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Uracil DNA Glycosylase Disruption Blocks Ig Gene Conversion and Induces Transition Mutations

Huseyin Saribasak,* Nesibe Nur Saribasak,† Fatih M. Ipek,* Joachim W. Ellwart,‡ Hiroshi Arakawa,* and Jean-Marie Buerstedde*‡

Ig gene conversion is most likely initiated by activation-induced cytidine deaminase-mediated cytosine deamination. If the resulting uracils need to be further processed by uracil DNA glycosylase (UNG), UNG inactivation should block gene conversion and induce transition mutations. In this study, we report that this is indeed the phenotype in the B cell line DT40. Ig gene conversion is almost completely extinguished in the UNG-deficient mutant and large numbers of transition mutations at C/G bases accumulate within the rearranged Ig L chain gene (IgL). The mutation rate of UNG-deficient cells is about seven times higher than that of pseudo V gene-deleted (ψV−) cells in which mutations arise presumably after uracil excision. In addition, UNG-deficient cells show relatively more mutations upstream and downstream of the VJ segment. This suggests that hypermutating B cells process activation-induced cytidine deaminase-induced uracils with approximately one-seventh of uracils giving rise to mutations depending on their position. The Journal of Immunology, 2006, 176: 365–371.

The activation-induced cytidine deaminase (AID) gene tightly regulates all three B cell-specific processes of Ig repertoire formation: hypermutation, gene conversion, and switch recombination (1–4). Based on the findings that uracil DNA glycosylase (UNG)-deficient mice and human patients show reduced switch recombination and a shift of mutations at C/G bases toward transitions (5, 6), it was postulated that AID exerts its action within the Ig loci by the deamination of cytosines to uracils which are subsequently further processed by UNG.

One of the best cellular systems to study the mechanism of AID-induced gene conversion and hypermutation is the chicken B cell line DT40 (7). DT40 diversifies its rearranged Ig L chain gene (IgL) almost exclusively by ψV-templated gene conversions in an AID-dependent fashion (3). The gene conversion events typical of wild-type DT40 are, however, replaced by frequent single nucleotide substitutions when homologous recombination factors like the RAD51 paralogues XRCC2, XRCC3, and Rad51B are inactivated (8), or the nearby ψV locus is removed (9). The hypermutation activity elicited by the block of gene conversion shares many features with Ig hypermutation in mice and human B cells, but is limited to C/G bases. Expression of the dominant-negative inhibitor of UNGs, Ugi, shifted the mutation spectrum toward transitions efficiently and specifically (10), providing first evidence that uracil is a likely intermediate in the hypermutation process.

Since the studies cited above indicate that Ig gene conversion is initiated by AID-induced cytosine deamination in the rearranged V(DJ) segments, it is an interesting question whether gene conversion, in analogy to hypermutation and switch recombination, requires the processing of uracils by UNG. It was reported (11) that expression of an Ugi transgene in DT40 reduced gene conversion to 30% of the wild-type level, suggesting that UNG activity enhances gene conversion. However, this phenotype remains difficult to interpret, because, contrary to expectation, no evidence for an increased mutation rate was found, and it was unclear how efficiently and specifically Ugi transgene expression inhibited UNG activity.

We, therefore, decided to disrupt both copies of the UNG gene in DT40 and analyze the mutant phenotype with respect to Ig gene conversion and hypermutation.

Materials and Methods
Isolation of chicken UNG cDNA
The EST dkfz426_17p6r1 of the bursal EST database (12) shows significant homology to the murine UNG cDNA. The corresponding clone was sequenced by bidirectional primer walk and found to contain a 1.1-kb cDNA insert including the full-length UNG open reading frame of 299 aa. The amino acid sequence of the chicken UNG is 79 and 75% identical to the human and murine UNG, respectively.

Construction of gene targeting and cDNA expression constructs
Primers derived from the chicken UNG cDNA were used to amplify the genomic locus by long-range PCR using DNA from DT40 as template. The exon-intron structure deduced from the PCR fragments was verified when the chicken genome sequence, including the UNG locus on chromosome 15, was released (13). The targeting constructs were made by cloning appropriate genomic fragments upstream and downstream of floxed marker genes (14). The constructs were linearized by NotI before transfection. The UNG expression cassette was made by cloning the chicken UNG cDNA downstream of the β-actin promoter and upstream of an IRES-GPT sequence.

Cell culture
Cell culture, transfection, selection of stable transfecants, and marker re-centering by transient Cre induction were performed as previously described (3).

