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# Mice lacking Sμ tandem repeats maintain RNA polymerase patterns but exhibit histone modification pattern shifts linked to class switch site locations

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ABSTRACT

Antibody switching involves class switch recombination (CSR) events between switch (S) regions located upstream of heavy chain constant (C) genes. Mechanisms targeting CSR to S-regions are not clear. Deletion of Sμ tandem repeat (SμTR) sequences causes CSR to shift into downstream regions that do not undergo CSR in WT B-cells, including the Cμ-region. We now find that, in SμTR$^{-/-}$ B cells, Sμ chromatin histone modification patterns also shift downstream relative to WT and coincide with SμTR$^{-/-}$ CSR locations. Our results suggest that histone H3 acetylation and methylation are involved in accessibility of switch regions and that these modifications are not dependent on the underlying sequence, but may be controlled by the location of upstream promoter or regulatory elements. Our studies also show RNA polymerase II (RNAPII) loading increases in the Eμ/Iμ region in stimulated B cells; these increases are independent of SμTR sequences. Longer Sμ deletions have been reported to eliminate increases in RNAPII density, therefore we suggest that sequences between Iμ and Sμ (possibly the Iμ splicing region as well as G-tracts that are involved in stable RNA:DNA complex formation during transcription) might control the RNAPII density increases.



## 1. Introduction

The process of class switch recombination (CSR) alters antibody heavy (H)—chain constant ($C_H$) region gene segments through deletional DNA recombination events between switch (S) region sequences located upstream of each $C_H$ (except Cδ) (reviewed in Stavnezer et al., 2008). S-regions contain highly repeated tandem arrays of G-rich DNA. Activation-induced cytidine deaminase (AID) and transcription through donor and acceptor S-region sequences have been shown to be required for CSR (Muramatsu et al., 2000; Stavnezer et al., 1988; Stavnezer-Nordgren and Sirlin, 1986). AID deaminates dC residues in single stranded DNA and in transcribed double stranded DNA (Chaudhuri et al., 2003; Di Noia and Neuberger, 2002; Muramatsu et al., 2000; Petersen-Mahrt et al., 2002; Rada et al., 2002). In single-stranded DNA, AID preferentially deaminates within WRC sequence motifs (W = A or T and R = purine) (Beale et al., 2004; Pham et al., 2003; Yu et al., 2004) that are often enriched in S regions.

For CSR to occur, DNA breaks must be made in S-regions. Individual S-regions have different nucleotide sequences and vary from ~1 kb to 12 kb in length (Arakawa et al., 1993; Kataoka et al., 1981; Mowatt and Dunnick, 1986; Nikaido et al., 1981, 1982; Szurek et al., 1985). The basis for targeting of CSR to S regions is not understood. Transcription through S-regions can form stable R-loop structures that leave the non-template strand as a favorable single-stranded target of AID activity (Shinkura et al., 2004; Tian and Alt, 2000; Yu et al., 2003). However, substantial CSR can occur in S-regions where no R-loops have been found (Zarrin et al., 2004), and RNA transcription appears sufficient to provide AID access to double-stranded DNA (Chaudhuri et al., 2003).

Chromatin remodeling could be important for targeting CSR. Post-translational modifications alter DNA-histone interactions in the nucleosome (Kouzarides, 2007) and could regulate access of the CSR machinery to S-regions. Covalent modifications, such as acetylation (AcH3 and AcH4) and H3 K4 methylation (di-me H3K4) alter transcription by modifying chromatin structure to an active state (Kouzarides, 2007). Induction of SHM in a B-cell lymphoma correlates with increased histone acetylation of the transcribed and mutated V region but not of the equivalently transcribed, but

*Abbreviations:* AcH3, histone H3 acetylation; AID, activation-induced cytidine deaminase; $C_H$ region, antibody heavy (H)—chain constant region; ChIP, chromatin immunoprecipitation; CSR, class switch recombination; di-MeH3K4, histone H3 dimethylation; GLT, germline transcript; PST, post-switched transcript; RNAPII, RNA polymerase II; S, switch; S region, switch region; SμTR, Sμ tandem repeat; tri-MeH3K4, histone H3 trimethylation; WT, wild type.

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unmutated, Cμ region (Woo et al., 2003). Reports have indicated increases in active histone modifications of downstream switch regions that coincide with CSR, however these increases, if any, are modest in the Sμ region (Beale et al., 2004; Kuang et al., 2009; Li et al., 2004; Nambu et al., 2003; Wang et al., 2006, 2009).

