# Target Sequence Accessibility Limits Activation-Induced Cytidine Deaminase Activity in Primary Mediastinal B-Cell Lymphoma

Sergey W. Popov,[1] Gerhard Moldenhauer,[2] Beate Wotschke,[1] Silke Brüderlein,[1] Thomas F. Barth,[1] Karola Dorsch,[1] Olga Ritz,[1] Peter Möller,[1] and Frank Leithäuser[1]

[1]Department of Pathology, University of Ulm, Ulm, Germany and [2]Department of Molecular Immunology, German Cancer Research Center, Heidelberg, Germany

## Abstract

Activation-induced cytidine deaminase (AID) initiates somatic hypermutation (SHM) and class switch recombination (CSR) in activated B lymphocytes and is potentially implicated in genomic instability of B-cell malignancies. For unknown reasons, B-cell neoplasms often lack SHM and CSR in spite of high AID expression. Here, we show that primary mediastinal B-cell lymphoma (PMBL), an immunoglobulin (Ig)–negative lymphoma that possesses hypermutated, class-switched Ig genes, expresses high levels of AID with an intact primary structure but does not do CSR in 14 of 16 cases analyzed. Absence of CSR coincided with low Ig germ-line transcription, whereas high level germ-line transcription was observed only in those two cases with active CSR. Interleukin-4/CD40L costimulation induced CSR and a marked up-regulation of germ-line transcription in the PMBL-derived cell line MedB-1. In the PMBL cell line Karpas 1106P, CSR was not inducible and germ-line transcription remained low on stimulation. However, Karpas 1106P, but not MedB-1, had ongoing SHM of the Ig gene and BCL6. These genes were transcribed in Karpas 1106P, whereas transcription was undetectable or low in MedB-1 cells. Thus, accessibility of the target sequences seems to be a major limiting factor for AID-dependent somatic gene diversification in PMBL. [Cancer Res 2007;67(14):6555–64]

## Introduction

Primary mediastinal B-cell lymphoma (PMBL) is categorized as a subentity of diffuse large B-cell lymphoma (DLBCL) in the current WHO classification. The concept of PMBL as a distinct lymphoma type relies on its characteristic clinical, histomorphologic, immunologic, and molecular properties (1). The immunoglobulin (Ig) genes of PMBL are clonally rearranged and typically carry a high load of somatic hypermutation (SHM) with a distribution pattern that indicates selection of a functional B-cell receptor (2, 3). In combination with the finding of switched isotypes (3), this strongly suggests that PMBL originate from B cells that have been exposed to the germinal center reaction.

The maturation of a B-cell reaction is achieved by two distinct genetic alterations of the Ig gene. As a hallmark of the germinal center reaction, SHM hones the B-cell receptor for maximum binding affinity by introducing point mutations to the variable region of the Ig heavy and light chain gene. Class switch recombination (CSR) exchanges the Ig isotype via nonhomologous DNA recombination between the targeted switch regions, thereby determining the effector function of the B-cell response (4). For long, the molecular basis of SHM and CSR has remained elusive. Recently, however, it was shown that activation-induced cytidine deaminase (AID) plays a central role in SHM and CSR (5, 6). This finding has triggered intense research activity shedding some light on the initial molecular mechanisms of these two processes, although there is an ongoing debate on whether AID targets RNA or DNA. The DNA hypothesis, which has gained recently some acceptance, holds that AID directly deaminates cytosine residues in DNA (7). Because single-stranded, but not duplex, DNA is the substrate of AID, target sequences require transient denaturation by transcription (8). For CSR, this is achieved by germ-line transcription through the acceptor and donor switch region, flanking the sequence to be looped out (4). To facilitate SHM, AID must be phosphorylated and interact with replication protein A (9), whereas additional, as yet unidentified, cofactors are likely to complex with AID to initiate CSR (10). For this reason, it is obvious that the detection of AID gene products alone does not automatically proof AID to be active. Indeed, a dissociation of AID expression and activity is a well-recognized finding in B-cell neoplasms (11–13). This observation has been substantiated recently by an experimental approach showing that the induction of a mutator phenotype by AID overexpression *in vitro* critically depends on the targeted B-cell line (14).

Thus far, there has been only one report investigating AID expression in PMBL (15). This study, which included six PMBL cases, revealed AID mRNA levels in PMBL that were comparable with follicular lymphoma (FL) and to non-PMBL types of DLBCL. The presence of hypermutated and class switched Ig genes proves that AID has been active in PMBL (3). However, it is presently unclear whether AID continues to be active or shut down due to alterations of the molecule itself or a nonsupportive state of the lymphoma cells.

To address this question, we measured AID gene products in 16 primary PMBL cases and 2 PMBL-derived cell lines, confirming AID expression by mRNA quantification and on the protein level. We then searched for evidence of ongoing class switching as an indicator of AID activity. To elucidate the reason for the low rate of CSR despite the prevalent expression of a putatively functional AID molecule in PMBL, we determined the accessibility of the AID target sequences in the Ig switch regions and carried out *in vitro* experiments to assess whether physiologic stimuli could revert PMBL cells into a class switching mode. Finally, we examined ongoing SHM in PMBL cell lines and determined whether it correlated with target gene transcription.

## Materials and Methods

**Tissues and cell lines.** Sixteen cases of PMBL, 15 cases of other (non-PMBL) DLBCL subtypes, 11 cases of FL, and 5 reactive tonsils were included in this study. Lymphoma diagnosis was in accordance with the current WHO classification. All samples were drawn from our archive of snap-frozen tissues and pseudonymized to comply with the German law for correct usage of archival tissue for clinical research (Deutsches Ärzteblatt 2003; 100 A1632). Approval for these studies was obtained from the University of Ulm ethics board. One lymphoma sample was taken from a pulmonary recurrence of a patient who had received antitumor chemotherapy (PMBL case 16). A stable cell line generated previously from this tumor (MedB-1; ref. 16) and the PMBL cell line Karpas 1106P (17) were included in this study.

**Microdissection.** Seven-micrometer frozen tissue sections were mounted on membrane-covered glass slides, stained with hematoxylin, dried, and stored at $-70°C$. Microdissection and laser pressure catapulting was done as described (18) using a Robot Microbeam system (PALM Microlaser Technologies) equipped with an IX 50 microscope (Olympus). Several tissue areas comprising a minimum of $\sim 2 \times 10^3$ cells were catapulted into the cap of one PCR tube.

**mRNA isolation and reverse transcription.** Total RNA was extracted from microdissected tissue and from pelleted cells as described (18). RNA was annealed to 1 pmol hexamer (random) primer, denatured at $80°C$, and reverse transcribed with SuperScript reverse transcriptase (Invitrogen) as depicted elsewhere (18).

**Real-time reverse transcription-PCR.** mRNA expression was determined by real-time reverse transcription-PCR (RT-PCR) and relative quantification with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a reference gene in most essays. When exclusively cell lines were examined, *CD19* was used as a reference gene. A fresh aliquot of a cDNA preparation from a mixture of various human B-cell lines served as a calibrator in every PCR run. Amplification and quantification was carried out as described (18) on an iCycler iQ Real-time PCR instrument (Bio-Rad) with the iQ SYBR Green Supermix (Bio-Rad) and primer sets as shown in Supplementary Table S1.

**RT-PCR of the AID coding region.** We amplified 591 bp of the 594 bp AID coding region within a seminested PCR amplification protocol. Primer sequences are shown in Supplementary Table S1. AID-cod$_{right}$ plus AID-cod$_{left-ext}$ and AID-cod$_{right}$ plus AID-cod$_{left-int}$ were applied for the first and second PCR round, respectively, using 5 units/µL of the High Fideli Taq polymerase (USB Corp.).

**Amplification of the Ig heavy chain variable region, BCL6, PAX5, MYC, and RhoH/TTF.** Genomic DNA was extracted from cell lines as described (3). The rearranged MedB-1 Ig heavy chain gene, which included the variable (V), diversity (D), and joining (J) segment, was amplified with a VH4 leader primer (Supplementary Table S1) and a mixture of JH primers (19). The VDJ sequence of Karpas 1106P was amplified using published primer sets (19). The SHM-targeted 5′ regions (20) of BCL6, RhoH/TTF, MYC, and PAX5, respectively, were amplified with the High Fideli Taq polymerase using primer pairs as shown in Supplementary Table S1. The sizes of the respective amplicons were 713 bp (BCL6), 768 bp (RhoH/TTF), 708 bp (MYC), and 694 bp (PAX5).

