The EMBO Journal Vol.18 No.12 pp.3484–3490, 1999

Deficiency in Msh2 affects the efficiency and local sequence specificity of immunoglobulin class-switch recombination: parallels with somatic hypermutation

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During maturation of the immune response, IgM$^+$ B cells switch to expression of one of the downstream isotypes (IgG, A or E). This class switching occurs by region-specific recombination within the IgH locus through an unknown mechanism. A lack of switch recombination in mice deficient in components of the DNA-dependent protein kinase (DNA-PK)–Ku complex has pointed to a role for non-homologous end joining. Here we characterize a switching defect in mice lacking a protein involved in DNA mismatch recognition. Mice deficient in Msh2 give diminished IgG (but not IgM) responses following challenge with both T cell-dependent and T cell-independent antigens. This appears to reflect a B cell-intrinsic defect since B cells from Msh2-deficient mice also exhibit impaired switching (but not blasting or proliferation) on *in vitro* culture with lipopolysaccharide. Furthermore, those switches that do occur in Msh2-deficient B cells reveal a shift in the distribution of recombination sites used: the breakpoints are more likely to occur in consensus motifs. These results, which intriguingly parallel the effects of Msh2 deficiency on hypermutation, suggest a role for Msh2 in the mechanics of class-switch recombination.

Keywords: heavy chain switch recombination/ immunoglobulin class switching/mismatch repair/Msh2/ somatic hypermutation

Introduction

Class-switch recombination (reviewed in Lorenz and Radbruch, 1996; Stavnezer, 1996) allows a B lymphocyte that initially expresses an IgM antibody to develop into an IgG-, IgA- or IgE-expressing cell. It is exceptional amongst programmed gene rearrangements in that it is neither a homologous nor a site-specific recombination event; rather, it is region specific. Class switching occurs by the creation of a deletion in the heavy chain locus which removes the exons of Cμ and thereby brings the rearranged $V_HD_HJ_H$ segment into proximity with the C_H exons of one of the downstream isotypes. The 5′ end of the deletion lies either within or adjacent to the μ switch region (Sμ), an ~3 kb stretch of highly repetitive sequence which is located in the intron between J_H and Cμ. The 3′ end of the deletion lies at one of the switch regions located upstream of the C_H1 exon of each of the downstream isotypes. Analysis of a large number of switch junction sequences has revealed that the recombination itself occurs between non-homologous sequences without any clear consensus motifs defining either the donor or acceptor breakpoints (Dunnick *et al*., 1993; Kinoshita *et al*., 1998; Lee *et al*., 1998).

In an attempt to glean information about the molecular mechanism of class switching, various groups have monitored whether it is affected by deficiency in various generally expressed proteins involved in DNA recombination and repair. Such experiments revealed that switching is greatly diminished in B lineage cells from mice lacking components of the DNA-dependent protein kinase (DNA-PK)–Ku complex (Rolink *et al*., 1996; Casellas *et al*., 1998; Manis *et al*., 1998), arguing for a role for non-homologous end joining in switch recombination. During a similar screen looking at the effects of DNA repair deficiency on somatic hypermutation, we recently noted (Rada *et al*., 1998) that mice deficient in the mismatch repair protein Msh2 could make a fully satisfactory IgM response to a T cell-dependent antigen but exhibited greatly diminished IgG responses. Here we show that this defect reflects an intrinsic switching deficiency in the B cells themselves and leads to an altered pattern of switch recombination sites.

Results

Immunoglobulin heavy chain class switching can occur in Msh2-deficient mice: there is little difference between Msh2$^{+/-}$ and Msh2$^{-/-}$ siblings in the abundance of the various serum isotypes (Figure 1A). However, our previous work revealed a consistent 3- to 4-fold reduction in the titre of NP-specific IgG1 (but not IgM) antibody in NP–chicken γ-globulin (NP–CG)-immunized Msh2-deficient mice at day 8 and day 12 as compared with controls (Rada *et al*., 1998).

Impaired IgG (but not IgM) responses to NP–Ficoll

To discover whether the diminished IgG1 response to this T cell-dependent antigen might be attributable, for example, to impaired T cell help or whether it could reflect an intrinsic B cell defect, we compared the response of mice to the T cell-independent antigen NP–Ficoll. The results reveal that Msh2 deficiency leads to a greatly diminished IgG3 response to NP–Ficoll although the IgM-specific response is essentially unaffected (Figure 1B). A weak IgG1 anti-NP response was also found at day 14 in the control mice but was not detectable in the Msh2-deficient animals (not shown).

