***In vivo* and *in vitro* studies of immunoglobulin gene somatic hypermutation**

**Julian E. Sale^{1*}, Mats Bemark¹, Gareth T. Williams¹, Christopher J. Jolly²,
Michael R. Ehrenstein³, Cristina Rada¹, César Milstein¹ and Michael S. Neuberger¹**

¹*Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*

²*Centenary Institute, Locked Bag no. 6, Newtown, Sydney, New South Wales 2042, Australia*

³*Bloomsbury Rheumatology Unit, Department of Medicine, University College London, Arthur Stanley House, Tottenham Street, London W1 9PG, UK*

Following antigen encounter, two distinct processes modify immunoglobulin genes. The variable region is diversified by somatic hypermutation while the constant region may be changed by class-switch recombination. Although both genetic events can occur concurrently within germinal centre B cells, there are examples of each occurring independently of the other. Here we compare the contributions of class-switch recombination and somatic hypermutation to the diversification of the serum immunoglobulin repertoire and review evidence that suggests that, despite clear differences, the two processes may share some aspects of their mechanism in common.

Keywords: immunoglobulin; somatic hypermutation; class-switch recombination; terminal deoxynucleotidyl transferase; DNA-dependent protein kinase; translocation

1. INTRODUCTION

In mice and humans, the diversity of the primary repertoire of immunoglobulins (Ig) is generated by integrative recombination of germ line V gene segments. Following antigen encounter, two genetic alterations take place in the rearranged immunoglobulin genes: (i) somatic hypermutation allows further diversification of antigen-selected V genes and underpins affinity maturation; and (ii) class-switch recombination changes the constant region of the expressed immunoglobulin and hence its effector activity. Combined with antigen-mediated selection in the germinal centre, these two processes shape the repertoire of soluble antibodies.

The molecular mechanism of somatic hypermutation remains obscure. Much has been learned over the past decade of regulatory *cis*-elements within the immunoglobulin loci that influence the process. To date, studies of potential *trans*-acting factors in humans and mice carrying defined gene disruptions have told us much about gene products which are not involved or, at least, are redundant for hypermutation and a little about those which might be (reviewed in Wood 1998). An important development for the analysis of hypermutation has been the identification of cell lines that either inducibly or constitutively hypermutate their immunoglobulin genes such that mutations can be readily assayed by DNA sequencing after short periods of culture (Denépoux *et al.* 1997; Sale & Neuberger 1998; Wabl *et al.* 1985; Zan *et al.* 1999; Zhu *et al.* 1995). Such models are already providing clues as to the signals required to trigger hypermutation as well as insights into the molecular mechanism.

While the dissimilarities between hypermutation and class-switch recombination seem obvious, recent evidence points to some striking parallels. We review features of the two processes, which hint that they may share some aspects of their mechanism in common.

2. THE CONTRIBUTION OF SOMATIC HYPERMUTATION TO THE REPERTOIRE OF SOLUBLE IMMUNOGLOBULIN

The contribution of class switching to immunoglobulin repertoire has been analysed at both the cellular level and at the level of serum immunoglobulin. Class-switched isotypes account, in molar terms, for > 90% of serum immunoglobulin in adults but for < 10% of the total B-cell population in spleen and blood. In contrast, the contribution of somatic mutation to the immunoglobulin repertoire has routinely been analysed only at the cellular level, revealing that only a small fraction of murine splenic B cells carry mutated immunoglobulin genes, although mutation is a major contributor to V gene diversity amongst B cells in germinal centres (Berek *et al.* 1991; Gu *et al.* 1991; Jacob & Kelsoe 1992; MacLennan & Gray 1986). A cellular analysis of V gene diversity does not, however, take account of the fact that it is secreted antibody that mediates the protective arm of humoral immunity. Very little is known about the contribution of somatic mutation to this repertoire.

