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Somatic immunoglobulin hypermutation

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Abstract

Immunoglobulin hypermutation provides the structural correlate for the affinity maturation of the antibody response. Characteristic modalities of this mechanism include a preponderance of point-mutations with prevalence of transitions over transversions, and the mutational hotspot RGYW sequence. Recent evidence suggests a mechanism whereby DNA-breaks induce error-prone DNA synthesis in immunoglobulin V(D)J regions by error-prone DNA polymerases. The nature of the targeting mechanism and the *trans*-factors effecting such breaks and their repair remain to be determined.

Introduction

The generation of B lymphocytes capable of producing high-affinity antibodies occurs through the accumulation of mutations in the variable (V) regions of rearranged immunoglobulin (Ig) genes [1] and the selection for B cells whose Ig antigen-receptors (i.e. BCRs) have accumulated mutations that increase affinity for the immunizing antigen [2]. Hypermutation of antigen-receptor genes has been described for a wide variety of vertebrates including sharks, frogs, sheep, mice and humans (reviewed in [3]). Although some differences among species do exist in the pattern of mutations effected by the Ig hypermutation machinery, many similarities reveal a common mechanism. Particularly striking similarities among species include a vast preponderance of base substitutions, identical mutational hotspots and, in the absence of antigenic-selection, a bias to generate transitions over transversions. In most species, somatic Ig hypermutation is triggered upon exposure to antigen, although sheep additionally utilize Ig hypermutation to generate the primary repertoire — as do chickens, pigs and rabbits, but in the context of gene conversion (reviewed in [3]).

Many aspects of the molecular machinery responsible for Ig hypermutation remain unknown, but some studies have revealed the *cis*-acting elements within the Ig locus that are required for hypermutation. These include the intronic enhancer for the κ chain and the heavy chain, and the V-region promoter, although the Ig promoter itself is not necessary as it can be replaced by a heterologous promoter (reviewed in [4]). The V region of the sequence is not necessary to target hypermutation as it too can be replaced by heterologous sequence. A putative role for the transcriptional machinery is likely, but it remains controversial

whether transcription plays a direct role in hypermutation or whether it simply facilitates accessibility of the locus to the mutational machinery.

Recent advances have begun to reveal some of the molecular components and intermediate products of the Ig hypermutation molecular machinery. It now appears that DNA lesions, either double-strand breaks (DSBs) or single-strand breaks, are a feature of this mechanism [5,6••,7••,8]. Indeed, templated nucleotide additions in the IgV region were detected in the human B-cell line Ramos that hypermutates constitutively following transfection with a TdT (terminal deoxy-nucleotidyl transferase) transgene. The mutational rate and pattern suggest error-prone synthesis by one or more of the translesion synthesis polymerases typically involved in the mutagenic bypass of DNA lesions, and recent evidence strongly implicates their involvement [9•,10••,11••].

In addition, recent studies have revealed a mechanistic link between Ig hypermutation and Ig gene conversion [12••], as well as the existence of a germinal-center-restricted cytidine deaminase (activation-induced cytidine deaminase [AID]) that appears to play a critical role both in Ig hypermutation and class-switch recombination (CSR) [13••].

A critical but yet-unknown aspect of Ig hypermutation is the mechanism that ensures the targeting of lesions to the IgV region, while sparing the constant domains, which are only a few kilobases (kb) downstream from the V region. Addressing this feature of Ig hypermutation is likely to reveal molecules that are unique to this process.

Here, we will review these findings and integrate them into a multilayered model of Ig hypermutation that at least includes the following: targeting by Ig-hypermutation-specific elements; introduction of DNA lesions into IgV regions; and gap synthesis by error-prone DNA polymerases. We then examine a possible role for AID in the regulation of Ig hypermutation.

Targeting of hypermutation to the V(D)J regions of rearranged Ig loci

Ig hypermutation specifically targets a region of about 1.5 kb that includes the region containing the V, D (diversity) and J (joining) region — the V(D)J region — and part of the JC intron. The C (constant) domains of the heavy and the κ chains are largely spared, although some very low-grade mutation can be detected in the first C domain of the mouse λ chain, perhaps because of the short length of the JC intron.

