Published in final edited form as:

Mol Immunol. 2015 August ; 66(2): 284–289. doi:10.1016/j.molimm.2015.04.005.

Effect of CpG Dinucleotides within IgH Switch Region Repeats on Immunoglobulin Class Switch Recombination

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Abstract

Immunoglobulin (Ig) heavy chains undergo class switch recombination (CSR) to change the heavy chain isotype from IgM to IgG, A or E. The switch regions are several kilobases long, repetitive, and G-rich on the nontemplate strand. They are also relatively depleted of CpG (also called CG) sites for unknown reasons. Here we use synthetic switch regions at the IgH switch alpha (S α) locus to test the effect of CpG sites and to try to understand why the IgH switch sequences evolved to be relatively depleted of CpG. We find that even just two CpG sites within an 80 bp synthetic switch repeat iterated 15 times (total switch region length of 1200 bp containing 30 CpG sites) are sufficient to dramatically reduce both Ig CSR and transcription through the switch region from the upstream I α sterile transcript promoter, which is the promoter that directs transcripts through the S α region. De novo DNA methylation occurs at the four CpG sites in and around the I α promoter when each 80 bp I α switch repeat contains the two CpG sites. Thus, a relatively low density of CpG sites within the switch repeats can induce upstream CpG methylation at the IgH alpha locus, and cause a substantial decrease in transcription from the sterile transcript promoter. This effect is likely the reason that switch regions evolved to contain very few CpG sites. We discuss these findings as they relate to DNA methylation and to Ig CSR.

Keywords

immunoglobulin; isotype switch; class switch; recombination; DNA methylation; DNA rearrangement; B cell

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1. INTRODUCTION

The murine immunoglobulin heavy chain (IgH) locus consists of eight different constant (C) genes, most of which, except C δ , contain a cytokine-inducible promoter and a highly repetitive switch (S) region (1,2). Class switch recombination (CSR) replaces the default C μ exons with exons for a downstream constant chain (C α , C ϵ or C γ). Thus, instead of IgM, a switched IgH isotype (IgA, IgE or IgG) is produced. During this process, double-strand DNA breaks in the donor S μ and in the acceptor switch region are created, and the intervening DNA region is deleted. Mammalian switch regions are all long, repetitive, and G-rich on the nontemplate DNA strand, but there is no significant length of homology among different switch regions, and their similarity consists of having occasional WGCW motifs and occasional clusters of 3 or 4 G nucleotides (3). These two motifs are enriched in mammalian switch regions relative to the neighboring DNA regions, or elsewhere in the genome (3). Most of the recombination events occur within the portion of the switch region that is most repetitive, which is where 49 to 80 bp repeats are located. These switch repeats are the portion of the switch region that are most enriched for the WGCW and G-cluster motifs (3).

Transcription through switch regions is essential for IgH CSR (3). Transcription of the IgH S μ region is constitutive, but transcription of downstream IgH switch regions (S γ , S α , S ϵ) is only active when specific cytokines are present. A sterile transcript promoter is located upstream of each of the downstream switch regions, and the functions of this promoter and the resulting sterile transcript have been unclear for many years because this transcript is not translated (hence the designation ‘sterile’)(1,2). Transcription likely influences histone tail modifications and generates paused RNA polymerase complexes (4,5). We have previously proposed that an additional role of transcription during CSR is to induce R-loop formation in the switch regions, which provides stable single-stranded DNA substrates for deamination of C to U by activation-induced deaminase (AID) (6–8). In cell-free biochemical systems, the G-richness on the nontemplate DNA strand is critical for R-loop formation (9–11). Cellular studies at murine IgH S γ 3 and S γ 2b switch regions have shown that most R-loops terminate within the switch regions, and that the remaining ones terminate within 600 bp downstream of the switch regions (12). The downstream boundaries of R-loops correspond extremely well with the zone where G-density gradually falls to the level of genome average. However, only recently were direct experimental studies performed using a cellular system to evaluate the role of high G-density and G-clusters in mammalian switch region during R-loop formation and CSR (13,14). R-loop formation also has been suggested as a possible cause of RNA polymerase pausing in switch regions (4).

One notable feature of IgH switch repeats is their relative lack of CpG (also designated simply as CG) sites (approximately one CG per 400 to 1000 bp in the repetitive portions of the mammalian switch regions). In contrast, CpG sites have a genome-wide density of approximately one per 100 bp. Suppression of transcription is known to occur when the methylated CpG density approaches and exceeds one per 20 bp (15). A predilection for CpG methylation in zones of repetitive DNA may have evolved as a way to suppress transcription of transposons (16).

