

Switch Circles from IL-4-Directed ϵ Class Switching from Human B Lymphocytes

Evidence for Direct, Sequential, and Multiple Step Sequential Switch from μ to ϵ Ig Heavy Chain Gene¹

Ke Zhang,^{2*} Frederick C. Mills,[†] and Andrew Saxon*

*The Hart and Louise Lyon Laboratory, Division of Clinical Immunology/Allergy, Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024-1680; and the [†]Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892

Ig isotype switch via deletional recombination is accompanied by excision of the intervening DNA between the two switch regions. The excised DNA is looped out as extrachromosomal circular DNA or switch circle. Such switch circles have been isolated and characterized in mice. We investigated deletional recombination in human B cells undergoing Ig isotype switching to demonstrate whether switch circles are also excised, and to thereby gain insight into the processes involved in human isotype switching. We characterized the deleted switch circular DNA from IL-4 directed μ to ϵ switching in polyclonal human B lymphocytes. By using two sets of specially designed PCR primers, we amplified switch circle fragments representing switch circles resulting from μ to ϵ direct switching and μ - γ - ϵ sequential switching. The PCR-amplified products were subcloned by a TA cloning strategy and resulting clones were screened by hybridization with a 5' S ϵ probe. Sequence analysis of the positive clones revealed that all clones representing μ to ϵ direct switching indeed had 5' S ϵ directly joined to 3' S μ . Most clones representing μ - γ - ϵ sequential switching showed 5' S ϵ joined to 3' S γ as expected. However, two clones contained S μ and S α 1 sequences interposed between 5' S ϵ and 3' S γ , respectively. These data demonstrate that switch circles are excised during human B cell isotype switching, and that IL-4 directed ϵ class switching is accomplished by 1) direct μ to ϵ switching, 2) sequential μ - γ - ϵ switching, and 3) double sequential μ - α - γ - ϵ switching. *Journal of Immunology*, 1994, 152: 3427.

Ig isotype switching is an important process whereby B cells generate different types of effector Ab molecules without changing Ag specificity (1, 2). As B lymphocytes differentiate into memory or plasma cells, μ heavy chain gene expression may change to expression of other isotypes, a process called Ig isotype switching (2, 3). DNA rearrangement between two different isotype

switch regions (S)³ has been found in switched myelomas, lymphomas, and hybridomas as well as induced fresh B lymphoblastoid cells (4–9), indicating that deletional recombination of the Ig C_H genes is a mechanism for isotype switching. This deletional rearrangement, called S-S recombination, joins the μ S with a downstream S to create a new transcriptional unit encompassing VDJ sequences (originally expressed with C μ) and the targeted downstream C_H gene (3). According to the proposed mechanism for deletional recombination, intervening DNA between two recombining S should loop out and ligate through its ends to form an extrachromosomal circular DNA molecule or Ig switch circle (10, 11). Switch circles would not be created by sister chromosome exchange, an alternate mechanism for switch recombination (12).

Received for publication November 2, 1993. Accepted for publication January 10, 1994.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by U.S. Public Health Service Grants AI-15251, AI-34567, and CA-12800 from the National Institutes of Health, a grant from the Allergy Research Foundation, and gifts from the Dorothy Foundation and Robert and Sara LeBien.

² Address correspondence and reprint requests to Dr. Ke Zhang, Division of Clinical Immunology/Allergy, Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024-1680.

³ Abbreviations used in this paper: S, switch region.

Isolation and characterization of Ig switch circular DNA from mouse B lymphocytes during cytokine-directed isotype switch demonstrated directly that deletional recombination occurs in mice (13–17). Switching to IgE (18, 19), the Ig class that mediates immediate hypersensitivity (allergic) diseases (20), is induced by the cytokine IL-4. In mice, analysis of Ig switch circles revealed that sequential switching from μ to ϵ via $\gamma 1$ predominated in B cells treated with IL-4 (16). For human IgE switching, analysis of only chromosomal (as opposed to switch circular) recombination products has been published, with both $S\mu$ - $S\epsilon$ and $S\mu$ - $S\gamma$ - $S\epsilon$ composite S being observed (6–9). Recombination between chromosomal $S\mu/S\gamma$ S and $S\epsilon$ may completely eliminate $S\gamma$ sequences between $S\mu$ and $S\epsilon$, creating composite $S\mu/S\epsilon$ S indistinguishable from chromosomal S arising from direct μ - ϵ switching. Therefore, one cannot estimate the frequency of direct μ - ϵ vs sequential μ - γ - ϵ switching in humans from published data on chromosomal recombination products. However, switch circles resulting from direct μ - ϵ switching will always have a different structure from circles created by sequential μ - γ - ϵ switching (Fig. 1) and thus can in principle be used to assess the frequency of direct and sequential IgE switching.

For these reasons we sought to 1) determine whether switch circular DNAs are excised via looping out and deletional recombination from human B cells undergoing isotype switching and 2) provide incontrovertible evidence of direct and sequential switching from μ to ϵ in cytokine-induced human B lymphocytes. We used PCR to amplify and analyze the S fragments from switch circles derived from human B lymphocytes stimulated by IL-4 and CD40 mAb (21–23). Our results demonstrate that isotype switch to the ϵ locus can be achieved by direct switch from μ to ϵ , sequential switching from μ to ϵ via γ , and triple step sequential switching from μ to ϵ via an α and γ gene.

Materials and Methods

Cells and cell cultures

Human B lymphocytes were purified from normal PBMC by the AET-rosette method (24). For induction of ϵ switch, purified B cells (1×10^6 cells/ml) were cultured in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 2 mM glutamine, 50 U/ml of penicillin, and 50 μ g/ml of streptomycin in the presence of 200 U/ml of IL-4 (a gift from Dr. E. Medlock, Amgen, Thousand Oak, CA) and 0.1 μ g/ml of CD40 mAb G28-5 (a gift from Dr. E. A. Clark, University of Washington, Seattle, WA) (22). One set of cultures was incubated for 5 days before preparation and analysis of circular DNA, whereas another set of cultures was incubated for 14 days for measurement of IgE production. In the stimulated cultures on day 14, IgE was 48.5 ng/ml, whereas control cultures IgE was below 0.2 ng/ml.

