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Somatic Hypermutation of Immunoglobulin Genes: Merging Mechanisms for Genetic Diversity

Review

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Somatic hypermutation is critical for the generation of high-affinity antibodies and effective immune responses, but its molecular mechanism remains poorly understood. Recent studies have identified DNA strand lesions associated with the hypermutation process and suggested the involvement of specific repair molecules and pathways. Particularly exciting has been the discovery of a putative RNA editing enzyme, the activation-induced cytidine deaminase (AID), that is required for all immunoglobulin gene-specific modification reactions (somatic hypermutation, class switch recombination, and gene conversion). Parallels between these three reactions are considered in light of recent advances.

In his landmark monograph "The Specificity of Serological Reactions," Landsteiner (1936) documented how the immune system could produce antibodies to almost any foreign substance. This amazing ability to generate diversity is one of the hallmarks of acquired immunity. At the same time, immune reactions are specific for the particular antigen. The observation of an increase in the specificity of serum to a substance over time was not rigorously tested until Siskind and Eisen (1965) clearly demonstrated that, after injecting small amounts of antigen in rabbits, there was a gradual increase in the intrinsic affinity of serum antibodies to the antigen.

We now understand that immunoglobulin (Ig) and T cell receptor genes are generated by the somatic recombination of a relatively small number of gene segments in a process known as V(D)J recombination (Tonegawa, 1983). In many species, including primates and rodents, this assembly reaction generates much or all of the preimmune diversity in these antigen receptors, with combinatorial diversity alone enough to generate up to 10^7 different antibody specificities. Ig molecules from the preimmune repertoire, however, generally bind antigens with only modest affinity and specificity, and fine tuning of the antibody response is driven by another lymphocyte-specific process known as somatic hypermutation (SHM). This reaction is triggered when the Ig on the surface of B cells engages antigen and involves the introduction of point mutations into the variable regions of Ig genes. Some of the mutagenized antibodies will have a higher affinity for the antigen, and cells harboring these higher affinity antibodies proliferate and survive preferentially. Successive cycles of mutation and selection lead to the generation of B cells with very high-affinity antibodies, a phenomenon that Siskind and Benacerraf (1969) termed "affinity maturation."

Talmage (1957), Burnet (1957), and Lederberg (1959) were the first to propose that the variety of different specificities is a consequence of a high rate of mutation confined to a few genes in antibody-producing cells and to postulate that the increase in affinity might be the result of selection of clones by antigens. A theory to explain how such mutation might be achieved was originally put forward by Brenner and Milstein (1966), who proposed a model in which an unknown targeting mechanism directed a specific nuclease to the antibody genes. After DNA cleavage, error-prone repair would resolve the lesion into a mutation. Weigert, Cohn, and colleagues first observed hypermutation at work by studying variability in mouse λ light chains at the protein level. In their landmark paper (Weigert et al., 1970), they inferred some basic features of the process: the mutant λ chains were all derived from one germline sequence; variability was not randomly distributed, but instead amino acid changes tended to cluster in "specificity" regions; and replacements in such regions were sequentially selected for by antigen. Hypermutation was later confirmed at the DNA level (Bernard et al., 1978) and was causally linked to affinity maturation soon thereafter (Griffiths et al., 1984). Finally, advances in transgenic technology, coupled with the realization that light chain transgenes can ectopically hypermutate in vivo (O'Brien et al., 1987), ushered in a period of extensive studies into the molecular mechanism of SHM.

The prescient model of Brenner and Milstein still provides the rough outline of our current understanding of SHM. Mechanistically, we divide the reaction into at least three phases: targeting, DNA recognition and cleavage, and repair. Transcription is thought to play a key role in targeting of a nuclease to the Ig locus (Jacobs and Bross, 2001). It is not clear what is being recognized by the nuclease, although there is some sequence specificity to the process (Michael et al., 2002). The cleavage step is thought to result in the production of a DNA double strand break (DSB) (Bross et al., 2000; Papavasiliou and Schatz, 2000), although a single strand lesion might precede the DSB (Kong and Maizels, 2001). Finally, the DSB is probably repaired into a mutation through the action of a subset of error-prone polymerases (Gearhart and Wood, 2001).

While V(D)J recombination is used by all jawed vertebrates to assemble antigen receptor genes, diversity in the preimmune antibody repertoire can be generated by other means: SHM (in sheep), gene conversion (in pigs, cows, chickens), or by a combination of both reactions (in rabbits) (Diaz and Flajnik, 1998). Maizels (1995) was the first to propose that, despite their obvious differences (Figure 1), SHM and gene conversion are variations on a common mechanism, an idea strongly supported by recent findings (Sale et al., 2001). Perhaps more surprising still are the emerging links between