Employing an antibody which specifically recognizes the mutated form of a dominant hot spot in mouse V_κOx1, we found a striking difference between the accumulation of switched immunoglobulin in serum with age and the accumulation of mutated antibody (figure 1). Restricting attention to endogenous (rather than maternally

*Author for correspondence (jes@mrc-lmb.cam.ac.uk).

Figure 1. A serological assay for hypermutation using the monoclonal antibody JEN31. The majority of immunoglobulin at birth is IgM (upper solid line) with switched isotypes rapidly rising in titre such that they are practically at adult levels by the time of weaning (lower solid line). The mutation load in serum immunoglobulin (dashed line), however, rises with a much slower course, but continues to rise even past six months.

derived) immunoglobulin, this study revealed that neither mutation nor switching contributes to the primary (neonatal) antibody repertoire. However, after the first few days, and presumably following an encounter with environmental antigens, switched antibody rapidly appears in serum such that it practically reaches adult levels by the time of weaning. Mutated antibody, however, develops at a much slower rate, beginning to rise from the time of weaning and continuing to accumulate for a least six months. Thus, despite similar IgG titres, the immunoglobulin from a one-year-old mouse carries a mutation load some two orders of magnitude greater than that seen in an eight-week-old animal (Williams *et al.* 2000). The different kinetics with which switched and mutated immunoglobulins accumulate in serum could, at least in part, be due to switched-isotype memory B cells undergoing further rounds of mutation over time and thus developing an increased mutation load. This indeed is suggested by an analysis of mutation as monitored by V gene sequences in germinal centre B cells from Peyer's patches (González-Fernández *et al.* 1994). However, it is also possible that much of the switching in young mice occurs without concomitant hypermutation. That switching can occur without hypermutation is well documented: extrafollicular B cells are able to differentiate into antibody secretion following class switching without accompanying hypermutation (Jacob & Kelsoe 1992; Toellner *et al.* 1996) and human naive tonsillar B cells *in vitro* can be differentially triggered into isotype switching or somatic mutation (Razanajaona *et al.* 1997). Thus, switching and hypermutation, while linked, can clearly occur independently.

3. G:C-BIASED SOMATIC HYPERMUTATION AND ISOTYPE SWITCHING

A surprising finding in recent years has been instances of somatic hypermutation that exhibit a strong bias

towards targeting G and C nucleotides. 'Normal' hypermutation, as extensively characterized in mouse and human, exhibits a more-or-less even targeting of the bases with a slight bias towards mutating A on the coding strand (Milstein *et al.* 1998). G:C-biased hypermutation is seen in mice lacking the mismatch repair protein Msh2 (Frey *et al.* 1998; Phung *et al.* 1998; Rada *et al.* 1998). In these mice the overall mutation load is decreased and the pattern of mutation altered such that the targeting of G or C rises from *ca.* 48 to 73%. This observation prompted us to propose that hypermutation is a two-step process with the first step being G:C (or hot spot) focused and Msh2 independent with a second Msh2-dependent phase resulting in a more even spread of mutations (Rada *et al.* 1998).

In common with all cell line models so far studied, both inducible and constitutive (Bachl & Wabl 1996; Denépoux *et al.* 1997; Zan *et al.* 1999), the cell line model of somatic hypermutation ('Ramos', Klein *et al.* 1975) characterized in our laboratory exhibits a marked preference for mutating G and C nucleotides (Sale & Neuberger 1998). However, the explanation for this G:C bias is unlikely to be that Ramos is deficient in mismatch repair and, in particular, in Msh2. First, Msh2 protein can be readily detected by Western blotting in all Ramos clones so far analysed (Sale 1999). Second, Ramos exhibits no instability at the D3S11 microsatellite when analysed in 40 subclones (Sale 1999). Instability should be readily detectable in this number of clones in the face of most mismatch repair deficiencies with perhaps the exception of Msh6 (Edelmann *et al.* 1997; Parsons *et al.* 1995). Third, Ramos exhibits no increase in the background mutation rate as assayed by the spontaneous generation of hypoxanthine phosphoribosyl transferase (HPRT) mutants (Sale & Neuberger 1998). The rate observed at the HPRT locus in Ramos ($<1.0\text{--}2.8 \times 10^{-7}$ per locus per generation) is comparable with that determined for other cell lines (Kaden *et al.* 1989; Monnat 1989; Umar *et al.* 1998) and a variety of primary cell types (Green *et al.* 1995; Jacobs & Demars 1978). In contrast, cells deficient in mismatch repair proteins, including Msh2, exhibit mutation rates between ten- and 100-fold higher (Modrich & Lahue 1996). Thus, while G:C-biased hypermutation can be revealed through a deficiency in Msh2, such a deficiency is unlikely to be the cause of all G:C-biased hypermutation.

