

# Evidence for Class-specific Factors in Immunoglobulin Isotype Switching

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## Abstract

Immunoglobulin class switch recombination (SR) occurs by a B cell-specific, intrachromosomal deletional process between switch regions. We have developed a plasmid-based transient transfection assay for SR to test for the presence of transacting switch activities. The plasmids are novel in that they lack a eukaryotic origin of DNA replication. The recombination activity of these switch substrates is restricted to a subset of B cell lines that support isotype switching on their endogenous loci and to mitogen-activated normal splenic B cells. The factors required for extrachromosomal plasmid recombination are constitutively expressed in proliferating splenic B cells and in B cell lines capable of inducibly undergoing immunoglobulin SR on their chromosomal genes. These studies suggest that mitogens that induce switching on the chromosome induce accessibility rather than switch recombinase activity. Finally, we provide evidence for two distinct switching activities which independently mediate  $\mu \rightarrow \alpha$  and  $\mu \rightarrow \gamma 3$  SR.

Key words: immunoglobulin • isotype switch • plasmid assay • transient transfection • PCR

## Introduction

Ig isotype switching leads to diversification of the humoral immune response effector functions. Stimulation of mature B cells leads to expression of new Ig H chain C region genes, while maintaining the same antigen binding specificity. The murine IgH locus is composed of multiple C<sub>H</sub> genes,  $\mu$ ,  $\delta$ ,  $\gamma 1$ ,  $\gamma 2b$ ,  $\gamma 2a$ ,  $\gamma 3$ ,  $\epsilon$ , and  $\alpha$ , each of which are coupled with unique switch (S)<sup>1</sup> regions, except  $\delta$ . Isotype switching focuses on S DNA, and produces new hybrid DNA combinations. The composite S $\mu$ -S $\alpha$  DNA configuration is formed on the 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 switch recombination

(SR) on the DNA-dependent protein kinase catalytic subunit (DNA-PK<sub>Cs</sub> [6]), Ku80 (7), and Ku70 (8), components of the DNA-PK complex involved in DSB repair, strongly suggest that SR is resolved through a nonhomologous DNA end-joining process (for a review, see reference 9). However, the factors and processes that facilitate SR have not been defined.

Two views of the regulation of SR have been articulated. In one model, S region-specific recombinases have been postulated based on the unique character of each S region (10, 11). In the second model, specificity of the SR process is achieved through production of germline transcript, (gts) (12–16) and a single recombinase. Gts are RNA transcripts from specific unrearranged C<sub>H</sub> genes that are induced before SR, and initiate upstream of S DNA, terminate downstream of the C<sub>H</sub> gene, and appear not to be translated. The mechanism whereby gt production regulates isotype-specific SR is unknown.

Evidence suggests that gt expression is necessary but not sufficient for SR to occur. Treatment of B cells with activators and cytokines, or alteration of transcription factor expression can lead to changes in SR activity without corresponding changes in gt expression (17). The combina-

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<sup>1</sup>Abbreviations used in this paper:  $\alpha$ 8dex, dextran-conjugated anti-IgD antibodies;  $\beta$ -gal,  $\beta$ -galactosidase; CAT, chloramphenicol acetyl transferase; DC, digestion-circularization; DNA-PK<sub>Cs</sub>, DNA-dependent protein kinase catalytic subunit; DSB, double-stranded break; E, enhancer; gt, germline transcript; NF, nuclear factor; RAG, recombination activating gene; RRL, relative recombination level; S, switch; SR, switch recombination; TK, thymidine kinase gene.

tion of dextran-conjugated anti-IgD antibodies ( $\alpha\delta\text{dex}$ ), which stimulates B cells to proliferate, and IL-4, which induces  $\gamma 1$  gt expression, does not lead to an increase of  $\mu \rightarrow \gamma 1$  switched B cells (18, 19). The addition of IL-5 to these inducers stimulates  $\mu \rightarrow \gamma 1$  SR, suggesting that factors distinct from gts and cell proliferation are required to support SR (19). Furthermore, splenic B cells in which nuclear factor (NF)- $\kappa\text{B}$ /Rel transcription factors have been disrupted by targeted recombination have selective defects in gt expression and isotype switching (17). B cells deficient in the COOH-terminal transactivation domain of c-Rel ( $\Delta\text{c-rel}$ ) express  $\alpha$  and  $\epsilon$  gts, but switch only  $\mu \rightarrow \alpha$  and not  $\mu \rightarrow \epsilon$  (20). Similarly, B cells deficient in p105, which encodes the p50 of NF- $\kappa\text{B}$ , express  $\gamma 1$  and  $\alpha$  gts, but switch only  $\mu \rightarrow \gamma 1$  and not  $\mu \rightarrow \alpha$  (21). These findings suggest that there are specific factors that function in SR independent of gts, and that affect recombination to particular S regions.

Several plasmid-based recombination assays have been devised to study SR using both transient and stable transfection (22–32). Although many of these plasmid assays have shown some preference for recombination in B cells and B cell lines (26, 29, 31), recombination on these switch substrates is not restricted to B cells as is physiological SR (22, 24, 29), or is not completely dependent on S sequences for recombination (22, 24, 29, 31). While it is likely that some of the recombination events scored on these plasmids arise through SR, it is often difficult to distinguish between specific and nonspecific rearrangements. Previous analyses did not include a comparison of switch plasmid recombination in B cell lines capable of endogenous SR and those that do not support SR. In these earlier studies, plasmid-based SR was found in many mature B cell lines and led to the suggestion that switch recombinase activities are broadly expressed in B cells. A new switch plasmid was recently devised that displayed some specificity for B cell lines that switch, in stable transfection experiments (33). However, the use of stable transfection methods to study SR is cumbersome.

Here we report that a novel plasmid-based transient transfection assay for SR tests for the presence of transacting factors. The plasmid substrates lack a eukaryotic origin of replication (33). DNA sequence analysis of recombinant S–S junctions derived from switch plasmids indicates direct S–S joining. Thus, plasmid-based SR can occur in the absence of an origin of DNA replication. These switch plasmids undergo recombination only in cell lines capable of switching their endogenous genes and in activated splenic B cells. The switching cell lines do not require mitogen or cytokine activation to support SR on plasmid substrates, suggesting that the switching activities are constitutively expressed. Furthermore, comparison of the recombination potential of  $\mu \rightarrow \alpha$  and  $\mu \rightarrow \gamma 3$  specific switch substrates indicates that recombination activity on the plasmids strictly correlates with isotype-specific switching at the endogenous loci and suggests that isotype-specific factors may regulate SR.

## Materials and Methods

**Construction of Plasmids.** p273, p200, and p218 were described previously (33). To obtain pG3. $\Delta\text{I}\alpha\Delta\text{EP}$ , the complete 2.0-kb Balb/c genomic S $\gamma 3$  region was amplified (34). The S $\gamma 3$ -PCR product was cloned into the XbaI site of Bluescript KS<sup>-</sup>. The cloned S $\gamma 3$  was then excised as a BamHI–NotI fragment and cloned into gel-purified p218, from which the S $\alpha$  fragment had been excised by a BamHI–NotI digestion. To obtain pG3. $\Delta\text{I}\alpha$  from pG3. $\Delta\text{I}\alpha\Delta\text{EP}$ , EP was cloned in the forward orientation into the unique ClaI site located upstream of S $\mu$  in pG3. $\Delta\text{I}\alpha\Delta\text{EP}$ . EP is composed of the 0.7-kb core intronic enhancer and a 0.3-kb fragment containing the VH186.2 promoter (35). To obtain pG3. $\Delta\text{EP}$  from pG3. $\Delta\text{I}\alpha\Delta\text{EP}$ , the germline  $\alpha$  promoter (I $\alpha$ ) was PCR-amplified from I.29 $\mu$  DNA using forward (–489/–469; 5' GGC CGC GGA CCC TGT GAC CTC AAA GCT CT 3' and reverse (+33/+13; 5' GGG CGG CCG CTC TGT GTG TGA TTG CCT GTG 3') primers (36) and cloned into the SmaI site of pUC 18. The I $\alpha$  segment includes nucleotides –488 to +30, relative to the first RNA initiation site (sequence data are available from EMBL/GenBank/DBJ under accession no. L04145 [36]). I $\alpha$  was excised as an XbaI–NotI fragment (the primer carries a NotI site), and was cloned in the physiological orientation into the BamHI site of pG3. $\Delta\text{I}\alpha\Delta\text{EP}$ . To obtain pG3.1, EP fragment was cloned in the forward orientation into the ClaI site of pG3. $\Delta\text{EP}$ . The plasmid p208 is identical to p218, except for the addition of the I $\alpha$  fragment. To obtain p208, the I $\alpha$  fragment was cloned in the physiological orientation into the BamHI site of p218. To obtain pG3.1.01s, two partially complementary oligonucleotides were synthesized such that the sense oligo was representative of 2439–2508 and the antisense was representative of 2568–2488 of the Balb/c genomic S $\gamma 3$  sequence (MUSIGHANA). The oligos were annealed, filled-in with Klenow DNA polymerase (Promega) and cloned. A plasmid containing thymidine kinase gene (TK), I $\alpha$ , and the S $\gamma 3$  cloned oligos was constructed. This insert, TK.I $\alpha$ .S $\gamma 3$  oligo, was isolated as a cassette and cloned into pG3.1, which had been digested with EcoRI and NotI.

For pGL3-SV40, the SV40 enhancer/promoter was excised from pRL-SV40 vector (Promega) and cloned upstream of the firefly luciferase gene of the pGL3 basic vector (Promega). For pI $\alpha$ -Luc, the I $\alpha$  fragment, was cloned into pGEM-T Easy according to the manufacturer's instructions (Promega). The cloned I $\alpha$  segment was excised from pGEM-T Easy by EcoRI digestion, isolated from an agarose gel, and the ends were filled in using Klenow DNA polymerase. The resulting I $\alpha$  fragment was ligated into pXP2, a luciferase reporter vector that lacks a promoter or enhancer (37, 38), which had been digested with SalI and treated with Klenow DNA polymerase. Nucleotide sequencing was performed to verify the correct promoter sequence. For pI $\alpha$ E-Luc, the Ig  $\mu$  intron enhancer (E), was obtained by digestion of p273 with HindIII to excise the EP fragment, which was isolated from an agarose gel, and ligated into the HindIII site in pUC19. pUC-EP was then digested with HindIII and BamHI to excise the E fragment alone. The ends were filled in using Klenow DNA polymerase, and the E segment was inserted at the filled-in HindIII site of pXP2 upstream of the I $\alpha$  promoter segment.

