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Immunology

Immunoglobulin gene rearrangements and deletions in human Epstein–Barr virus-transformed cell lines producing different IgG and IgA subclasses

(isotype switching/B-cell differentiation)

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ABSTRACT **During differentiation B lymphocytes may switch from the expression of surface IgM to the synthesis of IgG, IgA, or IgE isotypes by using a different heavy chain constant region (C_H) gene. The molecular mechanisms by which switching occurs remain controversial. Rearrangements and deletions of C_H genes 5′ to the expressed gene have often been observed in the mouse and, more recently, in human cells that have switched isotypes. We have used human J_H, C_μ, C_γ, and C_α probes to examine the extent of the deletions and rearrangements in clones of Epstein–Barr virus-transformed human cells that produce IgG1, IgG3, IgG4, or IgA1. Though deletions of C_H genes 5′ to the expressed C_H gene were consistently observed, the rearrangement process appeared to be highly variable for the nonproductive C_H gene locus: deletion or persistence of 5′ C_H genes, combinations of deletion and duplication of 5′ genes, and deletions extending to 3′ C_H genes. Our results reveal an unexpected lack of specificity in the DNA deletions in cells that have undergone isotype switching.**

The immunoglobulin heavy chain constant region (C_H) genes in humans are aligned on chromosome 14 in the order 5′ C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\psi\varepsilon}$-$C_{\alpha 1}$-$C_{\psi\gamma}$-$C_{\gamma 2}$-$C_{\gamma 4}$-C_ε-$C_{\alpha 2}$ 3′ (1, 2). During B-lymphocyte differentiation cells may switch from the synthesis of IgM to the synthesis of IgG, IgA, or IgE (3, 4). This occurs when the functionally rearranged variable exon (V_H) used in conjunction with C_μ to produce the IgM heavy chain is subsequently transcribed with a different C_H gene. Although the exact molecular mechanisms by which switching occurs are not yet fully understood, several have been proposed. One possibility is that switching is mediated through pairing and recombination between regions of homology in the DNA sequences 5′ to each of the C_H genes. These switch sequences exist about 1.5 kilobases (kb) 5′ to each C_H gene except C_δ and are composed of short repeated sequences (5, 6). Recombination could occur either between switch sites on one chromosome, resulting in formation of a loop and deletion of intervening DNA sequences, or through unequal sister chromatid exchange (4, 7–9). Alternatively, it has been proposed that switching may occur via processing of a long RNA transcript containing V_H and all of the C_H genes (10–12).

Most of the evidence to date has been obtained from analyses of mouse plasmacytomas and supports a deletion mechanism on either one or both chromosomes (7, 13, 14). Studies using lipopolysaccharide-stimulated mouse spleen cells have also shown C_μ deletion in cells that have switched to IgG3 (15). Evidence obtained from unstimulated mouse B cells favors the possibility that RNA processing is the initial event in the switching process before B cells begin plasma cell differentiation (11, 12).

Fewer studies have been performed using human B cells. Rearrangements of C_μ, C_λ, and C_κ occur in chronic lymphocytic leukemia cells (16, 17), and both rearrangements and deletions of C_H genes have been observed in several Epstein–Barr virus (EBV)-transformed lines (18). However, there has been no systematic study to determine the rearrangements and the extent of the deletions in cell lines producing defined IgG and IgA subclasses. We have established from four individuals a panel of EBV-transformed cell lines producing IgM, IgG3, IgG1, IgA1, and IgG4 antibodies and have analyzed DNA from these lines for rearrangements and deletions of C_μ, the four C_γ, and both C_α genes. Although we have observed one interesting exception in which genes 5′ to the functionally rearranged C_H are retained, possibly due to duplication, most of our data are consistent with a deletion model for isotype switching. The data also suggest that the switching process is not a precise, well-defined, orderly deletion confined to the functional chromosome.

