

Ordered rearrangement of immunoglobulin heavy chain variable region segments

Frederick W. Alt*, George D. Yancopoulos, T. Keith Blackwell, Charles Wood¹, Elise Thomas^{1,2}, Michael Boss^{1,2}, Robert Coffman³, Naomi Rosenberg⁴, Susumu Tonegawa¹ and David Baltimore^{1,2}

Department of Biochemistry and Institute for Cancer Research, Columbia University, College of Physicians and Surgeons, New York, NY 10032, ¹Center for Cancer Research and Department of Biology, and ²Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA 02139, ³DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304, and ⁴Cancer Research Center, Tufts University Medical School, Boston, MA 02111, USA

*To whom reprint requests should be sent
Communicated by D. Baltimore

The immunoglobulin heavy chain variable region is encoded as three separate libraries of elements in germ-line DNA: V_H, D and J_H. To examine the order and regulation of their joining, we have developed assays that distinguish their various combinations and have used the assays to study tumor cell analogs of B-lymphoid cells as well as normal B-lymphoid cells. Abelson murine leukemia virus (A-MuLV) transformed fetal liver cells – the most primitive B-lymphoid cell analog available for analysis – generally had DJ_H rearrangements at both J_H loci. These lines continued DNA rearrangement in culture, in most cases by joining a V_H gene segment to an existing DJ_H complex with the concomitant deletion of intervening DNA sequences. None of these lines or their progeny showed evidence of V_HD or DD rearrangements. Heavy chain-producing tumor lines, representing more mature stages of the B-cell pathway, and normal B-lymphocytes had either two V_HDJ_H rearrangements or a V_HDJ_H plus a DJ_H rearrangement at their two heavy chain loci; they also showed no evidence of V_HD or DD rearrangements. These results support an ordered mechanism of variable gene assembly during B-cell differentiation in which D-to-J_H rearrangements generally occur first and on both chromosomes followed by V_H-to-DJ_H rearrangements, with both types of joining processes occurring by intrachromosomal deletion. The high percentage of J_H alleles remaining in the DJ_H configuration in heavy chain-producing lines and, especially, in normal B-lymphocytes supports a regulated mechanism of heavy chain allelic exclusion in which a V_HDJ_H rearrangement, if productive, prevents an additional V_H-to-DJ_H rearrangement.

Key words: immunoglobulin/heavy chain/V_H, D and J_H gene segments/B-lymphoid cells

Introduction

The variable region of an immunoglobulin heavy chain is encoded by three separate germ-line DNA elements, the V_H gene segment, the D (diversity) segment and the J_H (joining) segment (Brack *et al.*, 1978; Max *et al.* 1979; Sakano *et al.* 1979, 1980; Early *et al.*, 1980). These elements are encoded in three separate clusters in the genome, requiring at least two somatic recombination events to produce the complete V_HD-

J_H heavy chain variable region gene. These recombination processes are apparently mediated by a set of highly conserved recognition sequences which consist of a palindromic heptamer and a characteristic nonamer separated by a spacer region (Early *et al.*, 1980; Sakano *et al.*, 1980). A complete recognition sequence, starting with the heptamer, lies flush with the 3' border of each V_H and D segment and the 5' border of each D and J_H segment (Early *et al.*, 1980; Sakano *et al.*, 1980, 1981; Kurosawa *et al.*, 1981; Kurosawa and Tonegawa, 1981). The spacer region is characteristically 23 bp long for V_H and J_H recognition sequences (Early *et al.*, 1980; Sakano *et al.*, 1980) and 12 bp for the 3' and 5' D recognition sequences (Sakano *et al.*, 1981; Kurosawa *et al.*, 1981). It is believed that the recombination process can only occur between recognition sequences containing 12 and 23 bp spacers (Early *et al.*, 1980; Sakano *et al.*, 1980). Thus, the V_H-to-J_H joining process for heavy chains appears to be obligately mediated by V_H-to-D and D-to-J_H joins. Although most D segments have been rigorously proved to lie 5' to the four J_H segments (Wood and Tonegawa, 1983), neither the relative location of V_H segments nor their transcriptional orientation relative to the D and J_H segments has been shown. It is also unclear whether the joining process always deletes the intervening DNA. For D-to-J_H joining, the relative orientation of the segments implies that deletion occurs. It is possible, however, that inversion rather than deletion might be involved in V_H-to-D joining as has been shown for one aberrant D-to-J_H join (Alt and Baltimore, 1982) and has been suggested for light chains (Lewis *et al.*, 1983).

Because cells are diploid, two heavy chain genes could be functionally joined in any one cell. One mechanism that could restrict a cell to only one functional joined gene would be a cessation of the joining process once a functional join is made. Such a model was previously suggested for light chains, where only V-to-J joining is involved (Alt *et al.*, 1980a; Hieter *et al.*, 1981; Coleclough *et al.*, 1981), but for heavy chains the two step joining process makes interpretation more difficult. The suggestion has been made that productive synthesis of a μ chain may signal the termination of further rearrangement (Alt *et al.*, 1981, 1982a) but it was based on the assumption that DJ_H rearrangements are preferred or obligatory intermediates in the V_HDJ_H gene assembly process. Thus, by this model, the DJ_H rearrangements that are often observed in immunoglobulin-producing cells (Sakano *et al.*, 1981; Kurosawa *et al.*, 1981) would represent intermediates in the V_H gene construction process that were frozen by the production of μ chains encoded by the other heavy chain allele (see Alt *et al.*, 1982a for details).

To analyze further the regulation of V_H gene assembly, we have assayed for potential V_HDJ_H, DJ_H, V_HD and DD rearrangements in the various types of Abelson murine leukemia virus (A-MuLV) transformed pre-B cells, in tumor cell lines representing more mature stages of the B-lymphoid pathway, and in normal B-lymphocytes. We have analyzed in greatest detail the nature of primary and secondary J_H-associated