**Cell Culture, Transfection, Luciferase Assays, DNA Isolation, and Hirt Extracts.** Splenic B cells were prepared and grown as described previously (39). In brief, spleens were removed from 8–12-wk-old Balb/c nu/nu mice and a single cell suspension was prepared. The cells were washed twice in HBSS or RPMI 1640 supplemented with 2% FCS (Hyclone). Cells were resuspended

in 1 ml of HBSS and underlaid with 3 ml of Ficoll-Paque (Amersham Pharmacia Biotech) and centrifuged at 1,200 *g* for 20 min at room temperature. Cells were recovered from the Ficoll, washed extensively, and then seeded at  $5 \times 10^5$  cells/ml and cultured in RPMI 1640, 20% FCS, 4  $\mu$ M glutamine, penicillin-streptomycin,  $5 \times 10^{-5}$  M 2-ME and cultured in 6% CO<sub>2</sub>. Cells were activated for a minimum of 40 h in either 50  $\mu$ g/ml LPS (*Salmonella typhimurium*, phenol extract, Westphal; Sigma Chemical Co.), or in 3 ng/ml anti-IgD-coupled  $\alpha$ dex beads (a gift from C. Snapper, Uniformed Service, University of Health Sciences, Bethesda, MD) before electroporation. I.29 $\mu$  and CH12.LX were grown as described (40, 41). The conditions for 1B4.B6 cell growth were identical to those for CH12.LX, except that serum was specifically screened for its ability to support SR in this cell line (our unpublished results). The cell lines A20, M12, BW5147, EL4, and P388D1 were grown as described previously (39). Bal17 was grown as described (42). Cells were electroporated in the presence of 10  $\mu$ g of plasmid DNA at 0.28–0.33 kV at 960  $\mu$ F in a Gene Pulser (Bio-Rad), cultured for 2 d, and total genomic DNA was isolated from  $5 \times 10^6$  cells using the Genomic DNA isolation kit (Genra Systems), or from nuclei prepared using the Blood and Cell Culture DNA Preparation kit (Qiagen). Hirt extracts were prepared by isolating nuclei and obtaining low molecular weight DNA using the Qiaprep Spin Mini Prep Kit (Qiagen). Cells cotransfected with pGL3-SV40 were cultured for 48 h, and an aliquot of cells ( $0.5 \times 10^6$ ) was analyzed for luciferase activity (Promega).

Two luciferase reporter plasmids, p $\alpha$ -Luc and p $\alpha$ E-Luc, were used to assay transcriptional activity in cell lines. Transfections were performed as described previously (36). Two different internal control plasmids were used as controls for transfection efficiency: pSV2-CAT (43), containing the chloramphenicol acetyl transferase (CAT) gene, and pPGK $\beta$ -gal, containing the  $\beta$ -galactosidase ( $\beta$ -gal) gene driven by PGK promoter (44), obtained from P. Dobner (University of Massachusetts Medical School). Cells were then cultured at  $1-2 \times 10^6$ /ml for 24 h, and assayed for luciferase activity and either CAT (45) or  $\beta$ -gal activity (46).

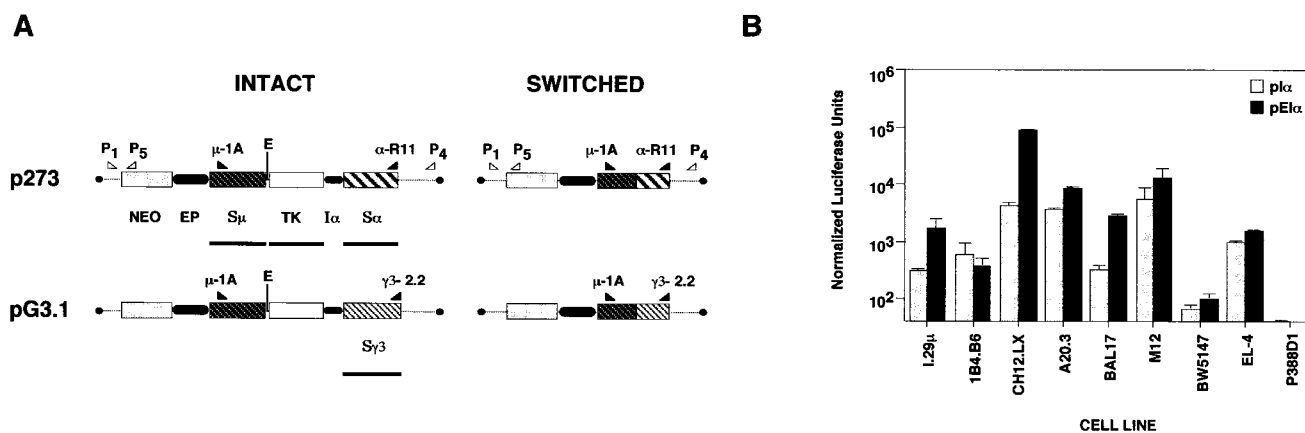
**PCR of S-S Recombinant Molecules and Southern Analysis.** Total DNA isolated from transfected cell lines was normalized for plasmid concentration based on luciferase activity values. S $\mu$ -S $\gamma$ 3 recombinant molecules were amplified using the  $\mu$ -1A primer, 5' CTC TAC TGC CTA CAC TGG ACT GTT CTG 3', and  $\gamma$ 3-2.2 primer, 5' CCT GGG ACC CTG TGA TCT GAT AGC C3', located 3' of S $\gamma$ 3, as described (34) with modifications. S $\mu$ -S $\alpha$  recombinant molecules were amplified using the  $\mu$ -1A primer and the  $\alpha$ -R11 primer, 5' CTC TAT CTA GGT CTG CCC GGT CTA GGT AAG 3'. The Expand Long Template PCR system (Boehringer Mannheim) was used according to manufacturer's instructions with modifications. PCR was a total of 36 cycles using a thermocycler (model 480; Perkin Elmer). Primers were annealed at 64°C for 40 s and elongation time was 4 min at 68°C for 10 cycles. A further 26 cycles were carried out with an elongation time of 4 min, with an additional 20 s added for each cycle. DNA recovered from transfected cells was digested with EcoRI before PCR to linearize intact plasmid. A segment of the neomycin gene was amplified using P1 primer (forward), 5' GTA ATA CGA CTC ACT ATA GGG C 3', and P5 primer (reverse), 5' ATG GCC GCT TTT CTG GAT TC 3'. PCR was performed using Taq polymerase (Boehringer Mannheim) and an initial denaturation at 94°C for 2 min was followed by 26 cycles with each cycle consisting of 93°C for 30 s, 55°C for 40 s, 72°C for 2 min 30 s, and a final elongation step at 72°C for 10 min. Amplification products were visualized by electrophoresis on 1%

agarose gels containing ethidium bromide. Southern analyses were performed by standard methods. The filters were sequentially hybridized with radioactive S $\mu$ , TK, S $\gamma$ 3, or S $\alpha$  probes prepared using the random labeling kit (New England Biolabs) and quantitated by PhosphorImager<sup>®</sup> analysis (Molecular Dynamics).

**Digestion-circularization PCR.** Digestion-circularization (DC)-PCR analysis for the switch plasmids was performed as described previously (25) with modifications. 1  $\mu$ g of genomic DNA, or 5 of 50  $\mu$ l of Hirt extracts from nuclei, was digested overnight with 20 U of SacI (Promega) in the presence of 2  $\mu$ M spermidine in a total volume of 50  $\mu$ l. After heat inactivation, 4  $\mu$ l of digested DNA was ligated overnight using 6 U of T4 DNA ligase (Promega) in a total volume of 40  $\mu$ l. After heat inactivation, 1  $\mu$ l of the ligated DNA was used for PCR amplification. The 510-bp fragment was amplified using primers P1, 5' GTA ATA CGA CTC ACT ATA GGG C 3', and P4, 5' CCC GAC TGG AAA GCG GGC AGT GAG CGC AAC 3'. PCR amplification was in the presence of 3  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dCTP and was carried out for 26 cycles with each cycle consisting of 93°C for 30 s, 60°C for 40 s, and 72°C for 1 min 30 s, followed by a final step at 72°C for 10 min. Amplification of the 180-bp fragment was as described above, except that PCR consisted of 36 cycles and was performed using the P2/P3 $\gamma$  or P2/P3 $\alpha$  primer sets: P2, 5' TTT CAG ATC AGC TCA CCG CAA CAC AGC AGC GTA GCA T 3'; P3 $\gamma$ , 5' GGG TTG GAT GGA AAT GTG AAT AAC CTG CCT GA 3'; and P3 $\alpha$ , 5' CCT AGA CCG GGC AGA CCT AGA TAG AGT TGC 3', which is the reverse sequence of the R11 primer. To control for efficiency of digestion and ligation, DC-PCR amplification of the 180- and 510-bp fragments was carried out from the same ligation reaction. To establish the linear range of detection, serial dilutions of plasmid DNA were mixed with 1  $\mu$ g of genomic DNA and analyzed by DC-PCR for the 510-bp fragment. PCR products were purified by a phenol and chloroform extraction and then 1/5 (10  $\mu$ l) of the sample was analyzed on 4% nondenaturing TBE polyacrylamide gels and quantitated by PhosphorImager<sup>®</sup> analysis. Further experimental details are available upon request.

## Results

**Structure of the SR Plasmid Substrates.** To identify factors that participate in the SR reaction, we devised a plasmid-based transient transfection assay (Fig. 1 A). The switch substrate, p273, is specific for  $\mu \rightarrow \alpha$  SR events (33; Fig. 1 A). Intact, p273 contains a neomycin-resistance gene (neo), the Ig intronic enhancer (E $\mu$ ), an IgH variable region promoter (P<sub>H</sub>), a TK, the TGF- $\beta$  inducible promoter for  $\alpha$  gts (I $\alpha$ ), and the S $\mu$  and S $\alpha$  regions, with an overall configuration of 5'-neo-E $\mu$ -P<sub>H</sub>-S $\mu$ -TK-I $\alpha$ -S $\alpha$ -3'. Transcription through the S $\mu$  and S $\alpha$  regions is initiated by the P<sub>H</sub> and I $\alpha$  promoters, respectively. The TK and I $\alpha$  segment would be deleted as a consequence of S-S recombination. This deletion will confer gancyclovir resistance to cells. However, as explained below, gancyclovir selection is found to be unnecessary. To assay  $\mu \rightarrow \gamma$ 3 recombination, pG3.1 was constructed from p273 by replacing S $\alpha$  with S $\gamma$ 3 DNA (Fig. 1 A). These switch substrates are distinguished from those devised by others (24, 29) by the absence of a polyoma origin of replication. The rationale for this omission is that this origin may cause a significant frequency of background rearrangements between S sequence and vector.



