7, VHX, and VHY regions. The Pyrimidine tract and the Heptamer, TATA Box, and Octamer sequences are also provided for reference.
Figure 19: Comparison of nucleotide sequence of hybridoma 1B3 VDJ gene and its promoter with nucleotide sequences of germline $V_{H4}$ and $V_{H9}$. Dots represent identity to the germline $V_{H4}$ sequence. Known promoter motifs, leader, $V_{H}$ region, $D_{H}$ and $J_{H}$ are indicated. Translated amino acid sequence of germline $V_{H4}$ is shown above $V_{H4}$ nucleotide sequence. Box denotes somatic gene conversion tract in which $V_{H9}$ or a $V_{H9}$-like gene served as a donor. The a2 allotype-associated residues that are derived by somatic diversification process are shown in bold letters; K, E and F in FR1 and E in FR3 above the deduced amino acid sequence of the germline $V_{H4}$ segment.
<table>
<thead>
<tr>
<th></th>
<th>Pyrimidin tract</th>
<th>Heptamer</th>
<th>TATA-like box</th>
<th>Octamer</th>
</tr>
</thead>
<tbody>
<tr>
<td>V₄</td>
<td>agcgtacccccccaggtaccacatctgccctgggccctgtccgtctctgagccatctgaccccacggtctggagcctggttat/tagcagcagcaca tgcaaatgcccccttc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>a.a. t. g. t. c. a.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₉</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Heptamer**

|    | | |
| V₄ | ctctgccccatgaaaccagccccctacctctgctgctggaccagagctccagccccaggtctccactctgtctgatccactcactcaaccaca | |
| B3 | | |
| V₉ | | |

**Leader**

|    | METGLRLWLLLVAVLK | | |
| V₄ | cgctccactGAGACTGCGCTGCGCTGCGCTAAAGgtatatgtgagacgcggcggcactgactgctggagaggtaggtgag | |
| B3 | | |
| V₉ | | |

**CDR1**

|    | TDLTLTCTVSGFLSSYGVIVWVRQAPNGNGLEYIG | |
| V₄ | CGGATACCTGCAGCTACCTACCTGCGCTGCGCTGCTCCTACCTCACGTAGTAATGATGATCTGCTGCGCTGCGCTGCGCTGGAATACCGGGCA | |
| B3 | G. T. GGA. A. A. A. GA. | |
| V₉ | | C.A.A.G. |

**CDR2**

|    | TIGSSGCAYYASWAKSRSTIITRTNTNLNTVTTLKMTS | |
| V₄ | ACCATTGGTAGAGTGGATGGCGACACCCGTCGCTGCGGCAAAAAGCCTCCACCACCACCAAAACACCCACCTGCGACTGCTGAATATGACCGAG | |
| B3 | TAT.C.G.GA.TGT.AT | |
| V₉ | T.A.AA.TA.A | |

**CDR3**

|    | AGAGAA | |
| V₄ | | |
| B3 | | |
| V₉ | | |

**DH**

|    | TCTGACAGGCCGCGCAACGCACGCCACCTATTTCTGCGAGA | |
| V₄ | | |
| B3 | | |
| V₉ | | |

**JH**

|    | GA.TTGTGGGATAGTACTTTCGCCCTTGACATCTGGGGCCCAGGCACCCTGGTC | |
| V₄ | | |
| B3 | | |
| V₉ | | |
Figure 20: Comparison of nucleotide sequence of hybridoma 1F7 VDJ gene and its promoter with nucleotide sequences of germline $V_{H^4}$ and $V_{H^9}$. Dots represent identity to the germline $V_{H^4}$ sequence. Known promoter motifs, leader, $V_{H}$ region, $D_{H}$ and $J_{H}$ are indicated. Translated amino acid sequence of germline $V_{H^4}$ is shown above $V_{H^4}$ nucleotide sequence. Box denotes somatic gene conversion tract in which $V_{H^9}$ or a $V_{H^9}$-like gene served as a donor. The a2 allotype-associated residues that are derived by somatic diversification process are shown in bold letters; K, E and F in FR1 and E in FR3.
Figure 21: Comparison of nucleotide sequence of hybridoma 4A4 VDJ gene and its promoter with nucleotide sequences of germline \( V_{\text{H}4} \) and \( V_{\text{H}9} \). Dots represent identity to the germline \( V_{\text{H}4} \) sequence. Known promoter motifs, leader, \( V_{\text{H}} \) region, \( D_{\text{H}} \) and \( J_{\text{H}} \) are indicated. Translated amino acid sequence of germline \( V_{\text{H}4} \) is shown above \( V_{\text{H}4} \) nucleotide sequence. Box denotes somatic gene conversion tract in which \( V_{\text{H}9} \) or a \( V_{\text{H}9} \)-like gene served as a donor. The a2 allotype-associated residues that are derived by somatic diversification process are shown in bold letters; K, E and F in FR1 and E in FR3.
Figure 22: Comparison of nucleotide sequence of hybridoma 6A1 VDJ gene and its promoter with nucleotide sequences of germline $V_{rrf}$ and $V_{H9}$. Dots represent identity to the germline $V_{rrf}$ sequence. Known promoter motifs, leader, $V_H$ region, $D_H$ and $J_H$ are indicated. Translated amino acid sequence of germline $V_{rrf}$ is shown above $V_{rrf}$ nucleotide sequence. Box denotes somatic gene conversion tract in which $V_{H9}$ or a $V_{H9}$-like gene served as a donor. The a2 allotype-associated residues that are derived by somatic diversification process are shown in bold letters; K, E and F in FR1 and E in FR3.
Figure 23: Comparison of nucleotide sequence of hybridoma 8E1 VDJ gene and its promoter with nucleotide sequences of germline $V_{Hr}^A$ and $V_{Hr}^9$. Dots represent identity to the germline $V_{Hr}^A$ sequence. Known promoter motifs, leader, $V_H$ region, $D_H$ and $J_H$ are indicated. Translated amino acid sequence of germline $V_{Hr}^A$ is shown above $V_{Hr}^A$ nucleotide sequence. Box denotes somatic gene conversion tract in which $V_{Hr}^9$ or a $V_{Hr}^9$-like gene served as a donor. The a2 allotype-associated residues that are derived by somatic diversification process are shown in bold letters; K, E and F in FR1 and E in FR3.
Figure 24: Comparison of nucleotide sequence of hybridoma 10A2 VDJ gene and its promoter with nucleotide sequences of germline $V_{H4}$ and $V_{H9}$. Dots represent identity to the germline $V_{H4}$ sequence. Known promoter motifs, leader, $V_{H}$ region, $D_{H}$ and $J_{H}$ are indicated. Translated amino acid sequence of germline $V_{H4}$ is shown above $V_{H4}$ nucleotide sequence. Box denotes somatic gene conversion tract in which $V_{H9}$ or a $V_{H9}$-like gene served as a donor. The a2 allotype-associated residues that are derived by somatic diversification process are shown in bold letters; K, E and F in FR1 and E in FR3.
Pyrimidine tract

Heptamer

TATA-like box

Octamer

Heptamer

Leader

FR1

FR2

CDR1

CDR2

FR3

DH

JH
Figure 25: Comparison of nucleotide sequence and its deduced amino acid sequence of hybridoma 1C3 VDJ gene with nucleotide sequence of germline $V_{HF}$ and its promoter. Dots represent identity to the germline $V_{HF}$ sequence. Known promoter motifs, leader, $V_H$ region, $D_H$ and $J_H$ are indicated.
### Pyrimidine tract

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>agcgtaaccccagttcacatctgctgcctgggccccctgctctgctcttgaggctgacctgccccatctacctgccctgctgttactcactgctgtttactaccaatggggcctcc</td>
<td></td>
</tr>
</tbody>
</table>

### Heptamer

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctctggcccatgaaaccagcccaacccctcaccctgcagcttggacagagctccagccccacaggactcccaggttctcactcagttgatctgctacactcaacacaga</td>
<td></td>
</tr>
</tbody>
</table>

### TATA-like box

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>cgctcaccATGGAGACTGGGCCGCTGGCTGGCTTCTCTCGCTGCTGCTCAAAAG_taatgatgggagacgcgccccactgtcttgagaggagatgtgagtgag</td>
<td></td>
</tr>
</tbody>
</table>

### Octamer

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>agacacagagagtgtgagtgacagtgtcttgacactgctgtgttgagctggctccagtGTTTGCAGTGTCAGTCGGTGGAGGAGTCCAGGGGAGGCCTGATCAAGCCAA</td>
<td></td>
</tr>
</tbody>
</table>

### CDR1

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGATACACCTGACACTCACTGCACAGTCTCTGGATTCTCCCTGAGTAGATTAGTGATCTGGCTGCGCCAGGACGGCGCTCAAAATATCGGA</td>
<td></td>
</tr>
</tbody>
</table>

### FR2

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCATTGGTAGTGGATGCGCATCTACCGCAATCCGCGGAATGACCTGGCCCGCAGACACCCTCCACCCAGGACCCGCTGCAAAATCTGACCAGFGTCGCTAAAGTGGATGGTATTGGTGCTCCGGACATCTGGGGCCCAGGCGCTGCAAAATCTGACCAG</td>
<td></td>
</tr>
</tbody>
</table>

### CDR2

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCTGACAGCCGGACGAGCCACCTATTTCTGGCAGAG</td>
<td></td>
</tr>
</tbody>
</table>

### FR3

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTAADTYFCAReeL</td>
<td></td>
</tr>
</tbody>
</table>

### D<sub>H</sub>

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTCGCTAAAGTGGATGGTATTGGTGCTCCGGACATCTGGGGCCCAGGC</td>
<td></td>
</tr>
</tbody>
</table>

### J<sub>H</sub>

<table>
<thead>
<tr>
<th>VH4</th>
<th>1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTAADTYFCAReeL</td>
<td></td>
</tr>
</tbody>
</table>
Figure 26: Comparison of nucleotide sequence and its deduced amino acid sequence of hybridoma 4C4 VDJ gene with nucleotide sequence of germline $V_H^4$ and its promoter. Dots represent identity to the germline $V_H^4$ sequence. Known promoter motifs, leader, $V_H$ region, $D_H$ and $J_H$ are indicated.
Pyrimidine tract

Heptamer

TATA-like box

Octamer

V\textsubscript{H}4

cagcgtacaccccgagtcaccacatctgcctgccctgtccttgtaggcatctgcacccagcgttttat/tgacagcagcacatgcaaatgaggcctc

