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Two New Isotype-Specific Switching Activities Detected for Ig Class Switching

Limei Ma,* Henry H. Wortis, † and Amy L. Kenter²*

Ig class switch recombination (CSR) occurs by an intrachromosomal deletion 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 mediating CSR in vivo. This cell line is the result of transfection with a plasmid-based S substrate. The 1.B4.B6 cell line supports CSR to γ5 and δ4, but does not support CSR to α2. By contrast, normal splenic B cells activated with LPS and IL-4 are capable of mediating CSR to α2 and δ4. The M12 and A20 cell lines were identified as possible candidates for supporting CSR. The M12 and A20 cell lines were found to be capable of supporting CSR to γ5 and δ4, but not to α2. 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. The Journal of Immunology, 2002, 168: 2835–2846.

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AID expression per se does not fully explain the isotype specificity detected in the S plasmid assay.

Materials and Methods

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, Bally7 (20), L29a (23), CHLX2 (24), TBI114 (25), TIB114(12) and 120.1 (26) were previously described elsewhere. 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), and 100 μM each primer. The PCR products were used to transfect the supernatant collected from untransfected J558L cells. FACS analysis was conducted using 1.B4.B6 cells washed twice in PBS with 1% FBS and 0.1% sodium azide). Cells were resuspended in 100 μl of PBS buffer and were incubated 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.ΔαλαEP were described previously (20, 28) and p218 and pG3.ΔαλααEP are referred to in this study as pα1Δα and pG3.Δα1Δα, respectively. To build additional S substrates, a 300-bp fragment corresponding to residues 1813 to 2101 of mouse Sγ3 (M12182.1) was excised from pSy3α (our unpublished data), a 0.78-kb fragment corresponding to residues 3878 to 4661 of Sγ3 was isolated from the pHy1/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 p3PK (a gift from J. Stavnezer). The S region fragments were individually cloned into the Xbal site of Bluescript KS−, orientation was determined, and then fragments were excised by a BamHI-NcoI digestion. The BamHI-NcoI fragments were cloned into gel purified pα1Δα from which the Sγ3 fragment had been excised previously. The viral DNA was amplified in 5 μg of viral DNA by NotI digestion and the new S substrates were excised from BglII-digested pG1.Δα and pE1.Δα by introduction of 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 by a final 7-min elongation at 72°C. All PCR contained 2.5 μl of Taq polymerase (Boehringer Mannheim, Indianapolis, IN), 0.2 mM each dNTP, 2.0 μCi [α-32P]dCTP (3000 Ci/mmol; NEN, Boston, MA), 1× PCR buffer with Mg2+ 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 radioative dCTP was quantified by phoshorimaging using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

