A Single Pre-B Cell Can Give Rise to Antigen-specific B Cells that Utilize Distinct Immunoglobulin Gene Rearrangements

By Andrew J. Caton

From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Summary

A group of hybridomas that express antibodies with related specificities for the influenza virus hemagglutinin (HA), that represent B cells that were the clonal progeny of a single pre-B cell, and that utilized distinct L chain gene rearrangements have been characterized. The clonal relationship was established by the sharing of H chain gene rearrangements at both the productive and the nonproductive alleles. Among these hybridomas, one group had rearranged only one of its κ alleles, having joined a Vk24 gene to the Jκ2 gene segment. The other group utilized the same Vk24 gene segment in productive rearrangement to the Jκ3 gene segment, and shared an aberrant rearrangement at the nonproductive κ allele. Accordingly, the expression of distinct Ig gene rearrangements among members of the same B cell clone can normally occur, and can contribute to the generation and diversification of the immune repertoire that is available for the recognition of foreign antigens. Mechanisms by which the distinct rearrangements expressed by the hybridomas might have been generated are discussed.

The generation of functional Ig H and L chain V regions through the assembly of distinct VH-D-JH and VL-JL gene segments occurs as an ordered process during B cell differentiation (reviewed in reference 1). The generally held scheme for assembly involves D to JH rearrangements occurring first on both chromosomes, following which a VH region is joined to one of these alleles. If this rearrangement generates a functional gene, H chain rearrangement ceases; if not, rearrangement takes place at the other allele. Once a productive rearrangement has been generated, cytoplasmic μ heavy chain is synthesized and the cell proceeds to L chain rearrangement. If rearrangement of the κ locus fails to produce a L chain polypeptide, then the pre-B cell undergoes rearrangements at the λ locus. Rearrangements cease once a complete Iγ heterodimer has been assembled, and it is most likely the expression of functional gene products that signals the end to rearrangement of each locus (2–4). A major consequence of how these events are ordered and regulated is that B cells normally express only one of their H and L chain alleles as a cell surface receptor for recognition of antigen, a process known as allelic exclusion (5, 6). Moreover, because of these mechanisms, it has been a common inference that individual members of a B cell clone utilize the same set of rearranged H and L chain alleles, and that structural differences among the clonal progeny of a single pre-B cell occur through somatic mutation.

In recent years, however, it has become apparent that in the case of certain B cell tumor lines, the individual members of a B cell clone can express distinct DNA rearrangements at both their H and L chain loci. For H chains, recombination between a rearranged VH gene and a germline VH gene segment, resulting in the replacement of the initial coding sequences by those of the germline gene segment, has been characterized in lymphomas and in Abelson murine leukemia virus (AMLV)1-transformed pre-B cell lines (7, 8). Several mechanisms have been identified by which clonal progeny that express different L chain alleles can be generated. AMLV-transformed pre-B cell lines have been identified that express productive H chain rearrangements but have unrearranged L chain alleles, and which following cell division can give rise to individual progeny cells that express different rearrangements of the κ locus (9, 10). Lymphoma and plasmacytoma cell lines have also been described in which productive Vκ/Jκ rearrangements have been replaced by further rearrangement of a germline Vκ gene to one of the unrearranged downstream Jκ gene elements on the expressed allele (11–14). Finally, the activation of excluded λ alleles has been observed among the progeny of lymphomas that previously expressed either κ of different λ alleles (15, 16). These studies demonstrate that an individual pre-B cell precursor has the capacity to give rise to clonal progeny that utilize distinct germline elements, and thus considerably increase the degree of structural diversity that can be expressed by members of a B cell

1 Abbreviations used in this paper: AMLV, Abelson murine leukemia virus; HA, hemagglutinin.
Distinct Light Chains Used by Clonally Related B Cells

Materials and Methods

Hybridomas. The hybridomas were generated as previously described by fusion of SP2/0-Ag14 myeloma cells with splenocytes from an adult BALB/c mouse that had been primed 24 d and boosted 3 d before fusion by the intraperitoneal and intravenous routes, respectively, with intact influenza virus PR8 [A/PR/8/34 (H1N1)]. The specificity of these hybridomas for the PR8 HA, and the nucleotide sequences of their H and L chain V regions have been described (17).

