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Analysis of immunoglobulin Sγ3 recombination breakpoints by PCR: implications for the mechanism of isotype switching

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ABSTRACT

The molecular mechanism of immunoglobulin switch recombination is poorly understood. Switch recombination occurs between pairs of switch regions located upstream of the constant heavy chain genes. Previously we showed that switch recombination breakpoints cluster to a defined subregion in the Sγ3, Sγ1 and Sγ2b tandem repeats. We have developed a strategy for direct amplification of Sμ/Sγ3 composite fragments as well as Sμ and Sγ3 regions by PCR. This assay has been used to analyze the organization of Sμ, Sγ3 and a series of Sμ/Sγ3 recombination breakpoints from hybridomas and normal mitogen-activated splenic B cells. DNA sequence analysis of the switch fragments showed direct joining of Sμ and Sγ3 without deletions or duplications. Mutations were found in two switch junctions on both sides of the crossover point, suggesting that template switching is the most likely model for the mechanism of switch recombination. Statistical analysis of the positions of the recombination breakpoints in the Sγ3 tandem repeat indicates the presence of two sub-clusters, suggesting non-random usage of DNA substrate in the recombination reaction.

INTRODUCTION

Immunoglobulin (Ig) variable regions are encoded by germline gene segments V, D and J, which are assembled during early B cell development by the process of V(D)J joining (1). The VDJ portion of the Ig protein is responsible for antigen binding while the constant (C) region is involved in biological effector function. The VDJ region is initially expressed with the Cμ region to produce IgM. The murine Ig heavy (IgH) locus is composed of eight constant region (C_H) genes which are arrayed as follows: 5′-VDJ-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′ (2). When B cells are stimulated by antigen and the appropriate lymphokines they produce new isotypes while maintaining the same antigen binding specificity. This phenomenon, the IgH chain class switch, permits expression of a variable region with new constant regions (3–5). The recombination event focuses on the switch (S) DNA, regions of repetitive sequence upstream of each C_H gene (with the exception of Cδ) and produces a new hybrid DNA combination, Sμ-Sx. The hybrid Sμ-Sx DNA configuration is formed on the chromosome while the intervening genomic material is looped out and excised as a circle, confirming the importance of switch regions in the recombination process (7–11).

Sequence analyses of the switch recombination joints of switched IgH genes from normal B cells and switch circles have revealed that for both the Sμ (donor) and acceptor S region breakpoints fall within the tandem repeats (7–10,12). No obvious consensus recombination signal sequences have been identified, though the pentamers GAGCT, GGGGT and GGTGG, components of all the switch regions, are often found at or close to recombination joints (6,12). Sequence comparison of S DNAs has shown that while all are highly repetitive, there has been significant sequence divergence between them (6). Several views on the mechanism of switch recombination have been articulated, which include switch-specific recombination, homologous recombination, illegitimate priming, template switching and illegitimate recombination (12–18). It is currently unclear which of these models best describes the mechanism of switch recombination.

Multiple proteins bind specific sites in the DNA and are required for alignment of DNA recombination regions in highly ordered structures (19,20). We have identified a DNA binding protein complex, SNUP, which specifically recognizes a site in the Sμ tandem repeat (21), and two DNA binding proteins, SNIP and SNAP, which specifically interact with motifs within the Sγ tandem repeats (22). SNIP is indistinguishable from NF-κB p50 homodimer, a member of the rel family of transcription factors, while SNAP contains epitopes in common with E47, a helix–loop–helix transcription factor (22,23). Recombination breakpoints cluster in the region spanning the SNIP and SNAP recognition motifs (22,24,25). Since this correlation is based on a statistical analysis, we sought to expand the sample size of available Sμ/Sγ3 recombination junctions to more fully analyze the recombination breakpoint distribution in Sγ3. We report here a PCR method which permits direct amplification of Sμ/Sγ3 composite molecules from genomic DNA without the necessity of using nested primers. Using this method we have derived nine new Sμ/Sγ3 recombination junction sequences from hybridomas and normal lipopolysaccharide (LPS)-activated splenic B cells. Statistical analysis of the positions of Sγ3 recombination breakpoints in the tandem repeat confirms the correlation of clustered breakpoints with the region spanning the SNIP and SNAP motifs and identifies two statistically significant subclusters of switch junctions. Based on these findings a new model

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describing the initiating cleavage steps in the recombination reaction is proposed. The presence of mutations surrounding the switch junctions is one of the most striking features of switch recombination. The illegitimate priming and the template switching models have been proposed to accommodate the presence of mutations in switching (13,14). The illegitimate priming model predicts that mutations will be found on only one side of the switch junction while the template switching model predicts the presence of mutations on both sides of the crossover site. We find that mutations occur on both sides of the crossover site in several switch junctions newly described here. These findings support the the template switching model of switch recombination.

MATERIALS AND METHODS

Cell lines and culture conditions

Single cell suspensions prepared from the spleens of 8–12-week-old female BALB/c *nu/nu* mice were stimulated in culture with LPS as previously described (26). The BALB/c *nu/nu* mice contain a T cell deficiency, thus T cell depletion to obtain B cells is unnecessary. Cells were maintained in RPMI-1640 (Gibco BRL Life Technologies Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine in 5% CO_2 at 37°C. The cell lines 8A5.4A5.II.88 (27), TIB 114 and HB 70 (28) were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine. 8A5.4A5.II.88 was suppplemented with 50 μM β mercaptoethanol and 1 mM sodium pyruvate and TIB 114 with 50 μM β mercaptoethanol. The cells were maintained in culture at densities of 0.5–2.0 × 10^6 cells/ml. The TIB 114 and HB 70 hybridomas were obtained from the American Type Culture Collection.