PCR amplification and cloning of Sα/Sγ3 hybrid molecules

The PCR amplification and cloning of Sα/Sγ3 molecules was conducted as previously reported (25) except that primer μ-1.2 (5′-GTCTGGGGTG AGCTCAGCTGCTAAGCC-3′), which anneals to positions 5307–5333 at the 5′ end of the germline Sα (MUSIGCD07), was used. The Cκ gene was analyzed by PCR amplification using Ce-1 (5′-CATCTGGGAGGTGCCTACGTCGTGTGC-3′) and Ce-2 (5′-ACATTTCCAAAAGCCCAGAGTCTCC-3′) primers, which anneal to positions 1030–1055 and 2398–2422 on the Cκ gene (MUSIGKF3), 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 Sγ3 coding DNA was conducted using primer Rel-1 (5′-TTACCTGCGT GATGCTCTACATTAAAGC-3′) and dc-μ1 or dc-μ2 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→ε 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 Sα primers were dc-μ1 and dc-μ2, and μ→α was minimally described (19). The Sε primers were dc-ε1 (5′-ACCACGGTCAGGATTACACCCCGACC-3′) and dc-ε′2 (5′-CCCCCA GACCTTTCAAGCTGTG-3′), and the Sα primer was dc-α1 (5′- CCCCCCGACAGACACCTTCACAGG-3′). In the second round of PCR for μ→α DC-PCR, the dc-μ2 and dc-α1 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 before (20) with modifications. Transfected genomic DNA (500 ng) was digested overnight with SacI (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 untransformed 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 5-bp fragment was amplified by PCR, with the primers TIB111-NcoI (5′-GGACTGAAGCAGCATGCACC-3′) and TIB111-P1 (5′-CGCCTTACAGCCTCCACCTC-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 described previously (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 JH 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 VH of 1.B4.B6 cells differed at only a single nucleotide residue from QS2, 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 Tγ32 type T cell line and Staphylococcus 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. 1A). 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 γ3, γ1, α, and ε gts (Fig. 1B, lane 1). Production of the γ3 gt was induced following treatment with CD40L and LPS in the presence or absence of TGFβ or IL-4 (Fig. 1B). Individual activators or CD40L control (CD40Lc) supernatant (see Materials and Methods) only slightly augmented γ3 gt expression (Fig. 1B). Stimulation of 1.B4.B6 cells with CD40L and LPS in the presence of IL-4 induced γ1 and ε gts expression (Fig. 1B, lane 8), whereas CD40L and LPS in the presence of TGFβ induced α gt (Fig. 1B, 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 $\mu$ and one of the downstream S regions to generate a complex mixture of hybrid $\mu$/Sx molecules of various sizes. To determine whether $\mu\rightarrow\gamma_3$...
CSR could be induced in 1.B4.B6 cells, a PCR assay for detection of composite Sμ/Sγ3 molecules was used (25). Multiple Sμ/Sγ3 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/Sγ3 composite molecules amplified by PCR represent bona fide CSR events, the Sμ/Sγ3 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 Sμ and Sγ3 sequences, and eight clones were confirmed to contain Sμ/Sγ3 S junctions (Fig. 1C). 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. 2A. 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 Sμ/Sγ3 DC-PCR product (Fig. 2B, lane 7). DC-PCR product was only evident in DNA from cells induced with LPS and CD40L (Fig. 2B). These findings directly demonstrate the induction of μ→γ3 CSR in 1.B4.B6 cells and are consistent with the observation of surface IgG3 expression, γ3 gt induction, and the presence of Sμ/Sγ3 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 and γ3, and the γ1 and e γts, 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 Sμ/α CSR product. The Sμ/α DC-PCR product was detected only in DNA from 1.B4.B6 cells stimulated with CD40L and LPS and TGFβ, indicating the induction of μ→α CSR (Fig. 2C). DNA from LPS and IL-4-treated splenic B cells, which switch μ→γ1 and μ→e, served as a positive control for the Sμ/γ1 and Sμ/e DC-PCR products (Fig. 2D). No Sμ/Sα and Sμ/Sγ1 DC-PCR products were detected in unstimulated 1.B4.B6 cells or cells stimulated with CD40L and LPS (Fig. 2D). 1.B4.B6 cells stimulated with CD40L and LPS and IL-4 gave rise only to Sμ/Se but not Sμ/Sγ1 DC-PCR products (Fig. 2D). No Sμ/Sα 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 Sμ/Sγ1 DC-PCR were intact in 1.B4.B6 cells because the primers dc-γ1.1 or dc-γ1.2 in combination with a downstream primer Rγ1 (see Fig. 2A) amplified a PCR product of the expected size and appropriate DNA sequence (data not shown). The priming sites located at 5′ end of Sα are intact because the dc-μ-1 and dc-μ-2 primers were able to participate in amplification of Sμ/Sγ3, Sμ/α, and Sμ/e DC-PCR products in activated 1.B4.B6 (Fig. 2, B–D). Genomic Southern analysis demonstrated that the overall Sγ1 region in