DNA Hybridization. The following probes were used in Southern hybridization analysis (19) and for the identification of recombinant clones. pjl1 (20), which contains a 1.8-kb BamHI/EcoRI fragment that includes the JH3 and JH4 gene segments and surrounding sequences, was obtained from Dr. K. B. Marcu, Department of Biochemistry, State University of New York at Stony Brook, NY. IVS, which is a 1-kb HindIII/XbaI fragment excised from the plasmid pECK (21), which contains sequences derived from the intron between the Jk and Ck loci, was obtained from Dr. M. Weigert, Fox Chase Cancer Center, Philadelphia, PA. py3/BglH2.5 (22), which contains a BglII/HindIII fragment corresponding to the Sy3 region, was obtained from Dr. W. Dunnik, University of Michigan Medical School, Ann Arbor, MI. The 0.3-kb EcoRI/BamHI probe was generated by subcloning the H37-311 nonproductive H chain rearrangement from a bacteriophage λ recombinant (see Results, and below). The 0.2-kb PstI/HindIII probe was excised from the pjl1 plasmid and purified by electrophoresis in a 3% NuSieve GTG agarose gel (FMIC Bioproducts, Rockland, ME). Hybridization probes were labeled with 32P by random priming, hybridized for 16 h at 68°C in 5 × SSPE (1 × SSPE = 0.18 M NaCl, 20 mM sodium phosphate, 1 mM EDTA, pH 7), 0.1 mg/ml denatured DNA, and either 1% SDS (for nylon membranes used in Southern analysis) or 0.5% SDS, 5 × Denhardt's solution (for nitrocellulose membranes used in screening bacteriophage libraries). After hybridization, filters were extensively washed in 2 × SSPE, 0.2% SDS at room temperature. Filters from library screening were given a final wash in 1 × SSPE, 0.2% SDS at 65°C for 1 h, and filters from Southern analysis were given a final wash of 30 mM NaCl, 3 mM sodium citrate, 0.2% SDS at 65°C for 30 min.

Analysis of Hybridoma H and L Chain DNA Rearrangements. High molecular weight DNA was extracted from hybridoma cells or from BALB/c liver using standard methods (23). For Southern analysis, 10 μg of DNA was digested with the appropriate restriction enzyme, electrophoresed in 0.8% agarose gels, and transferred to Hybond N+ membranes (Amerham Corp., Arlington Heights, IL) in 0.4 M NaOH. For construction of genomic libraries, 500 μg each of H37-311 and H37-45 DNA was partially digested with Sau3A and size fractionated by sucrose gradient centrifugation as described (23). Individual fractions were ligated into the BamHI site of the bacteriophage λ vector EMBL3 (generously provided by Dr. L. Showe, the Wistar Institute, Philadelphia, PA) and packaged into virus particles using the Packagene Lambda DNA packaging system (Promega-Biotech, Madison, WI). For the molecular cloning of the productive L chain rearrangements from H37-31 and H37-45 DNA was digested with BamHI and HindIII and purified by phenol extraction and ethanol precipitation. The DNA was methylated with EcoRI methylase (New England Biolabs, Beverly, MA), again purified, and then ligated to 1 μg each of the oligonucleotides 5’OH-5GATCC-GAATTCG-3’OH and 5’OH-5GATCC-GAATTCG-3’OH (synthesized at The Wistar Institute DNA synthesis facility). These self-complementary oligonucleotides can hybridize to form a short dsDNA fragment that contains an internal EcoRI site, and that has BamHI and HindIII cohesive ends. Accordingly, this oligonucleotide mixture was ligated to the BamHI and HindIII ends of the digested hybridoma DNA, and then in turn incubated with EcoRI to generate restriction fragments with EcoRI cohesive ends. The DNA was then electrophoresed on a 0.8% agarose gel, and DNA fractions whose size corresponded to the productive L chain rearrangements were purified by electrophoresis. Size fractionated DNA was then ligated to 5’-dephosphorylated bacteriophage λ GT11 arms (Promega-Biotech) and packaged into virus particles using the Packagene λ DNA packaging system. In all cases, bacteriophage plaques were plated, screened using 32P-labeled hybridization probes, and DNA was purified from positive phage by standard methods (23). Phage DNA was initially analyzed by Southern hybridization analysis, and restriction fragments containing the relevant DNA rearrangements were subcloned either into M13mp18 or into pGEM4 (Promega-Biotech) for sequence analysis using Sequenase (U.S. Biochemical Corp., Cleveland, OH) as directed by the manufacturer.
Results

