

Secondary Deletional Recombination of Rearranged Switch Region in Ig Isotype-Switched B Cells

A Mechanism for Isotype Stabilization¹

Ke Zhang,^{2*} Hai-Kit Cheah,* and Andrew Saxon^{*†‡}

*The Hart and Louise Lyon Laboratory, Division of Clinical Immunology and Allergy, [†]The Molecular Biology Institute, and

[‡]The Jonsson Comprehensive Cancer Center, University of California Los Angeles School of Medicine, Los Angeles, CA 90024-1680

Analysis of the Ig switch (S) region structure from many stable isotype-switched B cells reveals that the majority of the ultimate switch recombination sites are located at the 5' end, or even upstream of the 5' end of the $S\mu$ region. These findings strongly contrast with results from switch circle analysis which reveal that the primary switch recombination sites are preferentially distributed in the middle or at the 3' end of the $S\mu$ region. Secondary deletion within recombined chimeric S regions has been proposed as a mechanism to account for the different results. We directly tested whether secondary deletion of rearranged chimeric S regions indeed occurs in human cells. Circular DNAs representing secondary deletion events in γ switched cells were isolated and characterized by using a specially designed PCR-based approach. Nucleotide sequence analysis revealed that all clones had the $S\gamma 1-S\mu-S\gamma 1$ structure. Thus, these cloned fragments resulted from secondary deletion/recombination events within a chimeric $S\mu-S\gamma 1$ switch region, i.e., rearrangement between its 5' $S\mu$ and 3' $S\gamma 1$. Analysis of the $S\mu$ region from stimulated B cells also revealed that some $S\mu$ regions undergo an internal deletion/rearrangement between the 5' and 3' ends. These results definitively demonstrate that secondary deletion/recombination of chimeric S regions of isotype-switched B cells occurs. Such secondary deletion/recombination events potentially can be responsible for isotype stabilization of switched B cells, as the active Ig gene may have an insufficient amount of retained chimeric switch sequence to serve as a substrate for further S-S recombination. *The Journal of Immunology*, 1995, 154: 2237-2247.

Isotype switching in B lymphocytes functions to generate Abs with different effector activities, thereby expanding the capabilities of the humoral immune response. This process is achieved by the switching of the Ag-specific VDJ segment from μ to one (or more) of the downstream isotype(s). The actual switching occurs by deletional recombination between two Ig genes, with recombination specifically occurring at the switch regions (S),³ highly repetitive regions upstream of each cluster of con-

stant gene segments except for δ . This is believed to be the major mechanism for isotype switching (1), although several other mechanisms have also been proposed to interpret isotype switching occurring under certain circumstances (2-8). Such deletional recombination generates in the genome a chimeric switch region composed of the 5' $S\mu$ joined to the 3' portion of a targeted downstream S. The intervening DNA between the two involved S regions is looped out of the chromosome and excised as an extra chromosomal circular DNA, or switch circle (9-14). We have shown that such recombinational/deletional isotype switching occurs in humans and that switching to ϵ may proceed by multiple sequential switches.

Given that sequential switching occurs, an important question about Ig production is how can a specific Ig isotype be maintained after switching, i.e., what prevents cells undergoing continued sequential switching from switching to isotypes further downstream. Analysis of the structure of the chimeric switch region from many stable

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² Address correspondence and reprint requests to Dr. Ke Zhang, Division of Clinical Immunology and Allergy, Department of Medicine, University of California, Los Angeles, School of Medicine, 52-175 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90024-1680.

³ Abbreviations used in this paper: S, switch region; P5' $S\mu$, 5' $S\mu$ probe.

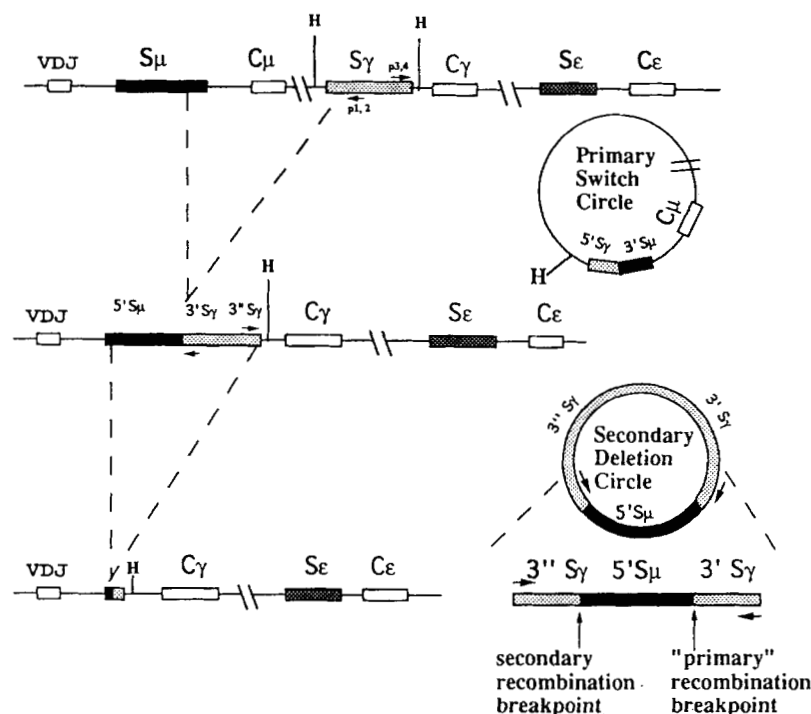


FIGURE 1. Schematic diagram demonstrating the formation of circular DNA representing primary and secondary recombination/deletion and the PCR primer position strategy for detection of secondary deletion circles. At top is a map of the disrupted human Ig C_H chain gene region related to this study. The typical switch regions with repetitive sequences are indicated by different shaded boxes and the constant regions are shown simply as an open box. The numbered arrows indicate position and orientation of PCR primers; arrows above the gene map are sense sequences and those below the map are antisense sequences. For initial μ to γ switching (primary switch), the 5' S_μ portion and 3' S_γ portion join together on the chromosome to generate a hybrid S_μ-S_γ region. The intervening DNA excised from the chromosome forms a primary switch circle as shown. The hybrid S_μ-S_γ region, when undergoing a secondary deletion as predicted, rearranges its most 3' end (3' S_γ) to the 5' end of the tandemly repeated sequences of the S_μ region. This leaves a very abbreviated retained S region with minimal chromosomal tandem switch region sequences while excising a circular DNA (secondary deletion circle) with a part of the initial hybrid S region joined directly to itself. Such a secondary deletion circle can be amplified by PCR using the primer set p2 and p3 (followed by p1 and p4 for a second-round PCR) to give a unique S_γ-S_μ-S_γ structure, as shown in the bottom part of the figure. The *Hind*III site, shown as H in the figure, is 5' and 3' to all of the S_γ sequences and was used to linearize and later digest all primary switch circles involved in any S_γ, including the possible switch circles derived from sequential switching between two S_γs, as discussed in the text.

