

Human Ig S γ Regions and Their Participation in Sequential Switching to IgE

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The Ig isotype switch from IgM to IgE is accompanied by a DNA recombination that joins S μ , the highly repetitive "switch" region upstream of the C μ gene, to the S ϵ region upstream of C ϵ , thereby creating a composite S μ -S ϵ region. In human B cells cultured in vitro with IL-4 to promote the switch to IgE, we previously described evidence for S μ -S γ -S ϵ structures, suggesting that some B cells can switch sequentially from μ to γ and then to ϵ ; similar sequential switching to ϵ occurs routinely in the mouse. To identify which of the four human γ genes might be involved in this μ - γ - ϵ switching pathway, we cloned and analyzed nine S μ -S γ -S ϵ composite switch regions and studied S ϵ -S γ junctions from reciprocal deletion circles. Since only the S γ 4 sequence had previously been described, our investigation required determination of the germline S γ 1, S γ 2, and S γ 3 sequences. This analysis showed that S γ 1 is the longest and most highly repetitive switch region, including nearly identical 79-bp repeats partially homologous to the 49-bp repeat of murine S γ sequences. Of nine cloned chromosomal S μ -S γ -S ϵ junctions, seven were derived from S γ 1, and one each from S γ 3 and S γ 4 (both of which were in inverted orientation). Analysis of reciprocal S ϵ -S γ junctions demonstrated contributions of S γ 1, S γ 2, and S γ 4. Thus, all four of the human γ loci can participate in sequential switching to IgE, arguing against a model of directed switching from a specific subtype, such as was proposed in the murine system. *The Journal of Immunology*, 1995, 155: 3021–3036.

B lymphocytes initially express IgM, but may mature to switch their H chain isotype to IgG, IgA, or IgE. Isotype switching results from a deletional DNA recombination event in which the C μ constant region, initially located downstream of the VDJ region, is replaced by a C γ , C α , or C ϵ constant region (1–8). The deletion end points fall within (or near) repetitive switch (S)² regions located upstream of each CH gene, except for the C δ gene. The switch recombination event thus forms a composite switch region that includes an upstream segment from the μ switch (S μ) region and a downstream segment from the S region of the targeted H chain. The DNA originally lying between the two recombined switch regions is excised from the chromosome and, at least in some cases, circularizes to form a reciprocal "deletion circle" (9–14). Switching to γ , α , and ϵ isotypes is directed

by cytokines (13, 15) that induce transcription of RNA species (germline or "sterile" transcripts) containing sequences of unrearranged C γ , C α , or C ϵ constant region genes (16–22); recombination to the transcribed constant region gene follows.

Switching to the C ϵ gene in humans is of clinical importance because of IgE involvement in allergic responses and parasitic infection (23, 24). In both human and mouse lymphocytes, switching to IgE is induced by the cytokine IL-4 (15, 25, 26). Since the C ϵ gene is adjacent to the most distal CH gene (in the human H chain cluster, C α 2) (27), isotype switching to IgE can, in principle, proceed either from C μ directly to C ϵ or sequentially from C μ to one of the CH genes lying between C μ and C ϵ (γ 3, γ 1, α 1, γ 2, or γ 4 in humans), followed by switching to C ϵ .

We previously showed that some composite switch regions from human IL-4-treated B lymphocytes switching to IgE have the structure S μ -S γ -S ϵ , suggesting that sequential switching (μ to γ , followed by γ to ϵ) can occur in human B lymphocytes at an appreciable frequency (28). Several lines of evidence suggest that the most frequent pathway to ϵ expression in the mouse is via a sequential switch through the γ 1 isotype. In splenic B cells, IL-4 induces germline message from both IgG1 and IgE genes (25, 29). This initial activation is followed by a peak in

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² Abbreviations used in this paper: S, switch (region); ECS, evolutionarily conserved sequence; MGE (clones), denotes μ - γ - ϵ switching.

frequency of switching to $\gamma 1$ about 1 day before the peak in switching to ϵ , with most of the IgE⁺ cells also staining for IgG1 (30). Incubation of such cultures with an Ab to IgG1 markedly reduces the yield of cells switching to ϵ , suggesting that $\gamma 1$ -expressing cells are precursors of ϵ expressors (30). Up to 30% of murine $S\mu$ - $S\epsilon$ composite switch regions contain an inserted segment of $S\gamma 1$ (31). Finally, DNA clones isolated from circular DNA representing the reciprocal recombination product of murine B cells switching to IgE include $S\gamma 1$ - $S\mu$ junctions and $S\epsilon$ - $S\gamma 1$ junctions, but none of the $S\epsilon$ - $S\mu$ junctions that would be expected from direct μ - ϵ switching (11). On the other hand, it is clear that switching to $\gamma 1$ is not absolutely required for ϵ expression, since switching to ϵ is not diminished in a mouse strain engineered with a defect in the $\gamma 1$ locus to prevent switching to that isotype (32).

In human cells, the details of switching to IgE have been less thoroughly investigated. IL-4 induces germline ϵ message (20) and induces germline message for $\gamma 3$ and $\gamma 4$ (22), but there are no published data that define which γ subclasses actually participate in sequential μ - γ - ϵ switching. In a study of switching induced by IL-4 plus hydrocortisone, Jabara et al. (33) analyzed the sequence of 10 unselected $S\mu$ - $S\epsilon$ composite junctions and identified one questionable and one unequivocal $S\gamma$ insertion, which they identified as deriving from $S\gamma 4$ on the basis of similarity to the published $S\gamma 4$ sequence. However, because published $S\gamma$ sequence has been available only for $S\gamma 4$ (34), it has not been previously possible to identify with certainty the γ subtype of any $S\gamma$ insert in a composite μ - γ - ϵ switch region. Zhang et al. (14) used PCR to amplify reciprocal composite switch regions expected from reciprocal circles and found evidence for sequential switching involving γ as well as direct μ - ϵ switching. Finally, van der Stoep et al. (35) sequenced 29 composite $S\mu$ - $S\epsilon$ junctions amplified from chromosomal DNA by PCR and found no $S\gamma$ inserts. They concluded that these junctions had been formed by direct μ - ϵ switching. However, the absence of $S\gamma$ inserts in a given $S\mu$ - $S\epsilon$ junction does not rule out a μ - γ - ϵ sequential switch in which an $S\mu$ - $S\gamma$ intermediate recombined with $S\epsilon$ upstream of the $S\gamma$ sequence so that the latter was deleted from the chromosome. The reciprocal deletion circle resulting from such a recombination would generally contain some $S\mu$ sequence between $S\epsilon$ and $S\gamma$; such composite $S\epsilon$ - $S\mu$ - $S\gamma$ junctions have been observed in circular DNA from B lymphocytes (14), demonstrating indirectly the occurrence of μ - γ - ϵ switching that leaves no $S\gamma$ insert in chromosomal $S\mu$ - $S\epsilon$ junctions.

The primary goal of the present work was to determine which precursor γ subtypes are involved in human μ - γ - ϵ sequential switching. To address this question we determined the sequence of the human $S\gamma 1$, $S\gamma 2$, and $S\gamma 3$ regions, making it possible to deduce the origins of $S\gamma$ sequences in both $S\epsilon$ - $S\gamma$ deletion circles and chromosomal $S\mu$ - $S\gamma$ - $S\epsilon$ switch regions resulting from a sequential switch to IgE. The sequence data also provided interesting

comparisons among the four $S\gamma$ regions and their flanking DNA segments.

Materials and Methods

Cell culture, B cell purification, and DNA preparation

Culture of PBL and preparation of B lymphocyte DNA from these cultures were previously described (36). In brief, PBL obtained from a normal donor by lymphapheresis were separated from residual RBC by centrifugation through Histopaque 77 (Sigma Chemical Co., St. Louis, MO), washed with PBS, and cultured for 4 days in Iscove's medium supplemented with 9% FCS, 2 mM glutamine, 50 μ M β -ME, and antibiotics. The PBL were stimulated with 20 U/ml human rIL-4 (a generous gift of G. D. Searle Laboratories, St. Louis, MO); as a negative control, an aliquot of cells was cultured in the absence of IL-4. B cells were purified from cultured the PBL by positive selection with anti-CD19 magnetic beads and used for DNA preparation.