A Group of HA-specific Hybridomas Represent Progeny of a Single Pre-B Cell. A group of hybridoma antibodies that recognize structurally overlapping epitopes on the influenza virus HA have previously been analyzed for the sequence of their Ig H and L chain V regions (17). 10 of the hybridomas were found to express VH genes derived from the VH7183 gene family in conjunction with very similar Vk genes derived from the Vk24 gene group. Five of these hybridomas, which were generated from an individual donor mouse, displayed sequence identity across their VH/D/JH junctions (Fig. 1). Comparison with the sequences of known D gene elements suggested that this junctional sequence was generated by N nucleotide addition to each side of a 10 nucleotide core sequence derived from the DFL16.2 gene segment (18, 25). This sharing of H chain junctional sequences, which have an enormous capacity for variability, strongly suggests that these hybridomas were generated by fusion of B cells that were the clonal progeny of a single pre-B cell (26).

In view of this likely clonal relationship established by sequence analysis, it was surprising that Southern blot analysis of the hybridomas using a JH-specific probe (pJ11) to assess the rearrangement status of both the productive (H+) and nonproductive (H-) H chain alleles revealed differences between the individual hybridomas (Fig. 2A). The hybridomas all share a 2.4-kb EcoRI rearrangement. However, H37-80 and H37-311 share an additional 2.2-kb rearrangement, whereas H37-45 and H37-84 share an additional 9.2-kb rearrangement. To further define the nature of these rearrangements, a genomic DNA library was constructed from H37-311 DNA, and a recombinant was isolated that contained the 2.2-kb EcoRI rearrangement. From this recombinant, a 0.3-kb BamHI/EcoRI fragment was isolated and sequenced, and found to contain a 33 nucleotide, non-germline-encoded sequence joined to the JH2 gene segment (Fig. 3). This 33 nucleotide sequence is identical to a recently identified D region sequence that lacks appropriate 5' recognition signals for recombination and is therefore a pseudogene (designated ψD; reference 27). Thus, the 2.2-kb rearrangement represents the H- allele in H37-311 (and in H37-43 and H37-80), and the 2.4-kb rearrangement that is shared by all the hybridomas corresponds to the common H+ allele identified by sequence analysis. When the 0.3-kb BamHI/EcoRI fragment was used to probe EcoRI-digested hybridoma DNA, it was found also to hybridize to the 9.2-kb rearrangement present in hybridomas H37-45 and H37-84 (Fig. 2B). Partial restriction maps for the shared H+ and different H- rearrangements were established using pJ11, the 0.3-kb BamHI/EcoRI fragment, and a 0.2-kb PstI/HindIII fragment derived from the intergenic sequence between JH3 and JH4 as probes in Southern analysis of hybridoma DNA that had been digested with a variety of restriction enzymes (Fig. 2). It is apparent that the hybridomas share the same ψD/ J2 aberrant rearrangement at their H- alleles, and that a further rearrangement of the H- allele occurred in a region ~0.5-1 kb 3' of the JH locus in hybridomas H37-45 and H37-84, in the vicinity of the IgH enhancer (28).

