A role for AID in chromosome translocations between c-myc and the IgH variable region

Yair Dorsett,[1] Davide F. Robbiani,[1] Mila Jankovic,[1] Bernardo Reina-San-Martin,[3] Thomas R. Eisenreich,[1] and Michel C. Nussenzweig[1,2]

[1]Laboratory of Molecular Immunology and [2]Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021
[3]Institut de Génétique et Biologie Moléculaire et Cellulaire, CNRS-INSERM-ULP, Illkirch, 67404 Strasbourg, France

Chromosome translocations between oncogenes and the region spanning the immunoglobulin (Ig) heavy chain (*IgH*) variable (V), diversity (D), and joining (J) gene segments (Ig V-J_H region) are found in several mature B cell lymphomas in humans and mice. The breakpoints are frequently adjacent to the recombination signal sequences targeted by recombination activating genes 1 and 2 during antigen receptor assembly in pre–B cells, suggesting that these translocations might be the result of aberrant V(D)J recombination. However, in mature B cells undergoing activation-induced cytidine deaminase (AID)-dependent somatic hypermutation (SHM), duplications or deletions that would necessitate a double-strand break make up 6% of all the Ig V-J_H region–associated somatic mutations. Furthermore, DNA breaks can be detected at this locus in B cells undergoing SHM. To determine whether SHM might induce *c-myc* to Ig V-J_H translocations, we searched for such events in both interleukin (IL) 6 transgenic (*IL-6* tg) and *AID*$^{-/-}$ *IL-6* tg mice. Here, we report that *AID* is required for *c-myc* to Ig V-J_H translocations induced by IL-6.

CORRESPONDENCE
Michel C. Nussenzweig:
nussen@rockefeller.edu

Abbreviations used: AID, activation-induced cytidine deaminase; IgH, Ig heavy chain; Ig V-J_H, genomic region spanning the heavy chain V(D)J; RSS, recombination signal sequences; SHM, somatic hypermutation; tg, transgenic; V(D)J, variable, diversity, and joining.

Chromosome translocations are products of unresolved double-strand DNA breaks (1–4) and therefore occur frequently at Ig genes because these loci undergo programmed DNA damage during antigen receptor gene diversification. In developing T and B cells, RAG1 and RAG2 proteins produce double-strand breaks at recombination signal sequences (RSS) found adjacent to variable, diversity, and joining (V(D)J) gene segments (5, 6). These RSS are found in close proximity or within translocation breakpoints in numerous B cell lymphomas (for review see references 7 and 8).

In mature B cells, expression of activation-induced cytidine deaminase (AID) (9–11) leads to deamination of cytidine residues in Ig V-J_H and Ig switch regions resulting in U:G mismatches that are processed to produce somatic hypermutations (SHMs) and initiate class switch recombination (for review see references 12–14). Double-strand breaks are obligate intermediates in the class switch reaction, and translocations involving switch regions are frequently found in sporadic Burkitt's lymphoma, diffuse large B cell lymphoma, and multiple myeloma, suggesting that AID is responsible for the lesions that lead to such translocations (15–22). Consistent with this idea, AID-induced breaks in the switch region activate the cellular DNA damage response machinery (23), and AID is essential for *c-myc* translocations to the Ig switch region in *IL-6* transgenic (tg) (24, 25). In addition, AID appears to target several oncogenes that are frequently mutated and often translocated to antibody genes in mature B cell malignancies (26–30). In agreement with these observations, deregulated expression of AID is associated with malignancy (31–34).

Double-strand breaks are not obligate intermediates in SHM of the Ig V-J_H (35). Nevertheless, duplications or deletions that would necessitate a double-strand break make up 6% of all the Ig V-J_H region–associated somatic mutations, and DNA breaks can be detected in this region in B cells undergoing mutation (36–42). In addition, endemic Burkitt's lymphoma, multiple myeloma, follicular lymphoma, and diffuse large B cell lymphoma contain mutated V genes as well as translocations to the Ig V-J_H or Ig V-J_L regions (for review see references 7, 8, and 43). This suggests that translocations in these malignancies may have occurred in mature B cells that express AID but not RAG1/2 (44), and that some Ig V-J_H region–associated

translocations are byproducts of lesions induced by AID during hypermutation. To ascertain the extent to which AID contributes to genomic instability at the *IgH* locus, we tested if AID is required for translocations between *c-myc* and the Ig V-J_H region in *IL-6* tg mice.