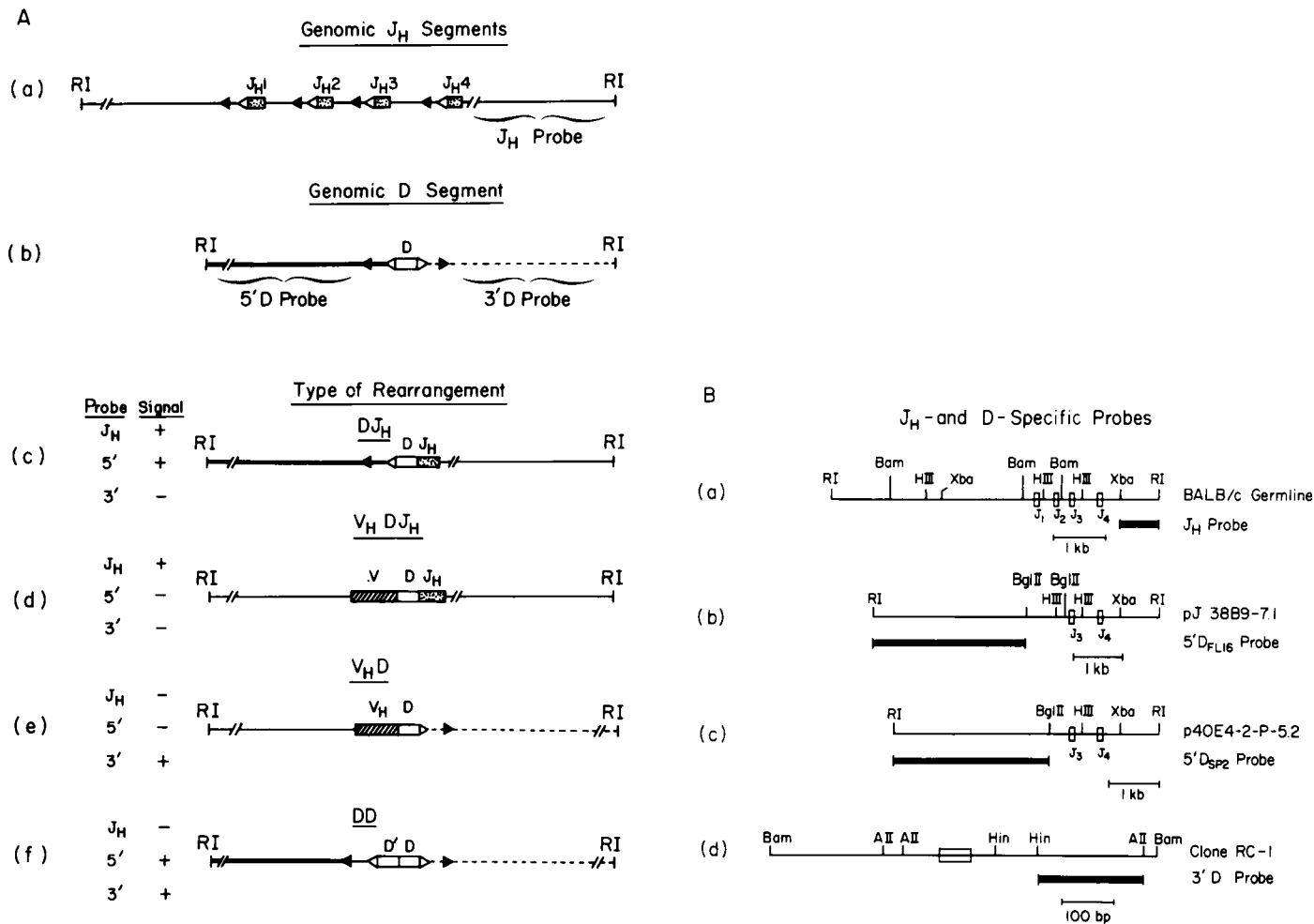


Fig. 1. Rearrangement assays. **Panel A:** an assay to determine the nature of D- or J_H -associated rearrangements. The details of the assay are described in the text. A detailed representation of the probes used is given in **panel B**. The sizes of the various *EcoRI* fragments and V_H D, and J_H segments are not drawn to scale. **Panel B:** J_H and D-specific probes. (a) A partial restriction map of the J_H -containing 6.2-kb *EcoRI* fragment of mouse genomic DNA is shown. The J_H probe was prepared by excising the 3'-most *XbaI/EcoRI* fragment from a plasmid containing the 6.2-kb *EcoRI* fragment (pR1- J_H , Alt *et al.*, 1981) and sub-cloning this segment into the *EcoRI* site of pBR322 (pJ_H800). The 770-bp insert from this plasmid was excised and used as the J_H probe as described previously (Alt *et al.*, 1981). (b) A partial restriction map of the insert of clone pJ_H38B9-7.1 is indicated. This DNA segment contains the 5' flanking sequence of an FL16-type D segment aberrantly rearranged to J_H 1 (Alt and Baltimore, 1982). The 5' D_{FL16} probe was made by dissecting out the indicated *EcoRI/BglII* fragment as previously described (Alt and Baltimore, 1982). (c) A partial restriction map of the insert of plasmid p40E4-2-P-5.2 is indicated. This plasmid was derived by cloning the 5.2-kb J_H -associated *EcoRI* fragment of line 40E4-subclone 2 (Alt *et al.* 1981) into the *EcoRI* site of Charon 16A and sub-cloning the insert into the *EcoRI* site of pBR322 as previously described (Alt *et al.*, 1982a). The 5.2-kb insert was shown to contain a $D_{Sp2.3}$ - J_H 3 join (Desiderio *et al.*, in preparation). The 5' D_{Sp2} probe was prepared by excising the indicated *EcoRI/BglII* fragment from this subclone. (d) A partial restriction map of the $D_{FL16.1}$ -containing *BamHI* fragment of phage lambda clone RC-1 (Kurosawa and Tonegawa, 1981) is shown. The 3' D probe was prepared by excising the indicated *HindIII/AvaII* fragment from this clone. Symbols: RI, *EcoRI*; Bam, *BamHI*; HIII, *HindIII*; Xba, *XbaI*; AII, *AvaII*.

rearrangements in fetal liver-derived A-MuLV transformants which undergo J_H -associated rearrangements during growth in culture (Alt *et al.*, 1981). The results of these studies suggest an ordered mechanism of V_H gene construction in which D-to- J_H rearrangements generally occur first and on both chromosomes followed by V_H -to- DJ_H rearrangements. These results also support a deletional process of joining and a regulated mechanism of heavy chain allelic exclusion.

Results

An assay for $V_H DJ_H$ and DJ_H rearrangements

To determine the nature of the J_H -associated rearrangements in a large number of B-lymphoid lines, we have devised a simple hybridization assay to distinguish DJ_H from $V_H DJ_H$ rearrangements. All the J_H segments lie within a single germ-line *EcoRI* fragment of 6.2 kb (Figure 1) and most J_H -associated rearrangements should generate an *EcoRI* fragment of novel

size which hybridizes to a probe prepared from the 3'-most portion of the 6.2-kb embryonic fragment (Figure 1A, panels a, c and d). If a rearrangement was the result of a normal DJ_H join (Alt and Baltimore, 1982) without an appended V_H gene segment, the novel *EcoRI* fragment would also contain a segment of 5' D-specific flanking sequence. Therefore, such a rearranged *EcoRI* fragment should also hybridize to an appropriate 5' D flanking sequence probe in addition to the 3' J_H -specific probe (Figure 1A, panels b and c). If, however, a V_H segment were appended to a DJ_H segment, the 5' D flanking sequence would be deleted and the novel fragment would hybridize to the 3' J_H -specific probe but not the 5' D flanking sequence probe (Figure 1A, panel d). Neither the novel *EcoRI* fragments generated from DJ_H nor those resulting from $V_H DJ_H$ joins should hybridize to a probe specific to the 3' D flanking sequences. DNA from cells with a $V_H DJ_H$ rearrangement may still show D-specific hybridization to some germ-line D-containing *EcoRI* fragments and/or

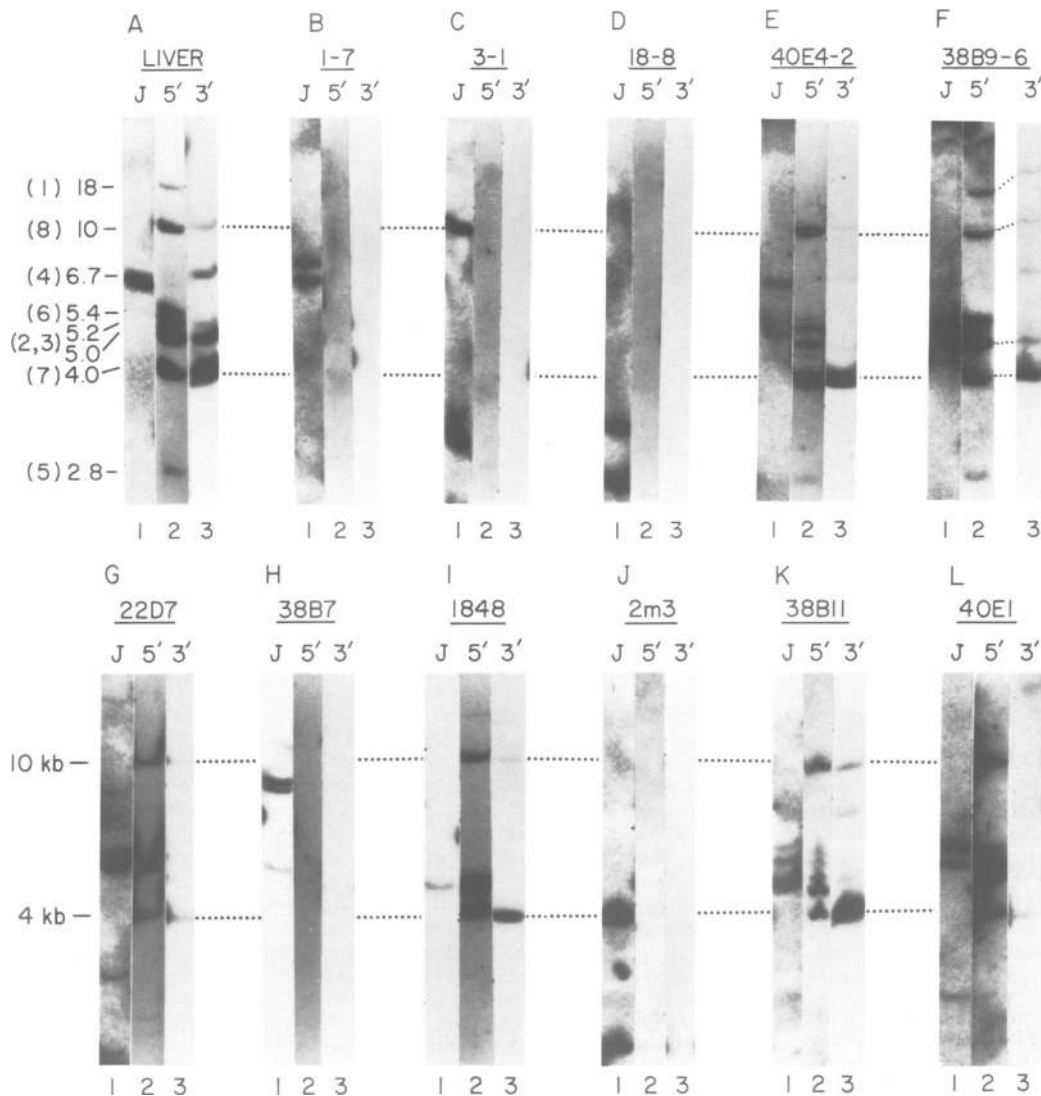


Fig. 2. Rearrangement of J_H and D segments in A-MuLV transformants. Approximately 10 μ g of genomic DNA from the indicated lines was digested with *Eco*RI, electrophoresed through 1% agarose gels, transferred to nitrocellulose paper and assayed for hybridization to either the J_H , 5'-D, or 3'-D probes (see Figure 1B), which were labelled by nick-translation with 32 P to a final specific activity of $\sim 5 \times 10^8$ c.p.m./ μ g. The 5' D probe shown contains a mixture of the 5' D_{FL16} and D_{SP-2} probes, although the FL16 probe predominates in this particular experiment. The size in kb of the various *Eco*RI fragments which contain sequences homologous to the 5' D probes is indicated to the left in panel A. The relative order from 3' to 5' of these *Eco*RI fragments in the genome as determined by Kurosawa and Tonegawa (1981) is indicated in parentheses next to the sizes. **Lane 1**, J_H -probe; **Lane 2**, 5' D-specific probe; **Lane 3**, 3' D-specific probe.

a DJ_H rearrangement if such a rearrangement occurred at the other allele (see below). If, however the D segments are located between the V_H and J_H segments, the deletional model of V_HDJ_H joining (Alt and Baltimore, 1982) would predict loss of hybridization to 5' D-specific probes if V_HD - J_H rearrangements occurred on both chromosomes.