PCR primers and amplification of Sμ, Sγ3 and Sμ/Sγ3 switch fragments

Primers were designed using Amplify 1.0 software (University of Wisconsin) with the default settings. Once primers which fit these criteria were identified they were further tested for cross-hybridization with their own switch region and all other switch regions in the IgH locus. Only primers which did not or only weakly hybridized to other sites in S DNA were chosen for use. Primer μ-1 (5′-CTCTACTGCCTACATGGACTGTTC-3′), located 5′ of Sμ and which anneals to positions 5269–5293 (MUSIGCD07) on the germline μ sequence, and primer μ-2 (5′-CTGGCTCACT-TAGCCTAATTGATTCTTGG-3′), located 3′ of Sμ and which anneals to positions 1058–1086 (MUSIGCD09) on the germline μ sequence, were used to amplify Sμ regions. Primer γ3-1 (5′-CAGGCTAAGATGGATGCTACAGGGA-3′), located 5′ of Sγ3 and which anneals to positions 403–428 (MUSIGHANA) on the germline γ3 sequence, and primer γ3-2 (5′-TACCCTGAC-CCAGGAGCTGCATAAC-3′), located 3′ of Sγ3 and which anneals to positions 2603–2627 on the germline γ3 sequence, were used to amplify Sγ3 regions. The combination of primers μ-1 and γ3-2 was used to amplify Sμ/Sγ3 hybrid fragments. The PCR reactions for amplification of Sμ, Sγ3 and Sμ/Sγ3 hybrid fragments from genomic Balb/c DNA contained 200 μM each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3, 0.01% gelatin, 0.2 mg/ml BSA, 10 pmol each PCR primer, 0.3–0.5 μg genomic template DNA, 2.5 U AmpliTaq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in a 50 μl volume under 50 μl mineral oil. The reaction was first heated to 95°C for 3 min and cycled in a Perkin-Elmer/Cetus DNA thermal cycler for 30 cycles each consisting of 1 min at 95°C, 45 s at 64°C and 2 min at 72°C. After the final cycle the reaction was incubated at 72°C for 5 min. Alternatively, Vent polymerase (New England Biolabs, Beverly, MA) was substituted for Taq where indicated and the buffer used was according to the manufacturer's instructions.

Cloning, screening and DNA sequence analysis of PCR products

PCR amplification products were purified using the QIAquick Spin PCR Purification Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The purified fragments were then cloned into plasmid pCR3.1 (Invitrogen Corp., San Diego, CA). Alternatively, PCR product ends were polished using T4 DNA polymerase (Promega, Madison, WI) and cloned into the *Sma*I site of pBlueScript KS and then transformed into STBL2 (Gibco BRL, Gaithersberg, MD) or DH5α competent cells. The clones that contained inserts were verified by minipreparation, digestion with *Eco*RI and the inserts visualized by gel electrophoresis. Clones containing inserts were randomly chosen for further analysis by DNA sequencing. DNA sequencing reactions were performed using Sequenase sequencing kits (US Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions and/or by automated DNA sequence analysis (Genetic Engineering Facility, University of Illinois, Urbana, IL). Cloned Sμ/Sγ3 fragments from TIB 114 and HB70 were sequenced from independent isolates derived from two individual PCR reactions or PCR products were directly sequenced (US Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions. The genomic Sγ3 region from Balb/c spleen DNA was PCR amplified using the Sγ3 locus-specific primers γ3-1 and γ3-2, described above. PCR was performed using Vent polymerase (New England Biolabs, Beverly, MA) for 30 cycles. Each cycle consisted of 1 min denaturation at 95°C, 45 s annealing at 65°C and 2 min elongation at 72°C. The amplified PCR fragment was gel purified and electroeluted from the agarose using Gene Capsule (Midwest Scientific, St Louis, MO) and blunt end cloned into the pBluescript KS cloning vector (Stratgene, La Jolla, CA); this is referred to as pSγ3. The 5′- and 3′-ends were submitted to DNA sequence analysis and the resulting sequence was found to match the published sequence for Sγ3 (MUSIGHANA) (29).

Preparation of genomic DNA and probes for Southern blot analysis

High molecular weight genomic DNA was prepared from splenic B cells and from the cell lines using the QIAamp Tissue Kit (Qiagen, Germany). pSγ3 described above and pM2-20 (a gift from K.Marcu; 30) were used as the sources of probes for hybridization with genomic Sγ3 and Sμ respectively. The plasmids were digested with appropriate restriction enzymes and the resulting DNA fragments were separated by gel electrophoresis. The Sγ3 and Sμ fragments were isolated from the gel and prepared as probes for Southern blot hybridization using a random primers kit (New England BioLabs, Beverly, MA) as recommended by the manufacturer.

RESULTS

Southern blot hybridization analysis of μ heavy chain- and IgG3-producing B cell lines

The 8A5.4A5.II.88 cell line is a μ heavy (H) chain-producing pre-B cell line while TIB 114 and HB 70 are IgG3-producing hybridomas; all these cell lines are derived from BALB/c mice (27,28). Southern analysis was carried out to determine the organization of the Sμ and Sγ3 regions in these cell lines. There may be four or more chromosomes containing the IgH locus in the hybridomas, making analysis of the switch DNA complex. Nonetheless, in DNA from TIB 114 digested with *Sac*I or with *Xba*I (data not shown) the Sμ-associated restriction fragments differ from the comparable fragment from BALB/c liver, indicating that these loci have undergone rearrangement (Fig. 1A, lanes 1 and 3). In DNA from HB 70 digested with *Xba*I all of the Sμ-associated restriction fragments are smaller than the 6.0 kb germline Sμ fragment (31), indicating that in HB 70 the Sμ loci have undergone rearrangement. Southern analysis of genomic DNA from 8A5.4A5.II.88 cells digested with *Xba*I or with *Sac*I (data not shown) demonstrates that the *Xba*I fragment spanning the Sμ region is 3.4 kb, as compared with the 6.0 kb fragment from BALB/c liver DNA (Fig. 1B; 31). This finding implies that the Sμ region in 8A5.4A5.II.88 contains an internal deletion.

When Sμ→Sγ3 switch recombination occurs a new composite switch region is formed such that Sμ and Sγ3 sequence will be located on a new restriction fragment (Fig. 2). Southern analysis indicates that in TIB 114 the Sγ3-associated restriction fragments have rearranged as compared with the germline Sγ3 found in BALB/c liver DNA (Fig. 1A, lanes 2 and 4). Moreover, the 3.6 and 2.3 kb fragments (which do not photograph well) hybridize with both the Sμ and Sγ3 probes. Similarly, in DNA from HB 70 the 4.9 and 4.2 kb fragments cross-hybridize with the Sμ and Sγ3 probes (Fig. 1B, lanes 3 and 4). When analyzed by genomic Southern blot, co-migration of two switch regions is suggestive of a switch event. In contrast, the Sγ3 region in DNA derived from 8A5.4A5.II.88 cells did not co-migrate with the Sμ sequence and was slightly smaller than the expected size of 4.0 kb for germline Sγ3 (Fig. 1B; 29). These findings suggest that the rearrangements which resulted in a shortened Sμ region in 8A5.4A5.II.88 cells did not affect the Sγ3 region. The restriction maps of the rearranged Sμ and Sγ3 regions in 8A5.4A5.II.88, HB 70 and TIB 114 have been compared with that of the germline BALB/c Sμ and Sγ3 genes (Fig. 1C).