RESULTS

IL-6 tg mice develop hyperplastic lymph nodes that contain large numbers of class-switched plasmacytes, a portion of which express GL7 and CD138 (24, 45, 46). Plasmacytosis is believed to develop in these mice because IL-6 attenuates apoptosis and promotes proliferation and differentiation of late-stage B cells, allowing for the accumulation of translocations between *IgH* and *c-myc* (45). Although a majority of the *c-myc* translocation breakpoints are at the Ig switch region, a small fraction occur at the V-J_H region (46) and therefore resemble the translocations found in endemic Burkitt's lymphoma (for review see references 7 and 8). To determine whether AID is required for translocations between the Ig V-J_H region and *c-myc*, we generated *AID*-deficient *IL-6* tg mice (*AID*$^{-/-}$*IL-6* tg) by breeding (24). *AID*$^{-/-}$*IL-6* tg mice developed lymph node hyperplasia and plasmacytosis with a slightly delayed onset compared with *IL-6* tg mice, and there was no detectable class switching in the *AID*$^{-/-}$*IL-6* tg mice (Fig. 1, A and B) (24).

To document translocations between the Ig V-J_H region and *c-myc*, we developed PCR assays for these events and examined cells from hyperplastic lymph nodes from *IL-6* tg and *AID*$^{-/-}$ *IL-6* tg mice (Fig. 2 A). Southern blotting and DNA sequencing were used to verify candidate translocations (Figs. 2 and 3). Assaying four different lymph node pools per mouse for derivative 12 and derivative 15 translocations (Fig. 2 A), we identified 37 unique translocations in 14 *IL-6* tg mice, but none in 12 *AID*$^{-/-}$*IL-6* tg mice (P = 0.0025) (Fig. 2 C). Derivative 12 and 15 translocations were similar in both the number of translocations identified (21 and 16, respectively)