Previous analyses have demonstrated that class switch–related DNA rearrangements can occur in this region between the JH and CH loci (22, 29). In order to establish whether class switching could have caused the DNA rearrangement observed here, DNA from hybridomas H37-45 and H37-84 was analyzed in Southern blots using a probe derived from the Cy3 switch region (termed Sy3; Fig. 4). The H+ alleles of H37-45 and H37-84 utilize the Cy3 and Cy2a isotype, respectively (17). Analysis with the Sy3 probe following digestion with BamHI or BglII identified unique rearrangements in H37-45, corresponding to the H+ allele. It also identified rearrangements shared by both hybridomas indicating that these hybridomas underwent a common Sy3-specific DNA rearrangement. It should be noted that the pJ11 and Sy3 probes have not been directly linked through hybridization to the same restriction fragment. The failure of these probes to hybridize to a common restriction fragment has previously been observed among hybridomas that express the γ3 isotype (22). Nevertheless, the sharing of rearrangements 3' of JH and in the vicinity of Sy3 is consistent with the further rearrangement that is observed on the H- alleles of H37-45 and H37-84 having occurred as a result of class-switching.

Distinct L Chain Rearrangements Among Clonal Progeny of a Single Pre-B Cell. Previous sequence analysis of L chain mRNA demonstrated that this group of hybridomas all express extremely similar Vk genes derived from the Vk24 gene group (17; Fig. 1). However, hybridomas H37-43, H37-80, and H37-311 were found to express their Vk gene in conjunction with the Jk2 gene segment, whereas H37-45 and...
Figure 1. Hybridoma H and L chain V region nucleotide sequences. The nucleotide sequences are shown relative to consensus sequences generated by their alignment and are numbered relative to the sequence encoding the mature polypeptide. The amino acid sequences deduced from the consensus sequences are also shown. Dashes indicate identity with the consensus sequence. Upper case letters indicate mutations that change the deduced amino acid, lower case letters indicate silent mutations. Nucleotide differences that are shared by individual hybridomas are circled (except for those shared by the nearly identical hybridomas H37-43 and H37-80). The H chain probable D and N region sequences are shown in full, and the portion that is identical to part of the DFL16.1 germline sequence is underlined; H37-311 shows an individual difference from the other hybridomas in this region, which is the result of somatic mutation. The L chain differences that are shared by H37-45 and H37-84 reflect normal differences between the Jk2 and Jk3 gene segments. The regions encoding complementarity determining regions and L chain J regions, and the Jk gene segments that are utilized are indicated.
Figure 2. Southern blot hybridization analysis of hybridoma H chain gene rearrangements. BALB/c liver, SP2/0 fusion partner and hybridoma DNA were digested with various restriction enzymes and hybridized with the pJII, the 0.3-kb EcoRI/BamHI probe, or the 0.2-kb PstI/HindIII probe as follows: (A) DNA digested with EcoRI, and hybridized with pJII; (B) DNA digested with EcoRI, and hybridized with the 0.3-kb BamHI/EcoRI probe; (D) DNA digested with BamHI, and hybridized with the 0.3-kb BamHI/EcoRI probe; (E) DNA digested with EcoRI and XbaI, and hybridized with the 0.3-kb BamHI/EcoRI probe; (F) DNA digested with PstI, and hybridized with the 0.2-kb PstI/HindIII probe. The sizes in kilobases of molecular weight markers are indicated. (C) The previously published restriction map of the unrearranged JH locus (H°) (20), and deduced maps for the shared productive rearrangement (H+), the aberrant rearrangement found in H37-43, H37-80 and H37-311 (H-), and the aberrant rearrangement found in H37-45 and H37-84 (H-). The location of sites for the restriction enzymes BamHI (B), EcoRI (E), HindIII (H), PstI (P) and XbaI (X) are indicated. Broad lines represent rearranged, non-germline sequences, and bars underneath each map indicate sequences that are detected by each probe. The locations of JH gene segments are indicated. Dashed lines indicate regions where boundaries have not been clearly defined.