MATERIALS AND METHODS

Establishment of EBV-Transformed Cell Lines. Blood mononuclear cells collected from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation, and T cells were depleted by rosetting with aminoethylisothiouronium bromide-treated sheep erythrocytes (19). This B-cell-enriched fraction was then resuspended at 2×10^6 cells per ml in RPMI 1640 medium, and 100 μl of EBV-containing supernatant per ml [toxic dose, 50% (TD_{50}) $>10^4$] from the B95-8 marmoset cell line was added (20). Cells were incubated at 37°C in 7% CO_2 in air overnight, washed, resuspended at 10^6 cells per ml, and grown in RPMI 1640 medium supplemented with 15% fetal calf serum, L-glutamine, 50 μM 2-mercaptoethanol, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 μg of Fungizone (Flow Laboratories) per ml. After 10 days cells were subcloned by limiting dilution onto mouse peritoneal feeder cells in 96-well plates (Costar, Cambridge, MA). Statistical clones (i.e., those obtained from plates showing growth in <30 wells) were screened after 4 wk for secretion of IgM, IgG, and IgA by ELISA. Sublines producing only one isotype were examined further for cytoplasmic immunoglobulin by immunofluorescence staining with a panel of monoclonal antibodies to human isotypes (21). Fixed cell smears were stained indirectly by using fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin as a second layer (22). At least 10^4 cells per slide were examined, and cell lines were used in molecular analyses only after they were recloned so that they

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Abbreviations: C_H, heavy chain constant region; EBV, Epstein–Barr virus; kb, kilobase(s); J, joining; V, variable.

produced only one heavy and one light chain isotype. Further analyses for clonal origin of these lines were performed by indirect immunofluorescence staining with a panel of 34 mouse anti-human V_H and anti-idiotype antibodies provided by H. Kubagawa (23, 24). Karyotype analysis was performed by Andrew Carroll (Department of Medical Cytogenetics, University of Alabama at Birmingham).

DNA Isolation and Analysis. High molecular weight DNA was isolated (25) and digested to completion with *Hin*dIII, *Bam*HI, or *Eco*RI, as suggested by the manufacturer (Boehringer Mannheim). Five micrograms of DNA was loaded per lane onto 0.7% agarose gels, and fragments were size-separated by electrophoresis at 0.5–1.5 V/cm for 36–48 hr. Southern blots (26) were prepared with Zetabind membranes as recommended by the manufacturer (AMF, Meriden, CT). Blots were hybridized at 42°C, washed under high-stringency conditions, and exposed to XAR-5 x-ray film (Kodak). For rehybridization, filters were stripped with 0.4 M NaOH.

Probes. A 1.3-kb *Eco*RI genomic fragment, containing the C_{H2}, C_{H3}, and C_{H4} domains and a portion of the 3′ untranslated sequence of human C_μ, and a 6.0-kb *Bam*HI/*Hin*dIII fragment, containing all six human heavy chain joining region (J_H) exons and the three $_\psi J_H$ gene segments, were provided by J. V. Ravetch (27). The 2.0-kb *Eco*RI/*Hin*dIII fragment containing the germ-line $C_{\gamma 4}$ exons was provided by J. Ellison (28). $C_{\gamma 4}$ included all three constant domains and the hinge sequence as well as ≈200 nucleotides 5′ and 3′ to the coding exons. In some cases a *Hin*fI fragment containing only the hinge, C_{H2} and part of C_{H3}, was used as a probe, and results were always identical to those obtained by using the entire probe. The $C_{\alpha 2}$ probe was a 4.4-kb *Xho* I/*Bam*HI fragment subcloned into pBR322 from a Charon 28 λ clone and containing part of the C_{H1} domain, the hinge region, domains C_{H2} and C_{H3}, and the 3′ flanking sequence also obtained from J. V. Ravetch (29). All probes were restricted with the appropriate endonucleases, separated from vector DNA by electrophoresis, and labeled with [^{32}P]dCTP (30).

Densitometric Analyses. Densitometric analyses were performed with a 2202 Ultrascan laser densitometer and GELSCAN software (LKB) in an Apple IIe computer (Cupertine, CA). To avoid discrepancies in intensity due to variations in quantities of DNA per lane or differences in transfer efficiency, analyses were performed on C_γ- and C_α-hybridizing bands within a single lane, and the ratios of these intensities were compared with ratios of the intensities obtained in a control cell line containing two germ-line copies of each gene. The comparison of peak ratios to ratios obtained from control lines also prevented misinterpretations that might occur due to preferential hybridization of the $C_{\gamma 4}$ and $C_{\alpha 2}$ probes to those particular subclasses.

