DNA repair: **Knockouts still mutating after first round**

Richard D. Wood

Recent studies have investigated whether particular DNA repair pathways are involved in the somatic hypermutation mechanism that increases antibody diversity. The primary mutation mechanism still functions in mice carrying knockouts of all repair genes examined, but mismatch repair defects affect the final outcome.

Address: Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK.

Current Biology 1998, 8:R757–R760
http://biomednet.com/elecref/09609822008R0757

In vertebrate cells, a huge variety of antibodies is generated by the familiar mechanism of recombination between the sets of a few hundred different variable (V), diversity (D) and joining (J) genes in the immunoglobulin locus that encode heavy and light antibody chains, before linking these rearranged segments to constant (C) genes. Through V(D)J recombination and its associated junctional variability, there are more than enough potential combinations to allow each human B cell to produce a distinct antibody. B cells that generate appropriate antibodies against an antigen survive and proliferate during a process of primary selection. To produce antibodies with higher specificity, a second cycle of 'affinity maturation' takes place. During this process, amino acid changes occur in the rearranged V regions. Cells that express antibodies with alterations that increase the affinity for a particular antigen are then selected.

How do these amino acid changes arise? In mammalian cells, the main process is the directed introduction of single-base point mutations throughout the V gene DNA. These occur with an estimated frequency of 10^{-3} to 10^{-4} per gene per cell generation, and accumulate as a memory B cell matures. The mutation process occurs when B cells migrate into and divide in germinal centers — regions of B-cell maturation that are concentrated in lymphoid tissues such as the spleen and the Peyer's patches scattered along the wall of the small intestine. B cells cycle in and out of the germinal centers many times, with the end result being that in a cell activated for mutation, 0.1% to 1 or 2% of the nucleotides in a V gene have been changed from the germ-line sequence. The process has been given the name 'somatic hypermutation' because of this high frequency of mutation (about a million-fold higher than the spontaneous mutation frequency for other genes) and its occurrence in cells other than those of the germ line.

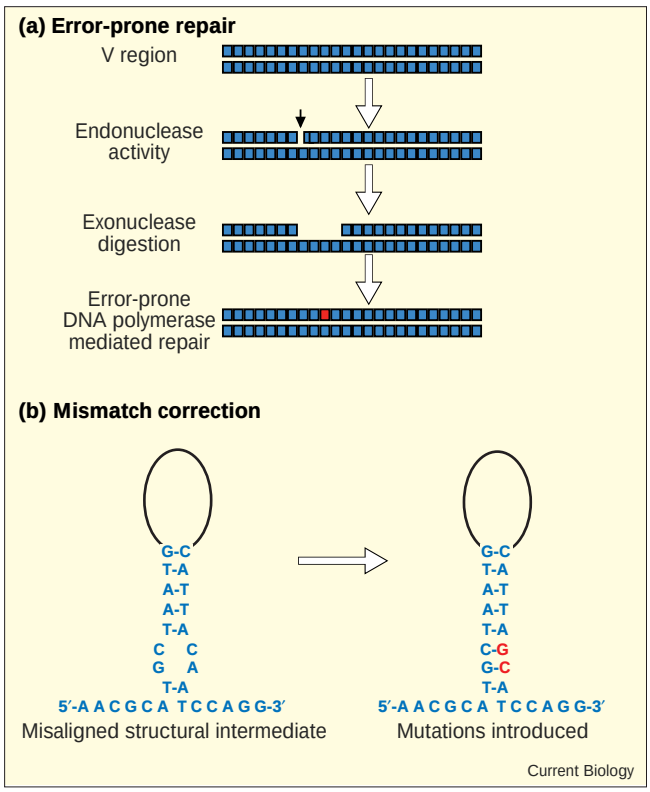
A remarkable enhancement of affinity for antigen can be achieved by hypermutation. For example, a recent structural study of the antigen-binding site of an antibody shows how the introduction of nine amino acid replacements by somatic hypermutation increases the affinity for antigen by 30,000-fold [1]. The unmutated germ-line antibody has to change its conformation significantly when binding antigen, while the mutated mature antibody binds with a tight 'lock-and-key' fit that does not require conformational adjustment.