isotype-switched B cells from mice and humans, including myelomas, lymphomas, hybridomas, and stimulated fresh B cells, revealed that the vast majority of their switch recombination sites cluster in the 5' end, or even upstream of the 5' end, of the tandemly repeated sequences comprising the S_μ region (15–28). These observations strongly contrast with the results from switch circle analysis, in which the recombination sites show a biased distribution in the middle or 3' end of the S_μ region (10–14).

As switch circles necessarily reflect the primary recombination sites, it has been proposed that the observed discrepancies in the switch recombination sites between switch circles and the chromosomal chimeric S regions in Ig-producing cells results from secondary recombination occurring in the primary rearranged switch region (10, 29). Such recombination may delete much or all of the remaining switch S_μ and leave minimal S sequences available as

a substrate for sequential switching to downstream isotype(s). In this manner, a secondary recombination process could act directly as a mechanism for isotype stabilization. However, the reciprocal products of such secondary S deletion have not been isolated and characterized.

To determine whether secondary S recombination occurs with isotype-switched B cells, we employed a specially designed PCR-based approach to amplify circular DNA specifically representing secondary (intra-S) deletion/recombination events. Analysis of these PCR products revealed that secondary deletion of primary rearranged chimeric switch regions indeed occurred in stimulated B cells. The structure of the excised secondary deletion circular DNA indicated that the secondary recombination rearranged the 5' end of the S_μ region (or regions even further than 5') to the 3' end of the S_γ1 region. These data add further evidence to the hypothesis that secondary S deletions

may act as an important mechanism for Ig isotype stabilization.

Materials and Methods

Cells and cell cultures

Human peripheral blood and tonsillar B lymphocytes were isolated and purified by the AET-rosette method as described (30). For induction of switching to $C_{H}\gamma$, purified B cells (1×10^6 cells/ml) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin in the presence of 200 U/ml IL-4 (a gift from Dr. E. Medlock, Amgen, Thousand Oaks, CA) or IL-4 plus IL-10 (100 U/ml; a gift from Dr. K. Moore, DNAX Institute, Palo Alto, CA) and 0.1 μ g/ml of α CD40 mAb G28-5 (a gift from Dr. E. A. Clark, University of Washington, Seattle, WA) (31). One set of cultures was incubated for 5 days before preparation and analysis of circular DNA, and another set of cultures was incubated for 12 days for measurement of production of IgG, IgM, and IgE in the supernatant.

Preparation of circular DNA fractions

Circular DNA fractions were prepared from 1×10^7 stimulated B cells as described (14). To analyze only switch circles representing excisional deletion of intra-S sequences, the precipitated circular DNA fraction was first digested with 20 U *Hind*III for 4 h at 37°C and then treated with 5 U ATP-dependent DNase (United States Biochemical Corp. (USB), Cleveland, OH) for 1 h at 37°C to digest the linearized switch circles as described (32). Because *Hind*III sites are located both 5' and 3' to $S\gamma$ for all four γ subclasses, as shown for $S\gamma 4$ (33; and F. C. Mills and E. E. Max, personal communication), this procedure destroys any switch circles resulting from switch recombination between two separate switch regions involved in $S\gamma$ and from sequential switch recombination between two $S\gamma$ subclasses. However, this linearization and digestion does not destroy circular DNAs resulting from secondary (intra-S) recombination, e.g., excised from within a $S\mu$ - $S\gamma$ chimeric switch region, because no *Hind*III sites are contained in the latter structure (see Fig. 1 and below). The remaining circular DNA fraction was precipitated and used as DNA templates for PCR amplification.

PCR strategy for amplification of circular DNA representing secondary S recombinations

PCR was performed in a 25- μ l vol/reaction with 5% (v/v) DMSO, 50 mM KCl, 20 mM Tris-HCl, pH 9.1, 2.5 mM $MgCl_2$, 50 pM primer/reaction, and 2.5 U Taq polymerase (Promega Corp., Madison, WI). The 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 15 min (without Taq polymerase) for complete DNA denaturation, 65°C for 5 min for annealing (Taq polymerase was added to the tube at this step), and 72°C for 5 min for extension. PCR was continued at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min for 40 cycles with a 15-min extension in the final cycle.

A second-round PCR using nested primers was run on the DNA templates derived from the first PCR reaction. The same conditions were employed as in the first-round PCR except for changes in primers. The first-round PCR products (2 μ l) from primer pair p2 and p3 were reamplified with nested primer pair p1 and p4 (see Fig. 1). The PCR primer sequences that are homologous to all four subclasses of the $S\gamma$ region are shown in the following list:

p1: 5'-ACTGCTCAGTGGGATGGACTCACTGGGT' (complementary to 3536–3507 of $S\gamma 4$ (33) and 3994–3965 of $S\gamma 1$ (GenBank, under the accession name Humigcc4; F. C. Mills and E. E. Max, personal communication)).

p2: 5'-AAGCACACGTTCCTCCTGCAGCCACACCT3' (complementary to 3635–3607 of $S\gamma 4$ (33) and 4093–4065 of $S\gamma 1$).

p3: 5'-AGGGCCTTGGAGGGCAGAGGGCAGGC3' (corresponding to 4391–4416 of $S\gamma 4$ (33) and 4874–4889 of $S\gamma 1$).

p4: AAGTCAGGCCAACTACTAGGGGAG3' (corresponding to 4436–4460 of $S\gamma 4$ (33) and 4920–4944 of $S\gamma 1$).

