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Activation-induced cytidine deaminase fails to induce a mutator phenotype in the human pre-B cell line Nalm-6

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Activation-induced cytidine deaminase (AID) plays a key role in the induction of somatic hypermutation and class switching at the immunoglobulin loci of B lymphocytes. AID overexpression can induce a mutator phenotype in lymphoid and nonlymphoid cell lines, suggesting that AID by itself is sufficient to trigger hypermutation and class switching. AID expression *in vivo* is considered to be restricted to germinal center B lymphocytes, yet AID expression is also seen in many B cell lymphomas, hinting at a potential role for the development of these malignancies. We used a GFP-based reversion assay to efficiently evaluate the activation of mutator phenotypes. As expected, AID overexpression in the human Burkitt lymphoma cell line BL70 caused hypermutation. Surprisingly, AID overexpression in the human pre-B cell line Nalm-6 failed to induce a detectable mutator phenotype, indicating that Nalm-6 cells are probably lacking an essential factor(s) to confer AID-induced mutagenesis. This finding supports the concept that AID overexpression by itself must not automatically lead to the onset of a mutator phenotype. In addition, treating Nalm-6 transfectants with thymidine, a potential mutagenic drug, caused profound mutation rates on the *GFP* transgene. Thus, the GFP-based mutation assay might prove a powerful tool to study protein- and chemical-induced mutator phenotypes in cell lines.

Key words: Human · B lymphocytes · Repertoire development · Antibodies

Introduction

The human body is constantly exposed to a vast variety of harmful microorganisms and pathogens. In order to cope with this broad spectrum of antigens, the immune system has developed two major strategies to diversify its antibody repertoire: V(D)J recombination and somatic hypermutation. During somatic hypermutation (SHM), point mutations are introduced at an estimated frequency of 10^{-3} mutations per base pair (mut/bp) into the variable region of the immunoglobulin (Ig) genes [1]. Somatic hypermutation is induced in an antigen-dependent mode in germinal center B lymphocytes and may increase the affinity of an antibody for a specific antigen. B lymphocytes expressing high-affinity antibodies are positively selected and mount the antibody defense against the microorganism or pathogen [2].

The enzyme activation-induced cytidine deaminase (AID) plays a key role in the induction of somatic hypermutation and class switch recombination (CSR), another B lymphocyte-specific mechanism [3]. AID-deficient mice fail to undergo somatic hypermutation and class switching, although their B lymphocytes are not affected in their ability to generate germinal centers [4], the physiological site where CSR and SHM are induced [5–7]. AID expression *in vivo* is usually restricted to germinal center B lymphocytes, yet it is also seen in some B cell lymphoma types, pointing to a potential role in the development of these malignancies

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Abbreviations: **AID:** Activation-induced cytidine deaminase · **CSR:** Class switch recombination · **GAPDH:** Glyceraldehyde 3-phosphate dehydrogenase · **HA:** Hemagglutinin · **hnAID:** Human AID · **IRES:** Internal ribosome entry site · **mut/bp:** Mutations per base pair · **mut/bp/gen:** mut/bp per generation · **NGFR:** Nerve growth factor receptor · **SHM:** Somatic hypermutation · **UDG:** Uracil-DNA glycosylase

[8]. Indeed, AID overexpression by itself can induce hypermutation in mature B lymphocytes of later differentiation stages [9], in nonlymphoid cell lines such as fibroblasts [10], and even in *E. coli* [11]. The Ig enhancers are generally considered to be required for the Ig-specific targeting of the hypermutation mechanism [12]. However, the reporter transgenes used in experiments of Yoshikawa et al. [10] and Martin and Scharff [13] lacked any Ig-specific DNA sequences, such as the Ig enhancers. In addition, AID overexpression in *E. coli* conferred a mutator phenotype on various loci in the *E. coli* genome [11]. Okazaki et al. [14] described a transgenic mouse ubiquitously overexpressing AID, which developed T cell lymphomas and micro-adenomas. Thus, overexpression of AID may result in a mutator phenotype lacking its specificity for Ig loci.

AID shares strong homology with the cytidine deaminase APOBEC-1 [3], an RNA-editing enzyme of the small intestine [15, 16]. According to the current model, AID directly deaminates single-stranded DNA [17–20], thereby initiating somatic hypermutation and/or CSR [11]. Indeed, AID-expressing cell lines predominantly show a GC mutator phenotype [10, 21, 22]. Cytidine residues, especially when embedded in the Ig hypermutation hot spot motif (RGYW), become deaminated to uracil by AID. Uracil can pair with adenosine and, consequently, cause a C to T transition. Elevated levels of uracil caused by AID are also suggested by the increased mutation frequency in AID-overexpressing *E. coli*, where uracil-DNA glycosylase (UDG) was inhibited [11].

