Two New Isotype-Specific Switching Activities Detected for Ig Class Switching[1]

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Abstract

Ig class switch recombination (CSR) occurs by an intrachromosomal deletional process between switch (S) regions in B cells. To facilitate the study of CSR, we derived a new B cell line, 1.B4.B6, which is uniquely capable of $\mu \rightarrow \gamma 3$, $\mu \rightarrow \varepsilon$, and $\mu \rightarrow a$, but not $\mu \rightarrow \gamma 1$ CSR at its endogenous loci. The 1.B4.B6 cell line was used in combination with plasmid-based isotype-specific S substrates in transient transfection assays to test for the presence of trans-acting switching activities. The 1.B4.B6 cell line supports $\mu \rightarrow \gamma 3$, but not $\mu \rightarrow \gamma 1$ recombination, on S substrates. In contrast, normal splenic B cells activated with LPS and IL-4 are capable of plasmid-based $\mu \rightarrow \gamma 1$ CSR and demonstrate that this S plasmid is active. Activation-induced deaminase (AID) was used as a marker to identify existing B cell lines as possible candidates for supporting CSR. The M12 and A20 cell lines were identified as AID positive and, following activation with CD40L and other activators, were found to differentially support $\mu \rightarrow \varepsilon$ and $\mu \rightarrow a$ plasmid-based CSR. These studies provide evidence for two new switching activities for $\mu \rightarrow \gamma 1$ and $\mu \rightarrow \varepsilon$ CSR, which are distinct from $\mu \rightarrow \gamma 3$ and $\mu \rightarrow a$ switching activities previously described. AID is expressed in all the B cell lines capable of CSR, but cannot account for the isotype specificity defined by the S plasmid assay. These results are consistent with a model in which isotype-specific switching factors are either isotype-specific recombinases or DNA binding proteins with sequence specificity for S DNA.

In B cells, the generation of different classes of Ig is achieved by class switch recombination (CSR),[3] in which the $C\mu$ gene is replaced by one of the downstream C_H genes while the Ag binding variable (V_H) region is maintained. The murine *IgH* locus is composed of multiple C_H genes, $C\mu$, $C\delta$, $C\gamma 3$, $C\gamma 1$, $C\gamma 2b$, $C\gamma 2a$, $C\varepsilon$, and Ca, which are each (except δ) coupled with unique switch (S) regions. Isotype switching focuses on S DNA and produces new hybrid S DNA combinations. The composite $S\mu$-Sx DNA configuration is formed on the

[3]Abbreviations used in this paper: CSR, class switch recombination; AID, activation-induced cytidine deaminase; DSB, double strand break; gts, germline transcripts; CD40L, CD40 ligand; DC-PCR, digestion circularization-PCR; CD40Lc, CD40L control; nAChR, nonrearranging acetylcholine receptor; S, switch.

chromosome while the intervening genomic material is looped-out and excised as a circle (1–4). The presence of double strand breaks (DSBs) in S DNA (5) and the dependency of CSR on the DNA-dependent protein kinase catalytic subunits (6), Ku80 (7) and Ku70 (8), components of the DNA-PK complex involved in DSB repair, strongly suggest that CSR is resolved through a nonhomologous DNA-end joining process (reviewed in Ref. 9).

Activation-induced cytidine deaminase (AID), a putative RNA editing enzyme, has been implicated in both CSR and somatic hypermutation in mouse and human (10, 11). AID expression is restricted to activated germinal center B cells that undergo CSR and somatic hypermutation. AID protein sequence is homologous to the mammalian RNA editing deaminase, APOBEC-1 (12, 13). APOBEC-1 is the catalytic component of the complex that edits apolipoprotein-B mRNA by changing a single ribo-cytidine to uridine (14). This RNA editing event generates a stop codon and causes the production of a truncated apoB polypeptide by cells of the small intestine. AID has deaminase activity when tested for deamination of deoxycytidine (13). It remains unclear whether AID functions directly or indirectly in mediating CSR and somatic hypermutation (15).

The isotype specificity of the CSR process is achieved in part through production of germline transcripts (gts; reviewed in Ref. 16). Accumulating evidence suggests that in addition to gts, there are other factors that contribute to CSR. For example, B cells lacking the transactivation domain of c-Rel are capable of expressing both α and ε gts, but are able to carry out only $\mu \rightarrow \alpha$ and not $\mu \rightarrow \varepsilon$ CSR (17). Similarly, B cells that are deficient in the p50 subunit of NF-κB can produce $\gamma 1$, α, and $\gamma 3$ gts, but switch only $\mu \rightarrow 1$ and not $\mu \rightarrow \alpha$ (18) or $\mu \rightarrow \gamma 3$ (19). Recent analyses that assay for CSR on extrachromosomal S plasmids demonstrate the existence of distinct switching activities that mediate $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \alpha$ CSR and are independent of gt expression (20). However, questions remain regarding the number of isotype-specific S factors that function in CSR, the nature of their recognition of S DNA, and their relationship to AID.

In this study, we report studies using a plasmid-based transient transfection assay for CSR to test for the presence of isotype-specific transacting factors. We provide evidence for two new switching activities that mediate $\mu \rightarrow \gamma 1$ and $\mu \rightarrow \varepsilon$ CSR. These activities are distinct from each other and from the previously described activities for $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \alpha$ CSR and they indicate a high degree of DNA sequence specificity in the mechanism of CSR. We found that in switching B cell lines, endogenous CSR was strictly correlated with the coordinate expression of the appropriate gt and isotype-specific switching activity, indicating that the transacting S factors are integral to the process of CSR. All the B cell lines capable of supporting CSR also express AID. Thus, AID expression per se does not fully explain the isotype specificity detected in the S plasmid assay.

Materials and Methods

Cell culture, FACS analysis, transfection, and genomic DNA isolation

Transformation of BALB/c splenocytes was conducted by coculturing LPS-activated B cells with a fibroblast line expressing the J2 virus as previously described (21, 22). Stable transformants were cloned by limiting dilution and were assayed for their phenotypes by

FACS staining (data not shown). One of these clones was designated 1.B4.B6. Culture conditions for 1.B4.B6, A20.3, M12, *Bal*I7 (20), I.29μ (23), CH12.LX (24), TIB114 (25), J558, J558L, and MPC11 (26) were previously described. Splenic B cells were prepared and activated with LPS as described previously (20) in the presence or absence of rIL-4 (1000 U/ml, a gift from C. Snapper). 1.B4.B6 cells were stimulated with LPS (50 μg/ml; Sigma-Aldrich, St. Louis, MO), CD40 ligand (CD40L), in the presence or absence of rIL-4 or human rTGFβ1 (1 ng/ml; R&D Systems, Minneapolis, MN). CD40L is the rCD40L-CD8α fusion protein secreted by the J558L mouse myeloma cell line (27). Supernatant containing CD40L was used at 1/2 dilution (v/v) for stimulation. CD40Lc is the supernatant collected from untransfected J558L cells. FACS analysis was conducted using 1.B4.B6 cells washed twice in ice-cold PBS buffer (1× PBS with 1% FBS and 0.1% sodium azide). Cells were resuspended in 100 μl of PBS buffer and were incubated on ice with rat IgG2b FcR block (anti-mouse CD32/CD16; BD PharMingen, San Diego, CA) for 5 min at a concentration of 5 μg/ml. Surface IgG3 was detected by incubating the cells with 5 μg/ml FITC-conjugated goat anti-mouse IgG3 Ab (Southern Biotechnology Associates, Birmingham, AL) on ice for 30 min. Flow cytometry analysis was performed on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA). Viable cells were gated by exclusion of propidium iodide and were analyzed for surface phenotype. Transfection and genomic DNA isolation was conducted as previously described (20).

Construction of plasmids

p218 and pG3. Iα EP were described previously (20, 28) and p218 and pG3. Iα EP are referred to in this study as pA.1 and pG3.1, respectively. T*o* build additional S substrates, a 300-bp fragment corresponding to residues 1813 to 2101 of mouse Sγ3 (M12182.1) was excised from pSγ3A (our unpublished data), a 0.78-kb fragment corresponding to residues 3878 to 4661 of Sγ1 was isolated from the pγ1/B.V1 plasmid (M12389; Ref. 29), and an 0.8-kb fragment corresponding to residues 405 to 1170 of Sε (M17012.1) was derived from pε 3PK (a gift from J. Stavnezer). The S region fragments were individually cloned into the *Xba*I site of Bluescript KS$^-$, orientation was determined, and then fragments were excised by a *Bam*HI-*Not*I digestion. The *Bam*HI-*Not*I fragments were cloned into gel purified pA.1 from which the Sα fragment had been excised in a *Bam*HI-*Not*I digestion. The new S substrates pG3.1 -S, pG1.1, and pE1.1 were generated by introduction of the cloned Sγ3, Sγ1, and Sε fragments into the prepared p218, respectively.

