Published in final edited form as:

Ann N Y Acad Sci. 2005 June ; 1050: 146–162. doi:10.1196/annals.1313.119.

DNA Lesions and Repair in Immunoglobulin Class Switch Recombination and Somatic Hypermutation

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Abstract

Immunoglobulin (Ig) gene somatic hypermutation (SHM) and class switch DNA recombination (CSR) are critical for the maturation of the antibody response. These processes endow antibodies with increased antigen-binding affinity and acquisition of new biological effector functions, thereby underlying the generation of memory B cells and plasma cells. They are dependent on the generation of specific DNA lesions and the intervention of activation-induced cytidine deaminase as well as newly identified translesion DNA polymerases, which are expressed in germinal center B cells. DNA lesions include mismatches, abasic sites, nicks, single-strand breaks, and double-strand breaks (DSBs). DSBs in the switch (S) region DNA are critical for CSR, but they also occur in V(D)J regions and possibly contribute to the events that lead to SHM. The nature of the DSBs in the Ig locus, their generation, and the repair processes that they trigger and that are responsible for their regulation remain poorly understood. Aberrant regulation of these events can result in chromosomal breaks and translocations, which are significant steps in B-cell neoplastic transformation.

Keywords

activation-induced cytosine deaminase (AID); class switch recombination (CSR); DNA lesion; double-strand break (DSB); error-prone DNA repair; lesion bypass or translesion DNA polymerase; pol ι ; pol θ ; pol ζ ; somatic hypermutation (SHM); V(D)J recombination

INTRODUCTION

Antibody diversity is generated through processes occurring at sequential B-cell developmental stages: (1) rearrangement of different germline V (variable), D (diversity), and J (joining) genes to yield an enormous variety of V_HDJ_H gene segments in the heavy (H) chain locus; (2) rearrangement of V and J genes in the light (L) κ and λ chain loci, also giving rise to an enormous assortment of rearranged $V_\kappa J_\kappa$ or $V_\lambda J_\lambda$ segments; and finally (3) random pairing of rearranged V_HDJ_H and $V_\kappa J_\kappa$ or $V_\lambda J_\lambda$ chains. The staggering number of $V_HDJ_H/V_\kappa J_\kappa$ or $V_HDJ_H/V_\lambda J_\lambda$ pairs generated at any time (in excess of 10^9) underlies the

high degree of diversity of the B-cell receptors (BCRs) for antigen and the primary antibody repertoire. This consists of IgM antibodies, in general, with a low to moderate affinity for antigens. All the gene rearrangement events that underlie the generation of the primary BCR repertoire depend on the expression of recombination-activating genes 1 (RAG1) and RAG2, which are critical for V(D)J gene rearrangement (Fig. 1).

The impact of antigen on the primary BCR repertoire results eventually in the production of antibodies of higher affinities and different classes, that is, IgG, IgA, and IgE. The shift to high-affinity and secondary isotypes is underpinned by two critical genetic processes: Ig somatic hypermutation (SHM)¹ and class switch DNA recombination (CSR)² (Fig. 1). Both SHM and CSR occur in the specialized micro-environment of the germinal center, and both contribute to the maturation of the antibody response, although in different ways. By diversifying the binding strength of the BCR, SHM provides the structural basis for clonal selection by antigen for higher affinity mutants and the affinity maturation of the antibody response. By changing the constant (C) region of the Ig H chain with a downstream C region, CSR changes the antibody effector functions, which will be better suited to the later stages of the antibody response. Ig V(D)J SHM is turned on at the germinal center centroblastic stage after mature B cells encounter antigen and in the presence of T-cell help. At the subsequent centrocytic stage of germinal center, B clones expressing BCRs with higher antigen-binding affinity undergo antigen-driven positive selection and develop into memory B cells or antibody-secreting plasma cells. Autoreactive B cells or low-affinity clones undergo negative selection through apoptosis, anergy, or receptor editing. Both *in vivo* and *in vitro* experiments have shown that SHM is induced in B cells that have received T-cell help and sustained BCR cross-linking, consistent with the role of T-cell and BCR engagement in germinal center formation.^{3–5} Although different in their induction requirements, both SHM and CSR depend on the activity of a recently characterized cytidine deaminase, referred to as activation-induced deaminase (AID).^{6,7} AID plays a central role in antigen-driven clonal expansion, selection, and differentiation of B cells in the peripheral lymphoid organs, eventually giving rise to memory B cells and plasma cells. Although dependent on AID, SHM and CSR are independent of RAG1 or RAG2.

