A Deletion Map of the Human Immunoglobulin Heavy Chain Variable Region
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Summary
Analysis of VH gene segments deleted in the process of immunoglobulin heavy chain (IGH) variable region assembly in three series of monoclonal B cell lines has been used to determine the human VH region organization. A deletion map of the relative positions of 21 different VH gene segments has been determined. The characterization of B cell lines from three unrelated adults of two racial groups yielded the same relative VH gene segment order, suggesting that the overall order of VH genes in the normal population is constant. This VH gene segment order was consistent with what we had previously generated from physical mapping techniques. DH segments from the second DH cluster, distinct from the major DH locus 3' of the VH region, were not observed to be used in 32 different rearrangements. Approximately 77% of the VH-(D)JH rearrangements involved VH gene segments within 500 kb of the JH region, indicating that human B cell lines preferentially rearrange JH-proximal VH gene segments. The switch, observed in mice, from the fetal use of JH-proximal VH gene segments to an adult VN usedependent upon VH family size may therefore not occur in humans. This detailed map of the VH gene segments is a necessary prerequisite for understanding VN usage in development and disease.

The human immunoglobulin heavy chain (IGH) gene complex is comprised of ~100 heavy chain variable (VH) gene segments, at least 24 diversity (DH) elements, six functional joining (JH) segments, and a constant (CH) region composed of nine genes and two pseudogenes. The IGH gene complex maps to the most distal band of chromosome 14, at 14q32.33 (1, 2). The order telomere-VH-JH-CH-centromere has been determined by analysis of Burkitt lymphoma cells having 8;14 translocations between the IGH locus and the oncogene c-myc (3).

The VH gene segments, coding for the first 95-101 amino acids of the heavy chain peptide, have been subdivided into six families (VH1 to VH6) based upon DNA homology (4–9). The organization of portions of the VH region has been determined from examination of cloned regions (10) and from long-range restriction mapping (4, 11). The human Ig VH gene families are interspersed. This is in marked contrast to the murine VH organization, which has some interspersion of the members of different VH families, but appears to be characterized predominantly by the clustering of VH families (reviewed in references 12 and 13).

The major DH region, between VH6 (the most 3' VH gene segment) and the JH region, is composed of four 9-kb intervals (14, 15). Each 9-kb repeating unit contains six different DH gene families (16). A second DH cluster is located within the VH region (17, 18), but its functional significance, if any, is unknown. VH, DH, JH, and CH gene segments are juxtaposed in the course of B cell development. IGH variable region assembly is an ordered process that begins with DH-to-JH rearrangement, usually at both IGH alleles (19). The DH-to-JH complex then may recombine with a VH gene segment. If the first IGH allele rearrangements does not produce an open reading frame, the second (D)JH rearrangement can become a substrate for VH-to-(D)JH joining (reviewed in reference 20). Site-specific recombination of an upstream VH gene segment into a Vn(D)Jn rearrangement, resulting in the replacement of the initially rearranged VH gene segment, also can occur (21, 22). The frequency of this occurrence during normal B cell development is unknown. As the frequency of VH-(D)JH rearrangement is greater than the frequency of direct VH to DH rearrangements (23), control of Ig variable region assembly could be influenced by the accessibility of the rearrangement components to the recombination machinery.

In addition to DH-to-JH followed by VH joining, direct D-D joining has also been suggested as potentially possible (14, 24). D-D joining was found infrequently by Meek et al. (60) in the analysis of IGH chain rearrangements from murine bone marrow. Ichihara et al. (25) found no evidence of direct D-D to-Dn joining. However, the same group discovered a novel type of Dn element, diversity segments with irregular spacer lengths (DIR), in sequencing 15 kb of the

1 Abbreviations used in this paper: AAT, α1-antitrypsin; DIR, diversity segments with irregular spacer lengths; PCFIA, particle concentration fluoroimmuno assays; PFGE, pulsed field gel electrophoresis.
Dn region, which could be involved in Dn-to-DIR or DIR-to-Dn joining (16). While both mechanisms would result in the generation of increased diversity in the CDR III region of human Ig heavy chains, the frequencies of the occurrence of Dn-to-Dn and of DIR-to-Dn joining are unknown.

IGH variable region rearrangements result in the deletion of the intervening DNA that separated the rearranging Vn gene segment from the (D)Jn sequences (26). Deletion mapping takes advantage of this process to determine the relative positions of Vn gene segments. Vn gene segments 5' of a selected Vn sequence remain unaltered by the rearrangement event, while those 3' are deleted. This method has been used to elucidate the murine Vn organization (27-31).

We have used deletion mapping to determine the human Vn organization. We have examined 21 Vn gene segments of the Vn2, Vn4, Vn5, and Vn6 families in three sets of monoclonal B cell lines from different human donors. This analysis resulted in the generation of a Vn gene segment order consistent with the physical map we have derived from long-range restriction mapping experiments. Our results suggest that there is a single Vn gene segment order in humans, that interspersion of Vn gene segments is extensive, and that the human IGH recombination machinery generating adult B cell repertoires shows strong 3' to 5' bias.

Materials and Methods

B Cell Transformation. EBV-transformed human B cell lines were generated from PBMC of three unrelated normal adults as described (32, 33). Fifth-week supernatants were analyzed for the presence of secreted human IgG, A, M, D, E, κ, or λ by automated particle concentration fluorimuno assays (PCFIA), using a Baxter-Pandex ScreenMachine (Baxter, Pandex Div., Mundelein, IL) (34). Data were captured and processed using MacPlate PCFIA software developed in our institution. The frequencies of transformable cells committed to a given Ig isotype were determined by Poisson analysis by both minimum χ² and maximum likelihood procedures as described (32, 35). Secretory cultures, calculated by Poisson analysis to be monoclonal (initial cell dose containing <0.3 transformable cells) and producing a single Ig heavy and light chain, were subcloned into 46 fresh microwells followed by recloning at limiting dilution (36). Clones were maintained in 100-cm² tissue culture flasks with complete medium. Phenotypic analysis by flow cytometry in a profile analyzer (Coulter Electronics, Hialeah, FL), followed standard procedures with commercial mAb reagents (Coulter Electronics) (32, 36).

Electrophoretic Analysis. High molecular weight DNA from peripheral blood samples of the B cell line donors was prepared in agarose plugs (37). DNA was prepared similarly from B cell lines, but cells were rinsed only once in PBS before embedding in agarose. Pulsed field gel electrophoresis (PFGE) analysis of DNA from HSC no. 1321 has been described (11; called there L1). HSC no. 1321 is of Caucasian descent; HSC no. 1001 and HSC no. 1322 are of Oriental descent. DNA was digested with 5-10 U of restriction endonuclease/μg of DNA in the manufacturer's recommended buffer (Boehringer Mannheim Biochemicals, Indianapolis, IN). Digested DNA samples (3 μg/lane) were fractionated through 0.7% agarose gels in a large submarine apparatus (Bethesda Research Laboratories, Bethesda, MD) at 60 V for 20-24 h. DNA was then transferred to Hybond N or N' membrane according to the manufacturer's recommendations (Amersham Canada, Oakville, ON). Blots were hybridized with ³²P-labeled DNA probes as described (37). After hybridization, blots were briefly rinsed in 2x SSC at room temperature and washed for 1 h in 0.1% SDS, 0.1x SSC at 52°C for the Vn2, Vn3, Vn5, and AAT probes, or at 65°C for the Jn, Dn, Vn1, Vn4, and Vn6 probes. Filters were exposed to Kodak XAR-5 film at ~70°C using intensifying screens (Lightning Plus, Du Pont Co., Wilmington, DE).