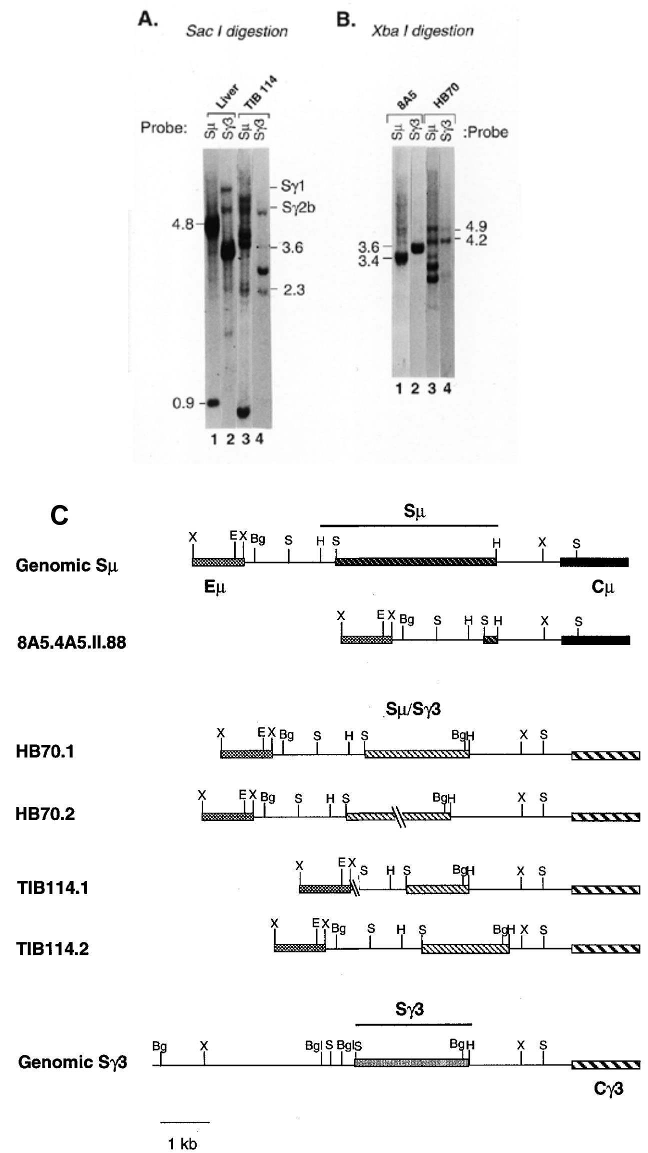

Figure 1. Organization of Sμ and Sγ3 DNA in μ heavy chain- and IgG3-producing cell lines and hybridomas. (**A** and **B**) Southern blot analysis of the Sμ and Sγ3 regions derived from the cell lines and BALB/c liver DNA. High molecular weight DNA was extracted and digested (20 μg) with either *Sac*I (A) or *Xba*I (B), fractionated on a 0.8% agarose gel, transferred to a nitrocellulose filter and assayed for the presence of Sμ or Sγ3 sequences by filter hybridization using the Sμ fragment from pM2-20 (30) or the Sγ3 fragment from pSγ3 (see Materials and Methods) as probe. Sizes of selected restriction fragments are shown in kilobases, indicated by the numbers along the sides. In (A) DNA samples in each lane are: lanes 1 and 2, liver; lanes 3 and 4, TIB 114. In (B) DNA samples in each lane are: lanes 1 and 2, 8A5.4A5.II.88 (8A5); lanes 3 and 4, HB 70. The fragments which cross-hybridize with Sγ3 include Sγ and Sγb and are indicated. (**C**) Restriction maps of the Sμ and Sγ3 regions in BALB/c liver, 8A5.4A5.II.88, TIB 114 and HB 70 are shown. The organization of the germline Sμ and Sγ3 DNA were previously derived (2,29,30). Restriction sites are indicated: *Bgl*I, Bg; *Bgl*II, Bgl; *Eco*RI, E; *Hin*dIII, H; *Sac*I, S; *Xba*I, X. Slash marks indicate a deletion relative to the germline configuration.

Direct PCR amplification of Sμ, Sγ3 and Sμ/Sγ3 switch fragments from B cell lines

Switch recombination is a deletional process in which the 5′-end of the Sμ region and the 3′-end of the targeted S region join together to form a new hybrid S region (Fig. 2). When composite Sμ/Sγ3 regions are formed by recombination, the distance between the PCR primers, located 5′ of Sμ and 3′ of Sγ3, is greatly shortened, permitting amplification of the hybrid switch DNA. The use of PCR to directly amplify germline and rearranged switch regions provides an opportunity to confirm the organization of the Sμ, Sγ3 and Sμ/Sγ3 fragments in specific cell lines and to characterize the switch/switch recombination junction sites by nucleotide sequence analysis.

Genomic DNA from 8A5.4A5.II.88, TIB 114, HB 70 and spleen cells was tested for the presence of Sμ/Sγ3 hybrid switch regions by amplification using primers μ-1 and γ3-2 (Figs 2 and 3). Amplification of DNA from HB 70 and TIB 114 cells reproducibly gave rise to one or two Sμ/Sγ3 fragments respectively (Fig. 3, lanes 6 and 9). Two Sμ/Sγ3 co-migrating fragments were

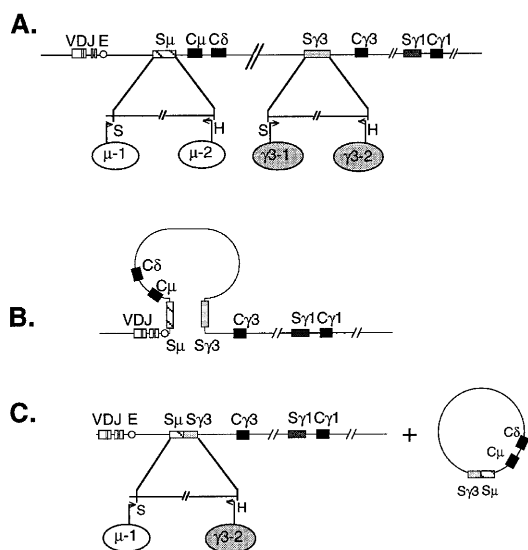

Figure 2. The deletion–looping out model of switch recombination and strategy for direct PCR amplification of Sμ, Sγ3 and rearranged Sμ/Sγ3 hybrid fragments. (**A**) A partial schematic map of the IgH locus following V(D)J joining and prior to switch recombination. The Sμ and Sγ3 regions are intact and separated by ~50 kb (2). The numbered arrows indicate position and orientation of the PCR primers. Primers μ-1 and μ-2 amplify genomic Sμ DNA while primers γ-1 and γ-2 amplify Sγ3 DNA. In each case the primers are positioned either just upstream or downstream of tandem repeats in Sμ and Sγ3 DNAs and are shown relative to the *Sac*I (S) and *Hin*dIII (H) sites in these switch regions. (**B**) During switch recombination the Sμ and Sγ3 regions are brought into close apposition, causing the intervening DNA to form a loop. (**C**) Following recombination, a hybrid S/S region is formed on the chromosome with the configuration 5′-Sμ/Sγ3-3′ while the intervening DNA is excised as a circle. Primer μ-1, located at the 5′-end of Sμ, and primer γ-2, located at the 3′-end of Sγ3, amplify Sμ/Sγ3 hybrid fragments. The priming sites for the downstream Sμ primer, μ-2, and the upstream Sγ3 primer, γ-1, have been deleted as a consequence of recombination.

detected by Southern analysis of genomic DNA from HB 70 cells (Fig. 1), whereas a single Sμ/Sγ3 fragment resulted from PCR amplification. This difference may be due to loss of one or more priming sites for this fragment. No Sμ/Sγ3 fragments were observed following amplification of DNA derived from either 8A5.4A5.II.88 or spleen cells (Fig. 3, lanes 3 and 12). These results suggest that hybrid Sμ/Sγ3 regions can be specifically amplified from genomic DNA using locus-specific primers.