Preparation of circular DNA fractions

Circular DNA fractions were prepared from stimulated B cells as described (25) with minor modification. The 5×10^6 cells from stimulated or control cultures were lysed with alkaline-SDS buffer (50 mM NaCl, 2 mM EDTA, 1% SDS, pH 12.4). The cell lysates were vortexed for 5 min and then incubated at 30°C for 1 h. The final lysates were neutralized with 0.05 vol of 1 M Tris-HCl (pH 7.1) and then with 0.1 vol of 5 M NaCl followed by treatment with proteinase K (100 μ g/ml in final) for 30 min at 37°C. After phenol extraction, the circular DNA-containing aque-

ous phase was precipitated with 2.5 vol of absolute ethanol. The precipitated DNA was digested with *Eco*RI to linearize circular DNAs and with RNase to remove RNA for 1 h at 37°C (Preliminary experiments showed that linearization of circular DNAs yielded better amplification results compared with uncut supercoiled DNA. Because the $S\mu$, $S\gamma 4$, and $S\epsilon$ regions do not contain *Eco*RI sites, digestion of circular DNAs would not destroy the switch fragments.) After *Eco*RI linearization, the circular DNA-enriched portion was used as PCR templates for amplification of switch circle fragments without further treatment.

PCR strategy

PCR was performed in a 25 μ l vol/reaction with 10% DMSO, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 50 pM primer/reaction, and 2.5 U of Taq polymerase. An amount of circular fraction DNA derived from 1×10^6 cultured cells was used as the DNA template for each reaction. The first cycle of PCR was conducted at 94°C for 10 min (without Taq polymerase) for complete DNA denaturation, 65°C for 10 min for annealing (Taq polymerase was added to the tube at this step), and 72°C for 10 min for extension. PCR was continued in 94°C for 1 min, 65°C for 1 min, and 72°C for 5 min for 40 cycles with 15-min extension in final cycle.

A second round of PCR using nested primers was run on the DNA templates derived from the first PCR reaction with the same conditions as the first round PCR except for the changes in the primers. The first round PCR products (2 μ l) from primer set 2 and 5 (μ and ϵ) were reamplified by primer set 1 and 6 and the products (2 μ l) from primer set 4 and 5 ($\gamma 4$ and ϵ) were reamplified by primer set 3 and 6 (Fig. 1). A second round of amplifications were conducted at 94°C for 1 min, 68°C for 1 min, and 72°C for 3 min for 40 cycles with a 15-min extension time for final cycle.

The primer sequences are shown in the following list:

1. 5' ATTGGCCAGTGCCATGCTCTCCAGTTCAT3', complementary to position 3738–3710 of $S\mu$ sequence.
2. 5' CCATCTATGTCCAACAAGATCATGA3', complementary to position 3766–3742 of $S\mu$.
3. 5' CTCTGGCCATCGGTGCCACCTCA3', complementary to 4620–4598 of $S\gamma 4$.
4. 5' AGTCAGCACAGTCCAGGCGCTCTA3', complementary to position 4656–4633 of $S\gamma 4$.
5. 5' AGGCTCCACTGCCGGCACAGAAAT3', corresponding to position 542–566 of $S\epsilon$.
6. 5' TGTCCCTTAGAGGACAGGTGGCCAAG3', corresponding to position 1156–1181 of $S\epsilon$ (26).

Cloning and screening

The final PCR products were cloned by the TA cloning method as provided by the manufacturer (Invitrogen, San Diego, CA). The TA cloning strategy for direct cloning of PCR products is based on the characteristic of Taq polymerase to incorporate an extra A nucleotide to the 3' end of the newly synthesized DNA chain in a DNA template-independent manner (27). PCR-generated DNA products thus frequently have 3' protruding ends with one A nucleotide in both DNA chains. Therefore, a PCR cloning vector with T nucleotide protruding ends (complementary to A nucleotide) was created to facilitate the direct cloning of PCR products. This cloning procedure, called TA cloning, does not require any modifications or further treatment of PCR products before cloning. Our comparison of this method with other cloning procedures for PCR products (i.e., blunt end ligation) revealed that the TA cloning always gave much higher cloning efficiency and also was simple and very easy to manipulate. PCR products were precipitated and reconstituted in H_2O . One of five of the PCR products was ligated to the commercially prepared vector, and the ligated materials were transformed into competent cells. The clones that contained inserts (white clones) were selected for miniprep and digested with *Eco*RI. After electrophoresis, the clones were transferred to nylon membrane and screened by hybridization with a PCR-generated 257-bp probe that is specific for 5' $S\epsilon$ sequence (P5' $S\epsilon$). P5' $S\epsilon$ was amplified by using primer 6 and a primer that is complementary to 1413–1390 of the germ line $S\epsilon$ sequence, 5' CAGCTTAACCAATCTGGACCACC3' (26). Hybridization was conducted according to the standard procedures except that the hybridization and blot washing were performed at 70°C (28). The clones that hybridized with P5' $S\epsilon$ were subjected to DNA sequence analysis.