DNA Probes. The hybridization probes used in this study were agarose gel--purified DNA fragments as follows: y4, the 2.2-kb Sacl fragment from the switch region of μ, derived from Abt8, originally isolated by P. Early and provided by R. Wall and L. Hood; Jn, a 6-kb BamHI/HindIII fragment derived from pHu(J)H spanning the Jn region, provided by P. Leder (38); Dn, the 9.5-kb ClaI fragment of cosmid C17p3 (15); Vn2, the 1.2-kb BamHI/EcoRI insert of VH2BE1.2 (39), derived from VCE-1 (40) from T. Honjo; Vn3f, the 2.2-kb EcoRI fragment of VHE2.2 (39), which flanks the Vn3 family gene VH26 (41); Vn4, a 245-bp Apa1/Eagl fragment derived from 58P2X, from H.W. Schroeder and R. Perlmuter (8); Vn5, a 221-bp PstI fragment derived from 2V, from F. W. Alt (4); Vn6, a 9.3-kb BamHI fragment derived from cosmid C17p3 (15), containing the Vn6 gene segment; Cla20, a ClaI fragment (only 20 kb of which was cloned into C17p3) containing the Vn6 gene segment and flanking regions, derived from cosmid C17p3 (15). AAT is a 1.6-kb PstI fragment, subcloned from pATMB6.5 (42) containing exon II of the α1-antitrypsin gene.

Densitometric Analysis. A computing laser densitometer (300A; Wise Molecular Dynamics) was used to compute fragment hybridization intensities in the B cell lines and controls, according to the manufacturer's suggested protocols. The hybridization intensities of 21 Vn gene segments in the DNA from each of the three sets of monoclonal B cell lines were compared with those in the donor's leukocyte DNA. Filters were rehybridized with a probe for the α1-antitrypsin gene (AAT) to control for different amounts of DNA between lanes. Dosage of nonpolymorphic Vn gene segments deleted only on one rearranged chromosome was determined by both visual and densitometric analysis, the latter by averaging a minimum of three densitometric scans of each autoradiogram. Visual comparison and densitometric measurements differed in Vn dosage estimates in ~10% of the measurements. The Vn gene segment dosages in these situations were reassessed visually by an experienced independent person and dosage was then determined by the agreement of two of the three dosages estimates (two visual, one densitometric).

Restriction Map of the Human Vn Region. The physical map positions of the 21 Vn gene segments analyzed here were determined from the restriction map of a 1,500-kb region of the human IGH Vn region reported in reference 11, and included in Fig. 6. Vn gene segment designations were those of Walter et al. (11), based upon the relative positions (largest to smallest) of restriction fragments hybridizing to Vn family probes, in human DNA digested with EcoRI (for Vn3f-1) or BglIII (for Vn2, Vn4, and Vn5 gene segments).

Results

Cell Lines. The culture efficiencies (sum of all transformable cells) in the three limiting dilution experiments (6.3-14.8% of B cells transformed) were within the range usually observed in our laboratory (33, 34, 43). Reflective of the Ig isotype commitment in circulating human B cell pools, ~85-90% of all transformants expressed IgM, ~5%...
each expressed IgG or IgA, and <10% of IgM producers cosecreted IgD with 1-4% of these IgD producers coexpressing IgE. Of 42 lines derived from the three normal donors, 26 showed stable growth 6 mo after initiation, nine randomly selected clones had normal karyotypes, all had doubling times of ~48 h, and secreted their Ig at rates of 1-3 x 10^6 molecules/cell/h to a concentration of 1-10 μg/10^6 cells. None of the lines expressed T cell surface markers and all had the CD20+/CD21+ B cell phenotypes (36). None of the clones expressed CD5 but all were CD23+. Therefore, the cell lines examined were EBV-transformed mature B cell clones.

Analysis of JH Rearrangements. 26 different B cell lines, derived from the three different donors, were examined to determine if they were stable, monoclonal B cell lines suitable for deletion analysis. The B cell lines were digested with the restriction enzyme HindIII, and hybridized with the JH probe (Fig. 1a). The JH probe hybridizes to a 9.6-kb HindIII fragment when not rearranged. Absence of this fragment or detection of novel fragments in DNA hybridized with the JH probe indicates rearrangement of the JH locus. By Southern hybridization, 19 of the 26 B cell lines examined appeared to have both JH alleles rearranged (summarized in Fig. 2), six had retained one nonrearranged JH allele. Information concerning the JH region of B cell line 21A12 was not obtained, although later Vn and Dn analyses were consistent with the hypothesis that 21A12 had undergone two JH rearrangement events. No lines showed identical patterns of rearranged IGH loci, demonstrating the independence of these B cell lines. Each B cell line contained only two JH hybridizing fragments, consistent with the clonal nature of these cells. The B cell lines analyzed here were therefore concluded to be independent clonal B cell populations with stably rearranged IGH loci.

Analysis of Dn-to-Dn Rearrangements. To analyze the Dn rearrangements that the B cell lines had undergone in the process of differentiation, the 26 B cell lines were hybridized with a Dn probe, and with the Cla20 probe that extends 5' of the Dn region. The B cell clones derived from HSC no. 1001 were hybridized with the Dn + AAT probes, and then with the Cla20 probe (Fig. 1b). The locations of the Dn, Cla20, Vn6, and Jn probes are shown in Fig. 2. The Dn and Cla20 probes, together with the Vn6 probe and the Jn probe, survey the ~75-kb interval extending from the Vn6 gene segment to the Jn sequences (Fig. 2). The Dn probe detects a region of ~30 kb, hybridizing to fragments corresponding to the four 9-kb repeat elements that constitute the Dn major cluster (16). Additional fragments, belonging to the Dn minor cluster (discussed below), were also detected. The Cla20 probe detects the 24-kb EcoRI fragment of the Dn major cluster, and detects the 6.1- and 7-kb EcoRI fragments that are 5' of the Dn major cluster (and also weakly hybridizes to the Dn minor region). However, only the 6.1-kb fragment hybridized with the Cla20 probe in a manner that allowed consistent determination of dosage. The Dn probe detects a common EcoRI polymorphism in the Dn region with alleles of 22 and 14 kb. The location of the polymorphic EcoRI site is indicated with an asterisk in Fig. 2. These 22- and 14-kb alleles have frequencies of 0.54 and 0.46, respectively, in the normal Canadian Caucasian population (Walter, M.A., and D.W. Cox, unpublished results). HSC no. 1322 is homozygous (22/22) for this Dn polymorphism, HSC no. 1001 is a heterozygote (22/14), and HSC no. 1321 is homozygous (14/14).

