Loyola University Chicago
Loyola eCommons

Dissertations

Theses and Dissertations

1996

V, D and J Gene Rearrangements on the Unexpressed Igh Allele in Rabbit B Cells

Chainarong Tunyaplin
Loyola University Chicago

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

Part of the Microbiology Commons

Recommended Citation

Tunyaplin, Chainarong, "V, D and J Gene Rearrangements on the Unexpressed Igh Allele in Rabbit B Cells" (1996). *Dissertations*. 3400.
https://ecommons.luc.edu/luc_diss/3400

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1996 Chainarong Tunyaplin

LOYOLA UNIVERSITY CHICAGO

V, D AND J GENE REARRANGEMENTS ON THE UNEXPRESSED IGH ALLELE IN RABBIT B CELLS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN PARTIAL FULFULLMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

BY

CHAINARONG TUNYAPLIN

CHICAGO, ILLINIOS

JANUARY, 1996

LOYOLA UNIVERSITY MEDICAL CENTER LIBRARY

Copyright by Chainarong Tunyaplin, 1995

All rights reserved.

ACKNOWLEDGEMENTS

I am greatly indebted to Dr. Katherine L. Knight, my mentor, for the best training one can ever have in their graduate education and for all the supports in the past several years. Despite her extremely busy schedule, she is always available to students when they need her. Her dedication to science and educating young scientists is exemplary.

I also like to thank my committee, Dr. C. F. Lange, Dr. P. M. Witte, Dr. P. T. Le, Dr. S. C. Baker and Dr. H. M. Jäck, for all the guidance and encouragement, and for enduring with the everchanging focus of my project over the years. I thank my best friend, Martha L. Friedman, who helped me tremendously when I first came to the United States and continues to do so after all these years. I also thank Helga Spieker-Polet and Pi-Chen Yam for their rabbit hybridomas and Shi-Kang Zhai for all the sequencing reactions. Lastly, I would like to acknowledge all the members of Dr. Knight's laboratory and the Department of Microbiology and Immunology, who together make this department such an ideal workplace.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

LIST OF ABBREVIATIONS xi

ABSTRACT xiii

INTRODUCTION 1

Chapter

I. LITERATURE REVIEWS 3

Basic Structure of Ab Molecules 4

Discovery of Somatic Gene Rearrangement 5

V_H, D and J_H Genes Organization 9

IgH Gene Rearrangement 24

Allelic Exclusion 36

V_H Gene Utilization in B-lineage Cells 49

II. MATERIALS AND METHODS 68

Animals 68

Chemicals 68

Purification of Rabbit B Cells 68

Hybridoma Cell Culture 70

Collection of Rabbit Fetuses 71

Genomic DNA Purification 72

Genomic Blot Analysis 73

PCR Amplifications and Cloning of VDJ, DJ and VD Genes 75

Nucleotide Sequence Analysis of V_H Promoters 82

III. RESULTS 84

Quantification of Germline J_H and V_H1 in Polyclonal Rabbit B Cells 84

Quantification of Germline J_H in Polyclonal Rabbit B Cells 84

Quantification of Germline V_H1 in Polyclonal Rabbit B Cells 91

Rearrangements of V_H1 and J_H in the B Cell Hybridomas 105

IgH Gene Rearrangement in Rabbit T Cell Lines 140

Intermediates of IgH Gene Rearrangement in Polyclonal B Cells 145

Fetal VDJ Genes 155

V_H Gene Usage in Fetal VDJ Gene Repertoire 156

V_H Promoter Regions 169

IV. DISCUSSION 176

REFERENCES 200

VITA 229

LIST OF TABLES

Table Page

1. Probes used in this study and their origins 74

2. PCR primers used in this study 76

3. The hybridization patterns of probe A and pJ5 probe for each possible status of IgH gene on the unexpressed allele 106

4. Summary of Southern analysis of B cell hybridomas derived from rabbit 363H1#2 113

5. Summary of Southern analysis of B cell hybridomas derived from rabbit 27H1 116

6. Summary of Southern analysis of B cell hybridomas derived from rabbit 196G2 120

7. Summary of Southern analysis of B cell hybridomas derived from rabbit 64J2 123

8. Distribution of DJ, VDJ genes and germline V_H, D and J_H gene segments on the unexpressed allele of 29 rabbit hybridomas 133

9. Frequency of V_H gene usage in fetal VDJ gene repertoire 170

LIST OF FIGURES

Figure Page

1. Maps of the mouse IgH locus 12

2. Map of human IgH locus on chromosome 14 16

3. Maps of the rabbit IgH locus 22

4. Diagram of IgH gene rearrangement products 26

5. Schematic representation of the location of PCR primers used in the nested-primer PCR 79

6. A partial map of the V, D and J chromosomal region from the a1 (C haplotype), a2 (E haplotype) and a3 (G haplotype) allotype rabbits 86

7. Quantitative Southern analysis of EcoRI/HindIII-restricted DNA for germline J_H in a1+ B cells of rabbit number 35G 88

8. Quantitative Southern analysis of EcoRI/HindIII-restricted DNA for germline J_H in a1+ B cells of rabbit number 138G6 92

9. Quantitative Southern analysis of BamHI-restricted DNA for probe A region in a1+ B cells from rabbit number 147J5 96

10. Quantitative Southern analysis of BamHI-restricted DNA for probe A region in a1+ B cells from rabbit number 147J6 99

Figure Page

11. Quantitative Southern analysis of HindIII-restricted DNA for probe A region in sIg+ B cells from rabbit number 64H1 102

12. Genomic analysis for the status of IgH genes in the fusion partner using probe A and pJ5 probe 108

13. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 363H1 using pJ5 probe 111

14. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 27H1 using pJ5 probe 114

15. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 196G2 using pJ5 probe 117

16. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 64J2 using pJ5 probe.. 121

17. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 363H1 using probe A 124

18. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 27H1 using probe A 126

19. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 196G2 using probe A 128

20. Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 64J2 using probe A. 131

21. Nucleotide sequence of the region immediately upstream of J_H locus 135

Figure Page

22. PAGE analysis of the PCR products from PCR amplification for DJ genes from the indicated hybridomas 138

23. Comparison of the nucleotide sequences of four PCR-amplified DJ genes with those of germline D and JH genes 141

24. Genomic analysis for the status of IgH genes in the 14 rabbit T cell lines using D1 probe 143

25. Genomic analysis for the status of IgH genes in the 14 rabbit T cell lines using pJ5 probe 146

26. PAGE analysis of the PCR products from PCR amplification for D2bJ and VD2b genes from rabbit splenic DNA 149

27. Comparison of the nucleotide sequences of the DJ genes isolated from splenic DNA with those of germline D2b and JH genes 151

28. Comparison of the nucleotide sequences of the VD2b genes isolated from splenic DNA with those of germline V_H1-*a3* and D2b genes 153

29. PAGE analysis of nested-primer PCR for fetal VDJ gene 157

30. Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 14 fetuses with germline V_H1-*a3* or V_H2-*a3* 159

31. Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 15 fetuses with germline V_H1-*a2* 161

32. Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 21 fetuses with germline V_H1-*a3* or V_H2-*a3* 163

Figure Page

33. Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 24 fetuses with germline V_H1-*a1* or V_H1-*a3* 165

34. Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 28 fetuses with germline V_H1-*a3* or V_H2-*a3* 167

35. Comparison of the nucleotide sequences of the promoter region of five germline V_H genes 172

36. Schematic representation of the "narrow window for rearrangement" model 181

37. Schematic representation of the "inefficient double-strand breaks repair" model 184

38. Schematic representation of the "suppression and desuppression" model... 187

39. Schematic representation of the "inactivation of one IgH allele" model 191

40. Comparison of the RSSs of all several functional germline V_H genes 195

LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AMuLV	Abelson Murine Leukemia Virus
bp	base pair
CD40L	CD40 ligand
D	D gene of heavy chain gene
EBV	Ebstein-Barr Virus
g	gram
HAT	Hypoxanthine-Aminopterin-Thymidine
HBSS	Hank's balance salt solution
hr	hour
Ig	Immunoglobulin
IgH	Heavy chain of immunoglobulin

J_H	J gene of heavy chain gene
kb	kilobase
LTBM	Long term bone marrow culture
μg	microgram
mg	milligram
μl	microliter
ml	milliliter
mAb	monoclonal antibody
MLN	Mesenteric lymph node
mM	millimolar
PCR	Polymerase chain reaction
RAG2	Recombination Activating Gene 2
TCR	T cell receptor
V_H	V gene of heavy chain gene

ABSTRACT

The status of gene rearrangement on the unexpressed IgH allele in rabbit B cells was studied by three approaches: quantitative Southern analysis of polyclonal B cells, Southern analysis of B cell hybridomas and PCR using appropriate combinations of V_H, D and J_H primers. Quantitative Southern analyses for the amount of germline J_H and V_H1 were performed with DNA of polyclonal rabbit B cells purified by fluorescence-activated cell sorter or panning. For the quantification of germline J_H, two independent quantifications showed that 75% to 95% of J_H on the unexpressed allele of rabbit B cells was in germline configuration. The amount of germline V_H1 was quantified indirectly by measuring the germline DNA between V_H1 and D, DNA that is deleted following V_H rearrangement. Three independent quantifications suggested that 48% to 98% of the germline region was present in rabbit B cells.

To confirm the finding that many rabbit B cells do not rearrange J_H and V_H1 on the unexpressed allele, clonal population of rabbit B cells were analyzed. These cells were obtained as hybridomas that resulted from fusion of rabbit spleen cells with a rabbit fusion partner. The status of the unexpressed IgH allele of 29 hybridomas were analyzed by Southern analysis using probe A, derived from the germline region between V_H1 and

the most upstream D gene, and J_H probe. Fifty percent of rabbit B cell hybridomas were found to have the unexpressed allele in germline configuration. Forty percent had rearranged DJ genes and the remainder (10%) had rearranged VDJ genes. The results of the quantitative Southern analysis of polyclonal B cells and the Southern analysis of the hybridomas together indicate that, unlike in other species, DJ genes do not occur on both IgH alleles before VDJ gene rearrangement can occur and that many B cells have unrearranged IgH genes on the unexpressed allele. Furthermore, only 10% of the hybridomas had two VDJ genes. One of these must be productive while the other is presumably nonproductive. This suggests that nonproductive VDJ genes are infrequently found in rabbit B cells.

The intermediates of IgH gene rearrangements in rabbit B cells were analyzed by PCR. DJ genes were demonstrated in the hybridomas as well as in splenocytes by PCR using 5'D and J_H primers. In addition to the DJ genes, VD genes were found in splenocytes by PCR using V_H and 3'D primers. The fact that many DJ and VD genes were isolated from splenic DNA suggests that both may be intermediates of IgH gene rearrangements although this possibility has not been persued in this study.

I sought to explain why nonproductive VDJ genes are infrequently found in rabbit B cells. The explanation can be that nonproductive VDJ genes are rarely generated in rabbits or that only one IgH allele can be rearranged to a VDJ gene. I excluded the first possibility by showing that many rearrangements in rabbit fetuses resulted in nonproductive VDJ genes. Therefore, I conclude that only one IgH allele in rabbit B cell

progenitors can be rearranged to a VDJ gene.

The analysis of V_H gene usage in the nonproductive VDJ genes showed that most of them had utilized V_H1. This result indicates that the preferential utilization of V_H1 resulted from its preferential rearrangement. I tested for the possibility that the promoter of V_H1 may be responsible for its preferential rearrangement by searching for any prominent difference between the promoter of V_H1 and those of other V_H genes. I found that the core transcriptional factor motifs were similar in all V_H promoters examined and therefore, the activity of the V_H1 promoter probably does not explain the preferential rearrangement of V_H1.

INTRODUCTION

Much of our knowledge of Ig structure and genetics was obtained from studies of rabbit Ig. In the 1960s, Todd (1963) and Feinstein (1963) reported the study of the inheritance and molecular localization of the allotypic specificities in the V_H and C_H regions of rabbit Ig, the study which led to the revolutionary idea that defied the "one gene one polypeptide" dogma (Dreyer and Bennett, 1965, Lennox and Cohn, 1967). Furthermore, allelic exclusion in plasma cells was first discovered in rabbit by Pernis *et al.* (1965) and Cebra *et al.* (1966). Other studies of rabbit Ig which contributed to our knowledge of Ig gene structure include the linkage of genes encoding the V and C regions (Dubiski, 1969, Mandy and Todd, 1970), the *cis*-association of V and C region genes (Landucci-Tosi *et al.*, 1970, Kindt *et al.*, 1970) and the high frequency of germline recombination which occurs between V_H and C_H genes (Mage *et al.*, 1971, Kindt and Mandy, 1972, Mage *et al.*, 1982, Kelus and Steinberg, 1991).

Rabbit immunoglobulin (Ig) is unique in that it has allotypic specificities not only in the constant (C) regions but also in the variable (V) regions of the heavy chains. For many years the allelic inheritance of the allotypes in the V_H region, V_Ha1, V_Ha2 and V_Ha3 allotypes, was the most perplexing aspect of rabbit Ig. Because 80%-90% of serum Ig molecules in rabbits bear the V_Ha allotypes and because the rabbit genome contains more than 100 V_H genes, most of the germline V_H genes must encode the V_Ha allotypic specificities assuming that many of these germline V_H genes are used to generate the VDJ gene repertoire. The question was then how was the rabbit V_H chromosomal region protected from meiotic recombination which would shuffle the V_H genes encoding the

different allotypes, such that the allelic inheritance of the allotypes would be lost. The answer to this puzzle is rather simple. Knight and Becker (Knight and Becker, 1990) found that 80%-90% of rabbit B cells preferentially utilized only one V_H gene, V_H1, the gene which encodes a prototypic V_Ha molecule. Therefore, the recombination event would not be detected phenotypically even if meiotic recombination occurred within the V_H locus of different alleles, which most likely it had. Up until now, the molecular mechanism for the preferential utilization of V_H1 is unknown.

Although rabbit Ig seized the early attention of immunochemists, whose motivation led to many studies on rabbit Ig which contributed significantly to the advance in our knowledge of Ig structure and genetics, the molecular study of rabbit IgH gene lags behind those of mouse and human. In this study, two aspects of IgH gene rearrangement in rabbit B cells were studied. The first aspect is how rabbit B cell rearranges its IgH gene. I approached this issue by examining the status of the IgH gene on the unexpressed allele in polyclonal and monoclonal rabbit B cells. The second aspect is whether the preferential utilization of V_H1 resulted from preferential rearrangement. This issue was approached by analyzing the V_H gene usage in nonproductive VDJ genes. Here, I report the findings.

CHAPTER I

LITERATURE REVIEW

The humoral immune system, mediated by the B cell compartment, plays an important part in immune response by producing antibodies (Abs) against foreign antigens (Ags). The hallmark of this response is that Abs can be generated against a practically infinite number of antigenic determinants. This raised a unique genetic mystery about how B cells generate such a large repertoire using the genome with limited coding capacity. Thanks to the discovery of restriction enzymes and the advent of molecular biology techniques, this mystery was solved in mid 1970s representing a major achievement in immunogenetics and marking the beginning of molecular B cell biology.

Because Ab is the effector of the humoral immunity, understanding how the Ab repertoire develops is crucial to our understanding of the humoral immune system. The discovery of somatic gene rearrangement, the process by which B cells generate the Ab repertoire, has raised many questions, for example, what is the enzymology of the recombinase, how the recombinase performs the gene rearrangement, what is the pattern of gene usage in the Ab repertoire and what is the mechanism that contributes to the tissue specificity of the rearrangement process. In the 20 years since the discovery of the somatic gene rearrangement, we have been accumulating information regarding the gene rearrangement process, some of which will be presented here. This literature review will summarize the basic structure of Ab molecules and the discovery of somatic gene rearrangement. Then I will discuss the organization of IgH gene organization, the process

of IgH gene rearrangement and the V_H gene utilization in three species, mouse, human and rabbit.

Basic Structure of Ab Molecules

Each Ab or immunoglobulin (Ig) monomer is made of four polypeptide subunits: two identical heavy chains (H-chains) and two identical light chains (L-chains) (reviewed in Cohen and Milstein, 1967, Potter and Lieberman, 1967). Much of the data regarding the basic structure of Ig molecules were also obtained from studying the homogeneous Ig secreted by plasmacytomas, the tumors of Ab secreting cells. Some of these proteins were of H-chain type and some were of L-chain type. From extensive analysis of the amino acid sequences of these myeloma proteins, it became clear that the amino-terminal regions of both H- and L-chains were highly variable while the carboxy-terminal regions were relatively constant. Thus each H-chain and L-chain could be divided into two regions, variable (V) and constant (C) regions (reviewed in Kabat *et al.*, 1991).

The variable regions, as the name implied, are different among different H-chains or L-chains. No two identical V regions have ever been found among the myeloma proteins. The constant regions of H-chains can be typed into five major isotypes, μ, δ, γ, ε and α. For L-chain, two major C regions were described, κ and λ. Some major isotypes could be further divided into subisotypes. For example, the Cγ region in mouse could be divided into 4 subisotypes, Cγ1, Cγ2a, Cγ2b and Cγ3.

At the end of 1960's, even before the studies of myeloma protein, papain and pepsin digestion studies of polyclonal Ab have provided the scientists the first glimpse of how an Ab molecule is organized (Porter, 1958, Nisonoff and Woernley, 1959, Nisonoff *et al.,* 1960). From these studies, an Ab molecule can be divided into two parts. One part is responsible for the Ag binding. This part is subsequently found that it consists of the

variable region and part of constant region (reviewed in Cohen and Milstein, 1967, Capra and Kehoe, 1975). The other part does not bind Ag but bears the antigenic specificity found in all Ig molecules and consists of only the constant region. (reviewed in Cohen and Milstein, 1967, Capra and Kehoe, 1975).

Discovery of Somatic Gene Rearrangement

Generation of Ab Diversity via V(D)J Gene Rearrangement

Because myeloma proteins with different V regions could be found in association with identical C region, the question was raised as how the H- and L-chains were encoded in the germline. According to the "one gene, one polypeptide" dogma, there was one gene in the germline for each different H- and L-chains. Because many different H- and L-chains must be generated to form the Ab repertoire, the genome required to encode all the possible H and L chain would be much larger than the genome of any organism. A compromising theory between these two facts was proposed in 1965 by Dryer and Bennett (1965).

Dryer and Bennett proposed that the V and C regions were encoded by two separate genes and that there were many V genes but only one gene for each of the C regions. During B cell genesis, one of the "pre-made" V genes in the germline V gene repertoire was selected and rearranged to the C gene to form an expressible gene. This proposal was revolutionary and remained hypothetical until a decade later when the first evidence of such somatic gene rearrangement was reported.

In mid 1970s, the discovery of restriction enzymes permitted the examination of Ig genes directly. Hozumi and Tonegawa (1976) tested whether the κ gene underwent gene rearrangement in κ producing myeloma. They performed solution hybridization of BamHI-digested genomic DNA from either a κ-producing plasmacytoma cell line or

BALB/c embryo using κ mRNA as probe. They found that in the embryonic configuration, the Vκ and Cκ genes were located on two different BamHI fragments but in MOPC321, both Vκ and Cκ genes were rearranged such that they were found on the same BamHI fragment. This experiment described for the first time the evidence to support the somatic gene rearrangement theory of Dryer and Bennett. What Dryer and Bennett had not predicted was that the V regions were not pre-made and encoded in the germline. But rather, they were generated by the gene rearrangement itself.

At the end of the 1970s, gene cloning techniques were available and many germline V and C genes of H-, κ- and λ- loci were cloned (Tonegawa *et al.*, 1977, Tonegawa *et al.*, 1978, Seidman *et al.*, 1978, Seidman and Leder, 1978, Brack *et al.*, 1978, Early *et al.*, 1980, Sakano *et al.*, 1980). It was then apparent that part of the V region as defined by the amino acid sequence analysis of the myeloma proteins was not encoded in the cloned germline V_H and V_L genes (Seidman *et al.*, 1978). The repetitive occurrence of the same missing sequence in different myeloma proteins suggested that they were encoded by another germline gene separated from the V genes. By gene cloning, these missing parts were shown to be encoded by germline genes, D (for diversity) and J (for joining). For L-chain, only J genes encoded for the missing parts (Seidman *et al.*, 1978, Sakano *et al.*, 1979, Max *et al.*, 1979). In the case of H-chain, the missing parts were encoded by both D and J genes (Early *et al.*, 1980, Sakano *et al.*, 1980, Sakano *et al.*, 1981). During B cell development then, one of each of the V, (D) and J genes rearranged to generate a functional expressible V_H and V_L genes. To generate a V_L gene, only one rearrangement is required, V to J, while the generation of V_H gene requires two separate rearrangements, V to D and D to J.

Recombination Signal Sequence

The comparison of nucleotide sequences of several germline V, D and J genes revealed a conserved DNA motif adjacent to all genes, at every locus known to undergo rearrangement (Tonegawa *et al.*, 1977, Tonegawa *et al.*, 1978, Seidman *et al.*, 1978, Seidman and Leder, 1978, Brack *et al.*, 1978, Sakano *et al.*, 1979, Max *et al.*, 1979, Early *et al.*, 1980, Sakano *et al.*, 1980, Ramsden *et al.*, 1994). This DNA motif was named Recombination Signal Sequence (RSS) because of its potential involvement in the rearrangement process. The motif consists of two stretches of conserved sequences separated by non-conserved sequence of either 12-bp or 23-bp in length (Tonegawa *et al.*, 1977, Tonegawa *et al.*, 1978, Seidman *et al.*, 1978, Seidman and Leder, 1978, Brack *et al.*, 1978, Max *et al.*, 1979, Early *et al.*, 1980, Sakano *et al.*, 1980, Ramsden *et al.*, 1994). The first conserved stretch is a heptamer, whose consensus sequence is a dyad symmetric CACAGTG and is found immediately adjacent to the coding elements. The second conserved stretch is a nonamer whose consensus sequence is ACAAAAACC. This nonamer is less-conserved than the heptamer. The consensus sequence of RSS and the frequency of occurrence of the consensus sequence at each position in the heptamer and nonamer are shown below (Ramsden *et al.*, 1994, Hesse *et al.*, 1989).

Heptamer								Nonamer								
C	A	C	A	G	T	G	....	A	C	A	A	A	A	A	C	C
100	**99**	**99**	87	82	85	76		72	86	83	73	**91**	**97**	87	84	76

Blackwell *et al.* (1984) and Lewis *et al.* (1984) made a major contribution to the studies of V(D)J gene rearrangement when they showed that pre-B cell lines could rearrange artificial rearrangement substrates. This finding permitted the analysis of factors or sequences which influence somatic rearrangement simply by testing the rearrangement ability of artificial substrates containing these factors in pre-B cell lines. Akira *et al.*

(1987) and Hesse *et al.* (1987) directly tested the involvement of RSS in the rearrangement process. They constructed several rearrangement substrates, each contained different combinations of RSS and found that the rearrangement could occur in the substrate with only one pair of RSSs. No sequence from the coding region was required. This result suggested that, indeed, the RSS was important in the rearrangement process. Upon examination of the arrangement of the RSS in germline V, D and J genes, Early *et al.* (1980) proposed that the rearrangement could occur only between the gene with a 12-bp RSS and the gene with a 23-bp RSS. Indeed, Akira *et al.* (1987) and Hesse *et al.* (1987) showed that rearrangement of the artificial substrates would only occur between the 12-bp RSS and 23-bp RSS.

The nucleotides in the RSS important for mediating the rearrangement have been studied by determining the ability of artificial rearrangement substrates with mutated RSS to undergo rearrangement in pre-B cell lines. Two different approaches were taken to measure the extent of rearrangement of the substrates with essentially the same result. One approach employed an *in vitro* recombination assay in which the rearrangement substrate was transiently transfected into pre-B cell line. After 48-76 hr, the rearrangement substrate was then recovered and the frequency of rearrangement was determined (Hesse *et al.*, 1989). The other approach was to determine the frequency of rearrangement of a stably integrated rearrangement substrate mediated by a retroviral vector in pre-B cells (Akamatsu *et al.*, 1994). Both approaches showed that mutations in the heptamer sequence were less tolerable than mutations in the nonamer. The most important nucleotides were the first three nucleotides of the heptamer where any single mutation resulted in abrogation of rearrangement. The most important nucleotides in the nonamer were the fifth and sixth adenine residues but rearrangement substrates containing single mutations at one of these two bases still underwent rearrangement at appreciable frequency. These results complemented the finding that the first three bases

of the heptamer and at the fourth, fifth and sixth bases of the nonamer are most conserved (Ramsden *et al.*, 1994, Hesse *et al.*, 1989).

V_H, D and J_H Genes Organization

This section of the review will summarize how the V_H, D and J_H genes are organized in the IgH locus of three species, mouse, human and rabbit. The organization of IgH locus in mouse, human and rabbit is similar in that the V_H, D, J_H and C_H genes are linked on the same chromosome. The most upstream region of the IgH locus is the V_H locus which generally contains >100 genes. The next regions in the 5' to 3' order are the D, J_H and C_H loci. The D locus contains as few as 11 D genes to as many as 30 D genes. Downstream of the D locus in all three species is the J_H locus which resides less than 90 kb downstream of V_H regions and contains 4 to 9 J_H genes. The C_H region is the most downstream region of the IgH locus residing 3' to the J_H locus and contains between 8-16 C_H genes.

The germline V_H genes are generally encoded in two exons. The 5' exon encodes for most of the leader region. The 3' exon is approximately 350 bp long with 23-bp RSS at the 3' end and encodes for the rest of the V_H gene. The V_H genes with more than 80% similarity are placed in the same V_H families. Initially, the categorization was based on the similarity in amino acid sequences of many myeloma proteins. Later, advances in molecular biology made the nucleotide sequence easily obtainable and therefore, the categorization is now based on the similarity of the nucleotide sequences between V_H genes (Brodeur *et al.*, 1988, Dildrop, 1984, Kabat *et al.*, 1991). It is also possible to place an unknown V_H gene to a described V_H family by its ability to cross-hybridize with a V_H-family specific probe. This type of grouping provides a fast method to place unknown V_H genes in a particular V_H family.

The categorization of V_H genes into several families has an implication on the study of V_H gene evolution (Tutter and Riblet, 1989, Schroeder *et al.*, 1990). On the basis of cross-species hybridization with family-specific V_H probes, the human V_H3 family, the mouse V_H7183 and S107 families, and the only rabbit V_H family, were found to be analogous (Tutter and Riblet, 1989). These families of V_H genes are thought to represent the primordial V_H gene because they are the only V_H families found in every species examined so far (Tutter and Riblet, 1989). In fact, the rabbit V_H genes are so similar to the primordial V_H gene that antibodies to the allotypic determinants in the variable region of rabbit Ig cross-react with V_H regions of Ig from several species, including human, shark, and toad, which are evolutionary distant from rabbit (Knight *et al.*, 1975, Rosenshein and Marchalonis, 1985).

Germline D genes are characterized by a 10-30 bp coding region flanked by two 12-bp RSS's on both 5' and 3' ends. The D genes could also be grouped into families based on cross-hybridization with germline D genes. Three to seven D families in mouse, human and rabbit were described based on such hybridization. In human, non-conventional D genes were also identified. These D genes, named DIR genes, are characterized by a rather long coding region (>120 bp long) and are flanked on both sides with multiple RSS's. Six DIR genes were described in human so far.

The J_H genes are approximately 30 bp long with one 23-bp RSS on the 5' end. Four to nine J_H genes were described in mouse, human and rabbit. Downstream of J_H region, eight to 16 C_H genes were identified. The C_H locus in mouse, human and rabbit all spans over 200 kb of DNA.

The V_H, D and J_H Genes Organization in Mouse

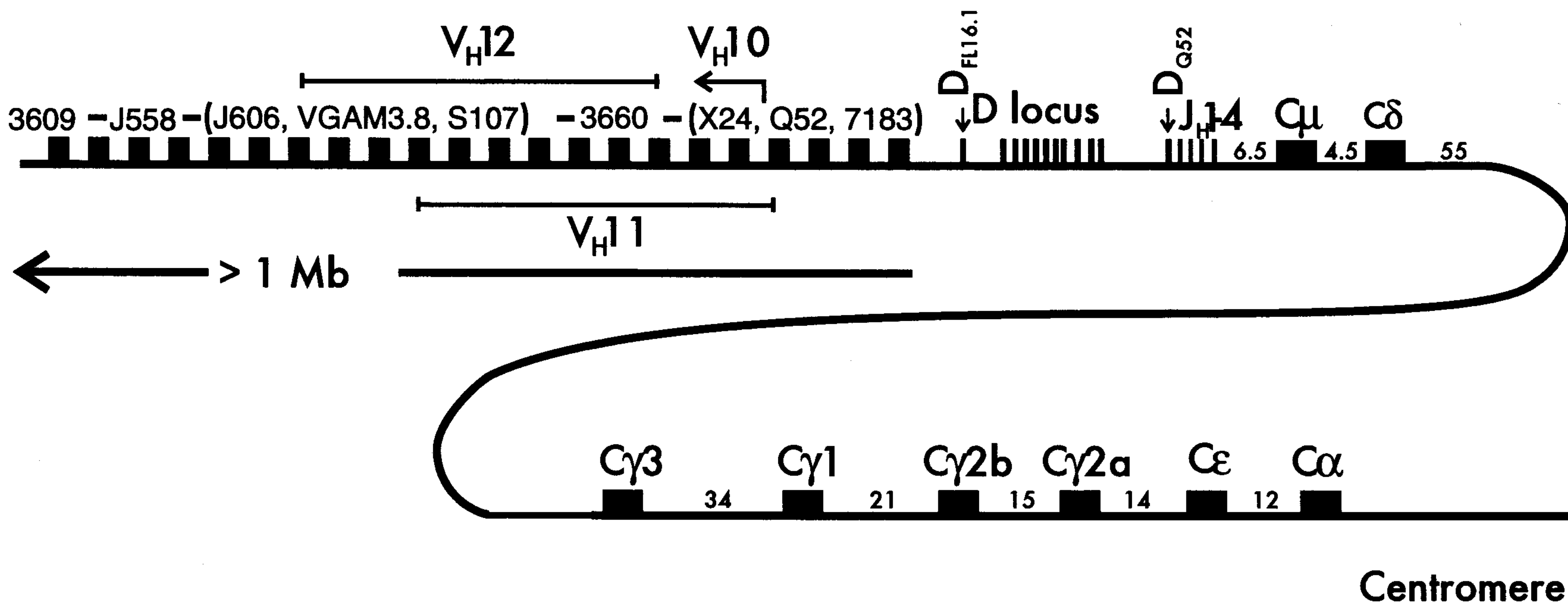
Mouse IgH locus mapped to chromosome 12 (Hengartner *et al.*, 1978) in the telomere—V_H—D—J_H—C_H—centromere orientation (Erikson *et al.*, 1985, Brueckner *et*

al., 1989). The locus contains approximately 100-1,000 germline V_H genes (Brodeur and Riblet, 1984, Livant *et al.*, 1986), 12 D genes (Sakano *et al.*, 1981, Kurosawa and Tonegawa, 1982, Wood and Tonegawa, 1983), 4 J_H genes (Gough and Bernard, 1981, Lai *et al.*, 1989) and 8 C_H genes (Tucker *et al.*, 1980, Roeder *et al.*, 1981, Shimizu *et al.*, 1981, Nishida *et al.*, 1981, Shimizu *et al.*, 1982). Most of the studies of IgH gene organization were performed in the BALB/c strain and its IgH locus is presented in Figure 1. The following discussion refers to the findings in BALB/c mice except where noted.

The V_H locus spans over 1 Mb of DNA (Tutter and Riblet, 1989) on chromosome 12 (Hengartner *et al.*, 1978). Unlike human, no V_H gene located outside this IgH locus has been described. From published germline V_H genes, approximately 30% of them are pseudogenes (Lai *et al.*, 1989). These V_H genes could be divided into 13 V_H families based on their similarity. These families are J558, VGAM3. 8, 3660, Q52, 7183, X24, J606, S107, 3609, V_H10, V_H11, V_H12 and V_H13 (Dildrop, 1984, Brodeur and Riblet, 1984, Brodeur *et al.*, 1984, Winter *et al.*, 1985, Kroemer *et al.*, 1987, Reininger *et al.*, 1988, Kofler, 1988, Lai *et al.*, 1989, Pennell *et al.*, 1989, Hardy *et al.*, 1989, Kirkham *et al.*, 1992). Most of the V_H families were named after the plasmacytomas or hybridomas from which the V_H genes were first described. Only recently, beginning with the V_H10 family, the V_H families were named numerically reflecting the order of their discovery.

Each V_H family has different degree of complexity (namely, number of V_H genes in the family) as determined by Southern hybridization of genomic DNA with V_H-family specific probes. The V_HJ558 family is the most complex family consisting of 45% of the germline V_H genes (Brodeur and Riblet, 1984) and is located at the 5' end of the V_H locus Brodeur *et al.*, 1988). The V_H3609, V_HQ52 and V_H7183 families are the next most complex families, each consisting of approximately 10% of the germline V_H genes

Figure 1 Maps of the mouse IgH locus (not to scale). The V_H families are shown in their germline order (Brodeur and Riblet, 1984, Brodeur *et al.*, 1988, Lai *et al.*, 1989). Some of the V_H families are clustered while the others are interspersed. The latter is shown in parentheses. The V_H10, V_H11 and V_H12 families have not been mapped in detail and their approximate positions are shown. Only one D locus has been described in mouse. The germline order of the D genes is $D_{FL16.1}$, $D_{SP2.3}$, $D_{SP2.4}$, ($D_{SP2.1}$, $D_{SP2.5}$, $D_{SP2.6}$, $D_{SP2.9}$), $D_{SP2.2}$, $D_{SP2.7}$, $D_{SP2.8}$ and D_{Q52} (Sakano *et al.*, 1981, Kurosawa and Tonegawa, 1982, Lai *et al.*, 1989). The relative order of the D genes in parenthesis is not known. The location of J_H and C_H genes is also shown (Tucker *et al.*, 1980, Gough and Bernard, 1981, Roeder *et al.*, 1981, Shimizu *et al.*, 1981, Nishida *et al.*, 1981, Shimizu *et al.*, 1982, Christoph and Krawinkel, 1989.). The numbers between the C_H genes indicate the distance in kb between the flanking genes.



Telomere
VH12
VH10
VH11
3609 –J558 –(J606, VGAM3.8, S107) –3660 –(X24, Q52, 7183)
DFL16.1
D locus
DQ52
JH1-4
Cμ
Cδ
6.5
4.5
55
> 1 Mb
Cγ3
Cγ1
Cγ2b
Cγ2a
Cε
Cα
34
21
15
14
12
Centromere

(Brodeur and Riblet, 1984). The V_HQ52 and V_H7183 families are the 3'-most V_H families in all the mice studied so far (Lai *et al.*, 1989). The rest of the V_H families are small and each consists of 1% -5% of the V_H genes. The V_HS107 family mapped to the middle of the V_H locus and is used to demarcate the 3' V_H families from the 5' V_H families. Using this guideline, the V_H7183 and V_HQ52 families are considered 3' families while V_HJ558 family is considered a 5' family.

The organization of V_H families in other inbred mice is similar to that of BALB/c (Lai *et al.*, 1989), although restriction fragment length polymorphism (RFLP) exists (Brodeur *et al.*, 1984, Atkinson *et al.*, 1993). All polymorphisms of the V_H locus among inbred mouse strains appeared to have occurred by recombination events rather than deletion or duplication of the locus (Tutter and Riblet, 1988). As a result of such recombination, V_H loci from 74 inbred strains could be typed into 13 haplotypes by RFLP (Brodeur *et al.*, 1984, Tutter and Riblet, 1988). However, such haplotype nomenclature was not generally used because almost all the studies regarding the V_H locus were performed in BALB/c mice.

The 3'-most functional V_H gene in BALB/c mouse is a member of the V_H7183 family, V_H81X (Yancopoulos *et al.*, 1984). This V_H gene was used preferentially in fetal and neonatal B-lineage cells in mouse (see V_H gene utilization section). Christoph and Krawinkel (1989) reported of a VGAM3. 8-like gene located in the D region. However, upon detailed deletion mapping analysis, no such gene was found (Atkinson *et al.*, 1993).

Unlike human, only one D region has been described in mouse. This region is located between the V_H and J_H regions spanning approximately 80 kb of DNA (Sakano *et al.*, 1981, Kurosawa and Tonegawa, 1982, Lai *et al.*, 1989). Three D families, D_{SP2}, D_{FL16} and D_{Q52}, were found at this D locus. The D_{SP2} family has 9 members which spread out over 62 kb of DNA (Kurosawa and Tonegawa, 1982). The D_{FL16} family has 2 members,

one of which is the 5'-most D gene (Kurosawa and Tonegawa, 1982). The last D family, D_{Q52}, has only one member and is located only 700 bp 5' of the J_H.

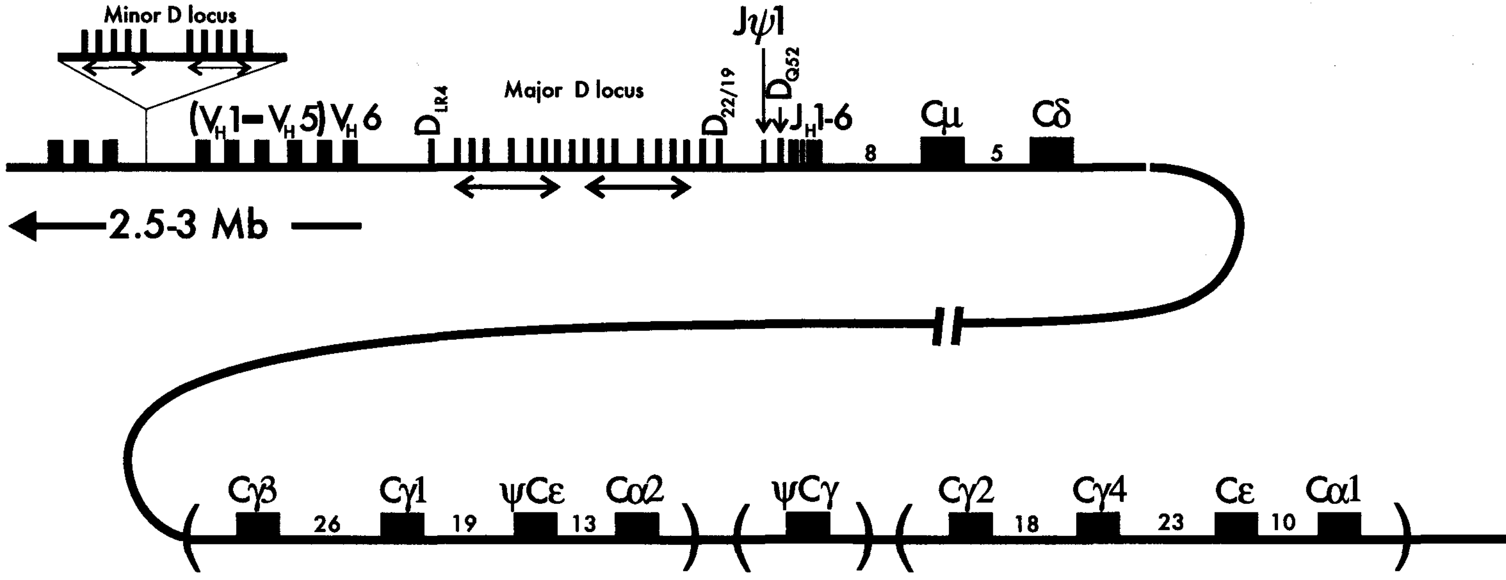
The J_H region is located 90 kb 3' of the V_H locus (Christoph and Krawinkel, 1989) and 6. 5 kb 5' of $C\mu$ gene (Gough and Bernard, 1981). Four functional J_H genes were found in this region, J_H1-4, with the most upstream J_H being J_H1. The C_H locus spans over 150 kb of DNA and contains eight C_H genes: $C\mu$, $C\delta$, $C\gamma1$, $C\gamma2a$, $C\gamma2b$, $C\gamma3$, $C\varepsilon$ and $C\alpha$ (Tucker *et al.*, 1980, Roeder *et al.*, 1981, Shimizu *et al.*, 1981, Nishida *et al.*, 1981, Shimizu *et al.*, 1982). The order of the C_H genes and distance between them is shown in Figure 1.

The V_H, D and J_H Gene Organization in Human

The human IgH locus maps to chromosome 14q32. 33 (Kirsch *et al.*, 1982, McBride *et al.*, 1982) in the same orientation in mouse, i. e. telomere—V_H—D—J_H—C_H—centromere (Erikson *et al.*, 1982). The locus contains 100-200 V_H , over 25 D, 9 J_H, 9 C_H and 2 C_H pseudogenes (Pascual and Capra, 1991). The organization of human V_H, D, J_H and C_H loci is presented in Figure 2. Because IgH locus in human is polymorphic (Benger and Cox, 1989, Sasso *et al.*, 1990, Bottaro *et al.*, 1991, Pascual and Capra, 1991, van Dijk *et al.*, 1991, Rubinstein *et al.*, 1994), this organization may vary between individuals.

The major V_H locus spans 2. 5-3 Mb of DNA (Berman *et al.*, 1988, Matsuda *et al.*, 1988, Walter *et al.*, 1990) and contains an estimated 100-200 germline V_H genes. From published V_H sequences, approximately 50% of them are functional (Kodaira *et al.*, 1986, Matsuda *et al.*, 1993). The V_H genes in human could be categorized into 7 families, V_H1-7, based on the order of discovery. The V_H1-3 families were described initially from amino acid sequences of several myeloma proteins (Capra and Kehoe, 1975). Since 1987,

Figure 2 Map of human IgH locus on chromosome 14 (not to scale). The 3'-most V_H gene is the only member of the V_H6 family (Schroeder *et al.*, 1988). Upstream of it are members of the V_H1-V_H5 families which are highly interspersed (Berman *et al.*, 1988). The major and minor D loci are shown. The repeating units within both D loci are indicated by double-headed arrows. In the major D locus, the order of the D genes from 5' to 3' is D_{LR4}, D_{XP4}, D_{A4}, D_{K4}, D_{N4}, DIR1, D_{M1}, D_{LR1}, D_{XP1}, $D_{XP'1}$, D_{A1}, D_{K1}, D_{N1}, DIR2, D_{M2}, D_{LR2}, D_{LR3}, $D_{22/19}$ and D_{Q52} (Matsuda *et al.*, 1988, Buluwela *et al.*, 1988, Ichihara *et al.*, 1988, Ravetch *et al.*, 1981). In the minor D locus, the order of the D genes is D_{M5a}, D_{LR5a}, D_{XP5a}, D_{A5a}, D_{K5a}, D_{M5b}, D_{LR5b}, D_{XP5b}, D_{A5b} and D_{K5b} (Matsuda *et al.*, 1988, Matsuda *et al.*, 1990). The 5' and 3' orientation of the D genes in the minor D lcus is not known. Nine J_H genes were found in the following order: $J\psi1$, J_H1, J_H2, $J\psi2$, J_H3, J_H4, J_H5, $J\psi3$ and J_H6 (Schroeder *et al.*, 1988). The D_{Q52} gene is located between the $J\psi1$ and J_H1 genes. The relative germline order of C_H genes is shown. The germline order of all the C_H genes is as shown although the C_H genes in brackets are not yet physically linked (Ravetch *et al.*, 1981, Flanagan and Rabbitts, 1982, Lefranc *et al.*, 1982, Chaabani *et al.*, 1985, Bottaro *et al.*, 1991, Pascual and Capra, 1991). The numbers between the C_H genes indicate the distance in kb between the flanking genes.


Telomere
Minor D locus
Major D locus
Jψ1
D_{LR4}
$D_{22/19}$
D_{Q52}
$(V_H1-V_H5)\ V_H6$
J_H1-6
8
Cμ
5
Cδ
2.5-3 Mb
Cγ3
26
Cγ1
19
ψCε
13
Cα2
ψCγ
Cγ2
18
Cγ4
23
Cε
10
Cα1
Centromere

V_H genes with less than 80% similarity to V_H1-3 at the nucleotide level were isolated and they were designated V_H4-7 (Berman *et al.*, 1988, Pascual and Capra, 1991, Rubinstein *et al.*, 1994). Unlike mouse V_H families, the V_H families in human are highly interspersed among each other (Berman *et al.*, 1988). The largest V_H family is the V_H3 family comprising 38% of the germline V_H genes (Berman *et al.*, 1988, Walter *et al.*, 1990). However, this family is larger than the V_H1 family by only a few genes (Berman *et al.*, 1988, Walter *et al.*, 1990). The other V_H families are small, comprising 1% to 10% of the germline V_H genes. The smallest family is the V_H6 family having only one member, identified as the 3'-most V_H gene (Schroeder *et al.*, 1988). Most of the V_H genes in human are polymorphic, as demonstrated by nucleotide sequences of the allelic V_H genes and RFLP (Sasso *et al.*, 1990, Pascual and Capra, 1991, van Dijk *et al.*, 1991, Rubinstein *et al.*, 1994). In contrast to the polymorphism in the mouse V_H locus which were generated mostly by recombination events (Tutter and Riblet, 1988), the polymorphisms in the human V_H locus appeared to have occurred through gene duplication and deletion events (Sasso *et al.*, 1990, Pascual and Capra, 1991, van Dijk *et al.*, 1991, Rubinstein *et al.*, 1994). However, the single-member V_H6 family appeared to be non-polymorphic (Schroeder *et al.*, 1988). Honjo's laboratory had analyzed the 3'-most 0.8 Mb region of the V_H locus. They identified 64 germline V_H genes (Kodaira *et al.*, 1986, Matsuda *et al.*, 1993, Matsumura *et al.*, 1994) and confirmed the previous finding that the V_H genes from different families were highly interspersed (Berman *et al.*, 1988, Buluwela *et al.*, 1988).

Not all V_H genes in human are located on chromosome 14. Matsuda *et al.* (1990) had performed Southern analysis of DNA from a panel of mouse-human hybrids and found V_H genes on human chromosome 15. They named these V_H genes orphons. Cherif and Berger (1990) had identified additional orphons on human chromosome 16 by *in situ* hybridization. Four orphon V_H genes on chromosome 15 were cloned and were found to belong to V_H1 and V_H3 families (Matsuda *et al.*, 1990). The members of the V_H3 family

appeared functional and could potentially be rearranged to generate VDJ genes. However, whether these orphons are associated with their own D, J and C_H genes and whether they undergo gene rearrangement is currently unknown.

In contrast to mouse and rabbit, D genes in human are found in at least 2 loci, major and minor D loci. The major D locus in human is located between V_H and J_H region on the chromosome 14q32. There are at least 17 D genes identified in this region. These D genes are divided into 7 families, D_{XP}, $D_{A,}$ D_K, $D_{N,}$ D_M, D_{LR} and D_{Q52} (Matsuda *et al.*, 1988, Buluwela *et al.*, 1988, Ichihara *et al.*, 1988, Ravetch *et al.*, 1981). D_{Q52} is the 3'-most D gene located only 25 bp upstream of J_H1 (Ravetch *et al.*, 1981). D_{LR4} gene is the 5' most D gene separated from the most downstream V_H by 20 kb (Matsuda *et al.*, 1988). The other 15 D genes reside between D_{Q52} and D_{LR4} genes. Except for D_{LR3} and $D_{21/9}$ genes, located at the 3' end of the D locus, the other 13 D genes were arranged in two repeating units of 9 kb in length (Buluwela *et al.*, 1988, Ichihara *et al.*, 1988), each unit having 6-7 D genes. These repeating units are shown in Figure 2 as double-headed arrows.

The minor D region was found to be in the V_H region (Matsuda *et al.*, 1988, Matsuda *et al.*, 1990). As in the major D region, the minor D region also exists as two repeating units of approximately 9 kb in length. Each unit contains 5 D genes which belong to the D_{XP}, $D_{A,}$ D_K, , D_M, and D_{LR} families. These two repeating units are physically linked to a V_H pseudogene on the chromosome 14 (Matsuda *et al.*, 1990). The exact location of this minor D region is unknown.

In the course of analyzing the D region, Ichihara *et al.* (Ichihara *et al.*, 1988) had identified two non-conventional D genes in the major D region, DIR1 and DIR2 genes. These genes are located between D_N and D_M in the two 9 kb repeats at the major D region (Ichihara *et al.*, 1988) and are flanked on both sides with multiple 12-bp and 23-bp RSSs.

The coding regions of the DIR genes are rather long, ranging from 130 to 150 bp depending on which RSSs were used. Additional DIR genes, DIR 3-6, are described (Shin *et al.*, 1993, Sanz *et al.*, 1994) but the location of these genes is unknown.

The entire J_H locus is located on a 3 kb region 77 kb downstream of V_H6 (Schroeder *et al.*, 1988), the 3'-most V_H gene, and 8 kb upstream of $C\mu$ gene (Ravetch *et al.*, 1981). In this 3 kb region, nine J_H genes were found. six of which are functional. As in other species, J_H1 was assigned to the most upstream functional J_H gene and J_H6 was assigned to the most downstream functional J_H gene.

The C_H locus on chromosome 14 spans approximately 200 kb (Bottaro *et al.*, 1991). This locus contains 9 C_H genes and 2 pseudogenes (Pascual and Capra, 1991). The organization of C_H genes in human is somewhat similar to that in mouse in that the $C\mu$ and $C\delta$ genes were found at the 5' end of the locus and the $C\varepsilon$ and $C\alpha$ genes were found at the 3' end (Ravetch *et al.*, 1981, Flanagan and Rabbitts, 1982). However, there is a large segment containing multiple C_H genes which has been duplicated in the middle of the C_H locus (Flanagan and Rabbitts, 1982). One cluster contains $C\gamma3$ — $C\gamma1$ —$\psi C\varepsilon1$ — $C\alpha1$ genes (Lefranc *et al.*, 1982) is found upstream of the second cluster which contains $C\gamma4$ — $C\gamma2$ —$C\varepsilon$ —$C\alpha2$ genes (Lefranc *et al.*, 1982). One $\psi C\gamma$ was described and mapped to between $C\alpha1$ and $C\gamma2$ (Chaabani *et al.*, 1985). The human C_H region has yet to be linked by cosmid clones but at least we now know that the entire C_H locus spans less than 210 kb (Bottaro *et al.*, 1991). The diagram of C_H locus is presented in Figure 2.