To directly determine whether Sμ and Sγ3 regions are intact in spleen and 8A5.4A5.II.88 cells these S regions were directly amplified with locus-specific primers (Fig. 2). Amplification of the Sμ and Sγ3 DNA regions from 8A5.4A5.II.88 cells yields a 0.3 and a 2.2 kb fragment respectively (Fig. 3, lanes 1 and 2). The 0.3 kb amplification product hybridizes with Sμ sequence, confirming the origin of this DNA fragment (data not shown), and is consistant with the size of the Sμ genomic restriction fragment observed by Southern blot analysis (Fig. 1B). The 2.2 kb Sγ3 fragment from DNA obtained from 8A5.4A5.II.88 cells was identical in size to the Sγ3 fragment amplified from spleen DNA (Fig. 3, compare lanes 2 and 10). To verify the specificity of the Sγ3 locus-specific primers, the 2.2 kb fragment from the Sγ3 region of Balb/c spleen DNA was cloned and the DNA sequence for the 5′- and 3′-ends (data not shown) was found to correspond to that previously reported for Sγ3 DNA (29). These findings confirm that the Sμ and Sγ3 regions in spleen and 8A5.4A5.II.88

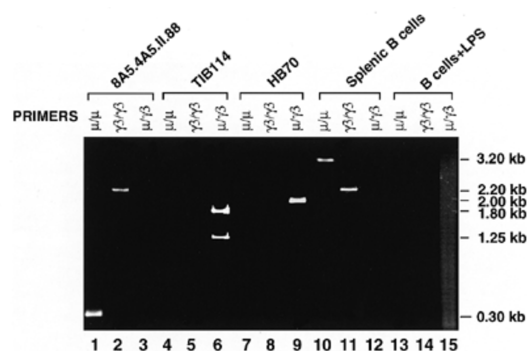

Figure 3. Direct PCR amplification of genomic Sμ, Sγ3 and hybrid Sμ/Sγ3 fragments. (**A**) Agarose gel electrophoresis of PCR products. Aliquots of DNA (300 ng) derived from 8A5.4A5.II.88 (lanes 1–3), TIB 114 (lanes 4–6), HB 70 (lane 7–9), spleen cells (lanes 10–12) and splenic B cells activated with LPS for 92 h (lanes 12–14) were amplified with locus-specific primers, μ-1, μ-2, γ3-1 and γ3-2. The Sμ region is amplified with the primers μ-1 and μ-2, which are indicated as μ/μ. The Sγ3 region is amplified with the primers γ3-1 and γ3-2, which are indicated as γ3/γ3. The Sμ/Sγ3 hybrid fragment is amplified with μ-1 and γ3-2, which are indicated as μ3/γ3. Resultant PCR fragments were subjected to 1.0% agarose gel electrophoresis. Sizes of selected DNA fragments are shown in kilobases.

cells have not undergone switch recombination and that the priming sites for the locus-specific primers are intact. No Sμ- or Sγ3-specific fragments were obtained using DNA from TIB 114, HB 70 or LPS-activated B cells (Fig. 3), confirming that rearrangements had occurred which involve these switch regions.

Sequence analysis of Sμ/Sγ3 fragments from IgG3-producing hybridomas

The three composite Sμ/Sγ3 switch fragments which were reproducibly amplified from DNA of TIB 114 and HB 70 cells were purified and cloned. Independent clones from two separate PCR reactions were analyzed and/or PCR amplification products were directly sequenced. The clones TIB 114.1 (1.2 kb), TIB 114.2 (1.8 kb) and HB 70.1 (2.0 kb) share the 5′-Sμ sequence starting from position 5268 of the germline Sμ sequence (MUSIGCD07). TIB 114.1 was sequenced from both the 5′- and 3′-ends and confirmed to contain Sμ and Sγ3 sequence. Restriction mapping of TIB 114.1 helped identify the region in which the crossover occurred and the Sμ/Sγ3 crossover point was located at position 2133 in genomic Sγ3 DNA (MUSIGHANA) by automated DNA sequence analysis (Fig. 4). Three sequences, germline Sμ, germline Sγ3 and the recombined Sμ/Sγ3 region, are shown in the area surrounding the breakpoint (Fig. 4B). TIB 114.1 contained a total of 735 bp of Sμ DNA and 480 bp of Sγ3 DNA. It is not possible to identify the actual genomic position of the Sμ breakpoint in TIB 114.1 since the sequence available for genomic Sμ in this region contains many internal deletions. However, a good match for the sequence immediately upstream of the crossover point, located at position 588, was found (MUSIGCD09). In clone TIB 114.2, 118 bp of DNA sequence were determined starting at the 5′-end; the Sμ crossover point is located at position 5351 (MUSIGCD07) in genomic Sμ while the Sγ3 breakpoint is located at position 1025 in genomic Sγ3 DNA (Fig. 4). There is a direct joining of Sμ with Sγ3 DNA in these cloned fragments around the Sμ/Sγ3 junctions (Fig. 4). At the Sμ/Sγ3 junction of the TIB 114.1 and TIB 114.2 clones, no

