Rabbit IgH Sequences in Appendix Germinal Centers: VH Diversification by Gene Conversion-Like and Hypermutation Mechanisms

Peter D. Weinstein,*† Arthur O. Anderson,* and Rose G. Mage†
*Applied Research Division
United States Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, Maryland 21702
†Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
Building 10 11N311
National Institutes of Health
Bethesda, Maryland 20892

Summary

Although the rabbit IgH locus contains approximately 100 VH genes, the majority of B cells rearrange VH1. To produce a primary repertoire containing a sufficient number of protective antibodies, rearranged VH1-DH-JH sequences may diversify within rabbit B cells in an organ that functions like a chicken bursa, sheep ileal Peyer's patch, or both. It was suggested many years ago that the rabbit appendix could be a bursal equivalent. To reexamine this possibility, we analyzed rearranged heavy chain variable region sequences in B cells from light and dark zones of appendix germinal centers from 6-week-old rabbits. Our findings indicate that antibody diversification occurs by gene conversion-like and somatic hypermutation mechanisms in appendix germinal centers of young rabbits.

Introduction

Early in life, different antibodies with unique binding specificities are generated that protect against a multitude of invasive antigens. In species such as humans, mice, and rats, combinatorial assortment contributes to antibody diversity. This random joining of one of many distinct germline variable region VH, diversity (DH), and joining (JH) gene segments for the heavy chain; VL and JL for the light chain (reviewed by Blackwell and Alt, 1988), has been described as the generation of a high copy number primary antibody repertoire (Cohn and Langman, 1990; Langman and Cohn, 1993). Diversification of the amino acid sequences in the third complementarity determining region (CDR3) during recombination occurs because of imprecise coding joint formation. The coding ends of VH, DH, and JH genes may be shortened by exonuclease activity or lengthened by P and N nucleotide additions (McCormack et al., 1989; LaFaille et al., 1989; Alt and Baltimore, 1982).

Not all species use combinatorial mechanisms to produce a diverse primary antibody repertoire. In chickens, with only one rearranging VH and VL gene, upstream VH and VL pseudogenes are used as DNA donors to diversify the rearranged V by gene conversion (Reynaud et al., 1987, 1989; Thompson and Neiman, 1987). Some junctional diversity and somatic hypermutation also occurs (Parvari et al., 1990). The diversification of rearranged chicken V genes occurs in the bursa of Fabricius, a primary lymphoid organ located in the hind gut (Glick et al., 1956). Due to a limited pool of functional VL genes, sheep diversify their rearranged light chain variable region genes in a peripherally located primary lymphoid organ, the ileal Peyer's patch (Reynaud et al., 1991). Unlike chickens, sheep appear to use only somatic hypermutation for VL sequence diversification (Reynaud et al., 1991).

The rabbit IgH locus contains one gene family, VHIII, with approximately 100 VH genes (reviewed by Knight, 1992; Mage, 1993). Many are pseudogenes, but several can and do rearrange (Allegrucci et al., 1991; Short et al., 1991; Chen et al., 1993). Of peripheral B cells in normal rabbits that have undergone a productive VH–DH–JH gene rearrangement, 80%–95% utilize the VHa allotype-encoding VH1 gene, which is located at the 3′ end of the VH cluster (Becker et al., 1990; Knight and Becker, 1990; Becker and Knight, 1990; Allegrucci et al., 1991). The utilization of VH1 by a majority of rabbit B cells thus limits the contribution of combinatorial diversity to production of the primary high copy number antibody repertoire (Langman and Cohn, 1993). The production of the vast array of antigen-binding sites that protect the rabbit may involve mechanisms to diversify rearranged VH1–DH–JH that share some similarity to those in chickens and sheep. Evidence presented by several laboratories has suggested that in peripheral B cells of the rabbit, donor DNA from upstream VH gene sequences was used in a gene conversion–like process to generate diversity of rearranged VH–DH–JH sequences (Becker and Knight, 1990; Roux et al., 1991). The lymphoid organs and compartments in which this diversification occurs in rabbits were not determined. In contrast with data on the chicken, where sequential segmental gene conversion events have been shown to leave donor sequences unaltered (Carlson et al., 1990), the nonreciprocal nature of the mechanism used by rabbits remains to be documented. Hence, we refer to the mechanism in rabbits as gene conversion-like.

Recently, we showed through a comprehensive immunohistochemical examination of the appendixes of 6-week-old rabbits that this lymphoid organ shares several morphological cellular distribution and cell surface glycosylation features with both the chicken bursa and sheep IPP (SIPP) (P. D. W. et al., submitted). For example, the germinal centers (GC) of the chicken bursa and SIPP are devoid of CD4 and CD8 T cells (Aleksandersen et al., 1990; Landsverk et al., 1991; Kon-Ogura et al., 1993), as are those of the appendix of the 6-week-old rabbit (P. D. W. et al., submitted). Histologically, GCs of secondary lymphoid tissues contain a peripheral dark zone (DZ) comprised of tightly packed large proliferating B cells with little surface immunoglobulin and a central light zone (LZ) thinly populated with small and large B cells, macrophages, and follicular dendritic cells (FDC). The GCs of the appendix from

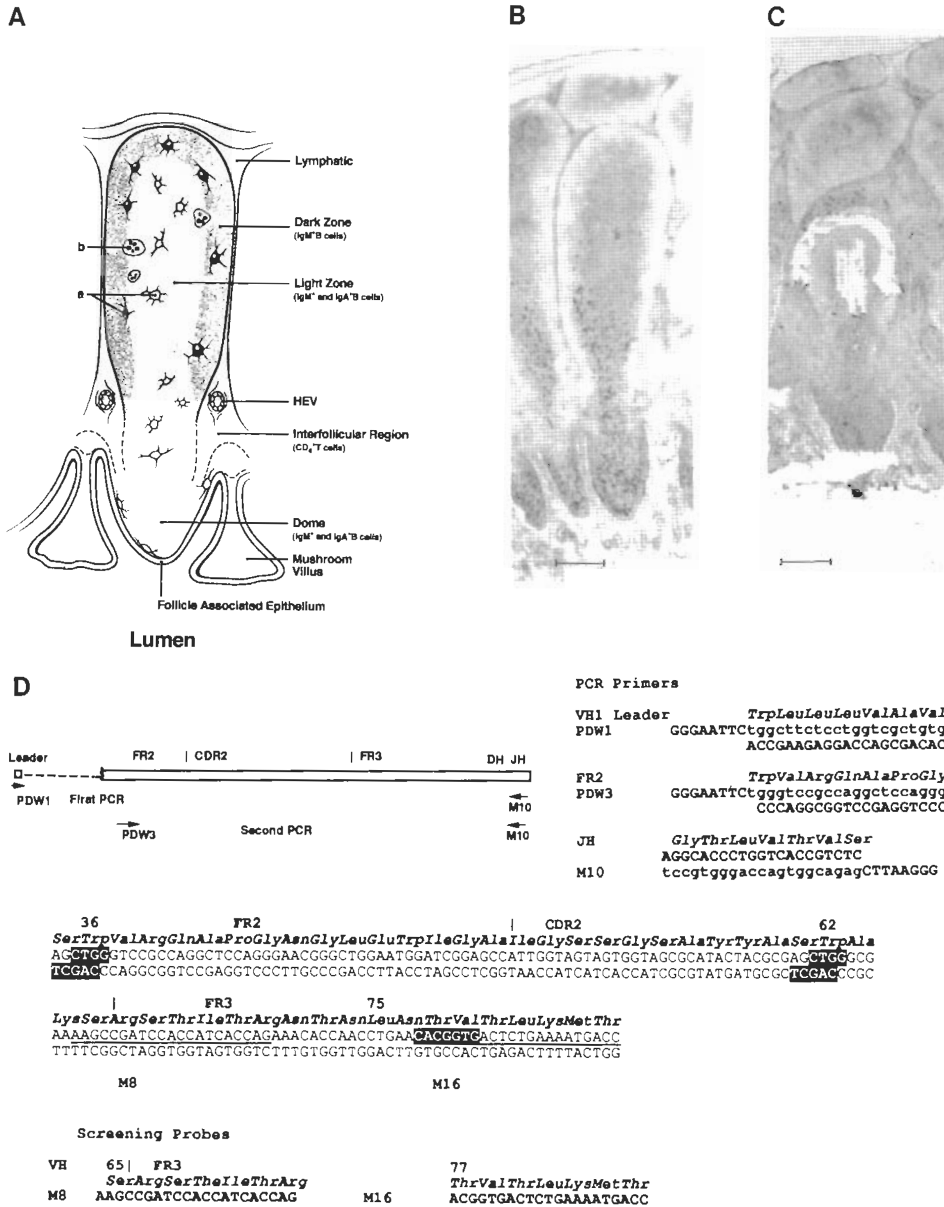


Figure 1. Summary of Methods Used to Obtain Rabbit Appendix Germinal Center B Cells and their Corresponding VH DNA for Sequencing

(A) The rabbit appendix is a gut-associated lymphoid tissue composed of several hundred B cell follicles arranged in a spiral with each surrounded by other follicles on all sides. Each follicle is composed of a follicle-associated epithelium that protrudes into the lumen and through which antigen enters the appendix; a dome region containing small IgM$^+$ and IgA$^+$ B cells and FDC; and a GC with an average cell number of $\sim 2 \times 10^5$ compared with $\sim 1 \times 10^4$ in a GC of human lymph node (Kuppers et al., 1993). The GC consists of a DZ with large proliferating B cells expressing IgM and a LZ with FDC (a), scattered macrophages (b), and small B cells expressing either IgM or IgA. Between each follicle is the interfollicular region containing primarily CD4$^+$ T cells, a few FDC, and high endothelial venules (HEV). In young rabbits, this is the only region containing CD4$^+$ or CD8$^+$ T cells. This follicular and interfollicular organization is similar to that found in the sheep ileal Peyer's patch and chicken bursa of Fabricius.

