The in vivo pattern of AID targeting to immunoglobulin switch regions deduced from mutation spectra in *msh2*$^{-/-}$ *ung*$^{-/-}$ mice

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Immunoglobulin (Ig) class switching is initiated by deamination of C→U within the immunoglobulin heavy chain locus, catalyzed by activation-induced deaminase (AID). In the absence of uracil-DNA glycosylase (UNG) and the homologue of bacterial MutS (MSH)–2 mismatch recognition protein, the resultant U:G lesions are not processed into switching events but are fixed by replication allowing sites of AID-catalyzed deamination to be identified by the resulting C→T mutations. We find that AID targets cytosines in both donor and acceptor switch regions (S regions) with the deamination domains initiating ~150 nucleotides 3′ of the I exon start sites and extending over several kilobases (the IgH intronic enhancer is spared). Culturing B cells with interleukin 4 or interferon γ specifically enhanced deamination around Sγ1 and Sγ2a, respectively. Mutation spectra suggest that, in the absence of UNG and MSH2, AID may occasionally act at the μ switch region in an apparently processive manner, but there is no marked preference for targeting of the transcribed versus nontranscribed strand (even in areas capable of R loop formation). The data are consistent with switch recombination being triggered by transcription-associated, strand-symmetric AID-mediated deamination at both donor and acceptor S regions with cytokines directing isotype specificity by potentiating AID recruitment to the relevant acceptor S region.

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Abbreviations used: AID, activation-induced deaminase; MSH, homologue of bacterial MutS; S region, switch region; Sμ, μ switch region; UNG, uracil-DNA glycosylase.

Isotype switching of immunoglobulins is achieved by recombinational deletion at the IgH locus such that Cμ is excised and the productively rearranged V_HDJ_H segment is instead brought into proximity with Cγ, Cε, or Cα (for review see references 1–4). The deletion is achieved by a form of nonhomologous, region-specific recombination with the 5′ endpoints of the deletion being located in proximity of the repetitive region of the μ switch region (Sμ) and the 3′ endpoint being within or close to the repetitive portion of the S region of one of the downstream isotypes.

Switching is initiated by activation-induced deaminase (AID)–catalyzed deamination of cytosine to uracil within the immunoglobulin locus DNA and likely depends on nonhomologous end joining. Thus, genetic evidence indicates that switching is dependent on AID and proteins involved in uracil excision (5–8), as well as on factors known to be implicated in nonhomologous end joining (e.g., Ku70, Ku80, 53BP1, and DNA ligase IV) (9–13). Furthermore, AID-dependent DNA double-strand breaks have been detected in the vicinity of Sμ in switching cells (14–16), but the detailed mechanism by which AID-triggered deamination leads to switch recombination remains uncharacterized.

Major unresolved problems also relate to the mechanisms by which AID is recruited to switch regions (S regions) as well as to how donor and acceptor S regions are brought together to facilitate recombination. Transcription regulatory elements have been shown to be major cis-acting DNA sequences controlling switch recombination and somatic hypermutation (for review see reference 3) and might somehow orchestrate AID recruitment. In addition, AID has been shown to be able to interact with replication protein A (an interaction that fits well with the fact that AID itself is active in single-stranded DNA in vitro) (17), and the homologue of bacterial MutS (MSH)–2 appears able to synapse donor and acceptor S

The online version of this article contains supplemental material.

