

Ig class switching: targeting the recombinational mechanism

Erik Selsing

Recent studies have provided insights into the mechanisms involved in targeting antibody gene class switch recombination (CSR) to various switch DNA regions located upstream of constant region genes. Targeting appears to involve sequence motifs that are favored for deoxycytosine deamination by the activation-induced deaminase enzyme that is required for CSR, together with transcription (and in some cases R-loop formation) to provide the single-stranded DNA needed for activation-induced deaminase activity. There is also another poorly understood mechanism that limits CSR to a specific length of DNA downstream of the switch-region transcriptional promoter.

Addresses

Department of Pathology, Tufts University School of Medicine, 150 Harrison Avenue, Boston MA 02111, USA

Corresponding author: Selsing, Erik (erik.selsing@tufts.edu)

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Introduction

Newly emerging B cells generally express immunoglobulin (Ig)M antibodies but, upon stimulation by antigen and T-cell help in the periphery, can proliferate and differentiate into cells that express antibodies of other isotypes. This isotype switching involves the replacement of C (exons within the antibody H-chain gene by other downstream CH exons. These C-region replacements occur by class switch recombination (CSR) events [1]. CSR results from DNA recombinations between switch (S) region DNA segments located upstream of each CH gene (Figure 1). The various CH genes are located downstream of C μ and reside within a genetic locus that stretches over approximately 180 kb of DNA.

S regions are sequence elements between 1 kb and 10 kb in length and are located ~1–5 kb immediately upstream of each CH gene. S regions include segments of highly repeated tandem arrays of short specific sequence units. For instance, the S μ region contains a tandem repeat region (S μ TR) that can be represented by a 20 bp consensus sequence, (GAGCT) $_3$ GGGGT, which repeats

approximately 150 times to make up a 3 kb element. Each S region has a distinctive repeat but all exhibit at least some GAGCT and GGGGT sequence motifs

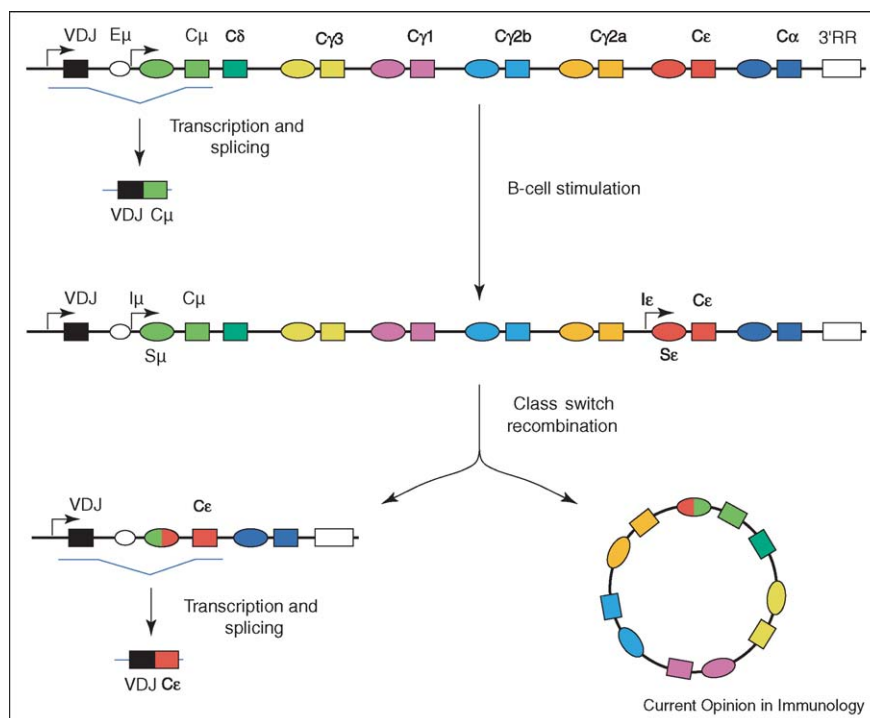
A number of proteins are important for CSR. One protein crucial for both isotype switching and somatic hypermutation (SHM) is the activation-induced deaminase (AID) protein [2]. This protein is specifically expressed in activated B cells and is the only B-cell-specific protein that is required for somatic hypermutation [3]. The AID protein is capable of deaminating cytosine residues in single-stranded DNA [4,5].