H37-84 utilize the Jk5 gene segment (Fig. 1). The expression of distinct Jk gene segments was unexpected in view of the clonal relationship between the hybridomas described above. The hybridoma L chain rearrangements were therefore analyzed in greater detail by Southern blotting, using a probe (termed IVS) derived from the intron between Jk and Ck to detect rearrangements at the Jk locus (Fig. 5). Sequence analysis of L chain mRNA had identified a BamHI restriction site in the 5' leader sequence of the Vx24 genes utilized by each hybridoma (data not shown; see Fig. 6). Digestion with both BamHI and HindIII gave rise to rearrangements consistent with the rearrangement of this identified BamHI site to either Jk2 or Jk5 (Fig. 5A). The presence of comigrating sequences derived from unrearranged κ al-
a. **H37-311 H chain non-productive allele**

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5'...GTTACGTCGAGCACTCCTGGCICTGACCTGCAGCTCA...3'
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b. **H37-45 L chain non-productive allele**

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G S S P K L W I Y S I S N L
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A S G L P A R F S G S G S G T S
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Y S L T I S V K A E D T A T Y
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'ACTGCC=ATT
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PTW K
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CAAGCIGGAAATAAAA...3'
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**Figure 3.** Nucleotide sequences of the H37-311 H chain (a) and H37-45 L chain (b) nonproductive alleles. In each case, the sequence from the restriction site used in molecular cloning (underlined) through the J gene segment is shown, and the junction between the rearranged sequence and the J gene segment is indicated. In b, the deduced amino acid sequence is also shown in the single letter code; (*) indicates a termination codon.

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**Figure 4.** Southern blot hybridization analysis of hybridoma S'y3 switch region rearrangements. BALB/c liver, SP2/0 fusion partner and hybridoma DNA were digested with BamHI or BglII and hybridized with the S'y3-specific p'y3/BgH2.5 probe. The two rearrangements of ~9.4 kb that are detected in H37-45 following digestion with BamHI alone (Fig. 5 B). No additional rearrangements (other than ones corresponding to those of the SP2/0 fusion partner) were observed in hybridomas H37-43, H37-80, or H37-311 following digestion with both BamHI and HindIII, or BamHI alone. Thus, the nonexpressed allele of these B cells was most likely in an unarranged (k+ configuration, since no additional rearrangements were identified in any of the hybridomas. After digestion with BamHI and HindIII, hybridomas H37-45 and H37-84 display a shared 1.9-kb rearrangement corresponding to the productive Vk24/Jk5 rearrangement, and an additional shared rearrangement of 2.5 kb. H37-45 also displays a k° allele, which derives from the SP2/0 fusion partner (and was not retained in H37-84).

**The L Chain Productive Rearrangements Utilize the Same Vk24 Gene Segment.** Sequence analysis of L chain mRNA revealed extreme similarity between the Vk24 genes utilized by this group of hybridomas, indicating their derivation from the same, or from very closely related germline gene segments. In an attempt to distinguish between these possibilities, the hybridoma L chains were analyzed for the sequence of the intron that is found within Vk leader exon sequences. Previous analysis of members of the Vk24 gene group demonstrated that individual family members display much greater sequence variability within these regions than is found within the coding sequences (31). Accordingly, if the Vk24 gene segments utilized here had derived from more than one gene segment, an analysis of these intron sequences might distinguish between these genes. DNA from H37-84 and H37-311 was digested with BamHI and HindIII and size-fractionated by agarose gel electrophoresis. Fractions corresponding in size to the k+ rearrangements were molecularly cloned, and recombinants containing these rearrangements were isolated and subjected to sequence analysis (Fig. 6). Based on these sequences, oligonucleotide primers were designed and used in the polymerase chain reaction (PCR; reference 24) to amplify the intron sequences from all the hybridomas, and also from BALB/c liver DNA. For amplification of the hybridoma k+ rearrangements, the Vk24 5' primer was used in conjunction with either a Jk2- or Jk5-specific oligonucleotide. The Vk24 5' and Vk24 3' primers were used to amplify the unarranged sequence from BALB/c liver DNA. PCR reaction products were subjected to direct sequence analysis, and the sequences obtained for H37-84 and H37-311 were found to correspond exactly to those obtained by molecular cloning. The analysis of the BALB/c liver DNA amplification products yielded a single sequence when analyzed as a popula-
tion, and the individual hybridomas were found to display either the same sequence, or individual differences from that sequence. In addition, the BALB/c reaction products were molecularly cloned and eight individual clones (four each from two independent amplification reactions) were analyzed for their sequence. Four of these clones had an identical sequence to that of the population, and the remainder had individual nucleotide differences from the population (data not shown).

