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Endogenous Expression of Activation-Induced Cytidine Deaminase in Cell Line WEHI-231[1]

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Because of its susceptibility to apoptosis on Ag receptor cross-linking, cells of the mouse cell line WEHI-231 have been classified as immature B cells. Surprisingly, however, the cell line expresses activation-induced cytidine deaminase, the enzyme that mediates hypermutation and Ig class switch recombination in activated B cells. Although both cDNA sequence and protein expression of activation-induced cytidine deaminase appear normal, the cell line does not hypermutate an indicator plasmid. For the readout, the indicator plasmid depends on the removal of deoxyuridine after transition from C to U and, therefore, on functional expression of uracil *N*-glycosylase 2, which is normal in WEHI-231. At the endogenous Ig locus, however, WEHI-231 does undergo the canonical hypermutation of G · C to A · T base pairs to some extent. The cell line also expresses the germline transcripts of the Ig γ2b, ϵ, and α loci, but it does not switch its IgM surface Ig. ***The Journal of Immunology*****, 2004, 173: 1858–1867.**

WEHI-231 is a B cell lymphoma of BALB/c × NZB F_1 origin, induced by mineral oil injection (1). Although it has been studied in great detail and revealed a wealth of information, the Ig loci of the WEHI-231 cell line and their potential to hypermutate or switch the Ig class have not yet been characterized. Cells of this line display IgM on its surface, and in vitro growth seems to be dependent on the continuous signaling through the Ag receptor (2, 3). Thus, even after chemical mutagenesis, mutants without surface receptor could not be isolated yet. Because of its propensity to apoptose on cross-linking, and because of the absence of IgD on its surface, WEHI-231 has been classified a tumor of immature B lymphocytes (4).

Recently, it has been shown that in fibroblasts, exogenous expression of activation-induced cytidine deaminase (AID)[3] is necessary and sufficient for both hypermutation (5) and class switching (6) of exogenous substrates. Presumably because no Ig genes are present in fibroblasts and because the processes are strictly transcription dependent (7–9), there is no hypermutation or switching at the endogenous loci. However, introduction of AID alone into hybridomas results in hypermutation of the active allele in hybridoma cells, which normally do not hypermutate their Ig genes (10). Although AID mediates both processes, the two functions can be dissociated in the appropriate AID mutants (11, 12).

For both hypermutation and switch recombination, repair enzymes are important. Pms-2 (13), msh2 (14, 15), mlh1 (16), and UNG (17) modify the incipient mutations introduced by AID, thereby extending the spectrum of the mutants generated; in class switch recombination they may increase the chance of introducing a double-stranded break into the DNA (18, 19)—a prerequisite for the recombination to occur. A deficiency in uracil *N*-glycosylase (UNG) in humans (20) and mice (21) is associated with impaired H chain class switch and perturbed hypermutation. For switch recombination, both Ku70 (22) and Ku80 (23) are required. It is envisaged that one or more Ku70/Ku80 proteins bind to Sμ and to another S region and bring them into close proximity, either with (24) or without (25, 26) the help of the catalytic subunit of DNA-protein kinase, a DNA-dependent phosphokinase that is recruited by Ku70/80 in conventional (nonhomologous) double-strand repair (27). Furthermore, B cells lacking H2AX show impaired class switch recombination (28, 29); H2AX, however, is not needed for hypermutation (30). In this report, we follow up on our surprising observation that WEHI-231 expresses AID. We investigate whether or not cells of this line hypermutate the endogenous Ig genes or exogenous substrates and whether or not they switch their endogenous Ig H chain gene.

Materials and Methods

PCR amplifications and subcloning in bacteria

RNA was extracted from cultured cells with an Oligotex Direct mRNA Mini Kit (Qiagen, Chatsworth, CA), genomic DNA with a DNeasy Tissue kit (Qiagen), and plasmid DNA with a QIAprep Spin Miniprep Kit (Qiagen). mRNA encoding AID was reverse transcribed and amplified with the SuperScript One-Step kit (Invitrogen Life Technologies, Carlsbad, CA), with the following primers: 5′-primer, 5′-CGC(CTCGAG)(GCCACC)ATGGACAGCCTTCTGATG; and 3′-primer, 5′-ATGGTT(CCAATTTAATGG)TCAAAATCCCAACATACGAAATGC; conditions were 40 cycles, of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. To confirm that AID has no mutation or deletion, first-strand cDNA was synthesized using oligodeoxythymidylate primers (40-mer) and Superscript II (Invitrogen Life Technologies). Amplification was done with Pfu and primers priming in the noncoding region: 5′-AIDNC, 5′-AGGGAGTCAAGAAAGTCACGCTG; 3′AIDNC, 5′-AGAGCATCATTCACGACCCAAAGTC; 35 cycles of 95°C for 1 min, 52.2°C for 1 min, and 72°C for 1 min.

cDNA for part of the VDJ segment and part of the cμ1 fragment were amplified using the SuperScript One-Step kit, 40 cycles of 94°C for 30 s, 53.4°C for 30 s, and 72°C for 1 min; with Vgen, 5′-AGGT(CG)(AC)A(AG)CTGCAG(CG)AGTC(AT)GG, and cμ1, 5′-GGTTCTGATACCCTGGATGACTTCAG. The VDJ segment DNA of the active allele from WEHI-231 was amplified with PfuTurbo (Stratagene, La Jolla, CA) and the primers V_HWEHI, 5′-CTTATGCCATACTATAGGAAAACAGGG; and the JH2 primer 5′-CCAGAGATTTATAGGGATCCTGGCCA. The amplification conditions were 40 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 1 min. Cμ1 DNA was amplified with PfuTurbo (Stratagene), with 5′-primer genCμ, 5′-GCAAGAAGACAGATTCTTACCCC; and 3′primer

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[3] Abbreviations used in this paper: AID, activation-induced cytidine deaminase; UNG, uracil *N*-glycosylase; EGFP, enhanced green fluorescent protein; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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genCμ, 5′-GGGAGGGTTTGGTTCTTACCTGG; 40 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 1 min.

UNG was amplified using a one-step RT-PCR kit (Invitrogen Life Technologies): 5′-primer mUNG, 5′-AGGTCCGTGCTCAGCGCAGGC; 3′-primer mUNG: 5′-GGCGGCGGTAGCTGAAAGCACC with 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min.

Ku70 and Ku80 first-strand cDNA was synthesized using oligodeoxythymidylate primers (40-mer) and Superscript II (Invitrogen Life Technologies). Amplification was done with Pfu and the following primers: 5′-primer Ku70, 5′-ACCTTCTGCTGTCCCAGTGG; 3′-primer Ku70, 5′-AAGTGGCTGG GCTTCTGAGC; 5′-primer Ku80, 5′-TCAAATCACCTGAGGACCAGC; 3′-primer Ku80, 5′-ACTCTTGGATTCCCCACACATC; with 30 cycles of 95°C for 45 s, 62.5°C for 45 s, and 72°C for 2 min 30 s.