The results of the deletion analysis of the Vn6-Jn region of the 26 B cell lines are summarized in Fig. 2. 22 of the 26 B cell lines were found to have deletions in the Dn
regions of both chromosomes when hybridized with the Dn and Cla20 probes (Fig. 2). Differences between the number of Jn rearrangements and Dn rearrangements were found in five B cell clones: TB10, 25C3, 6B12, 6H5, and 9F9. 9F9 appeared to rearrange only a single Dn allele, but two Jn alleles. 9F9 probably represents the rearrangement of two nonoverlapping portions of the Dn region, or alternatively, could have contained a rearrangement to the DHQ52 segment. DHQ52 is unusual as it lies within the Jn region (38). Nine B cell lines with portions of the Dn regions deleted on both chromosomes were found to retain DNA fragments that are 3' of related Dn region sequences (T1C10, T5F8, 2C11, T4B8, TB10, 3F11, 6B12, 6D3, and 6H5). This suggests that in the 26 B cell lines, at least one half had IGH alleles that underwent Dn rearrangements distinct from Dn-to-Jn joining, likely the result of D-D rearrangement events. Since four of the B cell lines that appeared to retain one Jn allele in germline configuration (TB10, 25C3, 6B12, and 6H5), had rearrangements involving both Dn alleles (Fig. 2), D-D rearrangement events appear to occur rather more commonly than expected, and before Dn-to-Jn joining in Ig variable region assembly.

While most Dn sequence-containing fragments are rearranged or deleted in the B cell lines, two EcoRI fragments, ~19 and ~16 kb in size, are not (Fig. 1 b). These fragments persist in B cell lines in which several Vn gene segments have been deleted on both chromosomes (e.g., B cell line T1B5, see below). Examination of the restriction map of the 75-kb region between Vn6 (the most Jn-proximal Vn gene segment) (15) and the Jn region indicates that EcoRI restriction fragments of these sizes containing Dn homologous sequences do not lie in this interval (Fig. 2). These Dn fragments, therefore, appear to belong to the minor Dn cluster reported to map within the human Vn region (11, 17, 18) and were not involved in any of the Ig rearrangements analyzed here.

Analysis of 32 Different Vn-to-(D)Jn Rearrangement Events. Further analysis of the Vn6-Jn regions of the B cell lines revealed that, of the B cell lines with rearrangements of both Dn alleles (22/26), only six had Vn-to-(D)Jn rearrangements at both IgH alleles (T1B5, T3E2, 21A12, 12A4, 6A9, and 6B13), shown in Fig. 2. Most B cell lines had Vn-to-(D)Jn rearrangements of only one IgH allele, with the remaining Vn allele in the germline configuration (for example, B cell

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**Table 1:** Summary of the Dn region deletions present in the B cell lines. The EcoRI restriction map of the Vn6-Jn region is indicated at the top, thin vertical lines indicate positions of EcoRI restriction sites. Sizes of EcoRI restriction fragments (kb) are indicated. (2) The position of a polymorphic EcoRI site in the Dn region. The positions of the Vn6 gene segment, Dn region, and Jn sequences within this DNA interval are indicated above the EcoRI restriction map, probes are mapped at the bottom. Thick lines indicate bi-allelic deletion of EcoRI fragments in a B cell clone. Dn region deletions on only a single chromosome in a B cell line were not determined. The horizontal dashed line indicates a region within the B cell line 9BS that was not surveyed. The number of rearranged Jn alleles in a particular B cell line was obtained from Fig. 1. The number of Vn-(D)Jn rearrangements within a B cell line (right panel) was determined from the deletion mapping of fragments 5' of the Dn region; in particular, the Vn6 gene segment and the 6.1-kb EcoRI fragment detected with the Cla20 probe.

| Clone   | # JH rearrangements | # DH rearrangements | # VH - DJH rearrangements |
|---------|---------------------|---------------------|---------------------------|
| T1B5   | 2                   | 2                   | 2                         |
| T3E2   | 2                   | 2                   | 2                         |
| T1C10  | 2                   | 2                   | 2                         |
| T2G2   | 1                   | 1                   | 1                         |
| T5F8   | 2                   | 2                   | 1                         |
| 2C11   | 2                   | 2                   | 1                         |
| T4B8   | 1                   | 1                   | 1                         |
| TB10   | 1                   | 1                   | 1                         |
| A12    | 2                   | 2                   | 1                         |
| 2C12   | 2                   | 2                   | 1                         |
| 25C3   | 2                   | 2                   | 1                         |
| 12A4   | 2                   | 2                   | 2                         |
| 3E12   | 2                   | 2                   | 1                         |
| 3F11   | 2                   | 2                   | 1                         |
| 6A9    | 2                   | 2                   | 1                         |
| 6B12   | 2                   | 2                   | 1                         |
| 6B13   | 2                   | 2                   | 1                         |
| 6D3    | 2                   | 2                   | 1                         |
| 6E11   | 2                   | 2                   | 1                         |
| 6F2    | 2                   | 2                   | 1                         |
| 6H5    | 2                   | 2                   | 1                         |
| 6G8    | 2                   | 2                   | 1                         |
| 8A9    | 1                   | 1                   | 1                         |
| 9A11   | 1                   | 1                   | 1                         |
| 9BS    | 1                   | 1                   | 1                         |
| 9F9    | 2                   | 2                   | 2                         |
Figure 3. Analysis of V\\textsubscript{H} gene segments of the B cell lines derived from HSC no. 1322. All Southern blots were of BglII-digested DNA, except for VH3f (EcoRI). Sources of DNA separated in each lane are identified at the top. Blots were hybridized with the indicated V\\textsubscript{H} family probe. The V\\textsubscript{H} gene segments analyzed in the B cell lines are identified to the right. (*) The polymorphic V\\textsubscript{H} loci. Positions of V\\textsubscript{H} gene segments that were not present in the leukocyte DNA of HSC no. 1322 are indicated with parentheses. Sizes of V\\textsubscript{H} gene segments analyzed in this study are indicated to the left. None of the B cell lines retained two nonrearranged IGH loci. We conclude that: (a) the 26 B cell lines analyzed here represent 32 different V\\textsubscript{H}-to-(D)\\textsubscript{n} rearrangement events; and (b) that single allele V\\textsubscript{H}-to-(D)\\textsubscript{n} joining seems to be common in human B cells.

