

# Influence of switch region length on immunoglobulin class switch recombination

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**The class and effector functions of antibodies are modulated through the process of Ig heavy chain class switch recombination (CSR). CSR occurs between switch (S) regions that lie upstream of the various Ig heavy chain constant region exons. Molecular analyses of S-region functions have been hampered by their large size and repetitive nature. To test potential relationships between S-region size and efficiency of CSR, we generated normal B lymphocytes in which the 12-kb S region flanking the C $\gamma$ 1 exons (S $\gamma$ 1) was replaced with synthetic or endogenous S regions of various lengths. Replacement of S $\gamma$ 1 with 1- and 2-kb synthetic sequences representing the S $\gamma$ 1 core repeats or a 4-kb portion of the core endogenous S $\gamma$ 1 region supported CSR frequencies that directly correlated with S-region length. These findings indicate that S-region size is an important factor in determining endogenous CSR efficiency. Moreover, these results also will allow the development of a systematic system to test the function of various S-region motifs by replacing endogenous S regions with synthetic S regions controlled for size effects.**

IgH switch region | activation-induced deaminase | synthetic switch region | IgG1

Immunoglobulin (Ig) genes undergo several genetic alterations during the course of B lymphocyte development and activation. During early stages of mammalian B cell development, Ig heavy (IgH) and light (IgL) chain variable region exons are generated from germ-line V, D, and J gene segments via V(D)J recombination (1). When B cells are activated in the context of an immune response, the IgH and IgL variable region exons acquire point mutations through a process known as somatic hypermutation (SHM). SHM allows selection of mutated B cell clones that produce higher-affinity antibodies (2). In addition, the constant region (C<sub>H</sub>) of the IgH chain can be changed through the process of IgH class switch recombination (CSR) (3). CSR is a deletional recombination reaction that allows the C $\mu$ , the first C<sub>H</sub> "gene" expressed during development, to be replaced with one of a number of downstream C<sub>H</sub> genes. In mouse, individual C<sub>H</sub> genes are organized in the order 5'-VDJ-C $\mu$ -C $\delta$ -C $\gamma$ 3-C $\gamma$ 1-C $\gamma$ 2b-C $\gamma$ 2a-C $\epsilon$ -C $\alpha$ -3'. Each germ-line C<sub>H</sub> gene that undergoes CSR is preceded by a long, repetitive target sequence for CSR termed a switch (S) region. CSR results from recombination between the most 5' S region (S $\mu$ ), which lies upstream of the C $\mu$  gene, and one of the downstream S regions (e.g., S $\gamma$ 3, S $\gamma$ 1, etc.).

Individual C<sub>H</sub> genes are organized into germ-line transcription units that consist of a transcriptional promoter, a noncoding I exon, the S region, and the C<sub>H</sub> coding exons. S regions span most of the intron between I exons and the corresponding C<sub>H</sub> gene. Individual S regions range in size from  $\approx$ 10 kb (S $\gamma$ 1) to only 1 kb (S $\epsilon$ ), and are characterized by highly repetitive ("core") sequences. The core repeats of S $\mu$  (4.0 kb) (4), S $\epsilon$  (1.0 kb) (5), and S $\alpha$  (4.2 kb) (6) are homologous to each other and rich in GGGGT, GAGCT motifs. The core S regions of S $\gamma$ 3 (2.5 kb) (7), S $\gamma$ 1 (8 kb) (8), S $\gamma$ 2b (5.0 kb) (9), and S $\gamma$ 2a (2.5 kb) (5) contain a characteristic 49-bp repeat that shares some of the repetitive elements (e.g., AGCT motifs) found in the S $\mu$ , S $\epsilon$ , and S $\alpha$  sequences. Although the four S $\gamma$  regions are

related by overall homology of consensus repeats, there also are consistent differences among individual S regions (8) that have been suggested to provide some specificity in directing CSR (10). Intronic sequences external to the core repeats also contain similar repetitive elements interspersed with other sequences. Gene-targeted mutational studies have demonstrated that S regions are specialized targets for CSR. Thus, deletion of the 5' donor S $\mu$  greatly diminishes CSR (11, 12), whereas deletion of S $\gamma$ 1, which is a 3' acceptor, essentially abolishes CSR to C $\gamma$ 1 (13). Moreover, replacement of S $\gamma$ 1 with a 4-kb *Xenopus* S $\mu$  region, which contains a region of AGCT repeats similar to those found in mammalian S regions, allowed substantial CSR. In contrast, replacement of S $\gamma$ 1 with a 4-kb intronic sequence from *Xpf* gene, which lacks abundant AGCT motifs, did not support high-level CSR (14).

