Regulation of AID expression in the immune response

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The B cell–specific enzyme activation-induced cytidine deaminase (AID) has been shown to be essential for isotype switching and affinity maturation of antibody genes during the immune response. Conversely, AID activity has also been linked to autoimmunity and tumorigenesis. Determining how AID expression is regulated in vivo is therefore central to understanding its role in health and disease. Here we use phylogenetic footprinting and high-resolution histone acetylation mapping to accurately demarcate AID gene regulatory boundaries. Based on this strategy, we identify a novel, positive regulatory element required for AID transcription. Furthermore, we generate two AID indicator mouse strains using bacterial artificial chromosomes that faithfully recapitulate endogenous AID expression. The first strain uses a green fluorescent protein reporter to identify B cells that actively express AID during the immune response. In the second strain, AID transcription affects the permanent expression of a yellow fluorescent protein reporter in post–germininal center and terminally differentiated lymphocytes. We demonstrate the usefulness of these novel strains by resolving recent contradictory observations on AID expression during B cell ontogeny.

The elimination of antigens by B lymphocytes is performed by antibody molecules that contain an N-terminal variable domain followed by a C-terminal constant domain. During the immune response, B lymphocytes express the activation-induced cytidine deaminase (AID) enzyme, which induces two major alterations in Ig gene loci to enhance antibody and B cell function. First, the process of somatic hypermutation introduces single point mutations at variable genes, which can increase antibody affinity for antigens (1). Second, the mechanism of class switch recombination replaces the Ig heavy chain constant region $C_\mu$ for $C_\gamma$, $C_\varepsilon$, or $C_\alpha$, thereby controlling the antibody effector function (2).

The importance of AID in the humoral immune response is highlighted in AID-deficient patients and animals, which are highly susceptible to infection and exhibit gut flora–dependent hyperplasia of intestinal villi (3, 4). Conversely, complex diseases such as autoimmunity have long been associated with AID–dependent hypermutation (5). Moreover, untimely, ectopic, or elevated AID expression results in translocations and malignant transformation of B cells (6, 7) and T cells (8). These considerations emphasize the need to understand the molecular pathways that regulate AID expression during B cell ontogeny.

Initially, AID expression was thought to be limited to follicular germinal center (GC) B cells (9). Conversely, recent evidence suggests that AID is also active outside of the GC microenvironment. In mice carrying a highly reduced V gene repertoire, hypermutation and AID transcripts were reported in a small subset of immature bone marrow B cells (10). In a lupus-prone mouse, hypermutation and affinity

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Abbreviations used: Ab-MLV, Abelson murine leukemia virus; AID, activation-induced cytidine deaminase; BAC, bacterial artificial chromosome; CNS, conserved noncoding sequence; GC, germinal center; ILF, isolated lymphoid follicle; NP, nitrophenol; PP, Peyer’s patch; QM, quasimonoclonal; SC-RT-PCR, single cell RT-PCR strategy; YFP, yellow fluorescent protein.
maturation occur at the T cell zone–red pulp border (11). Likewise, in rheumatoid arthritis patients, somatic hypermutation has been shown to occur in B cells residing in nonlymphoid synovial tissue (12). In normal mice, AID transcription and class switch recombination might occur in the gut lamina propria outside of Peyer’s patches (PPs) and isolated lymphoid follicles (ILFs) (13), although others have recently contradicted these findings (14). Large extrafollicular proliferating cells have also been shown to express AID in human lymphoid tissues (15, 16). Finally, recent data indicate that AID is induced in pre-B cells in response to retroviral infection, perhaps as part of an innate defense mechanism (17). These studies therefore underscore the need for experimental model systems to monitor AID expression in the context of the entire organism.

The development of indicator mouse strains using bacterial artificial chromosomes (BACs) has greatly facilitated the study of gene expression and the unraveling of gene regulatory elements in the past decade (18, 19). The success of gene reporter expression systems is based on the premise that transgene constructs must carry sufficient regulatory information to recapitulate tissue-specific and copy-dependent regulation of genes of interest. However, the in vivo demarcation of gene regulatory boundaries typically relies on the production of large cohorts of transgenic animals (20, 21), making this process expensive and time-consuming. We here provide an alternative approach that combines comparative genomic analysis with high-resolution histone H3 acetylation mapping. This strategy allowed us to predefine the minimal DNA segment capable of recapitulating the temporal and spatial expression patterns of endogenous AID and led to the development of two complementary AID reporter lines that efficiently track AID-expressing cells as well as their progeny. Our studies define AID expression during T cell–dependent and T cell–independent immune responses as well as upon retroviral infection of immature B cells.

RESULTS
Mapping AID gene regulatory boundaries
To identify the cis-regulatory elements governing AID transcription in vivo, we used phylogenetic footprinting to compare one megabase of human, dog, and mouse genomic DNA containing the AID (Aicda) gene. Using a combination of global and local alignment algorithms (http://family.caltech.edu/ and http://www-gsd.lbl.gov/vista/index.shtml), we generated alignment plots that revealed the conserved protein-encoding sequences (exons) of three syntenic genes within the one megabase of genomic DNA analyzed: Kiaa1238, Mfap5, and Aicda (Fig. 1, red peaks). Additionally, we found 11 highly conserved noncoding sequences (CNSs) that exhibited on average ≥75% identity in all three species (Fig. 1, black peaks).

