

# Analyses of Single B Cells by Polymerase Chain Reaction Reveal Rearranged $V_H$ with Germline Sequences in Spleens of Immunized Adult Rabbits: Implications for B Cell Repertoire Maintenance and Renewal<sup>1</sup>

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We used PCR to amplify rearranged  $V_HDJ_H$  genes in single cells collected by micromanipulation from splenic germinal centers of immunized adult rabbits. In the course of the study, the objective of which was to analyze diversification of rearranged  $V_HDJ_H$  sequences, we were surprised to find cells 7 and 10 days after immunization with rearranged  $V_H1a2$  as well as a-negative ( $\gamma33$  and  $x32$ ) sequences that were identical or close to germline (10 or fewer changes). About 58% (82/140) of the sequences had unique CDR3 regions and were unrelated. In seven different germinal centers, we found one to four different clones with two to seven members. Clonally related cells underwent diversification by hypermutation and gene conversion. We found that contrary to published reports, adult rabbits indeed have newly diversifying B cell receptors in splenic germinal centers. The attractive idea that the rabbit, like the chicken, develops its B cell repertoire early in life and depends upon self-renewing cells in the periphery to maintain its B lymphocyte pool throughout life, is challenged by the current finding. Although a major population of B lymphocytes may be generated early in life, diversified extensively, and maintained by self-renewal in the periphery, some sources of cells with sequences close to germline do exist in adult rabbits and appear in the developing germinal centers. Although considerable repertoire diversity is generated in young rabbits, mechanisms for continued generation of B cell receptor diversity are retained in adult life, where they may confer survival advantage. *The Journal of Immunology*, 1998, 161: 5347–5356.

In most mammals, lymphopoiesis occurs throughout life (1–4). In mice and humans, the fetal liver, omentum, and bone marrow serve as primary sites for B cell development (5–8), and rearrangement of Ig genes takes place continuously in the adult bone marrow from uncommitted progenitors. Rabbits are most similar to chickens in that there is rearrangement and diversification of mainly a single  $V_H$  gene ( $V_H1$ ) (9–12). Diversification occurs by gene conversion in the bursa of Fabricius in young chickens (13, 14) and the appendix and other gut-associated lymphoid tissues (GALT)<sup>3</sup> of young rabbits (15). There are both anatomic and functional similarities between the chicken bursa and the rabbit appendix (15–17). Surgical removal of the appendix, sacculus rotundus, and Peyer's patches from neonatal rabbits had a profound effect on B cell development (18), and such rabbits had reduced levels of somatic diversification in their VDJ sequences (19).

It has been proposed by Crane et al. that just as lymphopoiesis does not occur in adult chickens, little or no B lymphopoiesis occurs in adult rabbits (20). Recently, this group reported that by 4 wk of age, expressed VDJ genes from peripheral blood leukocytes had, on average, three nucleotide changes per  $V_H$  region, with approximately 75% of the genes showing some diversification. By 6–8 wk of age, all but 1 of 35 sequences analyzed were diversified, and the average number of nucleotide changes per  $V_H$  region increased to 12. Using an RNase protection assay, they reported that by adulthood, essentially all expressed VDJ genes in cells from appendix, peripheral blood, and bone marrow were diversified. In addition, reduced levels of recombination signal joints (VD and DJ excision circles) were found in bone marrow of adult rabbits compared with the levels found in bone marrow of newborn rabbits. This led them to conclude that B lymphopoiesis is limited in adults (20). The finding of only highly diversified VDJ sequences in the adult rabbit is in keeping with the notion that the rabbit, like the chicken, develops its B cell repertoire early in life and depends upon self-renewing cells in the periphery to maintain its B lymphocyte pool throughout life.

The present study was initiated to investigate  $V_H$  sequences in splenic germinal centers during T cell-dependent immune responses to a protein or a hapten in adult rabbits. We adopted an experimental approach that combined the techniques of microdissection of single Ag-specific B cells with a PCR-based sequencing strategy that avoids PCR artifacts. This approach is especially powerful to study rabbit germinal center B cells because rabbit B cells rearrange only a few  $V_H$  genes and the majority do not rearrange the second allele. In the course of this study, we were surprised to find that cells collected during early stages of the germinal center reaction from adult rabbit spleen had rearranged  $V_H$  sequences that were identical or nearly identical to germline sequences. Cells with predominantly rearranged  $V_H1a2$  as well as

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<sup>3</sup> Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; ABC-AP, avidin-biotin complex conjugated to alkaline phosphatase; BGG, bovine  $\gamma$ -globulin; CDR, complementarity-determining region; DNP-AP, dinitrophenylated alkaline phosphatase; HP, hypothetical precursor; TBS, Tris-buffered saline.

other sequences (V<sub>H</sub>4, x32, and y33) that were identical or close to germline sequences (10 or fewer changes) were found in spleens of the immunized rabbits.

## Materials and Methods

### Ag preparation and immunization

Dinitrophenylated bovine  $\gamma$ -globulin (DNP-BGG) was prepared as described by Eisen (21). The number of DNP groups per protein molecule was calculated spectrophotometrically. By measuring absorbance at 360 and 280 nm in 0.1 N NaOH, it was estimated that each protein molecule had bound 37 DNP groups. Rabbits 1–2 yr of age, homozygous for V<sub>H</sub>a2 (haplotype F-I) and Ckb5 allotypes, were given a priming immunization of 500  $\mu$ g of BGG in CFA injected s.c. Seven to 10 days later, 500  $\mu$ g of DNP-BGG was given i.v. Control rabbits received BGG at both time points. Rabbits were sacrificed 7 and 10 days after the second injection.

In an independent experiment, adult rabbits were immunized with 500  $\mu$ g of the fraction 1 (F1) component of *Yersinia pestis* capsular Ag precipitated in alum (22). The Ag was injected intradermally, intraappendix, i.p., and into one Peyer's patch. Rabbits were sacrificed 10 days postimmunization. Spleens in both experiments were removed and frozen in optimum cutting temperature (OCT) compound in dry ice or liquid nitrogen.

### Immunohistochemistry

Seven-micrometer serial sections were cut from the spleens in a cryostat microtome, kept for 30 min at room temperature, fixed at 4°C in acetone for 10 min, and stored at -70°C. DNP-binding cells from splenic germinal centers were revealed by incubation with dinitrophenylated alkaline phosphatase (DNP-AP), followed by incubation with an appropriate substrate, as detailed below. DNP-AP was prepared as follows: 5  $\mu$ l of 2,4-dinitrofluorobenzene (Sigma, St. Louis, MO) was dispersed in 50  $\mu$ l of dimethylformamide and added dropwise to 2 ml of 0.1 M borate buffer (pH 8.5) containing 500 U of alkaline phosphatase (Pierce, Rockford, IL). The solution was dialyzed extensively against Tris-buffered saline (TBS) and recovered in 2 ml of TBS. The optimal working dilution (1/100) was determined empirically. For staining, sections were allowed to reach room temperature, incubated for 20 min with TBS, and incubated further for 1 h at room temperature with DNP-AP. After thorough washing in TBS, sections were incubated with the VECTOR Blue Substrate kit (Vector Laboratories, Burlingame, CA) containing levamisole (to inhibit endogenous alkaline phosphatase activity) and counterstained with the VECTOR Nuclear Fast Red (Vector Laboratories). Spleens from control animals were also stained with DNP-AP to exclude any nonspecific binding. As an additional control, splenic sections from DNP-BGG-immunized rabbits were stained with unconjugated alkaline phosphatase. To identify proliferating cells within germinal centers, an adjacent section was stained with mouse anti-human Ki-67 mAb that cross-reacts with rabbit (Dako, Carpinteria, CA), followed by biotinylated horse anti-mouse IgG (Vector Laboratories) and avidin-biotin complex conjugated to alkaline phosphatase (ABC-AP) (Vector Laboratories). Color development was achieved as described for DNP-AP staining. In some experiments for double staining with both DNP-AP and anti-Ki-67, we used VECTOR Red Substrate kit (Vector Laboratories) in combination with the VECTOR Blue Substrate kit.