We have recently developed a GFP-based reversion assay to facilitate the search for hypermutating cell lines and to study factors involved in somatic hypermutation [23]. Here, we show that AID overexpression, as was to be expected, caused a mutator phenotype in the human Burkitt lymphoma cell line BL70, analyzed by a GFP-based reporter transgene. In contrast, AID overexpression in the human pre-B cell line Nalm-6 failed to induce somatic hypermutation on the *GFP* transgene. In this, Nalm-6 cells behave exceptionally, because in all other published cell systems, AID overexpression led to the onset of hypermutation [9–11, 13]. We reason that an unknown cellular factor is either missing or prevents AID function in Nalm-6 cells. This finding supports the concept that AID expression must not automatically lead to the onset of a mutator phenotype and that AID function could be modulated by additional factors.

Results

Mutator phenotypes were analyzed by a GFP reversion assay

To measure the induction of hypermutation, we used the reporter construct ptk-GFP*stop* (Fig. 1A). The *GFP* transgene contains a premature TAG stop codon, thus giving rise to a truncated nonfunctional GFP protein. The TAG stop codon is part of a Rogotsin/RGYW motif, generating an Ig mutation hot spot [24]. Point mutations in the GFP reporter gene that revert the premature stop codon can reconstitute the *GFP* gene. Revertant cells start to fluoresce and can be detected via flow cytometry [23].

For induction of the somatic hypermutation process, the construct pAID-ngfr encoding a hemagglutinin (HA)-tagged human AID (hnAID) protein was created (Fig. 1B). The *AID* coding region and the truncated coding region of the nerve growth factor receptor (*NGFR*) gene, linked via an internal ribosome entry site (IRES), are constitutively transcribed by the bos (elongation factor 1a) promoter [25]. The expression of the NGFR marker facilitates the detection of AID-expressing transfectant cells, because it can easily be detected by anti-NGFR surface immunostaining. The *NGFR* coding region used in the plasmid pAID-ngfr lacks the cytoplasmic signaling domain; thus, the NGFR protein fails to trigger intracellular signaling events.

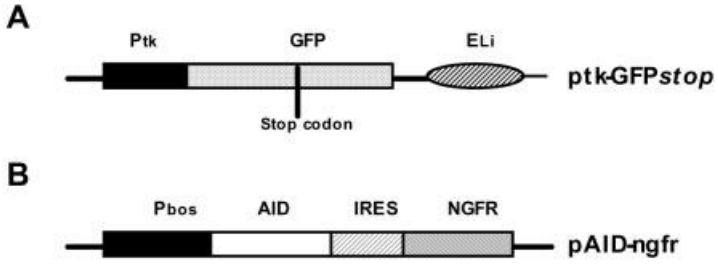

Fig. 1. GFP construct and AID expression construct to monitor and induce mutator activity. (A) Construct ptk-GFP*stop* contains the *GFP* coding region (GFP) with a premature TAG stop codon. The *GFP* coding region is driven by the thymidine kinase promoter (p_{tk}) and the Ig heavy-chain large intron enhancer (E_{Li}). (B) In construct pAID-ngfr, the bos promoter (p_{bos}) drives the transcription of a bicistronic transcript encoding HA-tagged hnAID (AID) and a truncated NGFR (NGFR). The *AID* and *NGFR* coding regions are separated by an IRES.