LPS blasts from Msh2$^{-/-}$ mice exhibit diminished switching to IgG3

That switching to IgG3 was greatly diminished even on day 8 of a T-independent response suggested that the

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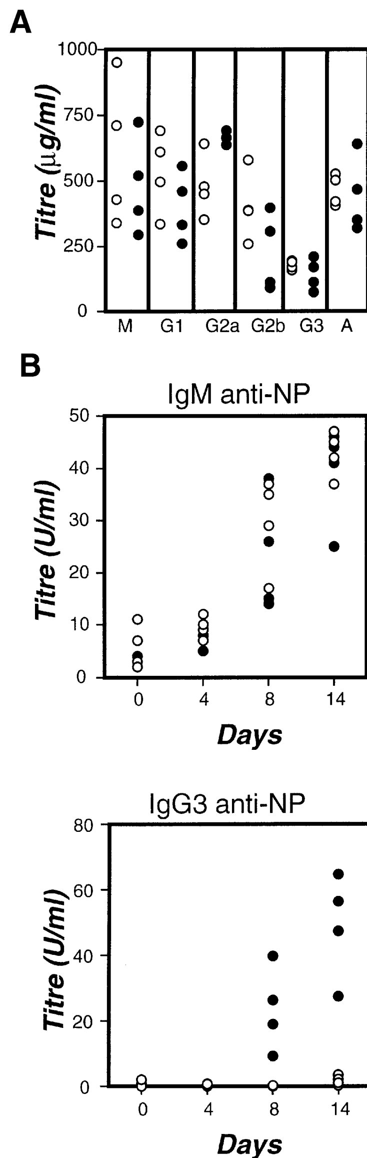


Fig. 1. Serum immunoglobulin titres in unimmunized animals and in mice challenged with a T cell-independent antigen. (**A**) Titres of serum immunoglobulin in unimmunized 8-week-old Msh2$^{+/-}$ (●) and Msh2$^{-/-}$ (○) mice. (**B**) Titres of NP-specific IgM and IgG3 antibody in mice challenged with NP_{13}–Ficoll. Specific antibody titres are given in units defined as in Ehrenstein *et al.* (1998).

switching defect in Msh2-deficient mice might be intrinsic to the B cells themselves. To test this, we compared the ability of splenic B cells from Msh2$^{+/-}$ and Msh2$^{-/-}$ mice to undergo class switching *in vitro* in response to lipopolysaccharide (LPS). The results reveal a 7-fold reduction in the proportion of blasting B cells that switch to IgG3 using B cells from Msh2-deficient mice as compared with control animals (from 5.3 ± 0.9% down to 0.7 ± 0.1%; Figure 2A). However, in keeping with our previous observation that Msh2 deficiency does not affect the proliferative response to LPS (Rada *et al.*, 1998), the proportion of B cells that have blasted in response to the LPS treatment is similar in the two sets of samples (Figure 2B). Furthermore, whereas the culture supernatants of the day 6 LPS-blasted B cells from Msh2-deficient mice contain a substantially diminished titre of IgG3, the concentration of secretory IgM is largely unaffected (Figure 2C). The effect of Msh2 deficiency on class switching is not peculiar to IgG3; a diminution in IgG1-expressing B cells (from 25 ± 2% to 9 ± 1%) is also seen following culture with LPS plus IL4 (Figure 2D). Thus, there is a deficiency in class switching exhibited by Msh2$^{-/-}$ mice which is, at least in part, a property of the B cells themselves.

Altered distribution of Sμ–Sγ recombination junctions in LPS blasts

These results led us to consider whether the deficiency in Msh2 might affect the mechanics of class switching rather than simply the induction of the process. Since switching can occur in the absence of Msh2 (albeit at significantly reduced efficiency), we asked whether lack of Msh2 affected the nature of the recombination junctions.

We used a PCR approach analogous to that used in other studies of switching (Du *et al.*, 1997; Lee *et al.*, 1998) to characterize the μ–γ3 switch recombination junctions in LPS-cultured B cells from Msh2$^{+/-}$ and Msh2$^{-/-}$ mice. Such an approach allows a reasonable database of switch junction sequences to be assembled, but the method will, nevertheless, introduce biases of its own. Thus, the distribution of the junctions characterized will be probably be biased in favour of those that yield smaller PCR amplification products. This should not, however, give rise to an artificial distinction between junctions detected in Msh2-proficient and -deficient B cells, and the size distribution of PCR products in the two sets of samples is indeed similar (see below). Another topic of concern, given the repetitive nature of the switch regions, is whether the distribution of sequences obtained by PCR could be distorted by the effects of polymerase slippage during the amplification itself or by non-specific oligonucleotide priming. The sequences of some of the PCR products could be modified by polymerase slippage between repeated sequences, but this is unlikely to distort the sequences of the actual μ–γ3 recombination junctions themselves since these do not show donor–acceptor homology (see below). To avoid non-specific priming, the amplification was performed using one oligonucleotide that primes forward from the unique sequence located in the 5′-flanking region of the repetitive region of Sμ with the other priming back from unique sequence located at the 3′ end of Sγ3, several hundred nucleotides distal of the tandemly reiterated 49 nucleotide repeats. This also has the advantage that information can be gleaned about recombination events that occurred in the Sμ 5′-flanking region which, since it is composed of interspersed rather than tandemly reiterated consensus pentamer motifs, can provide insight into the sequence specificity of the recombination.