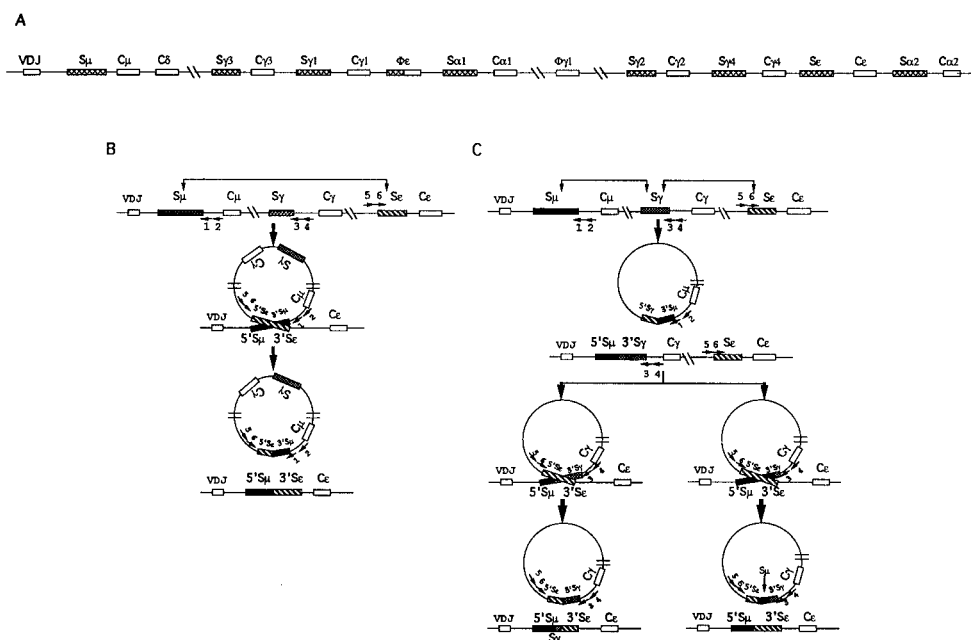


FIGURE 1. Schematic diagram showing the formation of switch circular DNA (switch circles) and our PCR primer position strategy for detection of switch fragments contained within such switch circles. **A:** Diagrammatic map of the C_{H1} genes of the human IgH gene cluster. **B:** Diagram of the occurrence and detection of μ to ϵ direct switch. A map of disrupted human Ig C_{H1} chain region genes that relate to this study is shown at the top. The S are indicated by shaded boxes and the constant region are indicated by an open box. The numbered arrows indicate the position and the orientation of the PCR primers, arrows above the gene map are sense sequences, and those below the map are antisense sequences. For μ to ϵ direct switching, after looping out and deletion, a hybrid S-S region with 5' S_{μ} -3' S_{ϵ} would be generated on the chromosome as shown in the linear diagram at the bottom. The excised DNA should join their two ends to form a switch circular DNA with 5' S_{ϵ} -3' S_{μ} sequence as shown in the circle. Primer sets 2 and 5 would amplify the switch fragments from switch circles if the deleted S-S regions indeed are joined to form a circular DNA. The primer sets 1 and 6 was used for the second round PCR reactions to increase the sensitivity and specificity of the procedure. **C:** Diagram of the occurrence and detection of μ to ϵ sequential switch via γ . The primers 4 and 5 are used for the first round PCR and the primer sets 3 and 6 for second round PCR for amplification of γ to ϵ switching. The μ to γ switch would generate a S_{μ} - S_{γ} switch circle as shown and a 5' S_{μ} -3' S_{γ} sequence would be generated on the chromosome as shown in the linear diagram. Subsequent γ to ϵ switch could generate two types of switch circles depending on where the recombination sites occur. A switch circle that has 5' S_{ϵ} directly joined to 3' S_{γ} would be generated when the switch takes place between the S_{γ} part of the hybrid 5' S_{μ} -3' S_{γ} region and S_{ϵ} as shown in the left hand side of **B**. The chromosome would retain some S_{γ} sequence as shown. A second type of switch circle, with S_{μ} sequences interposed between the 5' S_{ϵ} and 3' S_{γ} sequences, would result when switch recombination occurs between the remaining 5' S_{μ} part of the hybrid 5' S_{μ} -3' S_{γ} S and S_{ϵ} . This is shown on the right hand side of **B**. In this case, the retained chromosomal S-S region will have 5' S_{μ} joined to 3' S_{ϵ} .

DNA sequencing

Nucleotide sequences of the inserts were determined by the standard dideoxy chain termination method using a kit purchased from USB (USB, Cleveland, OH). Sp6 and T7 primers and PCR primers and several synthesized oligonucleotides were used as sequencing primers. The synthesized sequencing primers were: TTATCTGGGCAGGCAAAG (18 mer, 1346–1363 S_{ϵ}), TATTACCTGCTGAGTTACCT (20 mer, 1541–1560 S_{ϵ}), TCTTCTGTGCAGCAGAGGGG (20 mer, complementary to 4388–4369 $S_{\gamma 4}$), and TTTGGCTCAACCCAGCTTGG (20 mer, complementary to 3324–3305 S_{μ}) (26).

Results

PCR strategy for detection of specific switch circular DNA

During deletional recombination, the 5' part of the upstream S sequence and 3' part of the targeted S region

sequences join together on the chromosome to form a hybrid S. The 3' portion of the upstream S region sequence, the 5' sequences of the targeted S region, and all the intervening sequence should be looped out in those B lymphocytes undergoing isotype switch. We used PCR-based methods to assay for production of IgE switch circles in human IgE switching. As depicted in Figure 1, if deleted DNA forms a circular DNA by joining the 5' switch section of a downstream isotype to 3' switch sequences of a more proximal isotype, as demonstrated in mice (13–17), then the orientation of the PCR primer pairs 2 (antisense sequences) and 5 (sense sequences) should allow amplification of switch circle sequences formed by S_{μ} to S_{ϵ} direct switching (Fig. 1B). Primer pairs 1 and 6 were used

for second round PCR to increase the sensitivity and specificity of first round PCR. Switch circular DNA formed by μ to ϵ sequential switch via any intervening Ig C_H would not be amplified by this set of primers. This follows because the 3' part of S μ sequences where primer 2 and 1 are located would be part of a switch circle containing the 5' switch region of the intervening isotype (rather than ϵ) to which μ had initially switched (Fig. 1C, top switch circle).

Similarly, primer sets 4 and 5, with primer sets 3 and 6 used for second round amplification, were designed to detect only switch circles representing γ to ϵ switch events (Fig. 1C). Although primers 4 and 3 are derived from the published sequence of the γ 4 locus (26), the four human γ genes (γ 3, γ 1, γ 2, and γ 4) are more than 97% homologous to each other in this region (F. C. Mills and E. E. Max, unpublished observations). Therefore, it is likely that μ - γ - ϵ switch circles derived from any of the four γ genes would be amplified by these primer sets. Such γ - ϵ switching must follow an earlier direct or sequential switch from μ to one or more γ gene(s). Primer pairs 4 and 5 and 3 to 6 would not detect μ to ϵ direct switching because the 3' part of S μ would have been looped out and deleted during the prior switch recombinations between S μ and S γ .

The orientation of the primers ensures that the amplified switch fragments are from the excised circular DNA and not from the rearranged genomic DNA. Thus, these approaches enabled us to test the hypothesis that deleted intervening DNA excised indeed forms switch circular DNA, and to demonstrate in humans that both direct and sequential switch mechanisms are involved in μ to ϵ switch.

Amplification of the DNA switch circle fragments from IL-4 plus CD40 mAb-stimulated B cell is shown in Figure 2A. Multiple bands are visualized when amplified with the primer set for μ to ϵ direct switch (labeled p1 and 6) and the primer set for γ to ϵ sequential switch (labeled p3 and 6). No PCR bands were seen with unstimulated B cells (B+O). The multiple bands were consistent with the prediction that breakpoints for switch recombination would be scattered over both S regions involved in the joining (13). An example of cloning of the PCR products is shown in the Figure 2B where 17 randomly selected individual subclones from the minipreps are shown. Subclones that hybridized with the 5' S ϵ -specific probe (P5'S ϵ) in Figure 2C indicated that these subclones contained S ϵ sequences of interest. The various sizes of the inserts implies that the subclones represent switch sequences from different switch circles.