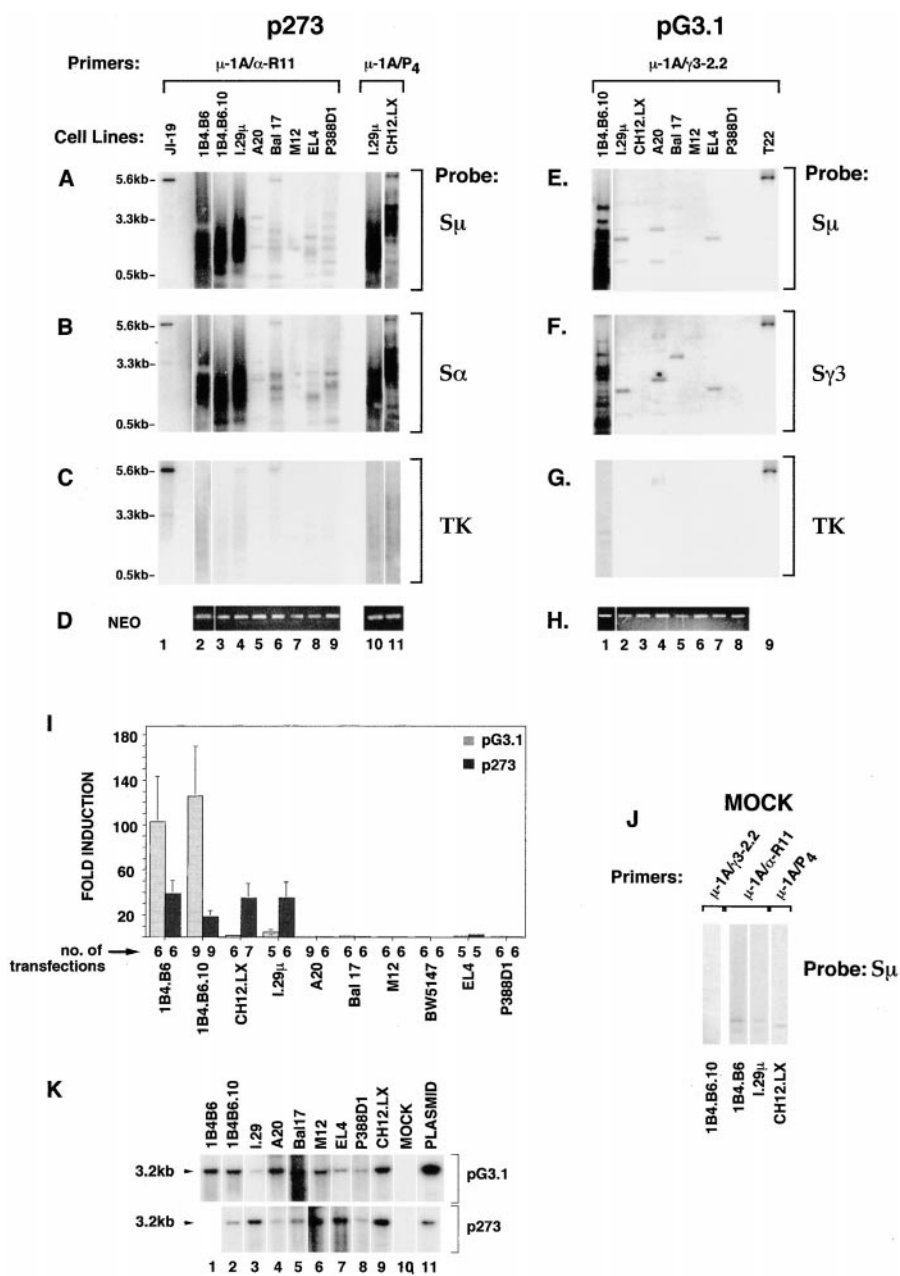
**Figure 1.** (A) Schematic diagram of extrachromosomal SR substrates, p273 and pG3.1, and PCR assay for SR. Transcription of the substrate S $\mu$  and S $\alpha$  regions is mediated respectively by the V<sub>H</sub> region gene promoter (P) and I $\alpha$ , a TGF- $\beta$ 1 inducible promoter associated with the  $\alpha$  gts (reference 36), and is induced by the IgH intronic  $\mu$  enhancer (E of EP). Probes (black horizontal bars) used in the Southern hybridization of PCR products are shown. The position and orientation of S region-specific forward ( $\mu$ -1A) and reverse ( $\gamma$ 3-2.2 and  $\alpha$ -R11) primers are shown by the filled triangles. Plasmid-specific forward (P1) and reverse (P4) primers, and neomycin-specific reverse (P5) primer (open triangles) are shown. When recombinant S-S regions are formed on p273 or pG3.1, the distance between the PCR primers,  $\mu$ -1A and  $\alpha$ -R11 or  $\gamma$ 3-2.2 located 5' of S $\mu$  and 3' of S $\alpha$  or S $\gamma$ 3, respectively, is shortened from 5.7 to 3.3 kb or less. The position of the unique EcoRI (E) site is shown. (B) Analysis of pI $\alpha$  and pI $\alpha$ E luciferase reporter plasmids in various cell lines. Luciferase reporter plasmids containing I $\alpha$  (-490/146) in the presence and absence of E $\mu$  were constructed and are referred to as pI $\alpha$ E and pI $\alpha$ , respectively. Luciferase activities were assessed 24 h after transfection. Two independent experiments were performed for all cell lines and the mean luciferase activities and their ranges are reported.

Transcription across S regions and expression of gts is necessary for isotype switching *in vivo* (12, 15, 47, 48). B cell-specific promoters, P<sub>H</sub> and I $\alpha$ , and the enhancer, E $\mu$ , were incorporated into the switch substrates to accommodate the possible dependence of SR on these transcriptional control elements (Fig. 1 A). In this context, it was important to establish that the promoters and enhancer used in the switch plasmids were active in all cell lines used in this study. Transcriptional activity of the I $\alpha$  promoter alone, or in combination with E $\mu$ , was analyzed using luciferase reporter plasmids, pI $\alpha$ -luc and pI $\alpha$ E-luc, respectively, and found to be present in the B cell lines I.29 $\mu$ , CH12.LX, 1B4.B6, Bal17, M12, and A20, and in the T cell line, EL4 (Fig. 1 B). These transcriptional elements were minimally active in the T cell line, BW5147, but not in the macrophage/monocyte line, P388D1. As these B cell lines produce Ig, we presume that the V<sub>H</sub> promoter and E $\mu$  are transcriptionally active. Cell lines that are able to support transcription at levels comparable to that found in I.29 $\mu$  are candidates for the analysis of plasmid-specific SR.

**Detection of S-S Recombinant Plasmids in Transient Transfections by Direct PCR.** SR occurs anywhere within the donor and acceptor S regions, and produces a population of S-S recombinant fragments heterogeneous in size and structure. On the switch plasmids, successful SR is predicted to result in the deletion of the TK and I $\alpha$  segment, and formation of a heterogeneous population of S $\mu$ -S $\alpha$  and S $\mu$ -S $\gamma$ 3 hybrid molecules (Fig. 1 A). As described previously, PCR assays for S-S composite molecules at endogenous loci (34) and on p273 (33) were modified and adapted to pG3.1 (Fig. 1 A). When S $\mu$ -S $\alpha$  hybrid molecules are formed, the distance between primers upstream of S $\mu$  ( $\mu$ -1A) and downstream of S $\alpha$  ( $\alpha$ -R11) is reduced. Compari-

son of the switching potential of p273 and pG3.1 in cell lines capable of SR relative to that in cell lines devoid of SR activity may allow definition of parameters that govern this process. The cell lines, I.29 $\mu$  (40), CH12.LX (41), and 1B4.B6 (Ma, L., H. Wortis, and A.L. Kenter, unpublished data) are capable of supporting endogenous  $\mu$ - $\alpha$  SR, and 1B4.B6 is also capable of endogenous  $\mu$ - $\gamma$ 3 SR. No other mouse cell line is known to be inducible for SR.

The switch substrates were transiently transfected into various cell lines and analyzed for formation of plasmid-specific S-S hybrid DNA by PCR amplification followed by Southern analysis. Cotransfection of a luciferase reporter plasmid, pGL3-SV40, and measurement of luciferase activity was used to control for transfection efficiency. To further confirm that the concentration of plasmid was equivalent in the normalized samples, a standard curve was set up for neomycin amplification. PCR amplification of the neomycin gene, which is unique to the switch plasmids, verified the presence of p273 and pG3.1 in all cell lines analyzed (Fig. 2, D and H). A standard curve for amplification of the neomycin gene indicated that the neomycin PCR products obtained were in the linear range of detection (data not shown). Thus, PCR amplification of the neomycin gene functioned to independently verify that the transfected samples were successfully normalized. A unique EcoRI site is located in the TK on the switch plasmid (Fig. 1 A). DNA recovered from transfected cells was digested with EcoRI before PCR amplification. This treatment destroys the linear integrity of intact or partially rearranged substrate, and renders these plasmids unavailable for amplification. Genomic DNA from JI-19 and T22, stable transfectants containing intact p273 and pG3.1, respectively, were used as positive controls for the PCR reaction and



**Figure 2.** Switch plasmid-derived S-S recombinant molecules are detected in a restricted set of B cell lines. (A-H) Cell lines were cotransfected with a switch plasmid (10  $\mu$ g) and pGL3-SV40 (10  $\mu$ g) and analyzed after 48 h of culture. One aliquot was taken for luciferase analysis while DNA was prepared from a second aliquot. The amount of DNA taken for PCR was normalized based on luciferase values to control for transfection efficiency. Before PCR, DNA was digested with EcoRI to eliminate intact or partially rearranged plasmid. A unique EcoRI site is located in the TK (see Fig. 1 A). (A-C) Cell lines transfected with p273 were analyzed by PCR amplification with primer sets  $\mu$ -1A/ $\alpha$ -R11 (lanes 1-9) or  $\mu$ -1A/P4 (lanes 10-12) for the formation of S $\mu$ -S $\alpha$  composite molecules. The primer set  $\mu$ -1A/ $\alpha$ -R11 nonspecifically amplified endogenous loci in CH12.LX cells and was thus replaced with the  $\mu$ -1A/P4 primer set. A representative example is shown of PCR products analyzed by Southern hybridization using the S $\mu$  (A), S $\alpha$  (B), or TK (C) probes. JI-19 (lanes 1 and 12) is a stably transfected I.29 $\mu$  clone carrying an intact copy of p273 and was not digested with EcoRI. (E-G) The formation of S $\mu$ -S $\gamma$ 3 composite molecules was analyzed by PCR amplification with primer set  $\mu$ -1A/ $\gamma$ 3-2.2 (lanes 1-9), followed by Southern hybridization using the S $\mu$  (E), S $\gamma$ 3 (F), or TK (G) probes. T22 is a stably transfected I.29 $\mu$  clone carrying an intact copy of pG3.1 and was not digested with EcoRI. Sizes of selected DNA fragments are shown. Presence of plasmid DNA p273 (D) and pG3.1 (H) after transfection was verified by amplification of a portion of the neomycin (neo) gene using the P1/P5 primers. (I) Quantitation of S-S recombinant molecules by PhosphorImager<sup>®</sup> analysis of Southern hybridizations for each cell line are derived from multiple independent transfected samples, from two to three experiments. The number of transfected samples tested for each cell line is indicated. The average hybridization signal, relative to M12, is shown by the histograms with SD indicated. (J) PCR amplification products are plasmid specific. The 1B4.B6, CH12.LX, and I.29 $\mu$  cell lines were (mock)

transfected in the absence of plasmid, and DNA was isolated from nuclei and tested for the presence of S-S recombinant products using the indicated primer sets. Two experiments were performed with identical results. (K) Plasmid recovery from transfected cell lines was verified by Southern analysis. Equal amounts of genomic DNA (4  $\mu$ g/sample) from the indicated cell lines (lanes 1-9) were digested with SacI and analyzed by Southern hybridization. As a control for probe specificity, DNA from mock-transfected 1B4.B6.10 cells was included in lane 10. Plasmid pG3.1 and p273 DNA was digested with SacI and is shown as a positive control for hybridization, as indicated (lane 11). The probe used was a 1,267-bp BglII restriction fragment from the Bluescript SK plasmid, containing the ampicillin-resistance gene.