DNA lesions include any alteration of the nucleotide composition of the double-strand that may lead to DNA informational or structural change. DNA lesions such as thymidine dimers, oxidized nucleotides, mismatches, and DNA strand breaks are caused by irradiation, oxidative chemicals, erroneous replication, and nucleolytic activities.⁸ Double-strand breaks (DSBs) caused by irradiation, retroviral integration, transposition, or V(D)J recombination entail the loss of genetic information due to the deletion of significant stretches of DNA.^{9,10} In general, DSBs are repaired through either non-homologous end joining (NHEJ) or homologous recombination (HR) in a faultless fashion. However, in hypermutating B cells, DNA lesions in the IgH as well as the κ and λ loci trigger a DNA repair process that is prone to insert mismatches, that is, mutations. DSBs are characteristic and critical DNA intermediates in CSR. These DNA breaks undergo processing by different protein complexes before being repaired. Some of the same protein factors and/or complexes may be involved in the repair of DSB occurring in the V(D)J region DNA, eventually leading to the insertion of mutations. Here, we discuss mechanisms of generation of DNA lesions in

the Ig H locus, particularly DSBs, and the processes that are involved in their repair and that affect CSR and SHM.

DNA LESIONS AND REPAIR IN CSR

In the mouse, there are eight different Ig heavy chain constant region (C_H) genes downstream of the Ig VDJ region genes: C_{μ} , C_{δ} , $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_{ϵ} , and C_{α} . In the human, the H-chain locus underwent a phylogenetically semiconservative duplication, resulting in two tandem clusters of C_H genes separated by a duplicated 3' enhancer (E_{α}): C_{μ} , C_{δ} , $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\alpha 1}$ - E_{α} - $C_{\gamma 2}$, $C_{\gamma 4}$, C_{ϵ} , and $C_{\alpha 2}$. In germinal center B cells, upon stimulation by CD40L expressed by activated $CD4^+$ T cells and cytokines, the IgH locus undergoes CSR to juxtapose the VDJ region to a C_H gene downstream of C_{δ} . The DNA sequences mediating the CSR process are referred to as switch (S) regions, and an S region is present 5' of each C_H gene with the exception of C_{δ} . In CSR, the 5' portion of the upstream S region is joined to the 3' portion of the downstream S region, thereby excising and circularizing the intervening genomic DNA (Fig. 1). The "looping-out" nature of CSR strongly suggests the existence of DNA DSB intermediates. CSR depends on transcription of the intervening (I), S, and C regions of the upstream (donor) and downstream (acceptor) C_H loci, which is driven by the I_H promoter lying upstream of each I_H region and under both positive and negative regulations by switch regulatory elements (SREs).^{11,12} Although variable in length, ranging from 1 (S $_{\epsilon}$) to 12 kb (S $_{\gamma 1}$), all S regions contain highly repetitive GC-rich sequences. The 3-kb S $_{\mu}$ region consists of iterations of the [(GAGCT) $_n$ (GGGGT)] sequence, which is believed to act as the recognition motif for the S $_{\mu}$ -S $_{\alpha}$ recombination. Interestingly, the *Xenopus laevis* S $_{\mu}$ region, which is AT-rich instead of GC-rich, also contains the "AGCT" repeats and can functionally replace the mammalian S $_{\mu}$ region,¹³ suggesting that the repeated AGCT motif is important for an evolutionarily conserved targeting mechanism of CSR (Fig. 2). The palindromic nature of these repeated AGCT motifs raises the possibility that a complex and stable secondary structure may form during the S $_{\mu}$ region transcription. This structure may be a substrate of CSR recombinases itself or may serve as a scaffold to recruit important cofactors.¹⁴ The S $_{\epsilon}$ and S $_{\alpha}$ regions consist of the "RRGCT" repeats, a variation of the GAGCT repeats. Both (R)RGCT and (G)AGCT are different iterations of the RGYW/WRCY motif (R = A and G, Y = C and T, and W = A and T), the hypermutation "hot spot".^{15–17} Indeed, more than 50% of the S $_{\mu}$, S $_{\epsilon}$, and S $_{\alpha}$ DNA is accounted for by RGYW/WRCY sequences, and RGYW/WRCY is highly represented in S $_{\gamma}$ regions (S $_{\gamma 3}$, S $_{\gamma 1}$, S $_{\gamma 2b}$, and S $_{\gamma 2a}$) as well, suggesting that CSR and SHM share a targeting mechanism and/or DNA intermediates. The top strand of all S-region DNA is G-rich, implying that there may exist another CSR targeting mechanism that entails the formation of stable RNA:DNA hybrid strands during the S-region transcription. The resulting single-stranded nontemplate DNA and possibly G-quartet structures formed within would be substrates of the CSR recombinase(s).^{2,18}