The V_H, D and J_H Genes Organization in Rabbit

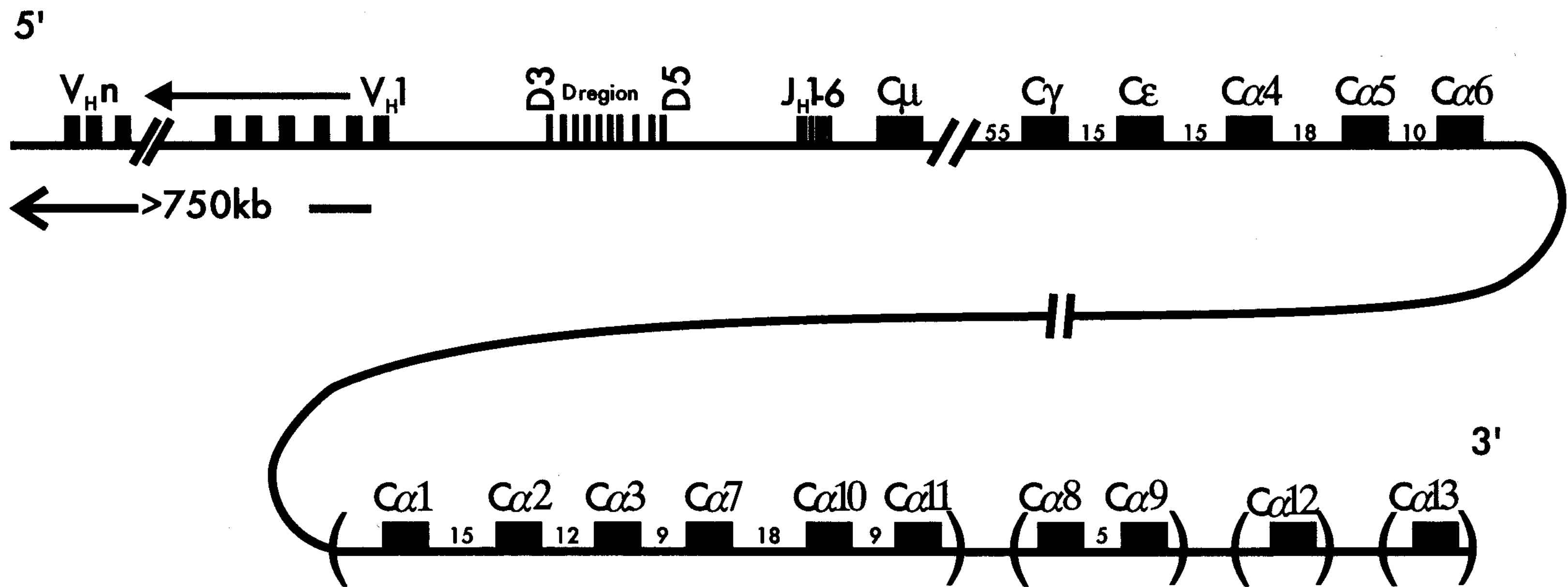
Rabbit IgH locus was reported to be on chromosome 16 (Medrano and Dutrillaux, 1984). The locus contains more than 100 V_H genes (Gallarda *et al.*, 1985, Currier *et al.*, 1988), at least 11 D genes (Becker *et al.*, 1990, Friedman *et al.*, 1994), 6 J_H genes (Becker

et al., 1989, Short *et al.*, 1991) and 16 C_H genes (Knight *et al.*, 1985, Burnett *et al.*, 1987, Burnett *et al.*, 1989). Laboratory rabbits have polymorphism at the V_H and C_H regions in their Ig. Based on the polymorphism at the V_H region, three allotypes were described, a1, a2 and a3. The V_H and J_H loci were studied in all three rabbit allotypes while the D locus was studied mainly in the rabbit of the a3 allotype. Diagram for rabbit V_H locus is presented in Figure 3.

The V_H locus spans over 750 kb of DNA (Currier *et al.*, 1988) and it appears that all V_H genes in rabbit are on a single chromosome (Tunyaplin C. and Knight KL. unpublished data). All rabbit V_H genes belong to a single family based on 80% similarity in nucleotide sequences of about 50 published rabbit germline V_H genes (Bernstein *et al.*, 1985, Currier *et al.*, 1988, Fitts and Metzger, 1990, Knight and Becker, 1990, Roux *et al.*, 1991, Short *et al.*, 1991). Many of the V_H genes on the a3 allele were cloned on overlapping cosmid clones (Currier *et al.*, 1988). These cosmid clones could be grouped into clusters, but many of these clusters are not linked to each other, and hence they do not span the entire V_H region. The V_H genes which were linked to the D region by cosmid or phage clones were given numerical numbers with V_H1 gene being the 3'-most V_H gene.

The entire germline D locus was cloned from a cosmid library construct from DNA of a rabbit of the a3 allotype (Becker *et al.*, 1990). Eleven D gene segments have been identified in this region (Becker *et al.*, 1990, Friedman *et al.*, 1994), and they are grouped into seven families—D1 to D7. The D1 family has four members, D1a-D1d. The D2 family has two members, D2a and D2b. The rest of the families are single-member. Upon analyzing VDJ genes, Friedman *et al.* (1994) found that several VDJ genes had the same D region which did not correspond to any of the known germline D gene segments.

Figure 3 Maps of the rabbit IgH locs (not to scale). The 3'-most V_H gene is V_H1 (Knight and Becker, 1990). Only one D locus has been described in rabbit. The germline order of the D genes is D3, D1a, D4, D1b, D6, D7, D2a, D1c, D2b, D1d and D5 (Becker *et al.*, 1990, Friedman *et al.*, 1994). The location of J_H and C_H genes is also shown (Knight *et al.*, 1985, Burnett *et al.*, 1987, Becker *et al.*, 1989, Burnett *et al.*, 1989). The 13 $C\alpha$ genes are shown in five unlinked clusters. The $C\alpha4$, $C\alpha5$ and $C\alpha6$ genes have been linked to each other and to the the rest of the IgH locus, while the rest of the $C\alpha$ genes have not been linked. Within each unlinked cluster, the $C\alpha$ genes are shown in their germline order. However, the germline order of these unlinked clusters is not yet known.



5'
VHn
VH1
D3
D region
D5
JH1-6
Cμ
Cγ
Cε
Cα4
Cα5
Cα6
55
15
15
18
10
>750kb
3'
Cα1
Cα2
Cα3
Cα7
Cα10
Cα11
Cα8
Cα9
Cα12
Cα13
15
12
9
18
9
5

This suggested that these D regions represented germline D genes that have not yet been identified.

The J_H region is contained within 2 kb of DNA located 63 kb downstream of *VH1* (Becker *et al.*, 1989) and 8 kb upstream of Cμ gene (Knight *et al.*, 1985). The nucleotide sequence of the entire region was determined and six J_H genes were identified, J_H1-J_H6 (Becker *et al.*, 1989, Short *et al.*, 1991). The J_H1 was assigned to the 5'-most J_H gene. All but J_H1 are functional genes.

The rabbit C_H chromosomal locus spans over 200 kb of DNA (Knight *et al.*, 1985, Burnett *et al.*, 1989, Burnett *et al.*, 1987). Sixteen C_H genes were identified: $C\mu$, $C\gamma$, $C\varepsilon$ and $C\alpha1$-$C\alpha13$ genes. In contrast to mouse and human, rabbit $C\delta$ gene has not been identified. Not all C_H genes were physically linked and those which were not linked are shown in brackets in Figure 3. Unlike mouse in which the order of its unlinked C_H genes was known for certain, the order of the unlinked C_H genes rabbit is not yet known.

IgH Gene Rearrangement

Combinatorial Rearrangement and Its Product

Combinatorial V(D)J gene rearrangement can generate a large number of unique variable regions from a finite number of genes. During B-cell development, one of the V, (D) and J genes are rearranged to form a functional V genes. If we consider that there are 200 V_H, 12 D and 6 J_H genes in the germline, the combinatorial rearrangement of these genes alone can generate many different VDJ genes. An even larger repertoire is possible following junctional diversity and random nuleotide insertion (N region). The Ab repertoire was further expanded after the rearrangement by random combination of V_H and V_L, and somatic diversification such as somatic gene conversion and somatic point

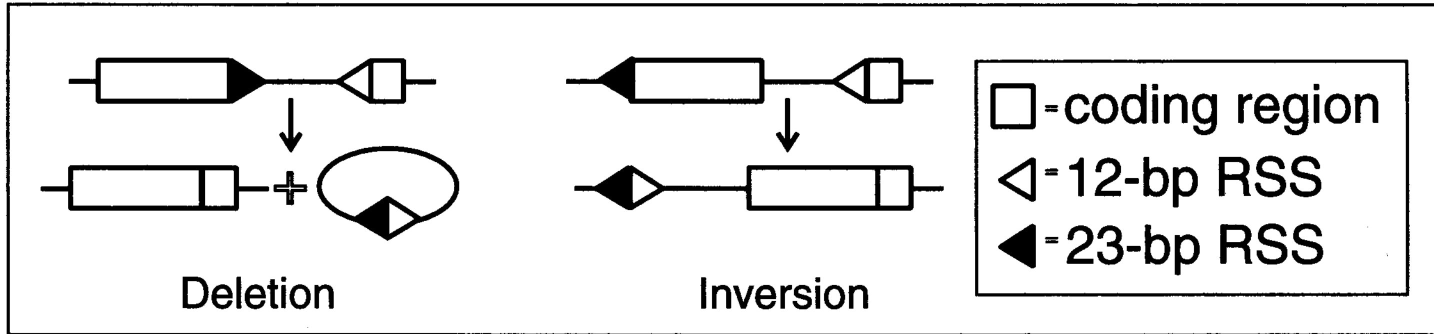
mutation. The following section will discuss the generation of Ab repertoire during the V(D)J gene rearrangement.

After the gene rearrangement, two joints are formed, coding and signal joints (Max *et al.*, 1979, Sakano *et al.*, 1979, Hochtl *et al.*, 1982, Van *et al.*, 1982, Tonegawa, 1983, Desiderio *et al.*, 1984, Selsing *et al.*, 1984, Feddersen and Van, 1985, Lewis *et al.*, 1985, Yancopoulos and Alt, 1986,). The formation of the coding joints resulted from fusion of the coding region of the V, D and J genes into one functional V region. The signal joints are formed by joining the RSSs in a head-to-head fashion and believed to be by-products with no biological significance. Gene rearrangement can be either deletional or inversional depending on the orientation of the rearranging RSSs. If the two RSSs are directed toward each other, the intervening DNA between the two genes will be deleted (Figure 4a) (Cory and Adams, 1980, Yamagishi *et al.*, 1983, Okazaki *et al.*, 1987, Fujimoto and Yamagishi, 1987, Hirama *et al.*, 1991, Shimizu and Yamagishi, 1992). After the signal joint formation, this deleted by-product persists as circular DNA. If the two RSSs are in the same orientation, the intervening DNA between the two genes will be inverted and retained in the chromosome (Figure 4a) (Hochtl *et al.*, 1982, Lewis *et al.*, 1982, Hochtl and Zachau, 1983, Feddersen and Van, 1985, Malissen *et al.*, 1986). The characteristic of coding and signal joints will be discussed in the next section.

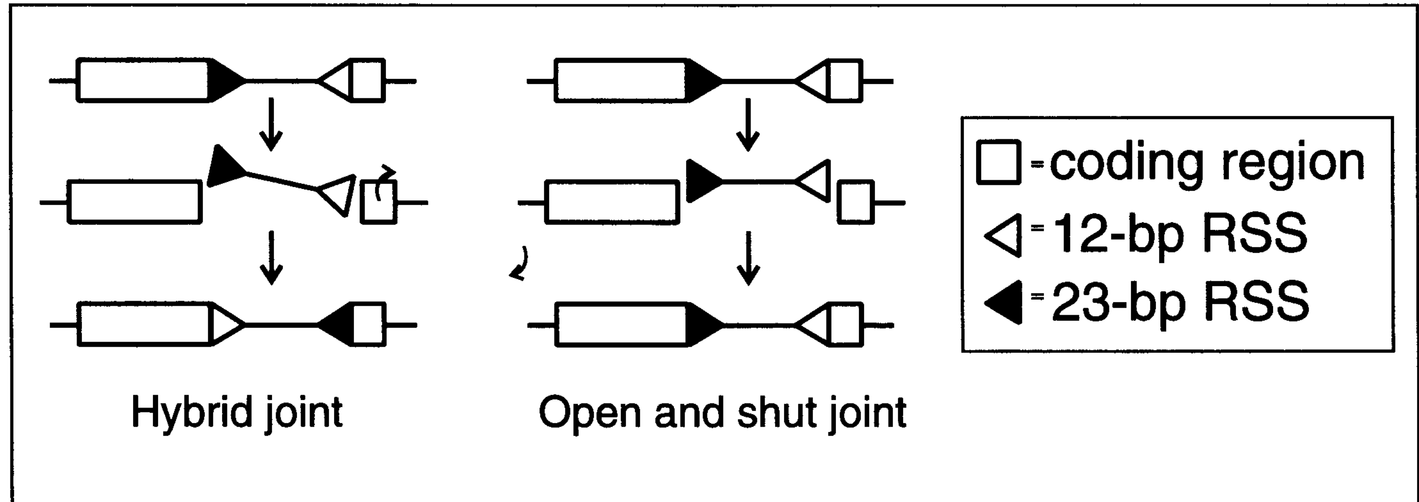
Most of the rearrangement has been shown to occur via deletion rather than inversion (Meek *et al.*, 1989, Gauss and Lieber, 1992). The underlying mechanism for this preference is currently unknown. It was originally thought that the bias for deletion is due to chromosomal topology. However, Gauss and Lieber (1992) showed by *in vitro* recombination assay using artificial rearrangement substrates which contained the mouse D_{FL16} and J_H genes that this may not be the case. Most of rearrangements which occurred between the D_{FL16} and J_H genes in the IgH locus preferentially rearranged the 3'-RSS of

Figure 4 Diagram of IgH gene rearrangement products: a) the products of deletion and inversion rearrangements (both are successful rearrangements) and b) the products of unsuccessful rearrangements, hybrid joint and, open and shut joint.

a)



b)



the D_{FL16} to the RSS 5' of J_H resulting in deletional rearrangements despite the fact that the 5'-RSS of D_{FL16} could also be rearranged to J_H. In the *in vitro* rearrangement assay, when the D_{FL16} was placed in the artificial rearrangement substrate in an inverted orientation such that the preferentially used 3'-RSS became a 5'-RSS, the authors found that most of the rearrangements still preferentially used that particular RSS (which is now a 5'-RSS) resulting high frequency of inversional rearrangements. This result suggested that the high frequency of the deletional rearrangements resulted from the specific recognition of the recombinase for the RSS, regardless of the 5' or 3' location of the RSS, rather than from topological preference. No qualitative differences between the RSSs which confer the deletional bias and those which confer the inversional bias has been studied.

Not all V, D, and J gene rearrangements are successful. Two unusual rearrangement products have been identified: a hybrid joint, and an open and shut joint (Lewis *et al.*, 1988, Morzycka *et al.*, 1988, Lewis and Gellert, 1989). The hybrid joint is characterized by the exchange of RSSs between the two rearranging genes (Figure 4b) (Lewis *et al.*, 1988, Morzycka *et al.*, 1988, Lewis and Gellert, 1989). The open and shut joint involves the disconnection and rejoining of the RSSs and their flanking sequences without any rearrangement (Figure 4b). These two unusual joints were identified in the rearrangement of artificial rearrangement substrates. We do not know whether these two joints also occur in the rearrangement of endogenous genes.

It has long been known that the coding joints are imprecise (Bernard *et al.*, 1978, Max *et al.*, 1979, Sakano *et al.*, 1979, Alt, 1987,). Sakano *et al.* (1980) reported, after they compared sequences of VDJ genes with the germline V_H and J_H genes, that the exact boundary of the V_H and J_H were unfixed between different VDJ genes. This imprecise joining generally resulted in loss of nucleotides encoded by the germline genes (Honjo,

1983, Tonegawa, 1983, Lewis *et al.*, 1985, Alt, 1987). It is thought that after the recombinase recognized the rearranging genes and made the excision, the coding ends were exposed to exonuclease activity before they were joined. Such flexibility allows an even larger Ab repertoire to be generated than from combinatorial rearrangement alone. However, the drawback is that the rearrangement could result in out-of-frame coding region (nonproductive gene) which, in theory, could be as many as two-thirds of the rearrangements. In support of this, many of the rearrangement products were found to be nonproductive genes (Max *et al.*, 1979, Altenburger *et al.*, 1980, Perry *et al.*, 1980). The signal joint formation, in contrast to the coding joint formation, is precise (Lewis *et al.*, 1985, Okazaki *et al.*, 1987, Fujimoto and Yamagishi, 1987, Hirama *et al.*, 1991, Shimizu and Yamagishi, 1992) and, in general, the heptamer of the two RSS's are joined head-to-head with no base loss.

Many of the VDJ genes had extra-nucleotides at the V/D and D/J junctions which were not part of the V, D or J genes. These nucleotide additions could be categorized into two groups. The first group is N-segment, a non-templated nucleotide addition. Although the sequences added are template independent, there is a bias toward the additon of G and C (Kurosawa*et al.*, 1981, Alt and Baltimore, 1982, Kurosawa and Tonegawa, 1982). The second group of nucleotide addition is P-elements which are templated addition. The sequence added is palindromic to 1-2 bp of the end of the coding region (McCormack *et al.*, 1985, Lafaille *et al.*, 1989).

In mouse, the capability to add N-segment to the juction is acquired after birth. This was determined by comparing the VDJ and DJ sequences at the IgH locus from B cells of fetal, newborn and adult mice and in general, the sequences derived from newborn mice do not have N-region. Only 10% or less of VDJ genes from fetal liver or newborn spleen have N-segments (Feeney, 1990). For those which do have N-segments,

they are shorter than 4 bp and presented only at one of the V/D or D/J junctions (Feeney, 1990, Gu *et al.*, 1990). In contrast, more than 85% of adult VDJ genes have N-segments. In most cases, N-segments are found at both V/D and D/J junctions. The N-segements at both junctions are generally longer than those found in VDJ genes from fetus and neonates, and the N-segments at the V/D junctions are longer on average than those at the D/J junctions (Gu *et al.*, 1990).

In human, it appears that N-segments are found in VDJ genes derived from liver of fetus as young as 105 days (Schroeder, 1987, Schroeder and Wang, 1990). More than 90% of the VDJ genes isolated from fetal liver and cord blood cDNA libraries contain N-segments, usually at both the V/D and D/J junctions (Schroeder, 1987, Schroeder and Wang, 1990, Mortari *et al.*, 1992). The average length of these N-segments in the VDJ genes from fetal or neonatal tissues is, however, shorter than that of adult (Schroeder, 1987, Schroeder and Wang, 1990, Sanz, 1991).

Signal joints were also shown to contain N-segments. Shimizu and Yamashigi (Shimizu and Yamagishi, 1992) isolated circular DNA generated from deletional rearrangements of D to J_H and V_H to D from mouse fetal liver or adult spleen and analyzed the signal joints in these circular DNA. They found that N-segments were also present in signal joints in the circular DNA generated from both V_H to D and D to J_H rearrangements. Like the N-segment at the coding joints in mouse VDJ genes, the N-segments of the D/J_H signal joints were sparse while those of V_H/D signal joint were abundant. The addition of N-segment at the signal joint was also demonstrated in the rearrangement of artificial rearrangement substrate in *in vitro* rearrangement assay (Lieber *et al.*, 1988).

P-elements, in contrast to N-segment, are templated nucleotide insertion initially described at the V/J junction of chicken λ chain gene (McCormack *et al.*, 1985) These

nucleotide insertions are 1-2 bp long and are palindromic to the very 1-2 bp of the ends of the coding region. The P-elements were not specific to chicken Ig gene and are subsequently identified in Ig genes of many species including mouse, human and rabbit (Feeney, 1990, Gu *et al.*, 1990, Chen and Alt, 1993, Pascual, 1993). Since P-elements are found only at junctions with no junctional diversity, it is thought that the addition of P-elements precedes the exposure of the coding end to the endonuclease.

Recombinase

The enzyme system that performs the V(D)J gene rearrangement is collectively called VDJ-recombinase. Although the products of the V(D)J gene rearrangement are now well-characterized, the exact nature of the recombinase is still obscure, although several genes that play a role in VDJ gene rearrangements have been identified. It is surprising to find that the expression of some of these genes is not lymphoid-specific. In fact most of the components of recombinase are probably expressed in many cell types, if not ubiquitously expressed (Schatz *et al.*, 1989, Oettinger *et al.*, 1990, Pergola *et al.*, 1993, Taccioli *et al.*, 1994). Only three components of recombinase, RAG-1, RAG-2 and Terminal deoxynucleotidyl transferase (TdT), have been found to be lymphoid specific thus far.

Two essential components of the recombinase are RAG-1 and RAG-2 (Schatz *et al.*, 1989, Oettinger *et al.*, 1990). The expression of these two genes alone in a mouse fibroblast cell line, which by itself is rearrangement-incompetent, conferred the cell line the rearrangement capability suggesting that RAG-1 and RAG-2 were indispensable components of the recombinase. The result also suggested that all other components of recombinase were expressed in the fibroblast cell line. Both RAG-1 and RAG-2 genes are conserved through out evolution and are expressed only in lymphoid tissue, pre-B and pre-T cell lines (Schatz *et al.*, 1989, Oettinger *et al.*, 1990). Mice deficient in either RAG-

1 or RAG2 have no mature B and T cells were found (Mombaerts *et al.*, 1992, Shinkai *et al.*, 1992) and AMuLV-transformed B cell lines generated from these knock-out mice showed no sign of Ig or TCR gene rearrangements. These data suggested that the whole process of IgH gene rearrangement was halted in the absence of RAG-1 or RAG-2, confirming the importance of RAG-1 and RAG-2 in the rearrangement process.

The function of RAG-1 and RAG-2 proteins in the rearrangement process is still obscure. In order for DNA rearrangement to occur, one would expect the recombinase to bind DNA, cleave it via endonuclease activity and then ligate the DNA via ligase activity. RAG-1 and RAG-2 have none of these expected properties. Because RAG-1 contained a topoisomerase-I domain, which was found to be important for its function, the possibility that RAG-1 protein might be responsible for cleaving DNA during the rearrangement was proposed (Kallenbach and Rougeon, 1992, Kallenbach *et al.*, 1993, Silver *et al.*, 1993, Sadofsky *et al.*, 1994a, Sadofsky *et al.*, 1994b). However, since a mutation at the active conserved tyrosine in the topoisomerase I domain did not abolish the rearrangement activity of RAG-1, this domain must not function in this manner. Therefore, if RAG-1 is responsible for cleaving the DNA during the rearrangement process, the mechanism must be different from the conventional mechanism used by other topoisomerases of type I. Unlike RAG-1, RAG-2 does not have any recognizable functional domains (Oettinger *et al.*, 1990). The only prominent feature of RAG-2 is an acidic-residue rich domain which was found to be dispensable for its activity (Silver *et al.*, 1993, Sadofsky *et al.*, 1994a, Sadofsky *et al.*, 1994b). It is disappointing that scientists have been attempting to study the role of RAG-1 and RAG-2 in the rearrangement process since they were cloned, but yet this goal has not been realized. Now that monoclonal Abs to RAG-1 and RAG-2 proteins have been developed (Lin and Desiderio, 1993, Silver *et al.*, 1993), the protein chemistry and enzymology studies of RAG-1 and RAG-2 should appear shortly.

Another lymphoid-specific component of recombinase is the TdT enzyme. TdT is a DNA polymerase which catalyzes the polymerization of dNTPs to the 3'-end of DNA molecule in a template-independent manner (Bollum, 1974). Although any nucleotide could be incorporated, the TdT is biased toward incorporating dGTP and dCTP. The level of TdT expression in a pre-B cell line correlated well with the extent to which N-segments are found (Desiderio *et al.*, 1984, Landau *et al.*, 1987). Conclusive evidence that TdT is responsible for the N-segment addition was reported by Komori *et al.* (1993) and Gilfillan *et al.* (1993) who generated TdT knock-out mice. The VDJ genes from these mice showed minimal N-segment addition, with most of the VDJ genes having no N-segment. Therefore, TdT is the enzyme responsible for most of N-segment additions in the V(D)J gene rearrangements. The fact that V(D)J gene rearrangement occurs normally in TdT-deficient mice suggests that, unlike the other components of the recombinase, TdT is dispensable.

The rest of the recombinase enzyme components have not yet been studied. Studies which identified these components could be divided into two groups. One group of studies is based on the assumption that after the cleavage of DNA, the DNA repair machinery would be required to repair the DNA lesion. Four mutants of chinese hamster ovary cell line defective in DNA-repair were found to be incapable of performing V(D)J gene rearrangement even after RAG-1 and RAG-2 were supplied suggesting that the defective genes in these four mutants were essential component of the recombinase. These genes are xrs-6, XR-1, V3 and XR-V9B (Pergola *et al.*, 1993, Taccioli *et al.*, 1993). Mouse SCID mutant was also defective in DNA-repair pathway and was found to be defective in V(D)J gene rearrangement (Bosma and Carroll, 1991). The SCID gene was found to be a homolog of the V3 gene in chinese hamster (Taccioli *et al.*, 1994). Taken together, these results suggested that four genes involved in DNA-repair pathway, xrs-6, XR-1, V3 or SCID and XR-V9B, are components of the recombinase enzyme. Of

these four genes, only xrs-6 gene has been cloned. It was shown to encode the 80kb subunit of the DNA-dependent protein kinase or Ku Ag (Taccioli *et al.*, 1994) but how it participates as part of the recombinase is unknown.

The other group of studies approached the identification of recombinase components based on the assumption that there should be DNA binding proteins which bind specifically to the RSS and assist the recombinase to recognize the V, D and J genes. Several RSS-binding proteins have been described in nuclear lysate of lymphocytes, some of which have been cloned (Aguilera *et al.*, 1987, Halligan and Desiderio, 1987, Hamaguchi *et al.*, 1989, Matsunami *et al.*, 1989, Shirakata *et al.*, 1991). Not much is yet known about their proposed function in the recombinase complex except that these proteins bind the RSS.

It is apparent that the V(D)J gene rearrangement is not a trivial process, illustrated by the complexity of the recombinase enzyme. Although many of the its components have been identified, their enzymology and how they function as a recombiase has been only minimaly studied. Since monoclonal Abs to many of these components are now available and many components of the recombinase have been described, the time has arrived for scientists to start deciphering these intriguing aspects of the recombinase.

Order of IgH Gene Rearrangement

The V region of H-chain is encoded in the germline by three non-contiguous genes, V_H, D and J_H. To generate a V region, two separate rearrangements, V_H to D and D to J_H, are required. The assembly process occurs in an orderly fashion and at least one rearrangement occurs at each IgH allele. The order of IgH gene rearrangement has been established in mouse by Alt *et al.* (1984), and their finding has been regarded as dogma for the order of IgH gene rearrangement in all species.

The order of IgH gene rearrangement in mouse could be established primarily due to the ability of AMuLV to transform mouse B cells, especially immature B cells or pre-B cells (Rosenberg *et al.*, 1975, Rosenberg and Baltimore, 1976). Furthermore, the AMuLV-transformed fetal liver pre-B cells continued to rearrange their IgH genes *in vitro* (Alt *et al.*, 1981). Alt *et al.* found that most fetal liver derived AMuLV-transformants contained rearranged DJ_H on both alleles. These cell lines then rearranged V_H to the DJ to form VDJ genes. None of the cell lines rearranged VDJ genes without first rearranging DJ genes on both alleles. These results suggested that DJ genes are the intermediates in the IgH gene rearrangement process and that the first step of IgH gene rearrangement is DJ on both alleles. Neither VD gene nor D-D fusion were found in these cell lines suggesting that DJ gene was the major, if not exclusive, intermediate. This conclusion was supported by the finding that AMuLV-transformed B cell lines from J_H gene knock-out mice showed no sign of the IgH gene rearrangement (Chen *et al.*, 1993).

The order of IgH gene rearrangement in mouse B cells was established based on the finding that J_H on both alleles were rearranged to DJ or VDJ genes. Such analysis in human B cells has not been determined due to several factors. First, human equivalent of AMuLV-transformed cell in mouse is not available. Therefore, the sequential progress of the IgH gene rearrangement in human B cells can not be followed *in vitro*. Second, the IgH gene rearrangement in human appears to be more complex than mouse. Shin *et al.* (1993) had isolated a genomic phage clone containing a VD gene rearrangement suggesting that the order of IgH gene rearrangement in human may occur via an alternative intermediate not found in mouse. Third, many VDJ genes from human B cells contained D-D fusions suggesting that D-D fusion occurred rather frequently in human. Finally, human D_{Q52} gene is located only 25 bp 5' of J_H1. The $D_{Q52}J_H1$ rearrangement would result in 25 bp deletion and therefore, this rearrangement is difficult to distinguish from germline J_H on Southern analysis. These factors, in addition to the polymorphism of

human IgH locus, made the evaluation of the status of IgH gene on the unexpressed allele in human B cells complex and therefore, the order of IgH gene rearrangement in human has not been conclusively determined.

Allelic Exclusion

The term allelic exclusion describes a phenomenon in which the Ig and TCR proteins are made from only one of the two homologous chromosomes in individual B and T cells. In the mid 1960's, several groups of investigators reported that each individual Ig producing plasma cell from rabbits and mice, which are heterozygous at the heavy (H) or light (L) chain genes, produced H and L chains from only one of the two alleles (Cebra *et al.*, 1965, Weiler, 1965, Pernis *et al.*, 1965). After the IgH and IgL genes were cloned, the evidence of allelic exclusion of IgH and IgL genes accumulated at both serological and molecular levels (Coleclough *et al.*, 1981, Gathings *et al.*, 1982, Alt *et al.*, 1984). After the TCR genes were cloned, they too were assumed to be under allelic exclusion control. But, so far, only TCRβ genes were conclusively shown to be allelically excluded (Goverman *et al.*, 1985, Chou *et al.*, 1986, Dembic *et al.*, 1986, Leiden *et al.*, 1986, Malissen *et al.*, 1988, Uematsu *et al.*, 1988).

The reason why B and T cells develop allelic exclusion mechanism is not known. One may speculate that, to minimize the error associated with the VDJ gene rearrangement, any unnecessary rearrangements are prohibited after a productive VDJ gene was generated. Not only could non-functional genes result from the rearrangement process, but much evidence suggest that chromosomal translocations could also occur (Tsujimoto *et al.*, 1985a, Tsujimoto *et al.*, 1985b, Finger *et al.*, 1986). These translocations may cause tumors and may explain why chromosomal translocations are prevalent in tumors of lymphoid origin (Foon and Piro, 1992, Hagemeijer, 1992, Mirro, 1992). Another possible purpose of allelic exclusion is to prevent autoreactive B and T

cells from escaping clonal deletion. In one H-chain transgenic mouse model in which the heavy chain transgene was derived from an autoreactive hybridoma, autoreactive B cells could be generated if the transgenic H-chain was paired with a certain V_L gene (to be derived from endogenous rearrangement) (Iliev *et al.*, 1994). B cells which paired the H-chain transgene with other VL genes would be non-autoreactive. Iliev *et al.* (1994) found that autoAb producing B cells in these mice always co-expressed non-autoreactive endogenous IgM while the non-autoreactive B cells did not. This result suggested that the expression of endogenous IgM allowed the autoreactive B cells, which normally would have been clonally deleted (Nossal, 1994), to escape the clonal deletion. These speculations of the significance of allelic exclusion are still hypothetical and remain to be proven. To gain insight into the significance of allelic exclusion, it is desirable to be able to disrupt the allelic exclusion in normal mice and observe the outcome. As a steppingstone toward that goal, many investigators have studied how the allelic exclusion is regulated.

The allelic exclusion can possibly be regulated at two, not mutually exclusive, steps. One controlling step is at the rearrangement (or genetic) level by inhibiting the rearrangement of the second allele so that it would remain in the un-expressible state. Otherwise, allelic exclusion can be implicated post-rearrangement at the level of gene expression and/or protein assembly,or at the cellular level by negative selection for B cells which simultaneously express H-chains from both IgH alleles. This review will present the studies regarding allelic exclusion as two groups: studies which addressed the genetic level and those which addressed the post-rearrangement control.

Allelic Exclusion at the Rearrangement (Genetic) Level

Although there are seven Ig and TCR loci which undergo somatic rearrangement and are theoretically subjected to allelic exclusion phenomenon, the allelic exclusion at

the rearrangement level was studied virtually in four of the seven loci: IgH, Igκ, TCRβ and TCRα. These studies will be discussed in the following section. The allelic exclusion at the rearrangement of the IgH and TCRβ genes will be discussed together because their rearrangements bear similar characteristics. These characteristics are that the IgH and TCRβ genes are the first gene to rearrange in pre-B and pre-T cells (Siden *et al.*, 1981, Raulet *et al.*, 1985, Snodgrass *et al.*, 1985a, Snodgrass *et al.*, 1985b) and that they required two separate rearrangements, D to J and V to DJ, to generate a functionally rearranged gene. Then, the allelic exclusion at the rearrangement level of Igκ and TCRα genes will be discussed.

Two theories were proposed to explain the allelic exclusion at the rearrangement step: stochastic and regulated theories. The stochastic theory assumes that the rearrangement of Ig and TCR genes is so prone to error that many rearrangements result in non-productive genes and, therefore, the probability of having two functional genes in one cell is remote (Coleclough *et al.*, 1981). The regulated theory explains that the rearrangement of Ig and TCR genes on the second allele are inhibited when a productive gene is achieved (Bernard *et al.*, 1981, Kwan *et al.*, 1981, Alt *et al.*, 1984). Many experiments support the idea that the allelic exclusion is better explained by the regulated theory.

The first evidence to support the regulated theory was obtained from the study of IgH genes. In AMuLV-transformed pre-B cell lines which actively rearranged their IgH genes, the rearrangement of the unexpressed IgH allele ceased after a functional VDJ gene was made (Alt *et al.*, 1984). Furthermore, Reth *et al.* (1987) had transfected a VDJ-Cμ gene into two AMuLV-transformed pre-B cell lines and the active ongoing rearrangement of the endogenous IgH genes was halted. It appeared as if once a μ protein was made, the rearrangement of the IgH locus was turned off and the unexpressed allele

stably existed as either DJ or non-functional VDJ genes. At the TCRβ locus, the allelic exclusion appeared to be regulated in a similar fashion because only DJ or non-functional VDJ genes were found on the unexpressed TCRβ allele (Tsukamoto *et al.*, 1984, Malissen *et al.*, 1992). These results suggested that the rearrangement of the unexpressed IgH and TCRβ alleles were inhibited, presumably by the μ and TCRβ chains.

To directly test the assumption that a productive rearrangement turns off the rearrangement at each locus, transgenic mice were generated with functionally rearranged μ and TCRβ transgenes (Grosschedl *et al.*, 1984, Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Fenton *et al.*, 1988, Imanishi-Kari *et al.*, 1988, Manz *et al.*, 1988, Nussenzweig *et al.*, 1988, Uematsu *et al.*, 1988, van Meerwijk *et al.*, 1991, Rothe *et al.*, 1993). As predicted, rearrangement of the endogenous IgH genes was inhibited in the μ transgenic mice and rearrangement of the endogenous TCRβ genes was inhibited in the TCRβ transgenic mice. Only the constant regions of the μ and TCRβ genes were required to mediate allelic exclusion because endogenous gene rearrangement was also inhibited in transgenic mice generated from only the constant region portions (Krimpenfort *et al.*, 1989, Corcos *et al.*, 1991). The ability of other heavy chain genes to inhibit the rearrangement was also tested using transgenic mice. Allelic exclusion of the endogenous IgH gene was found only in δ but not γ2a or γ2b transgenic mice (Iglesias *et al.*, 1987, Offen *et al.*, 1992, Tsao *et al.*, 1992) suggesting that the δ chain could also mediate the inhibition of rearrangement.

The fact that both the functionally rearranged μ and δ, but not γ2a or γ2b, transgenes could mediate allelic exclusion of IgH genes suggested that the regulatory region responsible for the allelic exclusion should be present in both the μ and δ genes. Subsequently, several studies have shown that the membrane exon (μm) of the μ gene, which shares high homology with the membrane exon of the δ gene, was essential for

allelic exclusion of endogenous IgH genes (Manz *et al.*, 1988, Nussenzweig *et al.*, 1988, Kitamura and Rajewsky, 1992). Transgenic mice with a VDJ-Cμ gene lacking the μm exon (Manz *et al.*, 1988, Nussenzweig *et al.*, 1988) or mice in which the μm exon has been disrupted (Kitamura and Rajewsky, 1992) showed high incidence of double-Ig producers that expressed IgH from more than one allele simultaneously. Furthermore, Reth *et al.* (1987) showed that the ability of the transfected functionally rearranged μ gene to inhibit the rearrangement of the endogenous IgH gene depended on the presence of μm exon in the transfected gene. These results suggested that the μm exon was important for the regulation of allelic exclusion at the IgH locus. Similar studies have not been performed for the TCRβ gene.

Allelic exclusion at the rearrangement level of IgH and TCRβ loci was proposed to be mediated at the V to DJ gene rearrangement step (Alt *et al.*, 1984, Malissen *et al.*, 1992). If this were the case, it would be expected that D to J rearrangement of the endogenous IgH and TCRβ genes could occur normally in the IgH and TCRβ transgenic mice but the V to DJ rearrangement would be blocked. Indeed, analysis of T cell lines or thymocytes from the TCRβ transgenic mice showed that all rearrangements of the endogenous TCRβ genes were DJ gene rearrangements (Uematsu *et al.*, 1988, Krimpenfort *et al.*, 1989, van Meerwijk *et al.*, 1991). No germline Jβ or VDJβ genes were found. One interpretation of these results is that allelic exclusion of TCRβ gene by the functional TCRβ protein was mediated solely at the V to DJ rearrangement step. Analysis of IgH genes in hybridomas generated from μ and δ transgenic mice also revealed DJ gene rearrangements (Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Iglesias *et al.*, 1987, Muller *et al.*, 1989, Iacomini *et al.*, 1991). However, in contrast to the TCRβ transgenic mice model, complete endogenous VDJ genes as well as unrearranged D and J_H genes were found. The VDJ genes represented 15%-33% of the endogenous IgH alleles while the unrearranged J_H genes were found in 10%-20% of the

alleles (Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Iglesias *et al.*, 1987). These results explain why μ and δ transgenic mice expressed endogenous Ig in their serum (Grosschedl *et al.*, 1984, Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Stall *et al.*, 1988, Rath *et al.*, 1989, Forni, 1990, Vos and Hodes, 1992). They also suggested that, unlike TCRβ genes, both D to J as well as V to DJ gene rearrangements at the IgH locus could be inhibited by allelic exclusion process.

Since endogenous IgH genes in the μ and δ transgenic mice could be rearranged to functional VDJ genes, this raised the possibility that allelic exclusion was not complete and that the endogenous VDJ genes and the transgene were co-expressed in the same B cell. In fact, mixed-IgM molecules, which contained μ chains from both the transgene and the endogenous gene, were found in the serum of the μ transgenic mice (Stall *et al.*, 1988, Rath *et al.*, 1989, Vos and Hodes, 1992). Furthermore, some hybridomas derived from the μ transgenic mice produced and secreted both transgenic and endogenous μ chains (Manz *et al.*, 1988, Kenny *et al.*, 1989, Muller *et al.*, 1989). In addition, B cells from these mice expressed on the surface both transgenic and endogenous μ chains on the surface (Kenny *et al.*, 1989, Lamers *et al.*, 1989, Forni, 1990, Grandien *et al.*, 1990). However, another group of investigators reported that they could never find such double-producers (Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Nussenzweig *et al.*, 1988, Imanishi-Kari *et al.*, 1993). These contradictory results were not due to the differences in the transgenic lines because some were in common among the studies (Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Stall *et al.*, 1988, Forni, 1990, Grandien *et al.*, 1990, Imanishi-Kari *et al.*, 1993). Imanishi-Kari and co-workers suggested that the double-producers were experimental artifacts caused by anti-allotypes specific for Ig from the endogenous genes shown to cross-react with the variable region of the transgenic μ protein (Imanishi-Kari *et al.*, 1993). In this way, it would appear in studies which used these Abs that the cells which expressed only transgenic μ chain also expressed the endogenous IgH gene

(Rusconi and Kohler, 1985, Stall *et al.*, 1988, Rath *et al.*, 1989, Forni, 1990, Grandien *et al.*, 1990). This proposal remains to be tested.

One possible mechanism which mediates the allelic exclusion effect of functional IgH and TCRβ gene rearrangements is that the expression recombinase is down-regulated. It is an unlikely mechanism, however, because the pre-B and pre-T cells which had successfully rearranged the IgH and TCRβ genes have yet to rearrange IgL and TCRα loci. An alternative explanation would be that after a successful VDJ gene rearrangement at the IgH or TCRβ loci, the rearrangement at the respective locus is suppressed. This explanation is attractive for the following reasons. First, it has been shown that a constant production of μ chain is required to maintain the inhibition of rearrangement at the IgH locus in pre-B cells. Maeda *et al.* (1989) had identified a subclone of a pre-B cell line which stopped rearranging its IgH genes after it had functionally rearranged a VDJ gene and expressed the μ chain. However, the rearrangement of the IgH gene was reinitiated in the subclones which lost the functional VDJ allele. Similar results were obtained by Beck-Engeser *et al.* (1987) who reported that IgH gene rearrangements were re-initiated in a pre-B cell line after it switched from expressing μ chain to expressing γ2b chain. The inhibition of rearrangement at the TCRβ locus also depended on the expression of TCRβ protein. Pircher *et al.* (1990) examined the expression of the endogenous TCRβ gene in several TCRβ transgenic lines and found that the expression of the endogenous gene was inhibited only in the lines which expressed high level of the transgene. This result suggested that the high level of transgenic TCRβ chain could inhibit the rearrangement of the endogenous TCRβ gene. Taken together, these data suggest that inhibition of IgH and TCRβ gene rearrangement is an active process which requires the production of μ and TCRβ proteins.

The biochemical basis of the inhibitory effect on the rearrangement process by μ and TCRβ chains is currently unknown but some evidence suggests that $p56^{lck}$ may be involved. Transgenic mice which over-expressed $p56^{lck}$ proteins showed a reduced level of VDJβ rearrangement while the level of DJβ rearrangement was normal (Anderson *et al.*, 1992). Furthermore, by interfering with the function of $p56^{lck}$ in TCRβ transgenic mice, using a dominant negative mutant of $p56^{lck}$, Vβ to DJβ gene rearrangements were no longer inhibited (Anderson *et al.*, 1993). In both experiments, the rearrangement of TCRα occurred at levels equivalent to normal mice. These results suggest that $p56^{lck}$ is involved in delivering an allelic exclusion signal specific for the Vβ to DJβ gene rearrangements. Whether $p56^{lck}$ exerts its inhibitory effect via the same pathway it uses in the activation of peripheral T cells remains to be determined. Because $p56^{lck}$ is expressed in B-lineage cells and involved in B-cell activation pathway (Gold *et al.*, 1994), it is possible that $p56^{lck}$ is also involved in μ–chain mediating allelic exclusion of the IgH gene. To answer this question, it is necessary to examine the effect of $p56^{lck}$ over-expression as well as that of the dominant negative mutant of $p56^{lck}$ on the allelic exclusion by μ gene and to determine the level of $p56^{lck}$ expression at different stages of B cell development.

Susceptibility of endogenous IgH and TCRβ genes to the allelic exclusion process may also be dependent on chromosomal location. In μ transgenic mice, more endogenous VDJ gene rearrangements involve V_H genes in the 3' V_H families than those in the 5' V_H families (Iacomini *et al.*, 1991, Costa *et al.*, 1992). In mice double-transgenic for a functionally rearranged TCRβ gene and a germline Vβ, Dβ, Jβ and Cβ minilocus, van Meerwijk *et al.* (1991) found that the expression of the functionally rearranged TCRβ transgene could inhibit the rearrangement of the germline TCRβ minilocus. However, the inhibition of the rearrangement of the minilocus occurred in only one of the five transgenic lines they have generated. The fact that the five double-transgenic mice all had

the functionally rearranged TCRβ gene integrated at the same chromosomal location but had the minilocus integrated at different location suggested that the location where the minilocus transgene integrated into the chromosome determined the susceptibility of the minilocus to the allelic exclusion machinery. It is possible that the reason why TCRβ transgene can completely inhibit the rearrangement of Vβ to DJβ (Fenton *et al.*, 1988, Uematsu *et al.*, 1988, van Meerwijk *et al.*, 1991, Rothe *et al.*, 1993) is that the endogenous TCRβ gene is located in the chromosomal region which is highly susceptible to allelic exclusion machinery. One way to test this hypothesis is by generating a mouse in which the TCRβ locus is shuffled to the IgH locus and observe the allelic exclusion effect of the TCRβ transgene in the rearrangement of TCRβ gene in its new location. However, such an experiment is not feasible with current technology and awaits the development of the technique to shuffle genetic loci.

Allelic exclusion at the Igκ locus is less complicated than those at IgH and TCRβ loci. Many plasmacytomas rearranged both κ alleles, only one of which was functional (Perry *et al.*, 1980, Coleclough *et al.*, 1981, Kwan *et al.*, 1981) so that it appeared that allelic exclusion at the κ locus could be explained by a stochastic theory. However, subsequent studies in polyclonal B cells showed that a substantial amount of κ gene in B cells was not rearranged (Nottenburg and Weissman, 1981, Tsukamoto *et al.*, 1984). This result suggested that the rearrangement of the unexpressed Igκ allele may also be inhibited. The analysis of the κ locus in the functionally rearranged κ transgenic mice supports this idea. Ritchie *et al.* (1984) analyzed transgene-expressing hybridomas derived from transgenic mice and found that their endogenous κ genes were in germline configuration. This inhibition was not found in the hybridomas which did not express the transgene or the μ gene suggesting that the inhibitory process depended on both κ and μ chains. These results, together with the finding that μm exon of the μ gene was important for the allelic exclusion, suggest that the inhibitory process is mediated by sIgM

molecules rather than the κ chains alone. Because the expression of RAG-1 and RAG-2 genes was terminated after the expression of sIgM molecules (Ma *et al.*, 1992), it is unclear whether Igκ rearrangement is actively suppressed, or simply due to the lack of recombinase enzyme.

The expression of TCRα was assumed to be allelically excluded, similar to Ig and TCRβ genes. However, this idea is challenged by recent findings. Approximately 30% of T cell lines have been found to functionally rearrange VJα on both alleles (Champagne *et al.*, 1988, Furutani *et al.*, 1989, Casanova *et al.*, 1991, Couez *et al.*, 1991, Kuida *et al.*, 1991, Malissen *et al.*, 1992, Heath and Miller, 1993), both of which could be transcribed (Marche and Kindt, 1986, Casanova *et al.*, 1991, Couez *et al.*, 1991, Kuida *et al.*, 1991, Hu *et al.*, 1993). Padovan *et al.* (1993) used Ab to Vα2, Vα12 and Vα24 to show that one in 10^3 to 10^4 human peripheral blood T cells express two different Vαs on the surface. T cell clones established from these cells stably expressed the two different Vαs and were shown to contain two functional VJα genes. T cells expressing two different VJα genes were further demonstrated in mice transgenic for a functionally rearranged TCRα gene (Borgulya *et al.*, 1992). These results together suggest that neither the expression nor the rearrangement of TCRα gene were allelically excluded. However, *in vivo*, approximately 70% of T cell clones contain a non-functional rearrangement on the unexpressed allele (Malissen *et al.*, 1992) and these T cells would thus express only one species of T cell receptor. The TCRα genes probably continue to rearrange until the expression of RAG-1 and RAG-2 genes is down-regulated after the cells are positively selected in the thymus (Turka *et al.*, 1991).

The lack of allelic exclusion at the TCRα locus raised a question whether the allelic exclusion at Igκ locus was an active suppression. Because the allelic exclusion at the κ locus is mediated by sIgM (Ritchie *et al.*, 1984) and because the expression of

RAG-1 and RAG-2 genes is down-regulated after the cross-linking of sIgM (Ma *et al.*, 1992), it is possible that the inhibition of the rearrangement of the unexpressed Igκ allele is simply due to the lack of recombinase enzyme. Evidence from bcl-2 transgenic mice supports this view (Rolink *et al.*, 1993). sIgM$^+$ B cell lines derived from these transgenic mice continue to express RAG-1 and RAG-2 genes and also continue to rearrange their light chain genes. This suggests that there was no active suppression of κ gene rearrangement following the expression of sIgM. Rather, Igκ gene rearrangement continues as long as recombinase enzyme is expressed.

Although we currently do not know for certain, the supposition that there is no active suppression of the rearrangement at the Igκ and TCRα loci makes the allelic exclusion of Ig and TCR genes fairly straight forward to implement in B and T cells. After pre-B and pre-T cells have a functionally rearranged VDJ gene at the IgH and TCRβ genes, further rearrangement at these loci are inhibited by an active inhibitory process which requires the expression of the μ or TCRβ chain and p56^{lck}. The rearrangements at these loci remain suppressed while the pre-B and pre-T cells rearrange their Igκ and TCRα genes, respectively. Then Igκ and TCRα genes continue to rearrange until the expression of RAG-1 and RAG-2 genes is turned off. In B cells, the expression of RAG-1 and RAG-2 genes could be turned off rapidly after the expression of sIgM. Therefore, the chance of having a second rearrangement of κ gene is small. In T cells, RAG-1 and RAG-2 genes continue to be expressed until the cells undergo positive selection. Therefore, both TCRα alleles are usually rearranged. This scenario, although simple, is my hypothetical view and remains to be tested.

Post-Rearrangement Control of Allelic Exclusion

The idea that the allelic exclusion can be mediated through post-rearrangement control originated from the findings that some B and T cells had more than one functional VDJ or VJ genes but only one of the two functional genes was expressed. For example, Nottenburg *et al.* (1987) showed that some mouse peripheral B cells have two functional VDJ genes yet only one was expressed on the surface. Bernard *et al.* (1981) found plasmacytoma cell lines with functional VJ genes on both κ alleles, both of which were transcribed but only one assembled into Ig molecule. T cell clones which had two functionally rearranged TCRα genes but only one Vα on the surface have also been reported (Malissen *et al.*, 1988, Furutani *et al.*, 1989, Couez *et al.*, 1991, Kuida *et al.*, 1991). All these results suggest that there must be some post-rearrangement control of allelic exclusion.

Most of the post-rearrangement control has been studied at the level of protein assembly. It was found that some κ and TCRα chains fail to properly pair with the heavy and TCRβ chains (Bernard *et al.*, 1981, Cherepakhin *et al.*, 1987, Malissen *et al.*, 1988, Berinstein *et al.*, 1989, Furutani *et al.*, 1989) thus, these κ and TCRα chains were not expressed on the surface of the cells or secreted. However, we do not know how frequently these improper pairings between heavy and light chains and between TCRα and TCRβ chains occur *in vivo.*

One potential step of post-rearrangement control is at the level of transcription. However, this possibility has been addressed only to a limited extent. Gerondakis *et al.* (1984) showed that the fusion of a mouse pre-B cell line with a mouse plasmacytoma cell line could induce the expression of a previously silent functional VDJ gene and this event

correlated with the demethylation of the IgH allele. In liver cells which do not express Ig genes, the IgH and IgL loci are hypermethylated and DNaseI hyposensitive. Both loci became increasingly demethylated and acquire DNaseI hypersensitivity as B cells mature (Storb *et al.*, 1981, Storb and Arp, 1983). These results raise the possibility that, in cells which had functionally rearranged two genes, one allele might be hypermethylated and is therefore not expressed. However, the methylation status of Ig or TCR loci has not been examined in cells which contained two functional genes.

The allelic exclusion can also be explained by cellular selection theory (Wabl and Steinberg, 1982). The theory proposes that when B cells simultaneouly produce heavy chains from both IgH alleles, they are overloaded by the extra heavy chain proteins being produced from the second allele. This, in turn, results in free heavy chain proteins in the cells and the cells die from toxicity of the free heavy chain proteins. The theory was based on the observation that, while it is relatively easy to isolate a variant of hybridoma which produces only light chain, rarely a variant which produces only heavy chain can be isolated (Köhler, 1980). At the present time, the extent to which this cellular selection contributes to the allelic exclusion has not been conclusively determined.

In summary, allelic exclusion can be regulated at both the rearrangement (genetic) and post-rearrangement levels. At the rearrangement level, the expression of the μ and TCRβ chains, in turn, inhibits further rearrangement of the respective loci. It appears that the rearrangement of Igκ locus was also inhibited by the κ chain. However, at present it is still unclear whether the inhibition of Igκ rearrangement is an active inhibitory process. TCRα locus is different from all the mentioned genes in that it was not allelically excluded. While many experiments studied the allelic exclusion at the rearrangement level, studies of allelic exclusion at the post-rearrangement level have been limited. The

best characterized mechanism is the failure of the κ and TCRα chains to pair with the H-chain and TCRβ chain. At present, the phenomenon of allelic exclusion has been well-described and characterized. Although the involved biochemical pathway is still largely unknown, $p56^{lck}$ has been shown to be involved in the allelic exclusion at the TCRβ locus. Ultimately, we would like to understand the biochemistry of the allelic exclusion. The study of the involvement of $p56^{lck}$ in the allelic exclusion process should bring us toward that goal.