RESULTS

Absence of Isotype Switching in Clones of EBV-Transformed B Cells. Since the interpretation of any molecular data relies heavily upon the clonality of the cell lines examined, care was taken to document the clonal origin of each line. At least two rounds of subcloning were performed since immunofluorescence staining after the initial limiting dilution cloning procedure revealed small numbers of cells (from 0.3% to as many as 30% in one instance) producing a second isotype. Cells producing the minority isotype were not observed after subsequent recloning, and in no instance was isotype switching indicated by the appearance of an expanding subpopulation of cells producing a second isotype.

Two of the clones reacted with the anti-V_H subtype monoclonals; ME-G1 reacted with SAFD44, whereas IL-G4 reacted with MH4410. None of the clones examined so far has reacted with any of the 30 anti-idiotype antibodies despite the fact that small numbers of cells that react with these reagents are present in transformed cultures from the same individuals before cloning (unpublished results). After final subcloning, >70% of the cells in each of the clones stained brightly for cytoplasmic immunoglobulin of the appropriate isotype. Although the cell types present in these lines ranged from B lymphocytes to mature plasma cells, most of the cells had a plasmablast morphology.

Deletion of C_μ from Cell Lines Expressing IgG1, IgA1, and IgG4. Hybridization of *Bam*HI- and *Hin*dIII-digested DNA from two IgG1-, one IgA1-, and one IgG4-producing cell line with a C_μ probe is shown in Fig. 1. In the *Bam*HI-digested lane, the IgM line (ME-M) shows one rearranged band and one unrearranged germ-line copy of C_μ. C_μ has been deleted from both chromosomes in the IgA1 and IgG4 lines as well as in IL-G1; however, ME-G1 shows a single C_μ-hybridizing band. Results from three other cell lines, an IgA1 line from another individual and an IgG1 and an IgG3 line from IL, also show deletion of C_μ from both chromosomes (data not shown).

Rearrangement and Deletion of C_γ Genes in Cell Lines Expressing IgG and IgA Isotypes. Fig. 2 shows the results of hybridization of DNA from the same cell lines with the $C_{\gamma 4}$ probe. All four C_γ genes and the $C_{\psi\gamma}$ are sufficiently homologous for the $C_{\gamma 4}$ probe to detect each of these genes, and digestion with *Bam*HI allows the detection of each gene as a discrete band on genomic DNA blots. We assigned C_γ genes to a particular *Bam*HI-hybridizing band according to size, as described by Bech-Hansen *et al.* (31). Due to polymorphic differences in the introns between the C_γ genes among individuals (31), IgM lines from each individual were used as controls for the germ-line configuration of the C_γ genes. Only one IgM-producing control is shown, since both individuals ME and IL have identical germ-line configurations in the *Bam*HI digests.

Deletion from both chromosomes of $C_{\gamma 3}$ and $C_{\gamma 1}$, which are 5′ to $C_{\alpha 1}$, is apparent in ME-A1, and deletion of both $C_{\gamma 3}$ genes has occurred in IL-G1. An IgA1 line from a third

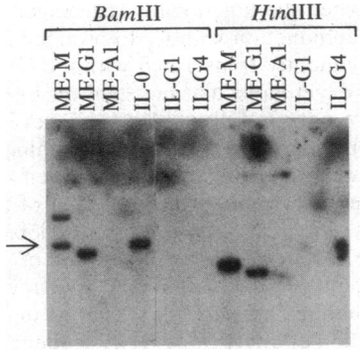
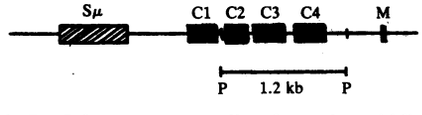


FIG. 1. Hybridization of *Bam*HI- and *Hin*dIII-digested DNA from two individuals (ME and IL) with the human C_μ probe. The heavy chain isotype produced by each cell line is indicated after the individual's initials. IL-0 is an immunoglobulin negative line. The arrow designates the germ-line *Bam*HI C_μ fragment. All size determinations were calculated by using a 1.0-kb ladder (Bethesda Research Laboratories) and *Hin*dIII-digested λ DNA as size markers. *Bam*HI cleaves the genomic DNA 5′ of J_H and 3′ of C_μ, such that J_H, S_μ, and all of C_μ including the membrane exon remain on the same restriction fragment. The C_μ probe



was a 1.2-kb *Pst* I fragment extending from the middle of the C_{H2} domain through the 3′ flanking sequences.