RT-PCR and digestion circularization (DC)-PCR

RT-PCR for gts was conducted as described (18) with modifications. Primers for γ3, γ1, ε, and α gts and GAPDH were previously described (18, 19, 30). RT-PCR primers for *AID* were previously described (13). A PCR protocol for AID amplification of 30 cycles consisting of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min was followed by a final 7-min elongation at 72°C. All PCR contained 2.5 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN), 0.2 mM each dNTP, 2.0 μCi [α-^{32}P]dCTP (3000 Ci/mmol; NEN, Boston, MA), 1× PCR buffer with Mg^{2+} provided by the manufacturer, and 0.2 μM each primer. The PCR products were purified by phenol and chloroform extraction and were separated by electrophoresis on 7% polyacrylamide gels. The incorporation of radioactive

dCTP was quantified by phosphorimaging using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

PCR amplification and cloning of Sμ/Sγ3 hybrid molecules

The PCR amplification and cloning of Sμ/Sγ3 molecules was conducted as previously reported (25) except that primer μ-1.2 (5′-GCTGGGGTGAGCTCAGCTATGCTACGC-3′), which anneals to positions 5307–5333 at the 5′ end of the germline Sμ (MUSIGCD07), was used. The Cκ gene was analyzed by PCR amplification using Cκ-1 (5′-CATCTGGAGGTGCCTCAGTCGTGTGC-3′) and Cκ-2 (5′-ACATTCCCCAAGCCCCAGA GTTTCC-3′) primers, which anneal to positions 1030–1055 and 2398–2422 on the Cκ gene (MUSIGKAF3), respectively, and gave rise to a 1.4-kb product. The Cκ PCR began with denaturation at 95°C for 3 min, followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min, and a final incubation at 72°C for 7 min. PCR amplification of the Sγ1 *cis* flanking DNA was conducted using primer Rg1 (5′-TTCAGCCTG GATGCCTACATTAAGAC-3′) and dc-μ.1 or dc-μ.2 primers (19) using the PCR amplification conditions as for Cκ amplification.

Endogenous and plasmid specific DC-PCR and the bacterial transformation assay

DC-PCR analysis for endogenous μ→γ3 and μ→γ1 CSR was performed as described (19). DC-PCR analysis for endogenous μ→ε and μ→α CSR was performed as described (18) with modifications. Two rounds of PCR using nested primer sets were performed where the Sμ primers were dc-μ.1 and dc-μ.2, as previously described (19). The Sε primers were dc-ε.1 (5′-ACACCGATGCAGGATACACCCCAGACC-3′) and dc-ε.2 (5′-CCCCA GACCTTCCAAGACTATG-3′), and the Sα primer was dc-α.1 (5′-CCCTCTGATGCACACCCTCACAGG-3′). In the second round of PCR for μ→α DC-PCR, the dc-μ.2 and dc-α.1 were used. The DC-PCR products for μ→ε and μ→α CSR were 585 and 499 bp, respectively. DC-PCR analysis for the S plasmids was performed as described previously (20) with modifications. Transfected genomic DNA (500 ng) was digested overnight with *Sac*I (MBI Fermentas, Amherst, NY) in the presence of 1 μM spermidine in a total volume of 100 μl. Digested DNA (10 ng) was ligated overnight in the presence of 200 ng of untransfected genomic DNA in a total volume of 100 μl using 6 U of T4 DNA ligase (MBI Fermentas), and 2 μl of the ligated DNA was used for PCR amplification. The 510-bp fragment was amplified with primers P1 and P4 in the presence of 3 μCi of [α-^{32}P]dCTP for 26 cycles each consisting of 95°C for 1 min, 60°C for 40 s, and 72°C for 1 min 30 s, followed by a final step at 72°C for 7 min. The 81-bp fragment was amplified using plasmid-specific primers P2 and P3γ1 (5′-CCTGTTACCCAGGTTACCTTGT-3′) and the 110-bp fragment was amplified using primers P5γ1 (5′-CCTGTTACCCAGGTTACCTTGT-3′) and P6 (5′-GACCGACGCCGTTCTGGCTCCTCATA-3′). Amplification of the 81- and 110-bp fragments was as described above, except that PCR programs consisted of 36 and 34 cycles, respectively. The bacterial transformation assay was conducted as previously described (20). Further experimental details are available upon request.

Results

B cell lines generated by J2 viral transformation

There is a paucity of B cell lines that can be induced to undergo CSR. We have derived B cell lines by transforming mitogen-activated murine splenic B cells with the J2 retrovirus carrying *v-raf-v-myc* oncogenes (see *Materials and Methods*; Refs. 21 and 22). One cell line, 1.B4.B6, was taken for further analysis and was found to express surface markers characteristic of mature, naive B cells; IgM$^+$, IgD$^+$, and B220$^+$. Two rearranged J$_H$ bands were observed in each of the subclones of 1.B4.B6 by Southern analysis indicating clonality. Clonality was verified by sequencing the VDJ regions from PCR products obtained from cDNA templates generated from each of the subclones. The V$_H$ of 1.B4.B6 cells differed at only a single nucleotide residue from *Q52*, the D is identical with *DFL16.2*, and the J is identical with *J3*. There were several N insertions at both the VD and DJ joins (data not shown).

CSR can be stimulated in 1.B4.B6 cells

J2-transformed B cell lines were previously shown to respond to the presence of a T$_H$2 type T cell line and Staphylococcal enterotoxin B by proliferation and secretion of very low amounts of IgG1 (30–70 ng/ml) (21). We reasoned that T cell signals such as soluble CD40L alone or in combination with other mitogens and cytokines might stimulate robust isotype switching in 1.B4.B6. To test this hypothesis, 1.B4.B6 cells were grown in the presence or absence of inducers and were analyzed for membrane IgG3 expression by FACS analysis. Surface IgG3 expression was essentially undetectable in unstimulated 1.B4.B6 cells and was only marginally detectable in cells stimulated for 4 days by CD40L or LPS (Fig. 1*A*). In contrast, 15.3% of the cells stimulated with CD40L and LPS expressed membrane IgG3, demonstrating a synergistic induction by this combination of stimuli.

To further characterize CSR in 1.B4.B6 cells, the induction of gts by mitogen and cytokines was assessed using RT-PCR. *GAPDH* was used as an internal control for cDNA template input. Unstimulated 1.B4.B6 cells did not express the $\gamma3$, $\gamma1$, α, and ε gts (Fig. 1*B*, *lane 1*). Production of the $\gamma3$ gt was induced following treatment with CD40L and LPS in the presence or absence of TGFβ or IL-4 (Fig. 1*B*). Individual activators or CD40L control (CD40Lc) supernatant (see *Materials and Methods*) only slightly augmented $\gamma3$ gt expression (Fig. 1*B*). Stimulation of 1.B4.B6 cells with CD40L and LPS in the presence of IL-4 induced $\gamma1$ and ε gts expression (Fig. 1*B*, *lane 8*), whereas CD40L and LPS in the presence of TGFβ induced α gt (Fig. 1*B*, *lane 7*). These observations demonstrate that gt production is inducible in 1.B4.B6 cells and parallels the cytokine requirements for gt induction found in normal splenic B cells (reviewed in Ref. 16).

CSR is a deletional process that occurs between Sμ and one of the downstream S regions to generate a complex mixture of hybrid Sμ/Sx molecules of various sizes. To determine whether $\mu \rightarrow \gamma3$ CSR could be induced in 1.B4.B6 cells, a PCR assay for detection of composite Sμ/S$\gamma3$ molecules was used (25). Multiple Sμ/S$\gamma3$ hybrid molecules were detected in DNA isolated from 1.B4.B6 cells following stimulation with LPS and CD40L, but were not found in unstimulated cells (data not shown). To verify that the Sμ/S$\gamma3$

composite molecules amplified by PCR represent bona fide CSR events, the $S\mu/S\gamma3$ hybrid molecules were cloned and 12 clones containing inserts of different sizes were randomly chosen for automated DNA sequence analysis. All 12 clones were found to contain $S\mu$ and $S\gamma3$ sequences, and eight clones were confirmed to contain $S\mu/S\gamma3$ S junctions (Fig. 1*C*). In all of these clones, the S junctions showed the same characteristics as previously described breakpoints found in physiological CSR events (31).

1.B4.B6 cells were analyzed for their capacity to switch to each of four isotypes using the DC-PCR analysis (32) summarized in Fig. 2*A*. The nonrearranging acetylcholine receptor (*nAChR*) gene was used as a control for digestion and ligation reactions, and all samples tested were positive for the *nAChR* DC-PCR product (Fig. 2, *B–D*). DNA from TIB114 served as a positive control for the $S\mu/S\gamma3$ DC-PCR product (Fig. 2*B*, *lane 7*). DC-PCR product was only evident in DNA from cells induced with LPS and CD40L (Fig. 2*B*). These findings directly demonstrate the induction of $\mu\rightarrow\gamma3$ CSR in 1.B4.B6 cells and are consistent with the observation of surface IgG3 expression, $\gamma3$ gt induction, and the presence of $S\mu/S\gamma3$ composite fragments in LPS- and CD40L-stimulated cells.