UNG activity assay
Exponentially growing cells were washed twice with PBS and resuspended at a concentration of ~10⁶ cells/μl in a buffer containing 25 mM
HEPES, 5 mM EDTA, 1 mM DTT, 10% glycerol, and a mixture of protease inhibitors (10). The cells were then freeze-fractured by liquid nitrogen and centrifuged at 6500 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and then centrifuged again at 5000 rpm for 5 min at 4°C. The supernatant of this second centrifugation was stored as cell extract at −20°C. UNG activity was measured by incubating 5 μl of cell extract with 10 pmol of a 5′ FITC-labeled oligonucleotide containing a single uracil at position 23 (5′-TCACCGTGCTCCGGGGGTGGCAGUTA CGGCTGAGGTGACTATTAGGGTCTG-3′) in a final volume of 10 μl for 2 h at 37°C. Control reactions were incubated with an oligonucleotide in which the uracil was replaced by cytidine. Because AP endonuclease activity in the extracts was found to be insufficient for complete cleavage of the abasic site after uracil excision, 5 μl of 0.5 M NaOH was added to the reaction and incubation was continued for 1 h at 37°C. The DNA was then precipitated by ethanol and resuspended in 10 μl of formamide-loading buffer before being analyzed on a 15% Tris-borate-EDTA-urea PAGE gel. After electrophoresis, gel images were visualized using a Fuji Film (FLA-3000) phosphor imager.

Ig reversion assays and sequence analysis
Quantification of surface IgM (sIgM) expression by FACS and sequence analysis of L chain VJ regions have been described previously (3, 9).

Results
UNG inactivation does not affect cell viability in DT40
The 5′ and 3′ arms of the UNG knockout constructs were designed to insert an early in-frame stop codon at codon 66 and delete the region encoding the highly conserved codons 67–120. Two versions of the constructs, named pUngBsr and pUngGpt, included a floxed blasticidin S resistance gene and a floxed guanine phosphoribosyl transferase gene (14), respectively (Fig. 1A). Targeted integration was detected by PCR using a primer located upstream of the 5′ targeting arm of the construct (ung1, Fig. 1A) along with a primer from the resistance marker.

Transfection of pUngBsr into a sIgM (−) variant of the DT40 clone AIDRk (9) yielded a targeting frequency of ~1 in 10. One of the heterozygous UNG+/− clones was then transfected by pUngGpt. This construct integrated frequently into the already targeted UNG allele, but only one clone with targeted integration into the other allele was found upon screening of >300 transfectants. The identified UNG-deficient clone showed normal cell viability and cloning efficiency.

**FIGURE 1.** UNG gene disruption. A, Aligned maps of the chicken UNG locus, the targeting constructs, and the disrupted locus after targeting integration of the constructs and marker excision. The locations of the primers used to confirm the UNG gene disruption are indicated. B, Analysis of the UNG locus by PCR. The AIDk precursor clone is compared with the AIDk/UNG−/− mutant. C, UNG assay using cell extracts. The structure of the oligonucleotide substrate is shown below the gel image.
suggesting that the low recovery of homozygous clones was not related to a growth defect of UNG-deficient cells. Excision of the bsr and gpt marker cassettes from the double-targeted clone yielded a clone named AID8/UNG−/− that was used for further analysis. Genomic PCR of AID8/UNG−/− confirmed the homozygous deletion of part of the UNG loci (Fig. 1B).

AID8/UNG−/− was transfected by an UNG cDNA expression vector in which the UNG gene is expressed as a bicistronic message along with the gpt transgene. One of the mycophenolic acid resistant transfectants was named AID8/UNG8 and retained for further analysis.

UNG activity is strongly reduced in UNG-deficient cells

Cell extracts of the AID8, AID8/UNG−/−, and AID8/UNG8 clones were incubated with a uracil-containing oligonucleotide. Excision of the uracil by UNG leads to an abasic site, which can be detected by the appearance of a shorter oligonucleotide after NaOH-mediated strand cleavage (Fig. 1C). In the absence of in vitro UNG treatment, the amount of cleavage product was strongly reduced in extracts of AID8/UNG−/− cells compared with extracts of wild-type and reconstituted AID8/UNG8 cells. This confirms that the UNG activity is severely compromised by the UNG gene disruptions and that most of the activity in wild-type cell extracts is linked to UNG. Nevertheless, a low level of cleavage was still detectable after incubation with AID8/UNG−/− extract (Fig. 1C, lane 2), but not in the “No extract” control (Fig. 1C, lane 5) upon prolonged incubation time. Because the UNG gene disruption most likely behaves like a null mutation, the residual activity may reflect the presence of one or more backup UNGs.