**Estimation of the Taq error.** To estimate the Taq error, 300 ng DNA from the nonlymphoid cell line ENSA and 0.3 pg pGEM-T Easy vector carrying the rearranged VDJ sequence of Karpas 1106P as an insert were mixed and submitted to a single-round PCR using $V_H2$ and a $3′_{JH}$ primer combination as described (19). PCR conditions were identical to those that served to amplify VDJ rearrangements and BCL6 from genomic DNA.

**Cloning and sequencing.** PCR bands were cut out from the agarose gel. Subsequent gel purification, cloning in a pGEM-T Easy vector, and plasmid DNA preparation were as described (3). Sequencing of plasmid DNA was accomplished by MWG Biotech.

**Sequence analysis.** Sequences of VDJ rearrangements were aligned to published germ-line sequences of the human Ig heavy chain gene using the IMGT/V-QUEST software (21).[3] Multiple alignment of VDJ and BCL6 sequences was achieved with the ClustalW software (22).[4]

**Circle transcript PCR.** For detecting circle transcripts (CT) in the small amount of tissue obtainable by microdissection, a sensitive nested RT-PCR assay was carried out using primers as shown in Supplementary Table S1. Oligonucleotide sequences of IεF1, IεF2, and CγR were as published by Takhar et al. (23). To amplify Iα-Cγ CT, Iα plus Cγ (first round) and Iα$_{int}$ plus Cγ$_{int}$ (second round) were used. To amplify Iε-Cγ CT, IεF1 plus CγR (first round) and IεF2 plus CγR (second round) were used. The specificity of representative PCR products was verified by sequencing.

**Amplification of mature Ig transcripts.** Mature VDJ-Cε IgE and VDJ-Cγ IgG transcripts were amplified by a nested RT-PCR and by a conventional one-round RT-PCR, respectively. The primer sequences are given in Supplementary Table S1. The first PCR round was carried out with FR2a (24) and IgE$_{ext}$ or Ig G$_{ext}$, respectively. For the second round, FR2a was combined with IgE$_{int}$.

**Transfection of MedB-1 cells with human AID.** The pCFG5-AID expression vector, generated by cloning the full-length AID coding sequence into a pCFG5 IEGZ vector (25), was transiently transfected into MedB-1 cells using the Nucleofector kit V (Amaxa GmbH) with the program U-01 on the Nucleofector device (Amaxa GmbH). Per transfection, we applied $2 \times 10^6$ cells and 2 µg expression vector.

**Protein kinase A induction and extraction of nuclear protein.** MedB-1 cells transfected with human AID or an empty vector, respectively, and Karpas 1106P cells were treated with forskolin (50 µmol/L; Sigma-Aldrich) and 3-isobutyl-1-methylxantine (100 µmol/L; Sigma-Aldrich) to induce protein kinase A (PKA; ref. 26). After 1 h, cells were harvested, washed twice in ice-cold PBS, incubated for 15 min in three packed cell volumes of buffer A (27), and aspirated 20 times with a 26-gauge needle. Nuclei were pelleted, washed twice with buffer A, and incubated for 1 h in two packed cell volumes of radioimmunoprecipitation assay buffer (28). Next, nuclear membranes were removed by centrifugation, and the protein concentration in the supernatant was quantified as described (29). Equal amounts of nuclear protein were used for Western blotting.

**Western blotting.** Cell lysates or nuclear protein extracts, respectively, were separated by 12% SDS-PAGE under reducing conditions and transferred onto an Immobilon-P membrane (Millipore) in a semidry blotting system. Membranes were blocked with 5% milk powder in PBS-Tween and incubated with the recently generated mouse antihuman AID monoclonal antibody (mAb) clone C12.38 (30) or CD19 (clone CD19.13; DAKO), respectively. After washing, bound mAb was labeled with goat anti-mouse IgG/Fc conjugated with alkaline phosphatase (Dianova) and visualized by nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Roche) substrate reaction. A mouse β-actin mAb served as a control.

**Immunomorphology.** Immunostaining with the anti-AID mAb C12.38 was done as described (30) on cryosections or cytospin preparations fixed in methanol/acetone (ratio of 1:1) at $-20°C$ for 5 min. Sections were incubated with C12.38 diluted 1:10. Bound mAb was labeled using the envision antimouse system (DAKO) and visualized by the substrate 3-amino-9-ethyl-carbazole. Slides were counterstained in hematoxylin. Sections were examined under a Zeiss Axiophot microscope using a 40× objective lens (numerical aperture, 0.75). Picture acquisition was done with a KY-F75U digital camera (JVC) and the DISKUS software (version 4.5, C.H. Hilgers Technisches Büro). Images were processed using the Photoshop software (version 5.5, Adobe).

**Stimulation of PMBL cells.** Cells were incubated for 5 days in the presence of 40 ng/mL interleukin-4 (IL-4; Sigma-Aldrich) and/or $4 \times 10^4$/mL murine fibroblasts (L-cells) stably transfected with human CD40L (American Type Culture Collection no. CRL-12095 hCD40L, obtained from DNAX Research Institute of Molecular and Cellular Biology). Subsequently, RNA was extracted from pelleted cells as depicted above.

---

[3] http://imgt.cines.fr/

[4] http://www.ebi.ac.uk/clustalw/

## Results

**AID is expressed in PMBL.** To determine whether and to what extent AID is produced in PMBL, we first measured the level of AID mRNA expression in 16 PMBL lymphoma cases along with the PMBL cell lines MedB-1 and Karpas 1106P by quantitative real-time PCR. Tissue was obtained by microdissection to minimize contamination by nonneoplastic cells. To relate our measurements to other lymphoma types with known AID expression (12, 31) and to the AID production found in a normal germinal center reaction, we additionally analyzed 15 cases of DLBCL, 11 cases of FL, and microdissected germinal centers of 5 reactive tonsils. AID mRNA was detected in 15 (94%) PMBL cases and in the 2 PMBL cell lines, covering a wide range of expression levels (Fig. 1; Supplementary Table S2). The median AID expression in PMBL was higher than in DLBCL and FL but lower than in germinal center cells. Our quantitative PCR data are in line with a recent study reporting AID message in PMBL (15). Yet, it was unclear whether mRNA expression reflected AID production on the protein level. To address this question, we carried out immunohistochemistry on frozen lymphoma tissue. Applying a semiquantitative score, we found that AID immunostaining largely correlated with the mRNA
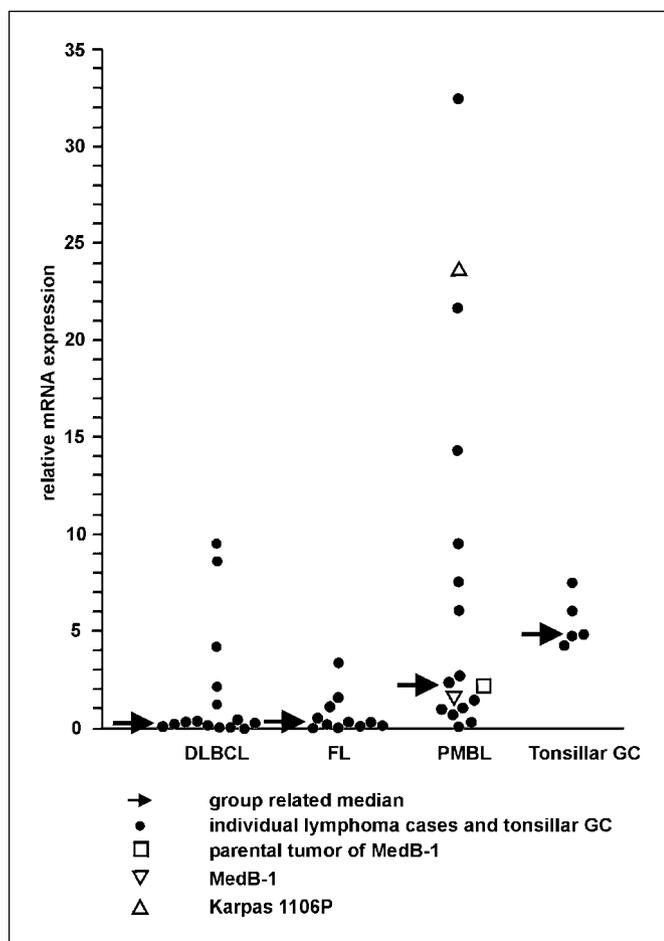