Direct switch from μ to ϵ

To demonstrate that those clones hybridizing with P5'S ϵ truly represent the switch circle fragments from switch circular DNAs, determination of their exact nucleotide sequences and identification of the recombination breakpoints between the isotype switch segments was accom-

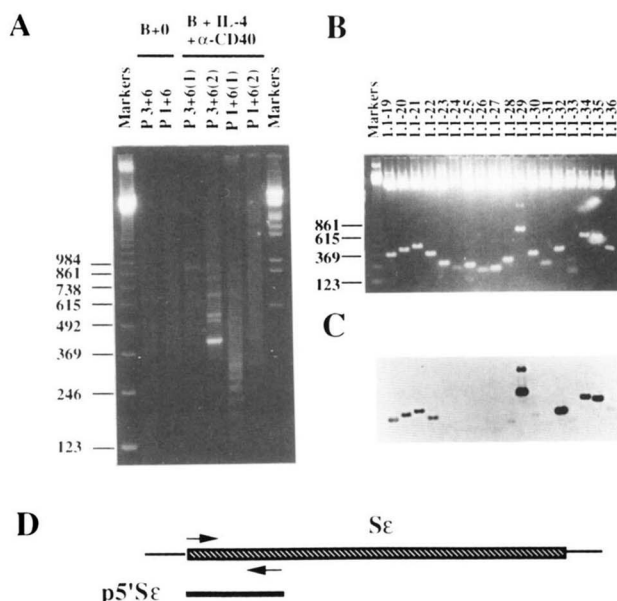
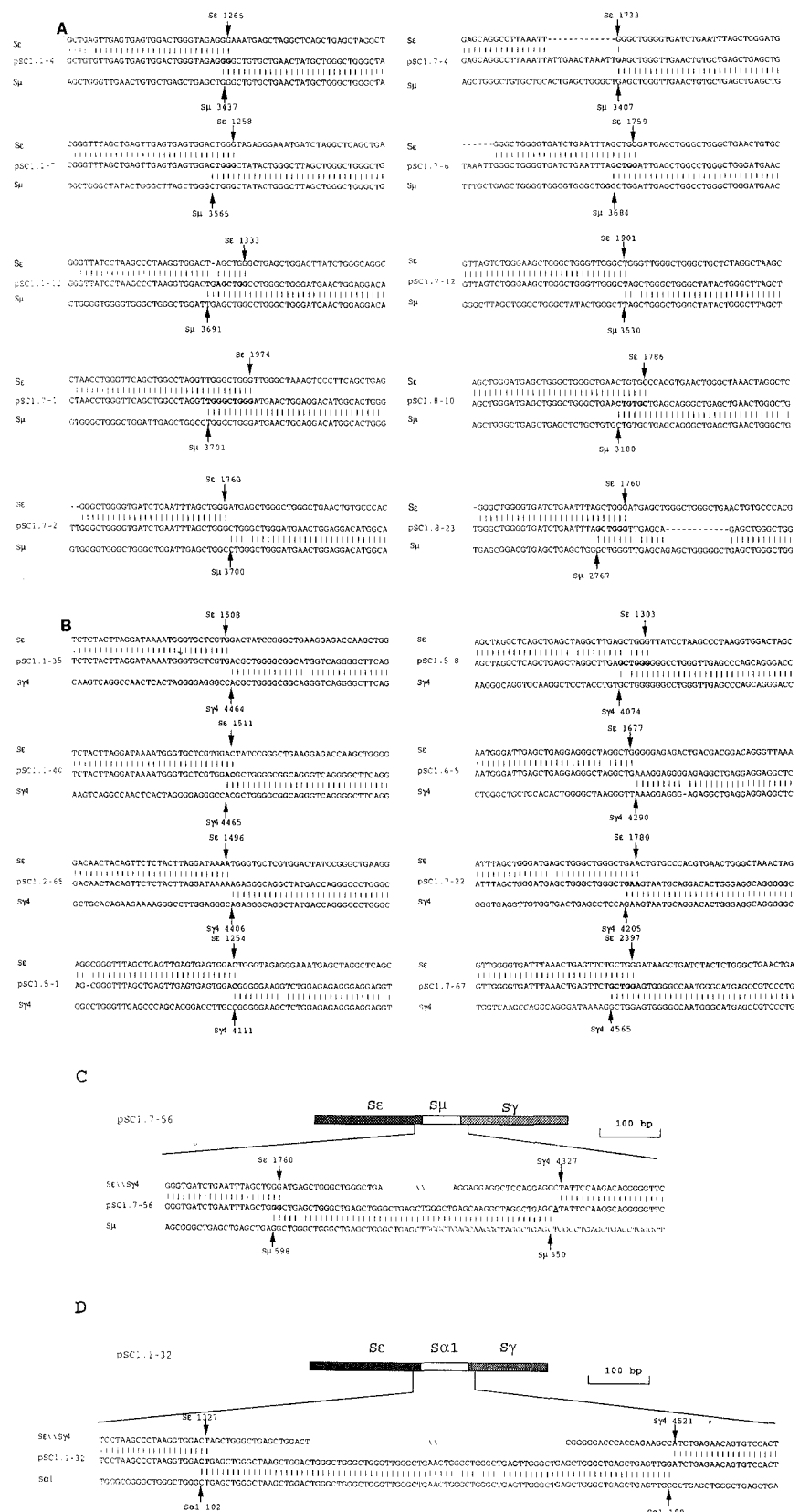


FIGURE 2. Detection and cloning of PCR-amplified recombinant S from switch circles containing S ϵ sequences. **A:** Gel electrophoresis of PCR-amplified switch fragments from switch circles using μ - ϵ primers (P1+6) or γ - ϵ primers (P3+6). Multiple DNA bands of differing sizes can be seen with the stimulated cells. DNA from control (B+O) and IL-4 plus CD40 mAb-stimulated B cells (B+IL-4+ α CD40) served as templates for amplification of switch fragments by different primer sets (P). Two independent amplifications (indicated with the numbers in the parenthesis) were performed with the induced B cells with each set of primers. **B:** Subcloning of the PCR products. The 5 μ l of the second round PCR products (shown in A) were ligated into the TA cloning vector as described in *Materials and Methods*. Clones with inserts were randomly picked up for miniprep analysis. The different sized inserts derived from these clones are shown. **C:** Southern blot analysis of the isolated subclones showing that contain 5' S ϵ sequence. The subclones from B were hybridized with a 257-bp PCR-generated probe representing 5' S ϵ sequences as shown in D. The strongly hybridizing or positive clones (clones 19-22, 29, 32, 34, and 35 were considered positive) were subjected to nucleotide sequencing analysis because they probably were derived from switch circles of interest. Some of the clones (i.e., 1.1-23, 25, 30, and 36) that were considered negative were also sequenced. The results indicated that all the negative clones were PCR-generated nonspecific amplification products. The weak hybridization signals observed are probably due to the probe (P5'S ϵ) hybridizing to primer sequences in these clones because these clones contained the sequences of primer 6, which had also been used for generation of the probe by PCR amplification (see *Materials and Methods*). **D:** Diagram for PCR-generated probe P5'S ϵ that was used for Southern blot in 2C. A 257-bp fragment was amplified by PCR (see *Materials and Methods*). This fragment was cloned and the sequences was confirmed by nucleotide sequencing.