Ig frameshift repair by gene conversion is blocked in the absence of UNG

The three clones, AID8, AID8/UNG−/−, and AID8/UNG8, carry a frameshift mutation in their rearranged L chain V segment, which can be corrected by gene conversion giving rise to slgM− revertants. To determine how inactivation of the UNG gene affects frameshift repair by gene conversion, slgM(+) subpopulations were measured for 24 subclones of each of the three clones and the stable slgM(−) control clone DT40 IgL−. Representative FACS profiles and the calculation of the average percentages of gated events reveal a striking reduction of slgM(+) cells for AID8/UNG−/− (Fig. 2A). It remains uncertain whether slgM reversion still occurs at a low level in UNG−/− cells or whether it is entirely blocked, since the average of slgM(+) events in AID8/UNG−/− subclones (0.35%) is only slightly above the background noise seen in DT40 IgL− (0.24%). The lack of slgM reversion is partially complemented in the AID8/UNG8 clone and sequence analysis of slgM(+) subpopulations confirmed that the L chain frameshift was indeed repaired by gene conversions (data not shown). These results demonstrate that inactivation of UNG severely compromises Ig gene conversion.

UNG-deficient cells rapidly lose slgM

The Ig mutation rate is most conveniently quantified in slgM(−) clones by measuring the appearance of slgM(+) cells due to deleterious mutations (9). To study whether UNG inactivation induces Ig hypermutation, slgM(+) cells of an AID8/UNG8 subclone were sorted, exposed to different levels of tamoxifen, and subcloned. This procedure allowed the isolation of the following slgM(+) clones: 1)
AID\(^R\) retaining both floxed AID and UNG expression cassettes, 2) AID\(^{-/-}\) UNG\(^{-/-}\) lacking the AID and the UNG cassettes, and 3) AID\(^R\)ΨV\(^/-\) clone 1–3 retaining the AID, but lacking the UNG cassette. The appearance of sIgM(\(^/-\)) subpopulations was measured in parallel in subclones of these clones, as well as in subclones of control sIgM(\(^/-\)) AIDR and AIDR\(^/-\)ΨV\(^/-\) (9) clones (Fig. 2B). This analysis revealed dramatically increased percentages of sIgM(\(^/-\)) cells for all three AID\(^R\)UNG\(^/-\) clones (49.00, 53.93, and 34.57%, respectively) compared with the AIDR precursor clone (1.50%). AID\(^R\)UNG\(^/-\) subclones displayed on average twice as many sIgM(\(^/-\)) cells than AIDRΨV\(^/-\) clones (23.84%), which accumulate L chain hypermutations due to the loss of ΨV gene conversion donors. The rise of sIgM(\(^/-\)) was significantly reduced in the AID\(^R\)UNG\(^/-\) clone (7.18%) upon reintroduction of an UNG transgene. The AID\(^R\)ΨV\(^/-\) clone remained stably sIgM(\(^/-\)), showing that UNG disruption only causes sIgM(\(^/-\)) loss in the presence of AID expression.

UNG inactivation induces a high rate of Ig transition mutations

To determine the cause of the rapid sIgM loss in AID\(^R\)UNG\(^/-\) cells, the rearranged Ig L chain genes were sequenced for two AID\(^R\)UNG\(^/-\) and two AID\(^R\)ΨV\(^/-\) subclones 6 wk after subcloning. As expected from the results of the sIgM analysis, sequences of AID\(^R\)UNG\(^/-\) cells showed an extraordinarily high number of single nucleotide substitutions at G/C bases and only few gene conversion tracts (Fig. 3). The average number of mutations per sequence was 7-fold higher in sequences of AID R UNG\(^/-\) than that of AID RΨV\(^/-\) cells (Table I). Almost all mutations of AID\(^R\)UNG\(^/-\) cells (98%), but only about one-third of the mutations of AID RΨV\(^/-\) cells (40%) were transitions (Fig. 4A). Mutations in both cell populations occurred preferentially at the known hypermutation hot spots (~50%, data not shown), almost exclusively at G/C bases and in about equal numbers at G and C bases.