Sequencing of Ku70/Ku80. Primers for Ku70: 70-top forward (topfor), 5′-GACATCATCACCACCGCTGAG; 70-top reverse (toprev), 5′-CAGCGGT GGTGATGATGT; 70-bottom forward (btmfor), 5′-TCCTCTGGG TACAC GAAC; 70-bottom reverse (btmrev), 5′-TGTTCGTGTACCCAGAGGAG. Primers for Ku80: 80-topfor, 5′-GCCCCAACTTGTCTATAAAG; 80-toprev, 5′-CAATGGTCAGTTGGCAGG; 80-btmfor, 5′-CTCCTGGAGATGTAAG GCT; 80-btmrev, 5′-AGCCTTACATCTCCAGGAGC. Ku70 and Ku80 DNA from WEHI-231 and WEHI-HM, respectively, were subcloned in bacteria, isolated, and sequenced.

GAPDH was amplified with Pfu under the following conditions: 22 cycles of 95°C for 45 s, 55°C for 1 min, 72°C for 1 min.

GAPDH cDNA was amplified using one-step RT-PCR kit (Invitrogen), with 22 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min; 5′ GAPDH primer 5′-TGAAGGTCGGTGTGAACGGATTTGGC, 3′ GAPDH primer: 5′-CATGTAGGCCATGAGGTCCACCAC.

cDNA encoding rearranged ϵ H chain was amplified using the OneStep RT-PCR kit (Qiagen), with 40 cycles of 94°C for 45 s, 51°C for 45 s and 72°C for 1 min. Primers were 5′Vgen; and 3′IgE-CH2: 5′-CTCCGAGTGTGGGCCAAATA.

Germline transcript amplifications were done as follows: ϵ germline transcript, from the IGEL 177 b4–2 cell line: 5′ Iϵ: 5′-TGGGCATGAAT TAATGGTTACTAG; 3′ IgE-CH2-Primer (see above). Amplification was done by using OneStep RT-PCR kit (Qiagen). Amplification conditions were 40 cycles of 94°C for 45 s, 51°C for 45 s and 72°C for 1 min. γ2b germline transcript: 5′ Iγ2b: 5′-CTTCACGCGGCTCCACATGTGA; 3′ IgGγ2b-CH1: 5′-TGGGTGAGCAACGCTGCAGGT. Iγ2b was amplified using OneStep RT-PCR kit (Qiagen). Amplification conditions were 40 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 1 min. α germline transcript: 5′ Iα: 5′-ACTCTCTACCATAGGGAAGATAGCC; 3′ IgGα-CH2: 5′-CAGGATTTCTCAGGCCATTCAGAG; Iα was amplified using OneStep RT-PCR kit (Qiagen), with 42 cycles of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 1 min.

Enhanced green fluorescent protein (EGFP): YFP-XF, 5′-AATTCTC GAGTCGCCACCATGGTGAGCAAG-3′; YFP-XB, 5′-AATTCTCGAG TTACTTGTACAGCTCGTCCATGCC-3′. Amplification conditions: 30 cycles of 95°C for 1 min, 60.6°C for 1 min, and 72°C for 1 min using native Pfu (Stratagene).

Ig allotypes. To analyze the allotypic differences in WEHI-231, we performed PCR on genomic DNA with Pfu (Invitrogen Life Technologies). For the α locus, we used the following primers; 5′-IgA, 5′-AGTCTGC GAGAAATCCCACC; 3′-IgA, 5′-ATGTTGCACGGAACATTTCACG. Amplification conditions: 35 cycles of 95°C for 1 min, 55.9°C for 1 min, and 72°C for 1 min.

For the γ2a and γ2b loci, two sets of primers were used, both yielding amplicons containing γ2a and γ2b sequences. For exons 1 and 2: 5′-IgG2–1/2, 5′-ACACATTCTCCTCTCTTGCAG; 3′-IgG2–1/2: 5′-AGTGGAGC TCTGGTAGTGAC. Amplification conditions: 35 cycles of 95°C for 1 min, 55.9°C for 1 min, 72°C for 2 min. For exons 3 and 4: 5′-IgG2–3/4, 5′-ATTTCCATCTCTCCTCATCAGC; 3′-IgG2–3/4, 5′-TACCTTGGAA AGAACCAGGAC. Amplification conditions: 35 cycles of 95°C for 1 min, 57.2°C for 1 min, and 72°C for 2 min.

For cloning into bacteria, 3′-A overhangs were added to the amplification product by incubation for 10 min at 72°C with *Taq* polymerase. TOPO TA cloning (Invitrogen Life Technologies) was done according to the manufacturer's manual using the pCR2.1-TOPO vector. Bacteria were transformed with the TOPO vector containing the amplification product; single colonies were picked; plasmid DNA was extracted and sequenced.

Quantitative PCR

Quantitative PCR was conducted using ABI 7700 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Probes for real time PCR were purchased from Applied Biosystems; at the 5′-end, they were conjugated to the fluorochrome FAM; and at the 3′-end to the quencher TAMRA. First-strand cDNA was synthesized using random hexamer primers. The cycle conditions for real time PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. In the following, I primers and I probe denote (part of) the germline transcript that includes the I exon and the C region exons. 5′-Iγ2b, 5′-GAAGAGT TCAGAGTTCTCACACACAGA; 3′-Iγ2b, 5′-AGTTGTATCTCCACAC CCAGGG; Iγ2b probe, 6-FAM-ACCACCAAAACAACACCCCCAT CAGTCTAT-TAMRA. 5′Iϵ, 5′-CCACTCACTTATCAGAGGACCTCA; 3′Iϵ, 5′-AGCAGTGCCTTTACAGGGCTT, Iϵ probe, 6-FAM-CCTCTAT CAGGAACCCTCAGCTCTACCCC-TAMRA; 5′-hypoxanthine-guanine phosphoribosyltransferase (HPRT; ME)-PRI), 5′-TGGAAAGAATGTCTT GATTGTTGAA; 3′-HPRT, 5′-AGCTTGCAACCTTAACCATTTTG; HPRT probe: 6-FAM-CAAACTTTGCTTTCCCTGGTTAAGCAGTA CAGCTAMRA.

Western blot

Cells were washed twice with PBS, resuspended in 1 ml of lysis buffer (1.5 ml of 1 M Tris-HCl, pH 7.4; 4.5 ml of 1 M NaCl; 1.5 ml of 100 mM EDTA; 1.5 ml of 10% Triton X; and 21 ml H_2O) and incubated for 20 min on ice. Samples were spun down, and the protein concentration of the supernatant was determined using the Bradford assay. Of each sample, 30 μg of whole protein lysate were loaded on a 12.5% SDS gel. The gel was blotted onto a nitrocellulose membrane and blocked with 5% milk overnight. First Ab was a rabbit affinity-purified anti-AID Ab against the C terminus of AID (kindly provided by F. Alt); and second Ab was goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL). After development and recording, the blot was stripped twice in buffer containing 140 μl of 2-ME, 6.25 ml of 1 M Tris buffer at pH 6.8, and 5 ml of 20% SDS in 88 ml H_2O and incubated with an anti-actin (Ab-1) Ab (Oncogene Research Products, San Diego, CA) and the same second Ab as above.

Abs and control cells used for Ig class identification

For flow cytometry, the following FITC-coupled Abs were used: anti-IgM^b (AF6-78; BD Pharmingen, San Diego, CA); goat anti-IgG2a (Fisher Scientific, Hampton, NH); anti-IgG1 (BD Pharmingen), anti-IgG2b (R12-3; BD Pharmingen); anti-mouse IgG3-FITC (R40-82; BD Pharmingen); anti-IgE, (R35-72; BD Pharmingen); anti-IgA (R5-140; BD Pharmingen); and polyclonal anti-IgD was PE coupled (Southern Biotechnology Associates). As positive control, served cells from GCL 28-22 (IgD), PC1-56.0 (IgG3), OKT3.5 (IgG2a), GK14-1 (IgG2b), IGELb4-2 177), ALFA2.16 (IgA), and spleen cells from a C57BL/6 mouse were cultured with 50 μg/ml LPS and 100 ng/ml IL-4 for 4 days.

