Error-prone Candidates Vie for Somatic Mutation

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Antibodies play a major role in the resistance of higher organisms to disease. This is made possible because mice, humans, and other species have evolved three unusual molecular mechanisms that allow the generation of an enormously diverse repertoire of antibodies from a relatively small amount of genetic material. First, each antibody-forming B cell assembles different combinations of variable (V), diversity (D), and joining (J) minigenes to create an antibody with a unique antigen-binding site. Second, to generate the high-affinity antibodies that are required for survival, B cells target many point mutations to the V(D)J regions of the H and VJ region of the L chain immunoglobulin (Ig) genes. Those B cells that are making higher affinity antibodies are selected for further growth and differentiation, resulting in the affinity maturation of the antibody response. Third, B cells rearrange the H chain V region to various downstream C regions that encode the different isotypes. This makes it possible for each of the many antigen-binding sites to mediate the effector functions that are encoded in the different C region genes and to be distributed throughout the body (1).

Even though the somatic hypermutation of antibody V regions was first described in 1970, the mechanisms responsible for its regulation, targeting, and biochemistry have been remarkably elusive. This is especially surprising because the sequences of thousands of mutated H and L chain V regions have been determined and the general characteristics of the mutational process are known. The rate of mutation of antibody V regions is estimated to be one million times higher than the rate of mutation in most other genes, with V regions accumulating 5–10 mutations during the secondary antibody response. Somatic mutation begins a few hundred bases downstream from the promoter of rearranged V regions and continues for ~1.5 kb (2) but not further downstream to the intronic enhancer and the constant region. Mutations are largely single base changes, although deletions and insertions occur (3). Transitions occur more frequently than transversions, and hot spot motifs such as RGYW (A/G, G, C/T, A/T) and its complementary sequence on the other strand are preferentially targeted. Although mutations are targeted to both strands, there is some controversy about whether there is strand bias (4).

Some of the cis-acting sequences responsible for the regulation and targeting of V region hypermutation have been identified through deletion analysis of Ig transgenes. In ectopically integrated L chain transgenes and in endogenous H chain genes in mice, promoters and enhancers that regulate transcription are required for mutation, although the promoter and the V(D)J target for mutation and can be replaced by non-Ig elements without affecting the mutational process (5). The requirement for transcriptional regulatory elements has led to the belief that transcription, or at least accessibility, is required for the activation of V region hypermutation (6).

Proteins that participate in V region mutation have been sought by studying mice and humans that are genetically defective in a wide variety of repair processes, including those that are linked to transcription. It appears that transcription-associated base and nucleotide excision repair is not involved in V region mutation (7). However, mismatch repair (MMR) does play a role, as V regions in mice that lack the MutS homologue (MSH)2 and MSH6, as well as postmeiotic segregation (PMS)2 and MutL homologue (MLH)1 that act downstream from them, have mutations mostly in G and C bases within hot spots, whereas almost no mutations are seen in A and T (8, 9). This has led to the suggestion that G and C are initially targeted for mutation and that the mismatches created by those changes are then recognized by the MMR proteins, which cause secondary mutations in A and T through some error-prone process (10). It has also been suggested that MMR proteins play a more direct role in the primary mutational event (3, 11).

As V(D)J rearrangement, somatic V region mutation, and isotype switching are all linked to transcription and thought to require DNA breaks, many studies have sought trans-acting proteins and biochemical mechanisms that might be shared by these three processes. Even though V(D)J rearrangement occurs early in B cell development in primary lymphoid organs, whereas both isotype switching

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and somatic V region mutation occur later in the germinal centers of secondary lymphoid organisms, there has been a recurrent interest in whether the RAG1 and RAG2 endonucleases could play a role in V region hypermutation. This has been difficult to test because Ig expression and B cell development is blocked in mice that are lacking these enzymes. Even if B cells were provided with already rearranged H and L chain genes, somatic mutation requires a T cell–dependent response, but both TCRs and T cell development are also blocked in mice that lack the RAG proteins. In this issue, Bemark et al. (12) have overcome this problem by creating $Rag1^{-/-}$ mice that have rearranged Ig and TCR transgenes. Although these mice are monoclonal for both antibody and TCR, they develop Peyer's patches with germinal centers. The important observation is that V region mutation occurs in the L chain transgenes of the $Rag1^{-/-}$ mice. Though there may be some subtle differences, the frequency and characteristics of the mutations were indistinguishable from the $Rag1^{+/+}$ mice bearing the same transgenes (12)