**Figure 1.** AID mRNA expression in PMBL. AID mRNA expression was determined by quantitative RT-PCR on microdissected tissue and cell pellets, respectively. *Points,* mean of three measurements. *Arrows,* median of each group. In the PMBL group, the cell lines Karpas 1106P and MedB-1 and the parental tumor of MedB-1 have been assigned to specific points. The relative AID expression was determined using the housekeeping gene *GAPDH* and is given in arbitrary units. *GC,* germinal center.

measurements (Fig. 2A; Supplementary Table S2). Again, the expression intensity varied over a broad range between the individual lymphoma samples. AID expression of the PMBL cell lines MedB-1 and Karpas 1106P was assessed by Western blotting, yielding a weak signal in MedB-1 and a strong band in Karpas 1106P (Fig. 2B), in accordance with the mRNA expression data. An analogous result was obtained by immunostaining cytospin preparations of both cell lines (Fig. 2C).

**AID is potentially functional in PMBL.** We then asked whether genomic aberrations and/or alternative splicing might impede AID function in PMBL. To this end, we sequenced AID transcripts generated by RT-PCR with oligonucleotide primers spanning 591 bp of the 594 bp AID coding region. From 15 (94%) PMBL samples and both cell lines, specific PCR products were amplified (Fig. 3A). Sequence analysis of dominant bands revealed full-length AID transcripts in all 15 cases as well as MedB-1 and Karpas 1106P. No replacement mutations were observed. In addition to a preserved primary structure, phosphorylation of the AID protein catalyzed by PKA is crucial for AID activity (32). We therefore measured the expression of PKA α-catalytic subunit mRNA by quantitative RT-PCR. In all PMBL samples and both PMBL cell lines, PKA mRNA levels were comparable with or higher than in the tonsillar germinal center (Fig. 3B). Because nuclear translocation of AID has been suggested to be phosphorylation dependent (32) and may thus be indicative of active AID, we determined whether AID protein localized to the nucleus of MedB-1 and Karpas 1106P cells. Western blotting of nuclear protein yielded a specific band in Karpas 1106P cells (Fig. 3C) but no signal in MedB-1 cells (data not shown). Because only trace amounts of AID are translocated to the nucleus (33), the failure to detect nuclear AID in MedB-1 cells might be explained by the moderate AID expression level in this cell line. In fact, when overexpressed by transient transfection, nuclear AID could be detected in MedB-1 cells but not in mock-transfected controls (Fig. 3C). In neither cell line did PKA induction increase the amount of nuclear AID. All samples were negative when probed with a CD19 mAb (data not shown), showing the purity of the nuclear protein preparation. Taken together, these results indicate that AID is potentially functional in the majority of PMBL.

**Class switching is an infrequent event in PMBL.** To further elucidate whether AID is active in PMBL, we addressed CSR as one AID-dependent process in human B cells. During class switching, the DNA located in between two activated switch regions is excised and converted into circular DNA (switch circle). Switch circles are transcribed, but both circular DNA and circle transcripts are unstable and rapidly become degraded. Hence, circle transcripts provide a suitable marker for recent CSR (34). We conducted a sensitive nested PCR to search for circle transcripts indicative of secondary class switching from IgG to IgA (Iα-Cγ) and IgG to IgE (Iε-Cγ). We did not analyze switching from IgM because it has been shown that the Ig genes in PMBL have already undergone CSR (3). As shown in Fig. 4A, no Cγ→Cα specific circle transcripts were found in the PMBL lymphoma samples and cell lines. In contrast, a strong signal was obtained from microdissected germinal center tissue that served as a positive control. From 2 (13%) cases, Cγ→Cε specific circle transcripts could be amplified, whereas the remaining 14 (87%) cases and both PMBL cell lines were negative. Thus, secondary CSR occurs only rarely in PMBL and targets Cε, but not Cα.

**Switch region transcription correlates with Ig isotype recombination in PMBL.** Because there were no clues for functional incompetence of AID in PMBL, we hypothesized that the status of the target gene accounts for the lack of switch
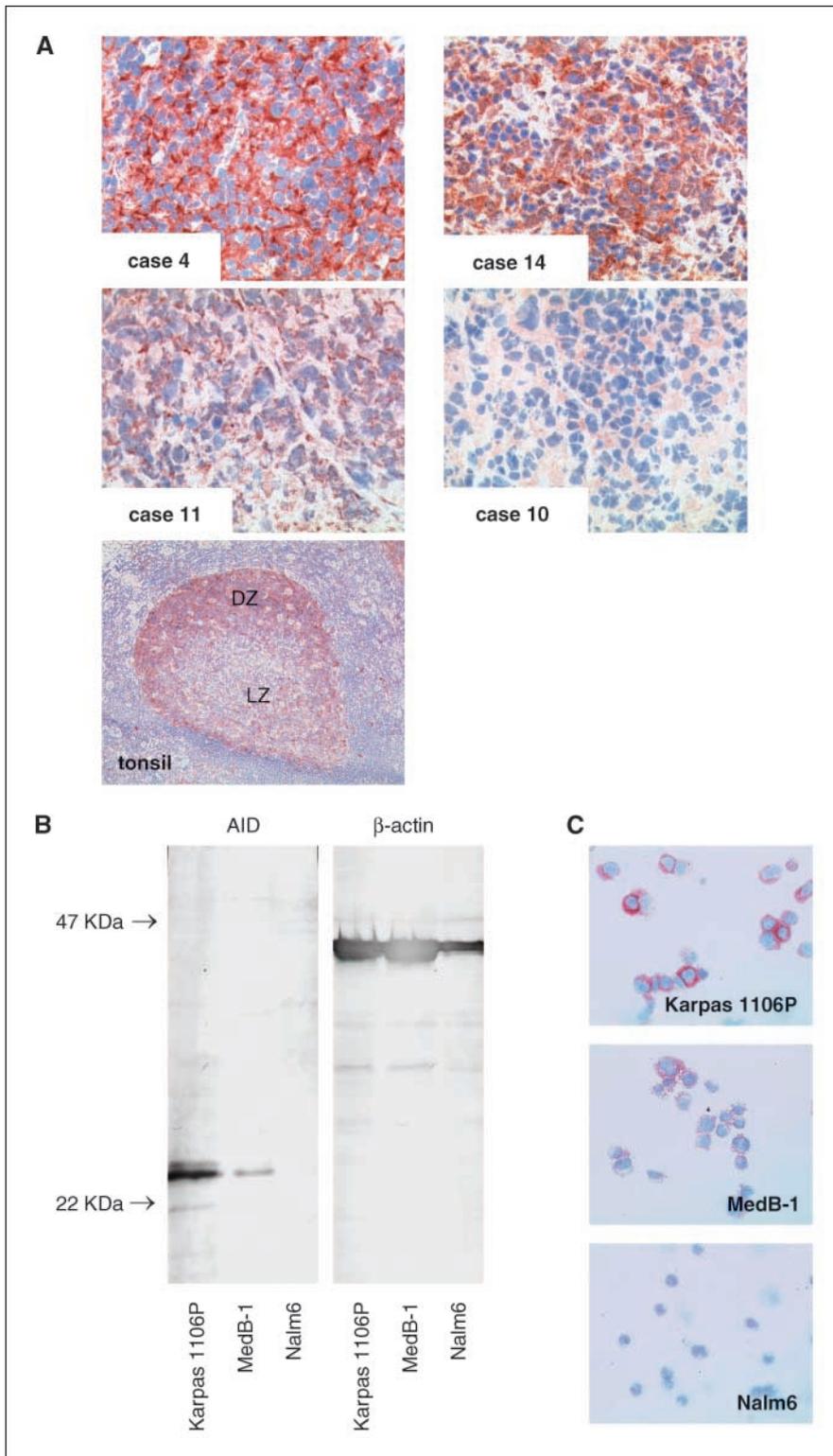
**Figure 2.** AID protein expression in PMBL. *A,* AID protein expression as determined by immunohistochemistry using the AID-specific mAb clone C12.38. Four different staining intensities observed in PMBL (see Supplementary Table S2) are exemplified by case 4 (strongly positive), case 14 (positive), case 11 (weakly positive), and case 10 (negative). A tonsillar germinal center is shown for comparison. Strong AID expression is present in the dark zone (*DZ*), but not in the light zone (*LZ*), of the germinal center. *B,* Western blotting of cell lysates with anti-AID (*left*) revealed an intense, AID-specific band of ~24 kDa in Karpas 1106P and a weak signal in MedB-1, whereas no specific band was seen with the immature B-cell line Nalm6, which served as a negative control. β-Actin probing was used as a loading control (*right*). *C,* an analogous expression pattern was found in AID immunostainings of cytospin preparations. Note that AID staining in (*A* and *C*) is predominantly cytoplasmic. Original magnification, ×40 (*A* and *C*).