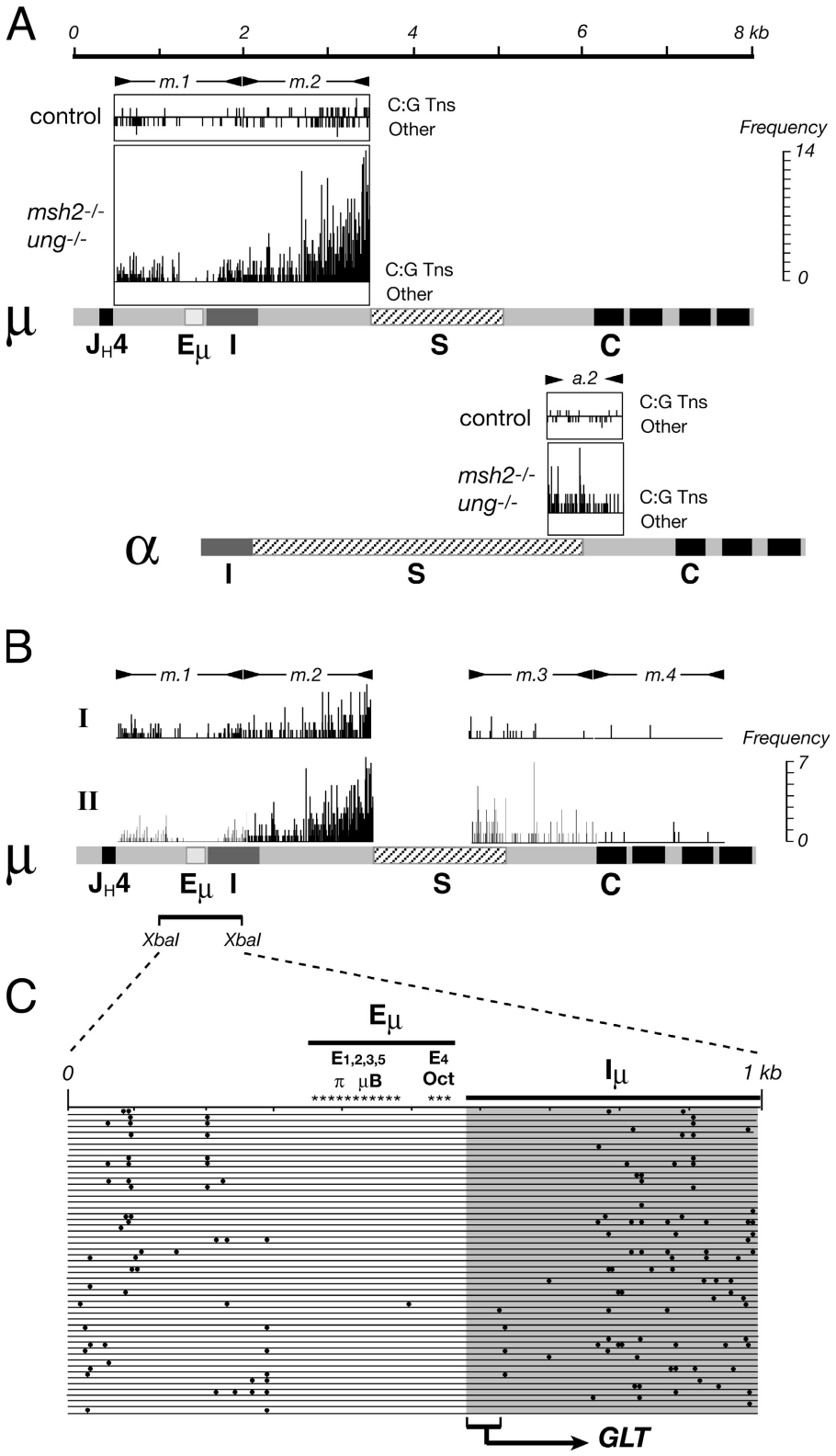

Figure 1. Distribution of Sμ-associated mutations. (A) Comparison of switch-associated mutations in wild-type and *msh2*$^{-/-}$ *ung*$^{-/-}$ mice. Mutations in Sμ-associated regions m.1 and m.2 and in Sα-associated region a.2 were compared with Peyer's patch germinal center B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$ and wild-type mice. Mutations were categorized as either transitions at C:G pairs (plotted above the midline) or other (transversions at C:G or transitions/transversions at A:T, plotted below the midline). Mutations are plotted as vertical bars, with the height representing the frequency of mutations at each nucleotide calculated as the number of cases per set of 21 sequences in which that position is mutated. (B) Distribution

Table I. Mutations in the vicinity of the IgH S regions

Region	Length (nt)	Sequences		Mutations		Mutation frequency[c]	%Mutations on NT strand[d]	%Mutations at WRC[e]	%WRC on NT strand[f]
		Total[a]	Mutated	Tns@C:G[b]	Other				
m.1	1,520	52	50	296	0	36	46	59	43
m.2	1,407	31	27	929	5	213	55	70	46
m.3	1,477	27	22	158	1	40	59	79	50
m.4	1,484	18	6	10	0	4	-	-	-
g3.1	739	22	2	2	1	<1	-	-	-
g3.2[g]	1,400	25	22	242	1	69	48	76	54
g3.3	1,300	10	6	30	0	23	63	57	48
g1.1	1,280	12	1	1	0	<1	-	-	-
g1.2	1,700	27	15	56	1	12	38	73	49
g1.3	793	16	5	9	1	7	-	-	-
g1.4	813	11	0	0	0	<1	-	-	-
g2b.1	1,480	15	3	3	2	1	-	-	-
g2b.2	930	20	18	77	1	1	42	69	44
g2b.3[h]	767	20	15	105	1	68	36	79	44
g2a.1	852	12	5	10	0	10	-	-	-
g2a.2	752	12	2	10	0	11	-	-	-
g2a.3	1,008	23	8	17	0	7	53	71	43
e.1	969	22	1	1	0	<1	-	-	-
e.2	809	21	0	0	0	<1	-	-	-
a.1	733	48	0	0	0	<1	-	-	-
a.2	934	23	20	122	2	57	52	60	37
a.3	1,393	15	9	23	1	11	61	56	56

[a]The total number of sequences in each dataset was computed after removing all but the most mutated sequence in any set of dynastically related sequences.
[b]Transition mutations at C:G pairs.
[c]Overall mutation frequency is given as the average number of transitions at C:G pairs per 10 kb sequenced.
[d]Percentage of C→T transition mutations that are on the nontemplate DNA strand.
[e]Percentage of C→T transition mutations that occur within a WRC consensus, where W = A / T and R = A / G.
[f]Percentage of the WRC motifs in the germline sequence that occur on the nontemplate (as opposed to template) DNA strand.
[g]58 nt at the 3′ end of g3.2 contribute to R loop structures in vivo (reference 31) with 35% of the 49 mutations observed within this region being on the NT strand.
[h]This segment forms R loops in vivo (reference 59).

regions through binding to G quartet structures formed within transcribed S regions (18). However, the mechanism by which specific molecular interactions lead to locus specificity of AID action has not been elaborated in any detail.