V_H Gene Utilization in B-lineage Cells

In many species, Ab diversity is generated primarily through combinatorial rearrangement of V, D and J gene segments. In the adult mouse, scientists have found that all the 13 described V_H gene families contribute to the diversity of the VDJ gene repertoire. However, before the adult pattern of V_H gene usage is reached, B cells in fetal and neonatal mice preferentially utilized the 3' V_H gene families (defined as the V_H gene families that are 3' to the V_HS107 family). The shift to the adult pattern of V_H gene usage occurs gradually after birth via an unknown mechanism. In human, the Ab diversity is also generated largely via the combinatorial rearrangement. Unlike mouse, however, the pattern of V_H gene usage does not change from as early as an 8-week fetus through adulthood. The characteristic of this V_H gene usage is that V_H families are utilized independently of their germline location. For example, the V_H3 family, located at the 5' end of the IgH locus, is used frequently as are the V_H5, V_H6 and V_H7, which are located toward the 3' end of the locus. The V_H3 family is the biggest V_H family and is used most frequently. The V_H5, V_H6 and V_H7 are small V_H families and are used less frequently than the V_H3 family. At present, it has not been determined whether the V_H gene usage would be different in fetus earlier than 8-weeks of gestation.

In some species, the combinatorial rearrangement does not appear to contribute much to the Ab diversity. For example, in rabbit, the entire VDJ gene repertoire is generated from as few as four V_H genes in spite of the presence of more than one hundred V_H genes in the germline, half of which appear to be functional. Each of the four utilized V_H genes are used at different frequencies. One of them, V_H1, is the 3'-most V_H gene and is utilized in 70%-80% of the VDJ genes. Unlike the preferential utilization of the 3' V_H families in fetal and neonatal mouse, the preferential utilization of these V_H genes in rabbit starts from as early as neonatal period and continues through adulthood. Another species which does not use combinatorial rearrangement to generate Ab diversity is chicken. Chicken has only one functional V_H gene and therefore, the generation of Ab diversity through combinatorial rearrangement of different V_H genes is not possible.

The following review will present our current knowledge regarding the V_H gene usage in B-lineage cells of three species: mouse, human and rabbit. The V_H gene usage in chicken, which is limited to the one and only functional germline V_H gene, will not be discussed. The V_H gene usage in mouse pre-B and B cells will be presented separately. The V_H gene usage in human and rabbit will be discussed as V_H gene usage in B-lineage cells because the studies of V_H gene usage in human and rabbit were generally performed in B-lineage cells, not in separate pre-B and B cell populations.

V_H Gene Utilization in Mouse Pre-B Cells

Several approaches have been employed to examine the V_H gene usage in newly generated mouse pre-B cells. These approaches are directed toward examining the pre-B cells in fetal liver or adult bone marrow at monoclonal or polyclonal levels. At the monoclonal level, V_H gene usage has been determined in individual untransformed pre-B cells (Blackwell, 1984, Malynn, 1987, Jeong, 1988, Yancopoulos, 1988, Freitas *et al.*, 1989, Freitas *et al.*, 1990, Jeong and Teale, 1990, Malynn *et al.*, 1990), pre-B cells

generated in long-term bone marrow (LTBM) culture (Reth, 1986, Yoshida, 1987, Jeong and Teale, 1989), transformed pre-B cell lines (Yancopoulos *et al.*, 1984, Lawler *et al.*, 1987, Kleinfield and Weigert, 1989) and pre-B cell hybridomas (Yancopoulos *et al.*, 1984, Perlmutter, 1985) using *in situ* hybridization (Jeong, 1988, Freitas *et al.*, 1989, Jeong and Teale, 1989, Jeong and Teale, 1990, Freitas *et al.*, 1990), northern analysis (Blackwell, 1984, Perlmutter, 1985, Reth, 1986, Lawler *et al.*, 1987, Malynn, 1987, Yoshida, 1987, Yancopoulos, 1988, Kleinfield and Weigert, 1989, Malynn *et al.*, 1990) and Southern analysis (Yancopoulos *et al.*, 1984, Yoshida, 1987). At the polyclonal level, V_H gene usage in polyclonal pre-B cells was determined using quantitative northern analysis (Blackwell, 1984, Yancopoulos, 1988, Kleinfield and Weigert, 1989). The study at the monoclonal level informs us how frequently each V_H family is used in fetus and adult, but the number of clones that can be assayed is limited. On the other hand, the study at the polyclonal level allows the examination of many clones but it only informs us of the levels to which various V_H families are used in fetus relative to those in adult or vice versa. Using these approaches, the fetal and neonatal pre-B cells are found to be biased toward the usage of the 3' V_H gene families. In adult pre-B cells, however, the studies in different laboratories have yielded contradictory results so no definite conclusion can be drawn.

V_H Gene Utilization in Fetal and Neonatal Pre-B Cells

Preferential V_H gene usage in fetal pre-B cells was first reported by Yancopoulos *et al.* (Yancopoulos *et al.*, 1984) who studied V_H gene usage in fetal liver pre-B cell hybridomas and in AMuLV-transformed fetal liver pre-B cell lines, which rearranged their IgH genes *in vitro*. They found that these fetal pre-B cells preferentially rearranged V_H genes in the V_H7183 family, one of the 3' V_H gene families, and concluded that the preferential usage of 3' V_H gene family is characteristic of fetal pre-B cells. Since then,

many studies regarding the preferential V_H gene usage in fetal pre-B cells have been reported. Like the study of Yancopoulos *et al.* (1984), most studies were performed in BALB/c fetuses, although the V_H gene usage in fetuses from C57Bl/6, NFS/N, Columbia inbred mice and NIH/Swiss outbred mouse has also been examined (Blackwell, 1984, Yancopoulos *et al.*, 1984, Perlmutter, 1985, Reth, 1986, Lawler *et al.*, 1987, Jeong, 1988, Yancopoulos, 1988, Freitas *et al.*, 1989, Jeong and Teale, 1989, Jeong and Teale, 1990). All the studies showed that fetal pre-B cells preferentially utilize the two 3'-most V_H families, V_H7183 and V_HQ52 but the frequency at which each V_H family was used varied among different studies.

One difficulty of drawing any firm conclusion regarding how often each V_H family was used is that different studies and experimental approaches resulted in different levels of usage. For example, Yancopoulos *et al.* (1984) examined the V_H gene usage by northern analysis in AMuLV-transformed fetal pre-B cell lines and reported that 92% (11 of 12) of the lines used the V_H7183 family. But when they examined the hybridomas generated from fetal pre-B cells, only 40% (2 of 5) of the lines used V_H7183 family. Yet in another study, Jeong and Teale (Jeong and Teale, 1989) found by *in situ* hybridization that only 30% of fetal pre-B cells generated in LTBM culture used V_H7183 family. The differences in these numbers are not due to the difference in mouse strain used in the studies because all of these studies were performed with BALB/c mice. It is evident in all the studies mentioned above that different experimental approaches, and even the same approach performed in different laboratories, resulted in different frequency of V_H gene usage.

The studies mentioned above showed that members of the V_H7183 and V_HQ52 are used preferentially in the fetal pre-B cells of all mouse strains examined. However, both V_H families are utilized at a different level in different mouse strains. The majority of

AMuLV-transformed fetal liver pre-B cell lines derived from BALB/c prefer to use the germline V_H7183 gene family (Yancopoulos *et al.*, 1984). On the other hand, most pre-B cell lines derived from NFS/N mouse (Kleinfield and Weigert, 1989) and NIH/Swiss (Reth, 1986) preferred to use the V_HQ52 gene family. Currently, we do not know whether the differences in V_H gene usage among mouse strains are factual because, as discussed earlier, it is difficult to compare data from different experimental approaches used in these studies.

In addition to the studies mentioned above which determined V_H gene usage in monoclonal pre-B cells, V_H gene usage in polyclonal fetal pre-B cells has also been studied by quantitative RNA blot analysis of μm mRNA. In these experiments, RNA blots of fetal liver and adult spleen RNA were sequentially hybridized with V_H -family specific and Cμ probes. The amount of radioactivity was measured and the relative utilization of each V_H gene family in μm mRNA was calculated in each RNA sample. This approach is based on the fact that μm mRNA is produced by pre-B and B cells and that the B-lineage cells in fetal liver are mostly pre-B cells. Therefore, the V_H gene usage in fetal liver μm mRNA would reflect the V_H gene usage in fetal pre-B cells. Using this approach, Yancopoulos *et al.* (1988) and Blackwell *et al.* (1984) reported that the relative utilization of the V_H7183 family in fetal liver RNA from BALB/c and C57Bl/6 mice is much higher than that of adult spleen RNA. In a similar experiment performed in NFS/N mice, Kleinfield & Weigert (1989) found that neonatal mice used V_HQ52 at a much higher level than adult mice. These results from polyclonal pre-B cells in fetus and neonate are similar to those of monoclonal pre-B cells in that V_H7183 is preferentially utilized in fetuses of BALB/C and C57Bl/6, and V_HQ52 is preferentially utilized in neonates of NFS/N mice.

One genetic mutation that abrogates the preferential usage of 3' V_H genes in fetal liver pre-B cells has been reported. Mice with xid (X-linked immune deficiency) mutation have a partial defect of B cell compartment. They have a decreased number of B cells, a decreased serum IgM level and a failure to respond to type II thymus-independent polysaccharide antigen (Sher, 1982, Rawlings, 1994). Using homozygous xid mice (phenotypically xid), Osman *et al.* (1992) derived fetal liver pre-B cell lines and examined the V_H gene usage in these cell lines. The authors found that these cell lines do not display the biased usage of 3' V_H genes. Rather, these cell lines used the 5' V_H gene families, V_HJ606 and V_HJ558, at a high level. When the authors derived pre-B cell lines from fetal liver of heterozygous female mice (phenotypically normal), these cell lines displayed the normal bias for the 3' V_H gene families. These results suggested that the xid or closely linked locus can influence the V_H gene usage in fetal pre-B cells. Xid gene in mouse was shown to be the homologue of human Btk (Bruton's tyrosine kinase) gene (Rawlings, 1994). Because Btk protein is involved in B cell developmental and activation pathways, it is likely that xid gene product affects the V_H gene usage in fetal pre-B cells by being involved in proper development of pre-B and B cells rather than in the rearrangement process *per se*.

Currently we do not yet understand the mechanism of preferential usage of the 3' V_H families in fetal pre-B cells in mouse. Several hypotheses have been put forward to explain this observation (Wood and Tonegawa, 1983, Alt, 1987, Malynn, 1987, Perlmutter, 1987, Wu *et al.*, 1990, Cancro *et al.*, 1991, Chukwuocha and Feeney, 1993, Grandien, 1994). The position-dependent hypothesis, suggests that these genes are easily accessible to recombinase because of their 3' location and, therefore, they can be readily rearranged. Several lines of evidence suggested that this hypothesis might not be the explanation. Atkinson *et al.* (1993) mapped the location of the 3' V_H families and found that the preferentially utilized V_H genes were not always the 3'most genes, suggesting that

the 3' location is irrelevant to preferential utilization. The irrelevance of the 3' location is further supported by the finding that preferentially utilized $V\kappa$ genes in fetal liver in mouse were dispersed throughout the κ locus (Kaushik *et al.*, 1989, Lawler *et al.*, 1989). and that the two V_H genes in V_H5 family that were equally over-represented in human fetal B cell repertoire were located 500 kb apart in the genome (Pascual, 1993). In addition, Guass and Lieber (1992) showed in an *in vitro* recombination assay of an artificial recombination substrate containing several recombination signal sequences that the more 5' recombination signal sequence was used at the same frequency as the 3' one. Taken together, these data do not support "position-dependent hypothesis. " The second hypothesis suggests that the preferential usage of V_H7183 and V_HQ52 in fetus and neonates is associated with CD5 B cells, the predominant B cell population during fetal and neonatal period. After birth, the CD5 B cell population continues to decline resulting in a decreased usage of V_H7183 and V_HQ52 gene families. It is unlikely that this hypothesis is the explanation because the CD5 B cells preferentially utilize neither V_H7183 nor V_HQ52 families (Hardy, 1994) and because the preferential usage of V_H7183 family in neonate was shown to be associated with the $CD5^-$ B cell subpopulation (Jeong and Teale, 1990). Another hypothesis explains that the Ab specificity encoded by the 3' V_H genes are required to initiate the immuno-regulatory network and that the interaction of this network ultimately leads to the development of the adult-pattern of V_H gene usage. This hypothesis is supported by the finding that many poly-specific natural Abs secreted by newborn B cell hybridomas utilized V_H7183 and V_HQ52 gene families (Holmberg, 1987). Furthermore, the V_H7183 family is conserved in different mammalian species. This conservation of V_H genes suggested that there might be a special function for these genes besides encoding for the variable region. To test the immuno-regulatory hypothesis, we need to examine the VDJ gene repertoire in a mutant mouse that lacks both V_H7183 and V_HQ52 families but such mutant mouse has not yet been identified (Tutter and Riblet,

1988). A recently proposed hypothesis is based on the finding that most of the rearrangement of V_H81X, a member of V_H7183 family, in fetus resulted in productive VDJ genes (Chukwuocha and Feeney, 1993). This is believed to be due to homology-directed rearrangement, a process in which the homologous sequences at the 3' end of V_H and the 5' end of D gene segments target the rearrangement such that most of the junctions would occur at the overlapping sites. From nucleotide sequence analysis, such homology directed rearrangement of V_H7183 and V_HQ52 families with the D gene segments would result in productive VDJ genes. The higher yield of productive VDJ genes then appears as if these genes are preferentially utilized. This hypothesis is attractive, but requires further investigation. Other hypotheses proposed to explain the preferential usage of 3' V_H genes include the variation in recombination signal sequences, the properties of recombinase and selection. Because the knowledge of how VDJ gene repertoire develops is important to our understanding of the humoral immune response, the mechanism of the preferential V_H gene usage will attract many investigations for quite some time.

V_H Gene Utilization in Adult Pre-B Cells

The V_H gene utilization in newly generated pre-B cells in adult has been studied although no firm conclusion can be drawn from these data. The findings, from several laboratories, can be divided into 2 groups. One group of findings shows that, unlike fetal pre-B cells, adult pre-B cells do not preferentially use the 3' V_H families. Rather, they use the V_HJ558, the 5'-most V_H family more frequently. Yancopoulos *et al.* (1984) showed that most AMuLV-transformed adult pre-B cell lines did not utilize the V_H7183 family, but the authors did not further characterize which V_H genes were utilized. In subsequent studies, Jeong *et al.* (1989) and Yoshida *et al.* (1987) cultured bone marrow from adult mice in LTBM cultures and found that the majority of pre-B cells generated in this *in*

vitro system use the V_HJ558 family. In contrast to the first group of findings, the second group showed that the V_H gene usage in adult pre-B cells was similar to that of fetal pre-B cells. Freitas *et al.* (1990) performed an almost identical experiment to that of Jeong *et al.* (1989) and Yoshida *et al.* (1987) and found an opposite result in that the pre-B cells generated *in vitro* still preferred to use the V_H7183 family. Likewise, Lawler *et al.* (1987) examined the V_H gene usage in adult pre-B cell hybridomas and found that the V_H7183 family was preferentially used. Furthermore, Malynn *et al.* (1990) showed by quantitative northern analysis that adult bone marrow RNA contained a higher level of μm mRNA that hybridized to a V_H7183 family-specific probe than adult spleen RNA. These results suggested that B-lineage cells in adult bone marrow, presumably newly generated pre-B cells, still preferentially utilized the V_H7183 family. The conclusive experiment, in my opinion, would be to examine sIg-, Cμ+ bone marrow cells for the V_H gene usage by *in situ* hybridization or single-cell PCR. However, this approach presents a technical challenge and, so far, has not been undertaken.

V_H Gene Utilization in Mouse Resting B Cells

Several methods have been used to study V_H gene usage in resting sIg+ B cells. These studies were performed with peripheral B cells, especially those in spleen. Since plasma cells are found in spleen and the inclusion of plasma cells in the evaluation could significantly skew the V_H gene usage due to the fact that V_H gene usage in plasma cells is antigen-dependent, care must be taken to exclude these cells from the analysis. The most well-characterized and accurate method to evaluate the V_H gene usage in resting B cells was to examine LPS-stimulated B cells. The interference of contaminating plasma cells can be greatly reduced after the LPS stimulation because only B cells proliferate and produce μ mRNA at high level in response to LPS (Wu *et al.*, 1990). The V_H gene usage in stimulated cells can then be determined directly by using *in situ* hybridization (Dildrop,

1985, Schulze, 1987, Jeong, 1988, Wu, 1988, Freitas *et al.*, 1989, Jeong and Teale, 1989, Feng and Stein, 1991, Viale *et al.*, 1992, Ravichandran *et al.*, 1994). V_H gene usage in these LPS-stimulated cells was also determined indirectly, by RNA blot analysis of the B cell hybridomas generated from the stimulated splenocytes (Perlmutter, 1985, Holmberg, 1987, Jeong, 1988, Bos and Meeuwsen, 1989, Osman *et al.*, 1992) or by constructing a cDNA library from LPS-stimulated B cells and examining the V_H gene usage of all μm cDNAs represented in the library (Sheehan and Brodeur, 1989, Sheehan *et al.*, 1993). The advantage of these approaches is that they study the V_H gene usage at the monoclonal B cell level. The V_H gene usage in resting B cells has also been examined at the polyclonal level by quantitative northern analysis (Yoshida, 1987, Lawler *et al.*, 1987, Yancopoulos, 1988, Malynn *et al.*, 1990). This approach was based on the fact that resting B cells produce mostly μm mRNA while plasma cells produce little or no μm mRNA. By examining the V_H gene usage in μm mRNA, we are able to determine the V_H gene usage in resting B cells.

V_H Gene Utilization in Fetal and Neonatal Resting B Cells

V_H gene usage in fetal and neonatal resting B cells has not been examined as intensely as that in fetal pre-B cells. The results from limited studies have been shown that resting B cells in fetal and neonatal mice, like pre-B cells, also preferentially utilized the 3' V_H families, the V_H7183 and V_HQ52. Among fetal B cell hybridomas, Perlmutter *et al.* (1985) found that 66% of them used the V_H7183 family while Jeong and Teale (1988) and Holmberg *et al.* (1987) found that the V_H7183 and V_HQ52 families were used at the same level, approximately in 25% of the hybridomas. Subsequently, Jeong and Teale confirmed their finding in normal resting B cells using *in situ* hybridization (Jeong and Teale, 1989). The differences in the number of V_H7183 utilizing hybridomas may reflect the difference in the mouse strains used in the studies. In Perlmutter's study, the fetal B

cell hybridomas were derived from several mouse strains while in Jeong's and Holmberg's studies, the hybridomas were derived from only BALB/c. More experiments must be conducted for a firm conclusion to be drawn.

V_H Gene Utilization in Adult Resting B Cells

The V_H gene usage in resting B cells in adults was examined in several mouse strains by several laboratories and the findings varied among the reports. Although the frequency that each V_H family being utilized differed among the studies, it is clear that V_HJ558 family was the most utilized V_H family in adult resting B cells (Dildrop, 1985, Lawler *et al.*, 1987, Schulze, 1987, Yoshida, 1987, Jeong, 1988, Wu, 1988, Yancopoulos, 1988, Bos and Meeuwsen, 1989, Freitas *et al.*, 1989, Jeong and Teale, 1989, Sheehan and Brodeur, 1989, Malynn*et al.*, 1990, Feng and Stein, 1991,Osman *et al.*, 1992, Viale *et al.*, 1992, Sheehan *et al.*, 1993, Ravichandran *et al.*, 1994). The V_H families that were also utilized frequently were the V_H7183 and V_HQ52, the two preferentially utilized V_H families in fetal VDJ gene. At birth, the V_H7183 and V_HQ52 families were preferentially utilized. After birth, the V_HJ558 family was increasingly utilized and by 1-2 weeks of age, the adult pattern of V_H gene usage was reached (Perlmutter, 1985, Malynn *et al.*, 1990). The frequent usage of V_HJ558 in adult resting B cells led to the suggestion that the V_H gene usage in adults is "normalized" such that it reflects the complexity of each V_H gene family in the genome. Although this may appear so for the large V_H families, the usage of V_H families other than the V_H J558, V_H7183 and V_HQ52 may not reflect their complexity. For example, in BALB/c mice, 7%-10% of resting B cells used V_HX24 family which comprises of only 1-2 genes while V_H3660 family, which has 10 genes, was used at lower level, 2%-6% (Freitas *et al.*, 1989). However, the frequency of small V_H families being utilized is usually low and statistical analysis of the results is required.

Although the usage of large V_H families is normalized in adults, each V_H family is used to a different extent in resting B cells in different mouse strains (Schulze, 1987, Jeong, 1988, Yancopoulos, 1988, Wu, 1988, Kastner *et al.*, 1989, Sheehan and Brodeur, 1989, Viale *et al.*, 1992). The most apparent differences were in the frequency at which the two 3'-most V_H families and the 5'-most V_H family were used in resting B cells. The two 3'-most V_H families, the V_H7183 and V_HQ52, were used more frequently in BALB/c and C.AL20 mice than in C57Bl/6, CBA and B.AL20 mice. The 5'-most V_H family, V_HJ558, was used more frequently in C57Bl/6 and B10.D2 than in BALB/c, CBA and C.AL20 mice (Jeong, 1988, Wu, 1988, Sheehan and Brodeur, 1989, Viale *et al.*, 1992). This hierarchy of V_H gene usage is highly reproducible. It may simply reflect differences in the number of functional V_H genes among mouse strains or may be the result of an actively controlled process governed by environmental or genetic factors. The fact that germline V_H gene repertoires in inbred mice are similar (Brodeur and Riblet, 1984, Riblet *et al.*, 1986) makes the first possibility less attractive. However, all germline V_H genes must be characterized to exclude this possibility. Regarding the latter proposal, at present, there is evidence to suggest that the different level of each V_H family being utilized in different mouse strains may be due to genetic factors. Wu and Paige (1988) showed that the preference of resting B cells in BALB/c for V_H7183 and those in C57Bl/6 for V_HJ558 is controlled by a genetic locus different from the IgH locus suggesting that the mechanism mediating the V_H gene usage is encoded in the genome. The influence of genetic loci outside the IgH locus on the V_H gene usage in BALB/c and C57Bl/6 mice has been verified by Viale *et al.* (1992). No further characterization of this locus has been reported.

Why do B cells in adult mice switch to use the more 5' V_H families, especially V_HJ558? One could argue that it is because the usage of a large V_H family would ensure a larger size VDJ gene repertoire. Although this correlation seems apparent, a large

germline V_H family may not be necessary for generating a diverse VDJ gene repertoire. Wagner *et al.* (1994) reported that transgenic mice, generated with a human IgH minilocus containing 2 to 6 germline V_H, D and J_H genes plus the Cμ gene, were capable of mounting human Ab response to two irrelevant Ags, phenyl-oxazolone and hepatitis core protein Ag. This result suggested that the D and J_H gene segments, and other mechanisms, such as junctional diversity, may be sufficient to generate a large VDJ gene repertoire. Another possibility to explain the switching to utilize the V_HJ558 family is that rearrangement of the 3' and 5' V_H families are regulated by different mechanisms. This is supported by the finding in VDJ-Cμ transgenic mice (Iacomini *et al.*, 1991, Atkinson *et al.*, 1993). Even with the expression of the μ transgene, the 3' V_H families were rearranged and expressed in B cells from transgenic mice, but not the 5' V_H families (Iacomini *et al.*, 1991, Atkinson *et al.*, 1993). This result suggested that rearrangement of the 5' V_H families is under an allelic exclusion mechanism mediated by μ chain while rearrangement of the 3' V_H families is not. Other explanations to explain switching to use 5' V_H families in adult mice include chromosomal topology and competence of recombinase, although we do not yet know how these two factors affect rearrangement. Our current knowledge about how VDJ gene repertoire develops is still in its infancy. As new experimental tools are developed and the mutant mice generated by gene targeting are at hand, the experiments that probe into a more mechanistic view of how VDJ gene repertoire develops are now possible.

V_H Gene Utilization in human B-lineage cells

Most studies of V_H gene usage in the human VDJ gene repertoire do not distinguish usage in pre-B from that in B cells. Therefore, this review will present these results altogether as V_H gene usage in B-lineage cells. All experimental approaches used to examine V_H gene usage in human B-lineage cells were similar to those that were

employed in the mouse studies. These approaches include northern analysis (Cuisinier *et al.*, 1989, Logtenberg *et al.*, 1989a, Logtenberg *et al.*, 1989b, Nickerson *et al.*, 1989, Cuisinier *et al.*, 1990, Guigou *et al.*, 1990, Timmers *et al.*, 1991), PCR (Huang *et al.*, 1992, Huang and Stollar, 1993, Pascual, 1993), cDNA library construction (Schroeder, 1987, Perlmutter, 1988, Mortari *et al.*, 1992) and *in situ* hybridization (Guigou *et al.*, 1990, Zouali and Theze, 1991) in both untransformed cells (Schroeder, 1987, Perlmutter, 1988, Cuisinier *et al.*, 1989, Guigou *et al.*, 1990, Zouali and Theze, 1991, Huang *et al.*, 1992, Mortari *et al.*, 1992, Huang and Stollar, 1993, Pascual, 1993) and transformed cell lines(Logtenberg *et al.*, 1989a, Logtenberg *et al.*, 1989b, Nickerson *et al.*, 1989, Cuisinier *et al.*, 1990, Guigou *et al.*, 1990, Timmers *et al.*, 1991) .

V_H gene usage in fetal B-lineage cells in human was studied to some extent. Cuisinier *et al.* (1989) examined the V_H gene usage of pre-B cells in 49-day human fetal liver using RNA dot-blot analysis. At this early stage of development, B-lymphopoiesis has just begun and only pre-B cells can be detected in fetal liver (Gathings *et al.*, 1977). The authors found that only the V_H5 and V_H6 families were expressed in 49-day fetal liver. Because the study did not distinguish the μ mRNA from the germline V_H transcript, we cannot conclude that these V_H genes were rearranged in pre-B cells. Several subsequent studies of V_H gene usage in fetal liver or cord blood cells analyzed μm mRNA, but did not examine pre-B and B cells separately. The fetal liver cells were from fetuses ranging from 56-day to 130-day (Schroeder, 1987, Perlmutter, 1988, Logtenberg *et al.*, 1989a, Logtenberg *et al.*, 1989b, Nickerson *et al.*, 1989, Berman and Alt, 1990, Pascual, 1993). and the cord blood was from a full-term infant (Mortari *et al.*, 1992). All these studies consistently showed that V_H3 family was used most frequently in human fetal B-lineage cells. The V_H 3 family was also utilized most often in fetal resting B cells as shown in the majority of IgM-secreting mature B cell lines derived from fetal liver (Logtenberg *et al.*, 1989b, Cuisinier *et al.*, 1990).

In the human fetal VDJ gene repertoire, certain V_H families are over-represented. The V_H3 family, although it is the largest V_H family, has a complexity of only 38% (Berman *et al.*, 1988), but it was found in to be utilized in over 50% of fetal VDJ genes (Schroeder, 1987, Perlmutter, 1988, Logtenberg *et al.*, 1989a, Logtenberg *et al.*, 1989b, Nickerson *et al.*, 1989, Berman and Alt, 1990, Mortari *et al.*, 1992, Pascual, 1993). In some studies, the V_H5 family (Cuisinier *et al.*, 1990, Pascual, 1993), the V_H6 family (Schroeder, 1987, Schroeder *et al.*, 1988, Schroeder and Wang, 1990) or the V_H7 family (Mortari *et al.*, 1992) were also over-represented. Except for the V_H3 family, different V_H families were over-represented in different studies. The differences, again, may result from different experimental approaches, from variation between experiments or from differences in the number of functional V_H genes between individuals.

Only adult resting B cells have been examined for their V_H gene usage (Logtenberg *et al.*, 1989, Logtenberg *et al.*, 1989, Guigou *et al.*, 1990, Timmers *et al.*, 1991, Zouali and Theze, 1991, Huang *et al.*, 1992,Huang and Stollar, 1993). Most of studies were performed with peripheral blood mononuclear cells using *in situ* hybridization. All studies showed that V_H3, V_H5 and V_H6 families were over-represented. Only the report from one group of investigators found that V_H gene utilization correlated with the complexity of each V_H family in the genome (Logtenberg *et al.*, 1989a, Logtenberg *et al.*, 1989b). Even though the approach was the same as in other publications, no obvious explanation was given as to why the finding in this group of reports was different from all the others.

In summary, it appears that V_H gene usage in B-lineage cells of human fetus is similar to that of adult. The V_H3 family is consistently utilized more often than other families and is over-represented in both the fetal and adult VDJ gene repertoire. Three other families, V_H5, V_H6 and V_H7, are also over-represented in some studies. This pattern

of V_H gene usage starts early during fetal development, as early as day 56 of gestation (Nickerson *et al.*, 1989). Although the exclusive expression of V_H5 and V_H6 families in 49-day fetal liver (Cuisinier *et al.*, 1989) may suggest that the 3' V_H families may be preferentially utilized in early human fetus, experiments with different experimental approaches are required to draw such a conclusion.

V_H Gene Utilization in Rabbit B-lineage Cells

The study of V_H gene usage in rabbit is more difficult than in other species due to the fact that all rabbit V_H genes belong to a single V_H family (Gallarda *et al.*, 1985, Currier *et al.*, 1988). This, in effect, prohibits the determination of V_H gene usage based only on hybridization. To assign the utilized germline V_H genes, the nucleotide sequences of VDJ genes must be determined. Two methods have generally been used: examining VDJ genes in genomic library (Knight and Becker, 1990) and PCR (Short *et al.*, 1991, Becker and Knight, 1990, Friedman *et al.*, 1994). For PCR, analysis has been performed using DNA from leukemic B cell lines (Becker *et al.*, 1990), mouse X rabbit B cell heterohybridomas (Raman *et al.*, 1994), and normal bone marrow cells and splenocytes (Knight and Becker, 1990). In the neonatal rabbit, the VDJ genes are undiversified (Short *et al.*, 1991, Friedman *et al.*, 1994) and the utilized germline V_H gene can be determined from the nucleotide sequence analysis of the rearranged VDJ, usually obtained by PCR. In adult rabbit, the VDJ genes are highly diversified and the utilized germline V_H genes cannot be assigned with certainty by the nucleotide sequences of the VDJ genes. To determine the V_H gene usage in adult rabbit B cells, the examinination of the promoter region of the VDJ genes is required. Studies regarding V_H gene usage in rabbit do not distinguish the V_H gene usage in pre-B, B cells and plasma cells. Therefore, in the following discussion, no attempt will be made to distinguish these possibilities.

The analysis of rabbit VDJ genes showed that the 3'-most V_H gene, V_H1, was preferentially utilized. Analysis of the VDJ genes cloned from genomic libraries of leukemic B cell lines (Becker *et al.*, 1990, Knight and Becker, 1990), splenocytes (Becker and Knight, 1990) and mouse X rabbit heterohybridomas (Raman *et al.*, 1994) showed that most of them utilized V_H1. Since the splenocytes used to generate heterohybridomas or genomic library were from adult rabbits (Becker and Knight, 1990, DiPietro *et al.*, 1990), it appears that V_H1 continues to be preferentially utilized during rabbit adulthood.

Not only was the discovery of the preferential usage of V_H1 important to understanding the development of rabbit VDJ gene repertoire, it had solved the puzzle regarding the allelic inheritance of the V_Ha allotypes of rabbit Ig. Serum Ig in rabbit can be grouped serologically into 2 groups: V_Ha^+ and V_Ha^- molecules, using Abs directed against the V_Ha allotypes. The a^+ molecules account for 70-80% of serum Ig (Dray *et al.*, 1963, Kim and Dray, 1973). The a allotypes are polymorphic and they are inherited as if controlled by allelic genes (Dray *et al.*, 1963, Kim and Dray, 1973). The finding by Knight and Becker (1990) that V_H1 is preferentially utilized simplifies the explanation. Further, the authors showed that V_H1 on all three a alleles, a^1, a^2 and a^3 encodes the prototypic V_Ha molecules of the respective allotype and the deletion of V_H1 on the a2 allele (V_H1-*a2*) correlated with the loss of the expression of a^+ molecules in a mutant Alicia rabbit (Knight and Becker, 1990, Allegrucci *et al.*, 1991).

The preferential V_H gene usage was also found among the a^- molecules. The a^- molecules can be grouped serologically into 2 groups, x and y, each of which is present at an equivalent level in the serum (Kim and Dray, 1973). The x and y allotypic specificities, like the a specificities, are polymorphic and are inherited by allelic genes. The molecular characterization of VDJ genes which encode the a^- molecules was mostly performed in a2 and mutant Alicia rabbits (DiPietro *et al.*, 1990, Short *et al.*, 1991, Chen

et al., 1993, Friedman *et al.*, 1994), both of which expressed Ig bearing x32 and y33 determinants. The results showed that as few as three V_H genes, V_Hx32, V_Hy33 and V_Hz, are preferentially utilized (DiPietro *et al.*, 1990, Short *et al.*, 1991, Chen *et al.*, 1993, Friedman *et al.*, 1994). The frequency that V_Hx32 and V_Hy33 were found in the VDJ genes correlated well with the serum levels of x32 and y33 molecules. V_Hz has only been identified by molecular cloning and, currently, we do not know whether V_Hz encodes any allotypic determinants. A limited study of the a^- molecules in a3 rabbits has also been reported (DiPietro *et al.*, 1992). In the a3 rabbit, the x group of molecules bore the x32 allotypic determinant, but the y group did not have the y33 determinant (Kim and Dray, 1973). From molecular cloning, the VDJ genes encoding these a^- molecules in a3 rabbits can also be divided into 2 groups. One group had sequence very similar to the V_Hx32 suggesting that they may encode the x32 molecules in the serum. The nucleotide sequences of the other group were similar to each other, but not to any utilized genes previously reported.

The study of the V_H gene usage in a^- molecules further undermines the position-dependent hypothesis that was proposed to explain the preferential utilization of the 3' V_H families in mouse fetus. In normal neonatal a2 rabbits, although V_H1 was preferentially used in 70%-80% of the VDJ genes, V_Hx32, V_Hy33 and V_Hz that were used to generate the rest of the repertoire (Friedman *et al.*, 1994) are at least 50 kb upstream of V_H1 (Knight and Becker, 1990). In Alicia rabbits, that do not have V_H1, instead of generating the VDJ gene repertoire from V_H4, the next upstream functional V_H gene, they used V_Hx32 and V_Hy33 to a high level. These data suggest that the preferential V_H gene usage in rabbit did not depend on their location at the 3' end of the IgH locus. At present, the mechanism governing the preferential V_H gene usage in rabbit is still unknown.

It is interesting how all three species discussed above have evolved to use combinatorial rearrangement differently. Many observations regarding V_H gene utilization were made and many questions are still unanswered. We do not yet know what the purpose is for the 3' V_H families being utilized at the beginning of mouse development just to be replaced by a repertoire which use the 5' V_H families later on. We do not know why rabbit preferentially rearranges as few as four V_H genes while the germline contains many more functional V_H genes which can be used to generate the Ab repertoire. These are examples of the problems which we expect to solve in the future. In perspective, we made a considerable progress toward the understanding of humoral immunity since the first gene rearrangement in mammals was discovered, and yet we still have much to learn.

CHAPTER II

MATERIAL AND METHOD

Animals

Colony rabbits of known V_Ha allotypes were maintained at the animal facility of Loyola University Chicago Medical Center, Maywood, IL 60153.

Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

Purification of Rabbit B Cells

Fluorescence-Activated Cell Sorting: Single cell suspension was prepared from mesenteric lymph node (MLN) and spleen from four a1/a2 heterozygous rabbits, 138G6, 35G, 147J5 and 147J6. Cells were released into suspension by pressing the tissues between the frosted end of two glass slides. For splenocytes preparation, cells were spun down at 250 g for 5 minutes and red blood cells were lysed by hypotonic PBS solution before they were stained. The splenocytes were resuspended in 1 ml of 1X PBS, then 9 ml of deionized water was added such that the final concentration of PBS is 0.1X. One milliliter of 10X PBS was added after the cells were incubated in 0.1X PBS for 15 seconds. The cells were washed once in 1X PBS before staining. For MLN cell preparation, the cells were used directly without hypotonic lysis. For staining, 2-3 x 10^8

splenocytes and MLN cells were resuspended in 10 ml of 1X HBSS (Hank's Balance Salt Solution) (Biologos, Naperville IL) and stained with biotinylated anti-a1 antibody at a final concentration of 1 μg/ml. The incubation was done at 4°C for 90 minutes, the cells were washed twice in 10 ml of cold 1X HBSS. The cells were resuspended in 10 ml of 1X HBSS and stained with avidin-Phycoerythrin at 1 μg of avidin-Phycoerythrin per 1 x 10^6 cells and incubated at 4°C for 90 minutes. Then the cells were washed twice in 10 ml of cold 1X HBSS and resuspended in 3 ml of buffered HEPES solution (1X HEPES buffered with saline, 1mM Ca^{2+}, 0. 5 mM Mg^{2+}, 10 mg/ml BSA). Ten million $a1^+$ B cells were sorted by FACS to a purity of more than 95%. In the sorting of MLN cells from 147J6, propidium iodide was added before the sorting (final concentration 50 μg/ml) to exclude non-viable cells which had taken up the fluorescent dye non-specifically.

Panning: Single cell suspension was prepared from MLN of rabbit 64H1 and kept in Modified RPMI-1640 (see hybridoma cell culture section) overnight at 4°C. Three plastic petri-dishes (100 x 20 mm) as well as 5 small plastic pieces were coated with 1 mg/ml of goat anti-rabbit light chain in 1X PBS overnight at 4°C. The morning after, the petri-dishes and the plastic pieces were rinsed extensively with 1X PBS before use. The sIg^+ cells were positively selected by incubating 1 x 10^8 MLN cells with each anti-light chain coated petri-dish. After the incubation at room tempearture for 60 minutes, non-adhering cells were removed. Cells adhering non-specifically were dislodged from the petri-dishes by vigorous swirling with large volume of 1X HBSS. Cells that adhered to the small plastic pieces were stained with 9AE10 (monoclonal anti-rabbit T cells) and 2C4 (monoclonal anti-rabbit MHC class II) to check for purity. All panned cells were positive for 2C4 but negative for 9AE10 as determined by fluorescent microscope suggesting that most, if not all, the panned cells were B cells.

Hybridoma Cell Culture

Generation of B cell hybridomas: B cell hybridomas were generated by fusing splenocytes of four rabbits, 363H1-2, 27H1, 196G2 and 64J2, with the rabbit fusion partner, 240E1-1, as described (Spieker-Polet H. *et al.* submitted). The hybridomas were cultured in modified RPMI supplemented with 15% fetal calf serum (FCS). The content of modified RPMI is as follows: 1X RPMI-1640 (Biologos), 1mM HEPES, 0. 3 mg/ml L-Glutamine, 110 μg/ml Sodium pyruvate, 50 units/ml Penicillin, 50 μg/ml Streptomycin, 0.5 μg/ml Fungizone® (GibcoBRL, Gaithersburg MA), 30 μg/ml Gentamicin and 5 mM 2-ME. In addition, the following solution mixtures were added to a concentration of 1X: Vitamin supplement (GibcoBRL), MEM-essential amino acid supplement (GibcoBRL), and MEM-nonessential amino acid supplement (GibcoBRL).

Cloning and culturing of B cell hybridomas: B cell hybridomas were cloned by limiting dilution at the cell density of 0. 5 to 1 cell/well in modified RPMI medium supplemented with 15% FCS. Fifty to 100 hybridoma cells were mixed with 50 ml of 5 x 10^5 cells/ml of HAT-sensitive 240E1-1 cell line (used as feeder cells). Then 0. 5 ml of the cell suspension mixture was seeded into each well of two 48-well tissue culture plates. After 2 days of incubation at 37°C 6% CO_2, the feeder cells were selected against by adding 0. 5 ml of modified RPMI containing 15% FCS and 2X HAT (GibcoBRL) to each well such that the final concentration of HAT was 1X. The medium was changed with modified RPMI containing 15% FCS and 1X HAT every 4-5 days until the hybridomas clones were visible, which generally took 2-3 weeks from the time when the limiting dilution was performed. Generally, less than 30% of the wells contained hybridomas after the cloning suggesting that they are monoclonal. The supernate was then collected and tested for the presence of rabbit Ig bearing the V_Ha allotypes of the spleen donors by ELISA. Two Ig-secreting subclones were selected from each parental hybridoma and grown up to 1-2 x

10^7 cells for genomic DNA isolation. Some of the clonings yielded only one subclone. In this instance, only one subclone was analyzed.

Collection of Rabbit Fetuses

Isolation of fetal liver DNA: Rabbits of our V_Ha allotype-defined colony maintained at Loyola University Chicago were mated, and fetal liver was obtained from 12- to 28-day-old fetuses. The 12- and 24-day-old fetuses were offspring from matings between a1 and a1/a3 allotype rabbits; the 13-, 14-, 21- and 28-day-old fetuses were offspring from matings between a3 allotype rabbits; and the 15-day-old fetuses were offspring from mating between a2 allotype rabbits.

At days 12 to 15, 21, 24 and 28 after mating, the female rabbits were sacrificed and the uteri were retrieved. To collect the fetuses from the uterus, a small incision was made to uterine wall on the opposite side from the site of implantation. The incision was then extended to expose the amnion which was then cut open and the fetus was collected. The use of stereoscope facilitates the collection of each fetus. Fetuses from day-12 and -13 were not easily seen. To collect fetus at these early gestation stages, the uterus was immersed in a large volume of RPMI-1640 medium to detach the fetus from the placenta, after which the fetus could be easily identified. After the fetuses were collected, they were rinsed extensively in RPMI-1640 to remove any trace of maternal blood. The fetal livers were then collected. The liver in 21-, 24- and 28-day-old fetuses could be readily identified. At early stages of fetal development (day 14 and 15), the livers are identified as the largest organ in the peritoneal cavity. The liver in fetuses younger than 14-day-old could not be identified and therefore, the middle-third of the fetal bodies were collected in place of the livers. All livers from the same litter were pooled; from them genomic DNA was prepared as outline below.

Genomic DNA Purification

Nuclei preparation: Genomic DNA was prepared from several tissue sources (spleen, kidney, liver and fetal liver), FACS-purified B cells, B cells purified by panning and B cell hybridomas. For DNA preparation from tissue sources, 0. 2 to 0. 4 gm of tissue were homogenized using tissue homogenizer (Polytron®) in 5 ml of sterile isotonic Saline solution containing TE buffer at a final concentration of 10 mM Tris•HCl pH 7. 4 and 1 mM EDTA. For fetal liver DNA preparation, all fetal liver from each litter of fetuses were pooled and homogenized as above. For DNA preparation of cells in suspension, cells were used directly without homogenization. The homogenated tissues or the B cell suspensions were then spun at 250g for 5 minute and the supernate was decanted. The cell pellet was resuspended by tapping and then 10 ml of cold nuclei lysis buffer (0. 32M Sucrose, 0. 01M Tris•HCl pH 7. 6, 5mM $MgCl_2$ and 1% Triton X-100) was added. The nuclei were harvested by centrifugation at 1,500g for 15 minutes. For DNA preparation from panned B cells, the cells were directly lysed on the petri-dishes by adding 3 ml of cold nuclei lysis buffer onto each petri-dish. The nuclei were harvested by scraping the cells off the dishes using cell scraper and pelleted as outlined above.

Genomic DNA extraction: Nuclei were resuspended in 300 μl of proteinase K buffer (0. 025 M EDTA, 0. 15 M NaCl and 0. 04 M Tris•HCl pH 7. 6). Subsequently, proteinase K and SDS were added to a concentration of 200 μg/ml and 0. 05% respectively. The proteinase K digestion was carried out overnight at 37°C. After the overnight digestion with proteinase K, the RNA in the samples was digested with RNaseA at a final concentration of 75μg/ml for 4 hrs at 37°C. The samples were then extracted twice with Phenol, twice with 1:1 mixture of Phenol:Sevag (24:1 mixture of Chloroform:Isoamyl alcohol (Sevag, 1935)) and twice with Sevag. The purified genomic DNA was then dialyzed against large volume of 1X TE overnight at room temperature. All the DNA used in this study was

prepared in Dr. Knight's laboratory except for the genomic DNA of rabbit T cell lines which was kindly provided by Dr. Thomas J. Kindt (NIAID, Bethesda MA).

Genomic Blot Analysis

Five to ten micrograms of genomic DNA were restricted with appropriate enzymes(s) at 2-3 units of enzyme(s)/μg of DNA at 37°C for 2 hrs to overnight. The completion of the digestion was analyzed on a mini-agarose gel. The completely restricted DNA was electrophoresed in 1% agarose gel in 1X E buffer at 1V/cm overnight, transferred overnight onto 0. 2 μm pore-size nitrocellulose membrane by standard capillary force method using 20X SSC and fixed to the nitrocellulose membrane by baking at 80°C for 1-2 hrs. The 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 10μg/ml of sheared salmon-sperm DNA) at 42°C for at least 6 hrs before 10 ng of ^{32}P-dCTP labelled probe was added (the probes used in this study are listed in Table 1 and were labelled by standard random priming to a specific activity of at least 1 X 10^8 cpm/μg). After overnight hybridization, the blot was washed in 1X blot wash solution (1X SSC, 0. 1% SDS and 0. 1% Sodium pyrophosphate) at 68°C and autoradiography was performed. For quantitative genomic analysis (quantification for the amount of germline J_H and probe A region in polyclonal B cells), the radioactivity of the hybridized probe was measured on the phosphoimager (Betagen®) overnight before autoradiography was performed.

In quantitative Southern analysis experiments, the radioactive probe A and pJ5 probe were removed from the blots before the blots were rehybridized with RAG2 or CD40L probes. This was done by pouring boiling 0.1X blot wash over the Southern blot. After the blot wash cooled to room temperature, the procedure was repeated one more time. Then the blots were rehybridized as outlined above.

Table 1 Probes used in this study and their origins

Probe	Germline origin	Derived as	References
p181	FR2 (codon 7-66) of pRV14, a ψ isolated from an a3 allotype rabbit	181 bp BamHI/PstI fragment of p181 plasmid	Garlarda J L. *et al.*, 1985 Currier S J. *et al.*, 1988
pJ5	germline J_H region encompassing J_H4-J_H6	1.2 kb BamHI/PstI fragment of pJ5 plasmid	Becker R S. *et al.*, 1989
probe A	germline region located between V_H1 and D3, the 5'-most D	400 bp HindIII/XbaI fragment of pD13 plasmid	Becker R S. *et al.*, 1989
D1 probe	germline D1b	800 bp EcoRI/XbaI fragment of pD7 plasmid	Knight K L., unpublished data
RAG2	germline RAG2 gene	1.5 kb PstI/HindIII fragment of RAG2 plasmid	Short J A. and Knight K L., unpublished data
CD40L	CD40 ligand cDNA	1.4 kb EcoRI fragment of CD40L cDNA plasmid	Boonthum A. and Knight K L., unpublished data

PCR Amplification and Cloning of VDJ, DJ and VD Genes

DJ from the hybridomas: DJ genes were PCR-amplified from DNA of the hybridomas in which the unexpressed allele was rearranged to a DJ gene as indicated by Southern analyses. Because the D gene used in the rearrangements was not known, a degenerate primer which recognizes all known D genes, o5'panD (Table 2), was used as 5'-primer in combination with $J_H$prB, which was used as 3'-primer. The PCR reaction was performed, using 0.5 to 1.0 μg of genomic DNA from hybridomas as templates, in 1X PCR buffer (Boehringer Mannheim, Indianapolis IN), 0.2 μM o5'panD primer, 0.2 μM $J_H$prB primer, 50 μM each of dATP, dCTP, dTTP and dGTP, and 25 units/ml of Taq polymerase. The volume of the reaction was adjusted to 50 μl and the reaction was carried out in thin wall PCR tubes for 30 cycles. Each cycle of the reaction consists of incubation at 94° for 45 seconds, at 60° for 45 seconds and at 72° for 45 seconds. Taq polymerase was not added to the reaction until the temperature of the reaction reached 80° during the first cycle. After the completion of the PCR reaction, 10 μl of the PCR products were analyzed on 5% or 12% PAGE. As negative controls, DNA from hybridomas which have unexpressed IgH gene in germline configuration (Hyb 11-1, Hyb 15-3, Hyb 21-1 and Hyb 68-1) or from hybridomas which have 2 VDJ genes (Hyb 31-11 and Hyb 2-1) was used. No PCR product was observed in these negative controls. The amplified DJ genes from Hyb 25-1, Hyb 36-1, Hyb 23-18 and Hyb 37-1 were cloned into pGEM-T vector (Promega, Madison WI). J_H probe-hybridizing clones were selected by standard dot-blot or standard colony lift hybridization using the pJ5 probe. The hybridization was performed overnight in 3X

Table 2 PCR primers used in this study

Name	Sequence (5' to 3')
$V_H$prB	CTGCAGCTCTGGCACAGGAGCTC
V_HRI	CTCGAGAATTCTGCGCTGGCTTCTCCTGGTCGCTG
$J_H$prB	GAGCTCACCTGAGGAGACGGTGACCA
J5-6ivs	GTTGTCTAGATAGCCGATACCCCAGAACAG
o5'panD	GGA(A/T)TTTGT(A/G/C)(A/C)(A/G/T)G(A/G)(G/T)C(C/T)(A/G)(A/C/T)(A/G/C)(A/G/C)CAC
5'D2b	AGAGACTCACCACACTTCACCATGCTGCAG
3'D2b	CACTAGGATCCTGGGCCGCTGAGAATCTGT

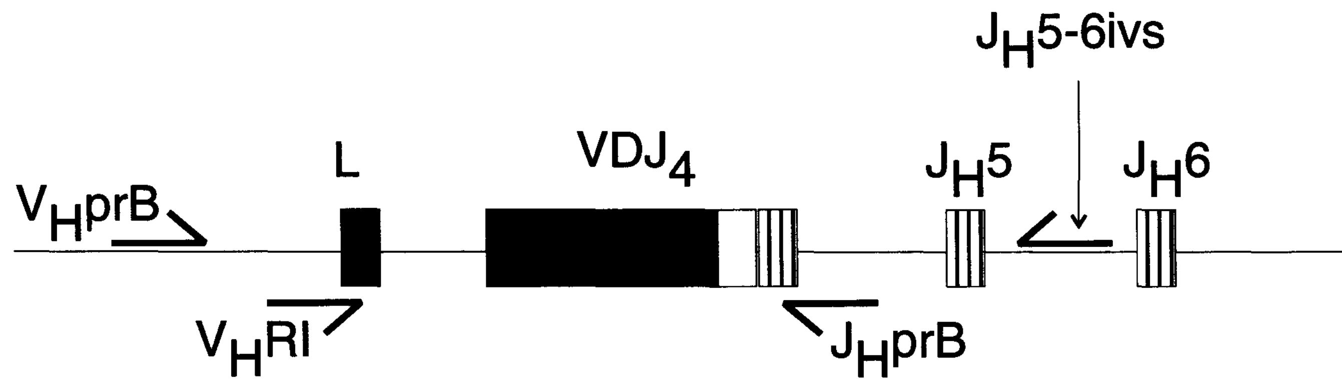
hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C. Nucleotide sequencing of pJ5-hybridizing clones was performed by using Sequenase (USB, Cleveland OH) and SP6 promoter primer.