Hypermutation indicator plasmid experiment

Phoenix E cells (2×10^5) were transfected, using FuGENE 6 (Roche Diagnostic, Indianapolis, IN), with a plasmid containing sequences of the Moloney virus and either EGFP or EGFP with an amber stop codon (31). Two days after transfection, cells of the lines 18-81, WEHI-231, and 70Z were infected with the Phoenix E cell supernatant, which contained the replication-defective virus. Twenty four hours after infection, cells were selected with puromycin (WEHI-231, 0.25–0.5 μg/ml; 18-81, 2.5 μg/ml; 70Z, 1.5 μg/ml). In some experiments, on day 3 after transduction of cells, EGFP-positive cells (i.e., pre-existing mutants) were selected against by FACS. Cells were grown and analyzed by flow cytometry at different time points.

Results and Discussion

WEHI-231 expresses nonmutated AID

AID is generally considered a marker of activated B cells in the germinal center (32). Indeed, in the National Center for Biotechnology Information est data base, only cells at this location express est encoding (parts of) AID. However, in a routine RT-PCR test for the presence of AID cDNA, we found it expressed in the WEHI-231 line. Fig. 1*A* compares the expression of cDNA encoding AID in cell line 18-81 (Fig. 1*A*, *lane 3*), a cell line that hypermutates and switches its Ig genes (33–35), WEHI-231 (Fig. 1*A*, *lane 4*), its subclone WEHI-HM (Fig. 1*A*, *lane 5*), and the pre-B cell line 70Z (Fig. 1*A*, *lane 6*), which is active in neither hypermutation nor class switching. Surprisingly, although the 70Z does not express it, the WEHI cells had at least as much AID cDNA amplified as 18-81. Because WEHI-231 has been classified an immature B cell line, we thought that RNA expression at the wrong differentiation stage might be accompanied by mutations in the

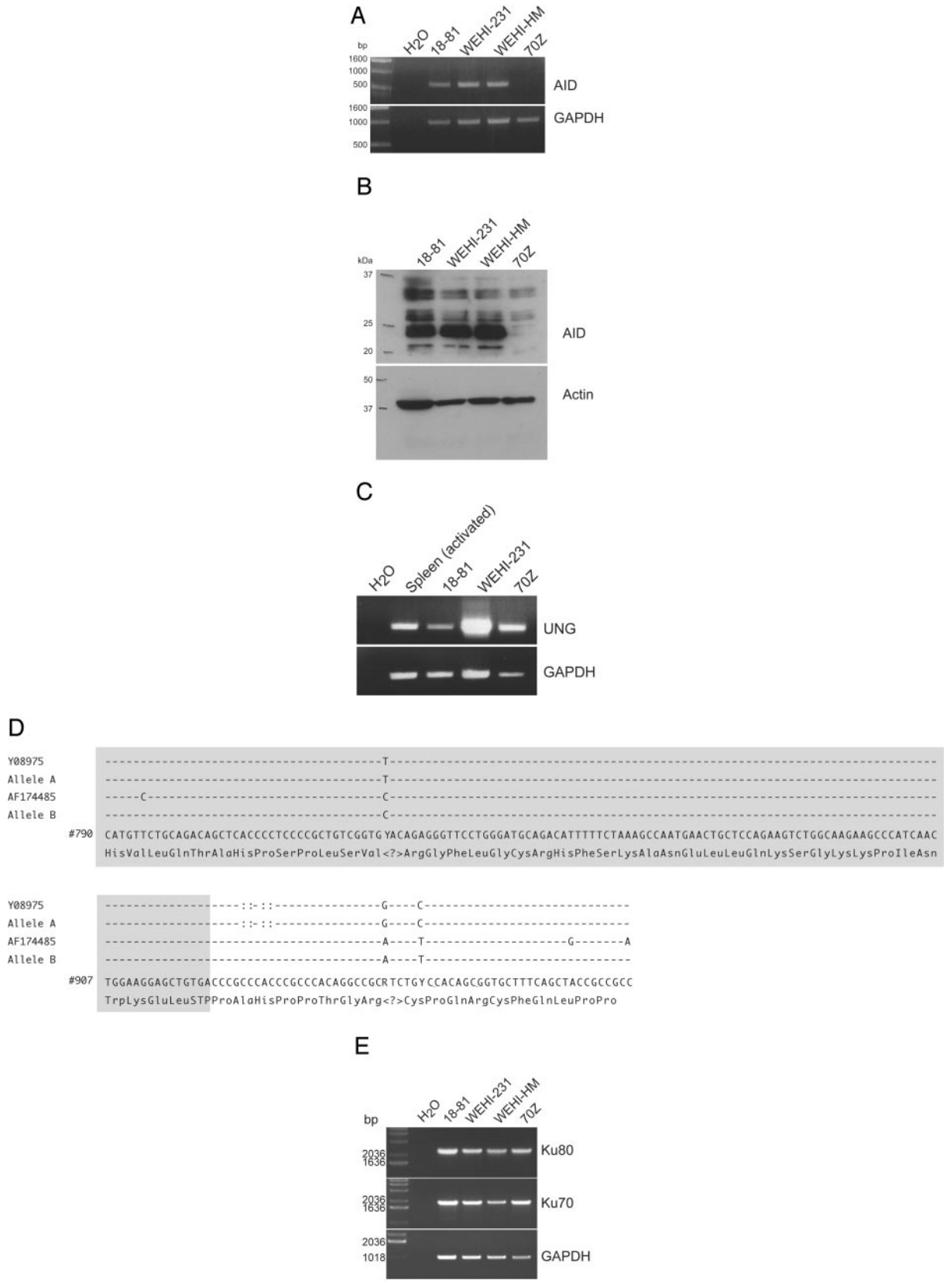


FIGURE 1. AID, UNG, and Ku70/Ku80 expression in WEHI-231. *A,* RT-PCR amplification of AID transcripts and GAPDH transcripts as mRNA amplification and loading controls. H_2O, amplification without template, but with all the other reagents. The size of the amplified AID segment was 596 bp. *B,* Western blot of AID expression; actin as loading control. *C,* RT-PCR amplification of UNG2 transcripts and GAPDH transcripts as mRNA amplification and loading controls. The size of the amplified UNG segment was 997 bp. *D,* Sequences of UNG2 alleles in WEHI-231. Although the complete cDNA encoding UNG was sequenced, only the part of the sequence starting with nucleotide 790 is shown here. Allele A of WEHI-231, as in database sequence Y08975; allele B of WEHI-231, as in database sequence AF174485. Below the consensus sequence, the translation is shown (shaded part). *E,* RT-PCR amplification of Ku70/Ku80 transcripts; the sizes of the amplified segments were: Ku70, 1905 bp; Ku80, 2248 bp; GAPDH, 983 bp.