4C4

Heptamer

V\textsubscript{H}4

cctctggcccatgaaaaaccagccagccctcaacctgcttcagctgcacaggactccagccccaggactcccaggttgccactcagtgatgcactcataacacag

4C4

V\textsubscript{H}4

dcgcttaccATGGAGACTGGGCTGCGCTGGCTTCTCGTGCTGCTCAAAGgtatactgtggagaagcggcggggcatgacgcactgcttggtgaggagagattgata

4C4

FRI

cctctg.c.c.catgaaaaccagcccagccctcaccctgcagctctggcacaggagctccagccccaggactcccaggttgccactcagtgatgcactcataacacag

4C4

V\textsubscript{H}4

gagacacagagagtgtgagtgacagtgctctgatggatacgtgcctgtgttgcaagGTGTCCAGTGATCTGGGTCCGCCAGGCTCCAGGGAACGGGCTGGAATATATCGG

4C4

CDRI

FR2

ACGGATACCTGACACTCCTGACAGTCTCTGGATTCTCCTCCTAGTAGCTATGAGTGATCTGGGTCCGCCAGGCTCCAGGGAACGGGCTGGAATATATCGG

4C4

G....AG..........................A.GAGTATA..........................A.G..G.GG

V\textsubscript{H}4

TDTTLTTLTCTLTVSGFSLSSYGVWVRQAPNGLEYIG

4C4

CDR2

FR3

AAACATTGGTAGTGGATGCGCATACTACGCAGCTGGCCGAAAACCGATCACATCCACCAGAAACACACCTGAAACGGTGATCTCGTGAATATAGCCA

4C4

.TA...A.TA.C...TAAT..........................GGA..................................G....TG

V\textsubscript{H}4

TIGSAGCAYASAWSAKRSRSTITTRNNTNLNTVTLKLMT

4C4

V\textsubscript{H}4

GTCTGACAGGCCGGACACGCACCCCTATTCTGTGGAGA

4C4

DH

JH

AGCCTGTCCTTCTGGATATCCATATTTCTCGATGTCCTGGGCGGACCGACCCCTGCCCTGGTCACCCTGCTCC

V\textsubscript{H}4

SLTAAADTATYFCAR

4C4

G

113
Figure 27: Somatic gene conversion in FR1 of $6V_{H4}$-utilizing hybridomas.
Gene Conversion in FR1 of VDJ Genes of Hybridomas

V\text{H},4
tcttgaccatgtcgtctgtttgcagGTGTCCAGTGCAGTCGGTGGAGGAGTCCAGGGGAGGCCTGATC

1B3       c...g.a........................................A.A........GA......CT..
1F7       c...g.a........................................A.A........GA......T..CT..
4A4       c...g.a........................................A.A........GA......T..CT..
6A1       c...g.a........................................A.A........GA......T..CT..
8E1       c...g.at...c....................................A.A........GA...........
10A2      c...g.a........................................A.A........GA......CT..
V\text{H},9
c...g.a........................................A.A........GA......T..CT..
rearrangements (Knight and Becker, 1990; Friedman et al., 1994; Raman et al., 1994). The study on $V_H$ gene usage in nonproductive VDJ genes showed that $V_{HJ}$ is preferentially used (Tunyaplin and Knight, 1995). One explanation for this finding is that preferential utilization of $V_{HJ}$ in normal rabbits is due to preferential rearrangement. Because the rearrangement of several genes has been observed in Alicia rabbits, in which sequences 5’ and 3’ to the germline $V_{HJ}$ as well as the $V_{HJ}$ segment itself are deleted, I proposed that the preferential rearrangement of $V_{HJ}$ in normal rabbits is due to a recombination enhancer linked to $V_{HJ}$, and that its absence in Alicia rabbits abolishes preferential $V_H$ gene usage resulting in rearrangement of multiple $V_H$ genes. Such a recombination enhancer could be in the region deleted in Alicia rabbits, either 5’ or 3’ of $V_{HJ}$. If the enhancer was 5’ of $V_{HJ}$, one would expect that the adjacent upstream gene, $V_{H2}$, could rearrange at the same frequency as $V_{HJ}$. However, analysis of $V_H$ gene usage in nonproductive VDJ genes showed that $V_{H2}$ is seldom rearranged (Tunyaplin and Knight, 1995). I therefore hypothesized that if a recombination enhancer was present, it would be 3’ of $V_{HJ}$ (Figure 28). Accordingly, I searched for a recombination enhancer from the region 3’ of $V_{HJ}$ by using an in vitro recombination assay.

To determine whether such a recombination enhancer exists in the region 3’ of the germline $V_{HJ}$ gene, the DNA fragments 3’ of $V_{HJ}$ were cloned into an extrachromosomal recombination substrate, pWTSJD (Figure 29), and assayed for the ability to increase the recombination frequency in an in vitro recombination assay. The negative control
Figure 28: Recombination enhancer hypothesis.
Recombination Enhancer

promote rearrangement
of $V_H1$

$V_n$ $V_H4$ $V_H3$ $V_H2$ $V_H1$

$V_H1DJ$
Figure 29: Recombination substrates containing the 3' $V_{H\ell}$ DNA fragments.
Recombination Substrate

[Diagram of pWTSJD plasmid with various restriction sites and cloning sites.]
construct contained non-relevant rabbit germline DNA of the same size as the 3' $V_{HJ}$ DNA, cloned from the IL-10 genomic region. These plasmid constructs were transfected into 103/BCL-2, a pre-B cell line expressing inducible recombinase. The transfected cells were first cultured at 34°C for 20-24 hrs, and then maintained at 39.5°C to induce recombinase activity. After 24 hrs, the plasmids were harvested from the pre-B cells and transformed into *E. coli* SURE cells. The transformants were subsequently assayed on agar plates containing ampicillin (Amp) and on plates containing Amp plus chloramphenicol (Amp + Cam). If the plasmids undergo recombination in the pre-B cell line, the prokaryotic transcriptional terminator flanking the recombination signal sequence (RSS) will be deleted and as a result the chloramphenicol acetyltransferase (CAT) gene will be expressed. Therefore, *E. coli* harboring recombined plasmids are able to grow on both Amp and Amp plus Cam (Amp$^f$ + Cam$^f$), whereas those having unrecombined plasmids are able to grow only on Amp (Amp$^f$). The frequency of recombination (R) was calculated by dividing the number of Amp$^f$ + Cam$^f$ colonies by the number of Amp$^f$ colonies, then multiplying by 100. If any fragments of 3' $V_{HJ}$ germline DNA contain a recombination enhancer, the recombination frequency (R) of that particular fragment will be higher than the frequency of the negative control.

The first 3' $V_{HJ}$ region that I tested was a 2-kb HindIII fragment (fragment 5 in Figure 29) because it spans most of the 3' $V_{HJ}$ region that is deleted from the Alicia IgH locus and its HindIII restriction site is convenient for cloning into the pWTSJD
recombination vector. The negative control was a 2-kb Hind III germline DNA fragment adjacent to rabbit IL-10 gene. The results of two experiments are shown in Table 3. In both experiments, the recombination frequencies of the 3' $V_{HJ}$ construct were higher (26% and 31.5%) than those of the IL-10 control (11% and 0.3%), although the recovery of plasmid DNA from the transfected cells was poor in the first experiment. When these two constructs were cotransfected into the pre-B cells, the recombination frequency was less than the frequency of the 3' $V_{HJ}$ construct alone. These data suggested to me that a recombination enhancer might reside in this 2-kb of DNA 3' of the $V_{HJ}$ gene segment.

To specifically locate the recombination enhancer region, I cloned smaller fragments from the region 3' of $V_{HJ}$, designated fragment 2 to 4 (Figure 29), and tested each construct in the recombination assay. As in previous experiments, the 3' $V_{HJ}$ construct had higher R values (45.6%) than the IL-10 control (0.2%) (Table 4). Thus, the assays appeared to be reproducible because the R values of 3' $V_{HJ}$ construct in all experiments tested were regularly higher than those of IL-10 negative control. Similarly, the R values of constructs 2 and 3.1 (75.7% and 87.1%) which were cloned into the recombination substrate in the same orientation as the original 2-kb Hind III construct (3' to 5'), were also significantly higher than the control (0.2%). However, the construct 3.2 which was cloned into the recombination substrate plasmid in the opposite orientation to the 2-kb Hind III 3' $V_{HJ}$ construct had lesser R value (2.0%). Because some bacterial colonies containing fragment 4 were not isolated from each other on ampicillin plates, the
Table 3 Recombination frequencies of recombination substrates containing 3' \(V_H\) or IL-10 as negative control.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Expt.No.</th>
<th>No. of Amp(^f) colonies</th>
<th>No. of Amp(^f) Cam(^r) colonies</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' (V_H)</td>
<td>1</td>
<td>100</td>
<td>26</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12500</td>
<td>3940</td>
<td>31.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>1</td>
<td>200</td>
<td>22</td>
<td>11.0</td>
</tr>
<tr>
<td>(negative control)</td>
<td>2</td>
<td>10500</td>
<td>30</td>
<td>0.3</td>
</tr>
<tr>
<td>3' (V_H) + IL-10</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20200</td>
<td>2023</td>
<td>10.0</td>
</tr>
</tbody>
</table>


Table 4  Recombination frequencies of 3' $V_h$ subclones, fragment 2, 3 and 4.

<table>
<thead>
<tr>
<th>Construct</th>
<th>No. of Amp$^f$</th>
<th>No. of Amp$^f$ Cam$^f$</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' $V_h$ 1 (3' to 5')</td>
<td>20850</td>
<td>9500</td>
<td>45.6</td>
</tr>
<tr>
<td>IL-10</td>
<td>9750</td>
<td>23</td>
<td>0.2</td>
</tr>
<tr>
<td>3' $V_h$ 1 + IL-10</td>
<td>8350</td>
<td>2460</td>
<td>29.5</td>
</tr>
<tr>
<td>3' $V_h$ 1-2 (3' to 5')</td>
<td>10700</td>
<td>8100</td>
<td>75.7</td>
</tr>
<tr>
<td>3' $V_h$ 1-3.1 (3' to 5')</td>
<td>6150</td>
<td>5360</td>
<td>87.1</td>
</tr>
<tr>
<td>3' $V_h$ 1-3.2(5' to 3')</td>
<td>5750</td>
<td>115</td>
<td>2.0</td>
</tr>
<tr>
<td>3' $V_h$ 1-4 (5' to 3')</td>
<td>&gt;7000</td>
<td>603</td>
<td>UD</td>
</tr>
</tbody>
</table>
R value of this fragment could not be determined. This set of experiments needed to be retested so that the existence of the recombination enhancer could be verified. In particular, I needed to confirm that the higher R values of the 2-kb Hind III 3' VHJ construct and its subclones over the control were truly due to the effect of a cis-acting element residing in the region 3' of VHJ.