![FIGURE 2. DC-PCR analysis of μ→γ3, μ→γ1, μ→e, and μ→α 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. EcoRI sites (denoted RI) that flank the 5′ end and the 3′ end of the Sμ and Sγ3 regions, respectively, are preserved following CSR. After digestion with EcoRI, 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 Sγ3 amplify the region spanning the circle joint and yield a specific DC-PCR product, indicating the presence of Sμ/Sγ3 composite molecules. A portion of the IgH locus is depicted before and after μ→γ3 and μ→γ1 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 μ→γ3 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 μ→γ3 switching. The IgG3 producing cell line, TIB114 (lane 7), was used as a positive control for the Sμ/Sγ3 DC-PCR product. C, DC-PCR analysis for μ→α 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 μ→α CSR. The IgA-producing cell line, J558 (lane 4), was used as a positive control for the Sμ/α DC-PCR product. D, DC-PCR analysis for the μ→γ1 and μ→e 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 μ→γ1 and μ→e CSR.](http://www.jimmunol.org/DownloadedFrom/CN487541E.pdf)
1.B4.B6 cells is intact (data not shown). Taken together, these findings indicate that 1.B4.B6 cells can be induced to switch $\mu \rightarrow \gamma 3$, $\mu \rightarrow \alpha$, and $\mu \rightarrow \epsilon$, but not $\mu \rightarrow \gamma 1$. However, it is possible that other combinations of stimuli are capable of inducing $\mu \rightarrow \gamma 1$ switching in 1.B4.B6 cells.

**Distinct factors mediate $\mu \rightarrow \gamma 3$ and $\mu \rightarrow \gamma 1$ plasmid-based CSR**

One hypothesis to explain the absence of endogenous $\mu \rightarrow \gamma 1$ CSR in 1.B4.B6 cells is that these cells fail to express the transacting $\mu \rightarrow \gamma 1$ switching activity. We previously demonstrated that distinct switching activities independently mediate $\mu \rightarrow \gamma 3$ 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 $\Delta$ (Fig. 3A). CSR on pG1.1 $\Delta$ and pG3.1 $\Delta$, 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 EcoRI restriction site, and those plasmids that are resistant to EcoRI digestion are likely to be S/S recombinants (Fig. 3A). To control for possible S/S recombination in bacteria, in the same experiments, pG3.1 $\Delta$ and pG1.1 $\Delta$ DNA were untreated or digested with EcoRI, and the frequency of ampicillin resistant colonies was determined. The efficiency of transformation with intact plasmid was 5 $\times$ 10$^7$ colonies/$\mu$g. Following transformation, we found no ampicillin-resistant colonies, indicating that EcoRI restriction 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 $\Delta$ 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 $\Delta$ 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 1.29$\beta$, A20, or M12 cells (Table I). In contrast, 10 S/S recombinants derived from pG1.1 $\Delta$ were recovered from LPS and IL-4-activated splenic B cells. The $\chi^2$ 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 $\Delta$ plasmids were taken for DNA sequence analysis and the S junctions were demonstrated to lie within S region DNA. The S$\mu$/SyI junctions were located for four plasmids (4BG-121, 142, 157, and 190; Fig. 3B) and showed direct joining of S$\mu$ and SyI DNA without deletion, duplication, or nucleotide insertion at the breakpoints. The microhomology at the S$\mu$/SyI 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 $\Delta$ 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 SyI in pG1.1 $\Delta$ is 0.78 kb as compared with the 2.0 kb of Sy3 DNA in pG3.1 $\Delta$. To determine whether the length of S DNA in the S plasmid contributes to the frequency of CSR events, we constructed pG3.1 $\Delta$-S, which contains 300 bp of Sy3 DNA (Fig. 3A) and compared its switching frequency to that of pG3.1 $\Delta$ in 1.B4.B6 cells. S/S recombinant pG3.1 $\Delta$-S and pG3.1 $\Delta$ were recovered at essentially equal frequencies (Table I). This result demonstrates that 300 bp of Sy3 containing only five tandem repeats are sufficient to support plasmid-based CSR and suggests that the length of SyI present in pG1.1 $\Delta$ 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 $\Delta$ (20) and is shown in Fig. 3C. The linear range of detection for the vector-associated 510-bp fragment was established using 2-fold serial dilutions of pG1.1 $\Delta$ into 0.5 $\mu$g of genomic DNA followed by DC-PCR analysis in the presence of radiolabeled deoxynucleotides (Fig. 3D). The addition of increasing amounts of pG1.1 $\Delta$ (0.25–2 ng/ml) resulted in a linear increase of vector-specific 510-bp product (Fig. 3D). 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 S$\mu$/SyI-associated 81-bp DC-PCR product, we tested for the presence of this fragment using a range of concentrations of plasmid digested with SacI (Fig. 3D). At high concentrations of input plasmid (2 ng/ml), intramolecular 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 $\Delta$ plasmid was undertaken in activated splenic B cells and in switching and nonswitching B cell lines. The intact standard sample of pG1.1 $\Delta$ 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. 3E, lane 1). A S/S recombinant derivative of pG1.1 $\Delta$ (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. 3E, 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 $\Delta$ is recombinogenic in B cells that undergo physiological switching. Recombination of pG1.1 $\Delta$ 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 $\Delta$ and pG1.1 $\Delta$ 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 $\mu \rightarrow \alpha$ and $\mu \rightarrow \epsilon$ recombination**