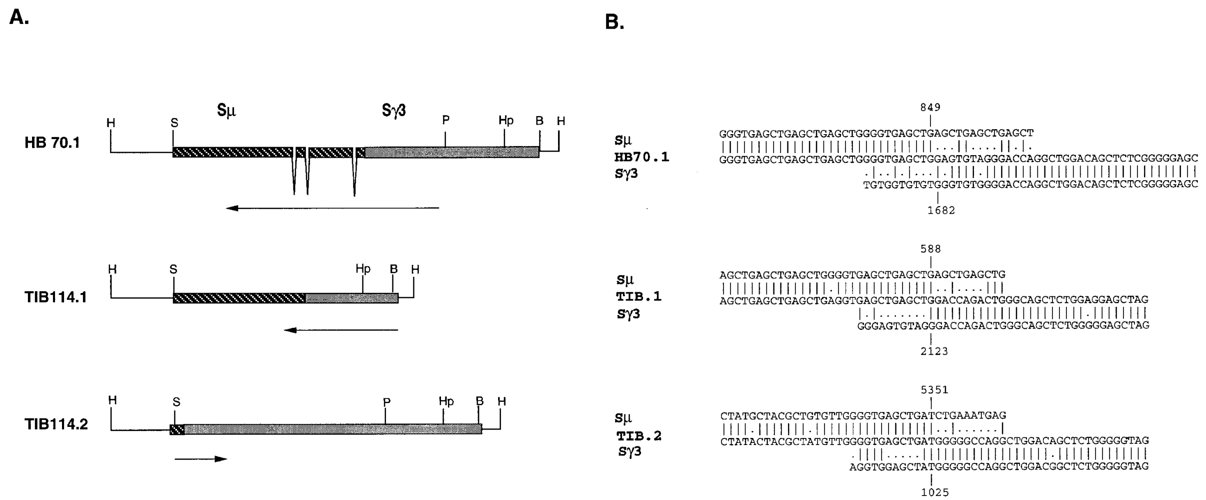

Figure 4. Analysis of the Sμ/Sγ3 switch junctions derived from IgG3-producing hybridomas. Three Sμ/Sγ3 switch fragments originating from TIB 114 and HB 70 DNA were analyzed by restriction mapping and DNA sequence analysis. (**A**) Restriction sites are indicated: *Bgl*II, B; *Hin*dIII, H; *Hpa*II, Hp; *Pvu*II, P; *Sac*I, S. The S regions are represented by the stippled and shaded boxes. DNA sequence analysis was carried out across the region indicated by the arrow(s). (**B**) Automated DNA sequence analysis was used to identify the crossover site which resulted from Sμ/Sγ3 recombination. The DNA sequence was compared with germline Sμ (MUSIGCD07 and MUSIGCD09) and Sγ3 (MUSIGHANA) sequences. Recombination sites are indicated by the vertical lines. Nucleotide positions of the database sequence corresponding to the breakpoint are also shown.

nucleotide addition is seen adjacent to the recombination site. The Sμ-Sγ3 homology of the germline sequence at the breakpoint was one base for TIB 114.1 and two bases for TIB 114.2.

Mutations are frequently found in the DNA flanking the switch junctions (12–14). The Sμ sequence in TIB.1 diverges from the genomic Sμ sequence at three positions (two of which are not shown) and from the genomic Sγ3 sequence at one position (Fig. 4). Although the mismatch located 14 bp upstream of the switch junction changes the sequence from the common Sμ pentameric repeat, it is still not possible to be sure that these changes are real mutations since the genomic sequence in this region is incomplete. The sequence surrounding the crossover point in TIB.2 diverges from the genomic sequence at three positions, of which two are in the Sμ and one in the Sγ3 sequences. These mutations were confirmed in three separate PCR products by direct sequencing. The genomic Sμ sequence was confirmed (unpublished data) by direct sequencing of the Sμ PCR product (Fig. 3). The genomic Sγ3 sequence surrounding the switch junction was independently confirmed by others (9). Thus the mutations reported in TIB.2 clearly diverge from the genomic switch sequences and are located on both sides of the crossover site.

DNA sequence analysis of HB 70.1 from the 5′- and 3′-ends confirmed the presence of Sμ and Sγ3 sequence respectively. HB 70.1 was further analyzed by automated DNA sequencing and 800 bp of Sμ sequence were determined from the 5′-end. There were a number of mismatches starting 65 bp upstream from the crossover site in the Sμ sequence. However, since the Sμ genomic sequence is incomplete in this area it is difficult to confirm the authenticity of these mutations. The Sμ/Sγ3 breakpoint was located at position 1682 in genomic Sγ3 DNA (Fig. 4). The HB 70 sequence diverged from the genomic Sγ3 sequence in two of seven nucleotides at the Sμ/Sγ3 junction. The genomic Sγ3 sequence at this site has been independently confirmed by others (32). These transitions were unlikely to have arisen as a PCR artifact since two independent isolates of these clones from two separate PCR reactions were analyzed. There is a direct joining of Sμ with Sγ3 DNA at the Sμ/Sγ3 junction and no homology of germline Sμ and Sγ3 at the crossover site.

PCR amplification of Sμ/Sγ3 switch fragments from LPS-activated normal splenic B cells

LPS is a specific B cell mitogen causing proliferation and differentiation of murine splenic B cells from surface IgM-positive to IgG-secreting cells (33–35). B cells were activated with LPS for 92 h, DNA isolated and hybrid Sμ/Sγ3 switch fragments amplifed. Switch recombination can occur anywhere within the Sμ and Sγ3 regions and produces a population of Sμ/Sγ3 switch fragments which are mixed with respect to size and structure. Amplification of DNA from LPS-activated B cells gives rise to a complex mixture of PCR products, whereas no fragments were amplified from DNA isolated from unstimulated splenic B cells (Fig. 3). The PCR products derived from DNA of LPS-activated B cells which fell in the size range 0.3–2.5 kb were purified and cloned. Twenty transformants containing inserts were randomly chosen for further analysis. These clones were shown by partial DNA sequence analysis to contain Sγ3 and Sμ DNA sequence. Restriction maps were deduced for six clones, (B-1–B-6) containing Sμ/Sγ3 fragments of ≥500 bp (Fig. 5A). These six clones were taken for automated DNA sequence analysis. In all of these clones the switch junctions showed direct joining of Sμ and Sγ3 DNA without deletion, duplication or insertion at the recombination breakpoint (Fig. 5B). The Sμ-Sγ3 homology of germline sequence at the breakpoint was one base for B-2, B-5 and B-6 and two bases for B-4. In clone B-6 a Sγ3→Sγ3 rearrangement was also found, which resulted from an internal deletion in Sγ3 downstream of the switch junction (Fig. 5A). Mutations can be introduced as an artifact of PCR amplification. Since the B-1–B-6 clones were obtained from a heterogeneous population of Sμ/Sγ3 recombinant molecules it is not possible to analyze them in independent PCR amplifications. Consequently,