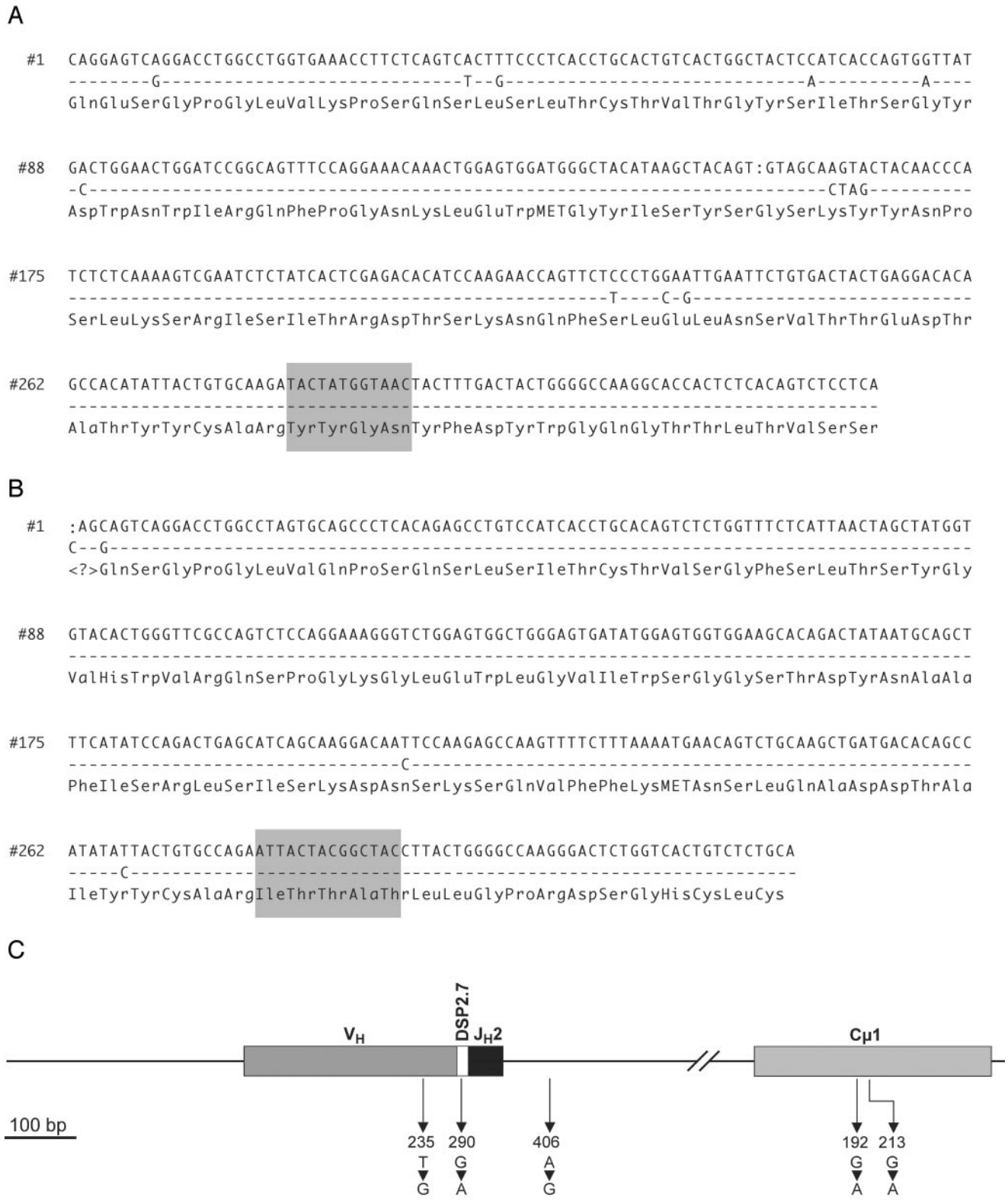


FIGURE 2. Sequences of the VDJ and Cμ1 gene segments of active (*A*) and silent (*B*) alleles. In *A* and *B*, the most similar germline sequence is given above the translation into polypeptide; dashes indicate identity of the WEHI-231 sequence with the sequence in the database. Shaded box, D region. DNA and cDNA sequences were determined for both alleles, A and B, but only A DNA and B cDNA are shown. *C*, Summary of the somatic mutations between cell line WEHI-231 and subclone WEHI-HM. Although not relevant for the work here, the productive κ allele is Cs1 joined to Jκ1.

gene encoding AID. However, the fragment sizes of the amplified cDNAs did not show any aberration, nor did the 900-nt cDNA sequence from the WEHI-231 line and from its subclone, WEHI-HM, display any mutation (not shown). Therefore, it was likely that normal AID protein is made in this line. Indeed, when we developed a Western blot from lysed cells of the WEHI-231 line with rabbit Ab to AID, we detected the predicted 25-kDa band that ran at the same position as in the 18-81 line (Fig. 1*B*, *lanes 1–3*).

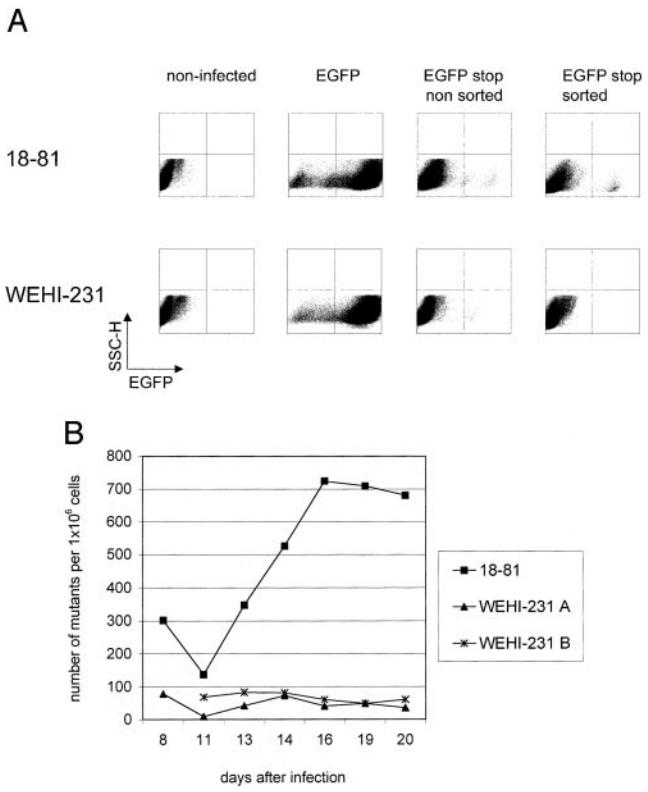


FIGURE 3. *A*, Flow cytometry profiles of cells transduced with an exogenous indicator substrate for hypermutation 13 days after infection. *y*-axis, side scatter (SSC-H); *x*-axis, fluorescence intensity, on a logarithmic scale, of EGFP. First column, 18-81 and WEHI-231 cells, not transduced; second column, transduced with the indicator substrate containing an *EGFP* gene without a premature stop codon; third column, with premature stop codon; fourth column, sorted *EGFP*-negative cell population, with premature stop codon, and expanded for 11 days. *B*, Time course of accumulation of mutations in the EGFP gene with a premature stop codon. *y*-axis, number of revertants per 1 million cells; *x*-axis, days. ■, 18-81 cells; ▲ and ∗, two independent cultures of WEHI-231 cells.

No such band is seen in the 70Z line (Fig. 1*B*, *lane 4*). On the Western blot, the bands in the WEHI-231 clones are as strong as in the 18-81 cell line (Fig. 1*B*).