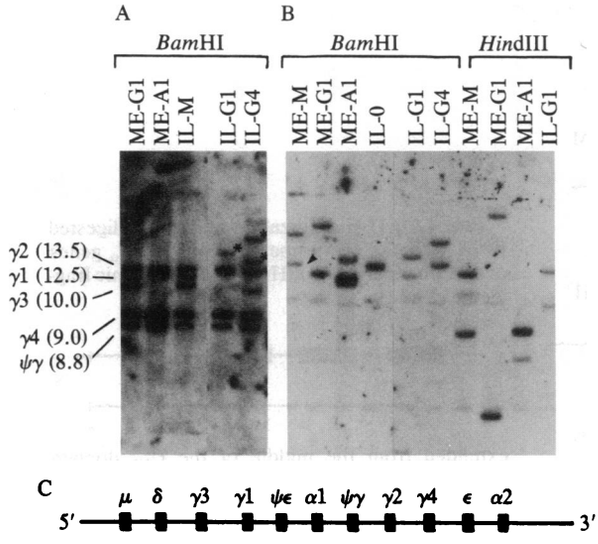
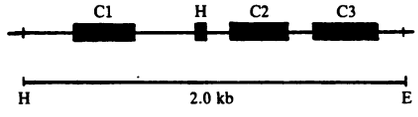
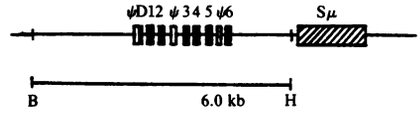


FIG. 2. (*A*) Hybridization of *Bam*HI-digested DNA from five clones with a $C_{\gamma 4}$ probe



containing the entire $C_{\gamma 4}$ coding region and about 200 bases in both 5′ and 3′ flanking sequences. Sizes were calculated by using the same markers described in the legend to Fig. 1, and γ subclasses were assigned to bands according to results described by Bech-Hansen *et al.* (31). Asterisks designate rearranged C_γ bands that also hybridize with the J_H probe in *B*. Unlabeled bands represent rearranged genes that are not linked to a J_H. Hybridization of *Hin*dIII-digested DNA from each of these clones with the $C_{\gamma 4}$ probe yielded similar results (data not shown). (*B*) Hybridization of *Bam*HI- and *Hin*dIII-digested DNA with the human J_H probe. The J_H probe includes 5′ flanking sequences, all of the known human J_H genes, and 3′ sequences approaching but not including the S_μ region



An arrow has been placed in the *Bam*HI ME-M lane to indicate the germ-line *Bam*HI fragment. (*C*) Germ-line organization of the human C_H locus.

individual has also deleted $C_{\gamma 3}$ and $C_{\gamma 1}$ genes from both chromosomes (data not shown). Each of the IgG-producing cell lines contains at least one rearranged C_γ-hybridizing band as well as several bands in the germ-line configuration. Rehybridization of the same blot with J_H (Fig. 2) shows at least one of the J_H-hybridizing bands coinciding with one of the rearranged C_γ bands in Fig. 2. Therefore, these rearranged bands must represent the productively rearranged genes.

Although both IgG1-producing lines show only one rearranged C_γ band hybridizing with J_H, these two lines differ markedly in the extent of the deletions that have occurred within the C_H locus. ME-G1 retains at least one copy of C_μ and appears to have undergone rearrangement on only one chromosome. Comparison of densitometric intensities obtained from the control cell line with those obtained from ME-G1 (Fig. 3) showed that the C_γ-hybridizing bands at the $C_{\gamma 3}$ and $C_{\gamma 1}$ positions in ME-G1 are probably representative of only one allele and confirmed that deletion of C_H genes occurred on only one chromosome in this instance. IL-G1, on the other hand, shows deletion on both chromosomes of C_μ and $C_{\gamma 3}$ genes, which are 5′ to $C_{\gamma 1}$, and appears to have