The products of the PCR amplification were cloned into Bluescript vector and sequenced. A database of recombination events was assembled that is derived from clones obtained from independent LPS cultures performed on splenic B cells from multiple mice (Figure 3). Typically, only 1–3 distinct clones were sequenced from each PCR amplification.

The μ breakpoints identified were scattered both within the main repetitive region of Sμ as well as within its 5′ flank, agreeing with the conclusions from other studies

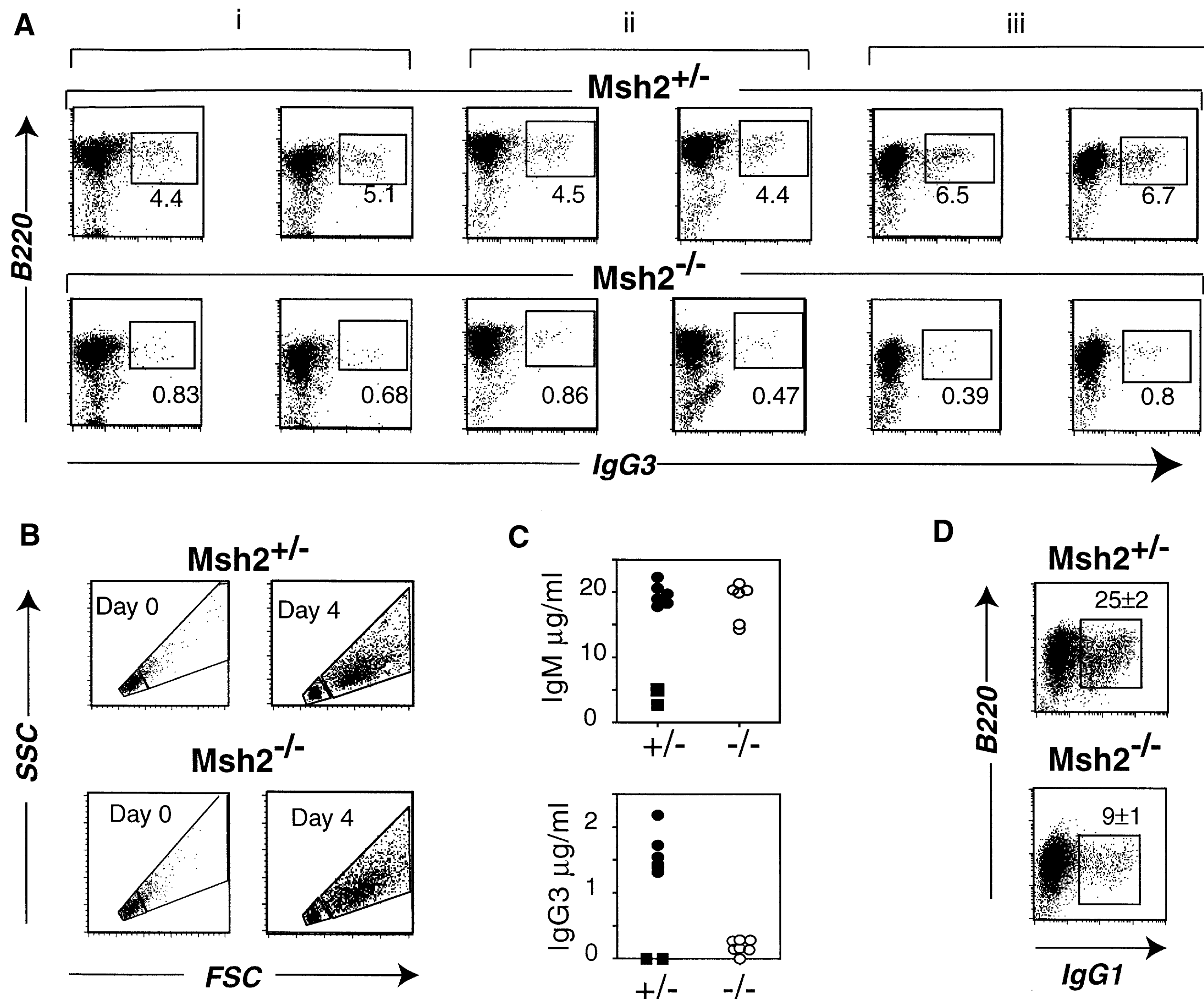


Fig. 2. Diminished class switching on LPS stimulation of B cells from Msh2-deficient mice. (**A**) Expression of surface IgG3. Spleen cells from six $Msh2^{-/-}$ mice and six litter-matched $Msh2^{+/-}$ controls were incubated in medium containing 50 µg/ml bacterial LPS for 4 days prior to cell-surface staining for IgG3 and CD45R(B220) and flow cytometric analysis. The analyses were performed on three separate litters (i, ii and iii) and the boxed areas indicate the proportion of $CD45R(B220)^+$ B cells that stain $IgG3^+$ (averaging 5.3 ± 0.9% in the $Msh2^{+/-}$ mice and 0.7 ± 0.1% in the Msh2-deficient animals). (**B**) Blasting response to LPS. The proportion of blasts that had developed during the 4 day culture with LPS was determined by analysing light scattering (FSC, forward scatter; SSC, side scatter). Results are shown for representative $Msh2^{+/-}$ and $Msh2^{-/-}$ mice; the proportion of blasts at day 4 was 60 ± 7.1% in the Msh2-deficient animals and 58 ± 10.7% in the $Msh2^{+/-}$ controls. (**C**) IgG3 expression in the culture supernatant of LPS-blasted cells. Titres of IgM (top panel) and IgG3 (bottom panel) in the day 6 culture supernatants (circles) were determined by ELISA. The filled squares indicate titres measured in the culture supernatants of the $Msh2^{+/-}$ controls at day 6 without stimulation. (**D**) Expression of surface IgG1. Spleen cells from $Msh2^{+/-}$ and $Msh2^{-/-}$ mice were incubated with 50 µg/ml LPS + 50 ng/ml IL-4 and analysed for surface IgG1 expression on day 4. Only one representative litter pair is presented, but the numbers above the boxed areas depict the mean percentage (±SEM) of $B200^+$ cells that are $IgG1^+$ as analysed on four litter pairs.

(Dunnick *et al.*, 1993; Lee *et al.*, 1998). However, comparison of the sequences of the µ breakpoints identified in Msh2-deficient and -proficient B cells reveals a striking difference (contrast the proportion of open symbols in the left and right hand panels of Figure 3B). Whereas only five of the 28 µ breakpoints identified in Msh2-proficient B cells occurred at a GAGCT or TGGGG motif, 17 of the 22 µ breakpoints from Msh2-deficient cells occurred at such a motif (mostly GAGCT), with four of the remaining five clones containing a GAGCT at the µ–γ3 switch junction created by nucleotide insertion (Figure 3).

Analysis of the γ3 breakpoints also reveals marked skewing. The γ3 switch region (like that of the other γ subclasses) is not composed of tandemly reiterated short motifs but rather exhibits a somewhat longer (49 nucleotide) repeat structure. By virtue of the PCR strategy used here, we will not have obtained information about biases in recombination junctions occurring within these 49 nucleotide repeats. However, even within the range of breakpoints detected, there is nevertheless clear preferential clustering of the γ3 breakpoints in the B cells from different Msh2-deficient mice as compared with controls (Figure 3D). The reason for this clustering is a matter for speculation. We note that the sequence at this cluster site bears homology to the sequence in the upstream repeat region of Sγ3 that has been proposed as a site of occurrence of double strand breaks (Wuerffel *et al.*, 1997). Furthermore, secondary structure predictions indicate that palindromic sequences in the vicinity of the cluster site would place it at the neck of a stem–loop, the same