WEHI-231 expresses nonmutated nuclear UNG

Because enzymatic activity of AID may be followed by UNG activity in class switching and hypermutation (20, 21, 35), we wanted to assess the integrity and expression level of the gene encoding this enzyme. There are two splice variants, UNG1 and UNG2, which localize to the mitochondria and to the nucleus, respectively (36, 37). The nuclear form, UNG2, takes part in class switching and hypermutation. Using RT-PCR, we determined that WEHI-231 expresses mRNA of apparently normal size and no lower steady state levels than the cell line 18-81, 70Z/3, or activated splenic B cells (Fig. 1*C*). Because WEHI-231 was derived from a cross between two inbred strains, one might expect allelic forms of the enzyme. Indeed, when we sequenced the cDNA encoding UNG2, we found two different alleles. The A allele is as present in the cell line 70Z/3 and is 100% identical with the sequence Y08975 in GenBank; the other is as in the 129SV mouse strain and, except for a T at position 796 (a noncoding mutation), which is shared with Y08975, is identical with the sequence AF 174485 (GenBank). Apart from the differences in the noncoding regions (nonshaded in Fig. 1*D*), the two alleles differ by one mutations in the coding region (shaded in Fig. 1*D*). At position 829, the allele A has a T, where the allele B has a C; this changes the amino acid residue from tyrosine to histidine (Fig. 1*D*). We do not know whether this changes the activity of the enzyme; but because they are present in standard mouse strains, we assume that at least one of these alleles is functional, and so we further assume that there is functional UNG2 enzyme present in the WEHI-231 line.

WEHI-231 expresses nonmutated Ku70/Ku80

The Ku70 and Ku80 proteins of the nonhomologous end joining repair pathway are both required for switch recombination (22, 23). Using RT-PCR, we determined that WEHI-231 expresses mRNA of apparently normal size and no lower steady state levels than the cell line 18-81 or 70Z/3 (Fig. 1*E*). Furthermore, the DNA sequences of the Ku70 and Ku80 alleles did not contain any mutations. It is, therefore, likely that Ku70/Ku80 function normally in this cell line.

Some canonical mutations at the H locus

Because we found no evidence for mutations in AID or UNG2 that would make these enzymes nonfunctional, we assayed the variable (V) region gene segments at the H chain (H) locus for hypermutation. Because the H alleles (and the L alleles) of WEHI-231 were not known, we first amplified the V region and Cμ1 of WEHI-231 and its subclone WEHI-HM with a generic primer for the V region via RT-PCR, subcloned the amplification products, and sequenced them. We then designed a primer specifically for the V region. This primer primes 361 nt 5′ of the V exon. Fig. 2, *A* and *B*, shows the sequences of the two alleles, from genomic DNA for the active allele and from cDNA for the silent allele. Underneath the nucleotide sequences are given the amino acid sequences. The shaded area represents the D region, after which the JH region needs to be in frame for the allele to contribute to surface Ig. This is the case for the sequence in Fig. 2*A*, which therefore is the active allele; the JH region in the sequence of Fig. 2*B* is out of frame, which therefore represents the silent allele. Although not perfect, the best fit for the active allele corresponds to nucleotide position 123800–124080 in the Ensembl database; for the silent allele it corresponds to position 46223–46501.

The WEHI-231 cell line has been in culture for some time. Because it expresses AID at a very high level, one would assume that it has mutated its V regions. When we compared this with the Ensembl database, we found 13 nucleotide differences for the active allele and 2 for the silent allele (not counting the 2 differences at the very beginning of the sequence). We do not know to what extent these differences represent mutations or polymorphism. As mentioned above, the WEHI-231 line originated in a BALB/c × NZB F_1 mouse. Because the surface IgM in this line is detected with an Ab to μ of b allotype, the active allele must be derived from the NZB mouse, and the silent allele from BALB/c mice. The changes in two positions on the silent allele are both from T to C. These transitions do not bear the signature activity of AID. One might argue that transcription dependent activity of AID ought to be similar at the rearranged active and silent alleles. Although in general, due to nonsense-mediated mRNA degradation the levels of mRNA encoding the silent allele are much lower (38), transcription levels are similar to the one of the active allele. It is also possible that mRNA is involved as well in the hypermutation process, which may decrease the rate at the silent allele.

Some of the 13 nucleotide differences between the active allele and the database may represent mutations; however, only 5 of these would be in a G · C base pair, and only 2 of them are embedded in the RGYW motif (39, 40). To be able to trace mutations, we compared the sequences of a subclone, WEHI-HM, to those of