Here we varied the sequence of the IgH S α repeats by replacing the natural S α switch region with synthetic repeats that do or do not have two CpG sites per 80 bp repeat. We find a strong reduction in transcription and IgH CSR when the two CpG sites per repeat are present. The sterile transcript promoter transcription start site is 290 bp upstream of the first switch repeat. In addition, presence of two CpG sites per 80 bp repeat causes four CpG sites which are located 90 to 389 bp upstream to become methylated. We discuss the significance of these findings for IgH CSR and for DNA methylation in repetitive regions.

2. EXPERIMENTAL PROCEDURES

2.1 Cell Culture and CSR Assay

Mouse B lymphoma cell line, CH12F3.2a, and its derivative cell line, 2B9, were cultured in 10% FCS RPMI medium supplemented with 50 μ M β -mercaptoethanol. 2B9 cells were constructed as previously described (15). For the CSR assay, cells in log phase were seeded at 5×10^4 cells/ml in medium with 1 μ g/ml anti-CD40 (eBioscience #16-0404-86), 5 ng/ml IL-4 (R&D #404-ML-010) and 0.5 ng/ml TGF- β 1 (R&D #240-B-002), and grown for 72 h. Cells were stained with FITC-conjugated anti-mouse IgA antibody (BD #559354) and analyzed by flow cytometry. CSR efficiency was determined by the percentage of IgA⁺ cells.

2.2 Plasmid Construction

The 80 nt oligonucleotides (ZZ351: ATGAGCTGGGATGAGCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTG GTGTGAGCTGGGTTAGGCTGAGCTGAGCTGGA/ ZZ352: GCTCATTCCAGCTCAGCTCAGCCTAACCCAGCTCACACCAGCCCAGCCCA GCCTATTCCAGCCTAGCTCAGCTCATCCCA for the cellular construction without CpG sites or ZZ353: ATGAGCTGGGATGAG- CTGAGCTAGGCGTGAATAGGCTGGGCTGGGCTGGTGTGAGCTGGGTTAGGCGTA GCTGAGCTGGA/ZZ354: GCTCATTCCAGCTCA- GCTACGCCTAACCCAGCTCACACCAGCC- CAGCCCAGCCTATTACGCCTAGCTCAGCTCATCCCA for the cellular construction with CpG sites) were 5'-phosphorylated and purified on a denaturing polyacrylamide gel. A pair of complementary oligonucleotides was annealed and self-ligated at a final concentration of 4 μ M with T4 DNA ligase (Roche Diagnostics). Ligated DNA of expected length was recovered after the ligation products were resolved on agarose gel, blunted with Klenow fragment of DNA polymerase I (New England Biolabs), and cloned into the exchange vector backbone described previously (13,14).

2.3 Cellular Screening

For RMCE-mediated knock-in of synthetic switch regions, five micrograms of exchange vector and one microgram of Cre-expression vector were cotransfected into 2B9 cells by electroporation (Lonza). Transfected cells were serially diluted and seeded in 96-well plates. After 72h, ganciclovir (Sigma-Aldrich #G2536-100MG) was added to a final concentration of 2 μ g/ml. At 7 days after transfection, single clones were picked for puromycin sensitivity test at a final concentration of 1 μ g/ml. Puromycin-sensitive clones were screened by PCR

near the upstream and downstream boundaries of LoxP sites and across the entire switch region. A subset of clones was further examined by Southern blot. At least five clones were used for the CSR assay.

For random integration experiments, five micrograms linearized vector with DNA of interest and one microgram linearized hygromycin expression vector (pHYG) were cotransfected into 2B9 cells. Corresponding procedures were performed as above except that hygromycin B (RPI Cat. No. H75000) at a final concentration of 1mg/ml was added as a selection, instead of ganciclovir.

2.4 Bisulfite Sequencing

Two micrograms of genomic DNA from cells of interest was incubated with the bisulfite solution (Lightning Conversion Reagent by Zymo Research, Cat. No. D5030) for 8 min at 98°C and 2 h at 54°C, and processed according to the instructions by the manufacturer. The I α exon region was amplified with one native and one converted primer using PCR (ZZ491: GTGGTTATGTATGTGGAGTTTGG and ZZ508: AACTCAACTC-ATCCCAACTCATAC), and subcloned into the pGEM-T Easy vector (Promega A1360). Multiple clones from each cellular construct were randomly chosen and sequenced, and multiple PCR reactions were carried out for each sodium bisulfite experiment. CpG sites are located 90, 368, 377 and 389 bp upstream of the start of the first synthetic switch repeat. For orientation, the I α sterile transcript promoter transcription start site is 290 bp upstream of the first S α synthetic switch repeat.