CSR absolutely requires the activation-induced deaminase (AID), a single-stranded-DNA-specific cytidine deaminase with no activity on double-stranded DNA (15, 16). AID initiates both CSR and SHM by deaminating cytidine residues in target S region or variable region DNA (3). Germ-line transcription through S regions also is required for CSR (17). Such germ-line C<sub>H</sub> gene transcription runs through the S region and is terminated downstream of the corresponding C<sub>H</sub> exons. Germ-line C<sub>H</sub> transcription of a given C<sub>H</sub> gene directly correlates with CSR potential of that C<sub>H</sub> gene and, thereby, serves to direct the CSR process. Transcription through S regions may contribute to generating a DNA substrate for AID in two manners. First, mammalian S regions are GC-rich and G-rich on their nontemplate strand, a property that allows them to generate single-stranded DNA in the form of R loops when transcribed (18, 19). Such R loops are effective AID substrates *in vitro* (20, 21). Second, in activated B cells, AID is phosphorylated, allowing it to interact with replication protein A (RPA) (22). *In vitro*, AID/RPA complex preferentially binds to and deaminates sequences, such as variable regions and S regions, which are rich in AGCT and related motifs. Together, these two properties, the ability to form R-loops and a high density of AGCT and related motifs, may help render transcribed S regions effective targets for AID in activated B cells.

Despite recent advances in our understanding of the role of AID and transcription in CSR, there are still many unknown issues related to potential S-region functions. For example, as mentioned above, S regions vary substantially in size, raising the possibility that size may increase the potential for AID targeting and contribute to effectiveness as a CSR substrate. In this regard, a synthetic 1-kb, G/C-rich, R-loop-forming sequence that lacks repetitive S-region motifs, provided  $\approx$ 10% of the activity of S $\gamma$ 1 when inserted in place of the 12-kb S $\gamma$ 1 region in normal B cells (13). In these experiments, it was not clear whether reduced CSR activity of this synthetic sequence reflected either the lack of important elements in S $\gamma$ 1 or the short length of the synthetic sequence (or both). With respect to S region length, it is notable

Abbreviations: AID, activation-induced deaminase; CSR, class switch recombination; ES, embryonic stem; IgH, Ig heavy chain; S, switch.

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that mouse IgG1, which derives from CSR to the very long S $\gamma$ 1 region, constitutes the highest level of IgG subtypes in the serum (23). In addition, CSR to IgG1 predominates in B cells activated *in vitro* in the presence of IL-4, even though both C $\gamma$ 1 and C $\epsilon$  germ-line transcription is induced by treatments involving IL-4 (24). Although the high level of C $\gamma$ 1 CSR might be attributed to various causes, it is possible that the large size of S $\gamma$ 1 region may be an important factor. Consistent with this possibility, increasing the number of S $\gamma$ 3 consensus repeats from 1 to 6 in a transient CSR reporter substrate resulted in increased levels of recombination (25).

To further elucidate potential roles of S-region size with respect to targeting CSR and to develop a system to systematically assess the role of various S-region motifs in CSR, we now have replaced the endogenous S $\gamma$ 1 regions with S $\gamma$ 1 core sequences of various lengths and assayed their ability to support CSR *in vivo*. We find that, at least up to 4 kb, the length of S region directly influences CSR efficiency *in vivo*. We discuss the implications of this finding for the mechanism of CSR and also with respect to implications for the development of a systematic approach to assess functions of S-region motifs *in vivo* by using synthetic S regions that are controlled for size effects.