The finding of G:C-biased hypermutation in several exothermic vertebrates including *Xenopus* (Wilson *et al.* 1992) and the nurse shark (Hinds-Frey *et al.* 1993) suggests that it may represent an evolutionarily more primitive form, mammals having evolved additional or alternative mechanisms to produce a wider spread of mutations. Intriguingly, however, while mutation of the nurse shark IgM is G:C biased, mutation of the recently described NAR (nurse shark, or new antigen receptor) is not (Diaz & Flajnik 1998). It will be interesting to know whether IgM and NAR are simultaneously mutated in the same cells.

Both we and others have noticed that in addition to a defect in hypermutation, Msh2-deficient mice exhibit impaired IgG responses to a T-cell dependent antigen while mounting a normal IgM response to the same (Rada *et al.* 1998; Vora *et al.* 1999). This led us to ask

Figure 2. The V gene of the constitutively hypermutating cell line Ramos is accessed by TdT. A schematic summary of the results presented in Sale & Neuberger (1998). The human Burkitt lymphoma cell line Ramos (Klein *et al.* 1975) performs constitutive V gene hypermutation. A subset of mutations within V_H inactivates Ig expression. Thus, after expansion of a single cell, an Ig-negative population can be demonstrated (upper fluorescence-activated cell sorting (FACS) panel). Following enrichment in a single round of sorting (lower FACS panel), V_H was amplified by polymerase chain reaction and cloned. The numbers at the centre of the pie charts show the number of V_H clones for which a case of V_H inactivation was identified. The segments of the pie show the causes of that inactivation: stop codons (stop) deletions (del) and sequence duplications (dup) are seen in Ig negatives from wild-type Ramos. When TdT is ectopically expressed, an additional class of inactivating mutations is seen—non-templated insertions. These insertions have the characteristics of being mediated by TdT and have no counterpart in the database of mutations from non-transfected cells.

whether this reflected an intrinsic defect in the switch recombination process. It does. Resting splenic B cells from *Msh2*^{-/-} mice show a marked diminution of *in vitro* switching to downstream isotypes in addition to an *in vivo* switching defect. Furthermore, *Msh2*^{-/-} mice revealed an altered distribution of switch junctions compared with their wild-type litter-mates: for both $\mu/\gamma 3$ and μ/α switches, *Msh2*^{-/-} mice show a bias in favour of the

μ -junction occurring within one of the GAGCT repeat motifs (Ehrenstein & Neuberger 1999).

Thus, hypermutation and switch recombination are region-specific events that exhibit a preferential localization at a consensus sequence. The major switch region repeat element (GAGCT) falls within the hypermutation hot spot consensus (in its most refined form GARYW (R', a purine base, G or A; Y', a pyrimidine base, T or C; W', a weak base, A or T) (Milstein *et al.* 1998)). Furthermore, under certain circumstances, exemplified by *Msh2* deficiency, both somatic hypermutation and switch recombination reveal enhanced focusing to these consensus sequences.

