

Evolution of the Immunoglobulin Heavy Chain Class Switch Recombination Mechanism

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Abstract	157
1. Overview of Genetic Alterations in B Lymphocytes	158
2. Activation-Induced Cytidine Deaminase.....	162
3. Role of Germ Line Transcription and Switch Regions in CSR.....	169
4. Posttranscriptional Regulation of AID	180
5. Mechanisms Involved in Synapsis of AID Initiated DSBs in Widely Separated S Regions.....	184
6. General DNA Repair Systems in the Joining Phase of CSR	189
7. Evolution of CSR.....	197
References	198

Abstract

To mount an optimum immune response, mature B lymphocytes can change the class of expressed antibody from IgM to IgG, IgA, or IgE through a recombination/deletion process termed immunoglobulin heavy chain (IgH) class switch recombination (CSR). CSR requires the activation-induced cytidine deaminase (AID), which has been shown to employ single-stranded DNA as a substrate in vitro. IgH CSR occurs within and requires large, repetitive sequences, termed S regions, which are parts of germ line transcription units (termed "C_H genes") that are composed of promoters, S regions, and individual IgH constant region exons. CSR requires and is directed by germ line transcription of participating C_H genes prior to CSR. AID deamination of cytidines in S regions appears to lead to S region double-stranded breaks (DSBs) required to initiate CSR. Joining of two broken S regions to complete CSR exploits the activities of general DNA DSB repair mechanisms. In this chapter, we discuss our current knowledge of the function of S regions, germ line transcription,

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AID, and DNA repair in CSR. We present a model for CSR in which transcription through S regions provides DNA substrates on which AID can generate DSB-inducing lesions. We also discuss how phosphorylation of AID may mediate interactions with cofactors that facilitate access to transcribed S regions during CSR and transcribed variable regions during the related process of somatic hypermutation (SHM). Finally, in the context of this CSR model, we further discuss current findings that suggest synapsis and joining of S region DSBs during CSR have evolved to exploit general mechanisms that function to join widely separated chromosomal DSBs.

1. Overview of Genetic Alterations in B Lymphocytes

B lymphocytes are critical components of the mammalian immune system as they can generate antibodies against almost any foreign pathogen. This enormous diversity is achieved by the unique ability of a B cell (and a T cell) to somatically modify its genome. Somatic diversification of immunoglobulin genes in B cells occurs in several developmental stages and involves three distinct processes. First, developing B cells in fetal liver and adult bone marrow assemble the variable regions of the immunoglobulin heavy (IgH) and light (IgL) chain genes from component variable (V), diversity (D), and joining (J) gene segments by the process of V(D)J recombination. This site-directed recombination reaction is initiated by the lymphoid-specific RAG-1 and RAG-2 endonuclease and completed by components of the nonhomologous end-joining (NHEJ) machinery (Dudley *et al.*, 2005; Jung and Alt, 2004; Jung *et al.*, 2006; Schatz and Spanopoulou, 2005). Productive V(D)J recombination at the IgH locus leads to the generation of a transcription unit in which the complete variable region exon lies upstream of a series of C μ heavy chain (HC) constant region exons. Processing of the resulting primary transcripts generates a mature mRNA that encodes the V(D)J-C μ protein known as a μ HC (Fig. 1). The μ HC pairs with a κ or λ light chain (LC) synthesized from a productively recombined κ or λ locus to form an IgM molecule, which leads to the generation of a surface IgM⁺ B cells. The mature IgM⁺ B cell then migrates to the secondary lymphoid organs such as the spleen and lymph nodes, where it may encounter a cognate antigen and be activated to undergo two additional diversification reactions, namely IgH class switch recombination (CSR) (Chaudhuri and Alt, 2004; Dudley *et al.*, 2005; Honjo *et al.*, 2002; Kenter, 2005; Maizels, 2005; Manis *et al.*, 2002b; Min and Selsing, 2005; Stavnezer, 1996) and somatic hypermutation (SHM) (Goodman *et al.*, 2007; Li *et al.*, 2004; Martomo and Gearhart, 2006; Neuberger *et al.*, 2005; Papavasiliou and Schatz, 2002a).

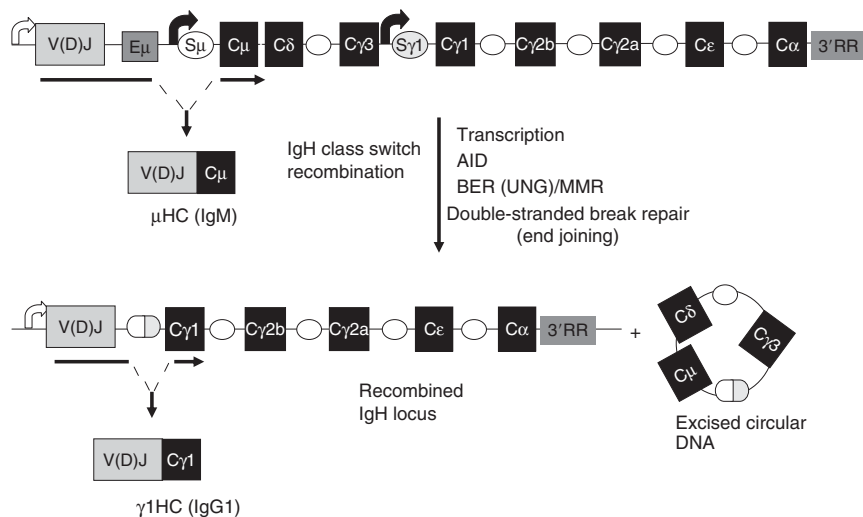


Figure 1 IgH CSR. The assembled V(D)J variable region segment together with C μ (or C δ) exons constitutes a transcription unit (arrows depict promoters) which synthesizes a μ HC that pairs with a κ or λ LC to generate an IgM-expressing mature B cell. On encountering antigens in the peripheral lymphoid compartments, B cells undergo IgH class switching. CSR replaces C μ with one of the downstream C H genes so that a new antibody isotype (IgG1 is shown) can be expressed. CSR occurs between repetitive S region sequences (ovals) that precede each C H gene and requires transcription, AID and components of several DNA repair pathways including BER, MMR, end-joining, and general DNA DSB response proteins. UNG, a component of BER plays an essential role in CSR downstream of AID activity. The IgH enhancers E μ and 3' regulatory region (3'RR) are shown. The 3' regulatory region is represented as 3'RR. See text for more details.

CSR is a process that, within a clonal B-cell lineage, allows the assembled V(D)J exon to be expressed first with C μ exons and then with one of several sets of downstream C H exons (referred to as C H genes) (Honjo and Kataoka, 1978). Thus, CSR allows production of different IgH isotypes or antibody classes (e.g., IgG, IgE, and IgA), which are determined by the different IgH constant region genes (e.g., C γ , C ϵ , and C α), with maintenance of the same variable region antigen-binding specificity. The C H region of the HC molecule determines the effector function of the antibody molecule, for example where the antibody goes in the body or what types of downstream antigen elimination pathways are activated once cognate antigen is bound (Chaudhuri and Alt, 2004; Honjo *et al.*, 2002). The mouse IgH locus consists of eight C H genes (Chaudhuri and Alt, 2004; Honjo and Kataoka, 1978; Stavnezer, 1996), each of which is preceded by long switch (S) regions. Unrearranged C H genes are also organized into

germ line transcription units in which transcription is initiated from a promoter lying upstream of individual S regions (see below). CSR is a deletional recombination event that occurs via the introduction of DNA double-stranded breaks (DSBs) into two participating S regions followed by joining of the broken S regions to each other accompanied by deletion of all of the intervening sequences, including the various C_H genes (Cory and Adams, 1980; Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; von Schwedler *et al.*, 1990). Thus, CSR juxtaposes the expressed V(D)J exon to a new, downstream C_H gene (Fig. 1).

We have learned that the initiation of the normal CSR process has several required components, including S regions, transcription through the participating S regions, and activation-induced cytidine deaminase (AID) (Chaudhuri and Alt, 2004; Honjo *et al.*, 2002) (Fig. 2). The functions of each of these components will be discussed in detail below. In our current model for the initiation of CSR, germ line transcription is required to allow AID to access targeted S regions and, thereby, to generate lesions that are ultimately turned into DSBs. This model for CSR also suggests that once DSBs are generated in two S regions, that, at least in large part, they are synapsed (e.g., brought

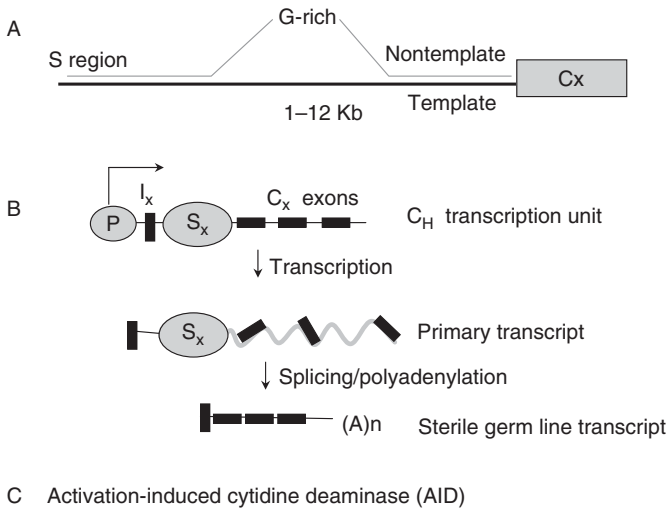


Figure 2 Basic requirements for the initiation of IgH CSR. (A) Switch regions are characterized by repetitive DNA sequences with a G-rich nontemplate strand. (B) C_H genes are transcription units with transcription initiating from a cytokine-inducible promoter (P) upstream of an I exon. The primary transcript undergoes splicing to remove the S region (S_x) and is polyadenylated, but the mature product, referred to as germ line transcript, does not encode a protein. (C) The activated B-cell-specific protein AID is essential for CSR and somatic hypermutation as outlined in the text.

together over large distances) and joined by general DNA repair processes with the actual joining employing forms of general cellular DNA end-joining and DNA repair pathways (Zarrin *et al.*, 2007). We will also discuss briefly the synapsis and joining pathways employed for CSR.

The mechanism by which CSR is initiated is intimately related to that of SHM, and, as we discuss later, CSR well may have evolved from SHM. For comparison to CSR in this chapter, we provide here only a very brief introduction to SHM. More detailed discussions of SHM can be found elsewhere (Goodman *et al.*, 2007; Li *et al.*, 2004; Neuberger *et al.*, 2005; Odegard and Schatz, 2006). SHM introduces point mutations, and sometimes, small insertions and deletions, at a very high rate ($\sim 10^{-3}$ to 10^{-4} per bp per generation) into the variable region exons of both IgH and IgL genes to allow selection of B cells that generate mutated variable regions that have increased affinity for antigen (Crews *et al.*, 1981; Griffiths *et al.*, 1984; McKean *et al.*, 1984; Rajewsky, 1996; Sablitzky *et al.*, 1985). SHM can occur throughout the assembled variable region exon; however, a majority of the mutations are focused to the so-called RGYW “hot spot” sequence (where R = purine, Y = pyrimidine, and W = A or T nucleotide) (Rogozin and Diaz, 2004; Rogozin and Kolchanov, 1992). While other nomenclature is used, for example the RGYW complementary sequence WRCY or the subset WRC (Goodman *et al.*, 2007; Pham *et al.*, 2003; Yu *et al.*, 2004), we will use RGYW as a generic term to cover all SHM hot spot motifs.

RGYW motifs are particularly abundant in the complementarity determining regions of IgH and IgL variable region genes that encode the antigen-binding domains (Wagner *et al.*, 1995). SHM requires AID and transcription through the assembled variable region exons, with mutations being observed from 100 to 200 bp from the promoter and extending 1.5- to 2-kb downstream of it, sparing the constant regions (Gearhart and Bogenhagen, 1983; Lebecque and Gearhart, 1990; Pech *et al.*, 1981; Rada *et al.*, 1994; Rothenfluh *et al.*, 1995; Winter *et al.*, 1997). In transgenic SHM substrate studies, variable region exons or their promoters could be replaced with various other sequences (Michael *et al.*, 2002; Peters and Storb, 1996; Tumas-Brundage and Manser, 1997; Yelamos *et al.*, 1995) and non-Ig promoters (Betz *et al.*, 1994; Tumas-Brundage and Manser, 1997), respectively, without markedly affecting the mutability of the sequence, indicating that the primary sequence of the V genes are not unique in ability to target the SHM machinery. SHM can also function to varying degrees on certain non-Ig genes (Gordon *et al.*, 2003; Muschen *et al.*, 2000; Muto *et al.*, 2006; Pasqualucci *et al.*, 1998, 2001; Shen *et al.*, 1998). At this time, the precise sequence requirements and *cis*-acting elements that target SHM *in vivo* have not been defined fully. Potential factors that influence targeting of SHM have been discussed elsewhere (Odegard and

Schatz, 2006; Yang, 2007) and will be covered only in part here, mainly in the context of the topic of factors that help target AID activity in CSR.

2. Activation-Induced Cytidine Deaminase

The discovery of AID represented a huge advance in our understanding of mechanisms of immunoglobulin gene diversification (Muramatsu *et al.*, 1999, 2000). AID was discovered in a subtractive hybridization screen for genes that are upregulated in a B-cell line (CH12-F3) undergoing CSR (Muramatsu *et al.*, 1999). AID is a 24-kDa protein expressed almost exclusively in activated B cells (Muramatsu *et al.*, 1999). Targeted deletion of AID in mice or mutations of the gene in hyper IgM patients led to a complete block in both CSR and SHM (Muramatsu *et al.*, 2000; Revy *et al.*, 2000). While the function of AID in CSR and SHM is a subject of debate (see below), the most widely accepted view is that AID functions as a single-stranded (ss)-specific DNA deaminase that catalyzes the deamination of cytidine residues in transcribed S regions and V genes during CSR and SHM, respectively (see below; Goodman *et al.*, 2007). In this regard, biochemical data indicates that efficient deamination of chromosomal DNA by AID may rely, at least in part, on structural features of transcribed double-stranded (ds) DNA substrates that allow them to form ssDNA structures or on additional factors that interact with AID to promote its access to transcribed dsDNA substrates (Chaudhuri *et al.*, 2004; see below). The deaminated DNA then appears to be processed by components of several DNA repair pathways, that for CSR lead to the generation of DSBs required to initiate CSR. In chicken B cells, AID also is required to initiate the gene conversion process that is involved in diversifying assembled Ig variable region genes in this species (Arakawa *et al.*, 2002; Harris *et al.*, 2002b; Yang, 2007).