plished. Sequencing of 10 clones reactive with P5'S ϵ and derived from primer pair 1 and 6 amplification revealed that all these clones had 5' S ϵ sequences directly joined to 3' S μ sequences in a 5'-3' orientation. As shown in Figure 3A, the

FIGURE 3. Nucleotide sequences surrounding breakpoints of switch fragments derived from switch circles. The clones from μ to ϵ direct switch and μ to ϵ sequential switch via γ were analyzed by nucleotide sequencing. The arrows indicate the switch recombination breakpoint sites. The vertical lines indicate the identical nucleotide bases. Switch circle sequences at the breakpoint with homology to both germ line sequences are indicated by the bold letters because it is not possible to assign the homologous sequence to one donor germ line S region. The dashed horizontal lines represent nucleotides absent from either switch circles or from the published germ line sequences. The nongerm line nucleotide insert at the breakpoint in pSC1.7-56 is underlined. **A:** Nucleotide sequences of the recombination sites for the clones derived from μ to ϵ direct switch circles. The nucleotide sequences around the breakpoints from 10 clones were shown. The breakpoints of the switch recombination sites were precisely defined by comparing with the germ line $S\epsilon$ and $S\mu$ region sequences. **B:** Nucleotide sequences around the breakpoints of eight clones from γ to ϵ sequential switch were shown. The breakpoints were defined by alignment of these clones with published germ-line $S\epsilon$ and $S\gamma$ S sequences. **C:** The nucleotide sequences across the $S\epsilon$ - $S\mu$ and $S\mu$ - $S\gamma$ junctions in switch circle clone pSC1.7-56 showing its derivation from $S\epsilon$, $S\mu$, and $S\gamma$. The recombination breakpoints shown were determined by alignment of the clone with germ-line $S\epsilon$, $S\mu$, and $S\gamma$ 4. **D:** The nucleotide sequences across the $S\epsilon$ - $S\alpha 1$ and $S\alpha 1$ - $S\gamma$ 4 (or $S\gamma$ 2) junctions in clone pSC1.1-32 showing its derivation from $S\epsilon$, $S\alpha 1$, and $S\gamma$ 4 (or $S\gamma$ 2). The breakpoints for $S\epsilon$, $S\alpha 1$, and $S\gamma$ 4 were defined by alignment of the clone with germ-line $S\epsilon$, $S\alpha 1$, and $S\gamma$ 4 sequences. The 87-bp sequence interposed between $S\epsilon$ and $S\gamma$ 4 (or $S\gamma$ 2) has complete homology with the stretch of $S\alpha 1$ sequences shown.



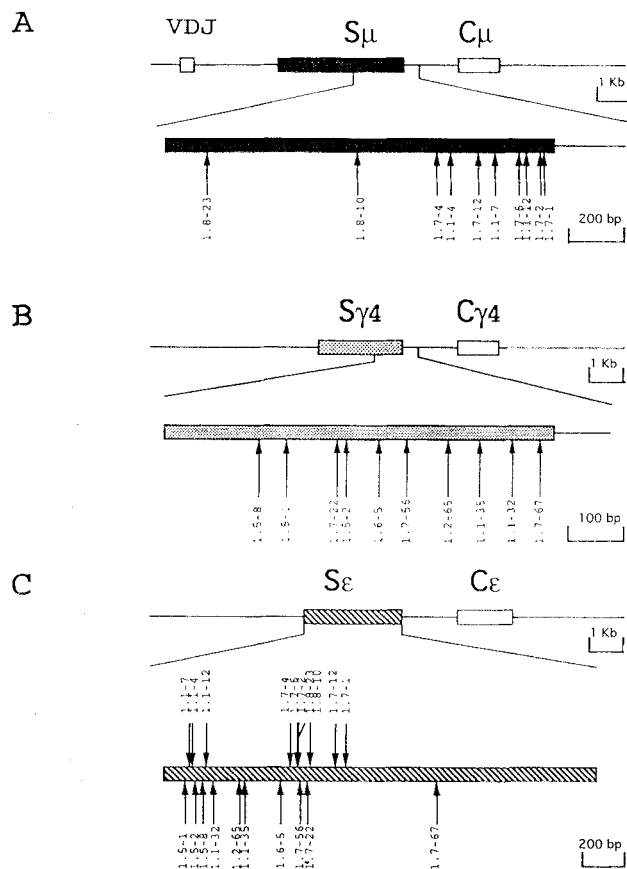


FIGURE 4. Assignment of the position of recombination breakpoints defined from the switch circles in the various S regions. The breakpoints in S_μ and S_ϵ from the μ to ϵ direct switch circle clones are indicated in the S_μ and S_ϵ regions in (A) and (C, upper set of arrows), respectively. The breakpoints of S_γ and S_ϵ from γ to ϵ switch clones are shown in (B) and (C, lower set of arrows), respectively.

recombination breakpoints for each clone are defined by alignment of the switch circle fragments with published germ-line S_ϵ and S_μ region sequences.

The breakpoints of these clones were assigned to positions within the germ-line S_μ and S_ϵ regions as shown in Figure 4A and C. Of the 10 clones, the homology between the two germ-line S sequences at the recombination site was only nine bases for one clone (pSC1.7-1), eight bases for one clone (pSC1.1-12), six bases for two clones (pSC1.8-10 and 1.8-23), five bases for two clones (pSC 1.1-7 and pSC 1.7-6), three bases for one clone (pSC1.1-4), one base for two clones (pSC1.7-4 and pSC1.7-12), and none for one clone (pSC1.7-2) (Fig. 3A). These observations, along with the data from the breakpoints of γ to ϵ sequential switching shown in the next section where non-homologous junction was also observed in some of the S_ϵ/S_γ switching recombination junction sites (Fig. 3B), are consistent with the idea that DNA recombination between two S regions is not mediated by the homologous recombination (4).