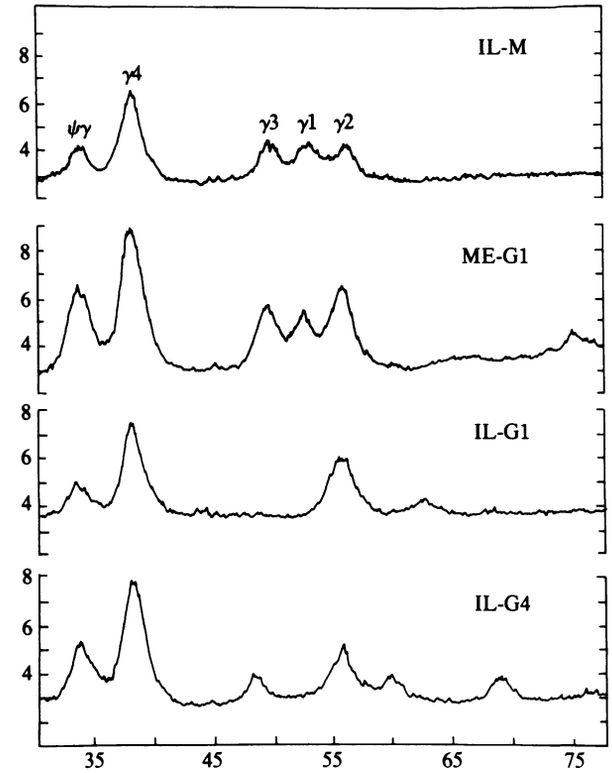


FIG. 3. Densitometric scans of autoradiograms of *Bam*HI-digested DNA hybridized with the $C_{\gamma 4}$ probe in Fig. 2. Scans were performed within a single lane and the peaks that represent the individual bands are labeled accordingly.

deleted $C_{\gamma 1}$ as well as C_H genes 3′ to $C_{\gamma 1}$ on the nonfunctional chromosome. Densitometric analyses of C_γ-hybridizing bands from IL-G1 (Fig. 3) indicate that $C_{\psi\gamma}$ as well as $C_{\gamma 1}$ may have been deleted on one chromosome, in which case one might also expect that $C_{\alpha 1}$, which is between $C_{\gamma 1}$ and $C_{\psi\gamma}$, would have been deleted on that chromosome. Hybridization of DNA from this line with a $C_{\alpha 2}$ probe and densitometric analyses (Fig. 4) show that there is indeed only one copy of $C_{\alpha 1}$ in this line. Thus, in this instance, deletions have occurred on the nonfunctional chromosome 3′ to the C_H gene that is expressed on the functional chromosome.

Possible Duplication of C_H Genes. The IL-G4 line is of particular interest. Although it has deleted both copies of C_μ and shows no germ-line bands for $C_{\gamma 3}$ and $C_{\gamma 1}$, as might be expected for a line that is actively producing IgG4, it contains three rearranged C_γ bands. It is also surprising that the $C_{\psi\gamma}$ and $C_{\gamma 2}$ genes are retained and that there is a very intensely hybridizing band in the germ-line $C_{\gamma 4}$ position. Although two of the rearranged bands hybridize with J_H, only one of these is likely to be the productive $C_{\gamma 4}$ allele. These data have been obtained consistently from this line and cannot be explained by artifacts or variations in procedure. Eighty percent of these cells stain brightly for IgG4 and with the anti-V_H antibody MH4410, suggesting that contamination with another IgG4-producing line is unlikely. Clonality is also borne out by the molecular data, since hybridization with J_H (Fig. 2) shows only two specifically hybridizing bands rather than the three or more that might be expected from clonal contamination. Karyotypic abnormalities cannot explain these results, since the cells in this line appear to be diploid for all chromosomes, have no apparent translocations such as those seen in Burkitt lymphoma cell lines, and have no detectable extrachromosomal fragments (data not shown).