It seemed more likely that the DNA protein kinase catalytic subunit (DNA-PK$_{cs}$) might play a role in V region mutation, as in addition to its role in V(D)J rearrangement, it is required for isotype switching that occurs in the microenvironment of germinal center B cells at about the same time as V region mutation. Although V region mutation and isotype switching can occur independently, a mechanistic relationship was suggested by the finding that the MSH2 protein that recognizes single base pair mismatches in DNA was involved in both processes and might even be targeting the same hot spot motifs (13, 14). In addition, as it has been suggested that double-stranded DNA breaks that can recruit terminal deoxynucleotidyl transferase (TdT) occur during V region mutation (15), it seemed possible that proteins such as DNA-PK$_{cs}$ that are involved in repairing such lesions might also play some role in V region mutation. Using the same transgenic system described above, Bemark et al. (12) have also shown that SCID mice lacking DNA-PK$_{cs}$ activity can carry out somatic V region mutation in an apparently normal manner. This not only shows that DNA-PK$_{cs}$ is not required for V region mutation but also raises the possibility that double-stranded DNA breaks are not required for the mutational process (12).

A continuing examination of the basic characteristics of V region mutation has led to suggestions for specific biochemical mechanisms such as mutation associated with transcriptional pausing (6), polymerase slippage (3), error-prone polymerization recruited by MMR (10), or error-prone reverse transcription (2). Within the last few months, an unusual cytidine deaminase (AID) that resembles an mRNA editing enzyme was found to be highly expressed in germinal center B cells and to be required for isotype switching (16, 17). Mice lacking AID and patients with mutations in the gene encoding this enzyme have a 10-fold, or perhaps greater, decrease in V region hypermutation. Although the mechanism of action of AID is unclear, and V region mutation still seems to be occurring in mice

and humans with defects in this protein, these studies again suggest that the mechanisms underlying V region mutation and isotype switching may be related.

Thus, through a process of elimination and the identification of some trans-acting proteins that are involved, it is possible that we are beginning to close in on some of the mechanisms that could play a role in V region mutation. There is even reason to hope that we may be closer than we think. Many of the studies described above invoke some sort of error-prone process and thus harken back to the suggestion by Brenner and Milstein in 1966 (18) that an error-prone DNA polymerase must be involved in the generation of antibody diversity. By studying a transgene with an artificial substrate for mutation, Bertocci et al. (19) concluded that mutation resulted from nonreplicative error-prone short patch DNA synthesis, again pointing to a central role for an error-prone polymerase. Unfortunately, at that time, only a few error-prone DNA polymerases that might contribute to the mutational process had been identified in animal cells. One prime candidate was pol β, which can fill small gaps in DNA and is quite error prone. However, Esposito et al. (20) have shown that B cells lacking pol β carry out normal V region mutation in vivo, thus eliminating one more possibility.
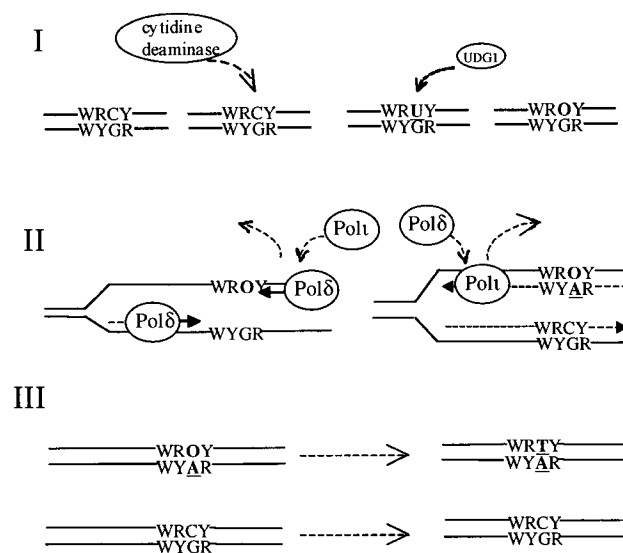

Figure 1. A speculative mechanism for somatic V region hypermutation. I. An unusual cytidine deaminase might play a role in the introduction of abasic lesions (O) in DNA via conversion of C to U, followed by the removal of the U by uracil glycosylase (UDG1). RGYW/WRCY represent a hot spot template sequence (A/G, G, C/T, A/T), with a mutation occurring most frequently opposite the G/C. II. A high-fidelity processive replicative polymerase (pol δ) stalls at the abasic lesion, dissociates, and is replaced by a promiscuous nonprocessive polymerase, e.g., pol ι (shown in sketch), or by some other errant polymerase. After translesional synthesis resulting in a mutation opposite the lesion, pol ι dissociates, and processive synthesis resumes with pol δ. III. A subsequent round of replication results in a mixed population of B cell clones, some mutated and some not. Alternative mechanisms can be envisioned that do not involve the introduction of a lesion. Instead, an undamaged DNA template could be copied by a low-fidelity polymerase such as pol ι, causing mutations that are favored in the RGYW hot spot sequence.