Genetic Differences in V\\textsubscript{H} Gene Segment Repertoires. Knowledge of the polymorphisms present in the V\\textsubscript{H} region is crucial to the interpretation of deletion mapping results. The degree of genetic polymorphism in the human V\\textsubscript{H} region is high. Restriction fragment length polymorphisms (RFLPs) of the V\\textsubscript{H}2 (39, 44), V\\textsubscript{H}3 (39, 45-49), V\\textsubscript{H}4 (50; Walter and Cox, unpublished data), and V\\textsubscript{H}5 (51) families have been de-
| Clone | V_{α}2-1 | V_{α}2-2 | V_{α}2-3 | V_{α}2-4 | V_{α}2-5 | V_{α}3f-1 | V_{α}3f-2 | V_{α}3f-3 | V_{α}3f-5 | V_{α}3f-6 | V_{α}3f-7 | V_{α}4-2 | V_{α}4-4 | V_{α}4-5 | V_{α}4-8 | V_{α}4-11 | V_{α}4-12 | V_{α}5-1 | V_{α}5-2 | V_{α}5-3 | V_{α}6 | Rearranged to: |
|-------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|-------|
| T1B5  | 2       | 2       | 1       | 0       | *       | 1       | 2       | 2       | *       | 2       | ND      | ND      | 2       | 0       | 1       | 1       | 2       | 2       | 0       | ND    |
| T3E2  | 1       | 2       | 1       | 0       | *       | 0       | 2       | 2       | *       | ND      | 2       | ND      | ND      | 0       | 2       | 2       | 0       | ND      | ND      | ND    |
| T1C10 | 2       | 2       | 1       | 1       | 1       | 2       | 2       | *       | ND      | 2       | 2       | 1       | 1       | 1       | 2       | 2       | 0       | ND      | ND      | ND    |
| T2G2  | 2       | 2       | 1       | 1       | 1       | 0       | 1       | 2       | 2       | ND      | 1       | 2       | 1       | 0       | 2       | 2       | 0       | ND      | ND      | ND    |
| T5F8  | 2       | 2       | 1       | 1       | 1       | 2       | 2       | *       | ND      | 2       | 2       | 1       | 1       | 1       | 2       | 2       | ND      | ND      | ND      | ND    |
| 2C11  | 2       | 2       | 1       | 1       | 1       | 0       | 1       | 2       | 2       | ND      | 2       | 2       | 1       | 0       | 2       | 2       | 0       | ND      | ND      | ND    |
| T4B8  | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 1       | ND      | ND      | ND      | ND      | 0       | ND      | ND      | ND      | ND      | ND      | ND    |
| 2B10  | 2       | 2       | 1       | 1       | 1       | 1       | 1       | 2       | 2       | ND      | 2       | 2       | 1       | 0       | 2       | 2       | 0       | ND      | ND      | ND    |

HSC no.

| 1322  | 2       | 2       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | 2       | 2       | 2       | 1       | 2       | 2       | 2       | ND      | ND      | ND      |

A12

| 2     | 1       | 2       | 2       | 1       | 1       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

21A12

| 2     | 1       | 2       | 2       | 1       | 0       | 1       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

25C3

| 3     | 1       | 2       | 2       | 0       | *       | 1       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

12A4

| 3     | 1       | 2       | 2       | 2       | 1       | 1       | 2       | 2       | 2       | 2       | 2       | ND      | ND      | 0       | 2       | 2       | 2       | ND      | ND      | ND      |

1321

| 2     | 2       | 2       | 1       | 1       | 1       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

3E12

| 2     | 1       | 2       | 2       | 1       | 1       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

3F11

| 2     | 1       | 2       | 2       | 1       | 2       | 1       | 1       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

6A9

| 2     | 2       | 2       | 0       | *       | 2       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

6B12

| 2     | 2       | 2       | 1       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | 2       | 2       | 1       | 1       | 2       | 2       | 1       | 2       | 2       | 2       |

6B13

| 2     | 2       | 2       | 1       | *       | ND      | 2       | 2       | 2       | 2       | ND      | ND      | 2       | 0       | 1       | 2       | 2       | ND      | ND      | ND      | ND      | ND      |

6D3

| 2     | 2       | 2       | 1       | *       | 2       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      | ND      |

6E11

| 2     | 2       | 1       | 1       | *       | 1       | 2       | 2       | 1       | ND      | ND      | 2       | 1       | 1       | 1       | 2       | 2       | 0       | 2       | 2       | 2       |

6F2

| 2     | 2       | 2       | 0       | *       | 2       | 2       | 2       | 2       | ND      | ND      | 2       | 1       | 1       | 1       | 2       | 2       | 0       | 2       | 2       | 2       |

6H5

| 2     | 2       | 1       | 0       | *       | 1       | 1       | 2       | 1       | ND      | ND      | 2       | 1       | 0       | 1       | 2       | 2       | 1       | 1       | 1       | ND      |

6G8

| 2     | 1       | 1       | 0       | *       | 1       | 1       | 2       | 1       | ND      | ND      | 2       | 1       | 0       | 1       | 2       | 2       | 1       | 1       | 1       | ND      |

8A9

| 2     | 2       | 2       | 1       | *       | 2       | 2       | 2       | 2       | ND      | ND      | 2       | 1       | 1       | 1       | 2       | 2       | ND      | ND      | ND      | ND      |

9A11

| 2     | 2       | 2       | 0       | *       | 1       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | 2       | 1       | 1       | 1       | 2       | 2       | 1       | 2       | ND      |

9B5

| 2     | 2       | 2       | 0       | *       | 2       | 2       | 2       | 2       | ND      | ND      | ND      | ND      | 2       | 1       | 1       | 1       | 2       | 2       | 1       | 2       | ND      |

9F9

| 2     | 2       | 2       | 1       | *       | 2       | 2       | 2       | 2       | ND      | ND      | 2       | 2       | 1       | 1       | 2       | 2       | 1       | 2       | 2       | ND      |

HSC no.

| 1001  | 2       | 2       | 2       | 1       | *       | 2       | 2       | 2       | ND      | ND      | 2       | 2       | 1       | 1       | 2       | 2       | 1       | 2       | 2       | ND      |

Percent DEL | 8 | 0 | 4 | 33 | 50 | 0 | 38 | 15 | 0 | 25 | 0 | 11 | 2 | 55 | 40 | 5 | 2 | 2 | 55 | 10 | 62 |
scribed. Most (13/15) of the characterized \(V_\mu\) region polymorphisms are insertion/deletion polymorphisms, indicating that the actual germline number of \(V_\mu\) gene segments differs between individuals. Examination of the DNA of the three donors reveals that no one donor has all human \(V_\mu\) gene segments (Table 1). Positional information of many of the polymorphic \(V_\mu\) gene segments could therefore be determined only through comparison, within each set of lines derived from each of the three donors.

**Determination of \(V_\mu\) Gene Segment Dosage.** DNA from three series of independent B cell clones, and from leukocytes of each donor, was analyzed for the presence or absence of \(V_\mu\) gene segments of five different human \(V_\mu\) families. The 21 \(V_\mu\) gene segments analyzed are shown in Fig. 3. The hybridization intensities of the 21 \(V_\mu\) gene segments in the DNA from each of the three sets of monoclonal B cells lines were compared by visual and densitometric examination with those in the donor's leukocyte DNA. Filters were rehybridized with another chromosome 14 probe, that for AAT, to control for different amounts of DNA between lanes. The results of the deletion analysis of 21 \(V_\mu\) gene segments detected with the \(V_\mu2\), \(V_\mu3\), \(V_\mu4\), \(V_\mu5\), and \(V_\mu6\) probes of all three series of B cell lines and their germline donors are summarized in Table 1. As 20 of the B cell lines had undergone only a single \(V_\mu\)-(D)\(J_\mu\) rearrangement event, in many cases a \(V_\mu\) gene segment was deleted on one IGH allele but not on the other. The regions deleted in the B cell lines are shown schematically in Fig. 4. B cell clones in which \(V_\mu\) probes detected novel fragments (discussed below) probably represent the \(V_\mu\) gene segments selected for \(V_\mu\)-(D)\(J_\mu\) joining (Table 1). The \(V_\mu\) gene segments that were rearranged in the other B cell clones could not be determined by the Southern hybridization analysis conducted here. In the B cell lines analyzed here with probes from the five \(V_\mu\) families, only the single member \(V_\mu6\) family was not involved in any \(V_\mu\)-(D)\(J_\mu\) rearrangements.