recombination. According to the prevailing model of CSR (35), simultaneous germ-line transcription through the donor and acceptor switch region of the Ig gene is an essential requirement for CSR. After splicing, the resulting germ-line RNAs comprise the sterile "I" exon and the adjacent constant (C) region exons. The presence of germ-line transcripts indicates accessibility of the Ig gene locus for downstream events of class switching (4). We set up

a real-time PCR assay to quantify the donor and acceptor germ-line transcription preceding secondary CSR from IgG to IgA and IgE, respectively. As shown in Fig. 4*B*, both Iμ-Cγ and Iε-Cε germ-line transcripts were highly expressed in lymphoma no. 2 and 5 (i.e., precisely those two cases that displayed evidence of active class switching from IgG to IgE). Although isolated germ-line RNA production of either donor or acceptor site was occasionally

detected in other PMBL samples, there was no simultaneous high level donor and acceptor germ-line transcription in any additional case and in none of the PMBL cell lines. We conclude that the failure to establish switch region accessibility contributes to the lack of CSR in the majority of PMBL.

**Cγ→Cε class switching is inducible in the PMBL cell line MedB-1.** T-cell–derived signals play a pivotal role in controlling germ-line transcription and targeting CSR (34). Therefore, we investigated whether IL-4 and CD40L (i.e., potent inducers of class switching to IgE in normal B-cells) could trigger CSR in the PMBL cell lines MedB-1 and Karpas 1106P. We incubated PMBL cells for 5 days in the presence of IL-4 and/or CD40L, the latter provided by murine fibroblasts transfected with human CD40L. Figure 5*A* shows that although neither IL-4 nor CD40L stimulation alone was effective, the combination of both signals induced Cγ→Cε CSR in MedB-1 cells, as indicated by the presence of Iε-Cγ circle transcripts and mature Ig VDJ-Cε transcripts. No Cγ→Cα switching was observed with any stimulus (data not shown). In contrast to MedB-1, Karpas 1106P cells were refractory to stimulation (data not shown). Along with the induction of class switching, Iμ-Cγ and Iε-Cε germ-line transcription through the donor and acceptor switch region, respectively, was strongly up-regulated in MedB-1 cells exposed to IL-4 and CD40L but not with either stimulus delivered alone (Fig. 5*B*). An analogous result was obtained for AID

expression, which was markedly increased by the combined treatment with IL-4 and CD40L, whereas IL-4 or CD40L provided alone showed no substantial effect (Fig. 5*B*). In Karpas 1106P cells, Iμ-Cγ and Iε-Cε germ-line transcription remained low after stimulation, and AID expression was essentially unaltered (Fig. 5*B*). To rule out that AID induction by itself is sufficient to elicit CSR in MedB-1, cells were transiently transfected with the full-length coding sequence of human AID. Although AID mRNA was strongly induced in these cells, no Iε-Cγ circle transcripts and thus no evidence of Cγ→Cε class switching was detected 5 days after transfection (Fig. 5*C*). Collectively, these findings suggest that in PMBL, deficient T-cell–dependent signaling contributes to the low rate of secondary CSR.

**Ongoing SHM is absent from MedB-1 but present at a low level in Karpas 1106P.** In an earlier study, no intraclonal heterogeneity of the Ig heavy chain gene was detected in PMBL tissue (3). To determine SHM activity in PMBL cell lines, we sequenced the Ig heavy chain gene rearrangement and the BCL6 5′ region of MedB-1 and Karpas 1106P and compared cell cultures at day 0 and after *in vitro* propagation for at least 180 days, respectively (Fig. 6*A*). BCL6 was chosen because it is the most intensely hypermutated non-Ig locus (20). In MedB-1, the Ig heavy chain gene was highly mutated, which agrees with published data (3). BCL6 displayed one mutation (a single bp deletion in the first