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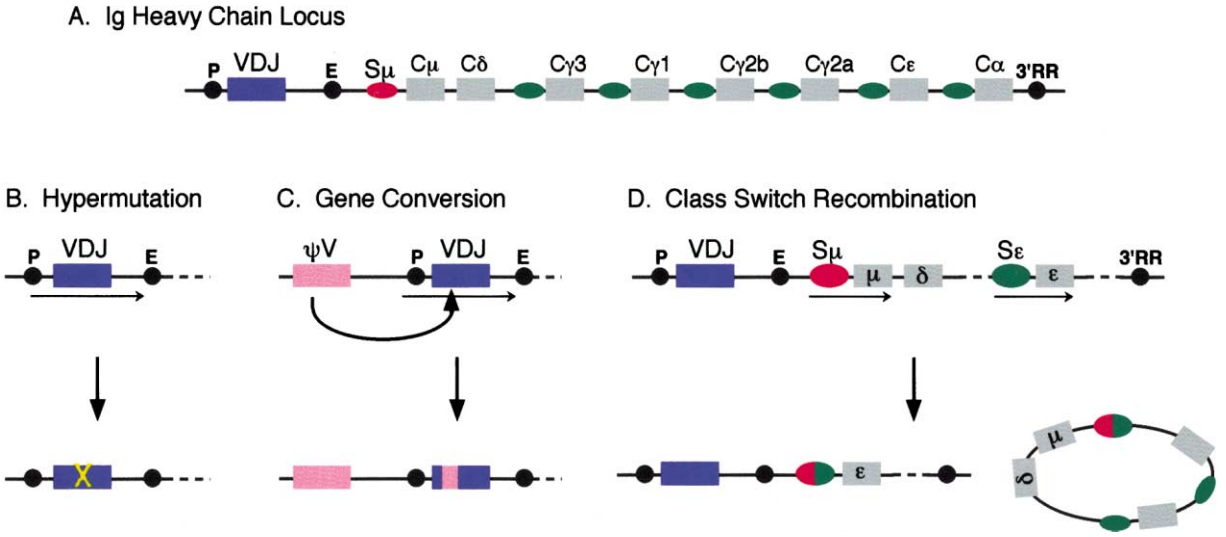

Figure 1. AID-Dependent Mechanisms that Diversify Ig Genes

(A) Schematic diagram of the Ig heavy chain locus, with the variable (VDJ) and constant (C) region exons represented as blue and gray rectangles, respectively, switch regions as ovals, and promoter (P), enhancer (E), and 3′ regulatory regions (3′RR) regions as black circles. Not to scale.

(B) Somatic hypermutation causes point mutations (x) in the vicinity of the V exon. Thin arrows represent transcription in (B), (C), and (D).

(C) Gene conversion involves the transfer of sequence information from a pseudogene (ψV) into the variable region exon.

(D) Class switch recombination involves looping out and deletion of DNA between two switch regions (in this case, between Sμ and Sε), thereby swapping the constant region of the expressed heavy chain.

SHM, gene conversion, and class switch recombination (CSR) (Honjo et al., 2002), most dramatically illustrated by the discovery of the activation-induced cytidine deaminase (AID), whose function is essential for all three reactions (Arakawa et al., 2002; Harris et al., 2002; Muramatsu et al., 2000; Revy et al., 2000). CSR alters the constant region and hence effector functions (but not the antigen specificity) of antibodies through a looping out and deletion process (Figure 1). With the discovery of AID and gathering evidence of mechanistic overlap between SHM, CSR, and gene conversion, these fields appear poised for major advances.

The Targeting Phase of SHM

Transgenic experiments from several laboratories have shown that mutations occur in the variable region exon and the surrounding 1 to 2 kb (Winter and Gearhart, 1998). The variable region promoter forms an upstream boundary to hypermutation (Lebecque and Gearhart, 1990), and mutations peak over the rearranged variable region. However, when the V gene is replaced with artificial substrates (bacterial sequences, the human β-globin gene, etc.), those substrates hypermutate successfully, and so the sequence of the V gene itself does not play a role in targeting the mutation process (Yelamos et al., 1995). A particularly important observation is that the probability of mutation declines with increasing distance from the promoter, with the decline being reasonably well fit by an exponential (Rada and Milstein, 2001). As a result, the frequency of mutation is highest in the upstream portion of the V exon (encoding complementarity determining region 1) and declines thereafter, with mutations rarely if ever found in the downstream constant region exons. If the promoter is removed by gene targeting, hypermutability of the locus is vastly diminished (Fukita et al., 1998), and if the promoter is duplicated upstream of the constant region, then that region begins to hypermutate (Peters and Storb, 1996). The link between mutation and transcription is very tight, with the frequency of mutation directly correlated to the rate of transcription (Bachl et al., 2001; Goyenechea et al., 1997).

These data might argue for a specific role of the Ig V gene promoter in targeting, but additional experiments have shown that this is not the case. The variable region promoter can be replaced with heterologous promoters (Betz et al., 1994; Tumas-Brundage and Manser, 1997), and hypermutation is not unduly compromised as long as the heterologous promoters are transcriptionally active. It is not yet clear whether these findings translate into a general requirement for a pol II promoter or whether the need for a promoter is more generic; replacement of the V region promoter with pol I (Fukita et al., 1998) or pol III (Shen et al., 2001) promoters yielded inconclusive results. In these experiments, hypermutation was not compromised, but the pol I and pol III promoters generated pol II-type transcripts (capped and polyadenylated). Thus it is not clear whether hypermutation can utilize a non-pol II promoter or whether in these experiments the transgenic promoters were simply hijacked by cryptic pol II elements.