their amplification products are included as hybridization controls (Fig. 2, A-C, lane 1, and E-G, lane 9). These DNAs were not digested with EcoRI before PCR amplification.

When DNA from p273 transfectants was analyzed, a complex mixture of PCR products that hybridize with S $\mu$  and S $\alpha$ , but not TK, were derived from DNA of 1B4.B6, subclone 1B4.B6.10, I.29 $\mu$ , and CH12.LX (Fig. 2, A-C). Few PCR products were obtained from the other cell

lines. When DNA from pG3.1 transfectants was analyzed, a heterogeneous population of amplified fragments that hybridize with S $\mu$  and S $\gamma$ 3, but not TK, were derived from DNA from 1B4.B6.10, but not from any other cell line (Fig. 2, E-G). Quantitation of hybridized S $\mu$  probe by PhosphorImager<sup>®</sup> analysis from two to three experiments, which included five to nine individual transfected samples, was averaged for each cell line and the SD was calculated (Fig. 2 I). This analysis indicates that the amounts of p273-

derived recombinant molecules are >20–40-fold in 1B4.B6, 1B4.B6.10, I.29 $\mu$ , and CH12.LX than in M12 and the other non-switching cell lines tested. The amount of pG3.1-derived S–S recombinant molecules is >125-fold in 1B4.B6.10 than in M12 and other cell lines analyzed (Fig. 2 I). As primers used for amplification of plasmid-derived S–S recombinant fragments are also capable of detecting fragments from the endogenous loci, DNA samples from mock-transfected cells were also tested in these same experiments. No S $\mu$ –S $\alpha$  recombinant fragments that hybridized with S $\mu$  were detected in DNA amplified from 1B4.B6, I.29 $\mu$ , and CH12.LX cells, and no S $\mu$ –S $\gamma$ 3 recombinant fragments were detected in DNA amplified from 1B4.B6.10 cells (Fig. 2 J). The cell lines 1B4.B6, 1B4.B6.10, I.29 $\mu$ , and CH12.LX require treatment with mitogen and/or cytokines to induce SR at endogenous loci. Thus, the absence of S–S recombinant fragments in DNA from these mock-transfected cells is consistent with their requirements for SR induction.

Southern analysis was used to independently verify the presence and integrity of the switch plasmids in representative samples from each of the transfected cell lines. Genomic DNA was isolated from cell lines transfected with pG3.1 or p273 and the luciferase reporter plasmid, digested with SacI, and analyzed by Southern hybridization using a probe specific for the plasmid backbone (Fig. 2 K). A DNA sample from mock-transfected 1B4B6.10 cells was analyzed and showed no hybridization with the plasmid-specific probe, whereas pG3.1 and p273 DNAs hybridized well, confirming the specificity of the probe (Fig. 2 K, lanes

10 and 11). A 3.2-kb band was evident in all of the DNA samples, indicating the presence of transfected switch substrate. The varying intensities of plasmid-specific hybridization represent the varying transfection efficiencies of the cell lines as equal amounts of genomic DNA were taken for analysis. These results indicate that the absence of S–S recombinant fragments detected by the direct PCR assay shown in Fig. 2 cannot be attributed to a failure of plasmid recovery from transfected cell lines. Together, these findings suggest that S–S recombination on the plasmid occurs selectively in B cells capable of switching their endogenous S genes (Table I).

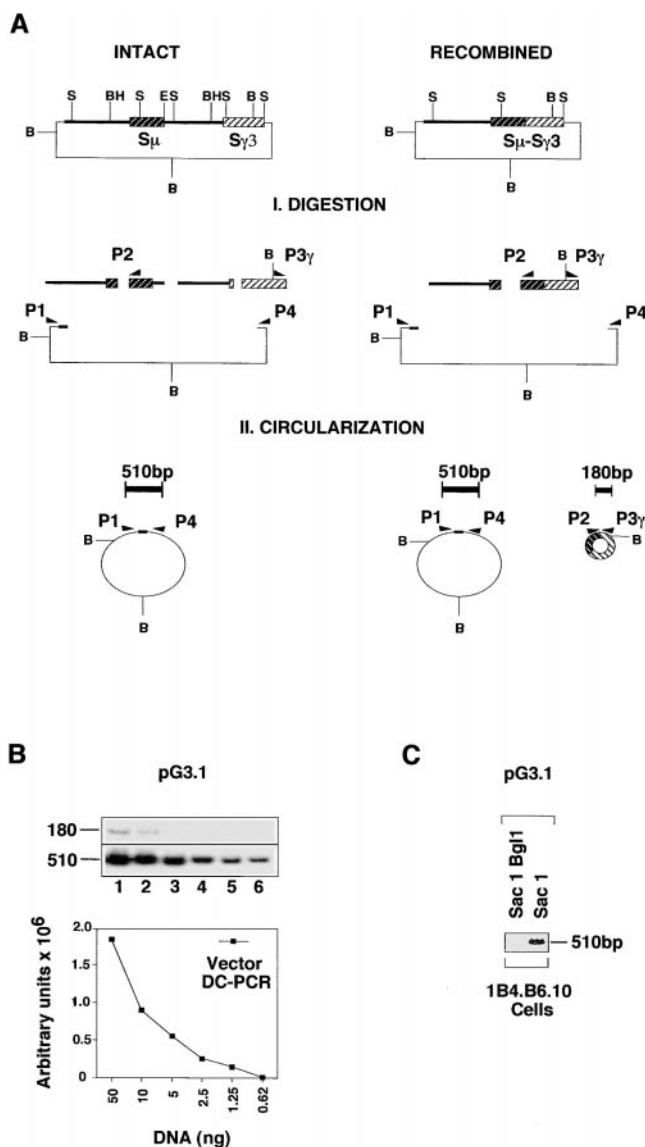
*DC-PCR Confirms the Presence of Class-specific Factors for SR.* SR may initiate through formation of DSBs in S DNA (5). S regions contain short regions of homologous sequence (49). Damaged DNA has been shown to promote formation of gene hybrids during PCR amplification (50, 51). Thus, it is possible that the S–S recombinant fragments produced by direct PCR amplification of the switch plasmids may arise through cleavage of S DNA followed by PCR-mediated S–S hybrid formation. A semiquantitative DC-PCR assay (25, 52) was used to determine whether the apparently composite S–S molecules are physically contiguous and to provide a more quantitative evaluation of the relative levels of recombination on the plasmids. On the switch substrate, recombination between two S regions will cause the deletion of intervening TK and I $\alpha$  gene segments and loss of an internal SacI site but conservation of SacI sites situated in the 5' region of S $\mu$  and 3' of S $\alpha$  and S $\gamma$ 3 (Fig. 3 A). The recombined S–S hybrid will be located

**Table I.** Summary of SR Activity at Endogenous Loci and on Switch Substrates in Various Cell Types

Tissue	Literature designation	Cell line or type	Endogenous SR		Plasmid* SR		
			$\mu \rightarrow \gamma 3$	$\mu \rightarrow \alpha$	$\mu \rightarrow \gamma 3$	$\mu \rightarrow \alpha$	
B lymphoid lineage							
Mature B cell	B cell	LPS- and $\alpha$ dex-stimulated	+	+	+	+	
		splenic B cells, Balb/c nu/nu					
	1B4.B6	J2 virus-transformed B cell line	+	+	+	+	
	1.29 $\mu$	Lymphoma	– <sup>‡</sup>	+	–	+	
	CH12.LX	Lymphoma	– <sup>‡</sup>	+	–	+	
	M12	Lymphoma	–	–	–	–	
	A20	Lymphoma	–	–	–	–	
	Bal17	Lymphoma	–	–	–	–	
T lymphoid lineage							
	BW5147	Lymphoma	–	–	–	–	
	EL4	Lymphoma	–	–	–	–	
Monocytic lineage							
	P388D1	Macrophage/monocyte	–	–	–	–	

\*Plasmid-based SR activity assessed by direct S–S PCR and DC-PCR.

<sup>‡</sup>Rare  $\mu \rightarrow \gamma 3$  events have been detected for 1.29 $\mu$  (reference 61) and CH12.LX (reference 80).



**Figure 3.** Semiquantitative DC-PCR analysis of switch substrate-derived S-S composite molecules. (A) Schematic diagram of the DC-PCR assay is shown for pG3.1 and is essentially identical for p273. SacI digestion of pG3.1 releases S $\mu$  and S $\gamma$ 3 as separate restriction fragments, whereas in the recombined substrate the S-S recombinant molecule is released as a single restriction fragment. Ligation is performed such that intramolecular restriction fragment circularization is favored. PCR using the P1/P4 primer set amplifies a 510-bp fragment derived from the vector backbone. PCR with primer set P2/P3 $\gamma$  leads to the amplification of a 180-bp fragment derived from the S-S recombinant fragment. (B) DC-PCR products are in the linear range of detection. Twofold serial dilutions of the pG3.1 were titrated into 1  $\mu$ g of genomic DNA and 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. Two samples (1.25 and 2.5 ng), which are in the linear range of detection, were included in every subsequent analysis. (C) Verification of the intramolecular ligation for the vector DC-PCR assay. The pG3.1 plasmid was transfected into 1B4.B6.10 cells, nuclei were isolated, DNA was recovered and digested with either SacI or SacI plus BglI, and the DC-PCR assay was performed.

on a new SacI fragment, whereas the unrecombined S regions would be located on two different SacI fragments. After digestion with SacI, DNA is ligated under dilute conditions that favor intramolecular ligation, resulting in the circularization of individual restriction fragments. The region spanning the circle "joint" is amplified using reverse primers specific for sites at the 5' end of S $\mu$  and the 3' end of S $\alpha$  or S $\gamma$ 3. PCR amplification of the S-S composite fragment after SacI digestion and ligation yields a 180-bp fragment (Fig. 3 A). The size of this DC-PCR product is plasmid specific and could not be derived from the endogenous locus based on the position of the genomic SacI sites (53, 54).

