Published in final edited form as:

Mol Immunol. 2008 April ; 45(7): 2062–2068.

AID-mediated diversification within the IgL locus of chicken DT40 cells is restricted to the transcribed IgL gene

Anjali R. Gopal¹ and Sebastian D. Fugmann^{*}

Molecular Immunology Unit, Laboratory of Cellular and Molecular Biology, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA

Abstract

Somatic hypermutation (SHM) and gene conversion (GCV) are closely related processes that increase the diversity the primary immunoglobulin repertoire. In both processes the activation-induced cytidine deaminase (AID) converts cytosine residues to uracils within the DNA of the immunoglobulin (Ig) genes in a transcription-dependent manner, and subsequent error-prone repair processes lead to changes in the antigen recognition site of the encoded receptors. This activity is specifically recruited to the Ig loci by unknown mechanisms. Our analyses of the chicken B-cell line DT40, and derivatives thereof, now revealed that even the closest neighbors of the Ig light chain (IgL) gene are protected from AID activity, albeit being transcribed and thus acting as potential targets of AID. Our findings are in support of a model in which *cis*-acting DNA boundary elements restrict AID activity to the IgL locus and guard the genome in the vicinity of the IgL gene from deleterious mutations.

Keywords

Somatic Hypermutation; Gene Conversion; Immunoglobulin; DT40

Introduction

The primary diversity of the immunoglobulin (Ig) repertoire is generated in B-cell progenitors by a somatic gene rearrangement process, named V(D)J recombination. The antigen specificity of each Ig can subsequently get further diversified by somatic hypermutation (SHM) and gene conversion (GCV). While SHM occurs in all jawed vertebrates, GCV has thus far only been described in chicken, rabbits and cattle.

Both secondary diversification processes, as well as the related class switch recombination, share an essential initiating factor, the activation induced cytidine deaminase (AID) (Arakawa et al., 2002; Harris et al., 2002; Muramatsu et al., 2000; Revy et al., 2000). This protein is thought to act on cytosine residues in single-stranded DNA that is transiently generated during transcription of the Ig heavy (IgH) and light chain (IgL) genes (reviewed in Longerich et al., 2006). The resulting U:G mismatch activates error prone DNA repair mechanisms involving uracil DNA glycosylase (UDG), general mismatch repair factors, and error-prone DNA

^{*}Corresponding author at: Laboratory of Cellular and Molecular Biology, Gerontology Research Center, National Institute on Aging, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA, phone: +1-(410)-558-8379, fax: +1-(410)-558-8386, email: fugmanns@grc.nia.nih.gov.

¹Current address: Medical University of South Carolina, 96 Jonathan Lucas Street, 807 CSB, PO Box 250623, Charleston, SC 29425

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

polymerases (reviewed in Maizels, 2005; Martomo and Gearhart, 2006). The final products either are individual point mutations, as in the case of SHM, or multiple nucleotide changes in the case of GCV, as homologous pseudo V (ψ V) elements are used as sequence donors during the repair phase.

The molecular rules that govern the restriction of SHM and GCV specifically to Ig genes are unclear, and “mistargeting” to a few other transcribed genes has been reported (Muschen et al., 2000; Pasqualucci et al., 1998; Pasqualucci et al., 2001; Shen et al., 1998). Non-Ig promoters are able to promote SHM of Ig genes in several mouse models (Betz et al., 1994; Fukita et al., 1998; Tumas-Brundage and Manser, 1997), and thus a popular model invokes cis-acting DNA elements that recruit AID to the Ig loci, possibly via interaction with distinct transcription factors (Conlon and Meyer, 2006; Michael et al., 2003; Schoetz et al., 2006). An alternative hypothesis is that AID acts genome-wide, while error-prone DNA repair processes are the preferentially employed in Ig gene loci.

Either scenario places genes that are physically closely linked to the Ig genes as strong candidates for being diversified in an AID-dependent fashion. We thus performed an extensive sequence analysis of two genes that flank the IgL locus in chicken DT40 cells, a B-cell line constitutively undergoing GCV (Buerstedde et al., 1990), and/or SHM (Arakawa et al., 2004). We found that both genes, VPS29 and VpreB3, were transcribed, however neither of them was subject to AID-mediated sequence diversification in wild-type DT40 cells. Furthermore, the genes remain protected in the absence of IgL transcription, as well as when the distance between them and the IgL gene is reduced from approximately 24 kb down to 5 kb. This suggests that an yet unknown mechanism prevents the spreading of AID-mediated Ig gene mutation onto neighboring genes.