First, I had to rule out the possibility that the process of cloning the rabbit DNA into the Hind III site of the recombination substrate did not interfere with the integrity of the RSSs. The nucleotide sequences of the RSSs of all 3' VHJ constructs and IL-10 control were therefore determined. There were no nucleotide changes from the RSS of the original recombination substrate, indicating that the difference in recombination frequency was not due to changes in RSSs introduced during cloning. Second, because the detection of the recombination enhancer relies on the difference in recombination frequency of 3' VHJ and IL-10, I needed to compare the result of the IL-10 DNA fragment with another negative control construct. I therefore cloned a 2-kb Hind III fragment from DNA region adjacent to the rabbit bcl-2 gene into the recombination substrate and tested it together with the former constructs. I performed the recombination assay as described above except that the screening for recombined and nonrecombined plasmids was done by colony hybridization (Figure 30). Transformants growing on Amp plates were randomly picked on to grids and hybridized with a probe derived from the prokaryotic transcriptional terminator, which would be deleted during RSS-mediated
Figure 30: An example of in vitro recombination assay detected by differential hybridization.
Tx terminator probe

IL10 probe
rearrangement. Therefore, hybridization would not be observed from bacterial colonies containing recombined plasmids (Figure 30). By differential hybridization of 300-500 Amp\(^f\) colonies from each construct, I found that R values varied from 16.5% to 23.8% (Table 5). Thus, I did not detect any significant differences in recombination frequencies between 3' \(V_{HJ}\) constructs (19.5%), including its subclones (23.8% and 21.8% for clone3, 18.0% for clone 4), and the two negative controls (16.5% for IL-10 and 21.9% for bcl-2) (Table 5). This result conflicted with the data obtained from chloramphenicol selection (Table 3-4) in which the R values of 3' \(V_{HJ}\) and clones 2, 3 and 4 were higher than the R value of the control. To ascertain that the contradictory result of chloramphenicol selection and hybridization was not due to the variation in tested DNA samples, I transformed the same DNA recovered from transfection experiment no.2 and 3 (Table 3 and 4) and analyzed the transformants by colony hybridization. As shown in table 6, the R values of 3' \(V_{HJ}\) constructs obtained from hybridization method (30.4% and 38.2%) were not different from those of the IL-10 control (35.0% and 39.2%) even though this 3' \(V_{HJ}\) DNA previously showed a higher recombination frequency as determined by selection on chloramphenicol plates. Because the hybridization method directly tests the recombination event, I conclude that the recombination enhancer does not reside on the DNA fragments 2, 3, 4 and 5. Because I had not tested the entire region 3' of \(V_{HJ}\) that is deleted from the \(ali\) chromosome, I further analyzed the region immediately downstream of \(V_{HJ}\) (see fragment 1 in Figure 29). Similar to the results of fragments 2, 3 and 4, no difference in frequency of recombination from both negative control was observed as determined by the hybridization method (Table 7).
Table 5 Analysis of recombination substrates by hybridization with transcription terminator probe.

<table>
<thead>
<tr>
<th>Recombination substrate</th>
<th># of tested colonies</th>
<th># of Non-hybridizing colonies (recombinants)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' V_H1</td>
<td>287</td>
<td>56</td>
<td>19.5</td>
</tr>
<tr>
<td>3' V_H1-3.1</td>
<td>471</td>
<td>112</td>
<td>23.8</td>
</tr>
<tr>
<td>3' V_H1-3.2</td>
<td>390</td>
<td>85</td>
<td>21.8</td>
</tr>
<tr>
<td>3' V_H1-4</td>
<td>473</td>
<td>85</td>
<td>18.0</td>
</tr>
<tr>
<td>IL10</td>
<td>472</td>
<td>78</td>
<td>16.5</td>
</tr>
<tr>
<td>Bcl2</td>
<td>411</td>
<td>90</td>
<td>21.9</td>
</tr>
</tbody>
</table>

1 Both 3' V_H1-3.1 and 3'V_H1-3.2 constructs represent fragment 3 but in opposite orientation.
Table 6  Analysis of recombination substrate containing 2-kb Hind III DNA region 3' of \( V_\text{\(\alpha\)}} \) by chloramphenicol selection and hybridization with transcription terminator probe.

<table>
<thead>
<tr>
<th>Recombination substrate</th>
<th>Experiment number</th>
<th>Chloramphenicol method</th>
<th>Hybridization method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td># of Amp(^{\text{r}})</td>
<td># of Amp(^{\text{r}}) Cam(^{\text{r}})</td>
</tr>
<tr>
<td>3' ( V_\text{(\alpha)}} )</td>
<td>2</td>
<td>12,500</td>
<td>3,940</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20,850</td>
<td>9,500</td>
</tr>
<tr>
<td>IL 10</td>
<td>2</td>
<td>10,500</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9,750</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 7 | Analysis of recombination substrate containing 400 bp Pst I/ Hind III DNA region 3’ of $V_H$ (fragment 1) by hybridization with transcription terminator probe.

<table>
<thead>
<tr>
<th>Recombination substrate</th>
<th># of tested colonies</th>
<th># of Non-hybridizing colonies (recombinants)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’ $V_H$</td>
<td>103</td>
<td>13</td>
<td>12.6</td>
</tr>
<tr>
<td>3’ $V_H$-1.1</td>
<td>103</td>
<td>18</td>
<td>17.4</td>
</tr>
<tr>
<td>3’ $V_H$-1.2</td>
<td>103</td>
<td>20</td>
<td>19.4</td>
</tr>
<tr>
<td>IL 10</td>
<td>103</td>
<td>18</td>
<td>17.4</td>
</tr>
<tr>
<td>Bcl 2</td>
<td>103</td>
<td>22</td>
<td>21.3</td>
</tr>
<tr>
<td>Vector</td>
<td>103</td>
<td>19</td>
<td>18.4</td>
</tr>
</tbody>
</table>
CHAPTER IV
DISCUSSION

The discovery of Ig gene rearrangement in the mid 1970's has shed light on the generation of antibody diversity and the development of the antibody repertoire. Most studies on Ig genes and the generation of antibody diversity have been performed in the mouse because of the availability of inbred strains and hybridomas as well as gene targeting technology. Not surprisingly, much of our knowledge in this area has been obtained from the studies in mouse and it is believed that the major mechanism to generate the primary antibody repertoire is combinatorial joining of multiple V, D and J gene segments. However, the discovery of somatic gene conversion and somatic hypermutation used by chicken and sheep, respectively, to generate their primary antibody repertoires, has identified additional molecular mechanisms by which the primary antibody repertoire is generated. Rabbits have also provided important insight into the generation of the primary antibody repertoire. By learning from different species, our knowledge can be integrated into a more complete and unified view of the immune system.

In the past, the studies of Alicia, the a2 allotype mutant rabbit, provided important
evidence that allelic inheritance of $V_H^a$ allotypes in normal rabbits could be explained by the preferential utilization of the $V_H^a$ allotype-encoding gene, $V_H^I$. Of major significance is the finding that Alicia rabbits lack $V_H^I-a2$, the gene that encodes $V_H^a2$ Ig molecules in normal rabbits (Knight and Becker, 1990). This finding explained the observation that most serum Ig molecules in young Alicia rabbits do not have $V_H^a2$ allotypic specificity (Kelus and Weiss, 1986). These Ig molecules are encoded by $V_H^{a^-}$ genes, $V_H^x$ and $V_H^y$ (DiPietro et al., 1990 and Chen et al., 1993). Interestingly, as Alicia rabbits age, up to 50% of their Ig molecules are $V_H^a2$ allotype. What is the explanation for the appearance of $V_H^a2$ Ig molecules in aged Alicia rabbits, given that they lack the preferentially utilized $V_H^a2$-encoding gene, $V_H^I-a2$? To answer this question, I set out to determine which $V_H$ genes are used by Alicia to produce $V_H^a2$ Ig molecules. I discuss below the experimental approaches that I used to investigate $V_H$ gene usage in $V_H^a2$ Ig molecules of Alicia rabbits. Subsequently, I will discuss the findings obtained from this study.

Experimental Approaches Used to Identify $V_H$ Genes

Encoding $V_H^a2$ Ig Molecules in Alicia Rabbits

Serologic studies by Kelus and Weiss showed that $V_H^a2$ Ig molecules in young Alicia rabbits were barely detectable in their blood circulation but that these molecules were readily detected in serum of adult Alicia rabbits (Kelus and Weiss, 1986). Molecular studies by DiPietro et al (1990) and Chen et al (1993) supported this finding. They found that the majority of the cDNA cloned from young Alicia rabbits encoded $V_H^{a^-}$
molecules rather than V<sub>H</sub>a2 Ig molecules. In order to identify V<sub>H</sub> genes encoding V<sub>H</sub>a2 Ig molecules by Alicia B cells, I needed to analyze a large number of VDJ genes isolated from older Alicia rabbits. The simplest way to approach this would be to PCR-amplify VDJ genes from adult Alicia rabbits and determine their nucleotide sequences. However, one problem with this strategy was that essentially all rabbit VDJ genes undergo extensive somatic diversification by 6 to 8 weeks of age. As a consequence, I could not identify with certainty the utilized V<sub>H</sub> gene by analyzing the nucleotide sequence of the VDJ gene alone. Therefore my initial approach was to clone VDJ genes as genomic fragments containing the region upstream of the VDJ gene. Since this region does not undergo somatic diversification, it could be used to unequivocally identify the utilized V<sub>H</sub> gene. At the time I started the project, the only available technique for cloning genomic VDJ genes was to construct a genomic library. This meant that I needed at least 50 µg of genomic DNA prepared from approximately 10<sup>7</sup> V<sub>H</sub>a2 Ig-expressing B cells in order to detect a single VDJ gene. To accomplish this, I originally proposed to generate V<sub>H</sub>a2 Ig-producing B cell lines from adult Alicia rabbits, clone the genomic VDJ genes from these cell lines and then identify the V<sub>H</sub> gene segments used in the VDJ gene rearrangements. When I began this project, there was no rabbit hybridoma fusion partner for generating stable rabbit hybridomas. Moreover, there were no known viral agents capable of inducing B lymphoid tumors in rabbits. I therefore attempted to generate rabbit B cell clones by stimulating B cells with CD40 ligand in culture (see appendix section). I cloned and determined the nucleotide sequence of rabbit CD40 ligand (CD40L) and IL-10 genes from cDNA and genomic libraries, respectively. At the same time, I optimized the
culture conditions for growing rabbit B cells in a “CD40 culture system” as described by Banchereau et al (1991). Because mouse CD40L (mCD40L) was shown to stimulate human B cells, I tested whether mCD40L would also activate rabbit B cells. I cultured rabbit mesenteric lymph node cells with irradiated Chinese Hamster Ovarian (CHO) cells that expressed membrane bound mCD40L (Spriggs et al., 1992). I found that the rabbit B cells were able to grow in this culture system, but they could be maintained for only one month and I could not obtain a sufficient number of B cells from this approach to do the experiment that I had planned.