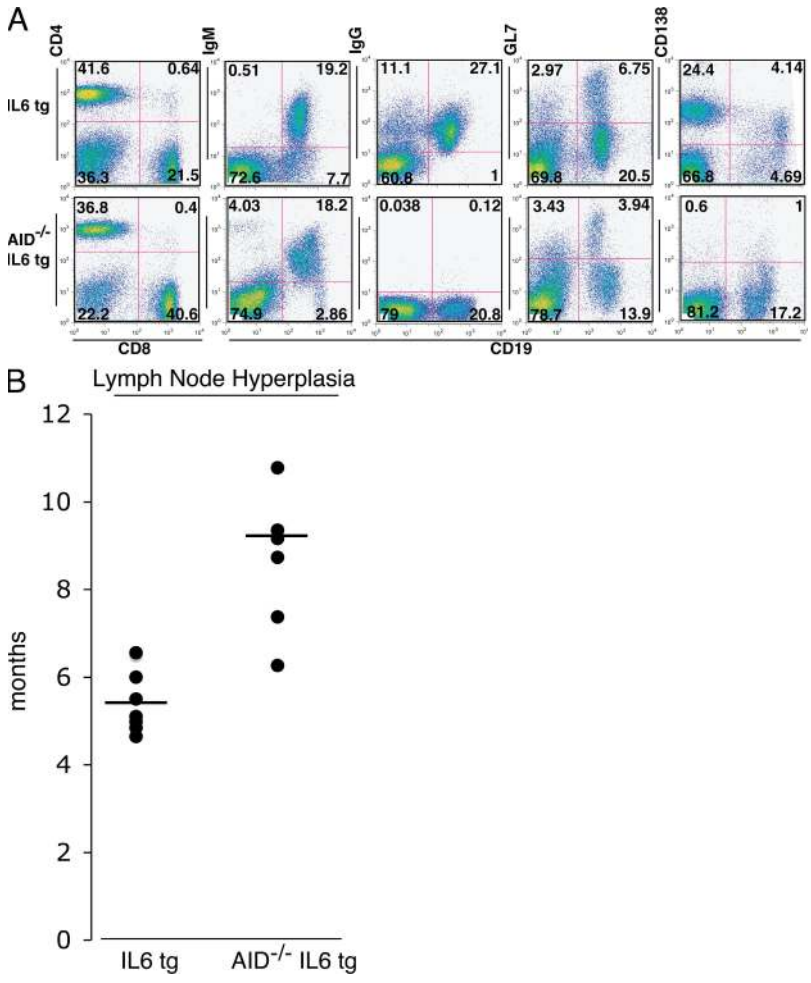

Figure 1. Characterization of IL-6 tg and AID$^{-/-}$ IL-6 tg mice. (A) Flow cytometry analysis of cells from hyperplastic lymph nodes from IL-6 tg and AID$^{-/-}$IL-6 tg mice. Numbers indicate percentages of cells in a given quadrant. (B) AID accelerates the development of disease in IL-6 tg mice. IL-6 tg mice and AID$^{-/-}$IL-6 tg mice were killed when they developed enlarged lymph nodes. The average time of death for IL-6 tg was 5.5 mo (n = 8) and 9.2 mo for AID$^{-/-}$IL-6 tg mice (n = 8; P = 0.0001476 using a two-tailed Student's t test assuming unequal variance). Each point represents one mouse, and the black bars indicate the average time of death.

as well as in their breakpoint distribution along the chromosome (Fig. 3), providing strong evidence that variable region translocations are reciprocal. As expected, *c-myc* to Ig switch region translocations were far more frequent (Fig. 2 B) (24, 46). We conclude that translocations between *c-myc* and the Ig V-J_H region are AID dependent in *IL-6* tg mice.

To gain further insight into the etiology of the identified translocations, we analyzed and mapped the breakpoints. Sequence analysis revealed that the majority of the breakpoints were in or around J_H segments, as commonly seen for oncogenic translocations in B cell non-Hodgkin's (Fig. 3) (for review see references 7 and 8). Junction sequences resembled those previously characterized for *c-myc* to Ig switch region translocations from *IL-6* tg mice (24) in that they involved either blunt ends or 1–3 nucleotide stretches of microhomology or nucleotide insertions (Tables I and II), implying that nonhomologous end joining resolves these breaks. Finally, the translocation breakpoints in *c-myc* were all in the first intron, which differs from those to the Ig switch region in *IL-6* tg mice, where a majority of the breakpoints are in the first exon of *c-myc* (Fig. 3) (24).

Although there was no correlation between the position of the breakpoints and the RGYW motifs, which are the preferred targets of AID, the translocated Ig V-J_H region was somatically mutated at a frequency of $\sim 0.6 \times 10^{-3}$ mutations per basepair (Fig. 4). This rate of mutation is similar to that reported for the Ig V-J_H region in B cells undergoing hypermutation (47). Derivative 12 and 15 *IgH* sequences had a similar frequency of hypermutation, suggesting that SHM must have occurred before or during translocation. Interestingly, the overall position of the mutations mirrored the positions of the translocation breakpoints (compare Figs. 3 and 4), supporting the idea that regions prone to SHM are susceptible to translocations. Excluding nucleotide insertions at the breakpoint, the rate of mutation within 20 bp of either side of the translocation breakpoints was almost 10-fold higher (5.4×10^{-3}) than that seen overall for *IgH* in our translocations. This implies that *c-myc/IgH* translocations are

