Ectopic Expression of AID in a Non-B Cell Line Triggers A:T and G:C Point Mutations in Non-Replicating Episomal Vectors

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Somatic hypermutation (SHM) of immunoglobulin genes is currently viewed as a two step process initiated by the deamination of deoxycytidine (C) to deoxyuridine (U), catalysed by the activation induced deaminase (AID). Phase 1 mutations arise from DNA replication across the uracil residue or the abasic site, generated by the uracil-DNA glycosylase, yielding transitions or transversions at G:C pairs. Phase 2 mutations result from the recognition of the U:G mismatch by the Msh2/Msh6 complex (MutS Homologue), followed by the excision of the mismatched nucleotide and the repair, by the low fidelity DNA polymerase η, of the gap generated by the exonuclease I. These mutations are mainly focused at A:T pairs. Whereas in activated B cells both G:C and A:T pairs are equally targeted, ectopic expression of AID was shown to trigger only G:C mutations on a stably integrated reporter gene. Here we show that when using non-replicative episomal vectors containing a GFP gene, inactivated by the introduction of stop codons at various positions, a high level of EGFP positive cells was obtained after transient expression in Jurkat cells constitutively expressing AID. We show that mutations at G:C and A:T pairs are produced. EGFP positive cells are obtained in the absence of vector replication demonstrating that the mutations are dependent only on the mismatch repair (MMR) pathway. This implies that the generation of phase 1 mutations is not a prerequisite for the expression of phase 2 mutations.

INTRODUCTION

Affinity maturation of the humoral immune response arises from the stepwise introduction of single nucleotide substitutions into the variable regions of immunoglobulin genes during B cell proliferation in germinal centers. This process is known as somatic hypermutation (SHM) and depends on the expression of AID, the activation induced cytidine deaminase whose expression is restricted to centroblast B cells [1,2].

Analysis of the altered mutation pattern in mice deficient in MSH2, a mismatch repair (MMR) protein, led to the proposal that SHM is a two step process. SHM is initiated by deamination of deoxycytidine (C) to deoxyuridine (U) in single-stranded DNA, produced during the transcription of the variable (V) gene. Phase 1 mutations are introduced during replication across the G:U mismatch and result in G:C to A:T transitions. If the U base is removed before replication by uracil-DNA glycosylase, the replication of the abasic site, created by a translesion DNA polymerase, gives rise to both transitions and transversions. Phase 2 mutations are mainly restricted to A:T pairs surrounding a U:G mismatch and involve the mismatch repair machinery. The recognition of the U:G mismatch by the Msh2/Msh6 complex results in a mutagenic patch repair mechanism involving exonuclease I and the low-fidelity DNA polymerase η (POLH) [3–5]. In activated B cells, G:C and A:T pairs are equally targeted at V genes. However, in B cell lines, as well as in non-B cell lines in which AID is ectopically expressed, mutations at G:C pairs are mainly found [6–8]; why mutations at A:T pairs are almost always absent remains unclear. In activated B cells, A:T mutations are strictly dependent on the Msh2/Msh6 pathway and are presumed to be introduced during patch repair by POLH, in the absence of DNA replication [9,10]. The function of MMR is to ensure the fidelity of DNA replication by removing mismatches produced during DNA synthesis [11,12]. The absence of mutations at A:T pairs in B or non-B cell lines expressing AID could be explained either by the prevalence of phase 1 mutations at G:C pairs preventing MMR from occurring or, alternatively, by recruiting a high fidelity DNA polymerase during the patch repair.

In order to examine if AID is able to trigger mutations in the absence of DNA replication in a non-B cell line, we developed a highly sensitive assay based on the reversion of nonsense mutations of the EGFP gene cloned in a non replicating vector. We show that the appearance of EGFP positive cells in non-B cells is dependent on the expression of AID and that, even in the absence of vector replication, mutations are found both at A:T and at G:C pairs.