(B) A 7 μm semithin section of 6 week rabbit appendix stained with succinylated wheat germ agglutinin. The dark staining of cells in the LZ and faint staining of cells in the DZ was the basis for isolation of B cells from the two GC regions. Scale bar represents 2.5 mm.

(C) A 7 μm semithin section of 6 week rabbit appendix stained with succinylated wheat germ agglutinin showing regions where B cells were collected using an Eppendorf micromanipulator. Two scrapings were taken from each GC DZ or LZ. Note a border of two or three cells left between the LZ and the DZ. Scale bar represents 2.5 mm.

(D) Summary of PCR strategy and oligonucleotide primers and probes. The primers PDW1, PDW3, and M10 had added bases and EcoRI restriction enzyme sites (shown in upper case letters) used to clone PCR products. The initial PCR of 35 cycles used the 5′ primer, PDW1, and the 3′ primer, M10, (Chen et al., 1993). The second set of 35 cycles used a new 5′ primer, PDW3, corresponding to a highly conserved sequence in framework region 2 (FR2) and the same 3′ primer (M10). Two oligonucleotide probes (M8 and M16), whose sequences are underlined, were used to identify bacterial colonies containing the cloned PCR-amplified VH DNA. The final amplified product collected after the second PCR was ~ 250 bp. A portion of the germline VH1a2 sequence is shown with the overlapping sequence motifs of Betz et al. (1993) and Chou and Morrison (1993), highlighted in reverse font near amino acid positions 36 and 62; also highlighted is a recombination signal sequence heptamer motif near Leu 75 (amino acids numbered according to Kabat et al., 1991).

a 6-week-old rabbit are similarly organized (Figure 1A) (Waksman et al., 1973; Nieuwenhuis et al., 1974; P. D. W. et al., submitted). In the GCs of secondary lymphoid tissues, changes in individual nucleotides of V region sequences resulting from a process of somatic hypermutation and antigen-directed selection lead to emergence and expansion of B cell clones producing antibody proteins with increased affinity for a specific antigen. The GCs that arise in primary lymphoid organs may also be formed by B cells stimulated by antigens, mitogens, or other mediators of proliferation; these reactions may not necessarily be specific for one particular antigen (Reynolds and Morris, 1984; Ekino, 1993; Weinstein et al., 1993). In the SIPP and the chicken bursa, initial GC formation can appear prenatally before exposure to environmental antigens. In contrast, GC formation in the rabbit appendix appears to be completely dependent on antigens sampled from the gut (Stepánková et al., 1980; Tlaskalová-Hogenová and Stepánková, 1980). We did not detect organized rabbit appendix GCs until approximately 2 weeks after birth (P. D. W. et al., submitted).

About 30 years ago, before analysis of the molecular genetic events that occur during B cell development was possible, several groups suggested that the rabbit appendix might serve a function analogous to that of the chicken bursa as a peripheral primary lymphoid organ (Archer et al., 1963; Sutherland et al., 1963; Cooper et al., 1968). Among the lines of reasoning that contributed to this belief were its location in the gastrointestinal tract, the depressed levels of immunoglobulin in the serum of appendectomized newborn rabbits, and the inability of appendectomized newborn rabbits to populate completely known secondary lymphoid organs such as spleen and lymph nodes.

For the reasons outlined above, we set out to determine whether development of a diversified primary antibody repertoire occurs in the GCs of the young rabbit appendix. Rearranged heavy chain variable region genes from B cells that were isolated from both the LZ and the DZ of several 6-week-old rabbit appendix GCs were cloned and sequenced. Examination of these sequences strongly suggests that the generation of a primary antibody repertoire occurs early in rabbit B cell development within appendix GCs, probably through both gene conversion-like and somatic hypermutation mechanisms.

Results

Lectin Staining, Cell Collection, and PCR Amplification of Rearranged VH Genes from the Rabbit Appendix

Through the selective binding of the lectin succinylated wheat germ agglutinin, we distinguished two GC regions from each other by the intensity of their staining following histochemistry; darkly staining cells in the LZ and faintly staining cells in the DZ (Figure 1B). This allowed us to use an Eppendorf micromanipulator to isolate LZ and DZ B cells from 7 μm semithin sections from tissue taken about 1 cm from the tip of the rabbit appendix (Figure 1C). Two samples were taken from each DZ and LZ of an individual GC and extracts were prepared in a manner similar to that described for studies of somatic hypermutation in mouse splenic GC (Jacob et al., 1991) and human lymph node GC (Kuppers et al., 1993). PCR primers corresponding to conserved sequences were used in a two step DNA amplification reaction, in which the second PCR relied on heminesting with an internal primer at the 5′ end of the intended DNA product (Figure 1D). Rearranged genes (VH–DH–JH) in DNA extracts from the DZs and LZs of several appendix GCs were amplified. Of the extracts, 50%–75% yielded PCR products. This yield compares favorably with results obtained by other laboratories using similar conditions (Jacob et al.,1991). PCR products were purified, subcloned, and clones positive when hybridized with two oligonucleotide probes (M8 and M16, Figure 1D) with specificity for VHa-positive sequences, were selected for sequencing. All the cloned DNA products that we sequenced appear to have rearranged the VH1a2 gene segment, several different DH genes, and two JH genes, JH4 (Becker et al., 1989) and JHx (Chen et al., 1993); JHx may correspond to the recently reported JH6 (Friedman et al., 1994).

Diversification within the Dark and Light Zones of Individual Germinal Centers

The rearranged VH gene sequences we obtained from 6 week rabbit appendix GCs originated from relatively few progenitors (Figure 2; see Figure 4, see Figure 5). Although as many as 10^3 cells may have been present in the original scraping, PCR amplification of 5 μl from a 25 μl DNA extract yielded one to four unique sequences before identical sequences began to recur, at which time we initiated examination of another DZ or LZ. The sequences can be traced back to one or more unique progenitors based on similarities in use of DH and JH gene sequences and the nucleotides added at the coding junctions. This allowed us to draw evolutionary trees showing clonal relationships based on the changes in the DNA sequences from B cells collected from a single GC. Our results show that an ongoing diversification process occurs within the rearranged heavy chain variable region sequence in B cells of appendix GCs.

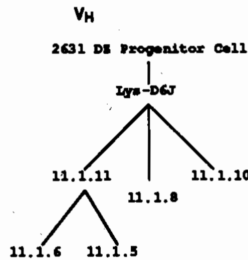
The sequences obtained from B cell DNA from the DZ of GC 2631 indicate that they all originated from a common progenitor B cell. Each sequence had a replacement of Arg with Lys at the V–D junction position 94 and identical DH–JH (Figure 2A). The VH sequences were highly diversified in CDR2, with three distinct blocks of changes; one clone, 2631 DZ 11.1.10 contained a three bp insertion (TAT–Tyr) in CDR2 (Figure 2A). This type of change is not associated with somatic hypermutation, but could be accounted for by gene conversion. Although a search of the available database of germline VH gene sequences for possible donor genes did not reveal an identical donor sequence, several sequences with high similarity to the altered region were found (Figure 3A). RVH705 from a VHa2 rabbit (Roux et al., 1991), as well as germline genes VH3a1 (from the different VHa1 allelic type) and RVH832 (from a rabbit of unknown VHa allotype) (McCormack et al., 1985), each carry the Lys 43 codon and the silent

A

2631DZ

```
                             FR2                       |50             CDR2              60               |   FR3
                                  AsnGlyLeuGluTrpIleGlyAlaIle---GlySerSerGlySerAlaTyrTyrAlaSerTrpAlaLysSerArgSerThrIl
GL VH1a2      gggtccgccaggctccagggAACGGGCTGGAATGGATCGGAGCCATT---GGTAGTAGTGGTAGCGCATACTACGCGAGCTGGGCGAAAAGCCGATCCACCAT
                                                       Ile   ---AspValTyr   GlyThr         Asn
11.1.11       ..........................................AT...---.A.GT.TA...G..A............A.........................
11.1.6        ..........................................AT...---.A.GT.TA...G..A............A.........................
11.1.5        ..........................................AT...---.A.GT.TA...G..A............A.........................
                                                             ---TyrThrAsp      Thr
11.1.8        ...............................................---TA..C.GA......A......................................
                                  Lys                  Asp   TyrAlaGly         Thr               ValAsnGly   Phe
11.1.10       ......................G........G..........A....TAT.C.G...........A..................T...TG.....T.......
                70                            80      82A B  C
              eThrArgAsnThrAsnLeuAsnThrValThrLeuLysMetThrSerLeuThrAlaAlaAspThrAlaThrTyrPheCysAlaArg
GL VH1a2      CACCAGAAACACCAACCTGAACACGGTGACTCTGAAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGA
                              Glu                                                               Lys
11.1.11       ................GA.................................................................A.
                  ***
11.1.6        ....T...........GA.................................................................A.
Pro
11.1.5        ................GA.............................................................C...A.
                                     Ala
11.1.8        .......................GC..........................................................A.
               SerSer
11.1.10       .T....C............................................................................A.

                    DH                   103   JH
                                   ATCTGGGGCCCAGGCACCCTGGTCACCGTCTCCTCA
3' Primer                                      tccgtgggaccagtggcagagcttaag
GL D6              GTATGCTGGTAGTAGC
              GlySerProAspGlySerGlyIleTrpGlyPro
11.1.11       GGGTCGCC..A.......G.............
11.1.6        GGGTCGCC..A.......G.............
11.1.5        GGGTCGCC..A.......G.............
11.1.8        GGGTCGCC..A.......G.............
11.1.10       GGGTCGCC..A.......G.............
```