Table 1. Mutation comparisons

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Culture Time (wk)</th>
<th>Region</th>
<th>Total Mutations</th>
<th>Total Sequences</th>
<th>Mut/ bps(^{-10^{-3}})</th>
<th>PM</th>
<th>GC</th>
<th>Del</th>
<th>Dup</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>AID(^R)UNG(^/-)</td>
<td>1</td>
<td>LVJ-intron (1360 bp)</td>
<td>610</td>
<td>374</td>
<td>1.15</td>
<td>583</td>
<td>24(^c)</td>
<td>2</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>AID(^{-/-})UNG(^/-)</td>
<td>6</td>
<td>LVJ-intron (1360 bp)</td>
<td>963</td>
<td>95</td>
<td>7.38</td>
<td>954</td>
<td>5</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AID(^{-/-})ΨV(^/-)</td>
<td>6</td>
<td>VJ-intron (480 bp)</td>
<td>2</td>
<td>78</td>
<td>0.05</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AID(^R)ΨV(^/-)</td>
<td>6</td>
<td>LVJ-intron (1372 bp)</td>
<td>603</td>
<td>376</td>
<td>1.14</td>
<td>592</td>
<td>—</td>
<td>7</td>
<td>4</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Mut, mutation; PM, point mutation; GC, gene conversion; Del, deletion; Dup, duplication; I, insertion.

\(^b\) Only point mutations were counted.

\(^c\) Conversion events seem to have occurred early during clone expansion from one subclone.
number of mutations at A and T is so low that it is difficult to rule out artifacts due to PCR amplification. Likewise, very few mutations were detected in sequences of rearranged VJ regions from AID\(^{+/−}\)/H11002/UNG\(^{+/−}\)/H11002 cells and sequences of the EF1\(α\) gene from AID\(^{+/−}\)/ΨV\(^{+/−}\) cells (Table I), confirming that the mutation activity induced by UNG disruption is AID dependent and Ig locus specific.

**FIGURE 4.** Comparison of the mutations from AID\(^{+/−}\)/UNG\(^{+/−}\) and AID\(^{+/−}\)/ΨV\(^{+/−}\) cells. A, Analysis of the mutation pattern of Ig L chain sequences 6 wk after subcloning. Only single nucleotide substitutions are included. The ratios of transition (trs) to transversion (trv) are shown in numbers and percentages. B, Distribution of mutations along the rearranged L chain gene. The number of mutations occurring within adjacent 10-bp intervals is plotted along the rearranged L chain gene sequence.
Mutations from AID<sup>R</sup>UNG<sup>−/−</sup> and AID<sup>R</sup>ΨV<sup>−</sup> cells are differently distributed

If uracils are not removed in the absence of UNG until the next replication cycle, transition mutations of UNG-negative cells will mark the positions of all AID-induced uracils. In contrast, hypermutations of UNG-positive cells indicate the positions of only those uracils that escaped error-free repair after UNG processing. To compare the location of AID<sup>R</sup>UNG<sup>−/−</sup> and AID<sup>R</sup>ΨV<sup>−</sup> mutations, their respective numbers within windows of 10 bases were plotted against the rearranged Ig L chain gene sequence (Fig. 4B). This display shows that AID<sup>R</sup>UNG<sup>−/−</sup> mutations are more scattered and less focused on the VJ regions than AID<sup>R</sup>ΨV<sup>−</sup> mutations. To rule out that the difference is an artifact due to the divergent mutation load, AID<sup>R</sup>UNG<sup>−/−</sup> sequences were determined 1 wk after subcloning. The average number of mutations per sequence in this culture is similar to the average in the 6-wk

![Diagram](https://example.com/diagram.png)

**FIGURE 5.** A model explaining the regulation of Ig gene conversion and Ig hypermutation by AID and UNG.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Sequences</th>
<th>Upstream V (560 bp)</th>
<th>VJ (313 bp)</th>
<th>Downstream J (487 bp)</th>
<th>Total (1360 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AID&lt;sup&gt;R&lt;/sup&gt;UNG&lt;sup&gt;−/−&lt;/sup&gt; (1 wk)</td>
<td>374</td>
<td>141&lt;sup&gt;a&lt;/sup&gt; (24%)</td>
<td>381 (65%)</td>
<td>61 (11%)</td>
<td>583</td>
</tr>
<tr>
<td>AID&lt;sup&gt;R&lt;/sup&gt;UNG&lt;sup&gt;−/−&lt;/sup&gt; (6 wk)</td>
<td>95</td>
<td>140 (15%)</td>
<td>609 (64%)</td>
<td>205 (21%)</td>
<td>954</td>
</tr>
<tr>
<td>AID&lt;sup&gt;R&lt;/sup&gt;ΨV&lt;sup&gt;−&lt;/sup&gt; (6 wk)</td>
<td>376</td>
<td>22 (4%)</td>
<td>540 (91%)</td>
<td>30 (5%)</td>
<td>592</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of mutations
<sup>b</sup> The percentage of mutations out of total.
The different distribution of AIDRUNG from the two cultures are similarly spread along the L chain gene (Fig. 4B), indicating that the distribution is not influenced by the mutation average. The different distribution of AID\(^{\text{UNG}^{-/-}}\) and AID\(^{\text{ψ}\text{V}^{-}}\) mutations is also evident from the relative number of mutations located upstream of the V segment’s start, within the VJ coding region and downstream of the J segment’s end (Table II). Whereas the VJ coding region encompasses 91% of the AID\(^{\text{ψ}\text{V}}\) mutations, this region contains only 65 and 64% of the AID\(^{\text{UNG}^{-/-}}\) mutations from the 1- and 6-wk cultures, respectively (Table II).