To further investigate the switching potential of the 1.B4.B6 cell line, the cells were stimulated with CD40L and LPS and TGFβ or CD40L and LPS and IL-4, which induce the α gt, and the $\gamma1$ and ε gts, respectively, and they were then analyzed by DC-PCR (Fig. 2, *C* and *D*). DNA from the IgA-producing myeloma cell line, J558, served as a positive control for the $S\mu/S\alpha$ DC-PCR product. The $S\mu/S\alpha$ DC-PCR product was detected only in DNA from 1.B4.B6 cells stimulated with CD40L and LPS and TGFβ, indicating the induction of $\mu\rightarrow\alpha$ CSR (Fig. 2*C*). DNA from LPS and IL-4-treated splenic B cells, which switch $\mu\rightarrow\gamma1$ and $\mu\rightarrow\varepsilon$, served as a positive control for the $S\mu/S\gamma1$ and $S\mu/S\varepsilon$ DC-PCR products (Fig. 2*D*). No $S\mu/S\varepsilon$ and $S\mu/S\gamma1$ DC-PCR products were detected in unstimulated 1.B4.B6 cells or cells stimulated with CD40L and LPS (Fig. 2*D*). 1.B4.B6 cells stimulated with CD40L and LPS and IL-4 gave rise only to $S\mu/S\varepsilon$ but not $S\mu/S\gamma1$ DC-PCR products (Fig. 2*D*). No $S\mu/S\gamma1$ DC-PCR product was detected from CD40L and LPS and IL-4-stimulated 1.B4.B6 cells even after additional cycles of PCR (data not shown). Priming sites for $S\mu/S\gamma1$ DC-PCR were intact in 1.B4.B6 cells because the primers dc-$\gamma1.1$ or dc-$\gamma1.2$ in combination with a downstream primer R$\gamma1$ (see Fig. 2*A*) amplified a PCR product of the expected size and appropriate DNA sequence (data not shown). The priming sites located at 5′ end of $S\mu$ are intact because the dc.μ-1 and dc.μ-2 primers were able to participate in amplification of $S\mu/S\gamma3$, $S\mu/S\alpha$, and $S\mu/S\varepsilon$ DC-PCR products in activated 1.B4.B6 (Fig. 2, *B–D*). Genomic Southern analysis demonstrated that the overall $S\gamma1$ region in 1.B4.B6 cells is intact (data not shown). Taken together, these findings indicate that 1.B4.B6 cells can be induced to switch $\mu\rightarrow3$, $\mu\rightarrow\alpha$, and $\mu\rightarrow\varepsilon$, but not $\mu\rightarrow\gamma1$. However, it is possible that other combinations of stimuli are capable of inducing $\mu\rightarrow\gamma1$ switching in 1.B4.B6 cells.

Distinct factors mediate μ→γ3 and μ→γ1 plasmid-based CSR

One hypothesis to explain the absence of endogenous $\mu\rightarrow\gamma1$ CSR in 1.B4.B6 cells is that these cells fail to express the transacting $\mu\rightarrow\gamma1$ switching activity. We previously demonstrated that distinct switching activities independently mediate $\mu\rightarrow\gamma3$ and $\mu\rightarrow\alpha$ CSR using extrachromosomal S substrates that are capable of detecting transacting factors (20).

To test the hypothesis that a $\mu \rightarrow \gamma 1$ switching factor is absent in 1.B4.B6 cells, a new S plasmid was constructed to assay for $\mu \rightarrow \gamma 1$ CSR and is referred to in this study as pG1.1 (Fig. 3*A*). CSR on pG1.1 and pG3.1, which assays $\mu \rightarrow \gamma 3$ switching, was compared in various cell lines by transient transfection, isolation of DNA from nuclei, and transformation of bacteria to recover S/S recombinant plasmids as previously described (20). Recombination between S regions leads to deletion of *TK* and loss of a unique *Eco*RI restriction site, and those plasmids that are resistant to *Eco*RI digestion are likely to be S/S recombinants (Fig. 3*A*). To control for possible S/S recombination in bacteria, in the same experiments, pG3.1 and pG1.1 DNA were untreated or digested with *Eco*RI, and the frequency of ampicillin resistant colonies was determined. The efficiency of transformation with intact plasmid was 5×10^7 colonies/μg. Following transformation, we found no ampicillin-resistant colonies, indicating that *Eco*RI resistance arises as a consequence of transfection into cell lines. Table I shows the cumulative numbers of colonies screened and S/S recombinant plasmids recovered and verified by restriction mapping from several transfected samples, as indicated. PG3.1 supported $\mu \rightarrow \gamma 3$ switching in 1.B4.B6 cells and LPS-activated splenic B cells, confirming previous studies (Table I) (20). No S/S recombinant pG1.1 were recovered from LPS-activated splenic B cells, unstimulated 1.B4.B6, 1.B4.B6 stimulated with the combination of CD40L and LPS and IL-4, or unstimulated I.29μ, A20, or M12 cells (Table I). In contrast, 10 S/S recombinants derived from pG1.1 were recovered from LPS and IL-4-activated splenic B cells. The χ^2 analysis indicates that all the cell lines and LPS-activated B cells are significantly different from the LPS and IL-4-activated B cells with respect to the incidence of S/S recombinant plasmids recovered (Table I). The 10 S/S recombinant pG1.1 plasmids were taken for DNA sequence analysis and the S junctions were demonstrated to lie within S region DNA. The Sμ/S$\gamma 1$ junctions were located for four plasmids (4BG-121, 142, 157, and 190; Fig. 3*B*) and showed direct joining of Sμ and S$\gamma 1$ DNA without deletion, duplication, or nucleotide insertion at the breakpoints. The microhomology at the Sμ/S$\gamma 1$ junctions was zero to four bases and is similar to that usually observed (31). In the remaining six clones, CSR occurred in the S regions; however, the junctions were too far from the ends to be located by automated DNA sequencing. These findings indicate that pG1.1 is capable of CSR and that the $\mu \rightarrow \gamma 1$ switching factor is induced by IL-4 in LPS-activated B cells, whereas the $\mu \rightarrow \gamma 3$ switching factor is found in B cells activated with LPS alone. These results also demonstrate that 1.B4.B6 cells can produce the $\mu \rightarrow \gamma 3$ but not the $\mu \rightarrow \gamma 1$ switching factor, and they confirm our hypothesis that the $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ switching factors are distinct.

The length of S$\gamma 1$ in pG1.1 is 0.78 kb as compared with the 2.0 kb of S$\gamma 3$ DNA in pG3.1. To determine whether the length of S DNA in the S plasmid contributes to the frequency of CSR events, we constructed pG3.1-S, which contains 300 bp of S$\gamma 3$ DNA (Fig. 3*A*) and compared its switching frequency to that of pG3.1 in 1.B4.B6 cells. S/S recombinant pG3.1-S and pG3.1 were recovered at essentially equal frequencies (Table I). This result demonstrates that 300 bp of S$\gamma 3$ containing only five tandem repeats are sufficient to support plasmid-based CSR and suggests that the length of S$\gamma 1$ present in pG1.1 is sufficient for plasmid-based CSR.

To further evaluate the expression profile of $\mu \rightarrow \gamma 1$ switching activity, a previously described semiquantitative DC-PCR assay was adapted to assess CSR on pG1.1 (20) and is shown in Fig. 3*C*. The linear range of detection for the vector-associated 510-bp fragment was established using 2-fold serial dilutions of pG1.1 into 0.5 μg of genomic DNA followed by DC-PCR analysis in the presence of radiolabled deoxynucleotides (Fig. 3*D*). The addition of increasing amounts of pG1.1 (0.25–2 ng/ml) resulted in a linear increase of vector-specific 510-bp product (Fig. 3*D*). One standard sample (0.5 ng/ml) was taken from this titration and was included in all subsequent studies to assure that the amplification product derived from the plasmid backbone was always in the linear range of detection. To confirm that the conditions chosen for intramolecular ligation were also valid for the Sμ/ Sγ1-associated 81-bp DC-PCR product, we tested for the presence of this fragment using a range of concentrations of plasmid digested with *Sac*I (Fig. 3*D*). At high concentrations of input plasmid (2 ng/ml), intermolecular ligation occurred and produced the 81-bp product, whereas at dilute plasmid concentrations (<1 ng/ml), no 81-bp product was detected, demonstrating that fragment ligation was intramolecular. A standard sample, 0.5 ng, was taken from this titration and was included in all subsequent studies to assure that the amplification product derived from the plasmid backbone was always in the linear range of detection.

DC-PCR analysis of CSR on the pG1.1 plasmid was undertaken in activated splenic B cells and in switching and nonswitching B cell lines. The intact standard sample of pG1.1 plasmid (0.5 ng/ml) was included as a control and showed no evidence of the 81- or 110-bp DC-PCR products, but was positive for the 510-bp DC-PCR product as expected (Fig. 3*E*, *lane 1*). A S/S recombinant derivative of pG1.1 (0.5 ng/ml) was included as a positive control and gave rise to the 510-bp fragment associated with the vector backbone and the 81-bp fragment associated with S/S recombinant molecules, but no 110-bp fragment associated with the circular excision product because it was previously lost (Fig. 3*E*, *lane 2*). The 81- and 110-bp fragments were detected in B cells induced with LPS and IL-4, but not with LPS alone and this detection confirms that pG1.1 is recombinogenic in B cells that undergo physiological switching. Recombination of pG1.1 is undetectable in all the B cell lines tested, including 1.B4.B6. Taken together, these results demonstrate isotype-specific CSR profiles of the pG3.1 and pG1.1 plasmids and support the conclusion that $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ CSR are mediated, at least in part, by S region-specific factors.

S plasmid assays for μ→α and μ→ε recombination

Sa and Sε regions share considerable homology to each other. This raises the question whether $\mu \rightarrow \alpha$ and $\mu \rightarrow \varepsilon$ recombination are mediated by distinct factors. A S plasmid, pE.1, carrying Sε was constructed and is identical with pG3.1, pG1.1, and pA.1 in all respects except for the identity of the downstream S region (Fig. 3*A*). The facility with which pE.1 and pA.1 support CSR in LPS-activated splenic B cells and a panel of B cell lines was compared using the bacterial transformation assay (Table II). Both pE.1 and pA.1 were found to recombine at similar frequencies in LPS-activated B cells, I.29μ, and 1.B4.B6 cells, suggesting that factors supporting $\mu \rightarrow \alpha$ and $\mu \rightarrow \varepsilon$ CSR are present. In contrast, very rare recombinant pA.1 and pE.1 were found in unstimulated A20 and M12 cells, indicating that the activities supporting plasmid-based CSR are very poorly expressed in these cells. χ^2

analysis indicates that pA.1 and pE.1 S frequencies in 1.B4.B6, I.29μ cells, and LPS-activated splenic B cells are essentially identical and significantly different from those found in M12 and A20 cells. Furthermore, switching on the pE.1 and pA.1 plasmids was constitutive in 1.B4.B6 and I.29μ cells, confirming previous observations for pA.1 (20). DNA sequence analysis of S junctions derived from the pE.1 and pA.1 plasmids and the S junctions are shown (Fig. 4, *A* and *B*). Six recombinant pE.1 plasmids were sequenced, and four S junctions were found. Sixteen recombinant pA.1 plasmids were sequenced, and six S junctions were found. All the junctions had characteristics associated with previously defined CSR breakpoints (31). The simultaneous presence of the $\mu \rightarrow \alpha$ and $\mu \rightarrow \varepsilon$ switching activities in I.29μ and 1.B4.B6 could arise from coexpression of two distinct activities or from a single activity with specificity for both Sε and Sα DNA. To distinguish between these alternatives, additional B cell lines are required that can be induced to undergo $\mu \rightarrow \varepsilon$ or $\mu \rightarrow \alpha$ CSR.