Since neither the Ig V region nor its promoter is essential for hypermutation, what accounts for the specific targeting of the process to Ig genes? Ig enhancer elements appear to provide at least part of the answer. The well-defined Igκ intronic and 3′ enhancers are individually necessary (Betz et al., 1994) but together not sufficient (Klix et al., 1998) to target hypermutation to trans-

genic substrates, and it appears that other, poorly defined sequences in the vicinity of the enhancers play an important role (Klix et al., 1998; Winter et al., 1998). The 3′ λ enhancer is sufficient to support hypermutation of a λ1 transgene (Kong et al., 1998), but the Ig heavy chain intronic enhancer does not drive efficient hypermutation of Ig heavy chain transgenes, despite the fact that the transgenes are heavily transcribed (reviewed in Storb et al., 1998b). It remains unclear what sequences are responsible for the targeting of hypermutation, especially for the Ig heavy chain locus. What is clear is that the targeting function of enhancers and other *cis*-acting sequences can be uncoupled from their role in driving transcription. For example, in a construct containing a strong, enhancer-independent promoter, transcription was constitutive but mutation was absolutely dependent on the presence of the Igκ enhancers (Papavasiliou and Schatz, 2000). It is unclear whether "mutational enhancers" are position dependent (unlike conventional transcriptional enhancers) (Bachl et al., 1998; Winter et al., 1998) or position independent (Klix et al., 1998; Papavasiliou and Schatz, 2000). It is possible that there is a maximum allowed distance between enhancers and promoters beyond which they cannot effectively "communicate," but that within these boundaries a fair degree of flexibility is allowed (which might account for the different experimental results).

Taken together, these data indicate that the transcriptional promoter determines the precise region that will mutate, in a locus that has been "licensed" for mutation by the enhancer. One influential model to emerge from these observations, put forward by Peters and Storb (1996; Storb et al., 1998b), proposes that a "mutatorsome" is recruited to the vicinity of the variable region by the Ig enhancer (Figure 2). The complex, which is proposed to contain a nuclease, is then loaded onto the DNA through an interaction with the transcription machinery. It is postulated that this interaction can only happen between the enhancer and the preinitiation complex, such that when the nuclease is deposited in the vicinity of and cleaves the DNA, it cannot reestablish contact with the transcription machinery (which would explain the decay in the rates of hypermutation as a function of the distance from the promoter; Rada and Milstein, 2001).

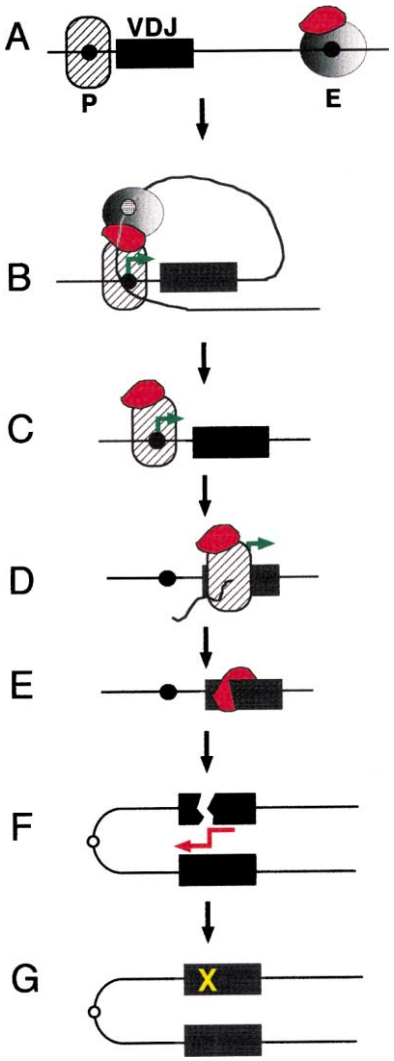

Figure 2. A Model for Somatic Hypermutation

(A) Proteins (gray oval) bound to the Ig enhancer recruit a nuclease (red) to the locus.

(B) Enhancer-promoter interactions deposit the nuclease in the vicinity of the transcription initiation complex (hatched oval).

(C) Transcription (green arrow) of the Ig locus is critical for the initiation of the reaction.

(D) The nuclease travels along the gene with the transcription complex.

(E) The initiating event is the introduction of an asymmetric DSB into one of the two sister chromatids.

(F) 5′ end resection exposes a single stranded 3′ extension that invades the intact sister chromatid.

(G) New DNA synthesis involves an error-prone polymerase and results in the introduction of point mutations (yellow x) in the sister chromatid that sustained the DSB. This model is a synthesis of models proposed previously (Brenner and Milstein, 1966; Papavasiliou and Schatz, 2000; Peters and Storb, 1996; Maizels, 1995).