$\alpha$ and $\epsilon$ regions share considerable homology to each other. This raises the question whether $\mu \rightarrow \alpha$ and $\mu \rightarrow \epsilon$ recombination are mediated by distinct factors. A S plasmid, pE1 $\Delta$, carrying Se was constructed and is identical with pG3.1 $\Delta$, pG1.1 $\Delta$, and pA1 $\Delta$ in all respects except for the identity of the downstream S region (Fig. 3A). The facility with which pE1 $\Delta$ and pA1 $\Delta$ 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 pE1 $\Delta$ and pA1 $\Delta$ were found to recombine at similar frequencies in LPS-activated B cells, 1.29$\mu$, and 1.B4.B6 cells, suggesting that factors supporting $\mu \rightarrow \alpha$ and $\mu \rightarrow \epsilon$ CSR are present. In contrast,
FIGURE 3. The pG1.1Δ 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.1Δ and pG3.1Δ were previously referred to as p218 and pG3.1ΔneoΔEP, respectively (20, 28). The S substrates pG1.1Δ, pE1.1Δ, and pG3.1Δ—S are new constructs (see Materials and Methods). The pG3.1Δ—S plasmid contains a 0.3-kb fragment of Sy3, whereas pG3.1Δ contains the full-length 2.0-kb Sy3 region. The overall structure of the S substrates is 5' neo gene (neo), Sy3, thymidine kinase gene (TK), and Sy3 or Sy3 or Sy1 or Sy4. A unique EcoRI restriction site (denoted RI) is located at the junction of Sy3 and TK. B, DNA sequence analysis demonstrates direct S/S joining in pG1.1Δ recombinant plasmids. Four S/S/S/S recombinant plasmids were recovered by bacterial transformation from LPS + IL-4-activated normal splenic B cells transfected with pG1.1Δ. Automated DNA sequence analysis was used to determine the S/S breakpoints. Nucleotide position 1 of the plasmid S/S sequence corresponds to position 5330 of the germline Sy3 sequence (GenBank accession number M12182.1). C, Schematic illustration of a DC-PCR assay for detection of μ→γ1 switching on the pG1.1Δ plasmid. Re combination between two S regions of pG1.1Δ will cause the deletion of TK, loss of two internal SacI sites (denoted S), and conservation of SacI sites situated in the upstream portion of Sy3 and downstream of Sy1. The recombined S/S hybrid molecules will be located on a new SacI fragment, whereas the intact S regions would be located on...
very rare recombinant pA.1Δ and pE.1Δ were found in unstimulated A20 and M12 cells, indicating that the activities supporting plasmid-based CSR are very poorly expressed in these cells. χ² analysis indicates that pA.1Δ and pE.1Δ 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.1Δ and pA.1Δ plasmids was constitutive in 1.B4.B6 and I.29μ cells, confirming previous observations for pA.1Δ (20). DNA sequence analysis of S junctions derived from the pE.1Δ and pA.1Δ plasmids and the S junctions are shown (Fig. 4, A and B). Six recombinant pE.1Δ plasmids were sequenced, and four S junctions were found. Sixteen recombinant pA.1Δ 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 μ→α and μ→ε 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 Se and So DNA. To distinguish between these alternatives, additional B cell lines are required that can be induced to undergo μ→ε or μ→α 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. 5A). In contrast, 70Z3 and 8A5,4A5.II.88, pre-B cell lines, TIB114 and B1.8-6, 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. 5A, lanes 2 and 4) was used to prepare standard curves (Fig. 5B). 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. 5A, 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. 5C) 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 two different SacI fragments. Following digestion with SacI, 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 P3y1 specific for sites at the 5′ end region of Se and 3′ end of Sy1, 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 SacI 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 P5y1 specific for sites at the 3′ end region of TK and 5′ of Sy1, 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 SacI sites. The plasmid backbone is contained in a single SacI 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 SacI-digested pG1.1Δ 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 pG1.1Δ and were analyzed by DC-PCR. Intact and S/S recombinant pG1.1Δ were used as negative (−) and positive (+) controls for the DC-PCR in lanes 1 and 2, respectively.