To control for transfection, plasmid recovery, and ligation efficiency, a DC-PCR assay for the plasmid backbone was devised. The plasmid backbone is contained in a single SacI restriction fragment that will circularize when ligated under dilute conditions. The DC-PCR of the plasmid backbone circle joint yields a 510-bp amplification product (Fig. 3 A). The linear range of detection was established using twofold serial dilutions of p273 (data not shown), and pG3.1, into 1  $\mu$ g of genomic DNA followed by DC-PCR in the presence of radiolabeled deoxynucleotides. Addition of increasing amounts of template ranging from 1.25 to 10 ng/ml resulted in a linear increase of vector-specific DC-PCR product, as shown for pG3.1 (Fig. 3 B). Two standard samples, 1.25 and 2.5 ng, were taken from this titration and included in all subsequent studies to assure that the amplification product deriving from the plasmid backbone was always in the linear range of detection.

To confirm that the DC-PCR assay was being carried out under intramolecular ligation conditions, various concentrations of pG3.1 plasmid were digested with SacI or SacI plus BglI. There are two BglI sites located in the plasmid backbone (Fig. 3 A). Digestion of the plasmid backbone with SacI and BglI will preclude the circularization of this DNA under dilute ligation conditions. We found that at 1.25 and 2.5 ng/ml of input plasmid DNA, the 510-bp DC-PCR product was present after digestion with SacI, but not with SacI and BglI, verifying the intramolecular character of this ligation reaction (data not shown). To further verify that the DC-PCR assay was being carried out under intramolecular ligation conditions, the same experiment was performed with pG3.1 DNA recovered from transfected 1B4.B6.10. The 510-bp DC-PCR product was detected using plasmid digested with SacI, but not with SacI and BglI, confirming the intramolecular character of this ligation reaction (Fig. 3 C).

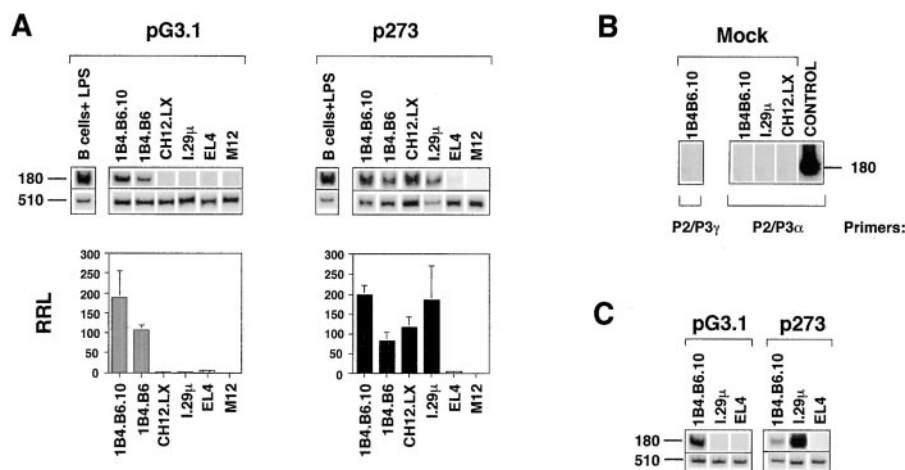
To confirm that the conditions chosen for intramolecular ligation were also valid for the 180 bp DC-PCR product, we tested for the presence of the 180-bp DC-PCR product from pG3.1 using a range of concentrations of plasmid that had been digested with SacI. At high concentrations of input plasmid, intermolecular ligation occurred and produced the 180-bp DC-PCR product (Fig. 3 B, lanes 1 and 2). As expected, under dilute plasmid concentrations (0.62–5 ng) no 180-bp product was detected, demonstrating that fragment ligation was intramolecular (Fig. 3 B, lanes 3–6).

DC-PCR analysis of switch substrates transiently transfected into a panel of cell lines was conducted. To compare plasmid-based SR between cell lines, a relative recombination level (RRL) is calculated for each cell line from the ratio of radioactivity associated with the 180-bp fragment to that of the 510-bp fragment and by arbitrarily setting the RRL for M12 at 1.0 (Fig. 4 A). Recombination of pG3.1 is undetectable in I.29 $\mu$ , CH12.LX, M12, and EL4, whereas switching activity is found in 1B4.B6 and 1B4.B6.10 with RRLs of 100–200-fold above background (Fig. 4 A). Switching activity for p273 is undetectable in M12 and EL4, whereas activity is found in 1B4.B6, 1B4.B6.10, I.29 $\mu$ , and CH12.LX with RRLs 80–200-fold above background (Fig. 4 A). As a control for plasmid specificity of the 180-bp DC-PCR product, cell lines that supported SR on pG3.1 and p273 were mock transfected in the absence of plasmid, and then DNA was isolated and analyzed by DC-PCR using the indicated primer sets (Fig. 4 B). No 180-bp product was detected in the mock-transfected cell lines, indicating the plasmid specificity of this PCR product. A p273 plasmid containing a recombinant S-S region was included as a positive control for ligation and PCR amplification. This recombinant plasmid gave rise to the 180-bp product as expected (Fig. 4 B). The DC-PCR analysis verifies that S-S fragments amplified by direct PCR are organized as covalently joined contiguous units before PCR. This analysis also demonstrates that  $\mu \rightarrow \gamma 3$  switching activity is present only in 1B4.B6 cells, whereas p273  $\mu \rightarrow \alpha$  recombination occurs in I.29 $\mu$ , CH12.LX, and 1B4.B6 cells.

To determine if the plasmids could undergo SR in normal B cells, p273 and pG3.1 were transfected into LPS-activated splenic B cells (55) from nu/nu mice, which lacked T cells, and were analyzed by DC-PCR. LPS is a well-characterized B cell mitogen that causes B cell proliferation,

Ig secretion, and SR (55). The DC-PCR analysis indicates the presence of S-S recombinant fragments in p273 and pG3.1, confirming that the plasmids are recombinogenic in cells that undergo physiological SR (Fig. 4 A).

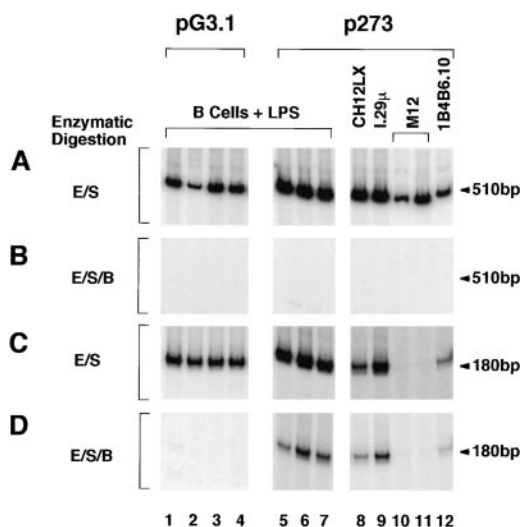
Incomplete digestion of the switch plasmid DNAs with restriction enzymes could lead to false positive results in the DC-PCR assay (Fig. 3 A). Several additional controls were performed to test for incomplete digestion. DNA isolated from CH12.LX, I.29 $\mu$ , M12, 1B4B6.10, and from LPS-activated splenic B cells transfected with pG3.1 or p273 was digested with both EcoRI and SacI, instead of only SacI, before analysis by DC-PCR (Fig. 5). The addition of EcoRI has no effect on the presence of the 510-bp fragment (Fig. 5 A), as there are no EcoRI sites in the vector backbone (Fig. 3 A). However, digestion of the DNAs with EcoRI, SacI, and BglII abolished the 510-bp fragment in all samples (Fig. 5 B) because there are two BglII sites located in the vector backbone (Fig. 3 A). The absence of the 510-bp fragment under these conditions indicates that the switch plasmid DNA can be digested to completion and that ligation was exclusively intramolecular. Intact switch substrates contain an EcoRI site in the TK, whereas after SR the recombinant plasmids contain no EcoRI sites (Fig. 1 A). Digestion of the DNA samples with EcoRI has no effect on the presence of the 180-bp fragment from either pG3.1 or p273 (Fig. 5 C). The pG3.1 plasmid contains a BglII site at the 3' end of the S $\gamma$ 3 region, whereas a comparable site is absent from p273. Digestion of the DNA samples with EcoRI, SacI, and BglII abolished the 180-bp fragment in the pG3.1 samples but not in the p273 samples, indicating that these switch plasmid samples can be cut to completion by restriction enzymes. The specificity of the DC-PCR reaction was further confirmed by the absence of the 180 bp fragment in DNAs derived from M12 cells transfected with p273 (Fig. 5 C, lanes 10 and 11). Taken



**Figure 4.** The differential activity of p273 and pG3.1 in B cell lines that support endogenous switching as analyzed by DC-PCR. (A) DC-PCR analysis of DNA from nuclei derived from cells transfected with either pG3.1 or p273 is shown in the upper panels. PCR using the P1/P4 primer set amplifies a 510-bp fragment derived from the vector backbone. PCR with primer set P2/P3 $\gamma$  or P2/P3 $\alpha$  amplifies the 180-bp fragment representing the S-S recombinant fragments from pG3.1 and p273, respectively. Normal splenic B cells from Balb/c nu/nu mice were activated with LPS, transfected with the switch plasmids, and analyzed by DC-PCR as indicated. The average RRL of four transfections in two independent experiments for each cell line for p273 and pG3.1 is plotted (bottom) and SDs are shown. (B) As a control for plasmid

specificity of the 180-bp S-S DC-PCR product, cell lines (1B4.B6.10, I.29 $\mu$ , and CH12.LX) exhibiting high levels of SR on pG3.1 and p273 were (mock) transfected in the absence of plasmid, and nuclei were prepared and analyzed by DC-PCR for presence of the 180-bp fragment. A recombinant p273 plasmid mixed in with genomic DNA was included as a positive control. (C) DC-PCR analysis of Hirt extracts indicates that SR occurs in the absence of DNA replication. Switch plasmids, pG3.1 or p273, were transiently transfected into various cell lines and after 48 h of cell culture, nuclei were harvested and Hirt extracts were made. DC-PCR analysis for vector and S-S recombinant fragments was carried out on the Hirt supernatants as indicated.