Material and Methods

Tissue culture

All DT40 cell lines were grown at 41°C and 5% CO₂ in RPMI (Mediatech) supplemented with 10% Fetal Bovine Serum (Invitrogen), 1% Chicken Serum (Invitrogen), 10mM HEPES, 2mM L-Glutamine, and Penicillin/Streptomycin.

Gene conversion assays

Cells were seeded out as single cells by limiting dilution. Multiple independent cell clones of each cell line were grown continuously for 4 weeks. Afterwards genomic DNA was prepared by standard procedure using SDS/Proteinase K and subsequent phenol/chloroform extraction. All genomic fragments of our genes of interest were amplified using Phusion DNA polymerase (NEB) using the primers listed in Table 1 and the following PCR conditions: 2min at 98°C, 30 cycles of 30sec at 98°C, 20sec annealing at 64°C for VpreB3 and IgL or 58°C for VPS29, and 30sec at 72°C; and a final incubation for 5min at 72°C. Gel purified DNA fragments were treated with Taq polymerase (Invitrogen) in the presence of dATP and cloned into a pCR4-TOPO vector (Invitrogen). At least 48 individual clones were sequenced using a gene specific primer (Table 1). To identify mutation/gene conversion events, nucleotide sequences were aligned using CLUSTALW (<http://align.genome.jp/>). In case of IgL sequence changes that could not be derived from a ψ V were counted as non-templated mutations. Identical nucleotide changes found within multiple sequences from an individual cell clone were only counted as a single event.

Gene expression analysis

Total RNA was isolated using RNeasy (Qiagen) according to the manufacturer's protocol. One microgram RNA aliquots were used to prepare cDNA with random hexamer primers using

the Superscript II Kit (Invitrogen). The transcripts were amplified by PCR using Taq polymerase using gene specific primers (Table 1) and the following cycling program: 2min at 95°C, 35 cycles of 30sec at 95°C, 30sec at 55°C, 30sec at 72°C, and a final extension of 5min at 72°C.

Results

Identification of neighboring genes in the chicken IgL locus

In order to identify genes that are flanking the IgL locus in chicken we compared the genomic sequence upstream of the 5'-most pseudo-V, ψ V25, and downstream of the exon encoding the IgL constant region (C_L) to the complete GenBank database using the BLAST alignment algorithm. We identified two genes residing upstream of ψ V25, VPS29 (vacuolar sorting protein, GenBank accession no. **XP_415222**) and VpreB3 (a VpreB receptor like molecule, GenBank accession no. **XP_415223**) (Fig. 1). VPS29 is part of the retromer complex responsible for the retrieval of mannose 6-phosphate receptors from the endosomal system to the Golgi (reviewed in Seaman, 2005). The chicken VpreB3 gene is thought to play a role in the maturation and secretion of IgL chain (Rosnet et al., 2004). Downstream of the C_L exon we identified one predicted gene with similarity to carbonic anhydrase XV (CAXV, GenBank accession no. **XP_415218**) and an additional gene (GenBank accession no. **XP_415217**) representing a putative chicken homolog of the DiGeorge Syndrome Critical Region gene 2 (DGCR2) (Fig. 1).

As AID can only exert its deaminase activity on transcribed genes (Chaudhuri et al., 2003; Pham et al., 2003; Ramiro et al., 2003; Sohail et al., 2003), we determined whether any of these genes were expressed in DT40 cells. Using random hexamer primed cDNA prepared from total RNA of wild-type DT40 cells, the expression of VpreB3, VPS29, IgL, CAXV, DGCR2, GAPDH, and AID was analyzed by RT-PCR using gene-specific primers (Fig. 2). Bands corresponding to transcripts for VpreB3, VPS29, IgL, and the control genes, GAPDH and AID, were readily detectable (Fig 2A–E). However, neither CAXV nor DGCR2 showed any signal (Fig. 2F+G), even when additional primer pairs were tested (data not shown). We conclude that the two downstream genes are either transcribed below the level of detection, or that the gene prediction models are incorrect. Thus we focused on VpreB3 and VPS29 for subsequent experiments.