## Materials and Methods

**Generation of Synthetic S $\gamma$ 1 Regions.** S $\gamma$ 1 consensus (8) oligonucleotides 5'-GATCC GGG AGC CAG GAC AGG TGG AAG TGT GGT GAC CCA GGC AGA GCA GCT CCA GG-3' and 5'-GATCT CTG GAG CTG CTG CTC TGC CTG GGT CAG GAC ACT TCC ACC TGT CCT GGC TCC CG-3' were phosphorylated by T4 polynucleotide kinase (New England Biolabs). Restriction sites outside of the S $\gamma$ 1 repeat consensus are italicized. The phosphorylated oligonucleotides were annealed and ligated into BamHI site of S85 plasmid. Subsequent ligations of the same oligonucleotide resulted in the unidirectional cloning of desired length confirmed by sequencing analyses. A 6-kb S $\gamma$ 1 fragment containing the core S $\gamma$ 1 repeat was cloned into the SmaI site of S85 vector. The 5' end of this insert corresponds to S $\gamma$ 1 site at position 4,130 of the genomic sequence under GenBank accession no. D78344. To obtain a 4-kb fragment, this fragment was subject to exonuclease treatment from the 3' end.

**Generation of Targeting Vectors and Gene Targeting.** Synthetic (1- or 2-kb) and 4-kb WT S $\gamma$ 1 repeat regions were cloned into *NotI* and *SalI* sites of the targeting vector reported in ref. 14 (Fig. 2). These constructs were transfected into  $\Delta$ S $\gamma$ 1 embryonic stem (ES) cells in which the S $\gamma$ 1<sup>a</sup> region was deleted (13). Correctly targeted clones were identified by Southern analyses. The configurations of the targeted alleles were further confirmed by *PstI* digest with internal I $\gamma$ 1 probe. The deletion of the *neo*<sup>r</sup> gene and subsequent inversion of the inserts were achieved by using adenovirus Cre-expressing vector. The ES cells were injected into RAG-2-deficient blastocysts to obtain chimeric mice with mature lymphocytes. Animal work was approved by the Institutional Animal Care and Use Committee of Children's Hospital.

**Assays for Class Switching.** All assays are described in detail in refs. 13 and 14. Briefly, splenocytes were stimulated with anti-CD40 and IL-4 for 4 days to generate hybridomas or for 6 days to perform ELISA. The monoclonal anti-IgG1<sup>a</sup> (Igh-4a, Pharmin-gen) was used to detect IgG1<sup>a</sup> (mutated alleles). Total IgG1 was measured by polyclonal anti-IgG1 (Southern Biotechnology Associates).

## Results

**Generation of Synthetic S $\gamma$ 1 Sequences.** To elucidate S-region functions, we developed a cloning strategy to generate synthetic S regions bearing repeat motifs similar to those of endogenous

regions. In BALB/c mice, the core S $\gamma$ 1 sequence in the intron between I $\gamma$ 1 and C $\gamma$ 1 consists of 120 tandem 49-bp repeats and is, thereby,  $\approx$ 6 kb in length (8). In 129/Sv mice, the core S $\gamma$ 1 repeat region is 2 kb larger (8 kb) than the core BALB/c S $\gamma$ 1 (data not shown). The longest tandem repeat in the central part of S $\gamma$ 1 consists of 79 repeat elements that show extensive (93.4%) homology to the consensus S $\gamma$ 1 49-mer (8). To reproduce this structure, we generated 20-mer or 40-mer repetitive synthetic S regions by concatenating a consensus S $\gamma$ 1 oligonucleotide that was flanked by *Bam*HI and *Bgl*II restriction enzyme sites. Based on alignment of 540 bp of the synthetic S $\gamma$ 1 with the endogenous S $\gamma$ 1, the synthetic S $\gamma$ 1 closely resembles the organization of endogenous S $\gamma$ 1 repeats, both with respect to overall nucleotide content and to repeat structure and organization (Fig. 1). Also, similar to the endogenous S regions, all synthetic repeats are unidirectional. The only difference between synthetic and endogenous S $\gamma$ 1 repeats is the presence of a 6-bp linker (AGATCC) between the 49-bp repeat units.