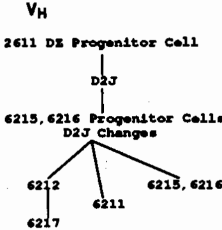


B

2611DZ

```
                            FR2                        |50             CDR2           60               |   FR3
                                  AsnGlyLeuGluTrpIleGlyAlaIleGlySerSerGlySerAlaTyrTyrAlaSerTrpAlaLysSerArgSerThrIl
GL VH1a2      gggtccgccaggctccagggAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTACGCGAGCTGGGCGAAAAGCCGATCCACCAT
                                                       Trp   SerAspGly   IleThr
6215,16       .........................................TGG...A..GA.G.....T.A......................................
                                              Tyr      Ile
6211          .................................AT......AT.........................................................
                                                             AspIle            Cys
6217          ................................................A..TC.............G.................................
6212          ................................................A..TC...............................................
                 70                            80       82A B  C                        90
              eThrArgAsnThrAsnLeuAsnThrValThrLeuLysMetThrSerLeuThrAlaAlaAspThrAlaThrTyrPheCysAlaArg
GL VH1a2      CACCAGAAACACCAACCTGAACACGGTGACTCTGAAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGA
6215,16       .....................................................................................
6211          .....................................................................................
6217          ..........................................T..........................................
6212          ..........................................T..........................................

                    DH                         103    JH
                                      TTTAACATCTGGGGCCCAGGCACCCTGGTCACCGTCTCCTCA
GL D2a        GTTACTATACTTATGGTTATGCT
              ArgTyrSerThrTyrGlyTyrAlaLeuAsnIleTrpGlyPro
6215,16       C......CA..............CC................
                      Pro   Ala
6211          C......CAC......C......CC................
              HisTyrSerSerTyrSerTyrAlaLeu
6217          CA.....CA.G....A.......CC................
6212          CA.....CA.G....A.......CC................
```



C

2611LZ

```
                            FR2                        |50             CDR2           60               |   FR3
                                  AsnGlyLeuGluTrpIleGlyAlaIleGlySerSerGlySerAlaTyrTyrAlaSerTrpAlaLysSerArgSerThrIl
GL VH1a2      gggtccgccaggctccagggAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTACGCGAGCTGGGCGAAAAGCCGATCCACCAT
                                                             Val
5246,38,47    ................................................T...................................................
5231          ................................................T...................................................
5227          ................................................T...................................................
                                                          MetThrGlyGly
5236          ..............................................GAC.G..G..............................................
                 70                            80      82A B  C                       90
              eThrArgAsnThrAsnLeuAsnThrValThrLeuLysMetThrSerLeuThrAlaAlaAspThrAlaThrTyrPheCysAlaArg
GL VH1a2      CACCAGAAACACCAACCTGAACACGGTGACTCTGAAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGA
5246,38, 47   .....................................................................................
                                                                                                Ser
5231          ....................................................................................T
5227          ........................................................................C............
5236          .....................................................................................

                    DH                              JH
                                                    103
GL JH                                 TTAACATCTGGGGCCCAGGCACCCTGGTCACCGTCTCCTCA
GL D5            GTTATGCTGGTAGTAGTTATTAT
              ProGlyTyrAspGlySerSerThrAlsLysIleTrpGlyPro
5246,38,47    CCTG.....A.........AC.GC...A...........
5231          CCTG.....A.........AC.GC...A...........
5227          CCTG.....A.........AC.GC...A...........
5236          CCTG.....A.........AC.GC...A...........
```

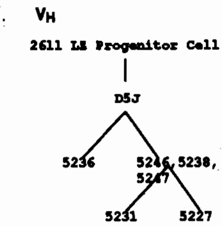


Figure 2. Sequences of Rearranged VH Genes from Two DZ and One LZ from a Single GC Compared with Germline VH1a2, DH, and JH

Amino acids are numbered according to Kabat et al. (1991). Dots signify identity to germline DNA sequence and only base changes and amino acid replacements are shown. Lower case letters show the sequences of the PCR primers at the 5′ end in FR2 and the 3′ JH primer is also shown in (A). 2611DZ and 2611LZ were isolated from the same GC tissue section; 2631DZ came from a section of the same GC 7 μm from 2611. In each GC region, isolated cells appear to have arisen from a common progenitor as diagrammed next to each set of sequences.

change in the Glu codon in FR2 (Figure 3A). RVH705 encodes the Tyr, Ala, Gly, and Thr codons in CDR2, and the Phe 67 codon in FR3 (Figure 3A); the Val 63 and Asn 64 codons are not found in this gene. However, another germline gene, RVH832, has the Val, Asn, and Gly codons at amino acid positions 63–65. A potential donor gene similar to these known genes might be identical to 11.1.10, beginning with the changes in FR2 and extending into FR3; alternatively, 11.1.10 may have acquired additional changes during one or more rounds of gene conversion or

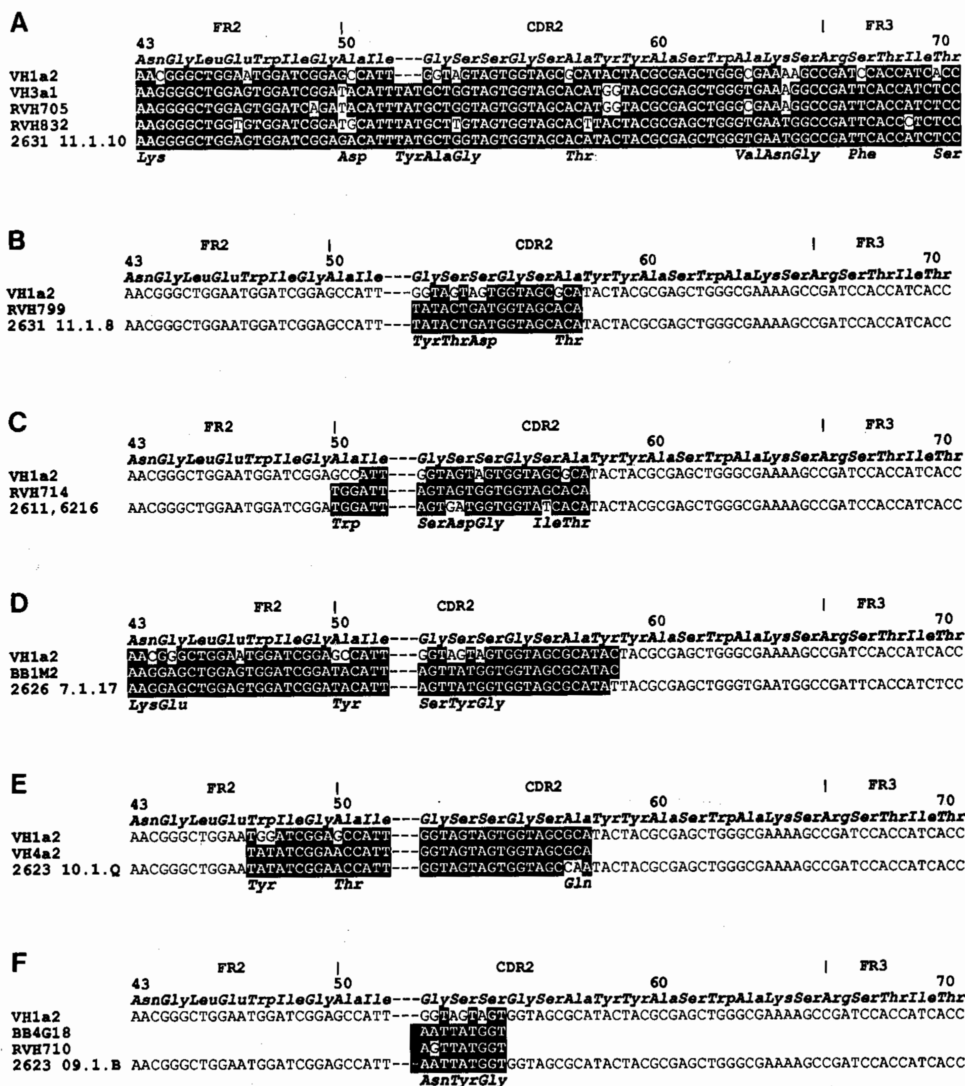


Figure 3. Possible DNA Donors for Gene Conversion of Different Diversified VH Sequences

(A–F) Amino acids are numbered according to Kabat et al. (1991). The germline VH1a2 sequences are shown at the top, and the cloned PCR-amplified rearranged VH gene sequences at the bottom of each panel. Between these, possible gene conversion donors are shown with identical sequences contained in the black box. As discussed in the text, two cDNA sequences from Chen et al. (1993), BB1M2 (D) and BB4G18 (F) (GenBank accession numbers M99548 and M99534), are included. We suggest these may be examples of sequences that shared a common donor with the diversified sequence we found.

somatic hypermutation. A potential DNA donor sequence was also identified for the gene conversion–like changes in CDR2 of clone 11.1.8 (Figure 3B). RVH799 (Roux et al., 1991) can account for all the changes found in CDR2 of this clone.