4. DNA BREAKS IN HYPERMUTATION AND ISOTYPE SWITCHING

The generation of DNA strand breaks is a feature of many models of somatic hypermutation, dating back to the earliest proposed mechanism (Brenner & Milstein 1966). Indeed, in the absence of any enzyme that is able to substitute a base directly by using, for example, a base-flipping strategy like the methyltransferases, cleavage of the sugar-phosphate backbone must be a necessary intermediate in mutagenesis. However, it is not clear whether such breaks would be single or double stranded and, furthermore, whether the break sites would be placed at one or a few stereotyped sites or widely scattered over the mutation domain.

The evidence for DNA breaks in class-switch recombination is clear since the process involves the excision of intervening DNA between the upstream and downstream isotypes with the excised DNA detectable in the form of switch circles (Iwasato *et al.* 1990; Matsuoka *et al.* 1990; Von Schwedler *et al.* 1990). The resolution of these breaks, necessary for the successful completion of switching, is dependent upon components of the DNA-dependent protein kinase (Casellas *et al.* 1998; Manis *et al.* 1998; Rolink *et al.* 1996).

We exploited a cell line model of somatic hypermutation, the human Burkitt's lymphoma, Ramos, to show that hypermutation is indeed accompanied by the creation of scattered breaks (Sale & Neuberger 1998). Ramos constitutively diversifies its immunoglobulin V regions by hypermutation. This hypermutation generates immunoglobulin-negative variants through the introduction of nonsense mutations as well as deletions and duplications, as described *in vivo* (Goossens *et al.* 1998; Wilson *et al.* 1998; Wu & Kaartinen 1995). When transfected with terminal deoxynucleotidyl transferase (TdT) this repertoire of immunoglobulin-negative cells is increased by the presence of mutants that carry short non-templated insertions in V_H (but not C _{μ}). Furthermore, the presence of TdT within the cell does not increase the background mutation rate supporting the selective targeting of the V region and suggesting that it is not acting as a global mutator (figure 2).

We have interpreted this TdT accessibility to indicate strand breakage within the hypermutation domain. It is not possible to tell whether such breaks are single or double stranded. Although TdT is able efficiently to tail single-stranded DNA *in vitro* (Sambrook *et al.* 1989) and can probably tail a nicked template (Grawunder &

Figure 3. Sites of TdT accessibility within Ramos V_H correlated with mutation spectrum. Schematic representation of data from Sale & Neuberger (1998). The arrows indicate the estimated border between V gene sequence and TdT inserted sequence. It is usually not possible to infer with complete accuracy the site of V gene incision from the TdT insert because the latter are often associated with sequence deletions.

Lieber 1997), its only known *in vivo* action is the tailing of double-strand breaks during V(D)J recombination (Alt & Baltimore 1982; Desiderio *et al.* 1984; Landau *et al.* 1987). *In vitro* studies suggest that this action on double-strand breaks is less efficient if the break is flush or has a recessed 3'-end (Bollum 1974).

Whether the TdT accessible breaks are intermediate steps in the mutation process or whether they reflect a by-product of it is also not clear. The TdT-mediated insertions are almost always found in association with adjacent sequence deletions or, more rarely, duplications. While we cannot exclude that the enzyme only tags the rare events that are destined to give rise to deletions or duplications, it seems more likely that the deletions and duplications seen in association with TdT insertions are simply the result of rejoining a tailed break.

It is likely, therefore, that the sites of TdT access in the V gene are giving clues to the position of the breaks within the V gene. The data is suggestive: 67% of the TdT insertions occur within or adjacent to an RGYW hot-spot sequence on either strand, compared with the 28% expected for a random tetranucleotide. Plotting the sites of TdT activity against the V_H gene mutation pattern of Ramos also shows a broad correlation of mutational activity and TdT accessibility (figure 3). Such a bias towards hot-spot consensus sequences has also been suggested following an analysis of sequence deletions and duplications in normal hypermutation (Wilson *et al.* 1998). Taken collectively, these data suggest that an early event in the mechanism of hypermutation is the creation of a DNA break within or near hot-spot consensus sequences within the V region.