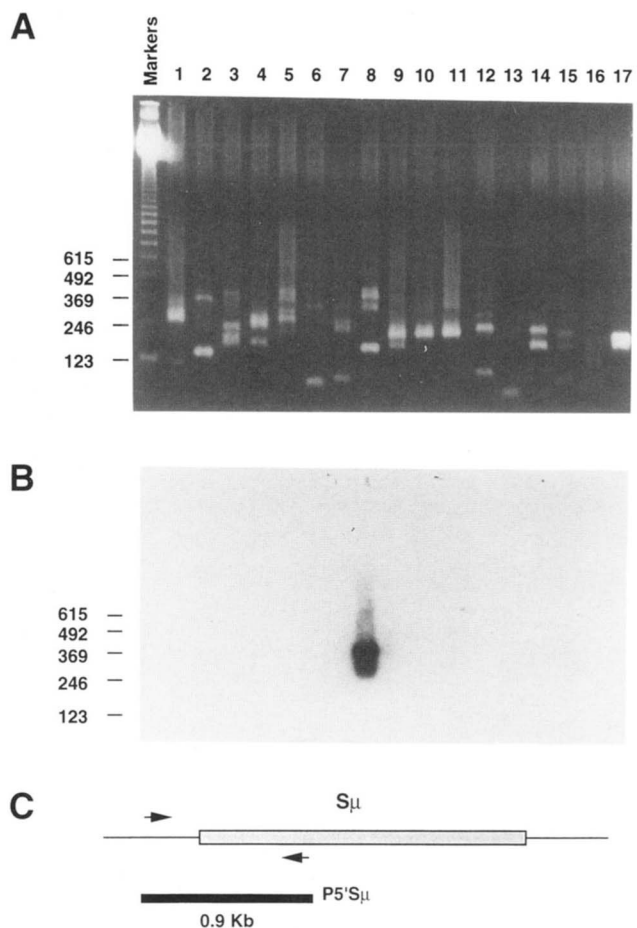


FIGURE 2. Detection of PCR-amplified DNA fragments representing secondary deletion circles. (A) Gel electrophoresis of PCR-amplified DNA fragments from the circular DNA fraction after *Hind*III linearization and digestion to destroy primary switch circles. Multiple DNA bands of differing sizes can be seen in the PCR reactions using DNA from IL-4 + IL-10 + α CD40 stimulated B cells. (B) Southern blot analysis of PCR-generated products from (A). Following transfer to a nylon membrane and hybridization, the samples were probed with a 0.9-kb, PCR-generated probe representing 5' $S\mu$ sequences (shown in C) to detect amplified DNA fragments that contained 5' $S\mu$ sequences. The products showing positive signal hybridization to the probe (lane 8) were subcloned and the positive subclones were sequenced (see *Materials and Methods*). (C) Diagram of the PCR-generated probe P5' $S\mu$ that was used for the Southern blot in (B). A 0.9-kb fragment was amplified by PCR (see *Materials and Methods*). This fragment was cloned and the sequence was confirmed by nucleotide sequencing.

PCR analysis of $S\mu$ internal deletion/rearrangement from genomic DNA

Genomic DNAs were prepared for the DNA templates of PCR according to the method described (34). The 5' primer (p $S\mu 1$, 5'-TCTAGA CAAGGGGACCTGCTCATTT3') corresponded to base pairs 1–25 of $S\mu$ and the 3' primer (p $S\mu 2$, 5'-CCATCTATGTCCAACAAGAT CATGA3') was complementary to base pairs 3767–3742 of the $S\mu$ sequence (33). For amplification, 1 μ g/reaction of DNA was used as template. The first cycle of PCR was conducted at 94°C for 15 min (without

Taq polymerase) for complete DNA denaturation, 65°C for 5 min for annealing (Taq polymerase was added to the tube at this step), and 72°C for 5 min for extension. PCR was continued at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min for 40 cycles with a 15-min extension in the final cycle.

Cloning and screening

The final PCR products (10 μ l) were electrophoresed on 1% agarose gels with Tris-Borate-EDTA buffer before transfer to a nylon membrane under the conditions described previously (14). Blots were then hybridized with a 0.9-kb probe derived from a 5' $S\mu$ region sequence that was prepared by PCR amplification using pS μ 1 (see above) as the 5' primer and a 3' primer (5' AGCACAGCACGCTCAACCCGGTTCAGCTTG3') which was complementary to 914–885 of the $S\mu$ sequence (33). PCR products that demonstrated a positive signal with the 0.9-kb 5' $S\mu$ probe (P5' $S\mu$) were then subcloned by the TA cloning method as described previously (14). The TA cloning vectors were prepared with the phagemid Bluescript SK⁺ in the *EcoRV* site according to the methods described (35). Clones that contained inserts (white clones) were selected for miniprep and digested with *EcoRI* plus *HindIII* and rescreened with P5' $S\mu$. Clones that hybridized were subjected to DNA sequencing.

DNA sequencing

Nucleotide sequences of the inserts were determined by the dideoxy chain termination method using a kit purchased from USB. T3 and T7 primers as well as the second-round PCR primers were used as sequencing primers.

Results

A PCR strategy for detection of the circular DNA resulting from secondary (intra-S) deletion

During deletional recombination occurring with Ig isotype switch, the 5' part of the upstream S sequence and 3' part of the targeted S sequences join together on the chromosome to form a hybrid switch region (primary switch recombination). We designed a PCR-based methodology to amplify circular DNA representing the secondary deletion products excised from the primary rearranged S region so as to test whether some isotype-switched B cells would further delete/rearrange such primary rearranged switch regions. As depicted in Figure 1, if the proposed secondary deletion/recombination occurs with rearrangement of the 3' end of the chimeric switch region to the very 5' end of $S\mu$, then the orientation of the PCR primer pair p2 and p1 (antisense sequences) and p3 and p4 (sense sequences)

would flip head to head. As a result, the primers would have the opportunity to amplify circular DNAs (other than the S region in genomic DNA) representing the secondary deletion excised from the primary switched chimeric S region. Employing such a strategy, detection of a product consisting of 5' $S\mu$ sequences interposed between two $S\gamma$ fragments derived from a single $S\gamma$ region would have to be derived from circular DNA representing secondary deletion circles (see Fig. 1).