While I was developing the “CD40 culture system”, Spieker-Polet et al (1995) successfully developed a rabbit plasmacytoma that can be fused with rabbit lymphoid cells to generate stable rabbit x rabbit hybridomas. Because the hybridoma technique is the best approach for generating antibody producing clones, I decided to use this technique to investigate V\textsubscript{H} gene usage in Alicia rabbits. In fact, this technique allowed me to develop 52 hybridomas from adult Alicia rabbits, of which 37 were V\textsubscript{H}a2 Ig producers. Since these hybridomas were derived from adult Alicia rabbits, their VDJ genes were most likely extensively diversified and the V\textsubscript{H} genes used in the VDJ gene rearrangements could not be unequivocally identified by nucleotide sequences of the VDJ genes. However, the regions immediately 5’ of the VDJ genes do not undergo somatic diversification and I hypothesized that this region could serve as a useful marker for identifying the V\textsubscript{H} gene segment used in a VDJ gene. One approach for obtaining the 5’ region was to construct a genomic library from each of these hybridomas and clone the
rearranged VDJ gene with its 5’ flanking region. Identification of the utilized $V_H$ gene was then performed by comparing restriction fragment length polymorphisms of the 5’ flanking region with the same regions of known germline $V_H$ genes. From my experience, this approach can be used to definitively identify the utilized $V_H$ genes but it is extremely labor-intensive and time-consuming to construct a genomic library for each hybridoma. I therefore searched for an alternative method by which the 5’ flanking region can be used to identify the utilized $V_H$ gene.

Because Tunyaplin and Knight (1995) showed that the nucleotide sequences of the promoter regions of several germline $V_H$ genes from various haplotypes were unique for each $V_H$ gene, I thought I could use the 5’ promoter regions to identify the genes utilized in my Alicia hybridomas. Consequently, I cloned the promoter regions of the known germline $V_H$ genes of the a2 haplotype and determined their nucleotide sequences. As expected, the promoter regions of these germline $V_H$ genes were indeed distinct from one another, suggesting that the utilized $V_H$ genes could be identified from diversified VDJ genes by analysis of promoter regions. This method is novel and has not been used for any other species.

**$V_H$ Gene Usage in Alicia Rabbits**

In this study, I identified the $V_H$ genes used in $V_H$ a2 Ig-producing hybridomas by analyzing nucleotide sequences of the promoter regions. As evident from Figure 18, I
found that a total of five \(V_H\) gene segments are used to encode \(V_{H\alpha2}\) Ig molecules in 17 hybridomas and one of these \(V_H\) genes is \(V_{H4}\). This is the first definitive evidence for the utilization of the \(V_{H4}\) gene segment in VDJ gene rearrangements of \(V_{H\alpha2}\) Ig-expressing B cells in Alicia rabbits. At present, \(V_{H4}\) has never been found in VDJ gene rearrangements in normal \(\alpha2\) allotype rabbits. Possible explanations for the usage of \(V_{H4}\) in Alicia rabbits but not in normal rabbits will be discussed in the section regarding the differential usage of \(V_H\) genes.

By RT-PCR using leader and \(J_H\) primers, Chen and co-workers (1993) reported that \(V_{H4}\)-like clones appeared in 2- to 8-week-old Alicia rabbits. Surprisingly, when I compared the nucleotide sequences of their VDJ genes with that of germline \(V_{H4}\), I found that the clone isolated from the 2-week-old Alicia rabbit was more diversified from germline \(V_{H4}\) than were the clones isolated from 6- and 8-week-old Alicia rabbits. This is surprising because Short et al and Crane et al showed that VDJ genes from rabbits up to 3 weeks old were undiversified and the VDJ genes from 6- to 8-week-old rabbits were highly diversified (Short et al., 1991; Crane et al., 1996). Therefore, the pattern of somatic diversification of the genes identified by Chen et al (1993) was quite the opposite from that seen in normal rabbits. How could that be? Because the utilized \(V_H\) genes in the study by Chen et al study were identified by analysis of nucleotide sequences of the VDJ genes alone, the utilization of \(V_{H4}\) was uncertain. Considering the fact that somatic diversification of rabbit VDJ gene usually occurs approximately 4 weeks of age, it is most
likely the VDJ gene isolated from 2-week-old Alicia has not yet undergone diversification. If so, this VDJ gene may use a \( V_H \) gene other than \( V_H^A \) in its VDJ gene rearrangement. Thus, I concluded that their study has not clearly defined which \( V_H \) genes are used to produce \( V_H^{a2} \) Ig molecules in Alicia rabbits.

By analysis of promoter regions, I showed conclusively that, in addition to \( V_H^A \), four, as yet unidentified, \( V_H \) genes are used in VDJ gene rearrangements of \( V_H^{a2} \) Ig-producing hybridomas. Initially, I PCR-amplified the promoter and VDJ regions from 25 \( V_H^{a2} \) Ig producing hybridomas using a 5' primer derived from 216-236 bp upstream of the ATG start codon and a 3' primer from the conserved region of \( J_H \) genes. However, I obtained PCR products from only 17 hybridomas. I have not been able to identify the genes of the other 8 hybridomas even though I attempted to set up PCR reactions using two different promoter primers derived from different conserved regions of known rabbit IgH promoters. These hybridomas presumably represent other different \( V_H \) genes used in the VDJ gene rearrangements. If so, the five \( V_H \) genes that I found in VDJ gene rearrangements of 17 hybridomas probably represent a minimum number of \( V_H \) genes used to encode \( V_H^{a2} \) Ig molecules in Alicia rabbits. In fact, DiPietro et al analyzed VDJ genes cloned from 8-week-old Alicia rabbits and found that 1 of 7 VDJ genes encoding \( V_H^{a2} \) Ig have 9 of the 11 \( V_H^{a2} \) allotype-associated amino acids (DiPietro et al, 1990). Although, the germline counterpart of this cDNA has not been identified, the authors suggested that an as yet unidentified \( V_H \) gene rather than \( V_H^A \) was used. As discussed
above, Chen et al. (1993) also isolated a VDJ gene that might use a $V_H$ gene other than $V_{H4}$ from a two-week-old Alicia rabbit. Collectively, these data suggested that Alicia rabbits use several $V_H$ genes that are not used in normal rabbits. The interpretation of this notion will be further discussed.

**Molecular Mechanism for the Appearance of $V_{H42}$ Ig in Alicia Rabbits**

Analysis of promoter regions clearly showed that $V_{H4}$ was used in VDJ gene rearrangements of $V_{H42}$ Ig-producing hybridomas. Because the nucleotide sequence of germline $V_{H4}$ was known, it provided an opportunity to investigate whether somatic diversification plays a role in the generation of $V_{H42}$ allotypic determinants in Alicia rabbits. By comparing the nucleotide sequences of $V_{H4}$-utilizing VDJ genes to that of germline $V_{H4}$, I identified clusters of nucleotide changes in FR1 and FR3 that resulted in the appearance of $V_{H42}$ allotype-associated amino acids. For instance, lysine (K), glutamic acid (E) and phenylalanine (F) were present in FR1 and glutamic acid (E) was found in FR3 of diversified $V_{H4}$-utilizing VDJ genes (Figure 19-24 and 27). This finding suggested to me that $V_{H42}$ Ig molecules were generated in Alicia rabbits lacking the prototypic $V_{H42}$-encoding gene by somatic diversification. This could be a consequence of either somatic point mutation, somatic gene conversion or $V_H$ gene replacement. I think it is unlikely that the appearance of these four $V_{H42}$ allotype-associated amino acids resulted from somatic mutation and my explanations are as follows. First, most $V_{H4}$-utilizing VDJ genes had almost identical nucleotide changes in FR1 and FR3 in spite of
the fact that they were independent clones, as evident from the difference in nucleotide sequences of N regions. Second, much of diversification in FR1 and FR3 occurred as clusters of nucleotide changes. It is therefore difficult to envision how point mutations could generate the same pattern of nucleotide changes in different VDJ clones. However, clusters of nucleotide changes are typical of somatic gene conversion events. To examine whether somatic gene conversion can contribute to the generation of VH a2 allotypic determinants, I searched for potential gene conversion donors that might be responsible for the diversified regions in FR1 and FR3. By comparing the nucleotide sequences of the diversified regions in FR1 and FR3 with those of all known germline VH genes in the a2 haplotype, I found a stretch of nucleotides from the leader intron through codon 11 in FR1 of six VDJ genes that was identical to the corresponding region of VH9. (Figure 20-25, 28). These data suggest that VH9 or a gene similar to VH9 donated the stretch of 66 nucleotides to these six VH-utilizing VDJ genes. However, the other possibility was that the diversification in these VDJ genes was due to a VH gene replacement mechanism in which the VH region of the VDJ gene was replaced by VH9. If so, the nucleotide sequence of the region 5' of the diversified VDJ gene would be identical to the sequence 5' of VH9. But the nucleotide sequences of the promoter regions 5' of the VDJ genes did not match with VH9 promoter, rather they were identical to the VH4 promoter. Taken together, these data strongly support that VH a2 Ig in aged Alicia rabbits can be generated by a somatic gene conversion-like process.
Is Preferential Usage of $V_H^A$ due to Preferential Rearrangement or Selection?