PCR amplification

For analytical PCR, 500 ng of B cell DNA dissolved in a 25- μ l volume was amplified for a total of 32 cycles as previously described (34). We used a 5' $S\mu$ primer (ggcc TCTAGACAAGGGGACCTGCTCATTTT TATC) containing a four-nucleotide spacer followed by a germline *Xba*I site (italicized) and 24 downstream nucleotides (nucleotides 1–30; Fig. 2 in Ref. 34); and a 3' $S\epsilon$ primer (ggcc gaattcAGTTGCGGTCTGTA CAGCGTGGC) containing a four-nucleotide spacer followed by an artificial *Eco*RI site (italicized) and antisense nucleotides 527–508 from the germline $S\epsilon$ sequence (Fig. 3 in Ref. 37). Amplified samples were electrophoresed, blotted, and hybridized with either a 5' $S\mu$ probe or a $S\gamma$ -specific probe as previously described (28, 34).

A similar procedure was used for preparative amplification of μ - γ - ϵ recombinant switch region DNA prepared from B cells purified from IL-4-stimulated PBL. Two micrograms of DNA was amplified in a 100- μ l volume using 2 cycles with 58°C annealing followed by 40 cycles with 63°C annealing to increase the yield of amplified $S\epsilon$ ($S\gamma$) $S\mu$ chromosomal switch regions.

Amplification and analysis of $S\epsilon$ - $S\gamma$ reciprocal products

A PCR strategy was also used to amplify junctions from $S\epsilon$ - $S\gamma$ deletion circles that would arise as a result of sequential $S\mu$ - $S\gamma$ - $S\epsilon$ switching (see Fig. 9A). A primer 5' to $S\epsilon$ (ggcc tctagaGCTGATCTTGGAAGTC CGAGCTGGGCGACTG) containing a four-nucleotide spacer followed by an artificial *Xba*I site and positions 1041 to 1072 on the germline ϵ sequence; Fig. 4 in Ref. 34) and a consensus primer for a sequence 3' to all the human $S\gamma$ regions (ggcc gaattc CCTGCCTCCCAGTGCTCTG CATTACTTCTG) containing a four-nucleotide spacer followed by an *Eco*RI site and antisense nucleotides 776 to 747 of the consensus 3' $S\gamma$ region (Fig. 2B, below) were used at 0.1- μ M concentrations to amplify 1 μ g of B cell DNA in a 50- μ l volume containing 5 U AmpliTaq DNA polymerase. The reaction buffer was the same as that used for $S\mu$ - $S\gamma$ - $S\epsilon$ chromosomal switch junction amplifications, except that the concentration of $MgCl_2$ was 1 mM instead of 1.1 mM. Two amplification cycles consisting of 1 min at 94°C, 2 min at 68°C, and 6 min at 72°C, were followed by 30 cycles consisting of 1 min at 94°C and 8 min at 72°C.

Following amplification, samples were diluted to 2 ml with water, and DNA was concentrated by centrifuging the solution through a Centricon filter until the volume was 50 μ l. Ten microliters of each sample was then digested for 90 min with *Pst*I, *Pst*I plus *Avr*II, or *Pst*I plus *Hha*I in a final volume of 20 μ l. Following digestion, samples were electrophoresed using Tris-borate-EDTA buffer in a 1.5% agarose gel for approximately 20 h at 50 V. DNA was alkaline-blotted onto Genescreen Plus (DuPont-NEN, Boston, MA) according to the manufacturer's instructions and hybridized at 65°C with a 338-bp *Pst*I fragment located 3' to $S\gamma 2$ (positions 158–496 on the aligned 3' $S\gamma$ sequences; Fig. 2C). The sequence of this probe fragment was greater than 98% homologous among the four human γ subtypes. The blot was washed at 65°C in 2X SSC, 1% SDS, and 10

mM EDTA and autoradiographed at -70°C . The intensities of hybridizing bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or an Ambis two-dimensional ^{32}P scanner (Ambis Systems, San Diego, CA).

Cloning and screening of μ - γ - ϵ switch regions

Preparative amplification products from IL-4-stimulated B cell DNA were digested with *Xba*I and *Eco*RI to expose the restriction sites created by the 5' $S\mu$ and 3' $S\epsilon$ PCR primers, respectively, and electrophoresed on a 0.7% low melting point agarose gel in Tris-borate-EDTA buffer. The region of the gel containing DNA fragments in the size range 0.8 to 4 kb was excised, and amplified DNA fragments were purified, ligated into Bluescript plasmid vector digested with *Xba*I and *Eco*RI, and transformed into *Escherichia coli* HB101 by standard methods (38). Of the transformants, nine distinct clones (designated MGE clones) were purified and restriction mapped to establish the approximate positions of the $S\mu$ - $S\gamma$ and $S\gamma$ - $S\epsilon$ junctions.

Sequencing and sequence analysis

Switch junctions in MGE clones were sequenced using either oligonucleotide primers that correspond to the 5' border of $S\mu$ or antisense primers internal to $S\epsilon$. For some cases (MGE1, MGE24, and MGE42) it was also necessary to clone fragments into M13 mp18 or M13 mp19 to sequence across the $S\mu$ - $S\gamma$ and $S\gamma$ - $S\epsilon$ junctions.

Genomic λ phage clones containing germline human $S\gamma 1$ ($\lambda 89A$), $S\gamma 2$ ($\lambda 38B$), and $S\gamma 3$ ($\lambda 34B$) were generous gifts from Dr. Ilan Kirsch. *Hind*III fragments containing the $S\gamma$ repeat regions plus flanking sequences were isolated from the λ phage DNAs and cloned into Bluescript. To facilitate sequencing of the germline $\gamma 1$, $\gamma 2$, and $\gamma 3$ switch regions, restriction fragments from these *Hind*III plasmid clones were further subcloned. Restriction fragments inserted into Bluescript were used in conjunction with appropriate oligonucleotide primers for sequencing the regions 5' and 3' of the $S\gamma$ repeats, while it was generally necessary to place fragments containing $S\gamma$ repeats into M13 mp18 or 19 to obtain sequence data from these regions. Ordered deletants of M13 subclones created using an Erase-A-Base kit (Promega Corp., Madison, WI) were used to sequence the $S\gamma 1$ repeat region.

All sequencing reactions were performed using Taqsequence sequencing kits (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions, with the modifications that chain extensions and chain terminations were performed at 55 and 70°C , respectively. These modifications were necessary particularly when sequencing in the 5' to 3' direction because of the high GC density of the $S\gamma$ switch regions. All germline $S\gamma$ sequence data were confirmed by analysis of both strands, except in the 1000-bp densely repetitive 3' region of $S\gamma 1$; for this region, sequencing was performed only in the 3' to 5' direction because reliable sequence data could not be obtained in the opposite direction even with Taqsequence.

Dot matrix analysis of sequences was performed using the MacVector program (IBI), while homology searches and alignments were performed using the University of Wisconsin GCG sequence analysis programs (39) implemented at the NCI Frederick Computer Research Facility (Frederick, MD).

Results

Sequences of human germline $S\gamma$ regions

In principle, any of the four human γ genes could function as precursors for μ - γ - ϵ sequential switching as they all lie between the μ and ϵ loci. Moreover, none of the previous reports (14, 28, 33) of sequential μ - γ - ϵ switching in human lymphocytes provide evidence that distinguishes which of the four γ subtypes was involved. To design strategies for identifying $S\gamma$ regions found in chromosomal DNA or in deletion circles resulting from sequential switching, it was necessary to obtain sequence data for all human $S\gamma$ regions.