**Figure 3.** Amplification of the AID coding region cDNA, PKA expression, and nuclear AID detection. *A,* 591 bp of the 594 bp AID coding region were amplified by a seminested RT-PCR. Reaction products were separated on a 2% agarose gel. The housekeeping gene *GAPDH* was amplified in a single-round RT-PCR. Except for case 10, specific AID PCR products were generated from all samples and both cell lines. The dominant upper bands were cloned and submitted to sequence analysis. The sequence of one representative PCR product has been submitted to the EMBL/Genbank database under accession no. DQ431660. *B,* mRNA expression of the PKA α-catalytic subunit was determined by quantitative RT-PCR in triplicate and normalized to GAPDH. *Columns,* mean (arbitrary units); *bars,* SD. *C,* MedB-1 cells transfected with full-length AID cDNA (*Me/AID*) or with an empty vector (*Me/mock*) and Karpas 1106P cells were treated for 1 h with forskolin and 3-isobutyl-1-methylxantine to induce PKA (+) or kept in medium alone (−). Western blotting of nuclear protein extracts was done with anti-AID mAb. *Me,* MedB-1; *Ka,* Karpas 1106P; *M,* molecular size marker; *I* to *V,* microdissected tonsillar germinal centers.
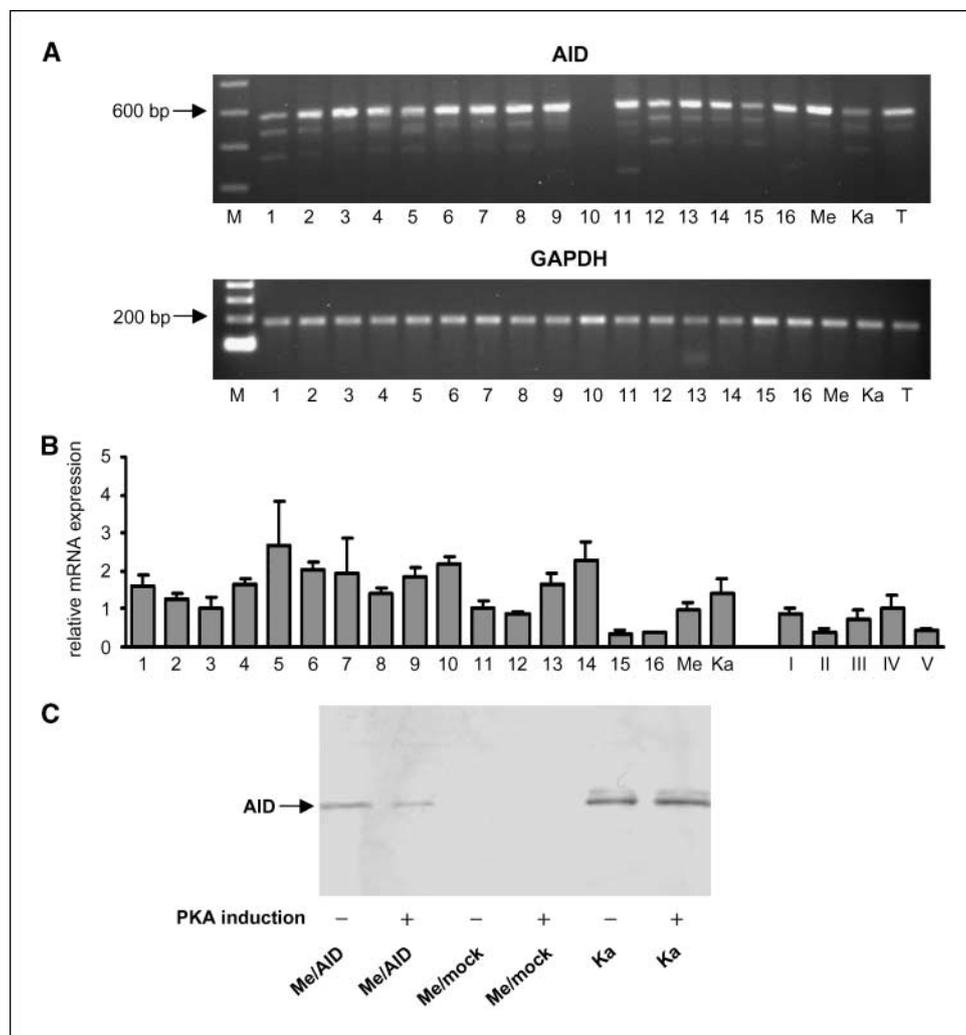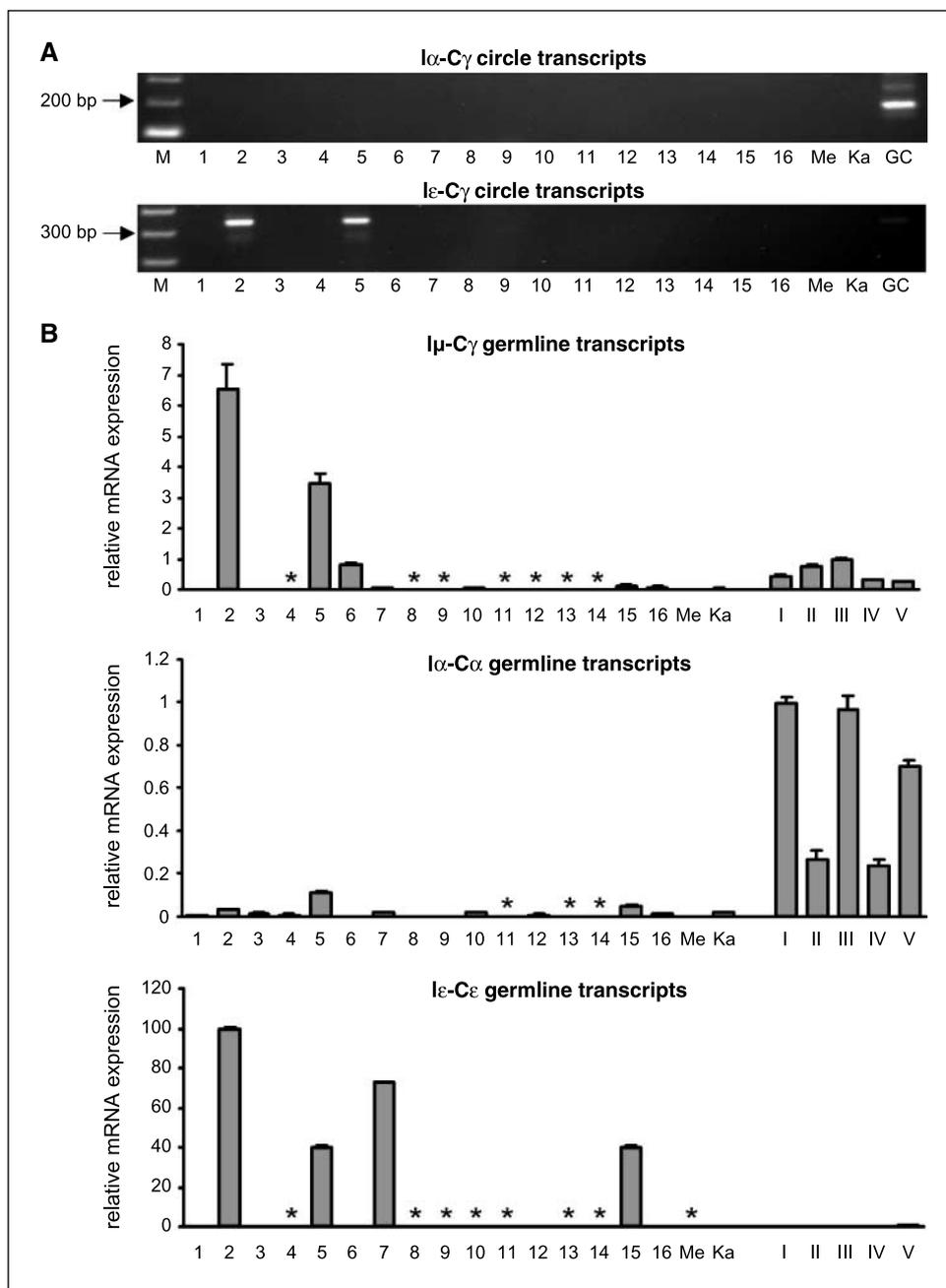
**Figure 4.** Secondary class switching and germ-line transcription in PMBL. *A,* circle transcripts indicative of secondary class switching from IgG to IgA (Iα-Cγ) and IgG to IgE (Iε-Cγ), respectively, were amplified by a nested RT-PCR. No switching from IgG to IgA was detected in PMBL. In cases 2 and 5, switching from IgG to IgE was observed, and all other lymphoma samples and both cell lines were negative. The specificity of a representative Iα-Cγ (generated from a tonsil) and a Iε-Cγ circle transcript was confirmed by sequencing [EMBL/Genbank accession nos. DQ083946 (30) and DQ431663, respectively]. *B,* transcription through switch regions was measured by quantitative RT-PCR. Primers were designed to amplify Iμ-Cγ donor site germ-line transcripts (*top*) as well as Iα-Cα (*center*) and Iε-Cε (*bottom*) acceptor site germ-line transcripts. The specificity of representative PCR products was confirmed by sequencing (EMBL/Genbank accession nos. DQ431661, DQ431662, and DQ431664, respectively). The relative expression was determined in triplicate using *GAPDH* as a reference gene. *Columns,* mean (arbitrary units); *bars,* SD. *, no detectable expression.

intron at position 235). Karpas 1106P had rearranged a VH2 Ig heavy chain gene element (VH2-5), which was substantially mutated (mutational rate, 11.1%). A biallelic mutational pattern was seen at the BCL6 locus of Karpas 1106P. On both alleles, *in vitro* propagation for 180 days led to one additional mutation. All BCL6 mutations were single nucleotide substitutions located in the first intron (G→C, position 201; A→T, position 221; G→A, position 371; and T→C, position 449 on allele A and A→G, position 219; A→G, position 227; and A→G, position 366 on allele B). Mutations were biased for transitions over transversions (5 transitions versus 2 transversions), consistent with an AID-dependent mechanism. To assess intraclonal diversity as an additional indicator of ongoing hypermutation, multiple PCR clones were sequenced (Fig. 6*B*). In MedB-1 cells, no intraclonal diversity of the Ig heavy chain gene

was found. This diverges from an earlier study, which yielded intraclonal diversity at two positions in this cell line (3). Stochastic effects might account for this difference, as a low level of ongoing SHM affecting small subpopulations could escape detection. Intraclonal diversity of the *BCL6* gene did not substantially exceed the Taq error in MedB-1 (Fig. 6*B*). In Karpas 1106P, a low level of intraclonal diversity was observed in the Ig heavy chain gene and BCL6 (Fig. 6*B*). Thus, ongoing SHM is absent from MedB-1 and detectable in Karpas 1106P, albeit at a low level.

**Ongoing SHM correlates with transcription in MedB-1 and Karpas 1106P cells.** The mRNA expression of SHM targets was assessed by RT-PCR. A weak Ig VDJ-Cγ band was amplified after one PCR round from Karpas 1106P, but not from MedB-1 (Fig. 6*C*). BCL6 transcription was ~20-fold higher in Karpas 1106P than in

MedB-1 (Fig. 6*C*). Therefore, the transcriptional rate correlates with SHM activity in MedB-1 and Karpas 1106P.