Recent progress to identify factors involved in CSR has established an essential role for AID, which is expressed in B cells activated by antigen and $CD4^+$ helper T cells.^{6,7} AID is a sequelog¹⁹ of the RNA-editing cytidine deaminase APOBEC1, suggesting that AID may edit an mRNA to code for a protein factor involved in CSR, possibly a DNA recombinase.^{20–23} Although this hypothesis has been supported by the finding that *de novo*

protein synthesis is required for the AID function in CSR,²⁰ it has recently lost much ground. A different but nonmutually exclusive possibility is that AID functions directly as a DNA deaminase, converting cytosine bases to uracil in germinal center B cells. This model is supported by the demonstration that AID can deaminate chromosomal DNA in *Escherichia coli* as well as cytosine bases in the WRCY motif in single-stranded DNA *in vitro*.^{24,25} The resulting U:G mismatch is either replicated over to introduce transition mutations (C to T and G to A) or dealt with in two ways: (1) The U:G mismatch activates the mismatch repair (MMR) pathway, which involves the MutS homolog 2 (MSH2), MSH6, MutL homolog 1 (MLH1), postmitotic segregation protein 2 (PMS2), and exonuclease 1 (Exo1). Both MSH2 and Exo1 are critical for CSR, perhaps because of their role in generating and/or repairing DNA DSBs during the MMR.^{26,27} (2) The U:G mismatch triggers the base excision repair (BER) pathway, which is dependent on UNG2, a uracil-DNA glycosylase (UDG). This removes the uracil base from the DNA to yield an abasic site, which is then excised by apyrimidinic endonuclease to generate a nick, resulting in a single-strand break. A similar nearby lesion on the opposite strand would generate a DSB, and DSBs in both an upstream and a downstream S region would lead to excision of the intervening DNA segment²⁸ (Fig. 3). Consistent with this AID-dependent DNA deamination and DNA lesion repair model, CSR is essentially abolished in *Msh2*^{-/-}*Ung2*^{-/-} mice.²⁹ This model also entails the formation of RNA:DNA hybrid strands and exposed single-stranded DNA on the non-template strand (R-loop) as the potential AID substrate during the S-region transcription, which was also observed *in vivo*.³⁰⁻³²

Other trans-factors critical for CSR include Nijmegen breakage syndrome protein (Nbs1), Mre11, phosphorylated H2A histone family member X (γ -H2AX), ataxiatelangiectasia-mutated (ATM) kinase, p53-binding protein 1 (53BP1), and the Ku70/ Ku80 heterodimer.³³⁻⁴¹ That these proteins all form complexes with other proteins and are involved in the DSB repair further strengthen the notion that DNA DSBs are important CSR intermediates and are processed and repaired by different protein complexes. DNA DSBs have been detected in the S μ region in activated human B cells, whereas no DSB is detectable in the C μ region.^{42,43} In resting B cells, such S μ region DSBs are blunt-ended; but in activated B cells undergoing CSR, S μ DSBs are resected through an AID-dependent process.⁴² The critical role of AID in processing blunt-end DSBs to yield resected ends is emphasized by the finding that in activated B cells from an AID^{-/-} patient, DNA DSBs are still detected in the S μ region⁴³ and such DSBs are blunt-ended (unpublished data). In this patient, failure to generate resected DSBs associates with failure to undergo CSR. This is consistent with the recent finding that the *c-Myc* gene is translocated to S regions at a similar frequency in AID-deficient mice as in wild-type mice, suggesting that the generation of S-region DSBs that are responsible for these translocations is independent of AID,⁴⁴ further favoring a model in which the “inherent fragility” of the S μ region and *c-Myc* gene makes them substrates of yet to be determined DNA cleaving proteins to yield blunt-ended DSBs.^{1,45} Our preliminary findings suggest that the upregulation and recruitment of AID to DSBs, possibly with a DNA helicase cofactor, lead to cytosine deamination near the free ends. The resulting U:G mismatches would activate the MMR and/or BER pathway, as described above, to generate resected DSBs (Fig. 3).