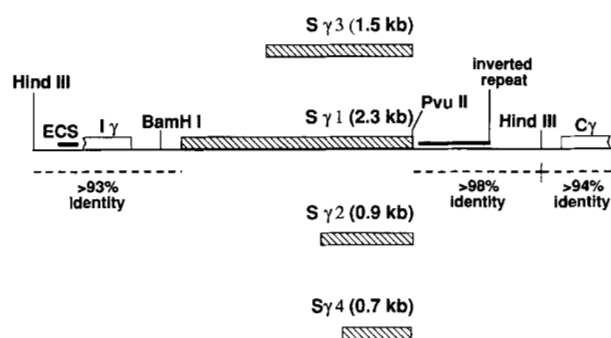


FIGURE 1. Schematic structure of human Ig $S\gamma$ regions, showing sequence similarities. Also shown are the positions of the $I\gamma$ exons (the 5' borders of the $I\gamma$ exons have only been defined for $\gamma 1$ and $\gamma 3$) (19), the evolutionarily conserved sequence (ECS) thought to function as a promoter for $I\gamma$ transcription (19, 34), and an approximately 700-bp segment that is duplicated in an inverted orientation 1600 to 2300 bp upstream from the $S\gamma$ repeats.

The $S\gamma 1$ and $S\gamma 3$ regions and flanking DNA were sequenced and linked to previously published upstream segments (19) to complete the sequence of the 5.0-kb ($\gamma 1$) and 4.2-kb ($\gamma 3$) *Hind*III fragments (40) (Fig. 1). The corresponding 3.6-kb *Hind*III fragment containing $S\gamma 2$ was sequenced in its entirety. In addition to $S\gamma$ sequences, these *Hind*III fragments included the $I\gamma$ exons of the germline γ transcripts that initiate 5' to each $S\gamma$ (22), and the inter- $S\gamma$ - $C\gamma$ regions. Sequence comparisons revealed that the flanking DNA 5' and 3' of the $S\gamma$ regions are highly conserved among the γ subtypes (Fig. 2, A and C, and Fig. 3). The conserved sequences 3' of $S\gamma$ included an approximately 700-bp segment that was also present in an inverted orientation 1600 to 2300 bp upstream from the $S\gamma$ repeats (34, 41) (F. Malissian, unpublished observations). However, the $S\gamma$ regions themselves were less similar, in that they were of different lengths due to different numbers of repeat units and varied in extent of conservation between repeat units. These differences were apparent from visual inspection of the aligned repeat units (Fig. 2B). Furthermore, it was clear from dot matrix comparison plots of each switch region with itself (Fig. 3) that the density of dots corresponding to repeats of similar sequences varied in the order $S\gamma 1 > S\gamma 4 > S\gamma 3 > S\gamma 2$. The 1-kb sequence comprising the 3' portion of $S\gamma 1$ contained an array of identical or nearly identical 79-bp repeats. Although the three other human $S\gamma$ regions had similar repeat units, they were not present in such a regular array. The regular $S\gamma 1$ repeats appeared to be prototypic for units in the other $S\gamma$ regions, as indicated by the fact that dot matrix comparisons of $S\gamma 1$ with $S\gamma 2$, $S\gamma 3$, and $S\gamma 4$ gave denser dot patterns than dot matrix comparisons for any of these three $S\gamma$, either with itself or with any other $S\gamma$ (Fig. 3).

A

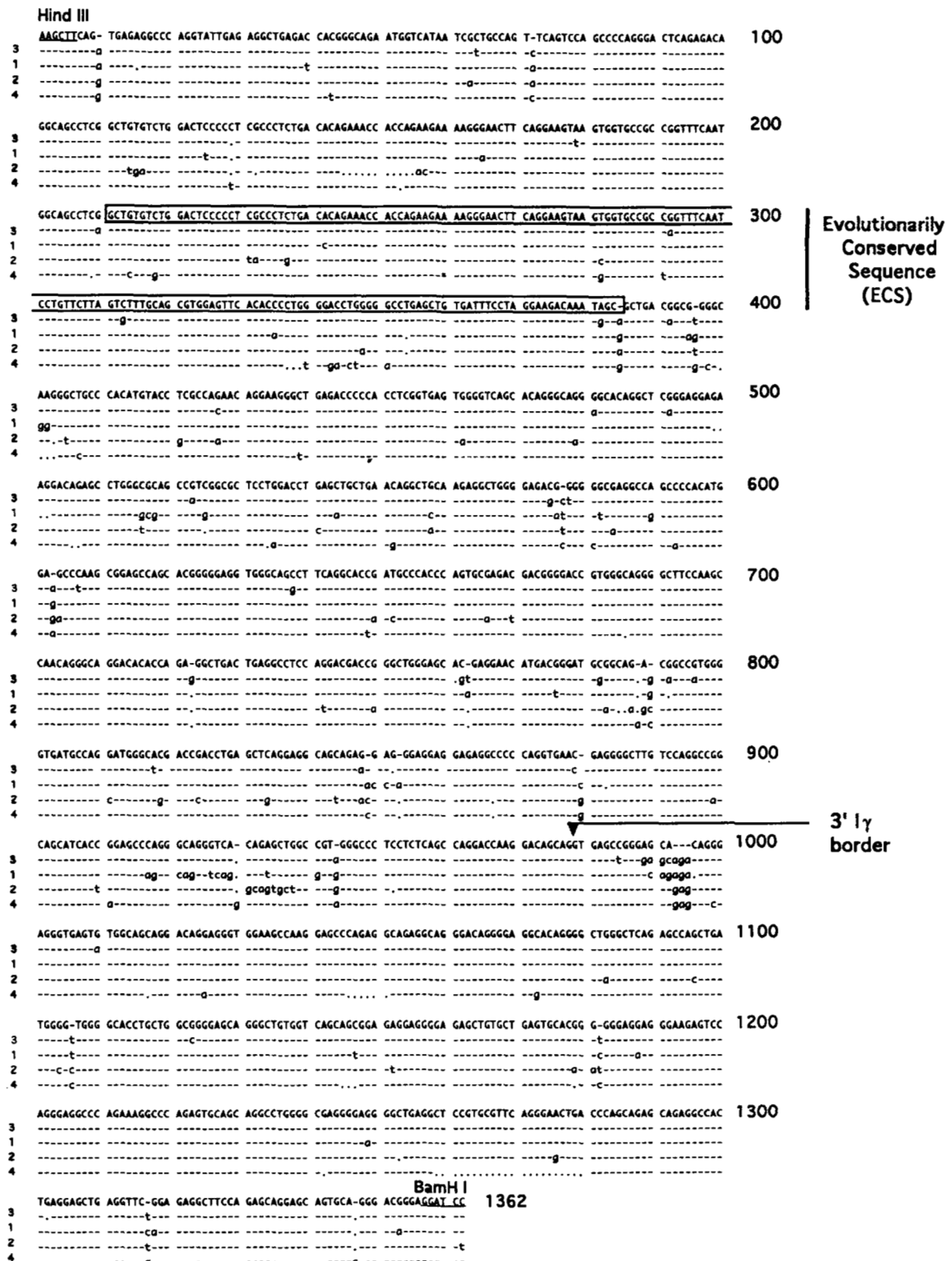
5' Human γ sequences:Hind III-BamH I

FIGURE 2. Aligned human 5' γ sequences, γ repeats, and 3' γ -C γ sequences. A, 5' γ sequences, including I γ exons, and putative promoters for sterile transcripts; B, γ regions, showing aligned repeat units; C, 3' γ -C γ sequences.