**Deletion Analysis of \(V_\mu2\) Gene Segments.** The \(V_\mu2\) probe detects three polymorphic \(V_\mu2\) loci in BgIII-digested DNA (39, 44). \(V_\mu2\)-2 is an insertion/deletion polymorphism, with the presence or absence of a 12-kb fragment (absence indicated as 0 kb). \(V_\mu2\)-5 is also an insertion/deletion polymorphism, with alleles of 3.4 and 0 kb. \(V_\mu2\)-4 is a polymorphic locus with alleles of 7.3 and 7.0 kb. The \(V_\mu2\) genotypes (\(V_\mu2\)-2, \(V_\mu2\)-4, \(V_\mu2\)-5) of the B cell line donors are: HSC no. 1322 (0/0, 7.0/7.0, 3.4/3.4); HSC no. 1321 (12/0, 7.0/7.0, 0/0); and HSC no. 1001 (0/0, 7.0/7.0, 3.4/0). \(V_\mu2\)-2, and
Figure 5. Comparison of deletion and physical mapping results in the \( V_n \) region. \( V_n \) gene segments in bold were characterized in both deletion and physical mapping experiments. Lines above the physical map indicate the alignment of the B cell line deletions from Fig. 4. Only the 26 linear \( V_n-(D)_n \) rearrangements were included. Single nonlinear rearrangements in 3F11 and 6G8, and both rearrangements present in T3E2 and 6B13 were excluded, since they could not be determined which rearrangement in the latter two B cell lines were nonlinear. Physical mapping information is from Walter et al. (11). Bs, N, and S indicate positions of BssHII, Ncol, and SfiI sites, respectively (parentheses indicate restriction sites that only partially digest in leukocyte DNA). The location of the common 80-kb insertion/deletion polymorphism, involving \( V_{2-2} \) and \( V_{2-3} \), is indicated with a dotted line on the restriction map. The scale (in kb) is indicated at the top. Thicker horizontal lines indicate the locations of \( V_n \) gene segments further refined by deletion mapping results. \( V_{2-3} \) positioning was determined from deletion mapping only. \( V_{2-3} \) gene segments also detected with the \( V_n \) probe are indicated in parentheses. (*) \( V_n \) polymorphic loci, usually of the insertion/deletion type.

\( V_{3f-1} \) (discussed below) are polymorphic \( V_n \) loci located within an 80-kb insertion/deletion polymorphism in the \( V_n \) region (11).

The deletions in the three sets of B cell lines are summarized in Fig. 4. The number of times that a \( V_n \) gene segment was deleted in the B cell lines, divided by the number of germline copies of the particular \( V_n \) gene segment, was used to determine relative \( V_n \) order. These values, expressed as a percentage, are shown in Table 1. In the \( V_n \) region rearrangements in the 26 B cell lines, \( V_{n2-5} \) was deleted in 50% (15/30) of the opportunities in which this could have been detected. The \( V_{n2-5} \) gene segment is only present on one chromosome of HSC no. 1001, and is not present at all in HSC no. 1321). \( V_{n2-4} \) was deleted in 33% (17/52) of IGH alleles, \( V_{n2-1} \) in 10% (5/52) of IGH alleles, and \( V_{n2-3} \) was deleted in 4% (2/52) of IGH alleles. \( V_{n2-2} \) was not observed to be deleted in any of the B cell lines but was present on only 4 of the 52 germline chromosomes examined. \( V_{n2-2} \) was only present in HSC no. 1321, and in this individual, only heterozygously. Since these four B cell lines did not delete \( V_{n2-2} \) or \( V_{n2-3} \) (Table 1), the relative position of the \( V_{n2-2} \) gene segment, 5' of \( V_{n2-4} \), could not be determined. Therefore, the \( V_{n2} \) gene segment order, as determined by deletion analysis, is: 5'\( V_{n2-2} \)-3'\( V_{n2-1} \)-2'\( V_{n2-3} \)-2'\( V_{n2-1} \)-3'\( V_{n2-4} \), with \( V_{n2-2} \) located 5' of the \( V_{n2-4} \) gene segment. The deletion map obtained from the analysis of the 21 different \( V_n \) gene segments investigated in the 26 B cell lines is shown in Fig. 5.

The B cell line 6G8 appeared to delete \( V_{n2-3} \), but not \( V_{n2-1} \). This deletion order is not consistent with \( V_n \) gene segment order derived from the other B cell lines from HSC no. 1001 (the donor of 6G8) or from the other two donors. Therefore, either the dosage of these two \( V_{n2} \) gene segments
was incorrectly determined in 6G8, or a nonlinear rearrangement event occurred in this B cell line (discussed below).

Novel fragments, not observed in the DNA of HSC no. 1322, were detected in the B cell lines T1C10 and T5F8 (Fig. 4). These novel V,3 fragments were also detected with the Jn probe, suggesting that these B cell lines contained rearrangements to V,3 gene segments. A novel fragment was also detected with the V,2 family probe in the B cell line A12 (derived from HSC no. 1321), consistent with a rearrangement in this B cell line to a V,2 gene segment (data not shown).

Deletion Analysis of V,3 Gene Segments. Hybridization of B cell line DNA with V,1 and V,3 family probes yielded complex patterns of hybridizing fragments, precluding the identification of deleted Vn gene segments using these probes (data not shown). To simplify the hybridization pattern detected with the V,3 family probe, we derived the V,3f probe from the 5' flanking region of the V,3 gene segment V,26 (11). V,3f detects 7 of the ~28 V,3 gene segments (11). The status of these seven V,3 gene segments in the B cell lines was determined using the V,3f probe. V,3f-2 is a polymorphic locus with alleles of 6.0 and 4.6 kb in EcoRI-digested DNA (39). V,3f-1, V,3f-3, and V,3f-4 are insertion/deletion polymorphisms, with alleles of 6.7 and 0 kb, 4.3- and 0 kb, and 3.5 and 0 kb, respectively in EcoRI-digested DNA. The V,3 genotypes (V,3f-1, V,3f-2, V,3f-3, V,3f-4) of the B cell line donors are: HSC no. 1322 (0/0, 4.6/4.6, 4.3/4.3, 0/0); HSC no. 1321 (6.7/0, 6.0/6.0, 4.3/4.3, 0/0); and HSC no. 1001 (0/0, 6.0/6.0, 4.3/4.3, 0/0). In HSC no. 1322, the deletion status of the V,3f-6 gene segment was not determined as it appeared that an additional Vn,3 containing fragment comigrated with V,3f-6 in this individual. V,3f-5 was not deleted in any of the B cell lines, consistent with the previous suggestion that V,3f-5 could lie outside of the V,3 region (11). The intensities of V,3f-2, V,3f-3, and V,3f-6 hybridizing fragments in the B cell lines derived from HSC no. 1321 were therefore determined by comparison with the hybridization intensity of the V,3f-5 fragment. The insertion allele of the V,3f-4 polymorphism (11) did not appear in the germline chromosomes of any of the three donors, precluding the ordering of this V,3 gene segment.