AID expression is used to identify B cell lines with the potential for CSR

Recent studies show that the *AID* gene is specifically expressed in mature normal B cells and is required for CSR in vivo (10, 11). We reasoned that cell lines that express *AID* might have the ability to undergo CSR, and we screened a panel of B cell lines to assess *AID* expression by RT-PCR. *GAPDH* expression was used as an internal control for cDNA template input for all samples tested (Fig. 5, *A* and *C*). The *AID* RT-PCR product was detected in LPS-activated splenic B in the presence or absence of IL-4, as previously reported (13), and in the mature B cell lines, 1.B4.B6, CH12.LX, I.29μ, M12, and A20 (Fig. 5*A*). In contrast, 70/Z3 and 8A5.4A5.II.88, pre-B cell lines, TIB114 and B1.8-δ, hybridomas, and J558, a myeloma, did not express detectable levels of *AID* transcript. In the same experiment, a 2-fold serial dilution of cDNA from LPS and IL-4 B cells and CH12.LX cells (taken from the same cDNAs used in Fig. 5*A*, *lanes 2* and *4*) was used to prepare standard curves (Fig. 5*B*). The arrows shown in the linear regression plots indicate that the signal intensities for the AID RT-PCR products (for the same dilution of cDNA used in Fig. 5*A*, *lanes 2* and *4* and *C*, *lanes 6*, *14*, and *15*) are within the linear range of detection.

Because AID was identified in CH12 cells activated to undergo CSR (13), we sought to determine whether increased AID expression was generally correlated with activation of isotype switching. The splenic B cells and B cell lines were induced with activators known to stimulate CSR, and the level of AID expression was assessed by RT-PCR. It is interesting to note that in normal splenic B cells and the B cell lines, the induced expression of *AID* transcripts (Fig. 5*C*) was not correlated with the induction of CSR (for M12 and A20 see below) because some stimulation mixtures led to CSR and to a concomitant reduction of AID expression. For example, CD40L and LPS induction of 1.B4.B6 cells leads to stimulation of $\mu \rightarrow \gamma 3$ CSR (Fig. 1), but to reduction of AID expression (Fig. 5*C*, compare *lanes 7* and *8*). Similarly, in normal B cells stimulated with LPS or LPS and IL-4, CSR was induced $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$, respectively, (data not shown), but the level of the AID transcript decreased in response to LPS as compared with cells induced with LPS and IL-4 (Fig. 5*C*, *lanes 5* and *6*). Thus, the level of AID transcript is not directly related to the facility with which CSR occurs. In contrast, gt expression is directly related to the level of CSR (16).

Among the *AID*-expressing cell lines, M12 and A20, IgG$^+$ B cell lymphoma cell lines have not been reported to switch. To explore the switching potential of M12 and A20, these cell were stimulated with various combinations of activators and they were tested for their ability to express gts and to engage in CSR. Un-stimulated I.29μ cells, which constitutively express α and ε gts, were used as positive controls in the RT-PCR analysis (Fig. 6*A*, *lane 8*). In M12 cells, ε gts were induced by each combination of activators tested, but were not found in unstimulated cells (Fig. 6*A*, *lanes 2–4*). In contrast, α gts were expressed in unstimulated M12 cells (Fig. 6*A*, *lane 1*), and treatment of the cells with any combination of inducers reduced gt expression (Fig. 6*A*, *lanes 2–4*). DC-PCR was used to assess CSR status, and the IgA expressing J558 and splenic B cells induced with LPS and IL-4 were used as positive controls for Sμ/Sα and Sμ/Sε DC-PCR products, respectively, (Fig. 6*B*, *lanes 8* and *9*). In M12 cells, no μ→α switching could be detected under any conditions tested (Fig. 6*B*, *lanes 1–4*), whereas μ→ε switching was induced with CD40L and IL-4 or CD40L and LPS and IL-4, but not in unstimulated cells or cells treated with CD40L alone (Fig. 6*B*, *lanes 1–4*). In A20 cells, the α gt was not detected under any of the conditions tested, but the ε gt was found in all of the conditions analyzed, albeit at different levels (Fig. 6*B*, *lanes 5–7*). However, neither μ→α nor μ→ε CSR was evident under any circumstances (Fig. 6*B*, *lanes 5–7*).

Distinct switching activities mediate μ→α and μ→ε recombination

To examine the profile of plasmid-based switching activities, M12 and A20 were transiently transfected with either pA.1 or pE.1 and were analyzed using the bacterial transformation assay. In M12 cells, the pA.1 plasmid underwent CSR following CD40L activation only (Table II). In contrast, the pE.1 plasmid underwent CSR following CD40L, LPS, and IL-4 stimulation, but not in unstimulated cells or cells activated with CD40L (Table II). The *p* values derived from the χ^2 analysis indicate that the μ→α and μ→ε switching factors are differentially inducible in M12 cells. It is notable that in M12 cells, the same activation conditions that gave rise to endogenous μ→ε CSR (Fig. 6*B*) also supported pE.1 recombination.

In A20 cells, S/S recombinant pA.1 was detected in cells activated with CD40L and TGFβ or with CD40L and IL-4, but not in unstimulated cells or cells activated with CD40L alone (Table II). Furthermore, in A20 cells, no significant pE.1-associated recombination events were found in any activation condition tested. The χ^2 analyses confirm these conclusions, which are summarized in Fig. 6, *C* and *D*. In A20 cells, the absence of endogenous μ→α CSR following induction with CD40L and IL-4 or CD40L and TGFβ is most likely due to the absence of the α gt (Fig. 6*A*, *lanes 5–7*) because S/S recombinant pA.1 was detected under these conditions. In these cells, the absence of endogenous μ→ε CSR is correlated with the absence of pE.1-associated recombination because ε gts are present in these cells (Fig. 6*A*, *lanes 5–7*).

It is important to note that our S plasmids do not contain an origin of replication (28), whereas the reporter plasmids for VDJ recombination undergo episomal replication (33). Switching frequencies derived from the bacterial transformation assay for the pE.1 and pA. 1 S plasmids transfected into M12 and A20 cells ranged from 8.3×10^{-4} to 2.3×10^{-4}

(Table II) and are similar to previously reported results (20). The frequency of S/S recombinant plasmids found in switching B cells is similar to that found for a VDJ plasmid that carries a defective origin of replication and is higher than the incidence VDJ recombination in the absence of an origin of replication (33).

Discussion

Although it is clear that S regions are targeted for CSR, essentially nothing is known regarding the basis of molecular recognition of S DNA. The studies presented in this paper demonstrate that each of four isotype specific S substrates detect distinct isotype-matched transacting activities for CSR in transient transfection experiments. Our earlier study that focused on $\mu \rightarrow \gamma 3$ and $\mu \rightarrow a$ CSR demonstrated that unique switching activities mediated these CSR events. However, the tandem repeats in the $S\gamma 3$ and Sa regions are relatively unrelated and there are numerous sequence differences and overall length differences between them. Our new studies indicate that two distinct activities mediate $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ switching events based on the differential capacity of the $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ S substrates to support recombination in 1.B4.B6 cells and in normal mitogen-activated splenic B cells. These findings are striking because there are only 12 nucleotide differences between the 49-bp consensus $S\gamma 1$ and $S\gamma 3$ tandem repeats (34). The $S\gamma 3$ and $S\gamma 1$ regions differ structurally where $S\gamma 1$ is the longest and the most complex. However, the $S\gamma 1$ and $S\gamma 3$ DNA segments located in the pG3.1 and pG1.1 S substrates are composed only of simple tandem repeats (34). Thus, global structural differences are unlikely to contribute to the differential recognition of $S\gamma 1$ and $S\gamma 3$ regions by the $\mu \rightarrow \gamma 1$ and $\mu \rightarrow \gamma 3$ switching activities found in 1.B4.B6 cells and in mitogen-activated splenic B cells. Our studies also indicate that there are distinct activities that mediate $\mu \rightarrow a$ and $\mu \rightarrow \varepsilon$ S events. This is based on the differential induction of plasmid-based $\mu \rightarrow a$ and $\mu \rightarrow \varepsilon$ switching in the IgG$^+$ B cell lymphoma cell lines, M12 and A20. The Sa and $S\varepsilon$ regions are members of the same subfamily of S regions and are most similar to $S\mu$ (34). Therefore, it is surprising that $S\varepsilon$ is uniquely recognized as compared with both $S\mu$ and Sa. Our studies demonstrate that relatively small differences in S DNA sequence can confer isotype specificity to the switching reaction.