2.1. AID Is the Major Specific Factor for CSR and SHM

In addition to being absolutely required for initiation of CSR and SHM, ectopic AID expression in nonlymphoid cells promoted CSR and SHM of artificial substrates, indicating that AID is also sufficient to induce these processes in nonlymphoid cells (Martin *et al.*, 2002; Okazaki *et al.*, 2002, 2003; Wang *et al.*, 2004; Yoshikawa *et al.*, 2002). However, in these studies, the rate of CSR was low, the spectrum of SHM was limited (primarily transition mutations at G:C bases), and there was no target or region specificity, as several transcribed genes were found to be mutated independent of chromosomal location. Thus, while current studies clearly identify AID as the key factor for B-cell-specific

initiation of CSR and SHM, reminiscent of the key role of the RAG endonuclease in initiating V(D)J recombination in developing lymphocytes (Jung *et al.*, 2006), the possibility remains that other B-cell-specific factors, yet to be identified, may still function to mediate efficiency and specificity of CSR and SHM *in vivo*.

2.2. AID Is Required Upstream of DNA Lesions

Several studies suggested that AID is required for the generation of DNA lesions in S regions. First, foci of phosphorylated histone H2AX (γ H2AX), which normally form around chromosomal DNA DSBs (Rogakou *et al.*, 1998), were observed around S regions in an AID-dependent fashion (Petersen *et al.*, 2001). Second, DSBs detected by ligation-mediated PCR (LM-PCR) in S regions of B cells undergoing CSR (Wuerffel *et al.*, 1997) were found to be AID dependent (Catalan *et al.*, 2003; Schrader *et al.*, 2005). In addition, S region somatic mutations, which may be related to AID function in initiation of CSR, are abrogated in AID-deficient mice (Nagaoka *et al.*, 2002; Petersen *et al.*, 2001). Also internal deletions of S regions, that most likely reflect joining of DSBs on individual S regions during CSR (see below), are largely AID dependent (Dudley *et al.*, 2002). We note, however, that LM-PCR assays were reported to detect AID-independent DNA breaks in V genes leading to the suggestion that AID may have functions downstream of DSBs (Bross and Jacobs, 2003; Papavasiliou and Schatz, 2002b; Zan *et al.*, 2003); however, it is not clear that these DSBs actually represent lesions directly related to SHM (Faili *et al.*, 2002). Thus, the generally accepted view is that AID is required upstream of the cleavage reaction in CSR.

2.3. DNA Deamination Versus Putative RNA Editing Activities of AID

The role of AID in generating DNA lesions in S regions has been a subject of intense debate and significant controversy. Early on, both DNA and RNA were proposed to be potential substrates of AID with respect to SHM and CSR (Martin *et al.*, 2002; Muramatsu *et al.*, 2000; Neuburger and Scott, 2000; Petersen-Mahrt *et al.*, 2002; Tian and Alt, 2000a). Here, we will discuss current evidence in support of each model. Evidence argued to support an RNA editing model for AID activity will be presented first and is covered in more detail elsewhere (Muramatsu *et al.*, 2007). Subsequently, evidence in support of the DNA deamination model will be presented along with a critical evaluation of data that has been proposed to argue against the DNA deamination model.

2.4. The RNA Editing Hypothesis

There are two major lines of evidence that have been used to argue for the hypothesis that AID effects CSR via RNA editing. The original line of evidence for RNA editing was based on the homology of AID to an RNA editing cytidine deaminase APOBEC1 (Muramatsu *et al.*, 1999). In this regard, AID was proposed to edit a cellular mRNA to generate a new mRNA that encodes a novel endonuclease required to activate CSR and SHM (Muramatsu *et al.*, 2000). This proposal was later extended to include the proposal that there are specific SHM and CSR mRNA targets of AID, based on observations that certain mutations in AID block CSR but not SHM and vice versa (Shinkura *et al.*, 2004; Ta *et al.*, 2003). Thus, it was argued that AID could associate with particular proteins so that the resulting complex edits one mRNA that encodes a factor required for SHM and another mRNA that encodes a factor required for CSR (Honjo *et al.*, 2005). To date, however, no SHM or CSR-specific AID cofactor or mRNA substrates have been identified. Moreover, it is now clear that APOBEC1 is a fairly recent evolutionary arrival and is apparently derived from AID. The only other descendants of AID whose function is known are the APOBEC3 family, members of which deaminate the DNA of retroviral replication intermediates (Conticello *et al.*, 2007; Gourzi *et al.*, 2006; Petersen-Mahrt, 2005; Rosenberg and Papavasiliou, 2007).

A second line of evidence that has been argued to support the RNA editing hypothesis versus a DNA deamination model of AID activity was based on the observation that *de novo* protein synthesis is required for generation of AID-dependent binding of γ H2AX to the IgH S regions in activated B cells (Begum *et al.*, 2004b) and for CSR (Doi *et al.*, 2003). However, an alternative explanation of these findings, that would not be inconsistent with the DNA deamination model, is that CSR requires highly labile proteins that function downstream of deamination to generate DSBs.

Thus, the RNA-editing model is still based largely on a presumed analogy with APOBEC1 function, without direct evidence in its support. In particular, no biochemical RNA editing activity has been described for AID. However, it is conceivable that if AID does indeed edit a particular RNA, the specific RNA sequence and/or specific cofactors would be required to demonstrate the editing activity. Finally, to date, no one has found any edited mRNA sequences that encode the putative SHM and CSR lesion-generating enzymes.

2.5. The DNA Deamination Model for AID Function

The DNA deamination model forms the basis for much of our current thinking and understanding of how AID initiates the CSR and SHM reactions. Thus, the basic tenets of this proposal, as put forward by Neuberger and colleagues for

SHM and CSR (Petersen-Mahrt *et al.*, 2002), are outlined below. There is also a significant body of data that relates the AID deamination models to potential functions of AID in initiating gene conversion as a mechanism to diversify chicken variable region genes that is discussed in detail elsewhere (Arakawa *et al.*, 2002; Harris *et al.*, 2002b; Yamazoe *et al.*, 2004; Yang, 2007).

It has been proposed that AID functions in SHM to convert dC to dU in variable region exons (Petersen-Mahrt *et al.*, 2002). Subsequently, during DNA replication, dU is read as dT by DNA polymerase, resulting in transition mutations that change dC:dG base pairs to dA/dT base pairs. In addition, dU in DNA can be converted into an abasic site by removal of uracil base by uracil DNA glycosylase (UNG), an isoform of which, UNG2, is expressed highly in proliferating B cells (Kavli *et al.*, 2005). This reaction is normally an early step in the repair of mismatches by the base excision repair (BER) pathway. However, if UNG activity, instead, is directly followed by replication across the abasic site, then any of the four nucleotides could be inserted, thereby leading to both transition (purine to purine, pyrimidine to pyrimidine) and transversion (purine to pyrimidine and vice versa) mutations at dC:dG bases. Finally, the mismatch repair (MMR) pathway also could process the dU:dG mismatch. The Msh2/Msh6 mismatch recognition complex, and its associated proteins such as Pms2/Mlh1 and exonuclease 1 (EXO1), can introduce nicks or single-stranded gaps distal to the initial site of deamination (Li *et al.*, 2004). Subsequently, gap-filling by recruited error-prone polymerases, such as polymerase η (Wilson *et al.*, 2005), could lead to additional mutations. Thus, AID-mediated DNA deamination of dCs, followed by activities of UNG and MMR proteins, theoretically can explain the entire spectrum of mutations observed during SHM *in vivo* (Petersen-Mahrt *et al.*, 2002).

For CSR, the DNA deamination model predicts that UNG activity followed by activity of the apurinic/apyrimidinic endonuclease (APE1), another normal BER component, will create a nick in S region DNA (Petersen-Mahrt *et al.*, 2002). Two closely spaced nicks on opposite strands of S regions could then lead to a DNA DSB. In addition, gaps and nicks created by MMR proteins as described for SHM, if overlapping on opposite strands of S region DNA, could also lead to DSBs in S regions (Chaudhuri and Alt, 2004; Li *et al.*, 2004; Min and Selsing, 2005). More details about the role of S regions as substrates for generating multiple AID-initiated DSBs required for CSR will be discussed below. However, we note that several key aspects of the DNA deamination model as applied to CSR remain to be elucidated, in particular, the mechanism by which AID-initiated lesions are turned into DSBs rather than just being repaired by the BER or MMR pathways (see below). A very intriguing question is why these lesions are turned into mutations in variable region exons during SHM and DSBs (as well as mutations) in S regions during CSR. One simple

possibility may be just the increased density of AID target motifs in S regions versus variable regions (Chaudhuri and Alt, 2004); while another, not mutually exclusive, possibility could be the existence of specific cofactors that might direct one process versus the other (Barreto *et al.*, 2003; Shinkura *et al.*, 2004; Ta *et al.*, 2003). Evidence relevant to consideration of the DNA deamination model of AID function is discussed in the next section.

2.6. Experimental Findings Relevant to Consideration of the DNA Deamination Model for AID Function

2.6.1. AID Mutates DNA in Bacteria

The first evidence in support of the DNA deamination model was based on the observation that enforced expression of AID in *Escherichia coli*, which is not expected to harbor an mRNA substrate for AID, led to mutations of several bacterial genes (Petersen-Mahrt *et al.*, 2002; Ramiro *et al.*, 2003). It is to be noted, however, that DNA deamination activity appears to be a function of all polynucleotide cytidine deaminases, as other members of this family, including APOBEC3 and even APOBEC1, had similar mutagenic activities in bacteria (Harris *et al.*, 2002a). Still, the finding that AID can mutate DNA in bacteria (Petersen-Mahrt *et al.*, 2002) provided the impetus to test and prove whether AID indeed functions by deaminating DNA during CSR and SHM. Below, we describe the various experimental approaches employed to test predictions of the DNA deamination model and also discuss results that have been presented in the context of challenging some of the interpretations of these findings.

2.6.2. AID Is an ssDNA-Specific Cytidine Deaminase In Vitro

Biochemical assays, using either recombinant or purified B-cell AID, showed that AID efficiently deaminates dC to dU on ssDNA but not dsDNA (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003, 2004; Dickerson *et al.*, 2003; Pham *et al.*, 2003; Sohail *et al.*, 2003; Yu *et al.*, 2004). More details about biochemical analyses of AID activity in relationship to transcription of dsDNA substrates will be discussed below and elsewhere (Goodman *et al.*, 2007). Notably, RGYW SHM hot spot motifs served as preferred sites for deamination *in vitro* (Chaudhuri *et al.*, 2004; Pham *et al.*, 2003; Yu *et al.*, 2004). Therefore, the *in vitro* biochemical activity of AID provides a striking recapitulation of the *in vivo* targets for SHM and CSR. In this regard, in addition to variable region exons, mammalian S regions and *Xenopus* S regions, the latter of which unlike mammalian S regions cannot form R-loop structures that are AID targets (see below), are very rich in RGYW sequences (Zarrin *et al.*, 2004). Thus, the

matching pattern of biochemical activity of AID with its *in vivo* SHM and CSR target sequence preferences provides very strong support that AID directly works on DNA substrates *in vivo*. The alternative RNA editing view would need to make the cumbersome argument that AID edits two different transcripts to generate two different DNA-modifying enzymes with the same general DNA substrate specificity as found, by biochemical analyses, for AID itself.

2.6.3. Binding of AID to S Regions

When overexpressed as a tagged protein in B cells, AID was found to specifically localize to S γ and S ϵ regions via chromatin immunoprecipitation (ChIP) assays performed with B cells activated for CSR (Nambu *et al.*, 2003). The association of AID with S regions strongly supported the notion that AID functions directly on DNA. However, this conclusion was questioned by another study that argued tagged AID did not go to the S μ region based on ChIP analyses of activated B cells (Begum *et al.*, 2004a). However, subsequent studies, in which ChIP experiments were carried out on the authentic endogenous AID protein in primary B cells activated for CSR, revealed a clear and definitive correlation between activation-dependent transcriptional activation of specific C_H genes and AID binding to the appropriate downstream regions (Chaudhuri *et al.*, 2004), confirming the earlier findings with the tagged AID proteins (Nambu *et al.*, 2003). Thus, in splenic B cells activated with lipopolysaccharides (LPS) (that directs CSR to IgG2b, see below), AID associated primarily with S γ 2b as opposed to S γ 1, while in B cells activated with LPS + interleukin 4 (IL-4) (that promote CSR to IgG1, see below), AID was bound primarily to S γ 1 as opposed to S γ 2b. In studies of the normal cellular AID protein, binding of AID to S μ was also clearly observed (Chaudhuri and Alt, unpublished observations). Thus, the association of AID with appropriate CSR target sequences in activated B cells strongly supports the notion that AID acts directly on DNA *in vivo*.

2.6.4. UNG/MMR Mutations Alter the Spectrum of SHM and Severely Impair CSR

Genetic studies have provided very strong support for the DNA deamination model. Inhibition or mutation of UNG in chickens, mice, and humans leads to a pronounced skewing of SHM to transitions at dC (Di Noia and Neuberger, 2002; Imai *et al.*, 2003; Rada *et al.*, 2002b; see also, Durandy *et al.*, 2007), as would be expected if most of the mutations incorporated are due to replication at dU. Furthermore, UNG deficiency led to a severe block in CSR in mice and humans (Imai *et al.*, 2003; Rada *et al.*, 2002b). Moreover, the majority of residual

CSR activity in UNG-deficient mice could be eliminated in UNG/Msh2-double-deficient B cells, indicating that both UNG-related and MMR pathways contribute to CSR (Rada *et al.*, 2004). In addition, in the double mutant, SHM was primarily restricted to transition mutations at dG:dC base pairs as one would predict if all mutations are generated due to replication at dG:dU bases (Rada *et al.*, 2004). It is very difficult to explain the skewing of the SHM pattern and the severe block in CSR without invoking the presence of dU in DNA. This evidence provided very strong support for the DNA deamination model. In this regard, the unrelated uracil-excision enzyme SMUG1 can restore CSR to UNG-deficient B cells (Di Noia *et al.*, 2006) suggesting that the CSR defect caused by UNG deficiency somehow results from a failure to recognize/excise uracil rather than some speculative nonuracil-related function of UNG.

