Isotype switching of immunoglobulins is achieved by recombinational deletion at the IgH locus such that Cμ is excised and the productively rearranged VHDJH 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′ end-points 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 DNA and likely depends on 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 regions.
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 Sex-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
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 $\text{S}_\mu$, 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 $\text{S}_\mu$ far upstream of transition mutations at C:G pairs in germinal center B cells from Peyer’s patches of a pair of $\text{msh}^2\text{−}/\text{msh}^2\text{−}$ and $\text{ung}^+\text{−}/\text{ung}^+\text{−}$ mice (I and II). Four regions of ~1.5 kb (m.1 to m.4) spanning from immediately downstream of $\text{J}_H$ to within the $\text{C}_\mu^4$ 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 $\text{E}_\mu$ 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 $\mu$ germline transcripts (GLT) is indicated. The asterisks designate the extents of the segments highlighted.

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

| Region | Length (nt) | Sequences Mutations | Mutation frequency | % Mutations on NT strand | % Mutations at WRC | % WRC on NT strand |
|--------|-------------|---------------------|--------------------|--------------------------|-------------------|-------------------|
| 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   | 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  | 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             |

*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.

†Transition mutations at C:G pairs.

Overall mutation frequency is given as the average number of transitions at C:G pairs per 10 kb sequenced.

‡Percentage of C→T transition mutations that are on the non-template DNA strand.

§Percentage of C→T transition mutations that occur within a WRC consensus, where $W = A / T$ and $R = A / G$.

| Region | Sequences | % Mutations on NT strand | % Mutations at WRC | % WRC on NT strand |
|--------|-----------|--------------------------|-------------------|-------------------|
| m.1    | 50        | 36 46 59 43              |
| m.2    | 27        | 213 55 70 46             |
| m.3    | 22        | 40 59 79 50             |
| m.4    | 6         | 4  -  - -                |
| g3.1   | 2         | <1  -  - -                |
| g3.2   | 22        | 69 48 76 54             |
| g3.3   | 6         | 23 63 57 48             |
| g1.1   | 1         | <1  -  - -                |
| g1.2   | 15        | 12 38 73 49             |
| g1.3   | 5         | 7  -  - -                |
| g1.4   | 0         | <1  -  - -                |
| g2b.1  | 3         | 1  -  - -                |
| g2b.2  | 18        | 1 42 69 44             |
| g2b.3  | 15        | 68 36 79 44             |
| g2a.1  | 5         | 10  -  - -                |
| g2a.2  | 2         | 11  -  - -                |
| g2a.3  | 8         | 7 53 71 43             |
| e.1    | 1         | <1  -  - -                |
| e.2    | 0         | <1  -  - -                |
| a.1    | 0         | <1  -  - -                |
| a.2    | 20        | 57 52 60 37             |
| a.3    | 9         | 11 61 56 56             |
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.
Peyer’s patch B cells obtained from a pair of 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. 1A) (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 Jμ4 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. 1B, 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, 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 Vμ1Dμ1 mutation domain and upstream of the Sμ mutation domain (Fig. 1C).

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, Sδ, and Sε 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 1γ start sites. The mutations were all transitions at C:G pairs, focused on a WRC consensus (where W = A/T and R = A/G).

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 nontranscribed 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. 3A 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 nontranscribed 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 nontranscribed DNA strand (Fig. 3B).
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 Sμ5′ 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 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 IgH and Cμ 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.

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,
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 IgVH, DJH, and IgVK regions (32–37).

The switch-associated mutation domains appear considerably longer than the IgVH, DJH domain, extending over several kb. In the case of the IgVH, DJH 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 PNAβ+ CD45R(B220)+ phenotype from Peyer’s patches of 6–10-mo-old mhc2−/− ung−/− mice on a C57BL/6 × CBA background as previously described (28). 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. Germ-line 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–CD43–CD19–CD25+CD11c+CD135−CD27+CD23–CD69+CD95−CD21+CD43− phenotype from Peyer’s patches of CBA background as previously described (28).

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.

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