**Figure 5.** The authenticity of the DC-PCR products was verified by restriction enzyme analysis. DC-PCR analysis of DNA from nuclei derived from LPS-activated splenic B cells transfected with either pG3.1 (lanes 1–4) or p273 (lanes 5–7) and from cell lines transfected with p273 is shown (lanes 8–12). The samples shown in lanes 1–12 were derived from independent transfections. Genomic DNA from transfected cells was digested with combinations of enzymes including EcoRI plus SacI (A and C, E/S), or EcoRI plus SacI plus BglII (B and D, E/S/B) before ligation and DC-PCR analysis. (A and B) PCR using the P1/P4 primer set amplifies a 510-bp fragment derived from the vector backbone from pG3.1 and p273. (C and D) PCR with primer set P2/P3 $\gamma$  or P2/P3 $\alpha$  amplifies the 180 bp fragment representing the S–S recombinant fragments from pG3.1 and p273, respectively. Restriction enzymes used in this analysis were EcoRI (E), SacI (S), and BglII (B).

together, the use of additional restriction enzyme combinations confirms that the 180-bp fragment, representing the presence of S–S recombinant plasmid substrates, arises from bona fide recombination events.

*SR on the Plasmid Substrate Is Not Dependent on an Origin of DNA Replication.* The requirement for cycling cells in the SR reaction was previously noted (56–59). The switch plasmid cannot autonomously replicate at high levels, as it does not contain an origin of replication (Fig. 1 A). If high-level DNA replication is an intrinsic requirement for SR, then extrachromosomal switch substrates will not support plasmid-based SR and must integrate into genomic DNA in order to replicate and recombine. Isolation of low molecular weight DNA from transfected cells by Hirt extraction allows examination of the recombination state of nonintegrated plasmid DNA. DC-PCR analysis of extrachromosomal switch plasmids recovered from Hirt extracts provides evidence for SR on p273 transfected into I.29 $\mu$  and 1B4.B6 cells, and for pG3.1 in 1B4.B6.10 cells (Fig. 4 C). Again, we find that p273 does not recombine in EL4, and that pG3.1 does not recombine in I.29 $\mu$  or EL4. These results indicate that SR on the switch substrate can occur in the absence of a DNA origin of replication.

*Structural Analysis of p273 Recovered from Cell Lines by Bacterial Transformation.* Recovery of recombinant switch substrates provides an opportunity to examine the full range of structures that arise in switching and nonswitching cell lines. We found that switch plasmid could be most easily recovered from preparations of genomic DNA by bac-

**Table II.** Recovery of p273 from Transfected DNA by Bacterial Transformation

Cell line	Endogenous SR activity	<i>n</i> <sup>‡</sup>	Bacterial Amp <sup>r</sup> * transformants					<i>P</i> value <sup>§§</sup>
			Total <sup>§</sup>	EcoRI <sup>r</sup> <sup>¶</sup>	% EcoRI <sup>r</sup> <sup>¶</sup>	EcoRI <sup>r</sup> -S-S <sup>**</sup>	% S-S Rec <sup>††</sup>	
A20	–	4	23,920	16	0.067	0	<0.004	NS
EL4	–	4	31,074	18	0.058	0	<0.003	NS
M12	–	5	51,456	7	0.014	0	<0.002	NS
CH12.LX	+	4	13,898	16	0.115	10	0.072	
1B4.B6.10	+	3	9,584	13	0.136	4	0.041	<0.001
I.29 $\mu$	+	4	20,968	39	0.186	28	0.133	<0.001
B cells <sup>    </sup>	+	2	8,340	21	0.252	12	0.144	<0.001

Plasmid was transfected into the indicated cell lines. The DNA recovered from nuclei of p273-transfected cells was either left untreated or digested with EcoRI and then transformed into bacteria. The EcoRI<sup>r</sup> colonies were prepared as minipreps and analyzed by restriction mapping to identify S–S recombinant plasmids.

\*Amp<sup>r</sup> denotes ampicillin resistance.

<sup>‡</sup>Number of individual experiments.

<sup>§</sup>The total number of Amp<sup>r</sup> transformants obtained.

<sup>¶</sup>EcoRI<sup>r</sup> denotes plasmid resistant to EcoRI digestion. Intact plasmids recovered despite EcoRI digestion were excluded.

<sup>¶¶</sup>The percentage of EcoRI<sup>r</sup> is obtained by dividing the total number of EcoRI<sup>r</sup> transformants by Amp<sup>r</sup> transformants and then multiplying by 100.

<sup>\*\*</sup>EcoRI<sup>r</sup>-S-S denotes bacterial recombinants that carry S $\mu$ -S $\alpha$  recombinant plasmid resistant to EcoRI digestion.

<sup>††</sup>The percentage of S–S recombinants (S–S Rec) is obtained by dividing the total number of EcoRI<sup>r</sup>-S-S transformants by Amp<sup>r</sup> transformants and then multiplying by 100.

<sup>§§</sup>The *P* value indicates the probability that the percentage of S–S recombinants obtained for each cell line is significantly different from that obtained for CH12LX, the cell line of reference. *P* values were derived by  $\chi^2$  analysis (see Materials and Methods). NS, not significant.

<sup>||||</sup>B cells were stimulated with 50  $\mu$ g/ml LPS.

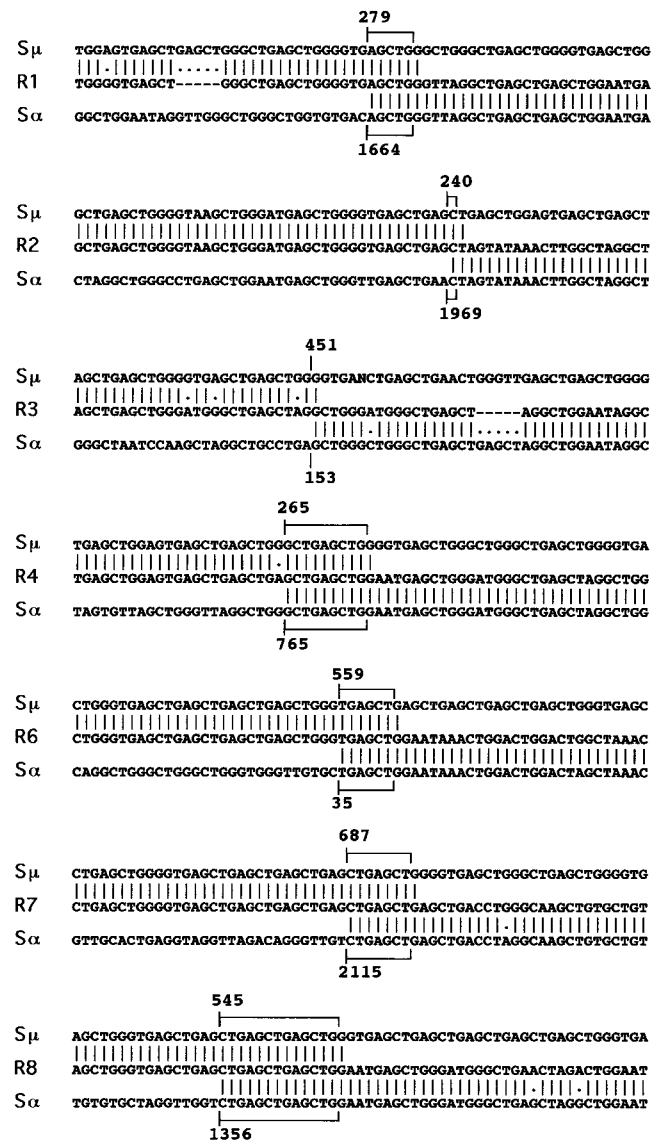
terial transformation. SR on p273 was analyzed in various cell lines by transient transfection, isolation of DNA from nuclei, and transformation of bacteria. Recombination between S regions leads to deletion of TK and loss of a unique EcoRI restriction site. To identify plasmids that are S-S recombinant, all recovered plasmids were digested with EcoRI and resistant plasmids were further analyzed by restriction enzyme mapping. S-S recombinants were recovered from I.29 $\mu$ , CH12.LX, 1B4.B6 cells, and LPS-activated splenic B cells, whereas none of the p273 recovered from A20, M12, or EL4 contained S-S recombination events (Table II). The fraction of recombinant plasmids was calculated for each cell line and compared with that for M12 using  $\chi^2$  analysis. The *P* values derived from the  $\chi^2$  analysis indicate that the switching cell lines are not significantly different from each other and are significantly different than the nonswitching cell lines with respect to the incidence of S-S recombinant plasmids recovered (Table II).

Although the loss of the EcoRI site located in the TK gene is diagnostic of an S-S recombinant plasmid, the absence of this restriction site may also occur when the TK gene is partially or completely deleted by other types of recombination events. The ratio of ampicillin-resistant colonies obtained after digestion with EcoRI, compared with undigested genomic DNA, indicates the total relative recombination frequency. We found that  $\leq 0.25\%$  of the recovered plasmids were EcoRI resistant in all the cell lines analyzed (Table II). Thus, nearly all the plasmids were intact 2 d after transfection.

Restriction enzyme mapping demonstrated that deletion of the region spanning the neo gene through the S $\alpha$  region was the most frequent event leading to EcoRI resistance in plasmids from all cell lines tested with the exception of CH12.LX, I.29 $\mu$ , and normal LPS-activated splenic B cells, where S-S recombination was more frequent (data not shown and Table II). The neo-deleted plasmids would not be detected by direct S-S PCR or by DC-PCR analysis, as the primer annealing sites were lost. These findings indicate that S-S recombination occurs on p273, which was transfected into switching cell lines or B cells but not into other cells. However, our studies also demonstrate that there is an inherent instability in the S plasmid such that the region spanning the neo gene through S $\alpha$  can be deleted in all the cell types analyzed.

To control for possible S-S recombination in bacteria, in the same experiments p273 DNA was untreated or digested with EcoRI and transformed into *Escherichia coli*. The efficiency of transformation for intact plasmid was  $5 \times 10^7$  colonies/ $\mu$ g. After transformation with 10 ng of EcoRI-treated p273, the equivalent of  $5 \times 10^5$  colonies, we found no ampicillin-resistant colonies, indicating that EcoRI resistance arises as a consequence of transfection into cell lines, and that EcoRI resistance does not arise in bacteria. Taken together, these results demonstrate that SR on the p273 plasmid recovered from transfected cell lines is detectable only in cells that switch, but also that the switch plasmids are inherently unstable. This instability may arise from the repetitive character of the S regions.