Clones 11.1.11, 11.1.6, and 11.1.5 share replacement changes in CDR2 that probably occurred through gene conversion; however, the potential DNA donor sequence has not yet been identified. These clones also contain a 2 bp change at amino acid position 75, leading to replacement of Leu with Glu that was not shared by clonally related 11.1.8 and 11.1.10 (see Figure 2A). This change is important because it was found in one or more of the DNA clones in five of the eight GC regions we examined. The Glu codon has been observed at this position in a number of expressed cDNA sequences from animals of the VHa2 allotype (Bernstein et al., 1982, 1983; Chen et al., 1993) and was included as a possible VHa2 allotype–correlated amino acid (Mage et al., 1984). Clone 11.1.6 also acquired an A to T change not found in 11.1.11 and 11.1.5, converting Arg 71 to a stop codon within FR3, and clone 11.1.5 picked up a single replacement change in FR3 (G to C; Ala to Pro). Neither involve the A to G or T to C transitions commonly caused by Taq polymerase. Since all three VH sequences shared a common block of changes in CDR2, it is likely that these changes were due to point mutations or further gene conversion after the initial gene conversion event.

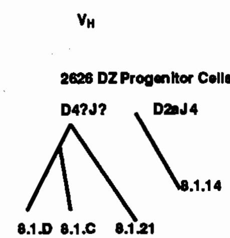
A

2626DZ

```
                              FR2                           |50                 CDR2         60                   |  FR3
                                    AsnGlyLeuGluTrpIleGlyAlaIleGlySerSerGlySerAlaTyrTyrAlaSerTrpAlaLysSerArgSerThrIl
GL VH1a2         gggtccgccaggctccagggAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTACGCGAGCTGGGCGAAAAGCCGATCCACCAT
                                                                AlaAsn                             Glu
8.1.14           ................................................C..A..............................CG................
                                                                AsnThrTyr      Thr
8.1.21           ...............................................AA..C.TA.......A...................C.................
                                                                                  Cys
8.1.D            ...............................................AA..C.TA.......A...G.................................
8.1.C            ...............................................AA..C.TA.......A.....................................

                   70                            80      82A B  C                        90
                 eThrArgAsnThrAsnLeuAsnThrValThrLeuLysMetThrSerLeuThrAlaAlaAspThrAlaThrTyrPheCysAlaArg
GL VH1a2         CACCAGAAACACCAACCTGAACACGGTGACTCTGAAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGA
8.1.14           .....................................................................................
                                 Glu                                                              Thr
8.1.21           ................GA.................................................................CT
8.1.D            ................GA.................................................................CT
                                      Ala
8.1.C            ................GA....G............................................................CT

                      DH                         103 JH
GL D2a JH4 TTATGGTATGCTGGTTATGCTTATGCT     TTAACATCTGGGGCCCAGGCACCCTGGTCACCGTCTCCTCA
           AspProIleTyrAsnTyrAlaAspThrGlnValAsnIleTrpGlyPro
 8.1.14    GACCC.AT.TA.AA.......G..A..CAGG...............
GLD4?J? TATAGTAGT------TGGCTGGG
           GlySerArgTrpTrpLeuTyr            SerIleTrpGlyPro
 8.1.21    G.....CGTTGG......TAT            .G............
 8.1.D     G.....CGTTGG......TAT            .G ...........
 8.1.C     G.....CGTTGG......TAT            .G............
```



B

2626LZ

```
                              FR2                           |50                 CDR2         60                   |  FR3
                                    AsnGlyLeuGluTrpIleGlyAlaIleGlySerSerGlySerAlaTyrTyrAlaSerTrpAlaLysSerArgSerThrIl
GL VH1a2         gggtccgccaggctccagggAACGGGCTGGAATGGATCGGAGCCATTGGTAGTAGTGGTAGCGCATACTACGCGAGCTGGGCGAAAAGCCGATCCACCAT
7.1.a;13;16      ....................................................................................................
                                              Asp
7.1.11           ...............................T....................................................................
                                      LysGlu                  Tyr    SerTyrGly
7.1.17           ......................G.A......G.........TA....A..TA.G.............T................................
                                                                Asn   Asp      ThrGly                  Arg
7.1.1;2          ...............................................AA....GA.......A..GG.................G...............

                   70                            80      82A B  C                        90
                 aThrArgAsnThrAsnLeuAsnThrValThrLeuLysMetThrSerLeuThrAlaAlaAspThrAlaThrTyrPheCysAlaArg
GL VH1a2         CACCAGAAACACCAACCTGAACACGGTGACTCTGAAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGA
7.1.a;13;16      .....................................................................................
7.1.11           .....................................................................................
7.1.17           .....................................................................................
                                 Glu
7.1.1;2          ................GAA..................................................................

                           DH                          JH
GL D3/D2b+    GCTAGT                                   103
GL D1/D7? JH4          CTATGGTGATTAC     TTAACATCTGGGGCCC
              AlaSerValGlyGlyAspTyrAlaThrAsnIleTrpGlyPro
7.1.a;13;16   ......GTTGG.........TGCTAC.................
7.1.11        ......GGTGG.........TGCTAC.................     103
     JH4                                               TTTAACATCTGGGGCCC
GL D2a           TACTATACTTATGGTTATGCTGGTTATGCTTAT
              ProTyrTyrThrTyrGlyTyrAl---aTyrAlaLeu   AsnIleTrpGlyPro
7.1.17        CCC.....................---.......CTG   ..............
                                   Leu-ProGlyTyrAsnSerGly
7.1.1;2                            ...-C........AA..C.GG..............
```

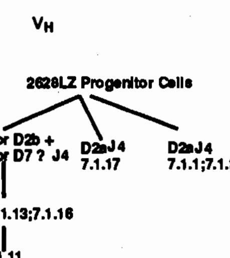


Figure 4. Sequences of Rearranged VH Genes from the DZ and LZ of a Single GC Compared with Germline VH1a2, DH, and JH

(A and B) Amino acids are numbered according to Kabat et al. (1991). Dots signify identity to germline DNA sequence and only base changes and amino acid replacements are shown. Lower case letters show the sequence of the PCR primer at the 5′ end in FR2. The sequences 8.1.21 and 8.1.D were found in both of two independently amplified aliquots of the same extract. The diagrams show the deduced clonal relationships of sequences from common and distinct progenitor cells.

We next examined the VH sequences from B cells in the same GC in a new tissue section (2611) located 7 μm, or approximately one to two cells away from the section that contained 2631DZ. B cells taken from the DZ yielded rearranged VH gene sequences with a high degree of diversity in CDR2 and CDR3 (see Figure 2B). The sequences differed from those in the 2631 section and, in contrast with 2631DZ, we found that a single rearranged DJ region had undergone further diversification, probably by somatic hypermutation. A simple evolutionary tree can be drawn showing how the cells with different DH sequences developed from a single progenitor. This is consistent with the three different patterns of alterations in CDR2 that led to as many as 6 out of 16 aa replacements in an individual clone (6215/6216) (see Figure 2B). Most of the changes in the CDR2 sequences were replacements that occurred in blocks that may be indicative of gene conversion. We found a germline gene, RVH714 (Roux et al., 1991), with similarity to the possible donor for gene conversion of clone 6216/6215 (Figure 3C). RVH714 encodes 4 of the 6 aa replacements in CDR2. Although some replacements may have been artifactual due to Taq polymerase errors, a few individual nucleotides, in addition to those in the DH region, may have been replaced by somatic mutation or another gene conversion event (see Figure 2B). In one example, clones 6212 and 6217 acquired identical changes in CDR2 and the DH region; then an A to G replacement of Tyr 55 with Cys may have occurred in the CDR2 of only 6217. Replacement of Tyr 55 with Cys was also found in an independent clone from another GC (2626DZ 8.1.D; Figure 4).

We found heterogeneity in the extent of diversification in the LZs (see Figure 2C, Figure 4B, Figures 5B and 5C) when compared with their corresponding DZ (see Figure 2B, Figure 4A, Figure 5A). Many LZ B cells had rearranged VH sequences that were identical or nearly identical to