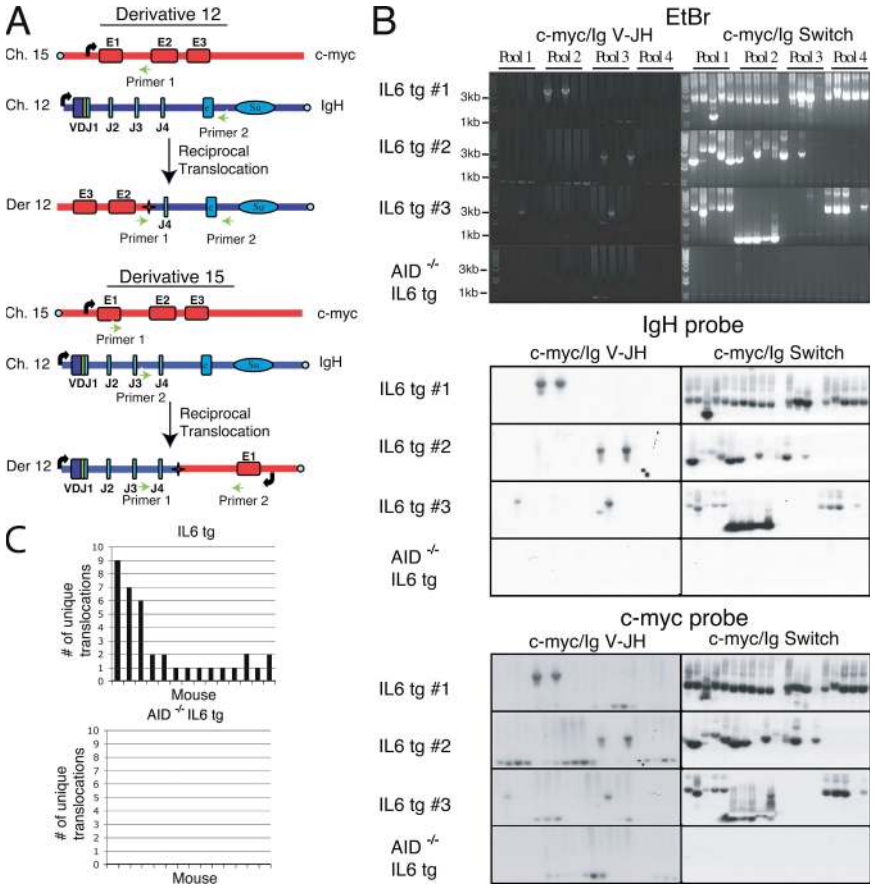

Figure 2. C-myc to Ig V–J_H region. (A) Diagram of translocation assay for detecting derivative 12 and 15 chromosomal translocations from c-myc to the Ig V-J_H region. Primer set 1 is indicated in the diagram for derivative 12 (see Materials and methods). The circles at the end of each chromosome represent the centromere. (B) Comparison of c-myc translocations to the IgH V-J_H versus switch region. Representative PCRs from three IL-6 tg and one AID$^{-/-}$IL-6 tg mouse. Primer set 1 was used to detect translocations to the variable region. Each lane represents amplification products from 10^5 cells. The ethidium bromide–stained gels at the top were probed for c-myc, stripped, and then probed for IgH to verify translocations. (C) Graph shows the number of unique c-myc to Ig V-J_H region translocations identified in 14 IL-6 tg mice. No translocations were identified in the same analysis of 12 AID$^{-/-}$IL-6 tg mice ($P = 0.00284$ using a two-tailed Student's *t* test assuming unequal variance.).

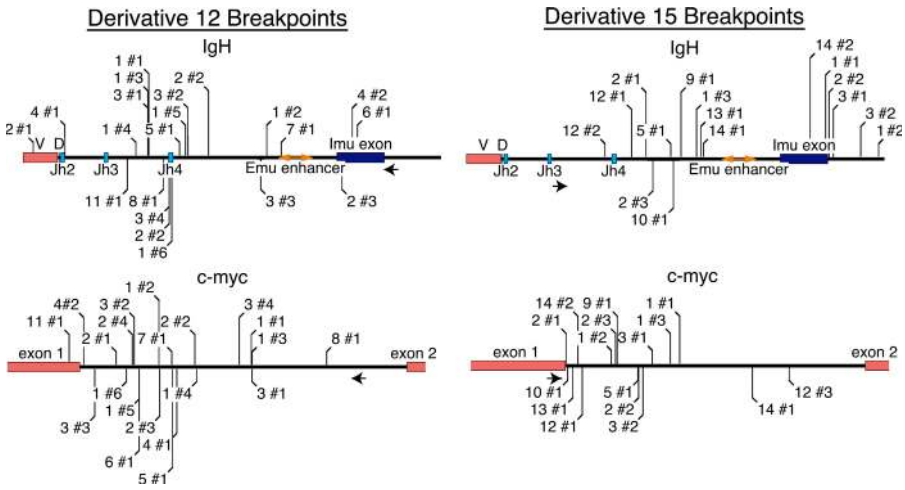

Figure 3. Translocation breakpoints. Diagram of translocation breakpoints from c-myc to the Ig V-J_H region. Only primer set 1 is indicated for derivative 12 (see Materials and methods). The translocations are annotated as the mouse number followed by the translocation number for that mouse. For example, "2 #2" is mouse 2 translocation number two amplified from that mouse.