2.5 Germ-Line Transcript Quantification

Two million healthy cells at a density around 1×10^6 cells/ml were supplemented with anti-CD40, IL-4 and TGF- β 1 for 6h, and total RNA was extracted with GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich Cat. No. RTN350). 10% of RNA was reverse transcribed into cDNA with M-MuLV Reverse transcriptase (New England Biolabs Cat. No. M0253S), and 10% of the products from this reaction was analyzed with quantitative PCR (qPCR) using TaqMan probe. β -Actin was used as an internal control. Each sample was done in duplicates, and at least three independent cellular clones were analyzed for each construct.

3. RESULTS

3.1 Experimental Strategy

To examine the importance of CpG sites in mammalian switch regions, we used a system that allows us to exchange out the genomic switch region and replace it with any desired synthetic switch sequence in a mouse B-cell line, CH12F3.2a (17). Such an exchange permits us to study how switch sequence variations affect R-loop formation and cellular CSR *in vivo*. This cell line is able to specifically and efficiently switch to IgA upon cytokine stimulation. Using this cellular assay system, the endogenous S α locus (sterile promoter, I α and S α) in CH12F3.2a was replaced with a positive-negative selection cassette (Puro TK, provides puromycin resistance and ganciclovir sensitivity) flanked by two different loxP sites (13,17). All of the CpG sites within this cassette (4 CpG around the I α promoter and any of the CpG sites within the synthetic switch repeats) are unmethylated at the time the

fragment is introduced into the genome. The resulting cell line is called 2B9. The sequence of interest is cloned into an exchange vector with the same loxP sites as the ones inserted into the 2B9 cells. This exchange vector is cotransfected into 2B9 cells along with a Cre-expressing vector. Cre mediates recombination between the exchange vector and the 2B9 chromosome at the corresponding loxP sites (Fig. 1). The successful replacement of the selection cassette by the sequence of interest gives rise to ganciclovir resistant and puromycin sensitive clones, which are screened with a puromycin sensitivity test and PCR analysis. The CSR assay is described in the Methods.

3.2 CpG Sites in the Ig Class Switch Regions Reduce CSR Markedly

We compared two synthetic S α switch regions for the effect of two CpG sites per repeat. The natural consensus S α switch repeat sequence (Fig. 2A, top line of sequence) was iterated 15 times for a total length of 1200 bp in the exchange construct and then inserted in place of the wild type 4.6 kb endogenous S α sequence at the IgH alpha locus of murine 2B9 cells as the control experiment. A parallel experimental construct was generated with GT dinucleotides in place of the TG nucleotides at two locations (Fig. 2A, red in lower line of sequence) to create two CpG sites in each of the 15 synthetic IgH S α switch repeat units, designated as 2CG-S α .

We generated and tested more than 5 cellular clones of each of these genomic arrangements for Ig CSR. We find that IgH class switch from IgM to IgA is ~55% for the natural synthetic switch repeat (indistinguishable from the 55% level of wild type CH12F3.2A), but is reduced to ~5% for the CpG-containing repeat, 2CG-S α at the same genomic location (Fig. 2B). This finding indicates that two CpG motifs in each 80 bp switch repeat are sufficient to reduce IgH CSR by nearly 10-fold.

3.3 CpG Sites in the Ig Class Switch Regions Reduce Transcription Markedly

Transcription through the switch regions is required for efficient IgH CSR (2,7,18,19). We tested transcription for the 2CG-S α versus the natural (without CpG) S α cell lines. Transcription through the switch region in cellular clones containing the 2CG-S α is reduced by ~10-fold compared with the cellular clones harboring the natural switch repeat (Fig. 3). The observed reduction of transcription is sufficient to account for the decrease in IgH CSR in these cells (Fig. 2).

3.4 CpG Sites in the Ig Class Switch Regions Cause DNA Methylation at the Sterile Transcript Promoter of the S α Locus

The marked reduction in transcription raised the possibility that the CpG sites in the synthetic switch region somehow caused methylation around the I α promoter located upstream of the switch region. We did genomic bisulfite sequencing to assess the methylation status of the four CpG sites around the IgH I α promoter. (Because of its repetitive nature, it is not possible to reliably examine the methylation status of the CpG sites within each repeat of the synthetic S α .) We find that there is a distinctly higher DNA methylation frequency (60 to 70% methylation) for these sites relative to the exact same sites in the cellular clones that harbor the natural switch repeat that lack CpG sites (< 20%

methylation) (Fig. 4). This finding strongly suggests that the inclusion of CpG sites in the switch repeat leads to methylation of the upstream DNA region.