There are many unanswered puzzles about somatic hypermutation. First, how are the mutations initially introduced? Some kind of error-prone DNA replication or DNA repair process that has gone awry is usually invoked (Figure 1a). Second, how are changes targeted to the V region? The mutations are remarkably focused, occurring in a region of about 2 kilobases centered on the V gene coding sequence and including the surrounding untranslated region [2]. The nearby C region downstream is apparently not mutated, and other genes in B cells which have been examined either are not mutated or accumulate mutations at a 100-fold lower frequency [3]. There are broad correlations between the mutability of a V region, transcription rate and distance from the promoter, as deduced from experiments in which relevant DNA sequence elements such as initiation sequences and enhancers are removed or altered [4,5]. Finally, if base changes are initially introduced into one strand of the DNA, how do they escape the defense system that cells normally use to eliminate mismatches from DNA?

Hypermutation in excision-repair-defective animals

The probable involvement of localized gap-filling of sections of DNA, resembling final steps in various DNA repair or recombination pathways has encouraged investigators to ask whether mammals defective in specific DNA repair enzymes can still carry out the hypermutation process. The nucleotide excision repair pathway was of interest because it involves filling a gap of ~30 nucleotides and because this type of repair occurs more quickly on actively transcribed genes than on the bulk of the genome. Consequences of defects in this repair pathway have now been examined using circulating B cells from individuals with the disorder xeroderma pigmentosum — an inherited condition associated with acute sunlight sensitivity and defects in nucleotide excision repair — or with the aid of mice that lack expression of proteins associated with nucleotide excision repair. Humans and mice defective in expression of the xeroderma pigmentosum genes *XPD* and *XPB* still undergo somatic hypermutation [6–8]; this does not completely exclude an involvement of nucleotide excision repair, however, because both of these genes are required for basal RNA polymerase II transcription as well

Figure 1



Two models for somatic hypermutation involving DNA repair, adapted from Male *et al.* [16]. (a) Error-prone repair of a strand break caused by a repair or recombination endonuclease. Following nicking of DNA and creation of a gap, a DNA polymerase in an error-prone mode introduces mutations during repair synthesis. Nuclear DNA polymerases implicated in various repair processes include DNA polymerases β, δ, ε, and ζ [17]. (b) Mismatch correction of misaligned templates. One strand of DNA is shown in this model. Imperfectly matched complementary secondary structures form transiently in a V region and are then acted upon by DNA mismatch correction, thereby changing the primary sequence.

as for the repair process, and so only mutations allowing partial activity are tolerated in humans. Mice with a disrupted *XPC* gene also show hypermutation, but such animals can still perform nucleotide excision repair in its faster, transcription-coupled mode [9].

Cells specifically disabled in transcription-coupled repair have been examined via disruptions of the human *CSA* [7] and mouse *CSB* [8] genes that are affected in Cockayne syndrome, another inherited human disorder associated with sensitivity to sunlight. The hypermutation mechanism was also found to be intact. The firmest evidence ruling out an involvement of the whole nucleotide excision repair process is provided by experiments with mice carrying disruptions of the *XPA* gene. Such mice have absolutely no residual nucleotide excision repair but are fully capable of somatic hypermutation, as shown by Winter *et al.* [10] and by Jacobs *et al.* [8]. Finally, cells from a xeroderma pigmentosum group V (variant) patient, harboring an unknown defect, can also carry out somatic hypermutation.

Mice with defects in enzymes related to other pathways of DNA repair and recombination were also checked by Jacobs *et al.* [8], including those lacking poly-ADP-ribose polymerase, the Rad54 protein which functions in homologous recombination and double-strand break repair, and the base excision repair enzyme 3-methyladenine-DNA glycosylase. All mice were found normal for somatic hypermutation.

Hypermutation in mice defective in DNA mismatch repair

Most research activity has been focused on the role of mismatch repair, the process that repairs DNA base–base mismatches (Figure 2). Mice with disruptions of several genes involved in the initial steps of mismatch recognition have allowed a definitive investigation of the role of mismatch repair in somatic hypermutation. There were several possible outcomes. First, mismatch repair might be intimately involved and required for the mechanism of somatic hypermutation. For example, it has been suggested that this type of repair could actually cause the mutations by processing heteroduplex DNA secondary structures that might form transiently in the V region [11] (Figure 1b). More likely, perhaps, one would expect that the absence of mismatch repair would cause the mutation frequency to increase even further in V genes, as any mismatches formed during the primary mechanism would escape correction. So what were the results? Taken together, the studies show clearly that somatic hypermutation can still occur at high frequencies in mice that lack the expression of mismatch-repair proteins, and rule out any models that involve these proteins as primary factors in the V gene mutation mechanism.