A critical implication inherent in a model entailing the occurrence of DSBs upstream of AID is that the blunt ends at different S regions are precluded from re-ligation of the intra-S region through NHEJ, a favored process because of the low energy requirement for directly ligating two adjacent ends. This re-ligation of blunt ends could partially be inhibited if AID is overexpressed in the nucleus. The current lack of quantitative study of AID expression in activated B cells makes it difficult to assess this possibility. Another question is how AID-generated staggered ends are tethered together for repair and ligation. It is possible that staggered-end generation and Sx-Sx region ligation are coupled and are effected within one large protein complex containing different enzymatic activities. However, this would pose significant spatial problems for the synapsis of different S regions. Alternatively, a post-resection complex, which may contain AID itself, could be recruited to prevent further end processing until it is later displaced by downstream recombinases while the intervening region “loops out” (Fig. 4c). The putative post-resection complex may also modify DSB ends, which would then be repaired only by CSR-specific machinery. This mechanism is reminiscent of the hairpin intermediates during the V(D)J recombination. Such a hairpin is generated at the coding joint after RAG-mediated DNA cleavage and is subsequently opened by DNA-PK-activated Artemis.^{46–48} The AID-dependent γ -H2AX foci formation at the CSR sites strongly suggests the recruitment of multiple protein factors to the proximity of the IgH gene⁴⁹ in response to AID-generated staggered ends. It is worthwhile to note that γ -H2AX foci formation does not necessarily reflect the generation of initial DSBs, but rather a later step in the DSB repair process,^{50,51} and factors other than γ -H2AX may carry out the end-protecting and/or modification tasks.

To gain further insights into how the DSB ends are processed and into the temporal and spatial regulation of protein factors involved during CSR, we utilized an *in vitro* system to identify proteins specifically bound to those ends. The “S-wt” double-stranded oligonucleotide contains three repeats of palindromic AGCT, which is the most abundant RGYW motif in the S μ region, separated by the TTTT motifs. A control double-stranded oligonucleotide, S-mt, consists of a similar sequence, except that the AGCT repeat is replaced by its reverse TCGA, which is not an iteration of RGYW (Fig. 4a). Incubation with the total protein extracts from a subclone of B-lymphoma cell-line CL-01, which was selected for the high rate of spontaneous CSR, results in the formation of three S-wt-specific DNA-protein complexes, which shifted the mobility of the radiolabeled DNA oligo (Fig. 4b). These DNA-protein complexes may rely on different protein-DNA binding sites, in either the internal or the 3'-terminal AGCT motif. Since the S-wt and S-mt only differ in the presence or absence of the RGYW motif, these findings strongly suggest that the RGYW motifs play a role in targeting certain trans-factors to S regions. Specifically, these S-region RGYW motifs may function not only as the initial cleavage signal of endonucleases, but also as the recognition and binding sites for the end-protecting and processing complex(es) (Fig. 4c).

DSBs at the CSR sites would recruit Mre11/Rad50/Nbs1 complex and activate H2AX phosphorylation, which in turn catalyzes foci formation and the repair process. Free DNA ends can be rejoined with little or no regard for sequence homology by NHEJ or they can be “rejoined” through HR, a homologous template-directed recombination process. AID-

dependent γ -H2AX foci formation in the IgH locus primarily occurs in the G1 phase,⁴⁹ suggesting that the repair process occurs through NHEJ, which also requires 53BP1 and Ku70/80. This process is mechanistically different from V(D)J recombination. In V(D)J recombination, DNA-PKcs, Artemis, XRCC4, and DNA ligase IV, but not 53BP1, are necessary.⁵² How staggered ends are resected by exonuclease or filled in by DNA polymerases to make ligation-compatible ends during CSR NHEJ is not clear. The frequent mutations found at the Sx-Sx junction suggest that an error-prone DNA polymerase is recruited to insert mismatched nucleotides and extends the strand to fill in the gap near the DSB ends (Fig. 4c). Although the available data strongly suggest that NHEJ is the major mechanism effecting Sx-Sx DNA repair, they do not rule out a possible role for HR in the resolution of S region DSBs. An abortive CSR repairing process can result in chromosomal breakage, which in turn can lead to chromosomal translocation involving protooncogenes, such as *Bcl-6*, *c-Myc*, and *Bcl-2*, as frequently occurring in germinal center B cells.