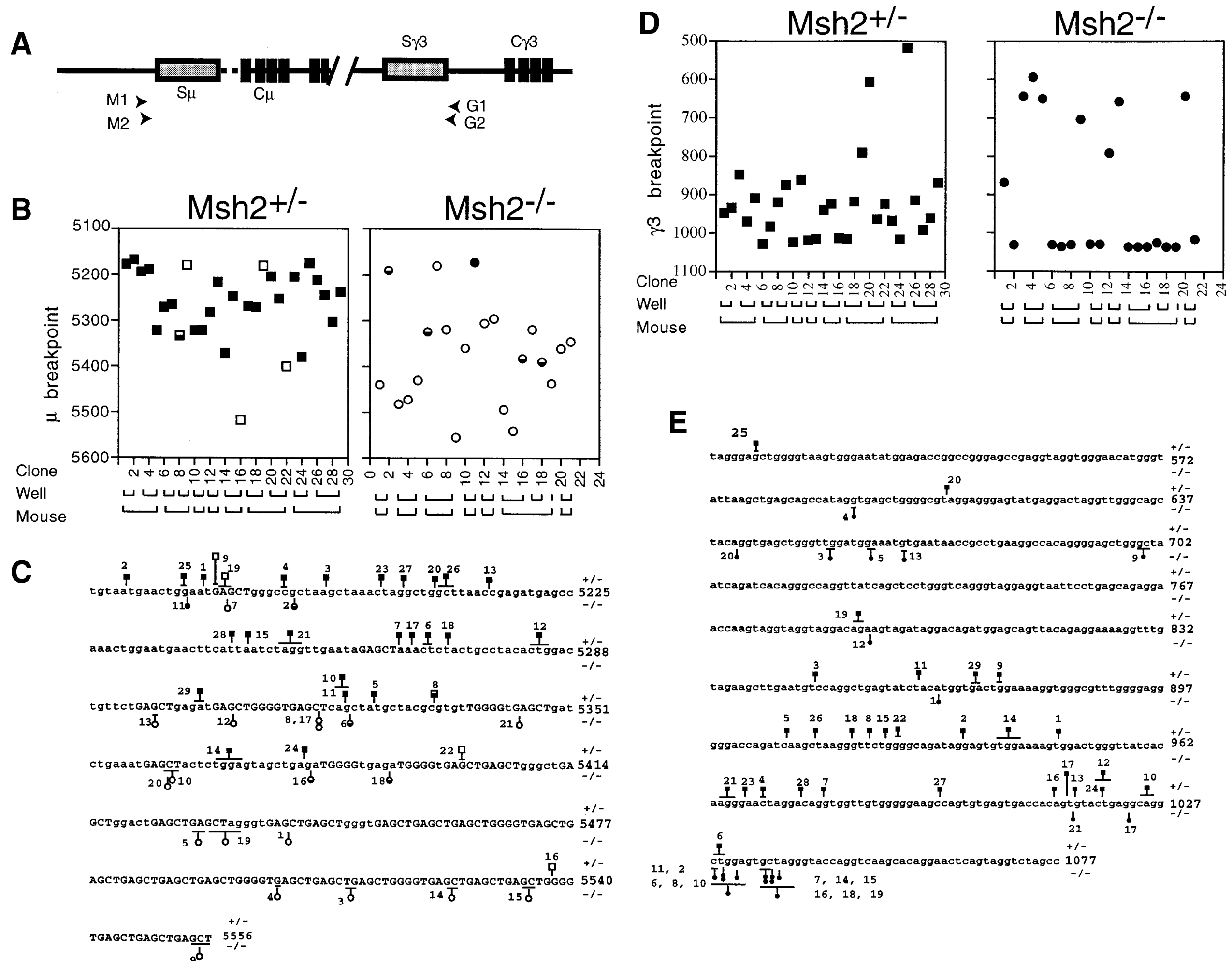


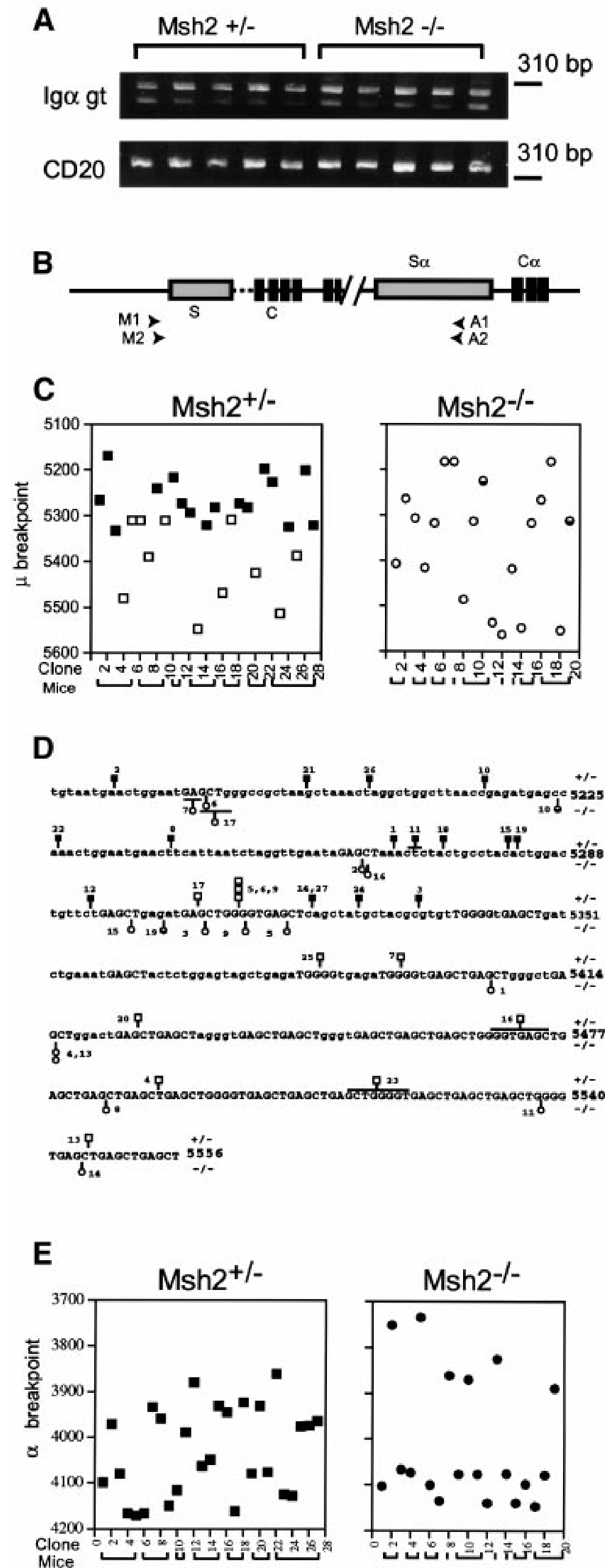
Fig. 3. Distribution of μ and γ3 breakpoints in IgG3 switch junctions in LPS blasts. DNA spanning μ–γ3 switch junctions was PCR amplified from individual wells of day 4 LPS splenic B cell cultures from seven Msh2$^{+/-}$ and seven Msh2$^{-/-}$ mice, cloned into Bluescript and sequenced. (**A**) PCR strategy used. The exons of Cμ and Cγ3 are depicted by filled boxes, the repeat regions of Sμ and Sγ3 by hatched boxes. The positions of the priming (M1 and G1) and nesting (M2 and G2) oligonucleotides used for the PCR are indicated. (**B**) Scatter analysis of the μ breakpoints. The charts depict the locations of the μ breakpoints of individual clones whose provenance is indicated on the *x*-axis. Nucleotide numbering on the *y*-axis is according to DDBJ/EMBL/GenBank accession No. MISIGCD07. Squares denote breakpoints from Msh2$^{+/-}$ mice and circles from Msh2$^{-/-}$ mice; open symbols denote breaks that occurred at a GAGCT or TGGGG consensus, those denoted by filled symbols did not. Half-filled symbols denote switches in which a GAGCT consensus had been created by nucleotide insertion at the switch junction: Msh2$^{-/-}$ clone 2 had AGCTGAGCTGA inserted, clone 6 had GAGCTGAGCTGAGCTGC inserted, clone 16 had a 149 nucleotide insertion that initiated CTAAGC, thereby forming a GAGCT consensus on juxtaposition with the μ breakpoint, and clone 18, whilst containing no insertion, contained a GAGCT at the breakpoint junction through abutment of μ and γ3 switch sequences. With regard to clones from Msh2$^{+/-}$ mice, clone 8 had a 44 nucleotide