resolved through error-prone repair, or that translocation breakpoints occur at or immediately adjacent to hypermutated sequences. Discounting mutations within 20 bp of the breakpoint, we identified three mutations in *c-myc*, resulting in a mutation rate that is above background ($\sim 0.2 \times 10^{-3}$) but approximately threefold lower than the rate observed for *IgH* (Fig. 4). These results suggest that *c-myc* translocations to the Ig V-J_H region in *IL-6* tg mice occur during or after Ig V-J_H region SHM (28, 47, 48).

DISCUSSION

Molecular characterization of translocations found in lymphomas showed that in addition to Ig switch regions, many breakpoints are near RSS, suggesting a role for aberrant V(D)J

Table I. Derivative 12

Translocation	IgH	Jnc	c-myc
4 #2	GAGTGTCCTCTCAACCACCA	A	GATCTGGTGGTCTTTCCCTG
2 #3	GAATACTCAGAAAGTGGTCT		CTTTTGCGTTTGGGAGCGAG
3 #3	TTAAACTTTAAGTAATGTCA	*t*	TTAGGACAGTCTTTCTTCCA
2 #4	CTTTTATTCTAAAAACTGAA		CGGCCGGTTGGACATTATTG
1 #2	TTAAAAGTCAGTTCTGAATA	G	ATTACCTTTTGCGTTTGGGA
1 #3	CCCCTGGACCCCTCTTTAGT		AGGGAAGACGCCCTGCACCC
3 #1	CCTGGACCCCTCTTTAGT		AGGGAAGACGCCCTGCAC
1 #5	GACAGTTTATTTCCCAATTT	CT	TTGAGGGGCAAACCGGGAG
4 #1	GTTGTAAGGACTCACCTGAG	G	CATTTCTGACAGCCTGGG
3 #2	CAGCCGGTTCCCTCAGGGAC		TTGGACATTCTTGCTTTGCTAt
1 #1	CCCCTGGACCCCTCTTAAGT		AGGGAAGACGCCCTGCAC
1 #6	GGAGACGGTGACTGAGGTT		TCCCGAGGTTACTATGGGC
5 #1	GTCTGCAATGTTCAGAAAAC	T	AAATTTCAGCTTGGTGCATT
1 #4	CCCTAATTCTCACAAGAGTC		TTTTGGCTTTAAAAATAGTG
2 #2	GTTCCTTGACCCCAGAAGTC		AAGAAGTTGCTATTTTGGCT
6 #1	CACAGAGCATGTGGACTGGC	TT	AATTGATATGTGTCCTTTG
7 #1	CCAAATAGCCTTGCCACATG		GGGCATTTAAATTTCAGCTT
8 #1	ATACCCGACAAAAACCCCAG	*t*	CTTTCTCCTTCAGGTGGCGC
2 #1	GCCTTGCAGGACAGCTTCAC		ATGACAGAGGAAAGGGGAAG
3 #4	CTTGACCCCAGTAtTCCATAG		GAAGACTGCGGTGAGTCGTGA
11 #1	CCTTCtTCATACTTCAGgTCT		CCTGAAAAGAGCTCCTCGAGC

Junction sequences are those that cannot be definitively assigned to c-myc or IgH. Lower case letters indicate mutations, and insertions are in italics. The translocations are annotated as the mouse number followed by the translocation number for that mouse. For example "2 #2" is mouse 2 translocation number two amplified from that mouse. Jnc., junction.