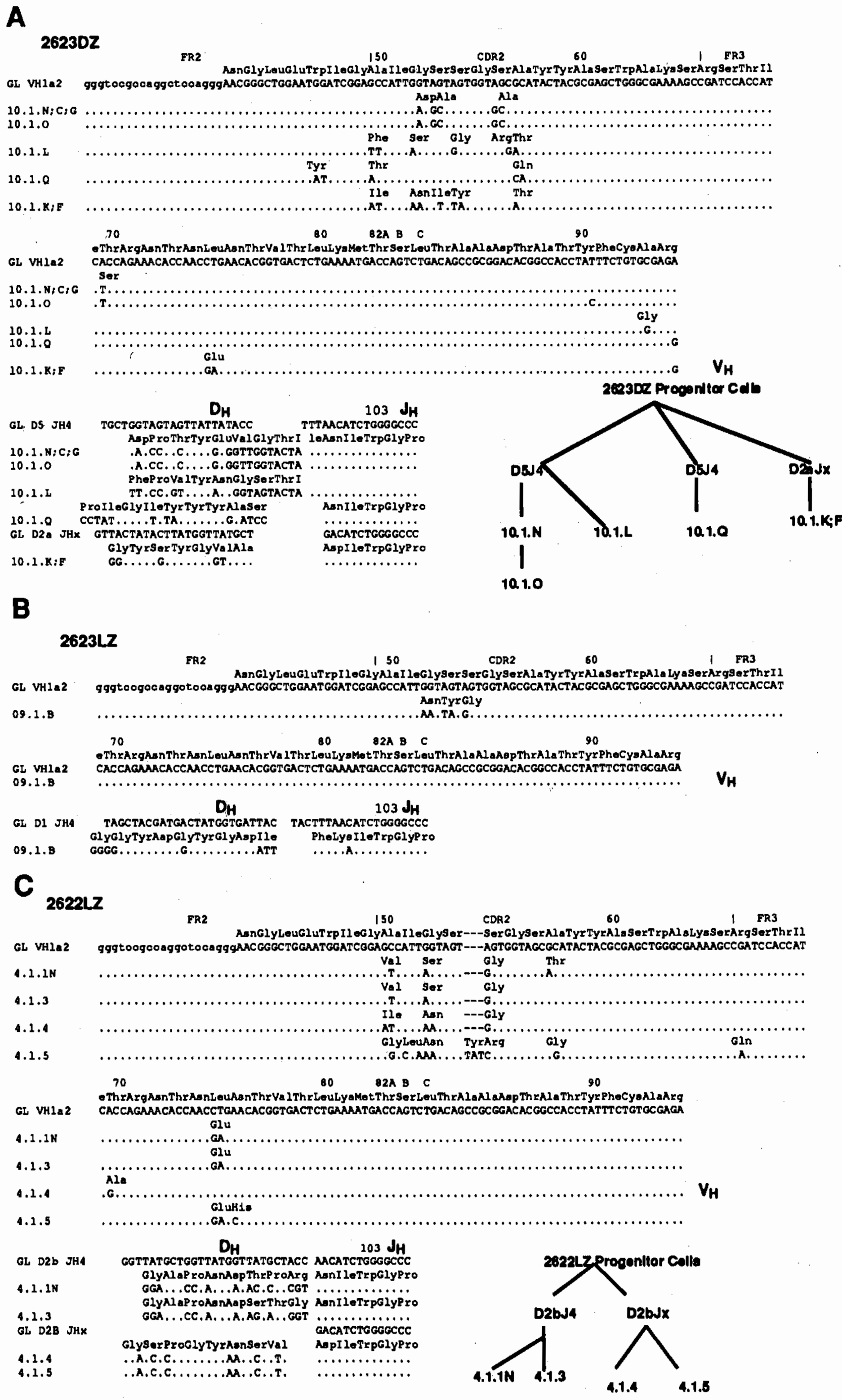


Figure 5. Sequences of Rearranged VH Genes from Two Different GC Compared with Germline VH1a2, DH, and JH

(A–C) Amino acids are numbered according to Kabat et al. (1991). Dots signify identity to germline DNA sequence and only base changes and amino acid replacements are shown. Lower case letters show the sequence of the PCR primer at the 5′ end in FR2. The diagrams next to (A) and (C) show the deduced clonal relationships of sequences from common and distinct progenitor cells. Sequences 4.1.1N and 4.1.3 were recovered from clones from two independently amplified aliquots of the same extract done more than 1 year apart. The sequence 4.1.4 was found in both independently amplified aliquots.

germline VH1a2. In 2611LZ, all six clones came from a common progenitor B cell, as shown by the complete identity of their DH–JH regions (see Figure 2C). The rearranged VH sequences from 2611LZ B cells are close to the germline VH1 sequence. In all but one clone, a single change in CDR2, (G to T), led to replacement of Gly with Val. Two sequences, 5231 and 5227, also picked up single base pair changes in FR3, one of which led to replacement of Arg 94 by Ser. The silent change in Tyr 90 that involved T to C was also found in sequence 2623DZ 10.1.0 from another GC (Figure 5). One clone, 5236, contained a track of five changes in an 8 bp stretch leading to replacement of 4 aa in CDR2. This type of track is similar to those seen in 2631DZ and 2611DZ, again suggesting that gene conversion–like events occurred using upstream donor sequences. Even though many LZ sequences tend to be close to the germline VH sequence, 5236 shows that there is no exclusion from the LZ of B cells that have extensively diversified the heavy chain variable region. A simple evolutionary tree can be drawn showing the relationships between B cells of 2611LZ with different VH sequences (see Figure 2C).

Sequences from B Cells in Two Other Germinal Centers Confirm Most of the Trends Found in 2611LZ and 2611DZ

DNA sequences of rearranged VH genes cloned from B cells from three other GCs, 2626, 2623, and 2622, maintained many of the trends seen for B cells from 2631DZ, 2611DZ, and 2611LZ (see Figure 4; Figure 5). B cells in both 2626DZ (see Figure 4A) and 2623DZ (Figure 5A) had rearranged VH sequences that were highly diversified, with mainly replacement changes in CDR2. In contrast with 2631 and 2611, not all isolated B cells from 2626DZ came from a single clonal precursor; they appear to have arisen from at least two distinct progenitor cells that were able to grow out (see Figure 4A). The degree of diversity of VH sequences from 2626DZ varies with replacement of 4–7 nt and 3–5 aa in CDR2. Once again, we found that the majority of the changes occurred in blocks suggestive of gene conversion, with a few scattered replacements that could have been due to somatic hypermutation. For example, 8.1.C, 8.1.D, and 8.1.21 of 2626DZ shared gene conversion–like changes in CDR2 and the 2 bp change at position 75 (CT to GA) in FR3, replacing Leu with Glu that was described for several clones from 2631DZ. Clones 8.1.D and 8.1.21 each picked up a unique single base pair change in CDR2 (see Figure 4A). These point mutations may have occurred through somatic mutation or a secondary gene conversion after the occurrence of a shared gene conversion event. The changes were probably not introduced by PCR because we recovered clones with 8.1.D and 8.1.21 sequences from independent PCR amplifications of the same extract done more than 1 year apart.

The LZ of GC 2626 contained B cells that can be divided into two groups (see Figure 4B). The first group, represented by DNA clone 7.1.a, had rearranged heavy chain variable region sequences identical to that of germline VH1, or had only a single replacement change in FR2 (A to T; Glu to Asp) (clone 7.1.11). The second group had sequences that were highly diversified, with long tracks of changes found in DNA clones 7.1.17, 7.1.1, and 7.1.2, most likely acquired by gene conversion. Most of the changes were located within CDR2, but 7.1.17 also had 3 bp changes in FR2 that led to replacement of two germline-encoded amino acids as well as six changes in CDR2 that led to four additional replacements. Although a donor for the postulated gene conversion of this stretch is not known, the entire set of silent and replacement changes from amino acid positions 43–54 was also found in a cDNA clone, BB1M2, obtained from splenic mRNA of a 2-week-old *ali/ali* rabbit (Chen et al., 1993) (see Figure 3D). Rabbits of this mutant strain lack VH1a2 (Allegrucci et al., 1990; Knight and Becker, 1990). The expressed mRNA resembled VH4a2 but had acquired each of these base changes. This may be explained if gene conversion had occurred in BB1M2 and 2626LZ 7.1.17, utilizing the same or a similar donor gene. BB1M2 and 7.1.17 differed downstream of amino acid position 54 (see Figure 3D) and had completely different DJ regions. Two identical clones, 7.1.1 and 7.1.2., contained GAA in FR3, leading to replacement of Leu at position 75 with Glu (see Figure 4B). Once again, we found a GC region that developed from multiple B cells with at least three progenitor cells forming 2626LZ (Figure 4B).

All DNA clones from 2623DZ were highly diversified from the germline VH1 DNA sequence, primarily in CDR2; it appears that progeny of at least three different progenitor cells were expanding in the 2623DZ (Figure 5A). A few single base pair changes were found in the framework regions and two identical clones, 10.1.K and 10.1.F, had the CT to GA change described for clones from 2631DZ and 2626DZ. We found 2–5 aa replacements reflecting 3–8 bp changes in the CDR2 region in clones from 2623DZ. For one sequence, 10.1.Q, we found a germline VH gene related to a possible gene conversion donor, VH4a2 (see Figure 3E). A new germline gene with similarity to 10.1.K;F in CDR2 and FR3 has recently been identified in our laboratory (H. T. Chen et al., unpublished data).

We were able to identify only one unique DNA sequence from 2623LZ (Figure 5B). The DNA sequence of 2623LZ 09.1.B was identical to the germline sequence in FR2 and FR3. However, a stretch of five changes in 7 bp in CDR2 led to the replacement of three consecutive amino acids, most likely through gene conversion. These identical changes have been found in a cDNA clone BB4G18 from splenic mRNA of an 8-week-old *ali/ali* rabbit (Chen et al., 1993); a germline gene, RVH710, encoded two of the three codons (see Figure 3F) (Roux et al., 1991).

Four B cell clones from 2622LZ arose from two different founders. Two, 4.1.4 and 4.1.5, from a common progenitor were diversified differently in CDR2, with 4.1.5 picking up a 3 bp insertion (TAT) (Figure 5C). An independent PCR amplification of the 2622LZ extract done more than 1 year later yielded a second clone with a sequence identical to 4.1.4. In addition, we recovered a clone, 4.1.1N, with a new sequence that was clearly derived from the same progenitor as 4.1.3. Sequence 4.1.1N had one additional replacement change in CDR2 and three in the DH region.

Clones 4.1.5, 4.1.3, and 4.1.1N contained the CT to GA change at position 75 described earlier (Figure 5C).

Discussion

Development of a Primary Antibody Repertoire Occurs in the Rabbit Appendix and Involves Gene Conversion and Somatic Hypermutation of Rearranged VH

Our data suggest that in the young rabbit appendix, rearranged VH genes undergo a somatic diversification process similar to that found in the bursa of Fabricius of young chickens (Thompson and Neiman, 1987; Reynaud et al., 1987, 1989). We found changes in rearranged VH sequences in 6 week rabbit appendix GCs that may have been generated by both gene conversion and somatic hypermutation. This diversification may compensate for the limited number of VH genes that rearrange in rabbit B cells. The long tracks of multiple base pair changes in the CDR2 of rearranged VH genes from GC DZ B cells do not appear to have arisen by the stepwise acquisition of single base pair changes observed during somatic hypermutation (Jacob et al., 1991; Kuppers et al., 1993). The unique blocks of changes with little or no similarity to each other in VH sequences from B cells derived from a common GC progenitor do not suggest a fine tuning process such as affinity maturation by somatic hypermutation, where the stepwise changes in sequence may alter antibody affinity for a specific antigen (for discussion, see Berek and Ziegner, 1993; Maizels, 1993). These large blocks of changes may reflect a process of genetic diversification that results in a highly diverse repertoire with specificities for different antigens. We found as many as 7 out of 16 aa replaced in CDR2. The large number of changes in the antibody-combining site would most likely lead to a new antigen specificity, distinct from that of other B cells that are part of the same clonal outgrowth in an individual GC zone.