Surprisingly, 9 of 17 (53%) $V_{H,a^2}$ Ig-producing hybridomas derived from aged Alicia rabbits use $V_H^A$, the 3’ most $V_H$ gene on the ali $V_H$ locus. Based on the fact that B cells of normal rabbits preferentially rearrange the 3’ most $V_H$ gene, $V_HJ$, one might argue that $V_{H,a^2}$ Ig-expressing B cells in Alicia rabbits frequently use $V_H^A$ as a result of the preferential rearrangement of $V_H^A$. I think this possibility is unlikely because $V_H^A$ is less frequently used, if ever, in young Alicia rabbits (DiPietro et al., 1990, Chen et al., 1993). Rather, during the first two months of life, they preferentially use $V_{H,a^1}$ genes, $V_H^x$ and $V_H^y$ located at least 50 kb upstream of $V_HJ$ (DiPietro et al., 1990; Chen et al., 1993). Moreover, I found 5 of 17 hybridomas used the same unidentified $V_H$ gene to encode $V_{H,a^2}$ Ig molecules. Thus, from my data, not only was $V_H^A$ found to be preferentially used to encode $V_{H,a^2}$ Ig in aged Alicia rabbits, but an as-yet-unidentified $V_H$ gene was also used frequently. Because the nucleotide sequence of this unidentified $V_H$ gene’s promoter region did not match the promoters of known germline $V_H$ genes located within 50 kb 5’ of $V_HJ$, I believe that this gene is at least 50 kb upstream of $V_HJ$. To directly test whether $V_H^A$ and this unidentified $V_H$ gene are preferentially rearranged, $V_H$ gene usage in nonproductive VDJ genes, which are not affected by a selection process, should be further investigated in Alicia rabbits.

The previous study by DiPietro et al showed that 8-week-old Alicia rabbits frequently use $V_{H,a^1}$ genes in VDJ gene rearrangements (DiPietro et al., 1990). In accord
with DiPietro et al, Chen et al reported that only 1 of 159 cDNA clones detected by hybridization analysis appears to encode a $V_{H}a2$ Ig molecule in 2-week-old Alicia rabbits (Chen et al., 1993). In the present study, I found approximately 71% (37 of 52) of hybridomas derived from three-year-old Alicia rabbits produce $V_{H}a2$ Ig molecules and 53% of these hybridomas use $V_{H}A$. Taken together, these findings suggest that early in life, only a small number of B cells use $V_{H}A$ but the utilization of $V_{H}A$ increases as Alicia get older.

One explanation for the increase in the utilization of $V_{H}A$ in aged Alicia rabbits is that during ontogeny B cells expressing $V_{H}a2$ Ig molecules are selectively expanded and that because the rearranged $V_{H}A$ had undergone somatic gene conversion to generate a $V_{H}a2$ allotype-encoding VDJ gene, it is now selected (Figure 31). Support for this idea came from the results of the present study together with the studies by Crane et al (1996) and Pospisil et al (1995). Crane et al reported that rabbit B cells are generated early in the ontogeny and presumably are maintained throughout life as a self-renewing population (Crane et al., 1996). By flow cytometric analysis of appendix cells stained with anti-a2 and anti-µ antibodies, Pospisil et al showed that the majority of appendix B cells in 6-week-old normal rabbits expressed $V_{H}a2$ allotype (Pospisil et al., 1995). In contrast, less than 10% of appendix B cells in 6-week-old Alicia rabbits displayed $V_{H}a2$ allotypic specificities. By 11 weeks of age, the number of $V_{H}a2$ Ig-expressing B cells in appendix of Alicia rabbits had increased to levels comparable to those detected in 6-week-old
Figure 31: Model for the appearance of $V_{\mu a2}$ Ig molecules in adult Alicia rabbits.
Selective Expansion of $V_H a_2$ Ig-expressing B Cells

$V_H a^-$ Ig

or

$V_H 4$-encoding Ig

$V_H a_2$ Ig

Unknown ligand

or

Somatic Gene Conversion
normal rabbits. Further, cell cycle analysis showed that approximately 34-48% of $V_{H}a2$ Ig-expressing B cells were in the proliferative phase whereas only 20-30% of $V_{H}a^{-}$ Ig-expressing B cells were in mitotic phase. Additionally, the $V_{H}a2$ Ig-expressing B cells were undergoing apoptosis to a lesser extent than the $V_{H}a^{-}$ Ig-expressing B cells (Pospisil et al., 1995). Thus, although both $V_{H}a2$ and $V_{H}a^{-}$-expressing B cells were proliferating, B cells expressing $V_{H}a2$ allotype appeared to be preferentially expanded and positively selected. Because the differences between $V_{H}a2$ and $V_{H}a^{-}$ are the $V_{H}a$ allotypic residues in FR1 and FR3, they speculated that interaction with the $V_{H}a$ allotypic determinants in FR1 and FR3 mediates positive selection for B cells expressing $V_{H}a2$ Ig molecules. Most recently, Pospisil et al (1996) reported that F(ab')$_2$ fragments from $V_{H}a2$ Ig interact with CD5 molecules, cell surface glycoproteins expressed on rabbit B cells, better than F(ab')$_2$ fragments from $V_{H}a^{-}$ Ig. On the basis of these findings, the authors proposed that B cells expressing $V_{H}a2$ Ig are positively selected in rabbit appendix through the interaction of $V_{H}a$ allotypic determinants and CD5 molecules. However, many relevant issues remain poorly understood. For example, why do a2 allotype rabbits positively select B cells expressing $V_{H}a2$ allotype Ig molecules? Do CD5 molecules bind to $V_{H}a1$ Ig and $V_{H}a3$ Ig molecules in a1 and a3 allotype rabbits, respectively? In other words, does this type of selection occur in a1 or a3 allotype rabbits which have different $V_{H}a$ allotype-associated residues in FR1 and FR3? Do CD5 molecules alone mediate this type of selection? Do other molecules, e.g. microbial products, also participate in selection of $V_{H}a2$ Ig-expressing B cells? These are questions that should be further investigated in order to
clarify the role of B cell selection in the formation of the antibody repertoire.

**What Amino Acids Determine V\_H\_a2 Allotypic Specificity?**

The V\_H\_a allotype, a1, a2 and a3, were initially identified by serological reaction (Oudin, 1956). Immunization of a genetically defined V\_H\_a allotype rabbit with rabbit Ig isolated from different allotype induces the production of anti-a allotype antibody. For example, immunization of a1 allotype rabbits with Ig prepared from a2 allotype rabbits leads to anti-a2 production. Amino acid sequence analysis of pooled serum a1, a2 or a3 Ig revealed that the particular amino acid residues in FR1 and FR3 correlated with serological reactivity and these amino acids were designated V\_H\_a allotype associated residues (Mage et al., 1984). Knight and Becker reported that V\_H\_I of a1, a2 and a3 allotype2 rabbits encodes V\_H\_a1, V\_H\_a2 and V\_H\_a3 allotypes, respectively (Knight and Becker, 1990). While V\_H\_I-a2 encodes 10 of 11 V\_H\_a2 allotype-associated amino acids, the germline V\_H\_A gene segment encodes 2 of 5 allotype associated residues in FR1 and 5 of 6 V\_H\_a2 residues in FR3 (Knight and Becker, 1990). Are the 7 V\_H\_a2 allotype-associated amino acids encoded by undiversified V\_H\_A enough to confer V\_H\_a2 allotypic specificity? At the present time, the answer to this question is not known. Nevertheless, analyses of the diversified V\_H\_A in this study clearly showed that the additional V\_H\_a2 allotype-associated amino acids (lysine, glutamic acid and phenylalanine in FR1 and glutamic acid in FR3) led to V\_H\_a2 allotypic reactivity. Currently, it is still unclear whether a complete set of V\_H\_a2 allotype-associated residues is required for reaction with
V<sub>Ha</sub>2 allotype antisera. It will be especially interesting to determine whether or not the IgH molecules encoded by undiversified V<sub>H</sub>4 have V<sub>Ha</sub>2 allotypic reactivity. This could be achieved by expressing the undiversified V<sub>H</sub>4-utilizing VDJ gene and testing its product for the ability to react with anti a2 allotype antibody.

If undiversified V<sub>H</sub>4-utilizing VDJ genes do encode IgH molecules that react with anti-a2 antibodies, then this could explain the trace amount of V<sub>Ha</sub>2 Ig molecules found in young Alicia rabbits. For example, it could be that early in ontogeny of Alicia rabbits, a small number of B cells use V<sub>H</sub>4 and that V<sub>H</sub>4-utilizing VDJ genes undergo somatic gene conversion to generate a complete set of V<sub>Ha</sub> allotypic residues and then are selectively expanded. On the other hand, the low amounts of V<sub>Ha</sub>2 Ig in young Alicia rabbits could be derived from unidentified V<sub>Ha</sub>2-encoding genes, instead of from undiversified V<sub>H</sub>4. One of these genes could be the utilized V<sub>H</sub> genes that Chen et al found in 2-week-old Alicia rabbit (Chen et al., 1993).

**Why is V<sub>H</sub>9 Frequently Used as a Gene Conversion Donor?**

Strikingly, 6 of 9 V<sub>H</sub>4-utilizing VDJ genes had undergone somatic gene conversion using V<sub>H</sub>9 as a donor gene. Why is V<sub>H</sub>9 used at such a high frequency? One possibility is that V<sub>H</sub>9 is preferentially used as a gene conversion donor because of its sequence similarity and close proximity to V<sub>H</sub>4. This explanation is based on findings previously reported in the studies of somatic gene conversion in chicken V<sub>lambda</sub> genes. It was
shown that pseudogenes with a high degree of homology or closer location to the rearranged $V_{\lambda}$ genes are more frequently used as donors (Reynaud et al., 1987; McCormack and Thompson, 1990). Another possibility is that the high frequency of $V_{H}9$ used as a donor gene was due to the selection of somatic gene conversion events that used $V_{H}9$. In fact, for the purpose of this study, I selectively analyzed VDJ genes from $V_{H}a2$ Ig-producing hybridomas. As mentioned above, $V_{H}A$ does not encode the $V_{H}a2$ allotype-associated residues, lysine, glutamic acid and phenylalanine in FR1, but these residues are encoded by $V_{H}9$. Thus, somatic gene conversion events of rearranged $V_{H}A$ using $V_{H}9$ as a donor are likely to give rise to $V_{H}a2$ allotype-encoding VDJ genes. Therefore, it is possible that most $V_{H}A$-utilizing VDJ genes in this study exhibit evidence of somatic gene conversion using $V_{H}9$ as a donor because of the selection based on the expression of $V_{H}a2$ allotype as discussed previously.