The presence of an intensely hybridizing band in the $C_{\gamma 4}$ germ-line position suggests that duplication of at least a

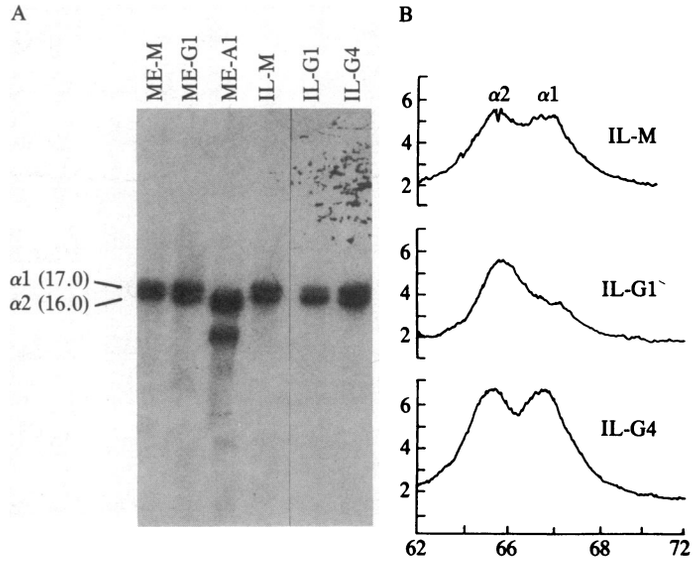
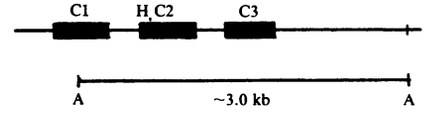


FIG. 4. (*A*) Hybridization of *Bam*HI-digested DNA with the $C_{\alpha 2}$ probe. Each of the C_α genes occurs on separate *Bam*HI-digested genomic fragments. The $C_{\alpha 2}$ probe



extended from the middle of the C_{H1} domain through all of the other domains and included 3′ flanking sequences. (*B*) Densitometric scans of the C_α-hybridizing bands in *A* were performed as described in the legend to Fig. 3.

portion of the C_H genes has occurred in this line resulting in two germ-line and one rearranged copy of $C_{\gamma 4}$. This is the simplest explanation for the results obtained from this cell line. It is unlikely that this reflects reintegration of a portion of the excised 5′ genes as may have occurred in an Abelson virus-transformed pre-B-cell line that switches from C_μ to $C_{\gamma 2b}$ and retains a C_μ no longer juxtaposed to J_H (32). Densitometric analyses (Fig. 3) did in fact show that there are probably at least three copies of $C_{\gamma 4}$ in this cell line, one of which is a rearranged band that hybridizes with J_H and represents the productive $C_{\gamma 4}$ gene. The other copies of $C_{\gamma 4}$ are represented by the germ-line-sized *Bam*HI fragment. A duplication of a fragment containing $C_{\gamma 1}$, $C_{\alpha 1}$, $C_{\psi\gamma}$, $C_{\gamma 2}$, and $C_{\gamma 4}$ could explain the detection of $C_{\gamma 2}$ and $C_{\psi\gamma}$ as well as the deletion of C_μ from both chromosomes. One possibility is that, although $C_{\gamma 4}$ is rearranged to J_H on one chromosome, the nonfunctional chromosome may have rearranged such that $C_{\gamma 1}$ is joined to J_H, thus retaining all of the 3′ C_H genes on that chromosome. The duplication of a large portion of the C_H gene locus on this chromosome and its reinsertion into the genome such that the duplicated $C_{\gamma 1}$ is reduced in size would account for all of our data. Since hybridization of the DNA from IL-G4 with a $C_{\alpha 2}$ probe (Fig. 4) showed identical numbers of $C_{\alpha 1}$ and $C_{\alpha 2}$ genes, the duplication probably does not extend through $C_{\alpha 2}$.

Our analyses of another two IgG1-, two IgG3-, and two IgA1-producing clones from these and other individuals have shown that deletion of genes 5′ to the expressed gene occurs on at least one chromosome in every case.

DISCUSSION

We have established a panel of EBV-transformed cell lines from which we have selected examples synthesizing different C_H isotypes and have analyzed the rearrangements and deletions that occurred in the C_H gene locus. After confirming the clonality of the EBV-transformed cell lines with a panel of monoclonal antibodies specific for isotype, V_H, and idiotype, we tested the clones for the production of any new heavy chain isotype. In the 15 cases studied in detail we found no indication of isotype switching *in vitro*, even after serial subclonings.