In clone pSC1.7-4 there was an additional 13-bp sequence, ATTGAATAAATT, between 1732 and 1733 of germ line S_ϵ , not present in the published germ line S_ϵ sequences. It is unlikely that this 13-bp fragment is due to an insertion induced by IL-4 and CD40 stimulation because the identical structure was present in all the isolated clones that go through this region (i.e., in pSC1.7-1, 1.7-2, 1.7-6, 1.7-12, 1.7-22, 1.7-67, 1.8-10, and 1.8-23), and the 13-bp fragment also existed in a switch circle isolated from another donor (data not shown). Therefore, it is most likely that this 13-bp stretch had been deleted from the clone that was served as DNA template for S_ϵ germline sequences published. Sequencing analysis also showed that there was a extra 30 bp of sequence (GCTAGGCT GGGCTGAGCTGGTCTGAGCTCA) between 3628 and 3629 of the published germline S_μ sequence (in pSC1.1-4, 1.1-7, 1.7-4, and 1.7-12). The clone pSC1.8-23 had a 12-bp deletion between 2779 and 2792 of S_μ (Fig. 3B). In addition, the sequences from switch circular DNAs also showed several minor point mutations, insertions, and deletions scattered in both S_μ and S_ϵ regions from the germ-line S_μ and S_ϵ sequences that could be due to polymorphisms, PCR-introduced mutations, and/or sequencing error.

Sequential switch from μ to ϵ via γ

The nucleotide sequence determination of 10 clones resulting from the primer set 3 and 6 and reacting with the P5'/ S_ϵ revealed that eight clones had 5' S_ϵ directly joined to 3' S_γ sequences in the predicted 5'-3' orientation. The S_ϵ partial sequences from all the sequenced clones shared more than 99% homology with the published S_ϵ germline sequence except for the 13-bp insert mentioned above. The S_γ partial sequences of the clones showed at least 97% homology with the published germ-line S_γ sequence (26), other than a 22-bp segment (GGGCTGAAGG GCGCCGACCCT) inserted at a position corresponding to coordinates 4355-4356 in the γ 4 locus. (Reexamination of the original γ 4 sequencing data used in reference 26 confirms the absence of the 22-bp segment in γ 4; however, this segment is found in the sequences of germ lines γ 1, γ 2, and γ 3 (F. C. Mills and E. E. Max, unpublished observations)).

It is not possible to rigorously define the S_γ origin of these sequences due to the high homology between the human γ genes; however, sequence comparisons using slight differences between the γ loci suggest that these clones arise from more than one γ gene (F. C. Mills and E. E. Max, unpublished observations). The sequences surrounding the recombination breakpoints defined by aligning the sequences of these clones with the germ-line S_ϵ and S_γ sequences are represented in Figure 3B. Determination of the sequences and alignment of them with germ line S_ϵ and S_γ sequences enable us to assign the breakpoints in germ line S_γ and S_ϵ sequences as indicated in Figure 4B and C.

One clone, pSC1.7-56, had 52 bp of sequence interposed between the $S\epsilon$ and $S\gamma$ sequences (Fig. 3C). All but one base of this sequence is homologous (98%) with a stretch of published germ-line $S\mu$ sequence, indicating that the interposed sequence originated from $S\mu$. This interposed $S\mu$ sequence is direct evidence for μ to ϵ sequential switch via γ gene as shown in Figure 1B. The origin of the extra 1 base located between at $S\mu$ and $S\gamma$ breakpoint is unclear. It could be due to a polymorphism, PCR-introduced mutation, a sequencing error, or an insertion of non-germ-line sequence by an error-prone DNA synthesis mechanism during the process of $S\mu$ to $S\gamma$ switch recombination (4).

Triple step sequential switch to ϵ via α and γ

One clone, pSC1.1-32, isolated from PCR products amplified by the γ - ϵ primer pairs contained a revealing 87-bp fragment interposed between the 5' $S\epsilon$ and 3' $S\gamma$ sequences (Fig. 3D). This fragment was 100% homologous with a stretch of $S\alpha 1$ sequence (F. Mills and E. E. Max, unpublished data). The placement of $S\alpha 1$ sequence in this orientation between 5' $S\epsilon$ and 3' $S\gamma$ segments is most readily interpreted as a evidence for a preceding recombination between $S\mu$ and $S\alpha 1$ before the switching to $\gamma 4$ or $\gamma 2$. Thus, at least a triple step sequential switch model would be invoked to explain generation of the switch circle that gave rise to this clone, i.e., from μ to $\alpha 1$ for the first step, from $\alpha 1$ to $\gamma 4$ or $\gamma 2$ for the second step, and from $\gamma 4$ or $\gamma 2$ to ϵ for the third step (Fig. 5).

Discussion

The PCR-based method for analysis of human switch circles described in this report provides an important approach for the investigation of the mechanisms of isotype switching. This is especially so for studying those low frequency switch events with relevant numbers of cells used such as polyclonal or Ag-specific activation of fresh human B cells, a situation where the classical approach for isolation and characterization of circular DNA cannot be expected to give satisfactory results. Proper selection of primers based on their predicted orientation in switch circular DNA enabled us to identify and study in a sensitive manner those specific switch events of interest (i.e., μ to ϵ or γ to ϵ) without being hindered by other types of switch circles or the S sequences in the retained genomic DNA. In addition, this analysis of the switch circles provides conclusive insights about events that cannot be directly revealed by the analysis of the retained switch junctions on chromosomal DNA.

Our findings definitively show that looping out and deletional recombination is one of the mechanisms by which human B lymphocytes achieve class switch from μ to ϵ . The simplest explanation for our PCR strategy's detection of switch fragments and their orientation is that the intervening DNA between two S is reciprocally looped out