RESULTS

The system we developed to study AID-dependent mutation is a simian virus 40 (SV40) -based vector containing a mutated EGFP gene to score mutations (SHM vectors: Figure 1A and B). We transfected the SHM vectors into cells that do not express the T antigen to prevent the plasmids from replicating, which was confirmed by a DpnI replication assay (data not shown). Because an essential component for plasmid replication is missing, only
mutations associated with mismatch repair will be detected [13]. The EGFP gene was mutated by introducing a premature stop codon, TAG or TAA (Figure 1C). The mutated EGFP protein is truncated and non-fluorescent. If the stop codon reverts fluorescence is restored and the cells can be detected by flow cytometry in the green fluorescence channel. The number of vector molecules present in transfected cells can not be evaluated precisely thus, it is not possible to estimate a mutation rate. The mutation level is, therefore, a relative value and corresponds to the percentage of fluorescent cells. This value depends on the percentage of cells transfected with an SHM vector. Consequently, the mutation level was expressed as the percentage of fluorescent cells relative to the transfection efficiency. Transfection efficiency was estimated using a plasmid containing a wild-type EGFP gene and was typically around 30–50% in Jurkat cells, 15–35% in Jurkat-AID cells.

SHM vectors were transfected into AID-expressing cell lines and compared to a control cell line that does not express AID. The cell lines used were a T lymphoma cell line, Jurkat, and its AID-expressing counterpart, Jurkat-AID. The expression of AID was tested by RT-PCR analyses of the Jurkat and Jurkat-AID cells (Figure 2). The Jurkat-AID clone used in this study over-expresses AID, as illustrated in Figure 2.

AID-dependent mutations are detected in SHM vectors less than 20 hours after transfection

To determine whether AID-induced mutations can be detected using SHM vectors, we first tested the pmutEGFP-TAG182 vector (depicted in Figure 1) in Jurkat and Jurkat-AID cells. As shown in Figure 3, the TAG-182 codon reverted significantly more frequently (0.3% ± 0.2 SD versus 8.1% ± 3.6 SD) in Jurkat-AID cells compared to Jurkat cells that do not express AID. To verify whether EGFP revertants are generated only by point mutations, a vector containing a 4-nucleotide deletion at position 52, which results in a stop codon, was transfected. The 4-nucleotide deletion substrate did not give rise to a functional EGFP gene in any of the cell lines transfected (data not shown). Thus, the mutations which

![Figure 1](https://example.com/figure1.png)

**Figure 1.** SHM vectors used to monitor SHM in AID-expressing cell lines (A) pmutEGFP vector: EGFP (enhanced green fluorescent protein), a variant of the GFP gene, is placed under the control of the CMV promoter (human cytomegalovirus immediate-early promoter/enhancer); pUC–prokaryotic origin of replication; AMPR –ampicillin resistance gene; SV40 ori–eukaryotic origin of replication; KanR/NeoR–kanamycin/neomycin resistance gene (B) pEM7-EGFP vector: EGFP is placed under the control of both an eukaryotic (CMV) and prokaryotic (EM7) promoter; pUC–prokaryotic origin of replication; SV40 ori–eukaryotic origin of replication; KanR/NeoR–kanamycin/neomycin resistance gene (C) Sequence contexts of premature stop codons in the EGFP gene. The upper row corresponds to the wild-type sequences and the lower row to the mutated sequences. Three variants of the EGFP gene inactivated with a premature termination codon introduced by site-directed mutagenesis were used 1) The G of the TAG termination codon at the position 182 is embedded within the RGYW hotspot motif and is thus a potential target of AID 2) The TAG stop codon at position 52 lies within two RGYW motifs 3) TAA codon at position 52 is used to monitor A:T mutations only. RGYW motifs are shown in bold letters. The mutated nucleotides are underlined.

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![Figure 2](https://example.com/figure2.png)

**Figure 2.** AID is transcribed in the Jurkat-AID and not in the control Jurkat cell line. AID transcription was monitored with RT-PCR in a control cell line, Jurkat, and in a Jurkat-AID cell line stably transfected with AID. A Burkitt lymphoma cell line Ramos constitutively expressing AID was used as a positive control for AID expression. G3PDH was used as an internal control. The bands corresponding to AID (380 bp) and G3PDH (1000 bp) are shown with black arrows.

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confer the fluorescent phenotype are point mutations of the TAG stop codon. This event is AID dependent, as illustrated in Figure 3.