In this paper, rather than focus directly on the molecular interactions involved in AID recruitment, we glean insight into the mechanism of switch recombination by addressing the nature of the targeting of AID-catalyzed deamination in the vicinity of immunoglobulin S regions (the distribution of deamination sites in and around Sμ, the targeting to acceptor S regions, and the relative targeting of the transcribed and nontranscribed DNA strands).

RESULTS

It was observed several years ago that mutations are often found close to the sites of switch recombination, and some of these mutations could be the consequence of the resolution of nucleotide mismatches arising in heteroduplexes formed during the switching process (19, 20). However, it has more recently become clear that mutations can also be detected in Sμ far upstream

of transition mutations at C:G pairs in germinal center B cells from Peyer's patches of a pair of *msh2*$^{-/-}$ *ung*$^{-/-}$ mice (I and II). Four regions of ~1.5 kb (m.1 to m.4) spanning from immediately downstream of J_H4 to within the $C_\mu4$ exon were sequenced (Table I). Mutations are plotted as vertical bars, with the height representing the frequency of mutations at each nucleotide calculated as the number of cases per set of 11 sequences in which that position is mutated. Position 1 in the sequence represents nucleotide 134,071 (available from GenBank/EMBL/DDBJ under accession no. AC073553). (C) Distribution of mutations in the vicinity of the Eμ enhancer. The data are represented as a composite of the transition mutations at C:G pairs identified in mice I and II, though similar distributions are evident when each mouse is analyzed individually. The region encompassing the heterogeneous start sites of the μ germline transcripts (GLT) is indicated. The asterisks designate the extents of the segments highlighted.

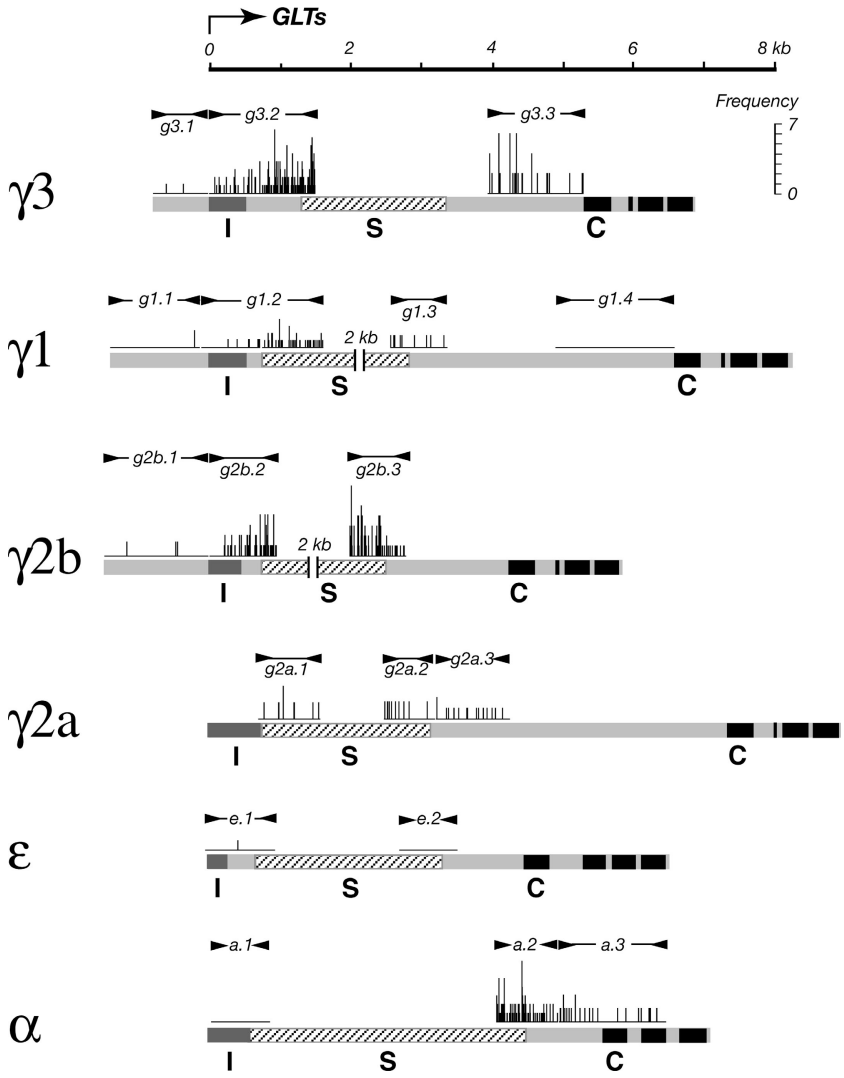

Figure 2. Distribution of Sγ-, ε-, and α-associated mutations. The distribution of transition mutations at C:G pairs around Sγ, ε, and α in the Peyer's patch germinal center B cells of *msh2*$^{-/-}$ *ung*$^{-/-}$ mice I and II are presented as a compilation. Mice I and II showed similar distributions of mutations across the IgH locus when analyzed separately and contribute 143 and 210 sequences, respectively, to the compilation. The regions analyzed (0.8–1.9 kb in length) are indicated by opposing arrowheads. The results for each isotype are aligned to the major start sites of the germline transcripts, which are taken as nucleotides 41,758 (γ3), 68,759 (γ1), 93,343 (γ2b), and 111,899 (γ2a; available from GenBank/EMBL/DDBJ under accession no. AC161365); nucleotide 6225 (ε; available from GenBank/EMBL/DDBJ under accession no. CAAA01071548); and nucleotide 126 (α; available from GenBank/EMBL/DDBJ under accession no. D11468). Mutations are plotted as vertical bars with the height representing the frequency of mutations at each nucleotide calculated as the number of cases per set of 22 sequences in which that position is mutated.