Although only a few mammalian error-prone DNA polymerases were known two years ago, recent studies in bacteria, yeast, and animal cells (21) shed new light on a class of enzymes that could be responsible for V region mutation. These are members of the UmuC/DinB/Rev1/Rad30 family of proteins that are required to replicate damaged DNA and are also responsible for many spontaneous mutations in *Escherichia coli* and *Saccharomyces cerevisiae*. Recent biochemical studies reveal that most members of the UmuC/DinB/Rev1/Rad30 family of DNA polymerases can be highly error prone when replicating normal undamaged DNA while also exhibiting the ability to tolerate damaged bases in a DNA template. Whereas most DNA polymerases stall when they encounter an aberrant base, these remarkable polymerases bypass lesions in damaged DNA by inserting one or a few bases across from the template stand (Fig. 1). These enzymes lack editing functions and, because they are relatively nonprocessive, they have to be replaced by replicative polymerases to fully extend the DNA. In the last two years, human and mouse homologues for this family have been identified based on their homology with five sequence motifs that are conserved in this family (22). The roles of these enzymes in vivo and the details of their tissue and cellular expression are largely unknown.

As additional members of this polymerase family such as pol ι have been characterized (23), it has been suggested that they might play a role in V region mutation. We have found that pol ι is expressed in lymphoid tissue. A role for pol ι is also suggested by its preference for incorporating G instead of A opposite T, creating transition mutations and mutating A rather than T (23), both characteristics of V region mutation. Other new polymerases have been discovered recently that are not members of the UmuC/DinB/Rad30/Rev1 family. The identification and characterization of pol ζ in yeast (24) and homologues in humans (25, 26) led Diaz et al. (27) to suggest that it might be playing a role in V region mutation. In a model system using human pol ι and yeast pol ζ, mismatches formed by pol ι were extended by pol ζ, and something akin to this could be occurring in vivo (28). It has also been suggested that pol μ, which is homologous to TdT and like pol ζ is not a member of the UmuC/DinB/Rev1/Rad30 family, might play a role in V region mutation (29). Pol μ can also carry out error-prone polymerization, and a large number of expressed sequence tags come from cells of germinal center origin, suggesting that it is highly expressed in B cells that are involved in V region mutation.

Thus, there is now an increasing abundance of error-prone DNA polymerases that, based on their expression and biochemical properties, could individually or in combination play a role in V region hypermutation. Even if studies that are now underway in many laboratories reveal that one or more of the errant polymerases plays a role in V region hypermutation, it will still be essential to understand how these molecules are targeted to Ig V regions at a particular stage in B cell differentiation. One possibility is that the relevant enzymes are induced in cells that are about to undergo V region mutation. They then might form a complex with B cell–specific factors and be targeted by cis–acting sequences to the Ig gene whose chromatin has been modified to make it accessible to this complex. This seems a likely possibility, as Bcl-6, which is also highly expressed in germinal center cells, is subjected to the V region mutational process (30, 31). As part of this process, the hot spots in the Ig gene and in Bcl-6 may be damaged, for example by the creation of abasic sites, and provide the signal for the recruitment and targeting of a mutation complex that may include pol ι and pol μ or other polymerases (Fig. 1). If these low-fidelity polymerases are to play a role in somatic hypermutation, it is unlikely that they act alone. For example, in *E. coli*, pol V acts in concert with Rec A, single-stranded DNA binding protein, and the β processivity binding clamp and γ clamp loading protein (that are also part of the replicative polymerase complex) to catalyze translesional synthesis (32). In an analogous manner, pol ι or one of the other error-prone polymerases might interact with B cell–induced factors that target these polymerases to variable gene loci. The important message is that these many error-prone DNA polymerases provide us with new opportunities to identify the major players responsible for V region hypermutation and then to see how they are regulated and targeted to the V region of Ig genes.

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