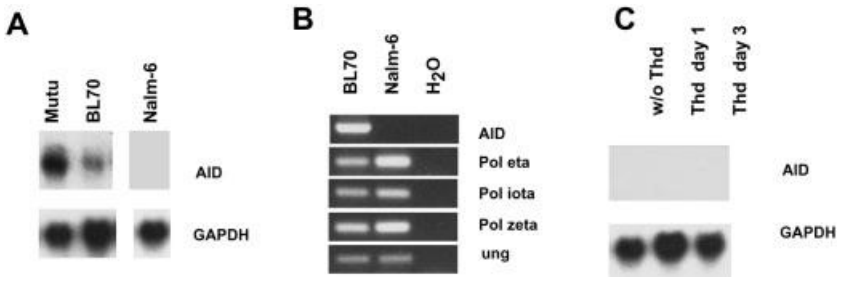

Fig. 2. Endogenous AID is expressed at various levels in Burkitt lymphomas, but not in Nalm-6 cells. (A) Total RNA was extracted from the Burkitt lymphoma cell lines Mutu and BL70 and from the pre-B acute lymphoblastic leukemia cell line Nalm-6. (B) The expression profile of various putative hypermutation *trans* factors was determined via RT-PCR for Nalm-6 and BL70 cells. H_2O served as negative control. (C) Total RNA from Nalm-6 cells without thymidine and after administering 1 mM thymidine + 0.1 mM cytidine (Thd) for 1 and 3 days. Both total RNA blots were sequentially hybridized with probes for the *AID* gene and the *GAPDH* gene.

The GFP reporter gene is not mutated in cell line Nalm-6 and very poorly in BL70

Two human B cell lines, Nalm-6 and BL70, were analyzed to study the effect of AID overexpression. BL70 is a Burkitt lymphoma cell line representing a germinal center-derived B cell lymphoma transformed by *c-myc* translocation into an Ig locus [26]. Nalm-6 is a human pre-B cell lymphoma of the acute lymphoblastic leukemia type [27]. Nalm-6 cells completely lack endogenous AID mRNA transcripts as determined by Northern blot analysis, whereas BL70 cells show very low levels of endogenous AID mRNA (Fig. 2A). The human group III Burkitt lymphoma cell line Mutu, which undergoes somatic hypermutation (data not shown), was used as a positive control for AID transcription. In addition, reverse transcription (RT)-PCR was performed for Nalm-6 and BL70 cells to address the expression of other *trans* factors, *i.e.* UDG and various DNA polymerases that have been shown to be involved in somatic hypermutation [28–31]. Besides AID expression, no striking differences were detectable (Fig. 2B).

For plasmid ptk-GFP*stop*, 24 independent, stably transfected clones were generated from the cell lines Nalm-6 and BL70, respectively, and analyzed by flow cytometry, to determine the revertant frequency per 10^5 viable cells. None of the 24 Nalm-6 transfectants contained GFP-expressing revertant cells, whereas a small number (2 out of 24 clones) of the BL70 transfectants contained very few (2 and 3, respectively) revertant cells per 10^5 cells (Fig. 3). These infrequent revertants in BL70 transfectants may be due to the very low endogenous AID expression, as seen by Northern blot analysis (Fig. 2A). Alternatively, the spontaneous mutation rate in the Burkitt lymphoma cell line BL70 may generally be slightly elevated by an AID-independent cellular mechanism. Nevertheless, both cell lines did not display a hypermutator phenotype.

Ectopic AID expression induces hypermutation in BL70 but not in Nalm-6 cells

To address whether expression of exogenous AID can induce somatic hypermutation in the cell lines Nalm-6 and BL70, the plasmids ptk-GFP*stop* and pAID-ngfr were stably cotransfected. Cotransfection provides two advantages: (i) stably transfected clones with various AID expression levels can be obtained, and (ii) genomic integration site effects potentially affecting the accessibility of the GFP reporter transgene will be minimized in the reversion analysis, because the GFP reporter transgene integrates at various genomic sites in independent clones.

Screening for AID-expressing transfectants was performed by anti-NGFR surface staining and intracellular immunostaining with anti-HA antibodies. We

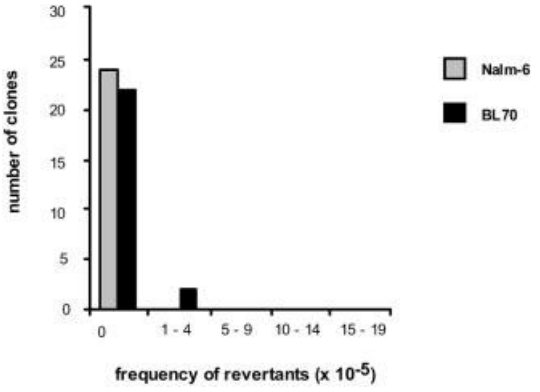

Fig. 3. Distribution of reversion frequencies for Nalm-6 and BL70 cells stably transfected with construct ptk-GFP*stop*. The frequency of revertants per 10^5 viable cells was determined by flow cytometry for 24 independent transfectant clones of each cell line, Nalm-6 and BL70. The increments on the x axis are: 0 revertants per 10^5 cells, 1–4 revertants per 10^5 cells, 5–9 revertants per 10^5 cells, 10–14 revertants per 10^5 cells and 15–19 revertants per 10^5 cells. Y axis: the accumulative number of independent transfectant clones (out of 24).