Spleens from F1-immunized rabbits were incubated with biotinylated F1 for 1 h at room temperature and subsequently incubated for 30 min with ABC-AP. Sections were washed and the color was developed using the VECTOR Blue Substrate kit containing levamisole. Spleens from control animals were also stained with biotinylated F1, to exclude any nonspecific binding.

### Micromanipulation of single cells

The stained sections were incubated with 5 mg/ml collagenase H (Boehringer Mannheim, Indianapolis, IN), as described (23). Single cells were collected from the sections by means of a hydraulic micromanipulator (Narishige, Greenvale, NY) assembled on an inverted microscope (Olympus, Lake Success, NY). Individual germinal centers were designated by capital letters, sections within a germinal center by lower case letters, and cells were numbered. Individual cells were each transferred to 0.2-ml microfuge tubes containing 5  $\mu$ l of an alkaline lysing solution (200 mM KOH/50 mM DTT). The tubes were incubated at 65°C for 10 min before adding 5  $\mu$ l of neutralizing solution (900 mM Tris-HCl, pH 8.3/300 mM KCl/200 mM HCl).

### Single cell PCR and DNA sequence analysis

We elected to use direct sequencing of PCR products from single cells without a cloning step to avoid two potentially serious artifacts. First, with-

Table I. Primers used for single cell PCR<sup>a</sup>

Primer	Sequence	Source
DSH1 <sup>b</sup>	GCTTCTCCTGGTCGTGTGCTC	Leader (L <sub>H</sub> )
DSH2 <sup>c</sup>	AGTGACAGTGTC (C/T) TGACCATGT	L <sub>H</sub> -V <sub>H</sub> intron
DSH5 <sup>d</sup>	AGGACTCACTGAGGAGACGG	J2/3/4
DSH7 <sup>e</sup>	GCAGCAAAGGCCCTCAGAGTTG	3' to J2
DSH8 <sup>f</sup>	GCAGAAAATCGCTCAGAGTTG	3' to J4

<sup>a</sup> The majority (80–90%) of rabbit B cells use the V<sub>H</sub>1 gene in their VDJ rearrangements. y33 is the most frequently used non-V<sub>H</sub>1 gene. J4 is used most frequently followed by J2 and J6.

<sup>b</sup> Corresponds to part of codon 7 through codon 14 of the leader sequences of V<sub>H</sub>1, 4, y33, and z genes.

<sup>c</sup> Corresponds to a conserved sequence in the L<sub>H</sub>-V<sub>H</sub> intron upstream of V<sub>H</sub>1, 4, and 7. Despite 3 mismatches it also amplifies y33 rearrangements.

<sup>d</sup> Binds the region encompassing the splice donor site 3' of J<sub>H</sub>2, 3, and 4. The reverse complement of donor GT sequence is underlined.

<sup>e</sup> Binds in the intron 30 bases 3' of the last codon of J<sub>H</sub>2.

<sup>f</sup> Binds in the intron 30 bases 3' of the last codon of J<sub>H</sub>4.

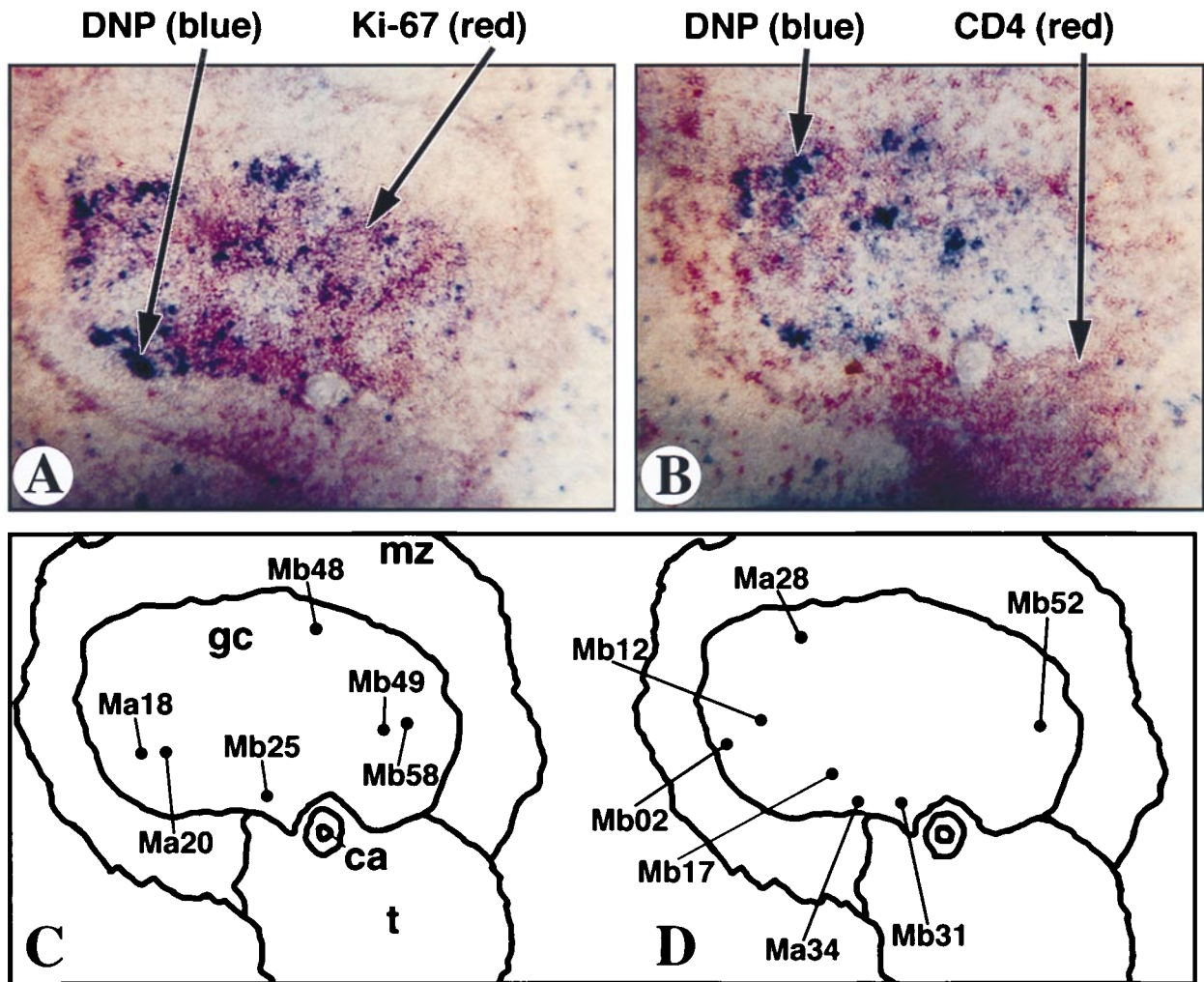
out a cloning step, the effect of *Taq* DNA polymerase errors is minimized (24–26) because *Taq* DNA polymerase-based errors are then detected in sequences only if they occur in the first few cycles of amplification. Second, by amplification of VDJ sequences from single cells, we avoided producing hybrid VDJ sequences that were found when DNA from several cells was amplified, especially during the somatic hypermutation stage of the germinal center reaction (27).