*Switch Junction Sequence Analysis Confirms Direct S-S Recombination.* The DC-PCR analysis demonstrates the presence of composite S $\mu$ -S $\alpha$  or S $\mu$ -S $\gamma$ 3 on a single SacI restriction fragment. However, it is still possible that a small number of nucleotides derived from either TK or I $\alpha$  are located between the apparently recombined S regions and would not be detectable by Southern analysis. S-S recombinant p273 plasmids, which had been transfected into



**Figure 6.** DNA sequence analysis demonstrates direct S-S joining in recombinant plasmids. Seven S $\mu$ -S $\alpha$  recombinant plasmids were identified from Hirt extracts of p273-transfected I.29 $\mu$  (R-1 to R-4) and 1B4.B6 (R-6 to R-7) cells after transformation of bacteria (STBL2; GIBCO BRL). Automated DNA sequence analysis using the  $\mu$ -1A and the  $\alpha$ -R11 primers allowed the determination of the precise S $\mu$ -S $\alpha$  breakpoints. Brackets around the breakpoints indicate DNA homology between the S $\mu$  donor and S $\alpha$  acceptor DNAs. Nucleotide position 1 of the plasmid S $\mu$  sequence corresponds to position 5330 of germline S $\mu$  sequence (sequence data available from EMBL/GenBank/DBJ under accession no. MUSIGCD07). Nucleotide position 1 of plasmid S $\alpha$  sequence corresponds to position 2121 of the germline S $\alpha$  sequence (available from EMBL/GenBank/DBJ under accession no. MUSIALPHA).

1B4.B6.10 and I.29 $\mu$  cells, were identified by restriction mapping (Table II). Nine S-S recombinant plasmids were taken for DNA sequence analysis and the S $\mu$ -S $\alpha$  switch junctions were located for seven plasmids (R-1 to R-7; Fig. 6). Several mutations were found in clones R1, R3, R4, R6, and R7. However, these mutations were not fully authenticated as it was only possible to sequence one DNA strand in the area of the switch junction. In all of these clones, the switch junctions showed direct joining of S $\mu$  and S $\alpha$  DNA without deletion, duplication, or nucleotide insertion at the breakpoint. Identity between the donor and acceptor S regions is frequently found in switch junctions (60). The microhomology at the S $\mu$ -S $\alpha$  junctions was 1-14 bases (Fig. 6). This level of homology at the junctions is slightly higher than usually observed (60), but may not be surprising given the degree of similarity between the S $\mu$  and S $\alpha$  regions (49). The recombination breakpoints for these S $\mu$ -S $\alpha$  switch junctions originate in different positions within the germline S $\mu$  and S $\alpha$  regions, demonstrating that the hybrid S $\mu$ -S $\alpha$  molecules arose from independent recombinational events. It should be stressed that the S-S recombinant plasmids recovered from switching cell lines were recombined only between S regions and contained no other rearrangements or deletions, suggesting that these recombination events arose through bona fide SR.

*SR on pG3.1 Is Switch Region Dependent.* SR demonstrates sequence specificity because it is focused on S region DNA at endogenous loci. To determine whether plasmid-based SR was specific for S DNA, a switch plasmid was constructed in which the S $\gamma$ 3 DNA was largely deleted. In this plasmid, named pG3.1.01s, all that remained of S $\gamma$ 3 DNA was 125 bp derived from the 3' end of the S region. The frequency of S-S recombinant plasmids arising from pG3.1 and pG3.1.01s was compared after transfection into normal splenic B cells activated with LPS. S-S recombinant

pG3.1 plasmids were recovered, whereas no S-S recombinant pG3.1.01s plasmids were found after transfection (Table III). The *P* values derived from the  $\chi^2$  analysis indicate that the frequency of S-S recombinant events for pG3.1 was significantly different from that for pG3.1.01s. In addition, deletion of the S $\gamma$ 3 region leads to enhanced plasmid stability as evidenced by the  $\sim$ 10-fold lower frequency of EcoRI-resistant pG3.1.01s compared with pG3.1. These results indicate that the presence of the S $\gamma$ 3 region is crucial for SR to occur on the switch plasmids and demonstrates the S region dependency of this recombination event.

*Transcriptional Elements Are Not Required for Recombination on the Switch Substrates.* Expression of *gts* is a prerequisite for SR in vivo. To determine whether transcription of S regions is required for SR on plasmid substrates, the RRLs of structural variants of pG3.1 and p273 that lack the I $\alpha$ , EP, or I $\alpha$  and EP segments were assessed in 1B4.B6 and I.29 $\mu$  cells, respectively. We found no consistent relationship between the presence or absence of the transcriptional elements and recombination (Fig. 7). Furthermore, recombination did not depend on the presence of the I $\alpha$  or EP segments in stable transfection studies of p273 and its I $\alpha$  and I $\alpha$  plus EP deletion variants (33). The lack of requirement for the presence of transcriptional elements may be due to a low level of transcription on the plasmid occurring in the absence of the I $\alpha$  and EP segments. Reverse transcriptase-PCR analysis indicates the occurrence of very low levels of read-through transcription from the TK promoter through the S $\alpha$  region in the absence of the I $\alpha$  promoter (33). However, read-through transcripts through the S $\mu$  region were not detected in the absence of the EP enhancer/promoter segment. We conclude that high levels of transcription are not necessary, and it is unclear whether any transcription is required for successful SR on the plasmid substrate.

**Table III.** Recovery of pG3.1 and pG3.01s from Transfected DNA by Bacterial Transformation

Plasmid	<i>n</i> <sup>‡</sup>	Bacterial Amp <sup>r</sup> * transformants					<i>P</i> value <sup>§§</sup>
		Total <sup>§</sup>	EcoRI <sup>†</sup> ‡	% EcoRI <sup>†</sup> ‡	EcoRI <sup>†</sup> -S-S**	% S-S Rec <sup>‡‡</sup>	
pG3.1	2	23,800	32	0.130	9	0.040	
pG3.01s	2	344,000	48	0.014	0	0.000	<0.0001

Plasmid was transfected into B cells from nu/nu mice stimulated with 50  $\mu$ g/ml LPS. The DNA recovered from nuclei of transfected cells was either left untreated, or digested with EcoRI and then transformed into bacteria. The EcoRI<sup>r</sup> colonies were prepared as minipreps and analyzed by restriction mapping to identify S-S recombinant plasmids.

\*Amp<sup>r</sup> denotes ampicillin resistance.

<sup>‡</sup>Number of individual experiments.

<sup>§</sup>The total number of Amp<sup>r</sup> transformants obtained.

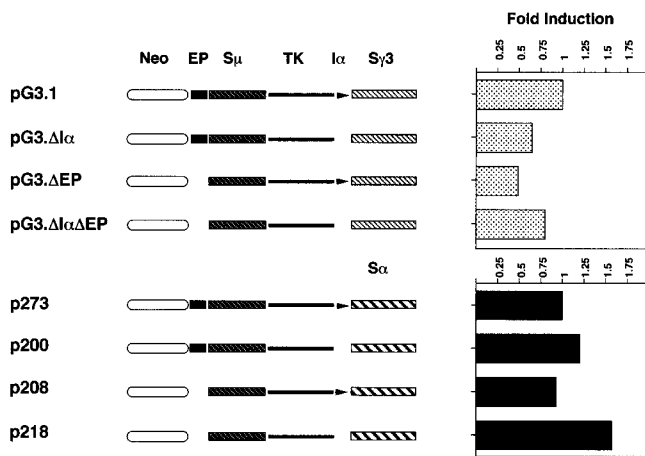
<sup>†</sup>EcoRI<sup>r</sup> denotes plasmid resistant to EcoRI digestion. Intact plasmids recovered despite EcoRI digestion were excluded.

<sup>‡‡</sup>The percentage of EcoRI<sup>r</sup> is obtained by dividing the total number of EcoRI<sup>r</sup> transformants by Amp<sup>r</sup> transformants and then multiplying by 100.

\*\*EcoRI<sup>r</sup>-S-S denotes bacterial transformants that carry S $\mu$ -S $\alpha$  recombinant plasmid resistant to EcoRI digestion.

<sup>‡‡</sup>The percentage of S-S recombinants (S-S Rec) is obtained by dividing the total number of EcoRI<sup>r</sup>-S-S transformants by Amp<sup>r</sup> transformants and multiplying by 100.

<sup>§§</sup>The *P* value indicated the probability that the percentage of S-S recombinants obtained for pG3.01s is significantly different from that obtained for pG3.1. *P* values were derived by  $\chi^2$  analysis (see Materials and Methods).



**Figure 7.** Deletion of the transcriptional elements, EP and I $\alpha$ , does not affect recombination frequency on the switch plasmids. A schematic of the pG3.1 and p273 and their respective deletion constructs in which EP (pG3.ΔEP, p208) or I $\alpha$  (pG3.ΔI $\alpha$ , p200) or EP+I $\alpha$  (pG3.ΔI $\alpha$ ΔEP, p218) have been removed is shown on the left. DC-PCR analysis of DNA from nuclei derived from 1B4.B6.10 cells transfected with pG3.1 or deletion constructs and I.29 $\mu$  cells transfected with p273 or deletion constructs is summarized on the right as indicated. Each of the plasmids was transfected in triplicate in at least three independent experiments and a representative experiment is shown. The RRLs for the parental plasmids pG3.1 and p273 are arbitrarily set at 1.0. The fold induction indicates the RRL of the deletion constructs compared with pG3.1 or p273. The histograms are aligned with the appropriate plasmid structure.