The Recognition and Cleavage Phase of SHM

Soon after researchers were able to construct transgenes that would hypermutate in vivo, a vast database of mutations began accumulating. Mathematical biologists mining this database have derived models that predict many of the features of the process. Rogozin and Kolchanov (1992) were the first to recognize that preferred hypermutation hotspots conformed to a certain nucleotide motif (RGYW or its complement, WRCY). More recently, Shapiro and Wysocki have ranked triplets from "hottest" to "coldest," according to a mutability index inferred from a database of unselected mutations (Shapiro et al., 1999). The preference of the mutational machinery for certain nucleotides is evolutionarily conserved (Diaz and Flajnik, 1998), and thus it is likely that it reveals an inherent feature of the reaction. Yet not all potential hotspots end up being mutated, suggesting that a loose sequence specificity is only partially descriptive of the mutator.

Because mutations do not seem absolutely sequence dependent, the possibility remained that perhaps the mutator was recognizing a structural motif. Gearhart was the first to postulate a role for DNA secondary structure as a recognition motif (Golding et al., 1987), but

experimental data did not seem to support that theory (mutations did not necessarily coincide with particular secondary structure elements (for review, see Winter and Gearhart, 1998). A role for RNA secondary structure was suggested recently by Storb and colleagues. Using an artificial transgene containing alternating restriction enzyme sites, they observed that the area of the transgene containing the repetitive sequence elements was more hypermutable than the rest of the transgene (Klotz et al., 1998). This led to the proposal that as the V region sequence is transcribed, the nascent mRNA folds into transient stem-loop structures that can cause pausing of the transcriptional machinery and render deposition of the mutator kinetically favorable (Storb et al., 1998a). While computer predictions of RNA secondary structure supported this theory (Storb et al., 1998a), a recent study by the same investigators did not (Michael et al., 2002). In transgenes containing predicted secondary structure elements and/or primary sequence hotspot elements, the strongest predictor of hypermutability was, in fact, primary sequence; the hottest nucleotide triplets were mutated most frequently, and if true hotspots were lacking, the least cold of the coldspots were preferred. Regions of high predicted secondary structure (either DNA or RNA) were not inherently highly mutable (Michael et al., 2002). In addition, there is no clear relationship between known RNA polymerase II pause sites and mutations in the human c-MYC gene (which can hypermutate when translocated into Ig loci) (Bemark and Neuberger, 2000). Overall, it is clear that the hypermutation machinery has local sequence preferences and that these preferences can be influenced by neighboring sequences. But what the mutator might actually recognize remains a mystery.

Equally mysterious, until recently, was the type of DNA cut the nuclease would make. The Brenner-Milstein model originally predicted a DNA single strand break (SSB) (Brenner and Milstein, 1966), as it is easy to envision how to introduce a point mutation during repair of a nick or a gap. In recent years, however, the possible involvement of DNA double strand breaks has been suggested by the finding that 5%–10% of the mutations associated with the SHM reaction are, in fact, insertions and deletions (Goossens et al., 1998; Wilson et al., 1998; Wu and Kaartinen, 1995). The first strong evidence for the involvement of DNA strand lesions (double or single stranded) in SHM was provided by Sale and Neuberger, who showed that TdT was capable of inserting nontemplated nucleotides into Ig V regions in a hypermutating cell line (Sale and Neuberger, 1998). Direct evidence for abundant DNA DSBs over hypermutating regions was independently obtained by two groups (Bross et al., 2000; Papavasiliou and Schatz, 2000) through the use of sensitive ligation-mediated (LM)-PCR techniques. Such DSBs were found to be restricted to the variable regions or to artificial hypermutating regions and to colocalize strongly with mutations. It is therefore tempting to speculate that mutational hotspots are also DSB hotspots, although proof of this is currently lacking. Furthermore, DSBs are promoter dependent (Bross et al., 2000; Papavasiliou and Schatz, 2000) and require Ig enhancer elements (Papavasiliou and Schatz, 2000). Surprisingly, the DSBs result in asymmetric DNA ends: the upstream end of the break (the end tethered to the promoter) is detectable by LM-PCR as a blunt end at levels one to two orders of magnitude higher than the downstream end (Bross et al., 2000; Papavasiliou and Schatz, 2000). Possible explanations are that the downstream end contains a modification that renders it inaccessible to LM-PCR or the upstream end is "protected" and remains blunt while the downstream end is further processed in anticipation of repair.

These data indicate that SHM results in the creation of DNA DSBs, although it remains to be proven that such DSBs are intermediates in the hypermutation process. If they are intermediates, the DSBs may not be the first, discrete step of the cleavage reaction. For instance, an SSB created before DNA replication could be converted into a DSB by the replication machinery in a fashion that would generate asymmetric ends (Haber, 2001). Indeed, Maizels and colleagues have amplified SSBs from hypermutating regions that also colocalize with mutational hotspots (Kong and Maizels, 2001). It remains unclear, however, whether these experiments detected the unique strand end of a SSB or, alternatively, one of the two strand ends generated by a DSB. Because of their abundance, location, and dependence on transcriptional control elements, DSBs are likely to be the end product of the cleavage step of the reaction and hence the substrate upon which DNA repair must act.