To examine potential contributions of CNSs to AID transcription, we explored their ability to attract histone acetyltransferase activity in B cells stimulated with LPS and IL-4 in vitro. Hyperacetylation of core histones in transcribed genes results in part from the interaction of transcription factors with regulatory elements, such as promoters and enhancers (22). To monitor changes in chromatin acetylation at high resolution, we assessed the percentage of acetylated histone H3 by chromatin immunoprecipitation and real-time PCR analysis (Fig. 2 A). In mature resting B cells, which do not express AID, we found H3 acetylation across Aicda at low levels (<10%; Fig. 2 B, blue circles). This acetylation was comparable to the calculated H3 acetylation in the mammalian genome (8%; see Materials and methods) as well as in neighboring genes such as Mfap5 (Fig. 2 B), which is not appreciably transcribed in B cells (not depicted).

![Figure 1. Phylogenetic analysis of Aicda.](https://example.com/figure1.png)

Figure 1. Phylogenetic analysis of Aicda. AID locus in human chromosome 12. Homology “peaks and valleys” graph showing percent conservation between human, dog, and mouse AID genomic loci. The nucleotide sequence is plotted on the horizontal axis. The vertical axis depicts the percent identity between the three species. Only conserved regions containing at least one peak with percent identity ≥75% within a shifting window of 100 bp in all three species are represented. Red peaks correspond to gene exons, whereas black peaks denote CNSs (I–XI). Genes and their orientation are represented by their RefSeq name and by arrows, respectively. As in the human, the mouse and dog Mfap5 and Kiaa1238 orthologs are located 5’ of Aicda and form a synteny group within the 1 MB interval analyzed. Conversely, the mouse and dog Clecsf9 and Clecsf8 genes are located beyond the 1 MB sequence analyzed here, and no homology is therefore assigned to these genes. Alignment was obtained using Family Relations (http://family.caltech.edu/) and VISTA software (http://www-gsd.lbl.gov/vista/index.shtml).
In contrast to resting lymphocytes, B cells activated with LPS and IL-4, which induces AID transcription, showed a dramatic increase in H3 acetylation at the Aicda locus. Nearly all nucleosomes of AID exon 1 and its vicinity (corresponding to CNS VIII) were acetylated (Fig. 2 B, red squares). This first peak of acetylation included an intronic regulatory element shown to bind E proteins during AID expression (23). Acetylation was decreased at exons 2–5 (50–70%), and a further decrease to basal acetylation levels was observed near Aicda’s polyA signal sequences. These results are consistent with the notion that acetylation signals are propagated over the chromatin of coding regions through interactions of histone acetyltransferases and transcription complexes (24).

Unlike CNS I–VII and XI, which exhibited no change in H3 acetylation upon B cell activation (Fig. 2 B and not depicted), a second acetylation peak occurred at CNS X. This element, located ~7 kb downstream of mouse Aicda (Fig. 2 A) or ~24 kb 3′ of human Aicda (Fig. 1), is composed of two highly conserved sequences between mouse and humans that correlate with the Southern blot bands obtained by DNasel hypersensitivity. Putative transcription factor binding sites in elements A and B, predicted using VISTA software, are shown.

Figure 2. H3 acetylation status at the AID locus. (A) Percent homology identity between the dog, human, and mouse genomes as a function of the mouse AID locus in chromosome 6. Homology peaks and genes are depicted as in Fig. 1. Black dots represent the position of quantitative PCR primers used to assess H3 acetylation by CHIP. AID exons are marked with yellow boxes. E, EcoRV; S, Sall; X, Xbal. (B) Absolute percentage of histone H3 acetylation from resting (blue circles) or LPS-activated (red squares) B cells. (C) DNaseI hypersensitivity at CNS VII and X. Nuclei prepared from LPS-activated mouse B cells were untreated (lane 1) or treated with increased concentrations of DNasel (lanes 2–6). DNA was extracted, digested, and analyzed by Southern blot hybridization using VII- or X-specific probes (PVII and PX, respectively). Horizontal arrows indicate DNaseI hypersensitive sites that correlate with CNS-X. (D) Detailed map of mouse CNS X. Elements A and B (red boxes) depict two highly conserved sequences between mouse and humans that correlate with the Southern blot bands obtained by DNasel hypersensitivity. Putative transcription factor binding sites in elements A and B, predicted using VISTA software, are shown.
and activated B lymphocytes. In contrast to CNS VII, which is not hyperacetylated during B cell activation (Fig. 2 C, left), we found clear evidence of protein binding at CNS X (Fig. 2 C, right), strongly suggesting that this element functions as a regulator of AID transcription.