DJ and VD genes from splenocytes: The D2bJ and VD2b genes were amplified by PCR from 0.5 to 1.0 μg of splenic DNA prepared from an a3 allotype rabbit (#357D2). For D2bJ gene amplification, 5'D2b and $J_H$prB primers (Table 2) were used. For VD2b gene amplification, V_HRI and 3'D2b primers (Table 2) were used. The PCR reaction was performed in 1X PCR buffer (Perkin-Elmer, Norwalk CT), 0.2 μM of 5'D2b or V_HRI, 0.2 μM of $J_H$prB or 3'D2b, 50 μM each of dATP, dCTP, dTTP and dGTP, and 25 units/ml of Taq polymerase. The reaction volume was adjusted 50 μl and carried out in regular PCR tube for 30 cycles. Each cycle consists of incubation at 94° for 1 minute, at 60° for 2 minutes and at 72° for 1 minute. The PCR products were analyzed on 5% PAGE. The amplified D2bJ genes were resticted with PstI/SacI and cloned into M13mp18 vector. J_H probe-hybridizing clones were selected by standard dot-blot hybridization using the pJ5 probe. The hybridization was performed overnight in 3X hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C. Nucleotide sequencing of J_H-hybridizing clones was performed as above using M13 universal primer. The amplified VD2b genes were resticted with BamHI/EcoRI and cloned into M13mp18 vector. V_H probe-hybridizing clones were selected by standard dot-blot hybridization using the p181 probe. The hybridization was

performed overnight in 3X hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C. Nuclotide sequencing of V_H probe-hybridizing clones was performed as above using M13 universal primer.

VDJ genes from fetuses: The VDJ genes in fetal liver were PCR-amplified by nested-primer PCR (except for 18-day-old fetus, see below) using 0.5 to 1.0 μg of fetal liver DNA template. The rationale for the nested-primer PCR is depicted in Figure 5. The primary PCR was performed with $V_H$prB and J5-6ivs primers. The final concentration of each component in the primary PCR was as followed: 1X PCR buffer (Boehringer Mannheim), 0.2 μM $V_H$prB, 0.2 μM J5-6ivs, 50 μM each of dATP, dCTP, dTTP and dGTP, and 25 units/ml of Taq polymerase. The reaction volume was adjusted 50 μl and carried out in thin wall PCR tube for 30 cycles. Each cycle consists of incubation at 94° for 45 seconds, at 60° for 45 seconds and at 72° for 45 seconds. Taq polymerase was not added to the reaction until the temperature of the reaction reached 80° during the first cycle. Ten microliters of the products from the primary PCR were used directly in the nested-primer PCR, which was performed in an essentially identical manner as the primary PCR except that the products from the primary PCR were used in place of fetal liver DNA, and that V_HRI and $J_H$prB primers were used in place of $V_H$prB and J5-6ivs primers. Ten microliters of the products from nested-primer PCR were analyzed on 5% PAGE. The PCR-amplified VDJ genes were either restricted with EcoRI/SacI and cloned into M13mp18 vector, or cloned directly into pGEM-T vector (Promega). V_H probe-

Figure 5 Schematic representation of the location of PCR primers used in the nested-primer PCR. The J_H4-utilizing VDJ gene is used in the diagram but the rationale is applicable to VDJ gene which had utilized any J_H genes.


J_H5-6ivs
L
VDJ_4
J_H5
J_H6
$V_H$prB
V_HRI
$J_H$prB

hybridizing clones were selected by standard dot-blot or standard colony lift hybridization using the p181 probe. The hybridization was performed overnight in 3X hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C.

The VDJ genes in 28-day-old fetal liver were PCR-amplified only by primary PCR reaction without nested-primer amplification using $V_H$prB and $J_H$prB as primers. The PCR-amplified VDJ genes were then cloned directly into pGEM-T vector (Promega, Madison WI). V_H probe-hybridizing clones were selected by standard colony lift hybridization using the p181 probe. The hybridization was performed overnight in 3X hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C.

The V_H probe-hybridizing clones were then subjected to nucleotide sequencing reactions. The nucleotide sequences of all VDJ clones in this study was partial and was determined in one orientation. The sequences obtained were all in anti-sense orientation (read from J_H upward to V_H) and therefore, the VD and DJ junctions in each VDJ genes can be determined with certainty. Any VDJ gene with identical VD and DJ junctions were considered as one clone. Five of the VDJ clones were randomly chosen for complete nucleotide sequence analysis (see legends for Figures 30, 31 and 34). Nonproductive VDJ genes were defined as the genes which 1) V_H and J_H are in different reading frames, 2) a

stop codon is present in the D region or N-segment and 3) a pseudogene was used in the rearrangement.

Nucleotide Sequence Analysis of V_H Promoters

V_H1 and V_H4 promoters: the 1. 2 kb HindIII/PstI fragments upstream of germline *V_H1-a3* and *V_H4-a3* were isolated from plasmids containing germline *V_H1-a3* and *V_H4-a3* previously described (Knight and Becker, 1990). The fragment from each V_H gene was further restricted with HpaI to generate two subfragments of 700 bp and 500 bp in size. Each of these fragments were cloned into M13mp18 and M13mp19 vectors. The clones which hybridized to the insert were selected by standard dot-blot hybridization. The hybridization was performed overnight in 3X hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C.

V_Hy33 promoter: because no HpaI site analogous to that found in the promoters of *V_H1-a3* and *V_H4-a3* was found in the *V_Hy33* promoter, an alternative strategy was employed. The plasmid containing germline *V_Hy33* promoter (20A1 clone) was restricted with AccI. After the restriction was completed, the 5' overhangs generated by AccI were filled with standard Klenow reaction. The Klenow was heat-inactivated by incubation at 68° for 15 minutes. The DNA was then further digested with SacI to generate several small fragments. The 200 bp, 350 bp and 550 bp fragments were gel-purified and cloned into M13 vector. The 200 bp fragment was cloned into M13mp19 vector while the 350 bp and

550 bp fragments were cloned into both M13mp18 and M13mp19 vectors. The clones which hybridized to the insert under standard dot-blot condition were selected. The hybridization was performed overnight in 3X hybridization solution (3X SSC, 5X Denhardt's, 0.1% SDS and 100 μg/ml of salmon-sperm DNA) at 68°C and washed in 1X blot wash at 68°C.

The promoter sequences were compared using Pileup module of GCG package (Genetics Computer Group, Inc. , Madison WI).

CHAPTER III

RESULTS

Quantification of Germline J_H and V_H1 in Polyclonal Rabbit B Cells

The order of IgH gene rearrangement during B cell development was established in mouse (Alt *et al.*, 1984). This order of "DJ on both IgH alleles before V to DJ" has since been regarded as dogma of how IgH genes are rearranged in every species. However, two findings regarding rabbit IgH gene suggested that rabbit may rearrange its IgH genes differently. First, Southern analysis of DNA of purified rabbit B cells showed that some of the J_H genes were in germline configuration (Allegrucci *et al.*, 1991). This finding is unusual in that similar study in mouse showed no J_H remained in germline configuration (Alt *et al.*, 1984). Further, Becker *et al.* found a VD gene rearrangement in a rabbit B cell line (Becker R. and Knight KL. unpublished data). This VD gene rearrangement has thus far never been found in mouse. To gain more insight into how rabbit B cells rearrange their IgH genes, I performed quantitative Southern analysis for the amount of J_H and V_H1 remaining in germline configuration in rabbit B cells.

Quantification of Germline J_H in Polyclonal Rabbit B Cells

To quantify the amount of germline J_H in polyclonal rabbit B cells, I purified $a1^+$ B cells from mesenteric lymph nodes of two a1/a2 heterozygous rabbits (#35G and 138G6, C/E haplotype) by FACS and prepared genomic DNA from the sorted $a1^+$ B cells.

The a1$^+$ B DNA as well as DNA prepared from livers of the two rabbits were then restricted with HindIII/EcoRI and subjected to quantitative Southern analysis using the J_H-specific probe, pJ5 and a probe from the non-rearranging gene, RAG2. I exploited the polymorphic EcoRI site in the J_H locus of C and E haplotype rabbits (Figure 6) so that I can distinguish the germline J_H on the a1 (expressed) allele from that on the a2 (unexpressed) allele.

Hybridization of the Southern blot of liver DNA of the 35G rabbit with pJ5 resulted in two hybridizing bands, a 3. 5 kb and a 5 kb fragment which corresponded to the germline J_H on a1 and a2 alleles, respectively (Figure 7a). The hybridization of DNA from purified a1$^+$ B cells showed that the 3. 5 kb fragment (a1-associated band) was absent (Figure 7a) while the 5 kb fragment (corresponding to the germline J_H gene on the unexpressed a2 allele) was present (Figure 7a). The amount of J_H on the a2 allele in both liver and purified a1$^+$ B cells DNA was quantified by measuring the radioactivity in the 5 kb hybridizing bands on a phosphoimager (Betagen®). The radioactivity measured in liver DNA represented the amount of J_H which should be present in a1$^+$ B cells if there had been no rearrangement of J_H on the a2 allele in these cells. Before comparing the amount of germline J_H on the a2 allele of purified a1$^+$ B cells with that in liver, I normalized the amount of DNA loaded in each lane by rehybridizing the Southern blot with RAG2 probe and comparing the amount of RAG2 hybridization of DNA from the a1$^+$ B cells with that of liver. The radioactivity in these samples was determined on the Betagen®. Because the RAG2 gene does not undergo rearrangement in either liver or B cells, the amount of RAG2 can be used as reference for the amount of DNA in each lane. From the amount of the radioactivity of pJ5 and RAG2 (Figure 7a), I calculated the amount of germline J_H on the unexpressed a2 allele of a1$^+$ B cells as shown in Figure 7b. I found that 75% of the theoretical amount of J_H on the a2 allele was in germline configuration in a1$^+$ B cells suggesting that 75% of a1$^+$ B cells did not rearrange J_H on their unexpressed IgH allele.

Figure 6 A partial map of the V, D and J chromosomal region from the a1 (C haplotype), a2 (E haplotype) and a3 (G haplotype) allotype rabbits. The regions from which probe A and J_H probes are derived are designated by brackets. The expected restriction fragments that hybridize with probe A and J_H probes for each haplotye are shown with double-headed arrows. The expected sizes are in kb.

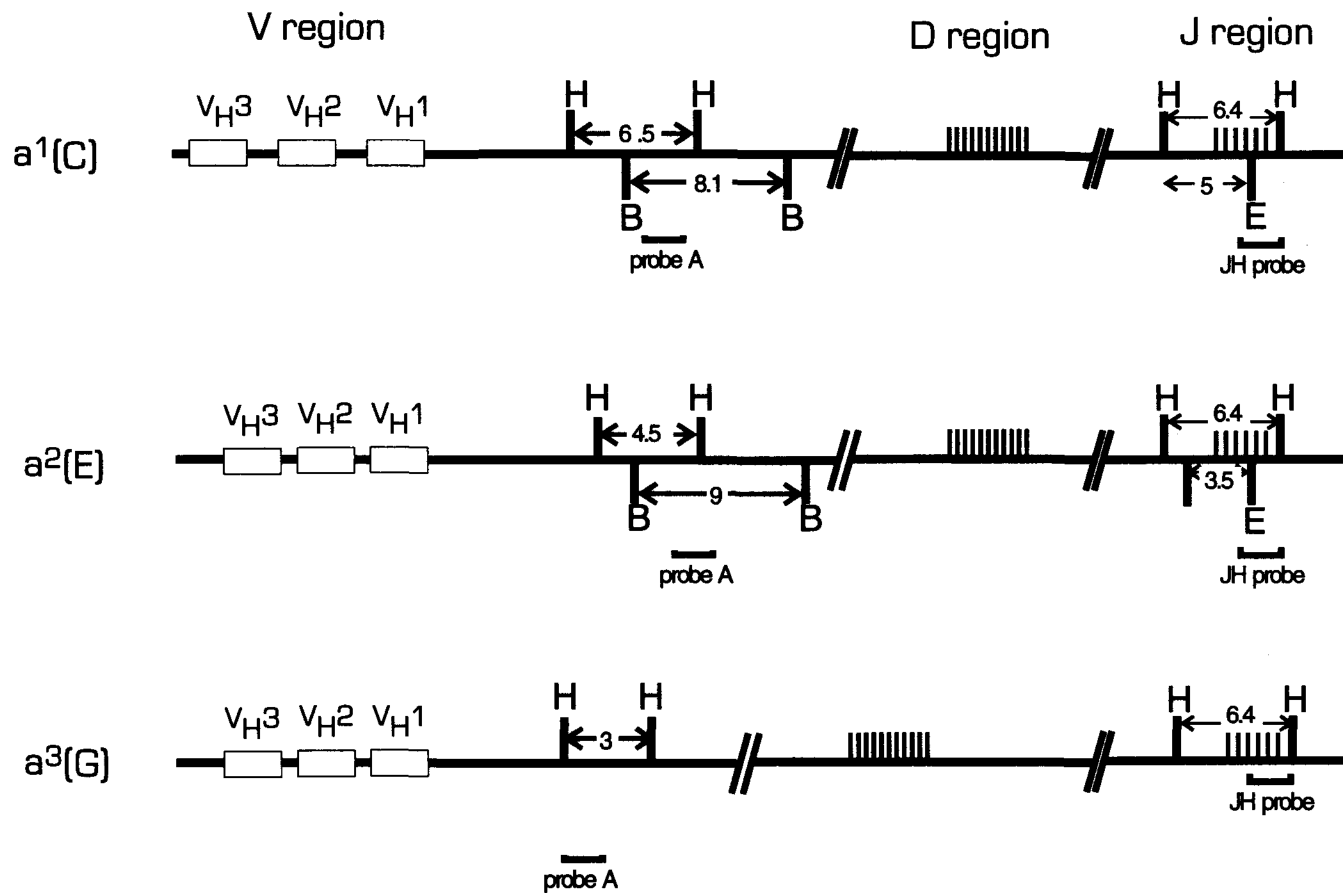
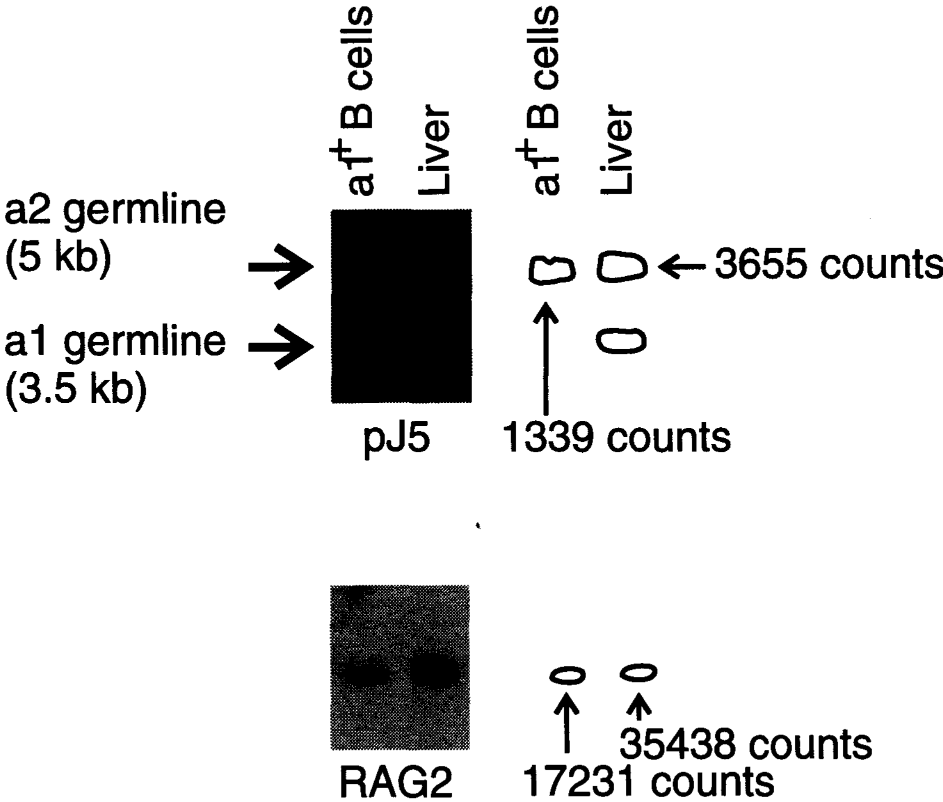

V region
D region
J region
a1(C)
VH3
VH2
VH1
H
H
6.5
8.1
B
B
probe A
H
6.4
H
5
E
JH probe
a2(E)
VH3
VH2
VH1
H
H
4.5
9
B
B
probe A
H
6.4
H
3.5
E
JH probe
a3(G)
VH3
VH2
VH1
H
H
3
probe A
H
6.4
H
JH probe

Figure 7 Quantitative Southern analysis of EcoRI/HindIII-restricted DNA for germline J_H in $a1^+$ B cells of rabbit number 35G. a) The autoradiogram of pJ5 (upper panel) and RAG2 (lower panel) hybridization. The hybridizing fragments associated with the germline J_H on the a1 and a2 alleles are indicated. The numbers in the diagrams next to the autoradiogram denote the amount of radioactivity of the indicated fragments (total counts). b) The calculation for the amount of germline J_H on the a2 allele of $a1^+$ B cells.

a) Quantification of germline J_H in B cells of rabbit #35G



b) Quantification for the amount of germline J_H in a1$^+$ B cells from 35G

pJ5:RAG2 ratio in Liver = *3655 / 35438* = *0.103*

Maximum ratio of pJ5:RAG2 in B cells = *0.103*

Observed ratio of pJ5:RAG2 in B cells = *1339 / 17231* = *0.078*

The percentage of remaining pJ5:RAG2 ratio to the maximum pJ5:RAG2 ratio

= *(0.078 / 0.103) x 100* = *75%*

Similar quantification was performed with the DNA of $a1^+$ B cells purified from rabbit 138G6. The hybridization of Southern blot of liver DNA from this rabbit with pJ5 probe resulted in a single 5 kb hybridizing band (Figure 8a) suggesting that this rabbit does not have the polymorphic EcoRI site in the J_H locus necessary to distinguish the J_H on the a1 allele from that on the a2 allele. Nevertheless, the hybridization of purified $a1^+$ B cells DNA showed, again, a pJ5-hybridizing fragment of the germline size suggesting that some of the J_H in purified $a1^+$ B cells were in germline configuration. The quantification was performed as outlined above and the result is shown in Figure 8b. In this experiment, I found that 93% of J_H in $a1^+$ B cells was in germline configuration. Taking the results from both rabbits together, I observed that 75% and 93% of J_H on the unexpressed allele of B cells are in germline configuration and that, in contrast to other species, many rabbit B cells did not rearrange J_H on their unexpressed IgH alleles.

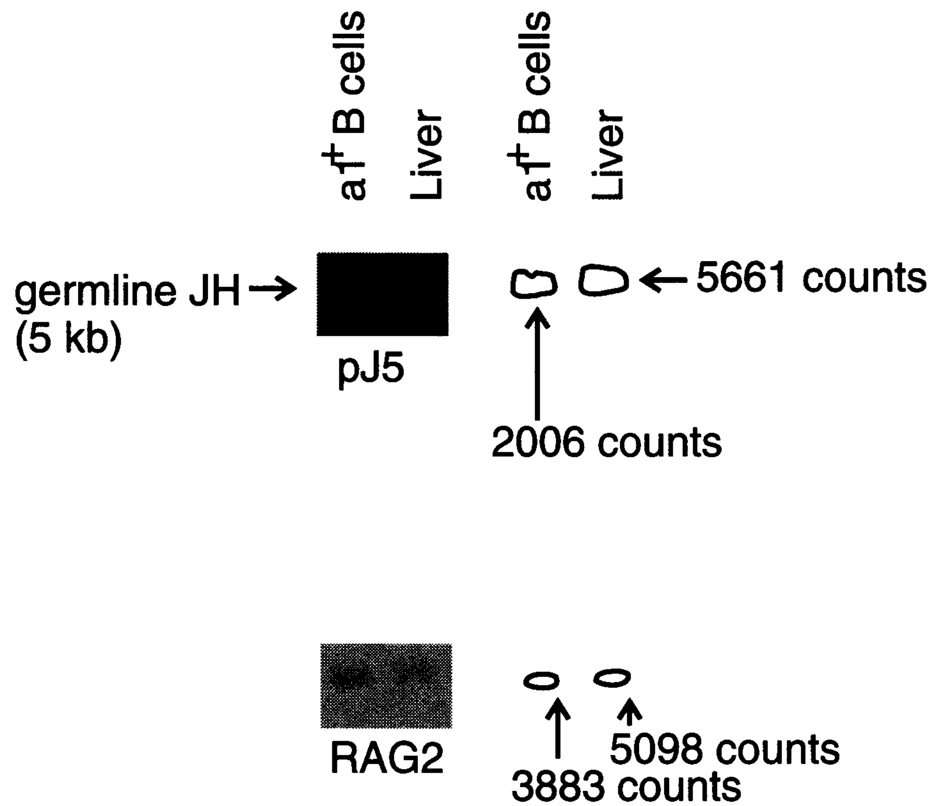
Quantification of Germline V_H1 in Polyclonal Rabbit B Cells

To determine whether V_H1, the preferentially utilized V_H gene, on the unexpressed allele of rabbit B cells is also unrearranged, like the J_H gene, I performed quantitative Southern analysis of DNA from purified B cells using probe A. Probe A was derived from a region between V_H1 and the 5'-most D gene segment (Figure 6) (Knight and Becker, 1990) which is deleted in V_H gene rearrangement. Therefore, quantification for the amount of probe A remaining in B cells would be equivalent to the amount of germline V_H1 in the B cells.

From two a1/a2 heterozygous rabbits, 147J5 and 147J6, I purified $a1^+$ B cells by FACS and prepared DNA from the sorted $a1^+$ B cells. The DNA preparations as well as DNA from livers of the two rabbits were restricted with BamHI and subjected to quantitative Southern analysis using probe A and either rabbit RAG2 or rabbit CD40L (CD40 ligand) probes. Because of polymorphic BamHI sites in the IgH locus, the probe A

Figure 8 Quantitative Southern analysis of EcoRI/HindIII-restricted DNA for germline J_H in a1$^+$ B cells of rabbit number 138G6. a) The autoradiogram of pJ5 (upper panel) and RAG2 (lower panel) hybridization. The hybridizing fragment associated with the germline J_H is indicated. The numbers in the diagrams next to the autoradiogram denote the amount of radioactivity of the indicated fragments (total counts). b) The calculation for the amount of germline J_H on the unexpressed allele of a1$^+$ B cells.

a) Quantification of germline J_H in B cells of rabbit #138G6



b) <u>Quantification for the amount of germline J_H in a1$^+$ B cells from 138G6</u>

pJ5:RAG2 ratio in Liver	=	*5661 / 5098*	=	*1.11*
Maximum ratio of pJ5:RAG2 in B cells	=	*1.11 / 2*	=	*0.56*

(because at least one allele of JH gene was rearranged to a VDJ gene)

Observed ratio of pJ5:RAG2 in B cells	=	*2006 / 3883*	=	*0.52*

The percentage of remaining pJ5:RAG2 ratio to the maximum pJ5:RAG2 ratio

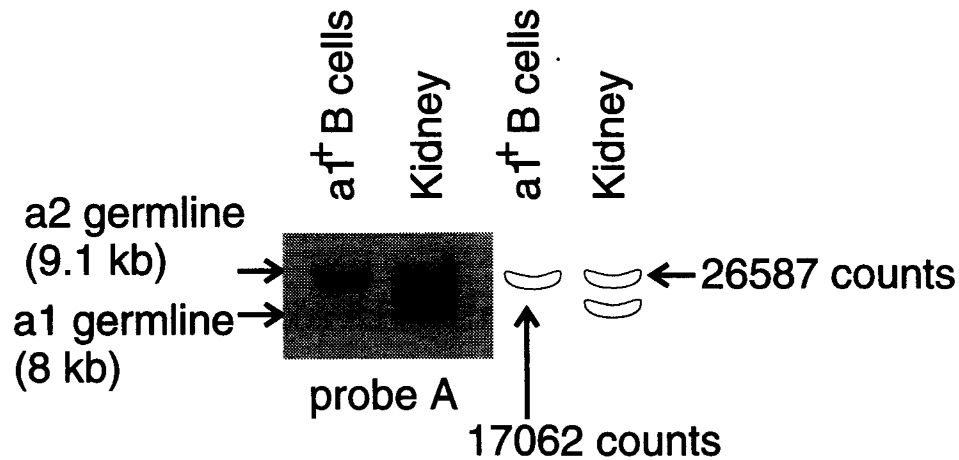
= *(0.52 / 0.56) x 100* = *93%*

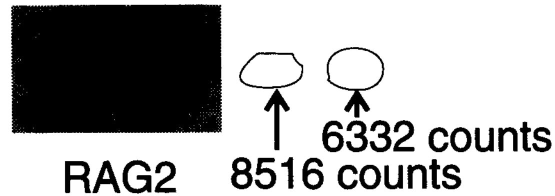
region on the a^1 allele could be distinguished from that on the a^2 allele in that the probe A region on the a^1 allele resides on an 8. 1 kb BamHI fragment while that on the a^2 allele resides on a 9 kb BamHI fragment (Figure 6). In kidney DNA of the two rabbits, the hybridization of probe A showed both the 9 kb and 8. 1 kb fragments as expected. In DNA of $a1^+$ B cells purified from both rabbits, I found the 9 kb (associated with unexpressed a2 allele) hybridizing fragment suggesting that many of the $a1^+$ B cells did not rearrange V_H1 on the unexpressed allele. To determine the amount of the probe A region remaining in these $a1^+$ B cells, the radioactivity of the 9 kb hybridizing fragment was measured on the Betagen®. To control for the amount of DNA loaded in each lane, I rehybridized the Southern blot with either CD40L or RAG2 probes and quantified the radioactivity of the hybridizing bands by using the Betagen® (Figure 9a and 10a). From the radioactivity count, I calculated the amount of probe A region remaining in purified $a1^+$ B cells (Figure 9b and 10b) and found that the levels of probe A region remaining on the unexpressed a2 allele of $a1^+$ B cells from 147J5 and 145J6 were 48% and 53%, respectively. This result suggested that many rabbit B cells did not rearrange their V_H1 on the unexpressed allele. However, a weak hybridizing fragment of 8. 1 kb in size (a1-associated) was present in both preparations of $a1^+$ B cell DNA suggesting that the $a1^+$ B cells preparation was contaminated by a2 B cells or T cells. Therefore, the actual amount of probe A region, and germline V_H1, in the $a1^+$ B cells may be somewhat lower than what the calculation showed.

Similar quantification for probe A was also performed with DNA of sIg+ B cells purified from rabbit 64H1 by positive panning with anti-L chain. Because this rabbit is an a3 homozygous rabbit, the hybridization of probe A with liver DNA showed only one hybridizing fragment of 3 kb in size (Figure 11a). The same 3 kb probe A-hybridizing fragment was found in hybridization of HindIII-restricted B cell DNA. The quantification for the amount of probe A region was performed by measuring the radioactivity of

Figure 9 Quantitative Southern analysis of BamHI-restricted DNA for probe A region in a1$^+$ B cells from rabbit number 147J5. a) The autoradiogram of probe A (upper panel) and RAG2 (lower panel) hybridization. The hybridizing fragments associated with the germline probe A region on the a1 and a2 alleles are indicated. The numbers in the diagrams next to the autoradiogram denote the amount of radioactivity of the indicated fragments (total counts). b) The calculation for the amount of probe A on the a2 allele of a1$^+$ B cells.

a) Quantification of germline 3' of V_H1 DNA in B cells of rabbit #147J5





b) Quantification for the amount of probe A region in $a1^+$ B cells from 147J5

probe A:RAG2 ratio in Kidney = 26587 / *6332* = *4.19*

Maximum ratio of probe A:RAG2 in B cells = *4.19*

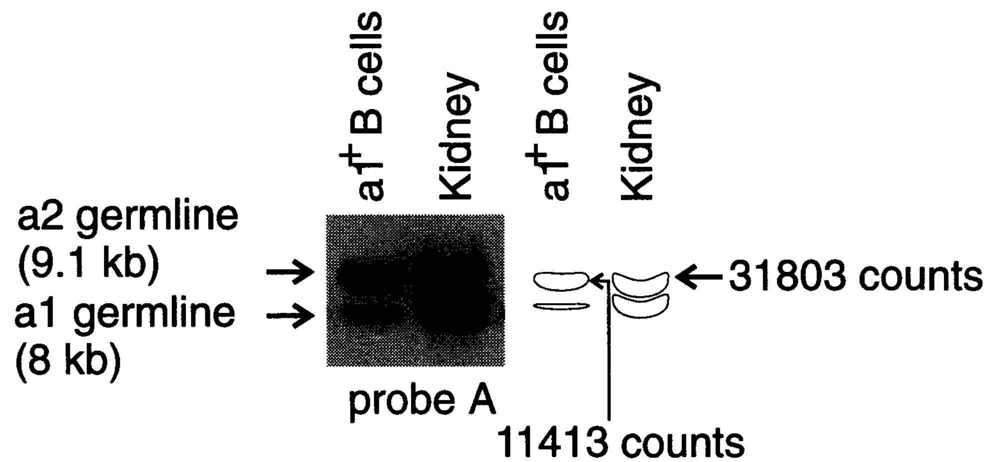
Observed ratio of probe A:RAG2 in B cells = *17062 / 8516* = *2.00*

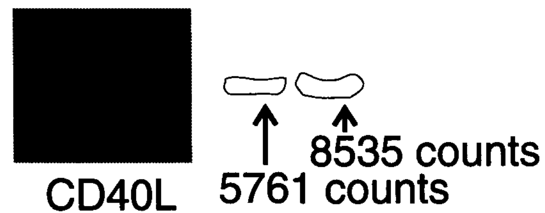
The percentage of remaining probe A:RAG2 ratio to the maximum probe A:RAG2 ratio

= *(2.00 / 4.19) x 100* = *48%*

Figure 10 Quantitative Southern analysis of BamHI-restricted DNA for probe A region in $a1^+$ B cells from rabbit number 147J6. a) The autoradiogram of probe A (upper panel) and CD40L (lower panel) hybridization. The hybridizing fragments associated with the germline probe A region on the a1 and a2 alleles are indicated. The numbers in the diagrams next to the autoradiogram denote the amount of radioactivity of the indicated fragments (total counts). b) The calculation for the amount of probe A on the a2 allele of $a1^+$ B cells.

a) Quantification of germline 3' of V_H1 DNA in B cells of rabbit #147J6





b) Quantification for the amount of probe A region in a1^{+} B cells from 147J6

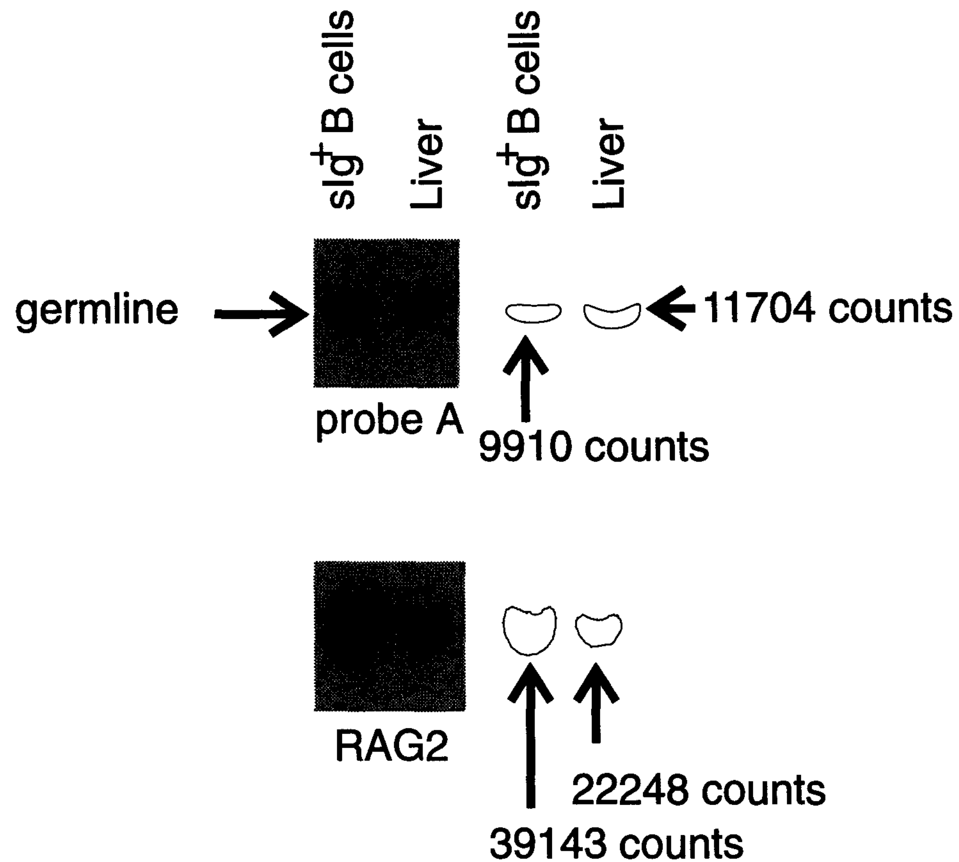
probe A:CD40L ratio in Kidney	=	31803 / 8538	=	3.72
Maximum ratio of probe A:CD40L in B cells			=	3.72
Observed ratio of probe A:CD40L in B cells	=	11413 / 5761	=	1.98

The percentage of remaining probe A:CD40L ratio to the maximum probe A:RAG2 ratio

$$= (1.98 / 3.72) \times 100 = 53\%$$

Figure 11 Quantitative Southern analysis of HindIII-restricted DNA for probe A region in sIg^+ B cells from rabbit number 64H1. a) The autoradiogram of probe A (upper panel) and RAG2 (lower panel) hybridization. The hybridizing fragment associated with the germline probe A region (3 kb) is indicated. The numbers in the diagrams next to the autoradiogram denote the amount of radioactivity of the indicated fragments (total counts). b) The calculation for the amount of probe A on the unexpressed allele of sIg^+ B cells.

a) Quantification of germline 3' of V_H1 DNA in B cells of rabbit #64H1



b) Quantification for the amount of probe A region in B cells from 64H1

probe A:RAG2 ratio in Liver = *11704 / 22248* = *0.53*

Maximum ratio of probe A:RAG2 in B cells = *0.53 / 2* = *0.26*

(because at least one allele of probe A was deleted in B cells during the rearrangement of the expressed IgH allele)

Observed ratio of probe A:RAG2 in B cells = *9910 / 39143* = *0.25*

The percentage of remaining probe A:RAG2 ratio to the maximum probe A:RAG2 ratio

= *(0.25 / 0.26) x 100* = *96%*

probe A hybridization using the Betagen® and by normalizing the value with the hybridization of the same blot using RAG2 probe (Figure 11b). I found that 98% of purified B cells retained probe A region suggesting that these cells did not rearrange the region 3' of V_H1.

Taking the quantification of germline J_H and 3' of V_H1 DNA in rabbit B cells together, the data suggest that many rabbit B cells do not rearrange their J_H and/or V_H1 on the unexpressed allele. However, the quantification was highly variable. Not only the quantification showed a high variability among rabbits, the repeated quantification of the same Southern blot can vary by as much as 100%. For instance, when quantification for the amount of probe A in the purified B cells of rabbit 147J5 was repeated twice as described above using the same Southern blot but in two different hybridizations, I found that the amount of probe A varied between 48% (Figure 9a and b) and 100% (not shown). Because of this high variability, I chose to analyze the status of IgH gene on the unexpressed allele in rabbit B cell hybridomas.

Rearrangements of V_H1 and J_H in B Cell Hybridomas

To determine the rearrangement of V_H1 and J_H genes in monoclonal B cells, I analyzed the IgH genes of rabbit x rabbit B cell hybridomas generated by fusing rabbit splenocytes with a Ig non-secreting rabbit fusion partner, 240E1-1 (Spieker-Polet H. *et al.*, submitted). Splenocytes and/or MLN cells from four rabbits were fused with 240E1-1. The four spleen donor rabbits have different V_Ha allotypes from 240E1, the rabbit from which 240E1-1 cell line was derived. The hybridomas which arose from the fusion were screened for secretion of rabbit Ig with appropriate V_Ha allotype and 29 of these Ig-secreting hybridomas were selected for the study. The status of IgH gene in the hybridomas was analyzed by Southern analysis of HindIII restricted DNA using probe A and pJ5 probes (Figure 6). From the pJ5 and probe A hybridization pattern, I can deduce

Table 3 The hybridization patterns of probe A and pJ5 probe for each possible status of IgH gene on the unexpressed allele

Status of the unexpressed allele	germline probe A	germline pJ5
VDJ genes[1]	-	-
VD gene and germline J_H	-	+
DJ gene and germline V_H	+	-
germline V_H, D and J_H	+	+

[1] Because of the preferential utilization of V_H1 and J_H4 in rabbit B cells, the two VDJ gene rearrangements may result in the same size fragment hybridizing to pJ5. In that case, I cannot distinguish between the possibility that there are two VDJ genes in this cell or one IgH-containing was lost after the fusion with the fusion partner.

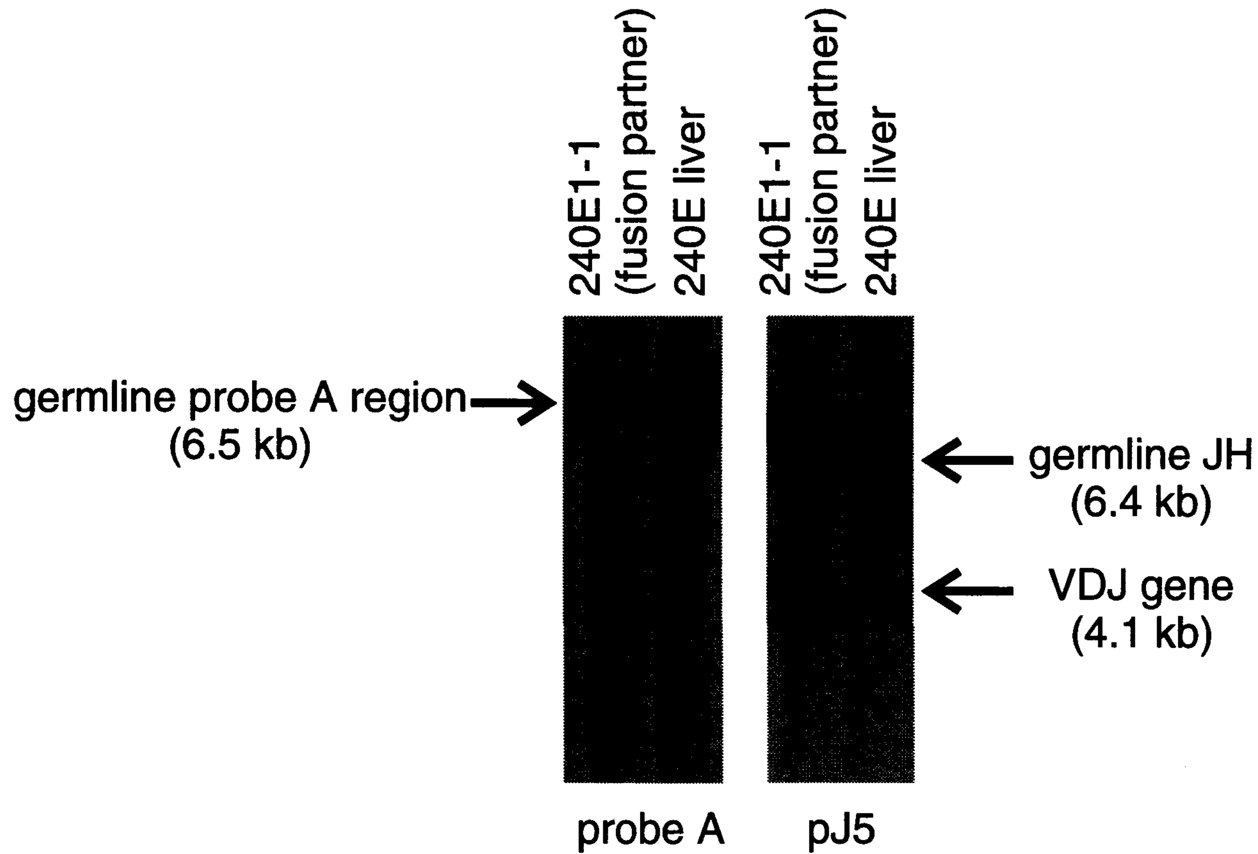
the status of the unexpressed IgH allele of the splenocyte as shown in Table 3. The status of the IgH gene in Table 3 represents theoretical status. In B cells of mouse, human and chicken (Alt *et al.*, 1984, Nickerson *et al.*, 1989, Reynaud *et al.*, 1987, Barth and Humphries, 1988, Benatar *et al.*, 1992) only two of these possible rearrangements, DJ and VDJ genes, were generally found on the second IgH allele.

First the status of IgH gene in the rabbit fusion partner was examined by Southern analysis of DNA from the fusion partner cell line as well as from liver DNA of rabbit #240E1, the rabbit from which the fusion partner was derived. The Southern blot of HindIII-restricted DNA was hybridized sequentially with the pJ5 probe and probe A. For the hybridization with the pJ5 probe, two hybridizing bands were detected in the rabbit fusion partner. One hybridizing fragment is 6. 2 kb in size (Figure 12), the same size as the germline J_H fragment seen in liver DNA, suggesting that the J_H on this unexpressed allele of the fusion partner was not rearrranged. Another pJ5-hybridizing fragment is a rearranged J_H of 4. 1 kb in size which must be a VDJ gene because the fusion partner initially secreted Ig in culture. The status of IgH gene on the unexpressed IgH allele was further examined by hybridization using probe A. The hybridization showed a hybridizing 6. 5 kb fragment, the germline size for probe A region as seen in DNA from liver of rabbit #240E1 (Figure 12). This result suggests that V_H1 on the unexpressed allele was not rearranged. From these results, I conclude that one IgH allele of the fusion partner was in germline configuration and the other allele was a VDJ gene.

Because the fusion partner retained its IgH gene in germline configuration on the unexpressed allele, the pattern of hybridization in the hybridomas is complex. Nevertheless, because of HindIII polymorphism in the the IgH locus, the IgH gene (both J_H and probe A regions) of the splenocytes can be distinguished from that of the fusion partner. The 29 hybridomas I chose to analyze were cloned by limiting dilution and

Figure 12 Southern analysis for the status of IgH genes in the fusion partner. DNA of fusion partner (240E1-1) was restricted by HindIII, Southern blotted and hybridized with either probe A (left panel) or pJ5 probe (right panel).

Southern analysis of the fusion partner using probe A and pJ5 probe



therefore, are monoclonal. Two subclones from each hybridoma were analyzed, if more than one subclone was obtained from the limiting dilution. In general, the two subclones derived from the same parental hybridomas gave the same result. From the hybridization with the pJ5 probe, each hybridoma had two or more hybridizing fragments, two of which were derived from the fusion partner. Because these hybridomas were selected for the secretion of Ig bearing V_Ha allotypic determinant, each of them must have at least one productive VDJ gene derived from the splenocytes. Because rabbit B cells preferentially utilized V_H1 and J_H4 genes (Knight and Becker, 1990, Friedman *et al.*, 1994) in their VDJ gene rearrangements, it is likely that the splenocytes utilized V_H1 and J_H4 which resulted in a rearranged 4. 1 kb HindIII fragment and the resulting VDJ gene would be indistinguishable from the VDJ gene of the fusion partner. The hybridization with the pJ5 probe confirmed this prediction (Figures 13-16). For example, Hyb 21-1 (Figure 13) did not have any rearranged J_H genes except for the 4. 1 kb HindIII. The hybridization was informative regarding the status of the J_H gene on the unexpressed allele, however. Fifteen of the 29 hybridoma clones examined had a 6. 4 kb (germline) J_H-hybridizing fragment suggesting that J_H genes on the unexpressed allele were in germline configuration (Figures 13-16 and Tables 4-7). The remaining 14 hybridomas did not have the germline-sized band that hybridized to the J_H probe suggesting that these hybridomas had rearranged a DJ or VDJ gene rearrangement on the second allele (Figure 13-16 and Tables 4-7).

To examine the rearrangement of V_H1 on the second IgH allele, I removed the pJ5 probe from the genomic blot by using boiled-0. 1X blot wash and rehybridized the blot with probe A. The 6. 5 kb probe A hybridizing fragment of the fusion partner was found in all the hybridomas I examined. In addition to this 6. 5 kb fragment, I found that in 26 of the 29 hybridomas, probe A hybridized to a fragment of 4. 5 kb or 2. 4 kb in size (Figures 17-20 and Tables 4-8). Because the 4. 5 and 2. 4 kb fragments which hybridized

Figure 13 Southern analysis of B cell hybridomas generated from rabbit # 363H1 using pJ5 probe. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with pJ5 probe. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Germline J_H of the splenocytes are indicated by star and rearranged J_H gene of the splenocytes that can be distinguished from J_H of the fusion partner are indicated by triangle. The status of these are further characterized by probe A hybridization (Figure 17) so that DJ can be distinguished from VDJ. Brackets indicate two subclones derived from the same parental hybridoma. The hybridoma names with circle are the two subclones from one parent which give different results.

Southern Analysis of B Cell Hybridomas Generatred from 363H1 using pJ5 Probe

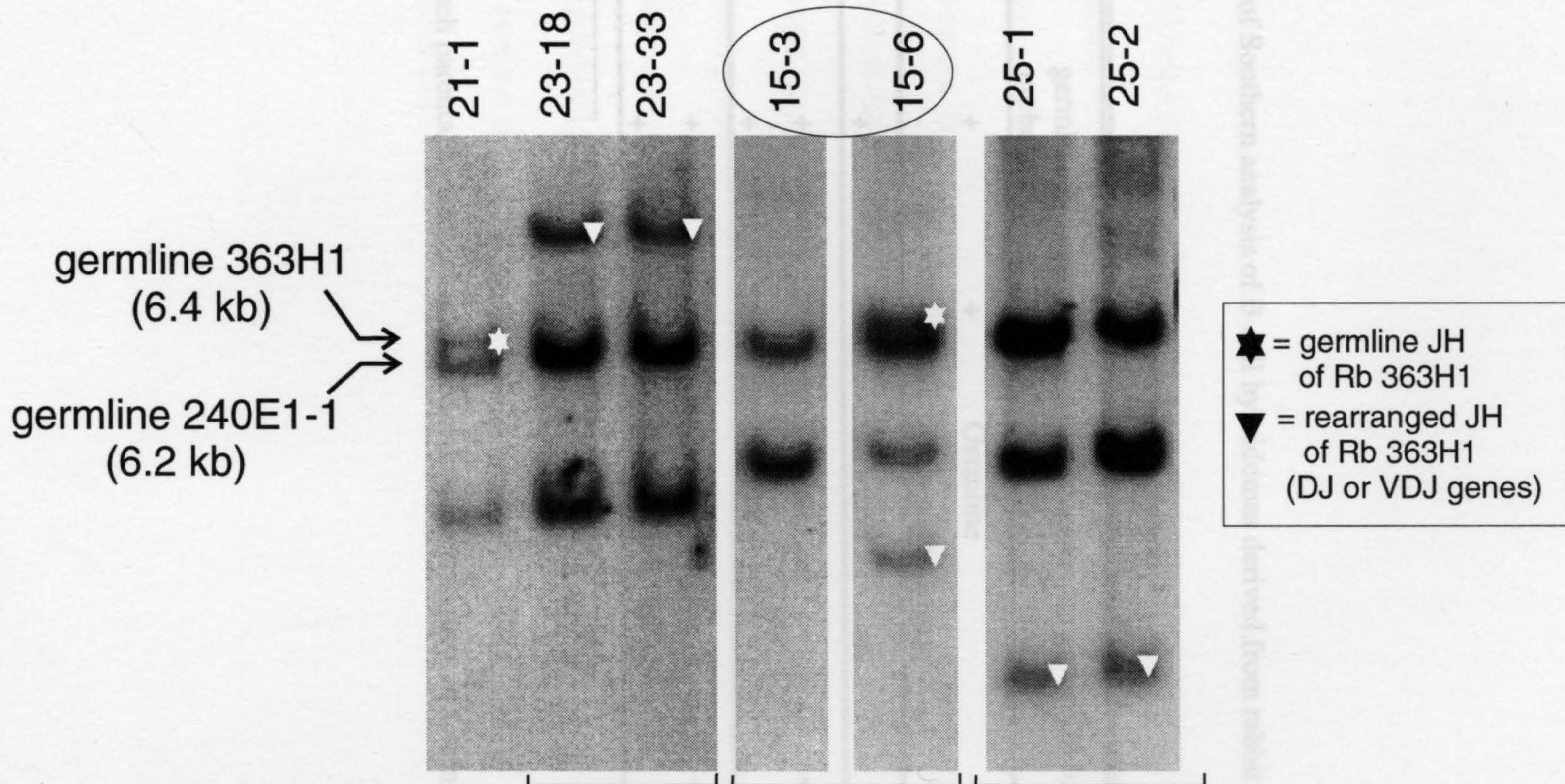


Table 4 Summary of Southern analysis of B cell hybridomas derived from rabbit 363H1#2

Rabbit #	Clones	germline probe A	germline J_H	Conclusion	PCR for DJ[1]
363H1#2	15-3[2]	+	+	Germline	-
	15-6	-	-	excluded (see text)	
	21-1	+	+	Germline	
	23-18	+	-	DJ gene	+
	23-33	+	-	DJ gene	
	25-1	+	-	DJ gene	+
	25-2	+	-	DJ gene	

[1] See Figure 22.

[2] The results from each parental hybridoma are separated from each other by a thin gray bar.

Figure 14 Southern analysis of B cell hybridomas hybridomas generated from rabbit # 27H1 using pJ5 probe. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with pJ5 probe. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Germline J_H of the splenocytes are indicated by star and rearranged J_H gene of the splenocytes that can be distinguished from J_H of the fusion partner are indicated by triangle. The status of these are further characterized by probe A hybridization (Figure 18) so that DJ can be distinguished from VDJ. Brackets indicate two subclones derived from the same parental hybridoma. The hybridoma names with circle are the two subclones from one parent which give different results.