FIGURE 2. *continued*

C

3' Human S γ sequences: Pvu II-Hind III

Pvu II				
3	CAGCTGAGGG	CTGGTGAAG	TGCAGTGCAG	CTCTCTGGTG CCGGGAAGGG AGTGTGAG-C CATCCCACTG AGCAGTTGGC AAGGG-GAGC TGGGATGGAG
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	AAGGGAAGGC	ATTCACGGGC	TCGGGGCTGA	GCTCTCAGGC AGGGGCAGGT GTGGCTGCAG GGGGAACGTG TGCTTGAGAC CAGGAGGGTC CCA-GGCTGG
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	CCACAGCGGA	CCCTGGGCG	GAAGGCTCT	GAGGCTGG-G CCCCAGAAGG AGCAAGATGG GCTGCCAGGA GCCAGGGCCA CCAGACAAT GAAGCTGAGT
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	GGAGTGGTG	CAGGGCAGTG	TAGCAGCAGA	GGGC-GCCAG AGGGGCCCAT TCAGGGCCTG GGCAGAGTCA GCCAGAGCCT GTGGTGCAGG TGAGGGGAAG
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	GGTGGTGAG	CGGGGCCCTG	GGGCTGAGCA	GAGGGGATGG CCTGGCTGAG GGCAGGGCGC TTAGCCTCTC CAGAGGTGAG GGGCACACCC CACCTGCAGT
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	GGGACTCCAG	GGCCACTGGG	CCAGCGGCAG	AGAGAAATGG GGCCTCCCTG TGCCCTGGGG GTCTGGCAC CA-GCAGGGT GGGGAGGGCC AAGGGCAGGT
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	GCAAGGCTCC	TACCTGTGCT	GGGGGGCCTG	GCTTGAGCCC AGCAGGGGAC TTGCCGGGGG AAGCTCTGGA GAGAGGGAGG AGGTGGGCTG GTGGCCGAGA
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	AGGCCAGGCC	AGGGCTGGGA	GGGTGAGGTT	GTGGTGAAGT AGCCTCCAGA AGTAATGCAG GACACTGGGA GGCAGGGGGC ATCCAGGCAC TCAGGGCCCT
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	-GACCTGGGC	TGCTGCACAC	TGGGGCTAAG	GGGAAAGGAG GGGAGAGGCT GAGGAGGAGG CTCC-GGGGC G-TATTCCAA GGCAGGGGGT TCCGGGGCCC
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	TGGGGCTGAA	GGGGCCCGA-	CCCTATGCAG	TGTCTGGCCC CTCTGTGCA CAGAAGAAAA GGGCCTTGGA GGGCAGAGGG CAGGCTATGA CCAGGGCCCT
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	GGGCAAGTCA	GGCCCACTCA	CTAGGGGAGG	GCCACGCTGG GCGGGCAGGG TCAGGGGCTT CAGGGGGGTC GGGGACCCCA CCAGAGGCCA TCTGAGAACA
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	GTGTCCACTG	GTCAAGCCAG	GCACCCATAA	AAGGCTGGAG TGGGGCCAAT GGGCATGAGC CGTCCCTGAG GTGGCACCGA TGGCCAGAGC TGAGGCCAAG
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	CTAGAGGCCC	TGGACTGTGC	TGACTCCCGG	CAGACA-AGA GCGCTGACCT GGCTCCGAG CCCCCTCTCC TAGGCTGCAG G-GGTGCTTG CAGAAGGGCA
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----
3	CCACAGGGCC	ACGGTCTCTG	CAAGCTT	1327
1	-----	-----	-----	-----
2	-----	-----	-----	-----
4	-----	-----	-----	-----

inverted
repeat of
5' S γ segment

FIGURE 2. continued

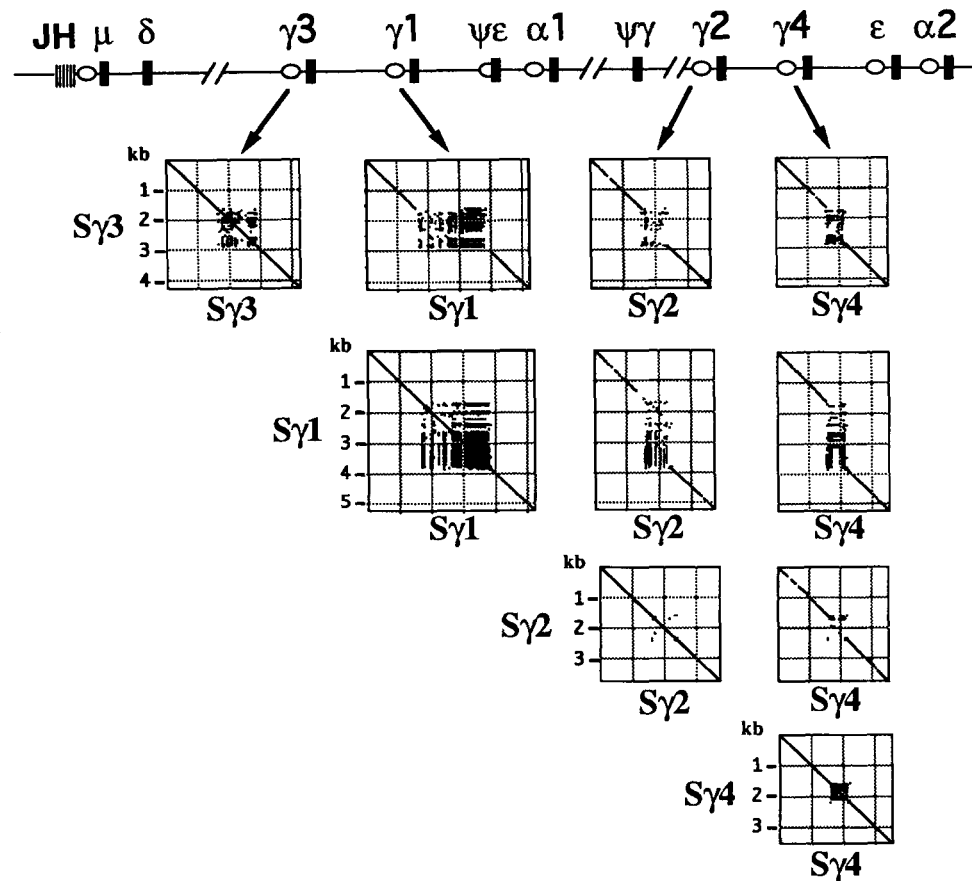


FIGURE 3. Dot matrix comparison of human $S\gamma$ regions. This analysis provides a visual pairwise comparison of the sequence similarities among the four human $S\gamma$ regions. In each matrix comparison, the central concentration of dots represents the $S\gamma$ repeat regions. The high density of dots in comparisons of $S\gamma 1$ with $S\gamma 3$, $S\gamma 2$, and $S\gamma 4$ suggests that the human $S\gamma 1$ repeats may be prototypic for repeats in the other three switch regions.

Cloning and characterization of recombined $S\mu$ - $S\gamma$ - $S\epsilon$ switch regions

To determine the γ subtype(s) participating in μ - γ - ϵ switching, we sequenced some $S\mu$ - $S\gamma$ - $S\epsilon$ composite switch regions and identified the $S\gamma$ inserts by comparison with the germline $S\gamma$ sequences. PBL were obtained from a normal donor by lymphapheresis and cultured with IL-4 (36). B cells were isolated from this culture, and DNA from the cells was subjected to a PCR strategy (Fig. 4A) designed to amplify recombined $S\mu(S\gamma)S\epsilon$ switch regions. Figure 4B shows blots of the amplification products hybridized with either a 5' $S\mu$ probe that detects all amplified switch regions or an $S\gamma$ probe that should detect amplified switch regions containing $S\gamma$ sequences interposed between $S\mu$ and $S\epsilon$. A subset of amplified switch regions hybridized to the $S\gamma$ probe, indicating that sequential $S\mu$ - $S\gamma$ - $S\epsilon$ switching occurred in this culture.

Chromosomal $S\mu$ - $S\gamma$ - $S\epsilon$ switch regions in DNA from the IL-4-treated culture analyzed in Figure 4 were amplified preparatively for 40 cycles of PCR, and products in the size range 0.8 to 4 kb were purified and cloned. About 80% of the random picks of the resulting transformants were shown by