In stimulated B cells that lack Sμ tandem repeat sequences (SμTR), CSR recombination break points shift downstream into regions where switch events are not found in wild-type (WT) mice, including in the Cμ region (Min et al., 2005). This shift demonstrates that these downstream sequences are fully capable of supporting CSR, but that, in WT cells, the sequences are inaccessible to the CSR machinery. We have compared the histone acetylation and methylation patterns within the μ locus in stimulated WT and SμTR$^{-/-}$ B cells to see if chromatin modifications associate with shifts in CSR locations. We find differences in acetylation and methylation in the μ locus that appear to designate accessible regions and boundaries in targeting CSR. Furthermore, our results show that removal of the SμTR results in increased active histone modifications in the Cμ region; suggesting that this increase allows the Cμ region to become CSR accessible. Our data indicate that histone H3 acetylation and methylation are involved in specific accessibility to switch regions and that these modifications are not dependent on the underlying sequence, but may be controlled by the location of a nearby promoter or regulatory elements. In addition, histone modifications of the WT Sμ region do not change when resting B cells are activated, indicating that other mechanisms control the activity of AID on accessible Sμ chromatin. We find that activated B cells show increased RNA polymerase density in the Eμ/Iμ region upstream of Sμ, suggesting that polymerase loading in this region could promote the entry of AID onto the accessible Sμ DNA target.

## 2. Materials and methods

### 2.1. Mice and cell culture

Animal experiments were approved by the Tufts University Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from Jackson Laboratories. All mice were 8–12 weeks of age. Splenic B cells were purified using StemSep B Cell Enrichment kit (StemCell Technologies). Splenic B cells were cultured at $5 \times 10^5$ cells/ml in RPMI-1640/10% FCS. Cultures were supplemented with LPS (25 μg/ml; Sigma Chemical Co.) and 10 ng/ml murine IL4 (Preprotech) to induce isotype switching to IgG1.

### 2.2. Chromatin immunoprecipitations (ChIPs)

Cells ($\sim 40 \times 10^6$ stimulated and $\sim 100 \times 10^6$ resting splenic B cells per ChIP) were fixed in 1% formaldehyde for 10 min at room temperature. Glycine (125 mM final concentration) stopped the reaction. Cells were washed 2× with cold PBS, then 3× with cold lysis buffer (10 mM Tris–HCl, pH 7.5/10 mM NaCl/3 mM $MgCl_2$/0.5% NP-40). The nuclear pellet was resuspended in 2 ml of micrococcal nuclease (MNase) reaction buffer (10 mM Tris–HCl, pH 7.5/10 mM NaCl/3 mM $MgCl_2$/1 mM $CaCl_2$/4% NP-40/1 mM PMSF) and incubated with 4 units of MNase (Sigma) for 10 min at 37 °C. MNase digestion was stopped with 3 mM EGTA. SDS (1% final concentration), NaCl (200 mM final concentration), and protease inhibitors were added. The samples were sonicated to lyse nuclei, and cellular debris was removed by centrifugation. Conditions were optimized to produce 100–500 bp DNA fragments. Chromatin samples were diluted with buffer (20 mM Tris–HCl, pH 8.0/2 mM EDTA/1% Triton X-100/150 mM NaCl/protease inhibitors) and precleared for 15 min at 4 °C with 50 μl of Protein A Sepharose (Sigma) resin. A DNA aliquot of this precleared sample was the "input" sample.