The Repair Phase of SHM

A plausible scenario is that SHM is initiated by a B cell-specific nuclease that inflicts a DNA break near a mutational hotspot. The break would then be repaired by ubiquitous factors in an error-prone fashion, introducing a point mutation in the immediate vicinity of the break. A statistical analysis done by Oprea and colleagues has shown that the mutational spectrum derived from SHM is not very different from that of meiotic ("spontaneous") mutation during evolution (Oprea et al., 2001), suggesting that these processes share repair factors. One way that meiotic mutations can arise is as a result of repair of unintended breaks, and so these authors and others have suggested that SHM can be viewed as an accelerated form of spontaneous mutation (accelerated in part as a consequence of an increased rate of break formation).

Mutations can arise "actively" from the error-prone processing of DNA breaks or "passively" from the absence of normal repair machineries. Many investigators have tried to find links between SHM and various repair pathways (reviewed in Wood, 1998), largely without success. Nucleotide and base excision repair factors do not appear to have a role in the reaction. Results from mice with deficiencies in mismatch repair factors have been somewhat contradictory and confusing (reviewed in Reynaud et al., 1999), perhaps because B cell responses are perturbed in these animals. For example, the reduced levels of mutation found in some mismatch repair-deficient mice may be a secondary consequence of B cells engaging in fewer rounds of mutation. The one relatively consistent finding is that Msh2-deficient B cells exhibit an altered spectrum of mutations, with preferential targeting of G and C nucleotides and of hotspots (Reynaud et al., 1999). These results led Neuberger and colleagues to propose a two-stage model

for SHM: a first phase that is hotspot focused, G/C biased, and Msh2 independent, and a second phase, triggered by events at hotspots, that introduces A/T biased mutations at non-hotspot regions and requires Msh2 (Rada et al., 1998). It is reasonable to think of the first phase as consisting of a DSB at a hotspot, followed by repair that leads to a mutation close to the site of the break. How the second phase of the reaction would widen the distribution of mutations, and what role Msh2 plays in this process, is unclear. It is interesting to note that SHM in frogs and of some Ig genes in sharks is strongly G/C biased and hence resembles that in mouse B cells deficient in Msh2 (Diaz and Flajnik, 1998).

Mismatch repair in yeast and higher eukaryotes is thought to involve initial lesion recognition by a heterodimer consisting of Msh2 and either Msh3 or Msh6, followed by recruitment of a Mlh1-Pms2 heterodimer (Kolodner, 1996). Does Msh2 influence SHM by virtue of its role in conventional mismatch repair? The answer appears to be no, because inactivation of mismatch repair by targeted disruption of *Pms2* does not alter the distribution of mutations in SHM (Ehrenstein et al., 2001; Frey et al., 1998). This finding, while somewhat controversial, suggests alternative roles for Msh2 (probably together with Msh6, not Msh3; Wiesendanger et al., 2000) in SHM. In yeast, Msh2 is involved in the recognition of a variety of DNA structures during DNA DSB repair (Paques and Haber, 1997; Sugawara et al., 1997), and it seems to serve as a recruiter of nucleases and an initiator of DNA repair. It is therefore conceivable that Msh2 and perhaps other mismatch repair factors have a role outside the normal mismatch repair pathway, perhaps in DNA DSB processing.

In vertebrates, the two main pathways of DSB repair are homologous recombination and nonhomologous end joining (NHEJ). These appear to operate predominantly in distinct phases of the cell cycle: G1/early S for NHEJ and late S/G2 for homologous recombination (Hendrickson, 1997). The DSBs associated with V(D)J recombination are repaired by NHEJ and are found almost exclusively in G1 phase cells (Lin and Desiderio, 1995). Mutation-associated breaks have been postulated to be repaired by homologous recombination (Maizels, 1995; Selsing et al., 1996), and indeed SHM DSBs are found almost exclusively in G2 phase cells (Papavasiliou and Schatz, 2000). It appears unlikely that NHEJ resolves any significant fraction of SHM-associated DSBs: in mice deficient for the NHEJ factor DNAPKcs, the rate of SHM is unperturbed and so is the rate of insertions and deletions associated with the process (Bemark et al., 2000). Further striking evidence linking SHM and homologous repair has come recently from Neuberger and colleagues, who disrupted either the XRCC2 or XRCC3 gene (Rad51 paralogs involved in homologous repair) in chicken DT40 cells. Wild-type DT40 cells diversify their Ig variable regions by gene conversion, with few associated untemplated mutations. By contrast, ΔXRCC2- or ΔXRCC3-DT40 cells exhibit a dramatic increase in the frequency of untemplated point mutations, to levels 10-fold above the gene conversion frequency in the wild-type cells (Sale et al., 2001). Interestingly, the frequency of gene conversion events is only modestly reduced in the mutant cells. The point mutations occur almost exclusively at G/C nucleotides and preferentially at classic SHM hotspots. The results strongly support the idea that gene conversion and SHM share a common, initiating DNA lesion (presumably a DSB) and suggest that recombinational repair of a DSB gives rise to gene conversion when templated from pseudogenes on the same chromosome, or SHM when templated off the sister chromatid (Figures 1 and 2). Presumably, error-free homologous repair using the sister chromatid occurs at a high rate in wild-type DT40 cells and is only revealed when the repair process is "perverted" (and made error prone) by disruption of XRCC2 or XRCC3 (Sale et al., 2001).