The rearranged VDJ sequences were amplified from single cells that were DNP<sup>+</sup>, Ki-67<sup>+</sup>, or both, collected from DNP<sup>+</sup> germinal centers using a nested PCR strategy. Throughout the entire procedure, care was taken to avoid contamination by DNA. The pre- and postamplification manipulations were done in separate working space using separate equipment. The gloves were changed frequently during the procedure, and aerosol-resistant pipette tips were used. Hot start was done to minimize the nonspecific binding of the primers during the initial cycle by making use of TaqStart Ab (Clontech, Palo Alto, CA). The temperature conditions for the first and second round of touchdown PCR were the same (28). There was an initial 95°C 2-min denaturation step. This was followed by five cycles of denaturation at 94°C for 1 min (for the first cycle, annealing was at 66°C for 30 s, which dropped down to 56°C at the end of the fifth cycle at the rate of 2° per cycle), and an extension step at 72°C for 1 min. The annealing temperature for the remaining 29 cycles was 56°C. The PCR ended with a 5-min extension at 72°C. The PCR was performed on a PTC-100 programmable thermal cycler (96-well model with hot bonnet) (MJ Research, Watertown, MA).

For the first round, MgCl<sub>2</sub>, Tris-HCl (pH 8.3), Triton X-100, and external primers (DSH1, DSH7, and DSH8) (see Table I) were added to the tube containing the neutralized cell lysate such that the final concentrations in a 40- $\mu$ l vol were 3.75 mM, 10 mM, 0.1% (v/v), and 0.75  $\mu$ M (of each primer), respectively. The DNA was denatured at 95°C for 10 min. Thereafter, 20  $\mu$ l of a mix containing 10 mM Tris-HCl (pH 8.3), 0.1% (v/v) Triton X-100, 600  $\mu$ M concentrations of each dNTP, and 5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ) was added, and the tube was set up for PCR.

From the first round PCR, 2.5  $\mu$ l of product served as the DNA template for the second round touchdown PCR (28). The other components in the 50- $\mu$ l PCR reaction were 1 $\times$  PCR buffer (containing MgCl<sub>2</sub>) (Perkin-Elmer), 200  $\mu$ M concentration of each dNTP, 0.5  $\mu$ M concentration of each primer (internal to those used in the first round) (DSH2 and DSH5) (see Table I), and 2 U of AmpliTaq DNA polymerase. The PCR parameters were the same as for the first round.

For the cells picked from F1<sup>+</sup> germinal centers, a whole genome amplification step using 15-mer random primers was included in the protocol (29, 30). This step was performed in the presence of neutralized cell lysate, 2.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.3), 0.1% (v/v) Triton X-100, 100  $\mu$ M concentration of each dNTP, 35  $\mu$ M 15-mer random primers, and 5 U of AmpliTaq DNA polymerase in a volume of 60  $\mu$ l. The thermocycling conditions were those described by Brezinschek et al. (29). For the VDJ amplification, 5  $\mu$ l of the globally amplified genomic DNA served as the template for the first round of PCR, which was done using DSH1 and DSH5 (used at a final concentration of 2.5  $\mu$ M each) in a 50- $\mu$ l reaction (Table I). The second round of PCR used 5  $\mu$ l of the first round PCR product as the input DNA. The two primers used for hemi-nesting were DSH2 and DSH5 (Table I). The



**FIGURE 1.** Splenic germinal center M from a DNP-BGG-immunized adult rabbit sacrificed 10 days after the DNP-BGG injection. *A* and *B*, Sections of germinal center M serial to the one from which cells were collected. *A*, DNP-binding B cells are stained in blue, and Ki-67<sup>+</sup>-proliferating cells are stained in red. *B*, DNP-binding B cells (blue) and CD4<sup>+</sup> T cells (red). *C* and *D*, An outline of the germinal center showing the microanatomy and the approximate location of the cells from which VDJ sequences were recovered from sections serial to the ones shown in *A* and *B*. From this germinal center, DNP<sup>+</sup>, Ki-67<sup>+</sup>, or double-stained cells were collected and analyzed. The approximate locations of cells collected from the germinal center with sequences that had zero to three nucleotide changes compared with the germline V, D, and J gene segments used in the VDJ rearrangement (*C*) and clonally related cells (*D*) are marked. Ma and Mb designate cells that were picked from two adjacent sections. Section Ma was double stained; section Mb was only stained for Ki-67. Cells Ma 20, Mb 25, Mb 48, and Mb 49 had V<sub>H</sub>1 gene-utilizing VDJ rearrangements, and Ma 18 and Mb 58 had used y33 (*C*). The nucleotide sequences obtained from the cells shown in *C* and *D* are available from GenBank under the accession numbers AF058604 (Ma 18-H), AF058605 (Ma 20-H), AF058619 (Mb 25-H), AF058625 (Mb 48-H), AF058626 (Mb 49-H), AF058629 (Mb 58-H), and AF058639-AF058645 (for those shown in *D*). Marginal and mantle zones (mz) (their demarcation in rabbits is not well defined in the absence of a marker like IgD); gc, germinal center; ca, central arteriole; t, T cell area. The original magnification was  $\times 250$ .

PCR conditions were similar to those used for the DNP samples, except that the touchdown protocols were different. Starting with an initial annealing temperature of 65°C, the annealing temperature was dropped at the rate of 1°C/cycle until it reached 55°C. The remaining 29 cycles were done at an annealing temperature of 55°C.

To check for amplification, 5  $\mu$ l of the second round PCR product was run on a 1% agarose gel. If a band of the expected size was observed, the remaining PCR product was gel purified with a QIAgen gel purification kit (Qiagen, Chatworth, CA). Alternatively, the PCR product was purified using the QIAquick PCR purification kit (Qiagen), according to the supplier's protocol. The Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used following the manufacturer's instructions to sequence both strands using the second round primers on ABI model 377 or 373 automated sequencers (Applied Biosystems) (31).

The sequences were analyzed by using Autoassembler version 1.3 (Applied Biosystems) and MacVector software versions 5.0 and 6.0 (Kodak Scientific Imaging Systems, Rochester, NY).

## Results

### *Immunohistochemistry, single cell collection, and sequencing*

In this work, we report an analysis of the rearranged VDJ sequences from rabbit splenic germinal centers collected at the seventh or tenth day of immune responses to a hapten (DNP) or a protein Ag (F1 capsular Ag of *Y. pestis*). Splenic sections were stained to detect regions within which B cells with soluble Ig receptors that bound to DNP or F1 Ag were localized. We also stained for other markers such as CD4 and Ki-67 in some sections. Fig. 1 shows an example of a splenic germinal center from which single cells were collected by micromanipulation and analyzed by nucleotide sequencing.

DNP-binding cells were detected by incubating the splenic sections with DNP-AP (blue). The sections were stained with



Table II. VDJ sequences from splenic germinal centers from DNP-BGG immunized animals

Day Postimmunization	Germinal Center <sup>a</sup>	Cells Collected <sup>b</sup> (VDJs Amplified)	Successful Sequences <sup>c</sup>	Independent Unique Sequences	Groups of Clonally Related Sequences (No. of Members)
7	Ca	28 (10)	6	4	1 (2)
	Da	14 (10)	10	3	2 (3,4)
	Fa, b, c	53 (21)	19	12	3 (2,2,3)
	Ga	23 (12)	12	2	4 (2,2,2,4)
	Jb, c	121 (44)	31	22	2 (3,6)
10	Ia, b	64 (25)	19	10	3 (2,2,5)
	Ma, b	166 (54)	43	29	4 (2,2,3,7)
Total		469 (176)	140	82	

<sup>a</sup> Individual germinal centers were designated by capital letters, sections within a germinal center by lower case letters, and cells were numbered.

<sup>b</sup> Cells from germinal centers C, D, F, G, J, and I were collected on the basis of DNP binding. Cells from section Ma and Mb of germinal center M were collected on the basis of binding to DNP and/or Ki-67 (Ma) and Ki-67 only (Mb).