Table II. Derivative 15

Translocation	IgH	Jnc	c-myc
2 #1	AGGCTCATTTGAGGGAcATG		GTTTCCAACGCCCAAAGAA
2 #3	AGTTGGAGATTTTCAGTTTT-AG		TCCAACCGGCCGGGTCAG
9 #1	GAGAGCTGTCTTAGTGATTG	*t*	AAGAATGTCCAACCGGCCG
14 #2	AGGTCTGAGACCAGGCTGCT	*aaaaat*	CGGCCGCTACATTCAAGACG
12 #2	ATGTCTGAGTTGCCCAGGGGT	G	GCGCCGCGTCGCTCGGCCCC
5 #1	TAAAACTTCATTTGTTGGAAG		ATCCCTTCTCCAAAGACCTCA
3 #2	AATTATTTCAtTTAAGTGTAT		CTTCTCGCTCCCAAACGCAAA
2 #2	TCCGAAACCAGGCACCGC	AAA	AGGTAATCCCTTCTCCAA
12 #1	GAAGTTGGGAAATAAACTGTC		GAtGAAAGACTGTCCTAACCG
13 #1	GTCCAAAATTTTTGTCAATC	A	AtACAGGGAAAGACCACCAG
10 #1	TTGCTGTCTAGAGAGGTCTG	G	CTTACCGGGGTTTCCAACGC
1 #1	GAACAATTCCACACAAAGACT	C	TTAAATTTTACTACGATCAC
1 #2	GAAGGAATTTAAATTGGAAG	C	CATAGTAACCTCGGGAACCC
1 #3	ACTTTAAAATGTGAGAGGG	TT	AAAAGGCTCAGGGACGGGT
3 #1	GCCACAGCTGTGGCTGCTGCT		GCTGAAATTTAAATGCCCTCT
14 #1	TCAATTTGAGGTCTTGTTTGT		AtAGCTCAGTCTCCGGCTATC

Junction sequences are those that cannot be definitively assigned to c-myc or IgH. Lower case letters indicate mutations, and insertions are in italics. The translocations are annotated as the mouse number followed by the translocation number for that mouse. For example "2 #2" is mouse 2 translocation number two amplified from that mouse. Jnc., junction.

recombination in their etiology. This idea is supported by experiments in mice deficient in DNA damage response and apoptosis pathways that develop spontaneous lymphomas harboring oncogenic translocations involving the *TCR* or *IgH* locus (1, 2, 49–53). For example, ataxia telangectasia mutated mice ($ATM^{-/-}$) develop thymic lymphomas with *TCR* gene translocations (54–58). These translocations can be ascribed to V(D)J recombination because they fail to occur in the absence of RAG expression (51, 53). Similarly, mice deficient in p53 and nonhomologous end joining factors or histone H2AX develop RAG-dependent translocations that lead to pro–B cell lymphomas (1, 2, 49, 50, 52, 59–63).

However, some translocation breakpoints in proximity of RSS are found in mature B cell tumors, such as endemic Burkitt's lymphoma, diffuse large B cell lymphoma, and multiple myeloma (for review see references 7, 8, and 43). These translocations are believed to arise during or after the germinal center reaction because the Ig genes involved in the translocations are usually somatically mutated (7). Nevertheless, they could be mediated by V(D)J recombination if RAG1/2 were reexpressed in the germinal center. An alternative possibility is that the translocations to the Ig V-J_H region in mature B cells are byproducts of double-strand DNA breaks created by AID during SHM.

Although double-strand breaks are not obligate intermediates in SHM, a measurable fraction of all Ig hypermutations involve deletions or insertions that require repair of double-strand breaks (36, 37). A region spanning 1–2 kbp downstream of Ig V_H genes undergoes somatic mutation at a rate of 10^{-3} per basepair per generation in germinal center B cells (for review see reference 12) (47). Given the large numbers of B cells in the germinal center and their rapid rates of division, AID-induced double-strand breaks in the Ig V-J_H region are not infrequent events (36–39). However, to date