Homologous recombination between sister chromatids is usually error free, so for DSB repair to lead to the generation of mutations, a highly error-prone polymerase should be involved in repair synthesis. Recently, a number of highly mutagenic polymerases have been discovered, four of which (pol ζ, η, ι, and μ) are plausible candidates for the mutagenic polymerase(s) of SHM (Gearhart and Wood, 2001). There are conflicting reports as to which polymerases are upregulated in germinal centers or in mutating B cell lines, and it has been difficult to implicate any of the polymerases definitively in the reaction because of potential functional redundancy and because some result in embryonic lethality when mutated in mice (e.g., pol ζ). To date, the evidence is strongest for pol ζ and pol η (Gearhart and Wood, 2001). Because of the significant functional redundancy between the polymerases in vitro, it is entirely possible that they are similarly redundant in vivo.

Trans-Acting Factors: AID

Our understanding of the molecular mechanism of SHM has been severely limited by the paucity of factors thus far shown to be involved in the reaction. Thus, the recent identification of the activation-induced cytidine deaminase (AID) has generated much excitement. AID was isolated in a screen for molecules differentially expressed after induction of CSR in the CH12F3-2 B cell lymphoma (Muramatsu et al., 1999). AID has highest sequence homology to RNA editing deaminases and can perform C-to-U deamination in vitro (Muramatsu et al., 1999). AID is preferentially expressed in secondary lymphoid organs and, most interestingly, mice and humans deficient in AID have no CSR and severely reduced (if not totally ablated) SHM (Muramatsu et al., 2000; Revy et al., 2000), even though germinal centers in $AID^{-/-}$ mice are large, and the initial (targeting) stages of the CSR reaction seem normal. AID deficiency has no other known consequences, and hence AID appears to be involved specifically in CSR and SHM. Strikingly, AID is also essential for the diversification of chicken Ig V genes (in the DT40 cell line) by gene conversion (Arakawa et al., 2002; Harris et al., 2002), which, as discussed above, appears to have strong mechanistic parallels with SHM (Maizels, 1995; Sale et al., 2001). Thus, in addition to the obvious functional significance of AID, its discovery has suddenly linked together these three distinct processes.

The role of AID in these reactions is far from clear, and its effect might not even be direct, as AID is thought to be an RNA-editing enzyme (Honjo et al., 2002). It has been postulated that AID edits the mRNA of the

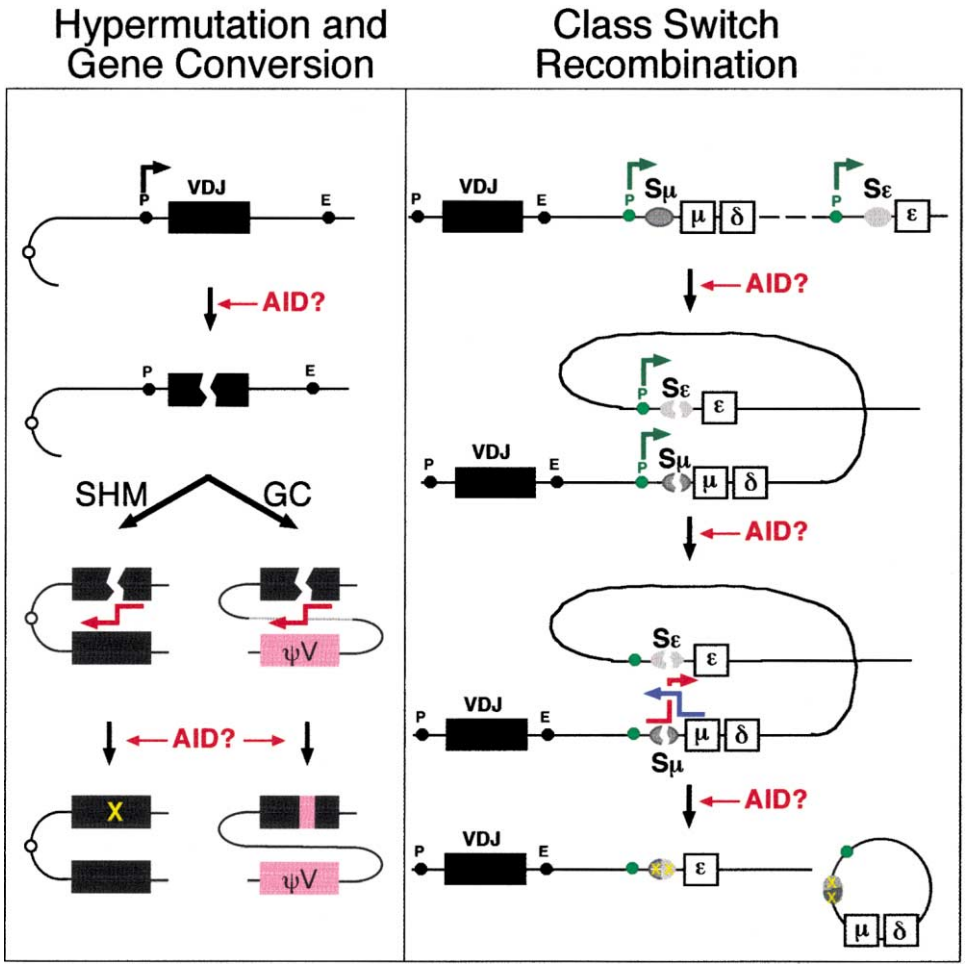

Figure 3. Similarities between Hypermutation/Gene Conversion and Class Switch Recombination