As in earlier studies that identified possible donor sequences for a gene conversion–like process of diversification of rearranged rabbit VH (Becker and Knight, 1990), we identified germline sequences similar to putative donor sequences for several of our diversified sequences (Figure 3). The length of the identified donated stretch varied, with the potentially longest one, 2631DZ 11.1.10 (Figure 3A), beginning in FR2, containing a one codon (TAT) insertion in CDR2, and ending in FR3. The length of a single gene conversion segment in the chicken VL region has been shown to vary from as little as 8 bp to as large as 249 bp (McCormack et al., 1991). There are probable reasons why we were unable to determine the DNA donor for every sequence. First, only a limited number of VH sequences from VHa2 rabbits have been published (Knight and Becker, 1990; Roux et al., 1991) and many were purposely selected because they had unusual sequences related to the VHa1 allotype (Roux et al., 1991); the few diversified VH sequences we obtained from four GC may represent only a small sample of a hundred or more possible donor sequences. In addition, alterations of the original converted sequences by subsequent rounds of individual point mutations, gene conversion events, or both might make identification of the original donors difficult.

Gene conversion may not be the sole mechanism responsible for all the changes we saw in the rearranged VH sequences. Somatic hypermutation probably also occurred. In fact, examination of the cloned DNA sequences suggests that diversification may be a stepwise process with an initial round of gene conversion, followed by round(s) of somatic mutation (or conversion that introduced point mutations). There were ten clonally related sequences, each ~200 bp long, that each had a single base change. This frequency of point mutations was at least twice that expected from errors introduced by Taq polymerase (Jacob et al., 1991, 1993) and only half of the changes were G to A and C to T transitions associated with Taq errors (Keohavong and Thilly, 1989). Therefore, we believe that many of the single base pair changes found were real.

Primary Localization of Mutations in CDRs

We found that most of the changes in VH sequence localized to the CDRs, with few mutations in either FR2 or FR3. One probable explanation for this is that there was greater sequence identity between the DNA donor and recipient sequences within these two FR than within their CDRs. In addition, since the FR regions are primarily responsible for the structure of the antibody molecule, some changes in the amino acid sequence of these regions may have disastrous effects on antibody conformation. B cells that acquired this type of change may have already undergone cell death.

Structural Motifs for Gene Conversion and a Recurrent Replacement Change

The frequent replacement of CT with GA or GAA leading to replacement of Leu with Glu at codon position 75 in FR3 may reflect a new conformation for which the FR is preferentially selected. It is commonly observed that during affinity maturation, a particular base pair change is selected during the later stages of the GC reaction (reviewed by Berek and Ziegner, 1993). However, the changes associated with affinity maturation have generally been localized in CDRs. The chance that antigen selection led to the two or three consecutive base pair changes we found at such a high frequency in FR3 appears remote. It is possible that the CT to GA or GAA change, which was so prevalent in VH sequences cloned from different GC regions, was mediated by a gene conversion–like event, since only sequences that had undergone diversification in CDR2 contained this change. The change may be linked to gene conversion–like events that occur in CDR2 or reflect a recombination hotspot; a sequence, CACGGTG, with similarity to the consensus heptamer of the immunoglobulin recombination signal sequence, CACAGTG (reviewed by Blackwell and Alt, 1988), is located only 5 bp downstream from the site of change (Figure 1D). We also searched for sequence motifs noted by others as intrinsic hotspots in somatic hypermutation (Betz et al., 1993) or occurring near nonhomologous recombination breakpoints involving immunoglobulin se-

quences (Chou and Morrison, 1993). The CTGG motif of Chou and Morrison (1993) and the CAGCT motif of Betz et al. (1993) colocalized in the rabbit VH1a2 sequence at the TGG of conserved Trp codons at positions 36 and 62 (Figure 1D). These positions enclosed most of the positions in CDR 2 that we propose were affected by gene conversion; possibly they acted as recognition signals during the gene conversion–like process.

Lack of Clonal Relationship Between DZ and LZ or DZ and DZ from the Same Germinal Center

We did not identify any sequences from the DZ of an individual primary GC that showed similarity to a sequence from the LZ of the same GC. However, the DNA sequences amplified from a single extract from B cells scraped from an individual DZ or LZ showed clonal relationships and could be traced back to a minumum of one to four progenitors. Kuppers et al. (1993), in similar comparisons of rearranged V genes from individual B cells from human lymph nodes (secondary lymphoid tissue), did find clonally related B cells in LZ and DZ. However, only a few of the B cells in the DZ had a homolog in the LZ, and several LZ B cells also did not appear clonally related to a B cell in the DZ. In rabbit gut-associated lymphoid tissue, the antigens may be complex mixtures that cause stimulation as microbes and foods are continually sampled from the gut. In the human lymph nodes, complex antigens related to infection(s) may have caused the chronic GC reactions that were studied. The GCs of 6 week rabbit appendix are very large when compared with those from lymph nodes, spleen, and Peyer's patch; they may commonly harbor multiple reactions in a single GC region initiated by specific antigens, mediators of proliferation, or both. The lack of clonally related B cells isolated from a nearby section of the same rabbit appendix GC (Figures 2A and 2B; 2631DZ and 2611DZ) may be similarly explained, especially if the cells that gave amplified products were isolated from distant points in the same DZ (see Figure 1C).

Similarities and Differences Between the Chicken and Rabbit

Chicken bursal development and diversification of VH and VL begin even before exposure to antigen. Most B cells probably arrive in the bursa with their VH–DH–JH and VL–JL gene segments already rearranged (Pink et al., 1985; Ratcliffe et al., 1986; Weill et al., 1986; Weill and Reynaud, 1987; Mansikka et al., 1990). After diversification occurs in the bursa, self-renewing populations of progenitor B cells seed the periphery and may be the source of protective antibodies during the lifetime of the chicken; bursal involution starts at about 12 weeks and by adulthood, the chicken bursa completely involutes (Ackerman and Knouff, 1959). The development of appendix GCs is probably an antigen-driven process in rabbits (Stepánková et al., 1980; Tlaskalová-Hogenová and Stepánková, 1980). V–D–J rearrangements in rabbit B cell progenitors probably occur in fetal and newborn liver and bone marrow and, as this process continues later in life, the majority of rearrangements probably occur in bone marrow (Hayward et al., 1978; Gathings et al., 1981, 1982). After completing an initial round of selection, cells with membrane μ heavy chains may exit and migrate to the appendix. Diversification of rearranged VH1 in the rabbit appendix is extensively occurring by 6 weeks of age but, unlike chicken bursa and SIPP (Ackerman and Knouff, 1959; Reynolds and Morris, 1983, 1984), the rabbit appendix does not involute and could conceivably be a site for continuing VH gene diversification in older animals (Mage, 1993). Instead of involuting, the rabbit appendix goes through a transformation during the first year of life that leaves a tissue with smaller GCs that resemble those in jejunal Peyer's patches (P. D. W. et al., submitted). However, older rabbits probably have a high proportion of cells with properties of self-renewal in the periphery and a low proportion of cells that are newly generated from bone marrow (reviewed by Mage, 1993). It remains to be determined whether both primary functions, such as production of the primary antibody repertoire, and secondary functions, such as affinity maturation, can occur concurrently in the young or adult rabbit appendix.

Implications for B Cell Development in Germinal Center Light and Dark Zones

We found that every DNA sequence from B cells collected from DZs of germinal centers was highly diversified, most likely by a gene conversion–like mechanism. In the LZs, many sequences were highly diversified but others were close to or identical to the germline VH1a2 sequence. These results do not fit with the simplistic idea that changes in the heavy chain variable region sequence occur in the DZ followed by some form of selection in the LZ (MacLennan et al., 1992; Hardie et al., 1993; reviewed by Berek and Ziegner, 1993). Perhaps this is because the migration pathway for germinal center B cells of both primary and secondary lymphoid tissue is complex. There is a possibility that B cells could repeatedly migrate back and forth between the DZ and LZ (Berek and Ziegner, 1993; Kepler and Perelson, 1993). Kuppers et al. (1993) also found that the GC reactions of human lymph nodes were not as neatly organized as had been predicted. They found clonally related highly diversified sequences in both the LZ and DZ of one human GC. In another GC from a second individual, DZ sequences were generally closer to germline and LZ sequences were significantly more diversified. However, they again found clonally related highly diversified sequences in both the DZ and LZ.