## Replacement of Endogenous S $\gamma$ 1 with Core S $\gamma$ 1 Repeats of Different Length.

To determine whether S-region length influences CSR frequency, we used gene-targeted mutation methods to replace the endogenous S $\gamma$ 1<sup>a</sup> allele in an F<sub>1</sub> ES cell line with 1 or 2 kb of synthetic S $\gamma$ 1 repeats, as well as 4-kb S $\gamma$ 1 of the endogenous S $\gamma$ 1 core repeat region. The F<sub>1</sub> ES cell was generated from the 129Sv-C57BL/6 mice in which the two *Igh* alleles represent the IgH<sup>a</sup> (from 129/Sv) or IgH<sup>b</sup> (from C57BL/6) allotypes, respectively (13, 26). The presence of allotypic markers and sequence polymorphism facilitates analyses of mutant mice. We have previously shown that the deletion of S $\gamma$ 1 region abolishes CSR to C $\gamma$ 1 (13, 14). We used our previous strategy (14) to target the different substitute S regions into the IgH<sup>a</sup> allele of an F<sub>1</sub> derivative in which the S $\gamma$ 1 region was deleted (referred to  $\Delta$ S $\gamma$ 1 allele) (Fig. 2). Subsequently, the inserted *neo*<sup>r</sup> gene was removed by Cre-mediated recombination through the flanking loxP sites. To analyze the effect of transcriptional orientation, the two loxP sites flanking the insert were placed in inverted orientation, allowing Cre-mediated recombination to invert the intervening sequence (Fig. 2). The size and integrity of the inserted synthetic S regions were confirmed by Southern blot analyses with a synthetic S $\gamma$ 1 probe (Fig. 6A, which is published as supporting information on the PNAS web site). The strong sequence homology between synthetic and endogenous S $\gamma$ 1 was evident by Southern blot, because under high stringency wash conditions, the synthetic S $\gamma$ 1 probe remains bound to the endogenous S $\gamma$ 1 (Fig. 6B).

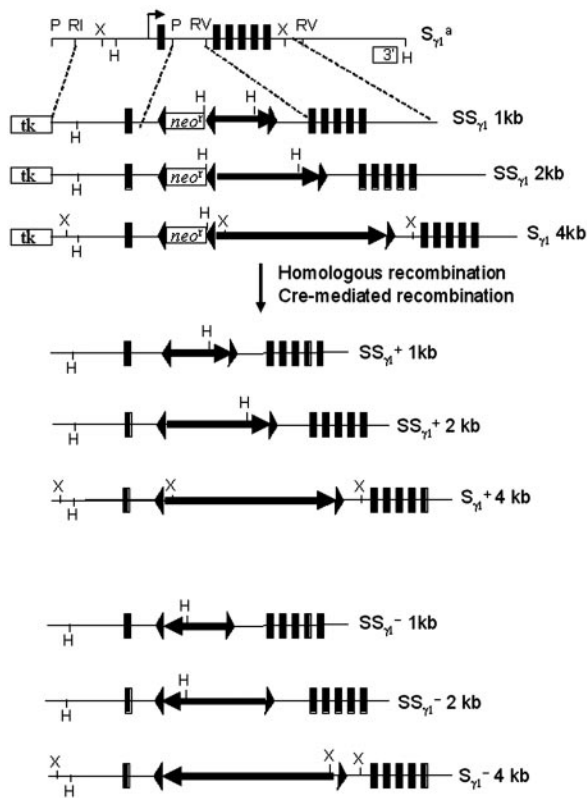
## Generation of B Lymphocytes with S $\gamma$ 1 Core Sequences of Various Lengths.