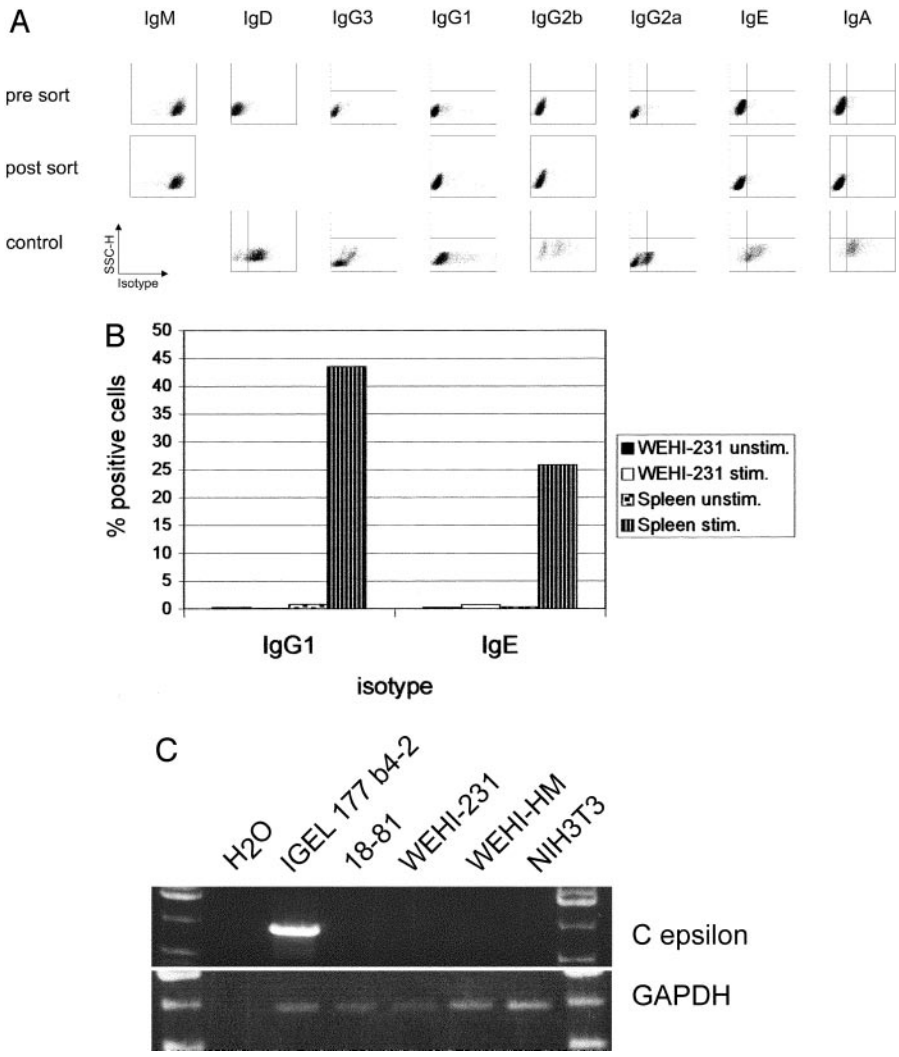


FIGURE 4. Absence of Ig class switched cells in the WEHI-231 line. *A*, Flow cytometry assessing membrane expression of the eight H chain isotypes. *y*-axis, side scatter (SSC-H); *x*-axis, fluorescence intensity on a logarithmic scale. The isotypes assayed are given above the respective flow profile. Pre sort, cells out of culture; post sort, fluorescence-positive cells sorted and grown in cell culture. Control, hybridomas synthesizing the respective isotype. *B*, Isotype switching on addition of IL4/LPS. *y*-axis, percentage of isotype positive cells; *x*-axis, IgG1 and IgE isotypes, in unstimulated (unstim.) and stimulated (stim.) WEHI-231 and spleen cells, respectively. *C*, PCR amplification of the ϵ gene in WEHI-231 and control cell lines. The *GAPDH* gene served as a method and loading control.

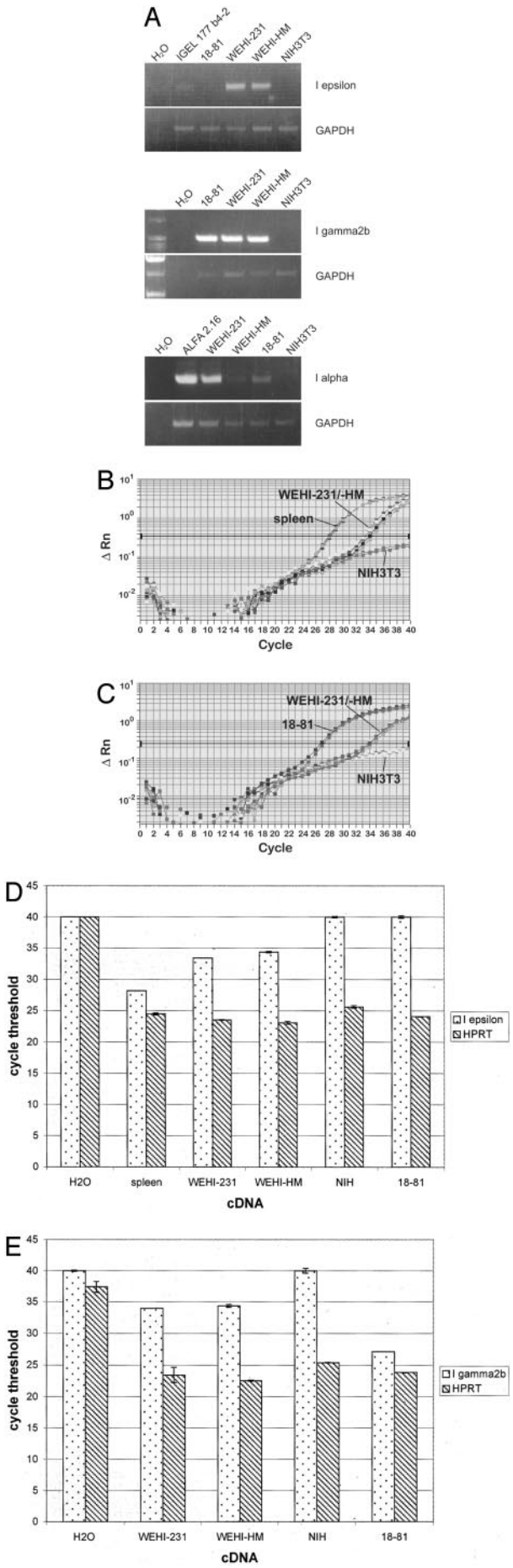
the cell line. The sequencing of the V region from genomic DNA was preceded by subcloning the amplified DNA into bacteria. In this way, the individual V region in different cells, and not just a composite of all possible sequences, was obtained. Between the five sequences of WEHI-231 and five sequences of the subclone WEHI-HM, we found three mutations (Fig. 2*C*). The T to G mutation at position 235 occurred in one of the cells of the line. That mutation could have been introduced by the method, but the other two mutations, at positions 290 and 406, respectively, could not have been; they are present in all five sequences, and they were also present when cDNA was amplified with *Taq* polymerase instead of Pfu. At position 290, in the D region, we found a G to A transition (the germline sequence has a G); at position 406, we found an A to G transition (the germline sequence has an A).

Because it is obvious that in general mutations are much less abundant in the constant region, we were quite surprised to find two mutations in the Cμ1 region (Fig. 2*C*), while surveying 12 sequences, 6 from WEHI-231 and 6 from WEHI-HM. Both nucleotide substitutions are silent mutations and therefore probably not selected for. One mutation, from G to A, embedded in a RGYW motif, occurred at position 192 in one of the six cells of the uncloned cell line (Fig. 2*C*). Because it occurred only once, it might have been introduced by the amplification. However, the other mutation, from G to A, outside of an RGYW motif, was found at position 213 in all sequences of the uncloned cell line and thus certainly has occurred in the cell line.

The Cμ a allele differs from the b allele in a single codon change, at nucleotide position 292 of exon Cμ1, where the b allele has an A, and the a allele has a G (41). Arginine, encoded by AGA, in this position prevents binding of the anti-μ^b Ab MB86 (42). In the six sequences of the subclone, three have the AAA codon (the b allele), and three have the AGA codon (the a allele). Of the six sequences of the uncloned line, none represented the a allele. We therefore think that a majority of the cells in the uncloned line have lost the silent allele (of a allotype); this is also confirmed by the fact that we did not find the silent VDJ allele in the uncloned line. At position 213, all six subclone cells (i.e., a and b alleles) have a G, as does the germline sequence. Therefore, it seems likely that the cells of the uncloned line surveyed underwent a mutation, from G to A. This would imply that a majority of the cells of the WEHI-231 line are derived from a single mutant that outgrew most of the sister cells, some of which, including the founder cell of subclone WEHI-HM, have retained the germline nucleotide. At position 192 one of the six WEHI-231 sequences bears a G to A mutation; this mutation could be method introduced.

An exogenous substrate is not hypermutated

From our limited sequencing, we cannot derive a mutation rate. However, the mutations we found were unselected and therefore are likely to be the consequence of a mutator activity. Because in fibroblasts (5) and in Chinese hamster ovary cells (43), expression of AID is both necessary and sufficient to cause



an exogenous substrate to hypermutate, we tested whether WEHI-231 would also hypermutate such a substrate. Our reporter gene consisted of an inactive EGFP that fluoresces on reversion of a premature amber stop codon (TAG) embedded in the canonical RGYW motif, which is the target of AID-mediated mutagenesis (31). Any single point mutation at the codon activates the reporter, except the unmodified transition from G to A, which creates only the stop codon TAA. We transduced the WEHI-231 and 18-81 lines with the retrovirus-based construct that contained the reporter and a gene encoding resistance to puromycin, with the cells being kept continuously under puromycin selection. As a control, the reporter gene contained functional EGFP, without stop codon. After 3 days, we performed flow cytometry on the lines (Fig. 3). Over 97% of cells in both cell lines, 18-81 and WEHI-231, expressed the EGFP control construct with high fluorescence intensity. However, the profiles were different for the two cell lines with the EGFP containing the stop codon: in the 18-81 line, two mutant cell populations accumulated, one with high fluorescence intensity and one with an intermediate intensity; in the WEHI-231 line, there was only the intermediate intensity mutant population. We know that the high intensity EGFP cells are generated by AID-mediated mutagenesis, and we confirmed this by sequencing (not shown) (31). In this population, reversions of the stop codon TAG is usually accomplished by a G to C, and, less frequently, by a G to T transversion (31, 33).