To be a valid assay, essentially all and particularly the most 5' genomic D segments must be detected by the 5' D probe. Most of the known D-segments fall into one of two families – the SP-2 type and the FL16 type D segments (Kurosawa and Tonegawa, 1981). Probes prepared from the 5' flanking sequences of individual members of this family detect an overlapping but non-identical set of embryonic *Eco*RI fragments, most of which contain individual D segments (Kurosawa and Tonegawa, 1981). Because essentially all of the known D segments found in DJ_H or V_HDJ_H joints can be accounted for by members of these two families or by the most 3' D segment (DQ52, Sakano *et al.*, 1981), it seems like-

ly that most genomic D segments have been identified (Tonegawa, 1983). For these experiments we have prepared 5' D flanking sequence probes from the insert 40E4-2-P-5.2 DJ_H which is of the SP-2 type and from the insert 38B9-7.1 DJ_H which is of the FL16 type (Figure 1B, panels b and c). Together these two probes detect all the embryonic *Eco*RI fragments that have been characterized as either D-containing or closely associated, including fragments of 2.8, 4.0, 5.0, 5.2, 5.4, 6.7 and 18 kb (Kurosawa and Tonegawa, 1981; Figure 2A, lane 2). Furthermore, the $D_{38B9-7.1}$ probe strongly detects an additional fragment of 10 kb which is apparently the most 5' of the fragments detected: it is the only fragment present in all cells which contain two DJ_H rearrangements or a DJ_H and V_HDJ_H rearrangement (see Figures 2 and 3). It is not yet known, however, whether this fragment also carries a functional D segment. The most 5' of the fragments detected by this probe were (5' to 3') 10, 4.0 and 5.4 kb (Kurosawa and Tonegawa, 1981).

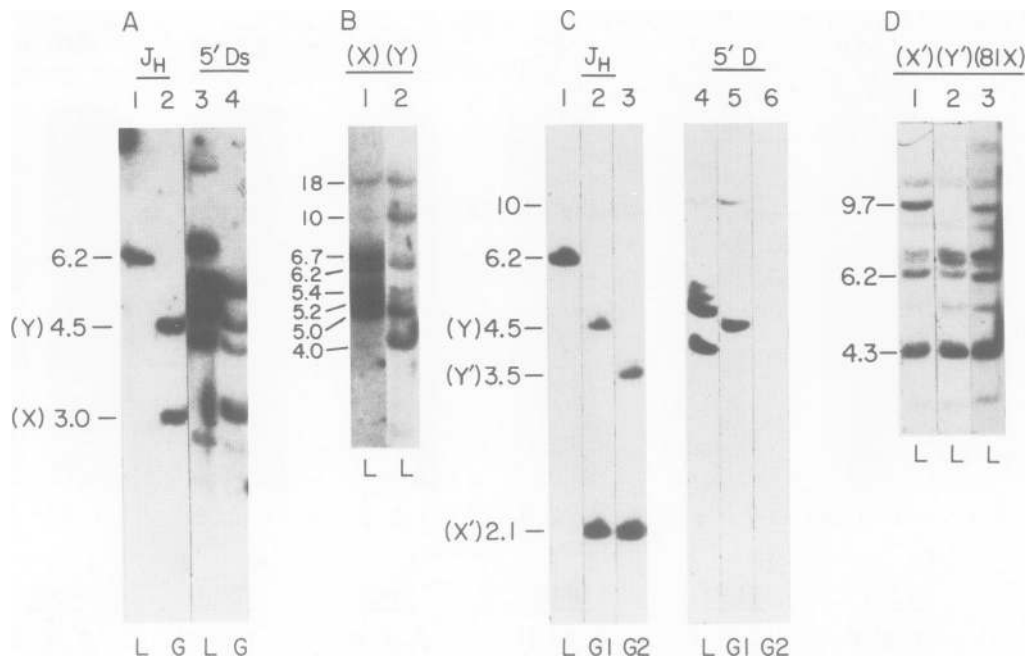


Fig. 3. Analysis of continued J_H -associated rearrangements in an A-MuLV transformant. The 22D6 line was cloned by limiting dilution into microliter wells as previously described (Alt *et al.*, 1981). One sub-clone (22D6-G) was characterized as described below and in the text and further sub-cloned to yield secondary sub-clones 22D6-G1 and 22D6-G2. Genomic DNA from various sources was digested with *EcoRI*, fractionated by electrophoresis through agarose gels, transferred to nitrocellulose, and assayed for hybridization to various probes which had been labeled with ^{32}P by nick-translation. **Panel A.** DNA was from liver (lanes 1 and 3) and 22D6G (lanes 2 and 4). Probes were J_H800 (lanes 1 and 2) and the $5' D_{Sp-2}$ (lanes 3 and 4). The putative 3.0 and 4.5 DJ_H rearrangements in the 22D6-G DNA are indicated as X and Y, respectively. **Panel B.** The 4.5(Y) and 3.0(X)-kb J_H -associated *EcoRI* fragments of the 22D6-G line were cloned into the *EcoRI* site of Charon 16A and sub-cloned into the *EcoRI* site of pBR322 as described previously (Alt *et al.*, 1982a, 1982b). The 3.0- and 4.5-kb inserts of the resulting plasmid clones (pDJ_H22D6X and pDJ_H22D6Y) were excised, labeled with ^{32}P , and hybridized to *EcoRI* digested liver DNA. Lane 1, probe DJ_H22D6X (3.0-kb insert); lane 2, probe DJ_H22D6Y (4.5-kb insert). The approximate sizes (in kb) of the hybridizing fragments are indicated to the left of lane 1. **Panel C:** DNA was from mouse liver (lanes 1 and 4), and 22D6 secondary sub-clones G-1 (lanes 2 and 5) and G-2 (lanes 3 and 6). Probes were the J_H800 (lanes 1, 2 and 3) and the $5' D_{FL16.1}$ (lanes 4, 5 and 6). The putative 3.5- and 2.1-kb V_H -to- DJ_H rearrangements of the 4.5 DJ_H (Y) and 3.0 DJ_H (X) rearrangements in the parental 22D6-G sub-clone are indicated as Y' and X'-kb, respectively. **Panel D.** The 3.5 (Y') and 2.1 (X')-kb J_H -associated *EcoRI* fragments of the 22D6-G2 line were cloned into Charon 16A and sub-cloned into pBR322. The inserts from the resulting plasmid clones [pV_H22D6X' and pV_H22D6Y'] and that from plasmid pV_H81X (Alt *et al.*, 1982a, 1982b)] were excised, labeled with ^{32}P and hybridized to *EcoRI*-digested mouse liver DNA. Lane 1, probe V_H22D6X'; lane 2, probe V_H22D6Y'; lane 3, probe V_H81X.

To test the validity of this assay, we analyzed *EcoRI*-digested DNA from lines with previously characterized rearrangements at their J_H alleles. The 3-1, 18-8 and 1-7 A-MuLV transformed lines, which are known to have $V_H D-J_H$ rearrangements at both J_H alleles (Alt *et al.*, 1982a), showed no hybridization to the combined $5' D$ probes (Figure 2B, C and D). A-MuLV transformed lines 40E4, 22D6 and 38B9-7, which are known to have DJ_H rearrangements (see below), all had *EcoRI* fragments hybridizing to the J_H -specific and $5' D$ probes as well as variable numbers of the *EcoRI* fragments containing the most $5'$ of the D segments (Figures 2E, F and 3A). In addition we have assayed five other A-MuLV transformants which were shown by molecular cloning and DNA sequencing to have either two DJ_H , a $V_H DJ_H$ and a DJ_H , or two $V_H DJ_H$ rearrangements. In all cases, the results of the blotting assay were consistent with the cloning and sequencing data (see Table I). These results strongly suggest that the D segments lie between V_H and J_H , and that V_H joining to DJ_H results in the deletion of intervening sequences.