The S $\gamma$ 1-replaced mutant F<sub>1</sub> ES cells were injected into recombination activating gene 2 (RAG2)-deficient blastocysts to generate chimeric mice to obtain mature B lymphocytes harboring the targeted mutation (27). Splenocytes from mutant or control animals were activated with antibody against CD40 (anti-CD40) plus IL-4, a treatment that induces germ-line transcription of the C $\gamma$ 1 gene and targets CSR to the S $\gamma$ 1 region. Based on ELISA of culture supernatant after 6 days of treatment, we found overall IgG1 secretion in the various splenocyte cultures to all fall within the expected range with cells harboring the  $\Delta$ S $\gamma$ 1 allele falling on the lowest end of the range (data not shown); we interpret this finding to indicate that all cultures were properly activated for CSR.

Because germ-line transcription is an important component of CSR, we assessed whether the targeted alleles were normally transcribed in B cells after anti-CD40 plus IL-4 treatment. For this purpose, we measured the relative levels of the steady-state germ-line transcripts from the WT  $\gamma$ 1<sup>b</sup> and targeted  $\gamma$ 1<sup>a</sup> alleles by reverse-transcription-mediated PCR (RT-PCR) (Fig. 3) (13, 14). Because the cultures were all similarly activated, the WT  $\gamma$ 1<sup>b</sup>





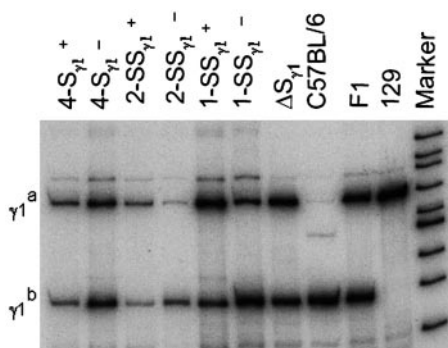


**Fig. 2.** Targeting of the  $S\gamma 1^a$  allele. Genomic organization of  $\Delta S\gamma 1$  is shown at the top. The design of targeting constructs is illustrated underneath. The structure of the targeted allele after Cre/loxP recombination is illustrated at the bottom.  $I\gamma 1$  and  $C\gamma 1$  are shown as rectangular black boxes. Other symbols are as follows: tk, gene encoding thymidine kinase; arrows, WT or targeted S regions with arrow direction indicating the physiological transcriptional orientation; triangles, loxP site; R1, *EcoRI*; RV, *EcoRV*; H, *HindIII*; P, *PstI*; X, *XbaI*; 1-SS $\gamma 1$ , 1-kb synthetic S $\gamma 1$ ; 2-SS $\gamma 1$ , 2-kb synthetic S $\gamma 1$ ; 4-S $\gamma 1$ , 4-kb endogenous S $\gamma 1$ ; 8-S $\gamma 1$ , 8-kb endogenous S $\gamma 1$ ; +, physiologic orientation; -, inverted transcriptional orientation.

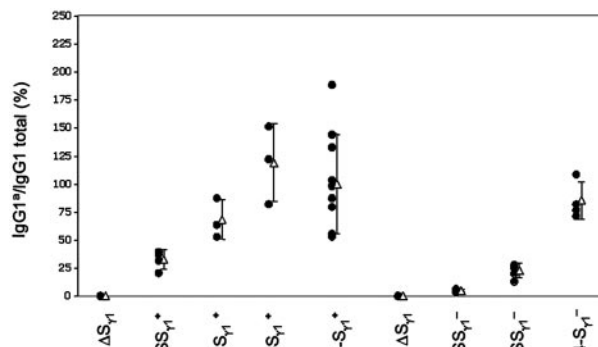
fully elucidated, although a recent study found little impact of a 6-fold reduction in germ-line transcription of  $\gamma 1$  locus on CSR (28).

**Discussion**

In this study, we have demonstrated the feasibility of replacing endogenous S regions with synthetic sequences to assess, *in vivo*, parameters associated with S-region function. Based on this ap-



**Fig. 3.** RT-PCR of germ-line  $\gamma 1$  transcripts. Germ-line transcripts were amplified by RT-PCR with  $I\gamma 1$  and  $C\gamma 1$  primers and subsequently subjected to primer extension. Representative data from a minimum of two separate stimulations employing cells from independent chimeras are shown.