3.5 Random Genomic Integration of the DNA Containing the I α and Synthetic S α

Since inclusion of two CpG sites in the switch repeat leads to hypermethylation of the upstream I α region in all of the cellular clones examined, we wondered whether the 2CG-S α is a sufficient signal for I α methylation. To test this, we randomly integrated the entire region encompassing I α to S α switch segment (I α – S α) of DNA in the murine CH12F3.2a genome other than the natural S α locus. We analyzed 7 independent cellular clones for DNA methylation. We find that while most of the clones containing the randomly integrated I α – S α fragment show I α promoter methylation, two clones have very little methylation (less than 20%) at these sites (Fig. 5). This finding suggests that the effect of CpG sites in the switch repeat on I α methylation is not entirely location-independent, and it may involve factors that are not completely within the I α -S α fragment. It is also possible that while the presence of CpG sites in the switch repeat has a very strong effect on I α methylation, the surrounding chromatin may overcome that effect in some instances. Thus, the I α – S α segment is not sufficient to dominantly determine the methylation around the upstream I α promoter, indicating that additional factors specific to the natural I α – S α locus are important in determining this.

4. DISCUSSION

It has been a mystery why the IgH class switch regions are remarkably depleted of CpG sites, even though the switch regions are relatively rich in G and C. One possible explanation could be that the switch repeats do not contain very many CpG dinucleotides purely by chance; but the extent of depletion in the repetitive portion of the switch regions far exceeds this, given that the switch repeats vary substantially in their sequence and given their extreme richness in GC base pairs (3,7,12,20).

Here we find that addition of only two CpG sites in each 80 bp synthetic IgH S α switch repeat is sufficient to have a dramatic effect on the sterile transcription from the I α promoter. This is surprising because the genome average of CpG sites is one per 100 bp (16), and one CpG site in 40 bp is still within the range of random DNA. In general, methylation of CpG sites substantially downstream of a mammalian promoter does not have a significant effect on transcription (15,21,22). We find here that the presence of CpG sites at a density of 1 in 40 bp in the synthetic switch repeat located 290 bp downstream appears to be sufficient to trigger DNA methylation at the four CpG sites located in and around the I α sterile transcript promoter when it is at its natural location in the murine genome. This I α promoter methylation appears to be sufficient to reduce transcription from the I α promoter, resulting in a substantial decrease in IgH CSR.

It does not appear that the 2CG-S α segment is sufficient to determine I α DNA methylation independent of its location. This is based on the variable I α methylation level observed in different cellular clones in the random integration study. This indicates that other factors such as adjacent chromatin play a role in I α promoter methylation, even though the presence

of CpG sites in the switch repeat has a very strong impact on the methylation of I α at its natural location.

While we are able to determine that switch regions evolved to have few CpG sites because these cause a loss of transcriptional activity from the upstream sterile transcript promoter, we acknowledge that our study highlights numerous longstanding questions in the DNA methylation field that remain unanswered regarding the interplay between CpG density, DNA methylation, and transcription.

5. CONCLUSIONS

Mammalian switch regions are depleted of CpG sites because even a moderate density of these would suppress the upstream sterile transcript promoter, resulting in a marked reduction in IgH CSR.

Acknowledgements

This work was supported by NIH grants to MRL and KY. The authors thank Dr. Raymond Mosteller for helpful comments on the manuscript. Use of cores for the project was supported in part by a general center grant, P30CA014089 from the National Cancer Institute.

Abbreviations

CpG or CG	deoxycytidine-deoxyguanylate dinucleotide
CSR	class switch recombination
IgH	immunoglobulin heavy chain
CIT	anti-CD40, IL4, TGF- β 1.

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Highlights

1. The CpG motif is rare within mammalian IgH class switch recombination repeats.
2. Two CpG sites per 80 bp synthetic S α switch repeat induces methylation at the I α , resulting in lower transcription.
3. CpG sites within the repetitive S α region trigger DNA methylation around the promoter.
4. Ig switch repeats evolved to have few CpG sites to avoid suppression of the promoter.

Figure 1. Construction of Chromosomal IgH $S\alpha$ locus Variants

A. Only 11 CpGs exist within the 4.6 kb of the murine IgH $S\alpha$ region, and only one of these is located within the repetitive core region, which means a CpG density of one per 2 kb in the natural repetitive $S\alpha$ region.

B. Genomic organization of the CH12F3.2a and 2B9 cells. L3 and 2L are mutant and wild type LoxP sites. Puro, puromycin selection cassette; TK, truncated thymidine kinase gene; DTA, diphtheria toxin chain. B, Bam HI.