The deletions in the three sets of B cell lines were analyzed to determine the V,3f gene segment order, as was done for the V,2 gene segments. This analysis indicates that the V,3f gene segment order is: 5'-(V,3f-5)-(V,3f-1/V,3f-7)-V,3f-3-V,3f-6-V,3f-2-3'. The relative order of the V,3f-1 and V,3f-7 gene segments could not be determined as no B cell line deleted one and not the other.

A novel V,3 fragment was observed in the DNA of the HSC no. 1322 B cell line T3E2, detected with the V,3 family probe. This novel fragment also hybridized to the Jn probe (data not shown), consistent with the rearrangement of the T3E2 B cell line to a V,3 family gene segment. The complex pattern of hybridizing fragments detected by the V,1 and V,3 family probes precluded the detection of other rearrangements to members of these Vn families by Southern hybridization. The question as to whether any of the other B cell lines were rearranged to members of Vn,1 or Vn,3 families could be addressed by sequencing amplified Vn-(D)Jn regions.

Deletion Analysis of V,A Gene Segments. The V,A probe detects 14 V,A gene segments, many of which are polymorphic, and several of which lie on similarly sized BgIII fragments (11, 50). Therefore, the deletion status of only the six clearly distinguishable V,A gene segments could be determined: V,A,4-2, V,A,4-4, V,A,5-5, V,A,8-8, V,A,11, V,A,12. Two of the V,A gene segments, V,A,4-8 and V,A,4-11, are insertion/deletion polymorphic loci, with alleles of 4.6 and 0 kb, and 3.8 and 0 kb, respectively, in BgIII-digested DNA. The V,A genotypes (V,A,4-8, V,A,4-11) of the B cell line donors are: HSC no. 1322 (4.6/0, 3.8/0); HSC no. 1321 (4.6/4.6, 3.8/0); and HSC no. 1001 (4.6/0, 3.8/0). Analysis of the deletions present in the B cell lines yielded a relative V,A gene segment order of: 5'-(V,A,4-4, V,A,4-12)-(V,A,4-2, V,A,4-11)-V,A,4-8-V,A,4-5-3'. The relative order of V,A,4-4 vs. V,A,4-12, and of V,A,4-2 vs. V,A,4-11, could not be determined, as no B cell clone had deletions between the members of these pairs of V,A loci. The V,A probe detected a novel V,A fragment in DNA from the HSC no. 1001 B cell line 6G8 (data not shown), consistent with a Vn rearrangement in B cell line 6G8 to a V,A gene segment.

Deletion Analysis of V,5 Gene Segments. The Vn,5 probe hybridizes to the two or three members of the Vn,5 family; the total number of V,A gene segments in humans is polymorphic (51). Vn,5-2 is an insertion/deletion polymorphism with alleles of 8.0 and 0 kb in BglIII-digested DNA. The V,A genotypes of the B cell line donors are: HSC no. 1322 (0/0); HSC no. 1321 (8.0/0.0); and HSC no. 1001 (8.0/0.0). Analysis of the B cell line deletions indicated a V,A gene segment order of: 5'-(V,A,5-1, V,A,5-3, V,A,5-2, V,A,5-3, 5-2'), as a novel fragment of 1.8 kb was detected by the V,A probe in BglIII-digested DNA from the HSC no. 1322 B cell line T2G2 (Fig. 4). This same fragment hybridized to the Jn probe, indicating that T2G2 is rearranged to a V,A gene segment. The particular V,A gene segment used is probably V,A,5-3, since HSC no. 1322 has only two V,A gene segments, and V,A,5-1 is not deleted.

Deletion Analysis of the V,6 Gene Segment. Hybridization of a Vn,6 probe to B cell line DNA indicated that all B cell lines had deleted the Vn,6 gene segment on at least one chromosome. In all B cell lines containing two Vn-to-(D)Jn rearrangement events, both Vn,6 alleles were found to be deleted (T1B5, T3E2, 21A12, 12A4, 6A9, and 6B13; shown in Fig. 4). Vn,6 was the Vn gene segment most often deleted: 63, 75, and 57% of the time in the B cell lines derived from HSC no. 1322, HSC no. 1321, and HSC no. 1001, respectively. None of the 32 different Vn-to-(D)Jn rearrangement events analyzed in the B cell lines were to the Vn,6 gene segment. As Vn,6 is the most 3' Vn gene segment (4, 5, 15), the deletion model of Vn rearrangement predicts that Vn,6 would be deleted whenever any other Vn gene segment is involved in a Vn-to-(D)Jn rearrangement. Analysis of the 32 different Vn-to-(D)Jn rearrangements in the 26 B cell lines
is consistent with this prediction. The V<sub>6</sub> data are also consistent with the D<sub>n</sub> region results, indicating that ~2/3 of the human B cell clones analyzed were rearranged for a single IGH allele only, surprisingly different from the observations in rodent B cell lines, which usually undergo bi-allelic V<sub>H</sub> region rearrangements (19, 23).

The Relative Order of V<sub>H</sub> Gene Segments. The analysis of 21 V<sub>H</sub> gene segments in the deletions generated through IGH rearrangements of the B cell lines indicates a relative order of V<sub>H</sub> gene segments. In Fig. 4, the extent of the V<sub>H</sub> region deletion present in each of the B cell lines are shown. The V<sub>H</sub> gene segment order for each of the three sets of B cell lines was consistent with a single relative V<sub>H</sub> gene segment order (Fig. 4). This deletion mapping V<sub>H</sub> gene segment order is also consistent with the order derived by PFGE physical mapping techniques (11). This indicates that EBV-transformed B cells appear to maintain the germline V<sub>H</sub> gene segment order, and that the V<sub>H</sub> region rearrangement/deletion process appears to be mainly linear for V<sub>H</sub> utilization. Exceptions to the linear rearrangement process are discussed below.

Comparison of the Results of Deletion and Physical Mapping Experiments in the V<sub>H</sub> Region. In four regions, the V<sub>H</sub> gene segment order from physical mapping studies (11) was further defined by deletion mapping results (Fig. 5). V<sub>H</sub>4-5, V<sub>H</sub>2-5, and V<sub>H</sub>5-2 were mapped to the same interval by PFGE methods (11). B cell lines 6B13, 6D3, and 9F9 have deleted V<sub>H</sub>4-5, but not V<sub>H</sub>2-5 or V<sub>H</sub>5-2. The order of these V<sub>H</sub> gene segments therefore must be 5'-(V<sub>H</sub>2-5, V<sub>H</sub>5-2)V<sub>H</sub>4-5-3'. Similarly, B cell lines 6H15 and TB10 deleted V<sub>H</sub>3F-3 but not V<sub>H</sub>4-2, indicating that V<sub>H</sub>3F-3 must be 3' of V<sub>H</sub>4-2. The B cell line T2G2 is deleted at the V<sub>H</sub>4-11 locus, but V<sub>H</sub>4-12 is retained. T2G2, as stated earlier, is rearranged to V<sub>H</sub>5-3. The order of these V<sub>H</sub> gene segments must be 5'-V<sub>H</sub>4-12-V<sub>H</sub>5-3-V<sub>H</sub>4-11-3'. The V<sub>H</sub>2-3 gene segment was mapped to a 100-kb NotI fragment, unlinked to the long range physical map of Walter et al. Deletion mapping results indicate that V<sub>H</sub>2-3 was deleted in ~4% of rearrangement opportunities (Table 1). This is a frequency comparable with V<sub>H</sub> gene segments V<sub>H</sub>4-4, V<sub>H</sub>4-12, V<sub>H</sub>5-1, located at the 5' end of the V<sub>H</sub> region (11). The deletion mapping data are consistent with the positioning of the 100-kb NotI fragment (containing the V<sub>H</sub>2-3 gene segment) near, and possibly adjacent to, the V<sub>H</sub>4-4, V<sub>H</sub>4-12, and V<sub>H</sub>5-1 gene segments.