**Table 1**
PCR primers.

| Primer name | Primer sequence reference |
|---|---|
| EμU | 5′-TGGCAGGAAGCAGGTCAT-3′ |
| EμD | 5′-GGACTTTCGGTTTGGTGG-3′ |
| 5Sμ1U | 5′-TGCTCTGTGTGAACTCCCTCTG-3′ |
| 5Sμ1D | 5′-AGCCACAACCATACATTCCCAGG-3′ |
| 5Sμ2U | 5′-GTAAATGTACTTCCTGGTTG-3′ |
| 5Sμ2D | 5′-GGTCTCTATTCTTTCTCAA-3′ |
| 3Sμ1U | 5′-ATAAGTTAGGCTGAGTAGGGC-3′ |
| 3Sμ2D | 5′-ACTGGCTGGGAGAACTATT-3′ |
| CμU | 5′-CACCATTTCCTTCACCTG-3′ |
| CμD | 5′-TGTTTTTGCCTCCGTAGT-3′ |
| 3Cμ1U | 5′-GAATGAGCAATAGGCAGTA-3′ |
| 3Cμ1D | 5′-GATGGTGAAGGTTAGGATG-3′ |
| 3Cμ2U | 5′-CCGAGAGGACCGTGGACA-3′ |
| 3Cμ2D | 5′-AGAGCAAGCAAAACACAACT-3′ |
| CδU | 5′-CCATCACTTTTTGTCCAT-3′ |
| CδD | 5′-AGCAAGAGGTGTAAGGTT-3′ |
| 5Sγ1U | 5′-TATGCCACCCACTGTCAATCCTGT-3′ |
| 5Sγ1D | 5′-TGGTCCTGCCCTTCTCTTGTCTTT-3′ |
| Sγ1U | 5′-GGTCCCAGGTTCAATCCCAGC-3′ |
| Sγ1D | 5′-TTTGCAGGTGCTCAGTCTTGTGTCCT-3′ |
| 3Sγ1U | 5′-ACAGGTCAAG GCTGAGTAGAAGCA-3′ |
| 3Sγ1D | 5′-TCCCACAACTCCCACTGGTTTAGTT-3′ |
| 3Sγ12ndU | 5′-GGAACTGCTGCAGGCACAAAGAAT-3′ |
| 3Sγ12ndD | 5′-CTCCAGCCTGTATGTTTCCACT-3′ |
| Cγ1U | 5′-AGCCAGCGGAGAACTACAAGAACA-3′ |
| Cγ1D | 5′-TGCTCTTCTGCACATTGAGCTTGC-3′ |
| Cγ12ndU | 5′-TCACACTGTCTGCTCATCTCGCTT-3′ |
| Cγ12ndD | 5′-CTTTGGTGCTGCTGTGATGGTGTT-3′ |
| β-GlobinU | 5′-GCCTTGCCTGTTCCTGCTC-3′ |
| β-GlobinD | 5′-CAGACCATAAACTGTATTTTTCTTATTGAGCCC-3′ |
| μGLTF | 5′-CTCGGTGGCTTTGAAGGAAC-3′ |
| μGLTR | 5′-TGGTGCTGGGCAGGAAGT-3′ |
| γ1GLTF | 5′-TCGAGAAGCCT-GAGGAATGTG-3′ |
| γ1GLTR | 5′-ATGGAGTTAGTTTGGGCAGCA-3′ |
| IgβF | 5′-CAGAAATGTGACAGCGCCAACCAT-3′ |
| IgβR | 5′-TGTCAAGTAGCAGGAAGATGGGCA-3′ |
| β-ActinF | 5′-AGGTATCCTGACCCTGAAG-3′ |
| β-ActinR | 5′-CACGCAGCTCATTGTAG-3′ |

Immunoprecipitations were rocked at 4 °C overnight with either 5 μg of anti-Acetyl H3 (K9/K14), Upstate Biotechnology, Lake Placid, NY (U.B.) catalogue no. 06-599, 5 μg of anti-di-Me H3-K4, Abcam Inc., Cambridge, Ma (Abcam) catalogue no. ab7766, 5 μg of anti-tri-Me H3-K4 (Abcam catalogue no. ab7766), 5 μg of anti-RNAPII CTD phosphorylated on serine 5 (Abcam catalogue no. ab5131), or 5 μg of anti-RNAPII CTD phosphoryated on serine 2 (Abcam catalogue no. ab5095), together with 50 μl of protein A sepharose resin. According to the manufacturer, RNAPII antibodies are specific for single-phosphorylated forms, neither antibody will bind to RNAPII phosphorylated on both serine 5 and serine 2. The resin was washed with IP dilution buffer, twice with IP dilution buffer plus 0.1% SDS, with IP dilution buffer plus 0.1% SDS and 500 mM NaCl, with ChIP wash 3 (10 mM Tris–HCl, pH 8.0/1 mM EDTA/250 mM LiCl/1% NP-40/1% deoxycholate), and twice with TE buffer (10 mM Tris/1 mM EDTA, pH 8.0). Chromatin was eluted and decrosslinked. DNA was phenol/chloroform extracted, ethanol precipitated, and quantified.

### 2.3. Real-time PCR analysis

Differences in amounts of specific DNA sequences enriched in an IP sample relative to an input sample were determined by SYBR green-mediated real-time PCR analysis using the Bio-Rad iCycler iQ. Triplicate reactions containing 1 ng input or IP DNA and 10 pmol of primers (Table 1) were amplified in SYBR Green PCR Master Mix (Applied Biosystems). Primers were tested in reactions containing α $^{32}$P dCTP, ensuring a single product with appropriate size. Fold enrichment values for amplified DNA sequences were determined