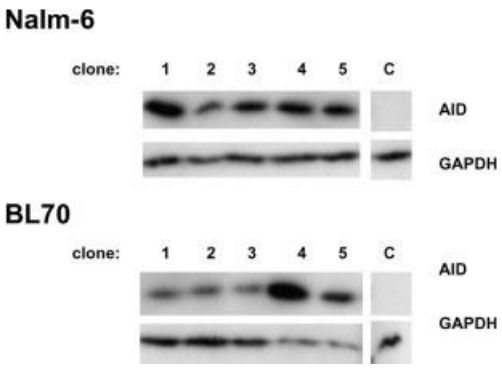

Fig. 4. Anti-HA Western blot analysis of ptk-GFP*stop* + pAID-ngfr cotransfectant Nalm-6 and BL70 clones. The expression level of HA-tagged hnAID protein for five independent Nalm-6 and five independent BL70 cotransfectants was determined by Western blot analysis using an anti-HA antibody. The Western blots for Nalm-6 and BL70 transfectants were generated independently. For quantitative comparison of the blots, GAPDH was used as loading control. C: Untransfected cells.

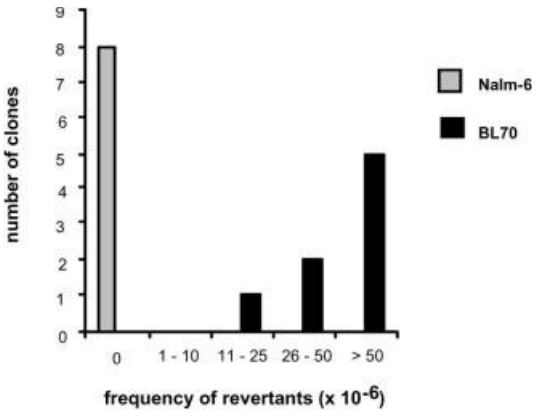

Fig. 5. Distribution of reversion frequencies for Nalm-6 and BL70 cells stably cotransfected with ptk-GFP*stop* and pAID-ngfr. The frequency of revertants per 10^6 viable cells was determined by flow cytometry for eight independent cotransfectant clones each of cell lines Nalm-6 and BL70. The analyzed Nalm-6 transfectant clones expressed HA-tagged hnAID and responded to thymidine treatment. The increments on the x axis are: 0 revertants per 10^6 cells, 1–10 revertants per 10^6 cells, 11–25 revertants per 10^6 cells, 26–50 revertants per 10^6 cells and >50 revertants per 10^6 cells.

obtained eight independent Nalm-6 clones with various AID protein levels, and eight independent BL70 clones. The AID protein levels of the Nalm-6 and BL70 transfectants were determined by Western blot analysis using an anti-HA tag-specific antibody (Fig. 4). None of the AID-expressing Nalm-6 transfectants showed GFP-revertant cells, regardless of the actual AID protein levels. In contrast, AID overexpression in BL70 cells significantly increased the mutation frequency. All (8 out of 8) of the AID-expressing transfectants contained GFP-expressing revertant cells (Fig. 5), compared to 8% (2 out of 24) in the absence of exogenous AID expression (Fig. 3). In both cell lines, the HA-tagged AID expression levels varied among the transfectants analyzed. In Nalm-6 cells, even GFP/AID transfectants with high AID levels did not display a mutator phenotype. This demonstrates that overexpression of AID in the cell line Nalm-6 cannot induce hypermutation on the *GFP* transgene, in contrast to AID overexpression in the BL70 cell line.

Thymidine induces a mutator phenotype in Nalm-6 cells

To exclude that the lack of GFP revertants in Nalm-6 AID transfectants is due to an impaired GFP reporter gene expression, Nalm-6 transfectants were exposed to various mutagenic drugs that might cause mutations in the *GFP* transgene, resulting in GFP-revertant cells. We observed that thymidine treatment (in combination with low levels of cytidine to avoid cell death) indeed triggered a profound mutator phenotype in Nalm-6 cells. However, not all ptk-GFP*stop* transfectants responded to the thymidine treatment, indicating that genomic integration sites may affect the susceptibility to thymidine-induced mutagenesis. The thymidine-induced mutation rates were determined for four independent ptk-GFP*stop* transfectant clones. Fig. 6A shows the result of an expansion analysis performed for 4 days in the presence or absence of supplemented thymidine, for one transfectant. The reversion rate varied between 1.9×10^{-5} and 7.9×10^{-5} mut/bp per generation (mut/bp/gen) among the ptk-GFP*stop* transfectants analyzed (Table 1). Revertant cells from four independent transfectants supplemented with thymidine were sorted and subsequently sequenced to analyze the reversion events. In all four cases, a point mutation from G to C within the TAG stop codon had caused the reversion.