<sup>c</sup> Refers to the subset of the amplified VDJ sequences from which reliable nucleotide sequence could be obtained. The Genbank accession numbers are: germinal center C, AF058506–AF058511; D, AF058512–AF058421; F, AF058522–AF058540; G, AF058541–AF058552; J, AF058553–AF058583; I, AF058584–AF058602; M, AF058603–AF058645.

anti-Ki-67, which recognizes a nuclear Ag found in proliferating cells (A, red), or with anti-CD4 (B, red). Histologic substructures visible in Fig. 1, A and B, are schematically depicted in C. Also shown in C are the approximate locations of the cells (before microdissection) with zero to three nucleotide changes from germline sequence from two sections serial to the ones shown in A and B. D displays for the same two sections the approximate locations of a set of clonally related germinal center B cells before microdissection, which will be discussed further below. The DNP-binding cells were found interspersed with proliferating (Ki-67<sup>+</sup>) cells (Fig. 1A). DNP-binding cells were also interspersed with CD4<sup>+</sup> cells within the germinal center (Fig. 1B).

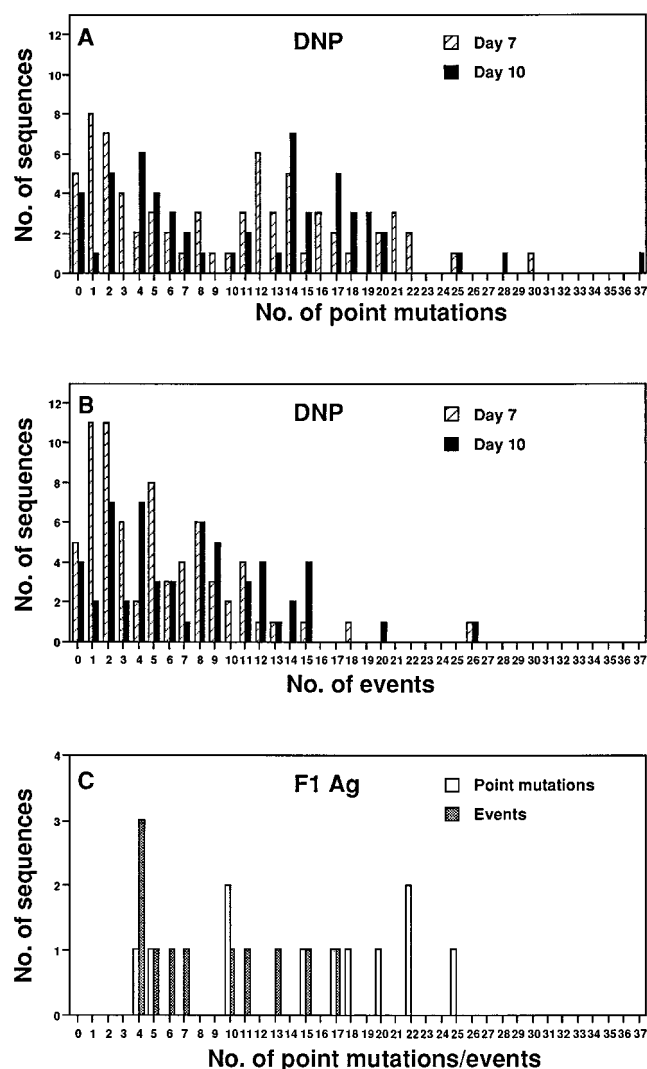
The VDJ portions of rearranged Ig heavy chain were PCR amplified and directly sequenced on both strands. To test the method for reliability, we performed a blind control experiment, in which we collected Ki-67<sup>+</sup> cells from a DNP<sup>+</sup> germinal center and CD4<sup>+</sup> T cells from the T cell area. In the PCR reactions run at the same time, we amplified 48 of 69 VDJ sequences from the cells collected from the germinal center; none of the 23 T cells gave a PCR product. The VDJ sequences obtained from single cells were aligned to the known rabbit germline V<sub>H</sub> gene sequences. A summary of the VDJ sequences obtained from single cells collected from DNP<sup>+</sup> splenic germinal centers on days 7 and 10 from DNP-BGG-immunized animals is shown in Table II. From day 7, 239 cells were collected from five different germinal centers, and from day 10, two different germinal centers contributed 230 cells. From a total of 469 collected cells, 176 VDJ sequences were amplified, giving an overall efficiency of 37.5%. We were able to determine the nucleotide sequence of 140 of the 176 (79.5%) VDJ sequences that were amplified. There were 15 nonfunctional sequences, 11 of which had a frameshift at the V-D or D-J junction, 2 had a stop codon in the D region, 1 in the V region, and 1 a frameshift in the J region. The majority of the cells obtained from a given germinal center contained unique VDJ rearrangements with unique CDR3 (Table II). Thus, at days 7 and 10 of this immune response, the germinal centers do not appear oligoclonal. However, in addition to the 82 independent unique VDJ rearrangements, we did find 12 and 7 groups of clonally related sequences from day 7 and 10 DNP-BGG-immunized animals, respectively. The groups were comprised of two to seven members, groups with two to three members being most common. By the fifteenth day, germinal centers became oligoclonal, were switching to IgG expression, and had dramatically fewer unique VDJ sequences. We detected no germline VDJ sequences, and 82 of 85 sequences had more than 10

changes from germline (the remaining three had 10, 9, and 9 changes; E. Schiaffella, D. Sehgal, A. O. Anderson, and R. G. Mage, manuscript in preparation).

#### *Germline or near germline VDJ sequences are present in the spleen of immunized adult rabbits*

The V<sub>H</sub> gene used in the VDJ rearrangement was determined by aligning the sequence with the rabbit germline V<sub>H</sub> genes in GenBank. As expected, the majority used the V<sub>H</sub>1a2 gene. The number of nucleotide changes (scored as single base mutations) relative to the V<sub>H</sub>1a2 sequence from all of the V<sub>H</sub>1 gene-utilizing VDJ rearrangements from day 7 and 10 DNP-BGG-immunized animals are shown in Fig. 2A. We found VDJ sequences identical to germline on the one hand, and sequences with as many as 37 nucleotide changes relative to V<sub>H</sub>1 on the other. Nine B cells had germline V<sub>H</sub>1 gene in their VDJ rearrangements (Fig. 2A). The overall pattern appeared bimodal, with a dip at about 9 mutations. The bimodal distribution was still observed when only the 82 unique sequences were analyzed. A closer examination of the nucleotide sequences revealed that several of the observed nucleotide changes could be accounted for by one or more gene conversion-like events, in which the donor for the block is a known germline V<sub>H</sub> gene. When the data in Fig. 2A were replotted as a function of number of events (counting introduction of a gene conversion block as one event), there was a general shift toward the y-axis (Fig. 2B). Although the data from animals immunized with the protein Ag F<sub>1</sub> are limited, the trend is strikingly similar to that seen in the case of DNP-immunized animals (Fig. 2C).

Although V<sub>H</sub>1 is rearranged in the majority of rabbit B lymphocytes, other V<sub>H</sub> genes are known that rearrange at low frequencies. Table III summarizes the 14 non-V<sub>H</sub>1 gene-utilizing VDJ sequences obtained from single cells collected from splenic germinal centers of DNP-BGG-immunized animals on days 7 and 10. In addition to the nine B cells with VDJ sequences with germline V<sub>H</sub>1 (Fig. 2A), there are three cells with germline y33 in their VDJ rearrangements and one cell with germline V<sub>H</sub>4 (Table III). Most of the non-V<sub>H</sub>1 gene-utilizing VDJ sequences have nine or fewer nucleotide changes compared with the corresponding V<sub>H</sub> gene used in the VDJ rearrangement, with the exception of one (Da 02-H) that has 12 nucleotide changes relative to the sequence of rearranging y33 gene (Table III). This and three other sequences contained blocks that could have been due to gene conversion-like events. Thus, in Table III (last column), the number of events to obtain the sequences from germline were 0 to 7.



**FIGURE 2.** Point mutations/events in VDJ sequences with rearranged  $V_H1a2$  from splenic germinal centers of DNP-BGG- and F1 Ag-immunized animals. Number of VDJ sequences from the DNP-BGG-immunized animals plotted as a function of number of point mutations (A) and as a function of number of events (B). The corresponding data from animals immunized with F1 Ag are shown in C. The nucleotide sequence obtained from cells collected from splenic germinal centers of rabbits immunized with DNP-BGG or F1 Ag is available from GenBank under accession numbers AF058506–AF058645 and AF058646–AF058656, respectively.