AID could function in the first step of all three reactions, initiating break formation. Alternatively, AID could be involved in later stages, either facilitating the synapsis of the substrates prior to repair or modifying the repair process itself. For SHM and gene conversion, the repair process is thought to involve homologous recombination (red arrows). For CSR, both nonhomologous end joining and single strand annealing repair pathways may be involved (red and blue arrows), and mutations are often found near the junctions (yellow x's). See text for details. Symbols and abbreviations as in Figure 1.

endonuclease responsible for the DNA lesions in both CSR and SHM (Honjo et al., 2002), and presumably in gene conversion as well. Given that CSR and SHM have been proposed to repair breaks by distinct mechanisms (NHEJ and homologous recombination, respectively), it is an attractive idea that the shared factor, AID, would be involved in the shared, upstream event (the creation of a DNA lesion; Figure 3).

The inference that CSR ends are repaired by NHEJ was initially supported by data demonstrating that (1) mice deficient in Ku70 or Ku80 are severely impaired for CSR both in vivo and in vitro (Casellas et al., 1998; Manis et al., 1998) and (2) pro-B cells from scid mice (deficient in DNAPKcs) cannot switch to IgE (Rolink et al., 1996). These results had to be interpreted cautiously, however, because Ku-deficient cells have proliferative and survival defects that could interfere with CSR indirectly, and the scid cells were only examined for switching to IgE. Very recently, these findings were extended by the surprising observation that DNAPKcs-deficient B cells (which do not have significant proliferative or survival defects) have a severe defect in CSR to all isotypes except IgG1 (Manis et al., 2002a). The Sμ/Sγ1 switch junctions from DNAPKcs-deficient B cells closely resemble those from wild-type B cells, and it remains unclear why CSR to Sγ1 is uniquely DNAPKcs independent. Hence, while NHEJ is strongly implicated in CSR, it remains uncertain whether it is the only repair process involved in the reaction. It is also curious that CSR junctions do not look like typical products of repair by NHEJ, in that a high frequency of point mutations is found in the region surrounding the repaired joint (Dunnick et al., 1989). This, together with other findings discussed in the next section, raises the idea that SHM and CSR are more closely related in their repair phase than previously thought, which in turn leads us to consider the possibility that AID edits the mRNA of a factor or factors responsible for orchestrating DSB repair (Figure 3).

In an attempt to distinguish whether AID plays its essential role in CSR in the generation of DNA lesions or in coordinating their repair, Nussenzweig and colleagues have looked at the association of repair factors

with switch region DNA in wild-type and AID$^{-/-}$ B cells (Petersen et al., 2001). The repair factors Nbs1 and phosphorylated histone H2AX (known as γ-H2AX) form distinct nuclear foci, easily visualized by immunofluorescence, in areas harboring DNA DSBs. These investigators found that such foci are detectable over switch regions in wild-type, but not AID-deficient, B cells stimulated to undergo CSR. This is consistent with a lack of CSR-related DSBs in the absence of AID and supports the conclusion that AID acts upstream of repair, most likely functioning in initiating DSB formation.

A similar attempt at deciphering the role of AID, this time in SHM, reached a seemingly opposite conclusion. We have examined DNA DSB formation in germinal center B cells from wild-type and AID-deficient mice and found that DSBs are detectable at equal frequencies in the two. Furthermore, in Ramos B cells overexpressing a dominant-negative form of AID, SHM is blocked but DSBs are, if anything, increased in abundance compared to control cells (Papavasiliou and Schatz, 2002). Even if DSBs are not direct intermediates in the SHM reaction, but are instead byproducts of the initiating lesion (e.g., a SSB), this result still indicates that the initiating lesion is present in AID-deficient B cells. We conclude that AID is not required for strand lesions in SHM and likely acts by editing the RNA of either a repair factor or a factor that recruits the repair machinery.

Taken together, these results indicate either (1) that AID plays different roles in CSR and SHM (presumably by editing different RNA species) or (2) that in CSR, as in SHM, AID acts in the repair phase of the reaction, perhaps to modify repair factor(s) and enable formation of the Nbs1/γ-H2AX foci. Clearly, our understanding of the mechanisms of SHM and CSR remains rudimentary, but significant advances may come quickly through a better characterization of the relevant DNA lesions (including identification of the nuclease(s) involved) and of course the discovery of the putative target RNA(s) edited by AID. Progress on these issues may come quickly, given recent findings that expression of AID is sufficient to activate SHM in B cell hybridomas (Martin et al., 2002) and CSR in 3T3 fibroblasts (Okazaki et al., 2002).

CSR and SHM: How Deep Are the Parallels?