### Table 1. pG3.1Δ, pG3.1Δ-S, and pG1.1Δ CSR in LPS B cells and B cell lines

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Stimulation</th>
<th>Plasmid</th>
<th>n</th>
<th>Total</th>
<th>S/S Recombinant</th>
<th>Switch Frequency (×10⁻⁴)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells/</td>
<td>LPS</td>
<td>pG3.1Δ</td>
<td>2</td>
<td>29,100</td>
<td>10</td>
<td>3.4</td>
<td>—/−</td>
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<tr>
<td></td>
<td>LPS</td>
<td>pG1.1Δ</td>
<td>4</td>
<td>132,360</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1.B4.B6</td>
<td>ILα + LPS</td>
<td>pG3.1Δ</td>
<td>7</td>
<td>59,550</td>
<td>21</td>
<td>3.5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pG3.1Δ-S</td>
<td>2</td>
<td>32,800</td>
<td>15</td>
<td>4.6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pG1.1Δ</td>
<td>5</td>
<td>151,350</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LPS + CD40L + ILα</td>
<td>pG1.1Δ</td>
<td>4</td>
<td>111,400</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>I.29μ</td>
<td>LPS + CD40L</td>
<td>pG1.1Δ</td>
<td>4</td>
<td>91,100</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
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<tr>
<td>M12</td>
<td></td>
<td>pG1.1Δ</td>
<td>6</td>
<td>102,500</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A20</td>
<td></td>
<td>pG1.1Δ</td>
<td>4</td>
<td>138,950</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*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.

**Number of individual experiments.

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

Switch frequency is obtained by dividing the number of S/S transformants by the number of total transformants and then multiplying by 10⁴.

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 χ² analysis.

Splenocytes were isolated from BALB/c nu/nu mice and were activated with the indicated stimuli for 3 days before transfection.

Reference switch frequency for pG1.1Δ was calculated.