Analysis of sequence diversification patterns in an around the IgL locus

The rearranged IgL allele in DT40 cells constitutively undergoes GCV and SHM, and these processes are strictly dependent on AID (Arakawa et al., 2002; Harris et al., 2002). In order to measure mutation frequency, we seeded cells by limiting dilution and cultured individual clones continuously for 4 weeks. The IgL gene from two individual clones was PCR-amplified from genomic DNA using a high-fidelity proof-reading DNA polymerase, cloned, and sequenced (Fig. 3). Within 72 sequences we identified 10 gene conversion and 5 non-templated mutation events, resulting in a mutation frequency of 4.07×10^{-4} events/bp (Fig. 3). In order to determine whether the VpreB3 and VPS29 genes are also targeted by AID, we looked for sequence diversification of both genes in the same DNA samples. As no corresponding pseudogenes of VpreB3 and VPS29 that could act as sequence donors are present within the entire chicken genome, only individual point mutations would be the expected. We amplified, cloned and sequenced a 600bp region downstream of their predicted transcription starts (Fig. 4), as AID activity is highest in the respective region of Ig genes (Rada and Milstein, 2001). In a total of 154 sequences (84 VpreB3 and 70 VPS29) we found only a single A to G point mutation in the VpreB3 gene (Fig. 3). This suggests that, although both genes are being transcribed and are close to the putative AID-targeting elements within the IgL gene, neither of the two genes is subject to frequent SHM-type sequence diversification. In addition, we

identified polymorphisms within both genes (VpreB3 and VPS29) that allow distinguishing between the two alleles of the genes (see below).

To determine the level of background noise in our sequencing approach, we used AID-deficient DT40 cells and their corresponding parental cell line DT40CreI (a DT40 clone stably transfected with a tamoxifen-inducible Cre recombinase expression construct) (Arakawa et al., 2002). The analyses of two independent subclones of each of these cell lines showed that the vast majority of the changes in the IgL gene itself are AID dependent (7.44×10^{-4} events/bp), and that the background level is approximately 5.14×10^{-5} events/bp (Fig. 3). Therefore, the frequency of events in the VpreB3 and VPS29 gene of the wild-type DT40 cells is clearly below the level of background in our experimental system.

Distance and promoter effects within the IgL locus

One obvious explanation for the absence of AID-mediated diversification within the VPS29 and VpreB3 genes could simply be the distance between them (measured from putative transcription starts) and the IgL gene, 23kb and 24.5kb respectively (Fig. 1). While this distance is extremely short with respect to the whole genome, it could still readily account for the differences in the efficiencies with which AID gets recruited to the three transcribed genes in this subdomain of the genome. To determine whether shortening this distance would significantly increase the rate of AID-dependent mutagenesis in VpreB3 and VPS29, we sequenced both genes from DT40 AID^RψV⁻ cells, a DT40CreI variant lacking all ψV elements (Arakawa et al., 2004). This deletion reduces the distances between the transcription start sites by approximately 18.5kb, down to 4.5kb and 6 kb for VpreB3 and VPS29, respectively. Note that the distance between the putative polyadenylation site of VPS29 and the transcription start of IgL gene is only 1.5kb. In these cells, more than 35% of the IgL amplicons show at least one point mutation for a total mutation frequency of 1.21×10^{-3} events/bp (Fig. 3). As described in the first report of this cell line (Arakawa et al., 2004), this frequency is higher than that found for wild-type DT40 cells, as some of the ψV elements used in the repair are a perfect sequence match and such events remain undetectable by our assays. The analysis of a corresponding AID-deficient cell clone, DT40 AID^{-/-}ψV⁻ confirmed again that this diversification was AID-dependent (Fig. 3). Interestingly, neither VpreB3 nor VPS29 were diversified by SHM in the cell line (Fig. 3). This suggests that there is a strong preference for AID to act on genes driven by Ig promoters, or that there are boundary elements that block the spreading of AID to neighboring transcribed genes.