Understandably, many possible secondary deletion circles might not be detected from the absence of $S\mu$ region sequences if the secondary deletion occurred entirely within the $S\gamma$ region. It was necessary to exclude the possibility that the amplified products were derived from $S\gamma$ - $S\gamma$ self-deletional circles, as the primer pairs were expected to amplify such events occurring either before or after the deletional recombination involved between $S\mu$ and $S\gamma$; therefore, the amplified DNA was screened by hybridization with a probe derived from the 5' $S\mu$ region.

Although primers p2 and p3 are derived from the published sequence of the $S\gamma$ 4 locus (33), the four human $S\gamma$ s (γ 3, γ 1, γ 2, and γ 4) are highly homologous to each other in the region where the primers were located. Therefore, it was critical to exclude the possibility that the products we detected derived from amplification of circles resulting from sequential switches occurring between two γ subclasses. To exclude such a possibility, we digested and destroyed the circular DNAs from the switch circles representing the sequential switch between two $S\gamma$ regions. Complete elimination of this type of circular DNA by this approach was verified by attempted PCR amplification of the $S\gamma$ - $S\mu$ switch circles with a set of primers designed for amplification of this type of switch circles (Ke Zhang et al., unpublished data). No products were amplified by PCR from the digested, APT-dependent DNase-treated DNA, whereas multiple specific products were detected from the untreated DNA portion as determined by Southern blot hybridization with a probe for the 3' $S\mu$ region (data not shown).

FIGURE 3. Nucleotide sequences surrounding break points of the secondary and primary recombination sites of the $S\mu$ - $S\gamma$ 1 segments. A schematic diagram of the structure of each of the clones is presented at the top of each section. The $S\gamma$ - $S\mu$ junction on the right and left represent the secondary and primary recombination sites, respectively. The original transcriptional alignment of each segment (3' $S\gamma$ 1, $S\mu$, and 3' $S\gamma$ 1) was from left to right. The actual sequences surrounding the break points are shown below the schematic diagram of each clone. The arrows indicate the precise position of the recombination break point (RB) sites defined by alignment of the cloned sequences with the germ-line $S\mu$ and $S\gamma$ sequences ($S\gamma$ 1 is shown). The vertical lines indicate the identical nucleotide bases. The sequences at the break points with homology to both $S\mu$ and $S\gamma$ 1 germ-line sequences are indicated by bold letters, as it is not possible to assign the homologous sequence to one germ-line S region. The dashed horizontal lines represent nucleotides absent from either the isolated clones or the S germ-line sequences. The non-germ-line nucleotide "insert" at the break point in clone B2.6–41 is underlined (A). The schematic diagram shown beneath each set of clone break point sequences represents the progression of the primary and secondary recombinations that can be inferred to have occurred to generate the results. (A) shows clone B2.6–41, in which secondary recombination occurred between the region 5' to $S\mu$ and the 3' end of $S\gamma$ 1. (B) shows the four clones in which the secondary recombination was in the 5' $S\mu$ sequences.