of the site of switch recombination, that such "preswitch" mutations are AID dependent and can occur even on IgH alleles that have not undergone evident switching recombination (21–26). The distribution of those AID-dependent mutations that are not generated as a consequence of the switch recombination itself could give insight into the nature of AID targeting.

Although mutations in the preswitch region have largely been described in immortalized B cell lines or in B cells that have been activated with LPS in vitro, they can also be detected in germinal center B cells sorted from mouse Peyer's patches (25, 26). Although the mutation loads in the preswitch region are often quite light, a considerably higher mutation load accumulates in germinal center B cells obtained from *msh2*$^{-/-}$ *ung*$^{-/-}$ mice (Fig. 1 A). The absence of both uracil-DNA glycosylase (UNG) and MSH2 in these animals means that the AID-generated U:G lesions are not recognized for processing into switch recombination and are also probably less likely to be repaired (25). Instead, the cells seem simply to replicate across the U:G lesions and, consequently, accumulate large numbers of C→T and G→A transition mutations upstream of Sμ. The *msh2*$^{-/-}$ *ung*$^{-/-}$ mice can therefore be used to obtain large databases of switch-associated mutations with no contribution to the database arising from mutations generated as a result of the switch recombination

itself. Furthermore, in contrast to what is observed in normal mice, there is no mutagenic patch repair of the U:G lesion in *msh2*$^{-/-}$ *ung*$^{-/-}$ mice, and all the observed mutations are likely to directly mark the sites of AID-catalyzed deamination events. Thus, the observed mutations are entirely restricted to transitions at C:G pairs with no substitutions accumulating at A:T pairs (Fig. 1 A) (25). In fact, this restriction to transitions at C:G pairs means that it is possible to check that individual databases are relatively free from contamination by PCR error, a considerable concern in lightly mutated databases, especially with highly repetitive target sequences.

The accumulation of mutations across a region extending from J_H4 through to Cμ4 was analyzed in germinal center Peyer's patch B cells obtained from a pair of *msh2*$^{-/-}$ *ung*$^{-/-}$ mice. Primers were designed so as to amplify the whole region in sections of ~1,500 base pairs. The results are shown in Fig. 1 B, excluding the highly repetitive part of Sμ, because we were unable to amplify this region using the same PCR strategy as was used for the rest of the region.

The two mice showed similar distributions of mutations. As expected for mutations attributable to error-free replication across the U:G lesion, >99% of the 1,399 mutations identified in the 130 Sμ sequences analyzed were transitions at C:G pairs (Table I). Consistent with the target site preference of AID, if all these mutations are computed as C deaminations, 69% of the mutations are found to fall within a WRC consensus. This is comparable with the 24.3% that would be anticipated if the mutations were randomly targeted to different C residues within the target (a calculation that takes account of the sequence of the Sμ target that is being analyzed).

The Sμ mutation domain begins ~150 nt downstream of the start sites of the Sμ germline transcripts and extends over 5 kb, tapering at a low level into Cμ exons. Interestingly, the Eμ enhancer is largely spared of mutations, apparently being located just downstream of the V_HDJ_H mutation domain and upstream of the Sμ mutation domain (Fig. 1 C).

To identify possible target sites of AID-catalyzed deamination in the acceptor S regions, we amplified and sequenced regions in and around the repetitive regions of the Sγs, Sε, and Sα from the same Peyer's patch germinal center B cell DNA samples that had been used for analysis of Sμ-associated mutations (Fig. 2 and Table I). Scarcely any mutations were observed associated with Sε, which might possibly reflect little or no switching to IgE in Peyer's patches. However, with all four Sγs, the results were similar to those obtained with Sμ, although the mutation loads were somewhat lower. Thus, mutations were observed both up- and downstream of the repetitive portions of the Sγs with the major mutation domains appearing to begin just downstream of the Iγ start sites. The mutations were all transitions at C:G pairs, focused on a WRC consensus (where W = A/T and R = A/G). With regard to Sα, although mutations were readily detected in the 3′ flanking region, we detected no mutations in the vicinity of the Iα exon. We do not know the reason for this, but one possibility is that there could be alternative start sites for the Sα transcripts in Peyer's patch germinal centers.