#### Clonal expansion, gene conversion-like and mutational changes in B cells with germline or near germline VDJ rearrangements

The bimodal distribution observed in Fig. 2 indicates the presence of two different populations of cells in the germinal centers. The first population comprises B cells that have germline or near germline VDJ sequences. The other population represents B cells that have 10 or more nucleotide changes relative to the rearranging  $V_H$  gene. We found that some B cells with germline or near germline VDJ sequences underwent clonal expansion. During the expansion, some members of the expanded clone underwent gene conversion-like events, in which the donated block could be accounted for by a known germline  $V_H$  gene. This occurred in clones identified on both day 7 and 10 of the immune response to the hapten DNP. Examples illustrating this point are shown in Fig. 3, A and B. The clone shown in Fig. 3A was initiated by a B cell with rearrangement of  $V_H1-D2x-J4$ . During clonal expansion, Jb 09-H

**Table III.** *Non* $V_H1$  gene utilizing VDJ sequences from single cells collected from DNP<sup>+</sup> splenic germinal centers from DNP-BGG immunized animals

Day Postimmunization	$V_H$ Gene Used	VDJ Sequence	No. of Mutations <sup>a</sup>	No. of Events <sup>b</sup>
7	$V_H4$ x32 y33	Jb 08-H <sup>c</sup>	0	0
		Fc 18-H	8	2
		Jb 19-H	0	0
		Ga 04-H	0	0
		Fb 12-H	3	3
		Fc 05-H	5	4
		Ga 11-H	5	5
10	$V_H4$ y33	Da 02-H	12	7
		Ia 24-H	2	2
		Ma 18-H	0	0
		Mb 58-H	3	3
		Mb 56-H	5	5
		Ma 12-H	6	6
		Mb 26-H	9	7

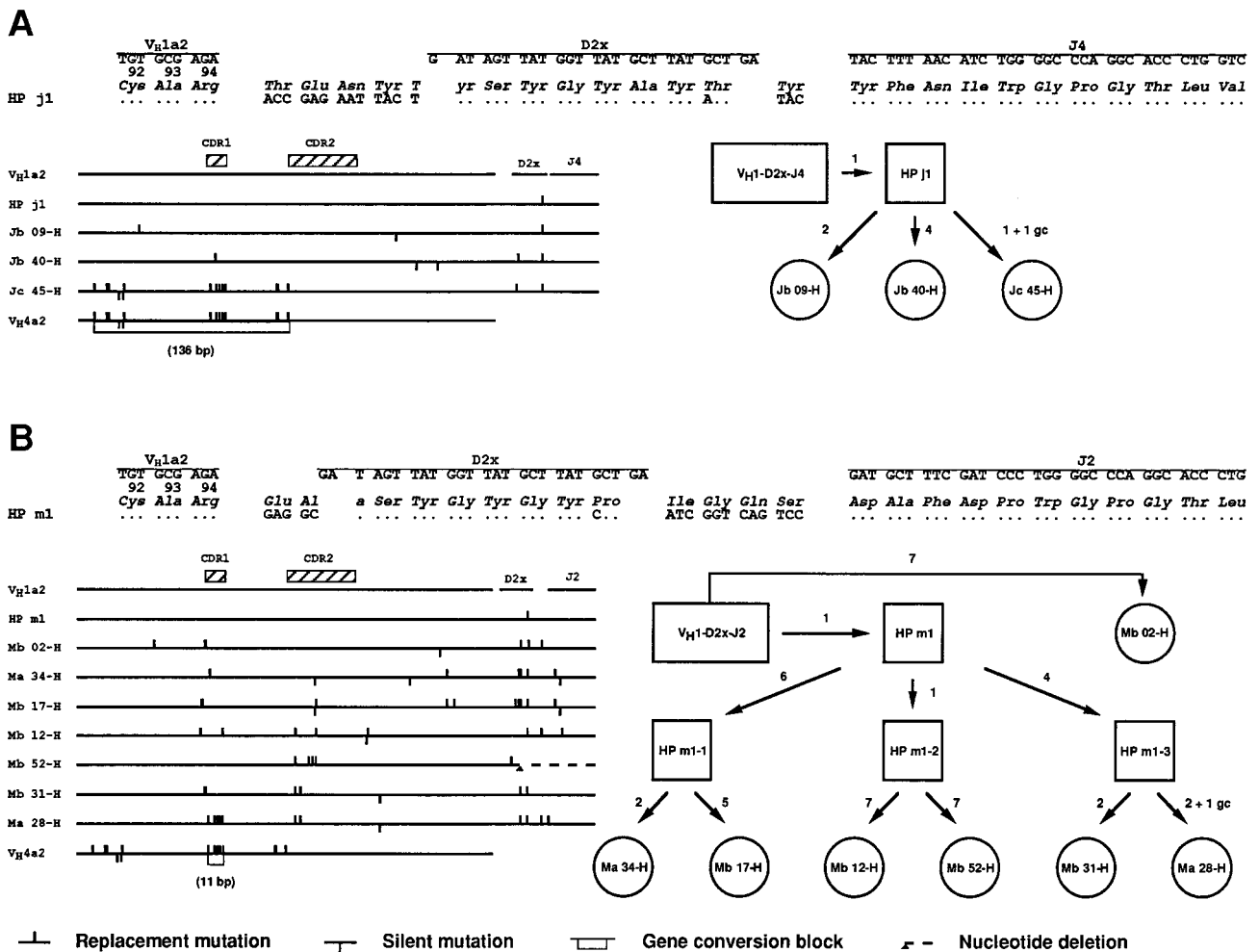
<sup>a</sup> Each nucleotide change was scored as an independent point mutation.

<sup>b</sup> Each gene conversion-like alteration was considered to be due to one event. Nucleotide changes other than the ones that were a part of the gene conversion block were considered as independent events.

<sup>c</sup> Individual germinal centers were designated by capital letters, sections within a germinal center by lower case letters, and cells were numbered. The suffix (–H) was added to designate heavy chain VDJ sequence. The GenBank accession numbers for the sequences are AF058553 (Jb 08-H), AF058531 (Fc 18-H), AF058558 (Jb 19-H), AF058543 (Ga 04-H), AF058524 (Fb 12-H), AF058527 (Fc 05-H), AF058544 (Ga 11-H), AF058512 (Da 02-H), AF058588 (Ia 24-H), AF058604 (Ma 18-H), AF058629 (Mb 58-H), AF058628 (Mb 56-H), AF058603 (Ma 12-H), and AF058620 (Mb 26-H).

and Jb 40-H acquired two and four point mutations, while Jc 45-H gained 14 nucleotide changes as a part of 136-nucleotide-long block of gene conversion. The donor for this block was  $V_H4$ . It is noteworthy that we found members of this clone (which shared the same CDR3) in two adjacent sections of the same germinal center. In addition, as previously observed by Kuppers et al. in human lymph node germinal centers (23), clonally related cells were not necessarily close to each other, but were dispersed. Gene conversion also occurred in clones obtained at day 10 of the response. The approximate locations of the collected cells from the clone depicted in Fig. 3B are shown in Fig. 1D. Again, the members of this clone were present in two adjacent sections of the same germinal center. The hypothetical precursor of the clone (HP m1) had a  $V_H1-D2x-J2$  rearrangement, and subsequent members of this clone accumulated mutations in their  $V_H$ , D, and  $J_H$  regions. The clonal tree has multiple branches. One of the members of this clone (Ma 28-H), which stained positive for DNP, acquired a block in CDR1 by a gene conversion-like event in which  $V_H4$  is the donor. During the clonal expansion, Mb 52-H acquired a frameshift mutation in the D region, making it nonfunctional. The examples shown in Fig. 3, A and B, demonstrate how, during clonal expansion, B cells with germline or near germline VDJ sequences acquired multiple nucleotide changes by gene conversion-like events, thus moving from left to right in the bimodal mutation distribution pattern (Fig. 2).