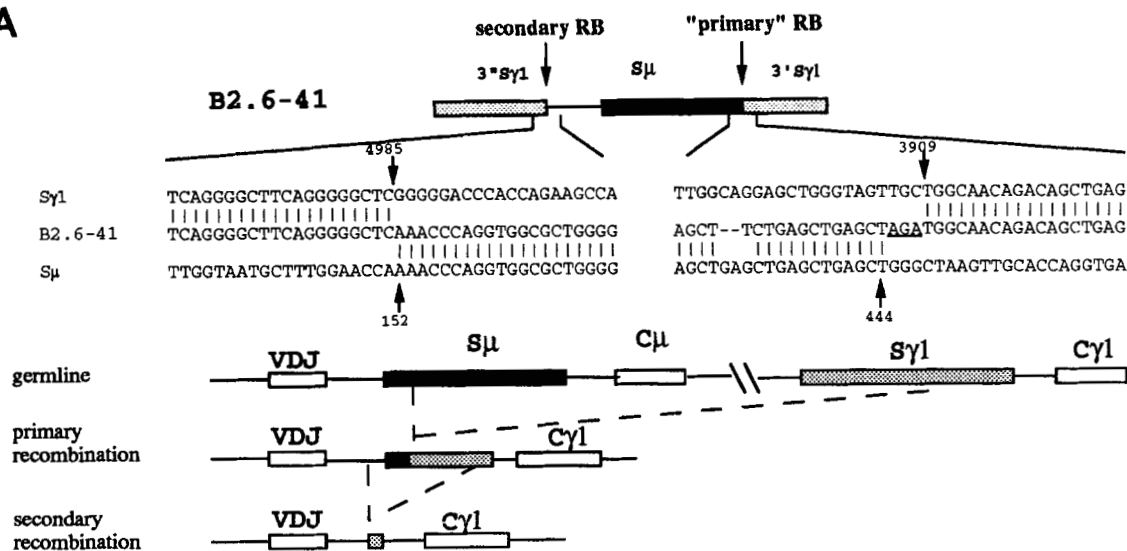
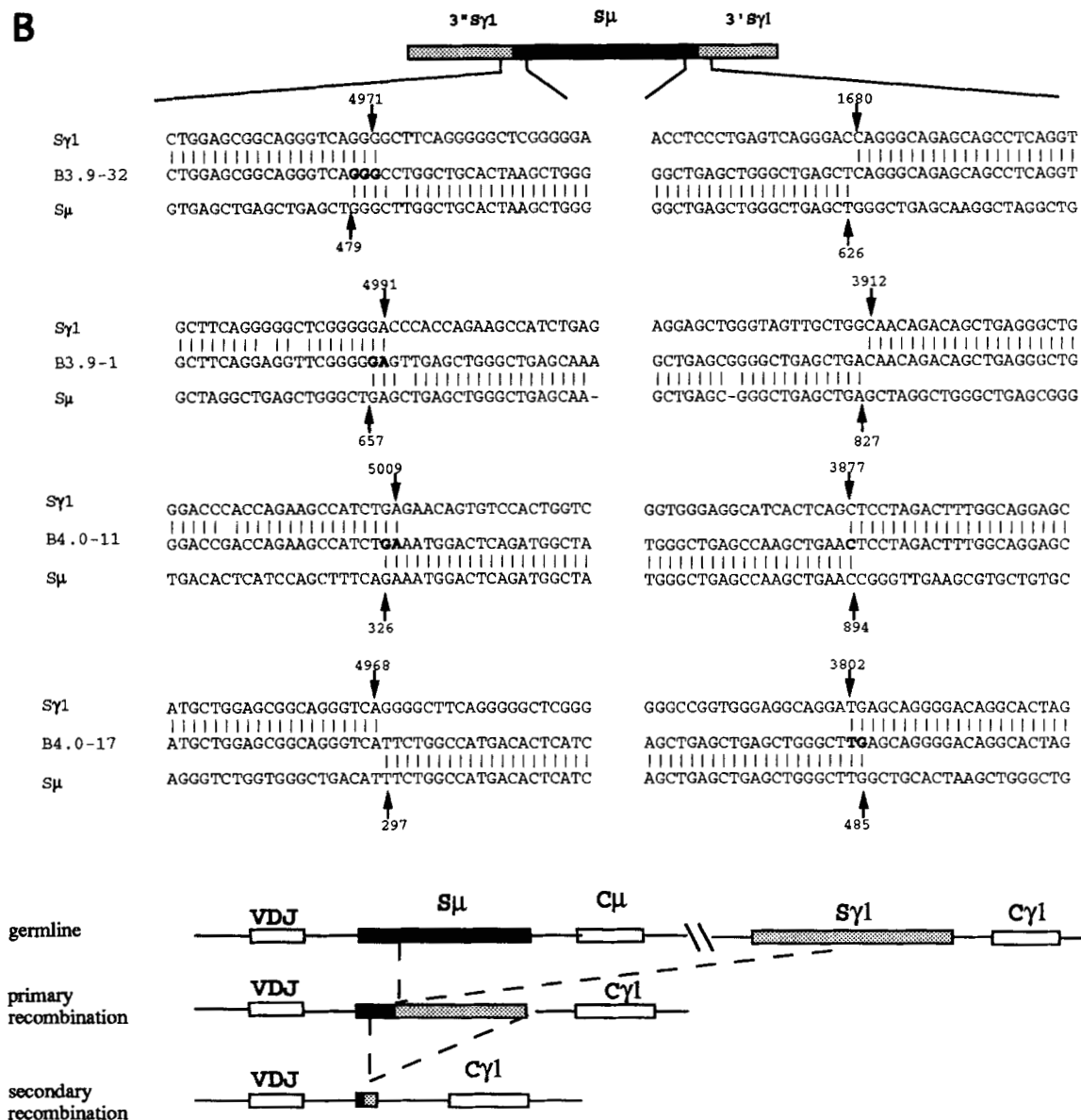
A**B**

Table I. Diagnostic nucleotides in the four *Sy*s and the base at these positions in isolated *Sy-S μ -Sy* clones

	Position of Diagnostic Nucleotides for <i>Sy</i> 1 ^a					
	3856	3857	3885	3914	4950	4956
Germ-line <i>Sy</i>						
<i>Sy</i> 1	C	T	C	A	T	A
<i>Sy</i> 2	C	C	T	G	C	G
<i>Sy</i> 3	A	C	C	G	C	G
<i>Sy</i> 4	A	C	T	G	C	G
Clones						
B2.6-41				A	T	A
B3.9-32	C	T	C	A	T	A
B3.9-1				A	T	A
B4.0-11			C	A	T	A
B4.0-17			C	A	T	A

^a The germ-line *Sy*1 sequence was used as the prototype for the comparison; therefore, position numbers for nucleotides listed in the table are from *Sy*1. The corresponding positions from *Sy*2, -3, and -4 are from the homologous region to *Sy*1.

A typical example of specific secondary deletion circle PCR-amplified products from IL-4, IL-10, and CD40 mAb stimulated B cells using the p2-3 then p1-4 primer sets is shown in Figure 2A. Multiple PCR-derived bands were visualized on agarose gel. Among the 17 amplifications shown, there was only one product from lane 8 (Fig. 2B) that hybridized to P5'*S μ* , indicating that this product contained a fragment of the 5' *S μ* region sequence. From a total of 186 PCR reactions, five positive clones were isolated. These P5'*S μ* -positive PCR products were subcloned, the resulting subclones were again screened with the same 5' *S μ* probe, and the positive subclones were then subjected to nucleotide sequence analysis.