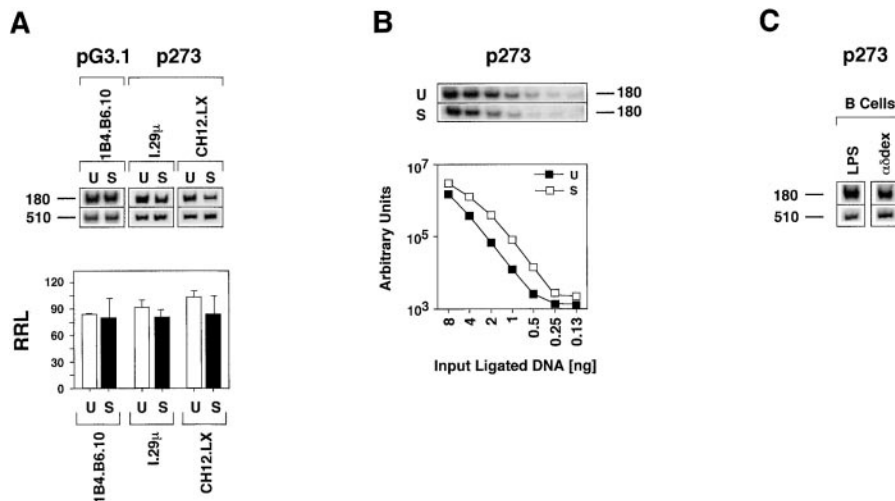
*Switching Factors Are Constitutively Expressed in Cell Lines Capable of SR.* SR at endogenous loci in the B cell lines I.29 $\mu$ , CH12.LX, and 1B4.B6 is dependent on cytokine and/or mitogen stimulation (40, 41, 61, 62). While the role of cytokines that specifically induce *gt* expression have been well defined, the function of mitogen activation is unknown. The fact that the switch substrates recombine in unstimulated B cell lines suggests that some of the factors

mediating SR are constitutively expressed. To determine if stimulation would increase recombination of the switch substrates, the RRLs for pG3.1 in 1B4.B6.10 cells, and p273 in 1B4.B6, I.29 $\mu$ , and CH12.LX cells, in both unstimulated and stimulated culture conditions were evaluated (Fig. 8 A). No significant difference in recombination levels was observed. To verify these results, a twofold serial dilution of ligated DNA taken from the DC-PCR analysis of p273 transfected into stimulated and unstimulated I.29 $\mu$  cells (Fig. 8 A) was further analyzed by DC-PCR (Fig. 8 B). The RRLs for unstimulated and stimulated cells are nearly identical, demonstrating the constitutive expression of switching factors detected by the switch substrates.

These results prompted us to ask whether splenic B cells induced to proliferate but not to undergo SR also express switching factors. To test this, splenic B cells were activated with  $\alpha$ dex, which causes B cell proliferation but not SR (63), and with LPS, which causes both proliferation and SR of endogenous loci. Activated B cells were transfected with p273 and analyzed for SR on the plasmid by DC-PCR. No difference was found between the levels of recombination induced by LPS or  $\alpha$ dex (Fig. 8 C). These findings demonstrate that plasmid-based SR activity is detectable in proliferating splenic B cells that are not undergoing SR at endogenous loci, and that all the factors required for exogenous SR reaction are expressed in these cells.

## Discussion

These studies demonstrate that a transiently transfected plasmid can specifically assay SR. B cell lines and splenic B cells capable of undergoing SR on their endogenous chromosomes support S-S recombination on the plasmid, whereas B cells that do not undergo endogenous SR do not support plasmid SR. The switch plasmids were ana-



**Figure 8.** Mitogen stimulation of cell lines has no effect on switch substrate recombination. (A) DC-PCR analysis of DNA from pG3.1- or p273-transfected cell lines, which were unstimulated (U) or stimulated (S) for 48 h (top). I.29 $\mu$  was stimulated with LPS and TGF- $\beta$ 1, CH12.LX with CD40L and IL-4 as described (references 40, 61), and 1B4.B6 with CD40L and LPS (our unpublished data). The average RRL and SD of four transfections in two independent experiments for each cell line is shown (bottom). (B) DC-PCR analysis of the 180-bp fragment from twofold serial dilutions of ligated DNA derived from nuclei of I.29 $\mu$  cells transfected with p273 and grown in unstimulated (U) and stimulated (S) conditions (top). Quantitation of the 180-bp fragment resulting from DC-PCR analysis of serial dilutions of ligated DNA (bottom). (C) DC-PCR analysis of Hirt extracts from normal splenic B cells activated with LPS or  $\alpha$ dex transfected with p273, as indicated.

lyzed by direct PCR for S-S recombinant molecules 2 d after transfection, revealing a heterogeneous population of substrate-derived S-S composite molecules in switching B cell lines. A semiquantitative DC-PCR assay confirmed the presence of S-S composite fragments, demonstrating that the level of SR activity in switching B cell lines is >80–200-fold in nonswitching B cell lines. This observation was confirmed in bacterial transformation studies in which S-S recombinant plasmids were detected after transfection into switching cells but not in other cell lines. Analysis of plasmid-derived S-S junctions isolated from stable transfectants (33) or recovered by bacterial transformation after transient transfection (Fig. 6) demonstrated direct S-S recombination. Finally, the specificity of the plasmid-based SR was demonstrated by the finding that SR required the presence of two S regions but was undetectable when one S region was deleted.

The  $\mu \rightarrow \alpha$  switching activity detected by the switch plasmid assay is found only in B cell lines capable of endogenous  $\mu \rightarrow \alpha$  SR, suggesting that this activity is not widely expressed in B cells. Comparison of the recombination potential of switch substrates specific for  $\mu \rightarrow \alpha$  and for  $\mu \rightarrow \gamma 3$  SR demonstrated the presence of two distinct switching activities. The  $\mu \rightarrow \gamma 3$  switching activity was observed in the single B cell line capable of endogenous  $\mu \rightarrow \gamma 3$  SR and in mitogen-activated normal B cells, but not in cell lines capable of only  $\mu \rightarrow \alpha$  SR, suggesting that the  $\mu \rightarrow \gamma 3$  switching activity is distinct from the  $\mu \rightarrow \alpha$  activity. It will be of significant interest to determine whether individual switching activities exist for each type of S DNA. It is also possible that switching activity detected by the switch plasmids results from the absence of a suppressor activity rather than a positive regulator. In this scenario, isotype- or class-specific suppressors regulate recombination and the selective expression of the suppressors determines recombination potential for individual S regions. In either case, these findings demonstrate the existence of transacting factors that influence the specificity of SR-independent of gt expression. Our studies clearly establish that ubiquitous recombination factors cannot be exclusively responsible for SR.

The hypothesis that DNA replication may be a requirement for SR was based on the observations that after mitogen stimulation several cell cycle divisions occur before isotype switching is detectable in B cells (56–59) and that DNA replication is required for V(D)J recombination on episomal plasmid substrates (64, 65). Consequently, polyoma origins of replication were included in switch plasmids previously constructed (24, 29). Our switch substrates lack a eukaryotic origin of replication. We find SR on our switch plasmids recovered in Hirt extracts, demonstrating that recombination can occur independent of high level DNA replication. However, it is currently unclear whether an origin of replication on the switch plasmids would augment SR. Moreover, our findings do not preclude the possibility that error-prone DNA synthesis occurs during the switching process at the site of recombination between the  $S_{\mu}$  donor and the downstream S DNA acceptor, as previously suggested (9, 66).

The expression of gts is always associated with SR *in vivo* (67). Studies in which the I region-associated promoter has been disrupted by targeted recombination indicate that transcription is necessary for SR to ensue (12, 15, 49). In addition, the splice donor located at the 3' end of the I region must be intact for SR to occur (68, 69). Our switch plasmids contain transcriptional elements upstream of the S regions but do not contain RNA splice donors, normally found upstream of each S region in the endogenous genes. Furthermore, removal of the EP and  $I_{\alpha}$  elements did not have a measurable effect on plasmid-associated SR, although deletion of EP and  $I_{\alpha}$  greatly reduces the level of transcripts detectable from the plasmid  $S_{\mu}$  and  $S_{\alpha}$  segments, respectively (33). These data suggest that transcriptional elements, a splice donor, and high level transcription are not intrinsic to the mechanism of SR. It is possible that expression of gts is a requirement for SR associated with endogenous genes that are under the additional constraints of chromatin.

An unanticipated finding was that switching factors that mediate plasmid-based SR are constitutively expressed in cell lines capable of SR at endogenous genes. This finding was also suggested when the p273 switch substrate was studied in stable transfection experiments (33). This is surprising, as these B cell lines require mitogen activation to initiate SR at endogenous loci (40, 41, 61, 62). These results imply that mitogen activation of the B cell lines induces factors that are distinct from the constitutively expressed activities detected by the switch substrates. These factors may regulate access of switching factors to the endogenous loci through modulation of chromatin configuration at S DNA. Our studies and the results of Kinoshita and co-workers (32) suggest that recombination is suppressed at endogenous loci in SR-proficient cell lines and is regulated by both mitogen-inducible factors and gt expression. Precedence for the involvement of chromatin in the regulation of recombination has been established in V(D)J joining. The recombination activating gene (RAG)-1 and RAG-2 proteins, which constitute the V(D)J recombinase, are constitutively expressed in B cells during early development, but specific chromatin changes are required to allow recombinase accessibility to the endogenous loci and to induce cleavage of DNA (70).

Using direct S-S PCR and DC-PCR assays, we have demonstrated that recombination on the switch plasmids occurs only in B cell lines capable of switching their endogenous genes (Table I). Previously reported switch plasmids recombined in many B cell lines that do not undergo SR of their endogenous genes and in some T cell lines (22, 25, 29). The discrepancy between these earlier findings and the results presented here may arise from several factors. Previous transiently transfected switch substrates contained polyoma origins of replication that are absent from the switch substrates reported here. These earlier studies relied on loss of a selectable marker to score for plasmid-associated SR. False positives could arise from deletion or partial deletion of this marker by recombination events other than SR. Our analysis of rearranged plasmid structures recov-

ered by bacterial transformation of genomic DNA from transfected cells indicates that in all cell lines tested, from 0.014 to 0.25% of the plasmids contain deletions of the entire nonvector segment of p273 (Table II). The instability of the switch plasmids may arise from the presence of tandemly repetitive DNA sequence in the S regions that is recombinogenic through homologous recombination pathways. Our PCR-based SR assays are focused on the detection of S-S recombinant fragments and thus filter out other unrelated recombination events. Finally, it may be most significant that B cell lines that are capable of endogenous SR were not previously tested with isotype-matched switch plasmids. Thus, the switching activity found with earlier switch plasmid assays may have resulted from low level expression of the switching factors in B cells. This low expression level may be just marginally above background and could account for the difficulty in separating bona fide switching events from other recombination events that affect the switch plasmids.

There are similarities between SR and V(D)J joining. Like SR, V(D)J joining occurs by an intrachromosomal deletion process (71, 72). SR is also similar to V(D)J joining in that DSBs appear to be intermediates in the recombination reaction (5, 73, 74) and both processes are dependent on the DSB repair protein complex, DNA-PK (6–8, 75–77). There are also striking differences between V(D)J joining and SR. RAG-1 and RAG-2, the enzymes that initiate V(D)J joining, are not involved in SR (78). Furthermore, V(D)J joining occurs between short, well-defined recombination signal sequences (79), whereas S regions are composed of arrays of tandem repeats that differ in length and sequence (49). Although the precise signals that mediate SR are unknown, the presence of DSBs in S DNA that is undergoing SR (5) suggest that DNA cleavage is the first step in the SR reaction. It is possible that the switching factors detected by our plasmid assay are involved in cleaving S DNA to form DSBs and to initiate SR. Further investigation is required to characterize the precise functions carried out by the switching activities detected by these assays.

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