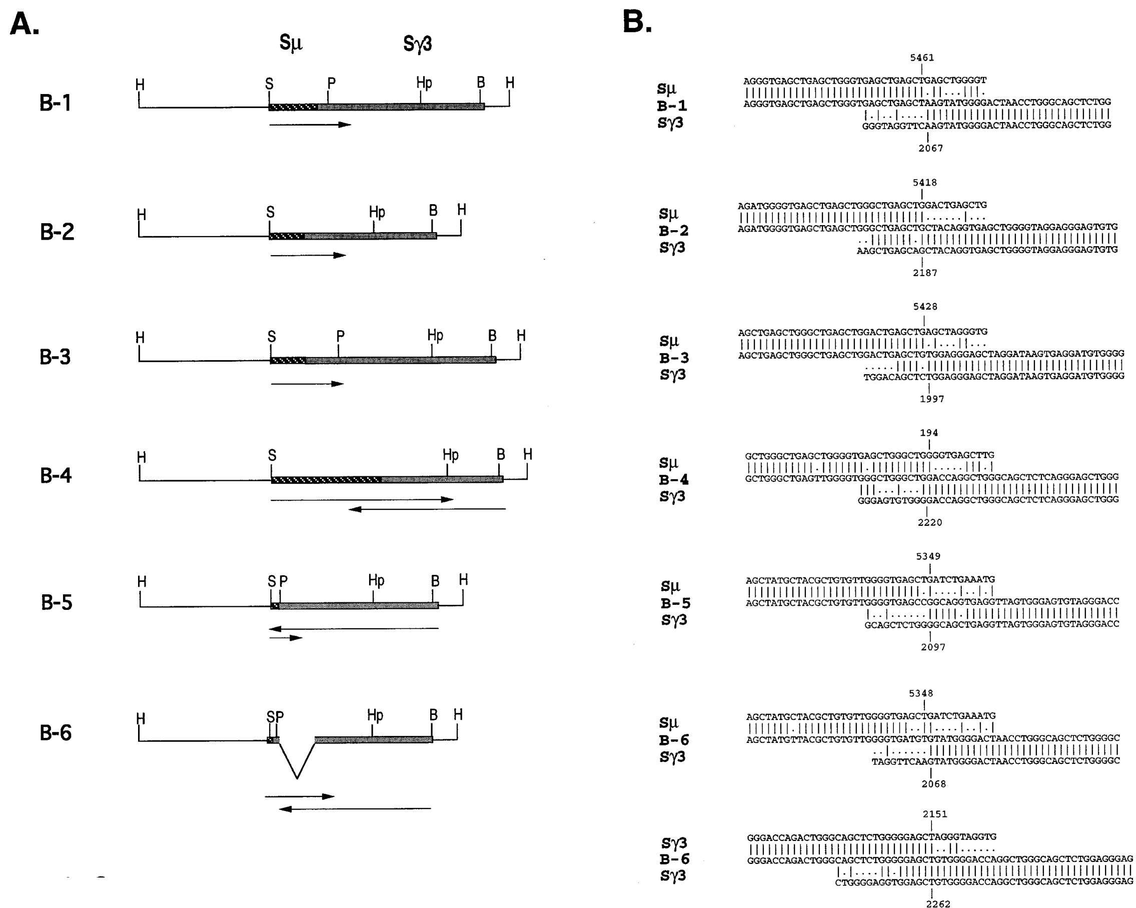

Figure 5. PCR amplification of Sμ/Sγ3 switch fragments from normal LPS-activated B cells. Splenic B cells from Balb/c *nu/nu* mice were unstimulated or stimulated with LPS for 92 h, DNA isolated and used as template (300 ng) for amplification of Sμ/Sγ3 fragments with the μ-1 and γ3-2 primers. (**A**) Restriction maps of the six Sμ/Sγ3 composite fragments isolated. Restriction sites are indicated: *Bgl*II, B; *Hin*dIII, H; *Hpa*II, Hp; *Pvu*II, P; *Sac*I, S. The S regions are represented by the stippled and shaded boxes. DNA sequence analysis was carried out across the region indicated by the arrow(s). The S regions are represented by the stippled and hatched boxes. (**B**) Automated DNA sequence analysis was used to define the precise Sμ/Sγ3 junctions. To identify the precise recombination site the sequence was compared with germline Sμ (MUSIGCD09) and Sγ3 (MUSIGHANA) sequences. Recombination sites are indicated by the vertical lines. Nucleotide positions of the database sequence corresponding to the breakpoint are also shown.

it is difficult to conclude that the apparent mutations reported in Figure 5 arose during the process of switching.

Analysis of the Sμ/Sγ3 recombination breakpoint position in the Sγ3 region

Examination of published switch recombination crossover sites in Sγ3 revealed a non-random distribution of breakpoints which focused on the region spanning the SNIP and SNAP recognition motifs (22,24,25). The SNIP binding site is an 11 bp recognition motif separated from the 15 bp SNAP binding site by a short spacer of 4 bp (Fig. 6). The Sγ3 germline sequence surrounding the nine newly derived Sμ/Sγ3 recombination breakpoints and one Sγ3/Sγ3 event from hybridomas and normal LPS-activated splenic B cells is shown (Table 1). These new switch junctions have been compiled together with a larger group of previously described breakpoints. The nine new Sμ/Sγ3 junctions (Table 1) have been compiled together with the 21 (primary acceptor and secondary donor) breakpoints previously analyzed (13,24) to make statistical analysis feasible (Fig. 6). The distribution of 31 breakpoints in the region spanning the SNIP and SNAP recognition motifs from a total of 33 Sγ3 breakpoints is shown to be significantly non-random, with $P = 0.005$ by χ^2 analysis (Fig. 6). The subclustering of breakpoints in the SNAP motif ($P = 0.001$) has been previously observed (22) and is confirmed here (Fig. 6). A statistically robust subcluster of Sγ3 breakpoints in the SNIP motif ($P = 0.001$) is also now observed (Fig. 6). The two secondary acceptor Sγ3 breakpoints formed in γ3→γ3 rearrangements were excluded from this calculation since they tend to cluster in the long spacer (25). These findings confirm and extend our previous results regarding the biased distribution of recombination breakpoints in the Sγ3 tandem repeat (22,24,25).

DISCUSSION

To study the mechanism of class switching we have sought to examine the distribution of switch recombination junctions in Sγ3 tandem repeats following Sμ→Sγ3 rearrangement. Southern blot analysis of DNA from the IgG3-producing hybridomas TIB 114 and HB 70 demonstrates that the Sμ and Sγ3 regions co-migrate, suggesting that these regions are now structured as composite Sμ/Sγ3 fragments. In contrast, the Sμ and Sγ3 regions do not co-migrate in DNA from μ heavy chain-producing 8A5.4A5.II.88

Table 1.