**Aberrant SHM im MedB-1 and Karpas 1106P cells.** PAX5, RhoH/TTF, and MYC are aberrantly targeted by SHM in some DLBCL (20). Sequencing of the 5′ regions of these loci revealed a single bp substitution in the first intron of PAX5 in MedB-1 cells (T→C at posi-

tion 173), a single bp substitution in the first intron of RhoH/TTF in Karpas 1106P (A→G at position 148), and two single bp substitutions in the first intron of MYC in Karpas 1106P cells (C→A at position 159 and C→A at position 214; data not shown). All sequences have been submitted to the European Molecular Biology Laboratory (EMBL)/Genbank database (accession nos. EF453361–6).

**Figure 5.** *In vitro* stimulation of PMBL cell lines. MedB-1 cells and Karpas 1106P cells were stimulated for 5 d in the presence of IL-4, murine fibroblasts transfected with human CD40L, IL-4 in combination with CD40L, or medium alone. *A,* a nested RT-PCR amplified Iε-Cγ circle transcripts and mature VDJ-Cε transcripts from MedB-1 cells. The specificity of the VDJ-Cε PCR product was confirmed by sequencing (EMBL/Genbank accession no. DQ680069). *B,* in MedB-1 cells (*left*) and Karpas 1106P cells (*right*), the expression of Iμ-Cγ donor site germ-line transcripts (*top*), Iε-Cε acceptor site germ-line transcripts (*center*), and AID mRNA (*bottom*) was measured by quantitative RT-PCR. *C,* MedB-1 cells were transiently transfected with the full-length coding sequence of human AID (*MedB-1/AID*). Five days after transfection, AID mRNA expression was confirmed by quantitative RT-PCR (*left*), and Iε-Cγ circle transcripts were amplified by a nested RT-PCR (*right*). The relative expression in (*B* and *C*) was determined in triplicate using *CD19* as a reference gene. *Columns,* mean; *bars,* SD. The expression level in microdissected tonsillar germinal centers served as a reference and was set as 1. *, no detectable expression.
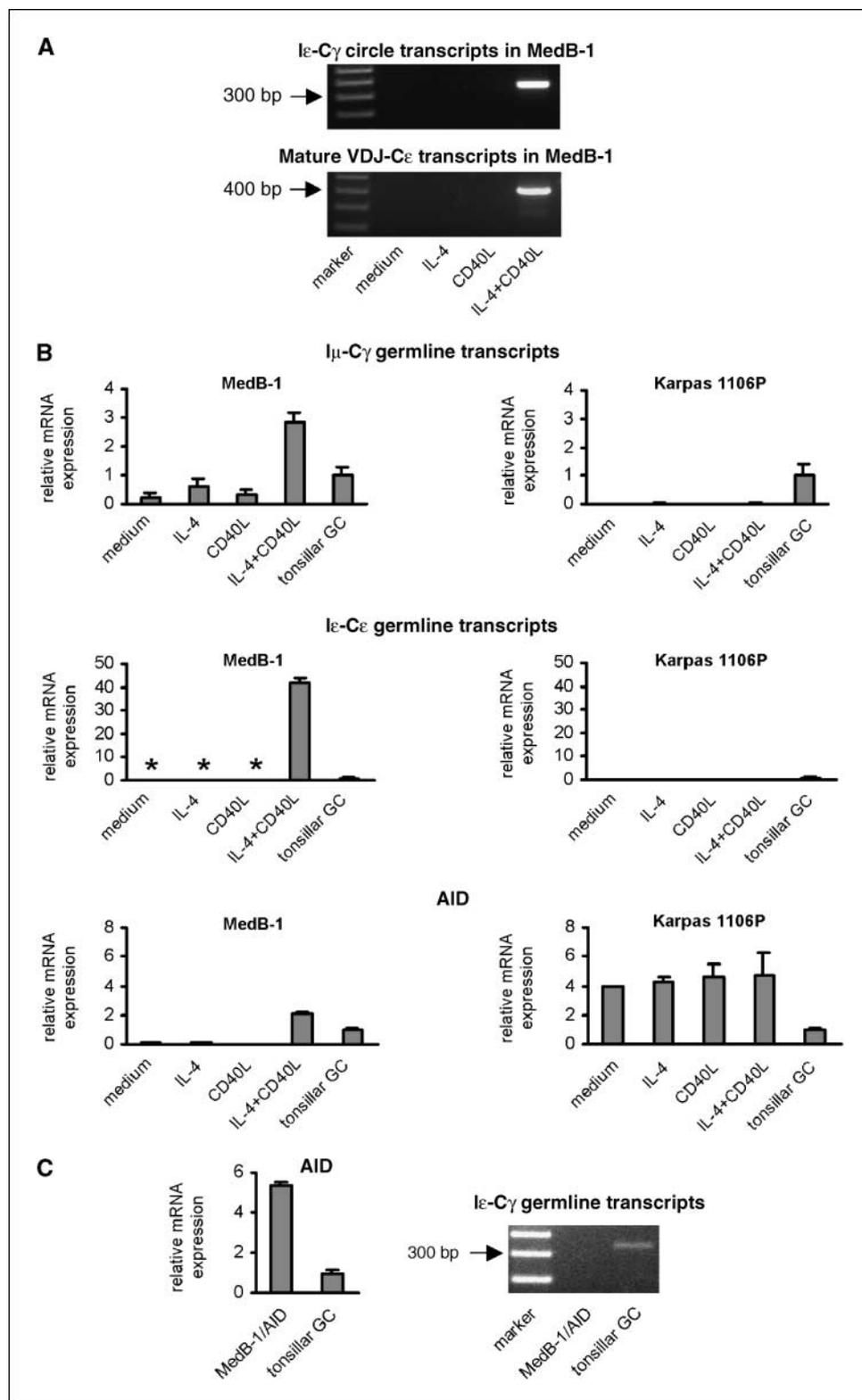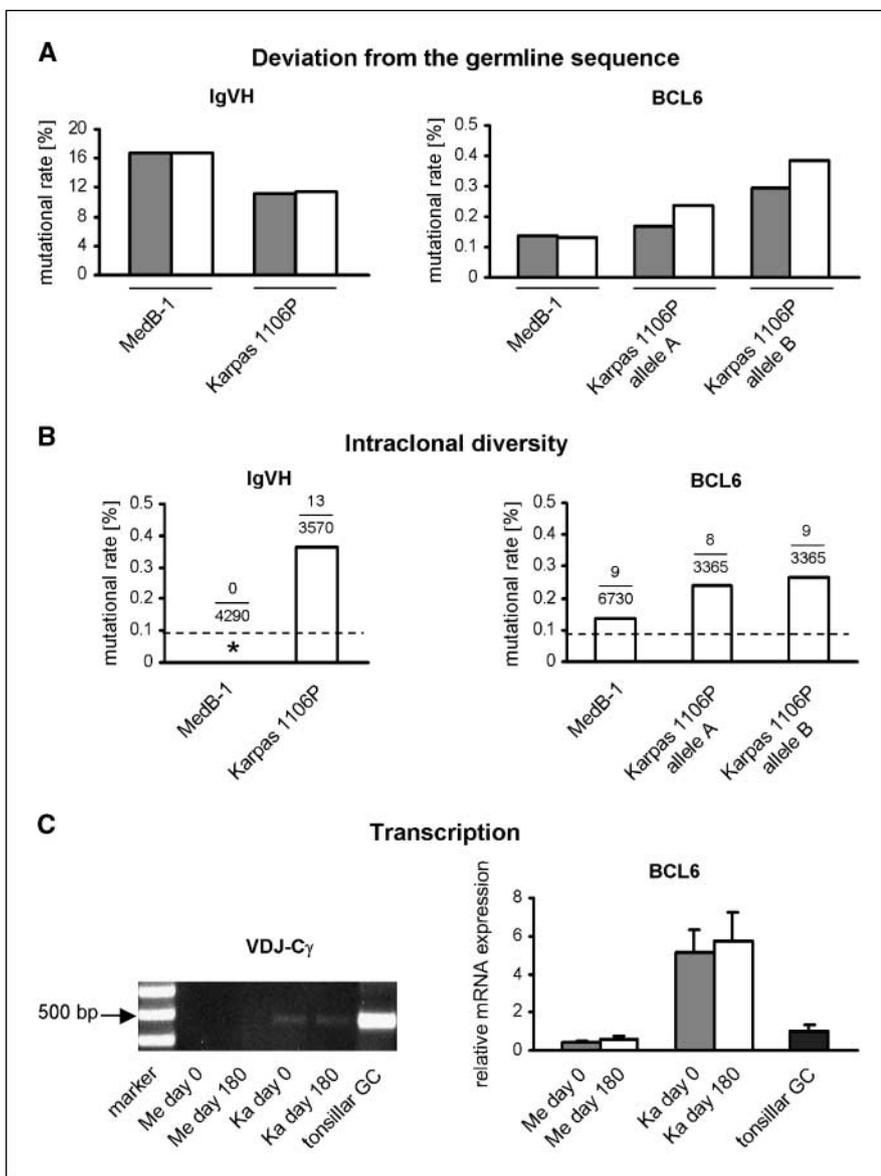
**Figure 6.** SHM and transcription of Ig gene and BCL6 in MedB-1 and Karpas 1106P. *A,* the Ig VDJ region and the BCL6 5′ region of MedB-1 and Karpas 1106P at day 0 (*gray columns*) and after *in vitro* propagation for at least 180 days (*white columns*) were amplified by PCR. *A,* deviation from the germ-line sequence. Note the different scale of the Y-axis in the left and right diagram. Sequences are available at EMBL/Genbank under the accession nos. EF192397-406. *B,* several PCR clones were sequenced to determine ongoing SHM. The ratio between the number of mutations and the total number of nucleotides sequenced is shown on top of each column. *Dashed lines,* Taq error (3/3222). *,* 0. *C,* mature Ig VDJ-Cγ transcripts were amplified by a conventional single-round RT-PCR from MedB-1 and Karpas 1106P cells at day 0 and after *in vitro* propagation for at least 180 d and from microdissected tonsillar germinal centers. BCL6 mRNA expression was measured in triplicate by quantitative RT-PCR using *CD19* as a reference gene. *Columns,* mean relative expression (arbitrary units); *bars,* SD.