All experiments published so far were performed with mice deficient in the mismatch repair proteins Msh2 and Pms2, although Msh6, Msh3 and Mlh1 are known to be other key players in this repair process (Figure 2). Two types of experiments have been described. To examine the primary immune response, Phung *et al.* [12] and Frey *et al.* [13] immunized mice with phenyl-oxazolone and several weeks later analyzed the rearranged VκOX1 gene, which is known to be mutated after exposure to this antigen. Both groups found that the average mutation frequency in *Msh2*$^{-/-}$ mice was 1.3–1.5%, the same level as in mice expressing wild-type Msh2. Jacobs *et al.* [8] examined the Vλ1 gene in *Msh2*$^{-/-}$ and wild-type mice and also found similar frequencies of mutation. Further, immunized mice carrying disruptions of the *Pms2* gene were examined by Winter *et al.* [10] and Frey *et al.* [13] and these also had high frequencies of mutation, but at about 1%, slightly reduced from corresponding wild-type mice.

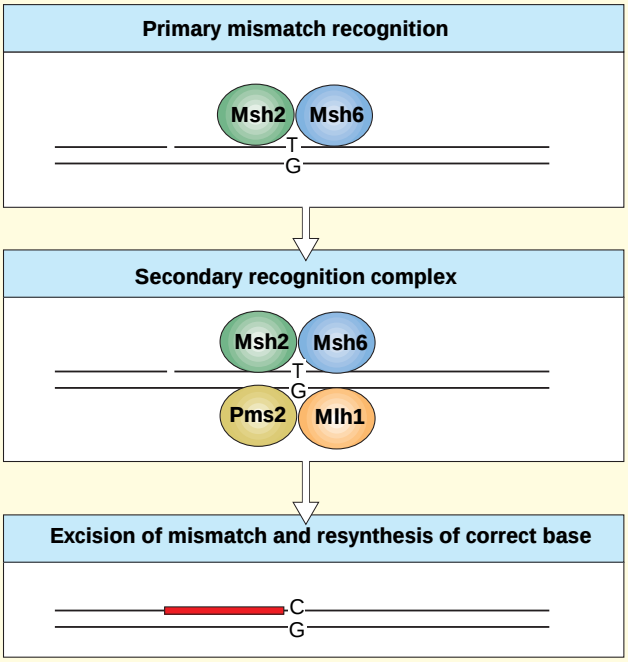
In an alternative approach, mice were not immunized, and genes in B cells from Peyer's patches were analyzed for mutations in variable regions of heavy-chain genes. This gives a picture of the immune response arising from chronic stimulation by diverse environmental antigens over the lifetime of the animal. Again, somatic hypermutation was observed, but here frequencies were clearly reduced in mismatch-repair-deficient mice, by about threefold in *Pms2*$^{-/-}$ mice [13] and by threefold [13] or fivefold [14] in *Msh2*$^{-/-}$ mice. Importantly, this lower frequency is readily accounted for in these deficient mice by the lack of clones having larger numbers of mutations (>9) per V gene. Investigation suggested a reasonable, if at first unexpected, explanation for the existence of fewer mutations per clone: mismatch-repair-defective mouse B cells show evidence of decreased maturity, and a diminished immune response [14].

Frey *et al.* [13] made the further telling observation that germinal center B cells of both *Pms2*$^{-/-}$ and *Msh2*$^{-/-}$ mice have high levels of instability of short repeated DNA sequences called microsatellites. Instability of microsatellite sequences is a consequence of DNA replication in mismatch-repair-defective cells and was found in 50–60% of germinal center B cells at the locus examined. This frequency is several times higher than that found in other cell types from mismatch-repair-deficient mice. The exceptionally high microsatellite instability is important because B cells can migrate in and out of germinal centers many times, with the possibility of accumulating a few additional somatic mutations in each cycle. The cells have a high proliferation rate, dividing every 6–8 hours during cycles of responses involving 20–40 divisions. A large number of chromosomal DNA alterations arising from microsatellite instability could readily cause problems with growth and division that would prematurely eliminate repeatedly stimulated B cells from the population. Cells would not survive long enough to accumulate larger numbers of mutations per clone, and this would explain the decreased frequency in chronically stimulated mismatch-repair-defective animals [13,14].