Recent biochemical data has suggested an interaction between TdT and two subunits of the DNA-dependent protein kinase (DNA-PKcs and Ku80) (Mahajan *et al.* 1999; Mickelsen *et al.* 1999), supporting the observation *in vivo* that mice deficient in Ku80 lack N-region additions in their heavy chain D-J joints (Bogue *et al.* 1997). In view of this link, we wondered whether DNA-PKs might be mechanistically involved in hypermutation.

Answering such a question experimentally *in vivo* is not easy since mice lacking components of the non-homologous end-joining pathway, including DNA-PKcs, Ku70 and

Ku80 do not form peripheral lymphoid compartments adequate for the study due to their inability to process recombination-activating gene(s) (RAG) induced breaks during V(D)J recombination. To circumvent this problem we analysed mutation in germinal centre cells from RAG1-deficient and SCID mice with transgenically reconstituted B- and T-cell compartments and found that mutation proceeds normally on both these backgrounds. Thus DNA-PKcs activity is not required for normal hypermutation (Bemark *et al.* 2000). Thus, although both hypermutation and switching are associated with DNA breaks, these data provide evidence of a clear distinction in the processing of those breaks.

5. HYPERMUTATION AND TRANSLOCATION: HYPERMUTATION OF THE TRANSLOCATED *c-MYC* GENE IN RAMOS

Many lymphoid tumours are characterized by translocations involving either the immunoglobulin or T-cell receptor loci. Both these loci are incised with physiological double-strand breaks during development of a functional antigen receptor and during isotype class switching in the case of B cells. Aberrant resolution of these breaks appears to explain the majority of translocations in such tumours (reviewed in Boehm & Rabbitts 1989). However, some translocations, where the break-point is within the V region itself, cannot be explained by these mechanisms (Kennedy 1991), suggesting that they may have arisen as a by-product of hypermutation. The finding that normal hypermutation is associated with V gene breaks and accompanied by deletion and insertion events supports this idea (Goossens *et al.* 1998; Sale & Neuberger 1998; Wilson *et al.* 1998; Wu & Kaartinen 1995).

So what role might hypermutation be playing in lymphomagenesis? Several non-exclusive possibilities exist. First, the somatic hypermutation mechanism may be responsible for initiating translocation events. DNA double-strand breaks are potent inducers of recombination in mammalian cells (Rouet *et al.* 1994). However, recent evidence has shown that while a single chromosomal break rarely results in translocation, two simultaneous breaks produce a strong stimulus to translocation

Figure 4. Hypermutation in the translocated *c-MYC* allele in Ramos. On the left is a schematic representation of the two *c-MYC* alleles. The translocated allele can be distinguished by virtue of an A523T mutation. The primers used (arrows) and regions sequenced (thin lines) are indicated under each allele. The sequence diversity in each of these polymerase chain reaction products is indicated in the pie charts. The figures in the centre of each pie chart indicate the number of sequences obtained and the segments represent the proportion of sequences carrying the number of mutations indicated on the circumference of the chart.

(Richardson & Jasin 2000; Richardson *et al.* 1998). Thus, it might be expected that both a hypermutation associated break and a simultaneous break outside the immunoglobulin locus is required to induce translocation. Although this clearly may happen by chance, the odds might be shortened by the second locus also being affected by a hypermutation-like mechanism. Such non-immunoglobulin hypermutation has been described in the *BCL-6* gene (Pasqualucci *et al.* 1998; Shen *et al.* 1998), a transcription factor highly expressed in germinal centre B cells and implicated in the immunoglobulin gene translocations of diffuse large-cell lymphomas (Kerckaert *et al.* 1993; Ye *et al.* 1993). That the Ramos *BCL-6* gene exhibits no mutations above polymerase chain reaction background (Bemark & Neuberger 2000) may at first appear to conflict with these findings. However, thus far the mutation load demonstrated *in vivo* within *BCL-6* has been some ten- to 100-fold lower than that seen in the immunoglobulin loci. Given that the mutation rate of the Ramos V_H is probably about tenfold less than that seen *in vivo*, it is likely that the mutation rate of Ramos *BCL-6* will fall below the detection threshold of our sequencing assay.