Fig. 4 confirms and extends the results in Fig. 3 by showing schematic representations of six other small clones that originated from cells with gene rearrangements that were entirely germline (A) or close to germline (B and C). The codon deletion and associated base changes in Ga 15-H (A, top) may have originated from a gene conversion, but we could not identify the putative donor sequence in the database. The cells clonally related to the germline rearrangement found in Jb 24-H (A, bottom) were found in two serial sections of the germinal center. Fa 08-H and Fc 17-H (B, top) were also found in serial sections. The cluster of changes in Ia 32-H (B, bottom) may also have arisen by gene conversion from an



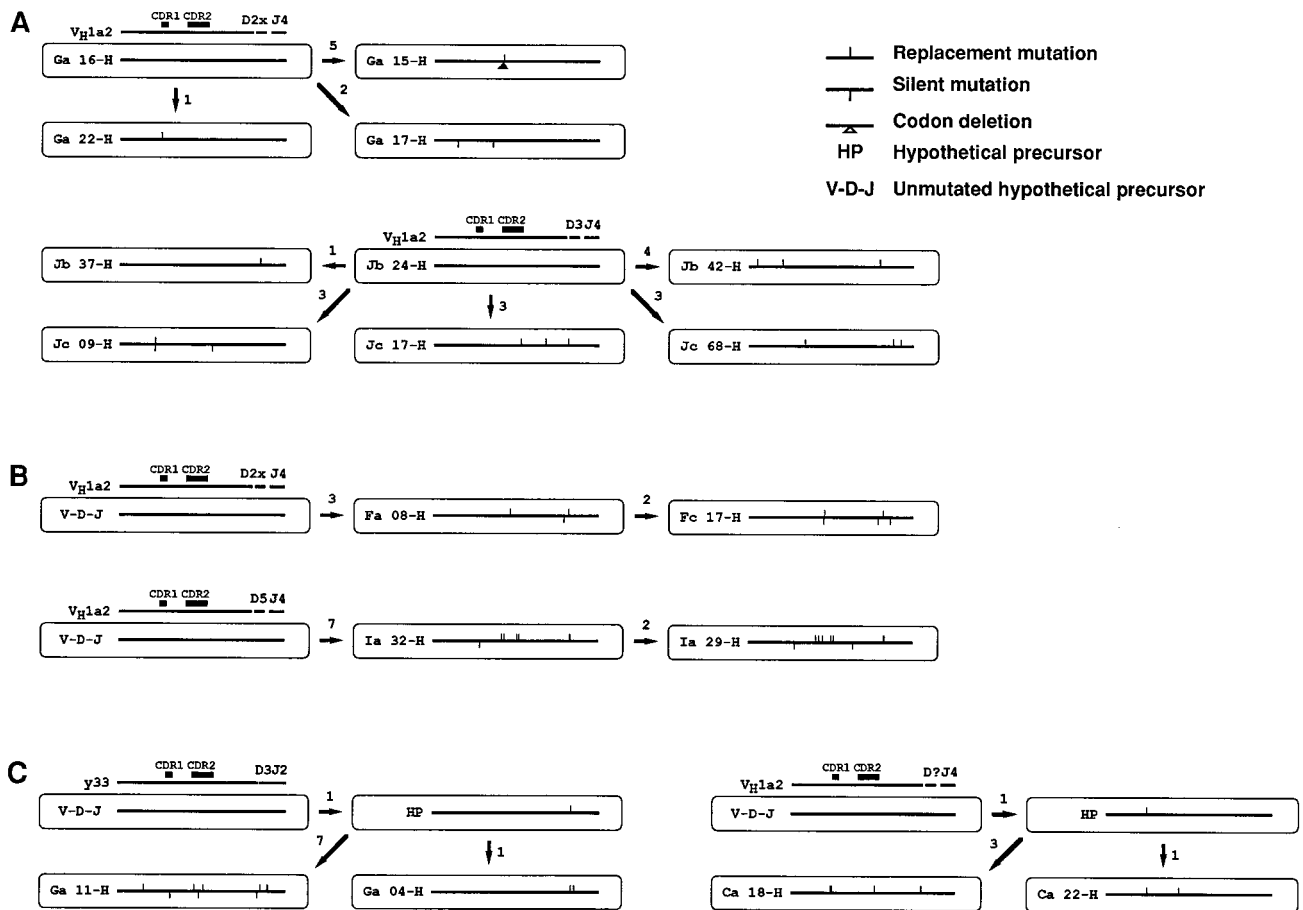
**FIGURE 3.** Examples of germline or close to germline VDJ sequences in clonally related B cells collected from splenic germinal centers of DNP-BGG-immunized animals on day 7 (A) and day 10 (B). The CDR3 and adjoining sequence from the rearranged VDJ of the hypothesized founder cell are shown on the top. Only the last three codons (92–94) of the V<sub>H</sub>1a2 sequence are shown. The V<sub>H</sub>, D, and J<sub>H</sub> gene segments used, and deduced amino acid sequence are indicated above the nucleotide sequence. Dots (.) indicate identity to the germline V<sub>H</sub>, D, or J<sub>H</sub> gene sequence. In the schematic alignments (shown below), the horizontal lines represent germline nucleotide sequences. Silent mutations are shown as vertical bars below the horizontal line, and mutations resulting in amino acid replacements as vertical bars pointing upward. Blocks of gene conversion are shown as open boxes beneath the schematic representation of the germline (V<sub>H</sub>4) donor sequence. The size of the gene conversion block (in nucleotides) is shown in parentheses next to the open box. In the clonal trees depicted on the right, rectangles represent the founder B cell (with unmutated VDJ rearrangement), squares represent the HP, and circles represent cells from which nucleotide sequence is available. The number of mutations that occurred from one member of the clonal tree to the following member are indicated beside the arrows. The postulated germline sequence D2x was described by Chen et al. (32). The sequences shown in A and B bear GenBank accession numbers AF058575–AF058577 and AF058639–AF058645, respectively. gc, Gene conversion event.

unknown donor. In Fig. 4C (left), the HP of Ga 04-H and Ga 11-H differs from germline y33-D3-J2 only at the V to D junctional position. Finally, in Fig. 4C (right), the HP differed by only one base from germline V<sub>H</sub>1a2 gene, but had a long D<sub>H</sub> region with no known germline counterpart, although the first nine bases were identical to D2a.

In contrast to the B cells with germline or near germline VDJ sequences, many of the clonally related B cells with 10 or more nucleotide changes relative to the rearranging V<sub>H</sub> gene shared blocks of nucleotide changes due to gene conversion-like event(s) involving known germline V<sub>H</sub> genes. The members of the clone acquired point mutations during a further expansion process. Two examples from the same germinal center section are shown in Fig. 5, A and B. In Fig. 5A, the founder cell (Da 04) had already undergone a 131-nucleotide block of gene conversion, in which V<sub>H</sub>7 was the donor. In addition, it differed from V<sub>H</sub>1 by five point mutations, of which two were superimposed on the gene conversion block. This founder cell underwent clonal expansion giving

rise to Da 14 and Da 07, which acquired two (different) additional nucleotide changes. In the example shown in Fig. 5B, we identified four cells, two (Da 06 and Da 13) with identical sequences, that originated from a common precursor with a rearranged V<sub>H</sub>1-D5-J<sub>H</sub>4 and a gene conversion-like stretch in CDR2 from V<sub>H</sub>9. These cells had undergone further mutations in the CDR1, CDR2, FR1, and FR3 regions.