Monitoring in vivo AID expression using AID-GFP reporter mice

The above studies predicted that AID proximal promoter elements and CNS X might be sufficient to direct physiological expression of an AID reporter gene. To evaluate this hypothesis, we generated transgenic mice with a 45-kb BAC carrying the mouse AID gene, including CNS X (Fig. 2 D and Fig. 3 A, top). By means of homologous recombination (25), we modified the BAC by fusing the GFP gene to AID exon 5. To define the role of CNS X in AID expression, we also created a second transgene construct in which CNS X was deleted by recombination (AID-GFP-ΔX; Fig. 3 A, bottom). Two AID-GFP and three AID-GFP-ΔX transgenic mouse strains were generated with two to five transgenic copies per line.

Transgenic mice and wild-type littermates were immunized in the front footpads with the conjugated hapten nitrophenol (NP)-CGG. 5 d after immunization, we developmentally staged axillary lymph node B lymphocytes by flow cytometry using a panel of cell surface markers, including the B cell GC marker CD95. Unlike nontransgenic littermates (Fig. 3 B, left), nearly all CD95<sup>high</sup> GC B cells in AID-GFP mice exhibited green fluorescence (Fig. 3 B, middle). AID-GFP was absent in resting B220<sup>+</sup> B cells (Fig. 3 B, middle, CD95<sup>low</sup>), bone marrow B cell populations (discussed below), and thymic or peripheral CD4<sup>+</sup> or CD8<sup>+</sup> T cells (not depicted). This GFP expression pattern matches the reported transcription profile of AID (9). The coordinate expression of AID-GFP and endogenous AID mRNA was validated by real-time PCR in resting and LPS plus IL-4–activated B lymphocytes (Fig. 3 C). In addition, RT-PCR analysis of seven mouse tissues revealed that AID expression is circumscribed to lymphoid organs both in wild-type and transgenic animals (Fig. S1). Analysis of the three transgenic AID-GFP-ΔX lines showed no green fluorescence in GC B cells in the absence of CNS X (Fig. 3 B). Furthermore, PCR analysis showed a five- to sevenfold decrease in AID-GFP transcripts in activated AID-GFP-ΔX lymphocytes compared with AID-GFP controls (Fig. 3 C). We conclude that our AID-GFP transgene recapitulates the spatiotemporal expression of endogenous AID and that CNS X is required for AID transcription during the immune response.

Activated B cells leaving the GC differentiate into plasma or memory B cells. To examine whether AID expression is maintained or down-regulated as B cells exit the GC microenvironment, we assessed green fluorescence in post-GC B cells during the NP response. We first analyzed pre-plasma memory B cells (NP<sup>+</sup>IgD<sup>-</sup>B220<sup>+</sup>CD138<sup>-</sup>) (26, 27) in AID-GFP mice immunized with NP-KLH. As described previously, these GC-derived B cells appear in the spleen 7–10 d after immunization, are somatically hypermutated, do not secrete antibody, and persist after primary challenge (27). We found that although NP<sup>+</sup>B220<sup>+</sup>CD95<sup>high</sup> GC cells express abundant AID-GFP (Fig. 4 A, right), pre-plasma memory lymphocytes lack AID-GFP expression (Fig. 4 A, middle) suggesting that AID is down-regulated as cells exit the GC microenvironment. To confirm this result, we assessed AID transcription in post-GC NP-binding cells recovered from the blood of nontransgenic animals. NP-specific IgG1<sup>+</sup> B cells carrying somatic mutations appear in the blood as early as 7 d after immunization (28). To determine AID mRNA levels in this small compartment, we developed a single cell RT-PCR strategy (SC-RT-PCR; see Materials and methods). Consistent with previous observations (28), few NP-specific B cells were found in unimmunized mice (Fig. 4 B, day 0), whereas 7 d after NP-CGG immunization, B220<sup>+</sup>IgG1<sup>+</sup>NP<sup>+</sup> B cells were clearly present in the blood (Fig. 4 B, day 7). We found that although nearly all (70 of 72) B220<sup>+</sup>IgG1<sup>+</sup>NP<sup>+</sup>CD95<sup>high</sup> lymph node GC cells were AID<sup>+</sup> at day 7, AID transcripts were absent in B220<sup>+</sup>IgG1<sup>+</sup>NP<sup>+</sup>blood lymphocytes (Fig. 4 B). In agreement with these observations, no AID-GFP<sup>+</sup> cells were observed in the blood of AID-GFP–immunized mice (not depicted).
To determine AID expression in T cell–independent immune responses, we analyzed AID-GFP mice carrying the NP-specific heavy chain B1-8 (B1-8hi) (29). 5 d after NP-Ficoll intraperitoneal injection, B1-8hi mice develop T cell–independent GCs (B220^+CD95^{high}CD138^{low} B cells) and large numbers of B220^-CD138^{high} plasma cells in the spleen (29). We found that GC cells express abundant AID-GFP after NP-Ficoll challenge (48% of B220^+CD138^{low} cells [Fig. 4 C], but nearly 100% of CD95^{high} cells within that population [not depicted]). AID-GFP was, however, absent in terminally differentiated B220^-CD138^{high} plasma cells (Fig. 4 C, population 4), as determined both by flow cytometry and SC-RT-PCR analysis, a result consistent with the reported down-regulation of AID mRNA in plasma cells (30). Yet, a subset of B220^-CD138^{high} plasmablasts expressed AID mRNA, AID-GFP, and high levels of CD95 (Fig. 4 C, population 3). These lymphocytes might represent an intermediate stage between B220^+CD138^{low}CD95^{high} GC cells and fully differentiated B220^-CD138^{high} plasmacytes (31). Collectively, our data demonstrate that AID is expressed both in T cell–dependent and –independent GCs, but it is extinguished as B220-FITC. Right: Analysis of AID transcription by SC-RT-PCR. NP^+ cells were recovered by cell sorting from blood (72 cells analyzed) or lymph node GCs (72 cells) isolated from wild-type or AID^-/- mice. (C) Left: AID-GFP animals carrying the pre-recombined B1-8hi heavy chain gene were immunized with NP-haptenated Ficoll and splenic B cells analyzed 5 d after immunization. AID-GFP expression in resting (B220^-CD138^{low}), T cell–independent GC (B220^-CD138^{low}), plasmablasts (B220^-CD138^{high}), and plasma cells (B220^-CD138^{low}) is indicated with histograms. Bottom histograms show CD95 expression in AID-GFP^+ cells from population 3 or population 4. Right: Analysis of AID transcription by SC-RT-PCR in the indicated populations isolated from B1-8i mice.