2.6.5. Studies That Have Challenged the Proposed Function of UNG in CSR

Several studies have challenged the proposed role of UNG in CSR. First, using accumulation of γ H2AX as a marker for DSBs, it was shown that inhibition of UNG (using a specific inhibitor Ugi) did not impair DNA cleavage at the IgH locus in B cells undergoing CSR (Begum *et al.*, 2004a). Second, UNG mutants that had apparently lost their U-removal activity rescued CSR in UNG-deficient B cells (Begum *et al.*, 2004a). Finally, B-cell hybridomas derived from UNG-deficient or UNG/Msh2-double-deficient mice revealed similar levels of internal deletions in S μ (a marker for AID-dependent DSBs in S regions, see below) as observed in wild-type B-cell hybridomas (Begum *et al.*, 2006). These observations led to the proposal that the U-removal activity of UNG is dispensable for the generation of the DSBs in S regions. Instead, UNG was proposed to participate in an as yet undefined step in CSR downstream of DNA DSBs (Begum *et al.*, 2004a). Since one of the basic tenets of DNA deamination model proposes UNG-mediated removal of AID-created dU residues prior to DSBs, the above results were interpreted as requiring a reevaluation of the DNA model (Honjo *et al.*, 2005). However, even if this interesting hypothesis that UNG has a novel function downstream of DSBs was proven correct, it would not directly argue against the DNA deamination model for CSR. Moreover, there are several alternative interpretations of these findings outlined in this paragraph that do not invoke a completely novel role of UNG in CSR.

It is possible that UNG inhibition does lead to decreased DSBs in S regions; however, the decrease may not be readily apparent in an indirect DSB assay such as accumulation of γ H2AX molecules. In this regard, a single DSB can cause the nucleation of γ H2AX over megabases of DNA (Rogakou *et al.*, 1999).

Indeed, a more direct LM-PCR assay revealed significantly reduced S region DSBs in UNG-deficient B cells from human patients and mice (Imai *et al.*, 2003; Schrader *et al.*, 2005). Another explanation offered for the finding of catalytically impaired UNG mutants that support CSR is that they still bind DNA dU-DNA and, in this way, may lead to a replication block severe enough to force resolution via MMR pathway (Lee *et al.*, 2004a). It has also been argued that the UNG catalytic mutants analyzed may have low levels of residual U-removal activity (Stivers, 2004). Thus, overexpression of such hypomorphic mutants in the retroviral transduction experiments (Begum *et al.*, 2004a) might result in sufficient UNG activity to restore CSR in UNG-deficient B cells. Another apparent set of contradictory findings involved a human UNG mutant that is severely defective in supporting CSR in a patient (Imai *et al.*, 2003) but which was found to complement the CSR defect of UNG-deficient mouse B cells (Begum *et al.*, 2004a). However, this difference appears to be explained by differential degradation of this mutant AID in human cells versus when overexpressed in UNG-deficient mouse cells (Durandy *et al.*, 2007; Kavli *et al.*, 2005). Also the observed internal deletions within S_μ in UNG or UNG/Msh2-deficient B cells might be explained by accumulation of AID-mediated deaminated residues in the mutant B cells that are channeled into DSBs and deletions on fusion via expression of UNG and/or Msh2 that is present in the hybridoma fusion partner.

2.7. AID Function: Conclusions

There is strong, direct genetic, and biochemical data to support the proposal that AID-mediated DNA deamination is an obligatory step in CSR and SHM. On the other hand, support for an RNA-based model essentially rests on drawing a parallel with apolipoprotein B RNA editing by APOBEC1. Thus, given the compelling body of evidence that supports AID function in CSR via DNA deamination, we will only focus on this working model of AID function in the remaining chapter.

3. Role of Germ Line Transcription and Switch Regions in CSR

3.1. Overview

Most current evidence supports the model that transcription through S regions provides DNA structures that allows AID to initiate CSR on genomic dsDNA. Here, we will review the current evidence that bears on the potential functions that S regions serve in the CSR along with a summary of findings that implicated a role for transcription in regulating and mechanistically targeting CSR.

Then, we will discuss our current understanding about how transcription of S regions makes them accessible to the CSR initiating activities of AID.

3.2. Organization of the IgH C_H Locus

The mouse IgH locus consists of eight sets of C_H exons that are arranged as 5'VDJ-C_μ-C_δ-C_{γ3}-C_{γ1}-C_{γ2b}-C_{γ2a}-C_ε-C_α (Chaudhuri and Alt, 2004; Stavnezer, 1996) (Fig. 1). Each set of C_H exons (except C_δ) is preceded by a 1- to 10-kb long switch (S) regions that are composed of repetitive sequences. In mammals, S regions are unusually G-rich on the template strand and are primarily composed of tandem repetitive sequences within which certain motifs, such as TGGGG, GGGGT, GGGCT, GAGCT, and AGCT, predominate (Davis *et al.*, 1980; Dunnick *et al.*, 1980; Kataoka *et al.*, 1981; Nikaïdo *et al.*, 1982; Obata *et al.*, 1981; Sakano *et al.*, 1980; Takahashi *et al.*, 1980) (Fig. 2A). The length and the distribution of the individual repetitive sequences vary among different S regions. S_μ is exceptionally high in the degree of repetitiveness and enriched in the GAGCT motifs, with the short AGCT palindrome representing the canonical form of the RGYW motif. S_{γ1}, at about 10 kb, is the largest S region. The S_{γ1} sequence, like other S_γ sequences, carries multiple repeats of a characteristic 49-bp unit that among other features is also rich in RGYW (including AGCT) motifs (Obata *et al.*, 1981). Notably, S regions precede all C_H genes that undergo CSR, although in some species the S region sequences can be quite divergent (see below). In addition, the vast majority of CSR junctions occur within (and occasionally just beyond) the S regions (Dunnick *et al.*, 1993; Kinoshita *et al.*, 1999). Thus, it has been reasonably assumed for decades that S regions are the main targets of CSR.

3.3. S Region Function in CSR

Formal proof for the role of S regions in CSR came from gene targeted mutation and replacement experiments. Thus, deletion of the majority of S_μ led to a severe block in CSR to all downstream C_H genes (Khamlichi *et al.*, 2004; Luby *et al.*, 2001). Likewise, deletion of S_{γ1} led to a nearly complete block in CSR to IgG1 but left CSR to other downstream C_H genes intact (Shinkura *et al.*, 2003). In addition, at least for the S_{γ1}, there is a direct correlation between length of the S_{γ1} 49-bp repeat sequence and its ability to promote CSR, as shown by replacing endogenous S_{γ1} with varying lengths of the core S_{γ1} 49-bp repeat units (Zarrin *et al.*, 2005). As CSR to IgG1 is the most frequent form of CSR in mice and S_{γ1} is by far the longest S region, the observed correlation between repetitive S region length and CSR frequency

may imply that S region length, at least in part, may have evolved to modulate CSR frequency (Zarrin *et al.*, 2005). Finally, it must be noted that the decrease in CSR associated with S region deletions or truncations does not prove a specific role for S region sequences per se in CSR, as it remained possible that any sufficiently long spacer sequence would be sufficient. However, additional gene replacement studies showed that non-S region sequences that lacked unusual characteristics of S regions (e.g., repeat structure, G/C rich structure, and so on) did not support CSR, strongly supporting the notion that S region structure, and not just having a certain length of intronic sequence flanking C_H genes, is important for mediating CSR (Shinkura *et al.*, 2003; Zarrin *et al.*, 2005). Together, these experiments provide compelling evidence that S region sequences evolved to serve as specialized targets for the CSR.

3.4. The Role of Germ Line C_H Gene Transcription in Targeting CSR

The germ line C_H genes (except C δ) are organized into characteristic transcription units (Fig. 2) whose induction directly correlates with CSR potential of the associated S region (Manis *et al.*, 2002b). Germ line transcripts that were considered “sterile” because they did not encode any detectable protein were first noted to occur for Abelson murine leukemia virus (A-MuLV) transformed pre-B cells that had an inherent capacity to switch from μ to γ 2b expression in culture; the transcripts also were considered to be germ line based on the fact that they lacked V_H sequences and “sterile” in that they did not encode proteins that cross-reacted with anti- γ 2b antibodies (Alt *et al.*, 1982a,b; Yancopoulos *et al.*, 1986). Similarly, a B-cell lymphoma line (I.29) was found to transcribe several C_H genes, again in an apparently germ line fashion, prior to CSR (Stavnezer-Nordgren and Sirlin, 1986). The transcription of germ line C_H regions in cell lines that were predisposed to switch to those IgH isotypes led to the proposal that germ line transcription of particular C_H genes rendered them “accessible” for CSR (Stavnezer-Nordgren and Sirlin, 1986; Stavnezer *et al.*, 1988; Yancopoulos *et al.*, 1986). This suggested mechanism of control was analogous to that previously suggested for differential control of Ig and TCR gene V(D)J recombination via accessibility to a common V(D)J recombinase (Yancopoulos and Alt, 1985, 1986). As we will discuss, this accessibility mechanism for controlling CSR has been born out by studies of AID function and we now know far more about how transcription of germ line C_H genes renders them accessible for AID function and CSR than we do about how germ line V, D, and J segments become accessible to the RAG endonuclease and V(D)J recombination.

Cloning and sequencing of a “germ line” transcript of the C μ gene provided the first structure of a germ line C_H transcript (Lennon and Perry, 1985).

The C μ transcript was found to be initiated from the intronic enhancer region between J H and C μ and the primary transcript was spliced to generate a “mature” transcript in which a novel noncoding exon was attached to the C μ exons. This transcript did not have any open reading frames that could encode a C μ -containing protein, agreeing with the notion that it was sterile (Lennon and Perry, 1985). Subsequently, cloning and sequencing of a germ line C γ 2b transcript derived from an A-MuLV transformant and comparing it to the structure of the genomic region encompassing the corresponding genomic sequences revealed a similar structure to that of the germ line C μ transcript (Lutzker and Alt, 1988). Subsequent studies of other germ line C H transcripts in mice and humans showed that all of the downstream C H genes were organized into similar transcription units (Manis *et al.*, 2002b; Stavnezer, 2000) (Fig. 2B). In particular, germ line C H transcripts are initiated from a promoter that lies just upstream of a noncoding 5' exon referred to as an “intervening” or I exon (Lutzker and Alt, 1988), proceed through the S region sequence, and are terminated/polyadenylated downstream at normal positions downstream of the C H exons. The primary C H transcripts undergo splicing which fuses the I exon to the C H exons and removes intronic sequences including S regions. However, the processed germ line transcripts are “sterile” because they lack an open reading frame and fail to encode any detectable protein (Manis *et al.*, 2002b). The inability of the germ line C H transcripts to encode proteins and the correlation of their expression with CSR potential provided the first support for the notion that they might function mechanistically to target CSR (discussed further below).

Treatment of B cells with various combinations of activators and cytokines induces them to switch to particular IgH isotypes (Snapper *et al.*, 1988b). For example, treatment of splenic B cells with bacterial LPS induces switching to IgG2b (and IgG3) (Kearney and Lawton, 1975); while also treating LPS-stimulated splenic B cells with IL-4 inhibits switching to IgG2b and induces switching to IgG1 and IgE (Calvert *et al.*, 1983; Kearney *et al.*, 1976; Snapper *et al.*, 1988a). In this regard, studies of normal LPS or LPS + IL-4 treated splenic B cells showed that germ line transcription through the S γ 2b and C γ 2b sequences was induced prior to actual CSR recombination to S γ 2b; while treatment of normal splenic B cells with LPS + IL-4 inhibited induction of γ 2b germ line transcription and CSR to γ 2b, while promoting germ line transcription of and CSR to C γ 1 and C ϵ (Lutzker and Alt, 1988; Rothman *et al.*, 1988; Stavnezer *et al.*, 1988). Similar findings were made for other C H genes and/or cytokines (Esser and Radbruch, 1989; Jabara *et al.*, 1993; Kepron *et al.*, 1989; Mandler *et al.*, 1993; Severinson *et al.*, 1990; Stavnezer, 1995), and subsequent studies showed that downstream germ line I region promoters had elements that allowed them to be specifically activated by particular cytokines

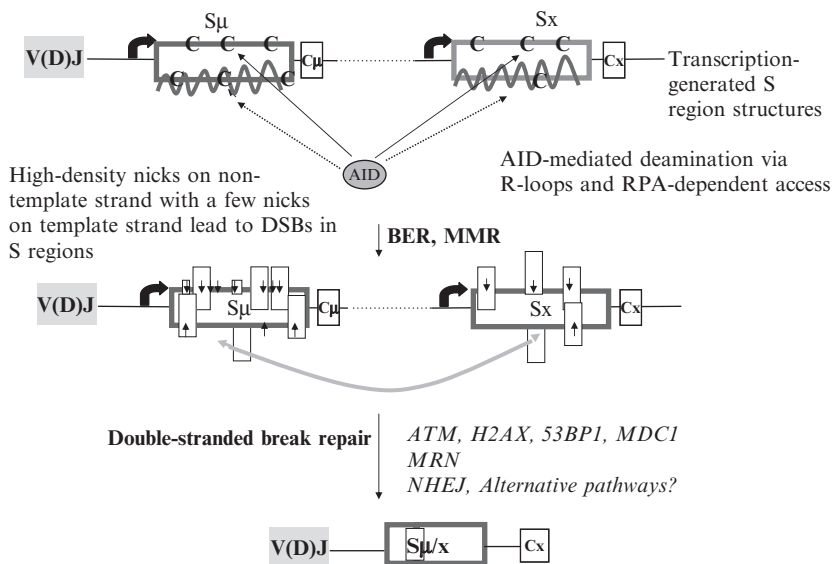


Figure 3 Overall model for CSR. Transcription through S regions generates higher-order RNA–DNA structures, including R-loops, that probably allow both RPA-dependent and -independent access to the nontemplate strand of S regions. The activities of BER and MMR proteins convert the deaminated residues into nicks, and closely spaced nicks on opposite strands of DNA are converted into DSBs. The mechanism that allows AID deamination on the template strand is not well understood, but it is possible that a high density of breaks on the nontemplate strand coupled with a few breaks on the bottom strand is sufficient to generate DSBs. The DSBs between S μ and a downstream S region are synapsed, probably by components of the general DSB response pathway, including ATM, the Mre11/Nbs1/Rad50 (MRN) complex, 53BP1, and MDC1. Subsequently, the DNA breaks are ligated, by the NHEJ or other alternative pathways discussed in the text. See text for more details.