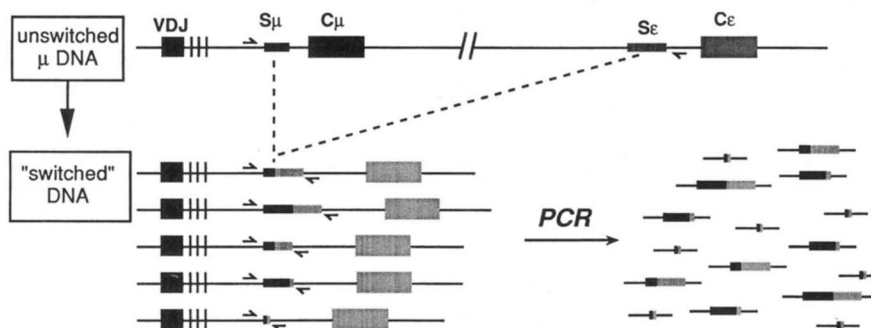
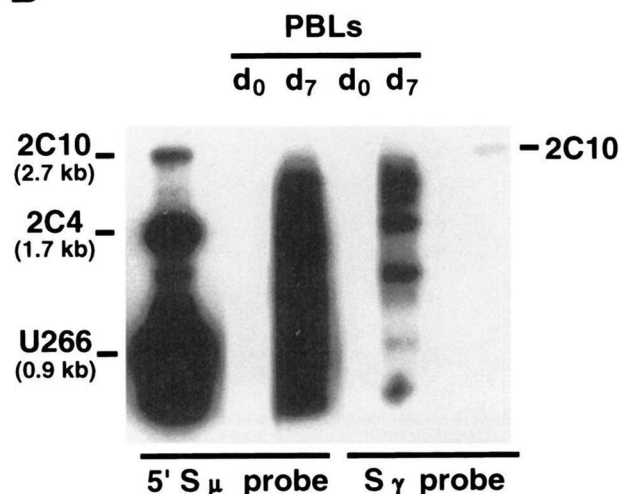
restriction mapping to have the predicted 5' $S\mu$ and 3' $S\epsilon$ ends. When 8000 colonies were plated and screened with the $S\gamma$ -specific probe (28, 34), approximately 1% hybridized, and nine distinct $S\gamma$ -hybridizing clones (designated MGE to denote μ - γ - ϵ switching) were purified for analysis.

For each MGE clone, the approximate positions of $S\mu$ - $S\gamma$ and $S\gamma$ - $S\epsilon$ junctions were determined by restriction mapping (Fig. 5), and all junctions were sequenced. All of the clones contained $S\gamma$ -like sequences. The 3' boundaries of the $S\mu$ sequence in six of the nine MGE clones fell within a 400-bp region near the 5' border of the $S\mu$ repeat sequences. This localization of switch junctions was similar (although slightly 5' to) a potential preferred region for human $S\mu$ - $S\epsilon$ switching described previously (7, 28, 35). In addition, two of the clones (MGE1 and MGE42) had $S\gamma$ sequences that were inverted relative to the $S\mu$ and $S\gamma$ sequences that flanked them, suggesting that these clones arose from initial inversional, rather than deletional, rearrangement from $S\mu$ to $S\gamma$, followed by a deletion switch between $S\gamma$ and $S\epsilon$.

To determine the germline origin of $S\gamma$ sequences at the $S\mu$ - $S\gamma$ and $S\gamma$ - $S\epsilon$ junctions of the MGE clones, these $S\gamma$

A**Direct PCR Assay: mixed cell population**

FIGURE 4. PCR amplification of chromosomal $S\mu(S\gamma)S\epsilon$ switch regions in human B cells purified from PBL cultures treated with IL-4 and harvested before culture (d_0) or after 7 days (d_7). **A**, Strategy for PCR amplification of recombined chromosomal switch regions; **B**, amplification products were hybridized as indicated with either a 5' $S\mu$ probe to detect all $S\mu(S\gamma)S\epsilon$ products or an $S\gamma$ probe to specifically detect $S\mu-S\gamma-S\epsilon$ sequential switch products. DNA from three IgE-producing cell lines (2C10, 2C4, and U266) was included as an amplification and hybridization control: products from all three cell lines hybridize with the 5' $S\mu$ probe, while only the 2C10 product contains $S\gamma$ sequence and hybridizes to the $S\gamma$ probe (28). The amplification products from these cell line DNAs also function as size markers.

**B**

segments were aligned with the four germline $S\gamma$ sequences. Slight differences between our germline $S\gamma$ sequences and the $S\gamma$ inserts in our MGE clones may be attributed to polymorphisms, mutations introduced during recombination (42), or PCR errors, but these differences did not prevent $S\gamma$ subtype assignment. In all but two cases, the highest similarity score was obtained with alignment to sequences in $S\gamma 1$ (Table I), indicating that these MGE $S\gamma$ sequences were derived from $S\gamma 1$. The $S\gamma$ sequence in MGE 42 was clearly derived from $S\gamma 3$, while the $S\gamma$ sequence in MGE 1 showed 89% similarity to $S\gamma 4$, but also showed 88% similarity to $S\gamma 1$, so the assignment to $S\gamma 4$ is equivocal. In addition, the $S\gamma$ sequence previously published for the switch region of the EBV-transformed cell line 2C10 (28) clearly was derived from $S\gamma 1$.

One $S\mu-S\gamma 3$ junction (MGE 42) and one $S\gamma 1-S\epsilon$ junction (MGE7) lay in the $S\gamma$ locus slightly 3' from the S repeat units. All of the other $S\mu-S\gamma 1$ and $S\gamma 1-S\epsilon$ junctions lay within the regular 79-bp repeat units found in the 3' portion of $S\gamma 1$ (Fig. 6). Although PCR is biased toward small products, this bias would be expected to limit the

total size of the $S\gamma 1$ segment interposed between $S\mu$ and $S\epsilon$, while the ends of these segments could still occur at random positions in $S\gamma 1$ if there were no preferred sites for switch recombination. Therefore, it is likely that the localization of both $S\mu-S\gamma 1$ and $S\gamma 1-S\epsilon$ junctions reflects a real preference for switching within the 3' $S\gamma 1$ region.

Features of the $S\mu-S\gamma$ and $S\gamma-S\epsilon$ junctions in MGE clones

Compiled in Figure 7 are $S\mu-S\gamma$ junctions and $S\gamma-S\epsilon$ junctions from the MGE clones as well the junctions from the 2C10 cell line. Except for the junction from 2C10, all of the $S\mu-S\gamma$ junctions represent a precise joining between $S\mu$ and $S\gamma$. On the other hand, five of 10 $S\gamma-S\epsilon$ junctions (including 2C10) have at least one intervening nucleotide not found in either the germline $S\gamma$ or $S\epsilon$ sequences (the identity of three nucleotides at the $S\gamma-S\epsilon$ junction for the MGE 11 clone could not be determined due to sequence compression).

The tetranucleotide CTGG/CCAG has been reported to occur within 7 bp of switch junctions more frequently than

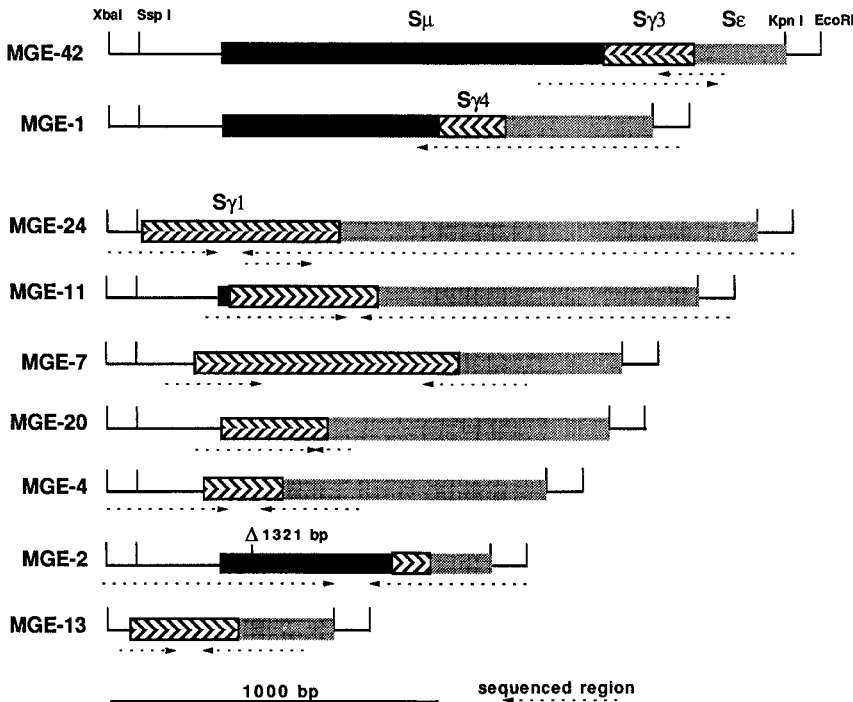


FIGURE 5. Structure of MGE clones. Black tone indicates $S\mu$ sequence, herringbone pointing right indicates $S\gamma$ sequence in a germline 5' to 3' orientation, herringbone pointing left indicates $S\gamma$ in an inverted orientation, and gray tone indicates $S\epsilon$.