One widely accepted model suggests that AID is recruited by *cis*-acting elements within or close to the Ig enhancer, and is subsequently loaded onto the transcription machinery that is assembled on the Ig promoter (Longerich et al., 2006). This raises the possibility that VPS and VpreB3 promoters could be far less likely to receive AID because the IgL promoter may be in closer proximity to such targeting elements. Therefore, we analyzed the VpreB3 and VPS29 genes in DT40-T7 cells, a DT40 derivative in which the original Ig promoter is replaced with the phage T7 promoter (Yang et al., 2006). These cells do not transcribe their IgL gene, and thus the VPS29 gene is now likely the gene closest to the putative AID targeting elements. Due to the lack of transcription in the IgL gene the diversification frequency in DT40-T7 is at background level (5.58×10^{-5} events/bp) (Fig. 3). Analyzing the level of sequence diversification in the VpreB3 gene in these cells revealed one of 2.85×10^{-5} events/bp, indistinguishable from that of the VpreB3 gene in wild-type cells (1.98×10^{-5} events/bp). The VPS29 gene shows slightly higher levels of mutations (6.52×10^{-5} events/bp) in DT40-T7 cells, but that is still not significantly above the background of our experimental system (5.14×10^{-5} events/bp), and even lower than that of the VPS29 gene in the AID-deficient DT40 ψV⁻ clone (7.87×10^{-5} events/bp). Thus we conclude that even in the absence of IgL transcription, VpreB3 and VPS29 remain protected from AID-induced mutations.

Allele specific nucleotide polymorphisms in VpreB3 and VPS29

The nucleotide sequence analysis of the VpreB3 and VPS29 genomic fragments from the wild-type DT40 cells revealed that the two alleles of both genes can be distinguished by sequence polymorphisms (Fig. 4). None of the sequence alterations leads to a change of the amino acids of the encoded genes, as they are either silent mutations or residing in non-coding regions (intron and UTR). The polymorphisms are, however, useful in distinguishing the two alleles of both genes in subsequent studies. Interestingly, a number of sequences showed permutations of the individual nucleotide polymorphisms in both genes (data not shown), suggesting an exchange of genetic information between individual alleles. As these permutations occur in both, AID-proficient and AID-deficient cells, they are, however, most likely not reflective of AID-dependent diversification mechanisms acting on these genes. It remains to be elucidated whether these events indicate ongoing DNA recombination in the vicinity of the IgL genes, or whether they reflect PCR artifacts.

Discussion

SHM and GCV are highly mutagenic processes that need to be tightly controlled to prevent genomic aberrations that could lead to cancer. To our current knowledge, the IgL and IgH genes are the only cognate targets of these two processes. Previous reports aimed solely at determining DNA elements and/or factors that recruit the SHM/GCV machinery to these loci (reviewed in Odegard and Schatz, 2006). In this study we now asked, for the first time, whether the same machinery, once recruited to the Ig loci, could move along the DNA to spread onto neighboring genes.

We identified four predicted genes in the chicken genome that are in close proximity to the IgL gene. Only two of them, VPS29 and VpreB3, are transcribed in DT40 cells, a prerequisite for being a target of AID-mediated mutagenesis. They are separated from the VJ exon of the IgL gene by a 18kb region that contains all 25 known ψ V elements. In wild-type DT40 cells, neither of the genes is mutated at levels above the background noise of our experimental system. Thus the diversification events are at least 20-fold less frequent when compared to the IgL gene in the ψ V-deficient cell line. This finding is unexpected as transgenic mouse models indicated that transcribed sequences in the proximity of Ig enhancers are subjected to SHM independent of their promoter (Betz et al., 1994). Although the RT-PCR analysis showed that the steady-state transcript level of VpreB3 is comparable to that of IgL (Fig. 2), two possibilities remain: (1) the actual rate of transcription is much lower leading to a strongly reduced AID-induced mutation rate, or (2) the promoters of VpreB3 and VPS29 are similar to the EF1 α promoter and do allow for efficient SHM and GCV (Yang et al., 2006). As all cell lines used in our experiments were derived from the same original DT40 clone and are now separated by countless generations, even low rates of mutation should be detectable when comparing the VpreB3 and VPS29 genes from different DT40-derived subclones (e.g. DT40CreI and DT40-T7). Nonetheless, the nucleotide sequences of both genes were absolutely identical within all DT40 cell lines analyzed for this study. Another formal possibility explaining the absence of nucleotide changes in the VPS29 and VpreB3 genes is that such mutations could be lethal or lead to a growth defect. In this scenario, however, one would expect an enrichment of replacement versus silent mutations in AID-positive compared to AID-negative cells; given the low number of events (1 of 7 for all AID⁺, and 0 out of 3 for all AID⁻ clones) there is, however, no evidence supporting this model.