DNA LESIONS AND REPAIR IN SHM

IgV gene SHM entails the insertion of mainly point mutations with occasional insertions and deletions at a rate of approximately 10^{-3} changes/base/cell division, which is about one million-fold higher than the spontaneous somatic mutation rate of the genome at large.⁵³ Similar to CSR, SHM depends on AID upregulation and is linked to transcription.^{6,54,55} It targets the V(D)J sequence immediately downstream of the IgV promoter, tapering off within the intronic DNA immediately upstream of Sp while sparing the constant region,^{56–58} and displays a striking preference for the RGYW/WRCY motif (mutational hot spot).^{15–17} Ig V(D)J is not the only gene targeted by SHM, as DNA outside the Ig H and κ and λ light chain loci, such as *Bcl-6* in the human and *c-Myc* in both human and mice, are also targeted.^{59–61} In antigen-selected antibodies, mutations are found mainly in the complementarity-determining regions (CDR). These regions dictate antibody specificity and comprise codons that are more inherently susceptible to replacement mutations than they would be for a random DNA sequence,⁶² suggesting that their nucleotide composition possibly evolved as a mutational strategy for the generation of higher affinity antibodies.

Somatic mutations would be inserted during error-prone repair of DNA lesions involving SSBs or DSBs, as first hypothesized by Brenner and Milstein⁶³ in 1966. The existence of DSBs in a hypermutating V region was first inferred by the presence of TdT-accessible DNA ends embedded in the Ig V(D)J DNA of hypermutating B cells *in vitro* and later confirmed by the ligation-mediated polymerase chain reaction (LM-PCR) in human *ex vivo* germinal center B cells as well as human B-cell lines *in vitro*.^{64–67} The occurrence of DSBs seems to be a highly specific function inherent only in the genes that can undergo SHM, such as those of the Ig locus, *Bcl-6* and *c-Myc*. By contrast, DSBs are not detectable in genes that cannot undergo SHM, including *C μ* , *PAX5*, *PIM1*, and alpha-fetoprotein (AFP). In the same gene, DSBs change in nature when starting the SHM process. In nonhypermutating germline V genes, DSBs are blunt, while DSBs occurring in hypermutating rearranged V(D)J genes are resected to yield 3' protruding free ends. Analysis of DNA from B cells at different stages of differentiation has shown that the resected DSBs appear at a germinal center stage when Ig V gene hypermutation is upregulated, but not in pre-germinal center or post-germinal center B cells, that is, memory B cells and plasma cells. Accordingly, in the

same hypermutating B cell, blunt-ended DSBs occur in both germline and translocated *c-Myc* alleles, whereas resected DSBs occur only in the translocated and hypermutating *c-Myc* allele.^{60,67} Hence, resected DSBs are characteristic of hypermutating genes and likely constitute crucial intermediates in the SHM process.⁶⁷ Processing of blunt-ended DSBs to generate resected ends would be mediated by AID. Indeed, although AID is dispensable for the generation of blunt-ended DSBs, it plays a critical role in the processing of blunt-ended DSBs to yield resected DSBs, possibly through deamination of cytosines within a few residues of free blunt ends. The appearance of U:G mismatches at these locations would trigger MMR or BER pathways, which would lead to the generation of staggered ends.

Staggered DSBs in hypermutating B cells appear predominantly in the S/G2 phase, suggesting that mutations are introduced during the DSB repair process through HR and involve error-prone DNA polymerases.⁶⁵ The role of resected DSB ends in strand invasion of sister chromatids and the subsequent events that lead to mismatches during HR have been further confirmed by ChIP assays, showing that γ -H2AX, Nbs1, Mre11, and Ku70/Ku80 are predominantly bound to the blunt ends. Rad 52 and Rad51, two Rad52 epistasis group proteins that are specific for HR, are bound only to resected DNA ends of rearranged V_HDJ_H genes.⁶⁷ This implies that (1) the blunt ends of nonhypermutating germline or rearranged V_H genes recruit Ku70/Ku80 and are re-ligated through the NHEJ pathway, and (2) after the assembly of Rad51 nucleoprotein filament around the 3'-resected ends of the hypermutating rearranged V_H DNA, other factors, possibly including a chromatin remodeling protein and a DNA helicase, unwind the sister chromatids or homologous chromosomes to make them accessible for strand invasion (Fig. 5).