**Genetic labeling of B lymphocytes after AID expression**

As stated above, the AID-GFP transgene and endogenous AID are activated in GC cells undergoing switching and/or hypermutation, but turned off during plasma and memory B cell differentiation. To permanently tag the progeny of AID–expressing B cells with a fluorescent reporter protein, we introduced the bacterial cre recombinase gene in lieu of AID exon 1 (Fig. 5 A). AID-Cre transgenic mice were bred to the ROSA26-EYFP reporter strain, in which the yellow fluorescent protein (YFP) is expressed indelibly upon Cre-mediated deletion of a fl oxed neomycin gene (Fig. 5 A) (32). To evaluate the interplay of the two transgenes in vivo, we measured yellow fluorescence in different B cell subsets from AID-Cre-YFP double transgenic mice. Six different founder lines with approximately one to five transgene copies were analyzed. We found no YFP expression in immature bone marrow B cells (see below) or other hematopoietic lineage cells (not depicted). In addition, the spleen or axillary lymph
AID variance and comparing values to mutation background determined in cal signifi cance was assessed by a two-tailed t test assuming unequal pie charts. Mutation frequency and P values are indicated below. Statisti-

indicated. The number of sequences analyzed is given in the center of the are sized in proportion to the number of sequences carrying mutations as CD95highGFP

AID-Cre-YFP, AID-GFP

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Figure 5. Permanent labeling of B cells expressing AID. (A) Upper schematics: molecular maps of the AID-Cre (top) and Rosa-Neo-EYFP (bottom) transgenes (S, SalI; P, PacI; E, EcoRI). Lower Southern blot: three independent AID-Cre transgenic lines were generated, with copy number ranging between 1 to 5 copies. (B) FACS pseudocolor plots showing CD95-PE and YFP expression in B cells (gated on B220 (PercP-Cy5.5)) from AID-Cre-YFP compound mice. Left panel: axillary lymph node cells from nonimmunized mice; right panel: PPs. (C) Immunofl uorescence analysis of gut lamina propria CD138+ plasma cells from AID-Cre-YFP mice. Scale bar = 20 μm. (D) Mutation analysis of the JH4 intron from CD95lowYFP−/− sequences. We found that B220+CD95lowYFP+ lymphocytes carry a similar amount of mutations to that measured in GC B cells from AID-GFP mice (Fig. 5 D). This result indicates that this population largely derives from activated follicular B cells. Collectively, our results demonstrate that the AID-Cre and ROSA-Neo-YFP transgenes efficiently tag the progeny of AID-expressing B cells.