The protection of neighboring genes from the mutagenic enzymes acting on Ig genes is a fundamental requirement for genomic integrity in B lymphocytes, and should as such be present in all jawed vertebrates, including chicken, mice, and most importantly humans. There are three obvious concepts to explain the protection of the VpreB3 and VPS29 genes in the IgL locus of chicken: 1) the distance between their promoters and the targeting elements is too

large, 2) the promoters compete inefficiently with the Ig promoter for being loaded with AID, and 3) the genes are protected by boundary elements preventing the spread of Ig gene-specific mutagenic activity. As reducing the intergenic distance down to 1.5kb did not result in any significant increase in mutation frequency of the VpreB3 and VPS29 genes, a simple distance effect seems unlikely to be the sole reason for their protection. The promoter competition model is also unlikely since even in the DT40-T7 cell line that lacks Ig transcription, no substantial increases in the level of mutations in VpreB3 and VPS29 were detected. This could only in part be explained by recent findings that different promoters drive SHM with different efficiencies that are not correlated to the transcription rates (Yang et al., 2006).

Altogether, this supports the boundary element concept as the most favorable possibility to limit any spreading of diversification activity from the IgL gene. Boundary elements are being found in other genomic loci, where they serve to separate genes that are under control of different regulatory elements, e.g. the chicken β -globin and the folate receptor genes (Prioleau et al., 1999). In some cases they separate active from inactive chromatin, and in they others block promoter enhancer interactions. It is conceivable that either one or both functions might exist in the IgL locus, as blocking promoter-enhancer interactions could prevent loading of AID onto RNA polII polymerase complexes assembled on the promoters of VpreB3 and VPS29. Alternatively, such element could restrict distinct chromatin modifications in the IgL gene that are responsible for the susceptibility to AID and/or error-prone DNA repair to the IgL gene itself. One candidate region in which such a boundary element could be located is an 800bp stretch between the polyadenylation site of VPS29 and the most upstream pseudo-V element, ψ V25. Interestingly, this region contains two putative binding sites for CTCF (SDF, unpublished data), a DNA-binding factor that plays a critical role in establishing chromatin boundaries (Burgess-Beusse et al., 2002). As gene-targeting is very efficient in DT40 cells, the deletion of such elements as well as the targeted integration of well-defined insulator and boundary elements are the logical step towards understanding how B-cells prevent the spreading of AID-activity to genes in close proximity to the Ig loci. Whether the same or similar concepts also apply to the corresponding gene loci in mice and humans will be the focus of future studies.

Acknowledgements

We are grateful to Dr. Arakawa and Dr. Jean-Marie Buerstedde for providing many of the DT40 cell lines used in this study. We thank Dr. Ranjan Sen and Dr. Michael Pazin for critical comments on this manuscript. This work was supported by the Intramural Research Program of the National Institute on Aging/National Institutes of Health.

References

- Arakawa H, Hauschild J, Buerstedde JM. Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. *Science* 2002;295:1301–6. [PubMed: 11847344]
- Arakawa H, Saribasak H, Buerstedde JM. Activation-induced cytidine deaminase initiates immunoglobulin gene conversion and hypermutation by a common intermediate. *PLoS Biol* 2004;2:E179. [PubMed: 15252444]
- Betz AG, Milstein C, Gonzalez-Fernandez A, Pannell R, Larson T, Neuberger MS. Elements regulating somatic hypermutation of an immunoglobulin kappa gene: critical role for the intron enhancer/matrix attachment region. *Cell* 1994;77:239–48. [PubMed: 8168132]
- Buerstedde JM, Reynaud CA, Humphries EH, Olson W, Ewert DL, Weill JC. Light chain gene conversion continues at high rate in an ALV-induced cell line. *EMBO J* 1990;9:921–7. [PubMed: 2155784]
- Burgess-Beusse B, Farrell C, Gaszner M, Litt M, Mutskov V, Recillas-Targa F, Simpson M, West A, Felsenfeld G. The insulation of genes from external enhancers and silencing chromatin. *Proc Natl Acad Sci USA* 2002;99 Suppl 4:16433–7. [PubMed: 12154228]
- Chaudhuri J, Tian M, Khuong C, Chua K, Pinaud E, Alt FW. Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* 2003;422:726–30. [PubMed: 12692563]