Southern analysis of B cell hybridomas generated from rabbit 27H1 using pJ5 probe

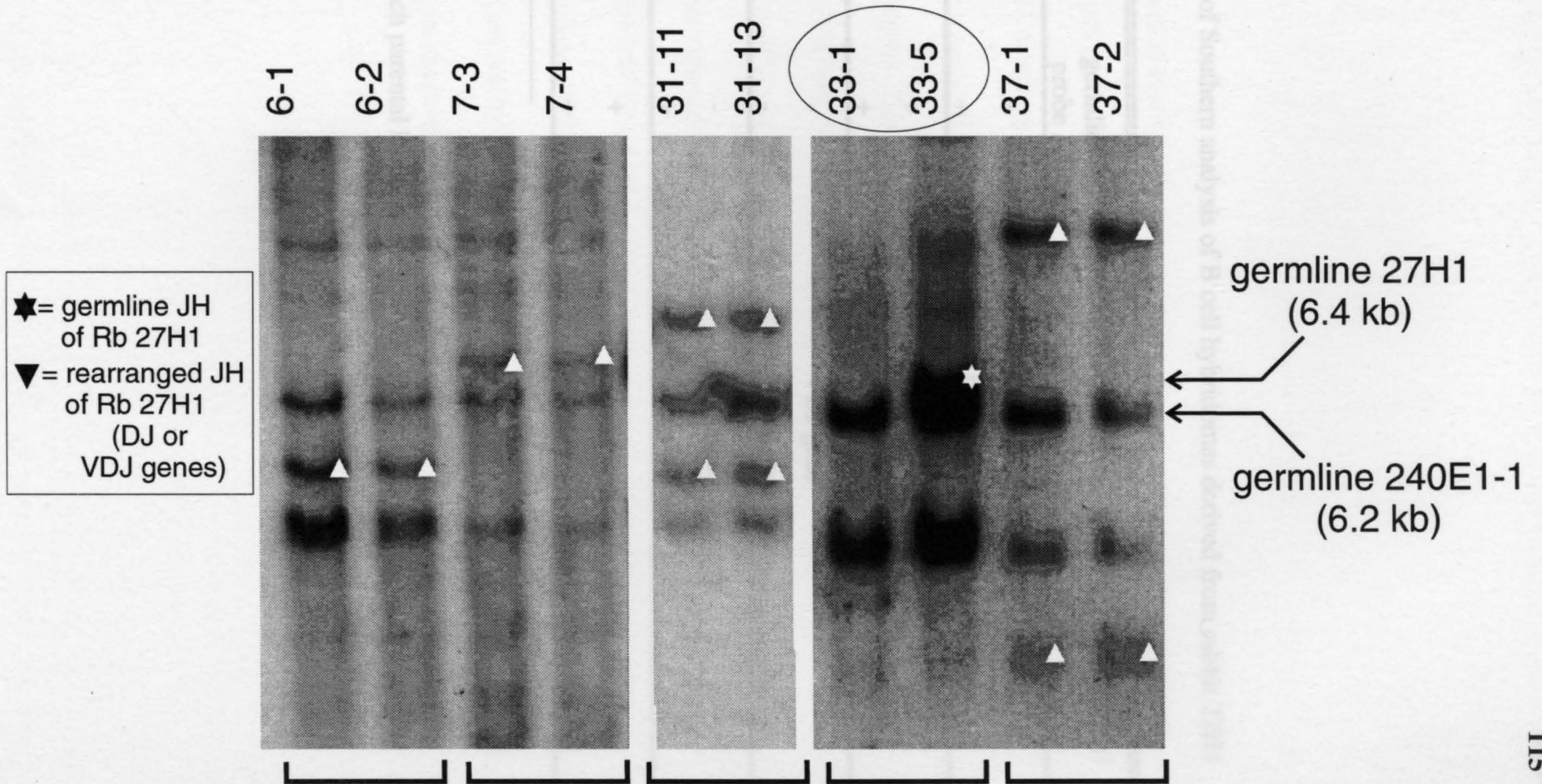


Table 5 Summary of Southern analysis of B cell hybridomas derived from rabbit 27H1

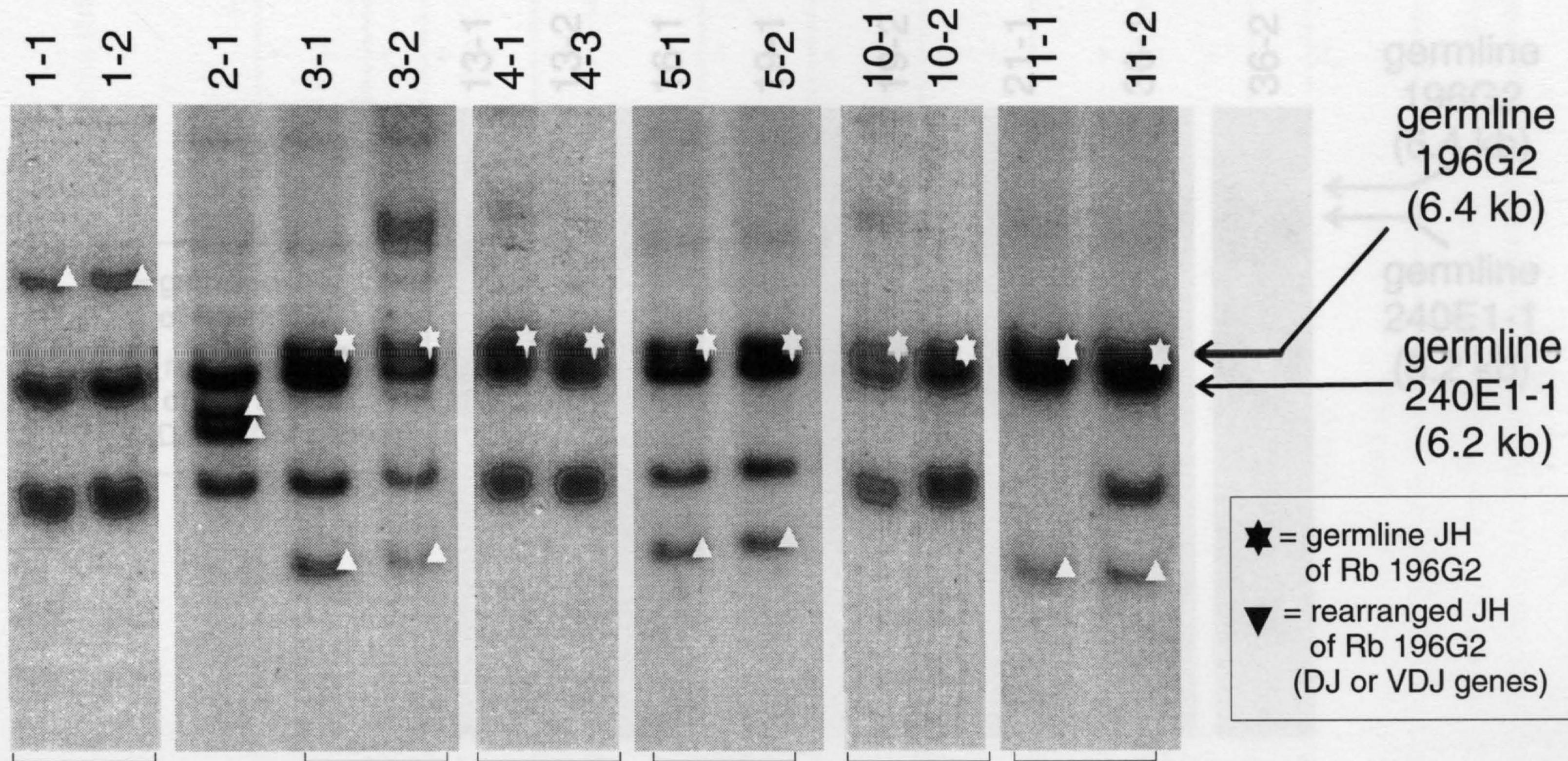
Rabbit #	Clones	germline probe A	germline J_H	Conclusion	PCR for DJ[1]
27H1	6-1[2]	+	-	DJ gene	+
	6-2	+	-	DJ gene	
	7-3	+	-	DJ gene	+
	7-4	+	-	DJ gene	
	31-11	-	-	VDJ gene	-
	31-13	-	-	VDJ gene	
	33-1	-	-	excluded (see text)	
	33-5	+	+	Germline	
	37-1	+	-	DJ gene	+
	37-2	+	-	DJ gene	

[1] See Figure 22.

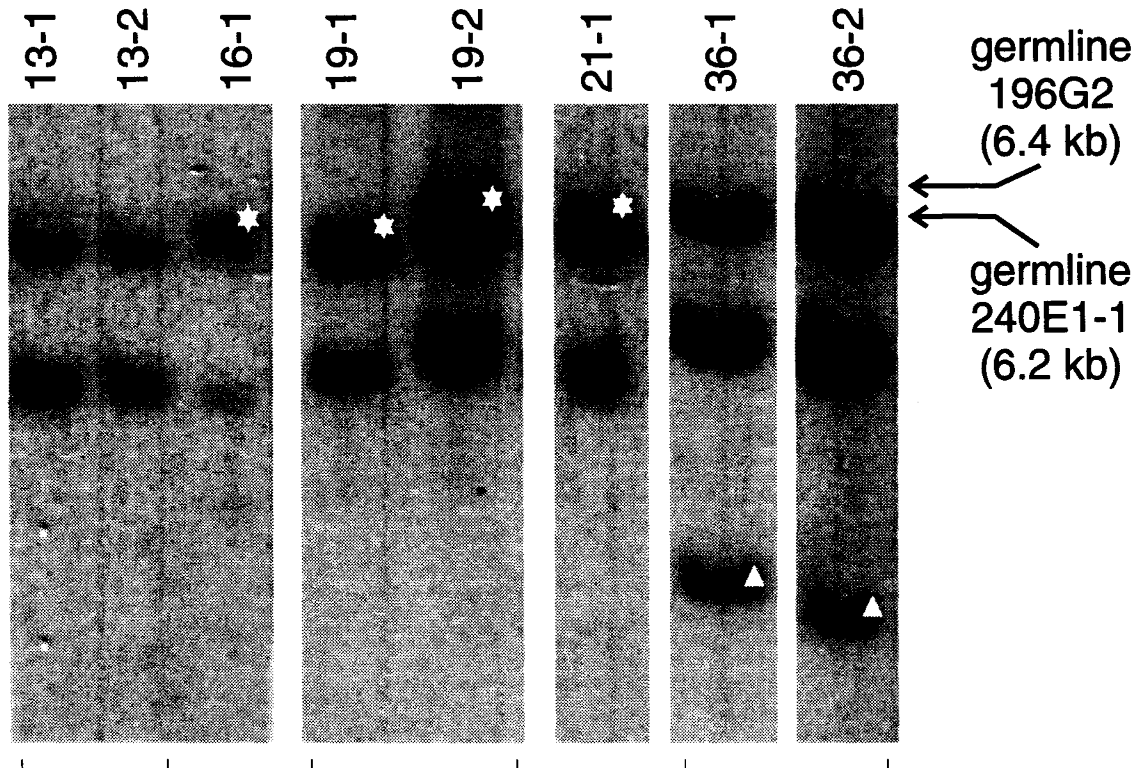
[2] The results from each parental hybridoma are separated from each other by a thin gray bar.

Figure 15 Southern analysis of B cell hybridomas generated from rabbit # 196G2 using pJ5 probe. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with pJ5 probe. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Germline J_H of the splenocytes are indicated by star and rearranged J_H gene of the splenocytes that can be distinguished from J_H of the fusion partner are indicated by triangle. The status of these are further characterized by probe A hybridization (Figure 19) so that DJ can be distinguished from VDJ. Brackets indicate two subclones derived from the same parental hybridoma. Hyb 11-1 does not have 4.1 kb pJ5-hybridizing fragment suggesting that it had lost the VDJ gene of the fusion partner.

Southern analysis of B cell hybridomas generated from rabbit 196G2 using pJ5 probe



Southern analysis of B cell hybridomas generated from rabbit 196G2 using pJ5 probe (cont.)



✱ = germline JH of Rb 196G2

▼ = rearranged JH of RB 196G2 (DJ or VDJ genes)

Table 6 Summary of Southern analysis of B cell hybridomas derived from rabbit 196G2

Rabbit #	Clones	germline probe A	germline JH	Conclusion	PCR for DJ[1]
196G2	1-1[2]	+	-	DJ gene	+
	1-2	+7	-	DJ gene	
	2-1	-	-	VDJ gene	-
	3-1	+	+	Germline	
	3-2	+	+	Germline	
	4-1	+	+	Germline	
	4-2	+	+	Germline	
	5-1	+	+	Germline	
	5-2	+	+	Germline	
	10-1	+	+	Germline	
	10-2	+	+	Germline	
	11-1	+	+	Germline	-
	11-2	+	+	Germline	
	13-1	-	-	VDJ gene	
	13-2	-	-	VDJ gene	
	16-1	+	+	Germline	
	19-1	+	+	Germline	
	19-2	+	+	Germline	
	21-1	+	+	Germline	-
	36-1	+	-	DJ gene	+
	36-2	+	-	DJ gene	

[1] See Figure 22.

[2] The results from each parental hybridoma are separated from each other by a thin gray bar.

Figure 16 Southern analysis of B cell hybridomas generated from rabbit # 64J2 using pJ5 probe. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with pJ5 probe. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Germline J_H of the splenocytes are indicated by star and rearranged J_H gene of the splenocytes that can be distinguished from J_H of the fusion partner are indicated by triangle. The status of these are further characterized by probe A hybridization (Figure 20) so that DJ can be distinguished from VDJ. Brackets indicate two subclones derived from the same parental hybridoma.

Southern analysis of B cell hybridomas generated from rabbit 64J2 using pJ5 probe

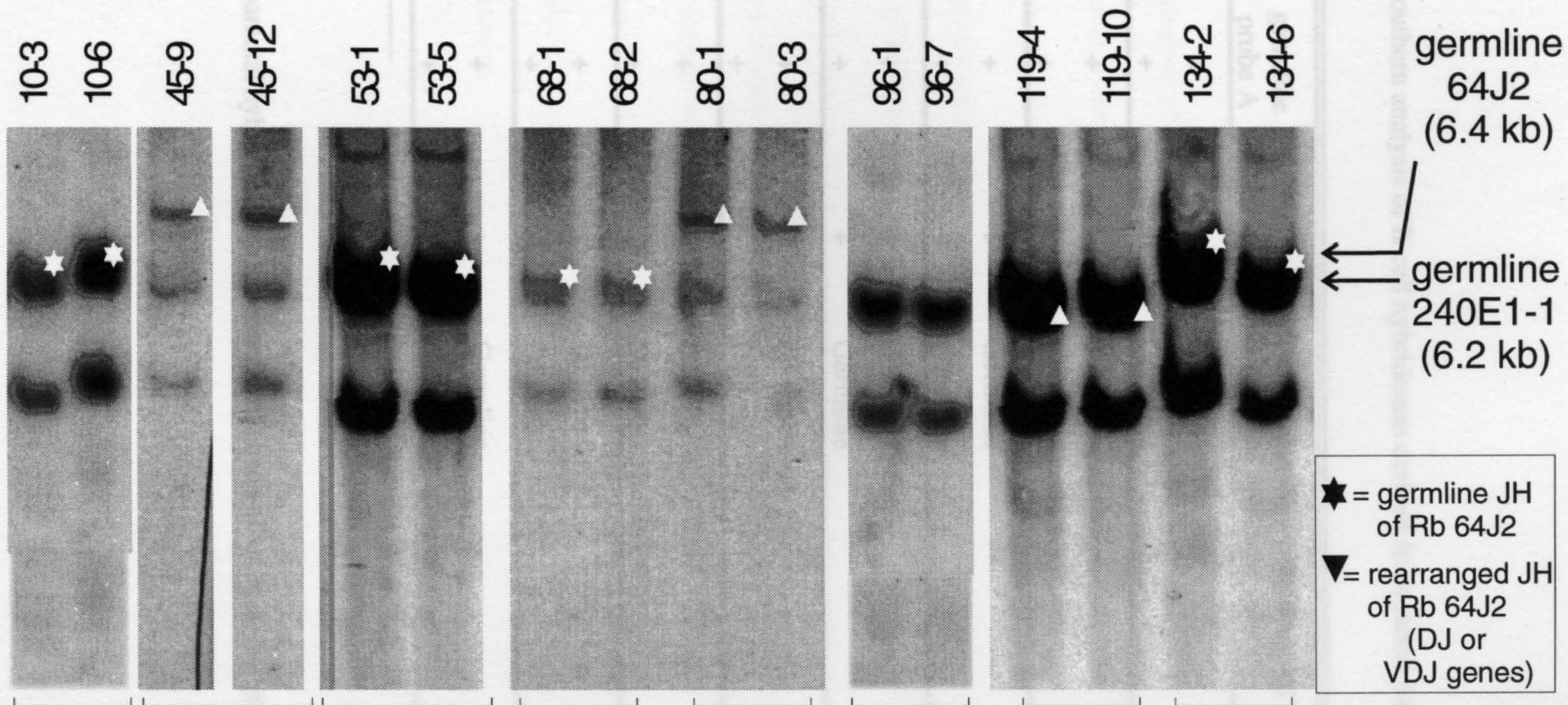


Table 7 Summary of Southern analysis of B cell hybridomas derived from rabbit 64J2

Rabbit #	Clones	germline probe A	germline J_H	Conclusion	PCR for DJ[1]
64J2	10-3[2]	+	+	Germline	
	10-6	+	+	Germline	
	45-12	+	-	DJ gene	
	45-9	+	-	DJ gene	+
	53-1	+	+	Germline	
	53-5	+	+	Germline	
	68-1	+	+	Germline	-
	68-2	+	+	Germline	
	80-1	+	-	DJ gene	+
	80-3	+	-	DJ gene	
	96-1	+	-	DJ gene	+
	96-7	+	-	DJ gene	
	119-10	+	-	DJ gene	
	119-4	+	-	DJ gene	+
	134-2	+	+	Germline	
	134-6	+	+	Germline	

[1] See Figure 22.

[2] The results from each parental hybridoma are separated from each other by a thin gray bar.

Figure 17 Southern analysis of B cell hybridomas generated from rabbit # 363H1 using probe A. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with probe A. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Polygon underneath the autoradiogram indicate the presence of germline J_H in the hybridomas (Figure 13). The status of IgH gene on the unexpressed allele of these hybridomas is summarized in Table 4. Brackets indicate two subclones derived from the same parental hybridoma. The hybridoma names with circle are the two subclones from one parent which give different results.

Southern analysis of B cell hybridomas generatred from rabbit 363H1 using probe A

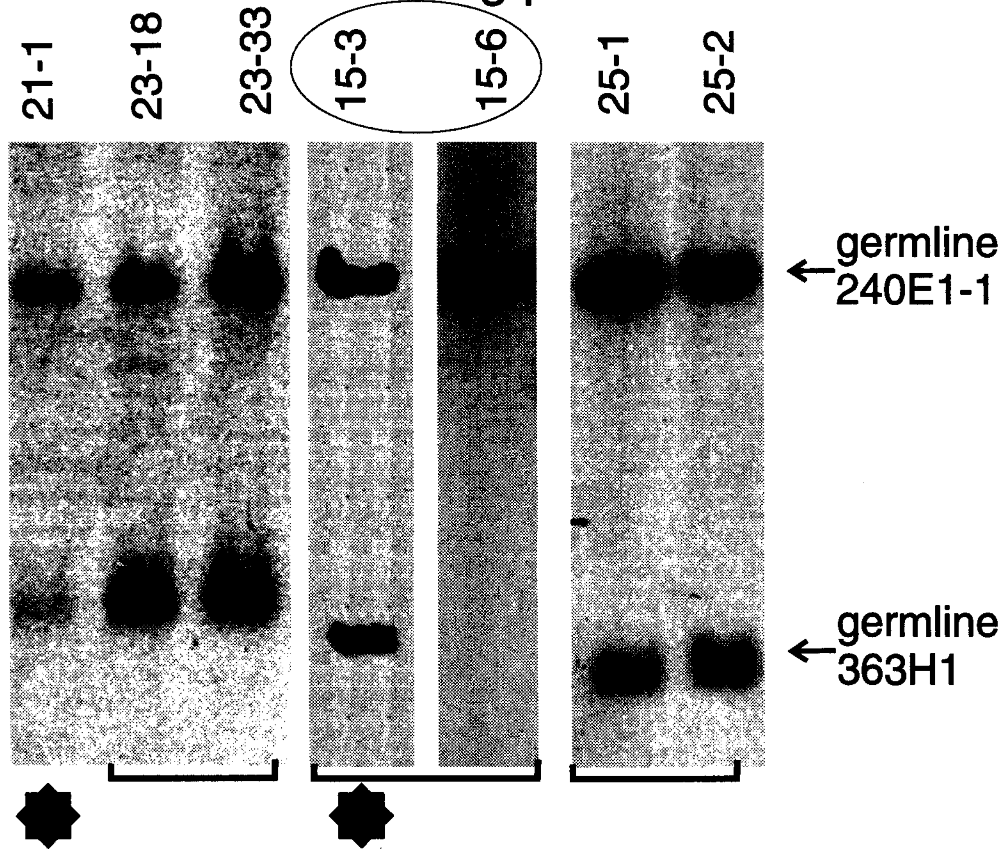


Figure 18 Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 27H1 using probe A. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with probe A. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Polygon underneath the autoradiogram indicate the presence of germline J_H in the hybridomas (Figure 14). The status of IgH gene on the unexpressed allele of these hybridomas is summarized in Table 5. Brackets indicate two subclones derived from the same parental hybridoma. The hybridoma names with circle are the two subclones from one parent which give different results.

Southern analysis of B cell hybridomas generated from rabbit 27H1 using probe A

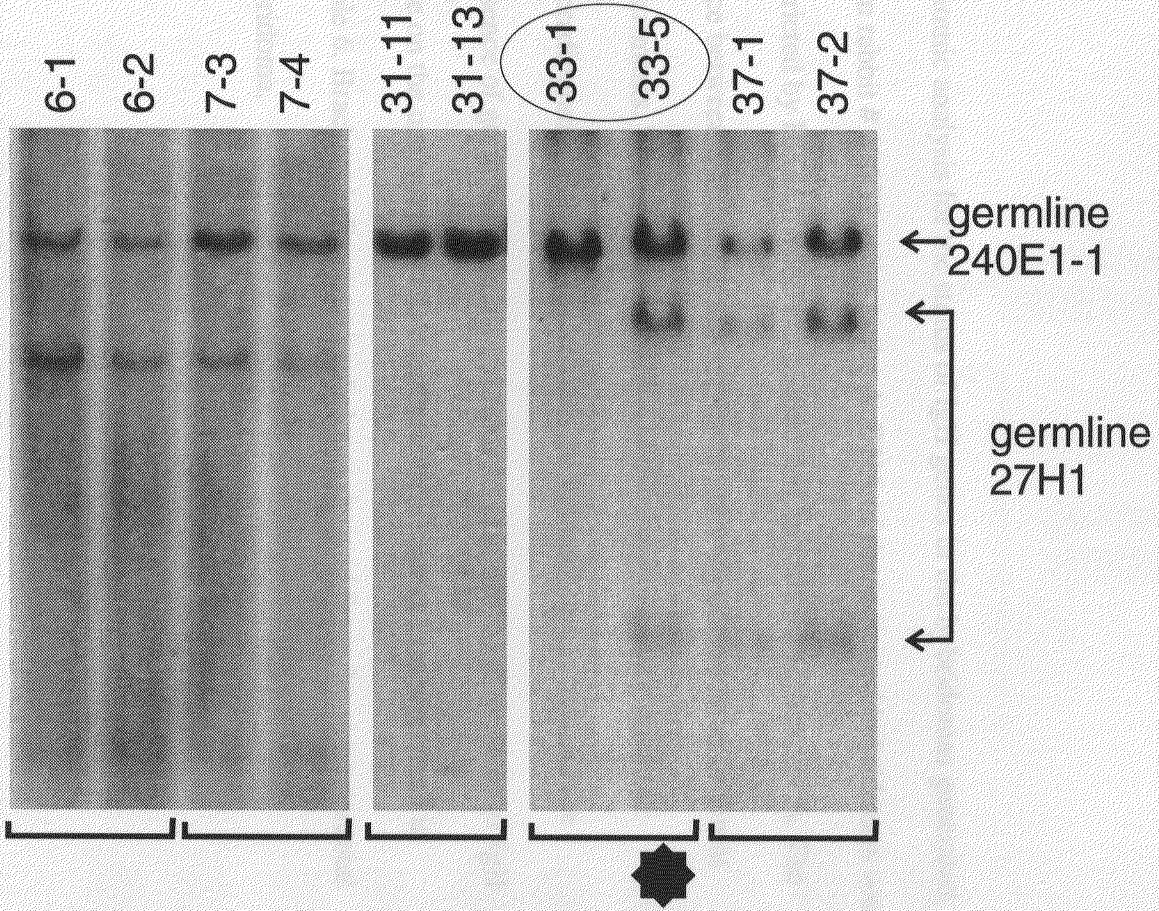
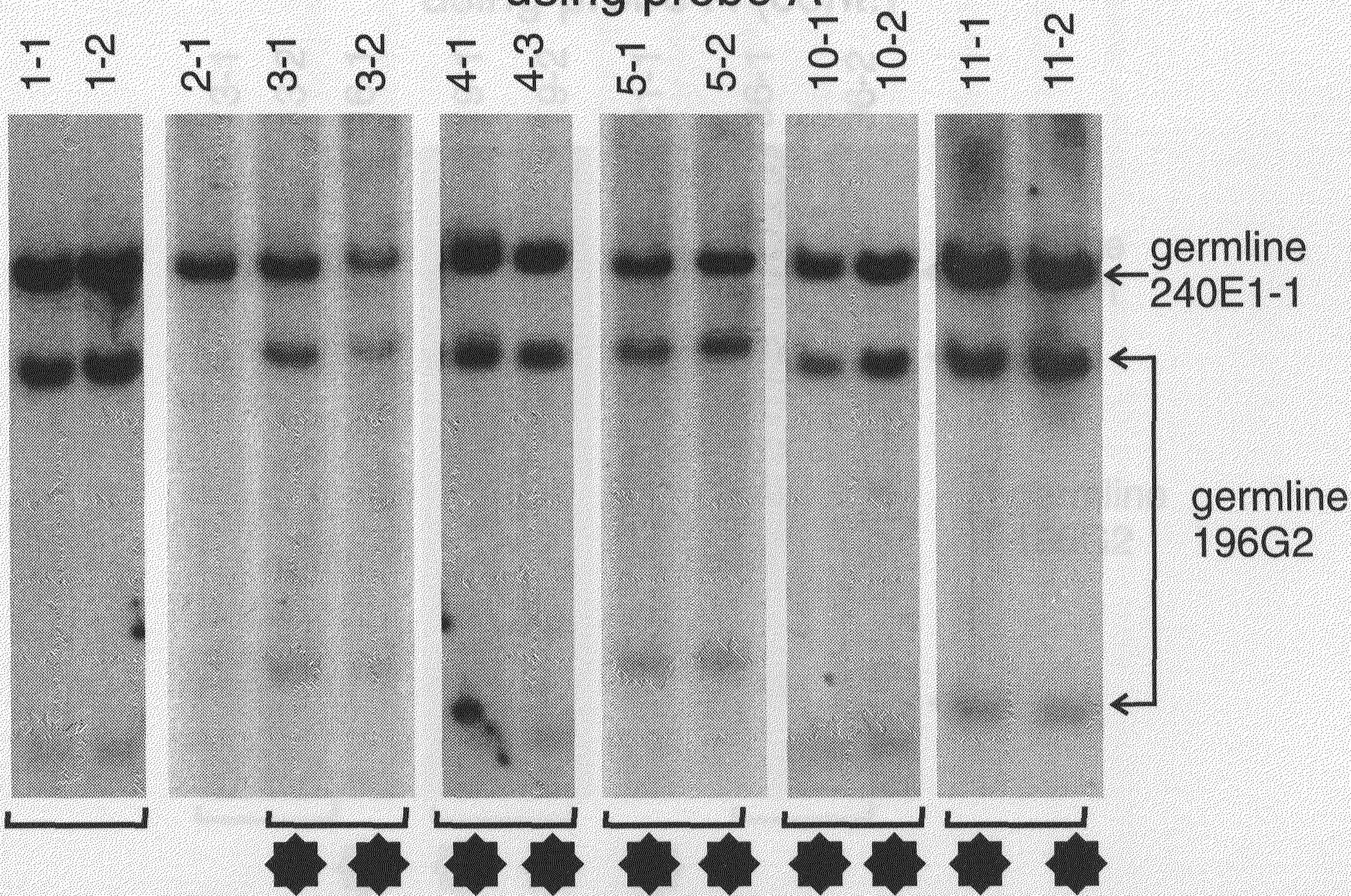


Figure 19 Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 196G2 using probe A. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with probe A. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Polygon underneath the autoradiogram indicate the presence of germline J_H in the hybridomas (Figure 15). The status of IgH gene on the unexpressed allele of these hybridomas is summarized in Table 6. Brackets indicate two subclones derived from the same parental hybridoma.

Southern analysis of B cell hybridomas generated from rabbit 196G2 using probe A



Southern analysis of B cell hybridomas generated from rabbit 196G2 using probe A (cont.)

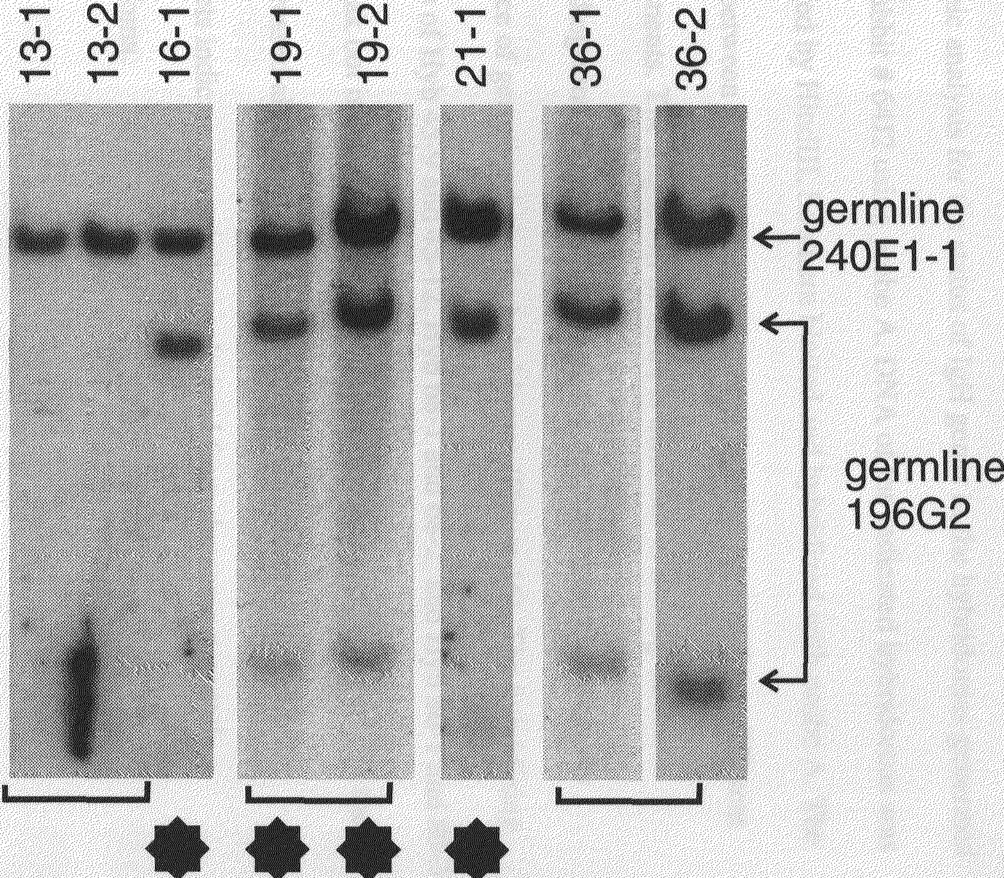


Figure 20 Genomic analysis for the status of IgH genes in the hybridomas generated from rabbit # 64J2 using probe A. DNA of the indicated hybridomas was restricted by HindIII, Southern blotted and hybridized with probe A. The space between autoradiograms separates autoradiograms from different experiments. The migration of DNA is different in among different autoradiograms. Polygon underneath the autoradiogram indicate the presence of germline J_H in the hybridomas (Figure 16). The hybridizing signals of Hyb 45-9 and 45-12, Hyb 68-1 and 68-2, and Hyb 80-1 and 80-3 are weak but is conclusively identifiable. The status of IgH gene on the unexpressed allele of these hybridomas is summarized in Table 7. Brackets indicate two subclones derived from the same parental hybridoma.

Southern analysis of B cell hybridomas generated from rabbit 64J2 using probe A

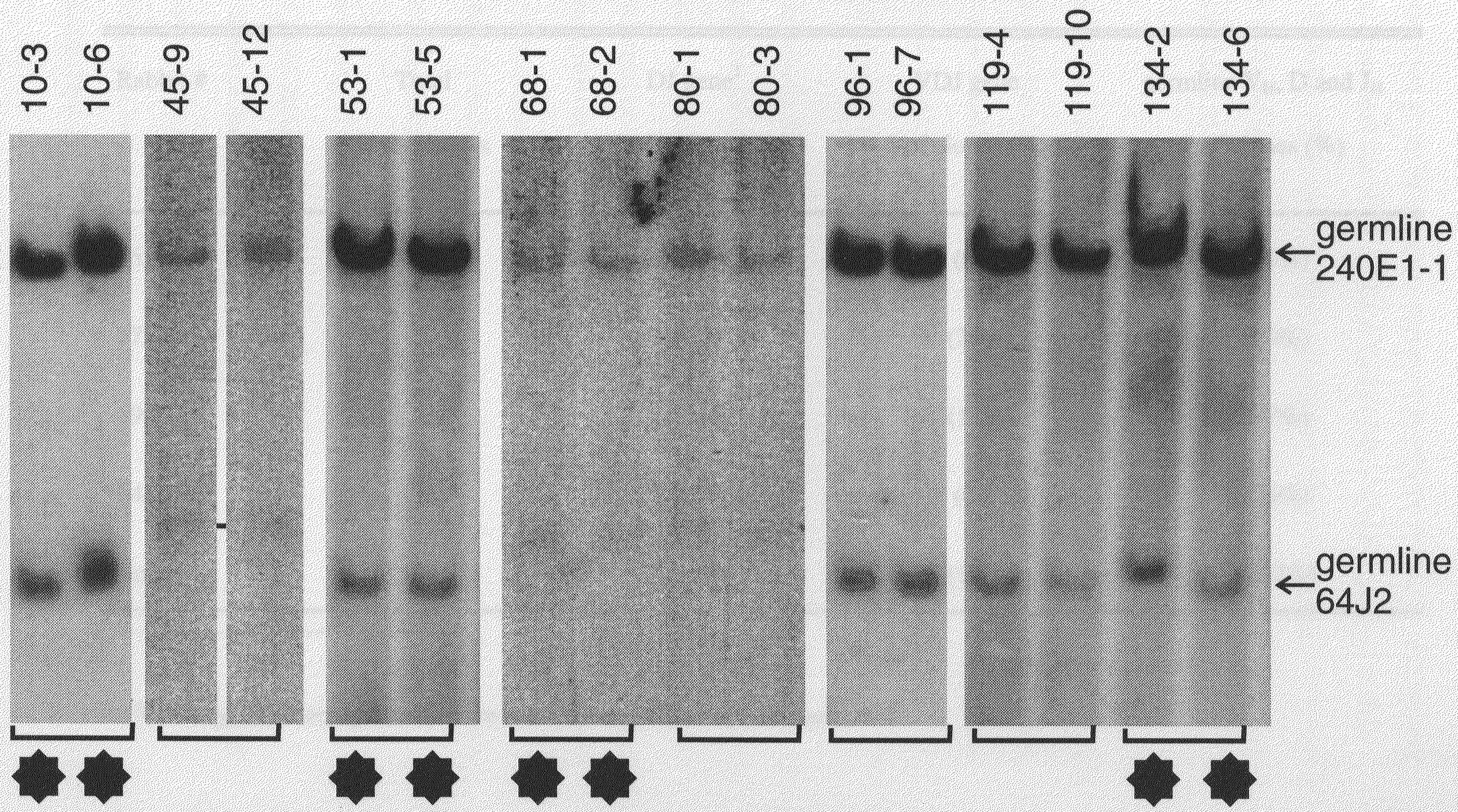


Table 8 Distribution of DJ, VDJ genes and germline V_H, D and J_H gene segments on the unexpressed allele of 29 rabbit hybridomas

Rabbit #	Total Hybridomas	DJ gene[1] # of clones (%)	VDJ gene # of clones (%)	germline V_H, D and J_H # of clones (%)
363H1#2	4	2 (50%)	0 (0%)	2 (50%)
27H1	5	3 (60%)	1 (20%)	1 (20%)
196G2	12	2 (16%)	2 (17%)	8 (67%)
64J2	8	4 (50%)	0 (0%)	4 (50%)
Total	29	11 (38%)	3 (10%)	15 (52%)

[1] No VD gene rearrangements were found in any of the hybridomas.

to probe A represent the germline probe A region of the rabbits which the splenocytes were taken from, I conclude that these 27 hybridomas did not rearrange V_H1 on their unexpressed IgH alleles. The remaining three hybridomas (Hyb 31-11 and 31-13; Hyb 2-1; Hyb 13-1 and 13-2) did not have the germline probe A region from the splenocytes (Figure 18 and 19, Table 8) suggesting that the V_H1 on the unexpressed allele was rearranged.

It is possible that the germline J_H I found in polyclonal and monoclonal B cells is actually DJ genes that had rearranged a D gene located in close proximity to a J_H gene such that the DJ gene is indistinguishable from germline J_H on Southern analysis. This possibility was ruled out when I examined a 340-bp region immediately upstream of J_H and found no indication of a D gene (Figure 21).

Taking the results of pJ5 and probe A hybridization together, I found that the 15 hybridomas which had the J_H gene in germline configuration also had germline probe A region (Tables 4-8). Therefore, these hybridomas had the IgH gene on the unexpressed allele in germline configuration, like that of the fusion partner. Eleven hybridomas had no germline J_H of the spleen donors but still had probe A region in germline configuration and hence, these hybridomas had DJ genes on the unexpressed IgH allele (Tables 4-8). The remaining three hybridomas had neither germline J_H and probe A region suggesting that IgH gene on both alleles were rearranged to VDJ genes (Tables 4-8).

From the cloning of the 29 hybridomas by limiting dilution, I obtained more than one subclone for 25 of them. For each of these 25 hybridomas, two subclones were analyzed for the status of the IgH gene on the unexpressed allele. The result I obtained from analyzing two subclones was generally identical suggesting that the hybridomas were already monoclonal before the cloning. Only two pairs of monoclonal hybridomas gave different results (Hyb 15-3 and 15-6 (Figure 13); Hyb 33-1 and 33-5 (Figure 14)). In

Figure 21 Nucleotide sequence of the region immediately upstream of J_H locus. No D-like element is found in this region. The diagram below the sequence indicates the approximate germline region from which the sequence was derived. The sequence of J_H1 (ψ) is underlined.

GGGAGCTGGAGGTAGGGAGCTAGAAGTGGGGGGCTAAGAGATGAAGGCTGGACAGAGGGTGGGGGAGGGGCTAGA

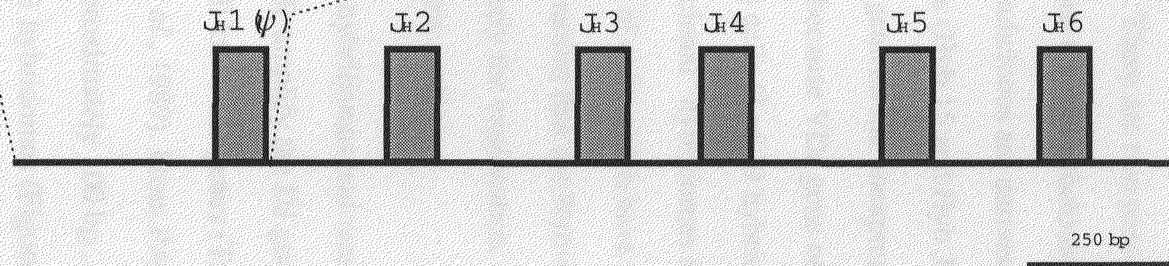
AGTGGATAATGAGTGCTGTTGGACTGGCTGGAGGTACAGCCCTGGCTGATACCCGAGGGCTCAGCTGACACCTCC

ACTGAGCAGGGAGCCCCACCCCGGGGGCCAATGAGAGGCCAACCTAGGAATCCCGGGCCCCAGATTGGCCAAGAT

GTCTAGGCAGTGGGTGCAGGCGCTCACGGTGCCTGCCTTTCCTTCTAGTCCTGGGCCAGGGTGCCTTGTCTGTGC

TGCAGAGACTATGGCCGGAGCCAAGGCCGGGGCAGGGGCTAGGGCCAGGGTGGGAGCCAGGGCCAGCCCGGGGCC

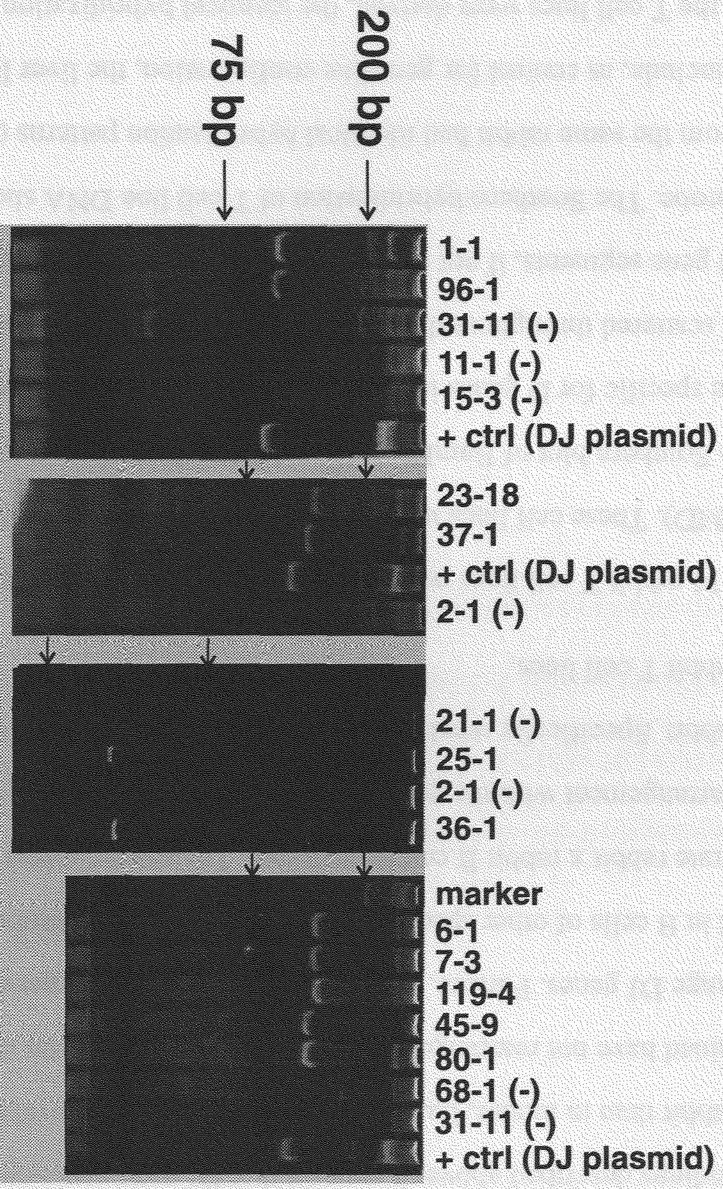
AGAGCTGGAGCTGTGCTATAAAGGGAGACTGAGGGAGGCAGAGGCTGTGCTACTGGTACCTGGATCCCTGGGGCA



both pairs, one subclone (Hyb 15-3 and Hyb 33-5) showed that the unexpressed IgH allele was in germline configuration while the other subclone in the pair (Hyb 15-6 and Hyb 33-1) showed that a VDJ gene was on the unexpressed allele. There are two possible explanations. First, it is possible that the difference between the two subclones was due to the polyclonality of the parental hybridomas and the two subclones are derived from different clones. In this case, both the parental hybridomas (Hyb 15 and Hyb 33) must contain one population of cells which have germline IgH gene on the unexpressed allele and another population which have VDJ gene. However, because VDJ gene on the unexpressed IgH allele was infrequently found in rabbit B cells (3 of 29 hybridomas), it is rather unlikely that both of the only two potentially "polyclonal" hybridomas in this experiment would coincidentally contain a subpopulation of cells which have VDJ gene on the unexpressed allele. I think that an alternative explanation that the Hyb 15-6 and Hyb 33-1 had lost their unexpressed IgH allele is more likely the explanation. Because of this reason, I have excluded these two hybridomas from this analysis.

To confirm the result of Southern analysis that 11 hybridomas had rearranged DJ genes on their unexpressed IgH alleles, I attempted to PCR-amplify the DJ genes in these hybridomas using a degenerate PCR primer specific for all known D genes (Table 2) and a $J_H$prB primer specific for all known J_H genes (Becker *et al.*, 1989). From PAGE analysis of the PCR products, an amplified product of approximately 100 bp , the size expected for a product of DJ gene, were observed in all 11 hybridomas (Figure 22). As a negative control, I used DNA from two hybridomas which had unexpressed IgH genes in germline configuration (Hyb 15-3; Hyb 11-1) and from two hybridomas which had VDJ genes on both IgH alleles (Hyb 31-11; Hyb 2-1) in the PCR reaction. No PCR-product was observed in these negative control DNA. To confirm that the PCR products from the 11 hybridomas are DJ genes, I chose at random the products from four of the 11 hybridomas (Hyb 37-1; Hyb 25-1; Hyb 36-1 ;Hyb 23-18), cloned and determined their

Figure 22 PAGE analysis of the PCR products from PCR amplification for DJ genes from the indicated hybridomas. The pictures shown are from four separate electrophoreses and therefore, the migration of DNA is different in each run. The migration sites of 200 and 75 bp DNA markers of each run are indicated by arrows. Minus signs indicate negative controls which are PCR using as template the DNA of hybridomas which have VDJ gene or germline IgH gene on the unexpressed allele.


200 bp
75 bp
1-1
96-1
31-11 (-)
11-1 (-)
15-3 (-)
+ ctrl (DJ plasmid)
23-18
37-1
+ ctrl (DJ plasmid)
2-1 (-)
21-1 (-)
25-1
2-1 (-)
36-1
marker
6-1
7-3
119-4
45-9
80-1
68-1 (-)
31-11 (-)
+ ctrl (DJ plasmid)

nucleotide sequences. The nucleotide sequence analysis showed that they are, indeed, DJ genes (Figure 23). I conclude that the PCR products from these four hybridomas as well as the remaining seven hybridomas were generated from amplification of DJ genes.

IgH Gene Rearrangement in Rabbit T Cell Lines

From the findings described above, it appears that DJ gene rearrangement occurs less frequently in rabbit than in mouse, human and chicken. Half of the rabbit B cell hybridomas I examined have not rearranged their unexpressed IgH alleles and for those that do, most rearrange DJ genes. Further, rabbit B cells rearrange VD genes, which was not regularly found in B cells of other species (see following section). Before we had the technology to generate rabbit x rabbit B cell hybridomas, I examined rabbit T cell lines for the IgH gene rearrangement with the goal of gaining insight into the IgH gene rearrangement in rabbit. Specifically what I asked is whether I can find DJ or VD gene rearrangement in rabbit T cell lines.

DNA from 14 rabbit T cell lines was kindly provided by Dr. Thomas Kindt (NIAID, Bethesda MD). These cell lines were derived from five rabbits: K30, K34, 6600, 6516 and RL5. The Southern blot of BamHI-restricted DNA from these 14 cell lines was probed with a probe specific for D genes in the D1 family (Table 1). Four members of the D1 gene family are scattered throughout the D region and therefore, most of the rearrangement of D gene segments, if not all, result in a non-germline fragment hybridizing to D1 probe. The Southern hybridization of T cell line DNA showed that T cell lines derived from the same rabbit had identical hybridization patterns (Figure 24). Although I did not include, as control for germline configuration, the liver DNA of rabbits from which the T cell lines were derived, the identical hybridization patterns in the T cell lines which were derived from the same rabbit suggests that the hybridization patterns represent the germline configuration. The alternative explanation for the identical

Figure 23 Comparison of the nucleotide sequences of four PCR-amplified DJ genes with those of germline D and J_H genes. Dots denote the similarity to the germline genes; the N regions are boxed; the degenerate PCR-primers specific for D genes are underlined.

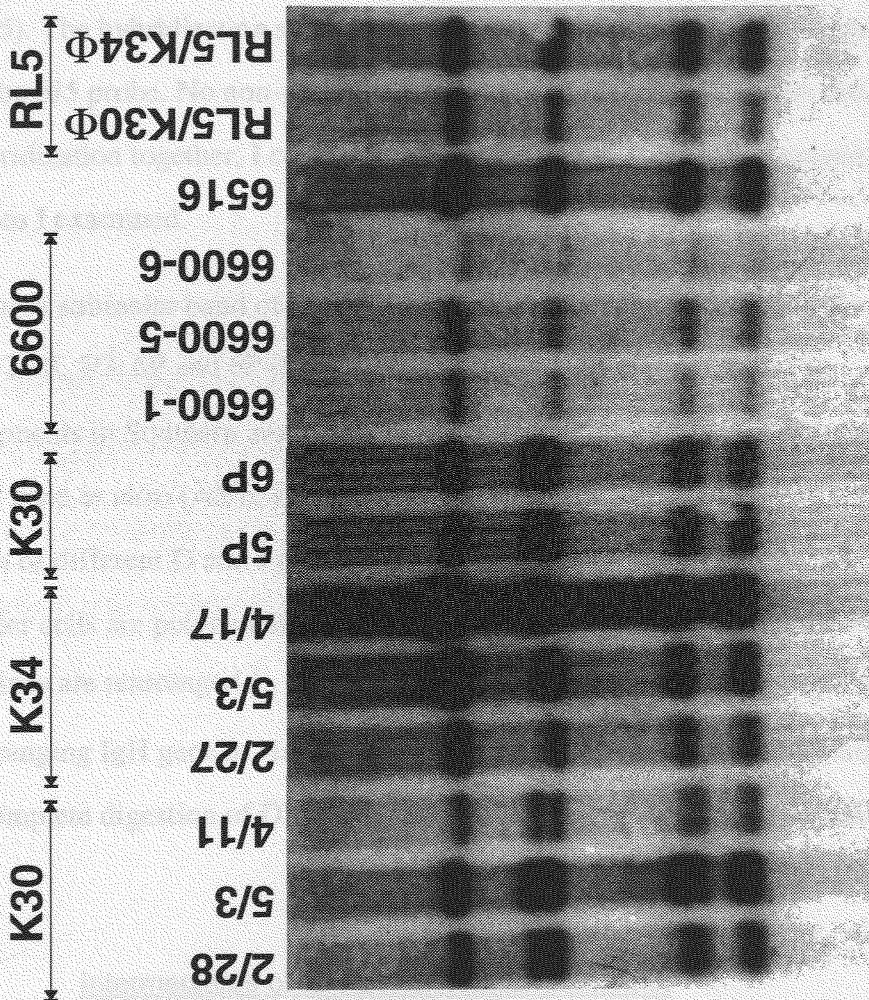
```
                                                D region                     N region                    JH region

                                   D4   GTGGTTACTATAGTAGTGGCTGGGGTG                    ACTTGTGGGGCCCAGGCACCCTGGTCACCGTCTCCTCAG   JH4
25-1 DJ   GATTGGATTTTGTGAGGGTCTGTGGCACC.................TA........      GGG        ....................................   D4JH4
36-1 DJ      TGGATTTTGTGCGGGTCTGTGACACC.....................             CAA       .....................................   D4JH4

                                   D3   GTGGCATATGCTAGTAGTAGTGGTTATTATA
23-18 DJ  GATTGGATTTTGTACTGGTCTGCGGCACT..............................   GGGGGATTTTTTA      ..............................   D3JH4

                                   D2b  GTGGTTATGCTGGTTATGCTGGTTATGGTTATG
37-1 DJ   GATTGGATTTTGTGCGGGTCTGCGGCACA................................     TCATA         ...............................   D2bJH4
```

Figure 24 Genomic analysis for the status of IgH genes in the 14 rabbit T cell lines using D1 probe, a probe specific for all four D gene in the D1 family. The rabbits from which the T cell lines were derived is indicated above the clone numbers.


K30
2/28
5/3
4/11
K34
2/27
5/3
4/17
K30
5P
6P
6600
6600-1
6600-5
6600-6
6516
RL5
RL5/K30Φ
RL5/K34Φ

pattern of hybridization that all T cell lines had rearranged the same D genes resulting in identical hybridization pattern is highly unlikely. I conclude that there was no rearrangement of the D region of IgH gene in any of the 14 T cell lines.