AID in the GALT

IgA, together with innate mucosal immunity, provides the fi rst line of defense against bacterial antigens in the GALT. AID expression and class switching to IgA have been proposed to take place either in situ in the gut lamina propria (13) or within GCs in PPs and ILFs of the small intestine (14). To ascertain the site of AID expression in mucosal surfaces, we determined green fl uorescence in AID-GFP lymphocytes isolated from small intestine PPs and large intestine lamina propria. Under specific pathogen-free conditions, the large intestine (which normally lacks PPs) fails to develop ILF GCs (unpublished data). We found little AID-GFP expression in B220+IgM+ PP B cells (Fig. 6 A, middle). In contrast, around 65% of all B220+IgA+ PP cells expressed AID and high levels of CD95 (Fig. 6 A, right panel and top histogram, respectively), indicating that PP GCs are primarily populated by IgA+ lymphocytes. As in PPs, B220+IgM+ B cells of the large intestine lamina propria displayed little green fl uorescence (Fig. 6 B, left). However, a population of B220+IgA+ B lymphocytes expressed AID–GFP and high levels of CD95 in the large intestine (Fig. 6 B, right, and Fig. 6 A, bottom histogram, respectively). IgA+CD95highAID–GFP+ plasma cells that have undergone affi nity maturation. To test these possibilities, we determined green fl uorescence in the lamina propria of OcaB–GFP–OcaB−/− mice. In the absence of OcaB, the capacity of B lymphocytes to form pauciclonal aggregates and differentiate into plasmacytes in T cell–rich areas of lymphoid organs is intact, whereas B cell translocation to follicles and GC formation is impaired (33–35). In consequence, OcaB−/− mice lack both PPs, ILFs in the gut, and GCs in mesenteric lymph nodes (Fig. 6 C, micrographs, and not depicted) (33–35). We found that OcaB−/− AID–GFP mice, although unable to form GCs, displayed IgA+CD95highAID–GFP+ B cells in the large intestine lamina propria (Fig. 6 C, right), although at lower levels compared with wild-type animals (Fig. 6 B, right). To determine whether
AID-GFP+ B cells showed ongoing AID transcription, we performed SC-RT-PCR. We found evidence of AID mRNA in nearly all CD95

levels of AID mRNA in pro-/pre-B cells corresponded to a

AID expression in IgM (APC) or IgA (biotin/Streptavidin PerCP-Cy5.5) B lymphocytes isolated from PPs (A) and lamina propria (LP; B) from AID-GFP mice (B220 was APC-Cy7 conjugated). Histograms show CD95 (PE) expression in the indicated populations. (C) Left micrographs: PNA staining of mesenteric lymph nodes (MLNs) isolated from OcaB−/− and OcaB+/− mice (bar, 150 μm). Right density plot shows AID-GFP expression of IgA+ cells isolated from the lamina propria of AID-GFP-OcaB−/− mice. Bottom gel shows analysis of AID expression by SC-RT-PCR in the indicated B cell populations recovered from OcaB−/− mice as indicated.

AID expression in immature B lymphocytes

Hypermutation in “GALT species,” including rabbit, sheep, and cattle, diversifies the preimmune repertoire before antigen B cell stimulation (36). In contrast, V(D)J hypermutation in normal mice and humans is widely believed to be restricted to the GC microenvironment. This view has been recently challenged by findings showing AID expression and hypermutation in immature bone marrow B cells in mice expressing a highly restricted Ig repertoire (quasimonoclonal [QM] mice) (10). To determine whether AID is present in developing lymphocytes from wild-type mice, we characterized bone marrow B cells from AID-GFP and AID-Cre-YFP mice. We found that AID-GFP was absent from pro-/pre-B (B220loIgM−), transitional (B220loIgM+), and mature recirculating bone marrow B cells (B220hiIgM+) recovered from AID-GFP transgenics (Fig. 7 A, left, pseudo-color plot, and middle). Likewise, YFP was undetectable in immature and transitional B cells (Fig. 7 A, right, 1 and 2). However, in all mice analyzed, we found a subset of mature recirculating B cells expressing YFP, which might represent post-GC lymphocytes (Fig. 7 A, right, 3). To investigate whether the apparent absence of AID expression in immature B cells was due to low expression of fluorescent proteins in our reporter animals, we quantified AID transcription in sorted bone marrow B cell populations using real-time PCR. Background levels of AID mRNA in pro-/pre-B cells corresponded to a 10−3 dilution of PCR template prepared from B220+CD95high GC B lymphocytes isolated by cell sorting from the lymph nodes of immunized mice (Fig. 7 B). In agreement with published semiquantitative PCR analysis (10), AID transcripts were consistently detected in CD93+IgM+ bone marrow lymphocytes and splenic mature B cells, but AID expression levels in these cells were 100–500-fold lower than those measured in GC lymphocytes (Fig. 7 B), and this transcription was not sufficient to promote efficient Cre-mediated recombination or GFP expression in our AID-Cre-YFP or AID-GFP indicator mice, respectively. Thus, unmanipulated mice show little AID expression in immature B cells under normal conditions.

Recent data indicate that AID transcription can be induced in pro- and pre-B cells upon infection with Abelson murine leukemia virus (Ab-MLV) (17), perhaps as part of an innate defense mechanism against viruses, as has been shown for other APOBEC protein family members (37, 38). To investigate whether retroviral infection leads to AID expression in immature B cells, we monitored YFP fluorescence in AID-Cre-YFP transgenic bone marrow cells infected with Ab-MLV in vitro. In these cultures, as in vivo, Ab-MLV infects with higher tropism a subset of developing B220loCD43+IgM− B lymphocytes, leading to preferential cell proliferation and transformation (Fig. 7 C, 1 and 2) (39). We found little or no YFP expression in day 1–infected CD43+ bone marrow B cells (Fig. 7 C, 3). However, between 5 and 10% of the cultured cells expressed YFP 7 d after infection (Fig. 7 C, 4; three independent experiments). The presence of YFP+ lymphocytes is unlikely due to preferential expansion of rare YFP+ clones, as few immature B cells express YFP after IL-7-S17 stromal cell culture (Fig. 7 C, 5). To confirm induction of AID expression upon infection, we measured endogenous AID mRNA levels in Ab-MLV/AID-Cre-YFP cultures by real-time PCR. We found that compared with uninfected controls, AID transcripts were present in B220+CD43+YFP+ and B220+CD43+YFP− B cells at levels comparable to those found in GC lymphocytes (Fig. 7 C, bar graph). We conclude that in wild-type bone marrow B cells, AID is not appreciably transcribed, but AID expression can be induced upon retroviral infection.