In B cell lines and in mitogen-activated normal B cells, the detection of isotype-specific switching activities using plasmid-based assays was paralleled by the ability of the endogenous loci to switch. There were two exceptions to this rule. A20 and M12 cells could each be induced to express plasmid-based $\mu \rightarrow a$ switching activities, but did not switch $\mu \rightarrow a$ at their endogenous loci. The absence of a gts in A20 and M12 cells under conditions that provide for the expression of $\mu \rightarrow a$ switching activities is the simplest explanation for this endogenous switching deficit. Our studies indicate that the $\mu \rightarrow \gamma 1$ switching activity is expressed only in B cells activated with LPS and IL-4, whereas $\mu \rightarrow \varepsilon$ activity is present in LPS B cells. However, at the endogenous locus, switching to ε is more sensitive to IL-4 induction than is switching to $\gamma 1$. The IL-4-dependent switching phenomenon most likely arises from the differential induction of the e and g1 gts by IL-4 (35). The absence of the $\mu \rightarrow \gamma 1$ switching activity in LPS B cells and its induction by IL-4 ensures that $\mu \rightarrow \gamma 1$ switching occurs only when both the $\gamma 1$ gt and the $\mu \rightarrow \gamma 1$ switching activities are coexpressed. Similarly, the absence of the ε gt in LPS B cells, even in the presence of the $\mu \rightarrow \varepsilon$ switching activity, assures the IL-4 dependence of this event. Together, our studies

indicate that CSR at endogenous loci is strikingly correlated with the coordinated expression of both isotype-specific gts and isotype-matched switching activities such that endogenous CSR is absent if either element is deficient or limiting. Furthermore, the expression of *AID* transcript led to the successful identification of M12 and A20 cells as containing CSR potential, suggesting that this marker may indicate a unique stage of B cell differentiation.

Several groups have previously reported DNA constructs designed to assay CSR. These S substrates and accompanying assay systems had a key limitation in that recombination was not limited to B cells in general or switching B cells in particular (36– 40). Recently, a S substrate assayed in stable transfection experiments and using a single switching cell line, CH12, reported that CSR was not dependent on S region identity (41). Although the CH12 cell line switches predominantly $\mu \rightarrow \alpha$, it is also capable of occasional $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ switching in a subclone-dependent fashion (24), implying that several isotype-specific switching activities can be expressed in these cells. Kinoshita et al. (41) did not further test the specificity of their S constructs in other switching cell lines with well-defined switching profiles. Therefore, the ability of several isotype-specific S substrates to undergo recombination in CH12 cells may not be inconsistent with our results. Additional work is required to clarify this issue.

S regions vary in length from 1 to 10 kb. Surprisingly, comparison of S substrates with full-length S$\gamma 3$ containing 44 tandem repeats or a short version of S$\gamma 3$ containing five tandem repeats indicated no discernable differences in CSR frequency as assessed by the bacterial transformation assay. Recently, a mouse was constructed in which the Sμ region, composed of $(GAGCT)_n GGGGT$ motifs, was removed by targeted homologous recombination (42). In the S$\mu^{-/-}$ mouse, 15 GAGCT motifs remained in regions flanking the original Sμ region and were sufficient to support endogenous CSR, albeit at a reduced frequency. These studies suggest that the number of S region tandem repeats may influence the efficiency of CSR, and are consistent with our findings. Together, these studies indicate that a limited S DNA target is sufficient to support CSR and they raise the interesting question of how many tandem repeats constitute a minimal S region.

Our studies indicate the existence of at least four independent isotype-specific switching activities. It is possible that switching activities detected by the S plasmids result from the absence of a suppressor activity rather than from a positive regulator. In this scenario, isotype-specific suppressors are present and are selectively turned off to give rise to isotype-specific switching. Alternatively, the switching activities could be positive regulators of CSR that function either at the cleavage step to generate nicks or DSBs in S DNA or in the resolution of the recombination intermediates. We favor a model in which the isotype-specific switching activities are either distinct S recombinases or complexes composed of a general recombinase and a docking protein with specificity for a specific S region. The docking protein would function as a DNA binding protein with specificity for a single S region and with the ability to recruit the S recombinase to the S region. This would provide for protection against inappropriate DNA cleavage because the endonuclease would be unable to directly recognize DNA except in the presence of the docking protein. We previously identified DNA binding proteins specific for the tandem repeats of Sμ, S$\gamma 3$, S$\gamma 2b$, and S$\gamma 1$ DNA (19, 43– 45); however, additional investigation is required to determine

whether these proteins are directly involved in CSR and function as the postulated docking proteins predicted in our model.

AID was originally identified in a subtractive hybridization screen for genes activated upon induction of CSR in a subclone of the CH12 B lymphoma cell line (13). In our studies, plasmid-based switching activities were documented in five mature B cell lines that express AID. Thus, AID expression in mature B cell lines may have strong predictive value with respect to a given cell lines switching potential. It is possible that AID is the endonuclease or regulates the expression of the endonuclease through its putative RNA editing activity. However, the AID-positive B cell lines differ with respect to their pattern of isotype specificity. This strongly implies the there are other factors in addition to AID that are important to CSR and that these additional activities confer isotype specificity to the CSR reaction.

Acknowledgments

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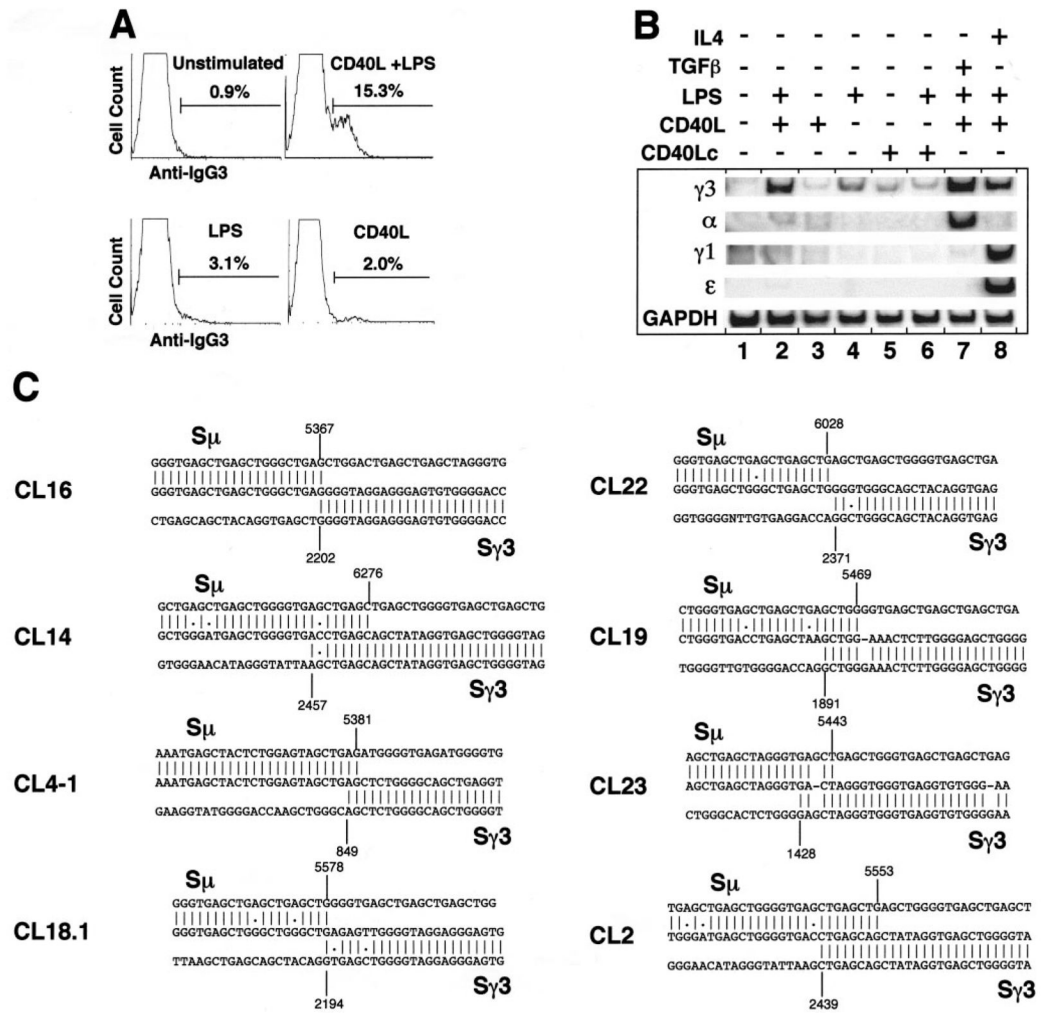
FIGURE 1.