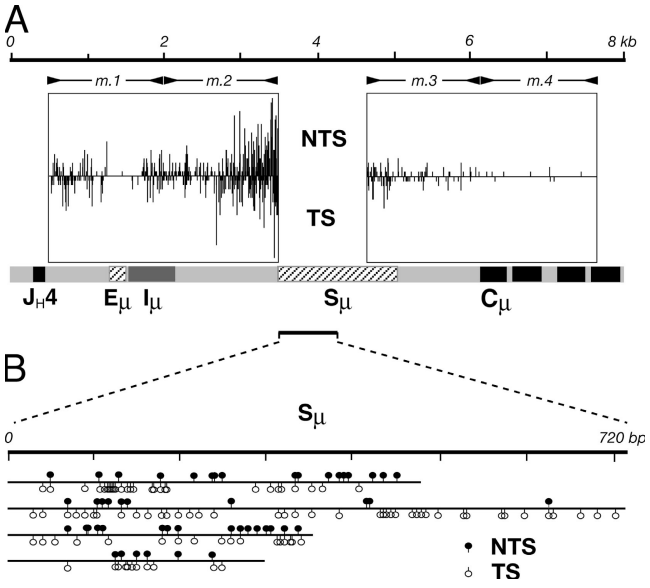

Figure 3. Absence of major strand polarity in Sμ-associated mutations. (A) Transition mutations at C:G pairs in regions m.1to m.4 were categorized as mutations at C on either the transcription template or nontemplate strand (TS or NTS, respectively) and accordingly plotted above or below a midline. (B) An expanded 720-bp region depicts in detail the distribution of NTS versus TS deaminations identified in four clones amplified by nested PCR from the repetitive region of Sμ. Position 1 corresponds to nucleotide 137,456 (available from GenBank/EMBL/DDBJ under accession no. AC073553). Of the 148 C→T transition mutations detected, 130 fell within a WRC motif, 49 were on the nontranscribed strand, and 99 were on the transcribed strand. Of the WRC motifs in this 720-bp region, 55% are found on the transcribed strand.

Several immunoglobulin S regions have been shown to be able to adopt R loop conformations during transcription (27–31), which could expose a single-stranded DNA substrate for AID action. We were therefore interested in ascertaining whether AID displayed any preference for deaminating the DNA strand that is not used as the template for transcription (the nontemplate strand) at the S regions in vivo. This, presumably, could be detected as a strand asymmetry in the pattern of the C→T transitions. No such asymmetry is evident from the m.1, m.2, or m.3 regions (Fig. 3 A and Table I). Although most of these sequences derive from regions that flank (rather than lie within) the highly repetitive portions of the S regions, no evidence of preferential deamination of the nontemplate DNA strand is observed even if attention is focused on sequences derived from parts of Sγ3 or Sγ2b that have been shown to be able to form R loops in vivo (Table I).

We extended the analysis to the repetitive portion of Sμ. Although we had failed to amplify this highly repetitive DNA in a single-stage PCR reaction using a variety of different primers, we did achieve success with a nested PCR approach. The Sμ clones obtained in this way were heavily mutated, forming part of a region that can form R loops in vitro (28), but certainly showed no evidence in favor of preferential deamination of the nontemplate DNA strand (Fig. 3 B).