Mice that are deficient in various DNA repair proteins have also been found to have defects in isotype switching. Deficiencies in Ku70, Ku80 or DNA-PK greatly decrease isotype switching [6–8]. Ku and DNA-PK are important for non-homologous end joining (NHEJ) in the repair of double-stranded DNA breaks, but do not appear to be involved in DNA repair that is mediated by homologous recombination. Thus, CSR appears to involve DNA double-stranded break repair using NHEJ processes.

An important question is the role of the AID protein in both CSR and SHM. It has been suggested that AID might either edit RNAs that encode factors important for CSR or SHM processes or deaminate DNA directly [2]. A series of studies [9–11] support the notion that AID is responsible for deoxycytosine (dC) deamination to dU in DNA and leads to DNA repair activities that could result in either SHM or CSR. One mechanism to repair dU residues introduced into DNA by AID deamination involves the protein uracil DNA glycosylase (UNG) and UNG-deficient mice and humans show large reductions in class switching [11,12]. Current models hypothesize that repair pathways for AID-induced dU residues in S regions lead to UNG removal of the dU base and subsequent cleavage of the abasic DNA residue; this creates single-stranded breaks that are important for DNA cleavages of S regions during the switching process [13].

The AID protein has limited sequence specificity, being able to deaminate most dC residues. The highest activity of AID is on dC residues within WRC motifs (in which W represents A or T and R represents a purine) [14,15]. In this review, I will focus on recent studies that have addressed the question of how AID activity (and CSR) is targeted to the S regions within the IgH locus. The induction of S-region transcription in stimulated B cells clearly plays a role in targeting CSR [16]. Whether the level of S-region transcription can quantitatively regulate

Figure 1



Overview of class switch recombination. The events leading to a μ -to- ϵ CSR event are depicted; for clarity, the exon and intragenic regions are not diagrammed to scale. Lines delineate portions of the IgH locus, and boxes and ovals indicate gene segments and switch regions, respectively. Bent arrows indicate active promoter elements and the white box and oval represent the location of important transcriptional regulatory elements. The top line represents the H-chain locus in a naïve resting B cell. After B-cell stimulation, induction of germline transcripts marks the initiation of the CSR process (middle line). CSR results in the juxtaposition of the VDJ gene segment with a downstream CH gene segment by excision/circularization of the intervening DNA (bottom lines).

the efficiency of CSR is not clear; studies have shown that a six-fold decrease in S-region transcription has little effect on CSR frequency [17] but larger decreases can dramatically reduce CSR [18]. Nevertheless, features other than transcription must contribute to CSR targeting because switch recombinations do not occur to all transcribed regions within H-chain genes.

Sequences that target switch recombination

The roles of specific S-region DNA sequences in CSR have been probed using gene targeting in mice. A mouse strain in which the $S\mu$ TR sequences are deleted [19] shows only a 2–3-fold reduction in CSR levels. This demonstrates that although $S\mu$ TR sequences are important for the highest efficiency of CSR, sequences outside of the $S\mu$ TR are sufficient for CSR targeting. Another mouse strain that lacks a larger portion of the JH– $C\mu$ intron (Δ/Δ mouse strain) has also been analyzed; this mutant shows a much more pronounced loss of isotype switching [20], which confirms that sequences that flank the tandem repeat region are important for switching.