In future experiments, it will be valuable to learn whether the young rabbit appendix produces different cell populations similar to those described by Paramithiotis and Ratcliffe (1993) as emigrating from the chicken bursa. B cells in the rabbit DZ that have highly diversified VH gene sequences may have surface immunoglobulin receptors specific for a wide array of antigens, and some of these might exit directly to the periphery and contribute to the virgin B cell repertoire; a role corresponding to that proposed for short-lived population 1 of the chicken. Once these cells exit to the periphery they might survive and expand only if they encounter antigen, but they would provide protective antibodies during the crucial period when maternal antibodies disappear, between 6–12 weeks after

birth. Some cells may leave the DZ and enter the LZ to develop into cells corresponding to populations 2 and 3 with moderate and long half-lives (postbursal stem cells). This development may include positive and negative selection by antigen. We find many of the cells implicated in the selection process at the luminal side of the LZ in 6 week rabbit appendix. These cells are CD11a$^+$, CD18$^+$, have high levels of expression of class II (Ia), and include large tingible body macrophages and FDC (P. D. W. et al., submitted). Some of the B cells that survive selection in the LZ may reenter the DZ as discussed earlier. In addition, it is likely that further diversification and antigen-selection steps occur in other sites. Future investigations will evaluate VH gene diversification in other sites, such as the sacculus rotundus, spleen, lymph nodes, and Peyer's patches.

Experimental Procedures

Animals

Mixed breed rabbits of the VH1a2 (F–I) haplotype were bred and raised in our own National Institute of Allergy and Infectious Diseases allotype-defined pedigreed colonies. Rabbits were sacrificed at 6 weeks after birth and appendix tissue was removed.

Histochemistry

Semithin 7 μm serial sections of rabbit appendix from 6-week-old rabbits were frozen in optimum cutting temperature (O. C. T.) compound (Miles Scientific, Naperville, Illinois), and cut in an International Equipment Company cryostat microtome. Tissues were air dried and stored in a desiccator cabinet (Sanpla Drykeeper, Fisher Scientific, Philadelphia, Pennsylvania) until used. Sections were rehydrated in phosphate-buffered saline (PBS) with 10% fetal calf serum for 30 min. This and subsequent incubations were at room temperature in a humid chamber. After rinsing with PBS, appendix sections were incubated for 1 hr with biotinylated succinylated wheat germ agglutinin (Vector Laboratories, Burlingame, California) followed by incubation with an avidin–biotin peroxidase complex (Vector Laboratories). The substrate solution of 3 μl 30% hydrogen peroxide and 15 μl 8% nickel chloride in 3 ml of 0.05% diaminobenzidine in PBS (Sigma, St. Louis, Missouri) was incubated on the tissue section for 10 min. After the final rinse, the slides were air dried and stored in a desiccator cabinet.

PCR Amplification and Sequencing of Rabbit Appendix B Cell Variable Region Gene DNA

Germinal center B cells were isolated from individual rabbit appendix GC, LZ, and DZ with an Eppendorf micromanipulator as described by Jacob et al. (1991). Cells were collected from an appendix tissue section by scraping the area containing the desired cellular population (LZ or DZ GC B cells, Figure 1C) with the fine pointed end of a pulled micropipette connected to the control arm of an Eppendorf micromanipulator, breaking off the fine pointed end into a microfuge tube and stored at −70°C. Two scrapings, either from the same region of the DZ or LZ or from different regions within these zones, were taken and each scraping was deposited into a separate microfuge tube. We avoided the region between DZ and LZ where high proportions of apoptotic cells are found. DNA extracts of cells from a single scraping were prepared by adding 5 μl PBS, 15 μl double distilled H_2O, and 5 μl proteinase K (Boehringer Mannheim, Indianapolis, Indiana) at a concentration of 2 mg/ml and incubating at 37°C for 5 hr. Extracts were then heated at 95°C for 10 min and 5 μl of individual DNA extracts amplified using a PCR amplification kit and recombinant Ampli Taq (Perkin Elmer Cetus, Norwalk, Connecticut). Two sets of PCR amplifications of 35 cycles each used primers shown in Figure 1D and the following conditions: 5 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by 30 cycles at 94°C for 1 min and 70°C for 4 min. The sets of VH sequences we report here were each from B cells from a single DNA extract. PCR amplified DNA was purified by agarose gel fractionation, cloned into the EcoRI site of pUC18 with T4 DNA ligase (Boehringer Mannheim), and the ligation mix used to transform library efficiency DH5α (Life Technologies, Gaithersburg, Maryland). Bacterial colonies were screened using two oligonucleotide probes, M8 and M16 (Figure 1D) (Chen et al., 1993). Positive clones were isolated and insert DNA sequenced on both strands by the dideoxy chain termination method (Sanger et al., 1977), with the Taq Track (Promega Biotech, Madison, Wisconsin) or the Circumvent DNA Sequencing kit (New England Biochemicals, Beverly, Massachusetts) labeling with [^{35}S]dATP.

To answer the question of whether we could recover VDJ sequences from the same progenitors in products of independent PCR, 5 μl aliquots from DNA extracts of 2626LZ, 2626DZ, and 2622LZ were independently amplified under conditions described above more than 1 year later. Amplified products were purified and cloned as described above and bacterial colonies were screened with a cocktail of oligonucleotide probes corresponding to germline FR2/CDR2 (AATGGATCGGAGCCATTGGTAGTAGTGGTAGCG) and to some of the changed regions of CDR2/FR3 shown in Figure 4A and Figure 5C. These probes were the following: PRGA, CACCAACGAGAACACGGTGA; 8.1.C, AATACTTATGGTAGCACATAC; 8.1.14, CTAATAGTGGTAGCGCATACTACGCCAGCTGGGCCG; 7.1.1;2, AATAGTGATGGTAGCACAGGCTACGCGAGCTGGGCGAG; 4.1.3, GTCATTAGTAGTGGTGGT; 4.1.4, ATCATTAATAGTGGTGGT. Sequences were obtained on both strands manually or using an Applied Biosystems Model 373A sequencer and their PRISM Dye Terminator Cycle sequencing kit. All DNA sequences were analyzed using MacVector Software (International Biotechnologies, Incorporated, New Haven, Connecticut).

Acknowledgments

We thank Drs. J. Jacob and G. Kelsoe (University of Maryland, Baltimore, School of Medicine) for teaching us the technique for isolation of cells from semithin sections for PCR and DNA sequence analysis, and Dr. R. Hunziker for providing access to the Eppendorf micromanipulator. We also thank G. O. Young-Cooper and C. Alexander for help with maintenance and preparation of animals, and S. Starnes and K. Kenyon for editorial assistance. We appreciate helpful comments from Drs. D. Camerini Otero, H. T. Chen, M. Fitts, N. Francis, P. Fuschiotti, P. Hsieh, and M. Mage.

Received March 31, 1994; revised September 9, 1994.

References

Ackerman, G. A., and Knouff, R. A. (1959). Lymphocytopoiesis in the bursa of Fabricius. Am. J. Anat. *104*, 163–205

Aleksandersen, M., Hein, E. R., Landsverk, T., and McClure, S. (1990). Distribution of lymphocyte subsets in the large intestinal lymphoid follicles of lambs. Immunology *70*, 391–397.

Allegrucci, M., Newman, B. A., Young-Cooper, G. O., Alexander, C. B., Meier, D., Kelus, A. S., and Mage, R. G. (1990). Altered phenotypic expression of immunoglobulin heavy-chain variable-region genes (V_H) in *Alicia* rabbits probably reflects a small deletion in the V_H genes closest to the joining regions. Proc. Natl. Acad. Sci. USA *87*, 5444–5448.

Allegrucci, M., Young-Cooper, G. O., Alexander, C. B., Newman, B. A., and Mage, R. G. (1991). Preferential 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. Immunol. *21*, 411–417.

Alt, F. W., and Baltimore, D. (1982). Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence for three D–JH fusions. Proc. Natl. Acad. Sci. USA *79*, 4118–4122.

Archer, O. K., Sutherland, D. E. R., and Good, R. A. (1963). Appendix of the rabbit: a homologue of the bursa in the chicken. Nature *200*, 337–339.

Becker, R. S., and Knight, K. L. (1990). Somatic diversification of immunoglobulin heavy chain VDJ genes: evidence from somatic gene conversion in rabbits. Cell *63*, 987–997.

Becker, R. S., Zhai, S. K., Currier, S. J., and Knight, K. L. (1989). Ig

VH, DH and JH germline gene segments linked by overlapping cosmid clones of rabbit DNA. J. Immunol. *142*, 1351–1355.

Becker, R. S., Suter, M., and Knight, K. L. (1990). Restricted utilization of V_H and D_H genes in leukemic rabbit B cells. Eur. J. Immunol. *20*, 397–402.

Berek, C., and Ziegner, M. (1993). The maturation of the immune response. Immunol. Today *14*, 400–404.

Bernstein, K. E., Reddy, E. P., Alexander, C. B., and Mage, R. G. (1982). A cDNA sequence encoding a rabbit heavy chain variable region of the V_Ha2 allotype showing homologies with human heavy chain sequences. Nature *300*, 74–76.

Bernstein, K. E., Alexander, C. B., and Mage, R. G. (1983). Nucleotide sequence of a rabbit IgG heavy chain from the recombinant F-I haplotype. Immunogenetics *18*, 387–397.

Betz, A. G., Neuberger, M. S., and Milstein, C. (1993) Discriminating intrinsic and antigen-selected mutational hotspots in immunoglobulin V genes. Immunol. Today *14*, 405–411.

Blackwell, T. K., and Alt, F. W. (1988). Immunoglobulin genes. In Molecular Immunology, B. D. Hames and D. M. Glover, eds. (Washington D. C.: IRL Press), pp. 1–60.

Carlson, L. M., McCormack, W. T., Postema, C. E., Humphries, E. H., and Thompson, C. B. (1990). Templated insertions in the rearranged chicken IgL V gene segment arise by intrachromosomal gene conversion. Genes Dev. *4*, 536–547.

Chen, H.-T., Alexander, C. B., Young-Cooper, G. O., and Mage, R. G. (1993). V_H gene expression and regulation in the mutant *Alicia* rabbit-rescue of V_Ha2 allotype expression. J. Immunol. *150* 2783–2793.