The 5′ boundary of the mutational track is sharp and it is near the transcriptional initiation site whereas the 3′ end tapers throughout a region downstream of the J clusters, within 1.5–2.0 kb of the transcription initiation site [14,15]. Interestingly, the mutational track maps with the location of the promoter as placing DNA segments, as spacers just downstream of the promoter displaces the mutational track upstream [16–18] prompting many investigators to propose a direct role of transcription in Ig hypermutation. Mutations are rarely seen upstream of the leader intron, suggesting that, although the location of the promoter is important for the local distribution of mutations, there is a paucity of mutations for a short track of about 200 bps (basepairs) just downstream of the promoter [19].

Hypermutation of the targeted region appears to be under the direction of transcription-related elements including the promoter and enhancer regions whereas sequences upstream of the promoter are dispensable as their deletion or replacement does not impact hypermutation [16]. Storb and colleagues found that duplicating the V-gene promoter upstream of the C region and moving the intronic enhancer downstream of the C region in a κ transgene conferred hypermutability to the C region [20], further emphasizing the role of these elements in hypermutation. Interestingly, the IgV promoter, as well as the V sequence itself, can be replaced by a heterologous promoter or heterologous sequence, respectively, without impacting hypermutability [21–23]. The Ig enhancer regions are critical (reviewed in [19]), particularly the intronic enhancer in the case of the κ locus, and much research is currently under way to determine the region within the enhancers that confers hypermutability. Therefore it appears that whereas the Ig enhancers and a promoter are required for hypermutation, the IgV promoter is not, suggesting that the region that targets the hypermutation machinery to the Ig locus is not within the promoter.

Recently, it was demonstrated that, in addition to the Ig locus, at least one other gene in humans, encoding BCL-6, can be targeted by the hypermutation mechanism [24,25]. The targeting of hypermutation in human BCL-6 requires the application to the B cells of the same stimuli that are critical for the induction of Ig hypermutation: T-cell/B-cell contact, involving CD40/CD154, CD80/CD28 engagement and crosslinking to the BCR [26•]. Nevertheless, because BCL-6 hypermutation occurs in human B cells but not in mouse B cells [19], because mutations are restricted to untranslated regions and because there is no obvious biological reason for BCL-6 to be hypermutated, human BCL-6 hypermutation is probably the accidental acquisition in the evolution of the human BCL-6 gene of sequences that help target the Ig hypermutation machinery. It will be interesting to identify regions within the human BCL-6 gene that are different from the mouse ortholog and that resemble enhancer regions in the Ig loci.

Introduction of DNA lesions

The discovery of B-cell lines that can either be induced to hypermutate [27] or that hypermutate constitutively [5] has enabled researchers to probe for the presence of DNA lesions in the IgV regions that correlate with hypermutation. Utilizing the Ramos human B-cell line, which hypermutates spontaneously, Sale and Neuberger [5] unveiled evidence for either DSBs or staggered nicks, throughout the IgV region. These authors introduced a TdT-expressing transgene into the Ramos B-cell line and found nontemplated nucleotide additions in the IgV regions; these breaks tended to be at, or near, known mutational hotspots.

Recently, Papavasiliou and Schatz [6••], and Bross and colleagues [7••] found evidence for DNA DSBs using ligation-mediated PCR in Ramos B cells and/or in mouse germinal-center B cells. In these studies, the DSBs tended to occur near the mutational hotspot RGYW. In the study by Papavasiliou and Schatz, the DSBs were found in the late-S and G2 phases of the cell cycle, prompting these authors to suggest that a homologous-recombination mechanism of break repair, with the sister chromatid as template, is operative in Ig hypermutation. The resolution of these breaks, however, remains undefined but it does not

require the nonhomologous DNA end-joining (NHEJ) mechanism of DSB repair [28••], strengthening the argument for homologous recombination. In addition, targeted deletion of molecules important to a variety of DNA-repair processes, such as nucleotide repair and base excision repair, did not impair hypermutation [29].