**Differential Usage of $V_{H}$ Genes in Normal and Alicia Rabbits**

In the present study, I found that Alicia rabbits use several $V_{H}$ genes to encode $V_{H}a2$ Ig molecules. These genes, including $V_{H}A$, have never been found in VDJ gene rearrangements of normal rabbits. One possible explanation for this observation is that normal rabbits preferentially use $V_{H}I$ in VDJ gene rearrangements and that $V_{H}I$-utilizing VDJ genes already encode 10 of 11 $V_{H}a2$ allotype-associated amino acids. Thus there is no pressure to select the rearranged $V_{H}A$ genes that had undergone somatic gene conversion to create $V_{H}a2$ allotypic epitopes or other $V_{H}a2$ like genes. At the current
time, it is not clear why a2 allotype rabbits attempt to maintain Ig carrying $V_{H2}$ allotypes.

The finding that Alicia rabbits use several $V_{H}$ genes to encode $V_{H2}$ Ig is striking. Why do Alicia B cells use multiple $V_{H}$ genes to encode $V_{H2}$ Ig but B cells in normal rabbits use only one $V_{H}$ gene, $V_{H1}$? This intriguing difference provides an opportunity to investigate the molecular mechanisms of preferential usage of $V_{H1}$. Because selection for $V_{H2}$ Ig expressing B cells are ongoing in both normal and Alicia rabbits, the difference in $V_{H}$ gene usage may result from the preferential rearrangement of $V_{H1}$ over rearrangements of other $V_{H}$ genes. In the following section, I will discuss the possible mechanisms for the preferential rearrangement of $V_{H1}$ in normal rabbits.

**Molecular Basis for Preferential Rearrangement of $V_{H1}$**

All studies of $V_{H}$ gene usage in normal rabbits clearly showed that $V_{H1}$ is preferentially used in VDJ gene rearrangements (Becker et al., 1990; Knight and Becker, 1990; Friedman et al., 1994; Raman et al., 1994; Tunyaplin and Knight, 1995). Further, analysis of $V_{H}$ gene usage in nonproductive VDJ genes demonstrated that 26 of 33 genes used $V_{H1}$ (Tunyaplin and Knight, 1995). Because nonproductive VDJ genes are derived from VDJ gene rearrangements in the absence of cellular selection, the preferential utilization of $V_{H1}$ can be explained by its preferential rearrangement. To date, the molecular basis for preferential rearrangement of $V_{H1}$ is poorly understood. I discuss
below the factors that might determine the molecular mechanisms underlying the preferential rearrangement of $V_H^l$.

Several factors have been reported to influence the frequency of V(D)J gene rearrangement. These include 1) proximity (Yancopoulos et al., 1984), 2) RSS (Hesse et al., 1989; Ramsden and Wu, 1991; Akamatsu et al., 1994; Connor et al., 1995), 3) coding end sequences and homology-directed recombination (Boubnov et al., 1993; Gerstein and Lieber, 1993, Chukwuocha and Feeney, 1993; Nadel and Feeney, 1995; Ezekiel et al., 1995), 4) accessibility (Storb and Arp, 1983; Yancopoulos and Alt., 1985; Engler et al., 1991; Schlissel et al., 1991; Hsieh and Lieber, 1992; Engler et al., 1993; Goodhardt et al., 1993), and 5) antisilencer (Ferradini et al., 1996). Which factor(s) are the explanation for the preferential rearrangement of $V_H^l$?

Proximity

The idea of proximity directing preferential rearrangement was proposed by Alt and associates who found that A-MuLV transformed pre-B cell lines preferentially use the D-proximal $V_H$ gene segments, $V_H81x$ and $V_H7183$ members (Yancopoulos et al., 1984). They suggested that $V_H$ genes located in close proximity to the D locus are more accessible to the recombinational machinery. In normal rabbits, $V_H^l$ is the most D-proximal $V_H$ gene segment and is preferentially used. If proximity to the D gene cluster determines the preferential utilization of $V_H$ genes, the 3' most $V_H$ gene in Alicia rabbits
should be frequently used. However, $V_{H4}$, the 3' most functional $V_H$ gene on the $ali$ chromosome, is not frequently used in B cells of young Alicia rabbits. Rather, they preferentially use $V_{Hx}$ and $V_{Hy}$ located more than 50 kb upstream of $V_{Hl}$. Therefore, close proximity of $V_{Hl}$ to the D gene segments is unlikely to be the explanation for the preferential utilization of $V_{Hl}$ in normal rabbits.

**RSS**

A number of laboratories have demonstrated that the difference in RSS, in particular the nucleotide sequences of heptamer and nonamer, affects the frequency of V(D)J gene rearrangement (Hesse et al., 1989; Ramsden and Wu, 1991; Akamatsu et al., 1994; Connor et al., 1995). However, at the present time, there is no evidence that the RSS plays a role in preferential rearrangement of $V_{Hl}$. Several known $V_H$ gene segments in rabbits have identical heptamer and nonamer. Particularly, the RSS of $V_{H4}$ is identical to the RSS of $V_{Hl}$. It is of interest to examine the RSS of the four unidentified $V_H$ genes that I found to be used in VDJ gene rearrangements of aged Alicia rabbits. This could be achieved once the germline counterpart of these $V_H$ genes are cloned.

**Coding End Sequences and homology-directed recombination**

There is substantial evidence that the nucleotide sequences of the two coding ends play an important role in V(D)J gene joining (Boubnov et al., 1993; Gerstein and Lieber, 1993; Chukwuocha and Feeney, 1993; Nadel and Feeney, 1995; Ezekiel et al., 1995).
Importantly, Chukwuocha and Feeney reported that preferential utilization of \( V_{H}81x \) in mouse pre-B cells during fetal and neonatal development, in which the enzyme terminal deoxy nucleotidyl transferase (TdT) is not yet expressed, can be explained by homology-directed recombination (Chukwuocha and Feeney, 1993). They showed that the 3’ end of \( V_{H}81x \) had short sequence homology with the 5’ end of D gene segment and alignment of the complementary strands of DNA at the short homology preferentially generated productive rearrangement. However, this factor probably does not explain the preferential utilization of \( V_{H}1 \) because most rabbit \( V_{H} \) gene segments, either those frequently used or those rarely used, have the same nine nucleotides at the 3’ end. Nevertheless, this does not preclude the possibility that the four unidentified \( V_{H} \) genes used in Alicia rabbits have some unfavored nucleotides at the coding ends resulting in infrequent use in normal rabbits. If the germline counterparts of these \( V_{H} \) genes are cloned and sequenced, this possibility can be reinvestigated.

Accessibility

Alt and colleagues proposed that antigen receptor loci are normally sequestered from V(D)J recombinational machinery. In lymphoid cells undergoing V(D)J recombination, the chromatin configuration of a particular V-region gene undergoes a biochemical alteration such that the recombinase can access that particular gene segment (reviewed in Alt et al., 1986 and 1987; Okada and Alt, 1995). The concept of accessibility has been proposed to explain the lineage-specificity as well as developmental state specificity of rearrangements. Although the precise molecular
mechanisms that modulate accessibility are still unknown, many studies have shown that accessibility correlates with active transcription and DNA hypomethylation (Storb and Arp, 1983; Yancopoulos and Alt., 1985; Engler et al., 1991; Schlissel et al., 1991; Hsieh and Lieber, 1992; Engler et al., 1993; Goodhardt et al., 1993). The effects of these parameters on VDJ gene rearrangements and $V_H$ gene usage are discussed below.

Transcription

Each V-region gene is transcribed from its own promoter. Transcription from a $V_H$ promoter can be detected even before VDJ gene rearrangement. It has been speculated that transcription is required to open the $V_H$ locus to become accessible to the V(D)J recombinational machinery (reviewed in Alt et al., 1986 and 1987). Because promoters regulate transcription that correlates with VDJ gene rearrangements and $V_H$ promoters are unique for each $V_H$ gene, the difference in transcriptional activity may determine the differential usage of $V_H$ genes. To test this possibility, Buchanan et al compared the transcription efficiencies of two murine $V_H$ promoters, V1 promoter from the $V_H$S107 family and BCL1 promoter from the $V_H$J558 family (Buchanan et al., 1995). They found that these two $V_H$ promoters were not transcribed with the same efficiency as measured by in vitro transcription assay. Further, they suggested that the spacing between the octamer and the heptamer elements affected the transcription efficiency resulting in differential use of $V_H$ genes.
To determine whether the difference in promoter elements determines the preferential utilization of $V_{\text{H}}I$ in normal rabbits, Tunyaplin and Knight cloned and sequenced 1.2 kb of promoter regions from several $V_{\text{H}}$ genes, including $V_{\text{H}}I$, $V_{\text{H}}4$ and $V_{\beta}y$ (Tunyaplin and Knight, 1995). By analyzing the regulatory elements of these $V_{\text{H}}$ promoters, they found that the $V_{\text{H}}$ genes examined had similar nucleotide sequences of the core promoter, known as the octamer, the heptamer, the pyrimidine-tract and TATA-box. Additionally, there is no difference in the spacing between the octamer and the heptamer elements that could determine the difference in transcription efficiency as previously shown in mouse $V_{\text{H}}$ promoters. Although the differences in the strength of these $V_{\text{H}}$ promoters have not been examined, transcriptional activity may not play an important role in the preferential utilization of $V_{\text{H}}I$. There are several examples in the literature that rearrangements can occur even in the absence of or very low level of transcription (Goodhardt et al., 1987; Engler et al., 1991; Kallenbach et al., 1993). It is not clear whether transcription is the determinant of accessibility or a by-product of accessible gene segments. Therefore, I decided not to test the effect of promoter activity on the differential utilization of $V_{\text{H}}$ gene.

DNA hypomethylation

Methylation of cytosine in DNA has been shown to be an important mechanism regulating tissue-specific gene expression (reviewed in Razin and Cedar, 1991). Hypomethylation of a recombination substrate, either endogenous or introduced plasmid
substrates, correlates with the efficient rearrangement of recombination substrate (Storb and Arp, 1983; Engler et al., 1991; Hsieh and Lieber, 1992; Engler et al., 1993; Goodhardt et al., 1993). It is possible that $V_H J$ is hypomethylated relative to other $V_H$ genes. However, at present, this possibility is very difficult to approach experimentally.