Most fetal liver-derived transformants have two DJ_H rearrangements

Fetal liver-derived A-MuLV transformants are like other B-lymphoid cell lines and normal B-cells in that none have an embryonic J_H region (Alt *et al.*, 1981). They are different,

however, in that more such lines are positive for hybridization to the $5' D$ probe (DJ_H -containing) than are negative (dual $V_H DJ_H$ rearranged lines) (Figure 2 and Table I). Transformants derived from fetal liver are also different from more mature pre-B-lymphoid cells in that most are immunoglobulin negative (null) and most of these exhibit continuing J_H -associated rearrangements during growth in culture (Alt *et al.*, 1981, 1982a). Significantly, DNAs from eight fetal liver transformants which exhibited continuing rearrangement were positive for hybridization with the $5' D$ flanking probe and most of these contained two (or more) novel *EcoRI* fragments that hybridized to both the $5' D$ and J_H -specific probes (DJ_H rearrangements) in addition to variable numbers of remaining embryonic D-containing *EcoRI* fragments (Figure 2E, F, G, K, L, representative lines are shown; Table I). This result suggests that these lines contained DJ_H rearrangements at one and usually both alleles. None of these lines produced significant levels of μ protein. Of the three fetal liver lines assayed which did not exhibit continuing rearrangement in culture (one was μ -positive, two null), none had *EcoRI* fragments that hybridized with the $5' D$ probe indicating $V_H DJ_H$ rearrangements of both heavy chain alleles within these lines (Figure 2H, Table I). Together, these results suggest that most fetal liver-derived A-MuLV transformants contain DJ_H rearrangements at both J_H alleles and that these are substrates for the further rearrangements observed in culture.

Table I. Rearrangement status of J_H alleles in various tumors or cell lines of B-lymphoid origin

Line	Heavy chain protein	Continued J _H rrmt.	5' D	Interpretation
A. Fetal liver (A-MuLV transformants)				
1. 38B7	-	-	-	VDJ ⁻ /VDJ ⁻
2. 38B9	-	+	+	DJ/DJ*
3. 38B11	-	+	+	DJ/DJ
4. 40E1	-	+	+	DJ/DJ
5. 40E4-2	-	+	+	DJ/DJ**
6. 40E4-2-26 ^a	-	-	-	VDJ ⁻ /VDJ ⁻ **
7. 22D7 ^a	±	+	+	DJ/DJ
8. 22D6-G	- ^b	+	+	DJ/DJ**
9. 22D6-G-2 ^a	-	-	-	VDJ ⁻ /VDJ ⁻ **
10. 22D10	-	+	+	DJ/DJ
11. 28C9	±	-	-	VDJ ⁺ /VDJ ⁻
12. 41B1	-	+	+	DJ/DJ
13. 40E3	-	+	+	DJ/DJ
B. Bone marrow (A-MuLV transformants)				
1. 298-13	+	-	+	VDJ ⁺ /DJ*
2. 220-8	-	-	-	VDJ ⁻ /VDJ ⁻ **
3. 2M3-M	-	-	-	VDJ ⁻ /VDJ ⁻
4. 300-18	+	-	-	VDJ ⁺ /VDJ ⁻ **
5. 300-19	+ ^c	+	+	DJ/DJ**
6. 230-37	+	-	-	VDJ ⁺ /VDJ ⁻
7. 230-238	-	-	-	VDJ ⁻ /VDJ ⁻ *
8. 223-18	+	-	+	VDJ ⁺ /DJ**
9. 18-8	+	-	-	VDJ ⁺ /VDJ ⁻ **
10. 18-48	-	-	+	DJ
11. 1-8	-	-	+	VDJ ⁻ /DJ
12. 1-7	+	-	-	VDJ ⁺ /VDJ ⁻ ^{d*}
13. 3-1	+	-	-	VDJ ⁺ /VDJ ⁻ ^d
14. 234-8	+	-	-	VDJ ⁺ /VDJ ⁻
15. 300-2	+	-	+	VDJ ⁺ /DJ
16. 298-8	-	-	-	VDJ ⁻ /VDJ ⁻ *
B. Cell lymphomas				
1. 231 E1	+	-	-	VDJ ⁺ /VDJ ⁻
2. 70Z	+	-	+	VDJ ⁺ /DJ ^e
Myelomas				
1. H2020	+	-	-	VDJ ⁺ /VDJ ⁻
2. SAPC 15	+	-	-	VDJ ⁺ /VDJ ⁻
3. M315	+	-	-	VDJ ⁺ /VDJ ⁻
4. CBPC 101	+	-	-	VDJ ⁺ /VDJ ⁻
5. M603	+	-	-	VDJ ⁺ /VDJ ⁻
6. LPC1	+	-	-	VDJ ⁺ /VDJ ⁻
7. y5830	+	-	-	VDJ ⁺ /VDJ ⁻
8. M460D	+	-	-	VDJ ⁺ /VDJ ⁻
9. M21 (P-3)	+	-	-	VDJ ⁺ /VDJ ⁻
10. ABPC 17	+	-	-	VDJ ⁺ /VDJ ⁻
11. MPC 11	+	-	-	VDJ ⁺ /VDJ ⁻

Line	Heavy chain protein	Continued J _H rrmt.	5' D	Interpretation
12. M 149	+	-	-	VDJ ⁺ /VDJ ⁻
13. y5444	+	-	-	VDJ ⁺ /VDJ ⁻
14. S107	+	-	-	VDJ ⁺ /VDJ ⁻
15. BEPC 52	+	-	-	VDJ ⁺ /VDJ ⁻
16. M70A	+	-	-	VDJ ⁺ /VDJ ⁻
17. M173	+	-	-	VDJ ⁺ /VDJ ⁻
18. M603	+	-	-	VDJ ⁺ /VDJ ⁻
19. TEPC 183	+	-	-	VDJ ⁺ /VDJ ⁻
20. BEPC 61	+	-	-	VDJ ⁺ /VDJ ⁻
21. M511	+	-	+	VDJ ⁺ /DJ
22. M195	+	-	+	VDJ ⁺ /DJ
23. J606	+	-	+	VDJ ⁺ /DJ
24. M141	+	-	+	VDJ ⁺ /DJ
25. y5606	+	-	+	VDJ ⁺ /DJ
26. M321	+	-	+	VDJ ⁺ /DJ
27. M141	+	-	+	VDJ ⁺ /DJ
28. S178A	+	-	+	VDJ ⁺ /DJ
29. TEPC 15	+	-	+	VDJ ⁺ /DJ
30. J558	+	-	+	VDJ ⁺ /DJ
31. ABPC 22	+	-	+	VDJ ⁺ /DJ
32. QUPC 52	+	-	+	VDJ ⁺ /DJ

A more detailed description of immunoglobulin-production by the A-MuLV transformed cell lines has been presented elsewhere (Alt *et al.*, 1981, 1982a) and a further description of most of the other tumors or cell lines listed is provided by Kabat *et al.* (1983). All lines have rearrangements of both J_H alleles. The presence (+) or absence (-) of continued J_H-associated rearrangement in the various cell lines during propagation was indicated by the presence or absence of novel, sub-molar J_H-positive *EcoRI* fragments in the population. This type of analysis might not detect a very low level of J_H rearrangement. All lines scored as 5' D positive contained, either the 10-kb DFL16-specific *EcoRI* fragment, one or more J_H-positive, 5' D-positive rearranged *EcoRI* fragment, or usually both (see Figures 1, 2 and 3). A fraction of the population of cells in fetal liver transformants that were interpreted as DJ/DJ may have replaced a DJ with a VDJ. In such lines the DJ/DJ description refers to our interpretation of the rearrangement status of the J_H alleles in the primary transformant. All of the myeloma lines scored as 5' D positive contained, at least, the 4.0 (or 10)-kb DFL16-specific *EcoRI* fragment (see Figures 2 and 3). The symbols VDJ⁺, VDJ⁻ refer to productive and non-productive V_HDJ_H rearrangements, respectively. A single or double asterisk following the interpretation of the J_H-associated rearrangements in a given A-MuLV transformant indicates that the status of one or both allele(s), respectively, has been confirmed by molecular cloning analyses. The rearrangement status of many of the myeloma lines has been determined by various investigators (for review, see Kabat *et al.*, 1983).

^aSub-clones with altered properties from parent lines due to V_H to DJ_H rearrangement in culture.

^bA low level of μ production is detected in this line, probably due to the occurrence of some productive V_H to DJ_H rearrangements during growth of the line (M. Reth, G. Yancopoulos and F. Alt, unpublished data).

^cProduces truncated μ chain (Alt *et al.*, 1982a) from a DJ_H rearrangement (M. Reth and F. Alt, unpublished observations).

^dIt has been determined that these two lines have VDJ rearrangement at both alleles using an independent method (Alt, *et al.*, 1982a).

^eThis interpretation is in agreement with the direct analysis of Nelson *et al.* (1983).