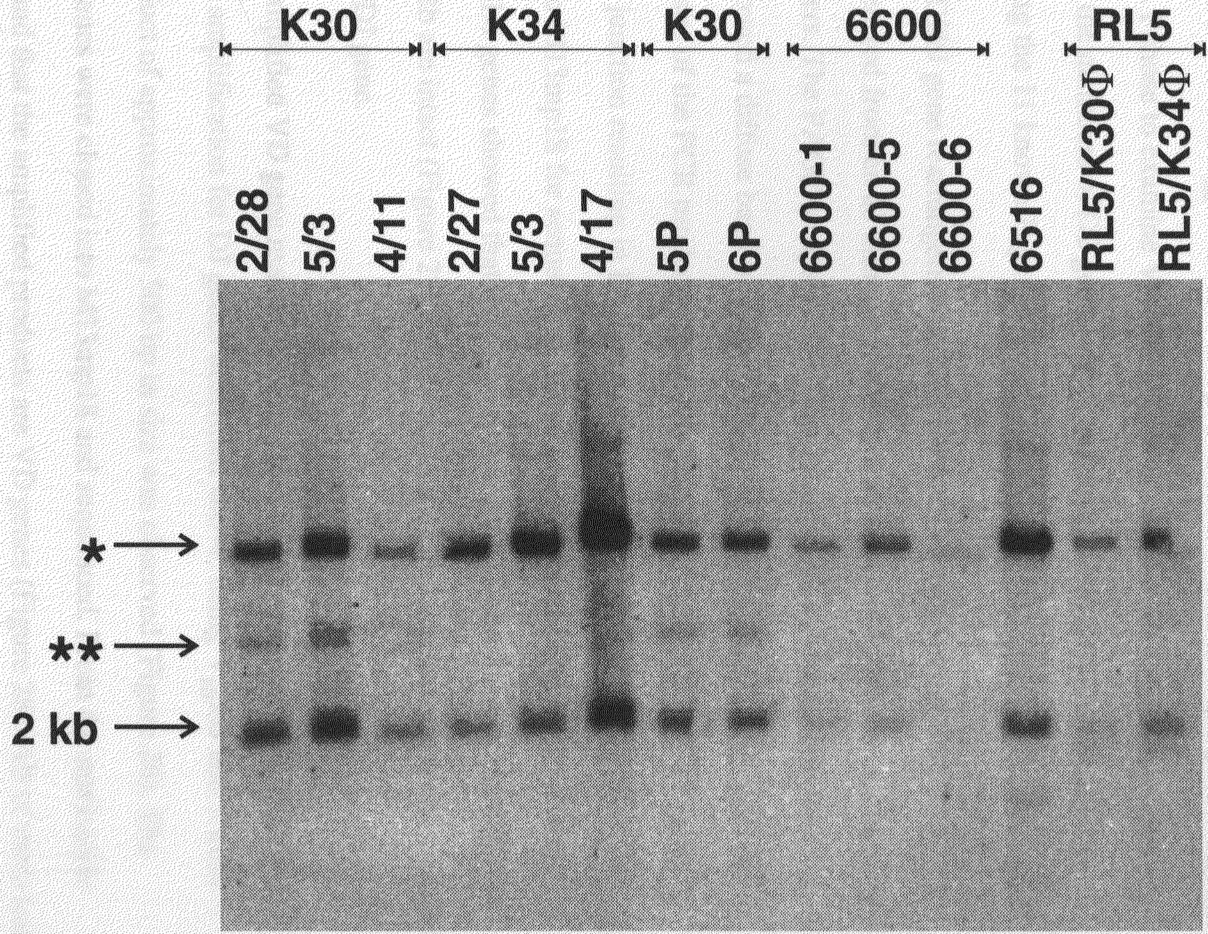
To further investigate whether the J_H gene was rearranged, the D1 probe was removed by using boiled-0. 1X blot wash and the blot was rehybridized with the pJ5 probe (Figure 25). The hybridization showed a non-polymorphic 2 kb BamHI-fragment hybridizing to the pJ5 probe. No non-germline fragment was found. Taking the result of D1 and pJ5 hybridization together, I conclude that the IgH gene was not rearranged in all the 14 T cell lines I examined.

There was a submolar band of approximately 3 kb in size hybridizing to the pJ5 probe in clones 2/28, 5/3, 5P and 6P (Figure 25). The submolar appearance of J_H hybridizing fragments in Southern analysis is characteristic of cell lines capable of rearranging IgH gene *in vitro* (Alt *et al.*, 1981). The submolar characteristic was a result of the utilization of different D and J genes in different rearrangements and therefore, the resulting daughter cells are polyclonal. However, I do not think that the submolar band in the four T cell lines are rearranged J_H genes because it is unlikely that T cell lines would be actively rearranging IgH gene *in vitro*. I conclude that these submolar bands resulted from either incomplete digestion of DNA of these cell lines or non-specific hybridization of pJ5 probe.

Intermediates of IgH Gene Rearrangement in Polyclonal B Cells

The analysis of the unexpressed IgH gene of rabbit B cell hybridomas suggests that DJ gene is an intermediate of IgH gene rearrangement in rabbit. Further, Becker R. and Knight K. L. analyzed the status of the IgH gene in a rabbit B cell line and identified a VD gene rearrangement (unpublished data). This finding suggests that a VD gene may

Figure 25 Genomic analysis for the status of IgH genes in the 14 rabbit T cell lines using pJ5 probe. The rabbits from which the T cell lines derived from are indicated above the clone numbers. The position of germline J_H (2 kb) was indicated. " * " indicates the 4.3 band which is superimposable to one of the D1 hybridizing fragment. The signal was likely from the D1 probe which was not completely removed. " ** " indicates the possible submolar band (see text).


K30
K34
K30
6600
RL5
2/28
5/3
4/11
2/27
5/3
4/17
5P
6P
6600-1
6600-5
6600-6
6516
RL5/K30Φ
RL5/K34Φ
*
**
2 kb

also be an alternative intermediate for IgH gene rearrangement in rabbit B cells. To determine whether the DJ and VD genes could be found in normal rabbit B cells, I PCR-amplified both DJ and VD genes from normal rabbit splenic DNA. In this experiment, instead of using the degenerate PCR-primer specific for all known D genes, I used D2 specific primers (Table 1). To amplify D2bJ gene rearrangements, 5'D2b and $J_H$prB primers were used whereas $V_H$prB and 3'D2b primers were used to amplify VD2b gene rearrangements. I chose to examine VD and DJ genes which utilized D2 gene family simply because the members of D2 gene family, especially D2b, were utilized most frequently among the 11 known D genes.

For the D2bJ gene rearrangements, I PCR-amplified two splenic DNA preparations prepared from an a3 allotype rabbit and an a1/a2 allotype rabbit using 5'D2b and $J_H$prB primers. The PAGE analysis of PCR products from both genomic DNA preparations showed a diffuse band of approximately 120 bp in size (Figure 26). The diffuse appearance of the PCR product is probably due to the addition of N-segment between the DJ junction resulting in DJ genes of varying length. These PCR products were cloned into M13mp18 for sequencing. The nucleotide sequence analysis of the PCR-amplified DJ genes showed that they are authentic DJ genes and that their RSSs located 5' of D2b are intact (Figure 27) suggesting that these DJ genes potentially can be rearranged to VDJ gene.

I PCR-amplified VD gene rearrangements from the a3 splenic DNA, which was also used in the amplification for DJ genes described above, using $V_H$prB and 3'D2b primers. A product of approximately 500 bp in size was observed (Figure 26). The amplified VD genes were cloned into M13mp18 and sequenced. From the nucleotide sequences, I found that the amplified products are VD genes (Figure 28). All of them had utilized V_H1-a3 and D2b in the rearrangements. The RSSs located 3' of D2b in these VD

Figure 26 PAGE analysis of the PCR products from PCR amplification for D2bJ and VD2b genes from rabbit splenic DNA. The 500, 400, 200 and 75 bp DNA markers are indicated by arrows.

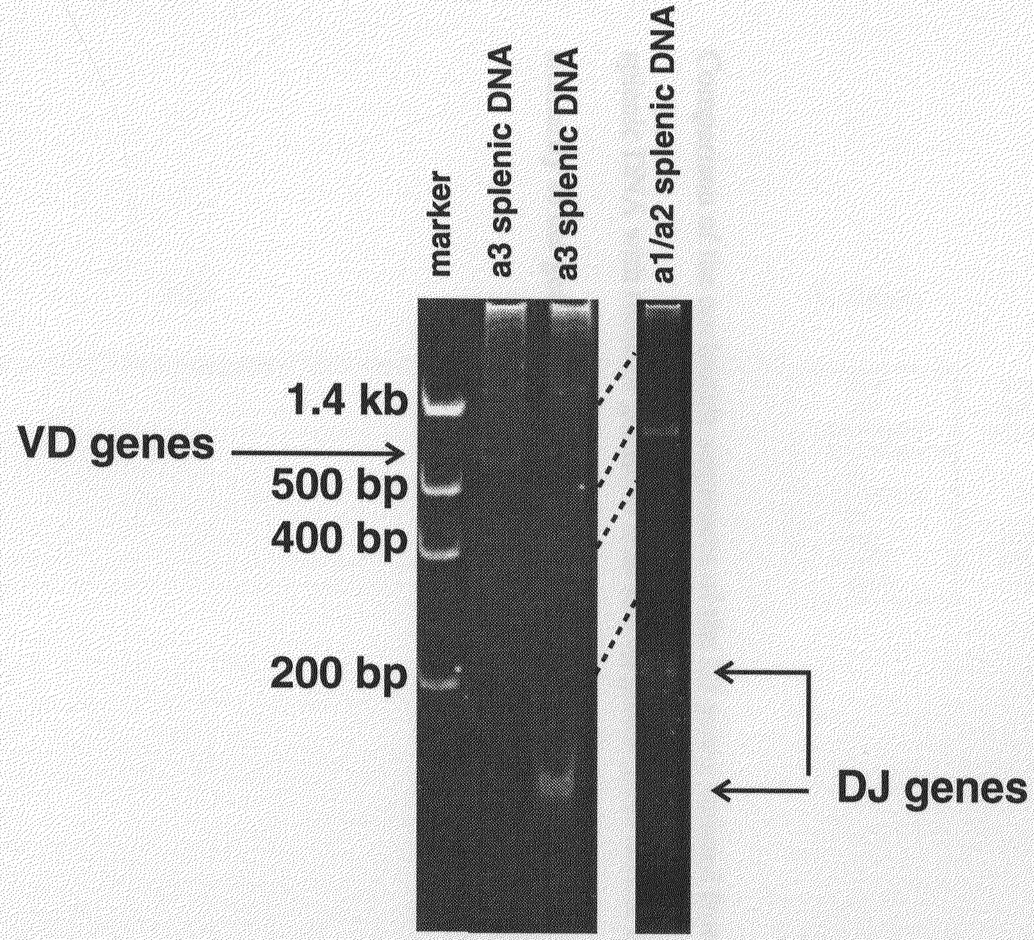

marker
a3 splenic DNA
a3 splenic DNA
a1/a2 splenic DNA
1.4 kb
VD genes
500 bp
400 bp
200 bp
DJ genes

Figure 27 Comparison of the nucleotide sequences of the DJ genes isolated from splenic DNA with those of germline D2b and J_H genes. Dots denote the similarity to the germline genes; the N regions are boxed.

```
                 D2b                                                                  N region          J_H

       CTGCAGGGGATTTTGTGCTGGTCTGAGACACAGTGGTTATGCTGGTTATGCTGGTTATGGTTATGCTACCCACAGTG
CT536  ....................................................................       GGT          ACTACTTTAACTTGTGGGGCCCAGGCACCC D2bJh4
CT537  .....................................................................      CAT          ACTACTTTAACTTGTGGGGCCCAGGCACCC D2BJh4
CT538  ...................................................................      TGAATGGGT     ACTCGGTTGGATCTCTGGGGCCAGGGCACCC D2bJh3
CT539  ................................................................         TCAAGATGAGG                  GGGGCCCAGGCACCC D2bJh4
CT540  .....................................................................      GA                  AACTTGTGGGGCCCAGGCACCC D2bJh4
CT1552 ...............................................................          CTACGCGGA       CTGGTTGGATCTCTGGGGCCAGGGCACCC D2bJh3
CT1553 .................................................................          GA                TTAACTTGTGGGGCCCAGGCACCC D2bJh4
CT1554 ....................................................................       GTT          ACTACTTTAACTTGTGGGGCCCAGGCACCC D2bJh4
CT1555 ............................................................C....        ATTGT         ACTACTTTAACTTGTGGGGCCCAGGCACCC D2bJh4
CT1556 ................................................................           GGGG           TACTTTAACTTGTGGGGCCCAGGCACCC D2bJh4
```

Figure 28 Comparison of the nucleotide sequences of the VD2b genes isolated from splenic DNA with those of germline V_H1-a3 and D2b genes. Dots denote the similarity to the germline genes; the N regions are boxed.

```
VH1-a3  GCGAAAGGCCGATTCACCATCTCCAAAACCTCGTCGACCACGGTGACTCTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGAGACACAGTG
VD290   .......................................................................................................
VD291   ......................................................................................................
VD292   .....................................................................................................
VD293   .......................................................................................................
VD295   .......................................................................................................
VD296   .......................................................................................................
VD297   .......................................................................................................
VD298   .......................................................................................................
VD299   .......................................................................................................
```

N-region　　　　　　　　　　D2b

```
                                   GTTATGCTGGTTATGCTGGTTATGGTTATGCTACCCACAGTGCCTCAGGCCTCACATAAAACTCAGCCACAGCC
VD290       GCCCC                    ..........................................................................
VD291      AGCCTGG                    .........................................................................
VD292       CTGCCCC                  ..........................................................................
VD293      GCTTCGATACTTA                  .....................................................................
VD295         G                         ...................................................................... 
VD296         G                            ....................................................................
VD297     GAGGATTACTATACTT                .....................................................................
VD298     GTGACTGGTGCTGGTGGC                          .........................................................
VD299    TTCCTACGCCCCAA              ..........................................................................
```

genes are intact suggesting that they are similar to the DJ genes in that they too potentially can be rearranged to VDJ genes.

Fetal VDJ Genes

It is rather unusual that only 10% of rabbit B cell hybridomas have 2 VDJ genes, one of which must be productive and the other, presumably, nonproductive. Because of the junctional diversity and N-nucleotide addition at the VD and DJ junctions, the generation of nonproductive VDJ genes is certain to occur. In mouse and human, more than 60% of B cells have rearranged VDJ genes on both IgH alleles due to the high frequency of nonproductive rearrangements. One possible explanation for the low frequency of nonproductive rearrangements in rabbit is that rabbit B cells, unlike mouse and human B cells, do not generate nonproductive VDJ genes. An alternative explanation would be that only one IgH allele can be rearranged to a complete VDJ gene.

I attempted to determine whether at any time during development, nonproductive VDJ genes can be found more frequently. In the case that I find many nonproductive VDJ genes, the benefit is two-fold. First, it will provide the evidence against the possibility that rabbit B cells do not generate nonproductive VDJ genes. Further, the V_H gene usage in these nonproductive VDJ genes will distinguish whether the preferential utilization of V_H1 resulted from its preferential rearrangement. Because nonproductive VDJ genes cannot be selected for or against by antigen selection process, the V_H gene usage in the nonproductive VDJ genes would reflect the V_H gene(s) utilized in the rearrangements.

In previous studies for VDJ genes in newborn and adult rabbits, nonproductive VDJ genes were found infrequently (Tunyaplin C. and Knight K. L. , unpublished data). I asked whether nonproductive VDJ genes might be found at higher frequency in the fetal VDJ gene repertoire, especially at the developmental stages preceding the appearance of

pre-B cells. Cytoplasmic μ^+ cells begin to appear in fetal liver between day 17 and day 23 of gestation (Gathings *et al.*, 1981, Hayward*et al.*, 1978). Hence, I attempted to PCR-amplify and clone VDJ genes from fetuses beginning at day 12 of gestation.

I determined the onset of VDJ gene rearrangement during fetal development by searching for VDJ genes in 12 to 28 day fetuses. Rabbits of known V_Ha allotype were mated and fetal livers were collected on day 12 to 15, 21, 24 and 28 after mating. The VDJ genes were PCR-amplified using nested-primer PCR. PAGE analysis of the PCR products showed that, in general, a product of 500 bp, the size expected from the amplification of VDJ genes, was routinely observed from 14-day onward (Figure 29). No PCR product was found in samples taken prior to day 14 of gestation. From this experiment, we conclude that VDJ genes in fetal liver appear around day 14 of gestation.

I cloned and determined partial nucleotide sequences of the PCR-amplified VDJ genes from the 14-, 15-, 21-, 24- and 28-day fetuses and compared them with that of V_H1 (Figures 30-34). From a total of 60 VDJ genes sequenced, I obtained 56 independent VDJ clones. The deduced amino acid sequence of these VDJ genes showed that 33 of them were nonproductive (Figures 30-34). The percentage of nonproductive VDJ genes was highest in 14-day fetuses and decreased as the fetus developed. I conclude that nonproductive VDJ genes are generated at high level and that VDJ gene rearrangement most likely occurs only on one IgH allele. If the rearrangement results in a nonproductive gene, the cell is selected against before the rabbit is born.

V_H Gene Usage in Fetal VDJ Gene Repertoire

The analysis of V_H gene usage in nonproductive VDJ genes provided me with evidence for the preferential rearrangement of V_H1. The preferential utilization of V_H1 in rabbit B cells is well established but we do not know whether the preferential utilization

Figure 29 PAGE analysis of nested-primer PCR for fetal VDJ genes. The gestational stage at which fetal liver was obtained are indicated. The white arrows indicate the position of the PCR product expected from a VDJ gene.

PCR amplification of fetal VDJ genes

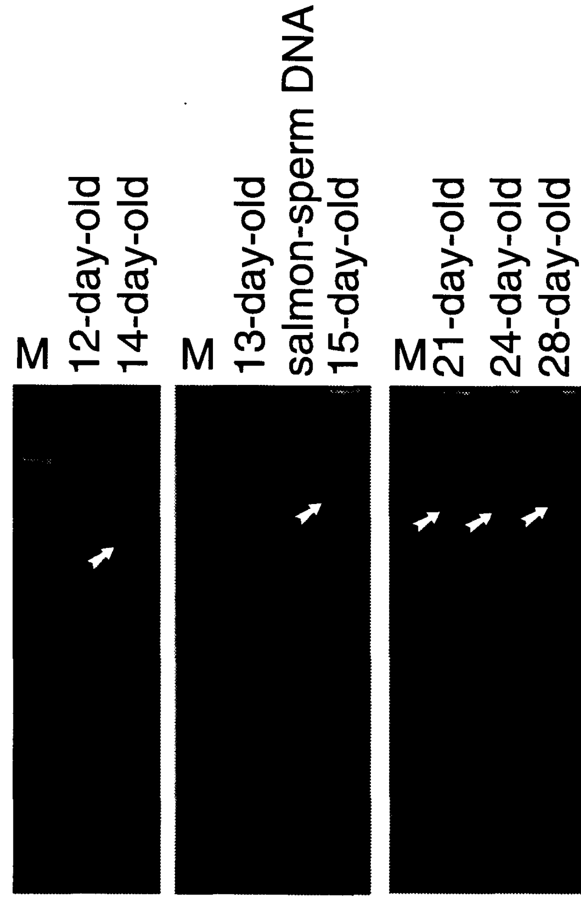


Figure 30 Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 14 fetuses with germline *V_H1-a3* or *V_H2-a3*. Dots and dashes denote identity to the sequence of *V_H1-a3* and *V_H2-a3*, respectively. N denotes a nucleotide that cannot be unequivocally determined. The utilized D and J_H genes, and functionality of the VDJ genes are indicated. The heptamer motifs of the recombination signal sequences of *V_H1-a3* and *V_H2-a3* are underlined. All clones were partially sequenced in one orientation from J_H to V_H. To test that these clones utilized *V_H1-a2*, clone 14-1808 was chosen for complete nucleotide sequence analysis. The analysis showed that the V region of this VDJ clone is identical to that of *V_H1-a3* (not shown).

A K G R F T I S K T S S T T V T L Q M T S L T A A D T A Y F C A R

V_H1-a3 GCGAAAGGCCGATTCACCATCTCCAAAACCTCGTCGACCACGGTGACTCTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGAGA***CACAGTG***

14-1525F ....

14-1526F ....

14-1527F ....

14-1579F ....

14-1580F ...............N...C..

14-1581F ....C....................................

14-1808F ....

V_H2-a3 GAATGGCCGAATCATCATCTCCAGGGACAACGCCACAACACGGTGTCTCGGCAAATGAACAGTCTGACAGCCAGGGATAGGGCCCTGTATTTCTGTGCAGGAGA***CACAGTG***

14-1523F --

14-1524F ---

14-1578F ---

	N region	D region	N region	J_H	
14-1525F	CAA	GTTATGCTGGTAGTAGTTATTATA	T	TTTAACTTGTGGGGCCCAGGCACCCTGG	D5 ,J_H4, non-productive
14-1526F	TCCGA	GCTGGTAGTAGTTATTA		TAACTTGTGGGGCCCAGGCACCCTGG	D5 ,J_H4, non-productive
14-1527F	GG	GTTACTATACTTATGGTTATGCTGGTTATGCTTATGCTA	GAGGAA	GGGGCCCAGGCACCCTGG	D2a,J_H4, non-productive
14-1579F	TCCCCC	TGCTGGTTATGCTGGTTATGGTTATG	GGGG	CTTGTGGGGCCCAGGCACCCTGG	D2b,J_H4, productive
14-1580F	GGT	GCTACGATGACTATGGTGATTA	GG	CGGTTGGATCTCTGGGGCCAGGGCACCCTGG	D1 ,J_H3, non-productive
14-1581F	CAA	TTACTATACTTATGGTTATGCTGGTTATGCTTATGCTACC		TTAACTTGTGGGGCCCAGGCACCCTGG	D2a,J_H4, non-productive
14-1808F	GTA	ATAACTACTACCAGCATAAC	GTGG	TGGTTGGATCTCTGGGGCCAGGGCACCCTGG	D5 (inverted), J_H5 non-productive
14-1523F	AG	GCTGGTTATGCTGGTTATGGTTATGC	CCT	ACTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D2b,J_H4, non-productive
14-1524F		TATGCTGGTAGTAGTTATTA		ACTTGTGGGGCCCAGGCACCCTGG	D5 ,J_H4, non-productive
14-1578F		TTACTATACTTATGGTTATGCTGGTTATGCTTATGCTAC	AAGG	ACTTGTGGGGCCCAGGCACCCTGG	D2a,J_H4, non-productive

Figure 31 Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 15 fetuses with germline V_H1-*a2*. Dots denote identity to the sequence of V_H1-*a2*. N denotes a nucleotide that cannot be unequivocally determined. The utilized D and J_H genes, and functionality of the VDJ genes are indicated. The heptamer motif of the recombination signal sequence of V_H1-*a2* is underlined. All clones were partially sequenced in one orientation from J_H to V_H. To test that these clones utilized V_H1-*a2*, clone 15-1486, 15-1487 and 15-1488 were chosen at random for complete nucleotide sequence analysis. The analysis showed that the V regions of these VDJ clones are identical to that of V_H1-*a2* (not shown).

```
             A  K  S  R  S  T  I  T  R  N  T  N  L  N  T  V  T  L  K  M  T  S  L  T  A  A  D  T  A  T  Y  F  C  A  R
VH1-a2    GCGAAAAGCCGATCCACCATCACCAGAAACACCAACCTGAACACGGTGACTCTGAAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGAGACACAGTG
15-1486F  ..........................................................................................
15-1487F  ..............................................................................................
15-1488F  .............................................................................................
15-1583F  ..............................................................................................
15-1584F                           .............N.....................................................
15-1585F  ..................................................................................A...........
15-1586F  .............................................................................................
15-1587F  .............................................................................................
15-1588F  .............................................................................................
```

	N region	D region	N region	J_H	
15-1486F	CT	TAGTAGTAGTGGTTAT	CC	CTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D3 , J_H4, non-productive
15-1487F	TC	GCTGGTAGTAGTTATTAT		TTAACTTGTGGGGCCCAGGCACCCTGG	D5 , J_H4, non-productive
15-1488F	C	CTATACTTATGGTTATGCTGGTCATGCTTATGCTAC	T	GGTTGGATCTCTGGGGCCAGGGCACCCTGG	D2a, J_H3, non-productive
15-1583F	GA	ACTATACTTATGGTTATGCTGGTTATGCTTATGC		TACTTTAACTTGTGGGACCCAGGCACCCTGG	D2a, J_H4, non-productive
15-1584F	A	TTATGCTGGTTATGCTGGTTATGGTTATGCT	CCC	CTTTAACTTGTGGGGCCCAGGCACCCTGG	D2b, J_H4, productive
15-1585F	C	CTATACTTATGGTTATGCTGGTTATGCTTATGCTAAC	TTA	TGGGGCCCAGGCACCCTGG	D2a, J_H4, productive
15-1586F	TAAAGG	ATACTTATGGTTATGCTGGTTATGCTTATGCTA	GG	TTTAACTTGTGGGGCCCAGGCACCCTGG	D2a, J_H4, productive
15-1587F	C	CTATACTTATGGTTATGCTGGTTATGCTTATGCTAC	T	GGTTGGATCTCTGGGGCCAGGGCACCCTGG	D2a, J_H3, non-productive
15-1588F	GGAG	TATGCTGGTTATGCTGGTTATGGTTATGCTAC		TACTTTAACTTGTGGGGCCCAGGCACCCTGG	D2b, J_H4, non-productive

Figure 32 Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 21 fetuses with germline *V_H1-a3* or *V_H2-a3*. Dots and dashes denote identity to the sequence of *V_H1-a3* and *V_H2-a3*, respectively. The utilized D and J_H genes, and functionality of the VDJ genes are indicated. The heptamer motifs of the recombination signal sequences of *V_H1-a3* and *V_H2-a3* are underlined. All clones were partially sequenced in one orientation from J_H to V_H. To test that these clones utilized *V_H1-a3,* clone 21-1489 was chosen at random for complete nucleotide sequence analysis. The analysis showed that the V region of this VDJ clone is identical to that of *V_H1-a2* (not shown).

```
        A  K  G  R  F  T  I  S  K  T  S  S  T  T  V  T  L  Q  M  T  S  L  T  A  A  D  T  A  T  Y  F  C  A  R
VH1-a3   GCGAAAGGCCGATTCACCATCTCCAAAACCTCGTCGACCACGGTGACTCTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGAGACACAGTG
21-1489F ............C...........................................................................................
21-1648F ......................................................................................................
21-1649F ........................................................................................................
21-1654F ........................................................................................................

VH2-a3   GAATGGCCGAATCATCATCTCCAGGGACAACGCCACAACACGGTGTCTCGGCAAATGAACAGTCTGACAGCCAGGGATAGGGCCCTGTATTTCTGTGCAGGAGACACAGTG
21-1491F --------------------------------------------------------------------------------------------------------
```

(***CACAGTG*** at the end of the V_H1-a3 and V_H2-a3 sequences is printed bold italic and underlined.)

	N region	D region	N region	J_H	
21-1489F	ATGG	TATGCTGGCTATGCTGGTTATGGTTATGCTA	GGG	CTTGTGGGGCCCAGGCACCCTGG	D2b, J_H4, non-productive
21-1648F	TGGGA	GCTGGTAGTAGTTAT	CAGGTCT	TTTAACTTGTGGGGCCCAGGCACCCTGG	D5 , J_H4, productive
21-1649F	GGG	CTATACTTATGG	CCACTGG	AACTTGTGGGGCCCAGGCACCCTGG	D2a, J_H4, productive
21-1654F	C	TATGCTGGTTATGCTGGTTATGGTTATG		TGGGGCCCAGGCACCCTGG	D2b, J_H4, non-productive
21-1491F	ATCG	GGTTATGCTGGTTATGGTTATGCTAC	AAA	TAACTTGTGGGGCCCAGGCACCCTGG	D2b, J_H4, non-productive

Figure 33 Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 24 fetuses with germline *V_H1-a1* or *V_H1-a3*. Dots and dashes denote identity to the sequence of *V_H1-a1* and *V_H1-a3*, respectively. N denotes a nucleotide that cannot be unequivocally determined. The utilized D and J_H genes, and functionality of the VDJ genes are indicated. The utilized D gene of clone 24-1799F is too short to be identified accurately. The utilized D gene of clone 24-1765F cannot be identified. The heptamer motifs of the recombination signal sequences of *V_H1-a1* and *V_H1-a3* are underlined. All clones were partially sequenced in one orientation from J_H to V_H.

```
                 A  K  G  R  F  T  I  S  K  T  S  T  T  V  D  L  K  I  T  S  P  T  T  E  D  T  A  T  Y  F  C  A  R
VH1-a1     GCGAAAGGCCGATTCACCATCTCCAAAACCTCGACCACGGTGGATCTGAAAATCACCAGTCCGACAACCGAGGACACGGCCACCTATTTCTGTGCCAGAGAGACAGTG
24-1492F            .........................................................................................
24-1493F   ........G......................................................................................
24-1655F   ...................................................................................................
24-1656F   .....................................................................................................
24-1658F   ...................................................................................................
24-1659F   ..................................................................................................
24-1660F   ...................................................................................................
24-1661F   ..................................................................................................
24-1662F   .....................................................................................................
24-1796F   N....NNN............................................G......T.................................
24-1797F   N....NNNN..........................................................................................
24-1798F            .......................................................N................................
24-1799F            .......................................................NN........N.........................
24-1810F               .................................N..................NN........N.........................
24-1811F            .......................................................NN........N........................
24-1813F            ...C.....................................................................................
24-1814F   ....................T................................................................................

VH1-a3     GCGAAAGGCCGATTCACCATCTCCAAAACCTCGTCGACCACGGTGACTCTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGAGACACAGTG
24-1657F   ------------------------------------------C-----------------------------------------------------------
24-1800F   -----G--N----------------------------------------------------------------T----------------------------
```

	N region	D region	N region	J_H	
24-1492F	ATATGAGGTA	TAGCTACGATGACTATGGTGATT	GGACT	GGTTGGATCTCTGGGGCCAGGGCACCCTGG	D1 , J_H5, non-productive
24-1493F	TAGGGGGG	TATGCTGGTAGTAGTTA		TTAACATCTGGGGCCCAGGCACCCTGG	D5 , J_H4, non-productive
24-1655F	C	CATATGCTAGTAGTAGTGGTT	CCCCC	GACTGGTTGGATCTCTGGGGCCAGGGCACCCTGG	D3 , J_H5, non-productive
24-1656F	A	TAGTGGCTGGGGTG	C	AACATCTGGGGCCCAGGCACCCTGG	D4 , J_H4, non-productive
24-1658F	TTA	GTATTGCTGGTTATGCTGGTTATGGTTATGCTA	ATCTGACT	GGTTGGATCTCTGGGGCCAGGGCACCCTGG	D2b, J_H3, non-productive
24-1659F	TTCCGG	ATCCTGGTTATAGTACT	GGTACC	GTTGGATCTCTGGGGCCAGGGCACCCTGG	Df , J_H3, productive
24-1660F	GG	GCTACGATGACTATGGTGA	GAGGTTGGTT	GGATCTCTGGGGCCAGGGCACCCTGG	D1 , J_H3, productive
24-1661F	TTGGG	GCTGGTAGTAGTTA		TTAACATCTGGGGCCCAGGCACCCTGG	D5 , J_H4, productive
24-1662F	C	CCTGGTTATAGTACT	AGG	AACATCTGGGGCCCAGGCACCCTGG	Df , J_H4, productive
24-1796F	TGCTAAGG	GATGACTATGGTGA	CCCAAAG	ATCTGGGGCCCAGGCACCCTGG	D1 , J_H4, non-productive
24-1797F	TGGACTT	ATCCTGGTTATAGTACTG	GTT	TACTTTAACATCTGGGGCCCAGGCACCCTGG	Df , J_H4, non-productive
24-1798F		TTACTATAGTAGTGGCTGGGGT	CTNGACT	GGTTGGATCTCTGGGGNCAGGGCACCCTGG	D4 , J_H3, productive
24-1799F		GGATAGG		CATCTGGGGCCCAGGCACCCTGG	D? , J_H4, productive
24-1810F	TG	ATCCTGGTTA	A	GTTGGATCTCTGGGGCCAGGGNACCCTGG	Df , J_H3, productive
24-1811F	CTTT	ATCCTGGTTATAGTACTG	GCTAT	ACTACTTTAACATCTGGGGCCCAGGCACCCTGG	Df , J_H4, non-productive
24-1813F	ATATGAGGTA	TAGCTACGATGACTATGGTGATT	G	GACTGGTTGGATCTCTGGGGCCAGGGCACCCTGG	D1 , J_H5, non-productive
24-1814F	GGG	GTTATGCTGGTTATGCTGGTTATGGTTATGC	CCCTG	AACATCTGGGGCCCAGGCACCCTGG	D2b, J_H4, productive
24-1657F		AATAGTGCTAGTTGGGGTGAAATGGG		CTTGTGGGGCCCCGGCACCCTGG	Unknown D, J_H4, productive
24-1800F	TCACT	TGGTGATTATGGTAGTTATGCTTATGC		CTTTAACTTGTGGGGCCCCGGCACCCTGG	D7-D5-D2a, J_H4, productive

Figure 34 Comparison of nucleotide sequence of VDJ genes (FR3 through J_H) from day 28 fetuses with germline *V_H1-a3* or *V_H2-a3*. Dots and dashes denote identity to the sequence of *V_H1-a3* and *V_H2-a3*, respectively. N denotes a nucleotide that cannot be unequivocally determined. The utilized D and J_H genes, and functionality of the VDJ genes are indicated. The heptamer motifs of the recombination signal sequences of *V_H1-a3* and *V_H2-a3* are underlined. All clones were partially sequenced in one orientation from J_H to V_H.

A K G R F T I S K T S S T T V T L Q M T S L T A A D T A T Y F C A R

V_H1-a3	GCGAAAGGCCGATTCACCATCTCCAAAACCTCGTCGACCACGGTGACTCTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGAGA***CACAGTG***
28-1305F	....
28-1306F	....
28-1603F	....
28-1605F	.......N..
28-1606F	....
28-1607F	....
28-1609F	....
28-1740F	....
28-1739F	....
28-1738F	....
28-1737F	....

V_H2-a3	GAATGGCCGAATCATCATCTCCAGGGACAACGCCACAACACGGTGTCTCGGCAAATGAACAGTCTGACAGCCAGGGATAGGGCCCTGTATTTCTGTGCAGGAGA***CACAGTG***
28-1602F	--
28-1742F	--

	N region	D region	N region	J_H	
28-1305F		TACGATGAC	C	CGGTTGGATCTCTGGGGCCAAGGCACCCTGG	D1 , J_H3 productive
28-1306F	TGG	TGCTGGTAGTAGTTATTATA	GG	GACTGGTTGGATCTCTGGGGCCAGGGCACCCTGG	D5 , J_H5 productive
28-1603F	TT	TGCTGGTTATGCTGGTTATGGTTATG	CCC	CCTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D2b, J_H4 productive
28-1605F	TG	GTTATGCTGGTTATGGTC	CC	TTTAACTTGTGGGGCCCAGGCACCCTGG	D2b, J_H4 productive
28-1606F	A	ATATGCTAGTAGTAGTGGTTATT	GGT	ACTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D3 , J_H4 non-productive
28-1607F	CGTGC	GCTGGTAGTA		ACTTGTGGGGCCCAGGCACCCTGG	D5/D6, J_H4 productive
28-1609F	GCGG	TATACTTATGGTTATGCTGGTTATGCTTATGCTACC	CTA	TTTAACTTGTGGGGCCCAGGCACCCTGG	D2a, J_H4 productive
28-1740F	GATTTAAAAA	TATGCTGGTAGTAGTTATTA	G	AACTTGTGGGGCCCAGGCACCCTGG	D5 , J_H4 non-productive
28-1739F	C	TTACTATACTTATGGTTATGCTGGTTATGC	CGAGC	ACTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D2a, J_H4 productive
28-1738F	TCCCC	GCTGGTTATGCTTATGTC	ACGG	CTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D2a, J_H4 non-productive
28-1737F	GG	AGCTACGATGACTATGG	G	TTGGATCTCTGGGGCCAGGGCACCCTGG	D1 , J_H3 productive
28-1602F	T	ATATGCTAGTAGT	CTT	TGACTGGTTGGATCTCTGGGGCCAGGGCACCCTGG	D3 , J_H5 non-fuctional
28-1742F	A	ACTATACTTATGGTTATGCTGGTTATGCT	TATACT	ACTACTTTAACTTGTGGGGCCCAGGCACCCTGG	D2a, J_H4 non-productive

is the result of preferential rearrangement or selection. Because the nonproductive VDJ genes cannot be translated into a mature IgH protein, their V_H gene usage reflects the V_H gene utilized in the rearrangement process.

Although only partial nucleotide sequences were obtained for most of the fetal VDJ genes, their V_H gene usage can be determined with certainty. Of the 33 non-productive VDJ genes I cloned from rabbit fetuses, the nucleotide sequences of 26 were identical to V_H1 (Table 9). From these V_H1 utilizing clones, three of the V_H1-a2 utilizing clones, clones 15-1486, 15-1487 and 15-1488, and two of V_H1 -a3 utilizing clones, clones 14-1808 and 21-1489, were selected randomly for complete nucleotide sequence analysis. The analysis of the complete V regions in these five VDJ clones showed that, indeed, these VDJ genes used V_H1 (data not shown). I conclude that all the 26 VDJ genes utilized V_H1. The nucleotide sequences of the other seven VDJ genes were identical to a pseudogene, ψV_H2-*a3* and I concluded that they used ψV_H2-*a3*. Because V_H1 was the only functional V_H gene utilized in non-productive VDJ genes, I conclude that the preferential utilization of V_H1 in the VDJ gene repertoire is due to preferential rearrangement. Among the 23 productive VDJ genes, all used V_H1 (Table 9). Taking the productive and non-productive VDJ genes together, regardless of the developmental stage of the fetuses, V_H1 was utilized in 88% of the VDJ genes (49 of 56).

V_H Promoter Regions

Because the rearrangement of V_H genes correlates with transcription of the germline V_H genes (Yancopoulos and Alt, 1985, Blackwell *et al.*, 1986), I searched the promoter region of V_H1 for transcription factor motifs that may be absent from the promoter regions of the other V_H genes. I determined the nucleotide sequences of the 1. 2 kb promoter regions of three V_H genes, the preferentially utilized V_H1-*a3*, the unutilized V_H4-*a3* and the less-frequently utilized V_Hy33 (Friedman *et al.*, 1994). I compared the

Table 9 Frequency of V_H gene usage in fetal VDJ gene repertoire.

Age of Fetus	Non-productive VDJ genes		Productive VDJ genes		% Productive[1]
(days)	V_H1	other[2]	V_H1[3]	other	
14	6	3	1	0	14
15	6	0	3	0	33
21	2	1	2	0	50
24	9	0	10	0	53
28	3	2	8	0	72
Total	26	7	23	0	56

[1] Considering only the VDJ genes which had utilized functional V_H gene.

[2] All VDJ genes which did not utilize V_H1 had rearranged the same pseudogene, $\psi V_H2\text{-}a3$.

[3] All productive VDJ genes had utilized V_H1.

nucleotide sequence of these promoters to each other and to two other rabbit V_H promoters (Figure 35) (Bernstein *et al.*, 1985).

One of the most important motifs for transcription factor is the highly conserved octamer motif (Eaton and Calame, 1987), which is found among the promoters of V_H, V_L, Eμ, Eκ and TCR genes (reviewed in McDougall *et al.*, 1989, Calame and Eaton, 1988). The octamer motif of all rabbit V_H promoters examined, except for the promoter of the pseudogene ψCL42. V_H25, are identical to each other and to the consensus sequence of the octamer motif (Figure 35). A potential TATA box was identified 10 bp upstream of the octamer motif of all the V_H promoters except for that of the pseudogene ψCL42. V_H34. Like the octamer motif, the TATA box of each V_H promoter (except for the promoter of the pseudogene, ψCL42.V_H34) is identical to each other (Figure 35).

Another conserved motif among the V_H promoter is the heptamer motif. Mutation studies showed that mutation of the heptamer reduced the activity of the V_H promoter by 30% to 80% suggesting that the heptamer is an important motif for the activity of V_H promoters (reviewed in Staudt and Lenardo, 1991). Two heptamer-like motifs were found in each of the five rabbit V_H promoters examined (Figure 35). One of them is located 13 bp downsteam of the octamer and all the V_H promoters have identical heptamer motif at this position. Another heptamer-like motif was found 20 bp upstream

of the octamer. The heptamer at this position differs considerably among the five V_H promoters. It is possible that these differences in the heptamer-like motif may result in different levels of promoter activity. However, these heptamer-like motifs themselves differ from the consensus sequence of the heptamer (CTCATGA) and therefore, the argument that the differences in the heptamer-like motifs may result in different promoter activity is not overly strong.

Figure 35 Comparison of the nucleotide sequences of promoter regions of five germline V_H genes. Dots denote identity to the sequence of V_H1-a3 promoter region. Gaps were introduced into the sequences to maximize the alignment of all V_H promoters. Known recognition motifs for DNA-binding proteins important for transcription of IgH genes are indicated. The position of the nucleotide in the promoter of V_H1 relative to the ATG start codon is shown.

```
VH1-a3 promoter   AAGCTTGTGGACTGCATATATAGCTAATGTCACATACTATGTGTCCACAAGTACCGGGAATATTTCTGTT   -1387
VHy33 promoter    ......................A.........A................TC....T.C.T..........
VH4-a3 promoter   ......................A.............C...........TC....T.. T..........
yCL42.VH34prom    ....G.................A..............G..........T.....T.....CA..TCT..

VH1-a3 promoter   AGCATATTATGCTCCACGGCTGACC CGGTTTCATAATGACAGAACAACCTCCTTTTTAATACTGAATAA   -1318
VHy33 promoter    ..............A.G.TTAAC..AA.A....C........A.....T......C....C.........
VH4-a3 promoter   ................G.CTAAC.. ACA....C.....T.....TG....G...C............G.
yCL42.VH34prom    TAATATG.........G.TTGAC...ACA....C......................C.............

VH1-a3 promoter   TATTCCATGTCCTAATGTG AAGAATCCATTCACTG TTCTATGAACACTTG    TTTATTGTAGACTG   -1249
VHy33 promoter    .......... .A.G...AAC...C.G.......C.T..T....C...T...GCTT.G.TG.AG......
VH4-a3 promoter   ..G.........A.....A.................................    C....A.........
yCL42.VH34prom    ............A....G.A.....G...........T....C..............CT.....A....

VH1-a3 promoter   AACTATTGTGAACAAAGCTGCATGACATGAACCCAGGACTGCGGGTGAATTGTGACACAGTGACTTC TA   -1186
VHy33 promoter    ............C........T..C...............T..........................A..
VH4-a3 promoter   ..G......A....................A......G..A.C.......................  ..
yCL42.VH34prom    ........G................C.....A.......G...............T............

VH1-a3 promoter   TCCAGTGCTTGACCTGAACTCAGAGTCATGGTTACTAGACACAGGGAAGAGGGGG  ACAGGGGACGGCT   -1116
VHy33 promoter    C..........G...................A....C...............A...  A....CA.......
VH4-a3 promoter   C......TG......C..A...C............  AGA..........A...ACACA ...  .....
yCL42.VH34prom    C..........G...C.............TA.........................GA.............

VH1-a3 promoter   GATACCAGGGCACAAAGCACCACTC ACAGGAAGGAATAATTCTGCCCACACCACAGTGGGCTGACTCAG   -1048
VHy33 promoter    .C.......A..........A....  .A..C..................C......... ...........
VH4-a3 promoter   ........................A.A...C......G.................................
yCL42.VH34prom    .........A...............C.A...C......................................

VH1-a3 promoter   TTCACCACAACACACGACAGGTTCCACGAATAAATAGAACTG TGCCTCCAGGCCTCCCA ACAGAGTGA    -979
VHy33 promoter    .....T.....C.......T.......A.....GC..T....AG.A.A..C.T.TC..A.C...A.....
VH4-a3 promoter   ...........................A.....C........A .................. ........
yCL42.VH34prom    ...........C..T............A.....C........AG.T..................T.....

VH1-a3 promoter   TGGAAAGGAGGTGGAAATGTGCATGACACTGAGTTGATCTGTGCCCATTTCT  ACCATGCACATTCCAA    -911
VHy33 promoter    .....C.......A.........A......G.......................GC.A..CA..G.......
VH4-a3 promoter   .....................................................  ...............
yCL42.VH34prom    .......................A......G.....C.................................

VH1-a3 promoter   TAGCACACTACACCCCACTACCATGTACAGTGTTCTGTGTTCAGGAACAGTTTTAAACAGTTTAAACAGT    -843
VHy33 promoter    .......................A....C...........A...A.T.............
VH4-a3 promoter   ........A.AC...ACTAC.AT ........... A.......C...............
yCL42.VH34prom    ........A.AC...AC.AC.ATC..........................................TT...

VH1-a3 promoter   TTTTAAACTTTAAGGCAATGGAT GAACAAGGAAACAAAACATAGTTAACCTT AAAAAATCCATTTGCA    -773
VHy33 promoter              A.....T.......  ...T..........  GCATAG..T....A..TG......C.....
VH4-a3 promoter               ...A.........  T..T...........G.............A..........C.....
yCL42.VH34prom    .......................GT..T...............C..........A.......A..C...A.

VH1-a3 promoter   GGCCTTCATCTGTTTAAAAGTGGGTGACAGGCATTAGGACAAAATAGGCTATAAGTTCTACTAGGGGCAC    -703
VHy33 promoter    .T.T.........G....TA....C.G.................T...  .A.GTCC.C.........A...
VH4-a3 promoter   .T.......................C.............AT......T....TA.GTCC.C.......A...
yCL42.VH34prom    .T.......................C..................T.T....TA.GTCC.C.......T...

VH1-a3 promoter   CCCTGTTCTCATCAGAGAAATTACTGAGTGTATCCTCATGGCTTCCCACA TTTTCCCTCACAATGTATC    -634
VHy33 promoter    ..G.....C.........TGC.GCT....CCC.G..A...........A...C.....T...G.T..CAC...
VH4-a3 promoter   ..G.....C..........C.G......CCC.G....................  ........T....C...
yCL42.VH34prom    ..G.....C..........C.G......CCC.G.....C...........C....T...G.T....C...

VH1-a3 promoter   ATGGGAAACTGTAGATGACGCTACACATGAATGAACCCTTACCCCTGAGGTTGGAAATACAGACAGAGTT    -564
VHy33 promoter    G...T.....AC......T....T. GC..TA..G...C...AA.C..CAAA.....C......GC.. .
VH4-a3 promoter   ............... .TT.......AG.......GG..C....A....CA......T........C....
yCL42.VH34prom    ...................T......AGCA..G..G...C....A.C..T...T...T....C...C....

VH1-a3 promoter   TCTTGCTCCCGGGTTCAGAAAGACTAACTCCTAGATGTCATAGGCATTTGGGATGTCTGGGGCTGTC AC    -495
VHy33 promoter    ...GAGCT.A..T..T.......G...............TG...........C.GA..T..........A.T
VH4-a3 promoter   ....  .C..AA........... .........C..GG............T.......A.......AA..
yCL42.VH34prom    .......T.A..........................GG........T.........................A..

VH1-a3 promoter   TCAAAGGCAGATATCTCCTATTGTCAACTTTTCTGTCCATCTCTACATTTCTGTA    TTCTCTCCTCT    -429
VHy33 promoter    C..........A......C...T...T....G..........A.....C..G...TTCTC.CAC.C.T...
VH4-a3 promoter   C..........A......C.......C.............T.............CTCTC.CAC.C.T...
yCL42.VH34prom    C..........A......C.......C...........................CTCTC.CAC.C.T...
```

```
VH1-a3 promoter   TCATCTATCACTGTCCCAATGTCTTTCATATAGATGATA                  AGGAACCAATGGT   -361
VHy33 promoter      C......G..A.T........................GAGAGGTCAATAGATGAT.T...A....T..
VH4-a3 promoter   CTC....................................                  ....G.T......
yCL42.VH34prom    CTC...........A...........................................A....T......

VH1-a3 promoter   TATTTAAATAAATGGGGGACATGATATTGCTAACTCTGGATATTAAGGGTACACACATATTAGAATGAAG   -291
VHy33 promoter    .....T.. .....AA...............T..A...........................A..G...A
VH4-a3 promoter   ..............A.....C.............A..................................A
yCL42.VH34prom    ..............A.................G.A.................................GA

VH1-a3 promoter   CAAGGTATTTGCCTTCAACTTTT    TAAAATAATGGTAACATAAAAATTCATATGATCTGAATCCTATC   -224
VHy33 promoter    ......CA...........G..ATAA....GC.ATAA.......................CA.........
VH4-a3 promoter   .......................    .........A.............................A....
yCL42.VH34prom    ......CA.........TC..............T..A..........C..................A....
yCL42.VH25prom                                                            ...............

                                                      Pyrimidine tract
VH1-a3 promoter   ACAGCCATCACTCTACATACCCAGGACA CCACATCTGCCCTGGGCCCTCTCCTGTCTGAGGCGTCT AC   -156
VHy33 promoter    .T....       ....CC.A.......TGT......A...........G...........A.....ACT
VH4-a3 promoter   .T...............C........T. .........T..........A...A..............G..
yCL42.VH34prom    .T..A.....GC.....A...T...........G...............G...A.........A...G..
yCL42.VH25prom    ............................ .......A............G...A..............G..

                  Heptamer  TATA-box      Octamer          Heptamer
VH1-a3 promoter   CTCATGCCTGCTATATAGGGGCAGCACATGCAAATAGGGCCTCCCTCTGCCCATGAAAACCAGCCCAGCC   -86
VHy33 promoter    .CAGG..TG..........A.........................C.............T..........
VH4-a3 promoter   .C....................................................................
yCL42.VH34prom    .C............C.......................................................
yCL42.VH25prom    .C............................A.G.....................................

VH1-a3 promoter   CTCACCCTGCAG                                                             -74
VHy33 promoter    ............
VH4-a3 promoter   .........
yCL42.VH34prom    ............
yCL42.VH25prom    ............
```

Lastly, a pyrimidine-rich tract was found 31 bp upstream of the octamer. This pyrimidine-rich tract is the least conserved motif amoung mouse V_H promoters (Eaton and Calame, 1987). In this 13-bp pyrimidine-rich tract, 1-to 2-bp differences were found among all the rabbit V_H promoters. Taking these findings together, I found that the five rabbit V_H promoters I examined are highly similar with the octamer and the downstream heptamer motifs are identical amoung the promoters of three functional V_H genes. I concluded that the preferential rearrangement of V_H1 is probably not explained by these core promoter elements. However, the functional assay for these promoters needs to be performed to draw a firm conclusion.

CHAPTER IV

DISCUSSION

I have shown in this study that the status of the IgH gene on the unexpressed allele of rabbit B cells is different from that in B cells of other species in two aspects. First, 50% of rabbit B cells do not rearrange their unexpressed IgH alleles (Table 8). The V_H and J_H genes on the unexpressed allele of these B cells remain in germline configuration. This is in contrast to mouse and human in which the unexpressed IgH alleles are rearrranged to a DJ or a nonproductive VDJ gene (Atkinson *et al.*, 1993, Alt *et al.*, 1984, Reth, 1986). In chicken, approximately 8% of bursal cells have J_H on the unexpressed allele in germline configuration (Barth and Humphries, 1988, Benatar *et al.*, 1992, Reynaud *et al.*, 1989). However, the status of V_H in these cells has not been determined. Second, for those B cells in rabbit which had rearranged the unexpressed IgH allele, most of them (85%) rearranged DJ (Table 8). Only 15% of them rearranged two VDJ genes, one of which is presumably nonproductive (Table 8). This is in contrast to mouse and human B cells in which nonproductive VDJ genes are such a common occurrence that 60% to 70% of B cells have nonproductive VDJ genes on the unexpressed IgH alleles. But, in this aspect rabbit B cells are similar to those in chicken where the incidence of nonproductive VDJ genes is low (Ferradini *et al.*, 1994). The possibility that rabbit B cells generate only productive VDJ genes was ruled out when I found that many VDJ genes isolated from early fetal development were nonproductive. Rather, it is likely that if a pre-B cell rearranges a nonproductive VDJ gene, it dies without rearranging a VDJ gene

on the second IgH allele. Regarding the intermediates of IgH gene rearrangements on the unexpressed IgH alleles, I found only DJ genes on the unexpressed allele of the hybridomas. For this reason, I think that the order of IgH gene rearrangement for most of the VDJ genes is that D rearranges to J and then V rearranges to DJ. Currently, it is no known what role VD gene rearrangement plays in the IgH gene rearrangements in rabbit.