Chou, C. L., and Morrison, S. (1993). A common sequence motif near nonhomologous recombination breakpoints involving Ig sequences. J. Immunol. *150*, 5350–5360.

Cohn, M., and Langman, R. E. (1990). The protecton: the evolutionarily selected unit of humoral immunity. Immunol. Rev. *115*, 1–131.

Cooper, M. D., Perey, D. Y., Gabrielsen, A. E., Sutherland, D. E. R., McKneally, M. F., and Good, R. A. (1968). Production of an antibody deficiency syndrome in rabbits by neonatal removal of organized intestinal lymphoid tissues. Int. Arch. Allergy *33*, 65–88.

Ekino, S. (1993). Role of environmental antigens in B cell proliferation in the bursa of Fabricius at neonatal stage. Eur. J. Immunol. *23*, 772–775.

Friedman, M. L., Tunyaplin, C., Zhai, S., and Knight, K. L. (1994). Neonatal V_H, D and J_H gene usage in rabbit B lineage cells. J. Immunol. *152*, 632–641.

Gathings, W. E., Mage, R. G., Cooper, M. D., Lawton, A. R., and Young-Cooper, G . O. (1981). Immunofluorescence studies on the expression of V_Ha allotypes by pre-B and B cells of homozygous and heterozygous rabbits. Eur. J. Immunol. *11*, 200–206.

Gathings, W. E., Mage, R. G., Cooper, M. D., and Young-Cooper, G. O. (1982). A subpopulation of small pre-B cells in rabbit bone marrow expresses light chains and exhibits allelic exclusion of *b* locus allotypes. Eur. J. Immunol. *12*, 76–81.

Glick, G., Chang, T. S., and Jaap, R. G. A. (1956). The bursa of Fabricius and antibody production. Poult. Sci. *35*, 224–225.

Hardie, D. L., Johnson, G. D., Khan, M., and MacLennan, I. C. M. (1993). Quantitative analysis of molecules which distinguish functional compartments within germinal centers. Eur. J. Immunol. *23*, 997–1004.

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*, 1366–1377.

Jacob, J., Kelsoe, G., Rajewsky, K., and Weiss, U. (1991). Intraclonal generation of antibody mutants in germinal centers. Nature *354*, 389–392.

Jacob, J., Przylepa, J., Miller, C., and Kelsoe, G. (1993). In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. J. Exp. Med. *178*, 1293–1307.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991). Sequences of Proteins of Immunological Interest, Fifth Edition. (Washington D. C.: United States Dept. of Health and Human Services).

Keohavong, P., and Thilly, W. G. (1989). Fidelity of DNA polymerases in DNA amplification. Proc. Natl. Acad. Sci. USA *86*, 9253–9257.

Kepler, T. N., and Perelson, A. S. (1993). Cyclic re-entry of germinal center B cells and the efficiency of affinity maturation. Immunol. Today *14*, 412–415.

Knight, K. L. (1992). Restricted VH gene usage and generation of antibody diversity in rabbit. Annu. Rev. Immunol. *10*, 593–618.

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*, 963–970.

Kon-Ogura, T., Kon, Y., Onuma, M., Kondo, T., Hashimoto, Y., and Sugimura, M. (1993). Distribution of T cell subsets in chicken lymphoid tissues. J. Vet. Sci. Med. *55*, 59–66.

Kuppers, R., Zhao, M., Hansmann, M.-L., and Rajewsky, K. (1993). Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. EMBO J. *12*, 4955–4967.

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 novel intermediate of V–(D)–J joining. Cell *59*, 859–870.

Landsverk, T., Halleraker, M., Aleksandersen, M., McClure, S., Hein, W., and Nicander, L. (1991). The intestinal habitat for organized lymphoid tissues in ruminants; comparative aspects of structure, function and development. Vet. Immunol. Immunopathol. *28*, 1–16.

Langman, R. E., and Cohn, M. (1993). A theory of the ontogeny of the chicken humoral immune system: the consequences of diversification by gene hyperconversion and its extension to rabbit. Res. Immunol. *144*, 422–446.

MacLennan, I. C. M., Liu, Y. J., and Johnson, G. D. (1992). Maturation and dispersal of B cell clones during T cell dependent antibody responses. Immunol. Rev. *126*, 141–161.

Mage, R. G. (1993). Rabbit facts and diversification of VH sequences by gene conversion. Res. Immunol. *144*, 476–485.

Mage, R. G., Bernstein, K. E., McCartney-Francis, N., Alexander, C. B., Young-Cooper, G. O., Padlan, E. A., and Cohen, G. H. (1984). The structural and genetic basis for expression of normal and latent V_Ha allotypes of the rabbit. Mol. Immunol. *21*, 1067–1081.

Maizels, N. (1993). Preimmune diversification creates a repertoire while somatic hypermutation fine tunes affinity: implications for the process of mutation. Res. Immunol. *144*, 459–463.

Mansikka, A., Sandberg, M., Lasilla, O., and Toivanen, P. (1990). Rearrangement of immunoglobulin light chain genes in the chicken occurs prior to colonization of the embryonic bursa of Fabricius. Proc. Natl. Acad. Sci. USA *87*, 9416–9420

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. Nucl. Acid Res. *13*, 7041–7054.

McCormack, W. T., Tjoelker, L. W., Carlson, L. M., Petryniak, B., Barth, C. F., Humphries, E. H., and Thompson, C. B. (1989). Chicken IgL gene rearrangement involves deletion of a circular episome and addition of single nonrandom nucleotides to both coding segments. Cell *56*, 785–791.

McCormack, W. T., Tjoelker, L. W., and Thompson, C. B. (1991). Avian B cell development: generation of an immunoglobulin repertoire by gene conversion. Adv. Immunol. 9, 219–241

Nieuwenhuis, P., Nouhuijs, C. E. van, Eggens, J. H., and Keuning, F. J. (1974) Germinal centres in the rabbit appendix: I. germinal centres in the rabbit appendix. Immunology *26*, 497–507.

Paramithiotis, E., and Ratcliffe, M. J. H. (1993). Bursa dependent subpopulations of peripheral B lymphocytes in chicken blood. Eur. J. Immunol. *23*, 96–102.

Parvari, R., Ziv, E., Lentner, F., Heller, D., and Schecter, I. (1990). Somatic diversification of chicken immunoglobulin light chains by point mutations. Proc. Natl. Acad. Sci. USA *87*, 3072–3078.

Pink, J. R. L., Ratcliffe, M. J. H., and Vainio, O. (1985). Immunoglobulin-bearing stem cells for clones of B (bursa-derived) lymphocytes. Eur. J. Immunol. *15*, 617–620.

Ratcliffe, M. J. H., Lassila, O., Pink, J. R. L., and Vainio, O. (1986). Avian B cell precursors: surface immunoglobulin expression is an early, possibly bursa-independent event. Eur. J. Immunol. *16*,129–133

Reynaud, C. A., Anquez, V., Grimal, H., and Weill, J. C. (1987). A hyperconversion mechanism generates the chicken light chain preimmune repertiore. Cell *48*, 379–388.

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*, 171–183.

Reynaud, C. A., Mackay, C. R., Muller, R. G., and Weill, J. C. (1991). Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches. Cell *64*, 995–1005.

Reynolds, J. D., and Morris, B. (1983). The evolution and involution of Peyer's patches in fetal and postnatal sheep. Eur. J. Immunol. *13*, 627–635.

Reynolds, J. D., and Morris, B. (1984). The effect of antigen on the development of Peyer's patches in sheep. Eur. J. Immunol. *14*, 1–6.

Roux, K. H., Dhanarajan, P., Gottschalk, V., McCormack, W. T., and Renshaw, R. W. (1991). Evidence for gene conversion at both the germline and somatic levels. J. Immunol. *146*, 2027–2035.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

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. Immunol. *147*, 4014–4018.

Stepánková, R., Kováru, F., and Kruml, J. (1980). Lymphatic tissue of the intestinal tract of germ-free and conventional rabbits. Folia Microbiol. *25*, 491–495.

Sutherland, D. E. R., Archer, O. K., and Good, R. A. (1963). Role of the appendix in development of immunologic capacity. Proc. Soc. Exp. Biol. Med. *115*, 673–676.

Thompson, C. B., and Neiman, P. E. (1987). Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. Cell *48*, 369–378.

Tlaskalová-Hogenová, H., and Stepánková, R. (1980). Development of antibody formation in germ-free and conventionally reared rabbits: the role of intestinal lymphoid tissue in antibody formation to E. coli antigens. Folia Biol. *26*, 81–93.

Waksman, B. H., Ozer, H., and Blythman, H. E. (1973). Appendix and M-antibody formation. VI. The functional anatomy of the rabbit appendix. Lab Invest. *28*, 614–626.

Weill, J. C., and Reynaud, C. A. (1987). The chicken B cell compartment. Nature *238*, 1094–1098.

Weill, J. C., Reynaud, C. A., Lassila, O., and Pink, J. R. L. (1986). Rearrangement of chicken immunoglobulin genes is not an ongoing process in the embryonic bursa of Fabricius. Proc. Natl. Acad. Sci. USA *83*, 3336–3340.

Weinstein, P. D., Mage, R. G., and Anderson, A. O. (1994). The appendix functions as a mammalian bursal equivalent in the developing rabbit. In Lymphatic Tissues and Germinal Centers in Immune Reactions. (New York: Plenum Press), in press.

The GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are U07070–U07082 (Figure 2); U07117–U07124 (Figure 4); and U07109–U07116, U07125, and U14139 (Figure 5).