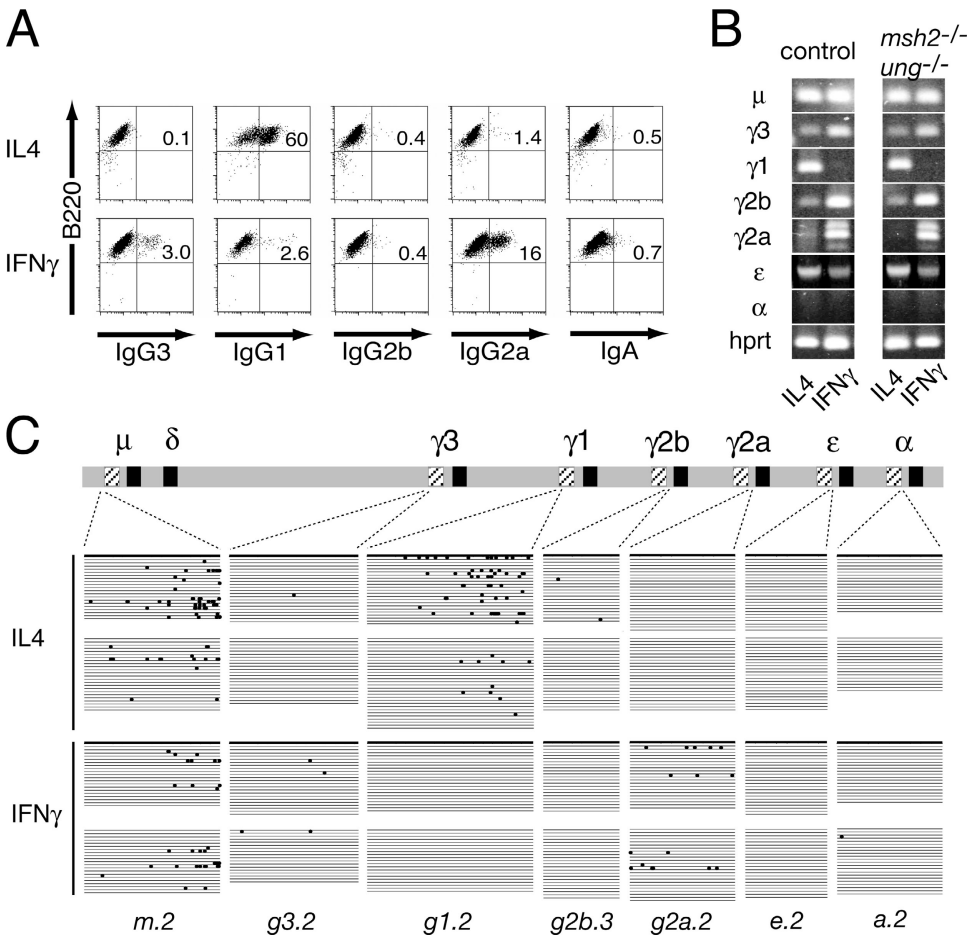

Figure 4. Cytokine induction of switch-associated mutations in LPS-cultured B cells. (A) In vitro class switching of splenic B cells from a wild-type (control) C57BL/6 mouse analyzed by flow cytometry after a 7-d culture with LPS plus either IL-4 or IFN-γ. The numbers indicate the percentage of events in the top right quadrants. Results are representative of two independent experiments. (B) Germline transcription in the in vitro–stimulated splenic B cells. Germline transcripts comprising I_H and C_H exons of each isotype were detected by RT-PCR from RNA extracted at day 3 of culture. hprt, hypoxanthine phosphoribosyltransferase control. (C) Distribution of transitions at C:G pairs in genomic DNA purified from splenic B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$ mice after a 7-d culture with LPS plus IL-4 or IFN-γ as indicated. For each isotype, a region that had been demonstrated to be targeted by AID in vivo (Fig. 2) was amplified and sequenced. The number of sequenced for each region vary as a consequence of sequencing failures. Data shown are from two independent experiments.

We wished to learn whether the cytokines that stimulate isotype-specific switching also play a role in recruitment of AID-catalyzed deamination. We therefore screened for S region mutations in B cells that had been activated with LPS in vitro in the presence of either IL-4 (to induce switching to IgG1) or IFN-γ (to induce switching to IgG2a). In all cases, mutations accumulated in the Sμ 5′ flank with mutations in Sγ1 being specifically enhanced in the IL-4 cultures and mutations in Sγ2a enhanced in the IFN-γ cultures (Fig. 4). No mutations were detected in Sγ1 or Sγ2a in the absence of the relevant cytokine.

Analysis of individual mutated S region sequences from LPS-activated B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$ mice reveals that several harbor clusters of linked C→T transition mutations on either the top or bottom DNA strand (Fig. 5 A). The abundance of contiguous same-strand deamination mutations is significantly higher than would be anticipated if each deamination mutation occurred as an independent event; the deviation from random is significant at the 97% level if the pool of sequences in Fig. 5 is analyzed using a χ^2 test. These clusters do not appear to reflect any strand asymmetry of the DNA target in respect of the location of intrinsic mutational hotspot motifs. Thus, examples can be seen where, in a specific segment of the S region, the top strand has been targeted for deamination in one clone, whereas it is the bottom strand of the same segment that has been targeted in another (Fig. 5).

The results suggest that, at least occasionally in B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$ mice, AID can introduce clustered deaminations on a single DNA strand, possibly through processive action. One might then expect to see clones with clustered deaminations even in sequences obtained from B cells that have been cultured for shorter periods with LPS. Indeed,