The overwhelming segregation of DSBs within the RGYW/WRCY hypermutation hot spots suggests that during DSB repair a putative error-prone DNA polymerase inserts incorrect bases and extends the newly generated mismatched terminus. Error-prone translesion, or lesion bypass, polymerases have evolved to effect continued strand synthesis opposite DNA lesions that would otherwise stall a replication fork.^{68,69} The growing family of recently identified or characterized polymerases adds to the high-fidelity DNA polymerases δ , ϵ , and α , which are responsible for DNA replication, and pol β , which performs faultless BER^{68,70} (Table 1). Several studies have shown that the translesion DNA polymerases μ , λ , and κ are not essential for SHM.^{71–74} In patients with the *Xeroderma pigmentosum* variant (XPV) disease, who are deficient in DNA polymerase η (pol η), somatic mutations were found to be normal in frequency.^{75–77} The profound downregulation of pol η by BCR cross-linking in actively hypermutating B cells suggests that this DNA polymerase does not play a significant role in SHM.⁷⁸ Rather, DNA polymerases ι and ζ likely play an important role in SHM. DNA polymerase ι (pol ι) possesses a low processivity and is highly error-prone when copying undamaged DNA, whereas it efficiently incorporates single nucleotides opposing DNA lesions. DNA polymerase ζ (pol ζ) efficiently extends from a mismatched terminus to stabilize the mutation. The concerted and sequential action of polymerase ι and polymerase ζ would mediate DNA repair during HR, thereby introducing mismatches (mutations).⁷⁹ Gene inactivation of pol ι in the human Burkitt's lymphoma cell line BL2 abolished inducible SHM, but did not affect the background mutation frequency, suggesting that inducible hypermutation depends on pol ι .⁸⁰ BCR cross-linking, which is necessary to

induce SHM, signals the upregulation of pol ζ , while blocking of pol ζ 's REV3 catalytic subunit activity impairs Ig V(D)J and *Bcl-6* SHM without affecting cell cycle or viability.⁷⁴ Inhibition of human pol ζ REV3 expression, however, results in a profound decrease of damaged-induced DNA mutagenesis,⁸¹ a function that is exquisitely mediated by this translesion polymerase in many cells of the body. This suggests that the machinery utilized by B cells for SHM, a sophisticated and highly specific function, is the same as that utilized to survive DNA damage, a critically basic process. The role of pol ζ in SHM has been verified *in vivo*. In transgenic mice generated to express antisense RNA to a portion of REV3, pol ζ REV3 is inhibited, and the accumulation of somatic mutations in the V_H genes of memory B cells is decreased and the generation of high-affinity antibodies is delayed.⁸²

Another recently characterized human DNA polymerase, pol θ , which is encoded by the *pol* θ gene and belongs to the DNA polymerase A family,^{83,84} possesses features that make it a good candidate in SHM. While an N-terminally truncated version of human pol θ has been shown to be a high-fidelity enzyme,⁸⁵ the full-length pol θ replicates undamaged DNA in an error-prone fashion. Interestingly, pol θ efficiently inserts "A", while strongly disfavoring "C", opposing an apurinic site, and effectually extends DNA synthesis, making it an efficient lesion bypass polymerase.⁸⁶ Because of its insertion and extension functions, its error-prone nature, and its upregulation in germinal center B cells,⁸⁷ pol θ may play a major role in the DNA lesion repair process that leads to SHM. Indeed, our recent data show that SHM is severely compromised in mice with either null *pol* θ alleles or mutated *pol* θ alleles, chaos 1,^{88–90} strongly suggesting that pol θ inserts mismatches during Ig V DNA synthesis and the newly generated mismatch termini are extended by either pol θ itself or pol ζ .

The functional features of pol θ , pol ζ , and pol ι suggest that these DNA polymerases act in concert to introduce mismatches. Pol ι would insert incorrect single nucleotides, whereas pol ζ would extend the DNA strand past the mismatch. Pol θ could both insert mismatched nucleotides and extend the DNA terminus past the mismatch. The mismatched terminus generated by pol θ could also be extended by pol ζ (Fig. 5). Although the concerted action of pol ζ and pol θ would play a central role in generating mutations during the HR repair process of DSBs that underlies SHM, we cannot rule out the possibility that these polymerases introduce mismatches (mutations) while participating in a patch repair process outside main DNA regulation.