Sequence analysis of the cloned PCR products

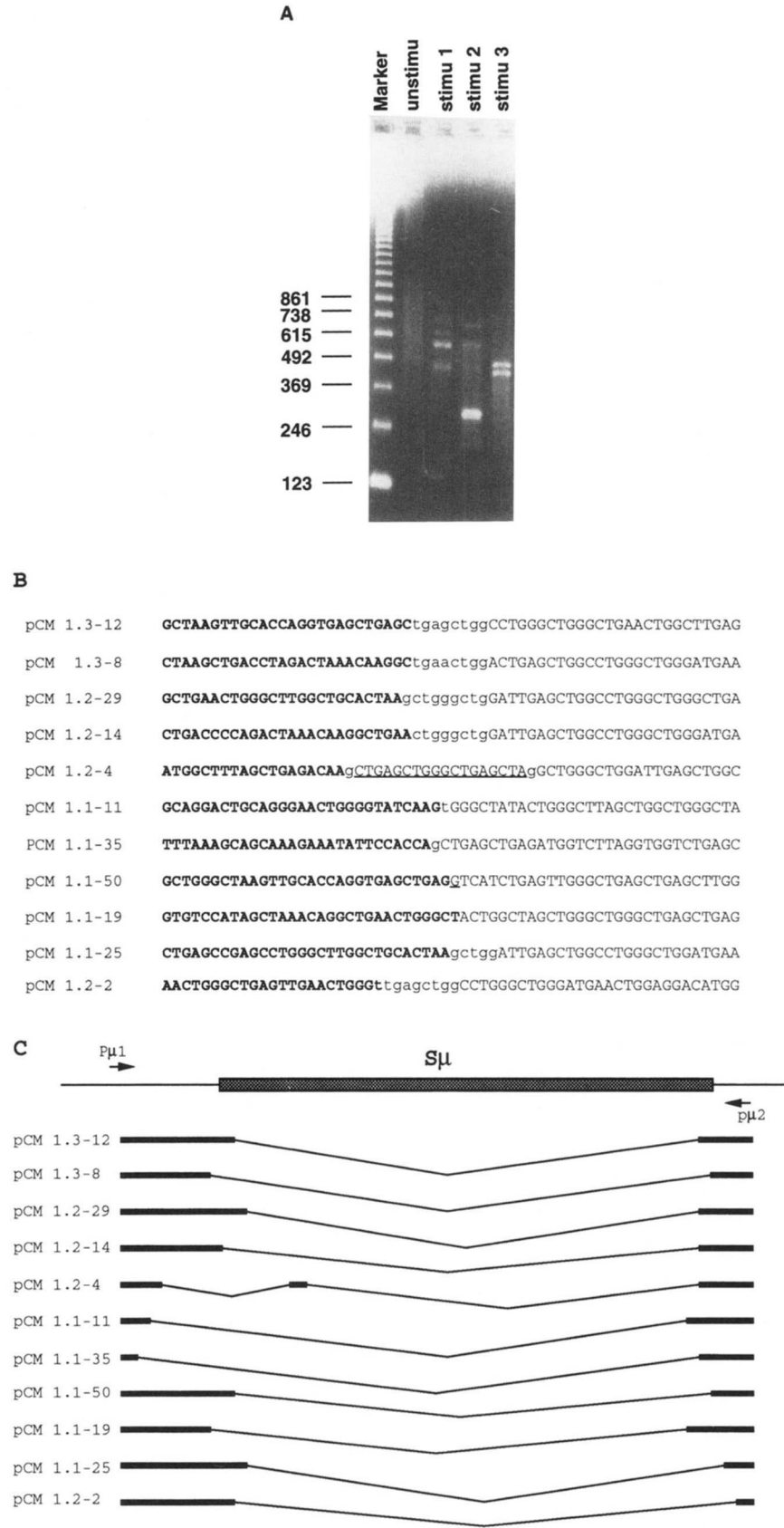
To demonstrate that those clones hybridizing with P5'*S μ* truly represent the circular DNAs from secondary deletion events, we determined the complete nucleotide sequences of those subclones and thereby identified the primary and secondary recombination break points. All five 5' *S μ* -positive clones revealed that they had a 3''*Sy*1-*S μ* -3'*Sy*1 structure in a 5'-3' orientation, where the left-side *Sy*1 sequences came from the very terminal 3' end of *Sy*1 (referred to as 3''*Sy*) and the right-side *Sy*1 sequences de-

rived from *Sy*1 sequences upstream or 5' to 3''*Sy*1 (referred to as 3'*Sy*1) but still derived from the 3' portion of the full *Sy*1. In Figure 3, the recombination break points for each clone were defined by alignment of the clone sequences with all four sets of germ-line subclasses of *Sy* and *S μ* region sequences. The γ sequence in the clones was determined to be derived from γ 1, as is discussed later. The left-hand *Sy*1-*S μ* junctions in those clones represented the secondary (intra-S) recombination break points, whereas the right-hand *S μ* -*Sy*1 junctions represented "primary" (inter-S) recombination break points. "Primary recombination break points" does not necessarily mean that these break points reflect the initial switch recombination sites occurring between the *S μ* and the *Sy*1 regions during the isotype switch from μ to γ 1 (see Discussion); the term "primary" here only indicates that the recombination event creating the break point occurred before the secondary recombination event.

Although in the S regions all four human subclasses of γ are highly homologous, there are regions close to the PCR primers that we used that contain γ subclass characteristic nucleotides. Therefore, the *Sy* portion sequences from all of the *S μ* -containing clones were subjected to special scrutiny to provide sufficient data to determine from which of the four *Sy* (γ 1-4) regions the γ sequences were derived. Important diagnostic nucleotides that distinguish between the four *Sy*s are listed in Table I. All of the *S μ* -bearing clones that we isolated had a T corresponding to position 4950 of *Sy*1, an A at 4956 of *Sy*1, an A nucleotide in position 3914 of *Sy*1, and a C nucleotide in position 3885 of *Sy*1. These diagnostic nucleotides define the *Sy* sequences of the clones as all being derived from the *Sy*1 region.

The nucleotide sequences surrounding the secondary and primary recombination break points in Figure 3 showed that the homology between the *Sy*1 and *S μ* sequences was minimal, varying from zero to three nucleotides. These results indicate that secondary recombination, as well as primary recombination, involve nonhomologous recombination, as observed previously (14, 28). There were three nucleotides at the primary recombination of clone B2.6-41 that could not be assigned to either of the two germ-line S sequences. In

FIGURE 4. Internal deletion in *S μ* following IL-4 plus α CD40 stimulation of B cells. (A) Gel electrophoresis of PCR amplified DNA fragments from genomic DNA with primer set p μ 1 and p μ 2. Multiple-DNA bands of differing sizes were amplified with DNA from the IL-4 plus α CD40 stimulated (lanes 3 through 5) but not unstimulated (lane 2) B cells. (B) The nucleotide sequences surrounding the recombination sites from the DNA cloned from (A). Bold letters represent the 5' *S μ* portion sequences; lighter letters represent the 3' *S μ* sequences. The lowercase sequences represent recombination junction sites homologous for both the 5' *S μ* and 3' *S μ* parts. The underlined portion of the clone pCM 1.2-4 were derived from an internal portion of the *S μ* region (see Fig. 4C) and therefore a two-step recombination event must have occurred to generate such a sequence. The underlined single nucleotide in the clone pCM 1.1-50 is an "inserted" nucleotide that could be assigned to either the 5' or 3' portion of the published *S μ* sequences. (C) The position of the μ PCR primers as related to the tandemly repeated sequences that comprise the *S μ* region; below that is a compilation of schematic diagrams representing the *S μ* internal recombinations defined from the clones shown in (B).



addition, clone B3.9–32 had an internal deletion in the nucleotide sequence of 1723–3827 of S γ 1 inside the 3' S γ 1 portion.