(Manis *et al.*, 2002b; Stavnezer, 2000). Of note, the germ line C μ transcripts appear constitutive and not specifically responsive to any form of B-cell activation or cytokine, perhaps in accord with the fact that S μ is the donor in all types of CSR to downstream C H genes (Li *et al.*, 1994). Therefore, these studies provided strong support that induction of germ line transcription through particular C H genes was somehow linked to determining accessibility for CSR in response to normal physiological stimuli (Figs. 1 and 3). Finally, while not discussed further here, the conserved structural organization and splicing patterns of all germ line C H units also suggest the possibility that transcripts themselves or splicing *per se* may have some role in the CSR process, a possibility with some experimental support (Lorenz *et al.*, 1995) but which mechanistically has remained unclear.

Direct evidence for the mechanistic requirement for germ line transcription in CSR came from gene-targeting studies. Deletion of I exon promoters severely impaired CSR to the corresponding IgH isotypes (Jung *et al.*, 1993; Lorenz *et al.*, 1995; Zhang *et al.*, 1993). Moreover, replacement of I exon promoters with heterologous constitutive promoters activated CSR to particular C_H genes under conditions where CSR to that C_H gene would not normally be induced (Bottaro *et al.*, 1994; Jung *et al.*, 1993; Kuzin *et al.*, 2000; Lorenz *et al.*, 1995; Qiu *et al.*, 1999; Seidl *et al.*, 1998; Zhang *et al.*, 1993); these studies provided the most direct evidence that germ line transcription per se and not just elements deleted with the germ line promoters somehow targeted CSR. Finally, other studies showed that the integrity of elements at the 3' end of the IgH locus, referred to as the 3'IγH regulatory region (Fig. 1; reviewed in Birshtein *et al.*, 1997), were necessary for the induction of germ line transcription from the various downstream C_H genes (Cogne *et al.*, 1994; Pinaud *et al.*, 2001) and led to a “promoter” competition models to, in part, explain how different stimulatory treatments could turn off as well as turn on different germ line promoters and, as a result, CSR to particular C_H genes (Cogne *et al.*, 1994; Manis *et al.*, 2002b). Together, these studies also led to the notion that induction of germ line transcription through S regions might somehow be directly mechanistically involved in targeting the CSR process and not just a correlate of S region “accessibility.” In contrast, it is commonly thought that germ line V segment transcription may not be required for V(D)J recombinational accessibility (Cobb *et al.*, 2006; Jung *et al.*, 2006; Sen and Oltz, 2006).

3.5. Mechanisms by Which Transcription Through S Regions Promotes CSR

The role of germ line transcription was a subject of intense speculation. Transcription was proposed to be required for several nonmutually exclusive steps in CSR, including modifying chromatin configuration (Stavnezer-Nordgren and Sirlin, 1986), providing accessibility to enzymes required for CSR (Stavnezer *et al.*, 1988; Yancopoulos *et al.*, 1986), or generating DNA structures that allow access to a CSR recombinase (Daniels and Lieber, 1995b; Lutzker *et al.*, 1988; Reaban *et al.*, 1994; Stavnezer *et al.*, 1988; Tian and Alt, 2000b). While none of these proposed mechanisms are mutually exclusive, it now appears that transcription through S regions functions, at least in large part, to provide AID access chromosomal S regions during CSR.

3.5.1. R-loops

As mentioned above, mammalian S regions consist of 1- to 12-kb-long DNA sequences that are characterized by a G-rich nontemplate strand and the presence of repeat sequences that, in the mouse, can vary in length between

5 bp ($S\mu$) and 80 bp ($S\alpha$). By virtue of the G-richness of the nontemplate strand, transcription through S regions leads to the generation of higher order DNA structures in which the transcribed RNA stably hybridizes to the template strand (Daniels and Lieber, 1995a, 1996; Mizuta *et al.*, 2003; Reaban and Griffin, 1990; Reaban *et al.*, 1994; Tian and Alt, 2000b). More detailed studies showed that the transcription of mammalian S regions *in vitro* leads to the generation of stable R-loops in which the nascent transcript hybridizes to the template strand and displaces the nontemplate strand as an ssDNA loop (Tian and Alt, 2000b; Yu *et al.*, 2003) (Figs. 2 and 3). Moreover, stable R-loops have also been shown to form on transcription of S regions *in vivo* (Yu *et al.*, 2003). The looped-out nontemplate strand can also assume additional structures such as stem-loops from the abundant palindromic repeats (Tashiro *et al.*, 2001) or can form G4 DNA in which four DNA strands associate via Hoogsteen bonding between planar arrays of G-residues (Dempsey *et al.*, 1999; Larson *et al.*, 2005; Sen and Gilbert, 1988).

Genetic support that R-loops may influence CSR came from the observation that inversion of the endogenous 12-kb $S\gamma 1$ inhibited R-loop formation *in vitro* and was accompanied by a significant decrease (to 25% of WT levels) in CSR to IgG1 in activated mutant B cells *in vivo* (Shinkura *et al.*, 2003). Furthermore, a 1-kb synthetic G-rich sequence that could form R-loops when transcribed *in vitro*, but which had no S region motifs, could replace $S\gamma 1$ to mediate CSR to IgG1, albeit at significantly lower levels than the 12-kb full length $S\gamma 1$ sequence (Shinkura *et al.*, 2003). Strikingly, the ability of the synthetic sequence, like the endogenous $S\gamma 1$ sequence, to mediate CSR was orientation dependent, with CSR observed only in the orientation that favored R-loop formation (G-rich on the nontemplate strand) (Shinkura *et al.*, 2003).

AID is an ssDNA deaminase with no activity on dsDNA (Bransteitter *et al.*, 2003; Chaudhuri *et al.*, 2003; Dickerson *et al.*, 2003; Sohail *et al.*, 2003). As most DNA in cells is in a duplex form, the mechanism by which AID can gain efficient access to dsDNA *in vivo* has also been a topic of considerable interest. The observation that transcribed S regions display ssDNA substrates in the context of R-loops, both *in vivo* and *in vitro* (Tian and Alt, 2000b; Yu *et al.*, 2003), suggested that AID could potentially gain access to S region DNA via such structures. The finding that purified AID efficiently deaminates ssDNA, but not dsDNA, *in vitro* also supports the proposed function for R-loops in the context of CSR. Moreover, purified AID did not deaminate T7 *in vitro* transcribed dsDNA sequences (such as V_H genes) that do not form R-loops but did efficiently deaminate nontemplate strands of an *in vitro* transcribed dsDNA substrates, including S regions, that could form R-loops (Chaudhuri *et al.*, 2003). In agreement with the proposed function of the R-loop, the *in vitro* studies showed that AID only efficiently deaminated the transcribed dsDNA when it

was transcribed in the R-loop forming but not in the reverse, non-R-loop forming orientation (Chaudhuri *et al.*, 2003). Thus, these findings suggested that mammalian S regions may have evolved the capability to generate R-loops on transcription as one means of optimizing AID access to double-stranded chromosomal S region DNA and provided the first clear mechanistic link between S regions, germ line transcription, and AID activity in the context of CSR (Chaudhuri *et al.*, 2003, 2004). Yet, these findings did not explain how AID accesses variable region exons, during SHM, since transcribed variable region exons are not predicted to and were not found experimentally to form R-loops. Additional studies of this issue revealed that AID access to S regions also likely employs additional mechanisms beyond R-loop formation (Fig. 3) as outlined below.

3.5.2. RPA Functions as a Cofactor to Allow AID to Access Certain Transcribed dsDNA Sequences That Do Not Form R-Loops

The R-loop mechanism does not provide an explanation as to how AID gains access to transcribed variable regions during SHM, which are not G-rich on their nontranscribed strand and which do not have a clear-cut propensity to form R-loops. To determine if cofactors could allow AID deamination of substrates that do not form R-loops, an *in vitro* assay was established that measured AID-mediated deamination of transcribed synthetic dsDNA substrates that consisted of repeated RGYW motifs (Chaudhuri *et al.*, 2004). Such substrates do not form R-loops when transcribed *in vitro*. These studies identified the trimeric, ssDNA-binding protein, RPA, as a potential cofactor that allowed AID to efficiently deaminate transcribed DNA, including the RGYW synthetic sequences and several tested variable region exons (Chaudhuri *et al.*, 2004) (Fig. 4). In this context, RPA interacts with AID purified from B cells via its 32-kDa subunit (the trimeric RPA complex consists of 17-, 32-, and 70-kDa subunits) and the AID/RPA complex, but not the two proteins separately, binds well to transcribed RGYW containing DNA *in vitro*, and leads to deaminations that occur near the known SHM hot spot motifs (Chaudhuri *et al.*, 2004). As AID preferentially deaminates RGYW sequences *in vitro* (Pham *et al.*, 2003; Yu *et al.*, 2004), and RPA can bind to ssDNA bubbles as small as four nucleotides (Matsunaga *et al.*, 1996), the AID/RPA complex was proposed to bind to and stabilizes ssDNA in the context of transcription bubbles, thus providing ssDNA substrate for AID-mediated deamination (Chaudhuri *et al.*, 2004) (Fig. 4).

While both AID and Ig gene SHM are found in bony fish, CSR is first observed evolutionarily in amphibians including *Xenopus*, suggesting that CSR evolved after SHM (Mussmann *et al.*, 1997; Stavnezer and Amemiya, 2004). Notably, the S regions of *Xenopus* are long, repetitive sequences that are

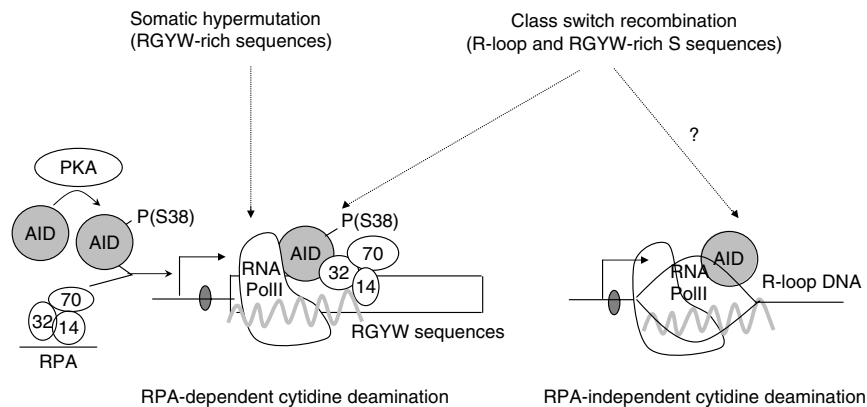


Figure 4 Model for access of AID to DNA during SHM and CSR. Phosphorylation of AID on Ser38 (P-Ser38) by PKA allows interaction with the 32-kDa subunit of the ssDNA-binding protein RPA. The AID/RPA complex can bind to and stabilize ssDNA in the context of small transcription bubbles of transcribed RGYW containing SHM substrates, allowing AID-mediated DNA deamination. Deamination of ssDNA in R-loops formed on transcription of S regions during CSR is probably RPA independent. However, S regions are also rich in RGYW sequences and RPA-dependent AID deamination could provide an additional mechanism to target S regions during CSR. See text for more details.

actually A/T rich and would not be predicted to form R-loops on transcription, a prediction that was confirmed by *in vitro* transcription studies (Zarrin *et al.*, 2004). The fact that *Xenopus* S regions are not R-loop forming structures raised the possibility that either amphibian AID gained access to transcribed dsDNA S region targets by a different mechanism than mammalian AID or that there were other mechanisms that can target AID, even to mammalian S regions. The latter possibility gained further support from the finding that the inverted S γ 1 sequences, that do not form readily detectable R-loops on transcription, possess residual CSR activity *in vivo*. Strikingly, the A/T rich *Xenopus* S μ region was able to replace mouse S γ 1 to promote substantial CSR to IgG1 in mouse B cells *in vivo* (Zarrin *et al.*, 2004). Analysis of CSR junctions showed that *Xenopus* S μ -mediated CSR in mice occurred primarily within AGCT sequences (a subset of the RGYW motif) that are present at a high density in the 3' portion of the *Xenopus* S μ employed and that when this sequence was inverted *in vivo*, CSR junctions tracked with the AGCT motifs (Zarrin *et al.*, 2004). Notably, the dense AGCT motifs within the *Xenopus* S μ were also the preferred sites of deamination by the AID/RPA complex when this sequence was transcribed *in vitro*, regardless of orientation (Zarrin *et al.*, 2004).

These findings support the notion that CSR activities of AID may have evolved from SHM functions of AID (Barreto *et al.*, 2005a; Wakae *et al.*, 2006), the latter of which may rely in part on ability of AID to access sequences rich in transcribed SHM motifs in the context of a complex with RPA (Fig. 4; see below). In this regard, the RGYW SHM motifs may also serve as primordial motifs for the targeting of AID/RPA during CSR (Zarrin *et al.*, 2004). Moreover, because mammalian S regions are particularly rich in RGYW sequences, these motifs are likely to have a role for AID targeting during CSR in mammals (see below; Fig. 3).