This deleterious effect of microsatellite instability on continued B-cell growth would have less of an impact on mutation frequency in experiments examining the primary immune response, as hypermutation is examined a few weeks to a month after immunization. In this instance, the number of cycles of mutation may be, on average, less than for chronic stimulation, as suggested by the rare occurrence in the immunization experiments of clones with very large numbers of mutations [10,12,13].

These considerations allow assimilation of a study from Wabl's group [15], which was the first of this string of reports to emerge. At first the results seemed to point towards a marked influence of mismatch repair on somatic hypermutation, with an average 10-fold reduction of frequency in a system involving a non-immunized *Pms2*$^{-/-}$ mouse [15]. Although mutation frequencies were calculated somewhat differently, it seems likely that the larger reduction of frequency may well be related to the particular system used in Wabl's study. The *Pms2*$^{-/-}$ mouse was crossed with a mouse which expresses only one heavy-chain V region and only one type of light chain. This very limited immunoglobulin repertoire causes an extraordinarily strong selective pressure on the available chronically stimulated B cells. Interference with cycles of proliferation because of microsatellite instability would therefore have more severe consequences. Wabl and colleagues [15] made the alternative suggestion that the mismatch repair

Figure 2



DNA mismatch repair in mammalian cells. Key features of mammalian mismatch repair are shown for the case of repair of a base–base mismatch. A heterodimer of Msh2 protein with Msh6 protein recognizes base–base mismatches as well as extrahelical loops of 1 or 2 nucleotides. This complex combines with a heterodimer of Pms2 and Mlh1. DNA between the mismatch and a nick on the newly synthesized strand is excised and replaced to correct the mismatch. This involves an exonuclease, a helicase, and a DNA polymerase holoenzyme. The repair of extrahelical loops of 2, 3, or 4 nucleotides is similar, except that Msh2 protein forms a heterodimer with Msh3 protein in the first step rather than with Msh6 protein. Repair of such loops is important for limiting microsatellite instability. Normally, mismatch repair is closely coupled to semiconservative DNA replication, so that mistakes made by DNA polymerase in a newly synthesized strand are corrected. The cue for recognizing which strand is the newly synthesized one may be a strand discontinuity (as indicated here), physical association of the mismatch-repair complex with the replication complex or the orientation of proliferating cell nuclear antigen PCNA (the DNA polymerase sliding clamp) on the DNA.

system is 'co-opted' for mutation and for some reason works in a direction opposite from normal on V genes, correcting the newly synthesized strand. Although this idea was received uncritically in an initial commentary, it does not easily accommodate the data and there is no evidence to support it.

At first consideration, the rate of somatic hypermutation might have been expected to increase in mice lacking the expression of mismatch-repair proteins. However, the microsatellite instability and lower maturity of mutating B cells in such mice indicates why this is not the case. Even when pressure for long-term B-cell survival is slightly relaxed, as in the immunized mice, it is possible that the mutation frequency does not go up any further because increases above the already high level of 1–2% are deleterious for antibody function. Furthermore, deletions and insertions, which would certainly inactivate antibody function, were found about five times more frequently in non-coding regions of the joining JH4 genes from mismatch-repair-deficient animals than in normal mice [13].

Altered spectra of hypermutation in mismatch-repair-deficient mice

There are some differences in the types of mutations seen in the various knockout mice examined. *Msh2*$^{-/-}$ mice clearly have more mutational changes from G and C residues on the coding strand than do normal mice, both for immunized [8,12] and chronically stimulated animals [13,14]. This indicates that mismatch repair actively influences the final result and is acting on some mismatches, even though the frequency does not increase for selective reasons. Perhaps the primary mutator mechanism tends to act preferentially at G and C nucleotides. Further, Winter *et al.* [10] note that mutations in immunized *Pms2*$^{-/-}$ mice include a somewhat higher proportion of tandem double-base changes. Such an increase in tandem changes was not observed in any of the studies of *Msh2*$^{-/-}$ mice. This hints at the existence of a previously unrecognized Msh2-independent but Pms2-dependent pathway for correction of tandem base changes, a possibility that deserves further investigation.

Selection for antibodies that bind antigen results in a preference for mutational changes in the complementarity-determining regions of V genes. These regions are hotspots for mutations and contain key G and C residues. In the Peyer's patch cells of mismatch-repair-deficient mice it appears that a higher proportion of mutations are found in these hotspots, which can account for some but not all of the G and C bias seen in *Msh2*$^{-/-}$ animals [13,14]. The increased proportion of hotspot mutations in *Msh2*$^{-/-}$ mice led Rada *et al.* [14] to propose a two-stage mechanism whereby hotspot mutations in the complementarity-determining regions are put in first, followed by later induction of mutations in surrounding regions. The later process may be impaired in mice defective in mismatch repair, and the reason for this may become clearer when the differences in immunological characteristics of these repair-deficient mice are more fully characterized. More is undoubtedly to be learned about why the spectra of mutations are altered in mice that lack the expression of different mismatch-repair proteins.