The other major result of my work is the analysis of V_H gene usage in nonproductive VDJ genes cloned from rabbit fetuses. Our laboratory has shown previously that rabbit B cells preferentially utilize V_H1 in their VDJ genes (Knight and Becker, 1990, Raman *et al.*, 1994). In this study, I found that many of the VDJ genes in fetus are nonproductive and the only functional V_H gene utilized is V_H1. Because the V_H gene utilization in these nonproductive VDJ genes cannot be influenced by the selection process, I conclude that the preferential utilization of V_H1 resulted from its preferential rearrangement.

Rabbit B Cells do not Rearrange DJ Genes on Both Alleles before Rearranging VDJ gene

During the development of B cells in mouse, human and chicken, the first rearrangement of IgH genes is D to J_H on both alleles. Therefore, the DJ gene rearrangement in B-lineage cells is believed the default step of IgH gene rearrangement without any regulation. In contrast, rabbit B cells do not rearrange DJ genes on both IgH alleles before VDJ gene rearrangement indicating that DJ gene rearrangement in rabbit is not a default step as it is in other species.

An alternative explanation for the lack of a DJ gene on the unexpressed IgH allele is that the DJ gene rearrangement is an inefficient process in rabbit. The IgH gene rearrangement has been shown to be dependent on the accessibility of the IgH locus to recombinase enzymes as suggested by the correlation of the rearrangement and the

transcription of germline genes (Yancopoulos *et al.*, 1984, Stiernholm and Berinstein, 1995). Regarding DJ gene rearrangement, the level of DJ gene rearrangements correlates directly with the level of germline J_H transcripts (μ^0 transcripts) which is initiated from upstream of DQ52, the most 3' D (Alessandrini and Desiderio, 1991, Schlissel *et al.*, 1991, Schlissel *et al.*, 1991). The low level of DJ gene rearrangement in rabbit B cells may be explained by the inefficient transcription of the μ^0 transcripts and subsequently, the accessibility of the J_H locus for recombinase enzymes is limited. To test this hypothesis, the transcription of germline J_H in rabbit pro-B or pre-B cells needs to be examined. The analysis of the region immediately upstream of J_H seems to support the hypothesis because it showed no indication of DQ52, the gene from which μ^0 transcription is initiated. However, rabbit DQ52 may be located in a region further upstream similar to mouse in which DQ52 is located approximately 700 bp upstream of J_H1. To rule out this possibility, the sequence of the region further upstream to J_H has to be determined.

It is desirable to develop rabbit pro-B or pre-B cell lines so that the IgH gene rearrangement can be observed *in vitro*. To date, no such cell line has been described for rabbit. One approach to develop such cell lines has been described in mouse and human which is to transform early B lineage cells by viruses, AMuLV or EBV. No such transforming virus for rabbit has been described. An alternative approach is to generate transgenic rabbits overexpressing cellular or viral oncogenes in a B cell specific manner. To date, transgenic rabbits overexpressing c-myc and v-abl have been developed (Knight *et al.*, 1988, Sethupathi *et al.*, 1994). However, none of these transgenic rabbits developed tumors of early B lineage cells. Hence, sequential IgH gene rearrangements of rabbit B lineage cells has not yet been observed *in vitro*.

Nonproductive VDJ Genes Occur Infrequently in Rabbit and Chicken B Cells

I report in this study that nonproductive VDJ genes are found infrequently in B cells from adult rabbit, which is similar to the finding in chicken. In chicken, DJ progenitors undergo V to DJ gene rearrangement only on one allele and essentially no second rearrangement is allowed if the first rearrangement results in a nonproductive VDJ gene (Ferradini *et al.*, 1994). I have considered the explanation, for the scarcity of nonproductive VDJ genes in rabbit, that nonproductive rearrangements may occur infrequently and therefore, the second rearrangement is generally not necessary. However, because I found many nonproductive VDJ genes in rabbit fetuses, I conclude that IgH gene rearrangement in rabbit generates nonproductive VDJ genes like in other species. Therefore,, I propose that the rearrangement in rabbit is similar to that in chicken in that no second rearrangement is allowed when the first rearrangement fails to generate a productive VDJ gene.

It appears as if nonproductive VDJ genes are not desirable in these cells. Although we do not know for certain why this is the case in rabbit and chicken B cells, we can speculate why chicken B cells do not have nonproductive VDJ genes on their unexpressed IgH allele. Chicken B cells diversify their VDJ genes by somatic gene conversion, the process in which the nucleotide sequences of donor genes are duplicated onto the coding region of the VDJ genes. Gene conversion in chicken occurs both in the V_H and the CDR3 regions. The reading frame of nonproductive VDJ genes can be restored if the gene conversion occurs in the CDR3 region. Such a gene conversion event could result in B cells expressing two VDJ genes simultaneously.

The reason why rabbit B cells do not have nonproductive VDJ genes is less clear. Although rabbit B cells, like chicken, diversify their VDJ genes by somatic gene conversion-like process, no gene conversion-like event has been documented in the

CDR3 region. Consequently, nonproductively rearranged VDJ genes in rabbit B cells cannot be converted to productive ones like in chicken.

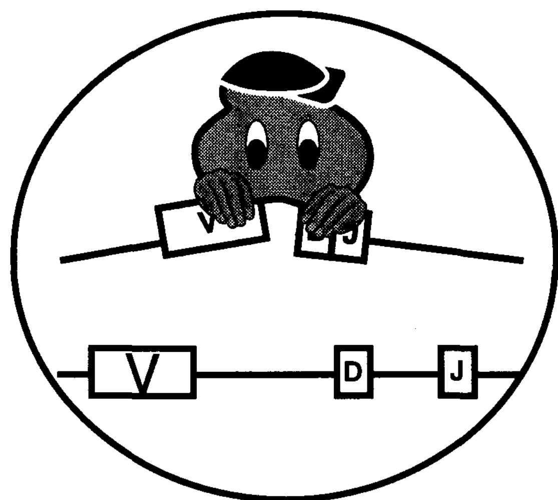
How rabbit confines the generation of VDJ gene to only one allele is not yet known. Several possibilities can explain the phenomenon and they are discussed below.

IgH Gene Rearrangement may Occur during a Narrow Window during B Cell Development

This model explains that, during differentiation of B cells, DJ gene rearrangements on each IgH alleles occur independently of each other (Figure 36). Once DJ is rearranged on one allele, VDJ gene rearrangement occurs rapidly regardless of the IgH gene rearrangement on the second allele. Productive VDJ gene on the first allele would provide the cell with survival and allelic exclusion signals while a nonproductively rearranged gene would not. These cells then die without rearranging the second allele because the period during which V(D)J gene rearrangement can occur has passed.

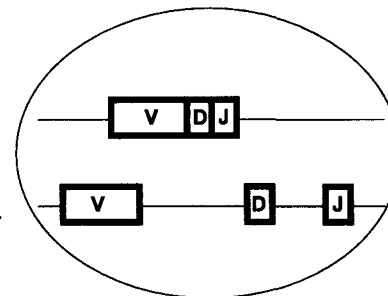
Although I think that this possibility is the most likely explanation, it is not without a shortcoming. The shortcoming of this model is that it cannot explain the allelic exclusion in cells which rearrange DJ and, subsequently, V to DJ on both alleles at the same time. Assuming that the chance of generating a productive VDJ gene is one-third (the chance that V_H is rearranged into the same reading frame of J_H), the probability of two productive VDJ genes to occur in one cell is (1/3 x 1/3) x 100 = 11%. If we take into account that the frequency of B cells with two VDJ genes in rabbit is 10% (Table 8), the frequency of B cells in the periphery with two productive VDJ genes would be 1.1% (10% of 11%). This concern of violating allelic exclusion is irrelevant, however, if there is a selection against B cells with two productive VDJ genes. But the finding of B cells in Ig transgenic mice expressing transgenic and endogenous Ig genes simultaneously discounts the negative

Figure 36 Schematic representation of the "narrow window for rearrangement" model.



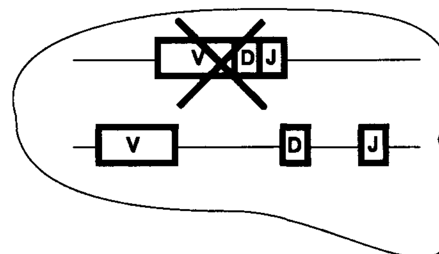
pre-B cell

productive



develops into mature B cell

non-productive



dies because the window for rearranging a VDJ gene on the second allele has passed

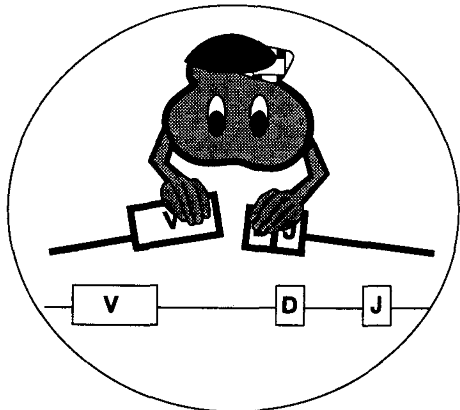
selection of such double producers (Grosschedl *et al.*, 1984, Rusconi and Kohler, 1985, Weaver *et al.*, 1985, Stall *et al.*, 1988, Vos and Hodes, 1992, Rath *et al.*, 1989, Forni, 1990).

Rabbit B Cells are Inefficient in Repairing the Double-Strand Breaks

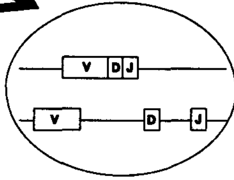
The existence of germline V_H, D and J_H genes on the unexpressed allele, although uncommon in mouse, human and chicken B cells, is common for B cells in SCID mice (Kotloff *et al.*, 1993). This is not unexpected, however, considering that SCID mice are defective in repairing the lethal double-strand-breaks (ds-breaks) generated during the rearrangement process. It is already so rare to find cells with a VDJ gene that the chance of findings cells with DJ on the unexpressed allele, which requires another successful DNA repair, is virtually none.

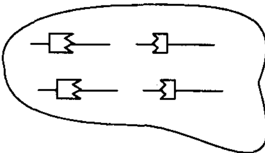
The finding that rabbit B cells have their IgH allele on the unexpressed allele in germline configuration is similar to the finding of SCID B cells and the above explanation for SCID mice may apply to rabbit (Figure 37). However, I do not think that the explanation in SCID mice is applicable to rabbit for three reasons. First, the DNA repair mechanism is a process highly conserved throughout evolution (reviewed in Jeggo, 1990). There is no apparent reason for rabbit to be an exception. Second, 50% of rabbit B cells have rearranged their unexpressed IgH allele to DJ or VDJ genes. And finally, rabbit B cells diversify their VDJ genes by gene conversion which also involves DNA repair machinery. If the DNA repair machinery in rabbit is not efficient, it is unlikely that the rabbit would evolve to use gene conversion as a mechanism to diversify their VDJ genes. The possibility that rabbit B cells may be inefficient in DNA-repair can be tested by determining the susceptibility of rabbit B cell lines to ds-breaks generated by X-irradiation, as compared to cell lines from other species.

Figure 37 Schematic representation of the “inefficient double-strand breaks repair” model.


V
D
J
pre-B cell with ds-break

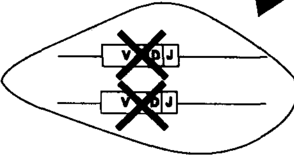
productive

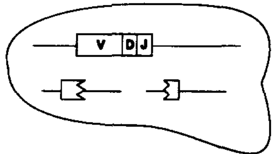

V
DJ
V
D
J
successful repair
develops into mature B cell


dies because of unsuccessful
ds-break repair on both alleles

nonproductive
on both alleles

productive


dies because of no productive
VDJ gene is generated


V
DJ
dies because of unsuccessful
ds-break repair on the second allele

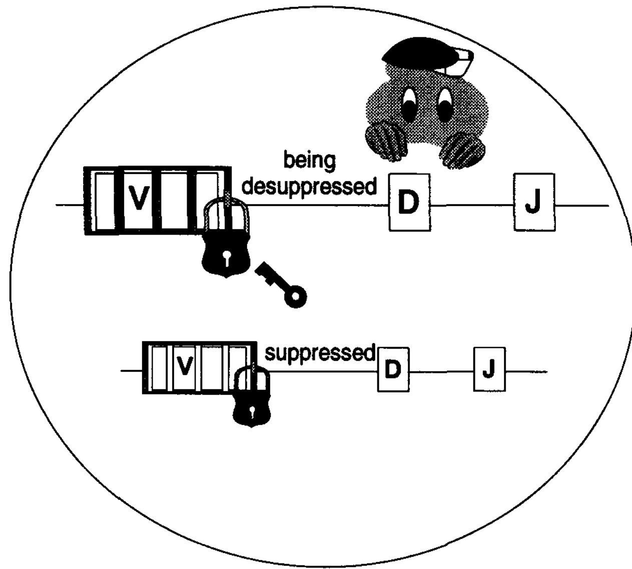
V(D)J Gene Rearrangement Requires a Desuppression

We can also speculate that the V to DJ gene rearrangement is actively suppressed and that a V to DJ gene rearrangement requires the IgH gene be released (desuppressed) from the suppression. If the factor(s) required for the desuppression is expressed in such

a low level that only one IgH allele can be desuppressed (Figure 38), as a result, only one allele can be rearranged to a VDJ gene. The suppression may be mediated by a silencer similar to the one reported by Luaster *et al.* (Lauster *et al.*, 1993) who found a silencer for the rearrangement process between $V\lambda$ and $J\lambda$ genes in chicken. This silencer, when included in the $V\lambda$ and $J\lambda$ transgenic construct, decreased the level of transgene rearrangements in transgenic mice. Abutting both 5' and 3' ends of the silencer are anti-silencers which counteract the function of the silencer thus allowing the rearrangement of the transgene. The report of a similar silencer—anti-silencer between V_H1 and D in chicken also supports this model (Ferradini *et al.*, 1994).

The definitive approach to determine whether such silencer—anti-silencer are present in rabbit IgH locus is to generate several transgenic mouse lines using artificial rearrangement substrates which contain germline V_H, D and J_H genes together with varying amount of the germline region between V_H and J_H genes and compare the level of rearrangement of the transgenes. However, this approach is cumbersome and not suitable as initial approach. Because the silencer in chicken λ gene also functions as a strong silencer and suppresses the expression of reporter gene in a heterologous promoter system (Lauster *et al.*, 1993), it is possible to locate the silencer in the rabbit IgH locus by searching for the germline region which possesses silencer activity.

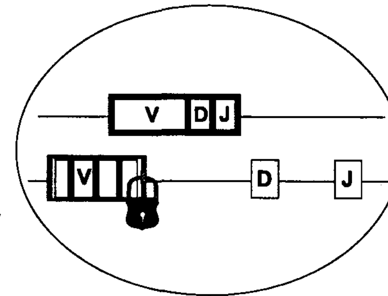
Figure 38 Schematic representation of the “suppression and desuppression” model.



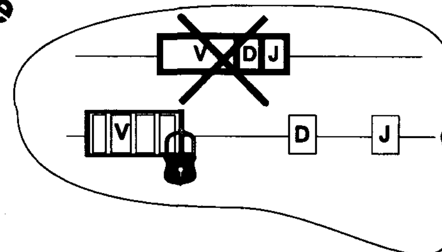
pre-B cell

productive

non-productive



develops into mature B cell



dies without rearranging
the second allele

One IgH Allele is Inactivated

An alternative explanation for the low frequency of VDJ genes on the unexpressed allele is that one IgH allele is inactivated and cannot be rearranged to a VDJ gene (Figure 39). Although there is no precedence for such inactivation of the IgH gene in any other species, we can speculate how this might occur in rabbit.

One IgH allele is hypermethylated

Methylation of DNA has been shown to affect several chromosomal activities. Hypermethylation results in suppression of gene expression (reviewed in Tilghman, 1993), condensation of the chromosome (reviewed in Tilghman, 1993) and reduction of V(D)J gene rearrangement (Goodhardt *et al.*, 1993, Hsieh and Lieber, 1992, Fernex *et al.*, 1994, Engler *et al.*, 1991). Therefore, it is not surprising that IgH genes are hypermethylated in non-B cells (Storb and Arp, 1983) and they become hypomethylated during the development of B cells (Storb and Arp, 1983). This hypomethylation correlates with the rearrangement and expression of the IgH genes. In rabbit, it is possible that during B cell development only one of the two IgH alleles was demethylated so that VDJ gene rearrangement can occur. If this rearrangement fails to yield a productive VDJ gene, the rearrangement on the second allele is prohibited because it was not demethylated. To test this possibility, we need to compare the methylation pattern of IgH genes in purified B-cell progenitors, pro- and pre-B cells, and B cells. This experiment is not feasible at the present time due to the lack of antibody to surface markers specific for early B lineage cells.

One IgH allele is genetically imprinted

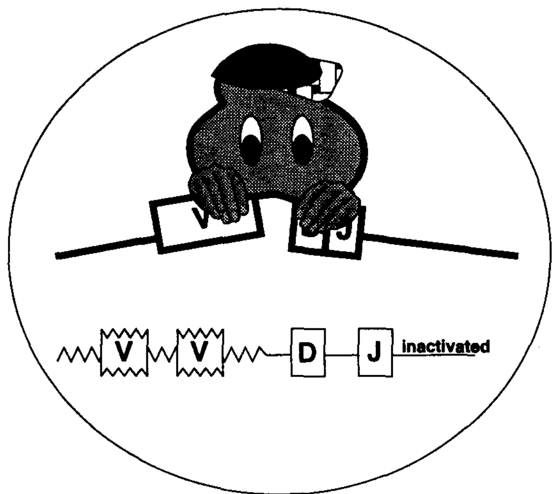
Genetic imprinting is an epigenetic phenomenon which results phenotypically in the expression of a gene on one of the two homologous alleles being inhibited (reviewed

in Varmuza and Mann, 1994). Five autosomal genes to date have been shown to be genetically imprinted (reviewed in Varmuza and Mann, 1994, Chess *et al.*, 1994). Rabbit may imprint one of its IgH alleles so even if productive VDJ genes are generated on this allele, B cells cannot express them. However, I doubt that rabbit genetically imprints its IgH genes because Kitsberg *et al.* (Kitsberg *et al.*, 1993) ruled out the possibility of genetic imprinting of IgH genes in mouse. If rabbit IgH genes are imprinted, this would probably be unique to rabbit. This possibility can be tested by determining whether the two rabbit IgH alleles replicate asynchronously. An imprinted allele was shown to replicate later in the cell cycle than the expressed allele (Kitsberg *et al.*, 1993). Asynchronous replication of rabbit IgH genes would then indicate that one of the IgH alleles is imprinted. Such asynchronous replication of the IgH gene must be found not only in non-B cells but also in every cell type because the imprinting process is independent of the expression of genes (Chess *et al.*, 1994, Kitsberg *et al.*, 1993).

Intermediates of IgH Gene Rearrangements in Rabbit

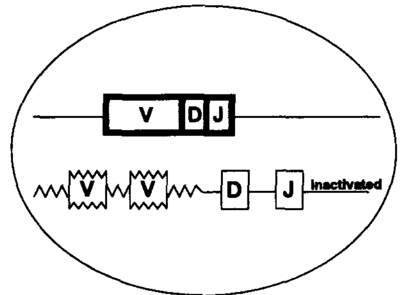
Rabbit appears to be unique regarding the intermediates of IgH gene rearrangement in that both VD and DJ genes are found. Rabbit is the first species to be reported on VD genes which utilized conventional D gene, D2b (Figure 28). (Shin *et al.* (1993) reported the cloning of a VD gene previously from a EBV-transformed human B cell line but that VD gene had used a nonconventional DIR gene). Further, the fact that I can PCR-amplify many different VD genes from splenic DNA suggests that VD genes may be the alternative intermediates to DJ genes in VDJ gene rearrangements. However, the extent to which DJ gene rearrangements occur must be much higher than that of VD gene rearrangements because most of the rearrangements on the unexpressed alleles of B cell hybridomas are DJ genes.

Figure 39 Schematic representation of the "inactivation of one IgH allele" model.



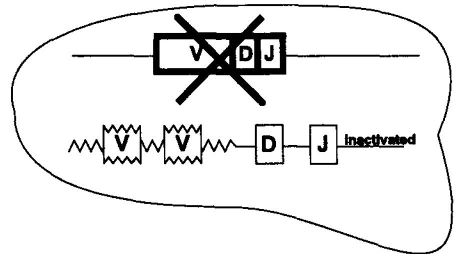
pre-B cell

productive



develops into mature B cell

non-productive



dies because the rearranement on the second allele is prohibited by the chromosomal inactivation

The VD gene rearrangement is probably not unique to rabbit. I predict that VD gene rearrangements also occur in other species, such as mouse, human and chicken, but they have not not detected because Southern analysis used in previous reports to study the IgH gene rearrangements (Alt *et al.*, 1984, Chen *et al.*, 1993, Nickerson *et al.*, 1989, Benatar *et al.*, 1992, Reynaud *et al.*, 1989) is not as sensitive as PCR, which I used in this study. I predict that VD genes are generated at low levels in these species, as well as in rabbit, and that VD genes could be detected in all species by using PCR.

Currently, we do not know whether the VD or DJ genes can be rearranged to form a complete VDJ gene. Although one of them may represent a misstep in the rearrangement process, the intact RSSs of D gene located 3' of VD or 5' of DJ genes (Figures 23, 27 and 28) argue against this possibility. Instead, they suggest that these intermediates could be further rearranged to a VDJ gene. This issue can be approached by examining the rearrangement of artificial rearrangement substrates containing either V and DJ, or VD and J in transgenic animals or *in vitro* rearrangement assay. The best approach is to generate gene-targeted mice using the DJ- or VD-containing substrates such that the rearrangement of these intermediates can be studied in the context of endogenous IgH locus.

V_H1 is Preferentially Rearranged

It has been previously shown that 70% to 80% of rabbit B cells preferentially utilized V_H1. However, previous studies did not discern whether the preferential utilization of V_H1 resulted from preferential rearrangement or selection for B cells in which V_H1 was rearranged. In this study, I found evidence to support the preferential rearrangement of V_H1 when the analysis of V_H gene usage in nonproductive VDJ genes showed that V_H1 was the only functional V_H gene utilized (26 of 33 nonproductive VDJ genes utilized V_H1, Table 9). However, the finding does not rule out the possibility of a

selection process for B cells which rearranged and expressed V_H1. At present, the molecular basis for the preferential rearrangement of V_H1 is not understood. I discuss several possible mechanisms for such preferential rearrangement below.

RSS

The variation of the RSS has been shown to affect the efficiency of V(D)J gene rearrangement (Hesse *et al.*, 1989, Akamatsu *et al.*, 1994). One obvious explanation for the preferential rearrangement of V_H1 is that its RSS is more suitable for the rearrangement than those of other V_H genes. By comparing the RSS of all the functional V_H genes we have cloned, I found that this explanation is unlikely to be the case. As shown in Figure 40, the RSS of the V_H genes are very similar, many of which are identical to that of V_H1. I conclude that the RSS alone is not sufficient to explain the preferential rearrangement of V_H1.

Promoters

Because Yancopoulos *et al.* (Yancopoulos and Alt, 1985) reported that the rearrangement of V_H genes correlate with transcription of the germline V_H genes, I attempted to correlate the preferential rearrangement of V_H1 with the activity of its promoter region by searching for transcription factor motifs which may be present in V_H1 promoter but absent from the promoter regions of the other V_H genes. By comparing the nucleotide sequences of the 1. 2 kb promoter regions of three V_H genes, the preferentially utilized V_H1-*a3*, the less-frequently utilized V_Hy33, the unutilized V_H4-*a3* (Friedman *et al.*, 1994) and two other rabbit V_H promoters (Bernstein *et al.*, 1985), I found that the four motifs important for the activity of mouse V_H promoters, the octamer, the heptamer, the pyrimidine-rich tract and the TATA-box (Eaton and Calame, 1987), were similar in all of the V_H promoters (Figure 35). I suggest that the preferential rearrangement of V_H1 is probably not explained by these core promoter elements. However, to draw a firm

Figure 40 Comparison of the RSSs of all several functional germline V_H genes. Dots denote identity to the RSS of V_H1-a1. Slashes indicate gaps which are introduced to maximize the alignment. The heptamer and nonamer elements of the RSS are indicated.

	Heptamer		Nonamer
*V_H1-a1**	CACAGTG	AGGGGCCCTCAGGCTGAGCCCAG	ACACAAACC
*V_H1-a2**	....	....	....
*V_H1-a3**	....	....	....
V_Hy33◊	....	.........AG......A.....	....
V_HZ◊	....	......./..../.........A	....
V_H4-a1	....	....	....
V_H4-a2	....	....	....
V_H3-a3	....	....T..................	....
V_H4-a3	....	....	....
V_H6-a3	....	..T./..................	....
V_H9-a3	....	....	....
PRV10	....	.......C.CA.G./..GT..TG	....

* Preferentially utilized V_H genes

◊ Less frequently utilized V_H genes

conclusion, we need to compare the ability of these promoters to drive the expression of reporter genes, such as CAT or Luciferase, in a transient transfection system.

Another experimental approach worth considering is the examination of the germline V_H transcript. If the higher activity of the V_H1 promoter, compared to that of other V_H genes, is the explanation for preferential rearrangement of V_H1, then we would expect to find a higher level of germline V_H1 transcripts than of other V_H genes in pre-B cells which are undergoing V(D)J gene rearrangement. But as mentioned earlier, the caveat of this experiment for the moment is the lack of technology to purify rabbit pre-B cells.

Proximity of V_H1 to D Region

Yancopoulos *et al.* (Yancopoulos *et al.*, 1984) found that murine fetal pre-B cell lines preferentially utilized the most D-proximal V_H gene, V_H81X, in their VDJ gene rearrangements. They proposed that the proximity of the V_H gene to the D rendered it more accessible to the rearrangement process. Because rabbit V_H1 is also the most D-proximal V_H gene (Knight and Becker, 1990), it may be readily accessible to the recombinase enzymes like the V_H81X in mouse. Although we could cite the utilization of the V_H gene immediately 5' to V_H1 (ψV_H2-*a3*) to argue in favor of this hypothesis, it is not likely the explanation for the preferential rearrangement of V_H1 for two reasons. First, the V_H genes other than V_H1 which are used most frequently in productive VDJ genes are not immediately upstream of V_H1. Rather, these genes, V_Hx, V_Hy and V_Hz, are located more than 50 kb upstream of V_H1, beyond V_H11 (Knight and Becker, 1990, Raman *et al.*, 1994). Furthermore, I found that ψV_H2 was used only in the fetal VDJ genes derived from a3 allotype fetuses and not in a1 or a2 fetuses.

Methylation Status of V_H1

The preferential rearrangement of V_H1 could also be explained by the methylation status of V_H1. Hsieh and Lieber (1992) demonstrated that a hypermethylated artificial rearrangement substrate became inaccessible for V(D)J recombination after it was transfected into a pre-B cell line. Furthermore, Fernex *et al.* (Fernex *et al.*, 1994) found in transgenic mice that hypermethylation of an Ig transgene correlated with decreased rearrangement. Possibly V_H1 in rabbit is undermethylated relative to other V_H genes and is therefore preferentially rearranged.

Chromosome Topology

Another possibility to explain preferential rearrangement of V_H1 is the presence of a matrix-associated region (MAR) in close proximity to V_H1. Chen *et al.* (1993) showed that the rearrangement of J_H genes was impaired after the MARs located upstream of Eμ was disrupted by gene targeting technology. It is possible that rabbit V_H1 may be located near a MAR sequence and that this proximity may render V_H1 susceptible to preferential rearrangement. At present, there is no good consensus sequence for MAR and therefore, the presence of MAR in any region of the chromosome has to be determined empirically. This involves determining whether the chromosomal DNA from the region of interest can bind to nuclear-matrix. By this method, four MARs have been identified in the mouse IgH locus between D genes and the 3'-most C_H gene, $C\alpha$ (Cockerill, 1990). However, the correlation between V_H gene usage and MARs has not yet been analyzed. If the association of V_H genes to MARs influences their usage in the VDJ gene repertoire, I would expect that rabbit IgH locus may contain as few as four MARs because only four V_H genes appear to generate the entire rabbit VDJ gene repertoire.

The study of V(D)J gene rearrangement is important for our understanding of humoral immunity. Rabbit presents a unique system to study the preferential V_H gene

utilization and rearrangement because of its limited V_H gene usage in the VDJ gene repertoire. The knowledge we obtain from studying rabbit V(D)J gene rearrangement will complement that obtained from other species. Ultimately, we hope to integrate all the knowledge about humoral immunity from every species so that a single picture of the humoral immune system with no species barrier may emerge.

REFERENCES

Aguilera, R. J., Akira, S., Okazaki, K. and Sakano, H. (1987). A pre-B cell nuclear protein that specifically interacts with the immunoglobulin V-J recombination sequences. *Cell* **51**:909.

Akamatsu, Y., Tsurushita, N., Nagawa, F., Matsuoka, M., Okazaki, K., Imai, M. and Sakano, H. (1994). Essential residues in V(D)J recombination signals. *J. Immonol.* **153**:4520.

Akira, S., Okazaki, K. and Sakano, H. (1987). Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. *Science* **238**:1134.

Alessandrini, A. and Desiderio, S. V. (1991). Coordination of immunoglobulin DJH transcription and D-to-JH rearrangement by promoter-enhancer approximation. *Mol. Cell. Biol.* **11**:2096.

Allegrucci, M., Young, C. G., Alexander, C. B., Newman, B. A. and Mage, R. G. (1991). Preferrential rearrangement in normal rabbits of the 3' VHa allotype gene that is deleted in Alicia mutants; somatic hypermutation/conversion may play a major role in generating the heterogeneity of rabbit heavy chain variable region sequences. *Eur. J. Immonol.* **21**:411.

Alt, F., Rosenberg, N., Lewis, S., Thomas, E. and Baltimore, D. (1981). Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: rearrangement of heavy but not light chain genes. *Cell* **27**:381.

Alt, F. W. (1987). Development of the primary antibody repertoire. *Science* **238**:1079.

Alt, F. W. and Baltimore, D. (1982). Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. *Proc. Natl. Acad. Sci. USA* **79**:4118.

Alt, F. W., Yancopoulos, G. D., Blackwell, T. K., Wood, C., Thomas, E., Boss, M., Coffman, R., Rosenberg, N., Tonegawa, S. and Baltimore, D. (1984). Ordered rearrangement of immunoglobulin heavy chain variable region segments. *Embo J.* **3**:1209.

Altenburger, W., Steinmetz, M. and Zachau, H. G. (1980). Functional and non-functional joining in immunoglobulin light chain genes of a mouse myeloma. *Nature* **287**:603.

Anderson, S. J., Abraham, K. M., Nakayama, T., Singer, A. and Perlmutter, R. M. (1992). Inhibition of T-cell receptor beta-chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56lck. *Embo J.* **11**:4877.

Anderson, S. J., Levin, S. D. and Perlmutter, R. M. (1993). Protein tyrosine kinase p56lck controls allelic exclusion of T-cell receptor beta-chain genes. *Nature* **365**:552.

Atkinson, M. J., Paige, C. J. and Wu, G. E. (1993). Map position and usage of 3' VH family members: usage is not position dependent. *Int. Immunol.* **5**:1577.

Barth, C. F. and Humphries, E. H. (1988). Expression of v-rel induces mature B-cell lines that reflect the diversity of avian immunoglobulin heavy- and light-chain rearrangements. *Mol. Cell. Biol.* **8**:5358.

Beck, E. G., Jack, H. M. and Wabl, M. (1987). Allelic inclusion in a pre-B-cell line that generates immunoglobulin heavy chain genes *in vitro*. *Proc. Natl. Acad. Sci. USA* **84**:1060.

Becker, R. S. and Knight, K. L. (1990). Somatic diversification of immunoglobulin heavy chain VDJ genes: evidence for somatic gene conversion in rabbits. *Cell* **63**:987.

Becker, R. S., Suter, M. and Knight, K. L. (1990). Restricted utilization of VH and DH genes in leukemic rabbit B cells. *Eur. J. Immonol.* **20**:397.

Becker, R. S., Zhai, S. K., Currier, S. J. and Knight, K. L. (1989). Ig VH, DH, and JH germ-line gene segments linked by overlapping cosmid clones of rabbit DNA. *J. Immonol.* **142**:1351.

Benatar, T., Tkalec, L. and Ratcliffe, M. J. (1992). Stochastic rearrangement of immunoglobulin variable-region genes in chicken B-cell development. *Proc. Natl. Acad. Sci. USA* **89**:7615.

Benger, J. C. and Cox, D. W. (1989). Polymorphisms of the immunoglobulin heavy-chain delta gene and association with other constant-region genes. *Am. J. Hum. Gen.* **45**:606.

Berinstein, N., Levy, S. and Levy, R. (1989). Activation of an excluded immunoglobulin allele in a human B lymphoma cell line. *Science* **244**:337.

Berman, J. E. and Alt, F. W. (1990). Human heavy chain variable region gene diversity, organization, and expression. [Review]. *Int. Rev. Immunol.* **5**:203.

Berman, J. E., Mellis, S. J., Pollock, R., Smith, C. L., Suh, H., Heinke, B., Kowal, C., Surti, U., Chess, L., Cantor, C. R. and et, a. l. (1988). Content and organization of the

human Ig VH locus: definition of three new VH families and linkage to the Ig CH locus. *Embo J.* **7**:727.

Bernard, O., Gough, N. M. and Adams, J. M. (1981). Plasmacytomas with more than one immunoglobulin kappa mRNA: implications for allelic exclusion. *Proc. Natl. Acad. Sci. USA* **78**:5812.

Bernard, O., Hozumi, N. and Tonegawa, S. (1978). Sequences of mouse immunoglobulin light chain genes before and after somatic changes. *Cell* **15**:1133.

Bernstein, K. E., Alexander, C. B. and Mage, R. G. (1985). Germline VH genes in an a3 rabbit not typical of any one VHa allotype. *J. Immonol.* **134**:3480.

Blackwell, T. K. (1984). Joining of immunoglobulin heavy chain variable region gene segments *in vivo* and within a recombination substrate. *UCLA Symp. Mol. Cell. Biol., New Series* **19**:537.

Blackwell, T. K., Moore, M. W., Yancopoulos, G. D., Suh, H., Lutzker, S., Selsing, E. and Alt, F. W. (1986). Recombination between immunoglobulin variable region gene segments is enhanced by transcription. *Nature* **324**:585.

Bollum, F. (1974). In "The Enzymes", vol. 10. p. 145. Academic Press, New York.

Borgulya, P., Kishi, H., Uematsu, Y. and von, B. H. (1992). Exclusion and inclusion of alpha and beta T cell receptor alleles. *Cell* **69**:529.

Bos, N. A. and Meeuwsen, C. G. (1989). B cell repertoire in adult antigen-free and conventional neonatal BALB/c mice. I. Preferential utilization of the CH-proximal VH gene family PC7183. *Eur. J. Immonol.* **19**:1811.

Bosma, M. J. and Carroll, A. M. (1991). The SCID mouse mutant: definition, characterization, and potential uses. [Review]. *Ann. Rev. Immunol***9**:323.

Bottaro, A., Cariota, U., DeMarchi, M. and Carbonara, A. O. (1991). Pulsed-field electrophoresis screening for immunoglobulin heavy-chain constant-region (IGHC) multigene deletions and duplications. *Am. J. Hum. Gen.* **48**:745.

Brack, C., Hirama, M., Lenhard, S. R. and Tonegawa, S. (1978). A complete immunoglobulin gene is created by somatic recombination. *Cell* **15**:1.

Brodeur, P. H., Osman, G. E., Mackle, J. J. and Lalor, T. M. (1988). The organization of the mouse Igh-V locus. Dispersion, interspersion, and the evolution of VH gene family clusters. *J. Exp. Med.* **168**:2261.

Brodeur, P. H. and Riblet, R. (1984). The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse. I. One hundred Igh-V genes comprise seven families of

homologous genes. *Eur. J. Immonol.* **14**:922.

Brodeur, P. H., Thompson, M. A. and Riblet, R. (1984). The content and organization of mouse IGH-V families. *UCLA Symp. Mol. and Cell. Biol., new series* **18**:445.

Brueckner, M., D'Eustachio, P. and Horwich, A. L. (1989). Linkage mapping of a mouse gene, iv, that controls left-right asymmetry of the heart and viscera. *Proc. Natl. Acad. Sci. USA* **86**:5035.

Buluwela, L., Albertson, D. G., Sherrington, P., Rabbitts, P. H., Spurr, N. and Rabbitts, T. H. (1988). The use of chromosomal translocations to study human immunoglobulin gene organization: mapping DH segments within 35 kb of the C mu gene and identification of a new DH locus. *Embo J.* **7**:2003.

Burnett, R. C., Hanly, W. C., Zhai, S. K. and Knight, K. L. (1989). The IgA heavy-chain gene family in rabbit: cloning and sequence analysis of 13 C alpha genes. *Embo J.* **8**:4041.

Calame, K. and Eaton, S. (1988). Transcriptional controlling elements in the immunoglobulin and T cell receptor loci. [Review]. *Adv. Immunol.* **43**:235.

Cancro, M. P., Kienker, L. J., Ferguson, S. E. and Allman, D. M. (1991). B-cell repertoire development. [Review]. *Immunol. Series* **55**:25.

Capra, J. D. and Kehoe, J. M. (1975). Hypervariable regions, idiotypy, and the antibody-combining site. *Adv. Immunol.* **20**:1.

Casanova, J. L., Romero, P., Widmann, C., Kourilsky, P. and Maryanski, J. L. (1991). T cell receptor genes in a series of class I major histocompatibility complex-restricted cytotoxic T lymphocyte clones specific for a Plasmodium berghei nonapeptide: implications for T cell allelic exclusion and antigen-specific repertoire. *J. Exp. Med.* **174**:1371.

Cebra, J. J., Colberg, J. E. and Dray, S. (1965). Rabbit lymphoid cells differentiated with respect to α–, γ– and μ– heavy polypeptide chains and to allotypic markers Aa1 and Aa2. *J. Exp. Med.* **123**:547.

Cebra, J. J., Colberg, J. E. and Dray, S. (1966). Rabbit lymphoid cells differentiated with respect to α–, γ–, and υ– heavy polypeptide chains and to allotypic markers Aa1, Aa2. *J. Exp. Med.* **123**:547.

Chaabani, H., Bech, H. N. and Cox, D. W. (1985). A multigene deletion within the immunoglobulin heavy-chain region. *Am. J. Hum. Gen.* **37**:1164.

Champagne, E., Sagman, U., Biondi, A., Lewis, W. H., Mak, T. W. and Minden, M. D. (1988). Structure and rearrangement of the T cell receptor J alpha locus in T cells and

leukemic T cell lines. *Eur. J. Immonol.* **18**:1033.

Chen, J. and Alt, F. W. (1993). Gene rearrangement and B-cell development. [Review]. *Curr. Op. Immunol.* **5**:194.

Chen, J., Trounstine, M., Alt, F. W., Young, F., Kurahara, C., Loring, J. F. and Huszar, D. (1993). Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. *Int. Immunol* **5**:647.

Chen, J., Young, F., Bottaro, A., Stewart, V., Smith, R. K. and Alt, F. W. (1993). Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. *Embo J.* **12**:4635.

Cherepakhin, V. V., Jackubov, L. Z., Ibraghimov, A. R. and Rokhlin, O. V. (1987). Allelic exclusion frequency analysis and molecular characteristics of immunoglobulins secreted by the hybridomas expressing both allelic genes. *Immunol. Lett.* **15**:33.

Cherif, D. and Berger, R. (1990). New localizations of VH sequences by *in situ* hybridization with biotinylated probes. *Genes, Chrom. Cancer* **2**:103.

Chess, A., Simon, I., Cedar, H. and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. *Cell* **78**:823.

Chou, H. S., Behlke, M. A., Godambe, S. A., Russell, J. H., Brooks, C. G. and Loh, D. Y. (1986). T cell receptor genes in an alloreactive CTL clone: implications for rearrangement and germline diversity of variable gene segments. *Embo J.* **5**:2149.

Christoph, T. and Krawinkel, U. (1989). Physical linkage of variable, diversity and joining gene segments in the immunoglobulin heavy chain locus of the mouse. *Eur. J. Immonol.* **19**:1521.

Chukwuocha, R. U. and Feeney, A. J. (1993). Role of homology-directed recombination: predominantly productive rearrangements of Vh81X in newborns but not in adults. *Mol. Immunol.* **30**:1473.

Cockerill, P. N. (1990). Nuclear matrix attachment occurs in several regions of the IgH locus. *Nucleic Acids Res.* **18**:2643.

Cohen, S. and Milstein, C. (1967). Structure and biological properties of immunoglobulins. [Review]. *Adv. Immunol.* **7**:1.

Coleclough, C., Perry, R. P., Karjalainen, K. and Weigert, M. (1981). Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin gene expression. *Nature* **290**:372.

Corcos, D., Iglesias, A., Dunda, O., Bucchini, D. and Jami, J. (1991). Allelic exclusion in

transgenic mice expressing a heavy chain disease-like human mu protein. *Eur. J. Immonol.* **21**:2711.

Cory, S. and Adams, J. M. (1980). Deletions are associated with somatic rearrangement of immunoglobulin heavy chain genes. *Cell* **19**:37.

Costa, T. E., Suh, H. and Nussenzweig, M. C. (1992). Chromosomal position of rearranging gene segments influences allelic exclusion in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**:2205.

Couez, D., Malissen, M., Buferne, M., Schmitt, V. A. and Malissen, B. (1991). Each of the two productive T cell receptor alpha-gene rearrangements found in both the A10 and BM 3. 3 T cell clones give rise to an alpha chain which can contribute to the constitution of a surface-expressed alpha beta dimer. *Int. Immunol* **3**:719.

Cuisinier, A. M., Fumoux, F., Moinier, D., Boubli, L., Guigou, V., Milili, M., Schiff, C., Fougereau, M. and Tonnelle, C. (1990). Rapid expansion of human immunoglobulin repertoire (VH, V kappa, V lambda) expressed in early fetal bone marrow. *New Biol.* **2**:689.

Cuisinier, A. M., Guigou, V., Boubli, L., Fougereau, M. and Tonnelle, C. (1989). Preferential expression of VH5 and VH6 immunoglobulin genes in early human B-cell ontogeny. *Scand. J. Immonol.* **30**:493.

Currier, S. J., Gallarda, J. L. and Knight, K. L. (1988). Partial molecular genetic map of the rabbit VH chromosomal region. *J. Immonol.* **140**:1651.

Dembic, Z., Haas, W., Weiss, S., McCubrey, J., Kiefer, H., von, B. H. and Steinmetz, M. (1986). Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* **320**:232.

Desiderio, S. V., Yancopoulos, G. D., Paskind, M., Thomas, E., Boss, M. A., Landau, N., Alt, F. W. and Baltimore, D. (1984). Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* **311**:752.

Dildrop, R. (1984). A new classification of mouse V_H sequences. *Immunol. Today* **5**:85.

Dildrop, R. (1985). VH-gene expression in murine lipopolysaccharide blasts distributeds over the nine known VH-gene groups and may be random. *Eur. J. Immunol.* **15**:1154.

DiPietro, L. A., Sethupathi, P., Kingzette, M., Zhai, S. K., Suter, M. and Knight, K. L. (1992). Limited repertoire of utilized VH gene segments in a VHa3-allotype-suppressed rabbit. *Int. Immunol* **4**:555.

DiPietro, L. A., Short, J. A., Zhai, S. K., Kelus, A. S., Meier, D. and Knight, K. L. (1990). Limited number of immunoglobulin VH regions expressed in the mutant rabbit "Alicia".

Eur. J. Immonol. **20**:1401.

Dray, S., Young, G. O. and Gerald, L. (1963). Immunochemical identification and genetics of rabbit γ-globulin allotypes. *J. Immunol* **91**:403.

Dreyer, W. J. and Bennett, J. C. (1965). The molecular basis of antibody formation: a paradox. *Proc. Natl. Acad. Sci. USA* **54**:864.

Dubiski, S. (1969). Immunochemistry and genetics of a "new" allotypic specificity Ae^{14} of rabbit γ-G immunoglobulins: recombination in somatic cells. *J. Immunol.* **103**:120.

Early, P., Huang, H., Davis, M., Calame, K. and Hood, L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell* **19**:981.

Eaton, S. and Calame, K. (1987). Multiple DNA sequence elements are necessary for the function of an immunoglobulin heavy chain promoter. *Proc. Natl. Acad. Sci. USA* **84**:7634.

Engler, P., Roth, P., Kim, J. Y. and Storb, U. (1991). Factors affecting the rearrangement efficiency of an Ig test gene. *J. Immonol.* **146**:2826.

Erikson, J., Finan, J., Nowell, P. C. and Croce, C. M. (1982). Translocation of immunoglobulin VH genes in Burkitt lymphoma. *Proc. Natl. Acad. Sci. USA* **79**:5611.

Erikson, J., Miller, D. A., Miller, O. J., Abcarian, P. W., Skurla, R. M., Mushinski, J. F. and Croce, C. M. (1985). The c-myc oncogene is translocated to the involved chromosome 12 in mouse plasmacytoma. *Proc. Natl. Acad. Sci. USA* **82**:4212.

Feddersen, R. M. and Van, N. B. (1985). Double recombination of a single immunoglobulin kappa-chain allele: implications for the mechanism of rearrangement. *Proc. Natl. Acad. Sci. USA* **82**:4793.

Feeney, A. J. (1990). Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J. Exp. Med.* **172**:1377.

Feinstein, A. (1963). Character and allotypy of an immune globulin in rabbit colostrum. *Nature* **199**:1197.

Feng, S. H. and Stein, K. E. (1991). VH gene family expression in mice with the xid defect. *J. Exp. Med.* **174**:45.

Fenton, R. G., Marrack, P., Kappler, J. W., Kanagawa, O. and Seidman, J. G. (1988). Isotypic exclusion of gamma delta T cell receptors in transgenic mice bearing a rearranged beta-chain gene. *Science* **241**:1089.

Fernex, C., Caillol, D., Capone, M., Krippl, B. and Ferrier, P. (1994). Sequences affecting the V(D)J recombinational activity of the IgH intronic enhancer in a transgenic substrate. *Nucleic Acids Res.* **22**:792.

Ferradini, L., Reynaud, C. A., Lauster, R. and Weill, J. C. (1994). Rearrangement of the chicken lambda light chain locus: a silencer/antisilencer regulation. [Review]. *Semin. Immunol.* **6**:165.

Finger, L. R., Harvey, R. C., Moore, R. C., Showe, L. C. and Croce, C. M. (1986). A common mechanism of chromosomal translocation in T- and B-cell neoplasia. *Science* **234**:982.

Fitts, M. G. and Metzger, D. W. (1990). Identification of rabbit genomic Ig-VH pseudogenes that could serve as donor sequences for latent allotype expression. *J. Immonol.* **145**:2713.

Flanagan, J. G. and Rabbitts, T. H. (1982). Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing gamma, epsilon and alpha genes. *Nature* **300**:709.

Foon, K. A. and Piro, L. D. (1992). Lymphocytic leukemias: new insights into biology and therapy. [Review]. *Leukemia* **4**:26.

Forni, L. (1990). Extensive splenic B cell activation in IgM-transgenic mice. *Eur. J. Immonol.* **20**:983.

Freitas, A. A., Andrade, L., Lembezat, M. P. and Coutinho, A. (1990). Selection of VH gene repertoires: differentiating B cells of adult bone marrow mimic fetal development. *Int. Immunol* **2**:15.

Freitas, A. A., Lembezat, M. P. and Coutinho, A. (1989). Expression of antibody V-regions is genetically and developmentally controlled and modulated by the B lymphocyte environment. *Int. Immunol* **1**:342.

Friedman, M. L., Tunyaplin, C., Zhai, S. K. and Knight, K. L. (1994). Neonatal VH, D, and JH gene usage in rabbit B lineage cells. *J. Immonol.* **152**:632.

Fujimoto, S. and Yamagishi, H. (1987). Isolation of an excision product of T-cell receptor alpha-chain gene rearrangements [published erratum appears in Nature 1987 Jun 4-10;327(6121):439]. *Nature* **327**:242.

Furutani, M., Yanagi, Y., Fujisawa, I., Nakayama, T., Kishimoto, H., Kuida, K., Asano, Y. and Tada, T. (1989). Post-transcriptional allelic exclusion of two functionally rearranged T cell receptor alpha genes. *Int. Immunol* **1**:281.

Gallarda, J. L., Gleason, K. S. and Knight, K. L. (1985). Organization of rabbit

immunoglobulin genes. I. Structure and multiplicity of germ-line VH genes. *J. Immonol.* **135**:4222.

Gathings, W. E., Lawton, A. R. and Cooper, M. D. (1977). Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. *Eur. J. Immonol.* **7**:804.

Gathings, W. E., Mage, R. G., Cooper, M. D., Lawton, A. R. and Young, C. G. (1981). Immunofluorescence studies on the expression of VH a allotypes by pre-B and B cells of homozygous and heterozygous rabbits. *Eur. J. Immonol.* **11**:200.

Gathings, W. E., Mage, R. G., Cooper, M. D. and Young, C. G. (1982). A subpopulation of small pre-B cells in rabbit bone marrow expresses kappa light chains and exhibits allelic exclusion of b locus allotypes. *Eur. J. Immonol.* **12**:76.

Gauss, G. H. and Lieber, M. R. (1992). The basis for the mechanistic bias for deletional over inversional V(D)J recombination. *Genes Dev.* **6**:1553.

Gerondakis, S., Boyd, A., Bernard, O., Webb, E. and Adams, J. M. (1984). Activation of immunoglobulin mu gene expression involves stepwise demethylation. *Embo J.* **3**:3013.

Gilfillan, S., Dierich, A., Lemeur, M., Benoist, C. and Mathis, D. (1993). Mice lacking TdT: mature animals with an immature lymphocyte repertoire [published erratum appears in Science 1993 Dec 24;262(5142):1957]. *Science* **261**:1175.

Gold, M. R., Chiu, R., Ingham, R. J., Saxton, T. M., van, O. I., Watts, J. D., Affolter, M. and Aebersold, R. (1994). Activation and serine phosphorylation of the p56lck protein tyrosine kinase in response to antigen receptor cross-linking in B lymphocytes. *J. Immonol.* **153**:2369.

Goodhardt, M., Cavelier, P., Doyen, N., Kallenbach, S., Babinet, C. and Rougeon, F. (1993). Methylation status of immunoglobulin kappa gene segments correlates with their recombination potential. *Eur. J. Immonol.* **23**:1789.

Gough, N. M. and Bernard, O. (1981). Sequences of the joining region genes for immunoglobulin heavy chains and their role in generation of antibody diversity. *Proc. Natl. Acad. Sci. USA* **78**:509.

Goverman, J., Minard, K., Shastri, N., Hunkapiller, T., Hansburg, D., Sercarz, E. and Hood, L. (1985). Rearranged beta T cell receptor genes in a helper T cell clone specific for lysozyme: no correlation between V beta and MHC restriction. *Cell* **40**:859.

Grandien, A. (1994). Positive and Negative selection of antibody repertoires during B-cell differentiation. *Immunol. Rev.* **137**:56.

Grandien, A., Coutinho, A. and Andersson, J. (1990). Selective peripheral expansion and

activation of B cells expressing endogenous immunoglobulin in mu-transgenic mice. *Eur. J. Immonol.* **20**:991.

Grosschedl, R., Weaver, D., Baltimore, D. and Costantini, F. (1984). Introduction of a mu immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody. *Cell* **38**:647.