3.6. Open Questions Regarding AID Access to Transcribed dsDNA

3.6.1. Template Versus Nontemplate Strand Activities

The generation of DSBs in S regions calls for AID activity on both the non-template and the template strand of DNA, as has been observed (Xue *et al.*, 2006). The issue of AID targeting both DNA strands is also pertinent for SHM where mutations occur on both DNA strands. Neither AID nor the AID/RPA complex (see below) deaminated the template strand of transcribed substrates *in vitro* (Chaudhuri *et al.*, 2003, 2004; Pham *et al.*, 2003; Ramiro *et al.*, 2003), thereby raising the question as to how deamination of both strands is achieved *in vivo*. For CSR, if a high density of nicks were generated on the nontemplate strand, only a few nicks would need to be generated on the template strand to make DSBs (Chaudhuri and Alt, 2004). In the context of an R-loop mechanism, AID might act on the ssDNA exposed at the transition between duplex DNA and the R-loop structure or RNaseH-mediated collapse of R-loop structures could lead to misalignment of repeats on opposite strands, resulting in ssDNA loops on both DNA strands (Yu and Lieber, 2003). Another possibility, for both SHM and CSR, is that there are other, as yet undefined, factors that allow AID to target both strands of transcribed DNA. It is also possible that bidirectional transcription through V genes or S regions could serve to target both strands. In this context, antisense transcription through rearranged variable region exons and S regions has been observed in naive and activated B cells from wild-type mice and in B-cell lines (Perlot and Alt, unpublished observations; Ronai *et al.*, 2007). Finally, it has been proposed that AID itself can target both DNA strands either in the context of supercoiled DNA (in the absence of transcription) (Shen and Storb, 2004) or in the context of transcription with *E. coli* RNA polymerase (Besmer *et al.*, 2006). In the latter context, it is conceivable that transcription by mammalian RNA polymerase may generate access to AID or the AID/RPA complex differently than what is found for the T7 RNA polymerase used in prior studies.

3.6.2. Targeting of AID Activity

A matter of continued debate is the mechanism that allows specific targeting of AID to the Ig locus during CSR and SHM (Barreto *et al.*, 2005b; Chaudhuri and Alt, 2004; Li *et al.*, 2004; Longerich *et al.*, 2006; Odegard and Schatz, 2006). The specificity of AID on S regions could potentially be explained by the uniqueness of S region sequence that predispose them to form higher order structures that could be targeted by AID (Chaudhuri *et al.*, 2003). The targeting of V genes during SHM, however, does not appear to rely on specialized structures, as the V genes could be replaced with non-Ig genes in the context of transgenic SHM passenger gene substrates without obvious effect on mutation rate (Betz *et al.*, 1994; Tumas-Brundage and Manser, 1997). Of all the transcribed genes analyzed in a B cell, the V genes and only a very small number of non-Ig genes (BCL6, CD95, mb1, B29) have been found to be mutated (Gordon *et al.*, 2003; Pasqualucci *et al.*, 1998; Peng *et al.*, 1999; Shen *et al.*, 1998, 2000; Yang, 2007). Other non-Ig genes that do undergo mutations, for example the proto-oncogenes cMYC, RHO/TTF, and PIM1 (Gaidano *et al.*, 2003; Montesinos-Rongen *et al.*, 2004; Pasqualucci *et al.*, 2001), probably represent mistargeted AID activity. Studies with ectopically expressed AID in transgenes have extended the range of genes and cell types in which AID was shown to function *in vivo* (Kotani *et al.*, 2005; Okazaki *et al.*, 2003).

What then allows AID to specifically target the Ig locus during SHM. It is possible that AID is recruited to the transcribing Ig locus via interaction with other protein(s) that interact specifically with *cis*-elements of the Ig locus. In this regard, transgenic studies on a rearranged Igk locus suggested that two enhancers in the Igk locus-iE κ (known as the intronic enhancer, located between J κ 5 and C κ) and 3'E κ (3' enhancers, located 9-kb 3' of C κ) are required for SHM of a transgenic Igk substrate (O'Brien *et al.*, 1987; Sharpe *et al.*, 1991). However, mutational studies of the enhancers in the endogenous locus gave somewhat different results as mice lacking 3'E κ (van der Stoep *et al.*, 1998) or iE κ (Inlay *et al.*, 2006) individually did exhibit major alterations in the frequency of SHM, although more subtle differences were not ruled out. In addition, deletion of either the core E μ enhancer or the 3'IgH enhancer does not appear to substantially impair SHM (Perlot *et al.*, 2005; Ronai *et al.*, 2005). In light of these findings, it will be necessary to further evaluate the putative roles of the enhancers in directing SHM and to assess whether there are additional endogenous elements that may have redundant activities (Odegard and Schatz, 2006). Overall, targeting of AID to the Ig locus could be due to a combination of several factors including transcription rate, presence of the *cis*-sequences in the DNA (such as transcription factor E2A-binding sites; Michael *et al.*, 2003), and the concentration of RGYW hot spot sequences. In the latter

context, it has been shown that efficiency of deamination by the AID/RPA complex is proportional to the density of RGYW (or AGCT) sequences *in vitro* (Chaudhuri *et al.*, 2004) and possibly *in vivo* (Zarrin *et al.*, 2004).

Elucidating the mechanism of AID specificity is of utmost relevance to our understanding of the ontogeny of mature B-cell lymphomas. Overexpression of AID converts it into a general mutator (Martin *et al.*, 2002; Okazaki *et al.*, 2003; Yoshikawa *et al.*, 2002), and deregulated AID activity has been implicated in the ontogeny of mature B-cell lymphomas that form the most common lymphoid malignancies in humans. A large number of diffuse large B-cell lymphomas (DLBCLs) as well as AIDS-related non-Hodgkins lymphoma harbor multiple mutations in several proto-oncogenes including *BCL-6* and *cMYC*, with a mutation pattern reminiscent of that observed during SHM of V genes (Pasqualucci *et al.*, 2001). Such aberrant SHM possibly results from a loss of AID target specificity and could thus be a major contributing factor toward DLBCL pathogenesis. Furthermore, a direct role for AID in the generation of MYC-IgH translocations has been shown in murine models B-cell malignancies (Ramiro *et al.*, 2004, 2006; Unniraman *et al.*, 2004).

It is clear that AID activity has to be stringently harnessed to prevent its loss of specificity and it is, thus, not surprising that AID expression and activity is regulated at multiple levels. These include transcription (Muramatsu *et al.*, 1999) (i.e., AID is expressed only in activated B cells), intracellular localization (Rada *et al.*, 2002a) (i.e., AID is primarily found in the cytoplasm, although it acts on DNA in the nucleus), and now, as described in detail below, AID phosphorylation has emerged as a major mode of regulating AID activity (Basu *et al.*, 2005; Chaudhuri *et al.*, 2004; McBride *et al.*, 2006; Pasqualucci *et al.*, 2006). Finally, it must be remembered that a further level in which AID activity could be controlled is via repair of deaminated cytidines via normal BER or MMR activities, as opposed to further, apparently, aberrant processing of these residues to initiate SHM or CSR. Thus, a further form of control might involve channeling AID-induced lesions into a normal repair pathway versus a pathway that leads to mutations and DSBs (Di Noia *et al.*, 2006). In this regard, the potentially high level of the latter pathway in B cells might also reflect specific factors.

4. Posttranscriptional Regulation of AID

4.1. Nuclear Versus Cytoplasmic Localization

Multiple reports indicate that the majority of AID present in B cells is sequestered in the cytoplasm of B cells (Basu *et al.*, 2005; Pasqualucci *et al.*, 2004; Rada *et al.*, 2002a; Schrader *et al.*, 2005). Considering that AID directly

deaminates DNA at the Ig locus, the cytoplasmic sequestration of AID may be a mechanism to harness its activity. In this regard, AID shuttling between the nucleus and the cytoplasm may be required for optimal regulation of CSR and SHM. AID possesses a defined nuclear localization signal (NLS) at its N-terminus, in addition to a nuclear export signal (NES) at its C-terminus (McBride *et al.*, 2004). Mutation of either of these motifs leads to the abrogation of nucleocytoplasmic shuttling of AID (Barreto *et al.*, 2003; McBride *et al.*, 2004; Shinkura *et al.*, 2004). Recent work also indicated that increased cellular AID levels in mouse B cells led to decreased CSR (Muto *et al.*, 2006). How the cellular level of the AID protein would alter AID activity in CSR is unclear. Perhaps, higher cytoplasmic AID levels may inhibit AID nuclear entry. Notably, phosphorylated AID also appears to be preferentially present in the nucleus of B cells as opposed to the cytoplasm (Basu *et al.*, 2005; McBride *et al.*, 2006). Moreover, studies that employed an antibody that recognizes a major form of phosphorylated AID (see below) have shown that phosphorylated AID is enriched in the chromatin-bound AID fraction (McBride *et al.*, 2006). Thus, phosphorylation-dependent interaction of AID with RPA may promote chromatin association of AID in B cells and, thereby, also contribute to its activity and nuclear localization.

4.2. AID Phosphorylation

Initial clues that AID may be phosphorylated were derived from *in vitro* deamination assays on transcribed SHM substrates (Chaudhuri *et al.*, 2004). It was observed that while AID purified from B cells could efficiently deaminate transcribed dsDNA SHM substrates in an RPA-dependent fashion, AID isolated from ectopically expressing 293 cells could neither interact with RPA nor efficiently promote deamination of transcribed dsDNA *in vitro* (Chaudhuri *et al.*, 2004). Metabolic labeling of activated splenic B cells with ^{32}P -orthophosphate revealed that AID is much more highly phosphorylated in activated B cells than in the line of 293 cells employed for these studies. Significantly, phosphatase treatment of B-cell AID led to a concomitant decrease in the ability of AID to mediate RPA-dependent deamination of transcribed SHM substrates, associated with a failure of dephosphorylated AID to interact with RPA (Chaudhuri *et al.*, 2004).

Mass spectrometric studies revealed that a portion of AID purified from B cells is phosphorylated at two sites, Ser38 and Tyr184 (Basu *et al.*, 2005; McBride *et al.*, 2006). Notably, Ser38 in AID exists in the context of a cAMP-dependent protein kinase A (PKA) consensus motif. In this regard, AID can be phosphorylated *in vitro* by PKA at Ser38 and coexpression of PKA with AID in fibroblast cells led to phosphorylation of AID at Ser38 (Basu *et al.*, 2005;

Pasqualucci *et al.*, 2006). Nonphosphorylated AID that has been phosphorylated by PKA *in vitro* gains ability to bind RPA and mediate dsDNA deamination of transcribed SHM substrates (Basu *et al.*, 2005). Notably, through use of an antibody that detects AID phosphorylated at Ser38, AID ectopically expressed in certain nonlymphoid cell lines was found to be phosphorylated to the same level as AID in activated B cells (McBride *et al.*, 2006). The difference between this result and the lack of phosphorylation observed in 293 cells observed in an earlier study (Chaudhuri *et al.*, 2004) most likely reflects cell type differences. As PKA is a ubiquitous kinase, it would not be surprising that there are cell type and growth condition variations that can alter its activity.

Mutation of AID(Ser38) to alanine AID(Ser38A) markedly decreased ability to phosphorylate AID *in vitro* by PKA and, correspondingly, impaired ability of AID to interact with RPA and mediate deamination of a transcribed SHM substrate *in vitro* (Basu *et al.*, 2005). However, the Ser38A mutation had no observed effect on the ssDNA deamination activity of AID(Ser38A) *in vitro*, indicating that this mutation did not affect the basic catalytic activity of the AID protein. Significantly, AID(Ser38A), expressed from retroviral vectors, was markedly reduced (10–20% of wild-type levels) in ability to restore CSR activity to AID-deficient B cells stimulated to undergo CSR in culture (Basu *et al.*, 2005; McBride *et al.*, 2006; Pasqualucci *et al.*, 2006), consistent with proposal that phosphorylation of AID at Ser38 plays an important role in allowing AID to function at optimal efficiency to effect CSR *in vivo* (Basu *et al.*, 2005). Pharmacological inhibition of PKA in B cells also decreased CSR, whereas conditional deletion of the regulatory subunit of PKA, so as to constitutively activate PKA, led to increased CSR (Pasqualucci *et al.*, 2006), providing additional *in vivo* evidence for the relevance of AID phosphorylation by PKA. Thus, work from multiple laboratories has strongly supported the model that AID phosphorylation is important for augmenting ability of AID to function in CSR, likely through an RPA-mediated mechanism for accessing transcribed S regions (Fig. 4).

In the types of retroviral transduction assays used to measure CSR activity of AID(Ser38A) mutants, expression of the transduced protein can significantly exceed wild-type levels in B cells (Ramiro *et al.*, 2006). Thus, for hypomorphic mutants, such as AID(Ser38A) which retain baseline catalytic activity in CSR, sufficient overexpression could potentially mask the effect of mutations that just decrease the overall activity of the protein in the CSR process. For example, given that the Ser38A mutant AID retained full catalytic activity on ssDNA, residual CSR activity observed for the mutant protein within the retroviral transduction studies might reflect CSR occurring via a non-RPA-dependent (e.g., R-loop) mechanism (Basu *et al.*, 2005; Longerich *et al.*, 2006).

In this regard, one study, in contrast to the findings of others outlined above, reported that the AID(Ser38A) mutation had little effect on CSR in a similar retroviral overexpression assay (Wang *et al.*, 2006). A potential explanation for this apparent discrepancy, supported by preliminary data (Basu *et al.*, 2007), is that retroviral expression vectors employed for the latter study may have led to even higher levels of AID expression than those obtained in the other studies and generated “saturating” expression levels of this hypomorphic mutant. Finally, because AID may be expressed at levels greater than those of the endogenous AID protein in all of the reported retroviral overexpression type assays used to measure ability of the mutant proteins to affect CSR, it remains possible that integrity of the Ser38A residue may prove even more important than estimated. In this regard, the relative contribution of phosphorylation-independent versus phosphorylation-dependent mechanisms to CSR likely will be most accurately evaluated via knock-in approaches that would allow mutant protein expression to occur at normal physiological levels.

It is notable that AID of bony fish, which undergo SHM but not CSR, lacks a Ser38-equivalent PKA-phosphorylation site. Yet, expression of zebra fish AID (zAID) could substantially restore CSR to AID-deficient mouse B cells (Barreto *et al.*, 2005a; Wakae *et al.*, 2006). This intriguing observation opened up several possibilities, including (1) zAID does not require RPA interaction or phosphorylation at a Ser38-equivalent site to efficiently catalyze CSR; (2) zAID acts via RPA, but through phosphorylation at a different amino acid; and (3) zAID acts via RPA, but through a phosphorylation-independent pathway. In the latter context, it was speculated that a D44 residue in zAID might act as constitutive mimic of a phosphorylation site that allows constitutive RPA interaction (Basu *et al.*, 2005). Indeed, preliminary studies suggest that zAID interacts constitutively with RPA and constitutively catalyzes transcription-dependent dsDNA deamination *in vitro*, with both activities being dependent on integrity of the D44 site (Basu and Alt, unpublished). In this regard, it will be of interest to determine whether mutation of D44 to an alanine impairs ability of zAID to mediate IgH CSR in B cells. If so, phosphorylation may have evolved in higher vertebrates as a means to regulate AID activity.