Table 1. Similarity of MGE $S\gamma$ segments to germline $S\gamma$ sequences

Clone		% Identity				Length segment
		$S\gamma 3$	$S\gamma 1$	$S\gamma 2$	$S\gamma 4$	
MGE13	5'	87	96	82	86	165
	3'	84	91	85	81	163
MGE2 (complete)		83	92	74	83	244
MGE4	5'	93	100	88	86	73
	3'	93	100	96	96	27
MGE20	5'	88	97	81	81	239
	3'	88	98	90	91	63
MGE7	5'	87	98	87	89	217
	3'	98	100	95	99	87
MGE1 (complete)		85	88	85	89	191
MGE11	5'	91	99	84	87	240
	3'	85	96	85	87	53
MGE24	5'	86	99	86	90	216
	3'	100	100	100	100	22
MGE42 (complete)		98	90	86	87	272

expected by chance (43). Examination of the MGE switch junctions (Fig. 7) revealed that the CTGG/CCAG motif was found at least once within 7 bp of the recombination site in seven of 10 $S\mu$ - $S\gamma$ junctions as well as 7 of 10 $S\gamma$ - $S\epsilon$ junctions. However, because of the high frequency of the CTGG/CCAG sequence in the human germline $S\mu$, $S\gamma 1$, and $S\epsilon$ switch regions, the occurrence of this motif in the MGE switch junctions was not significantly greater than would be expected by chance according to a statistical analysis similar to that performed by Chou and Morrison (43).

Our data indicate that the prototypic 79-bp repeats in the 3' $S\gamma 1$ region are highly favored as sites for human $S\mu$ - $S\gamma$ and $S\gamma$ - $S\epsilon$ switching. These 79-bp repeats include a region

that resembles the consensus mouse 49-bp $S\gamma 1$ and $S\gamma 3$ repeats (Fig. 8), including partial similarity to nuclear protein-binding sites (SNIP(NF κ -B)/SNAP) described in mouse $S\gamma$ regions (44, 45). However, in contrast to what has been reported for mouse switch junctions, the MGE junctions are not clustered within the putative human homologues of the SNIP/SNAP motifs.

Analysis of $S\epsilon$ - $S\gamma$ junctions from reciprocal circles

An $S\mu$ - $S\gamma$ switch recombination may be followed by a deletion that removes all of the $S\gamma$ sequence from the final composite switch region. Therefore, the $S\mu$ - $S\gamma$ - $S\epsilon$ products that we detected are likely to represent only a subset of the $S\mu$ - $S\epsilon$ junctions that recombined sequentially via an $S\gamma$ intermediate. Although the $S\gamma$ segments retained in our MGE clones were predominantly $S\gamma 1$ derived, it seemed possible that other $S\gamma$ regions participate in sequential switching to ϵ , but leave proportionately few $S\gamma$ inserts in the composite $S\mu$ - $S\gamma$ - $S\epsilon$ junctions. Therefore, we undertook an unrelated complementary strategy to assess the γ subtype involvement in switching to ϵ , i.e., analysis of the origin of $S\gamma$ segments in reciprocal $S\epsilon$ - $S\gamma$ deletion circles.

For this analysis, PBL were obtained (from the donor from whom our MGE clones were derived) and cultured with IL-4 for 6 days as described previously; DNA was then extracted from purified B cells and subjected to a PCR protocol (Fig. 9A) designed to amplify $S\epsilon$ - $S\gamma$ junctions characteristic of reciprocal deletion circles. To assess the relative contributions of the four human γ subtypes to sequential $S\mu$ - $S\gamma$ - $S\epsilon$ switching, we exploited subtype-specific restriction sites that our sequence analysis had

Positions of MGE S γ 1 Sequences

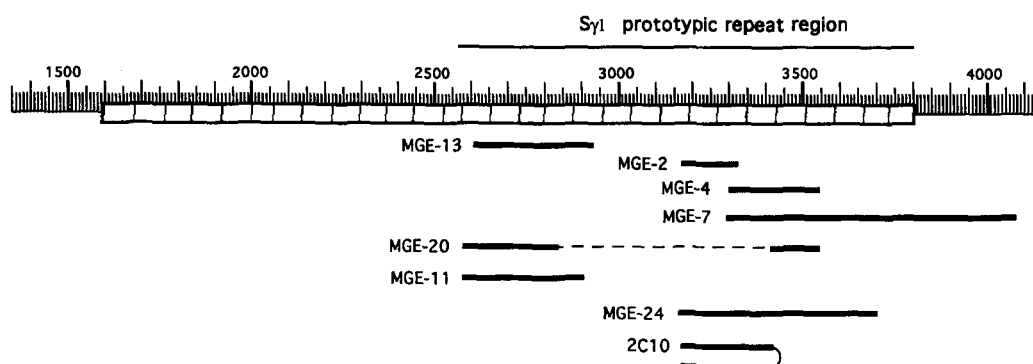


FIGURE 6. Location of MGE S γ 1 sequences within S γ 1. The scale indicates the distance in base pairs from the *Bam*HI site 5' of S γ 1. Open rectangles indicate individual repeat units within S γ 1.

detected within a $\sim$ 339-bp *Pst*I restriction fragment downstream of all four S γ regions (Fig. 9B). Figure 9C shows undigested amplification products as well as products digested with *Pst*I either alone or in combination with *Avr*II or *Hha*I. The completeness of the *Avr*II and *Hha*I digestion was verified by internal plasmid controls. Sequential switch circles arising from all four human γ subtypes were predicted to yield 337-bp (γ 4), 338-bp (γ 1, γ 3), or 339-bp (γ 2) *Pst*I fragments upon digestion with *Pst*I alone. S ϵ -S γ 3 circles should produce a unique 301-bp *Pst*I-*Hha*I fragment, S ϵ -S γ 1 circles should produce a 338-bp *Pst*I fragment that is resistant to digestion by either *Avr*II and *Hha*I, and S ϵ -S γ 2 circles should yield a unique 282-bp *Avr*II-*Pst*I fragment. Finally, both S ϵ -S γ 2 and S ϵ -S γ 4 circles should yield 251- and 219-bp *Hha*I fragments that result from heterozygosity for a polymorphic *Hha*I site within both the 339-bp γ 2 *Pst*I fragment and the 337-bp γ 4 *Pst*I fragment of this donor. The polymorphic nature of this *Hha*I site was demonstrated in DNA samples from 10 unrelated individuals by *Pst*I plus *Hha*I digestion of PCR products generated using subtype-specific primers located in the C γ hinge regions (22), and this methodology was also used to verify all subtype-specific restriction sites shown in Figure 9B for the particular donor analyzed in this paper (data not shown).

Based on radioimage analysis of the hybridized blot, approximately 10% of the 337–339 *Pst*I fragments were resistant to *Hha*I digestion, suggesting that S ϵ -S γ 1 deletion circles make only this small contribution to the amplified products. No 301-bp *Pst*I-*Hha*I fragment corresponding to γ 3 was detected, suggesting minimal participation of this subtype in sequential switching to ϵ . Ap-

proximately 50% of the $\sim$ 338-bp *Pst*I fragments were digested by *Avr*II, indicating that γ 2 contributes 50% of the *Pst*I fragments; since there is no detectable γ 3 contribution, the remaining 50% of *Avr*II-resistant *Pst*I fragments must arise from γ 1 and γ 4. Because only 10% of the total S ϵ -S γ products involve γ 1, roughly 40% of the S ϵ -S γ products must derive from γ 4.

The majority of the amplified bands were cut by *Pst*I, but yielded fragments larger than $\sim$ 338 bp. Such bands were probably produced by recombination events occurring downstream of the S γ switch repeats, deleting one or both *Pst*I sites that delimit the $\sim$ 338-bp fragment. Evidence of recombination downstream of the S γ regions has previously been seen in cloned human S ϵ -S γ reciprocal products (14). Our estimation of the γ subtype distribution above applies to the subset of amplification products containing an intact $\sim$ 338-bp *Pst*I fragment (see *Discussion*).