The discovery that CSR and SHM (and Ig gene conversion, which for simplicity we will consider a variant of SHM) depend on AID is the latest item on a growing list of similarities between the two reactions (reviewed in Manis et al., 2002b). How deep do the mechanistic parallels run, and might CSR and SHM be different repair outcomes of similar DNA cleavage reactions (Figure 3)? Both reactions take place in germinal center B cells in response to stimulation by antigen but, importantly, they are not obligate partners, since a number of in vivo and in vitro situations have been identified in which cells perform one reaction or the other, but not both (Honjo et al., 2002). The substrates for the two reactions have obvious differences, but also some intriguing overlap. CSR is inherently a two substrate process, requiring the synapsis of two nonhomologous switch sequences. SHM and gene conversion differ from CSR in that while they also may require the interaction of two DNA sequences (the V region to be mutated and the homologous/homeologous repair donor), only one of them is altered by the reaction (Figure 3). CSR is confined to the vicinity of switch regions while SHM focuses on the rearranged variable region. But as noted above, switch junctions are often mutated. Targeting of the two reactions to their different substrates is a particular challenge in the Ig heavy chain locus, where the variable region and Sμ switch sequence are only a few kilobases apart and are components of the same primary transcript, and hence both regions should be "accessible" to both reactions. Part of the explanation for target specificity may lie in differences in the enhancer elements that appear to be required for the two processes. For instance, the κ intronic enhancer is required for SHM whereas the 3′ IgH enhancer is important for CSR (Pinaud et al., 2001).

Both SHM and CSR are tightly linked to transcription, but the role played by transcription remains a mystery. Movement of the transcriptional apparatus along the gene is postulated to be necessary for SHM (Peters and Storb, 1996) (Figure 2), whereas for CSR it has been suggested that a transient RNA:DNA duplex (formed between the nascent transcript and the template strand of the switch region DNA) might be the secondary structure recognized by the CSR nuclease(s) (Manis et al., 2002b; Tian and Alt, 2000). SHM and CSR are thought to involve the generation of one or two DNA DSBs, respectively, but whether the initiating lesion is a DSB or a single strand nick that is converted into a DSB (through S phase-dependent DNA replication) is unknown.

Surprisingly, Msh2 deficiency creates a similar phenotype in SHM and CSR: a focusing on intrinsic hotspots in SHM, and a focusing of junctions on consensus switch motifs in CSR (Ehrenstein and Neuberger, 1999). The role of Msh2 in these reactions appears to be distinct from that of standard mismatch repair, because CSR junctions from Pms2-deficient mice do not show a similar focusing on consensus motifs and exhibit unusually long regions of microhomology (Ehrenstein et al., 2001; Schrader et al., 2002). Notably, the GAGCT switch consensus sequence matches the RGYW hotspot for SHM.

Finally, the repair of CSR DSBs is proposed to take place in the G1 phase of the cell cycle, as CSR repair foci are more frequent in G1 (Petersen et al., 2001). CSR breaks are thought to be repaired by NHEJ, but CSR-associated repair foci contain Nbs1 and Rad50 (Petersen et al., 2001), and these proteins along with Mre11 participate in the human single strand annealing (SSA) pathway (Karran, 2000) (a role for Mre11/Rad50/Nbs1 in NHEJ in higher eukaryotes remains uncertain [Huang and Dynan, 2002]). The involvement of a variant of SSA in CSR would not be inconsistent with the data at hand (Figure 3), as previously hypothesized (the "illegitimate priming model" [Dunnick et al., 1993], reviewed in Stavnezer [1996]). The longer regions of microhomology seen in some switch junctions from Mlh1- and Pms2-deficient B cells (Ehrenstein et al., 2001; Schrader et al., 2002) may reflect the participation of SSA.

In contrast, emerging data suggest that SHM DSBs are repaired in G2, and thus homologous recombination is an attractive mechanism for repair of such DSBs into mutations (Papavasiliou and Schatz, 2000; Sale et al., 2001) as well as for the generation of the small amounts of insertions and deletions associated with the process

(Bemark et al., 2000). Yet, the resolution products in CSR reactions are characterized by high levels of point mutations that surround the switch joint. This, along with the role of mismatch repair factors in both CSR and SHM, might suggest that some repair steps are identical between the two reactions.

CSR and SHM: Important Factors in Lymphomagenesis

In addition to their central role in the generation of lymphocyte diversity, CSR and SHM are of emerging significance to human disease. It is thought that most lymphomas are of B cell origin and most adult onset B cell lymphomas are of germinal center origin (Kuppers et al., 1999). In about half of these, the translocation breakpoints occur within the target sites for the CSR machinery and can thus be attributed to errors during switching. In others, the SHM machinery may be the culprit. Not only does SHM result in DSBs in Ig V regions but the reaction can also be targeted to a diverse set of protooncogenes, at least in tumor cells (Pasqualucci et al., 2001). Intriguingly, the mutagenized oncogenes are subject to chromosomal translocations in the same regions that incur mutations. The clearest example of mistargeting of SHM is the human BCL6 gene, which incurs a significant mutation load in normal human B cells (Shen et al., 1998) and is affected by translocations in some B cell lymphomas. Thus, errors in the targeting of the SHM machinery or errors in the repair of the DNA breaks could be a direct cause of translocations, oncogene activation, and subsequent malignant transformation. Aberrant targeting of CSR and SHM represents a novel and powerful mechanism of malignant transformation.

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