## Discussion

We show constitutive AID expression in PMBL. Based on a series of 16 lymphoma samples and two PMBL-derived cell lines, our data are in accordance with a previous study that analyzed six PMBL cases by quantitative RT-PCR (15). We additionally show that AID is not only present at the transcriptional level but is also translated to protein, as determined by immunohistology and Western blotting. According to recent reports, AID expression is restricted to germinal center-associated B-cell lymphomas (12, 13), such as Burkitt lymphoma (12, 36), FL (12), some types of DLBCL (11), and nodular lymphocyte predominant Hodgkin's lymphoma (36, 37). The constitutive AID expression in PMBL may therefore indicate that this lymphoma type originates from a germinal center B cell. However, one should consider that class switching (38) and even SHM (39) are not strictly confined to the anatomic structure of the germinal center. Correspondingly, AID-expressing B lymphocytes are detected at extrafollicular sites of the lymph node and tonsil (30, 40) as well as in the thymic medulla (30). The occurrence of an AID-positive B-cell neoplasm with a nongerminal center origin is exemplified by hairy cell leukemia (41), an AID-expressing malignancy with an immunophenotype closely related to memory B cells (42). In line with this, the detection of AID expression in PMBL does not contradict the widely recognized hypothesis that this tumor derives from B lymphocytes of the medullary thymus (1).

We found circle transcripts in only 2 of 16 (13%) cases and in none of the 2 PMBL cell lines, indicating that ongoing CSR is an exceptional event in PMBL. Therefore, a dissociation between AID expression and activity observed in some B-cell neoplasms (11–13) also prevails in PMBL. Although we cannot formally rule out a defective state of the AID molecule itself, we consider this as improbable because AID transcripts exhibited no deviation from the wild-type sequence, PKA was highly expressed in PMBL, and the machinery of AID translocation to the nucleus was intact in both PMBL cell lines, suggesting AID to be in a phosphorylated (i.e., functional) mode in this lymphoma type. Instead, accessibility of the switch region AID target rather than the availability of AID is likely to be the major limiting factor for CSR in most cases of

PMBL. This notion is based on the observation that a simultaneous germ-line transcription of both donor and acceptor switch region was restricted to exactly those two cases that also exhibited CSR. In addition, the induction of germ-line transcription by IL-4 and CD40L triggered CSR in the constitutively nonclass switching MedB-1 cell line. This was not simply attributable to AID up-regulation, as shown by the failure of ectopic AID overexpression to induce CSR in MedB-1 cells. Because IL-4/CD40L treatment did not increase Ig germ-line transcription in Karpas 1106P cells, target sequence inaccessibility could also explain why CSR was uninducible in this cell line. Ongoing SHM of the Ig heavy chain gene and BCL6 shows that AID is constitutively active in Karpas 1106P cells, whereas the absence of SHM in MedB-1 cells could be attributed to a low transcription level of these genes. In fact, impaired accessibility of AID target sequences due to insufficient transcription may not only explain the low frequency of CSR in PMBL but also account for the lack of ongoing SHM in this predominantly Ig-negative lymphoma type (1).

MedB-1 cells strongly responded to the combined treatment with IL-4 and CD40L but not to either stimulus delivered alone. In view of the constitutively activated mode of the IL-4–dependent signaling pathway in MedB-1 cells (43), the additional requirement for IL-4 receptor engagement is unexpected. Possibly, the level of signal transducer and activator of transcription 6 (STAT6) phosphorylation ascribable to endogenous factors, such as SOCS-1 mutations, is insufficient to promote switch region transcription, necessitating further enhancement by IL-4 receptor-mediated STAT6 activation. Stimulation by exogenous IL-4 and CD40L may also contribute to Ig germ-line transcription in those two cases that were doing Cγ→Cε class switching. A likely source of IL-4 and CD40L *in vivo* is CD4$^+$ T lymphocytes of the Th2 effector type infiltrating the lymphoma tissue. A potential role of T-cell–derived IL-4 has been discussed in the context of DLBCL (44), which is frequently associated with an overrepresentation of IL-4–producing Th2-polarized T lymphocytes in the peripheral blood (45). On the other hand, tumor-infiltrating CD4$^+$ T cells may be too few or display an inappropriate phenotype in most PMBL that do not exhibit CSR activity. In a subset of PMBL, exogenous T-cell–derived stimuli may not be sufficient to drive CSR, as suggested by the refractory state of Karpas 1106P cells to IL-4 and/or CD40L treatment.

CSR and SHM require the activity of several proteins acting downstream of AID-initiated deamination (35). The deficiency of some of these factors may lead to a severe impairment of CSR. We cannot rule out the possibility that defects of such proteins may additionally contribute to the low rate of CSR in PMBL. If this was the case, IL-4/CD40L stimulation would not only enhance germ-line transcription but also up-regulate as yet unidentified proteins controlling CSR. With the exception of nuclear uracil-DNA glycosylase being specifically expressed in activated B cells (46), there is, however, little data on the inducibility of CSR-associated cofactors by B-cell stimulation. Another aspect that needs to be considered is the recent finding that AID is rendered functionally inactive when overexpressed for a prolonged time range (47). This mechanism might affect the ability of constitutively AID-expressing lymphoma cells to do CSR.

In some B-cell lymphomas, including PMBL (15), AID activity is not strictly confined to the Ig locus but also targets particular non-Ig genes, such as the proto-oncogenes BCL6, RhoH/TTF, PAX5, and MYC (20, 48). Moreover, ectopic expression of AID in fibroblasts introduces mutations in an artificial GFP substrate (49), and a reporter construct randomly inserted in the genome is mutated in the presence of AID at sites unrelated to the Ig loci (50). Based on these findings, it has been suggested that AID may confer a general mutator phenotype (50), which could lead to genomic instability of the affected neoplasm. The high AID expression may render PMBL susceptible to a sustained accumulation of genetic aberrations in accessible target sequences, with possible implications on the biological behavior and the prognosis of this lymphoma type.