Gu, H., Forster, I. and Rajewsky, K. (1990). Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. *Embo J.* **9**:2133.

Guigou, V., Cuisinier, A. M., Tonnelle, C., Moinier, D., Fougereau, M. and Fumoux, F. (1990). Human immunoglobulin VH and VK repertoire revealed by *in situ* hybridization. *Mol. Immunol.* **27**:935.

Hagemeijer, A. (1992). Cytogenetics and oncogenes. [Review]. *Leukemia* **4**:16.

Halligan, B. D. and Desiderio, S. V. (1987). Identification of a DNA binding protein that recognizes the nonamer recombinational signal sequence of immunoglobulin genes. *Proc. Natl. Acad. Sci. USA* **84**:7019.

Hamaguchi, Y., Matsunami, N., Yamamoto, Y. and Honjo, T. (1989). Purification and characterization of a protein that binds to the recombination signal sequence of the immunoglobulin J kappa segment. *Nucleic Acids Res.* **17**:9015.

Hardy, R. R. (1994). CD5 B cells, a fetal B cell lineage. *Adv. Immunol.* **55**:297.

Hardy, R. R., Carmack, C. E., Shinton, S. A., Riblet, R. J. and Hayakawa, K. (1989). A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J. Immonol.* **142**:3643.

Hayward, A. R., Simons, M. A., Lawton, A. R., Mage, R. G. and Cooper, M. D. (1978). Pre-B and B cells in rabbits. Ontogeny and allelic exclusion of kappa light chain genes. *J. Exp. Med.* **148**:1367.

Heath, W. R. and Miller, J. F. (1993). Expression of two alpha chains on the surface of T cells in T cell receptor transgenic mice. *J. Exp. Med.* **178**:1807.

Hengartner, H., Meo, T. and Muller, E. (1978). Assignment of genes for immunoglobulin kappa and heavy chains to chromosomes 6 and 12 in mouse. *Proc. Natl. Acad. Sci. USA* **75**:4494.

Hesse, J. E., Lieber, M. R., Gellert, M. and Mizuuchi, K. (1987). Extrachromosomal DNA substrates in pre-B cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. *Cell* **49**:775.

Hesse, J. E., Lieber, M. R., Mizuuchi, K. and Gellert, M. (1989). V(D)J recombination: a functional definition of the joining signals. *Genes Dev.* **3**:1053.

Hirama, T., Takeshita, S., Yoshida, Y. and Yamagishi, H. (1991). Structure of extrachromosomal circular DNAs generated by immunoglobulin light chain gene rearrangements. *Immunol. Lett.* **27**:19.

Hochtl, J., Muller, C. R. and Zachau, H. G. (1982). Recombined flanks of the variable and joining segments of immunoglobulin genes. *Proc. Natl. Acad. Sci. USA* **79**:1383.

Hochtl, J. and Zachau, H. G. (1983). A novel type of aberrant recombination in immunoglobulin genes and its implications for V-J joining mechanism. *Nature* **302**:260.

Holmberg, D. (1987). High connectivity, natural antibodies preferentially use 7183 and QUPC52 VH families. *Eur. J. Immunol.* **17**:399.

Honjo, T. (1983). Immunoglobulin genes. [Review]. *Ann. Rev. Immunol***1**:499.

Hozumi, N. and Tonegawa, S. (1976). Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc. Natl. Acad. Sci. USA* **73**:3628.

Hsieh, C. L. and Lieber, M. R. (1992). CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. *Embo J.* **11**:315.

Hu, H., Queiro, M. R., Tilanus, M. G., de, W. R. and Schuurman, H. J. (1993). Expression of T-cell receptor alpha and beta variable genes in normal and malignant human T cells. *Brit. J. Haemat.* **84**:39.

Huang, C., Stewart, A. K., Schwartz, R. S. and Stollar, B. D. (1992). Immunoglobulin heavy chain gene expression in peripheral blood B lymphocytes. *J. Clin. Inv.* **89**:1331.

Huang, C. and Stollar, B. D. (1993). A majority of Ig H chain cDNA of normal human adult blood lymphocytes resembles cDNA for fetal Ig and natural autoantibodies. *J. Immonol.* **151**:5290.

Iacomini, J., Yannoutsos, N., Bandyopadhay, S. and Imanishi, K. T. (1991). Endogenous immunoglobulin expression in mu transgenic mice. *Int. Immunol* **3**:185.

Ichihara, Y., Matsuoka, H. and Kurosawa, Y. (1988). Organization of human immunoglobulin heavy chain diversity gene loci. *Embo J.* **7**:4141.

Iglesias, A., Lamers, M. and Kohler, G. (1987). Expression of immunoglobulin delta chain causes allelic exclusion in transgenic mice. *Nature* **330**:482.

Iliev, A., Spatz, L., Ray, S. and Diamond, B. (1994). Lack of allelic exclusion permits

autoreactive B cells to escape deletion. *J. Immonol.* **153**:3551.

Imanishi, K. T., Bandyopadhay, S., Busto, P. and Huang, C. A. (1993). Endogenous Ig production in mu transgenic mice. I. Allelic exclusion at the level of expression. *J. Immonol.*

Jeggo, P. A. (1990). Studies on mammalian mutants defective in rejoining double-strand breaks in DNA. [Review]. *Mut. Res.* **239**:1.

Jeong, H. D. (1988). Strain-dependent expression of VH gene families. *J. Immunol.* **140**:2436.

Jeong, H. D. and Teale, J. M. (1989). VH gene family repertoire of resting B cells. Preferential use of D-proximal families early in development may be due to distinct B cell subsets. *J. Immonol.* **143**:2752.

Jeong, H. D. and Teale, J. M. (1990). Contribution of the CD5+ B cell to D-proximal VH family expression early in ontogeny. *J. Immonol.* **145**:2725.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller, C. (1991). "Sequences of proteins of immunological interest", 5 Ed. U. S. Department of Health and Human Services.

Kallenbach, S., Brinkmann, T. and Rougeon, F. (1993). Rag-1: a topoisomerase? *Int. Immunol* **5**:231.

Kallenbach, S. and Rougeon, F. (1992). A V(D)J site-specific recombination model involving no compulsory double-stranded break formation at the coding segments. *Res. in Immunol.* **143**:873.

Kastner, D. L., McIntyre, T. M., Mallett, C. P., Hartman, A. B. and Steinberg, A. D. (1989). Direct quantitative *in situ* hybridization studies of Ig VH utilization. A comparison between unstimulated B cells from autoimmune and normal mice. *J. Immonol.* **143**:2761.

Kaushik, A., Shulze, D., Bona, C. and Kelsoe, G. (1989). Murine VK gene expression does not follow the VH paradigm. *J. Exp. Med.* **169**:1859.

Kelus, A. S. and Steinberg, C. M. (1991). Is there a high rate of mitotic recombination between the loci encoding immunoglobulin V_H and C_H regions in gonial cells? *Immunogenetics* **33**:255.

Kenny, J. J., Finkelman, F., Macchiarini, F., Kopp, W. C., Storb, U. and Longo, D. L. (1989). Alteration of the B cell surface phenotype, immune response to phosphocholine and the B cell repertoire in M167 mu plus kappa transgenic mice. *J. Immonol.* **142**:4466.

Kim, B. S. and Dray, S. (1973). Expression of the a, x, and y variable region genes of heavy chains among IgG, IgM, and IgA molecules of normal and a locus allotype-suppressed rabbits. *J. Immonol.* **111**:750.

Kindt, T. J. and Mandy, W. J. (1972). Recombination of genes coding for constant and variable regions of immunoglobulin heavy chains. *J. Immunol.* **108**:1110.

Kindt, T. J., Mandy, W. J. and Todd, C. W. (1970). Association of allotypic specificities of group a with allotypic specificities Aaa and A12 in rabbit immunoglobulin. *Biochem.* **9**:2028.

Kirkham, P. M., Mortari, F., Newton, J. A. and Schroeder, H. J. (1992). Immunoglobulin VH clan and family identity predicts variable domain structure and may influence antigen binding. *Embo J.* **11**:603.

Kirsch, I. R., Morton, C. C., Nakahara, K. and Leder, P. (1982). Human immunoglobulin heavy chain genes map to a region of translocations in malignant B lymphocytes. *Science* **216**:301.

Kitamura, D. and Rajewsky, K. (1992). Targeted disruption of mu chain membrane exon causes loss of heavy-chain allelic exclusion [see comments]. *Nature* **356**:154.

Kitsberg, D., Selig, S., Brandeis, M., Simon, I., Keshet, I., Driscoll, D. J., Nicholls, R. D. and Cedar, H. (1993). Allele-specific replication timing of imprinted gene regions. *Nature* **364**:459.

Kleinfield, R. W. and Weigert, M. G. (1989). Interspersion of the VHQ52 and VH7183 gene families in the NFS/N mouse. *J. Immonol.* **142**:4483.

Knight, K. L. (1992). Restricted VH gene usage and generation of antibody diversity in rabbit. [Review]. *Ann. Rev. Immunol***10**:593.

Knight, K. L. and Becker, R. S. (1987). Isolation of genes encoding bovine IgM, IgG, IgA and IgE chains. *Vet. Immunol. & Immunopath.* **17**:17.

Knight, K. L. and Becker, R. S. (1990). Molecular basis of the allelic inheritance of rabbit immunoglobulin VH allotypes: implications for the generation of antibody diversity. *Cell* **60**:963.

Knight, K. L., Burnett, R. C. and McNicholas, J. M. (1985). Organization and polymorphism of rabbit immunoglobulin heavy chain genes. *J. Immonol.* **134**:1245.

Knight, K. L., Johnson, A., Coligan, J. E. and Kindt, T. J. (1987). Partial amino acid sequence analysis of rabbit MHC class II molecules isolated from two-dimensional polyacrylamide gels. *Mol. Immunol.* **24**:449.

Knight, K. L., Malek, T. R. and Dray, S. (1975). Human immunoglobulins with a1 allotypic determinants of rabbit immunoglobulin heavy chains. *Nature* **253**:216.

Knight, K. L., Spieker, P. H., Kazdin, D. S. and Oi, V. T. (1988). Transgenic rabbits with lymphocytic leukemia induced by the c-myc oncogene fused with the immunoglobulin heavy chain enhancer. *Proc. Natl. Acad. Sci. USA* **85**:3130.

Kodaira, M., Kinashi, T., Umemura, I., Matsuda, F., Noma, T., Ono, Y. and Honjo, T. (1986). Organization and evolution of variable region genes of the human immunoglobulin heavy chain. *J. Mol. Biol.* **190**:529.

Kofler, R. (1988). A new murine Ig VH gene family. *J. Immonol.* **140**:4031.

Köhler, G. (1980) *Proc. Natl. Acad. Sci USA* 68:219.

Komori, T., Okada, A., Stewart, V. and Alt, F. W. (1993). Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes [published erratum appears in Science 1993 Dec 24;262(5142):1957]. *Science* **261**:1171.

Kotloff, D. B., Bosma, M. J. and Ruetsch, N. R. (1993). V(D)J recombination in peritoneal B cells of leaky scid mice. *J. Exp. Med.* **178**:1981.

Krimpenfort, P., Ossendorp, F., Borst, J., Melief, C. and Berns, A. (1989). T cell depletion in transgenic mice carrying a mutant gene for TCR-beta. *Nature* **341**:742.

Kroemer, G., Faessler, R., Strohal, R., Wick, G. and Kofler, R. (1987). Evidence for a new murine immunoglobulin heavy chain variable region gene family. *Immunol. Inv.* **16**:107.

Kuida, K., Furutani, S. M., Saito, T., Kishimoto, H., Sano, K. and Tada, T. (1991). Post-translational attainment of allelic exclusion of the T cell receptor alpha chain in a T cell clone. *Int. Immunol* **3**:75.

Kurosawa, Y. and Tonegawa, S. (1982). Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. *J. Exp. Med.* **155**:201.

Kurosawa, Y., von, B. H., Haas, W., Sakano, H., Trauneker, A. and Tonegawa, S. (1981). Identification of D segments of immunoglobulin heavy-chain genes and their rearrangement in T lymphocytes. *Nature* **290**:565.

Kwan, S. P., Max, E. E., Seidman, J. G., Leder, P. and Scharff, M. D. (1981). Two kappa immunoglobulin genes are expressed in the myeloma S107. *Cell* **26:**57.

Lafaille, J. J., DeCloux, A., Bonneville, M., Takagaki, Y. and Tonegawa, S. (1989). Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta

T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell* **59**:859.

Lai, E., Wilson, R. K. and Hood, L. E. (1989). Physical maps of the mouse and human immunoglobulin-like loci. [Review]. *Adv. Immunol.* **46**:1.

Lamers, M. C., Vakil, M., Kearney, J. F., Langhorne, J., Paige, C. J., Julius, M. H., Mossmann, H., Carsetti, R. and Kohler, G. (1989). Immune status of a mu, kappa transgenic mouse line. Deficient response to bacterially related antigens. *Eur. J. Immonol.* **19**:459.

Landau, N. R., Schatz, D. G., Rosa, M. and Baltimore, D. (1987). Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. *Mol. Cell. Biol.* **7**:3237.

Landucci-Tosi, S., Mage, R. G. and Dubiski, S. (1970). Distribution of allotypic specifities A1, A2, A14 and A15 among immunoglobulin G molecules. *J. Immunol.* **104**:641.

Lauster, R., Reynaud, C. A., Martensson, I. L., Peter, A., Bucchini, D., Jami, J. and Weill, J. C. (1993). Promoter, enhancer and silencer elements regulate rearrangement of an immunoglobulin transgene. *Embo J.* **12**:4615.

Lawler, A. M., Kearney, J. F., Kuehl, M. and Gearhart, P. J. (1989). Early rearrangements of genes encoding murine immunoglobulin kappa chains, unlike genes encoding heavy chains, use variable gene segments dispersed throughout the locus. *Proc. Natl. Acad. Sci. USA* **86**:6744.

Lawler, A. M., Lin, P. S. and Gearhart, P. J. (1987). Adult B-cell repertoire is biased toward two heavy-chain variable-region genes that rearrange frequently in fetal pre-B cells. *Proc. Natl. Acad. Sci. USA* **84**:2454.

Lefranc, M. P., Lefranc, G. and Rabbitts, T. H. (1982). Inherited deletion of immunoglobulin heavy chain constant region genes in normal human individuals. *Nature* **300**:760.

Leiden, J. M., Dialynas, D. P., Duby, A. D., Murre, C., Seidman, J. and Strominger, J. L. (1986). Rearrangement and expression of T-cell antigen receptor genes in human T-lymphocyte tumor lines and normal human T-cell clones: evidence for allelic exclusion of Ti beta gene expression and preferential use of a J beta 2 gene segment. *Mol. Cell. Biol.* **6**:3207.

Lennox, E. S. and Cohn, M. (1967). Immunoglobulins. *Annu. Rev. Biochem.* **36**:365.

Lewis, S. and Gellert, M. (1989). The mechanism of antigen receptor gene assembly. [Review]. *Cell* **59**:585.

Lewis, S., Gifford, A. and Baltimore, D. (1984). Joining of V kappa to J kappa gene segments in a retroviral vector introduced into lymphoid cells. *Nature* **308**:425.

Lewis, S., Gifford, A. and Baltimore, D. (1985). DNA elements are asymmetrically joined during the site-specific recombination of kappa immunoglobulin genes. *Science* **228**:677.

Lewis, S., Rosenberg, N., Alt, F. and Baltimore, D. (1982). Continuing kappa-gene rearrangement in a cell line transformed by Abelson murine leukemia virus. *Cell* **30**:807.

Lewis, S. M., Hesse, J. E., Mizuuchi, K. and Gellert, M. (1988). Novel strand exchanges in V(D)J recombination. *Cell* **55**:1099.

Lieber, M. R., Hesse, J. E., Lewis, S., Bosma, G. C., Rosenberg, N., Mizuuchi, K., Bosma, M. J. and Gellert, M. (1988). The defect in murine severe combined immune deficiency: joining of signal sequences but not coding segments in V(D)J recombination. *Cell* **55**:7.

Lieber, M. R., Hesse, J. E., Mizuuchi, K. and Gellert, M. (1988). Lymphoid V(D)J recombination: nucleotide insertion at signal joints as well as coding joints. *Proc. Natl. Acad. Sci. USA* **85**:8588.

Lin, W. C. and Desiderio, S. (1993). Regulation of V(D)J recombination activator protein RAG-2 by phosphorylation. *Science* **260**:953.

Livant, D., Blatt, C. and Hood, L. (1986). One heavy chain variable region gene segment subfamily in the BALB/c mouse contains 500-1000 or more members. *Cell* **47**:461.

Logtenberg, T., Schutte, M. E., Inghirami, G., Berman, J. E., Gmelig, M. F., Insel, R. A., Knowles, D. M. and Alt, F. W. (1989a). Immunoglobulin V_H gene expression in human B cell lines and tumors: biased V_H gene expression in chronic lymphocytic leukemia. *Int. Immunol* **1**:362.

Logtenberg, T., Young, F. M., van, E. J., Gmelig, M. F., Berman, J. E. and Alt, F. W. (1989b). Frequency of V_H-gene utilization in human EBV-transformed B-cell lines: the most J_H-proximal V_H segment encodes autoantibodies. *J. Autoimm.*

Ma, A., Fisher, P., Dildrop, R., Oltz, E., Rathbun, G., Achacoso, P., Stall, A. and Alt, F. W. (1992). Surface IgM mediated regulation of RAG gene expression in E mu-N-myc B cell lines. *Embo J.* **11**:2727.

Maeda, T., Sugiyama, H., Tani, Y. and Kishimoto, S. (1989). The DJ_H complex remains active in recombination to VH segments after the loss of mu-chain expression in mu-positive pre-B cells. *J. Immonol.* **142**:3652.

Mage, R. G., Dray, S., Gilman-Sachs, A., Hamers-Casterman, C., Hamers, R., Hanly, W.

C., Kindt, T. J., Knight, K. L., Mandy, W. J. and Naessens, J. (1982). Rabbit heavy chain haplotypes-allotypic determinants expressed by V_{H}- C_{H} recombinants. *Immunogenetics* **15**:287.

Mage, R. G., Young-Cooper, G. O. and Alexander, C. B. (1971). Genetic control of variable and constant regions of immunoglobulin heavy chain. *Nature New Biol.* **230**:63.

Malissen, M., McCoy, C., Blanc, D., Trucy, J., Devaux, C., Schmitt, V. A., Fitch, F., Hood, L. and Malissen, B. (1986). Direct evidence for chromosomal inversion during T-cell receptor beta-gene rearrangements. *Nature* **319**:28.

Malissen, M., Trucy, J., Jouvin, M. E., Cazenave, P. A., Scollay, R. and Malissen, B. (1992). Regulation of TCR alpha and beta gene allelic exclusion during T-cell development. [Review]. *Immunol. Today* **13**:315.

Malissen, M., Trucy, J., Letourneur, F., Rebai, N., Dunn, D. E., Fitch, F. W., Hood, L. and Malissen, B. (1988). A T cell clone expresses two T cell receptor alpha genes but uses one alpha beta heterodimer for allorecognition and self MHC-restricted antigen recognition. *Cell* **55**:49.

Malynn, B. A. (1987). Expression of the immunoglobulin heavy-chain variable gene repertoire. *Curr. Top. Microbiol. Immunol.* **135**:75.

Malynn, B. A., Yancopoulos, G. D., Barth, J. E., Bona, C. A. and Alt, F. W. (1990). Biased expression of JH-proximal VH genes occurs in the newly generated repertoire of neonatal and adult mice. *J. Exp. Med.* **171**:843.

Mandy, W. J. and Todd, C. W. (1970). Rabbit immunoglobulin allotype A12: A new agglutinating specificity. *Biochem. Genetics.* **4**:59.

Manz, J., Denis, K., Witte, O., Brinster, R. and Storb, U. (1988). Feedback inhibition of immunoglobulin gene rearrangement by membrane mu, but not by secreted mu heavy chains [published erratum appears in J Exp Med 1989 Jun 1;169(6):2269]. *J. Exp. Med.* **168**:1363.

Marche, P. N. and Kindt, T. J. (1986). Two distinct T-cell receptor alpha-chain transcripts in a rabbit T-cell line: implications for allelic exclusion in T cells. *Proc. Natl. Acad. Sci. USA* **83**:2190.

Matsuda, F., Lee, K. H., Nakai, S., Sato, T., Kodaira, M., Zong, S. Q., Ohno, H., Fukuhara, S. and Honjo, T. (1988). Dispersed localization of D segments in the human immunoglobulin heavy-chain locus. *Embo J.* **7**:1047.

Matsuda, F., Shin, E. K., Hirabayashi, Y., Nagaoka, H., Yoshida, M. C., Zong, S. Q. and Honjo, T. (1990). Organization of variable region segments of the human immunoglobulin heavy chain: duplication of the D5 cluster within the locus and

interchromosomal translocation of variable region segments. *Embo J.* **9**:2501.

Matsuda, F., Shin, E. K., Nagaoka, H., Matsumura, R., Haino, M., Fukita, Y., Taka, i. S., Imai, T., Riley, J. H., Anand, R. and et, a. l. (1993). Structure and physical map of 64 variable segments in the 3'0. 8-megabase region of the human immunoglobulin heavy-chain locus. *Nature Genetics.* **3**:88.

Matsumura, R., Matsuda, F., Nagaoka, H., Fujikura, J., Shin, E. K., Fukita, Y., Haino, M. and Honjo, T. (1994). Structural analysis of the human VH locus using nonrepetitive intergenic probes and repetitive sequence probes. Evidence for recent reshuffling. *J. Immunol.* **152**:660.

Matsunami, N., Hamaguchi, Y., Yamamoto, Y., Kuze, K., Kangawa, K., Matsuo, H., Kawaichi, M. and Honjo, T. (1989). A protein binding to the J kappa recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif. *Nature* **342**:934.

Max, E. E., Seidman, J. G. and Leder, P. (1979). Sequences of five potential recombination sites encoded close to an immunoglobulin kappa constant region gene. *Proc. Natl. Acad. Sci. USA* **76**:3450.

McCormack, W. T., Laster, S. M., Marzluff, W. F. and Roux, K. H. (1985). Dynamic gene interactions in the evolution of rabbit VH genes: a four codon duplication and block homologies provide evidence for intergenic exchange. *Nucleic Acids Res.* **13**:7041.

McDougall, S., Eaton, S., Peterson, C. L. and Calame, K. (1989). Transcriptional regulation of immunoglobulin heavy chain and T-cell receptor beta chain genes. *Adv. Exp. Med. & Biol.* **254**:77.

Medrano, L. and Dutrillaux, B. (1984). Chromosomal location of immunoglobulin genes: partial mapping of these genes in the rabbit and comparison with Ig genes carrying chromosomes of man and mouse. [Review]. *Adv. Cancer Res.* **41**:323.

Meek, K., Johansson, B., Schulman, J., Bona, C. and Capra, J. D. (1989). Nucleotide changes in sequential variants of influenza virus hemagglutinin genes and molecular structures of corresponding monoclonal antibodies specific for each variant. *Proc. Natl. Acad. Sci. USA* **86**:4664.

Mirro, J. J. (1992). Pathology and immunology of acute leukemia. [Review]. *Leukemia* **4**:13.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. and Papaioannou, V. E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**:869.

Mortari, F., Newton, J. A., Wang, J. Y. and Schroeder, H. J. (1992). The human cord blood antibody repertoire. Frequent usage of the VH7 gene family. *Eur. J. Immunol.*

22:241.

Morzycka, W. E., Lee, F. E. and Desiderio, S. V. (1988). Unusual immunoglobulin gene rearrangement leads to replacement of recombinational signal sequences. *Science* **242**:261.

Muller, W., Ruther, U., Vieira, P., Hombach, J., Reth, M. and Rajewsky, K. (1989). Membrane-bound IgM obstructs B cell development in transgenic mice. *Eur. J. Immonol.* **19**:923.

Nickerson, K. G., Berman, J., Glickman, E., Chess, L. and Alt, F. W. (1989). Early human IgH gene assembly in Epstein-Barr virus-transformed fetal B cell lines. Preferential utilization of the most JH-proximal D segment (DQ52) and two unusual VH-related rearrangements. *J. Exp. Med.* **169**:1391.

Nishida, Y., Kataoka, T., Ishida, N., Nakai, S., Kishimoto, T., Bottcher, I. and Honjo, T. (1981). Cloning of mouse immunoglobulin epsilon gene and its location within the heavy chain gene cluster. *Proc. Natl. Acad. Sci. USA* **78**:1581.

Nisonoff, A. and Woernley, D. L. Effect of hydrolysis by papain on the combining sites of an antibody. *Nature* 183:1325.

Nisonoff, A., Wissler, F. C., Lipman, L. N. and Woernley, D. L. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem. & Biophys.* 89:230.

Nottenburg, C., St, J. T. and Weissman, I. L. (1987). Unusual immunoglobulin DNA sequences from the nonexpressed chromosome of mouse normal B lymphocytes: implications for allelic exclusion and the DNA rearrangement process. *J. Immonol.* **139**:1718.

Nottenburg, C. and Weissman, I. L. (1981). Cmu gene rearrangement of mouse immunoglobulin genes in normal B cells occurs on both the expressed and nonexpressed chromosomes. *Proc. Natl. Acad. Sci. USA* **78**:484.

Nussenzweig, M. C., Shaw, A. C., Sinn, E., Campos, T. J. and Leder, P. (1988). Allelic exclusion in transgenic mice carrying mutant human IgM genes. *J. Exp. Med.* **167**:1969.

Oettinger, M. A., Schatz, D. G., Gorka, C. and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* **248**:1517.

Offen, D., Spatz, L., Escowitz, H., Factor, S. and Diamond, B. (1992). Induction of tolerance to an IgG autoantibody. *Proc. Natl. Acad. Sci. USA* **89**:8332.

Okazaki, K., Davis, D. D. and Sakano, H. (1987). T cell receptor beta gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion

in V-D-J joining. *Cell* **49**:477.

Osman, G. E., Brodeur, P. H., Rosenberg, N. and Wortis, H. H. (1992). The Ig VH repertoire of fetal liver-derived pre-B cells is influenced by the expression of a gene linked to X-linked immune deficiency. *J. Immonol.* **148**:1928.

Padovan, E., Casorati, G., Dellabona, P., Meyer, S., Brockhaus, M. and Lanzavecchia, A. (1993). Expression of two T cell receptor alpha chains: dual receptor T cells. *Science* **262**:422.

Pascual, V. (1993). Analysis of Ig H chain gene segment utilization in human fetal liver: revisiting the "proximal utilization hypothesis". *J. Immunol.* **151**:4164.

Pascual, V. and Capra, J. D. (1991). Human immunoglobulin heavy-chain variable region genes: organization, polymorphism, and expression. [Review]. *Adv. Immunol.* **49**:1.

Pennell, C. A., Sheehan, K. M., Brodeur, P. H. and Clarke, S. H. (1989). Organization and expression of VH gene families preferentially expressed by Ly-1+ (CD5) B cells. *Eur. J. Immonol.* **19**:2115.

Pergola, F., Zdzienicka, M. Z. and Lieber, M. R. (1993). V(D)J recombination in mammalian cell mutants defective in DNA double-strand break repair. *Mol. Cell. Biol.* **13**:3464.

Perlmutter, R. M. (1985). Developmentally controlled expression of immunoglobulin VH genes. *Sciences* **227**:1597.

Perlmutter, R. M. (1987). Programmed development of the antibody repertoire. *Curr. Top. Microbiol. Immunol.* **135**:95.

Perlmutter, R. M. (1988). Diversification of the human fetal antibody repertoire. *UCLA Symp. Mol. Cell. Biol., New Series* **85**:

Pernis, B., Chiappino, M. B., Kelus, A. S. and Gell, P. G. H. (1965). Cellular localization of immunoglobulins with different allotypic specificities in rabbit lymphoid issues. *J. Exp. Med.* **122**:853.

Perry, R. P., Kelley, D. E., Coleclough, C., Seidman, J. G., Leder, P., Tonegawa, S., Matthyssens, G. and Weigert, M. (1980). Transcription of mouse kappa chain genes: implications for allelic exclusion. *Proc. Natl. Acad. Sci. USA* **77**:1937.

Pircher, H., Ohashi, P., Miescher, G., Lang, R., Zikopoulos, A., Burki, K., Mak, T. W., MacDonald, H. R. and Hengartner, H. (1990). T cell receptor (TcR) beta chain transgenic mice: studies on allelic exclusion and on the TcR+ gamma/delta population. *Eur. J. Immonol.* **20**:417.

Porter, R. R. (1958) Separation and isolation of fractions of rabbit gamma-globulin containing the antibody and antigenic combining sites. *Nature* 182:670.

Potter, M. and Lieberman, R. (1967). Genetics of immunoglobulins in the mouse. [Review]. *Adv. Immunol.* 7:91.

Raman, C., Spieker, P. H., Yam, P. C. and Knight, K. L. (1994). Preferential VH gene usage in rabbit Ig-secreting heterohybridomas. *J. Immonol.* **152**:3935.

Ramsden, D. A., Baetz, K. and Wu, G. E. (1994). Conservation of sequence in recombination signal sequence spacers. *Nucleic Acids Res.* **22**:1785.

Rath, S., Durdik, J., Gerstein, R. M., Selsing, E. and Nisonoff, A. (1989). Quantitative analysis of idiotypic mimicry and allelic exclusion in mice with a mu Ig transgene. *J. Immonol.* **143**:2074.

Raulet, D. H., Garman, R. D., Saito, H. and Tonegawa, S. (1985). Developmental regulation of T-cell receptor gene expression. *Nature* **314**:103.

Ravetch, J. V., Siebenlist, U., Korsmeyer, S., Waldmann, T. and Leder, P. (1981). Structure of the human immunoglobulin mu locus: characterization of embryonic and rearranged J and D genes. *Cell* **27**:583.

Ravichandran, K. S., Osborne, B. A. and Goldsby, R. A. (1994). Quantitative analysis of the B cell repertoire by limiting dilution analysis and fluorescent *in situ* hybridization. *Cell. Immunol.* **154**:309.

Rawlings, D. J. (1994). Bruton's tyrosine kinase is a key regulator in B cells development. *Immunol. Rev.* **138**:105.

Reininger, L., Kaushik, A., Izui, S. and Jaton, J. C. (1988). A member of a new VH gene family encodes antibromelinized mouse red blood cell autoantibodies. *Eur. J. Immonol.* **18**:1521.

Reininger, L., Ollier, P., Poncet, P., Kaushik, A. and Jaton, J. C. (1987). Novel V genes encode virtually identical variable regions of six murine monoclonal anti-bromelain-treated red blood cell autoantibodies. *J. Immonol.* **138**:316.

Reth, M., Petrac, E., Wiese, P., Lobel, L. and Alt, F. W. (1987). Activation of V kappa gene rearrangement in pre-B cells follows the expression of membrane-bound immunoglobulin heavy chains. *Embo J.* **6**:3299.

Reth, M. G. (1986). VHDJH formation and DJH replacement during pre-B differentiation: non-random usage of gene segments. *EMBO J.* **5**:2131.

Reynaud, C. A., Anquez, V., Grimal, H. and Weill, J. C. (1987). A hyperconversion

mechanism generates the chicken light chain preimmune repertoire. *Cell* **48**:379.

Reynaud, C. A., Dahan, A., Anquez, V. and Weill, J. C. (1989). Somatic hyperconversion diversifies the single Vh gene of the chicken with a high incidence in the D region. *Cell* **59**:171.

Riblet, R., Tutter, A. and Brodeur, P. (1986). Polymorphism and evolution of Igh-V gene families. *Curr. Top. Microbiol. Immunol.* **127**:168.

Ritchie, K. A., Brinster, R. L. and Storb, U. (1984). Allelic exclusion and control of endogenous immunoglobulin gene rearrangement in kappa transgenic mice. *Nature* **312**:517.

Roeder, W., Maki, R., Traunecker, A. and Tonegawa, S. (1981). Linkage of the four gamma subclass heavy chain genes. *Proc. Natl. Acad. Sci. USA* **78**:474.

Rolink, A., Grawunder, U., Haasner, D., Strasser, A. and Melchers, F. (1993). Immature surface Ig+ B cells can continue to rearrange kappa and lambda L chain gene loci. *J. Exp. Med.* **178**:1263.

Rosenberg, N. and Baltimore, D. (1976). A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. *J. Exp. Med.* **143**:1453.

Rosenberg, N., Baltimore, D. and Scher, C. D. (1975). In vitro transformation of lymphoid cells by Abelson murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **72**:1932.

Rosenshein, I. L. and Marchalonis, J. J. (1985). Broad phylogenetic expression of heavy-chain determinants detected by rabbit antisera to V_Ha allotypes. *Mol. Immunol.* **22**:1177.

Rothe, J., Ryser, S., Mueller, U., Steinmetz, M. and Bluethmann, H. (1993). Functional expression of a human TCR beta gene in transgenic mice. *Int. Immunol* **5**:11.

Roux, K. H., Dhanarajan, P., Gottschalk, V., McCormick, W. T. and Renshaw, R. W. (1991). Latent a1 V_H germline genes in an a2a2 rabbit. Evidence for gene conversion at both the germline and somatic levels. *J. Immonol.* **146**:2027.

Rubinstein, D. B., Schwartz, R. S., Guillaume, T., Leblanc, P. and Stewart, A. K. (1994). Germline complexity, restriction fragment length polymorphism, and coding region sequences of the human V_H7 gene family identified with family-specific FR3 segment oligonucleotides. *Mol. Immunol.* **31**:713.

Rusconi, S. and Kohler, G. (1985). Transmission and expression of a specific pair of rearranged immunoglobulin mu and kappa genes in a transgenic mouse line. *Nature* **314**:330.

Sadofsky, M. J., Hesse, J. E. and Gellert, M. (1994). Definition of a core region of RAG-

2 that is functional in V(D)J recombination. *Nucleic Acids Res.* **22**:1805.

Sadofsky, M. J., Hesse, J. E., McBlane, J. F. and Gellert, M. (1994). Expression and V(D)J recombination activity of mutated RAG-1 proteins. *Nucleic Acids Res.* **22**:

Sakano, H., Huppi, K., Heinrich, G. and Tonegawa, S. (1979). Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature* **280**:288.

Sakano, H., Kurosawa, Y., Weigert, M. and Tonegawa, S. (1981). Identification and nucleotide sequence of a diversity DNA segment (D) of immunoglobulin heavy-chain genes. *Nature* **290**:562.

Sakano, H., Maki, R., Kurosawa, Y., Roeder, W. and Tonegawa, S. (1980). Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. *Nature* **286**:676.

Sambrook, J., Fritsch E. F. and Maniatis, T. Laboratory Manual for Molecular Biology. New York: Cold Spring Harbor Laboratory Press, 2nd edition, 1989.

Sanz, I. (1991). Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. *J. Immonol.* **147**:1720.

Sanz, I., Wang, S. S., Meneses, G. and Fischbach, M. (1994). Molecular characterization of human Ig heavy chain DIR genes. *J. Immonol.* **152**:3958.

Sasso, E. H., Van, D. K. and Milner, E. C. (1990). Prevalence and polymorphism of human VH3 genes. *J. Immonol.* **145**:2751.

Schatz, D. G., Oettinger, M. A. and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. *Cell* **59**:1035.

Schlissel, M., Voronova, A. and Baltimore, D. (1991). Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes Dev.* **5**:1367.

Schlissel, M. S., Corcoran, L. M. and Baltimore, D. (1991). Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. *J. Exp. Med.* **173**:711.

Schroeder, H. J., Hillson, J. L. and Perlmutter, R. M. (1990). Structure and evolution of mammalian VH families. *Int. Immunol* **2**:41.

Schroeder, H. J., Walter, M. A., Hofker, M. H., Ebens, A., Willems, van, Dijk, K, Liao, L. C., Cox, D. W., Milner, E. C. and Perlmutter, R. M. (1988). Physical linkage of a human immunoglobulin heavy chain variable region gene segment to diversity and

joining region elements. *Proc. Natl. Acad. Sci. USA* **85**:8196.

Schroeder, H. J. and Wang, J. Y. (1990). Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. *Proc. Natl. Acad. Sci. USA* **87**:6146.

Schroeder, H. W. (1987). Early restriction of the human antibody repertoire. *Science* **238**:791.

Schulze, D., H. (1987). Genotypic analysis of B cell colonies by *in situ* hybridization Stiochiometric Expression of Three VH families in adult C57BL/6 and BALB/c mice. *J. Exp. Med.* **166**:163.

Seidman, J. G., Edgell, M. H. and Leder, P. (1978). Immunoglobulin light-chain structural gene sequences cloned in a bacterial plasmid. *Nature* **271**:582.

Seidman, J. G. and Leder, P. (1978). The arrangement and rearrangement of antibody genes. *Nature* **276**:790.

Selsing, E., Voss, J. and Storb, U. (1984). Immunoglobulin gene 'remnant' DNA--implications for antibody gene recombination. *Nucleic Acids Res.* **12**:4229.

Sethupathi, P., Spieker, P. H., Polet, H., Yam, P. C., Tunyaplin, C. and Knight, K. L. (1994). Lymphoid and non-lymphoid tumors in E kappa-myc transgenic rabbits. *Leukemia* **8**:2144.

Sevag, M. S. (1935) *Biochem. Z.* 273:419.

Sheehan, K. M., Mainville, C. A., Willert, S. and Brodeur, P. H. (1993). The utilization of individual VH exons in the primary repertoire of adult BALB/c mice. *J. Immonol.* **151**:5364.

Sher, I. (1982). CBA/N immune defective mice: evidence of the failure of a B cells subpopulation to be expressed. *Immunol. Rev.* **64**:119.

Shimizu, A., Takahashi, N., Yamawaki, K. Y., Nishida, Y., Kataoka, T. and Honjo, T. (1981). Ordering of mouse immunoglobulin heavy chain genes by molecular cloning. *Nature* **289**:149.

Shimizu, A., Takahashi, N., Yaoita, Y. and Honjo, T. (1982). Organization of the constant-region gene family of the mouse immunoglobulin heavy chain. *Cell* **28**:499.

Shimizu, T. and Yamagishi, H. (1992). Biased reading frames of pre-existing DH--JH coding joints and preferential nucleotide insertions at VH--DJH signal joints of excision products of immunoglobulin heavy chain gene rearrangements. *Embo J.* **11**:4869.

Shin, E. K., Matsuda, F., Fujikura, J., Akamizu, T., Sugawa, H., Mori, T. and Honjo, T. (1993). Cloning of a human immunoglobulin gene fragment containing both VH-D and D-JH rearrangements: implication for VH-D as an intermediate to VH-D-JH formation. *Eur. J. Immonol.* **23**:2365.

Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M. and et, a. l. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**:855.

Shirakata, M., Huppi, K., Usuda, S., Okazaki, K., Yoshida, K. and Sakano, H. (1991). HMG1-related DNA-binding protein isolated with V-(D)-J recombination signal probes. *Mol. Cell. Biol.* **11**:4528.

Short, J. A., Sethupathi, P., Zhai, S. K. and Knight, K. L. (1991). VDJ genes in VHa2 allotype-suppressed rabbits. Limited germline VH gene usage and accumulation of somatic mutations in D regions. *J. Immonol.* **147**:4014.

Siden, E., Alt, F. W., Shinefeld, L., Sato, V. and Baltimore, D. (1981). Synthesis of immunoglobulin mu chain gene products precedes synthesis of light chains during B-lymphocyte development. *Proc. Natl. Acad. Sci. USA* **78**:1823.

Silver, D. P., Spanopoulou, E., Mulligan, R. C. and Baltimore, D. (1993). Dispensable sequence motifs in the RAG-1 and RAG-2 genes for plasmid V(D)J recombination. *Proc. Natl. Acad. Sci. USA* **90**:6100.

Snodgrass, H. R., Dembic, Z., Steinmetz, M. and von, B. H. (1985). Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature* **315**:232.

Snodgrass, H. R., Kisielow, P., Kiefer, M., Steinmetz, M. and von, B. H. (1985). Ontogeny of the T-cell antigen receptor within the thymus. *Nature* **313**:592.

Stall, A. M., Kroese, F. G., Gadus, F. T., Sieckmann, D. G., Herzenberg, L. A. and Herzenberg, L. A. (1988). Rearrangement and expression of endogenous immunoglobulin genes occur in many murine B cells expressing transgenic membrane IgM. *Proc. Natl. Acad. Sci. USA* **85**:3546.

Staudt, L. M. and Lenardo, M. J. (1991). Immunoglobulin gene transcription. [Review]. *Ann. Rev. Immunol* **9**:373.

Stiernholm, N. B. and Berinstein, N. L. (1995). A mutated promoter of a human Ig V lambda gene segment is associated with reduced germ-line transcription and a low frequency of rearrangement. *J. Immonol.* **154**:1748.

Storb, U. and Arp, B. (1983). Methylation patterns of immunoglobulin genes in lymphoid cells: correlation of expression and differentiation with undermethylation. *Proc. Natl.*

Acad. Sci. USA **80**:6642.

Storb, U., Wilson, R., Selsing, E. and Walfield, A. (1981). Rearranged and germline immunoglobulin kappa genes: different states of DNase I sensitivity of constant kappa genes in immunocompetent and nonimmune cells. *Biochem.* **20**:990.

Taccioli, G. E., Cheng, H. L., Varghese, A. J., Whitmore, G. and Alt, F. W. (1994). A DNA repair defect in Chinese hamster ovary cells affects V(D)J recombination similarly to the murine scid mutation. *J. Biol. Chem.* **269**:7439.

Taccioli, G. E., Cheng, H. L., Varghese, A. J., Whitmore, G. and Alt, F. W. (1994). A DNA repair defect in Chinese hamster ovary cells affects V(D)J recombination similarly to the murine scid mutation. *J. Biol. Chem.* **269**:7439.

Taccioli, G. E., Gottlieb, T. M., Blunt, T., Priestley, A., Demengeot, J., Mizuta, R., Lehmann, A. R., Alt, F. W., Jackson, S. P. and Jeggo, P. A. (1994). Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination. *Science* **265**:1442.

Taccioli, G. E., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P. A. and Alt, F. W. (1993). Impairment of V(D)J recombination in double-strand break repair mutants. *Science* **260**:207.

Tilghman, S. M. (1993). DNA methylation: a phoenix rises [comment]. *Proc. Natl. Acad. Sci. USA* **90**:8761.

Timmers, E., Kenter, M., Thompson, A., Kraakman, M. E., Berman, J. E., Alt, F. W. and Schuurman, R. K. (1991). Diversity of immunoglobulin heavy chain gene segment rearrangement in B lymphoblastoid cell lines from X-linked agammaglobulinemia patients. *Eur. J. Immonol.* **21**:2355.

Todd, C. W. (1963). Allotypy in rabbit 19S protein. *Biochem. Biophys. Res. Commun.* **11**:170.

Tonegawa, S. (1983). Somatic generation of antibody diversity. [Review]. *Nature* **302**:575.

Tonegawa, S., Brack, C., Hozumi, N. and Schuller, R. (1977). Cloning of an immunoglobulin variable region gene from mouse embryo. *Proc. Natl. Acad. Sci. USA* **74**:3518.

Tonegawa, S., Maxam, A. M., Tizard, R., Bernard, O. and Gilbert, W. (1978). Sequence of a mouse germ-line gene for a variable region of an immunoglobulin light chain. *Proc. Natl. Acad. Sci. USA* **75**:1485.

Tsao, B. P., Ohnishi, K., Cheroutre, H., Mitchell, B., Teitell, M., Mixter, P., Kronenberg, M. and Hahn, B. H. (1992). Failed self-tolerance and autoimmunity in IgG anti-DNA

transgenic mice. *J. Immonol.* **149**:350.

Tsujimoto, Y., Cossman, J., Jaffe, E. and Croce, C. M. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. *Science* **228**:1440.

Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P. C. and Croce, C. M. (1985). Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. *Nature* **315**:340.

Tsukamoto, A., Weissman, I. L. and Hunt, S. V. (1984). Allelic exclusion in rat kappa immunoglobulin chains: extent of Jk rearrangement in normal B lymphocytes. *Embo J.* **3**:975.

Tucker, P. W., Liu, C. P., Mushinski, J. F. and Blattner, F. R. (1980). Mouse immunoglobulin D: messenger RNA and genomic DNA sequences. *Science* **209**:1353.

Turka, L. A., Schatz, D. G., Oettinger, M. A., Chun, J. J., Gorka, C., Lee, K., McCormack, W. T. and Thompson, C. B. (1991). Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross-linking. *Science* **253**:778.

Tutter, A. and Riblet, R. (1988). Duplications and deletions of Vh genes in inbred strains of mice. *Immunogenetics* **28**:125.

Tutter, A. and Riblet, R. (1988). Selective and neutral evolution in the murine Igh-V locus. *Curr. Top. Microbiol. Immunol.* **137**:107.

Tutter, A. and Riblet, R. (1989). Conservation of an immunoglobulin variable-region gene family indicates a specific, noncoding function. *Proc. Natl. Acad. Sci. USA* **86**:7460.

Tutter, A. and Riblet, R. (1989). Evolution of the immunoglobulin heavy chain variable region (Igh-V) locus in the genus Mus. *Immunogenetics* **30**:315.

Uematsu, Y., Ryser, S., Dembic, Z., Borgulya, P., Krimpenfort, P., Berns, A., von, B. H. and Steinmetz, M. (1988). In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell* **52**:831.

van Meerwijk, J., Iglesias, A., Hansen, H. T., Bluethmann, H. and Steinmetz, M. (1991). Allelic exclusion of a T cell receptor-beta minilocus. *J. Immonol.* **147**:3224.

Van, N. B., Coleclough, C., Perry, R. P. and Weigert, M. (1982). DNA between variable and joining gene segments of immunoglobulin kappa light chain is frequently retained in cells that rearrange the kappa locus. *Proc. Natl. Acad. Sci. USA* **79**:262.

Varmuza, S. and Mann, M. (1994). Genetic Imprintings. *Trends in Genetics* **10**:118.

Viale, A. -C., Coutinho, A. and Freitas, A. A. (1992). Differential expression of VH gene

families in peripheral B cell repertoires of newborn or adult immunoglobulin H chain congenic mice. *J. Exp. Med.* **175**:1449.

Vos, Q. and Hodes, R. J. (1992). Immunoglobulin (Ig) mu, kappa transgenic mice express transgenic idiotype on endogenously rearranged IgM and IgA molecules by secretion of chimeric molecules. *J. Exp. Med.* **176**:951.

Wabl, M and Steinberg, C (1982). A theory of allelic and isotypic exclusion for immunoglobulin genes. *Proc. Natl. Acad. Sci USA* 79:6976.

Wagner, S. D., Williams, G. T., Larson, T., Neuberger, M. S., Kitamura, D., Rajewsky, K., Xian, J. and Bruggemann, M. (1994). Antibodies generated from human immunoglobulin miniloci in transgenic mice. *Nucleic Acids Res.* **22**:1389.

Walter, M. A., Surti, U., Hofker, M. H. and Cox, D. W. (1990). The physical organization of the human immunoglobulin heavy chain gene complex. *Embo J.* **9**:3303.

Weaver, D., Costantini, F., Imanishi, K. T. and Baltimore, D. (1985). A transgenic immunoglobulin mu gene prevents rearrangement of endogenous genes. *Cell* **42**:117.

Weiler, E. (1965). Differential activity of allelic gamma-globulin genes in antibody-producing cells. *Proc. Natl. Acad. Sci. USA* **54**:1765.

Winter, E., Radbruch, A. and Krawinkel, U. (1985). Members of novel VH gene families are found in VDJ regions of polyclonally activated B-lymphocytes. *Embo J.* **4**:2861.

Wood, C. and Tonegawa, S. (1983). Diversity and joining segments of mouse immunoglobulin heavy chain genes are closely linked and in the same orientation: implications for the joining mechanism. *Proc. Natl. Acad. Sci. USA* **80**:3030.

Wu, G., E. (1988). VH gene family utilization is regulated by a locus outside of the VH region. *J. Exp. Med* **167**:1499.

Wu, G. E., Atkinson, M. J., Ramsden, D. A. and Paige, C. J. (1990). VH gene repertoire. [Review]. *Semin. Immunol.* **2**:207.

Yamagishi, H., Tsuda, T., Fujimoto, S., Toda, M., Kato, K., Maekawa, Y., Umeno, M. and Anai, M. (1983). Purification of small polydisperse circular DNA of eukaryotic cells by use of ATP-dependent deoxyribonuclease. *Gene* **26**:317.

Yancopoulos, G. D. (1988). Developmentally regulated and strain-specific expression of murine VH gene families. *J. Exp. Med.* **168**:417.

Yancopoulos, G. D. and Alt, F. W. (1985). Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. *Cell* **40**:271.

Yancopoulos, G. D. and Alt, F. W. (1986). Regulation of the assembly and expression of variable-region genes. [Review]. *Ann. Rev. Immunol***4**:339.

Yancopoulos, G. D., Desiderio, S. V., Paskind, M., Kearney, J. F., Baltimore, D. and Alt, F. W. (1984). Preferential utilization of the most JH-proximal VH gene segments in pre-B cell lines. *Nature* **311**:727.

Yoshida, N. (1987). Ig gene rearrangement and expression in the progeny of B-cells progenitors in the course of clonal expansion in bone marrow culture. *EMBO J.* **6**:2735.

Zouali, M. and Theze, J. (1991). Probing VH gene-family utilization in human peripheral B cells by *in situ* hybridization. *J. Immonol.* **146**:2855.

VITA

The author, Chainarong Tunyaplin, was born in Bangkon, Thailand on September 5, 1963 to Sopoan and Suwanna Tunyaplin. After the completion of his high school education, he entered Chiang Mai University, Thailand, where he earned his MD degree in 1987.

After receiving his MD, he joined Department of Microbiology, Faculty of Medicine, Chiang Mai University as an instructor. He taught medical immunology course for medical, dental, nursing and pharmacy students. After two years as the member of the department, he joined the graduate program of Department of Microbiology and Immunology, Loyola University of Chicago as a graduate student. In 1991, he joined the laboratory of Dr. K. L. Knight where he studied the IgH gene rearrangements in rabbit B cells.

After the completion of his Ph.D. degree, he will return to Thailand where he will continue to teach immunology.

Publication:

Tunyaplin, C. and Knight, K. L. Fetal VDJ gene repertoire in rabbit: Evidence for preferential rearrangement of V_H1. Submitted.

Tunyaplin, C. and Knight, K. L. Gene rearrangement on the unexpressed IgH allele in rabbit B cells. In preparation.

Knight, K. L. and Tunyaplin, C. Rabbit IgH genes in Immunoglobulin Genes. In print.

Friedman, M. L., Tunyaplin, C., Zhai, S. K. and Knight, K. L. (1994). Neonatal VH, D, and J_H gene usage in rabbit B lineage cells. *J. Immonol.* **152**:632.

Sethupathi, P., Spieker, P. H., Polet, H., Yam, P. C., Tunyaplin, C. and Knight, K. L. (1994). Lymphoid and non-lymphoid tumors in E kappa-myc transgenic rabbits. *Leukemia* **8**:2144.

Approval Sheet

The dissertation submitted by Chainarong Tunyaplin has been read and approved by the following committee:

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

Charles F. Lange, Ph.D.
Professor
Department of Microbiology and Immunology
Loyola University Medical Center

Susan C. Baker, Ph.D.
Assistant Professor
Department of Microbiology and Immunology
Loyola University Medical Center

Hans-Martin Jäck, Ph.D.
Assistant Professor
Department of Microbiology and Immunology
Loyola University Medical Center

Pamela L. Witte, Ph.D.
Associate Professor
Department of Cell Biology, Neurobiology and Anatomy
Loyola University Medical Center

Phong T. Le, Ph.D.
Assistant Professor
Department of Cell Biology, Neurobiology and Anatomy
Loyola University Medical Center

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.

June 27, 1995
Date

Katherine L. Knight
Director's Signature