- Conlon TM, Meyer KB. The chicken Ig light chain 3'-enhancer is essential for gene expression and regulates gene conversion via the transcription factor E2A. *Eur J Immunol* 2006;36:139–48. [PubMed: 16342328]
- Fukita Y, Jacobs H, Rajewsky K. Somatic hypermutation in the heavy chain locus correlates with transcription. *Immunity* 1998;9:105–14. [PubMed: 9697840]
- Harris RS, Sale JE, Petersen-Mahrt SK, Neuberger MS. AID is essential for immunoglobulin V gene conversion in a cultured B cell line. *Curr Biol* 2002;12:435–8. [PubMed: 11882297]
- Longerich S, Basu U, Alt F, Storb U. AID in somatic hypermutation and class switch recombination. *Curr Opin Immunol* 2006;18:164–74. [PubMed: 16464563]
- Maizels N. Immunoglobulin gene diversification. *Annu Rev Genet* 2005;39:23–46. [PubMed: 16285851]
- Martomo SA, Gearhart PJ. Somatic hypermutation: subverted DNA repair. *Curr Opin Immunol* 2006;18:243–8. [PubMed: 16616477]
- Michael N, Shen HM, Longerich S, Kim N, Longacre A, Storb U. The E box motif CAGGTG enhances somatic hypermutation without enhancing transcription. *Immunity* 2003;19:235–42. [PubMed: 12932357]
- Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000;102:553–63. [PubMed: 11007474]
- Muschen M, Re D, Jungnickel B, Diehl V, Rajewsky K, Kuppers R. Somatic mutation of the CD95 gene in human B cells as a side-effect of the germinal center reaction. *J Exp Med* 2000;192:1833–40. [PubMed: 11120779]
- Odegard VH, Schatz DG. Targeting of somatic hypermutation. *Nat Rev Immunol* 2006;6:573–83. [PubMed: 16868548]
- Pasqualucci L, Migliazza A, Fracchiolla N, William C, Neri A, Baldini L, Chaganti RS, Klein U, Kuppers R, Rajewsky K, Dalla-Favera R. BCL-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci. *Proc Natl Acad Sci USA* 1998;95:11816–21. [PubMed: 9751748]
- Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R, Dalla-Favera R. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 2001;412:341–6. [PubMed: 11460166]
- Pham P, Bransteitter R, Petruska J, Goodman MF. Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. *Nature* 2003;424:103–7. [PubMed: 12819663]
- Prioleau MN, Nony P, Simpson M, Felsenfeld G. An insulator element and condensed chromatin region separate the chicken beta-globin locus from an independently regulated erythroid-specific folate receptor gene. *EMBO J* 1999;18:4035–48. [PubMed: 10406808]
- Rada C, Milstein C. The intrinsic hypermutability of antibody heavy and light chain genes decays exponentially. *EMBO J* 2001;20:4570–6. [PubMed: 11500383]
- Ramiro AR, Stavropoulos P, Jankovic M, Nussenzweig MC. Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. *Nat Immunol* 2003;4:452–6. [PubMed: 12692548]
- Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Labeau R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 2000;102:565–75. [PubMed: 11007475]
- Rosnet O, Blanco-Betancourt C, Grivel K, Richter K, Schiff C. Binding of free immunoglobulin light chains to VpreB3 inhibits their maturation and secretion in chicken B cells. *J Biol Chem* 2004;279:10228–36. [PubMed: 14670953]
- Schoetz U, Cervelli M, Wang YD, Fiedler P, Buerstedde JM. E2A expression stimulates Ig hypermutation. *J Immunol* 2006;177:395–400. [PubMed: 16785535]
- Seaman MN. Recycle your receptors with retromer. *Trends Cell Biol* 2005;15:68–75. [PubMed: 15695093]

- Shen HM, Peters A, Baron B, Zhu X, Storb U. Mutation of BCL-6 gene in normal B cells by the process of somatic hypermutation of Ig genes. *Science* 1998;280:1750–2. [PubMed: 9624052]
- Sohail A, Klapacz J, Samaranayake M, Ullah A, Bhagwat AS. Human activation-induced cytidine deaminase causes transcription-dependent, strand-biased C to U deaminations. *Nucleic Acids Res* 2003;31:2990–4. [PubMed: 12799424]
- Tumas-Brundage K, Manser T. The transcriptional promoter regulates hypermutation of the antibody heavy chain locus. *J Exp Med* 1997;185:239–50. [PubMed: 9016873]
- Yang SY, Fugmann SD, Schatz DG. Control of gene conversion and somatic hypermutation by immunoglobulin promoter and enhancer sequences. *J Exp Med* 2006;203:2919–28. [PubMed: 17178919]

Figure 1. The structure IgL locus in chicken

In this schematic representation of the chicken IgL locus the exons and pseudo V (ψ V) elements are indicated by filled boxes with the position and size drawn to scale. The genes and their transcriptional orientation are indicated by arrows.