insertion that included a single GAGCT pentamer, whereas clone 11 harboured an insertion of ACATA. Single nucleotide changes were also identified in the vicinity (50 nucleotides either side) of recombination junctions. From the Msh2$^{+/-}$ mice, six clones had one mutation, three had two mutations and 20 had no mutations. From the Msh2$^{-/-}$ mice, eight clones had one mutation, two had two mutations and 11 had no mutations. These mutations do not reflect germline polymorphism since the same regions were sequenced in multiple clones; however, we cannot exclude the possibility that they were generated during the PCR amplification. (**C**) Positions of the μ breakpoints. The μ breakpoints in PCR-amplified μ–γ3 switch recombination clones from the Msh2$^{+/-}$ mice are indicated above the sequence and those from Msh2$^{-/-}$ mice are below the sequence. Symbols as in (B). GAGCT and TGGGG motifs, which are characteristic of the Sμ core, are in upper case. Where there is donor–acceptor homology at the switch junction (and, therefore, ambiguity about the precise location of the breakpoint), the extent of ambiguity is indicated by a horizontal line attached to the lollipop symbol. (**D**) Scatter analysis of the γ3 breakpoints. The clone numbers, indicated on the *x*-axis, and symbols are the same as in (B) except that open and half-filled symbols are not used since, unlike Sμ, the γ3 switch region is not composed of a highly reiterated pentanucleotide repeat. Nucleotide numbering on the *y*-axis is according to DDBJ/EMBL/GenBank accession No. MUSIGCD18. (**E**) Positions of the individual γ3 breakpoints depicted as in (C).

