Sy3 Switch Sequences Function in Place of Endogenous Sy1 to Mediate Antibody Class Switching

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Sγ3 switch sequences function in place of endogenous Sγ1 to mediate antibody class switching

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Immunoglobulin heavy chain (IgH) class switch recombination (CSR) replaces the initially expressed IgH Cμ exons with a set of downstream IgH constant region (Cγ) exons. Individual sets of Cγ exons are flanked upstream by long (1–10-kb) repetitive switch (S) regions, with CSR involving a deletional recombination event between the donor Sμ region and a downstream S region. Targeting CSR to specific S regions might be mediated by S region–specific factors. To test the role of endogenous S region sequences in targeting specific CSR events, we generated mutant B cells in which the endogenous 10-kb Sγ1 region was replaced with wild-type (WT) or synthetic 2-kb Sγ3 sequences or a synthetic 2-kb Sγ1 sequence. We found that both the inserted endogenous and synthetic Sγ3 sequences functioned similarly to a size-matched synthetic Sγ1 sequence to mediate substantial CSR to IgG1 in mutant B cells activated under conditions that stimulate IgG1 switching in WT B cells. We conclude that Sγ3 can function similarly to Sγ1 in mediating endogenous CSR to IgG1. The approach that we have developed will facilitate assays for IgH isotype–specific functions of other endogenous S regions.
loci (9), correlating with the fact that IgG1, with the longest S region, is the most abundant IgH isotype. Most normal CSR junctions occur within and, occasionally, just beyond the S regions (10).

Individual C_H genes are organized into transcription units with transcription initiating from an intronic (I) promoter located upstream of each S region (11). In vivo, CSR is stimulated by T cell–dependent and independent antigens, which can be mimicked in vitro by activating B cells with anti-CD40 or bacterial LPS in the presence of cytokines such as IL-4 (1). Different activators and cytokine combinations appear to influence CSR to particular S regions by modulating germline transcription (11). Mechanistically, transcription through an S region may target CSR by generating optimal DNA substrates for AID. In this context, transcription through mammalian S regions, in association with their G-rich top strand, results in the formation of an R loop structure (7, 12, 13) that provides single-strand DNA that can serve as an AID substrate.

Various lines of evidence suggested that CSR to certain S regions (S3, S1, Se, and Sx) is mediated by S region–specific factors (15–24). In particular, plasmid-based switch substrates revealed several IgH isotype–specific CSR activities (18, 20). Notably, the recombination on particular switch plasmids (e.g., μ to α substrates) occurred only in lines that underwent CSR within the same endogenous S regions (e.g., μ to α but not μ to γ3). Comparison of switch substrates specific for μ to α and for μ to γ3 implicated Sy3- and Sax–specific CSR factors (18), and similar studies provided evidence for Sy1–specific CSR factors (20) (for review see reference 24). In addition, substrate studies showed that a single Sy3 or Sy1 consensus repeat (49 bp), respectively, supported specific μ to γ3 or μ to γ1 CSR, suggesting that IgH isotype specificity of CSR can be mediated by a single repeat unit (21). Point mutations of the Sy3 consensus repeat showed its activity to be dependent on the integrity of an NF-κB binding site (21, 22). In this regard, B cells deficient in the p50 subunit of NF-κB under certain conditions produce γ3 germline transcripts but are greatly impaired for switching from μ to γ3 (15, 19). The NF-κB p50 homodimer binds to specific motifs within the endogenous Sy3 (19, 21, 22), and mutation of these motifs within a synthetic Sy3 abolishes S region–specific CSR, supporting the notion that factor binding to these elements directs CSR to Sy3 (21). In contrast to transient CSR substrate studies, studies of stably integrated transcribed S region substrates suggested that individual

![Figure 1. Targeting and replacement of the Sy1a allele.](image-url)

(A) Genetic organization of Sy1 (top) and the design of targeting constructs (bottom) are shown. After gene targeting and Cre recombination, the neomycin (neo) gene will be deleted. Inverted loxP sites allow for changing the orientation of different sequences. I, Sy1a allele. (B) Southern blot analyses of genomic DNA digested with HindIII and hybridized with a 3′ probe. This probe on F1 ES cells detects 20- and 22-kb bands, which represent the endogenous γ1 locus from B6 and 129 alleles, respectively. A crosshybridizing band from the γ2b/γ2a regions is detected with the same probe because of strong sequence homology. The middle sample was a mix of two clones (2-SSy3) and was not used in the experiments. 2-SSy1 has been described previously (reference 9).
primary S region sequences may not play a critical role in directing CSR (25).