The vast majority of the amplified products from S γ 1-S γ 1 PCRs did not hybridize to the 5' S μ probe, as shown in Figure 2B. We cloned and sequenced 17 of these products to determine their structure and, thereby, the nature of the deletion/recombination events they represent. Thirteen were characterized by the 3' portion of S γ being directly ligated to the 3' portion of the S γ sequences (see Fig. 1), indicating that these clones were the looped-out products of internal S γ region rearrangements (sequence data not shown). Such clones could derive from either the internal rearrangement of the S γ region while the γ gene is in germ-line configuration or the secondary deletion/recombination of a rearranged S μ -S γ S region in which the secondary recombination occurred inside the S γ region. The structure of these S γ -S γ circles does not distinguish between these two possibilities.

Internal S μ region deletion: a mechanism for stabilization of IgM

The combination of B cells stimulated with cytokines that induce Ig switching (such as IL-4 and IL-10) plus CD40 mAb not only results in multiple switch recombination events (14), but also induces differentiation of IgM-secreting cells. We sought to determine whether this differentiation was associated with the internal rearrangement/deletion of the S μ region in a manner analogous to that which would result from the looping out of the secondary switch circles that we observed for S μ -S γ . We used a set of PCR primers located in the region 5' to the beginning of S μ (p μ 1) and 3' to the end of S μ (p μ 2; see Fig. 4C) to detect the S μ remaining after S μ internal deletion events. As shown in Figure 4A, multiple bands were amplified from the DNA sources of IL-4 plus α CD40 stimulated, but not from unstimulated, B cells. Positive hybridization of these bands to the 5' S μ probe indicated that these PCR products were not nonspecific amplification products (data not shown). Subsequent cloning and sequence analysis revealed that the S μ region amplified represented the true internal rearrangement of the S μ region, with direct recombination between 5' and 3' S μ sequences. As shown in Figure 4C, the sequence of the products revealed almost complete deletion of the tandemly repetitive S μ region sequences, with a number of clones rearranged at the very 3' end to the 5' end of the tandemly repetitive S μ region (clones pCM 1.3–8, 1.2–4, 1.1–11, 1.1–35, and 1.1–19). The sequences of the recombination sites of these clones are listed in Figure 4B and the nature of these clones, as related to the S μ region, are schematically diagrammed in Figure 4C. Interestingly, clone pCM 1.2–4 appears to have undergone two internal deletion/recombination events.

In other experiments, S μ -S μ PCR products were observed with unstimulated, freshly isolated B cells derived

from other donors. Cloning and sequencing of these products revealed that they too represented the internal recombination of the S μ region, as shown by direct recombination between 5' and 3' S μ sequences, and thus they appear to represent B cells that have undergone S μ internal deletional recombination *in vivo*.

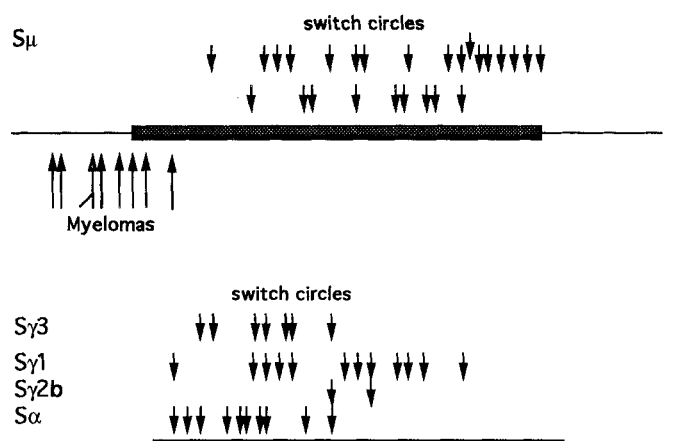
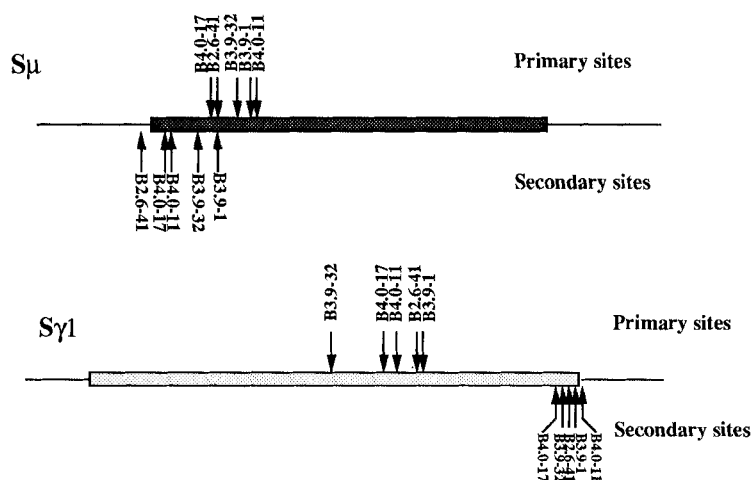
Comparison of the clones we sequenced with the published S μ sequences revealed that all of the S μ clones contain two segments not present in the published germ-line S μ (33). Because these sequences were present in S μ clones from all individuals examined, it appears likely that they were deleted during the cloning of the original published S μ germ line sequence. One segment, GCTAG GCTGGGCTGAGCTGGTCTGAGCTCA, has been deleted from the position between S μ 3628 and 3629 (14), and the other deleted segment, TTGAGCTGGCCTGGG CTGGGATGAACTGGA, is located between S μ 3749 and 3750 of published S μ sequences (33).