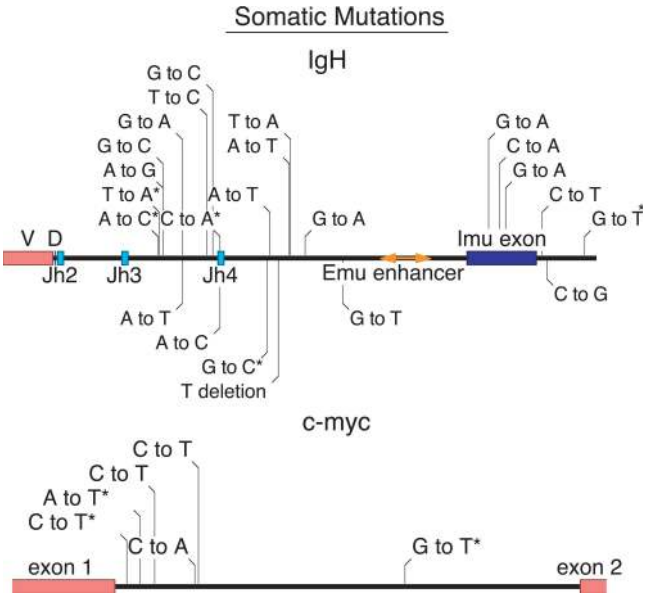

Figure 4. Somatic mutations in translocated IgH. Analysis of 32,654 bp of IgH sequence from c-myc/IgH translocations identified 20 different mutations (mutation frequency = 0.61×10^{-3}), excluding the two mutations found within 4 nts of breakpoints. Excluding the three mutations within 3 nts of the breakpoint, analysis of 16,960 bp of c-myc identified three different mutations (mutation frequency = 0.18×10^{-3}). The overall mutation rate within 20 nts of a breakpoint on either side of the translocation was 5.4×10^{-3}. Asterisks indicate mutations within 20 nts of a breakpoint.

there has been no direct evidence for the role of AID-dependent Ig V-J_H region breaks in chromosome rearrangement.

AID induces *c-myc* translocations to the Ig switch region by a mechanism that resembles class switching in that formation of the initial lesion requires cytidine deamination and uracil removal from DNA (25). However, resolution of the lesion proceeds by distinct pathways for switching and translocation (25). Factors that act in cis to promote switch region synapsis, such as 53BP1 and H2AX, have no impact on *c-myc* to Ig switch translocations despite their effects on genomic stability (25, 64). In contrast, factors that transmit damage signals to the nucleus, such as p53, do not appear to affect switching but are essential in suppressing Ig switch translocations, possibly by promoting the death of cells that overexpress *c-myc* (65, 66).

We have shown that mature B cells in *IL-6* tg mice develop translocations involving *c-myc* and the Ig V-J_H region and that they are AID dependent. These rearrangements closely resemble those found in endemic Burkitt's lymphoma, multiple myeloma, and diffuse large B cell lymphoma in that both of the translocated genes are hypermutated (for review see references 7 and 8). Ig V-J_H regions are direct targets of AID, which is likely to produce the DNA double-strand break intermediates in the translocation reaction. Although translocated *c-myc* was also mutated, germline *c-myc* is not thought to be a target for AID (28), and *c-myc* mutation was substantially lower than the Ig partner. We speculate that *c-myc* mutation may have occurred after the translocation when it was under the control of Ig regulatory elements (28, 48, 67, 68).

The higher level of mutation we found proximal to the translocation breakpoints is analogous to what is observed for switch region junctions (69). However, the mutation breakpoints proximal to the *c-myc* to Ig V-J_H region translocations are not RGYW hotspot biased and differ from breakpoint distal mutations in that they are not enriched for transitions. This suggests that the higher mutation frequency within 20 nts of the junction is the result of error-prone repair by nonhomologous end joining, whereas many of the mutations further away from the breakpoint are AID induced.

We conclude that translocations involving the Ig V-J_H region of the Ig locus can be attributed to lesions produced by AID.

MATERIALS AND METHODS

DNA preparation and PCR. Hyperplastic lymph nodes from individual male and female *IL-6* tg (14) and *AID*$^{-/-}$*IL-6* tg (12) mice were combined into four pools. Total DNA was prepared from 2×10^7 cells for each pool. 0.5×10^6 cells from each of the four pools for 12 different mice was assayed for derivative 12 translocations by PCR using primer set 1 and 2.5×10^6 cells using primer set 2 (see below). For derivative 15 translocations, 0.5×10^6 cells from each of the four pools from 12 different mice was amplified using set 3 (see below).