CONCLUSIONS AND PERSPECTIVES

Recent findings have defined the roles of DSBs, AID, and translesion DNA polymerases in CSR and SHM. The RGYW hot spot likely functions as the recognition and target sequence for the putative DNA enzyme involved in the generation of the DNA lesions that underlie CSR and SHM. Initial blunt-end DSBs are generated in Ig genes in an AID-independent fashion. AID-mediated resection of those blunt-end DSBs through cytosine deamination would yield U:G mismatches that trigger either the MMR or the BER pathway, which unfold through recruitment of multiple protein complexes. In CSR, synapsis of resected DSBs between upstream and downstream S regions and the subsequent DSB repair process, through either NHEJ or HR, lead to Sx-Sx DNA rejoining. In SHM, 3' free ends of such resected DSBs would initiate through Rad51 and Rad52 strand invasion to repair DSBs

through HR in an error-prone fashion. Concerted activities of translesion pol ι , pol ζ , and pol θ would incorporate incorrect single nucleotides and extend the mismatched terminus that effectually introduces mutations in Ig genes. Aberrant regulation and repair of such DSBs can lead to chromosomal breaks and translocations, which are significant steps in B-cell neoplastic transformation (lymphomagenesis).

Important issues that still need to be addressed in CSR and SHM are (1) identification of the inherent DNA features, nature of nucleolytic activities, and molecular mechanisms that effect blunt DSBs; (2) identification of the AID cofactors to deaminate ssDNA or blunt-end DSBs and reconstitution of an *in vitro* system to generate AID-dependent resected DSBs; (3) identification of the DSB end-binding proteins, their spatial and temporal regulation in germinal center B cells, and their precise role in shepherding DNA lesions to the proper DNA repair pathway, either NHEJ or HR; and (4) characterization of the precise mechanism by which the NHEJ or HR resolves DSBs and the mechanism of error-prone DNA polymerases that introduce mutations. Genetic studies using such powerful tools as knockout mice have generated significant progress towards understanding these issues and will continue to unveil critical factors in SHM and CSR. Detailed biochemical studies will be instrumental in unfolding the precise mechanisms that underlie functions of those proteins. They will also be indispensable in identifying proteins with regulatory functions that fine-tune SHM and CSR, thereby unraveling the functioning of these central mechanisms of adaptive immunity.

Acknowledgments

We thank Xiaoping Wu for insightful discussions and the sharing of unpublished data. This work was supported by National Institutes of Health Grant Nos. AR 40908 and AI 45011 (to P. Casali).

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FIGURE 1.

RAG and AID in B-cell differentiation. V(D)J recombination times B-cell development. It occurs in bone marrow and is dependent on the expression of RAG1 and RAG2, but not AID. Eventually, IgM⁺ B cells will leave the bone marrow and colonize the peripheral lymphoid organs. They will be activated by encounter with antigen and undergo SHM and CSR. Both processes are dependent on AID expression, but not RAG1/RAG2. SHM inserts mainly point mutations in the variable region, whereas CSR changes the constant region of the IgH chain with a downstream C_H region, thereby “looping-out” the intervening DNA.

FIGURE 2.

Phylogenetic emergence of SHM and CSR and development of adaptive immunity. From jawed fish to humans, SHM first emerges in cold-blooded vertebrates. CSR emerges first in amphibians. Both SHM and CSR are fully operational in mammals.

FIGURE 3.

Generation of DSBs in S regions. **(a)** AID-dependent generation of DSBs: deamination of cytosine on both strands generates U:G mismatches, which will activate either the mismatch repair pathway (MMR) or the base excision pathway (BER). The MMR complex, consisting of Msh2, Msh6, Mlh1, Pms2, and Exo1, would generate gaps in opposite DNA strands and eventually DSBs. In the BER pathway, uracil DNA glycosylase (UDG) removes the uracil base to yield an abasic site, which in turn is cleaved off by apyrimidinic endonuclease to generate nicks and, when cleaving on opposite strands, DSBs. **(b)** AID-independent generation of DSBs and their processing by AID. DNA strands are cleaved to form blunt-ended DSBs by a yet to be defined endonuclease. AID-dependent processing of the blunt ends leads to the U:G mismatches, which eventually yield resected DSBs. In either **a** or **b**, whether upstream or downstream of blunt-ended DSBs, AID would be critical in generating resected free DNA ends.