Sγ3 Germline Sequence with Breakpoints (▼)				Clone	Cell Type	Switch	Repeat No.
SNIP-motif		SNAP-motif					
GGGGACCAAGC	TGGG	CAGCTCTGGGGGAGC	TGGGGTAGGTTCAAGTAT	B-1	B Cell	Sμ/Sγ3	32
AGGATATTAAGC	TGAG	CAGCTACAGGTGAGC	TGGGGTAGGAGGGAGTGT	B-2	B Cell	Sμ/Sγ3	35
AGGAGCAGGC	TGGA	CAGCTCTGGAGGGAGC	TAGGATAAGTGAGGATGT	B-3	B Cell	Sμ/Sγ3	31
GGGGACCAGGC	TGGG	CAGCTCTGGGGGAGC	TGGGGAGGTGGAGCTGT	B-4	B Cell	Sμ/Sγ3	36
GGGGACTAACC	TGGG	CAGCTCTGGGGCAGC	TAGGGTAGGTGGAAGCAT	B-5	B Cell	Sμ/Sγ3	33
GGGGACCAAGC	TGGG	CAGCTCTGGGGGAGC	TGGGGTAGGTTCAAGTAT	B-6.1	B Cell	Sμ/Sγ3	32
AGGGACCAGAC	TGGG	CAGCTCTGGGGGAGC	TAGGGTAGGTGGAAGCAT	B-6.2	B Cell	Sγ3/Sγ3 donor	34
GGGGACCAGGC	TGGG	CAGCTCTCAGGGAGC	TGGGGAGGTGGAGCTGT	B-6.3	B Cell	Sγ3/Sγ3 acceptor	36
AGGGACCAGAC	TGGG	CAGCTCTGGGGGAGC	TAGGGTAGGTGGAAGCAT	TIB114.1	Hybridoma	Sμ/Sγ3	34
GGGGGCCAGGC	TGGA	CGGCTCTGGGGGTAGC	TAGGGTAAGTGAGGATGT	TIB114.2	Hybridoma	Sμ/Sγ3	11
AGGGAGCAGGC	TGGA	CAGCTCTGAGGGGAAGC	TAGAGTAAGTGTGGGTGT	HB70.1[a]	Hybridoma	Sμ/Sγ3	25
GGGGACCAAGC	TGGG	CAGCTCTGGGGGAGC	TGGGGTAGGTTCAAGTAT	**Sγ3 Consensus 49-mer[b]**			

[a]Sequences were aligned with genomic Sγ3 (MUSIGHANA) to evaluate the sequences flanking the SNIP and SNAP binding sites. In all cases single perfect matches were found. In six of nine Sμ/Sγ3 breakpoints evaluated here there was limited homology between Sμ and Sγ3, of one or two bases, at the site of crossover. In clone B-6 at the Sγ3→Sγ3 crossover site there was a six nucleotide homology. The homology between donor and acceptor DNA caused ambiguity in the assignment of the breakpoint location. In three of these cases the breakpoint could be positioned within the region of homology without changing the statistical analysis shown in Figure 6. For example, the TIB.1 breakpoint could be placed at either of two G residues but would still remain a SNIP site breakpoint. There is one case where the homology spans different subsites in the Sγ3 tandem repeat. In all cases where homology between Sμ and Sγ3 at the crossover junction causes ambiguity with regard to the precise breakpoint site the breakpoints were positioned at the most 5′ possible location.
[b]The concensus Sγ3 tandem repeat is based on the analysis of Gritzmacher (6).

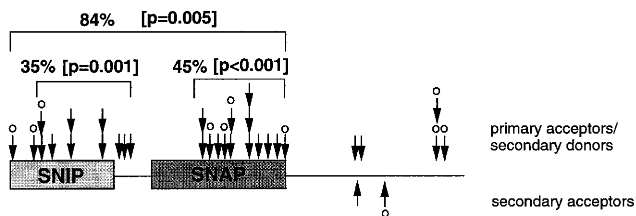

Figure 6. Summary of switch recombination breakpoints in Sγ3 and their positions with respect to the SNIP and SNAP binding sites. A schematic 49 bp repeat is shown in which the binding sites for SNIP and SNAP are boxed. The Sγ3 recombination breakpoints are indicated by the arrows. The Sγ3 breakpoint sequences derived in this report are indicated by the open circles and are shown in Table 1. Other Sγ3 breakpoints indicated by the arrows without circles were previously described (22,24). The levels of significance of breakpoint clustering are shown in the brackets, as determined by χ^2 analysis. Distinctions in switch DNA substrate use are found when primary (μ→γ3) and secondary (γ3→x) switch recombination events are considered. In primary switch recombination the Sγ3 DNA is always the acceptor substrate in the recombination reaction and the breakpoints focus on the region spanning the SNIP and SNAP binding sites (25). In secondary recombination events the Sγ3 DNA can function as either donor or acceptor substrate. The donor breakpoints focus on the region spanning the SNIP and SNAP recognition motifs, whereas the secondary acceptor breakpoints cluster in the long spacer (25). The arrows above the line indicate the breakpoints which were involved in the recombination reaction as primary acceptors and secondary donor sites. The arrows below the line indicate breakpoints involved in the recombination reaction as secondary acceptor sites.

cells. Using the direct PCR method, three Sμ/Sγ3 hybrid fragments were amplified and analyzed from TIB 114 and HB 70. The size of these Sμ/Sγ3 fragments was consistent with the organization of the rearranged genomic loci as observed by Southern blot analysis. DNA sequence analysis confirmed the origin of the Sμ/Sγ3 fragments and located the switch breakpoints. Using locus-specific primers we found that the Sμ and Sγ3 loci could be amplified in DNA from μ heavy chain-producing 8A5.4A5.II.88 cells and in splenic B cells but not from TIB 114 and HB 70, which had undergone isotype switching. Taken together, the absence of Sμ and Sγ3 regions and the presence of new Sμ/Sγ3 composite fragments provides direct evidence for switch recombination events. This PCR method was then applied to DNA derived from normal LPS-activated B cells. Twenty Sμ/Sγ3 fragments were analyzed by partial sequence analysis and confirmed to contain Sμ and Sγ3 DNA. Six of the Sμ/Sγ3 fragments were further characterized and the recombination junctions defined. This PCR-based method provides a means to study low frequency switch events with small numbers of normal B cells. The selection of primers based on their high specificity for sites proximal or distal of the tandem repeats of the Sμ and Sγ3 regions allows direct amplification of hybrid Sμ/Sγ3 fragments without the necessity of using nested primers.

Our studies previously demonstrated that Sγ recombination breakpoints were non-randomly distributed in the Sγ tandem repeat (22,24,25). Given that the significance of breakpoint clustering is dependent on statistical analyses, we wished to further examine this phenomenom using a larger sample pool of

Sγ3 breakpoints derived from B cells. Compilation of nine new Sμ/Sγ3 junctions with previously characterized Sγ3 recombination breakpoints revealed that 84% of Sγ3 breakpoints are localized in the region spanning the SNIP/SNAP binding sites and focused on two subclusters in the SNIP and SNAP bindings sites respectively. Statistical analysis of breakpoint position in the Sγ3 tandem repeat demonstrates the non-random character of this distribution and confirms our earlier findings (22,24,25). It should be noted that ~16% of Sγ3 recombination breakpoints fall outside the SNIP/SNAP region. Thus preferential usage of the SNIP/SNAP binding region for recombination does not reflect an absolute concordance of binding sites with recombination junctions.