### Discussion

Inactivation of the UNG gene in AID expressing DT40 almost completely abolishes Ig gene conversions and activates a very high rate of transition mutations at C/G bases in the rearranged L chain VJ region. This demonstrates conclusively that Ig gene conversion, like previously shown from hypermutation and switch recombination (5, 6), requires UNG downstream of AID. Nevertheless, a minor alternative pathway seems to be responsible for the few gene conversion tracts still seen in UNG-deficient cells.

The results strongly support a model in which AID first deaminates cytosine within the rearranged V(D)J segments, and the resulting uracils are then processed by UNG to give rise to either transition and transversion mutations or to gene conversions (Fig. 5). Inactivation of the UNG gene blocks the processing of uracils and leads exclusively to transition mutations when uracil pairs with adenine in the next replication cycle. So far, this model had been based on results showing that Ugi expression shifts Ig hypermutations toward transitions in a XRCC2-deficient mutant (10) and inhibits Ig gene conversion in wild-type DT40 (11). However, the phenotype of the Ugi gene transfectants differ from the phenotype of the UNG-deficient mutant in some features at odds with the model. For example, Ugi expression increased transition mutations only to 86% in the XRCC2-deficient mutant (10), whereas this number was 98% from the UNG-deficient mutant. Similarly, Ugi expression reduced Ig gene conversion to 30% of wild-type levels (11), whereas the UNG gene disruption decreased gene conversion almost 100-fold based on the L chain frameshift repair assay. The most striking discrepancy to the model is the lack of detectable hypermutation after Ugi expression in wild-type DT40. The difference between the results obtained after Ugi transfection and UNG disruption could be due to the fact that UNG-deficient cells express a transcribed AID, which may differ both in abundance and cell cycle regulation from endogenous AID. However, the most straightforward interpretation seems to be that Ugi expression in DT40 only partially inhibited UNG activity.

Murine and human B cells do not use gene conversion for Ig repertoire development and hypermutating chicken DT40 mutants differ from primary B cells, as they do not mutate A/T bases. Nevertheless, the initial steps leading to Ig gene conversion and hypermutations at C/G bases seem to be identical in all three cell systems. Thus, hypermutations at C/G bases are shifted toward transitions in UNG-deficient mice and human patients (5, 6), and B cells of the murine UNG/MSH2 double knockout which lack the A/T mutator due to the MSH2 defect show a mutation spectrum very similar to the DT40 UNG-deficient mutant (15).

If AID-induced uracils are not repaired in the absence of UNG, hypermutations in UNG-deficient DT40 cells will offer a unique glimpse at the in vivo cytosine deamination activity of AID without subsequent selection or repair bias. The similar numbers of mutations at C and G bases from UNG-deficient cells imply, for example, that the transcribed and the nontranscribed DNA strand are equally accessible to AID. A comparison of the transition mutations of UNG-deficient cells with hypermutations that accumulate in ψV cells after the UNG-mediated processing of uracils provides further insight into how the hypermutation process is initiated. UNG-deficient cells accumulate L chain mutation seven times faster than ψV cells, indicating that about one of seven AID-induced uracils is processed into a mutation. This relative frequent conversion of uracils into mutations suggests the presence of an unusual error-prone repair pathway that specifically recognizes AID-induced uracils. Interestingly, the distribution of mutations within the rearranged L chain gene varies between UNG-deficient and ψV-deleted cells. Although the majority of mutations arise within the VJ coding region in both cell types, relatively more mutations in UNG-deficient cells are located upstream of the V and downstream of the J coding regions. The most plausible explanation for this phenomenon is that UNG-mediated processing of uracils is more error prone within the VJ segment than in the flanking regions. Thus, the comparison of UNG and ψV mutations provides the first evidence that the characteristic distribution of Ig hypermutation over the V(D)J regions reflects not only AID-mediated cytosine deamination, but also UNG-mediated permutation of the resulting uracils in a position-dependent fashion.

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### Disclosures

The authors have no financial conflict of interest.

### References