To generate a physiologically relevant mouse model to test for S region specificity of CSR, we measured the activity of WT or synthetic Sy3 sequences inserted in place of the endogenous Sy1. We find that sized-matched Sy3, synthetic Sy3, and synthetic Sy1 all mediate endogenous CSR, suggesting that the particular sequence of the S region is not a predominant factor in targeting endogenous CSR to IgG1.

RESULTS AND DISCUSSION

We used our previously established strategy to replace the endogenous 10-kb Sy1 region of a γ1+/γ1b F1 embryonic stem (ES) cell line with a 2-kb portion of endogenous Sy3 (2-Sy3), a 2-kb synthetic Sy3 (2-SSy3), and a 2-kb synthetic Sy1 (2-SSy1; Fig. 1) (7, 9, 13). For generation of the 2-SSy3 sequence, we used linkers to concatemerize 40 copies (~2 kb) of the Sy3 consensus sequence (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080451/DC1).

The orientation of tandem repeats in the SSy3 is unidirectional, therefore mimicking the repeat structure and nucleotide content of the endogenous Sy3 (26). The 2-Sy3 sequence comprises 1946 bp of the endogenous Sy3 region from nucleotides 646 to 2592 (available from GenBank/EMBL/DDJB under accession no. M12182) (26). This sequence has been previously shown to mediate recombination in transient assays and has been used to assay for S region–specific factors (18, 20).

The purpose of testing synthetic Sy1 and Sy3 substrates was to determine if this approach would allow endogenous CSR assays of substrates in which the only variables were the few nucleotide differences within each repeat unit and also to be able to test potential functions of candidate motifs within a given S region by generation of synthetic sequences with differing repeat structures.

The F1 ES cell was derived from the hybrid 129Syv-C57BL/6 mice in which the two IgH alleles represent the IgH (from 129/Syv) or IgHP (from C57BL/6) allotypes, respectively. The presence of sequence polymorphisms and allotypic markers (antibodies to IgG1) facilitates comparison of the level of CSR on modified alleles to the internal control of the unmodified IgHP allele. After successful gene targeting, the inserted neo cassette was removed by loxp/Crerecombination (Fig. 1). To analyze the effect of transcription orientation, the two loxP sites flanking the insert were placed in inverted orientation, allowing Cre-mediated recombination to invert the test sequences. Southern blot analyses were performed to confirm the correct integration of the replaced sequences (Fig. 1 B) (9).

Figure 2. Germline transcripts from the Sy1 replacement alleles. Germline transcripts were RT-PCR amplified via the ly1 and Cy1 primers and subsequently subjected to primer extension. The final products were digested with MboI restriction enzyme to distinguish between C57B6 and 129 alleles. Representative data from a minimum of two experiments are shown. The black line indicates that intervening lanes have been spliced out.

Figure 3. ELISA on anti–CD40/IL-4–stimulated splenocytes. The ratio of IgG1/IgG1 total of the WT–Sy1 mice is set as 100% for the IgG1b allele. Data from WT and 2-SSy1 were adopted from a previous study (reference 9). Physiological (+) and inverted (−) orientations of each sequence were obtained by Cre/loxP. Error bars represent the standard deviation of the mean (triangles).
alleles (Fig. 3). On the other hand, stimulation of 2-Sy3 spleenocytes for up to 6 d by treatment with LPS, conditions that normally induce germline Cy3 gene transcription and IgG3 CSR, did not result in any significant increase in IgG1 production in either WT or mutant B cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20080451/DC1), consistent with the fact that the germline γ1 gene is not transcribed under LPS stimulation conditions. To quantify CSR at a single-cell level, we generated hybridomas from activated B cells. Hybridomas represent fusions of individual B cells to the myeloma partner cell line. We selected IgG1-producing hybridomas by ELISA and compared the level of IgG1a with IgG1b to score for recombination efficiency between WT and mutated alleles. The IgH locus is subject to allelic exclusion, and only one of the two IgH alleles in a given B cell is recombined functionally into a V(D)J coding region. Therefore, in an F1 control, half of the activated B cells should produce IgH α allotype antibodies, and the other half should produce IgH β allotype antibodies. Relative CSR frequency is defined by the ratio of IgG1a to IgG1b-producing hybridomas and is arbitrarily set as 100% for F1 cells (see Materials and methods). The data for WT and 2-Ssy1 were adopted from our previous study (reference 9).