\[ \text{Figure 5. Southern blot hybridization analysis of hybridoma L chain V region rearrangements.} \]

BALB/c liver, SP2/0 fusion partner and hybridoma DNA were digested with BamHI alone (A) or with both BamHI and HindIII (B), and hybridized with the Jx intron-specific probe IVS. The sizes in kilobases of molecular weight markers, and the sizes predicted for the Vx24/J2 and Vx24/J5 rearrangements are indicated.

\[ \text{Figure 6. Nucleotide sequences of the hybridoma and BALB/c liver Vx24C 5' introns.} \]

The intron sequence obtained by molecular cloning of the H37-84 Vx24C/J5 productive rearrangement is shown and numbered relative to the BamHI site used in cloning the V region exon sequences. The sequences of H37-311 obtained by molecular cloning, and H37-45, H37-80 and BALB/c liver (designated Vx24C) obtained by PCR are shown relative to this sequence; dashes indicate identity with the H37-84 sequence. Also shown is the corresponding sequence from the Vx24A germline gene that has previously been described (31). Gaps (indicated by periods) have been introduced into the sequences to maximize their alignment. The amino acid sequence encoded by mRNA is shown in the single letter code and is numbered relative to the sequence of the mature polypeptide. The location of RNA splice sites are indicated by arrows, and the sequences to which the PCR primers hybridize are boxed.
None of these differences, however, corresponded to any of the differences observed in the hybridomas, and were most likely the result of mutations introduced during amplification (24). No systematic differences were observed between the Vk genes that had been rearranged to Jk2 and those that had been rearranged to Jk5. This strongly suggests that the hybridomas all express the same Vk gene segment, and that their individual differences are the result of somatic mutation. In relation to the previously described Vk24 germ-line gene sequenced, this intron sequence displays 10 nucleotide substitutions, two nucleotide insertions and four nucleotide deletions relative to the Vk24A germ-line gene, and very many differences relative to the other described members (designated Vk24, Vk24B and Vkx24; reference 31). The Vk 5′ intron sequence described here is thus designated as deriving from the Vk24C gene segment.

Discussion

Detailed molecular characterization of a group of hybridomas that express antibodies with related specificities for the influenza virus HA has revealed that a single pre-B cell can give rise to antigen-specific B cells that utilize distinct L chain gene rearrangements. The clonal relationship of these B cells was established by the sharing of H chain gene rearrangements at both the productive and the nonproductive alleles (26). The hybridomas could, however, be subdivided into two groups based on their L chain rearrangements. One group had rearranged only one of its κ alleles, having joined a Vk24 gene to the Jk2 gene segment. The other group utilized an indistinguishable Vk24 gene segment in productive rearrangement to the Jk5 gene segment, and shared a nonproductive rearrangement of a Vk4 gene segment in an out-of-frame join to the Jk2 gene segment. This latter group also displayed an additional rearrangement near the nonproductive H chain allele, in the vicinity of the IgH enhancer, that may have been the result of class switching. The rearrangement status of each of the hybridoma H and L chain alleles is summarized in Fig. 7. The figure shows a common pre-B cell that contains the shared H chain rearrangements, and indicates three theoretical and simplified pathways by which B cells containing the different rearrangements observed in these hybridomas might have been generated. Analysis of these hybridomas has not yielded direct evidence to confirm or exclude any of these pathways. It nevertheless is informative to relate these findings to the processes of Ig rearrangement and B cell differentiation; based on current understanding of these processes, the results as discussed below seem most strongly to favor either pathway B or C.