DISCUSSION

Here we have described a rapid method to generate indicator transgenic mice that efficiently recapitulate endogenous expression of a gene of interest. This strategy, which relies on
phylogenetic footprinting and high-resolution histone acetylation mapping, delineates gene regulatory boundaries with great accuracy. In general, using chromatin acetylation would seem an obvious approach when designing reporter transgenic constructs, although it is rarely used to its full potential. One possible reason for its limited use might be the need to scan large segments of genomic DNA by chromatin immunoprecipitation and PCR, as cis-regulatory elements can be far removed from the genes they control (40). Such limitations could be easily overcome by high-throughput methods based on genomic DNA microarrays or similar ChiP-on-Chip technologies recently described (41, 42).

In this study, histone H3 acetylation mapping uncovered two elements at the mouse AID locus that undergo nearly complete acetylation upon B cell activation. The first element corresponds to _Aicda_ proximal promoter sequences, including transcription-enhancing E2A binding sites (23). High levels of acetylation at these sites correlate with the reported ability of E-box proteins to recruit p300 acetyltransferase activity to chromatin (43). Because evidence indicates that p300 and E2A collaborate in B cell ontogeny (44), it will be important to determine whether the p300–E2A complex regulates AID gene transcription in activated B lymphocytes. The second hyperacetylation element, termed CNS-X, is a putative

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**Figure 7. AID expression in immature, bone marrow B lymphocytes.** (A) AID expression determined by flow cytometry from bone marrow B220<sup>hi</sup>IgM<sup>−</sup> pro-/pre-B (population 1), transitional B220<sup>hi</sup>IgM<sup>+</sup> (population 2), and recirculating B220<sup>hi</sup>IgM<sup>+</sup> (population 3) B lymphocytes obtained from AID-GFP (left histograms) or AID-Cre-YFP (right histograms) mice. All immature B cells were also CD93<sup>+</sup> (not depicted). Antibodies were IgM-APC, B220-PercP-Cy5.5, CD93-biotin/Streptavidin-PE, and To-pro-3. (B) Real-time PCR analysis of AID transcripts in pro-, pre-, transitional, mature, and PP B lymphocytes cell sorted from wild-type mice. For comparative purposes, cDNA synthesized from GL7<sup>+</sup>CD95<sup>high</sup> GC B cells was serially diluted, as indicated below each column. cDNA was amplified using PCR primers annealing to AID exons 2 and 3. All B cell populations were sorted to >95% purity before RNA extraction and cDNA synthesis. Vertical bars represent the standard deviation of three independent experiments. (C) Bone marrow cells from AID-Cre-YFP mice were infected with Ab-MLV in vitro. The appearance of YFP<sup>+</sup> cells was monitored in the infected cultures as a function of time (panels 1–4; only days 1 and 7 after infection are shown). The images are representative of three independent experiments. Panel 5 represents bone marrow B220<sup>+</sup> cells isolated from AID-Cre-YFP mice and cultured for 7 d with IL-7 and in the presence of S17 cells. The following antibodies were used: CD43-PE, IgM-APC, B220-PercP-Cy5.5, and DAPI. Bar graph depicts the relative AID expression assessed by real-time PCR in Ab-MLV cultures.
transcriptional enhancer, and its deletion nearly entirely abolishes AID expression both in GC as well as in in vitro–activated B cells. DNaseI hypersensitivity assays demonstrate protein binding activity at CNS-X in activated B cells. Additional studies will be required to determine the nature of these factors and the mechanism by which they regulate AID gene transcription upon B cell activation.

Our reporter strains show that AID expression is induced in GC B cells both during T cell–dependent and –independent immune responses, and that memory and/or plasma cell differentiation leads to AID transcriptional down-regulation. These results are consistent with previous studies showing that the transition of GC cells to the terminally differentiated plasma cell stage is regulated by mutually exclusive transcription programs (30). These gene regulatory networks, which depend on the activity of Pax5, Bcl6, IRF-4, and Blimp1, directly control AID gene expression as B cells differentiate out of the GC microenvironment (45). However, as shown in mice that lack or have poorly developed PPs and ILFs, GCs are clearly not the only location for terminal B cell differentiation (46, 47). Molecular characterization of lamina propria B cells indicates active, ongoing class switch recombination outside of GCs (13). Our results using wild-type and OcaB−/−AID-GFP mice support the idea that AID expression is induced in situ in the GALT.