The relative V<sub>H</sub> gene order, as determined by deletion mapping results, had been further defined in the PFGE-generated physical map of Walter et al., in four regions. V<sub>H</sub>2-2 could not be positioned relative to V<sub>H</sub>2-3, V<sub>H</sub>2-1 and V<sub>H</sub>3F-1, V<sub>H</sub>3F-7; V<sub>H</sub>4-4, V<sub>H</sub>4-12; and V<sub>H</sub>4-2, V<sub>H</sub>4-11 could not be ordered by deletion analysis. The positions of these V<sub>H</sub> gene segments had been more precisely determined within the V<sub>H</sub> region though PFGE and 2D-DNA electrophoresis mapping to different large restriction fragments (11). The results of the physical and deletion mapping experiments were combined to generate the V<sub>H</sub> gene segment physical organization shown in Fig. 5.

Examination of the V<sub>H</sub> region organization presented in Fig. 5 reveals that the V<sub>H</sub> gene segments of different V<sub>H</sub> families are extensively interspersed. Many of the B cell lines (e.g., 9B5 and 21A12) deleted members of different V<sub>H</sub> families while retaining other members of the same V<sub>H</sub> families. No V<sub>H</sub> gene family was observed to be exclusively deleted in any B cell line.

Nonlinear V<sub>n</sub>-to-(DJ)<sub>n</sub> Rearrangements. A linear model of
IGH variable region assembly predicts that the V_n gene segments 3' of a selected V_n gene segment will be deleted by the rearrangement process. While most (28/32) of the different V_n-to-(D)J_n rearrangement events analyzed in the B cell lines appeared to be consistent with this model, four different B cell lines (T3E2, 3E11, 6B13, and 6G8) appeared to retain V_n gene segments indicated by other B cell line rearrangements to be 3' of V_n gene segments deleted in T3E2, 3E11, 6B13, and 6G8 (Fig. 4). For example, 3E12 is deleted for one copy of the V_n-2 gene segment, but has two copies of all V_n gene segments between V_n-3 and V_n-5. While errors in the determination of V_n gene segment dosage could have been made in these B cell lines, the nonlinear rearrangement events in T3E2, 3E11, 6B13, and 6G8 B cell lines could also be the result of other methods of V_n region rearrangement.

Utilization of 5' End of the V_n Region. The 26 linear deletions of the V_n gene segments characterized in the B cell clones were aligned in Fig. 5. 62 of the 76 V_n gene segments that hybridized to the known V_n family probes were positioned within the physical map described in reference 11. Therefore, the ~1,000-kb portion of the V_n region analyzed in the B cell clones contains the great majority (at least 80%) of the V_n gene segment repertoire. V_n-3 represents an approximately "half-way mark" in the portion of the V_n region analyzed, being ~500 kb from the J_n region (11). Of the 26 rearrangements (77%) characterized in the B cell lines with only linear rearrangements involve V_n gene segments 3' of, or in, the interval defined by V_n-3 and V_n-5. Only six use V_n gene segments 5' of V_n-3, and of these, only one extends beyond V_n-2, the most 5' V_n gene segment examined here. Thus adult-derived B cell lines preferentially use the V_n gene segments from the J_n-proximal V_n region.

Discussion

We have analyzed panels of monoclonal B cell lines from three different donors to elucidate the physical organization of the human Ig V_n region. The extent of the V_n region deletions that occurred during the rearrangement processes in the development of 26 independent B cell clones was characterized. This information was used to determine the relative positions of 21 V_n gene segments of the V_n2, V_n3, V_n4, V_n5, and V_n6 families, and allowed us to trace the search patterns of recombinase(s) involved in V_n-(D)J_n rearrangement processes in these B cells.

Honjo and Kataoka suggested a mechanism of IGH variable region assembly, later demonstrated by Cory and Adams, in which V_n gene segments 5' of a selected V_n gene segment remain in the rearranged IGH locus while those 3' are deleted. Deletion mapping takes advantage of this process. Several groups have used deletion mapping of V_n region rearrangements to generate a V_n family order for the murine V_n locus (27-31). Preliminary studies indicated that deletion analysis would also be possible in the human V_n region (17, 52). Our results indicate that deletion analysis is indeed a useful means of determining V_n gene segment organization in the large (1,500 kb) human V_n locus.

Our results are largely consistent with the deletion model of IGH region rearrangement (26, 53). Consistent with previous observations in several human B cell lines (54), most of our B cell lines (19 of the 25 B cell clones for which J_n results were obtained) had bi-allelic J_n rearrangements. Of these lines, however, only six clones rearranged both V_n alleles. A recent report of V_n expression in EBV-transformed B cell lines (55) found that human B cell clones expressed heavy chain mRNA containing only one V_n gene segment. In murine B cell lines that have two V_n-(D)J_n rearrangements, both rearrangements frequently express detectable mRNA (56). These findings, together with the observation here that only 6/26 clones had two V_n rearrangements, leads us to conclude that most human B cells, unlike murine B cells, only undergo a single V_n-(D)J_n rearrangement.

While 28 of the 32 different V_n-(D)J_n rearrangements observed in the 26 B cell lines were consistent with a simple deletion method of rearrangement, 4 of the 32 V_n-(D)J_n rearrangements appeared to involve a different, secondary V_n rearrangement process. In these B cell lines (T3E2, 3F11, 6B13, and 6G8), V_n gene segments 3' of deleted V_n regions were retained (Fig. 4). These rearrangements could be the result of an IGH V_n rearrangement method similar to, or the same as, the inversion/deletion processes observed in pre-B cells (57). An inversion rearrangement mechanism has also been shown to occur in the V_k region (58), which allows the use of V_k gene segments that are in the opposite transcriptional orientation to the J_k and C_k genes. The four nonlinear rearrangements that we observed in the B cell clones all involve the distal portion of the V_n region. The rearrangements in the B cell lines T3E2, 3F11, 6B13, and 6G8, therefore, could indicate that some of the V_n gene segments in the distal V_n region are in the opposite transcriptional orientation with respect to the C_n genes. However, at the level of Southern blot analysis, the majority (28/32) of the different V_n-to-(D)J_n rearrangement events appeared to be the result of single linear deletion events, suggesting that the transcriptional polarity of most V_n gene segments is the same as that of the C_n genes.