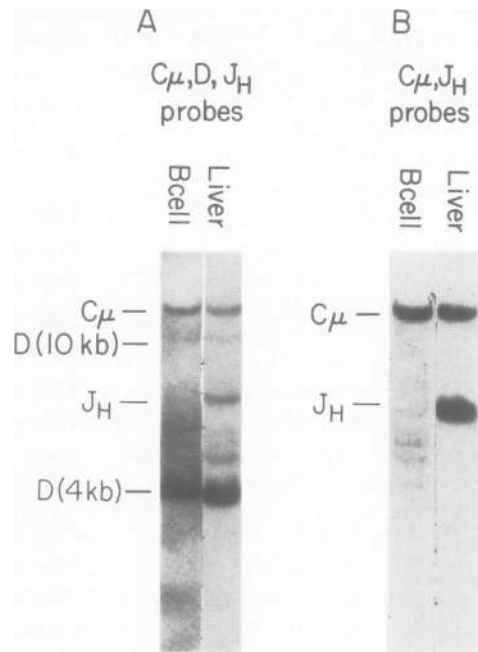


Fig. 4. The nature of heavy chain gene rearrangements in normal B-lymphocyte DNA. DNA (5 μ g) from surface \times light chain-positive splenic lymphocyte (B cells) and mouse liver, respectively, were digested with *Eco*RI, fractionated through a 1% agarose gel, transferred to nitrocellulose paper and assayed for hybridization to the indicated 32 P-labeled probes. The 1200-bp insert of pAB- μ 8 (C_{μ} probe, Alt et al. 1980b), the 2.5-kb *Eco*RI/*Bgl*II fragment of pJ38B9-7.1 (D probe, see Figure 1B) and the 3' most *Bam*HI/*Eco*RI fragment of pRI- J_H (J_H probe, see Figure 1B) were excised and labeled individually with 32 P by nick-translation to a final specific activity of 5×10^8 c.p.m./ μ g as previously described (Alt et al., 1981). A mixture of approximately equal quantities of all three probes was used in the assays shown in panel A while a mixture of the C_{μ} and J_H probes was used in the assay shown in panel B. Following hybridization, the nitrocellulose filters were washed in $0.1 \times$ SSC and 0.1% SDS at 68°C and exposed at -70°C to Kodak XR-S filter with a Dupont Cronex intensifying screen.

Fetal liver transformants undergo V_H -to- DJ_H joining during growth in culture

To define the continued rearrangement process in A-MuLV transformed fetal liver cells, we have further characterized the primary and secondary J_H -associated rearrangements. The results of a characteristic analysis are described below and others will be presented in more detail elsewhere (Desiderio et al., in preparation). Continued rearrangements in these lines were indicated by the occurrence of multiple novel J_H -containing *Eco*RI fragments within a clonal population of cells (Alt et al., 1981). The 22D6 line appeared to be quite active in this rearrangement process because DNA prepared from the parental line contained many such fragments (Alt et al., 1981). Sub-cloning of this line generated a series of secondary sub-clones which contained a subset of the fragments found in the parental line (data not shown), confirming the previous conclusion that most of the additional hybridizing fragments were generated after the line was established (Alt et al., 1981). Either a 4.5-kb or 3.0-kb J_H -positive *Eco*RI fragment or both were observed in the DNA from most of the daughter sub-clones (Figure 3A, lane 2; a representative sub-clone 22D6-G is shown), and these were predominant bands in the rearrangement pattern of the parental line (Alt et al., 1981). The unrearranged J_H -containing *Eco*RI fragment of liver DNA is shown for comparison (Figure 3A, lane 1). To test the nature of these two predominant rearrangements in

the 22D6 line, *Eco*RI-digested DNA from the 22D6-G sub-clone (as well as from other sub-clones) was analyzed for hybridization to the 5' D flanking sequence probes. DNA from 22D6-G line contained a subset of genomic D segments that hybridized to the FL16 or SP-2 type 5' D flanking sequence probes in addition to two novel 5' D-positive *Eco*RI fragments which co-migrated with the J_H -positive *Eco*RI fragments in this line (Figure 3A, compare lanes 2, 3 and 4; results shown are from SP-2 probe; novel bands are indicated as X and Y, respectively). This result strongly indicates that the 4.5(Y) and 3.0(X)-kb J_H -associated rearrangements in this line are DJ_H joins.

To confirm this conclusion, both the 3.0- and 4.5-kb *Eco*RI fragments were molecularly cloned into lambda phage Charon 16A, sub-cloned into pBR322, and further characterized. The 4.5- and 3.0-kb inserts (denoted Y and X, respectively) were labelled with 32 P by nick-translation, and assayed for hybridization to liver DNA that had been digested with *Eco*RI and fractionated by agarose gel electrophoresis. In addition to a 6.2-kb *Eco*RI fragment which hybridized to the J_H -associated portion of each insert, the 4.5-kb insert hybridized to a set of *Eco*RI fragments, characteristic of an FL16-type D (Figure 4B, lane 2) and the 3.0-kb insert hybridized to a set of fragments typical of an SP-2 type D (Figure 4B, lane 1; see Kurosawa and Tonegawa, 1981 for a description of the D families). Subsequent DNA sequencing analysis identified these rearrangements as a $D_{FL16.1}$ - J_H^4 and $D_{SP-2.5}$ - J_H^3 respectively (G. Yancopoulos and F. Alt, unpublished data).

To characterize the nature of secondary J_H -associated rearrangements (i.e., rearrangement of a previously rearranged J_H -segment), a series of secondary and tertiary cellular sub-clones of the 22D6-G sub-clones were generated, genomic DNA was prepared from each and then screened for secondary rearrangements. The results from two representative sub-clones that contained secondary rearrangements of one or both of the DJ_H rearrangements of the 22D6-G line are presented (Figure 3C). The 22D6-G-1 sub-clone retained the 4.5-kb J_H -positive *Eco*RI fragment observed in the parental clone, but the 3.0-kb fragment was replaced by a new fragment of 2.1 kb (Figure 3C, lane 2; denoted as X'). The DNA from this sub-clone contained only the 10-kb germ-line D-related *Eco*RI fragment; and significantly, the 2.1-kb J_H -positive *Eco*RI fragment did not hybridize with either the SP-2 or FL16 5' D flanking sequence probes (Figure 3C, lane 5, only results for FL16 probe are shown). These results suggest that the 2.1-kb *Eco*RI fragment was generated as a result of the appendage of a V_H gene segment to the DJ_H rearrangement contained within the 3.0-kb J_H -associated *Eco*RI fragment in the 22D6-G line, and that this rejoining resulted in the deletion of intervening D segments. The retention of only the 10-kb D-related fragment in this clone is consistent with the fact that the remaining D- J_H rearrangement in this line employs $D_{FL16.1}$ (see above), the most 5' characterized D-segment (Kurosawa and Tonegawa, 1981).

A similar analysis of the 22D6-G-2 sub-clone indicated that in this line both of the *Eco*RI fragments containing the parental DJ_H joins had undergone additional rearrangements. DNA from this sub-clone contained J_H -positive *Eco*RI fragments of 2.1 kb (which co-migrated with the secondary rearrangement of 22D6-G-1 line) and 3.5 kb (Figure 3C, lane 3; denoted as Y'). Analysis of *Eco*RI-digested DNA from this sub-clone for hybridization to the 5' D flanking sequence probes revealed that all of the embryonic D segments were

deleted (Figure 3C, lane 6). In addition, neither the 3.5(Y') nor the 2.1(X')-kb J_H -positive *EcoRI* fragments hybridized to either 5' D probe (Figure 3B, lane 10; only results of FL16 probe are shown). These results suggest that the 3.5 and 2.1-kb J_H -associated *EcoRI* fragments in this sub-clone were generated by appendage of V_H segments to both primary D- J_H joins contained in the 22D6-G parent with concomitant deletion of the remaining unrearranged D segments.

To confirm that the secondary rearrangements in these sub-clones were V_HDJ_H joins, the 2.1-kb and 3.5-kb *EcoRI* fragments from the 22D6-G-2 line were cloned into lambda phage Charon 16A, subcloned into pBR322, and each insert (denoted X' and Y', respectively) excised and assayed for hybridization to *EcoRI*-digested liver DNA as outlined above. Both of these probes hybridized to a nearly identical set of embryonic *EcoRI* fragments (Figure 3D, lanes 1 and 2) that were distinct from those containing the known D segments, but nearly identical to those which hybridize to V_H81X (Figure 3D, lane 3), a V_H segment that was previously cloned from a marrow-derived A-MuLV transformant (Alt *et al.*, 1982a). Nucleic acid sequence analyses have confirmed that the V_H segments used in these three V_HDJ_H rearrangements are very closely related (Yancopoulos, Desiderio, Paskind, Kearney, Baltimore, and Alt, in preparation). We have performed similar analyses on three independent fetal liver-derived transformants which undergo J_H -associated rearrangements during growth in culture (40E4, 38B9, and 38B11). In all of these lines, the majority of the characterized secondary rearrangements represented appendage of V_H segments to pre-existing DJ_H complexes, although other types of rearrangements have been observed at lower frequency (Alt *et al.*, unpublished data).