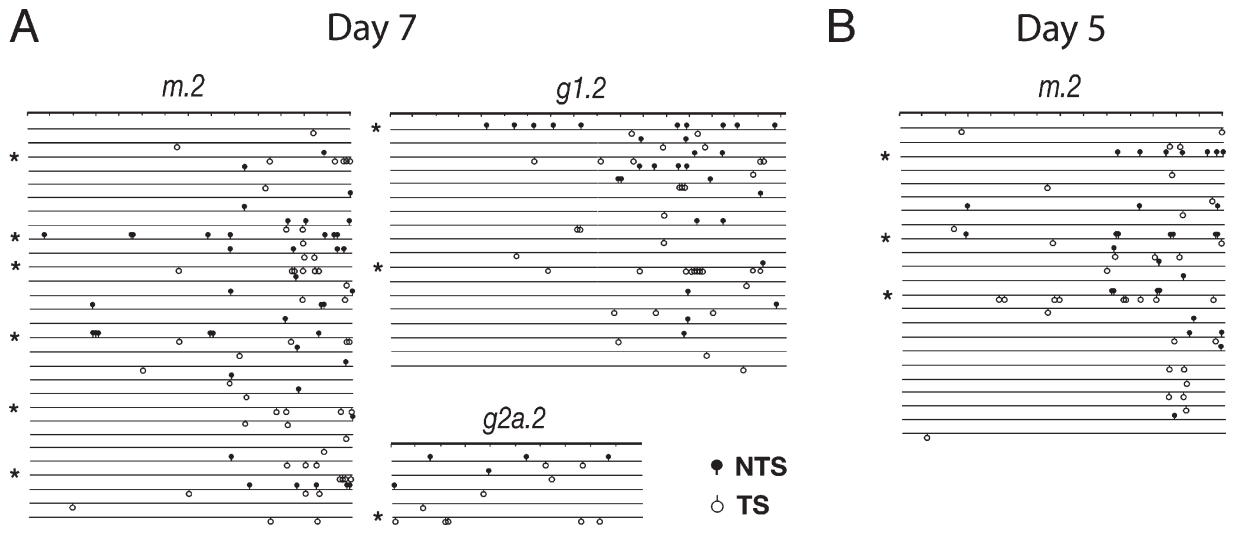

Figure 5. DNA strand polarity of switch-associated mutations in LPS-cultured B cells. (A) DNA strand polarity of C→T transition mutations in individual S region sequences amplified from splenic B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$ mice at day 7 of culture with LPS+IL-4/IFN-γ. (B) DNA strand polarity of C→T transition mutations in individual Sμ m.2 sequences amplified from splenic B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$ mice at day 5 of culture with LPS+IL-4/IFN-γ. All mutated sequences are illustrated with C→T transitions on the transcription template strand indicated by open circles below the line; those on the nontemplate strand are indicated by closed circles above the line. Asterisks mark sequences that harbor tandem C→T transitions on one strand in regions where other sequences in the database exhibit mutations on the opposite strand. Clusters of same DNA strand (linked) mutations were quantified by scoring the frequency of "same" (AA to TT) contiguous mutations (ignoring intervening distance) compared with the frequency of "different" (AT or TA) contiguous mutations in each sequence obtained from LPS-stimulated B cells. The bias in favor of same strand mutations was estimated using a χ^2 test ($P = 0.0277$).

although the overall mutation load is lower in Sμ m.2 sequences isolated from B cells on day 5 as opposed to day 7 of culture, the abundance of sequences with clustered same-strand deamination mutations appears similar (Fig. 5 B).

Although same-strand clustering is also discernable among some of the sequences from Peyer's patch B cells that cover the repetitive region of Sμ (Fig. 3 B), the heavy mutation loads evident in these sequences obtained from in vivo–activated B cells (probably reflecting an increased number of rounds of mutation) may mask the evidence of apparently processive deamination.

DISCUSSION

The results reveal that, at least with Sμ and most Sγs, the switch-associated mutation domains initiate ~150 nt downstream of the I exon start sites and extend over several kb through the repetitive S region and well into its 3′ flank. The relative positioning of the 5′ border of the mutation domain and that of the transcription start site parallels what has previously been described in the rearranged IgV_HDJ_H and IgV_KJ_K regions (32–37).

The switch-associated mutation domains appear considerably longer than the IgV_HDJ_H domain, extending over several kb. In the case of the IgV_HDJ_H mutation domain, it appears that it is the Eμ enhancer that forms its 3′ border, as well as providing promoter elements for Sμ transcription. It will be interesting to ascertain whether there are elements within Eμ that act as insulators for AID-catalyzed DNA deamination and, similarly, whether there are specific sequences at the 3′ ends of the S regions that determine the positions of the downstream borders of the deamination domains or if mutation simply tapers with distance from the promoter.

The characterization of the mutation domains associated with the unrearranged acceptor S regions in *msh2*$^{-/-}$ *ung*$^{-/-}$ mice extends on previous work from Reina-San-Martin et al. and Schrader et al. (23, 24), who noted mutations in the vicinity of the Sγ regions of switch-proficient mice. These results, together with those of Nambu et al. (38), who found by ChIP analysis that AID in LPS-activated B cells can be recruited to Sμ, Sγ1, and Sε, make it exceedingly likely that AID plays a role in inducing DNA strand breaks in both donor and acceptor S regions to potentiate switch recombination. It also appears that, in keeping with previous studies on switch recombination (39), AID can similarly target S regions on both functional and excluded IgH alleles because mutations were found in this work in >75% of the Sμ sequences amplified from the germinal center B cells.

Although studies of AID-catalyzed DNA deamination in vitro have revealed that the enzyme can act in a processive manner (40–42), there has been no evidence of such processive deamination in vivo where nucleotide substitutions during somatic hypermutation accumulate in a stepwise manner with only a small number (one to four) of usually unlinked mutations being fixed in each generation. The evidence that clustered same-strand deamination (compatible with processive AID action) has occurred in some S region sequences obtained after LPS stimulation of splenic B cells from *msh2*$^{-/-}$ *ung*$^{-/-}$

mice (Fig. 5) is therefore noteworthy. A possible explanation for the differential processivity observed in different situations is that although AID can indeed act processively, such processive action is normally curtailed in vivo through the action of UNG or MSH2 in early recognition of AID-generated U:G lesions.

The results provide further support for the accessibility model of switch recombination regulation in which a central feature is a linkage between switching and transcription (1, 43). Not only are the 5′ borders of the switch-associated mutation domains located just downstream of the I exon start sites but, in in vitro LPS cultures, cytokine induction of switch-associated mutation is associated with cytokine-induced sterile transcription (Fig. 4).