Several models for the mechanism of switching have been articulated (12–18). One model suggests that switch recombination is due to homologous recombination and is catalyzed by the short pentameric repeats common amongst all S regions (6,16,17). The homologous recombination hypothesis is poorly supported by the data since there is very limited homology found at the points of recombination. The lack of an identifiable recombination signal sequence in switch regions has led to the illegitimate recombination model, which involves joining of non-homologous DNA ends at random break sites (18). Observation of a clustered distribution of recombination breakpoints in Sγ DNA suggests an ordered switch mechanism. We propose that switching may be initiated by site-specific double-strand breaks and that cleavage may be followed by extensive processing which produces both blunt and staggered ends. This model would explain the absence of recombination signal sequences at switch breakpoint sites and focusing of breakpoints on discrete regions in the Sγ tandem repeat. The recognition site for endonucleolytic cleavage may occasionally occur at other sites in the tandem repeat, thus providing an explanation for the exceptional breakpoints which comprise ~15% of the total breakpoint population. One activity which could account for this processing is B cell-associated nuclease (BCAN) (36). BCAN is a B cell-specific 3′→5′ exonuclease which is non-processive and is expressed in the nuclei of normal mitogen-activated B cells and mature B cell lines but not in quiescent splenic B cells, pre-B cells, plasma cells, T cells or in other cell types (36). Further work is needed to test this model of switch recombination.

Two models which could account for mutations found immediately flanking switch junctions are illegitimate priming and template switching (13,14). Both models predict that single- or double-strand breaks occur in switch regions, that error prone DNA synthesis is intrinsic to the mechanism of switching and that mutations are generated around the switch junctions (13,14). The illegitimate priming model predicts that mutations will be found only on one side of the switch junction, while the template switching model proposes that mutations will be found on both sides of the crossover site. We found mutations on both sides of the switch junctions for both TIB.1 and TIB.2 and confirmed the authenticity of these mismatches relative to the germline sequence for TIB.2. This is the first example of a switch junction for which mutations have been confirmed on both sides of the crossover site and supports the template switching model for isotype switching. However, this is a limited data set and further examples of switch junctions containing mutations on both sides of the crossover site are needed to further confirm this conclusion.

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REFERENCES

1 Lewis,S.M. (1994) *Adv. Immunol.*, **56**, 27–149.
2 Shimizu,A., Takahashi,N., Yaoita,Y. and Honjo,T. (1982) *Cell*, **28**, 499–506.
3 Shimizu,A. and Honjo,T. (1984) *Cell*, **36**, 801–803.
4 Coffman,R., Lebman,D. and Rothman,P. (1993) *Adv. Immunol.*, **54**, 229–270.
5 Stavnezer,J. (1996) *Adv. Immunol.*, **61**, 79–146.
6 Gritzmacher,C.A. (1989) *CRC Crit. Rev. Immunol.*, **9**, 173–200.
7 Iwasato,T., Shimizu,A., Honjo,T. and Yamagishi,H. (1990) *Cell*, **62**, 143–149.
8 Iwasato,T., Arakawa,H., Shimizu,A., Honjo,T. and Yamagishi,H. (1992) *J. Exp. Med.*, **175**, 1539–1546.
9 Matsuoka,M., Yoshida,K., Maeda,T., Usuda,S. and Sakano,H. (1990) *Cell*, **62**, 135–142.
10 Yoshida,K., Matsuoka,M., Usuda,S., Mori,A., Ishizaka,K. and Sakano,H. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7829–7833.
11 von Schwedler,U., Jack,H.-M. and Wabl,M. (1990) *Nature*, **345**, 452–456.
12 Dunnick,W., Hertz,G.Z., Scappino,L. and Gritzmacher,C. (1993) *Nucleic Acids Res*., **21**, 365–372.
13 Dunnick,W., Wilson,M. and Stavnezer,J. (1989) *Mol. Cell. Biol*., **9**, 1850–1856.
14 Dunnick,W. and Stavnezer,J. (1990) *Mol. Cell. Biol*., **10**, 397–400.
15 Davis,M., Kim,S. and Hood,L.E. (1980) *Science*, **209**, 1360–1365.
16 Kataoka,T., Miyata,T. and Honjo,T. (1981) *Cell*, **23**, 357–368.
17 Nikaido,T., Yamawaki-Kataoka,Y. and Honjo,T. (1982) *J. Biol. Chem*., **257**, 7322–7329.
18 Li,J., Daniels,G. and Lieber,M. (1996) *Nucleic Acids. Res*., **24**, 2104–2111.
19 Echols,H. (1986) *Science*, **233**, 1050–1056.
20 Mathews,K. (1992) *Microbiol. Rev*., **56**, 123–136.
21 Wuerffel,R.A., Nathan,A.T. and Kenter,A.L. (1990) *Mol. Cell. Biol*., **10**, 1714–1718.
22 Wuerffel,R.A., Jameson,C., Morgan,L. Merkulov,G.V., Sen,R. and Kenter,A.L. (1992) *J. Exp. Med*., **176**, 339–349.
23 Ma,L., Hu,B. and Kenter,A.L. (1997) *Int. Immunol*., In press..
24 Wuerffel,R.A. and Kenter,A.L. (1992) *Curr. Topics Microbiol. Immunol*., **182**, 149–156.
25 Kenter,A.L., Wuerffel,R., Jamieson,C.E., Sen,R. and Merkulov,G.V. (1993) *J. Immunol*., **151**, 4718–4731.
26 Kenter,A.L., Watson,J.V., Azim,T. and Rabbitts,T.H. (1986) *Exp. Cell. Res*., **167**, 241–251.
27 Wabl,M., Beck-Engeser,G. and Burrows,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 867–870.
28 American Type Culture Collection (1992) *American Type Culture Collection*, 7th Edn. American Type Culture Collection, Rockville, MD.
29 Szurek,P., Petrini,J. and Dunnick,W. (1985) *J. Immunol*., **135**, 620–626.
30 Marcu,K., Banerji,J., Penncavage,N., Lang,R. and Arnheim,N. (1980) *Cell*, **22**, 187–196
31 Nikaido,T., Nakai,S. and Honjo,T. (1981) *Nature*, **292**, 845–848.
32 Petrini,J., Shell,B., Hummel,M. and Dunnick,W. (1987) *J. Immunol*., **138**, 1940.
33 Cebra,J.J., Komisar,J.L. and Schweitzer,P.A. (1984) *Annu. Rev. Immunol*., **2**, 493–548.
34 Kearney,J.F. and Lawton,A.R. (1975) *J. Immunol*., **115**, 671–676.
35 Kearney,J.F., Cooper,M.D. and Lawton,A.R. (1976) *J. Immunol*., **117**, 1567–1572.
36 Kenter,A.L. and Tredup,J. (1991) *Mol. Cell. Biol*., **11**, 4398–4404.