Discussion

In this report, we employed a PCR-based strategy to search for and analyze circular DNA representing secondary deletion of rearranged switch regions from cytokine-(IL-4 and IL-10) and CD40-stimulated human B cells. The results revealed that a chimeric S μ -S γ 1 switch region resulting from an isotype switch between μ and γ 1 could indeed undergo secondary recombination from the very 5' end of S μ to the very 3' end of S γ 1 in which the tandemly repeated switch region sequences (GGGCT, GAGCT, or GGGGT) were all or almost all removed from the corresponding chromosome. Indeed, in clone B2.6–41 (Fig. 3A), the recombination occurred in the region 5' to the beginning of the tandemly repeated S μ region and completely deleted the S μ sequence.

The approach we employed with PCR primers oriented away from each other in the genome required that the DNAs from secondary S region deletion events (as well as the DNA resulting from the deletional recombination of true isotype switching) ligate the ends of the excised DNA to form extrachromosomal circular DNAs. As such, our approach would only detect or amplify such excised circular DNAs. Our isolation and characterization of such circular DNA representing the S μ -S γ 1 secondary recombination provides the first direct evidence for secondary recombination occurring in isotype-switched B cells. This process has long been speculated to be implicated in the stabilization of an achieved Ig isotype production based on the analysis of the structure of the switch regions from stable isotype-switched B cells from a variety of sources, including plasmacytomas, myelomas, hybridomas, B cell lymphomas, normal B cell populations, and EBV-transformed human B cells (14–28).

Isotype switching through deletional recombination occurs at the tandemly repeated sequences that comprise the Ig S regions located upstream of each Ig heavy chain gene

**B**

The recombination sites defined by study of the excised switch circles, with very rare exception, will represent the original recombination sites, whereas the structure of the hybrid S regions on chromosomes of stable isotype-pro-

Because isotype switching from μ to a downstream isotype(s) is frequently accomplished by sequential switching through one or more intervening isotype(s) (10–14), a

mechanism that prevents isotype-switched B cells from undergoing subsequent switching to yet another, further downstream isotype(s) is required for maintaining or stabilizing the production of the achieved isotype. This is particularly true as the majority, if not all, of the definitive primary switch recombination sites defined by analysis of circular DNA are distributed in the 3' end of the donor S region and the 5' end of the acceptor S region (see Fig. 5A), the large chimeric switch regions generated retain a long stretch of tandemly repeated S region sequences suitable as the substrates for sequential switch recombination to a downstream isotype. Secondary deletion of the initially generated hybrid S region in such isotype-switched B cells, by leaving only minimal typical S region sequences available as the substrate for the further switch recombination, would be expected to prevent switching to a further downstream isotype(s). This should provide a means for stabilizing isotypes and prevent the majority of B cells from undergoing sequential switching to the most distal isotypes.

Although secondary deletion/recombination events can provide a mechanism for Ig isotype stabilization in isotype-switched B cells, it is likely that this is not the only mechanism available. In some EBV-transformed IgM- and IgG-secreting human B cell lines, we have observed that large portions of $S\mu$ were retained (Ke Zhang et al., unpublished observations). Similar phenomena have also been observed in some murine hybridomas derived from activated murine B cells (23). Both EBV-transformed B cell lines and murine hybridoma cells are generally unable to be induced to switch when stimulated in ways that induce primary B cells to undergo Ig switch. The inability of such "immortalized" cells to undergo isotype switching or to show secondary deletional recombination may be related to the turning off of the putative switch recombinase in these cells. Thus, alternative or multiple mechanisms may coexist and function in Ig isotype stabilization at various stages of B cell differentiation.

The so-called "primary" recombination sites defined in this report in the $S\mu$ region from our secondary deletion circular DNA are quite different from those defined by us from interisotype primary switch circles (14). In the latter, the switch recombination sites were predominately clustered in the 3' end of the $S\mu$ region. This discrepancy almost assuredly reflects a bias of our PCR approaches related to 1) the placement of the different primers employed to look for primary inter-S region vs secondary intra-hybrid S region generated circles, and 2) selective amplification of smaller size DNA fragments. For example, a secondary deletion circle generated from internal deletion of the hybrid switch sequences from the 5' portion of $S\mu$ to $S\gamma$ in a cell where the primary recombination occurred between the very 3' end of the $S\mu$ region and the 5' end of the targeted $S\gamma$ region would be too large to be effectively amplified.

Another possibility for the primary recombination sites from the secondary deletion products being located close to the 5' end, rather than the 3' end, of the $S\mu$ region might be due to a substantial secondary deletion(s) having already occurred, i.e., a deletion from the original hybrid S region that generated a "shorter" hybrid switch region. If this has been the case, the so-called primary recombination sites should actually be the secondary recombination sites for the first round of secondary deletion. Thus, as mentioned earlier, the primary break points described in this report do not necessarily represent the initial switch recombination break points; rather, they reflect that the recombination event(s) to create such "primary" break points occurred before the secondary recombination events.

In a manner analogous to the secondary deletion of the chimeric $S\mu$ - $S\gamma$ hybrid switch regions, $S\mu$ also was shown to undergo the internal rearrangement between the 5' end and the 3' end of the $S\mu$ region in stimulated B cells. As shown in Figure 4, such internal rearrangements of the $S\mu$ region removed almost all, if not all, of the tandemly repeated $S\mu$ sequences, the substrate for isotype switch recombination between $S\mu$ and any downstream targeted isotype(s). It is likely that the complete deletion of the tandemly repeated $S\mu$ region would block switching, i.e., the rearrangement between the remaining $S\mu$ and a downstream S. This would allow those μ heavy chain-producing cells to persist in producing IgM. Such a mechanism for the stabilization of IgM production parallels the situation observed in vivo and in vitro in which a given stimulus drove a portion of the IgM^+ cells to switch to a downstream isotype(s) while at the same time leaving the remaining IgM^+ B cells as the IgM-secreting cells.

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