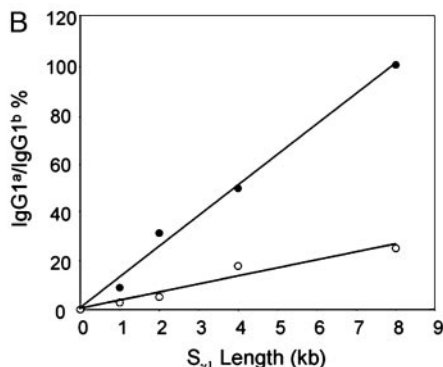
**Fig. 4.** Class-switching in mutant B cells. The ratio of IgG1<sup>H</sup>/IgG1 total of the WT mice is set arbitrarily as 100% for the IgH<sup>B</sup> allele. Each dot represents the average of at least two measurements of one mouse. Error bars represent standard deviation of the mean (open triangles). We have presented the endogenous S $\gamma 1$  region as 8 kb (8-S $\gamma 1^+$ ), which reflects the core repetitive segment of the intronic region.

proach, we have, for the first time, assessed the effect of the size of an S region on CSR efficiency *in vivo*. By replacing the endogenous S $\gamma 1$  sequence with “core” synthetic or endogenous S-region sequences of various sizes, we have found a clear relationship between the size of the core S region at the endogenous C $\gamma 1$  locus and efficiency of CSR. To directly measure the significance of the apparent relationship between core S $\gamma 1$  size and CSR efficiency, we performed a statistical analysis of CSR with respect to the CSR efficiency of the 1-, 2-, and 4-kb core replacement alleles and the normal S $\gamma 1$  S region. For this comparison, we considered the endogenous S $\gamma 1^a$  allele as 8 kb in length, because this is the size of its core 49-bp repeat region within the 12-kb  $I\gamma 1$ -C $\gamma 1$  intron. Our statistical analysis demonstrated that, at least for S $\gamma 1$ , there is a direct correlation between the length of the region of core S $\gamma 1$  49-bp repeats and the level of CSR that it supports (Fig. 5B). Therefore, the size of the core repeat S $\gamma 1$  region directly influences the ability to support CSR. In light of this correlation, the robust CSR to IgG1 and high serum levels of IgG1 in mice may, at least in part, be attributed to the exceptionally long endogenous S $\gamma 1$ .

Core S region length may play several different roles in influencing CSR. In this regard, S regions may target AID activity and CSR through several mechanisms. One apparent function of mammalian S regions is ability to form R loops when transcribed in physiological orientation because of the G-rich composition of their nontemplate strands. The length of R-loops formed within endogenous S regions has been shown to exceed 1 kb (19); therefore, longer S regions conceivably could contribute to increased CSR by generating more extensive R-loops. However, we also see a direct correlation of CSR frequency with length of inverted S $\gamma 1$  regions, which do not detectably form R-loops and likely target CSR by a non-R-loop mechanism (13, 14). We have previously argued that the ability of the 4-kb *Xenopus S $\mu$ , which does not generate detectable R-loops, to support CSR in murine B cells is associated with its high number and density of AGCT motifs (14). Likewise, the presence of substantial numbers of AGCT and related motifs might account for the ability of the inverted endogenous S $\gamma 1$  sequence to promote CSR. In this context, increasing the number of such motifs by increasing the length of S-region core sequence might increase CSR potential by providing more substrates for the AID/replication protein A complex.*

Although the present study was directed primarily at assessing the role of S-region length in CSR, it provides additional insights that may help interpret some unexplained differences in levels of CSR with respect to test sequences used in our prior replacement experiments (13, 14). A 1-kb random G-rich sequence, which forms R-loops but is devoid of standard S-region repeats and