We assume that these revertants in the population of intermediate intensity are generated by the reverse transcriptase of the retroviral vector. We have three reasons for why we think that this assumption is correct: 1) we found such a population also in the 70Z cell line, where no AID is expressed; 2) the intermediate fluorescence intensity of the revertants indicated to us that the stop codon had not reverted at the G nucleotide. Indeed, when we sequenced the revertants, we found that the TAG invariably was converted into a TGG, which encodes tryptophan (not shown). Apparently, a tryptophan in this position results in less than optimal fluorescence of EGFP. Because of AID acting on G · C, the mutation expected to be generated is TAT or TAC, which encode tyrosine; 3) because the reverse transcriptase is present only at the time of transduction, such revertants do not accumulate; obviously dependent on the error frequency of the reverse transcriptase, they are generated only when a sufficient number of cells is transduced. Indeed, when we sorted the EGFP-negative population of 18-81 and WEHI-231, respectively, the revertant population of intermediate fluorescence intensity did not return after 10 days in either cell line (Fig. 3*A*). However, the high intensity revertant population returned in the 18-81 cell line, but not in the WEHI-231 line. Fig. 3*B* shows a time course of accumulation of mutations in the reporter construct, as measured by flow cytometry. The duplicate cultures of WEHI-231, designated A and B, did not accumulate

FIGURE 5. Production of Ig germline transcripts in WEHI-231. *A*, RT-PCR amplification of ϵ (I epsilon), γ2b (I gamma2b), and α transcripts (I alpha). H_2O, amplification without template, but all other reagents. *B–E*, Taqman analysis of ϵ transcript (*B*) and of γ2b transcript (*C*); *D*, relative expression levels of ϵ and (E) γ2b transcripts in the various cell lines. *x*-axis in *B* and *C*, number of cycles needed to reach a preset value, on the *y*-axis; i.e., the lower the number of cycles, the higher is the expression level of the cDNA. HPRT, cDNA encoding the enzyme HPRT. Rn, Normalized reporter signal quotient of the fluorescence signal of the reporter dye, divided by the fluorescence signal of the passive reference dye; Delta Rn, normalized reporter signal minus the baseline signal established in the first few cycles of PCR.

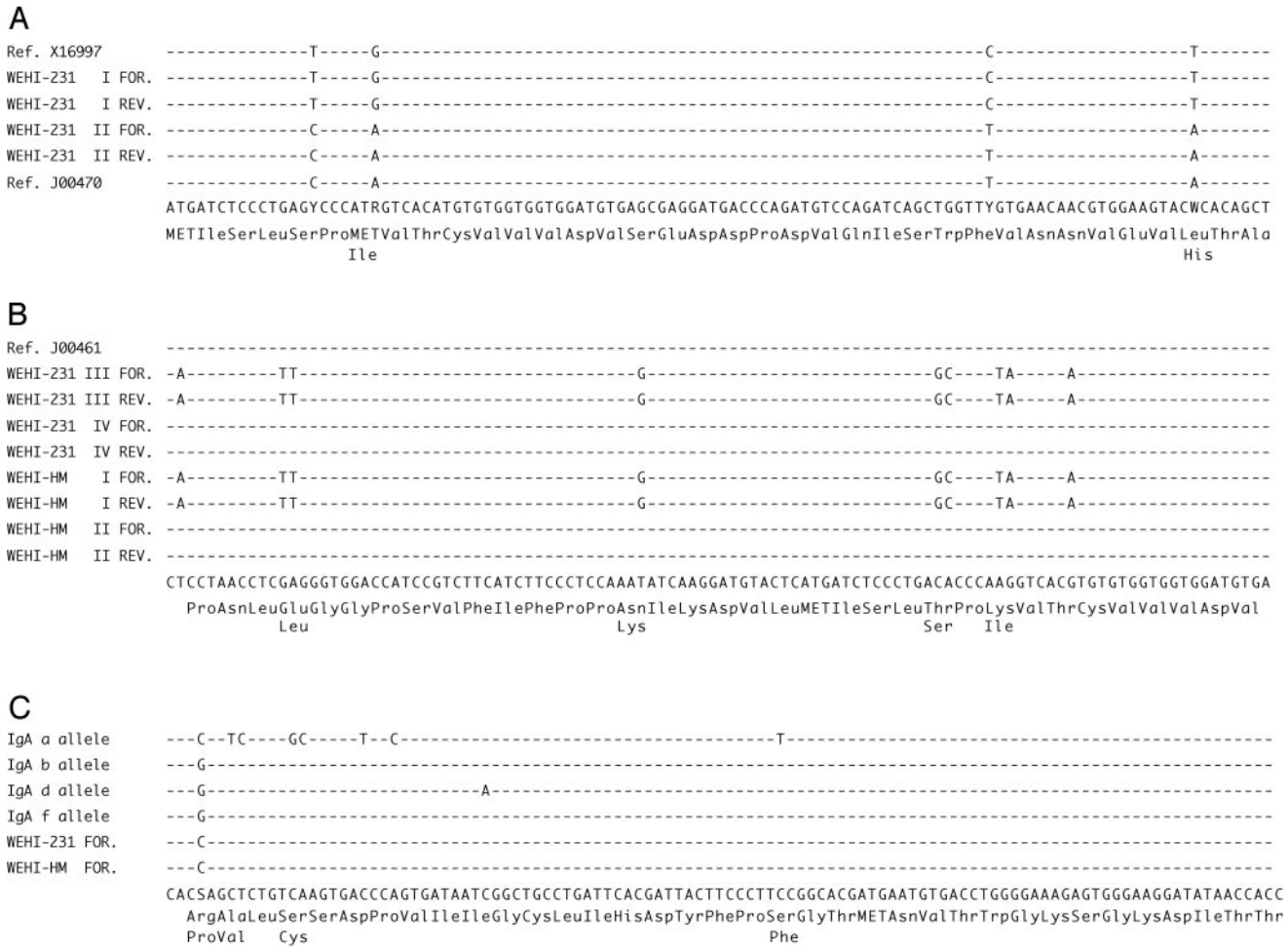


FIGURE 6. Partial sequences of the γ2a, γ2b, and α alleles. *A*, Alleles of cγ2a in WEHI-231 (sequences I and II), sequenced in forward (FOR.) and reverse (REV.) orientation. Ref. X16997, GenBank sequence of Ig^b; Ref. J00470, GenBank sequence for Ig^a. Underneath the consensus sequence, translation of the b allele into polypeptide and amino acid residue substitutions between the alleles. *B*, Alleles of cγ2b in WEHI-231 (sequences III and IV), and a WEHI-231 subclone (sequences I and II), sequenced in forward (FOR.) and reverse (REV.) orientation. Ref. J00461, GenBank sequence for Ig^a. *C*, α alleles of various allotypes and the non-Ig^a alleles in WEHI-231 and its subclone (WEHI-HM), sequenced in forward (FOR.) orientation. Translation as from the b allele.

any mutations over the time period assayed here, whereas the 18-81 line did.