The potential role of AID phosphorylation in SHM also was assessed by a similar retroviral transduction approach (McBride *et al.*, 2006). In these studies, AID(Ser38A) had significantly reduced capacity to mediate SHM on both physiological (S μ in B cells) and artificial substrates (in fibroblasts) as compared to wild-type AID. On the basis of the biochemical studies, it seems quite possible that the strong effect of the Ser38A mutation on SHM may also be due to an inability of AID to access its targets, possibility due to a failure to bind RPA. Indeed, AID phosphorylated on Ser38 was highly enriched in the chromatin fraction (McBride *et al.*, 2006). As for CSR, a more complete assessment

of the potential influence of AID(Ser38A) mutation on SHM of endogenous V genes awaits the generation of B cells with a knock-in mutation.

5. Mechanisms Involved in Synapsis of AID Initiated DSBs in Widely Separated S Regions

5.1. Overview

The completion phase of CSR requires the close juxtaposition of DSBs in two S regions, referred to as synapsis, followed by their ligation. The mechanism that synapses DSBs in S regions, that exists as far as 200 kb apart, is only beginning to be understood. Germ line V, D, and J segments are flanked by short conserved recombination signal sequences (RSs) that are targets for the RAG endonuclease (Jung *et al.*, 2006). During V(D)J recombination in progenitor lymphocytes, an RAG complex binds RS sequences of two participating V, D, or J segments and introduces DSBs between the RSs and the flanking coding sequences. Such RAG cleavage occurs only in the context of a preformed synaptic complex (Jung *et al.*, 2006). Thus, it is thought that RAG itself may be involved in synapsis of V, D, and J segments for V(D)J recombination (Jung *et al.*, 2006). Subsequent to cleavage, V, D, and J DSBs are joined by the NHEJ pathway of DNA DSB repair (Bassing *et al.*, 2002b; Rooney *et al.*, 2004), and RAG appears to be involved in shepherding broken V, D, and J segments specifically into the NHEJ pathway (Lee *et al.*, 2004b). By analogy, it seemed possible that AID and/or S regions may play specific roles in the synapsis and joining processes of CSR. However, as we discuss below, recent work suggests that mechanisms of S region synapsis and joining may predominantly exploit general cellular mechanisms and, at least in part, not rely on AID or S regions *per se*.

5.2. CSR Versus Internal S Region Deletions as Mechanisms to Resolve S Region DSBs

In contrast to RAG activity on V, D, and J segments, AID activity on S regions does not require coordinate interaction between two S regions, as AID can deaminate single S regions *in vitro* (Chaudhuri *et al.*, 2004). Moreover, constitutively transcribed S regions randomly integrated into the genome of a pro-B-cell line undergo high rate of AID-dependent internal deletions (Dudley *et al.*, 2002). In this regard, internal deletions in S regions have been extensively used in the interpretation of data related to synapsis. Internal deletions are readily observed in the endogenous S μ region in splenic B cells or B-cell lines activated in culture to undergo CSR (Alt *et al.*, 1982a; Dudley *et al.*, 2002; Gu *et al.*, 1993; Yancopoulos *et al.*, 1986). Such internal deletions are thought to reflect joining

of two DSBs within S_μ regions, perhaps in the absence of, or synapsis with, a DSB in a downstream S region (Dudley *et al.*, 2002) (Fig. 5). In addition, S_μ also undergoes mutations at a high rate, again possibly reflecting AID activity on S regions independent of CSR (Nagaoka *et al.*, 2002; Petersen *et al.*, 2001). Given that S region mutations and internal deletions are AID dependent (Dudley *et al.*, 2002; Nagaoka *et al.*, 2002; Petersen *et al.*, 2001; Schrader *et al.*, 2005), they probably represent the same general process as CSR, although this has never been formally proven.

While internal deletions in S_μ can be readily detected, those in the downstream S regions are observed less frequently (Dudley *et al.*, 2002; Reina-San-Martin *et al.*, 2003, 2004, 2007; Schrader *et al.*, 2003). One possible explanation for this observation is that S_μ may be a preferred AID substrate due to its sequence and/or proximity to the strong IgH intronic enhancer E_μ , with targeting of AID to downstream S regions constituting the rate-limiting step of CSR. In this scenario, S_μ would be the target of a higher level of DSBs, with some occurring simultaneously, than occurs in downstream S regions. Thus, a significant proportion of simultaneous S_μ breaks may be resolved via short-range intra-S recombinations leading to internal deletions (Fig. 5). This possibility

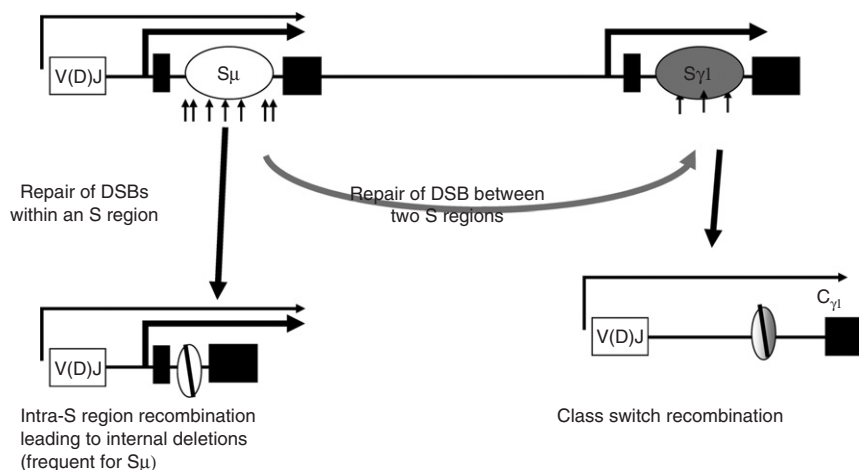


Figure 5 Internal S_μ deletions versus long-range CSR. In addition to productive CSR by long-range DSB repair between two distal S regions, B cells stimulated to undergo CSR also undergo a high rate of internal deletions in S_μ . Such intra-S recombination probably represents resolution of two DSBs (arrows) within S_μ . CSR represents resolution of two DSBs in different S regions. While internal deletions are readily observed in S_μ , those in the downstream S regions are less frequent, possible reasons for which are discussed in the text. See text for more details.

is consistent with the observation that when $\text{S}\mu$ was inhibited from being a donor, internal deletions in $\text{S}\gamma 1$ could be readily detected (Gu *et al.*, 1993). However, it must be acknowledged that there are other possible explanations for the appearance of increased internal $\text{S}\mu$ deletions versus downstream S region deletions, including different efficiencies or mechanisms (e.g., internal deletion versus simple rejoining) of repair of one versus the other. In the latter context, only deletions visible via Southern blotting have generally been assayed in prior studies for internal deletions. It is conceivable that DSBs in downstream S regions may occur more frequently but be repaired via smaller internal deletions/rearrangements, as have been observed in the sequence of an $\text{S}\gamma 2\text{b}$ region downstream of an $\text{S}\mu$ junction (Yancopoulos *et al.*, 1986). In this regard, it was shown that both the acceptor and donor S regions in B cells undergoing CSR accumulate mutations with comparable frequency in B cells undergoing CSR (Xue *et al.*, 2006).

5.3. CSR Has Adopted a General Repair Mechanism to Join/Synapse Distant DSBs

At least three different, not necessarily mutually exclusive, models have been put forward to explain how distal DNA DSBs are synapsed during CSR. First, by analogy to RAG proteins, AID itself may have a direct role in synapsis by binding to two participating S regions and bringing them together by its ability to dimerize (Prochnow *et al.*, 2006; Wang *et al.*, 2006). Certain C-terminal mutations in AID impair CSR without affecting SHM or $\text{S}\mu$ mutations, suggesting that AID activity and targeting to $\text{S}\mu$ is intact (Barreto *et al.*, 2003) and that the C-terminal portion of AID, itself or by recruiting other proteins, could contribute to such a synaptic role. Second, S regions themselves have been proposed to be involved in synapsis. For example, colocalization of transcribed S regions to transcription complexes assembled at the IgH locus may aid in the juxtaposition of S regions (Manis *et al.*, 2002b). In addition, an S region function in synapsis might also involve the binding of MMR complex Msh2/Msh6 to transcribed S regions and to itself (Larson *et al.*, 2005). Formation of S region higher order structures has been implicated in synapsis (Dempsey *et al.*, 1999; Larson *et al.*, 2005). Finally, DNA damage response proteins, such as ATM, 53BP1, and H2AX, have been proposed to facilitate synapsis and joining of distal DSBs by mediating changes in chromatin structure and potentially by physically anchoring distal DSBs (see below) (Bassing and Alt, 2004a; Reina-San-Martin *et al.*, 2003). The first evidence in support of the latter hypothesis came from observations that mutations in these proteins lead to CSR defects, without obviously affecting the rate of large internal $\text{S}\mu$ deletions that were visible via Southern blotting of IgM^+ hybridomas made from activated B cells (Reina-San-Martin *et al.*, 2003, 2004, 2007).

To elucidate potential mechanisms of synapsis during CSR, a novel system was established to test whether “recombinational IgH switching” could be established without S regions or AID when DSBs are artificially generated in place of S regions (Fig. 6) (Zarrin *et al.*, 2007). To accomplish this, S regions were replaced with cassettes containing an 18-bp target to generate for the rare-cutting yeast homing endonuclease I-SceI that recognizes its targets and introduces staggered DSBs (Colleaux *et al.*, 1988; Rouet *et al.*, 1994). B cells were generated via gene targeted mutation in ES cells in which the entire endogenous mouse $\text{S}\gamma 1$ sequence was replaced by two I-SceI sites separated by a 500-bp fragment of DNA that is inert with respect to ability to catalyze CSR *in vivo*. Replacement of $\text{S}\gamma 1$ with this cassette led to an essential inhibition of CSR to IgG1 on the mutant allele. However, on expression of I-SceI endonuclease from a retrovirus in activated mutant B cells, switching to IgG1 was stimulated to levels that in some experiments approached the lower range of wild-type levels (Zarrin *et al.*, 2007). Characterization of recombination junctions revealed that $\text{S}\mu$ was fused to one or the other I-SceI sites, consistent with the direct dependence of IgH class switching to IgG1 on I-SceI expression

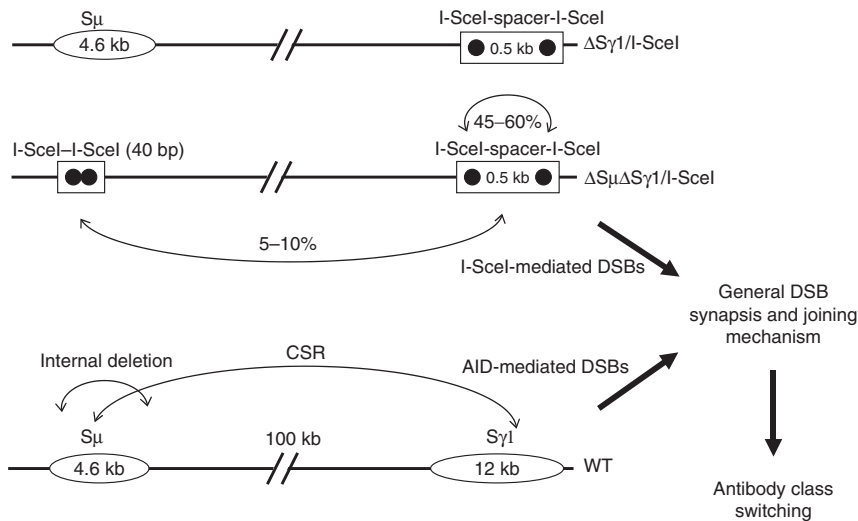


Figure 6 I-SceI-dependent class switching and a model for the role of general DNA repair during IgH CSR. In the absence of S regions and AID, the frequency of I-SceI-mediated DSBs can promote class switching. Short-range recombinations (0.5 kb apart) are joined more frequently (45–60%) than DSBs that are 100 kb away (5–10%). These results (discussed in details in the text) suggest that CSR utilizes the general DNA DSB repair pathway to synapse and join distal S region breaks during CSR. See text for more details. This figure is adapted from Zarrin *et al.* (2007).

in this system. These findings provided strong evidence that a DSB in an S region could bypass some of the requirements for an S region, at least for switching to $\gamma 1$. Moreover, the findings showed that AID-initiated DSBs in the $S\mu$ region, apparently unlike RAG generated DSBs during V(D)J recombination, can be joined efficiently to DSBs generated by an entirely different mechanism. This finding may also be relevant for mechanisms that lead to chromosomal translocations involving AID-initiated DSBs in S regions (Zarrin *et al.*, 2007).