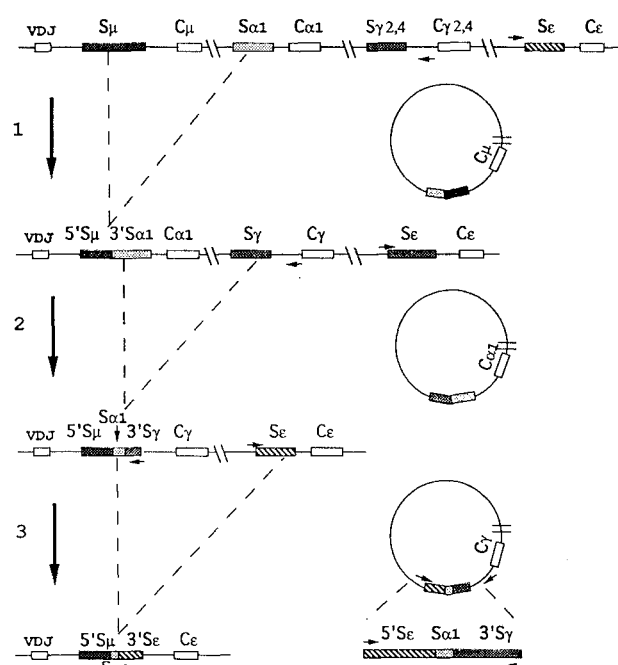


FIGURE 5. Schematic diagram of triple step sequential switch found in clone pSC1.1-32. The initial switch 1) between $S\mu$ and $S\alpha 1$ generated a hybrid S region with a 5'S μ -3'S $\alpha 1$ chromosomal junction. The second switch 2) occurred between the $S\alpha 1$ portion of the hybrid 5'S μ -3'S $\alpha 1$ region and $S\gamma 4$ or $S\gamma 2$, leading to formation of a complex 5' $S\mu$ - $S\alpha 1$ -3'(S $\gamma 4$ or S $\gamma 2$) chromosomal S. To generate the sequence found in clone pSC1.1-32, a switch event must have taken place between the $S\alpha 1$ sequences in the complex $S\mu$ - $S\alpha 1$ -(S $\gamma 4$ or S $\gamma 2$) region and $S\epsilon$. This led a switch circle containing 5'S ϵ - $S\alpha 1$ -3'(S $\gamma 4$ or S $\gamma 2$) sequences being excised as shown in the circle at the bottom right. Reciprocally, this switching step would create a chromosomal S containing 5'S μ - $S\alpha 1$ -3'S ϵ (bottom left) that allows for ϵ gene expression.

from the chromosome and deleted as an extrachromosomal switch circle. It remains to be conclusively shown that switch recombination is indeed reciprocal because we have not demonstrated the reciprocal recombination on the chromosome.

To gain insight into the processes involved in human Ig isotype switching, we have amplified and analyzed switch circles derived from IL-4 directed μ to ϵ switching. Our detection of direct 5' $S\epsilon$ -3' $S\mu$ junctions in the switch circles conclusively demonstrates that switching from μ to downstream isotypes such as ϵ can be achieved by direct switching in stimulated primary human B cells. Sequential switching to ϵ via γ genes between $S\mu$ and $S\epsilon$ also occurs but is not an obligatory step.

The evidence for sequential switching from both rearranged Ig S and switch circles have led to the proposal that class switching to C_H genes distal to $C\mu$ such as to the $C\epsilon$ and $C\alpha$ genes proceeds by obligate switch recombination via an $S\gamma$ intermediate (17). Our switch circle analysis does show that IL-4 directed ϵ switching in humans can be

achieved by sequential switching from μ to ϵ via γ . Most of the γ - ϵ switch circles sequences had 5' S ϵ sequence directly joined to 3' S γ . This situation represents sequential switching events from μ to γ and then to ϵ where γ to ϵ recombination site occurred the 3' S γ part of the hybrid 5' S μ -3' S γ switch regions and 5' S ϵ as indicated on the left side of Figure 1C. Such μ - γ - ϵ switching would result in a retained chromosomal hybrid S composed of 5' S μ - γ -3' S ϵ . Indeed, such μ - γ - ϵ sequential switching has been demonstrated in human IgE-secreting B cell lines and IL-4-induced primary B cells that have composite S with S γ sequences interposed between 5' S μ and 3' S ϵ (8, 9). Sequential μ - γ - ϵ switching is also clearly demonstrated by clone 1.7-56, which contains a stretch of S μ sequences interposed between 5' S ϵ and 3' S γ .

In addition to direct and two step sequential switching from μ to ϵ via γ , multiple step sequential switching to ϵ via more than one intermediate Ig C_H genes between C μ and C ϵ has been shown by the structure of the clone, pSC1.1-32. That clone revealed S α 1 sequences inserted between the 5' S ϵ and 3' S γ sequences. As depicted in Figure 5, generation of such a clone could be properly interpreted by a triple step sequential switching model from μ to ϵ via α 1 and γ 2 or γ 4. Whether such a multiple step sequential switching represents an unusual event in a common pathway to ϵ switching is not clear at this time. It is premature to conclude that triple step sequential switching is a rare event based on only a single clone of this structure being defined. If the frequency of direct or sequential switching were the same as for triple step sequential switching, the chance to detect the latter type of switch circles should be much lower. The switch circles reflecting multiple step sequential switching would be detected only in the case where a portion of the second S (i.e., S α 1) is retained in the chromosome during the next switching recombination (i.e., to S γ 4 or S γ 2) and then both downstream S sequences (S α 1 and S γ 4 or S γ 2) are excised as part of a subsequent switch circle. There are many opportunities for recombinations in multistep sequential switching to remove the switch remnants of earlier S-S joining so that a later switch circle will not contain the elements of earlier switches even though multistep sequential switching has occurred (Fig. 5). Interestingly, the notion of switching from μ to ϵ via α 1 is in agreement with the report that ϵ switching could be frequently induced from IgA1-positive B cells by IL-4 stimulation (29).

Breakpoints from nucleotide-sequenced clones are assigned to the S μ , S γ 4, and S ϵ germ-line sequences as shown in Figure 4. All the S μ and S ϵ recombination sites are located within the typical switch regions, the regions characterized by enrichment of tandemly repeated sequence GGGCT or GAGCT (26). Although recombination breakpoints cluster in the 3' parts of S μ and S γ and cluster in 5' part of S ϵ , one cannot conclude from this data that those breakpoints show a biased distribution, as has been described in LPS/TGF- β -stimulated α switching in mice where the S μ breakpoints

were clustered in the 3' portion (17). Our PCR amplification and cloning methods are not equally effective for amplifying and cloning different size switch circles. Thus, the clones we isolated may reflect those switch circles more readily amplified such that clones with larger sized switch fragments may have been excluded due to the limitations of the methodologies we used.

The discrepancies between the breakpoint distribution of our results and the breakpoint analysis for S μ -S ϵ junctions in chromosomal DNA, where the S μ breakpoints were clustered in the 5' region (6, 7), could also be explained by the methodology used. The relevant smaller switch fragments or regions would be better amplified by PCR in both systems (6, 7; and this report). More likely, the clustering of S μ breakpoints in the 5' region of the retained DNA may well result from possible secondary deletions occurring within the recombined switched sequences. Such secondary deletions have been suggested to account for the observation of the breakpoints clustering toward the 5' end of S μ from isotype-switched myelomas, lymphomas, and hybridomas (4). Excised switch circles are believed not to undergo further replication (13, 17), making the chance of secondary deletion occurring in the switch circles less likely than secondary deletions in retained switched loci on the active chromosome. Thus, breakpoints defined from switch circles should more closely resemble the original recombination sites than breakpoints from the switched chromosomes.

Acknowledgments

We thank Dr. Edward E. Max for sharing the unpublished data of human α 1 S sequences and for helpful discussions and Dr. Randy Wall for critical reading of the manuscript. We also thank Hai-Kit Heish for excellent technical assistance, Dr. Eugene Medlock of Amgen for the gift of human rIL-4, and Dr. Edward A. Clark for anti-CD40 mAb G28-5.