For derivative 12 translocations from c-myc to the IgH variable region, we performed nested PCR (Long Expand PCR system; Roche) using the following primers: primer set 1: first round with 5-GCAATGACTGAAGACTCAGTCCCTCTTAAG-3 (*IgH*) and ACTTAGCCCTGCAGACGCCCAGGAATCGCC (*c-myc*), followed by nested PCR with TACCATTTGCGGTGCCTGGTTTCGGAGAGG (*IgH*) and TTGGCTTCAGAGGCTGAGGGAGGCGACTG (*c-myc*); primer set 2: first round with GTGCCCCACTCCACTCTTTGTCCCTATGC (*IgH*) and GAAATAAAAGGGGAGGGGGTGTCAAATAATAAGAG (*c-myc*), followed by nested PCR with ATCATCCAGGGACTCCACCAACACCATCAC (*IgH*) and CCTCCCTTCTACACTCTAAACCGCGACGCCAC (*c-myc*). 500 ng DNA (10^5 cells) was amplified in each 20 μl first-round PCR reaction; 1 μl of the first reaction was template for the nested PCR reaction. PCR conditions for the first round were 94°C for the first 2 min, followed by 10 cycles of 94°C for 30 s, 61°C for 30 s, and 68°C for 7 min, followed by 19 cycles of 94°C for 30 s, 61°C for 30 s, 68°C for 7min plus 20 s/cycle. The conditions for the nested PCR were as follows: 94°C for 15 s, 61°C for 30 s, 68°C for 4 min, followed by 15 cycles of 94°C for 30 s, 61°C for 30 s, 68°C for 4 min plus 20 s/cycle. The combined total number of cycles of amplification was 45.

Conditions for amplification of derivative 15 translocations from c-myc to the *IgH* variable region were the same as those for derivative 12, with the exception of a 2-min extension time in the first-round PCR and a 1-min extension time in the nested PCR. Amplification of derivative 15 translocations was performed using the following primers: primer set 3: GTTGAGACATGGGTCTGGGTCAGGGAC (*IgH*) and ATCAGCGGCCGCAACCCTCGCCGCCGC (*c-myc*), followed by a nested PCR reaction with CTCTGCCTGCTGGTCTGTGGTGACATTAG (*IgH*) and GAAGGCTGGATTTCCTTTGGGCGTTGG (cmyc).

For amplification of derivative 12 *c-myc* to switch region translocations, primers described previously (24) were used following the PCR conditions described above for primer set 1.

All animal experiments were performed in accordance with the rules and regulations of The Rockefeller University Institutional Laboratory of Animal Care and Use committee.

Southern blot analysis. PCR products from primer set 1 were separated on 0.8% agarose gels and denatured for 15 min in 0.4 M NaOH before transfer to nylon membranes and probing with P32 radiolabeled primers. The *IgH* probe sequence is GGTGGCAGAAGCCACAACCATACATTCCCA, and the *c-myc* probe sequence is GCGCCTCGGCTCTTAGCAGACTGTAT.

Sequencing. The PCR reactions were separated on 0.8% agarose gels, and the bands were gel extracted using the QIAGEN gel extraction kit according to the manufacturer's instructions and sent for direct sequencing. Translocations were sequenced with the nested PCR primers. If the translocation breakpoint was not identified with the first round of sequencing, we designed new primers for sequencing to walk along the translocation until we reached the breakpoint.

Sequence analysis. PCR products were sent directly for sequencing without cloning so that we could discount the error rate of the polymerase in our analysis. Mutations that arose during early PCR cycles could be identified by chromatogram analysis by the presence of more than one base signal for the same nucleotide. Such nucleotides were discounted from our analysis. All sequenced translocations were aligned using both SeqMan from DNA Star and the Codon Code Aligner software. Overlapping traces from all the translocation sequences allowed for distinction between real mutations and single nucleotide polymorphisms. In the case of *c-myc*, we also amplified and sequenced the first intron from the *IL-6* tg mouse to verify that the *c-myc* mutations identified were not single nucleotide polymorphisms.

FACS analysis. FACS analysis was conducted as described previously (24). All antibodies were from BD Biosciences.

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