C. Southern blot of BamHI-digested genomic DNA. The 5'-probe detects only the productive (P) allele while the 3'-probe also detects the non-productive allele (NP).

D. Construction of synthetic switch regions and the genomic configuration of $S\alpha$ locus (17). Each 80 bp switch repeat unit was self-ligated and the products with the desired number of switch repeats were inserted into the exchange vector. An exchange vector and a Cre-expression vector were cotransfected into 2B9 cells. Triangles represent different loxP sites, and each arrow represents one 80 bp repeat unit. At least five independent cellular clones were made for each switch construct.

Figure 2. Murine S α Switch Repeats Containing Two CpG Sites have Reduced Ig CSR

A. Diagram showing the sequence difference between the IgH S α Loci with and without two CpG sites per switch repeat. Each green arrow represents one 80 bp synthetic switch repeat, and there are a total of 15 such repeats in each synthetic S α region. The nucleotide sequence of each repeat is shown in the lower portion of the panel. The nucleotides in red are different between the version with CpG sites and the version without CpG sites, and all other nucleotides are identical. These changes in red do or do not result in the presence of two CpG sites per 80 bp switch repeat.

B. FACS analysis of CSR is shown for the cellular clones with and without CpG sites. Each data symbol represents an independent clone. The bars represent the mean of each group. The Student's t test was used to analyze the effect of CpGs in the switch region. Wild type cells with the 4.6 kb unaltered S α region have an average CSR level of 55% (not shown). The puro delta TK insert alleles (seen in bottom line of Fig. 1B and top line of Fig. 1D) yield an average IgH CSR level of approximately 7% (not shown) because the puro delta TK insert has an abundance of G-clusters and ample WGCW sites.

Figure 3. Transcription is Inhibited by the CpGs within the Switch Region

Healthy cells grown to $10^6/\text{ml}$ were treated with $1\mu\text{g}/\text{ml}$ anti-CD40, $5\text{ng}/\text{ml}$ IL-4 and $0.5\text{ng}/\text{ml}$ TGF- β 1 (CIT) for 6h. Total RNA was extracted, reverse-transcribed into cDNA and quantified by real-time PCR. β -Actin was used as an internal control. The error bar represents the SEM of 3 independent clones. In parallel, the same cellular clones without CIT treatment were used to measure the background of germline transcription (GLT), which resulted in values that were approximately two thirds (without CpG sites) or one-tenth (with CpG sites) of the respective values shown, because the assay measures spliced mRNA, and such processed mRNA accumulates within the cell.

# of CpG sites in each switch repeat	Duplicate molecules included?	Site 1	Site 2	Site 3	Site 4	Overall
0	yes	5/19	1/19	0/19	2/19	11%(8/76)
	no	2/7	1/7	0/7	2/7	18%(5/28)
2	yes	16/21	19/21	11/21	13/21	70%(59/84)
	no	6/10	8/10	4/10	7/10	62%(25/40)

Figure 4. Methylation Around I α is Influenced by the CpGs within the Switch Region

Bisulfite genomic sequencing was done on the DNA around the I α region (sterile transcript promoter region) for which the transcription start site is located 290 bp upstream of the first synthetic switch repeat. This was done in order to determine the DNA methylation status at the four CpG sites located there (marked by inverted green triangles in the top line). The four CpG sites are numbered 1 to 4, and these are respectively located 389, 377, 368, and 90 bp upstream of the beginning of the first synthetic S α repeat. This region was sequenced between 40 and 84 times for the cellular clones with and without the CpG sites. Some sequences are identical to one another, and these can be excluded from the calculation of how many alleles from each type of cellular clone were analyzed (with or without CpG sites), as indicated in the second column. The ratios in the Site 1 to 4 columns indicate how many molecules were methylated (numerator) among all of the molecules sequenced (denominator). Regardless of whether duplicates are excluded, it is clear that the presence of CpG sites favors methylation of the four CpG sites in the I α promoter.

Figure 5. Random Integration of the I α -S α Segment in the Murine Genome Shows that Methylation around I α is Not Strictly Dictated by the Presence of CpG Sites in the IgH Switch Repeats

The I α -S α segment was randomly integrated by co-transfection with a linearized hygromycin expression vector (pHYG). Cellular clones were selected and expanded in culture. These clones were then analyzed using bisulfite genomic sequencing for DNA methylation. Some cellular clones do show methylation at the sites in and around the I α promoter and some do not, indicating that factors outside of the I α -S α segment influence methylation at the I α promoter.