A mutant mouse strain that contains a chromosome that lacks the $S\gamma 1$ region has also been produced [21]. In this

mutant chromosome, about 12 kb of the $I\gamma 1$ – $C\gamma 1$ intron has been removed (including about 8 kb that contains all of the $S\gamma 1$ tandem repeats). *In vivo* isotype switching to $C\gamma 1$ in the mutant chromosome was nearly abrogated [21], which demonstrates that S-region sequences are required for the switching process. Additional mouse strains that carry varying lengths of $S\gamma 1$ sequence exhibit levels of switching that correlate with the $S\gamma 1$ length [22], which indicates that tandem repeat regions carry multiple CSR sites that together provide for the highest efficiencies of successful chromosomal recombination.

What are the sequence motifs that are important for switch recombination? As mentioned above, WRC motifs are favored AID deamination sites and, within the $S\mu$ region in normal mice, the location of CSR sites follows the distribution of WRC motifs [23^{*}]. However, AID deaminates dC residues on single-stranded DNA and would, therefore, be expected to cause single-stranded nicks in the DNA duplex [24]. It is not clear how AID-induced nicks lead to DNA breaks that can be joined by the NHEJ process. Closely spaced nicks on opposite strands could lead to the generation of a DNA break suitable for NHEJ; such close nicks might result, for

instance, from AID activity at WGCW motifs that reflect the presence of overlapping WRC sequences on opposing DNA strands [14]. However, the process by which more widely spaced AID-induced nicks might lead to CSR is not known. DNA mismatch repair proteins such as Msh2, Mlh1, Pms2 and Exo1 are all important for about half of the CSR recombination events [25–27]. Although the roles of these mismatch repair (MMR) proteins in CSR are not clear, it has been suggested that MMR activity could process widely spaced AID-induced G:U mismatches or nicks to provide DNA ends that are also suitable for NHEJ [23*,28,29]. Consistent with this proposal, mice that lack Msh2 (and therefore lack the ability to process widely spaced nicks) exhibit CSR sites more frequently located in the WGCW or AGCT motifs that could provide DNA ends amenable to NHEJ without nick processing [23*,26].

Mutant mice in which a frog S μ segment has replaced the mouse S γ 1 region show CSR sites predominantly located within a frog S μ subregion that has the highest levels of AGCT motifs [30*]. By contrast, the distribution of CSR sites within the S μ region of normal mice more closely correlates with the distribution of WRC motifs than of AGCT motifs [23*]. *In vitro* deamination of transcribed sequences by AID (in concert with the single-stranded DNA-binding protein replication protein A [RPA]) shows a preference for AID activity on regions that have the highest levels of the SHM hotspot motif RGYW [31*]. Because all these motifs are related, the differences in the exact motifs suggested to be preferred might reflect the different DNA regions that were analyzed. For instance, within the S μ tandem repeats almost all WRC motifs are also AGCT motifs, whereas in sequences that flank the tandem repeats many WRC motifs are not AGCT. The more important point is that CSR is found to occur preferentially in sequences that are the best targets for AID deamination. These preferred sequences have been found in all S-region tandem repeats, and it seems probable that the tandem repeats reflect evolutionary duplication events selected, at least in part, to provide high concentrations of preferred AID target sites.

CSR targeting by DNA structures

Besides exhibiting multiple AID sites, many S-regions also exhibit high concentrations of guanine (G)-residues in the sequences of their non-transcribed DNA strands. Such G-richness has been shown to lead to stable RNA–DNA complexes after S-region transcription [32,33]; these complexes appear to reflect R-loops in which the G-rich RNA transcript pairs stably with the C-rich DNA template strand and causes the G-rich DNA strand to be displaced as a single-strand [34]. The presence of S-region R-loops has been demonstrated among B cells undergoing switching and it has been suggested that these R-loops provide favorable single-stranded targets for AID deamination [34].