Further evidence for DNA breaks in hypermutation comes from a recent study where evidence for the long-suspected mechanistic link between Ig hypermutation and Ig gene conversion [3,30,31] was detected in a chicken B-cell line, which normally undergoes gene conversion in the IgV region. Strikingly, Sale and colleagues [12••] found that chicken DT40 cell subclones that were defective for various molecules involved in the repair of breaks via homologous recombination (XRCC2, XRCC3 and RAD51B) underwent significantly higher rates of somatic hypermutation than gene conversion. Considering that gene conversion, like hypermutation, requires a rearranged V(D)J (the acceptor), and that transcription or transcription-related sequences are necessary [32], it appears likely that Ig hypermutation and gene conversion share both the targeting phase and the introduction of DNA breaks. The mechanism utilized to process those breaks, however, probably differs between the two mechanisms — perhaps one (gene conversion) utilizing a pseudogene and the other (hypermutation) utilizing a sister chromatid as donors, during break repair via homologous recombination [12••].

Error-prone synthesis

The frequency of mutations generated by the Ig hypermutation mechanism is estimated to be about one-million-fold over background with an estimated rate of 10^{-3}/bp/generation [2,33]. Furthermore, the generation of a mismatched pair during synthesis requires that a putative hypermutation polymerase be capable of commonly inserting an incorrect base and also of extending from the newly generated mismatched terminus. It is likely that such putative error-prone synthesis during somatic hyper-mutation occurs outside of global replication and in the context of short patch error-prone repair [34].

Interestingly, in the hypermutation of the novel antigen-receptor (NAR) in sharks — where the mutational pattern is otherwise very similar to mammalian hypermutation —and in a shark Ig light-chain locus (NS3), many mutations occur in doublets, suggesting two misincorporation and mismatch-extension events [35,36].

The unprecedented mutation frequency and the likely requirement for mismatch extension is very difficult, if not impossible, to attain if the involved polymerases synthesize DNA with high fidelity and have proofreading capabilities. It is instead much more likely that the hypermutation polymerase is one (or several) of the growing family of DNA polymerases that have evolved to allow continued synthesis opposite DNA lesions that would otherwise stall a replication fork: the translesion synthesis polymerases. These polymerases — which among others include DNA polymerases η, ι and ζ — are error-prone and capable of extending, with various efficiencies, from a mismatched terminus [37•,38,39].

Whereas DNA polymerases η and ι are highly error-prone when acting on undamaged DNA, DNA polymerase ζ may not be as error-prone but is very efficient at extending from a mismatched terminus. In fact, recent evidence suggests that these polymerases can act in

concert, where either η or ι inserts an incorrect base, and the ubiquitous polymerase ζ extends from the mismatched pair [40,41], thus stabilizing the mutation. Given the recent data suggesting a role for DSBs in Ig hypermutation, the finding in yeast that increased incidences of base-substitutions near DSBs are entirely dependent on DNA polymerase ζ [42] makes this polymerase a natural candidate for Ig hypermutation [3].

Several recent studies have provided evidence for at least two of these polymerases in Ig hypermutation. Zeng and colleagues [9•] found that in patients with the variant form of *Xeroderma pigmentosum*, who have been shown to have mutations in the DNA polymerase η gene, Ig mutations from A and T nucleotides were greatly reduced, prompting the investigators to suggest that polymerase η is an AT mutator in Ig hypermutation. Rogozin and colleagues [43•] further found that polymerase η tended to be more likely than other polymerases to generate mutations at Ig hyper-mutation hotspot dinucleotides. Zan and colleagues [10••] obtained a dramatic reduction in Ig and BCL-6 mutations when the hypermutation-inducible human B-cell line CL-01 was exposed to antisense oligonucleotides that target the RNA for the catalytic subunit of DNA polymerase ζ (Rev3). Such a reduction in Ig hypermutation was associated with a comparable reduction in UV-damaged induced mutagenesis, as typically seen for cells with low levels of this polymerase.