location as that proposed for the microsites at which switch recombination occurs in *Xenopus* (Mußmann *et al.*, 1997).

Altered distribution of Sμ–Sα recombination junctions in Peyer's patch cells

To ascertain whether Msh2 deficiency also affected the distribution of recombination sites in switches that occurred *in vivo* as well as switches to other isotypes, we analysed switches to Igα that had occurred in Peyer's patch lymphocytes. We initially confirmed that the Msh2 deficiency had not led to any major defect in the regulation of accessibility for class switching as judged by the presence of germline Sα transcripts (Figure 4A). We then amplified the μ–α junctions from Peyer's patch lymphocytes in order to detect whether the pattern of switch recombination sites was affected. Analysis of the

sequences of the μ breakpoints from Msh2-deficient mice again reveals a major bias in favour of the consensus motifs (Figure 4C and D); the two μ breakpoint identified in Msh2-deficient B cells that had not occurred at a GAGCT/TGGGG consensus had nevertheless acquired a nucleotide insertion at the μ–α junction creating a GAGCT.



With respect to the breakpoints within Sα (Figure 4E), there does appear to be a skewing in the distribution of breakpoints over the region analysed, but the database does not reveal markedly increased focusing on any short consensus motif. This may be attributable in part to the fact that the oligonucleotide for α amplification primes directly into a region comprising highly reiterated Sα short repeats, with the component Sα pentamers being identical to those found in Sμ.

Discussion

These experiments reveal that, in the absence of Msh2, class switching is less efficient both *in vivo* and *in vitro* and also exhibits an altered distribution of switch site breakpoints with a marked focusing towards consensus motifs.