### Table I. Ratio of IgG1a/IgG1b in hybridomas

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<thead>
<tr>
<th>Genotype IgG1a/IgG1b</th>
<th>CSR (%)</th>
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<tr>
<td>WT-Syγ1</td>
<td>63:42</td>
</tr>
<tr>
<td>ΔSy1</td>
<td>0:160</td>
</tr>
<tr>
<td>2-SSy1+</td>
<td>38:81</td>
</tr>
<tr>
<td>2-SSy1−</td>
<td>16:112</td>
</tr>
<tr>
<td>2-SSy3+</td>
<td>43:94</td>
</tr>
<tr>
<td>2-SSy3−</td>
<td>12:96</td>
</tr>
<tr>
<td>2-Syγ1</td>
<td>52:72</td>
</tr>
<tr>
<td>2-Syγ3</td>
<td>6:30</td>
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The numbers of IgG1a or IgG1b are indicated. Relative CSR frequency is defined by the ratio of IgG1a to IgG1b-producing hybridomas and is arbitrarily set as 100% for F1 cells (see Materials and methods). The data for WT and 2-Ssy1 were adopted from our previous study (reference 9).

inserted S region sequence resulted in substantially decreased IgH class switching to IgG1 (Fig. 3 and Table I), potentially caused by decreased R loop formation in the inverse direction (7, 9, 13). Finally, we used a nested PCR approach to map Sμ to SSy3 junctions in IgG1c-producing hybridomas. Junctions occurred throughout the SSy3 repeat, similar to WT Sy3 (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20080451/DC1) (10). Although four out of eight junctions (50%) fell into a GAGCT motif surrounded by G nucleotides, further mutational studies on synthetic S regions would be required to identify the motifs that are preferentially targeted during CSR in vivo.

Given the central role of IgH isotype class switching in the humoral immunity, it is of significant interest to identify the mechanisms that contribute to the specificity of this process. Past studies have led to the view that S region-specific factors may be required for targeting CSR to Sy3 versus Sy1, and vice versa (24). In this report, we show that synthetic or WT Sy3, or size-matched Sy1 sequences, when inserted in place of the endogenous Sy1, can mediate roughly similar levels of CSR under B cell activation conditions in which CSR to the endogenous Sy1, but not the endogenous Sy3, is induced. We have previously shown that the *Xenopus* Sμ sequence, when substituted for the mouse Sy1 region, can mediate CSR at substantial levels, even though it is AT rich and lacks the ability to form an R loop structure in vitro (13) and in vivo (Leiber, M., personal communication). Thus, our previous *Xenopus* Sμ replacement study (13) complements our current findings; collectively, these studies strongly indicate that nucleotide sequence differences between Sy1 and other S regions are not likely to be major determinants of endogenous IgG1 CSR targeting. Correspondingly, our current findings support germline transcriptional activation of the Sy1 region as the primary mechanism for targeting CSR to IgG1 (28, 29). In this context, the finding that Sy3 sequences in place of Sy1 sequences do not support CSR to IgG1 under conditions (LPS activation) in which IgG3 CSR is induced would reflect the fact that LPS fails to induce germline transcription of the Cy1 gene promoter. Finally, we note that our approach now can be used to test for potential roles of putative S region–specific factors in mediating specific CSR events to other S regions under other stimulation conditions (21, 24).

### MATERIALS AND METHODS

**Targeting constructs.** To generate synthetic Sy3, the consensus Sy3 (5′-GGATCCGGAGGAGCTGGGTTAGATTGAGTGCGGCACTGCCGGACGTCTCCGATCTC-3′; BamHI and BglII sites are underlined; reference 26) was oligomerized by sequential cloning into the BamHI site of S85 vector to generate 2-SSy3 (Fig. S1). After each cloning step, the insert orientation was confirmed by sequencing and restriction endonuclease digestion. The consensus repeats were confirmed to be unidirectional. The 2-SSy3 sequence was excised as a NotI and Sall fragment and cloned into the targeting construct previously described (9). The endogenous Sy3 region was excised as a BamHI/NotI fragment from pS5V plasmid (provided by A. Kenter, University of Illinois at Chicago, Chicago, IL) and cloned into pBluescript. Subsequently, the NotI/Sall fragment was ligated into the targeting vector as previously described (9). 2-SSy1 has been previously described (9).
Gene targeting, generation of RAG chimeras, and mutant B cells.