The identification of the IgG and IgA subclasses produced by our cell lines has allowed us to determine the extent of the deletions relative to the rearranged C_H gene on the productive allele. In most of the IgG and IgA clones examined (9/10), rearrangement and deletion appear to have occurred on both chromosomes. However, the data illustrate why there has been so much controversy in this area. Even among clones from the same individual we have found differences in the extent of the deletions occurring on the nonfunctional chromosome—i.e., deletion occurred on only one chromosome in the ME IgG1 line, whereas deletion has occurred on both chromosomes in the ME IgA1 line. There is also no strict correlation between the extent of the deletions on the nonfunctional chromosome and the expressed isotype, since one IgG1 line shows deletions on the nonfunctional chromosome that extend through C_H genes 3′ to the gene expressed on the functional allele. If the same switch mechanisms govern the DNA deletions and rearrangements on both chromosomes, then our data indicate a lack of isotype specificity in the actual recombination process.

The IgG4 line is especially interesting in that it has deleted C_μ and has retained $C_{\psi\gamma}$ and $C_{\gamma 2}$, both of which are between C_μ and $C_{\gamma 4}$ in the germ line. In addition, it appears to have duplicated at least a portion of the C_H locus containing $C_{\gamma 4}$, $C_{\gamma 2}$, $C_{\psi\gamma}$, and $C_{\alpha 1}$. Neither reintegration of 5′ C_H genes nor a simple sister chromatid exchange model will explain the apparent existence of three $C_{\gamma 4}$ genes within the line. Unfortunately, the extremely low frequency of IgG4, IgG2, and IgA2 cells in EBV-transformed cell cultures (33) makes it difficult to determine whether the unusual rearrangements occurring within this cell line are the result of a relatively rare event or whether they may occur more frequently among C_H genes that are more distal to the *V–D–J* exon (D = diversity).

Our previous studies suggested that direct, rather than sequential, isotype switches from IgM are the rule for normal B cells (34–36). Deletions of C_H genes 5′ to the expressed gene occur frequently within the lines in the present study, with deletion of C_μ, $C_{\gamma 3}$, and $C_{\gamma 1}$ occurring more often than deletions of the more 3′ C_H genes. Others have interpreted similar findings to indicate that switching occurs by the orderly, sequential 5′ to 3′ deletion of these genes (18). However, we do not believe that our data indicate sequential switching within the C_H locus. It is more likely that the infrequent occurrence of deletions of C_H genes 3′ to $C_{\gamma 1}$ in these lines reflects the low numbers of IgG4- and IgG2-producing cells present in EBV-transformed cultures (refs. 33, 35, 37; unpublished results), even though IgG2 is a major isotype on peripheral blood B cells.

It is possible that switching could occur by more than one mechanism. We cannot assess the contribution of intrachromosomal recombination versus unequal sister chromatid exchange in the deletions seen in these lines, since sister chromatids assort into separate cells at cell division and

antibody-secreting clones of cells were selected during subcloning. The ability to obtain cells producing each C_H isotype by EBV transformation of circulating B cells and the lack of spontaneous switching within these lines suggest that EBV transformation immortalizes cells that have already switched and that maintain their normal C_H rearrangements. This suggests that the population of switched cells transformable by EBV does not produce C_γ or C_α isotypes by an RNA processing mechanism, but rather by recombination and deletion of C_H genes. Thus, our data do not directly contribute to the resolution of the present controversy (10–12, 32, 38, 39) concerning whether or not RNA processing could be involved in cells representative of earlier stages in B-cell differentiation.

Our results clearly indicate that the recombination events that occur in conjunction with isotype switching are not restricted to the genes encoding the antibody isotype to which the cell will switch on the single functional chromosome. This is reflected by the diversity of rearrangements and deletions that have occurred on the nonfunctional chromosome. Although the ability to switch to the production of antibodies of every isotype is a biological advantage, the imprecision with which rearrangements occur may contribute to aberrant recombinations such as the translocations that are observed in many B-lymphocyte malignancies.

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