With regard to the diminished efficiency of switching, we cannot rigorously exclude the possibility that the defects seen are a consequence of unidentified effects of Msh2 deficiency on B cell maturation. However, it seems unlikely since the B cells from Msh2-deficient mice have been shown to behave normally with respect to both proliferation (Rada *et al*., 1998) and blasting with consequent antibody secretion (this work). Similar findings with respect to a diminished efficiency of class-switch recombination by LPS-stimulated B cells from Msh2-deficient mice have also been noted by Stavnezer *et al.* (C.E.Schrader, W.Edelmann, R.Kucherlapati and J.Stavnezer, unpublished data). However, in a very recent study, Vora *et al*. (1999), whilst noting (like we do) attenuated *in vivo* switch recombination following antigen challenge, failed to detect an effect of Msh2 deficiency on the efficiency of *in vitro* switching. The discrepancy between the results of Vora *et al*. and those of Stavnezer and ourselves probably rests in the assays used to detect *in vitro* class-switch recombination.

Fig. 4. Distribution of μ and α breakpoints in IgA switch junctions from Peyer's patch lymphocytes. (**A**) Abundance of Sα germline transcripts (Igαgt) in Peyer's patch lymphocytes from five Msh2$^{-/-}$ and five Msh2$^{+/-}$ mice was examined by RT–PCR (30 cycles) using the primers and protocol described by Qiu *et al*. (1999). The amplification was controlled using oligonucleotides for CD20 (O'Keefe *et al*., 1998). No distinction in the abundance of the Sα RT–PCR product obtained from the Msh2$^{-/-}$ and Msh2$^{+/-}$ mice was observed even if 10-fold less cDNA was used for the amplification (not shown). (**B**) Map to indicate the PCR strategy used for analysis of μ–α switch junctions. The exons of Cμ and Cα are depicted by filled boxes and the repeat regions of Sμ and Sα by hatched boxes. The positions of the priming and nesting oligonucleotides used for the PCR (adapted from Lee *et al*., 1998) are indicated. (**C** and **D**) Scatter analysis and positions of the μ breakpoints in IgA switch junctions are illustrated using the same symbols as in Figure 3B and C. Nucleotide numbering on the *y*-axis is according to DDBJ/EMBL/GenBank accession No. MUSIALPHA. The Sα region has a quite high homology with Sμ (Arakawa *et al*., 1993) and contains GAGCT and TGGGG motifs, although not in such a high density as found in Sμ. Of the α breakpoints, 10 out of 27 clones from the Msh2$^{+/-}$ mice occurred in these repeats compared with 11 out of 19 clones from the Msh2$^{-/-}$ mice. (**E**) Scatter analysis of α breakpoints in IgA switch regions. With regard to insertions, two clones from Msh2$^{-/-}$ mice had insertions that resulted in a GAGCT at the switch junction, clone 10 having an insertion of TAGGCTGAGCTGCGCTAAA and clone 19 having an insertion of CTAA. In contrast, three clones from the Msh2$^{+/-}$ mice had insertions at the switch junction, but these did not include or create a GAGCT consensus (the insertion in clone 2 was GTTC and that in clone 20 was TTGAATTGCAATGTCT).

Quite apart from the diminished efficiency of switch recombination, the most intriguing aspect of the Msh2 deficiency is the altered distribution of switch recombination junctions. The mechanism by which Msh2 deficiency leads to consensus-biased switching is, of course, a matter for speculation. If we assume that the initial DNA breaks can be made either at or removed from the pentamer consensus motifs, then a possible interpretation is that rejoining involving a break made at a consensus is Msh2 independent whereas joining of consensus-distal breaks is Msh2 dependent. The alternative explanation, which we favour, is that the initial DNA breaks are biased intrinsically towards consensus motifs and that, in the absence of Msh2, switch-recombination junctions are created preferentially by direct abutting of the donor and acceptor breaks. If present, however, we envisage that Msh2 plays a role in the migration of the final point of synapsis away from the consensus motifs.

What might such a role be? Although classically viewed as acting to prevent recombination between mismatched duplexes (Radman, 1988), it is clear from more recent work in yeast that Msh2 can actually facilitate recombination (Sarpabaev *et al*., 1996) and acts to assist the removal of non-homologous 3′ ends during double strand break-induced gene conversion, probably by recognizing branched DNA structures (Paques and Haber, 1997; Sugawara *et al*., 1997). In the context of heavy chain class switching, we note that there is little homology between the donor and acceptor sequences at switch recombination junctions, whether generated in the presence or absence of Msh2. We therefore favour models in which Msh2 performs a function in switching by virtue of its ability to recognize discontinuities in the DNA duplex, be they branched structures with free 3′ ends, as envisaged by Sugawara *et al*. (1997), or loops or junctions in continuous DNA duplexes, as noted by Alani *et al*. (1995, 1997) and Kirkpatrick and Petes (1997).