J_H-associated rearrangements in more mature B-lymphoid cell lines

Approximately 60% of the marrow-derived A-MuLV transformants produce μ heavy chain (Alt *et al.*, 1981) while most of the rest are null with respect to Ig production. When assayed as described above, both the μ^+ and μ^- A-MuLV transformants derived from bone marrow (Alt *et al.*, 1981) can be sub-divided into lines which had a DJ_H rearrangement in addition to a V_HDJ_H rearrangement and lines which had two V_HDJ_H rearrangements (Table I, some lines shown in Figure 2B, C, D, I and J). With one exception, none of these lines – including those that had a DJ_H rearrangement – show evidence of continuing rearrangement in culture (Table I). We have extended these studies to include two heavy chain-producing B-cell lymphoma or leukemia lines and 32 heavy chain-producing myeloma lines (Table I). As was observed with the heavy-chain-producing A-MuLV transformants, a significant percentage of these lines contained a DJ_H rearrangement as opposed to a V_HDJ_H rearrangement at the 'secondary' (non-productive) allele (Table I). For these more mature tumor lines, the ratio of cells with two V_HDJ_H rearrangements (i.e., D-negative) to those with a V_HDJ_H and a DJ_H was nearly 2 to 1 (Table I).

J_H-associated rearrangements in normal B-lymphocytes

Myeloma and B-cell lymphomas have been shown to contain rearrangements (deletions) of the Ig gene locus that are not associated with V_HDJ_H joining (i.e., oncogene rearrangements; for review, see Perry, 1983). Because any rearrangement which lead to the loss of D-segments would be interpreted as a V_HDJ_H rearrangement by our assay (Figure 1), such tumor-specific rearrangements could bias our estimate

of the relative number of Ig-producing cells with V_HDJ_H versus DJ_H rearrangements of the 'non-productive' allele. Therefore, we decided to quantitate the relative number of DJ_H versus V_HDJ_H rearrangements in normal surface Ig-positive B-lymphocytes. For these studies, surface Ig-positive splenic lymphocytes first were stained with a fluorescein conjugate of anti-mouse Ig antibody, subsequently isolated on the FACS (Coffman and Weissman, 1983) and then further analyzed.

As described above, clonal Ig-producing tumors of the B-lineage usually have two novel *EcoRI* fragments that hybridize to J_H -specific probe (e.g., Figure 2) and represent V_HDJ_H or DJ_H rearrangements of both J_H loci. Normal B-lymphocyte populations contain multiple, unique J_H -rearrangements each of which produces a novel J_H -specific *EcoRI* fragment. The dual rearrangement of both J_H loci in such populations is therefore evidenced by the disappearance of the germ-line 6.2-kb J_H -specific *EcoRI* fragment (e.g., Figure 4B). Likewise, since V_H -to- DJ_H rearrangements appear to be associated with the deletion of any intervening D segments (see above), the relative level at which the most 5' embryonic D segments are represented in a population of B-lymphocytes should allow an estimate of the relative numbers of V_HDJ_H and DJ_H rearrangements in that population (see legend to Table I). The most accurate method to measure a change in the relative level of a specific restriction fragment in DNA from a population of cells is to use appropriate DNA blotting procedures to compare the intensity of that fragment with the intensity of another single-copy DNA fragment whose level should remain constant (Coleclough *et al.*, 1981; Nottenberg and Weissman, 1981; Coffman and Weissman, 1983). In our experiments, we used the 12.5-kb *EcoRI* fragment which contains the C_μ gene (Coffman and Weissman, 1983) as the internal standard for a single copy sequence. Thus, DNA for mouse liver or surface Ig-positive B-lymphocytes was digested with *EcoRI*, fractionated by agarose gel electrophoresis, and assayed for hybridization to a ^{32}P -labelled combined probe that consisted of J_H -specific C_μ -specific, and 5' FL16-D flanking sequence-specific plasmid inserts (see legend to Figure 4). Visual comparison of the relative intensities of the 12.5-kb C_μ -specific, 6.2-kb J_H -specific, and 10-kb and 4-kb 5' D-specific *EcoRI* fragments in the liver DNA and B-cell DNA suggested that the extent of rearrangement within the J_H cluster was much greater than the extent to which the most 5' D segments were lost (Figure 4A, compare lanes 1 and 2). This interpretation was confirmed by densitometric tracings of these lanes (Table II). The B cells contain < 5% of the unrearranged J_H fragments found in liver DNA, a result that was confirmed by repeating these analyses with only the J_H and C_μ probes (Figure 4B, compare lanes 1 and 2; Table II). The level detected can be accounted for by contamination of the sorted cells with non-B lymphoid cells (see legend to Figure 4). The B-cell population, however, contained ~ 30% of the level of 4.0-kb D fragment found in liver DNA and similar levels of the 10-kb fragments. Assuming that all surface Ig-positive B-lymphocytes contain a productive V_HDJ_H rearrangement, the extent of retention of germ-line segments in this population is consistent with ~ 40% of cells containing two V_HDJ_H rearrangements and 60% of the cells containing one V_HDJ_H and one DJ_H rearrangement (see legend to Table II).

V_HD or DD rearrangements rarely occur in B-lymphoid cells
The frequent occurrence of DJ_H rearrangements in cells

Table II. Extent of J_H and D rearrangement in normal B cells

	B cell	Liver
A. Peak area ratio		
D/C _μ (from panel A)	4.1	12.9
% 4-kb D-specific EcoRI fragment	32	100
B. Peak area ratio		
J _H /C _μ (from panel B)	0.09	2.24
% germ-line J _H remaining	4	100

Autoradiograms from Figure 4A were analyzed with a Cary 219 spectrophotometer using a gel-scanner attachment. The relative intensity of the indicated bands was calculated by measuring peak areas with a Numonics Electronic Graphics calculator. The peak ratio for 4-kb D band to the C_μ band for the B-cell DNA was divided by the ratio obtained for the liver DNA on the same blot (panel A) to provide an estimate of the percentage of the unrearranged 4.0-kb D-FL16-containing EcoRI fragment remaining in the B-cell DNA (assuming 100% for liver DNA). The percentage of the unrearranged J_H-containing fragment remaining in B-cell DNA was similarly calculated from the data shown in Figure 4B. The data in Figure 4B and this table indicate that the vast majority of the B cells analyzed have rearrangements of both J_H alleles. Because the cells were purified on the basis of their surface immunoglobulin, each must have a productive V_HDJ_H rearrangement at one J_H allele. Assuming that the most 5' D segments are always deleted in the formation of a V_HDJ_H rearrangement, the level of retention of the most 5' segments in the DNA of the B cell population provides an estimate of the percentage of total J_H alleles in the DJ_H configuration (i.e., ~30%). Since, as indicated above, none of these cells should have DJ_H rearrangements of both alleles (one allele must be a productive V_HDJ_H rearrangement), the percentage of cells having a DJ_H rearrangement would be twice the percentage of alleles in DJ_H configuration or ~60%. The remaining 40% of the cells must have two V_HDJ_H rearrangements (presumably a productive and non-productive rearrangement).

representing various stages of B-cell differentiation and the predominance of DJ_H joins in lines derived from the most primitive sources suggested that in general, DJ_H recombinations may be the primary step in heavy chain variable region (V_HDJ_H) gene assembly. This further suggests that V_H-to-D or D-to-D joining *per se* might be infrequent events. To test this possibility, we employed the assay indicated in Figure 1A, panels e and f. Whereas a V_H-to-D join would delete the 5' flanking region of a D segment, if that D segment had not previously joined to a J_H segment, the newly created EcoRI fragment should retain hybridization to the 3' D flanking sequence probe (3' D probe) (Figure 1A, panel e). Such a fragment, however, should not hybridize to either the J_H-specific or 5' D flanking sequence probes since the 5' and 3' D probes do not cross-hybridize. Likewise, a D-D joint should create a novel EcoRI fragment which hybridizes to both the 5' and 3' D probes but not to the J_H-specific probe (Figure 1A, panel f). For this assay, we employed a 3' D flanking sequence probe prepared from the 4.0-kb embryonic EcoRI fragment containing D_{FL16.1} (Kurosawa and Tonegawa, 1981, Figure 1B, panel d). This probe detects the homologous 4.0-kb embryonic EcoRI fragment as well as additional, less strongly hybridizing, D-containing fragments of 5, 5.2, 6.6, 10, and 18 kb (Figure 2A, lane 3). We tested eight fetal liver-derived A-MuLV transformants, all of which contained multiple DJ_H rearrangements, as well as 81 sub-clones of these lines of which many formed additional V_H-to-DJ_H rearrangements in culture. None of these lines contained any novel EcoRI fragments hybridizing to the 3' D probe (Figure 2, representative lines are shown). In addition, similar results

were obtained when a combined SP-2/FL16.1 D probe which more strongly detected the other D segments was used (not shown). These results indicate that although these lines have made two DJ_H rearrangements and have continued to make DJ_H-associated rearrangements in culture, they have not produced V_HD or DD rearrangements at a detectable level. Similar analyses have demonstrated the general lack of DD or V_HD rearrangements in cell lines representing more mature B-lymphoid cells (data not shown). Although a few of the myeloma lines were found to contain novel EcoRI fragments which hybridize to 3' and 5' D probes but not to J_H probes, cloning and sequencing of these fragments has indicated that they did not result from V_H-to-D or D-to-D rearrangements (Wood and Tonegawa, unpublished results).