**Antisilencer**

Interestingly, Weill and associates identified cis-acting elements located in the V-J intervening sequences of chicken $\lambda$ locus and mouse $\kappa$ locus that regulate Ig gene rearrangement (Lauster et al., 1993; Ferradini et al., 1996). In chicken, this element, known as antisilencer, has been proposed to counteract the negative effect of the nearby regulatory element, silencer (Lauster et al., 1993). Weill and colleagues proposed that in B cell progenitors, antisilencer factors are transiently expressed and bind to antisilencer element(s) flanking the silencer. This binding results in transient inactivation of the silencer and allows VJ gene rearrangement to occur on one allele. The presence of the silencer on the other allele prohibits $\lambda$ gene rearrangement. Thus, these two regulatory elements are thought to function together to insure allelic exclusion at the $\lambda$ locus.

In the mouse $V_\kappa$-$J_\kappa$ intervening sequence, a homolog of the chicken antisilencer element was found to influence the efficiency of $\kappa$ gene rearrangement (Ferradini et al., 1996). By using a gene targeting approach, Ferradini et al showed that the mutation of this cis-acting element resulted in a significant decrease of $\kappa$ gene rearrangement. This element, designated recombination-enhancing element, affects the rearrangement process
without interfering with accessibility of κ locus, as measured by the germline transcription and the methylation status. Thus, it appears that the recombination-enhancing element regulates the rearrangement process through a mechanism other than by regulating transcription and the methylation. I think this novel mechanism is interesting and may explain the preferential rearrangement of $V_H^1$.

**Search for Recombination Enhancer**

As mentioned previously, I found that Alicia rabbits use several $V_H$ genes that are not used in normal rabbits. The mutation in Alicia rabbits is a 10 kb deletion spanning the region containing $V_H^2$, $V_H^1$, and 2 kb of DNA downstream of $V_H^1$. On the basis of differential $V_H$ gene usage, the DNA deletion in the mutant Alicia rabbits and the recombination-enhancing elements found in mouse and chicken, I hypothesized that the preferential rearrangement of $V_H^1$ in normal rabbits could be due to such a recombination-enhancing element residing in close proximity to $V_H^1$. Deletion of this element along with $V_H^1$ in Alicia rabbits then might lead to rearrangement of multiple $V_H$ genes. If this element was associated with $V_H^1$, it could reside in the region either 5' or 3' of $V_H^1$. However, I think it is most likely that the cis-acting recombination enhancer element resides in the region 3' of $V_H^1$. This is because if the recombination enhancer was 5' of $V_H^1$, it could equally promote rearrangement of both $V_H^1$ and $V_H^2$. However, the rearrangement of $V_H^2$ is rarely detected, suggesting to me that the recombination enhancer was not 5' of $V_H^1$. I therefore specifically searched for a cis-acting
recombination enhancer from the region 3’ of \( V_H J \).

My approach was to compare the recombination frequency of a recombination substrate containing DNA 3’ of \( V_H J \) with that containing an irrelevant DNA fragment. The first 3’ \( V_H J \) region that I tested was a 2 kb Hind III fragment and the negative control was a DNA fragment adjacent to rabbit IL-10 gene. Practically, the negative control DNA could be any non-relevant DNA fragment with the same size as the tested DNA. Because a 2 kb Hind III fragment germline DNA could be obtained from the rabbit IL-10 gene, I cloned this DNA fragment into a recombination substrate and used it as a negative control in the first three in vitro recombination experiments. If the region 3’ of \( V_H J \) contained the recombination enhancer, then I expected to detect a significantly higher recombination frequency with this substrate than with the negative control. All three experiments showed that the recombination frequencies of a recombination substrate containing DNA region 3’ of \( V_H J \) were higher than those of the IL-10 control. These exciting results led to an attempt to specifically locate the recombination enhancer region.

At the same time, I needed to confirm whether the recombination enhancer actually existed in the DNA 3’ of \( V_H J \) that I tested. Initially, I assessed the recombination frequencies by detecting the expression of chloramphenicol acetyltransferase (CAT) gene as an indicator of recombination events. Although this method is generally used to detect recombination in several studies, I needed to confirm the result of the IL-10 control by a different method (Hesse et al., 1987; Lieber et al., 1987; Wei and Lieber, 1993; VanDyk et al., 1996). Because the spacer DNA between the two RSS of recombination substrate
would be deleted during RSS-mediated rearrangement, I could use this DNA fragment as a probe to detect recombination events. Additionally, I needed to confirm the result of the IL-10 control with another negative control construct. Thus I cloned another 2 kb Hind III fragment, the germline DNA adjacent to rabbit Bcl-2 gene, into the recombination substrate and tested it together with the former constructs. By differential hybridization, I did not detect any significant difference in recombination frequencies of 3' \( V_HJ \) constructs, including its subclones from either of the negative controls. This result contradicted the data obtained from chloramphenecol selection in which the recombination frequencies of DNA from 3' of \( V_H \) and its subclones were higher than that of the control. How can I explain this discrepancy between results obtained from chloramphenicol selection and hybridization? To resolve this problem, I reanalyzed the DNA that were previously recovered from transfection experiment no.2 and 3 in which the recombination frequencies were detected by chloramphenicol selection. I retested these DNA by colony hybridization. No difference in frequency of recombination was observed even though this 3' \( V_HJ \) DNA construct previously showed a higher recombination frequency as determined by chloramphenicol selection (Table 6). Because the hybridization method directly tests the recombination event, I conclude that the recombination enhancer does not reside on the DNA fragments that I tested. However, this does not rule out the possibility that a recombination enhancer exists in other regions not tested in the present study. Alternatively, the recombination enhancer element might exist in the DNA region 3' of \( V_HJ \) that I tested but it could not be detected by the extrachromosomal recombination substrate assay. It is possible that the activity of this
recombination enhancer element can be observed only when it is present as the endogenous chromosomal substrate, rather than on an extrachromosomal substrate. If this is the case, a transgenic approach should be able to detect its activity. This is an area which needs further investigation.

In summary, I have examined the molecular mechanism for how $V_{H}a2$ Ig is generated in Alicia rabbits lacking the prototypic $V_{H}a2$-encoding gene. By generating and selecting for $V_{H}a2$ Ig-producing hybridomas from adult Alicia rabbits, I have shown that $V_{H}a2$ Ig molecules are derived, at least in part, from somatic gene conversion events. In addition, I found that, unlike normal rabbits that utilize predominantly one $V_{H}$ gene, Alicia rabbits use several $V_{H}$ genes to encode $V_{H}a2$ Ig molecules (Figure 32). Further studies of Alicia rabbits will likely provide an important insight into the molecular basis for preferential $V_{H}$ gene usage as well as into the molecular and cellular mechanisms that shape the antibody repertoire in normal rabbits.
Figure 32: Alicia rabbits use at least five $V_H$ genes to encode $V_{H a2}$ Ig molecules.
At Least Five $V_H$ Genes are Used to Encode a2 Ig in Adult Alicia rabbits
APPENDICES

Culture of Rabbit B Cells

The objective of my dissertation project is to definitively identify $V_H$ gene(s) used by Alicia rabbits to produce $V_H\alpha_2$ Ig molecules. To accomplish this, as described in result and discussion, I proposed to generate $V_H\alpha_2$ Ig-producing cell lines from adult Alicia rabbits and then identify the $V_H$ gene segments used in VDJ gene rearrangements of the $V_H\alpha_2$ Ig-producing cells. When I began this project, there was no rabbit hybridoma fusion partner for generating stable rabbit hybridomas. Furthermore, there are no viral agents capable of inducing B lymphoid tumors in rabbits. To develop long-term cultures of rabbit B cells, I modified the CD40 culture system developed by Banchereau et al. (1991). In their original culture system, CD40 on human B cells was cross-linked with anti-CD40 monoclonal antibody and after culturing in medium containing IL-4 or IL-10, the cells exhibited long-term growth. So, I tested whether rabbit B cells would also proliferate and exhibit long-term growth following CD40 ligation. In my experiments, I used CD40 ligand (CD40L) rather than anti-CD40 antibody to cross-link CD40 on rabbit B lymphocytes. I co-cultureed rabbit mesenteric lymph node (MLN) cells with irradiated cell line that stably expressed membrane bound mouse CD40L. After 3 weeks of culture, I observed many clusters of dividing lymphocytes. In contrast, MLN cells cultured at the same time, in the absence of CD40L, died. The clustered cells in the CD40L treated cultured reacted with FITC-conjugated anti-rabbit $\kappa$ chain antibody, suggesting that the
cells proliferating in culture are B lymphocytes. However, the rabbit B cells could be maintained in this culture for only one month.

Rabbit CD40L

Because rabbit B cells proliferated in response to mouse CD40L, I sought to optimize the culture condition by using rabbit CD40L and rabbit IL-10. I cloned rabbit CD40L from a rabbit splenic cDNA library in λ gt10 by using mouse CD40L as a probe. A clone with 1.4 kb insert was obtained. The DNA was restriction mapped and the nucleotide sequence was determined. Nucleotide sequence analysis reveals that this cDNA contains a long open reading frame of 785 bp which corresponds to those of mouse and human CD40L (Figure 33). It encodes a protein of 261 amino acid residues. Comparison of the nucleotide sequence with those of mouse and human CD40L cDNA showed 78.9% and 87.1% similarity, respectively. This level of DNA homology indicates that the isolated clone is rabbit CD40L cDNA.