The 1.B4.B6 cell line undergoes $\mu \rightarrow \gamma 3$ CSR. *A*, FACS analyses of surface IgG3 expression in 1.B4.B6 following induction of CSR. Cells were unstimulated or stimulated with CD40L + LPS, LPS, or CD40L for 5 days as indicated. Cells were then stained with FITC-conjugated anti-IgG3 Abs in the presence of Fc block. The percentages of IgG3^{+} cells are shown after gating on the indicated population. Data shown are representative FACS analyses after gating on viable cells. *B*, Analysis of gt expression by RT-PCR in 1.B4.B6 cells. cDNA was synthesized from total RNA isolated following 24 h of stimulation with the indicated inducers. *GAPDH* level was analyzed in each sample and was used to normalize cDNA template input for RT-PCR. Gts were analyzed in unstimulated 1.B4.B6 cells (*lane 1*), 1.B4.B6 stimulated with CD40L + LPS (*lane 2*), CD40L (*lane 3*), LPS (*lane 4*), CD40Lc supernatant (*lane 5*), LPS + CD40Lc (*lane 6*), the combination of CD40L + LPS + TGFβ (TGFβ was used at 1 ng/ml, *lane 7*), or the combination of CD40L + LPS + IL-4 (IL-4 was used at 1000 U/ml, *lane 8*). *C*, The Sμ/S$\gamma 3$ S junctions were PCR amplified from LPS + CD40L-activated 1.B4.B6 cells and the recombination breakpoints were determined using automated DNA sequencing. The DNA sequences were aligned with the germline Sμ
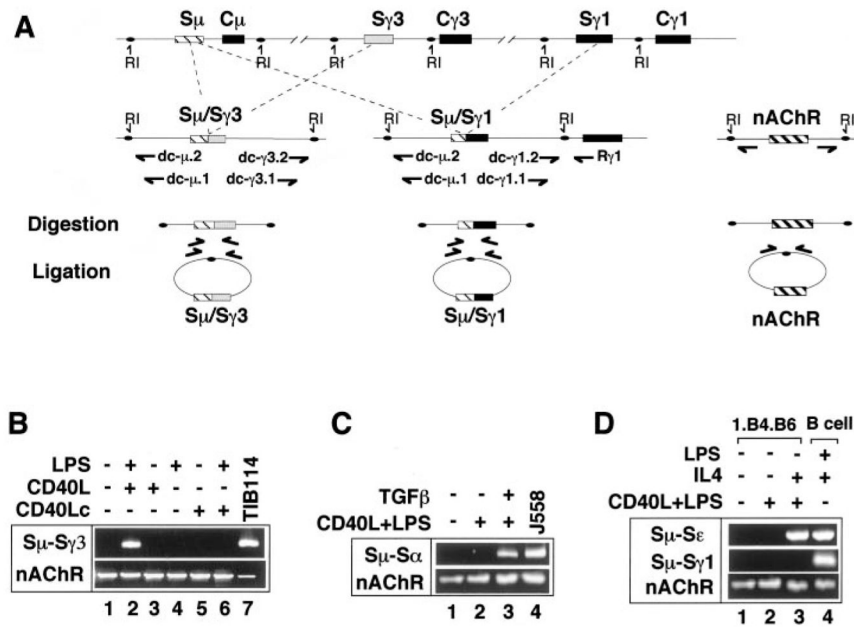
(MUSIGCD07) and Sγ3 sequences. Position 1 of Sγ3 corresponds to position 1578 in the GenBank sequence (D78343).

FIGURE 2.

DC-PCR analysis of $\mu \rightarrow \gamma 3$, $\mu \rightarrow \gamma 1$, $\mu \rightarrow \varepsilon$, and $\mu \rightarrow \alpha$ CSR in 1.B4.B6 cells. *A*, Schematic diagram of the DC-PCR strategy for endogenous loci. CSR results in the deletion of genomic DNA located between two S regions. *Eco*RI sites (denoted RI) that flank the 5′ end and the 3′ end of the Sμ and S$\gamma 3$ regions, respectively, are preserved following CSR. After digestion with *Eco*RI, the DNA is ligated under dilute conditions that favor circularization of the restriction fragments. Nested primer sets specific for sites at the 5′ end of Sμ and the 3′ end of S$\gamma 3$ amplify the region spanning the circle joint and yield a specific DC-PCR product, indicating the presence of Sμ/S$\gamma 3$ composite molecules. A portion of the *IgH* locus is depicted before and after $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ recombination. The nonrearranging *nAChR* gene serves as an internal control for digestion and ligation. The positions and the orientations of the primer sets are shown before and after ligation. *B*, DC-PCR analysis for the $\mu \rightarrow \gamma 3$ CSR. DNA from unstimulated 1.B4.B6 (*lane 1*) and 1.B4.B6 stimulated with the indicated activators (*lanes 2–6*) was analyzed by DC-PCR to detect $\mu \rightarrow \gamma 3$ switching. The IgG3 producing cell line, TIB114 (*lane 7*), was used as a positive control for the Sμ/S$\gamma 3$ DC-PCR product. *C*, DC-PCR analysis for $\mu \rightarrow \alpha$ CSR. DNA from unstimulated 1.B4.B6 (*lane 1*) and 1.B4.B6 stimulated with CD40L + LPS (*lane 2*) or CD40L + LPS + TGFβ (*lane 3*) was analyzed to detect $\mu \rightarrow \alpha$ CSR. The IgA-producing cell line, J558 (*lane 4*), was used as a positive control for the Sμ/Sα DC-PCR product. *D*, DC-PCR analysis for the $\mu \rightarrow \gamma 1$ and $\mu \rightarrow \varepsilon$ CSR. DNA from unstimulated 1.B4.B6 (*lane 1*) and 1.B4.B6 stimulated with CD40L + LPS (*lane 2*) or CD40L + LPS + IL-4 (*lane 3*) was analyzed. DNA from splenic B cells activated by IL-4 + LPS was used as a positive control for $\mu \rightarrow \gamma 1$ and $\mu \rightarrow \varepsilon$ CSR.
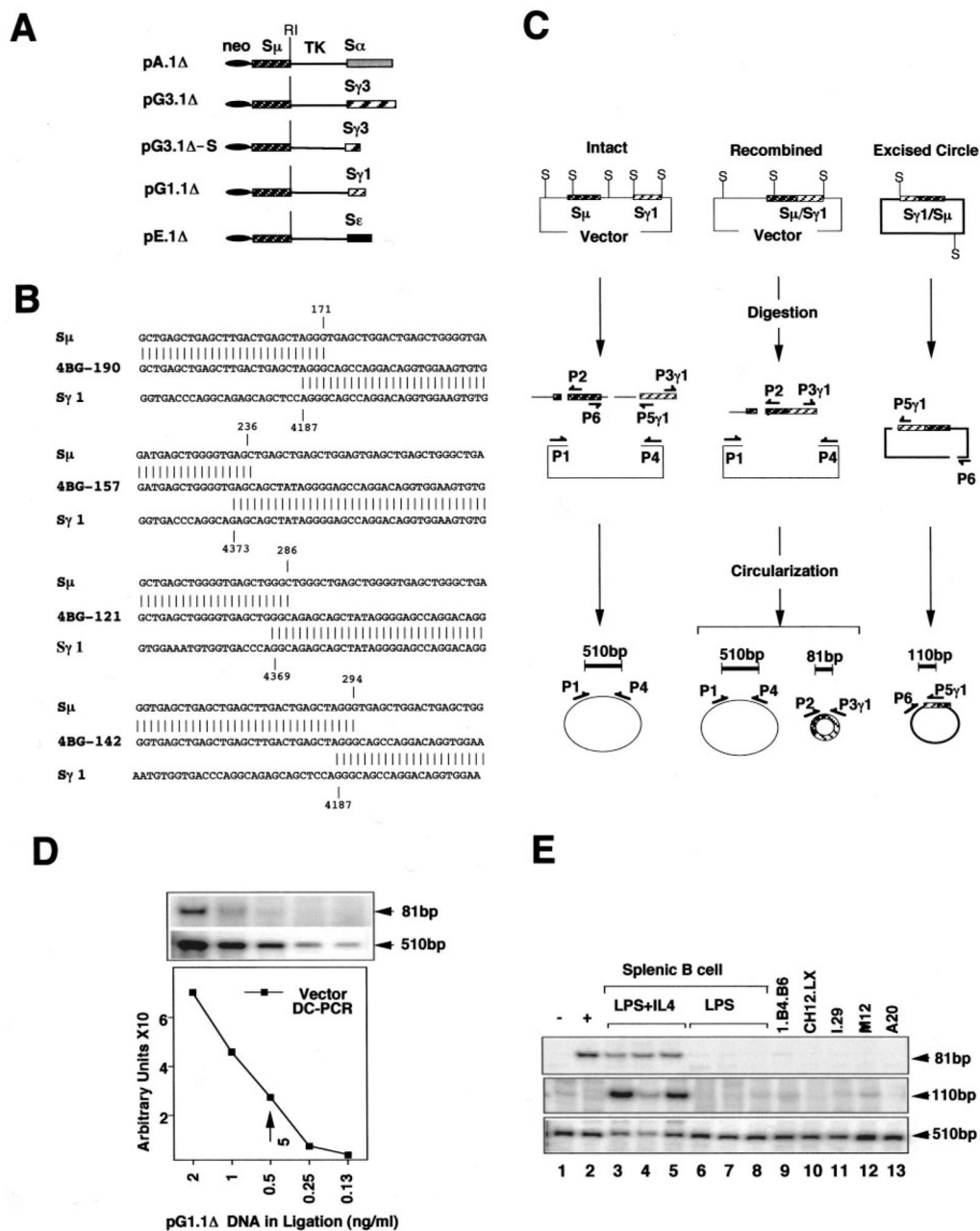
FIGURE 3.