From our flow cytometry experiments with the mutation reporter construct, it is clear that WEHI-231 does not show the full-range hypermutation spectrum that 18-81 shows. Specifically, it seems that the C to U transition cannot be modified by replacing the incipient U by another nucleotide. Because our reporter construct does not score unmodified C to U transitions, we cannot be certain whether the AID expressed in WEHI-231 performs this initial step in hypermutation. However, the G to A mutations at the endogenous locus indicate that this might be the case to some extent. If so, then it is interesting that because UNG2 seems to be functional in WEHI-231 (Fig. 1, *C* and *D*), the downstream events, thought to be set off by UNG2 removing the deoxyuridine (21), must differ between the 18-81 and WEHI-231 cell lines; while in 18-81, the U is replaced by a G or an A, this is not the case in WEHI-231.

Lack of class switching

Because of its high level expression of AID, it was possible that WEHI-231 switches its IgM to other subclasses. However, isotypes other than μ have not been reported in this line. Given the long life of this line in cell culture facilities all over the world, one would assume that if the line is actively switching that other isotypes would accumulate. When we stained WEHI-231 for all Ig subclasses, we found no evidence of switched cells (Fig. 4*A*). In our flow cytometry experiments, we used commercially available Abs to all eight classes. As positive controls, we stained various hybridoma cell lines. Hybridoma lines display very little Ig on their surface; the little there is presumably is on its way to be secreted; putative switched cells of WEHI-231, however, would be expected to show much higher intensity. We found that besides cells with IgM, in WEHI-231 there are no cells that express any other Ig isotype (Fig. 4*A*).

In flow cytometry profiles, there are always some cells with high intensities, where none is expected. Thus, we found a few such cells in our stainings for IgG1, IgG2b, IgE, and IgA. To decide whether these cells represent background or rare switched cells, we sorted them and grew them in cell culture, to be flow profiled again (Fig. 4*A*). The expanded cultures contained only cells with surface IgM, thereby confirming that WEHI-231 does not switch its IgM class, not even after stimulation with IL-4 and LPS for 65 h (Fig. 4*B*). Because we were particularly interested whether WEHI-231 would contain some IgE cells, we used PCR amplification between V and Cϵ primers. Although we could easily amplify the ϵ gene from the IgE-producing hybridoma IGELb4-2 (44), there were no

bands in WEHI-231 and its subclone WEHI-HM or in the 18-81 line, which switches from μ to γ2b, or in the NIH3T3 line with no Ig genes (Fig. 4*C*).

Germline transcripts

One can think of various reasons why there are no cells with switched Ig isotype in WEHI-231, despite the expression of apparently normal AID and UNG. WEHI-231 depends on the presence of signals from IgM for growth; thus, it is possible that cells with another isotype no longer provide this signal. However, because class-specific molecules analogous to the Ag receptor-associated signaling molecules Igα and Igβ have not yet been found, this may be less likely. Another possibility for why the cells do not switch would be the lack of an open configuration of the locus to which the cell is going to switch. Such an open configuration is indicated by the transcription of the so-called germline transcripts, which originate at a promoter upstream of the respective I exon, extend through the switch region and constant region, and are spliced so that the I exon is joined to the C region (45–47). Such germline transcripts are harbingers of the class switch recombination (48), and their splicing is necessary for switch recombination to occur (49). We therefore examined whether or not WEHI-231 produces germline transcripts of any of the six constant region loci to which the cells might switch. We RT-PCR amplified transcripts of γ2b, ϵ, and α and found that WEHI-231 expresses all of these, whereas the 18-81 cell line lacks the ϵ transcript and has a smattering of an α transcript (Fig. 5*A*), and NIH3T3 has none of them. Because class switching can occur on either one allele only or both alleles, hybridomas that are derived from switched cells may or may not contain a germline transcript. On switching an allele, the gene encoding the germline transcript is lost on that allele. Apparently, the ALFA hybridoma, which was derived from a fusion between the I29 cell line and the plasmacytoma Ag8.653, retained the silent allele in the germline configuration and therefore still synthesizes the α germline transcript (Fig. 5*A*). In the IGEL b4-2 hybridoma line, there is very little Iϵ transcript. We think that due to chromosome loss, there are few cells left with the silent homologue of chromosome 12, where the IgH is located. At any rate, we verified the identity of all these transcripts by sequencing.

The WEHI-231 line seems to express as much γ2b germline transcript as the switching line 18-81, and almost as much α transcript as the ALFA hybridoma. However, the quantitative outcome of RT-PCR can be very deceptive, and it is possible that the amount of transcript is much lower in WEHI-231 and simply not enough to start the recombination process. Indeed, when we performed Taqman analysis for the ϵ and γ2b transcripts, this proved to be the case (Fig. 5, *B–E*). In comparison with the stimulated spleen cells, expression of the ϵ transcript was 74 times lower in WEHI-231 and 195 times lower in the subclone WEHI-HM (Fig. 5, *B* and *D*). Expression of the γ2b transcript was 151 times lower in WEHI-231 and 370 times lower in the subclone WEHI-HM than the cell line 18-81 (Fig. 5, *C* and *E*). Thus, although it is not known how much transcription off the I promoter is needed to instigate the switch recombination process, there might not enough germline transcript expressed to enable WEHI-231 to switch.

There also remained the possibility that the gene segments downstream of Cμ might have been deleted on the active homologue of the WEHI-231 cell line. In that case, no switching to any of the other classes would be possible. To address this question, we analyzed the γ2b, γ2a, and α loci by PCR amplification and sequencing. Because WEHI-231 is of BALB/c $\times$ NZB F_1 origin, it has two different Ig haplotypes. The haplotype derived from BALB/c is Ig^a, and fully sequenced, whereas the NZB haplotype is less defined. However, the IgM expressed on the surface of this line reacts with an Ab to the b allotype (Fig. 4*A*), i.e., non-BALB/c; and by sequencing, we could assess it as being non-Ig^a as well. In some instances, sequences deposited in GenBank exactly matched allelic fragments of non-Ig^a allotypes. At least exons 1 to 4 of γ2b and γ2a were present on both alleles the WEHI 231 cell line, as well as some sequences of the active α allele (non-Ig^a) (Fig. 6). Although this does not exclude defects in the switch regions, or in sequences not covered, they would have to be present in the γ2b, γ2a, and α loci; but certainly, there is no wall-to-wall deletion downstream of cμ.

In this paper, we have shown that the WEHI-231 cell line expresses apparently normal AID and UNG2, at levels that in other cells mediate hypermutation and Ig class switching. Moreover, Ku70/Ku80 expression was also normal. However, we found no evidence for the two processes to be active in a complete form in this line. In the case of class switching, there might not be enough germline transcript produced, even though it is astonishing that there are such transcripts at all, considering that this line is thought to represent an immature B cell. Any of the unknown or known other necessary factors for class switching, such as mismatch repair proteins, and H2AX, might not be expressed or mutated. However, surely there is enough transcription off the V region promoter, needed for hypermutation. We think that the first step in hypermutation, the transition from C to U, is accomplished in the line but that despite the fact that UNG is functional, the U is not replaced by G or A. Whatever the case, the WEHI-231 cell line may be useful for cloning the putative missing cofactors in hypermutation and class switching, especially the factors that guide switch recombination to one Ig class over the other (50).

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