The V_n gene segment order as determined by deletion analysis was consistent with that determined by long-range physical mapping experiments (Fig. 5). This consistent V_n gene segment order also indicates that EBV-transformed B cell lines are suitable material for our studies. Different B cell clones use different V_n families, and rearrangements to gene segments of the V_n2, V_n3, V_n4, V_n6 families were observed (Table 1). The B cell lines deleted different portions of the V_n region (Fig. 5). Selection during the course of EBV transformation for particular B cell sublineages, resulting in a bias in these B cell clones, is therefore not very probable. The relative V_n gene segment order was the same for all three B cell line donors, despite racial and extensive polymorphic V_n region differences between the three donors.

Since the first report of the discovery of D_n sequences, it has been postulated that D-D joining could be an additional means of generating antibody diversity (14, 24). D-D joining has been suggested as an explanation for long N regions (59). However, D-D joining would break the 12/23 rule of Ig rearrangement (20). Meek et al. (60) found direct evidence of
D-D joining, and detected both direct and inverted D-D recombination events. They suggest that a new recombination system would not be required for D-D joining, or alternatively, D-D joining could occur at low efficiency or use cryptic secondary heptomer/nonamer sequences. Ichihara et al. (25) found no evidence of D-D joining involving the human Dn segments, but later reported the discovery of a new type of Dn segment, which they termed DIR, whose spacer lengths were found to be irregular (16). They suggest that these DIR segments may be involved in D-D joining.

In the analysis of the Dn regions in the B cell clones studied here, 13/26 B cell clones had evidence of D-D joining (e.g., T1C10). Regions 3' of portions of the Dn region that were deleted on both chromosomes of nine B cell lines were found to be present in at least one copy in the clones. Additionally, four of the clones (TB10, 25C3, 6B12, and 6H5) were observed to have two Dn region rearrangements, but only a single Jn region rearrangement, consistent with the occurrence of D-D joining events before D-J joining. The frequency of the occurrence of D-D joining observed in these human B cell lines is much higher than that found in mouse B cells (estimated at 1/33,000; [60]). This could be the result of DIR segments, which have not been found in mice, or could indicate that the rearrangement processes in these two species are not identical.

Hybridization of a Dn region probe to DNA extracted from the 26 B cell lines revealed that two of the fragments were not deleted in the B cell lines (Fig. 1b). As these fragments are homologous to the Dn region probe, but are not located within the major Dn region (between V6 and Jn), they define a second Dn locus. An additional Dn homologous region has been mapped to the IGH region of chromosome 14 (11, 17, 18), and may be the result of an unusual duplication event in the generation of the Vn locus in human evolution. However, a simple explanation of the events generating this second Dn locus is not possible, as neither the nearest Vn gene segment (V6), located 5' of the major Dn cluster, nor the Jn region, located 3' of the major Dn cluster, were duplicated in this putative event. It will be of interest to determine if, and when, any Dn sequences in this second Dn region are utilized in B cells, and if these cells define a distinct B cell lineage.

Extensive genetic polymorphism involving the human Vn gene segments has been observed (39, 44-49, 51). Three sets of isogenic cell lines were studied to avoid problems due to uncharacterized genetic polymorphism. The number of members of a given Vn family varies between individuals in the normal population, since 13/15 characterized Vn polymorphisms involve insertion or deletion of Vn gene segments. Interestingly, despite Vn number difference, the relative positions of the Vn gene segments in the three series of B cell lines are the same (Fig. 4). This implies that the order of Vn gene segments is the same for people with different sets of Vn polymorphic genes.

The order of Vn gene segments has been suggested to have functional significance. In mice, the Jn-proximal Vn gene segments were found to be preferentially used in fetal development (61-64). However, it has not been determined if the preferential use of the Jn-proximal Vn families is controlled by their position relative to the Jn region, or instead reflects fetal antigen selection of Vn families that might happen to be located in the 3' Vn region. The concept of Vn use based upon Vn position alone no longer adequately fits the data. Murine strain differences in the use of Vn families suggest that other loci outside of the IGH complex have a role in control of Vn gene segment expression (65, 66). However, any preferential use of specific Vn gene segments in murine fetal development is thought to disappear over the course of murine development. The adult mouse appears to use Vn families in a manner more reflective of the size of Vn gene families, although there are inconsistencies with this theory as well (67).

In contrast to the detailed studies in the mouse, the pattern of human Vn gene segment expression is not as well characterized. Analyses of human fetal Vn expression have shown that members of all six Vn families are utilized (8, 68-70). However, attempts to correlate Vn gene segment expression with Vn position relative to the Cn region have not been possible in humans as these studies require a detailed map of the Vn region which, until very recently, has been unavailable. The extensive interspersion of the members of the various human Vn families have complicated methods to elucidate Vn organization. The recent generation of a physical map of the Vn gene segments by PFGE and two-dimensional DNA electrophoresis methods (11), and by the deletion mapping techniques in this report, should make possible comparison of human Vn gene expression with Vn location.

The organization of Vn gene segments in mice and humans is very different (Fig. 6). The analysis of the deletions present in the 26 B cell lines (Fig. 4) is consistent with cosmid cloning data (10), long-range restriction mapping (4, 11), and genetic analysis (39), all indicating that the human Vn families are extensively interspersed. This is in sharp contrast to the organization of the Vn region in the mouse, where the members of a given Vn gene family are generally clustered within the locus (12, 13), although examples of limited interspersion of some mouse Vn families has been shown (28, 29).

The different organization of the Vn loci between mouse and humans indicates that the evolution of the IGH gene complex occurred differently in these two species. It is difficult to imagine a mechanism that would result in the clustered Vn family organization seen in mouse strains, from an initial organization of interspersed Vn families, as found in humans. As well, no simple mechanism would generate the human interspersed Vn organization from an initially clustered Vn organization. Since human and mouse Vn regions must have undergone very different evolutionary processes to generate their current Vn repertoires, the control of Vn gene expression could therefore also be different between these two species.

The change in mice from preferential use of Jn-proximal Vn families in fetal development to Vn family use that is
more reflective of $V_n$ family size in the adult, may not be necessary in humans, since the human $V_n$ families are not clustered. Fig. 5 is a "footprint" of the recombinase that was active in the development of the B cell clones. 26 of the $V_n$ region deletions characterized in the B cell lines have been aligned (the rearrangements present in the B cell lines with nonlinear rearrangements were not included). $V_n3f-3$ is located $\sim$500-kb from the $J_n$ region (11). The adult-derived B cell lines preferentially used $V_n$ gene segments 3' of, or in, the region containing $V_n3f-3$. $V_n$ gene segments mapping within the $J_n$-proximal $V_n$ region. This preference could represent selection for the expression of particular $V_n$ gene segments during the development of the B cell precursors of our clones, before their function. As discussed above, selection for the expression of particular $V_n$ gene segments in these B cell clones is unlikely. Instead, the B cell clones probably are representative of $V_n$ gene segment usage in the circulating human B cell population. Members of all six human $V_n$ families have members in the $J_n$-proximal $V_n$ region (Fig. 6). Murine $V_n$ organization, characterized by clustering of $V_n$ families, may require a "normalization" mechanism to change from fetal to adult patterns of $V_n$ use. In contrast, the recombination machinery in humans does not have to span large lengths of DNA in order to produce diverse antibody repertoires using $V_n$ region gene segments from all $V_n$ families. Preferential use of $J_n$-proximal $V_n$ gene segments may therefore be maintained throughout human development.

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