Discussion

Ordered rearrangements during B-lymphocyte differentiation

It is becoming increasingly apparent that the B-cell differentiation pathway involves a highly ordered sequence of immunoglobulin gene rearrangements and correspondingly, an ordered program of Ig gene expression. Previous work has indicated that the first cell in this pathway to produce detectable Ig, the cytoplasmic μ-positive 'pre-B' cell has rearranged its heavy chain genes, but not its light chain genes (Maki *et al.*, 1980; Perry *et al.*, 1981; Alt *et al.*, 1981; Korsmeyer *et al.*, 1981). Subsequent rearrangement and expression of light chain genes is one of the characteristic features of the next major stage of B-cell differentiation, the surface IgM-positive B-lymphocyte. The light chain gene rearrangement process also seems to be ordered in that kappa genes are generally rearranged before lambda genes (Alt *et al.*, 1980a; Hieter *et al.*, 1981; Coleclough *et al.*, 1981). This rearrangement pathway is summarized in Figure 5.

We have provided evidence that in murine cells this ordered program of rearrangement extends to the process by which individual segments of the heavy chain variable region gene (V_HDJ_H) are fused. We have assayed for DJ_H, V_HDJ_H, V_HD and DD rearrangements in cell lines representing various stages of the B-cell pathway. Lines representing the pre-B stages and beyond exhibit either two V_HDJ_H rearrangements or a V_HDJ_H plus a DJ_H rearrangement at their two heavy chain loci (Table I, Figure 5B). None of the lines examined showed evidence of V_HD or DD rearrangements. Furthermore, fetal liver-derived A-MuLV transformants – which are apparently the most primitive B-cell analog available for analysis – generally have DJ_H rearrangements at both J_H heavy chain loci. Such lines generally continue to rearrange their loci in culture, in most cases exhibiting the addition of V_H gene segment to an existing DJ_H complex (Figure 5B). Again, however, none of these lines or their progeny show evidence of V_HD or DD rearrangements.

Collectively, these results suggest that a new cell type should be added to those known to occur in the B-cell pathway – the null pre-B cell containing two DJ_H rearrangements. This cell would represent the most primitive known member of the pathway and have as an analog, the DJ_H-containing, fetal liver-derived A-MuLV transformants (Figure 5A and B). Such a null cell is distinct from the 'dead-end' null cell which may occur later in the pathway (Figure 5A). The former has not yet constructed a complete heavy chain variable region gene while the latter presumably has failed in both attempts to construct heavy chain genes (see below).

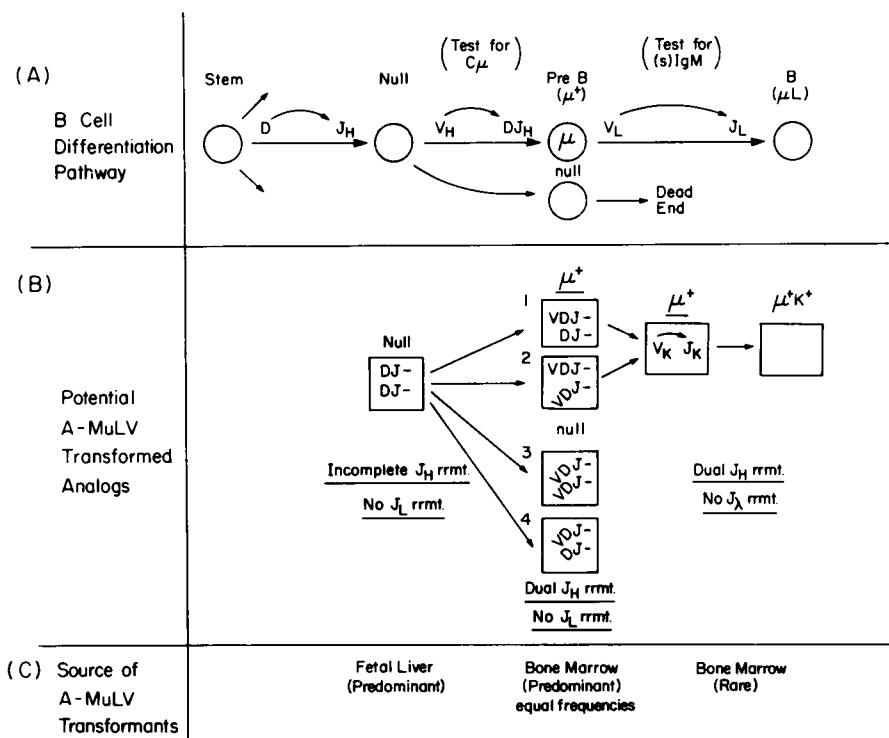


Fig. 5. Ordered rearrangement of Ig gene segments during B-lymphocyte differentiation. **Panel A.** B-cell differentiation pathway — details are described in text. **Panel B.** Types of Ig gene rearrangements found in A-MuLV transformants which correspond to various stages of the pathway outlined in panel A. Aberrant VDJ or DJ rearrangements are indicated by the skewed VDJ letters. Other details are in text. **Panel C.** Source of the various types of A-MuLV transformants. For details see Rosenberg and Baltimore, 1976; Alt *et al.*, 1981. According to the model for allelic exclusion outlined in the text, the ratio of μ -producing cell types 1 and 2 (see Figure) in a population of normal B-lymphocytes will depend on the accuracy of the V_H gene assembly mechanism as follows. We assume that D-to-J_H joining at both heavy chain alleles precedes joining of a V_H to a D segment (i.e., the pathway is D-to-J_H and V_H-to-DJ_H, in that order) and that a productive rearrangement leads to the cessation of further rearrangements. We also assume that a second V_H-to-DJ_H rearrangement does not occur until the first can be tested. Therefore, the percentage of cells with a productive V_HDJ_H and a DJ_H rearrangement would be P, where P equals the probability of a productive V_H-to-DJ_H rearrangement. The percentage of cells which first made a non-productive V_H-to-DJ_H rearrangement and subsequently made a productive V_H-to-DJ_H rearrangement at the second allele would be the probability of a non-productive rearrangement (1-P) times the probability of a productive rearrangement (P). Therefore, the ratio of Ig-producing cells with a V_HDJ_H and DJ_H rearrangement to those with two V_HDJ_H rearrangements can be calculated as follows:

$$\frac{V_HDJ_H/DJ_H}{V_HDJ_H/V_HDJ_H} = \frac{P}{(1-P)(P)}$$

It should be noted, however, that this is the most simple form of such a model and many factors — such as secondary rearrangements which could activate a non-productive V_HDJ_H rearrangement have not been considered. For a more detailed consideration of this model in the context of light chain gene rearrangement, see Coleclough, 1983.

The absence of V_HD or DD joining in B-lymphoid cell lines implies that these two joins *per se* do not occur at a high frequency relative to DJ_H joins. Thus, the construction of a complete heavy chain V_HDJ_H gene would be mediated by D-to-J_H and V_H-to-DJ_H joins in that order. The mechanism by which such an ordered joining process is effected remains to be elucidated. Preferential association of recombinases with the J_H recognition sequences possibly due to accessibility, coupled with the 23-12 recombination rule (Early *et al.*, 1980; Sakano *et al.*, 1980), could lead to preferential DJ_H recombination. Rearrangement of a D segment into the J_H region might then activate the 5' recognition sequence of a D for recombination with a V_H sequence. However, such a mechanism cannot fully explain the regulation of this process because, for example, T-lymphoid cell lines frequently produce DJ_H rearrangements, but apparently never produce V_HDJ_H joins (e.g., Kurosawa *et al.*, 1981). This observation suggests that these two rearrangement events may be independently regulated.

This model for V_H gene assembly is consistent with previous studies which have demonstrated rearrangements at

both J_H loci in the vast majority of normal B-lymphocytes (Coleclough *et al.*, 1981; Nottenberg and Weissman, 1981) and in normal pre-B cells (Coffman and Weissman, 1983) as well as with the large body of data which documents dual J_H rearrangements in a variety of immature (pre-B) and mature tumors of the B-lymphocyte series (e.g., Alt *et al.*, 1981; Coleclough *et al.*, 1981; Sakano *et al.*, 1981).

Ordered rearrangement and heavy chain allelic exclusion

The occurrence of DJ_H rearrangements on both chromosomes as the primary step in heavy chain V_H gene construction has important implications for the mechanism of heavy chain allelic exclusion. We have previously proposed that this process might be related in part to a high rate of aberrant rearrangement, but would ultimately rely on a signal that the cell received upon production of a heavy chain protein (Alt *et al.*, 1981, 1982a). Such a signal would feed back and lead to a shutdown of the heavy chain rearrangement process. Thus, given the above model for V_H gene construction, the V_H-to-DJ_H rearrangement process would be the regulated step. If the initial V_H-to-DJ_H rearrangement led to

the production of a heavy chain, an additional V_H -to- DJ_H join would be prevented, leading to the type 1 μ -positive pre-B cell (Figure 5B) which has a productive V_HDJ_H rearrangement and a 'frozen' DJ_H rearrangement. If the first V_H -to- DJ_H rearrangement were aberrant and did not lead to the production of a μ chain, an additional V_H -to- DJ_H rearrangement could occur. If productive, this join would generate a type 2 μ -positive pre-B cell (Figure 5B) with both a productive and an aberrant V_H -to- DJ_H rearrangement. If the second rearrangement was also aberrant, a 'dead-end', null pre-B cell with two V_H -to- DJ_H rearrangements would result (Figure 5B; type 3 cell).