We find that under physiological conditions, bone marrow–developing B cells do not significantly express AID. Previous experiments with animals expressing a limited repertoire (QM mice) suggested that AID may function in immature B cells (10). Similar conclusions were reached studying mMT−/− mice (48), where Igμ−δ− bone marrow B cells are arrested at the pro–B cell stage (49). In these animals, a small fraction of lymphocytes bypass the developmental block via class switch recombination, implying that AID is active at early stages of B cell development. However, both QM and mMT−/− differ from wild-type mice in that B lymphopoiesis occurs under strong selective pressure. Consequently, the physiological significance of bone marrow AID activity under wild-type conditions was unclear. Our reporter strains show no evidence of AID expression in the bone marrow of animals with a normal antibody repertoire, and sensitive real-time PCR analysis only detected AID transcripts in transitional CD93+IgM+ bone marrow B cells at levels 100–500-fold lower than those found in GC lymphocytes. Although we cannot rule out that under unique circumstances AID may play a role in early B cell lymphopoiesis, low levels of AID mRNA in the bone marrow of wild-type mice may be simply reminiscent of a common evolutionary past between mice and “GALT species” (36).

Although unperturbed bone marrow B cells show little AID expression, infection with a transforming retrovirus readily activates AID transcription in developing lymphocytes (17 and this study). Likewise, B cell infection with hepatitis C (50), Epstein–Barr virus (51, 52), or Moloney murine leukemia virus (53) leads to AID up-regulation, suggesting that AID induction is part of a general innate antiviral host response. It will be important to determine whether non–B cells upon viral infection can also trigger AID expression.

In conclusion, our AID indicator strains efficiently track B cells expressing AID and their progeny in the context of the humoral immune response. These animals along with similar B cell activation reporter mice, such as Blimp-EGFP (54) and Cy1–Cre (55) transgenics, will further our knowledge of the late stages of B cell differentiation both in the normal immune response as well as during autoimmunity and B cell tumorigenesis.

MATERIALS AND METHODS

Phylogenetic footprinting and histone H3 acetylation. Resting splenic B lymphocytes were isolated by negative selection using CD43 MACS beads and processed directly for chromatin immunoprecipitation or cultured at 7 × 10⁶ cells ml⁻¹ in the presence of 25 μg ml⁻¹ LPS (Escherichia coli 0111:B4; Sigma–Aldrich) for 3 d.

To immunoprecipitate acetylated histone H3–DNA complexes from resting and activated B cells, we used anti–acetyl-histone H3 antibodies (06-599; Upstate Biotechnology) according to the manufacturer’s instructions. On average, 85 ng of acetylated DNA was recovered per 10⁶ cells. To calculate the absolute percentage of acetylated histone H3 for a given genomic target, we amplified ng anti–acetyl-histone H3 immunoprecipitated (IM) and input (IN) DNA (normalized using PicoGreen, P7589; Invitrogen) in the presence of SYBR Green (4309155; Applied Biosystems) using an iCycler (Bio–Rad Laboratories). Primers were designed to scan the mouse AID locus at 100 and 150 bp in length. The fold difference between the two sets of samples for any given target sequence was calculated using the following PCR equation:

\[ X = X_0 (1 + E)^n \]  

where \( X_0 \) and \( X \) represent the DNA concentration before and after PCR amplification, respectively, \( E \) denotes the PCR efficiency and \( n \) represents the number of cycles necessary to achieve a specified threshold, which we set to cross a point at which amplification was linear (110 RFU using software from Bio–Rad Laboratories). Under these conditions, \( X \) becomes a constant and the ratio of the initial DNA abundance between the IM and the IN fractions can be calculated as follows:

\[ X_{IM}/X_{IN} = 2^{\text{IM}−\text{IN}} \]  

Thus, the fold difference between the immunoprecipitated and input samples is determined as the difference in number of cycles each fraction takes to reach the specified threshold and taking the resulting power of 2. The fold difference \( X_{IM}/X_{IN} \) (IM/IN) is also proportional to the percentage of acetylated histone H3 in the immunoprecipitated sample (%AcH3) over that of the input (%AcH3):

\[ X_{IM}/X_{IN} = 2^{\text{IM}−\text{IN}} = \%\text{AcH3}_{\text{IM}}/\%\text{AcH3}_{\text{IN}} \]  

Thus, the absolute percentage of histone H3 at any given genomic site can be assessed if %AcH3 is known. To empirically determined %AcH3, we measured the total fraction (\( f \)) of the genome that can be pulled down by immunoprecipitation in the presence of an excess of anti-acetylated H3 antibody as follows:

\[ \text{DNA}_{\text{im}} = f \times \text{DNA}_{\text{IN}} + B \]  