Genotype	IgG1 <sup>a</sup> /IgG1 <sup>b</sup>	CSR (%)
$\Delta S_{\gamma 1}$	0/160	0.0
1-SS <sub><math>\gamma 1</math></sub> <sup>+</sup>	16/112	8.9
2-SS <sub><math>\gamma 1</math></sub> <sup>+</sup>	38/81	31.2
4-S <sub><math>\gamma 1</math></sub> <sup>+</sup>	58/78	49.5
8-S <sub><math>\gamma 1</math></sub> <sup>+</sup>	63/42	100
1-SS <sub><math>\gamma 1</math></sub> <sup>-</sup>	4/92	2.8
2-SS <sub><math>\gamma 1</math></sub> <sup>-</sup>	7/91	5.1
4-S <sub><math>\gamma 1</math></sub> <sup>-</sup>	15/56	17.8
8-S <sub><math>\gamma 1</math></sub> <sup>-</sup>	34/105	25



**Fig. 5.** Linear correlation of S-region length and CSR frequency. (A) Ratio of IgG1<sup>a</sup>/IgG1<sup>b</sup>-producing hybridomas. The numbers of IgG1<sup>a</sup>- or IgG1<sup>b</sup>-producing hybridomas for each genotype are indicated. Relative CSR frequency is defined by the ratio of IgG1<sup>a</sup>- to IgG1<sup>b</sup>-producing hybridomas and is arbitrarily set as 100% for F<sub>1</sub> cells. WT S <sub>$\gamma 1$</sub>  is presented as 8-S <sub>$\gamma 1$</sub> <sup>+</sup> to illustrate size comparison. The data for inverted WT 8-S <sub>$\gamma 1$</sub> <sup>-</sup> were adopted from our previous study. (B) Linear correlation of S-region length and CSR frequency. The data for inverted WT S <sub>$\gamma 1$</sub>  (8-S <sub>$\gamma 1$</sub> ) were incorporated from our previous study (13). Data from hybridoma analyses (A) of 1, 2, and 4 kb and WT (8 kb) were used for statistical analyses.  $R^2 = 0.992$  for physiologic orientation, and  $R^2 = 0.9451$  for inverted orientation. Filled circles, physiologic orientation; open circles, inverted orientation.

motifs, was able to support CSR to a comparable level to the 1-kb synthetic S region (7% vs. 9%) analyzed in this study (13). This finding is consistent with the notion that R-loop formation can

contribute substantially to CSR activity of S regions. On the other hand, in inverted orientation, the CSR efficiency of the random G-rich sequence was reduced to background levels (13); whereas the inverted 1-kb S region still supported reduced, but readily detectable, levels of CSR. As the nucleotide composition of the random G-rich and synthetic S <sub>$\gamma 1$</sub>  sequences are comparable, the most obvious, major difference between the two is that the synthetic S <sub>$\gamma 1$</sub>  region contains a much higher number of AGCT motifs. Therefore, we would argue that the high density of AGCT motifs may be responsible for the CSR activity of the inverted S region. Similarly, the inverted 4-kb S <sub>$\gamma 1$</sub>  shows comparable CSR activity to the 4-kb *Xenopus* S region analyzed in ref. 14. Because the *Xenopus* S region, like the inverted endogenous S <sub>$\gamma 1$</sub>  region, does not make substantial levels of R-loops, the comparable activity of these two sequences might again be attributed to the abundance of AGCT motifs. Therefore, our current findings are consistent with the possibility that both R-loop formation and density of AGCT and related motifs contribute to CSR by providing targets for AID.

Our development of a system to analyze synthetic S <sub>$\gamma 1$</sub>  regions lays the groundwork for more defined analyses of the function of S regions and their component motifs. Previous analyses of S regions in CSR have relied on CSR recombination substrates, which do not always appear to reflect the normal mechanism and control of endogenous CSR. On the other hand, previous studies of endogenous S regions have necessarily viewed S regions as large DNA fragments, with no ability to readily dissect function of their component motifs. While R-loop-forming ability and the density of AGCT and related motifs may be major contributors to targeting CSR, Ig isotype-specific elements and structures [inverted repeats and stem-loop structures (29)], G4 quartets (30), and other factors (e.g., transcription levels, location, etc.) also have been argued to contribute to differential CSR targeting (25). By using synthetic S regions, controlled for size effects, we can now begin to systematically investigate the contribution of these different elements with respect to the function of individual S regions *in vivo*.

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