## Acknowledgments

## References

1. Barth TF, Leithäuser F, Joos S, Bentz M, Möller P. Mediastinal (thymic) large B-cell lymphoma: where do we stand? Lancet Oncol 2002;3:229–34.
2. Küppers R, Rajewsky K, Hansmann ML. Diffuse large cell lymphomas are derived from mature B cells carrying V region genes with a high load of somatic mutation and evidence of selection for antibody expression. Eur J Immunol 1997;27:1398–405.
3. Leithäuser F, Bäuerle M, Huynh MQ, Möller P. Isotype-switched immunoglobulin genes with a high load of somatic hypermutation and lack of ongoing mutational activity are prevalent in mediastinal B-cell lymphoma. Blood 2001;98:2762–70.
4. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 2002;20:165–96.
5. Muramatsu M, Sankaranand VS, Anant S, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem 1999;274:18470–6.
6. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 2000;102:553–63.
7. Petersen-Mahrt SK, Harris RS, Neuberger MS. AID mutates *E. coli* suggesting a DNA deamination mechanism for antibody diversification. Nature 2002; 418:99–103.
8. Chaudhuri J, Tian M, Khuong C, et al. Transcription-targeted DNA deamination by the AID antibody diversification enzyme. Nature 2003;422:726–30.
9. Chaudhuri J, Khuong C, Alt FW. Replication protein A interacts with AID to promote deamination of somatic hypermutation targets. Nature 2004;430:992–8.
10. Shinkura R, Ito S, Begum NA, et al. Separate domains of AID are required for somatic hypermutation and class-switch recombination. Nat Immunol 2004;5:707–12.
11. Lossos IS, Levy R, Alizadeh AA. AID is expressed in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas and is not correlated with intraclonal heterogeneity. Leukemia 2004;18:1775–9.
12. Smit LA, Bende RJ, Aten J, et al. Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin's lymphomas of germinal-center phenotype. Cancer Res 2003;63:3894–8.
13. Pasqualucci L, Guglielmino R, Houldsworth J, et al. Expression of the AID protein in normal and neoplastic B cells. Blood 2004;104:3318–25.
14. Rückerl F, Bachl J. Activation-induced cytidine deaminase fails to induce a mutator phenotype in the human pre-B cell line Nalm-6. Eur J Immunol 2005;35:290–8.
15. Bödör C, Bognar A, Reiniger L, et al. Aberrant somatic hypermutation and expression of activation-induced cytidine deaminase mRNA in mediastinal large B-cell lymphoma. Br J Haematol 2005;129:373–6.
16. Möller P, Brüderlein S, Sträter J, et al. MedB-1, a human tumor cell line derived from a primary mediastinal large B-cell lymphoma. Int J Cancer 2001; 92:348–53.
17. Nacheva E, Dyer MJ, Metivier C, et al. B-cell non-Hodgkin's lymphoma cell line (Karpas 1106) with complex translocation involving 18q21.3 but lacking BCL2 rearrangement and expression. Blood 1994;84: 3422–8.

18. Leithäuser F, Meinhardt-Krajina T, Fink K, et al. Foxp3-expressing CD103⁺ regulatory T cells accumulate in dendritic cell aggregates of the colonic mucosa in murine transfer colitis. Am J Pathol 2006;168:1898–909.

19. Küppers R, Zhao M, Hansmann ML, Rajewsky K. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. EMBO J 1993;12:4955–67.

20. Pasqualucci L, Neumeister P, Goossens T, et al. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 2001;412:341–6.

21. Giudicelli V, Chaume D, Lefranc MP. IMGT/GENE-DB: a comprehensive database for human and mouse immunoglobulin and T cell receptor genes. Nucleic Acids Res 2005;33:D256–61.

22. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.

23. Takhar P, Smurthwaite L, Coker HA, et al. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. J Immunol 2005;174:5024–32.

24. Trainor KJ, Brisco MJ, Story CJ, Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood 1990;75:2220–2.

25. Denk A, Goebeler M, Schmid S, et al. Activation of NF-κB via the IκB kinase complex is both essential and sufficient for proinflammatory gene expression in primary endothelial cells. J Biol Chem 2001;276:28451–8.

26. Pasqualucci L, Kitaura Y, Gu H, Dalla-Favera R. PKA-mediated phosphorylation regulates the function of activation-induced deaminase (AID) in B cells. Proc Natl Acad Sci U S A 2006;103:395–400.

27. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983;11:1475–89.

28. Harlow E, Lane D. Immunoprecipitation. In: Anonymous. Antibodies: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Press; 1988: p. 421–70.

29. Chapdelaine P, Vignola K, Fortier MA. Protein estimation directly from SDS-PAGE loading buffer for standardization of samples from cell lysates or tissue homogenates before Western blot analysis. Biotechniques 2001;31:478, 480, 482.

30. Moldenhauer G, Popov SW, Wotschke B, et al. AID expression identifies interfollicular large B cells as putative precursors of mature B cell malignancies. Blood 2005;107:2470–3.

31. Greeve J, Philipsen A, Krause K, et al. Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas. Blood 2003;101:3574–80.

32. Basu U, Chaudhuri J, Alpert C, et al. The AID antibody diversification enzyme is regulated by protein kinase A phosphorylation. Nature 2005;438:508–11.

33. Ito S, Nagaoka H, Shinkura R, et al. Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. Proc Natl Acad Sci U S A 2004; 101:1975–80.

34. Cerutti A, Zan H, Kim EC, et al. Ongoing *in vivo* immunoglobulin class switch DNA recombination in chronic lymphocytic leukemia B cells. J Immunol 2002; 169:6594–603.

35. Chaudhuri J, Alt FW. Class-switch recombination: interplay of transcription, DNA deamination, and DNA repair. Nat Rev Immunol 2004;4:541–52.

36. Mottok A, Hansmann ML, Bräuninger A. Activation induced cytidine deaminase expression in lymphocyte predominant Hodgkin lymphoma. J Clin Pathol 2005;58: 1002–4.

37. Greiner A, Tobollik S, Buettner M, et al. Differential expression of activation-induced cytidine deaminase (AID) in nodular lymphocyte-predominant and classical Hodgkin lymphoma. J Pathol 2005;205:541–7.

38. MacLennan IC, Toellner KM, Cunningham AF, et al. Extrafollicular antibody responses. Immunol Rev 2003; 194:8–18.

39. William J, Euler C, Shlomchik MJ. Short-lived plasmablasts dominate the early spontaneous rheumatoid factor response: differentiation pathways, hyper-mutating cell types, and affinity maturation outside the germinal center. J Immunol 2005;174:6879–87.

40. Cattoretti G, Buettner M, Shaknovich R, et al. Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. Blood 2006;107:3967–75.

41. Forconi F, Sahota SS, Raspadori D, et al. Hairy cell leukemia: at the crossroad of somatic mutation and isotype switch. Blood 2004;104:3312–7.

42. Basso K, Liso A, Tiacci E, et al. Gene expression profiling of hairy cell leukemia reveals a phenotype related to memory B cells with altered expression of chemokine and adhesion receptors. J Exp Med 2004;199: 59–68.

43. Guiter C, Dusanter-Fourt I, Copie-Bergman C, et al. Constitutive STAT6 activation in primary mediastinal large B-cell lymphoma. Blood 2004;104:543–9.

44. Lu X, Nechushtan H, Ding F, et al. Distinct IL-4-induced gene expression, proliferation, and intracellular signaling in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas. Blood 2005;105: 2924–32.

45. Mori T, Takada R, Watanabe R, Okamoto S, Ikeda Y. T-helper (Th)1/Th2 imbalance in patients with previously untreated B-cell diffuse large cell lymphoma. Cancer Immunol Immunother 2001;50:566–8.

46. Imai K, Slupphaug G, Lee WI, et al. Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. Nat Immunol 2003;4:1023–8.

47. Muto T, Okazaki IM, Yamada S, et al. Negative regulation of activation-induced cytidine deaminase in B cells. Proc Natl Acad Sci U S A 2006;103:2752–7.

48. Migliazza A, Martinotti S, Chen W, et al. Frequent somatic hypermutation of the 5′ noncoding region of the BCL6 gene in B-cell lymphoma. Proc Natl Acad Sci U S A 1995;92:12520–4.

49. Yoshikawa K, Okazaki IM, Eto T, et al. AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. Science 2002;296:2033–6.

50. Wang CL, Harper RA, Wabl M. Genome-wide somatic hypermutation. Proc Natl Acad Sci U S A 2004;101:7352–6.