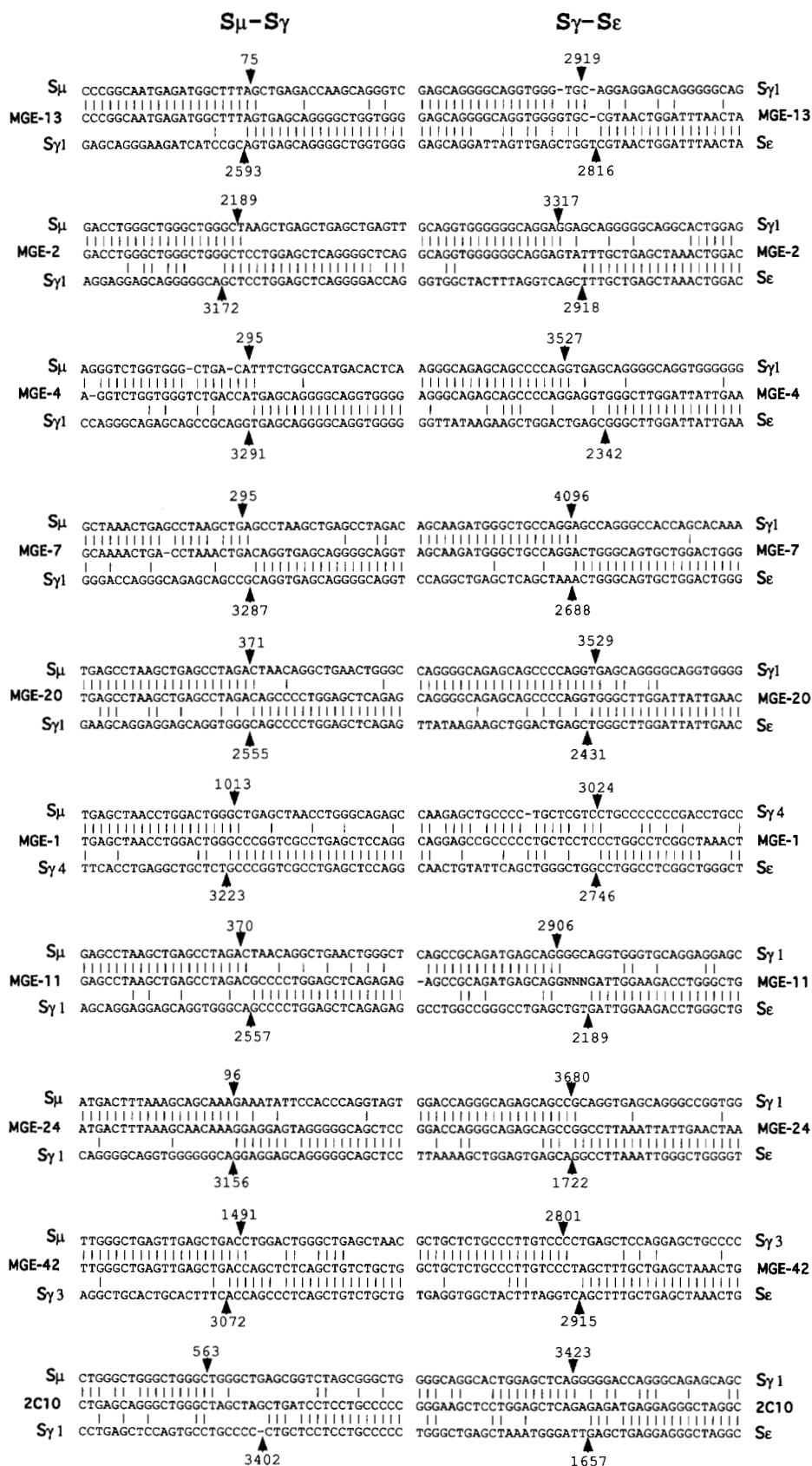
Discussion

Subtype comparisons among human S γ region sequences

Our sequence data on the human S γ 1, S γ 2, and S γ 3 regions along with the previously published S γ 4 sequence allow comprehensive comparisons among all four functional human S γ regions. A striking feature of human γ loci is that, excluding the S γ repeats, the four loci are highly conserved, with a region of 93% identity extending 1300 bp 5' to each S γ , greater than 98% identity in the region between S γ and C γ , and 94% identity in the constant regions (Figs. 1, 3, and 4). Moreover, for the γ 3 and

FIGURE 7. MGE switch junctions and junction breakpoints. Shown are S μ -S γ and S γ -S ϵ junctions for each MGE clone. Numbering for S μ starts with the *Xba*I site immediately 5' of the S μ pentameric repeats (34), numbering for S γ sequences starts with the *Hind*III site upstream from the S γ repeat units (Figs. 1 and 2, this paper), and numbering for S ϵ starts from the *Bam*HI site 5' of the S ϵ pentameric repeats (34).

MGE SWITCH JUNCTIONS



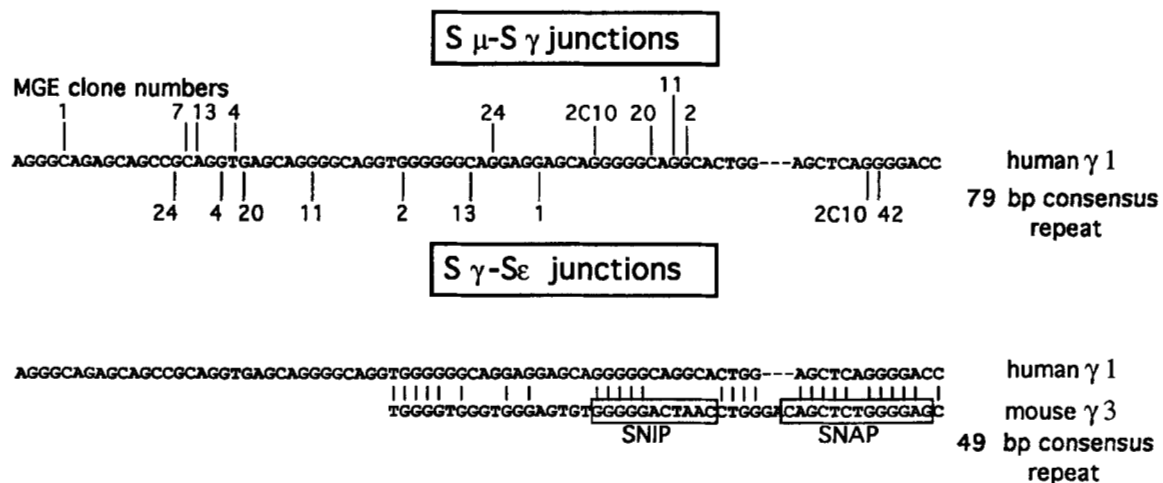


FIGURE 8. Location of MGE $S\mu$ - $S\gamma$ and $S\gamma$ - $S\epsilon$ junctions in the consensus 3' $S\gamma$ 1 repeat unit. Shown is an alignment with the mouse $S\gamma$ 3 repeat unit (50), which differs slightly from the consensus $S\gamma$ 3 repeat defined by Szurek et al. (51).

γ 4 loci, close similarity has been shown to extend more than 3000 bp 5' from $S\gamma$ (41), and published restriction maps indicate that similarity also extends far upstream from $S\gamma$ 1 and $S\gamma$ 2 as well as downstream from the $C\gamma$ (40).

It is puzzling that the regions containing the putative promoters for $I\gamma$ sterile transcripts (positions 211–385 in Fig. 2A) (19) are highly conserved among all γ subtypes, even though the individual γ genes show different levels of $I\gamma$ transcription in B cell tumors (46) and in primary B cells stimulated with cytokines (22). It is possible that the small differences within the putative $I\gamma$ promoters are sufficient to mediate subtype-specific transcriptional regulation. Alternatively, the switch regions themselves may mediate some control over human γ gene expression, or there may be subtype-specific control elements that lie outside both the $I\gamma$ promoters and the switch regions (46, 47).