CSI product and is used to control for plasmid recovery, ligation, and PCR amplification, efficiency. DC-PCR products are in the linear range of 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 pG1.1Δ and were analyzed by DC-PCR. Intact and S/S recombinant pG1.1Δ were used as negative (−) and positive (+) controls for the DC-PCR in lanes 1 and 2, respectively.
stabilized of \( \mu \rightarrow \gamma_3 \) CSR (Fig. 1), but to reduction of AID expression (Fig. 5C, 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. 5C, 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. Unstimulated L29 \( \mu \) cells, which constitutively express \( \alpha \) and \( \epsilon \) gts, were used as positive controls in the RT-PCR analysis (Fig. 6A, lane 8). In M12 cells, \( \epsilon \) gts were induced by each combination of activators tested, but were not found in unstimulated cells (Fig. 6A, lanes 2–4). In contrast, \( \alpha \) gts were expressed in unstimulated M12 cells (Fig. 6A, lane 1), and treatment of the cells with any combination of inducers reduced gt expression (Fig. 6A, lanes 2–4). DC-PCR was used to assess CSR status, and the IgA expressing 3558 and splenic B cells induced with LPS and IL-4 were used as positive controls for \( S_\mu/S_\alpha \) and \( S_\mu/S_\epsilon \) DC-PCR products, respectively, (Fig. 6B, lanes 8 and 9). In M12 cells, no \( \mu \rightarrow \alpha \) switching could be detected under any conditions tested (Fig. 6B, lanes 1–4), whereas \( \mu \rightarrow \epsilon \) switching was induced with CD40L and IL-4 or CD40L and IL-4, but not in unstimulated cells or cells treated with CD40L alone (Fig. 6B, lanes 1–4). In A20 cells, the \( \alpha \) gt was not detected under any of the conditions tested, but the \( \epsilon \) gt was found in all of the conditions analyzed, albeit at different levels (Fig. 6B, lanes 5–7). However, neither \( \mu \rightarrow \alpha \) nor \( \mu \rightarrow \epsilon \) CSR was evident under any circumstances (Fig. 6B, lanes 5–7).
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 μ→γ3 and μ→α CSR demonstrated that unique switching activities mediated these CSR events. However, the tandem repeats in the Sγ3 and Sα 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 μ→γ3 and μ→γ1 switching events based on the differential capacity of the μ→γ3 and μ→γ1 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 Sγ3 and Sγ1 tandem repeats (34). The Sγ3 and Sγ1 regions differ structurally where Sγ3 is the longest and the most complex. However, the Sγ1 and Sγ3 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 Sγ1 and Sγ3 regions by the μ→γ1 and μ→γ3 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 μ→α and μ→ω CSR.

**FIGURE 4.** Analysis of S/S recombinant pE.1α and pA.1Δ plasmids. A, DNA sequence analysis of S/S recombinant pE.1α recovered from I.29μ and 1.B4.B6 cells. Cells were transfected with pE.1α, 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 Se plasmid corresponds to position 1 in the Sμ sequence (M17012.1). B, Cells were transfected with pA.1Δ, and clones BA-19, 22, 29, and 36 were 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.
lo. The absence of α gts in A20 and M12 cells under conditions that provide for the expression of μ→α switching activities is the simplest explanation for this endogenous switching deficit. Our studies indicate that the μ→γ1 switching activity is expressed only in B cells activated with LPS and IL-4, whereas μ→ε 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 γ1. The IL-4-dependent switching phenomenon most likely arises from the differential induction of the γ1 and g1 gts by IL-4 (35). The absence of the μ→γ1 switching activity in LPS B cells and its induction by IL-4 ensures that μ→γ1 switching occurs only when both the γ1 gt and the μ→γ1 switching activities are coexpressed. Similarly, the absence of the ε gt in LPS B cells, even in the presence of the μ→ε switching activity, assures the IL-4 dependence of this event. Together, our studies indicate that CSR at endogenous loci is strictly 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 μ→α, it is also capable of occasional μ→γ3 and μ→γ1 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 Sy3 containing 44 tandem repeats or a short version of Sy3 containing five tandem repeats indicated no discernible differences in CSR frequency as assessed by the bacterial transformation assay. Recently, a mouse was constructed in which the Sμ region, composed of (GAGCT)₄GGGGT motifs, was removed by targeted homologous recombination (42). In the Sμ−/− 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 S/H9262/S/H9253, S/H9253/S/H9280 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.

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References


24. Shockey, P., and J. Stavnezer. 1991. Effect of cytokines on switching to IgA and γ1 and IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE IgE I