Rabbit IL-10

Because IL-10 is able to enhance the proliferation of mouse and human B cells stimulated with CD40L, I reasoned that addition of these cytokines to cultures of B cells stimulated with rabbit CD40L might enhance the long-term growth of rabbit B cells. Due to limitation in cross-species activity of these cytokines, I cloned rabbit IL-10 gene. According to Southern blot analysis of genomic DNA digested with BamHI, a single band of 9.5 Kb DNA was hybridized with a human IL-10 probe, suggesting that the rabbit IL-
Figure 33: Comparison of nucleotide sequence of a cDNA encoding RA-CD40L with that of human and mouse CD40 ligand. The nucleotide identities are indicated by dashed. The predicted amino acid sequence of RA-CD40L is given on the top of the nucleotide sequence.
<table>
<thead>
<tr>
<th></th>
<th>M</th>
<th>I</th>
<th>E</th>
<th>T</th>
<th>Y</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA-CD40L</td>
<td>TTGGTCAGAAATACCACTGCAACCCAGC ATG ATC GAA ACG TAC AGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>Q P T P R S V AT G P S V S M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>CAA CCT ACT CCT CGT TCT GTG GCC ACT GGA CCA TCT GTT AGC ATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>K I F M Y L L T V F L I T Q M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>AAA ATT TTT ATG TAT TTA CTT ACT GTT TTT CTT ATT ACC CAG ATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-CD40L</td>
<td>--G-- --C-- -C-- -C-- -A-- -G-- --G-- --G-- --A-- T-- --T--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>I G S A L F A V Y L H R R R L D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>ATA GGG TCA GCG CTT TTT GCT GTA TAT CTT CAT AGA AGG TTG GAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>K I E D E R N L H E D F V F M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>AAG ATA GAA GAT GAA AGG AAT CTT CAT GAG GAT TTT GTA TCC ATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>K T I Q R C N K G E G S L S L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>AAA ACG ATA CAG AGA TGC AAC AAA GGA GAA GGG TTC TTA TCC CTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-CD40L</td>
<td>--T-- --C-- -A-- A-- C-- -C-- -C-- -T-- --T-- --T-- --T-- --T--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>L N C K E I R S Q F E G F V K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>CTG AAC TGT AAG GAA ATT AGA AGC CAG TTT GAA GGC TTC GTC AAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-CD40L</td>
<td>--T-- --C-- -A-- A-- C-- -C-- -C-- -T-- --T-- --T-- --T-- --T--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>D I M L N K E E P K K E I N F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>GAT ATA ATG CTA AAC AAA GAG GAG CCG AAG AAA GAA ATA AAT TTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HU-CD40L</td>
<td>--T-- --C-- -A-- A-- C-- -C-- -C-- -T-- --T-- --T-- --T-- --T--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td>E N R K G V Q D P Q I A A H L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA-CD40L</td>
<td>GAA AAT CGA AAA GGT GTA CAG GAT CCT CAA ATT GCA GCA CAT CTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RA-CD40L</td>
<td>HU-CD40L</td>
<td>MU-CD40L</td>
<td>RA-CD40L</td>
<td>HU-CD40L</td>
<td>MU-CD40L</td>
</tr>
<tr>
<td>---</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>I</td>
<td>S</td>
<td>E</td>
<td>A</td>
<td>S</td>
<td>K</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>Q</td>
<td>W</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA</td>
<td>AGT</td>
<td>GCC</td>
<td>AGT</td>
<td>AGT</td>
<td>AAA</td>
<td>TCA</td>
</tr>
<tr>
<td>C-</td>
<td>-C</td>
<td>-A</td>
<td>-AC</td>
<td>-T</td>
<td>G-</td>
<td>-C</td>
</tr>
<tr>
<td>K</td>
<td>K</td>
<td>G</td>
<td>Y</td>
<td>Y</td>
<td>T</td>
<td>M</td>
</tr>
<tr>
<td>AAA</td>
<td>AAA</td>
<td>GGA</td>
<td>TAT</td>
<td>TAC</td>
<td>ACC</td>
<td>ATG</td>
</tr>
<tr>
<td>G-</td>
<td>-G</td>
<td>-C</td>
<td>-C</td>
<td>-T</td>
<td>G-</td>
<td>-C</td>
</tr>
<tr>
<td>N</td>
<td>G</td>
<td>K</td>
<td>Q</td>
<td>L</td>
<td>K</td>
<td>V</td>
</tr>
<tr>
<td>TAT</td>
<td>GCC</td>
<td>CAA</td>
<td>GTC</td>
<td>ACC</td>
<td>TTC</td>
<td>TGT</td>
</tr>
<tr>
<td>C-</td>
<td>-C</td>
<td>-T</td>
<td>-G</td>
<td>-C</td>
<td>-G</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>P</td>
<td>F</td>
<td>I</td>
<td>A</td>
<td>S</td>
<td>L</td>
</tr>
<tr>
<td>GCT</td>
<td>CCA</td>
<td>TTT</td>
<td>ATA</td>
<td>GCC</td>
<td>AGC</td>
<td>TTA</td>
</tr>
<tr>
<td>T-</td>
<td>-AG</td>
<td>-G</td>
<td>-C</td>
<td>-G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>R</td>
<td>I</td>
<td>L</td>
<td>L</td>
<td>R</td>
<td>A</td>
</tr>
<tr>
<td>GAA</td>
<td>CGA</td>
<td>ATC</td>
<td>CTA</td>
<td>CTC</td>
<td>AGA</td>
<td>GCA</td>
</tr>
<tr>
<td>A-</td>
<td>-A</td>
<td>T-</td>
<td>C-</td>
<td>T-</td>
<td>-A</td>
<td>-C</td>
</tr>
<tr>
<td>T-</td>
<td>-AG</td>
<td>-G</td>
<td>-A</td>
<td>-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>C</td>
<td>E</td>
<td>Q</td>
<td>Q</td>
<td>S</td>
<td>Y</td>
</tr>
<tr>
<td>ACT</td>
<td>TGT</td>
<td>GAG</td>
<td>CAG</td>
<td>CAA</td>
<td>TCC</td>
<td>TAC</td>
</tr>
<tr>
<td>T-</td>
<td>-T</td>
<td>G-</td>
<td>-C</td>
<td>-T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>A</td>
<td>V</td>
<td>A</td>
<td>S</td>
<td>V</td>
<td>F</td>
</tr>
<tr>
<td>CAA</td>
<td>GCG</td>
<td>GTA</td>
<td>GCT</td>
<td>TCG</td>
<td>GTG</td>
<td>TTG</td>
</tr>
<tr>
<td>C-</td>
<td>-T</td>
<td>GT</td>
<td>-C</td>
<td>-T</td>
<td>-C</td>
<td>-T</td>
</tr>
<tr>
<td>V-</td>
<td>N</td>
<td>H</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>F</td>
</tr>
<tr>
<td>GTG</td>
<td>AAC</td>
<td>CAC</td>
<td>GGG</td>
<td>ACC</td>
<td>GTT</td>
<td>ACA</td>
</tr>
<tr>
<td>G-</td>
<td>-T</td>
<td>C-</td>
<td>-T</td>
<td>C-</td>
<td>G-</td>
<td>-C</td>
</tr>
<tr>
<td>V-</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>GTT</td>
<td>-C</td>
<td>T-</td>
</tr>
<tr>
<td>TGA</td>
<td>acagtg</td>
<td>ttgc</td>
<td>cacacg</td>
<td>gactgc</td>
<td>agct</td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU-CD40L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
10 gene is on a 9.5 Kb BamHI fragment. Therefore, I screened for the IL-10 gene from 6-10 Kb BamHI genomic phage library using the human IL-10 cDNA as a probe and obtained one positive clone with a 9.5 Kb insert. The inserted DNA was subcloned into a plasmid vector for restriction mapping. One DNA fragment that hybridized with human IL-10 was further subcloned into M13 mp 18/mp 19 for DNA sequencing. Based on nucleotide sequence comparison with the published exon 1 of mouse IL-10 gene and human IL-10 cDNA, this clone is confirmed to be RA-IL-10 gene(Figure 34). Moreover, hybridization patterns with 5' and 3' human IL-10 probes reveal that this 9.5 Kb DNA contains a complete IL-10 gene.
Figure 34: Comparison of nucleotide sequences of the first exon of genomic RA-IL-10 clone, human and mouse IL-10. The nucleotide identities are indicated by dashed. The translation start codon is underlined.
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RA-IL-10</td>
<td>AAACCACAAGGCCTACGTAAGAGCAGGAGAGTTCACCATGCAGCTACGCTACGCTCTG</td>
</tr>
<tr>
<td>HU-IL-10</td>
<td>---A-A---T-C-A---A---//////////---A---CTG---A---</td>
</tr>
<tr>
<td>MU-IL-10</td>
<td>---C---G---T---</td>
</tr>
<tr>
<td>RA-IL-10</td>
<td>TATGTTCTGCTTTGCTGGGTGACAGGGCCAGCGAGGAGGACAGGACACCCCCCTCTG</td>
</tr>
<tr>
<td>HU-IL-10</td>
<td>-C-----C----AC----GTGA-----C-----G-----AG-----</td>
</tr>
<tr>
<td>MU-IL-10</td>
<td>-C-----C----A----AC----C-TGA----AT-----A-G-----T-----G-----GGAA</td>
</tr>
<tr>
<td>RA-IL-10</td>
<td>AGAACAGCTGCATTCCATTTCCAGGCCCTGGCCACATGCTCCGGAGCTCCGTCGNC</td>
</tr>
<tr>
<td>HU-IL-10</td>
<td>-C---C---C-----AA-----TA-----T----A----A-ATG</td>
</tr>
<tr>
<td>MU-IL-10</td>
<td>-C---T-A-----C-----C-----T-----A-AG-----TA-----G-----GACTG</td>
</tr>
<tr>
<td>RA-IL-10</td>
<td>GCTTGGCAAGGCTAAAGACTTTCTTT</td>
</tr>
<tr>
<td>HU-IL-10</td>
<td>C---CA---A---</td>
</tr>
<tr>
<td>MU-IL-10</td>
<td>C---CA---CA---</td>
</tr>
</tbody>
</table>
REFERENCES


VITA

Anusorn Boonthum was born in Thailand to Anuwat and Boonsri Boonthum. After the completion of her Bachelor of Science degree, Ms Boonthum joined the Department of Microbiology, Faculty of Medicine, Chiang Mai University as an instructor.

In 1990, she received a scholarship from the faculty of Medicine, Chiang Mai University to study for her Doctoral degree in the Department of Microbiology and Immunology, Loyola University Chicago. She joined the laboratory of Dr. Katherine Knight where she focused her efforts on studying the immunoglobulin genes of rabbits.

Upon completion of her Ph.D. degree, Ms Boonthum will return to Thailand where she will continue to teach Microbiology to Medical and graduate students as well as conduct her research on immunity to infectious diseases.

PUBLICATION

DISSETATION APPROVAL SHEET

The dissertation submitted by Anusorn Boonthum has been read and approved by the following committee:

Katherine L. Knight, Ph.D.
Professor and Chair
Department of Microbiology and Immunology
Loyola University Chicago

Charles F. Lange, Ph.D.
Professor Emeritus
Department of Microbiology and Immunology
Loyola University Chicago

Manuel O. Diaz, M.D.
Professor and Director
Program in Molecular Biology
Loyola University Chicago

Hans-Martin Jäck, Ph.D.
Associate Professor
Department of Microbiology and Immunology
Loyola University Chicago

John Nawrocki, Ph.D.
Assistant Professor
Department of Pathology
Edward Hines, Jr. Hospital

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 30, 1997

Date

Katherine L. Knight

Director's Signature