In contrast to their flanking sequences, the $S\gamma$ repeat regions differ significantly from one another in several ways. Whereas the 79-bp repeats of $S\gamma$ 1 are extremely precise, especially in the 3' part of the sequence, the precision of the repeats is considerably less in the other subtypes, in the order $S\gamma$ 1 > $S\gamma$ 4 > $S\gamma$ 3 > $S\gamma$ 2. The $S\gamma$ switch regions also vary greatly in length due to different numbers of repeats, from 27 repeats in $S\gamma$ 1 to eight repeats in $S\gamma$ 4. These differences in the character and number of repeats could strongly influence relative γ subtype participation in $S\mu$ - $S\gamma$ (and $S\gamma$ - $S\epsilon$) recombination both by altering the efficiency with which individual repeat units are used as substrates for recombination machinery and by altering the number of recombination targets. Moreover, as previously mentioned, it is conceivable that the structure of a given $S\gamma$ switch region affects transcription.

The contrast between the high conservation of DNA flanking the human $S\gamma$ regions and the relatively low conservation observed in the switch regions themselves suggests that the rate of sequence change in the $S\gamma$ regions

may be higher than that in the flanking regions, perhaps reflecting an enhanced mutation rate induced by the tandem repeat structure or selection for sequence divergence of the $S\gamma$ subtypes. We have also examined pairwise subtype comparisons between homologous DNA segments flanking the $S\gamma$ subtypes in the hope of deducing the evolutionary order of duplication, i.e., did a γ - γ - ϵ - α segment duplicate or did a γ - ϵ - α segment duplicate followed by two individual duplications (resulting in the γ 3- γ 1 and γ 2- γ 4 pairs). All of these pairwise comparisons show nearly identical degrees of sequence divergence, preventing an unequivocal determination of the order of duplications. This result is consistent with a scenario in which the two types of duplication occurred within a relatively short interval of evolutionary time.

S γ subtypes involved in sequential switch to IgE expression

When our earlier studies demonstrated the existence of $S\mu$ - $S\gamma$ - $S\epsilon$ composite switch regions in IL-4-treated human B cells switching to IgE, one attractive hypothesis was that the $S\gamma$ region involved was that of the γ 4, since expression of this subtype is also induced by IL-4 (48); this model would be similar to the murine system in which sequential switching to IgE clearly involves γ 1, the murine γ subtype whose expression is induced by IL-4 (11, 30, 31). Indeed, Jabara et al. (33) described a clone of the MGE type whose $S\gamma$ insert they attributed to $S\gamma$ 4. However, unequivocal attribution of $S\gamma$ inserts in composite switch regions requires knowledge of all four $S\gamma$ sequences, which is only now available. By comparing the sequences of cloned $S\mu$ - $S\gamma$ - $S\epsilon$ composite switch regions with the complete germline $S\gamma$ sequences, we can define $S\gamma$ 1 as the origin of $S\gamma$ sequences in seven of our nine MGE clones as well as in the previously described IgE-expressing EBV line 2C10.

Furthermore, the sequence of the S γ insert in the MGE-like clone HJ35 reported by Jabara et al. (33) is identical with our germline S γ 1 sequence (although differing by only 1 bp from either S γ 2 or S γ 4). These results suggest that γ 1 is the subtype most frequently involved in human μ - γ - ϵ switching.

As an alternative and independent strategy for obtaining information about the molecular pathways involved in sequential switching, we analyzed amplified reciprocal S ϵ -S γ composite switch regions that are formed by DNA deleted from the chromosome during recombination between an S μ -S γ composite switch region and S ϵ . A Southern blot strategy allowed a rough estimate of the γ subtype contribution to the S ϵ -S γ products: 0% γ 3, 10% γ 1, 50% γ 2, and 40% γ 4.

The different subtype estimates resulting from the two strategies we used highlight the importance of considering the limitations of the two methods. Both strategies require amplification of composite switch junctions and are, thus, affected by the bias for amplification of smaller templates. This bias could cause underrepresentation of the larger S ϵ -S γ reciprocal junctions that might be formed from the larger switch regions such as S γ 1, although it is not obvious how it could misrepresent the subtype distribution of S γ inserts in MGE clones. Another possible source of bias is that the MGE analysis does not see sequential switches in which the S γ segment is deleted from the final S μ -S ϵ product; such deletions might occur more frequently for some subtypes than others. The analysis of γ subtypes in S ϵ -S γ reciprocal junctions is subject to a different kind of bias, in that it depends on detecting deleted DNA segments that have rejoined their ends to form DNA circles. While such S ϵ -S γ reciprocal junctions are clearly seen in our studies and in other reports of reciprocal circles (11, 14, 30), it is not known how frequently the reciprocal ends actually join together to form DNA circles. In vitro ligation of naked DNA occurs less efficiently for larger DNA fragments, whose ends are less likely to "find each other," than for shorter fragments (49); if a similar bias affected the circularization of DNA segments generated by switch deletion, it might cause less efficient circularization of γ 3- ϵ deletion segments (~190 kb) and γ 1- ϵ segments (~160 kb) than γ 2- ϵ (~50 kb) or γ 4- ϵ (~30 kb). Such a bias might contribute to the lower representation of γ 3 and γ 1 that we observed in our S ϵ -S γ reciprocal products compared with that of γ 2 and γ 4. Small circles might also be more stable in the cell than larger circles. Finally, as mentioned above, our analysis of the subtype distribution of these products is based on the subset that has retained both of the *Pst*I sites used in our analysis.

These uncertainties make it impossible to derive from our data an accurate quantitation of the relative γ subtype involvement in sequential switching to ϵ . However, on a qualitative level, the available data are consistent with a model in which, for humans, multiple switching pathways lead ultimately to IgE expression, with switching occur-

ring from all four γ subtypes (our data) and α (14) as well as directly from μ (50) (N. Harindranath, unpublished observations); in the mouse, current literature supports a single predominant pathway (30, 31).

Inversions in μ - γ switching occur at an appreciable frequency

Two of our nine MGE clones (MGE1 and MGE42) contain S γ sequences that are inverted relative to S μ and S ϵ , indicating an initial S μ -S γ switch recombination resulting in inversion, rather than deletion of the region between S μ and S γ . In the inverted configuration, the H chain locus could not encode a functional protein. An inversional switch event between S μ and S γ has been documented previously in 2C10, an EBV-transformed human cell line that had undergone sequential μ - γ - ϵ switching (28). The S γ insert in that clone also showed a peculiar palindromic structure that is not seen in any of the MGE clones. The 22% frequency of inverted S γ regions in the MGE clones (two of nine) is similar to the 15% frequency of inversional S μ -S γ switch events observed in human chronic B cell leukemias (51) and reinforces the view that H chain class switching occurs via intrachromosomal recombination rather than by sister chromatid exchange (9). Furthermore, the finding that an appreciable number of S μ -S γ -S ϵ switch events resulted in the "rescue" of S μ -inverted S γ events by subsequent switching to S ϵ argues that the switch recombination machinery is flexible in its substrate requirements.

Timing of the S μ -S γ and S γ -S ϵ recombination events

The switch junction structures we studied provide evidence for two separate recombination events, but do not supply any information about the timing of their occurrence in relation to each other or other events in the cell. The MGE clone structures are compatible with an initial S γ -S ϵ recombination followed by joining of S μ to the S γ -S ϵ junction; the S ϵ -S γ reciprocal products are also compatible with such a scenario. However, no evidence for such a sequence of events has been reported for either mouse or human. The murine data clearly argue for an initial μ - γ switch, followed by recombination to ϵ (30), and we presume that this is the most common sequence in humans. A more relevant question is whether the B lymphocytes in our experiments began their incubation in tissue culture as unswitched IgM-producing cells and underwent two recombinations in culture to form S μ -S γ -S ϵ junctions or, alternatively, were IgG producers at the start of culture and underwent a single recombination to S ϵ . Published data indicate that the four human γ loci are activated for switching by combinations of cytokines and mitotic agents, which are different from the conditions used in our experiments (22), and that IgG-expressing cells are poor precursors for IgE production (50). However, these reports do not exclude the possibility of μ - γ

switching occurring under our conditions at low levels that our amplification and screening techniques would be capable of detecting. Additional experiments will be necessary to clarify these issues as well as to determine the relative frequency of direct vs sequential switching to ϵ .

Acknowledgments

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