In general, cells were analyzed by flow cytometry 20 hours after transfection. The maximum number of EGFP positive cells is observed between 12 and 24 hours. 24 hours after transfection 1.7% of Jurkat-AID cells reverted the TAG 182 codon. Surprisingly, we found that mutations appear very rapidly after transfection: we were able to observe EGFP positive cells within 3 hours of transfection (Figure 4). This suggests that mutations occur immediately after the DNA enters the cell.

Together these data demonstrate that AID dependent mutations can be detected with SHM vectors less than 24 hours after transfection.

Ectopic expression of AID triggers both G:C and A:T mutations

In order to characterize the molecular events responsible for the introduction of point mutations in the absence of DNA replication, we constructed two new vectors in addition to pmutEGFP-TAG182 : pmutEGFP-TAG52 and pmutEGFP-TAA52 which contain different stop codons at position 52 of the EGFP gene. These SHM vectors were transfected into Jurkat and Jurkat-AID cells and analyzed by flow cytometry 20 hours after transfection, as previously described. Figure 5 shows that all the three vectors, bearing different stop codons, were mutated more efficiently in the Jurkat-AID than in the Jurkat cell line. The TAG-52 mutation reverted in both transfected cell lines, at a higher level compared to the other mutations. It reverted at a sevenfold higher level in Jurkat-AID cells compared to Jurkat cells.

In order to identify which reverse mutation confers the fluorescent phenotype, we constructed a second series of vectors, pEM7 SHM vectors, with the EGFP gene under the control of both a prokaryotic (EM7) and a eukaryotic (CMV) promoter. These vectors allowed us to extract plasmid DNA after transfection and transform bacteria to analyze individual fluores-
cent clones by sequencing. We transfected the pEM7 SHM vectors into Jurkat and Jurkat-AID cells.

The vectors used were pEM7-EGFP-TAG182, pEM7-EGFP-TAG52 and pEM7-EGFP-TAA52 (Figure 1B and C). The reversion efficiency of EM7 SHM vectors (data not shown) is similar to those of SHM vectors (Figure 5).

To identify the nucleotide within the stop codon that is mutated in revertants of TAG-52 and TAG-182 SHM vectors, we first sorted out fluorescent colonies, then extracted plasmid DNA after secondary cloning in E.coli. Sequencing data demonstrate that the TAG-52 codon is mutated at the first T:A base pair (Table 1), TAG-182 is mutated at the G:C base pair in Jurkat-AID cells (Table 1). Interestingly, in the case of TAA-52, one revertant sequence bears a point mutation to TAC, thus mutated at the third A:T base pair, instead of the expected reversion to AAA or wild-type AAG (Table 1). This encodes a tyrosine instead of a lysine and despite these aminoacid differences, this restored the fluorescent phenotype. No mutations were detected outside the stop codon in any of the 24 revertant sequences analyzed. In order to examine the overall distribution of mutations in the EGFP gene, we sequenced the gene from 178 non-fluorescent colonies, 106 to examine the overall distribution of mutations in the EGFP gene. No mutations were detected outside the stop codon in any of the 24 revertant sequences analyzed. In order to examine the overall distribution of mutations in the EGFP gene, we sequenced the gene from 178 non-fluorescent colonies, 106 from Jurkat-AID cells and 72 from Jurkat cells. This analysis uncovered only two point mutations that were observed on plasmids from Jurkat-AID cells. The two mutations were the same: a G to A transition positioned near the end of the EGFP gene and seem to correspond to the same mutational event (data not shown).

DISCUSSION

In the present study we made use of non-replicating episomal vectors to study AID induced mutagenesis in a non-B cell context. Our system is based on the reversion of a nonsense mutation in an EGFP gene, cloned downstream of the CMV promoter/enhancer. The data demonstrate that transiently transfected DNA can be mutated in a cell type independent manner in non-B cells. Both G:C and A:T mutations were detected, suggesting that the lesion introduced by AID is sufficient for triggering both types of mutations. The high sensitivity of our transfected assay is probably due to the high plasmid copy number introduced in each cell (104-105 per cell) and to the fact that the reversion of only one stop codon per cell is sufficient to be detected on a FACS analyzer.