In Burkitt's lymphoma, translocations involving the heavy or light chain loci bring the proto-oncogene *c-MYC* under the control of immunoglobulin gene *cis*-acting sequences. Soon after these translocations were demonstrated, point mutations were noted in the translocated *c-MYC* allele in sequences isolated from these tumours (Rabbitts *et al.* 1983, 1984; Taub *et al.* 1984). Thus, a second possibility is that hypermutation introduces mutations into a translocated oncogene at high rate thereby providing a substrate upon which selection for growth advantage could act (Rabbitts *et al.* 1984). So, are the *c-MYC* mutations in these tumours rare sporadic but selected events or is there a major increase in mutation in the translocated allele? We employed Ramos to address this question.

Only the translocated *c-MYC* allele of Ramos is expressed, the two *c-MYC* alleles being distinguishable by a point mutation in the translocated allele, A523T. Significant levels of mutation are found solely in the translocated *c-MYC* allele and then only downstream of the promoter (figure 4) (Bemark & Neuberger 2000). Like immunoglobulin mutation in Ramos, the mutation in

c-MYC was heavily biased towards G and C nucleotides (32:33 as opposed to a random expectation of 62% for this sequence) and, in particular, to AGC hot spots (52% as opposed to a randomly expected 18%). Additionally, as in normal hypermutation, a small number of non-point mutation events (deletions or insertion duplications) are seen. Although this pattern of mutation shows no evidence of the skewing effect of selection, it remains fully possible that mutations lying outside the region of *c-MYC* analysed or mutations introduced into other proto-oncogenes that have translocated into the immunoglobulin loci could well contribute towards lymphomagenesis.

6. HYPERMUTATION, CLASS SWITCHING AND TRANSCRIPTION

A connection between hypermutation and transcription has been noted in a number of studies (Betz *et al.* 1994; Fukita *et al.* 1998; Goyenechea *et al.* 1997; Peters & Storb 1996). It has been proposed that the link may reside in the pausing of RNA polymerase II either gratuitously (Peters & Storb 1996) or at sites of pre-existing DNA damage (Neuberger & Milstein 1995) with the subsequent recruitment of DNA repair. The transcription pause sites of *c-MYC* have been extensively studied (Bentley & Groudine 1986; Chung *et al.* 1987; Kerppola & Kane 1988; Strobl & Eick 1992) and so it became possible to ask whether there is any obvious link between these documented pause sites and sites of mutation. Combining the mutations found in Ramos with *c-MYC* mutations documented in other Burkitt lymphomas, no clear relationship could be demonstrated. This suggests, but does not prove, that RNA polymerase II pause sites are not preferential initiators of hypermutation.

Transcription is also necessary but not sufficient for class-switch recombination (Lorenz *et al.* 1995). It has been recently shown that the germline switch transcripts, generated from an exon 5' of the switch region (I_H) and the C_H exons (Lutzker *et al.* 1988; Stavnezer-Nordgren & Sirlin 1986) are themselves required (Hein *et al.* 1998). Switch transcripts are able to form stable RNA:DNA triplex structures with the switch region *in vitro* (Reaban & Griffin 1990). It is possible that such structures are mechanistically involved in the process *in vivo*.

It is tempting to speculate that the parallels between hypermutation and switching may extend to the mechanistic significance of their transcription linkage. In other words, rather than reflecting a role for RNA polymerase in the recruitment of hypermutation and switching factors, the transcription linkage may instead reflect a direct involvement of the RNA itself in the initiation of the switching and hypermutation processes. For example, attempts to resolve an RNA–DNA hybrid structure could lead to the recruitment of factors involved in DNA breakage and/or error-prone repair—thereby triggering switching or hypermutation. Indeed, it may be significant that the formation of triple-helix structures has been shown to be associated with the introduction of point mutations in adjacent sequences (Wang *et al.* 1996).

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