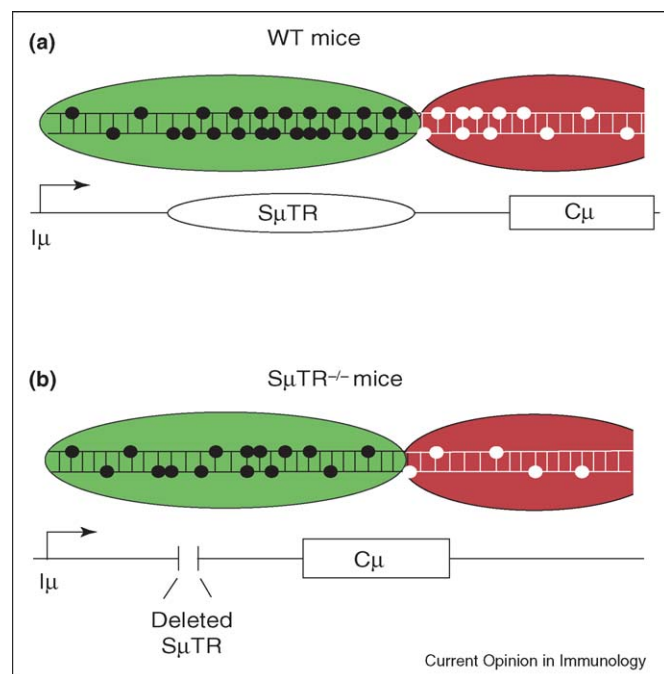
Although many transcribed S regions can form stable R-loop structures, S μ regions from frogs are not G-rich and do not appear to form R-loops upon transcription [30*]. Nevertheless, a frog S μ sequence is effective as a target for CSR even when used to replace the G-rich mouse S γ 1 region [30*]. These findings indicate that CSR does not require the presence of R-loops, probably because transcription of S-regions can provide sufficient transient single-stranded DNA for AID activity [5,35]. Even when not transcribed, double-stranded DNAs can be targets for AID deamination when they are supercoiled [36]; this is probably also owing to small regions of single-strandedness induced by supercoiling. Interestingly, when a synthetic DNA sequence that is G-rich on one strand but that has few preferred AID WRC target motifs is used to replace the mouse S γ 1 region, CSR depends strongly on the direction in which the synthetic sequence is inserted [21]. Much higher CSR levels are found when the synthetic G-rich strand is oriented to be the non-transcribed strand suggesting that, in this orientation, R-loop formation might be compensating for the low concentration of AID target sites. This could indicate that high WRC concentrations and R-loop-forming sequences are complementary in providing efficient AID targets for CSR.

S-region chromatin structure and CSR accessibility

Although AID motifs are important in CSR targeting, there are clearly higher levels of regulation that control whether any given AID motif will be accessible for CSR. Analyses of mutant mice that lack the S μ tandem repeat region show that sequences that flank the S μ TR region can clearly undergo CSR in the mutant mice but in wild-type mice exhibit no CSR events [23*]. Comparisons of S μ TR^{−/−} and wild-type mice indicate that CSR is constrained to a DNA region that is bounded upstream by the S-region transcriptional promoter (I μ), extends ~4–5 kb downstream, and is bounded downstream (in wild-type mice) near the end of the tandem repeat region (Figure 2). Previous studies also indicated that the I μ promoter represented the upstream boundary of CSR events [37]. Although wild-type and S μ TR^{−/−} mice both exhibit a 4–5 kb CSR domain [23*], the Δ/Δ mouse that has a larger S μ deletion shows not only greatly reduced CSR but also appears to have CSR limited to a much smaller domain [20]. This suggests that the additional S μ sequences deleted in Δ/Δ mice might have a role in defining the size and location of the CSR domain.

The mechanism that establishes this CSR ‘domain’ is not known. Previous studies have shown that DNA regions that undergo SHM are also established by the location of the upstream (V-region) promoter [38]. Although these SHM ‘domains’ are only 1–2 kb in length, this similarity in regulation adds to the numerous features that are shared between SHM and CSR. It has been suggested that SHM domains might reflect a transcriptional tracking mechanism whereby factors important for SHM associate with

Figure 2



Location of CSR-accessible domains in the JH-Cμ regions of (a) wild-type (WT) and (b) SμTR^{-/-} mice. The locations of the Iμ, Sμ and Cμ elements are indicated to scale. Simplified cartoons depict the double-stranded DNA in these regions with small circles indicating CSR motifs. Black circles indicate accessible motifs whereas white circles are not accessible. Regions accessible to CSR and regions that are not accessible for CSR are indicated by the green and red ovals, respectively. The deletion of the SμTR region in (b) causes some sequences, which in wild-type mice (a) are not accessible for CSR (e.g. the 5' end of the Cμ gene segment), to be within the accessible region in SμTR^{-/-} mice.