where DNA_{im} and DNA_{IN} are the amount of DNA measured before and after immunoprecipitation, respectively, and B represents nonspecific DNA pulled down by the antibody, which is constant when the immunoprecipitating antibody is in excess. To determine \( f \), we performed several
creasing amounts of 10 U μ−1154′ amplification using primer pairs 2B-5 fragments designated PI and PII. These probes were generated by PCR am-
30 min at 37 °C with RNase A (0.5 mg ml−1/1280, 1/2560, 1/5120 and 1/10240, respectively; 92877720; Roche).
R&D Systems) for 36 h. Resting and activated B cells were then washed, MACS beads and cultured in the presence of 25 g ml−1 DNaseI hypersensitivity.
μ using PicoGreen, the absolute concentration of DNA before and after dilutions of cross-linked DNA obtained from B lymphocytes and measured,
mice. BAC transgenes were constructed by modification of a 200-kb mouse AID and transgenic AID-GFP transcripts, splenic B cells from AID-GFP (and AID-GFP-ΔX) mice were isolated with CD43 microbeads and cul-
tured in the presence of LPS or LPS plus IL-4 for 48 h. RNA was extracted with RNAqueous-Micro (Ambion) and treated twice with DNaseI. cDNA was generated, and the level of endogenous AID transcripts were monitored by real-time PCR with primers: AIDWT-RT3′: CCCCCGGATCAGGTCACGCAGC, AIDWT-RT3′: ATACCGTTGACATTCCGAGGAG. AID-GFP transcripts were amplified with: AID-GFP-RT5′: GACCTGGCGAGATGCATTCTGATG and AID-GFP-RT3′: GCTGAACTGGTGGCCTTTAC. To determine the fold difference between different samples, cDNA obtained from AID-GFP–activated B cells was diluted twofold, fivefold, 10-fold, 50-fold, and 100-fold, and both endogenous AID and AID-GFP were amplified by real-time PCR. We normalized cDNA between samples by amplifying from each cDNA reaction the DNA-PK subunit Ku70 mRNA with the following primers: Ku70-5′: TGCCCTTTATCTGAAGAGTTGC and Ku70-3′: TGCTGCAAGACTGATTTCTC.
To quantify AID transcription in different B cell compartments (Fig. 4), mouse AID primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html): PrimerBank-mAID5′: GCCACCCTTGCCAAACAAGTCT and PrimerBank-mAID3′: CCGGCG-CACGTATAGCAC.
To analyze AID expression at the single cell level, B lymphocytes were stained as indicated and individual cells were sorted using a MoFlo (Dako
Cytometry) instrument into iCycler 96–well plates (Bio-Rad Laboratories) containing 10 μl of 10 mM RNAase-Free Tris buffer and 1 U/μl of RNAse inhibitor (Promega). RT-PCR was set up by adding to each well 15 μl of QIAGEN’s OneStep RT-PCR master mix plus 0.6 μM of PrimerBank primers (described above). The plates were incubated (iCycler; Bio-Rad Laboratories) at 50 °C for 30 min, followed by a 15-min cycle at 95 °C and 40 cycles of 10 s at 95 °C and 30 s at 55 °C. Samples were run in a 2% agarose gel.

Hypermutation analysis. GC or activated B cells were sorted using a MoFlo high-speed cell sorter (DakoCytometry). Genomic DNA was isolated, and the JH4 intron was amplified by nested PCR using KOD DNA polymerase (Novagen) and primers V1: AGGCTGACATCTGAGGAC and V2: TAGGTGTGGACATCTCCTCAC for 30 cycles at 55 °C. The second amplification step was performed with primers V3: CTCGACATCTGAGGAC-CTGTCG and V4: GCTGTACACAGGTTGGTCTCG for 30 cycles at 60 °C. PCR products were cloned using ZERO-blunt PCR cloning kit (Invitrogen) and sequenced using M13 universal primers. Sequence analysis was performed using SeqMan software (DNASTAR).

FACS analysis. Unless otherwise stated, all other antibodies were from BD Biosciences: B220-PerCP-Cy5.5, B220-APC-Cy7, B220-FlTC, IgM-APC (Jackson ImmunoResearch Laboratories), CD95-PE, CD95-PE-Cy7, GL7-FlTC, GL7-APC (APC conjugate was performed using Phycocyanin from Pronase), CD43-PE, CD95-biotin, CD138-PE, CD4-PE-Cy5, CD8-PE-Cy5, IgA-Biotin, IgG1-biotin, GR1-Alexa 647 and F4/80-Alexa 647 (eBioscience), IgD-Biotin, IgD-Alexa 647, Streptavidin-APC, Streptavidin-PE-Cy5, Streptavidin-PerCP-Cy5.5, Streptavidin-PE, NP-APC (provided by McHeyzer-Williams), and NP-PE (Biosearch Technologies). Apoptotic cells were gated out using To-Pro-3 (Invitrogen) or DAPI (Sigma-Aldrich). Flow cytometers used were FACS Calibur (Becton Dickinson) and Cyan (DakoCytometry) for acquisition and MoFlo (DakoCytometry) for sorting. FACS data was analyzed with FlowJo software.
Online supplemental material. Fig. S1 shows expression of AID mRNA by RT-PCR analysis in different tissues of wild-type, AID-GFP, and AID-Cre-YFP transgenic mice. It is available at http://www.jem.org/cgi/content/full/jem.20061982/DC1.

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