The pG1.1 S substrate undergoes CSR in LPS + IL-4-activated splenic B cells, but not in LPS B cells or in 1.B4.B6 cells. *A*, A schematic representation of the S plasmids used in these studies. The pA.1 and pG3.1 were previously referred to as p218 and pG3.1 Iα EP, respectively (20, 28). The S substrates pG1.1, pE1.1, and pG3.1 –S are new constructs (see *Materials and Methods*). The pG3.1 –S plasmid contains a 0.3-kb fragment of Sγ3, whereas pG3.1 contains the full-length 2.0-kb Sγ3 region. The overall structure of the S substrates is 5′ *neomycin* gene (*neo*), Sμ, *thymidine kinase* gene (TK), and Sα or Sγ3 or Sγ1 or Sε–3′, as indicated. A unique *Eco*RI restriction site (denoted RI) is located at the junction of Sμ and *TK*. *B*, DNA sequence analysis demonstrates direct S/S

joining in pG1.1 recombinant plasmids. Four Sμ/Sγ1 recombinant plasmids were recovered by bacterial transformation from LPS + IL-4-activated normal splenic B cells transfected with pG1.1 . Automated DNA sequence analysis was used to determine the Sμ/Sγ1 breakpoints. Nucleotide position 1 of the plasmid Sμ sequence corresponds to position 5330 of germline Sμ sequence (GenBank accession number MUSIGCD07). Nucleotide position 1 of plasmid Sγ1 sequence corresponds to position 1 of the germline Sγ1 sequence (GenBank accession number M12182.1). *C*, Schematic illustration of a DC-PCR assay for detection of μ→γ1 switching on the pG1.1 plasmid. Recombination between two S regions of pG1.1 will cause the deletion of *TK*, loss of two internal *Sac*I sites (denoted S), and conservation of *Sac*I sites situated in the upstream portion of Sμ and downstream of Sγ1. The recombined S/S hybrid molecules will be located on a new *Sac*I fragment, whereas the intact S regions would be located on two different *Sac*I fragments. Following digestion with *Sac*I, DNA is ligated under dilute conditions resulting in the circularization of individual restriction fragments. The region spanning the circle joint is amplified as an 81-bp product using reverse primers P2 and P3γ1 specific for sites at the 5′ end region of Sμ and 3′ end of Sγ1, respectively. Recombination also generates a circular deletion product that contains the reciprocal S/S hybrid fragment and *TK*. The reciprocal S/S region is on a single *Sac*I restriction fragment and will circularize after ligation. The region spanning the circle joint is amplified as an 110-bp product using reverse primers P6 and P5γ1 specific for sites at the 3′ end region of TK and 5′ of Sγ1, respectively. The 81- and 110-bp DC-PCR products are plasmid specific and could not be derived from the endogenous locus based on the position of the genomic *Sac*I sites. The plasmid backbone is contained in a single *Sac*I restriction fragment that will circularize when ligated under dilute conditions and will yield a 510-bp DC-PCR amplification product and is used to control for plasmid recovery, ligation, and PCR amplification efficiency. *D*, DC-PCR products are in the linear range of detection. Two-fold serial dilutions of the *Sac*I-digested pG1.1 were titrated into 200 ng of genomic DNA and were analyzed by DC-PCR (*top*). The PCR amplification signals for the 510-bp fragment were plotted against the concentration of input plasmid DNA (*bottom*) and served to determine a standard curve and the range of linear detection. One sample (0.5 ng/ml, marked by arrow), which is in the linear range of detection, was included in every subsequent analysis. *E*, Normal splenic B cells from BALB/c *nu/nu* mice activated with LPS in the presence (*lanes 3–5*) or absence (*lanes 6–8*) of IL-4 and B cell lines, as indicated (*lanes 9–13*), were transfected with pG1.1 were and analyzed by DC-PCR. Intact and S/S recombinant pG1.1 were used as negative (−) and positive (+) controls for the DC-PCR in *lanes 1* and *2*, respectively.
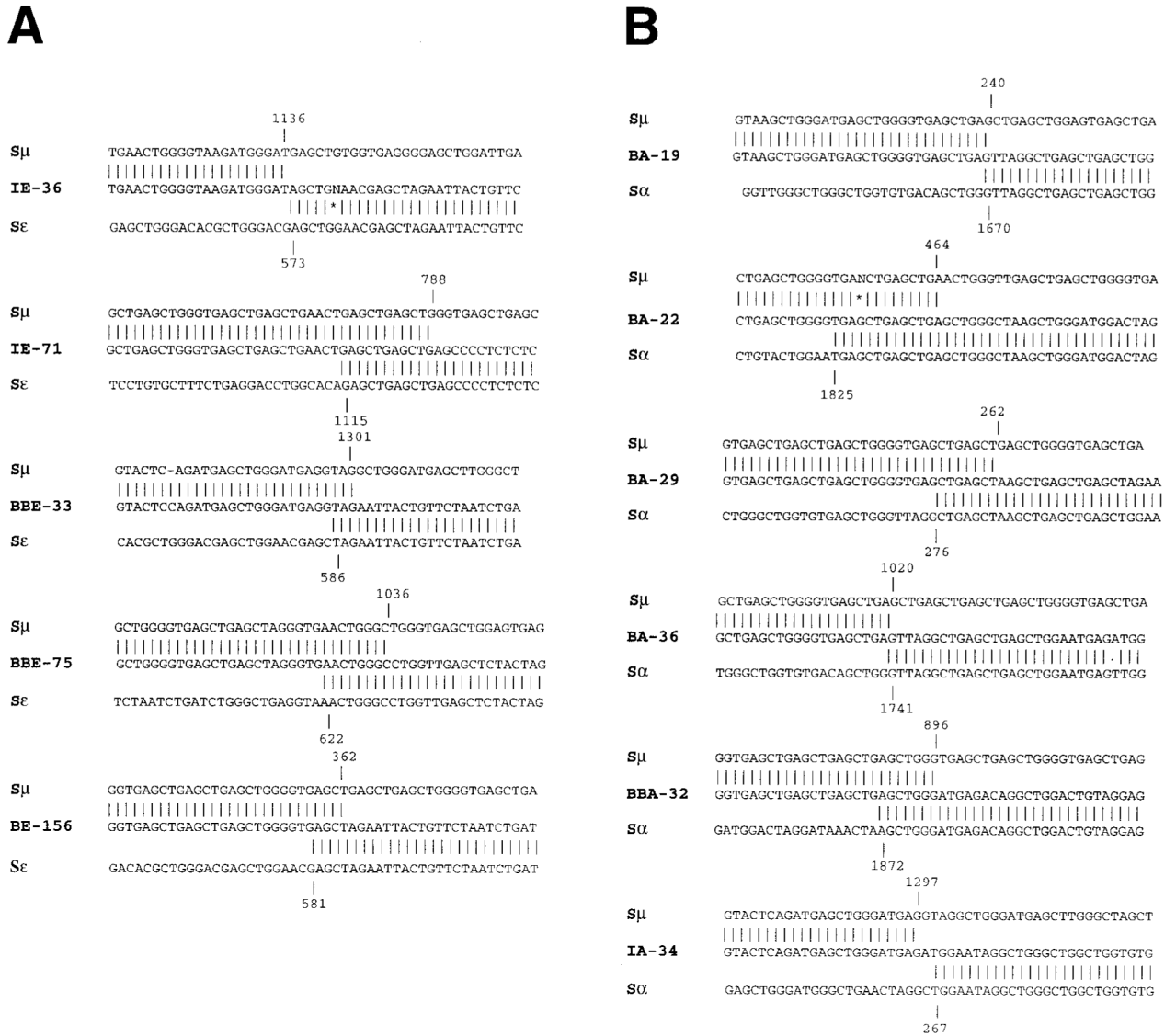
FIGURE 4.

Analysis of S/S recombinant pE.1ε and pA.1α plasmids. *A*, DNA sequence analysis of S/S recombinant pE.1ε recovered from I.29μ and 1.B4.B6 cells. Cells were transfected with pE.1ε, and clones IE-36 and IE-71 were isolated from I.29μ, and BBE-33, BBE-75, and BBE-156 were isolated from 1.B4.B6 cells. The DNA sequences were aligned with the germline Sμ and Sε sequences and nucleotide position 1 of the plasmid Sμ sequence corresponds to position 5330 of germline Sμ sequence (GenBank accession number MUSIGCD07). Nucleotide position 1 in the Sε plasmid corresponds to position 1 in the germline Sε (M17012.1) sequence. *B*, Cells were transfected with pA.1α, and clones BA-19, 22, 29, and 36 were isolated from LPS-activated splenic B cells, BBA-32 was isolated from 1.B4.B6 cells, and IA-34 was isolated from I.29μ cells. The DNA sequences were aligned with the germline Sμ and Sα sequences. Position 1 in Sα (D11468) corresponds to position 1 in the Sα plasmid.
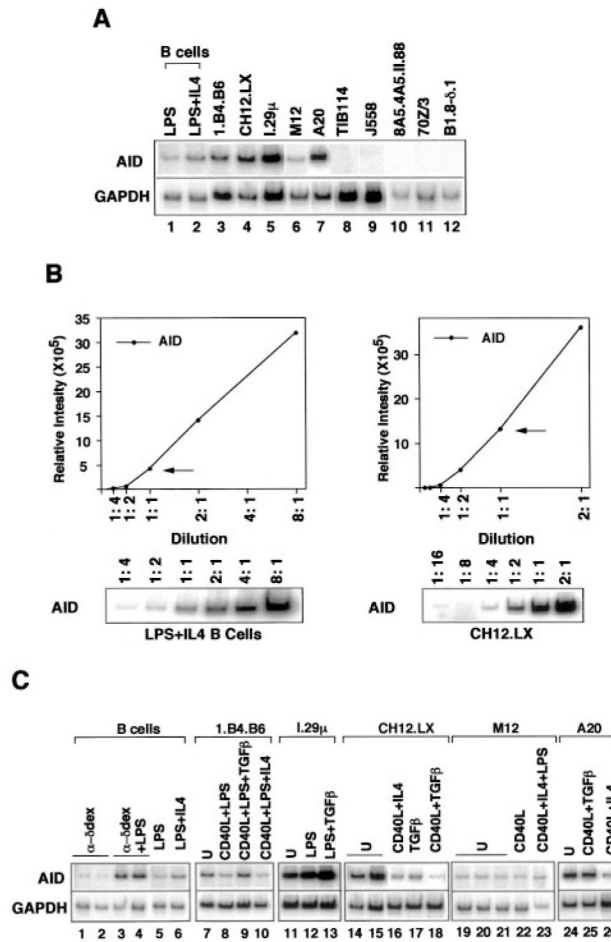
FIGURE 5.