In the experiments described above, only the downstream acceptor $\gamma 1$ region was replaced with an I-SceI site and AID was still present. To formally test potential roles of S regions, B cells were generated in which I-SceI replaced both the $\gamma 1$ and $S\mu$ sequences. The two I-SceI sites that replaced $S\mu$ were only 40 bp apart, compared to the 500 bp that separated the I-SceI sites in $\gamma 1$; however, these two pairs of I-SceI sites in the place of $S\mu$ and $\gamma 1$ were about 100 kb apart within the IgH locus (Fig. 6). The double mutant B cells showed essentially complete loss of ability to switch to IgG1 on the mutant allele that, again, was rescued to substantial levels via retroviral introduction of I-SceI endonuclease expression, showing that S region sequences were not necessary. In fact, I-SceI-dependent switching to IgG1 in B cells in which both $S\mu$ and $\gamma 1$ had been replaced with I-SceI sites occurred at levels as high or higher than in B cells with a full-length endogenous $S\mu$ and a 1-kb $\gamma 1$ (Zarrin *et al.*, 2005, 2007). To address the requirement for AID, IgM⁺ hybridomas were generated from B cells harboring $S\mu$ and $\gamma 1$ replacements. As expected from earlier studies, these hybridomas did not express detectable AID or germ line transcripts of the $C\gamma 1$ locus; nevertheless, infection with I-SceI retrovirus led to a substantial level of class switching to IgG1 (Zarrin *et al.*, 2007). Thus, it would appear that neither S regions nor germ line transcription (within the level detectable by this assay) are required for a relatively high level of recombinational IgH class switching mediated by two I-SceI generated DSBs in the IgH locus. These studies do not rule out roles for either AID or S regions in the joining phase of CSR, for example to promote the most efficient degree of synapsis and joining. However, the surprisingly high rate of recombinational IgH switching mediated via I-SceI DSBs in absence of these factors reveals an unanticipated general repair process that promotes the joining of widely separated IgH locus DSBs. Therefore, S regions and germ line S region transcription may have evolved, at least in large part, to provide structures for AID recognition and deamination *en route* to generation of DSBs with more general mechanisms leading to synapsis and joining (Fig. 6).

The I-SceI model system also allowed a comparative assessment of efficiency of joining between short-range (500-bp internal deletions) and long-range

(100-kb IgH class switching events) I-SceI-generated DSBs. Surprisingly, long-range DSBs were joined at a rate that was as much as 10–20% of the frequency of short-range joins between the two I-SceI sites in the downstream cassette. This pattern is somewhat reminiscent of internal deletions versus long-range CSR events observed in normal activated B cells (Figs. 5 and 6). These results led to the proposal that S regions may have evolved as structures to allow generation of a sufficiently high level of AID-initiated DSBs that CSR would be ensured by a general mechanism that promotes a relatively high frequency of long-range joins between DSBs within a chromosome with respect to the frequency of short-range joins or simple rejoining of a single DSB (Zarrin *et al.*, 2007). However, the question remains as to what mechanisms lead to the frequent synapsis and joining of chromosomal DSBs that are 100 kb or more apart. It is possible that there are features of the IgH locus structure in activated B cells that put S regions in proximity to promote such joining (Chaudhuri and Jasin, 2007; Zarrin *et al.*, 2007). On the other hand, it is also possible that aspects of the normal DSB response, including the generation of repair foci over large (greater than 100 kb) distances may function to join widely separated general DSBs (Zarrin *et al.*, 2007).

6. General DNA Repair Systems in the Joining Phase of CSR

6.1. DNA Damage Response

In mammalian cells, DSBs can be introduced into a cell due to external insults such as exposure to ionizing radiation (IR), metabolic processes such as oxidative stress, as well as via developmentally regulated processes which include V(D)J recombination and CSR in lymphocytes (Bassing and Alt, 2004b). If left unrepaired, DSBs can cause cell death or can give rise to potentially oncogenic translocations (Bassing and Alt, 2004b; Franco *et al.*, 2006a; Xu, 2006). Mammalian cells have therefore evolved a complex network to sense, signal, and repair DSBs. There are two major characterized pathways of DSB repair in mammalian cells. Homologous recombination is a postreplicative repair process that requires large regions of homology and provides accurate repair. The other major known mammalian repair pathway is NHEJ which joins ends that lack homologies or have only short homologies (Bogue *et al.*, 1997, 1998; Li *et al.*, 1995; Roth *et al.*, 1985) and appears to be most important in prereplicative phases of the cell cycle when HR is not operative (Mills *et al.*, 2004). Joining of DSBs via either of these pathways first involves the activation of a DNA damage response, which involves a large number of different proteins. There have been several comprehensive reviews of the DNA damage response (Bassing and Alt, 2004b; Burma *et al.*, 2006; Franco *et al.*, 2006a;

Povirk, 2006; Stucki and Jackson, 2006; Xu, 2006) and it is also covered more extensively by another chapter in this volume (Ramiro *et al.*, 2007). Here, we will introduce the DNA damage response as it pertains to the mechanism of CSR.

The exact sequence of events that lead to the recognition of a DSB and the assembly of proteins that effect cell cycle arrest and mediate repair is still emerging. One of the earliest responders to DSBs is the MRN complex (Lee and Paull, 2005) that consists of the proteins Mre11, RAD50, and Nbs1 (Carney *et al.*, 1998). The MRN complex associates with DSBs immediately following DNA damage (Lee and Paull, 2005) and recruits ATM, which is a serine/threonine kinase. ATM is considered to be one of the master regulators of the DSB response, via direct interaction between the C-terminal domain of Nbs1 and ATM (Falck *et al.*, 2005). ATM is a member of a large family of PI3-related kinases that include ATR (ATM and RAD3-related) and DNA-PKcs (DNA-dependent catalytic subunit catalytic subunit) (Shiloh, 2003). ATM and the related kinases can phosphorylate a large number of DNA repair and cell cycle checkpoint proteins, including the G1 checkpoint protein p53, as well as Nbs1 (Falck *et al.*, 2005), H2AX (Burma *et al.*, 2001; Paull *et al.*, 2000), 53BP1 (Anderson *et al.*, 2001; Rappold *et al.*, 2001), and MDC1 (Lou *et al.*, 2006). Activation of these downstream molecules leads to ATM-dependent cell cycle arrest in response to DSBs and the subsequent repair of the damage (Fig. 7).

An early and critical event in the ATM-dependent response to DSBs is the phosphorylation of DSB-proximal H2AX molecules on Ser139 to form γ H2AX (Rogakou *et al.*, 1998). H2AX phosphorylation leads to the rapid, hierarchical assembly of several repair factors, including MDC1, 53BP1, and NBS1, which via their ability to bind to γ H2AX form multiprotein complexes or foci at sites of DNA damage (Kobayashi *et al.*, 2002; Stewart *et al.*, 2003; Stucki *et al.*, 2005; Ward *et al.*, 2003a). Although H2AX phosphorylation is not required for the initial recruitment of these factors to the DSB (Celeste *et al.*, 2003b), it is necessary for the formation of normal, extended repair foci (Bassing *et al.*, 2002a; Celeste *et al.*, 2003a). Recent studies have highlighted a complex interplay between DSB repair factors and chromatin conformation around DNA breaks. For example, 53BP1 can be recruited to DSBs via its interaction with methylated lysine 79 of histone H3, a chromatin mark that may be exposed during chromatin remodeling following DNA damage (Huyen *et al.*, 2004). Likewise, chromatin-bound MDC1 facilitates the recruitment of additional molecules of ATM to the DSB. Such recruitment then leads to phosphorylation of H2AX molecules that are distal to the initial site of lesion, and generates a positive feedback loop that promotes the stable association of DNA damage response factors in large domains or foci that can spread up to a megabase on either side of the DSB (Lou *et al.*, 2006; Stucki and Jackson, 2006).

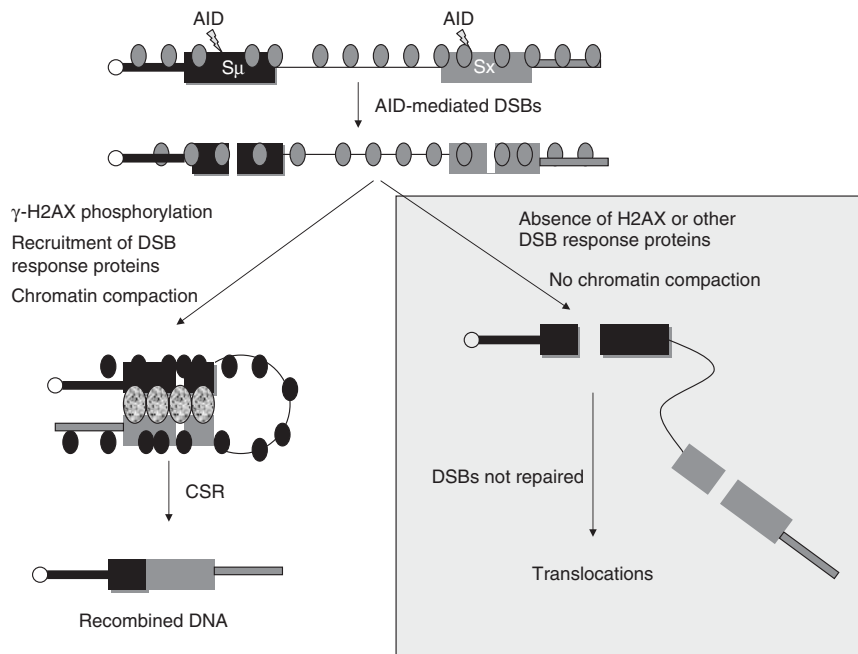


Figure 7 S region synapsis and roles of DSB response proteins during CSR. AID-dependent generation of DSBs induces modifications and mobilization of the DSB response pathway including the phosphorylation of the histone H2AX (gray ovals) to generate γ H2AX (black ovals). Recruitment of several DSB response proteins (ATM, MRN complex, MDC1, 53BP1) (speckled ovals) to the break alters chromatin structure which may facilitate synapsis between two distal DSBs, and hold broken S regions together for repair. In the absence of the DNA damage response proteins such as H2AX (as shown), the breaks are not held together and thus not repaired. Such broken S regions frequently participate in translocations. See text for details.

The formation of repair foci with the accumulated DSB response proteins may allow enforcement of cell cycle checkpoints while, potentially, simultaneously providing a molecular bridge between DNA ends, preventing their dissociation until repair ensues (Bassing and Alt, 2004a; Franco *et al.*, 2006a; Stucki and Jackson, 2006; Xu, 2006). In keeping with this notion, mice and cells deficient for ATM, 53BP1, Nbs1, Mdc1, and H2AX share a number of phenotypes potentially related to DSB repair deficiency, including IR sensitivity, DNA repair defects, and genomic instability (Bassing *et al.*, 2002a, 2003; Kracker *et al.*, 2005; Morales *et al.*, 2003; Reina-San-Martin *et al.*, 2005; Ward *et al.*, 2003b). Moreover, studies of CSR and V(D)J recombination have suggested that these proteins may have a direct influence on DNA DSB repair via end joining (Bredemeyer *et al.*, 2006; Franco *et al.*, 2006b).

6.2. DSB Response Proteins in CSR

Cumulative evidence suggests that the general response to deleterious DSBs also is operative at AID-initiated DSBs during CSR (Fig. 7). The first evidence to support a role for the DSB response in CSR came from the finding that AID-dependent foci of γ H2AX and NBS1 accumulate at the IgH locus of B cells activated *in vitro* (Petersen *et al.*, 2001). In addition, mice deficient for ATM, H2AX, MDC1, NBS1, or 53BP1 are impaired, to variable extents, for CSR (Franco *et al.*, 2006b; Kracker *et al.*, 2005; Lahdesmaki *et al.*, 2004; Manis *et al.*, 2004; Petersen *et al.*, 2001; Reina-San-Martin *et al.*, 2003, 2004, 2005; Ward *et al.*, 2004). In contrast, these DSB response factors are not required for SHM, a process that can occur without DSB intermediates (Manis *et al.*, 2004; Reina-San-Martin *et al.*, 2003). Moreover, mice that are doubly deficient for H2AX and p53 routinely develop B-cell lymphomas, a subset of which has S region translocations (Bassing *et al.*, 2003). How DSB response factors function in general chromosomal DSB repair and more specifically during CSR is not fully understood, but it could be by simply altering chromatin structure around DSBs to allow repair factor access. Alternatively, but not mutually exclusively, it could function by forming large complexes that would hold broken ends together or even help bring more widely separated ends together. In this context, it was proposed that the formation of H2AX-dependent foci of DSB response proteins, most of which also bind each other and/or DNA, might represent an “anchoring” activity that holds DSBs together in a synaptic complex to facilitate their ligation by end joining (Bassing and Alt, 2004a) (Fig. 7). Similar arguments have been made with respect to HR where phosphorylated histone H2A in yeast binds cohesin (Shroff *et al.*, 2004; Unal *et al.*, 2004).

Quantification of the frequency of AID-dependent chromosomal breaks within the IgH locus in single B cells activated for CSR has provided direct support of role of DSB response proteins in the end-joining phase of IgH CSR (Franco *et al.*, 2006b; Ramiro *et al.*, 2006). Specifically, analyses of H2AX-, MDC1-, ATM-, 53BP1-, or NBS1-deficient B cells activated for CSR via two color fluorescence *in situ* hybridization (FISH) with probes from upstream and downstream regions of the IgH locus revealed large numbers of metaphases containing chromosomal breaks within one and sometimes both IgH loci (Franco *et al.*, 2006b; Ramiro *et al.*, 2006) (Fig. 8). Such IgH locus chromosomal breaks were *bona fide* AID-dependent breaks, as they were fully rescued by AID-deficiency *in vivo* (Franco *et al.*, 2006b). Moreover, a substantial percentage of IgH locus breaks observed in these backgrounds participated in chromosomal translocations (Franco *et al.*, 2006b; Ramiro *et al.*, 2006). Therefore, the DSB response plays a major role in preventing DSBs introduced during IgH CSR, as well as general DSBs, from separating and

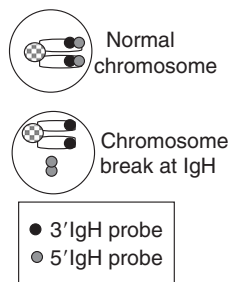


Figure 8 FISH assay commonly used to detect chromosome aberrations in the absence of DSB response proteins. FISH probes that detect the 5' or 3' regions of the IgH locus (gray or black ovals, respectively) can detect unrepaired DSBs in the IgH locus. The striped oval represents the centromere.

progressing into chromosomal breaks and translocations (Franco *et al.*, 2006b). These findings provided strong support for the notion that the DSB response is required to support normal end joining (Bassing and Alt, 2004a; Franco *et al.*, 2006a). Similar conclusions were subsequently made for the role of ATM in V(D)J recombination (Bredemeyer *et al.*, 2006), although the DSB response may not turn out to be as important overall in V(D)J recombination due to the role of RAG proteins in synapsis and/or channeling DSBs into NHEJ (Bassing and Alt, 2004b).