Fig. 6 confirms and extends the results in Fig. 5 by showing schematic representations of 10 remaining small clones that differed from the germline V<sub>H</sub>1 gene by containing one (A–F) or two (G–J) gene conversion-like blocks from known donor genes as well as additional base changes. It is possible that some of the other changes were also due to gene conversion-like events involving unknown donor genes. In D, cells containing V<sub>H</sub> sequences that were identical or differed at only one position were found in three serial sections; in E, two related cells were found derived from a HP that had a frameshift at the D–N–J junctional region; in F, five cells with the identical V<sub>H</sub> sequence were found



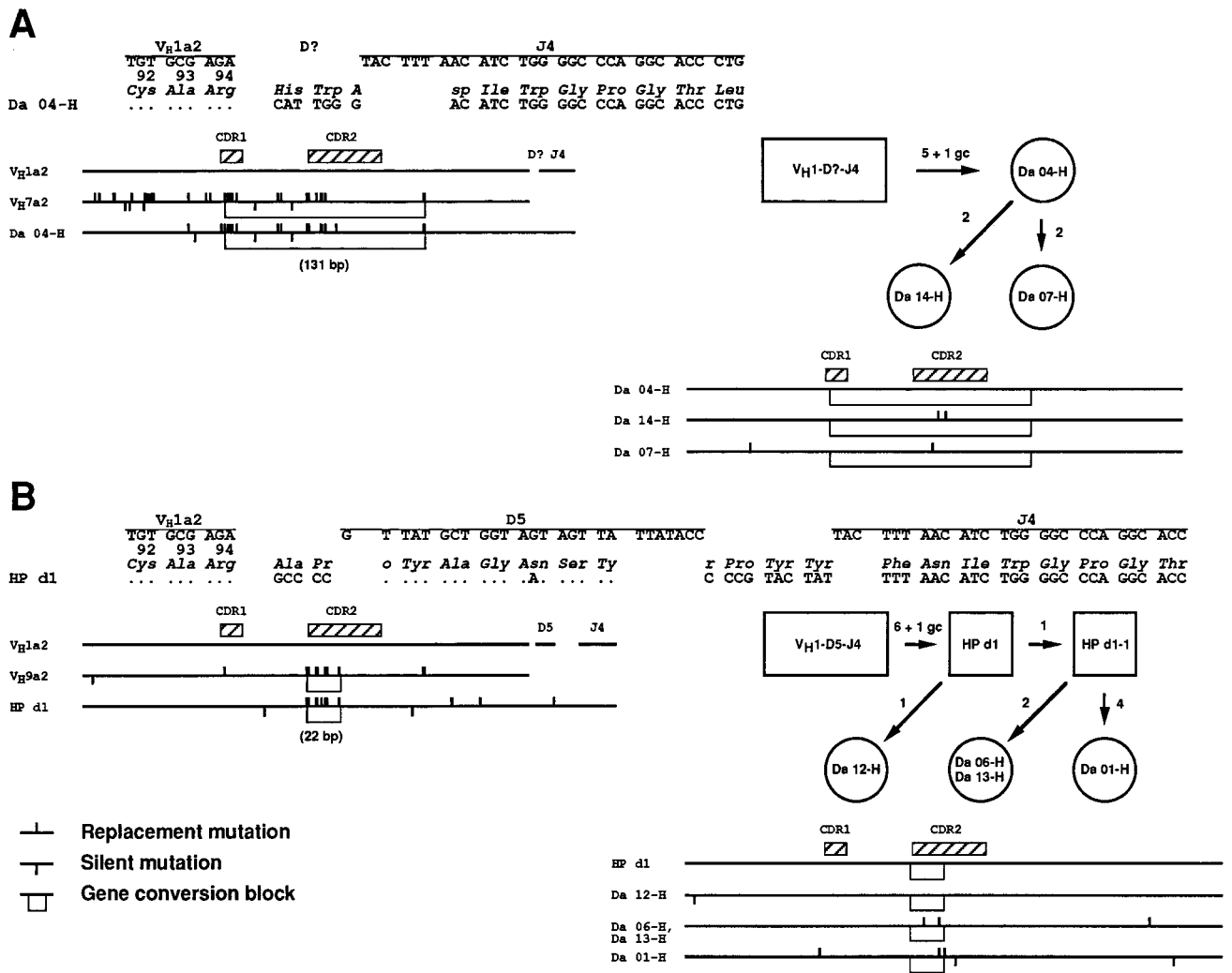
**FIGURE 4.** Genealogical trees originating from precursor B cells with germline or near germline VDJ sequences. In the schematic alignments, horizontal lines represent germline nucleotide sequences of the rearranging  $V_H$ , D, and  $J_H$  gene segment. Numbers of mutations are indicated next to the arrows. Clones initiated from cells with VDJ rearrangements that are identical to germline (A) or near germline (B and C). These sequences bear GenBank accession numbers AF058549–AF058552 and AF058578–AF058583 (A); AF058534, AF058535, AF058594, and AF058595 (B); AF058543, AF058544, AF058510, and AF058511 (C).

in two serial sections. *J* shows that two identical sequences were found in cells from a F1 Ag-specific germinal center. In the chicken, precursor cells with three to five gene conversion stretches initiated clones in splenic germinal centers that were identified 7 days after immunization (33). These were presumed to have been bursal derived. In the rabbit, we cannot presume that they were derived from GALT because we have shown that precursors with close to germline sequences within the spleen underwent gene conversions (Fig. 3, A and B). Further documentation of gene conversion-like events occurring during clonal expansion of germinal center lymphocytes at day 15 will be the subject of a separate report (E. Schiaffella, D. Sehgal, A. O. Anderson, and R. G. Mage, manuscript in preparation).

## Discussion

The finding of B cells with germline or near germline VDJ rearrangements utilizing  $V_{H1}$ ,  $V_{H4}$ , x32, or y33 genes in the splenic germinal centers of adult rabbits was surprising in view of a recent report that only highly diversified VDJ sequences were found in cells from appendix, peripheral blood, and bone marrow of adult rabbits, leading to the hypothesis that B lymphopoiesis is limited in adult rabbits (20). The conclusions (20) were based on studies of mRNA. Low levels of mRNA produced by immature B lymphocytes may not have been detected readily in the RNase pro-

tection assay. If the mRNA preparations mainly contained abundant transcripts from cells with diversified sequences, the results (20) can be explained. Even when we checked single cells from adult PBL using our method of DNA amplification and sequencing, we found one germline  $V_{H1}$  sequence, although the remaining 16 successfully sequenced gene rearrangements had an average of 11.62 differences from germline (range 5–21). Although this yield (6%) is consistent with the 7% germline splenic anti-DNP sequences, the similar percentages may be fortuitous. The previous study also did not examine splenic germinal centers from immunized rabbits, in which we found germline or near germline VDJ sequences in each of the five studied at day 7 and the two from day 10. Although there were no completely germline sequences from F1 protein-immunized animals obtained at day 10, they were surprisingly close to germline. It is important that we were able to identify the germline D segment that was used in the VDJ rearrangement and found them to also be close to germline. This contrasts with the results reported (20). We have preliminary data suggesting that in some of the single cells we collected, there were also  $V_{KJ}$  sequences close to germline. This is more difficult to document because there are very few published rabbit  $V_K$  germline gene sequences (our unpublished results; work in progress). One such cell also had a near germline  $V_H$  y33 (with three nucleotide changes) rearranged to D4 and  $J_{H2}$  that were totally unmutated.