FIGURE 4.

Protein factors bound to DNA DSBs. **(a)** The S-wt and S-mt oligonucleotide sequences. **(b)** The gel picture from an electrical mobility shift assay during which the ^{32}P -radiolabeled S-wt or S-mt was incubated without (*lanes 1 and 2*) or with (*lanes 3 and 4*) total CL-01 cell lysates on ice for 30 minutes. The DNA-protein complexes were separated through a 5% PAGE and then subjected to autoradiography. *Arrowheads* indicate three proteins or protein complexes specifically bound to the S-wt, but not S-mt; *Two proteins/protein complexes that are bound to both S-wt and S-mt. **(c)** Model depicting different proteins/ protein complexes involved in DSB repair during CSR. Processing of blunt-end DSBs by AID and its cofactors yields resected ends. A putative end-protecting factor prevents re-ligation of the ends intra-S regions until they are replaced by a recombinase complex while the intergenic region “loops out”. The recombinase either fills in or further resects the ends to undergo an NHEJ process. Alternatively, the resected ends are repaired by HR.

FIGURE 5.

DSBs and error-prone repair in SHM. Blunt-ended DSBs generated in a rearranged IgH variable region would recruit Mre11, Rad50, and Nbs1 as well as phosphorylated H2AX. They would be repaired by NHEJ through Ku70/Ku80 and DNA ligase. Alternatively, they would undergo AID processing to yield 3' protruding ends, which recruit Rad51/Rad52 to initiate HR through invasion of sister chromatids or homologous chromosomes. A chromatin remodeling protein or a DNA helicase is likely involved in making the homologous region accessible. Different translesion DNA polymerases, upregulated in germinal center B cells, participate in the error-prone repairing process. Pol η inserts incorrect single nucleotides, while polymerase ζ extends the DNA strand past the insertion. Pol θ could both insert mismatched nucleotides and extend the terminus. However, the mismatched terminus generated by pol θ could also be extended by pol ζ .

TABLE 1

Eukaryotic DNA polymerases

DNA polymerase	Class ^a	Enzymatic activities	Fidelity ^b	Proposed functions
α	B	Initiation	10^{-5} – 10^{-4}	Nuclear DNA primase
δ	B	Elongation, 3'→5' exonuclease	10^{-6} – 10^{-5}	Nuclear DNA replication
ε	B	Elongation, 3'→5' exonuclease	10^{-6} – 10^{-5}	Nuclear DNA replication
γ	A	Synthesis, 3'→5' exonuclease	10^{-6} – 10^{-5}	Mitochondria DNA replication
σ	X	Terminal transferase, 3'→5' exonuclease	10^{-6} – 10^{-4}	Sister chromatids cohesion
TdT	X	Terminal transferase		V(D)J-N insertion
λ	X	5'-Deoxyribose-5-phosphate lyase (dRP lyase)	10^{-5} – 10^{-4}	NHEJ
μ	X	Terminal transferase	10^{-5} – 10^{-4}	NHEJ
β	X	dRP lyase	10^{-5} – 10^{-4}	High fidelity repair, base excision repair
REV1	Y	Inserting "C" across abasic sites		Translesion synthesis
κ	Y	Inserting "A" across abasic sites	10^{-4} – 10^{-3}	Translesion synthesis, base substitution
η	Y		10^{-3} – 10^{-2}	Translesion synthesis, UV damage repair
ι	Y	Inserting mismatches	$\sim 2 \times 10^{-1}$	Translesion synthesis, base excision repair, SHM
ζ	B	Extending mismatch terminus	10^{-5} – 10^{-4}	Translesion synthesis, DSB repair, SHM
θ	A	Inserting "A" and other bases across AP sites	10^{-3} – 10^{-2}	Translesion synthesis, DSB repair, SHM

^aEukaryotic DNA polymerases are classified into four main classes based on phylogenetic relationships with *E. coli* polymerase I (class A), *E. coli* polymerase II (class B), human polymerase β (class X), and *E. coli* UmuC/DinB and eukaryotic *RAD30/Xeroderma pigmentosum* variant (class Y).⁹¹

^bFidelity is defined as base substitution error frequency during nucleotide incorporation on intact single-strand template.