Figure 2. Transcription profile of the IgL locus in DT40 cells

The steady-state transcript levels of VpreB3, VPS29, IgL, GAPDH, AID, CAXV, and DGCR2 in wild-type DT40 cells were determined by semi-quantitative RT-PCR. Using random hexamers cDNA was synthesized from total RNA using random hexamers. Individual transcripts were amplified using gene-specific primer pairs. Five-fold dilutions of template cDNA was added to individual PCR reactions (lane 1–3), and no template was added to the reactions in lane 4. Amplification from genomic DNA or unspliced primary transcripts primer pairs was excluded as primer pairs were chosen that span exonintron boundaries, and no PCR products of larger size were observed in any of the reactions.

Figure 3. Mutation analysis of the VpreB3, VPS29, and IgL gene in DT40 cells

The mutation profile of the VpreB3, VPS29, and IgL gene was determined after 4 weeks of culture. Two clones of each line (except for DT40 AID^{-/-} ψ V⁻ of which only one clone was available) were chosen and at least 48 amplicons of each gene were submitted for sequencing. The total number of obtained sequences is indicated in the middle of the pie chart, and the size of each slice corresponds to the number of clones with the respective number of mutation events (point mutations and or gene conversion events). The frequency of mutations per base pair is shown below each chart.

Figure 4. Sequence polymorphisms within the VpreB3 and VPS29 genes of DT40 cells

The genomic region surrounding the first two exons of the VpreB3 and VPS29 genes are shown schematically. Amplified regions are marked with a solid line below the graph. Exons are shown as boxes with white and grey indicating UTRs and coding regions, respectively. The positions and the types of each nucleotide polymorphism are indicated, and the conserved nucleotides of coding triplets are shown in grey. The top and bottom row of nucleotides correspond to individual alleles, respectively. It is still unknown which of the VpreB3, VPS29, and IgL alleles are linked.

Table I
Oligonucleotides for amplification genomic fragments and RT-PCR

Genomic fragments		
Gene	Name	Nucleotide sequence
VpreB3	PCR VPR3GF1	AGCACACAGAGCAGCCTGGGCTTGT
	PCR VPR3GR1	GTCAGGGATGTCCAGAGACTAAACA
	Seq. VPRBSEQR	GCTTCTACAGCCAGTTGATG
VPS29	PCR VSPGF1	GGCGTGCTACTTCCTCGTTACTGCA
	PCR VSPGR1	TGAGAAAGAGAGATACACAAGGTAG
	Seq. VSPSEQR	CCAGCTTTCAGTGGGACAA
IgL	PCR CVLF1	CAGTAAGCTTACCATGGCCTGGGCTCCTCTCCTCCTG
	PCR CLA2	CCTCCATTTCTAGACAGCACTTACCTGGACAGCTG
	Seq. CHVLSR	AGCCTGCCGCCAAGTCCAAG
RT-PCR		
Gene	Name	Nucleotide sequence
VpreB3	VPB3F1	CTCTTCCTCTGCTCCTGGATCCTAC
	VPB3R	TCCGTGTTGTAGTAGAGCAGG
VPS29	VSPF	AGTCTGAATTATCCTGAACAG
	VSPR	ATACATAAGTCACAACCTGTGG
IgL	CVLF1	CAGTAAGCTTACCATGGCCTGGGCTCCTCTCCTCCTG
	CCLR1	TCAGCTCCTCCTTGACGGTG
GAPDH	CHGAPDHF	ACCAGGGCTGCCGTCTCTC
	CHGAPDHR	TTCTCCATGGTGGTGAAGAC
AID	CAIDF	ACCTACCTCTGTTATGTTGTG
	CAIDR1	CGGGCAGTGAATGCGGAG
CAXV	CAXVF1	CCGTGCACTGCAGCTGCACTTC
	CAXVR1	AATGATGGTGTGTTAGTTGGAG
DGCR2	DGCR2F	ACTGTGATGCATCCCCACAGCTC
	DGCR2R	CTATCCATCCGCTTCATCACAC