Furthermore, Diaz and colleagues [11••] generated a mouse transgenic model where antisense RNA is made to the RNA encoding part of the REV3 gene. In this *in vivo* study, transgenic mice generated vigorous immune responses, and large and abundant germinal centers, but most failed to generate high-affinity antibodies; the accumulation of mutations in IgV regions was reduced, with a pronounced reduction in mutations that enhance affinity to antigen. In contrast to the polymerase η study, no appreciable alteration in the targeting of nucleotides was detected in the studies where the role for polymerase ζ was examined.

Perhaps this difference is explained by a mechanism whereby both polymerases act together, one (polymerase η) as a mispair inserter and the other (polymerase ζ) as a mispair extender. It is also possible that polymerase ι is involved in Ig hypermutation because the pattern of mutations it generates in undamaged DNA is strikingly similar to Ig hypermutation, particularly in its asymmetrical targeting of A rather than T nucleotides depending on the DNA strand that it is acting upon [44]. However, to date, no direct analysis of a putative role of this polymerase in Ig hypermutation has been examined. Finally, other error-prone DNA polymerases such as β, μ and λ have been shown not to be required for Ig hypermutation ([45]; C-A Reynaud, J-C Weill, personal communication).

Ig hypermutation regulation — a possible role for AID

Recently an RNA-editing enzyme that is specific to activated germinal-center B cells and/or cells undergoing CSR was discovered by Muramatsu and colleagues [13••]. Inactivation of this gene in mice resulted in abrogation of CSR and somatic hypermutation, strongly suggesting a mechanistic and/or regulatory link between Ig hypermutation and CSR [46]. Humans with mutations in the AID gene develop a type of hyper-IgM syndrome with absolute impairment in CSR and significant but not complete impairment in Ig hypermutation [47••]. Given its potential function as an RNA-editing enzyme, it is very

likely that AID plays a role in the modification of RNA transcripts that encode molecules critical to Ig hypermutation and CSR [46,48,49] (and perhaps Ig gene conversion?). Whether this molecule is involved in the regulation of hypermutation targeting, lesion introduction or error-prone repair remains a fascinating question that is likely to unveil novel molecules important for these mechanisms.

Conclusions

An emerging model of somatic hypermutation based on the most recent data from different laboratories, including ours, incorporates the targeted introduction of DNA breaks into Ig V(D)J regions, followed by error-prone repair, perhaps via homologous recombination using a sister chromatid as a template (Figure 1). It is likely that the mutational hotspots are the sites where the breaks occur, although one cannot rule out that they are a signature of the error-prone DNA polymerases involved. DSBs are likely to be necessary but not sufficient for the introduction of somatic mutations. However, a direct causal relationship between DNA breaks and mutation induction has not been determined and it is possible that these breaks are the by-products rather than the cause of hypermutation. The nature of the molecules responsible for effecting the DNA breaks is undefined but Rag-1 does not appear to be involved [28••].

In addition, the role of AID and the importance of mismatch-repair proteins in hypermutation remain unclear. Mismatch-repair proteins have been implicated in Ig hypermutation [49–51], but the potential for indirect effects, such as genomic instability and reduced proliferative potential from mismatch repair deficiency [52,53], has obscured their importance in Ig hypermutation. The fact that mismatch-repair-deficient mice display a significant alteration in the Ig mutational pattern (namely a bias for targeting of GC nucleotides) suggests a direct role in Ig hypermutation, because it is difficult to explain how a defect in proliferation would yield an alteration in the pattern of hypermutation. DNA polymerase ζ seems to play a critical role in Ig and BCL-6 hypermutation, but the nature of the break-repair mechanism and how high-fidelity polymerases are excluded from it remain to be better defined. DNA polymerase η may also play a role, although it may be minor ([54]; but see [55,56]).

Finally, the mechanism that targets hypermutation to the Ig locus and human BCL-6 remains unknown, although there is strong evidence for an important role of the Ig enhancers and perhaps transcription in this process [57•]. The spontaneously hypermutating and inducible mono-clonal B-cell lines that have become recently available should prove to be important transfectable vehicles for the definition of the role of different *cis* elements in hyper-mutation of the Ig and BCL-6 loci, as well as for the identification of the *trans*-factors and DNA polymerases that effect and mediate the DNA lesions and their repair.