Pathways A and C share in common the postulate that secondary rearrangements of one or more κ chain alleles took place during the proliferation of a B cell clone. Pathway A proposes that the B cells represented by H37-45 and H37-84 were generated by replacement of the alleles identified in H37-43, H37-80, and H37-311. In pathway C, an intermediate clonotype expressing undefined Vk/Jk rearrangements is postulated to have undergone clonal expansion before secondary rearrangement of its κ chain alleles. It has previously been established by analysis of B cell tumor models that rearranged κ chain alleles can be replaced by secondary rearrangements of upstream Vk gene segments to downstream, unrearranged Jk gene segments (11-14). Since the κ rearrangement in H37-45 and H37-84 involves a Vk4 gene segment joined to the Jk2 gene segment, pathway A would require that this aberrant rearrangement was generated from the unrearranged (κ°) allele of the original clone (the other Jk2 gene segment would already have been joined to the Vk24C gene segment). Accordingly, the generation of the Vk24C/Jk5 allele would necessarily require the replacement of the existing Vk24C/Jk2 allele with this new rearrangement. However, sequence analysis of intron sequences located within the leader exon of the Vk genes described here, as well of the corresponding genomic sequence, strongly suggests that the same gene segment was used in all these hybridomas. Pathway A, then, imposes the unfavorable requirement that two (or more) indistinguishable Vk24C genes are present in the BALB/c genome, and that one of these was used in the secondary rearrangement of Jk5. An alternate possibility that would allow the same Vk gene to be used is that the putative secondary rearrangement occurred by inter-
chromosomal recombination, although such recombinations are not thought to take place during Ig gene assembly (32, 33). If secondary rearrangements of k chains indeed led to the generation of the B cells represented by H37-45 and H37-84, it is more likely that this occurred by pathway C. Since this model allows for the generation of an intermediate clonotype that utilized unrelated V genes, it accommodates the conclusion that these hybridomas all express the same gene segment. Attempts to identify Jk-specific DNA sequences that might have been retained by H37-45 and H37-84 as remnants of an earlier joining event were unsuccessful. However, since many VK genes (including members of the Vk24 gene family) recombine by a deletional mechanism that does not give rise to such remnant sequences (13), their absence is uninformative. It is also significant that pathway C could allow for many different L chains to be generated by secondary rearrangements in individual members of the expanded clone, since this would permit antigen selection to have expanded those that used the Vk24C gene segment and could recognize the HA.

Pathway B proposes that a pre-B cell rearranged its H chain alleles, and then underwent cell division before L chain rearrangement. Among the clonal progeny, B cells that possess the k chain rearrangements identified here were generated. In view of the large number of different VK gene segments that are available for these independent rearrangements, however, the utilization of the same Vk24C gene segment by both sets of cells would be unexpected. This model might then require one of the following additional features. The first possibility is that the Vk24C gene is rearranged much more frequently than is the case for VK genes in general. This possibility is not supported by recent surveys of the frequency of VK gene rearrangements (34, 35). In addition, previous idioctypic analysis demonstrated that the L chain expressed by each of these hybridomas was expressed on 7 (1.5%) of 467 hybridomas generated by fusion of LPS-stimulated BALB/c fetal liver cells (17). This frequency estimate would suggest that there is a low probability (0.015) that two cells generated following a single division would both use this L chain. A second possibility is that the pre-B cell that gave rise to these B cells underwent clonal proliferation before L chain rearrangement, and generated a pool of B cells bearing different L chains. As in pathway C, antigen selection could then have expanded those B cells that express the Vk24C gene segment. Studies of pre-B cells in regenerating bone marrow in vivo have suggested that large pre-B cells expressing cytoplasmic µ chains, but not surface IgM (termed cm µ - sm µ -) can undergo several rounds of division before differentiation into small cm µ + sm µ + pre-B cells (36, 37). These cells then undergo L chain rearrangement and acquire the sm µ + phenotype. It was estimated that the large cm µ + sm µ + pre-B cells might undergo four to eight divisions prior to differentiation (37). Thus, if clonal expansion gave rise to 256 pre-B cells (from eight divisions) of which 100 underwent successful L chain rearrangement, then the probability of at least two cells expressing the Vk24C gene based on the above frequency becomes 0.5, which clearly is within a reasonable range to have been selected by antigen. This estimate is very approximate; nevertheless, it demonstrates that clonal proliferation by a cm µ + sm µ - large pre-B cell could account for the rearrangements observed here. The results may then indicate that proliferation among cm µ + sm µ - pre-B cells, and the consequent expression of particular H chain rearrangements with a variety of different L chain rearrangements makes a significant contribution to the diversification of the B cell repertoire.