The precise nature of the linkage between transcription and mutation remains obscure. The finding that cytokine induction of isotype-specific switching is associated with the induction of switch-associated mutation, as well as switch-associated sterile transcripts, suggests that it is the recruitment of AID itself that is at least one of the transcription-linked components of the switching process. However, transcription through the relevant acceptor S region does not appear to be sufficient to trigger either S region mutation or directed switching (Fig. 4) (44, 45). In this regard, there is a parallel between the transcription linkage of class switch recombination and that of IgV somatic mutation (46–48). Although in both cases there is a correlation between transcription of the DNA target and AID-dependent diversification, transcription is nevertheless insufficient to guarantee diversification in either case. An attractive interpretation is that, in switch recombination, cytokine stimulation triggers the formation, at the I exon promoters, of transcription initiation complexes whose composition makes them particularly effective in AID recruitment. Then, consistent with the findings of Nambu et al. (38), AID might track along the S region with RNA polymerase generating U:G lesions and thereby promoting class switch recombination. The location of the S region–associated mutation domain would, similarly to that of the IgV mutation domain (35, 49), then be defined by the position of the I exon promoter. Indeed, an analysis of switch breakpoints in mice lacking the Sμ tandem repeats led Min et al. (50) to similarly propose that the Iμ promoter defines the 5′ border of a 4–5-kb DNA switching domain, independent of the presence of the Sμ sequences themselves.

The parallels between the nature of the AID mutation domains associated with the IgV region and those associated with the S regions are striking. One notable difference is that, though not essential for switch recombination (51, 52), S regions comprise highly repetitive sequences that are prone to R loop formation. Nevertheless, we find in this paper that, even within these repetitive regions, AID-catalyzed deamination in vivo occurs in a strand-symmetric manner, as it does in the IgV domain. The in vivo situation therefore contrasts with what has been observed in many (17, 41, 53, 54) but not all (55, 56) artificial in vitro systems of transcription-linked AID-catalyzed DNA deamination. However, there are clearly also some major differences between AID targeting to S regions and to the IgV. It will be a challenge in future work to explain why deletional recombination rather than somatic mutation is only rarely observed in the IgV domain and why switching and somatic mutation show different requirements in respect of the AID COOH terminus (57, 58).

MATERIALS AND METHODS

The spectrum of mutations accumulating across the IgH locus in germinal center B cells was assayed by sequencing clones obtained by PCR amplification of DNA extracted from germinal center B cells that were sorted by virtue of their PNA^{high} $CD45R(B220)^{+}$ phenotype from Peyer's patches of 6–10-mo-old *msh2*$^{-/-}$ *ung*$^{-/-}$ mice on a C57BL/6 × CBA background as previously described (25). The PCR reactions were performed using oligonucleotides listed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20061067/DC1) using Turbo *Pfu* polymerase (Stratagene) under the following conditions: a 93°C hotstart for 3 min followed by 72°C for 1 min, 93°C for 30 s, a 64–56°C stepdown annealing over 5 cycles for 30 s, and 72°C for 4 min, followed by 24 cycles at 93°C for 30 s, 55°C for 30 s, and 72°C for 4 min. For the highly repetitive region of Sμ, sequences were obtained by a nested PCR amplification with the two rounds of amplifications performed using the Sμ external and internal oligonucleotides listed in Table S1. Both amplifications were performed using Turbo *Pfu*, comprising a 3-min 95°C hotstart followed by 30 cycles of 98°C for 20 s, 68°C for 30 s, and 72°C for 15 min. PCR products were gel purified and cloned into vectors (Zero Blunt TOPO; Invitrogen). To avoid repeat counting of individual mutational events, all but the most mutated sequence in any set of dynastically related sequences (as judged by >70% identity in mutation distribution) were removed before analysis.

For in vitro class switching, resting splenic B cells purified by virtue of their CD43 negative phenotype using a separator (MACS; Miltenyi Biotec) were cultured in RPMI medium supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol in the presence of 50 μg/ml LPS together with 50 ng/ml of recombinant mouse IL-4 (R&D Systems) or 25 ng/ml of recombinant mouse IFN-γ (R&D Systems). For analysis of sterile transcripts, cells were harvested on day 3 of cell culture, and RNA was isolated using reagent (TRIzol; Invitrogen) according to the manufacturer's instructions. Germline transcripts were then amplified by RT-PCR (5). For both flow cytometric and S region mutational analyses, cells were harvested on day 7 of culture. Flow cytometric analysis of surface immunoglobulin was performed on cells stained with PE-conjugated anti–mouse–CD45R(B220) (RA3-6B2) and biotinylated anti–mouse IgG3 (R40-82), IgG1 (A85-1), IgG2b (R12-3), IgG2a (R19-15), or IgA (all were purchased from BD Biosciences), together with FITC-streptavidin (DakoCytomation) and propidium iodide staining and scatter gating to exclude dead cells. S region mutation analysis was carried after PCR amplification was performed as described for the B cell samples from Peyer's patches.

Online supplemental material. Table S1 gives the sequences of the oligonucleotides used for the PCR amplifications. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061067/DC1.

We are grateful to Paul Dear for advice on statistical methods.

K. Xue thanks Trinity College and the James Baird Fund for support, and M.S. Neuberger is indebted to the Royal Society, David Tarlinton, and colleagues at the Walter and Eliza Hall Institute for discussion and support during the production of this work.

The authors have no conflicting financial interests.

Submitted: 18 May 2006
Accepted: 12 July 2006

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