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Abbreviations

AID	activation-induced cytidine deaminase
C	constant
CSR	class-switch recombination
D	diversity
DSB	double-strand break
Ig	immunoglobulin
J	joining
kb	kilobases
TdT	terminal deoxy-nucleotidyl transferase
V	variable

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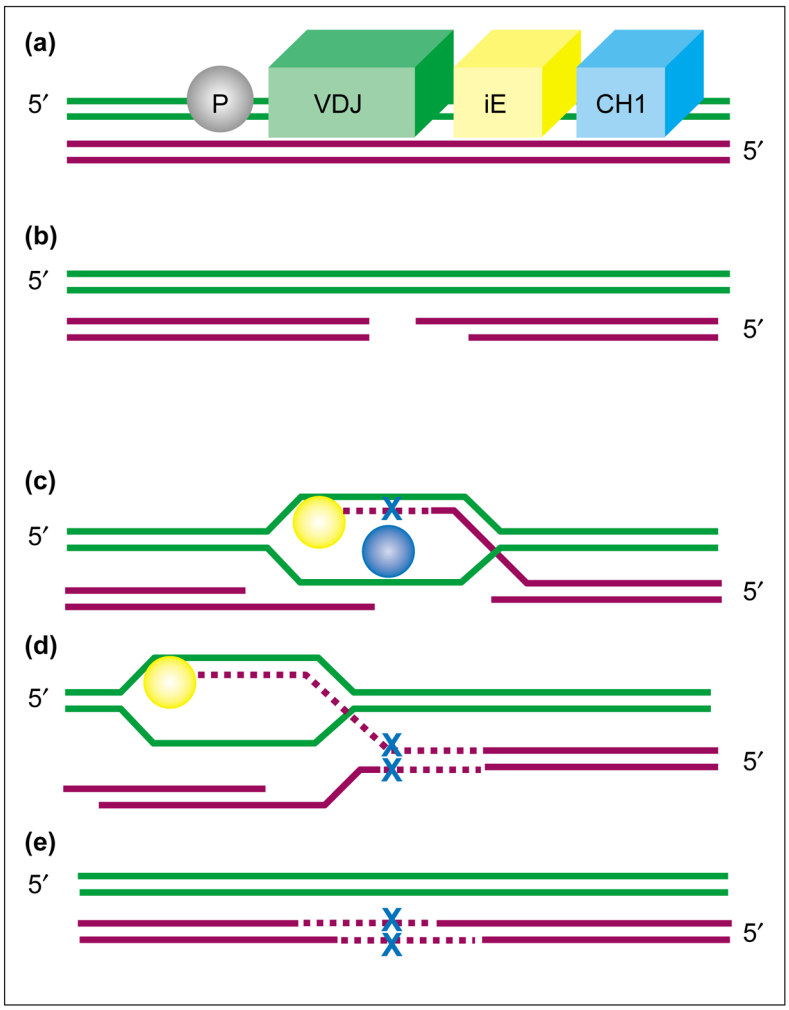

Figure 1.

The emerging model of somatic hypermutation. **(a)** Specific elements in the intronic enhancer (iE) target hypermutation to the V(D)J region (the heavy-chain is used as an example; CH1 is its first constant region). A promoter (P) (although not necessarily the Ig promoter) is required for hypermutation. **(b)** The introduction of DNA-breaks in the V(D)J region leads to the opening of a gap. **(c)** Homologous recombination is initiated and uses the sister chromatid as a template. BCR cross-linking regulates the expression of translesion DNA polymerases, including ζ and η. This results in error-prone gap DNA synthesis, including mispair insertion (blue cross) by one of several translesion polymerases (blue sphere). **(d)** Mispair extension by polymerase ζ (yellow sphere) will then occur. **(e)** The resultant hypermutation allows for high-affinity antibodies to be produced.