References

1. Wedemayer GJ, Patten PA, Wang LH, Schultz PG, Stevens RC: Structural insights into the evolution of an antibody combining site. *Science* 1997, 276:1665-1669.
2. Lebecque SG, Gearhart PJ: Boundaries of somatic mutation in rearranged immunoglobulin genes: 5′ boundary is near the promoter, and 3' boundary is approximately 1 kb from V(D)J gene. *J Exp Med* 1990, 172:1717-1727.
3. Shen HM, Peters A, Baron B, Zhu XD, Storb U: Mutation of ***BCL-6*** gene in normal B-cells by the process of somatic hypermutation of Ig genes. *Science* 1998, 280:1750-1752.
4. Neuberger MS, Ehrenstein MR, Klix N, Jolly CJ, Yelamos J, Rada C, Milstein C: Monitoring and interpreting the intrinsic features of somatic hypermutation. *Immunol Rev* 1998, 162:107-116.
5. Fukita Y, Jacobs H, Rajewsky K: Somatic hypermutation in the heavy chain locus correlates with transcription. *Immunity* 1998, 9:105-114.
6. Wagner SD, Elvin JG, Norris P, McGregor JM, Neuberger MS: Somatic hypermutation of Ig genes in patients with xeroderma pigmentosum (XP-D). *Int Immunol* 1996, 8:701-705.
7. Kim N, Kage K, Matsuda F, Lefranc MP, Storb U: B-lymphocytes of xeroderma pigmentosum or Cockayne syndrome patients with inherited defects in nucleotide excision repair are fully capable of somatic hypermutation of immunoglobulin genes. *J Exp Med* 1997, 186:413-419.
8. Jacobs H, Fukita Y, van der Horst TJ, de Boer J, Weeda G, Essers J, de Wind N, Engelward BP, Samson L, Verbeek S, *et al.*: Hypermutation of immunoglobulin genes in memory B-cells of DNA repair-deficient mice. *J Exp Med* 1998, 187:1735-1743.
9. Shen HN, Cheo DL, Friedberg E, Storb U: The inactivation of the XP-C gene does not affect somatic hypermutation or class switch recombination of immunoglobulin genes. *Mol Immunol* 1997, 34:527-533.
10. Winter DB, Phung QH, Umar A, Baker SM, Tarone RE, Tanaka K, Liskay RM, Kunkel TA, Bohr VA, Gearhart PJ: Altered spectra of hypermutation in antibodies from mice deficient for the DNA mismatch repair protein PMS2. *Proc Natl Acad Sci USA* 1998, 95:6953-6958.
11. Golding GB, Gearhart PJ, Glickman BW: Patterns of somatic mutations in immunoglobulin variable genes. *Genetics* 1987, 115:169-176.
12. Phung QH, Winter DB, Cranston A, Tarone RE, Bohr VA, Fishel R, Gearhart PJ: Increased hypermutation at G and C nucleotides in immunoglobulin variable genes from mice deficient in the MSH2 mismatch repair protein. *J Exp Med* 1998, 187:1745-1751.
13. Frey S, Bertocci B, Delbos F, Quint L, Weill J-C, Reynaud C-A: Mismatch repair deficiency interferes with the accumulation of mutations in chronically stimulated B cells and not with the hypermutation process. *Immunity* 1998, 9:127-134.
14. Rada C, Ehrenstein MR, Neuberger MS, Milstein C: Hot spot focusing of somatic hypermutation in MSH2-deficient mice suggests two stages of mutational targeting. *Immunity* 1998, 9:135-141.
15. Cascalho M, Wong J, Steinberg C, Wabl M: Mismatch repair co-opted by hypermutation. *Science* 1998, 279:1207-1210.
16. Male D, Cooke A, Owen M, Trowsdale J, Champion B: *Advanced Immunology*, 3rd Edn. London and Baltimore: Mosby; 1996.
17. Wood R, Shivji M: Which DNA polymerases are used for DNA repair in eukaryotes? *Carcinogenesis* 1997, 18:605-610.