In general, phase 1 mutations, located at the level of the U:G mispairs, are only found after the replication fork has passed the abasic site produced by uracil-DNA glycosylase and a dNTP has been inserted opposite the abasic site. The fact that the reporter gene can replicate independently of phase 1 mutations, before the passage of DNA strands and the creation of a gap by exonuclease 3’-5’.

How can we explain the mutability of the reporter gene in the absence of DNA replication in Jurkat-AID cells? Numerous studies have shown that the rate of mutation of V regions is proportional to the rate of transcription. The biochemical demonstration that AID deaminates C in single-stranded (ss) DNA led to the proposal that transcription triggers separation of the DNA strands, each ss DNA is then exposed to the action of AID [6,14-17]. The EGFP reporter gene is under the control of a strong promoter and this could explain its high mutability, caused by the formation of ss DNA created by the supercoiling of the DNA. It is interesting to observe that all three types of codons were reverted in the Jurkat-AID cell line and that the TAG-32 codon also reverted at a significant level in Jurkat cells without AID. The high mutability of the TAG-32 codon can be explained by the influence of secondary structures. Wright and coworkers showed that the position of the nucleotide within the stem loop structure determines the mutability of the nucleotide in prokaryotes [18] and in eukaryotes [19,20]. The most hypermutable bases are located immediately next to stems in stable DNA stem-loop structures (SLS). In light of this, we can assume that the background mutation for the TAG-32 codon, which lies within two adjacent hotspot motifs, is considerably higher compared to the other codons, due to a secondary structure effect (SLS effect). This phenomenon is amplified in AID expressing cells.

How can the formation of a U:G mismatch by AID trigger the reversion of the stop codons? Theoretically the elimination of a mismatch in non-dividing cells by MMR can operate without distinguishing between the two DNA strands [21]. In the case of a U:G mismatch this results, with equal probability, either in a G:C → A:T transition or in the maintenance of the sequence. Clearly none of the reversion of the three stop codons in the EGFP gene correspond to a G:C → A:T transition. We therefore postulate that the reversion of the stop codon is introduced during MMR.

MMR involves the recognition of the U:G mismatch by the Msh2/Msh6 heterodimer, endonucleotide cleavage of one of the DNA strands and the creation of a gap by exonuclease 3’. While gap repair is usually error-free due to the activity of high fidelity DNA polymerase δ & ε [12,22], during phase 2 of SHM, the gap could be repaired by error-prone DNA polymerases that insert mispaired nucleotides at A:T pairs [10,23]. A key question is why the mechanism that normally insures the fidelity of DNA repair in non-B cells seems ineffective in Jurkat-AID cells? DNA mismatch repair is normally used to correct mispairing occurring during DNA replication. Its efficiency is based on its ability to distinguish between parental and neosynthetized DNA strands. In the absence of DNA replication a mismatch will be repaired with equal probability to fix the mutation or to restore the wild type sequence. If theoretically mutagenic mismatch repair in the absence of DNA replication does not require a specialized DNA polymerase, the high rate of mutation observed during SHM is achieved by the recruitment of specialized error-prone DNA polymerases [23,24].

These results strongly support the view that, even in non dividing cells, mismatch repair can trigger mutations at distance from the initial mismatch. In addition, phase 2 mutations can be expressed independently of phase 1 mutations, before the passage of the replication fork.