The targeting constructs were transfected into ES cells in which the Syt1 was deleted (7). The targeted ES cells were identified by Southern blotting as described in Fig. 1 B (13). The deletion of the neo gene was achieved by infecting ES cells with cre-expressing adenovirus. Targeted ES cells were subcloned and injected into RAG2-deficient blastocysts to produce mature lymphocytes that all harbored the mutant allele (27). Splenic B cells from 6- to 8-wk-old chimeras were used in our experiments. Mouse protocols were approved by the Institutional Animal Care and Use Committee of Children’s Hospital.

Isotype switching assays. ELISA and hybridoma analysis were performed as previously described (13). Splenic cells from 6- to 8-wk-old chimeras were stimulated in vitro with 1 µg/ml anti-CD40 (HM40-3; BD Biosciences) plus 25 ng/ml IL-4, or 20 µg/ml LPS alone. 1.5 × 10⁷ cells were seeded in one well of a sixwell plate (0.5 × 10⁶ cells/ml) in RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin–streptomycin, and 100 µM β-mercaptoethanol. Stimulated B cells were used to generate hybridomas (after 4 d) or ELISA (after 6 d), as previously described (7, 13). Monoclonal anti-mouse IgG1 (Igh-4a; BD Biosciences) was used to detect IgG1 (from the mutated allele). Alkaline phosphotase–conjugated mouse anti-mouse IgG1 (SouthernBiotech) was used as the detection antibody. Purified mouse IgG1 (BD Biosciences) was used as the standard. Because an antibody specific for IgG1 b is not available, we normalized the production of IgG1 against IgG1 total for ELISA assays on splenic B cell stimulations. The ratio of IgG1/IgG1 total of the WT F1 chimeras was defined as 100% CSR efficiency for the WT γ1a allele. We measured the ratio of IgG1 to IgG1 for different chimeras. Relative CSR efficiency was calculated by the ratio of IgG1 a to IgG1 b–producing hybridomas. Hybridomas that produced only IgG1 and not IgG1 a were considered to produce IgG1 b. We defined the numbers of cells that switched γ1a on the α or β allele as γ1a and γ1b, respectively, and the numbers of total Igγ cells for the two alleles as Igγ and Igγ, respectively. The switching efficiency to γ1a was given as Sa = Sa/Sa + Sa = (γ1a/γ1a)/(γ1a/γ1a). The ratio of IgG1 a/γ1a was determined by the relative ratio of productive V(D)J recombination on the two alleles and was expected to be close to 1. Thus, Rα can be simplified as γ1a/γ1a. After mutation of Syt, Rα = γ1a/γ1b, and Rα/γ1a = (γ1a/γ1b)/(γ1a/γ1b) (7). For example, 2-Ssy1 produced 38 IgG1 a–producing and 81 IgG1 b–producing hybridomas. We normalized this ratio by dividing (38/81) to (63/42 = 1.5) to determine the CSR frequency of the mutated allele (31%).

CSR junctions. CSR junctions were amplified from hybridomas by nested PCR (13). Nested mouse Igα primers were 5′-CTCTGGGCCCCTGCTTATTTGTGTG-3′ followed by 5′-AGACCTGGGAAATGATGTAGGTG-3′. The reverse nested primers were located in exon1 of Cγ1, and they were 5′-CAATTTTCTGCTACCTTGTTGC-3′ followed by 5′-TGCCACACCCGTGGCAGG-3′. PCR products were gel purified and sequenced. S junctions were analyzed with the SeqMan program (DNAStar Lasergene) and the MEGABLAST program (National Center for Biotechnology Information; Fig. S3).

Online supplemental material. Fig. S1 shows the nucleotide alignment of the synthetic Syt3 and endogenous Syt3. Fig. S2 shows ELISA of LPS–stimulated splenocytes from 2-Sy3. Fig. S3 provides an analysis of CSR junctions. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080451/DC1.

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