The results presented here reveal intriguing parallels between heavy chain class-switch recombination and V region somatic hypermutation. The two processes evidently target distinct regions of the immunoglobulin loci and, whilst taking place at a similar stage of differentiation, can proceed independently. Nevertheless, we have proposed here that class switching, and have proposed elsewhere (Rada *et al*., 1998) that hypermutation, occurs in two stages. For switching, the first stage is the preferential creation of double strand breaks at consensus sequences; the second stage, which is Msh2 dependent, causes migration of the point of synapsis away from the original break site. For hypermutation, the first stage is also focused on intrinsic hotspots (Rada *et al*., 1998) and could well involve the introduction of DNA breaks (Sale and Neuberger, 1998); the second stage, which is Msh2 dependent (Rada *et al*., 1998), leads to mutation creation away from the hotspot sites. The GAGCT consensus of switch regions conforms to the consensus deduced several years ago for intrinsic mutational hotspots (RGYW; Rogozin and Kolchanov, 1992; Betz *et al*., 1993) and is indeed identical to the refined mutational hotspot consensus deduced in a more recent triplet analysis (Milstein *et al*., 1998). Furthermore, switching and hypermutation are both transcription-linked processes. Maybe in both cases this linkage reflects a role for the transcription complex in prompting the creation or detection of breaks formed in the vicinity of hotspots.

It is notable that the hotspot focusing of somatic hypermutation observed in mice deficient in Msh2 (Frey *et al*., 1998; Jacobs *et al*., 1998; Rada *et al*., 1998) was not evident in Pms2-deficient mice (Frey *et al*., 1998; Winter *et al*., 1998), suggesting that the role of Msh2 in hypermutation does not implicate the entire mismatch repair pathway. By analogy, it will be interesting to ascertain whether the distribution of switch recombination junctions is affected by deficiency in other components of the mismatch repair pathway apart from Msh2.

Materials and methods

Mice and immunizations

Msh2-deficient mice (de Wind *et al*., 1995) were kindly provided by Dr H.te Reile through Dr K.Brown (CRC Beatson Laboratories, University of Glasgow) and were bred and genotyped as previously described (Rada *et al*., 1998). For immunization, mice (8–12 weeks old) were challenged intraperitoneally with 5 μg of NP_{13}–Ficoll (Solid Phase Sciences, San Rafael, CA) in phosphate-buffered saline (PBS). Enzyme-linked immunosorbent assays (ELISAs) were performed on NP–bovine serum albumin (BSA)-coated plates using biotinylated anti-IgM and IgG1 and IgG3 antibodies from Pharmingen (San Diego, CA).

In vitro cultures

Single cell suspensions of spleen cells (after red blood cell lysis) were cultured at 1×10^6 cells/ml in RPMI medium supplemented with 10% fetal calf serum (FCS) and 50 μg/ml *Escherichia coli* LPS (Sigma, Poole, Dorset, UK) with or without 50 ng/ml interleukin-4 (IL-4) (R&D, Minneapolis, USA). Cultures were performed for 4 days for flow cytometric analysis and DNA extraction but for 6 days when monitoring immunoglobulin secretion into culture supernatants. Flow cytometric analysis of surface IgG1 or IgG3 expression was performed using biotinylated anti-immunoglobulin antibodies (Pharmingen), fluorescein isothiocyanate (FITC)-conjugated streptavidin (Dako, Denmark) and phycoerythrin-conjugated rat RA3-6B2 monoclonal anti-mouse CD45R(B220) (Gibco, Paisley, UK). Activated lymphocytes were gated on the basis of forward and side scatter.

Analysis of switch recombination junctions

Genomic DNA was prepared from day 4 LPS cultures or Peyer's patch lymphocytes following proteinase K digestion. First round PCR amplification [six cycles of 93°C (40 s), 64–55°C (touch-down annealing) (40 s) and extension at 72°C (120 s) followed by a further 24 cycles with these extensions performed at 55°C] was performed using *Taq* polymerase (Promega) and oligonucleotides Sμ1 (5′-TAGTAAGCG-AGGCTCTAAAAAGCAT; nucleotides 5031–5055 of MUSIGCD07) and either Sγ3.1 (5′-CTACTGAGTTCCTGTGCTTG; nucleotides 1050–1069 of MUSIGCD18) or Sα.1 (5′-CAGCAGTGAGTTTAACAATCC; nucleotides 4803–4823 of MUSIALPHA). PCR products were purified using a Qiagen kit (Crawley, Sussex, UK), and 1% of the sample was subjected to secondary amplification [24 cycles of 93°C (40 s), 55°C (40 s) and 72°C (120 s)] performed using Sμ2 (5′-ATCGAATTCGCTTG-AGCCAAAATGAAGTAGACT; nucleotides 5140–5163 of MUSIGC-D07) and either Sγ3.2 (5′-CCGGAATTCTTGACCTGGTACCCTAGC; nucleotides 1035–1052 of MUSIGCD18) or Sα2 (5′-CCGGAATTCCT-CAGTGCAACTCTATCTAGGTCT; nucleotides 4182–4205 of MUSI-ALPHA) prior to purification of the DNA cloning into Bluescript following digestion with *Eco*RI and sequencing.

Acknowledgements

We are indebted to Julian Sale, Cristina Rada and César Milstein for many helpful discussions during the course of this work and to Janet Stavnezer for permission to cite unpublished results.

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Received March 30, 1999; revised April 14, 1999; accepted April 15, 1999