An assumption that is implicit in the allelic exclusion model outlined above is that a cell undergoing V_H -to- DJ_H rearrangement has time to 'test' the first rearrangement before a second rearrangement occurs. Recently, we have found evidence of such selective rearrangement in certain A-MuLV transformants (M. Reth and F. Alt, unpublished data). A mathematical model which incorporates the considerations discussed above is outlined in the legend to Figure 5. In its simplest form, this model predicts that in a random population of Ig-producing cells a significant percentage of cells would contain frozen DJ_H rearrangements at the second allele. The exact ratio of cells with a frozen DJ_H to cells with an aberrant V_H -to- DJ_H at the second allele would depend on the efficiency with which the rearrangement process produces productive joins, and approach 1:1 as the probability that a given rearrangement would be non-productive became high. It is clear from what is known about the heavy chain joining process that it is inaccurate (for review, see Tonegawa, 1983); and no more than one-third and possibly less of the V_H -to- DJ_H joins lead to the production of a μ protein. Assuming that 33% of the V_H -to- DJ_H rearrangements were productive, the model outlined in the legend to Figure 5 predicts that ~30% of the rearrangements in a random population of Ig-producing cells would be DJ_H rearrangements. This number is consistent with the percentage of DJ_H rearrangements that we have estimated to occur in populations of FACS-purified B-lymphocytes (see legend to Table II). However, the relative number of heavy chain-producing cells and tumor lines which – by our assay – contained a frozen DJ_H rearrangement as opposed to an aberrant V_H -to- DJ_H rearrangement at the second allele was significantly less than the minimum predicted ratio of 1:1. There could be several reasons for this apparent discrepancy, including the relatively small number of lines assayed, the possibility of secondary rearrangements during growth in culture, or the possibility of tumor-specific rearrangements (particularly in the myelomas) which were scored as V_HDJ_H rearrangements by our assay. A similar discrepancy in the extent to which the non-productive κ allele is rearranged in permanent cell lines as opposed to normal B cells has been noted (Coleclough *et al.*, 1981; Coleclough, 1983).

The occurrence of the null, marrow-derived A-MuLV transformants with two V_H -to- DJ_H rearrangements suggests that such cells are a by-product of a relatively inaccurate joining mechanism. In several cases, we have cloned and sequenced both V_H -to- DJ_H rearrangements in such a line and confirmed that in both, the V_H and J_H sequences were in mutually incompatible reading frames. (G. Yancopoulos, N. Kohl and F. Alt, unpublished data). A cell type that is more difficult to explain in the context of the model described above is the null, marrow-derived transformants which have at least one (apparently stable) DJ_H rearrangement (Figure 5B, type 4 cell; Table I). Although the nature of these two lines remains

to be determined, it is possible that their DJ_H rearrangements are aberrant and not substrates for V_H recombinations (Alt and Baltimore, 1982), or that the lines once produced heavy chains and lost production during growth in culture (Alt *et al.*, 1982b). In the latter case, the rearrangement process might not have been reactivated. The observation that the 18-48 line has only a DJ_H rearrangement and has apparently lost the other allele (Figure 2; Table I) is consistent with this interpretation.

DJ_H and V_H-to-DJ_H joining occur by a deletional mechanism

The general absence of D segments in lines which have two V_H -to- DJ_H rearrangements (Table I, Figures 3 and 4) supports the concept that V_H -to- DJ_H recombination occurs intra-chromosomally by the deletion mechanism that has been previously outlined for D-to- J_H joining (Alt and Baltimore, 1982) and for V_λ -to- J_λ joining (Sakano *et al.*, 1979). A notable demonstration of the deletion mechanism is the loss of D segments associated with V_H -to- DJ_H joining in cultured fetal liver-derived transformants (Figure 3).

Materials and methods

Cell culture

The characteristics and growth conditions of the various lines have been previously described (Alt *et al.*, 1981; see also legend to Table II).

Isolation of surface κ light chain-positive splenic lymphocytes

Surface κ light chain-positive splenic lymphocytes (B cells) were isolated with a FACS II fluorescence-activated cell sorter as described by Coffman and Weissman (1983). The positive fractions were contaminated with 2–3% of κ negative cells.

Analysis of DNA rearrangements

DNA preparation, restriction enzyme digests, agarose gel electrophoresis, DNA blotting procedures, probe preparation, hybridization procedures, and genomic cloning procedures were performed as previously described (Alt *et al.*, 1981, 1982a).

Acknowledgements

We thank Drs. R. Schuurman and B. Pernis for critically reading this manuscript. This work was supported by American Cancer Society grants NP-393 (F.A.) and MV-34M (D.B.), NIH grants AI-22047 (F.A.), AI-17879 (S.T.), CA-14051 (a core grant to S. Luria) an award from the Searle Scholars Program (to F.A.), and a contribution from the Whitehead Charitable Foundation. F. Alt is a recipient of an Irma T. Hirschl Career Scientist Award, N. Rosenberg is the recipient of a Research Career Development Award, and D. Baltimore is an American Cancer Society Research Professor. G. Yancopoulos and T.K. Blackwell were supported by NIH training grant GM-07367.

References

- Alt, F.W. and Baltimore, D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4118–4122.
- Alt, F.W., Enea, V., Bothwell, A. and Baltimore, D. (1980a) *Cell*, **21**, 1–12.
- Alt, F.W., Botwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M. and Baltimore, D. (1980b) *Cell*, **20**, 283–291.
- Alt, F.W., Rosenberg, N., Lewis, S., Thomas, E. and Baltimore, D. (1981) *Cell*, **27**, 381–390.
- Alt, F.W., Rosenberg, N., Enea, V., Siden, E. and Baltimore, D. (1982a) *Mol. Cell Biol.*, **2**, 386–400.
- Alt, F.W., Rosenberg, N., Casanova, R., Thomas, E. and Baltimore, D. (1982b) *Nature*, **296**, 325–331.
- Brack, C., Hiram, M., Lenhard-Schuller, R. and Tonegawa, S. (1978) *Cell*, **15**, 1–14.
- Coffman, R. and Weissman, I. (1983) *Mol. Cell. Immunol.*, **1**, 33–38.
- Coleclough, C. (1983) *Nature*, **303**, 23–26.
- Coleclough, C., Perry, R., Karjalainen, K. and Weigert, M. (1981) *Nature*, **290**, 372–378.
- Early, P., Huang, H., Davis, M., Calame, K. and Hood, L. (1980) *Cell*, **19**, 981–992.
- Hieter, P.A., Korsmeyer, S.J., Waldmann, T.A. and Leder, P. (1981) *Nature*, **290**, 368–372.

- Kabat,E., Wu,T.T., Bilofsky,H., Reid-Miller,M. and Perry,H., eds. (1983) in *Sequences of Proteins of Immunological Interest*, NIH publication No. 80-20008.
- Korsmeyer,S.S., Hieter,P.A., Lavetch,S.V., Poplack,D.G., Waldmann,T.A. and Leder,P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7096-7100.
- Kurosawa,Y. and Tonegawa,S. (1981) *J. Exp. Med.*, **155**, 201-208.
- Kurosawa,Y., Boehmer,H., Haas,W., Sakano,H., Trauneker,A. and Tonegawa,S. (1981) *Nature*, **290**, 565-570.
- Lewis,S., Rosenberg,N., Alt,F. and Baltimore,D. (1983) *Cell*, **30**, 807-816.
- Maki,R., Kearney,J.K., Paige,C. and Tonegawa,S. (1980) *Science (Wash.)*, **209**, 1366-1369.
- Max,E., Seidman,J. and Leder,P. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3450-3454.
- Nottenberg,C. and Weissman,I.L. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 484-488.
- Nelson,K., Haimovich,J. and Perry,R.P. (1983) *Mol. Cell. Biol.*, **3**, 1317-1332.
- Perry,R. (1983) *Cell*, **33**, 647-649.
- Perry,R.P., Kelley,D.E., Coleclough,C. and Kearney,J.K. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 247-251.
- Rosenberg,N. and Baltimore,D. (1976) *J. Exp. Med.*, **143**, 1453-1463.
- Sakano,H., Huppi,K., Heinrich,G. and Tonegawa,S. (1979) *Nature*, **280**, 288-294.
- Sakano,H., Maki,R., Kurosawa,Y., Roeder,W. and Tonegawa,S. (1980) *Nature*, **286**, 676-683.
- Sakano,H., Kurosawa,Y., Weigert,M. and Tonegawa,S. (1981) *Nature*, **290**, 562-565.
- Tonegawa,S. (1983) *Nature*, **302**, 575-581.
- Wood,C. and Tonegawa,S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3030-3034.

Received on 20 March 1984