One feature that might have given support to one or other of the above models is the pattern of the individual somatic mutations that accumulated in each hybridoma. Previous analyses of the pattern of shared and unique mutations that have accumulated among members of an individual B cell clone have frequently allowed genealogic trees to be developed that indicate a progressive, stepwise accumulation of mutations among the clonal progeny (38–44). In this case, pathway C for example would have been supported by the presence of a number of shared mutations in the H chains of H37-45 and H37-84, but an absence of shared mutation in their L chains, consistent with these cells having undergone clonal expansion before a secondary L chain rearrangement. However, the pattern of mutation among this group of hybridomas is extremely unusual. With the exception of hybridomas H37-43 and H37-80 which display nearly identical sequences, the individual hybridomas for the most part display unique somatic mutations; thus, despite the presence of a large number of nucleotide differences between individual hybridomas (at least 57 nucleotide differences from H and L chain consensus sequences), there exist only four shared differences from these consensus sequences, all of which are found in the H chain (Fig. 1; due to its near identity with H37-80, H37-43 was not considered in this analysis). Strikingly, these shared mutations all contradict the genealogic relationships that are clearly established based on the DNA rearrangements observed at both the L and H chain alleles. One of the mutations is shared between H37-84 and H37-80 (and H37-43), and could readily be the result of a parallel mutation, selected perhaps for interaction with antigen. The other three, however, are found within a 32 nt region of CDR2, and the hybridomas fall into two groups according to the nucleotide they display at each position; two of these differences cause amino acid interchanges, but the third substitution is silent. In view of the different lineages that are firmly established based on DNA rearrangements, this pattern of mutation suggests that gene conversion events might have introduced a common 32 nt sequence into two of these VH regions (45, 46). The independent introduction of identical somatic mutations into individual members of the same and different B cell clones has recently been described, and was suggested to indicate the presence of "mutational hotspots" with V regions (44). Whatever the basis for the generation of these shared mutations, the results described here further demonstrate that the sharing of mutations need not necessarily indicate that the cells underwent mutation along a common pathway. Thus, the pattern of somatic mutations offers indirect support to pathway B described above, by failing to identify shared H chain mutations that might have accumulated prior to the secondary L chain rearrangements postulated in pathways A and C. However, in view of the shared
mutations that were actually accumulated, the pattern of mutation does not appear to be a reliable criterion for discriminating between these pathways. If gene conversion events indeed led to the introduction of some of the mutations that are observed among these hybridomas, then such events could have obscured any shared mutations that had accumulated during early stages of expansion of this B cell clone.

The analysis of this group of hybridomas has clearly demonstrated that an individual pre-B cell, following the productive rearrangement of one of its H chain alleles, can give rise to different B cell lineages that express distinct L chain rearrangements. Most significantly, these hybridomas were isolated because they produced HA-specific antibodies. The individual mouse from which these hybridomas were isolated gave rise to 47 HA-specific hybridomas, and because a large number of hybridomas from this individual mouse has been examined by sequence analysis. The identification and characterization of further groups of clonally related HA-specific hybridomas might allow these possibilities to be assessed.

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Address correspondence to Andrew J. Caton, The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, PA 19104.

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