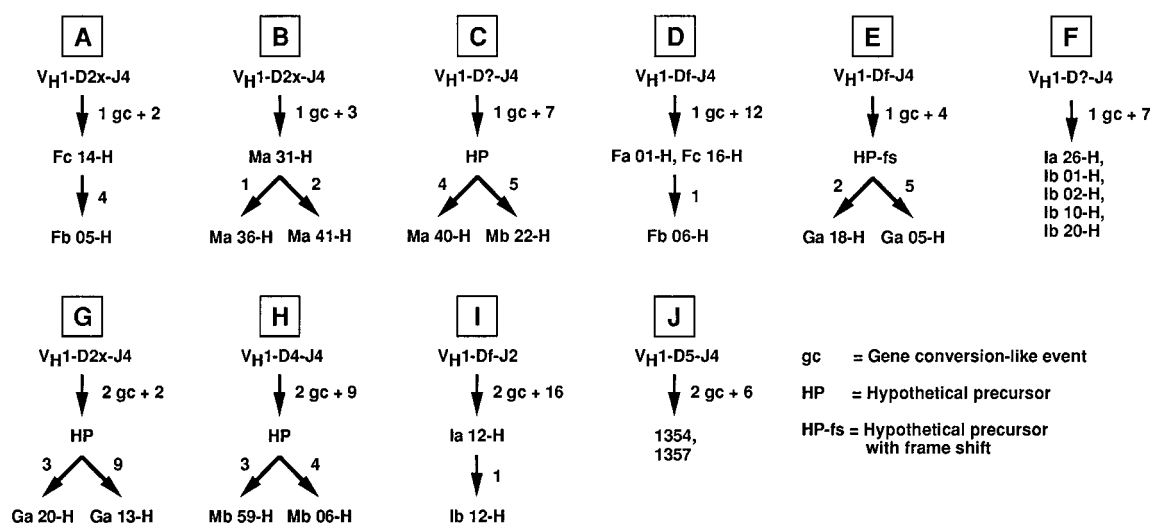
**FIGURE 5.** Examples of clonally related B cells with diversified VDJ sequences present in splenic germinal centers of DNP-BGG-immunized animals (day 7). The CDR3 and adjoining sequence from the rearranged VDJ of the (presumed) founder cell are shown on the top of the panel (A and B). Only the last three codons (92–94) of the V<sub>H</sub>1a2 sequence segment are shown. The V<sub>H</sub>, D, and J<sub>H</sub> gene segments used, and deduced amino acid sequence are indicated above the nucleotide sequence. Dots (.) indicate identity to the germline V<sub>H</sub>, D, or J<sub>H</sub> gene sequence. In the schematic alignments (shown below), the horizontal lines represent germline nucleotide sequences. Silent mutations are shown as vertical bars below the horizontal line, and mutations resulting in amino acid replacements as vertical bars pointing upward. The gene conversion event (represented as an open box) in the rearranged VDJ sequence of the presumed founder B cells is shown schematically on the left along with the possible germline donor for the block of gene conversion. The number in parentheses below the open box is the size of the gene conversion block in nucleotides. The relationship between the members of the genealogical tree is shown on the right. The unmutated VDJ rearrangements of the founder B cells are indicated as rectangles, squares represent HP, and circles represent cells from which nucleotide sequence is available. The number of mutations that occurred from one member of the clonal tree to the following member are indicated next to the arrows. Beneath the sketches of the clonal trees are simplified schematic alignments showing the nucleotide changes that occurred in the VDJ sequence of the clonally related members relative to that of the founder B cell. These sequences bear GenBank accession numbers AF058515–AF058521. gc, Gene conversion event.

There are several possible explanations for the finding of germline sequences in splenic germinal centers of adult rabbits. The simplest is that the peripheral B cell pool of adult rabbits contains a subset of immature B lymphocytes that recently underwent V<sub>H</sub>DJ<sub>H</sub> and V<sub>L</sub>J<sub>L</sub> rearrangements, arrived in the spleen from as yet unidentified locations, and initiated germinal center development. Old data on recovery from chronic allotype suppression in rabbits support this idea. Whereas anti-IgM treatment of 13-day-old chicken embryos leads to the complete suppression of B cells (34), anti-V<sub>H</sub> or anti-C<sub>κ</sub> allotype treatment of rabbits via maternal transfer or treatment initiated after birth leads to chronic, but not total suppression of expression of B lymphocytes with that allotype (35). One source of the cells that are found during recovery from

complete allotype suppression could be from residual B lymphopoiesis occurring in the rabbit after the neonatal period.

Although recently developed immature B cells are the most likely source of the cells that we found, there are some other possible explanations. At 6 wk of age, the rabbit appendix was found by Weinstein et al. to contain some cells with rearranged VDJ that were close to germline in sequence (15). Such cells that developed in GALT early in life could have exited to the periphery; contributed to a long-lived, recirculating, and self-renewing pool of rabbit B lymphocytes; and seeded primary follicles or the germinal centers. Finally, because many rabbit B lymphocytes have one allele in germline configuration (36), it is possible that some rearranged VDJ sequences that were close to





**FIGURE 6.** Genealogical trees originating from precursor B cells with VDJ sequences with one (A–F) or two (G–J) blocks of gene conversion. These sequences bear GenBank accession numbers AF058536 and AF058537 (A); AF058636–AF058638 (B); AF058632 and AF058633 (C); AF058538–AF058540 (D); AF058545 and AF058546 (E); AF058598–AF058602 (F); AF058547 and AF058548 (G); AF058634 and AF058635 (H); AF058596 and AF058597 (I); and AF058655 and AF058656 (J).

germline represented recent rearrangements of the second allele. Conceivably, some cells with a newly rearranged VDJ on the second allele were successfully sequenced, although cells with two fully rearranged VDJ might lead to mixed sequence. In fact, we were unsuccessful in obtaining nucleotide sequence from 36 of the 176 (20.5%) VDJ sequences that were amplified. If the B cell had both  $V_{H1}$  alleles at the Ig heavy chain locus rearranged and both the rearranged alleles contributed to the PCR product, this could have led to undecipherable sequence (the end result would also be the same if the original starting sample for amplification had more than one B cell).

Up-regulation of RAG gene expression has been observed in splenic germinal centers of the mouse, in which it appears in part to reflect receptor editing via new gene rearrangements in mature cells (37–42). Recent rearrangements and/or secondary receptor editing may explain the previously reported observation from this laboratory that there is RAG-2 protein expression in rabbit spleen (43). This RAG expression in rabbit spleen as well as some of the non- $V_{H1}$  sequences shown in Table III could conceivably reflect secondary events occurring in rabbit spleen.

Although predominant utilization of the  $V_{H1}$  gene limits combinatorial diversity in rabbits compared with mice and humans, the contributions of  $V_{\kappa}$  sequences to combining site structures and Ab affinities may be greater in rabbits, in which there is greater diversity in length of light chain CDR3 regions (44, 45). It will be of interest to learn whether diversification of rabbit light chain sequences can also occur by gene conversion-like mechanisms. The rabbit is strikingly efficient in diversification of heavy chain sequences through introduction of gene conversion blocks with replacement changes and without deleterious stop codons. The ratios of replacement to silent changes in the diversified sequences that we report in this work were remarkably high where there were blocks of gene conversion involved (Figs. 3 and 5). The donor sequences ( $V_{H4}$ ,  $V_{H7}$ , and  $V_{H9}$ ) contained mainly replacement changes.

Comparisons of rabbit B cell development and diversification with what is known to occur in germinal centers of humans and mice must be made with caution because there are no subsets defined by markers such as IgD, CD38, and CD5 (46, 47). Most, if not all, rabbit B lymphocytes bear CD5 (48), including those with

highly diversified sequences. Rabbits also lack the IgD marker (49). In mice, IgV genes derived from proliferating B cells from splenic extrafollicular foci were shown to be in germline configuration (50). We did not observe comparable foci in these studies. VDJ sequences with unmutated V gene segments have been found in B cells from the mantle zones of human lymph nodes and tonsils as well as in the germinal center (dark zone) (23, 51). Although the delineation of marginal and mantle zones in rabbit germinal centers is difficult in the absence of specific markers, the cells with germline sequences that we found were clearly from areas within the germinal center that contained proliferating cells (Fig. 1, A and C).

In conclusion, although a major population of B lymphocytes may be generated early in life, diversified extensively, and maintained by self-renewal in the periphery, some sources of cells with sequences close to germline do exist in adult rabbits and appear in developing germinal centers. There they undergo clonal expansion and V gene diversification by gene conversion-like and somatic hypermutation mechanisms. We hope that future investigations will reveal the source of the B cells with germline or close to germline VDJ sequences present in spleens of adult rabbits.

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