References

1. Wang, A. C., S. K. Wilson, J. E. Hooper, H. H. Fudenberg, and A. Nisonoff. 1970. Evidence for control of synthesis of the variable regions of heavy chains of immunoglobulin chains G and M by the same gene. *Proc. Natl. Acad. Sci. USA* 66:337.
2. Pernis, B., L. Forni, and A. L. Luzzati. 1977. Synthesis of multiple immunoglobulin classes by single lymphocytes. *Cold Spring Harbor Symp. Quant. Biol.* 41:175.
3. Cooper, M. D., J. F. Kearney, P. M. Lydyard, C. E. Grossi, and A. R. Lawton. 1977. Studies of generation of B-cell diversity in mouse, man, and chicken. *Cold Spring Harbor Symp. Quant. Biol.* 41:139.
4. Dunnick, W., G. Z. Hertz, L. Scappino, and C. Gritzmacher. 1993. DNA sequences at immunoglobulin switch region recombination sites. *Nucleic Acids Res.* 21:365.
5. Lutzker, S., and F. Alt. 1989. Immunoglobulin heavy-chain class switching. In *Mobile DNA*. D. E. Berg and M. M. Howe, eds. American Society for Microbiology, Washington, DC, p. 693-714.
6. Shapira, S. K., H. H. Jabara, C. P. Thienes, D. J. Ahern, D. Vercelli, H. J. Gould, and R. S. Geha. 1991. Deletional switch recombination occurs in interleukin-4-induced isotype switching to IgE expression by human B cells. *Proc. Natl. Acad. Sci. USA* 88:7528.
7. Shapira, S. K., D. Vercelli, H. H. Jabara, S. M. Fu, and R. S. Geha. 1992. Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. *J. Exp. Med.* 175:289.

8. Mills, F. C., G. Thyphronitis, F. D. Finkelman, and E. E. Max. 1992. Ig μ - ϵ isotype switch in IL-4 treated human B lymphoblastoid cells: evidence for a sequential switch. *J. Immunol.* 149:1075.
9. Jabara, H. H., R. Loh, N. Ramesh, D. Vercelli, and R. S. Geha. 1993. Sequential switching from μ to ϵ via $\gamma 4$ in human B cells stimulated with IL-4 and hydrocortisone. *J. Immunol.* 151:4528.
10. Honjo, T., and T. Kataoka. 1978. Organization of immunoglobulin heavy chain genes and allelic deletion model. *Proc. Natl. Acad. Sci. USA* 75:2140.
11. Jack, H.-M., M. McDowell, C. M. Steinberg, and M. Wabl. 1988. Looping out and deletion mechanism for the immunoglobulin heavy-chain class switch. *Proc. Natl. Acad. Sci. USA* 85:1581.
12. Obata, M., T. Kataoka, S. Nakai, H. Yamagishi, N. Takahashi, Y. Yamawaki-Kataoka, T. Nikaïdo, A. Shimizu, and T. Honjo. 1981. Structure of a rearranged $\gamma 1$ chain gene and its implication to immunoglobulin class-switch mechanism. *Proc. Natl. Acad. Sci. USA* 78:2437.
13. Von Schwedler, U., H.-M. Jack, and M. Wabl. 1990. Circular DNA is a product of the immunoglobulin class switch rearrangement. *Nature* 345:452.
14. Matsuoka, M., K. Yoshida, T. Maeda, S. Usuda, and H. Sakano. 1990. Formation of switch circular DNA in cytokine-treated mouse splenocytes: evidence for intramolecular DNA deletion in immunoglobulin class switch. *Cell* 62:135.
15. Iwasato, T., A. Shimizu, T. Honjo, and H. Yamagishi. 1990. Circular DNA is excised by immunoglobulin class switch recombination. *Cell* 62:143.
16. Yoshida, K., M. Matsuoka, S. Usuda, A. Mori, K. Ishizaka, and H. Sakano. 1990. Immunoglobulin switch circular DNA in the mouse infected with *Nippostrongylus brasiliensis*: evidence for successive class switching from μ to ϵ via $\gamma 1$. *Proc. Natl. Acad. Sci. USA* 87:7829.
17. Iwasato, T., H. Arakawa, A. Shimizu, T. Honjo, and H. Yamagishi. 1992. Biased distribution of recombination sites within S regions upon immunoglobulin class switch recombination induced by transforming growth factor β and lipopolysaccharide. *J. Exp. Med.* 175:1539.
18. Coffman, R. L., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, and W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.
19. Pene, J., F. Rousset, F. Briere, I. Chretien, J.-Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K.-I. Arai, J. Banchereau, and J. E. DeVries. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E2. *Proc. Natl. Acad. Sci. USA* 85:6880.
20. Ishizaka, K., T. Ishizaka, and M. M. Hornbrook. 1966. Physicochemical properties of human reagenic antibody. V. Presence of a unique immunoglobulin as carrier of reagenic activity. *J. Immunol.* 97:75.
21. Jabara, H. H., S. M. Fu, R. S. Geha, and D. Vercelli. 1990. CD40 and IgE: Synergism between anti-CD40 monoclonal antibody and interleukin 6 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* 172:1861.
22. Zhang, K., E. A. Clark, and A. Saxon. 1991. CD40 stimulation provides an IFN- γ -independent and IL-4-dependent differentiation signal directly to human B cells for IgE production. *J. Immunol.* 146:1836.
23. Rousset, F., E. Garcia, and J. Banchereau. 1991. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med.* 173:705.
24. Saxon, A., J. Feldhaus, and R. A. Robins. 1976. Single step separation of human T and B cells using AET-treated SRBC rosettes. *J. Immunol. Methods* 12:285.
25. Griffin, B. E., E. Bjorck, G. Bjursell, and T. Lindahl. 1981. Sequence complexity of circular Epstein-Barr virus DNA in transformed cells. *J. Virol.* 40:11.
26. Mills, F. C., J. S. Brooker, and R. D. Camerini-Otero. 1990. Sequences of human immunoglobulin switch regions: implications for recombination and transcription. *Nucleic Acids Res.* 18:7305.
27. Clark, J. M. 1988. Novel non-templated nucleotide additions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* 16:9677.
28. Sambrook, J., T. Maniatis, E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
29. Zhang, X., C. Werner-Faver, H. Tang, N. Brouwers, J.-Y. Bonnefoy, and R. H. Zubler. 1991. IL-4 dependent IgE switch in membrane IgA-positive human B cells. *J. Immunol.* 147:3001.