RNA polymerase during transcription and are carried along until they dissociate after a defined DNA distance [38]. A similar CSR model would only require the binding of an alternate factor that results in a longer complex retention to provide the longer CSR domain. However, because different S-regions have quite different lengths, this type of model might also suggest that isotype-specific factors provide the different CSR domain lengths. Factors important for switching to specific isotypes have been detected [39,40], but the roles of these factors are not yet known.

It is also possible that chromatin modifications or structure might define the DNA domain that is accessible to the CSR machinery. The induction of SHM within a H-chain VDJ region in a human B-cell line has been found to correlate with an increased level of H3 and H4 acetylation compared with the acetylation levels in the Cμ exon region downstream in the same gene [41]. This suggests that, for SHM, hyper-elevated levels of H3 and H4 acetylation might control targeting of AID. However, in normal mouse B cells, H3 and H4 hyper-acetylation did not appear to demarcate or correlate with the location of VDJ SHM [42].

A number of studies have investigated relationships between CSR and chromatin histone modification patterns. Increased acetylation of histones H3 and H4 within

the Sγ, Sε and Sα regions in stimulated B cells has been linked to the induction of germline transcription and CSR [16,42,43]; this increased acetylation appears to focus on the I and S elements associated with each CH exon region [43]. However, CSR appears to be more closely linked to germline transcription than to histone acetylation [16], and, for Sμ, acetylation and transcription are found in resting B cells prior to any stimulation or induction of CSR [42–44]. Furthermore, no increased acetylation of Sμ relative to Cμ was found when unstimulated B cells were compared to activated germinal center B cells [42]. By contrast, acetylation of H4 (and to a lesser extent H3) has been reported to increase in S-regions that are undergoing CSR, based on comparisons of unstimulated normal B cells with B cells activated in culture with mitogens and cytokines [43]. These somewhat discordant results might reflect the multiple H3 and H4 acetylation sites that could be reacting with the antibody reagents used to assess acetylation status, and could potentially be resolved using antibodies specific for each site. Nevertheless, the current findings leave open the question of whether histone acetylation might help define and target a DNA domain for CSR.

The presence of two other histone modifications — the phosphorylated histones γH2AX and H2B^{Ser14P} — appears to correlate with S-regions that are actively

undergoing AID-induced CSR [42]. However, these latter S-region chromatin modifications are AID-dependent and might be related to AID-induced DNA damage rather than to a mechanism that targets the S-region for CSR. Histone H4 acetylation also appears to be associated with AID-induced DNA damage in S-regions [43]. Overall, therefore, it is not yet clear whether any currently assayed histone modifications are responsible for targeting AID deamination to the CSR DNA domain.

The I μ promoter, the E μ enhancer and the intronic matrix-attachment regions are all elements that appear to be obvious candidates for regulating the CSR domain. However, studies of various mutant mice do not appear to favor these possibilities. Replacement of the I μ promoter with a phosphoglycerate kinase 1 promoter [45], deletion of the core E μ enhancer [46], or deletion of the intronic matrix-attachment regions [45] result in only modest effects on CSR. This suggests that sequences between I μ and C μ , but outside of the tandem repeat region, could play a role in defining the CSR domain. Future mutational studies should show the locations of these sequences.

Conclusions

Genetic mutation studies have greatly helped to illuminate some aspects of CSR targeting, and the involvement of AID motifs, transcription and R-loops in this process is becoming clearer. However, the mechanism that delineates the domain of CSR accessibility is still obscure. Furthermore, AID is active in both VDJ regions and S regions, but nothing is known about the regulation that limits CSR to S-region sequences.

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