Notably, while ATM-, H2AX-, and 53BP1-deficient B cells had a relatively major impairment in CSR, they still appeared to accumulate internal Sp deletions at normal or (for 53BP1, see below) increased frequency subsequent to activation, leading to the suggestion that such factors also may somehow be involved in long-range S region synapsis but are not necessary for shorter range deletions (Reina-San-Martin *et al.*, 2003, 2004, 2007). In contrast to H2AX-, MDC1-, or ATM-deficient B cells which have only a partial impairment of CSR, 53BP1-deficient B cells are severely impaired for CSR (Franco *et al.*, 2006b; Kracker *et al.*, 2005; Lahdesmaki *et al.*, 2004; Manis *et al.*, 2004; Petersen *et al.*, 2001; Reina-San-Martin *et al.*, 2003, 2004, 2005; Ward *et al.*, 2004). Yet, IgH FISH analyses demonstrated that the frequency of chromosomal breaks within the IgH locus does not differ significantly from those observed in H2AX- or ATM-deficient B cells (Franco *et al.*, 2006b). Moreover, activated 53BP1-deficient B cells proliferate and activate efficiently (Manis *et al.*, 2004; Ward *et al.*, 2004), and 53BP1-deficient cells and mice show only modest growth retardation and moderate cellular genomic instability (Manis *et al.*, 2004; Ward *et al.*, 2004). This group of findings led to the proposal that 53BP1 may have novel functions in CSR (Franco *et al.*, 2006a). In this context,

53BP1-deficient B cells exhibit markedly increased levels of internal deletions versus normal long-range CSR events, raising the intriguing possibility that 53BP1 might have a particularly important function in long-range synapsis (Reina-San-Martin *et al.*, 2007), although it will be important, as discussed for UNG experiments above, to ensure that such deletions happened in normal B cells as opposed to in hybridomas postfusion.

The requirement for DSB response factors to mediate normal levels of CSR, to suppress AID-dependent IgH locus translocations, and to promote long-range CSR reactions versus internal deletions has led to the suggestion that the DSB response may be a key factor involved in promoting the higher than expected joining frequency of widely separated chromosomal IgH locus DSBS (Bassing and Alt, 2004a; Zarrin *et al.*, 2007). To address this model, there are many questions that need to be answered. One would be whether the joining of widely separated versus proximal I-SceI-generated DSBs within the IgH locus would be altered in DSB response factor-deficient cells in a manner observed for internal S region deletions versus CSR events. Another would be whether joining of DSBs separated by 100 kb or more occurred at loci other than the IgH locus and in cells other than activated B cells. Yet another question would be the distance at which DSBs could be separated and still show increased frequency of joining. Moreover, one must ask why mammalian cells would have evolved mechanisms to mediate these long-range interactions in the first place, as opposed to resolving DSBs exclusively through religation of the broken DNA ends. In this context, the frequency of joining I-SceI-generated DSBs separated by 100 kb in the IgH locus is orders of magnitude greater than that observed for translocations between I-SceI-generated DSBs on separate chromosomes (Weinstock *et al.*, 2006). Given the greatly increased frequency of IgH locus translocations in DSB response-deficient B cells (Franco *et al.*, 2006b; Ramiro *et al.*, 2006), one notion would be that the long-range joining mechanisms might be a by-product of a mechanism that evolved to ensure DSB repair within a chromosome and suppress translocations (Bassing and Alt, 2004a; Zarrin *et al.*, 2007).

6.3. End Joining

CSR joins lack major regions of homology and, therefore, are catalyzed by some form of DNA end-joining pathway. The NHEJ pathway has been the prime candidate. In this context, NHEJ joins ends that lack homology or ends that have very short homologies to form junctions reminiscent of those observed for CSR (Lieber *et al.*, 2003; Rooney *et al.*, 2004). NHEJ has seven known components (Fig. 9). Ku70 and Ku80 bind DSBs as a complex (Ku). Ku bound to DNA ends binds DNA-PKcs and activates it to form the DNA-PK

holoenzyme (Lieber *et al.*, 2003; Rooney *et al.*, 2004). DNA-PK can phosphorylate Artemis to activate its endonuclease activity. XRCC4 and DNA Ligase 4 work together as a ligation complex which functions specifically in NHEJ (Lieber *et al.*, 2003; Rooney *et al.*, 2004). Cernunnos/XLF is a more recently identified NHEJ factor that appears to have some relationship to XRCC4 (Burma *et al.*, 2006; Sekiguchi and Ferguson, 2006). Ku70, Ku80, XRCC4, Ligase 4, and likely Cernunnos/XLF are conserved in evolution and are considered “core” NHEJ factors (Fig. 9). The activity of the NHEJ pathway has been characterized primarily in the context of V(D)J recombination. The RAG endonuclease generates V, D, and J coding ends as covalently sealed hairpins and RS ends a blunt 5′ phosphorylated DSBs (Fig. 9). The “core” NHEJ factors are required for both coding and RS joins where they have functions ranging

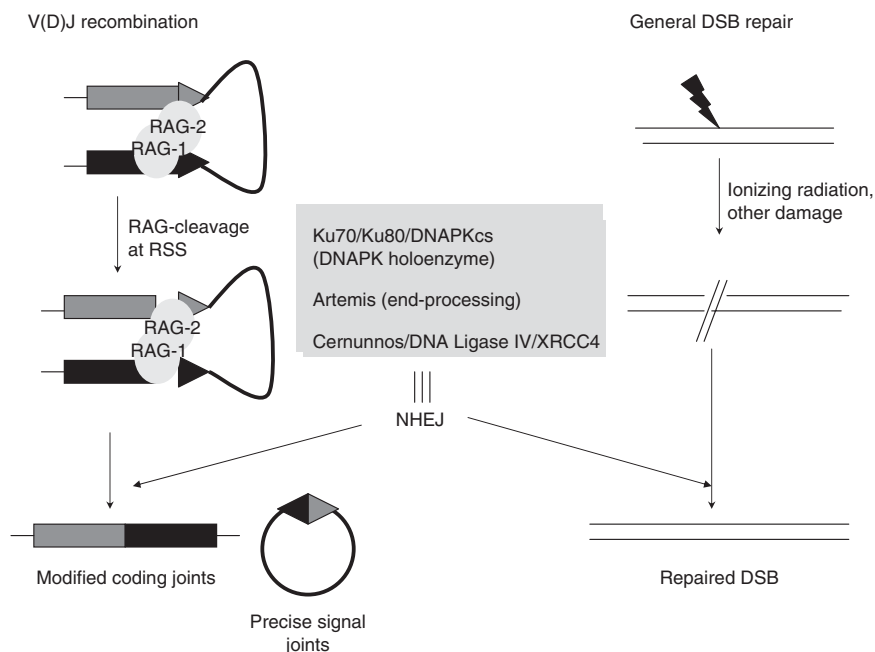


Figure 9 NHEJ is required for V(D)J recombination and for general DSB repair. The major components of the NHEJ pathway are shown. During V(D)J recombination, the RAG-1 and RAG2 proteins bind to and introduce DSBs at specific signal sequences (triangles) that flank the coding segments (rectangles). The RAG proteins cleave DNA mostly in the context of a preformed synaptic complex, and following cleavage, remain bound to the ends to shepherd in DNA repair proteins to ligate the DSBs. The NHEJ pathway also functions to join general DSBs. See text for more details.

from recognition of DSBs and recruitment of other factors to end ligation (Fig. 9) (Dudley *et al.*, 2005; Lieber *et al.*, 2003; Rooney *et al.*, 2004). In contrast, DNA-PKcs and Artemis are required for coding joins but are relatively dispensable for RS joins (Bogue *et al.*, 1998; Gao *et al.*, 1998a; Kurimasa *et al.*, 1999; Nicolas *et al.*, 1998; Rooney *et al.*, 2002; Taccioli *et al.*, 1998). In this context, the DNA-PKcs activated endonuclease activity of Artemis is necessary to open and process coding end hairpins (Ma *et al.*, 2002; Rooney *et al.*, 2002). It is thought that the various NHEJ factors play similar roles in the repair of general DSBs (Lieber *et al.*, 2003; Rooney *et al.*, 2004) (Fig. 9).

Evidence for a role for NHEJ components in CSR came from studies in which Ku 70 or Ku 80-deficient B cells were generated by rescuing B cell development with “knocked-in” productive V(D)J rearrangements at the IgH and Igk loci (Casellas *et al.*, 1998; Manis *et al.*, 1998; Reina-San-Martin *et al.*, 2003). Notably, both Ku70 and Ku80 deficient B cells were completely deficient for ability to undergo CSR. However, interpretation of these results was complicated by the fact that Ku-deficient cells have proliferation defects (Manis *et al.*, 1998; Reina-San-Martin *et al.*, 2003) and that proliferation is also required for CSR (Hodgkin *et al.*, 1996). Therefore, cause and effect were not unequivocal. Notably, DNA-PKcs deficient B cells or B cells homozygous for the *scid* mutation (a point mutation in the DNA-PKcs catalytic domain) were severely impaired for CSR but did show residual CSR (Cook *et al.*, 2003; Manis *et al.*, 2002; Rolink *et al.*, 1996). In the case of DNA-PKcs deficient B cells, CSR to IgG1 occurred relatively normally (Manis *et al.*, 2002). On the other hand, Artemis deficient B cells show relatively normal CSR to all IgH isotypes (Rooney *et al.*, 2005). Preliminary analyses of DNA-PKcs-deficient and Artemis-deficient B cells for chromosome breaks by the IgH locus FISH method suggest that both might have some IgH breaks and translocations (Franco and Alt, unpublished data). Thus, while more detailed characterization of the nature of the IgH locus breaks in the Artemis and DNA-PKcs mutant cells will be required for unequivocal interpretations, it is possible that both components may be necessary for joining a fraction of IgH locus breaks that occur in B cells activated for CSR, consistent with the role of these factors in joining a subset of normal DSBs. Overall, it appears that NHEJ joins, at least, a subset of CSR DSBs. However, given that Ku70 and Ku80 and DNA-PKcs have functions outside of NHEJ (Chu *et al.*, 2000; Cohen *et al.*, 2004; Sekiguchi *et al.*, 2001), some of the CSR defects observed, in particular the complete defect in Ku-deficient B cells, might be due to effects on other processes.

XRCC4 and DNA Ligase 4 are absolutely required for coding and RS joining during V(D)J recombination (Frank *et al.*, 1998; Gao *et al.*, 1998b; Li and Alt, 1996; Taccioli *et al.*, 1993) and have no known functions outside of NHEJ.

A role for Ligase 4 in CSR was suggested by the finding that hypomorphic human Ligase 4 mutations affected CSR, in particular by leading to alterations of certain S region junctions such as increased use of microhomologies by some ($S\mu$ to $S\alpha$) but not others ($S\mu$ to $S\gamma$) (Pan-Hammarstrom *et al.*, 2005). However, it is not clear whether such alterations resulted from decreased or altered activity of Ligase 4 or increased contribution by some alternative pathway (Pan-Hammarstrom *et al.*, 2005). To definitively assess the role of NHEJ in IgH CSR, it will be necessary to generate B cells completely deficient in XRCC4 or Ligase 4 by either IgH/Igk V(D)J knock-in approach or by a conditional gene inactivation approach. Preliminary analyses of XRCC4 deficient B cells generated by such approaches suggested that they proliferated relatively normally and show significant levels of CSR (C. Yan and F. Alt, unpublished data). It will now be of significant interest to employ IgH locus FISH assays to determine whether chromosomal breaks and translocations occur in such XRCC4-deficient B cells stimulated for CSR. In addition, it will be of substantial interest to determine the nucleotide sequence of the junctions of CSR events that occur in XRCC4-deficient B cells. In mice, normal CSR junctions are roughly equally divided between blunt joins (no homologies at the ends) and joins with short micro-homologies. While an alternative end-joining pathway that appears to prefer micro-homologies is known to exist, the pathway does not function in V(D)J recombination in the absence of NHEJ and there has been little evidence that it can function to join chromosomal sequences other than translocations (Lee *et al.*, 2004b; Zhu *et al.*, 2002). In this context, it will be of great interest to determine whether this pathway can catalyze chromosomal CSR in the absence of XRCC4 and, thereby, the classical NHEJ pathway.

7. Evolution of CSR

The process of SHM is an ancient reaction that exists in cartilaginous and bony fish (Cannon *et al.*, 2004). Fish AID has been identified and the fish mutational hot spot (AGC/T triplet) is similar to that in mammals, suggesting that SHM in fish is most likely AID dependent. Fish, however, do not have S regions and do not undergo CSR; yet fish AID can mediate CSR in mammalian cells, indicating that the molecule has already acquired most of the activities required for CSR (Barreto *et al.*, 2005a; Wakae *et al.*, 2006). CSR first appeared in amphibians, with *Xenopus* B cells being able to switch from IgM to IgX (Stavnezer and Amemiya, 2004). Unlike mammals, *Xenopus* S regions are AT rich (Mussmann *et al.*, 1997) and do not form R-loops (Zarrin *et al.*, 2004). Therefore, CSR is probably mediated via the ability of AID, perhaps in conjunction with an RPA or a similar molecule, to target the AGCT sequence

within *Xenopus* S regions in an SHM-like fashion (Zarrin *et al.*, 2004). As completion of CSR employs general DSB repair mechanisms, including a mechanism to synapse widespread chromosomal DSBs, it appears that what may have been needed for the evolution of CSR was the appearance target sequences that could allow AID activities to generate DSBs. In this regard, primitive S regions, such as those in amphibians, may be composed of a high density of RGYW motifs, allowing an SHM form of AID access, with the density of AID deaminations predisposing to DSBs. Evolution of R-loop forming ability may have evolved later to maximize AID-initiated DSB activity. Thus, one might imagine that the evolution of CSR beyond fish may have simply required the evolution of primitive S regions from SHM motifs along with their continued evolutionary refinement over time into specialized structures that allow generation of sufficient AID DSBs to allow physiological CSR levels by general joining and synapsis mechanisms (Zarrin *et al.*, 2007). While it is also possible that AID-specific cofactors evolved along the way to play a role in the diverse activities required for CSR, for example to direct the AID-induced lesions in to DSBs, the model outlined above is economical in that it would not require simultaneous evolution of S regions plus coevolution of AID-specific cofactors and/or potential AID structural modifications.

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