MATERIALS AND METHODS

Plasmid constructions

pmutEGFP-TAG182, pmutEGFP-TAG52, pmutEGFP-TAA52 were obtained as follows. First, an Asel MluI fragment from the pEGFP-C1 plasmid (Clontech) was inserted into an XhoI site (after Klenow fragment treatment) of the pBluescript sk+ plasmid (Stratagene) to create pSk+EGFP. A Bsu36I fragment from the pEGFPC1 plasmid was cloned into an EcoRV site of the pSK+EGFP vector to obtain the pEGFPconr vector. This vector was mutated by the site-directed mutagenesis kit (Stratagene) using the following primers: TAG-182 mutation, pTAG182a (gtgcggtcagcagacaccccc) and pTAG182b (gggggtgttctgcTGCT-}
The conditions used were: 260 V, 975 mA. Electroporation into 1 x 10^7 Jurkat and Jurkat-AID cells in 0.4 cm cuvettes using a Biorad Gene pulse transfection method. Cells were resuspended in 10 ml of fresh medium and cultured at 37°C, 5% CO2. In transfection experiments 5 ml of transfected cells was washed with PBS, centrifuged and resuspended at 10^7/ml. Viability (dead cell marker) and analysis by flow cytometry were performed. AID expression was monitored by RT-PCR (see below). Twenty hours after transfection 5 ml of transfected cells was washed with PBS, centrifuged and resuspended in 10 ml of fresh medium. Analysis of the acquired data was performed with the Cell Quest software (BD Sciences, Mountain View, CA). For plasmid sequencing, fluorescent colonies were sorted on a Fluoro-cell sorter (DakoCytomation) before extraction in order to concentrate fluorescent colonies (see below).

**Extraction of plasmid DNA from mammalian cells**

The NucleoSpin Plasmid (Macherey Nagel) kit for extraction of plasmid DNA from bacteria was adapted to extract plasmid from mammalian cells. Twenty hours after transfection 5 ml of transfected cells was washed with PBS, centrifuged and resuspended in 10 ml of fresh medium. Twenty hours after transfection 5 ml of transfected cells was washed with PBS, centrifuged and resuspended in 10 ml of fresh medium. After resuspension and lysis (according to manufacturer's instructions), the material was treated with 800 μg/ml proteinase K for 1 h to 2 h at 55°C. Proteinase K digestion was followed by neutralization, column fixation, washing and elution (according to manufacturer's instructions). In the DNA replication assay plasmid DNA was digested with Dpn1 2 h at 37°C.

**Transformation in E.coli and sequencing**

2 μl of extracted DNA was transformed in TOP10 bacteria (Invitrogen) and resuspended in 900 μl of SOC medium. The total suspension was plated on LB kanamycin (50 μg/ml) plates, 100–200 μl of bacteria suspension per plate. Plates were analyzed on a Lighttools Illuminatool Tunable Lighting System. Fluorescent colonies were grown in 4 ml LB kanamycin medium overnight. Plasmid DNA was extracted using NucleoSpin Plasmid (Macherey Nagel) kit according to manufacturer's instructions and sent for sequencing using a CMV primer (gtacgggagggtcttataagc).

**AID RT-PCR**

Total RNA was extracted from Jurkat, Jurkat-AID and Ramos cells using the Trizol reagent (Invitrogen) according to manufacturer's instructions. Two μg of RNA was denatured 5 minutes at 65°C with 0.5 μg of oligo dT and chilled on ice. Reverse transcription was performed in a 50 μl reaction for 90 minutes at 37°C with 1xMMLV buffer, 0.5 mM dNTPs, 10 mM dithiotreitol, 200 U of MMLV (all from Invitrogen) and 400 U/ml RNAsin (Promega). The MMLV was inactivated at 70°C for 15 min. 2 μl of the cDNA was used for each PCR reaction: AID and G3PDH. PCR was performed using Taq polymerase (QIagen), according to manufacturer’s instructions, in a 50 μl reaction using 0.4 μM of the following primers: AID1 (TAGAAGGTGGGCGCCTGTCATA-CC) and AID2 (CAAAAGGATGGCCGCGACTGTCCTGG-AG) for AID amplification, G3PDH1 (TGAAGTCCGAGT-CAACGGATTGTTGGT) and G3PDH2 (CATGTTGCATGAG-GTCCACCAAC) for G3PDH amplification. The cycling used for both PCRs was 94°C/2 min, 15 cycles of 94°C, 15s; 65°C, 30s; 72°C, 45s, 30 cycles of 94°C, 15s; 65°C, 30s; 72°C, 45s+1 s every cycle and 7 minutes at 72°C.

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**Author Contributions**

Conceived and designed the experiments: FR TJ. Performed the experiments: TJ GA BR OL. Analyzed the data: FR TJ GA BR OL. Contributed reagents/materials/analysis tools: FR. Wrote the paper: FR TJ.
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