RT-PCR analysis of *AID* expression in activated splenic B cells and mature B cell lines. *A*, Cells were either unstimulated or stimulated as indicated. Total RNA was prepared from 2×10^{-6} cells and cDNA was synthesized. *GAPDH* level was analyzed in each sample and was used to normalize cDNA template input for RT-PCR. *B*, Construction of a standard curve for *AID* RT-PCR. Two-fold serial dilutions of the cDNA from LPS + IL-4-activated splenic B cells and CH12.LX cells were analyzed by RT-PCR. The amount of cDNA used in the AID RT-PCR shown in Fig. 5*A* (*lanes 2* and *4*) was taken as the 1/1 dilution. Samples were titrated down (e.g., 1/2 relative to the 1/1 standard) or titrated up (e.g., 2/1). The intensity of the AID RT-PCR products obtained with the same amount of cDNA input used in *A*, *lanes 2* and *4*, are marked by arrows. *C*, AID transcript expression was analyzed in activated splenic B cells and in unstimulated and stimulated mature B cell lines, as indicated. All PCR were performed in the same experiment. The signal intensity of *AID* and *GAPDH* RT-PCR products was not manipulated with the exception of *lanes 18* and *23*, which were digitally enhanced.
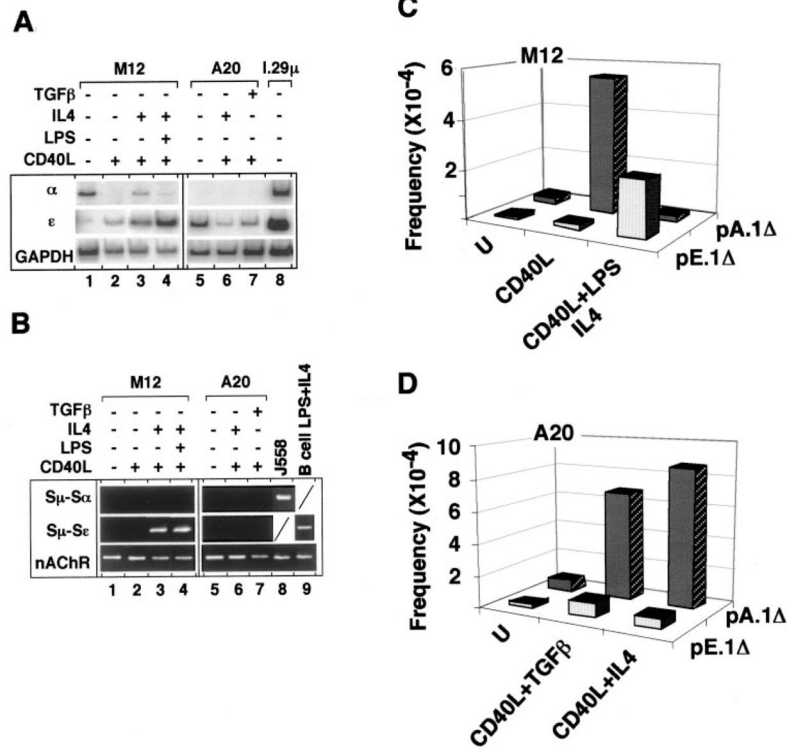
FIGURE 6.

M12 and A20 cells display switching activity. These cell lines are IgG[+] B cell lymphomas that have previously undergone $\mu \rightarrow \gamma$ switching on the expressed IgH chromosome. Secondary events will be $\gamma \rightarrow \alpha$ or $\gamma \rightarrow \varepsilon$ with S/S junctions composed of Sμ/Sγ/Sα or Sμ/Sγ/Sε, respectively. The DC-PCR assays that score for these secondary S events use the PCR priming sites upstream of Sμ and downstream of Sα or Sε. CSR is not allelically excluded, and the DC-PCR for the endogenous locus does not distinguish between the IgH-expressed and -nonexpressed chromosomes. Because switching at endogenous loci can occur on either chromosome, these events are referred to as $\mu \rightarrow \alpha$ and $\mu \rightarrow \varepsilon$ CSR. *A*, RT-PCR analysis of α and ε gts expression in M12 and A20 cells that were unstimulated or stimulated as indicated. I.29μ cells constitutively express the α and ε gts and were a positive control for the RT-PCR. *GAPDH* level was analyzed in each sample and was used to normalize cDNA template input for RT-PCR. *B*, DC-PCR analysis of $\mu \rightarrow \alpha$ and $\mu \rightarrow \varepsilon$ switching in M12 (*lanes 1–4*) and A20 (*lanes 5–7*) cells, which were untreated or activated, as indicated. DNA from J558 (*lane 8*) and from LPS + IL-4 B cells (*lane 9*) were positive controls for the Sμ/Sα and Sμ/Sε DC-PCR products, respectively. *C* and *D*, Summary of switching frequencies for the pE.1 and pA.1 plasmids in transfected into M12 (*C*) and A20 cells (*D*) and analyzed using the bacterial transformation assay shown in Table II. Cells were unstimulated or were stimulated as indicated.

Table I

pG3.1, pG3.1 -S, and pG1.1 CSR in LPS B cells and B cell lines[a]

Cell Type	Stimulation	Plasmid	n[b]	Total[c]	S/S Recombinant	Switch Frequency ($\times 10^{-4}$)[d]	p Value[e]
B cells[f]	LPS	pG3.1	2	29,100	10	3.4	—[g]
	LPS	pG1.1	4	132,360	0	0	<0.001
	IL4 + LPS		5	57,900	10	1.7	NS
1.B4.B6	—[h]	pG3.1	7	59,550	21	3.5	NS
		pG3.1 -S	2	32,800	15	4.6	NS
		pG1.1	5	151,350	0	0	<0.001
	LPS + CD40L + IL4		4	111,400	0	0	<0.001
I.29μ		pG1.1	4	91,100	0	0	<0.001
M12			6	102,500	0	0	<0.001
A20			4	138,950	0	0	<0.001

[a] Plasmids were transfected into the indicated cells. DNA recovered from nuclei of the transfected cells was either left untreated or digested with *Eco*RI and then transformed into bacteria. Miniprep DNA was prepared from the *Eco*RI-resistant colonies and analyzed by restriction mapping to identify S/S recombinant plasmids.

[b] Number of individual experiments.

[c] The total number of transformants obtained by transforming the bacteria with untreated DNA.

[d] Switch frequency is obtained by dividing the number of S/S transformants by the total number of transformants and then multiplying by 10^4.

[e] The p value indicates the confidence level that the plasmid switch frequency in a cell line is significantly different from that obtained for B cells. Values of p were derived from χ^2 analysis.

[f] Splenic B cells were isolated from BALB/c *nu/nu* mice and were activated with the indicated stimuli for 3 days before transfection.

[g] Reference switch frequency for χ^2 analysis of pG3.1.

[h] Unstimulated cells.

Table II

pA.1 and pE.1 CSR in LPS B cells and B cell lines[a]

Cell	Stimulation	Plasmid	n[b]	Total[c]	S/S Recombinant	Switch Frequency (×10⁻⁴)[d]	p Value[e]
Splenic B cells	LPS	pA.1	2	9,000	13	14.4	
		pE.1	4	59,800	23	3.8	
1.B4.B6		pA.1	3	83,100	20	2.4	
		pE.1	6	101,950	20	2.0	
I.29 μ		pA.1	7	32,050	14	4.4	
		pE.1	5	31,450	9	2.9	
M12	—[f]	pA.1	8	72,200	2	0.3	—[g]
	CD40L		7	34,800	19	5.5	<0.001
	LPS + CD40L + IL4		8	49,650	1	0.2	NS
		pE.1	8	87,250	1	0.1	—[h]
	CD40L		5	58,600	1	0.2	NS
	LPS + CD40L + IL4		8	66,744	15	2.3	<0.001
A20		pA.1	4	45,038	3	0.7	—[g]
	CD40L		4	70,100	0	0	NS
	TGFβ + CD40L		4	28,100	19	6.8	<0.001
	IL4 + CD40L		4	19,200	16	8.3	<0.001
		pE.1	3	79,900	2	0.3	—[h]
	TGFβ + CD40L		5	60,700	5	0.8	<0.005
	IL4 + CD40L		7	49,800	3	0.6	NS

[a] Plasmids were transfected into the indicated cells. DNA recovered from nuclei of the transfected cells was either left untreated or digested with *EcoRI* and then transformed into bacteria. Miniprep DNA was prepared from the *EcoRI*-resistant colonies and analyzed by restriction mapping to identify S/S recombinant plasmids.

[b] Number of individual experiments.

[c] The total number of transformants obtained by transforming the bacteria with untreated DNA.

[d] Switch frequency was obtained by dividing the number of S/S transformants by the total number of transformants and multiplying by 10^4.

[e] The p value indicates the confidence level that the plasmid switch frequency in the stimulated cells was significantly different from that obtained in unstimulated cells. Values of p were derived by χ^2 analysis.

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[f]Unstimulated cells.

[g]Reference switch frequency for χ^2 analysis of pA.1 .

[h]Reference switch frequency for χ^2 analysis of pE.1 .

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