We have previously shown that the GFP fluorescence intensity of revertant cells measured by flow cytometry directly correlates with the transcription rate of the GFP reporter transgene [32]. The GFP expression levels were comparable between revertant cells in cell lines BL70 (AID induced) and Nalm-6 (thymidine induced; Fig. 6B), indicating that the mutated reporter construct is equally well transcribed in both cell lines. Northern blot analysis and quantitative real-time PCR analysis were performed to determine the GFP transcript levels for the transfectant clones 1–5 derived from both cell lines Nalm-6 (without thymidine treatment) and BL70 (Fig. 7). The GFP transcript levels varied among the individual transfectant clones; however, there was no obvious expression difference regarding the parent cell line (Nalm-6 or BL70). Thus, the lack of GFP revertants in AID-overexpressing Nalm-6 transfectant clones (in the absence of thymidine) cannot be explained by insufficient GFP transcription levels.

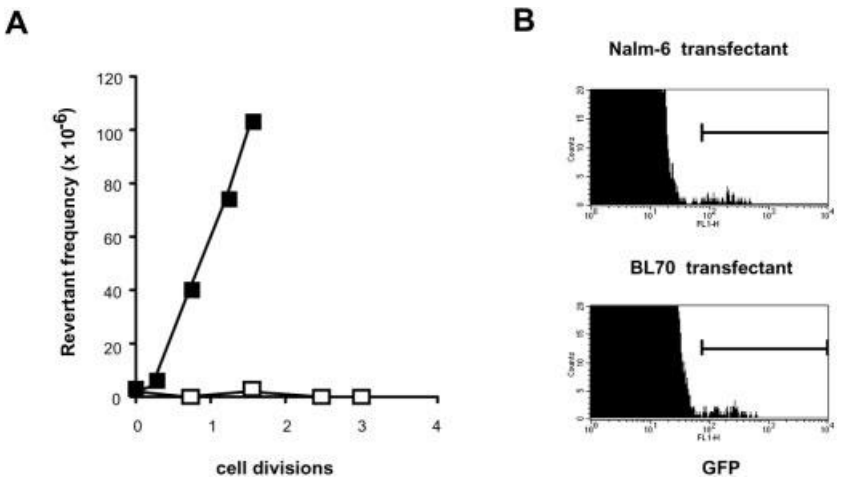

Fig. 6. Reversion analysis of Nalm-6 ptk-GFP*stop* transfectant clones in the presence or absence of thymidine. (A) The flow cytometry data obtained by a 5-day expansion analysis for one transfectant clone are plotted as a function of GFP-expressing revertant cells per 10^6 cells versus the number of cell divisions. Filled symbols: in the presence of thymidine. Open symbols: in the absence of thymidine. (B) Flow cytometry analysis for one Nalm-6 ptk-GFP*stop* transfectant clone after 3 days in thymidine-supplemented medium and one BL70 pAID-ngfr + ptk-GFP*stop* cotransfectant clone. X axis: relative fluorescence intensity of GFP; y axis: cell counts

Thymidine does not induce endogenous AID expression in Nalm-6 cells

Since the G to C transversion was reminiscent of previously observed G-C mutator phenotypes in hypermutating cell lines, it appeared conceivable that endogenous AID expression might be induced by thymidine treatment in Nalm-6 cells. Northern blot analysis was performed for a ptk-GFP*stop* transfectant at various time points during thymidine treatment (Fig. 2C). The Burkitt lymphoma cell lines Mutu and BL70 were used as controls for the detection of endogenous AID expression (Fig. 2A). Neither the untreated nor the thymidine-treated Nalm-6 cells expressed any detectable amounts of AID transcripts. Thus, the GC-biased reversion events were not caused by an AID-induced mutation mechanism, but rather by the intrinsic mutagenic property of thymidine.

Table 1. GFP reversion rates of thymidine-treated Nalm-6 transfectants

	Clone	Mutation rate ($\times 10^{-5}$)
w/o AID	1	7.9
	2	4.9
	3	6.3
	4	1.9
+ AID	1	2.8
	2	1.8
	3	6.5
	4	1.4
	5	5.2

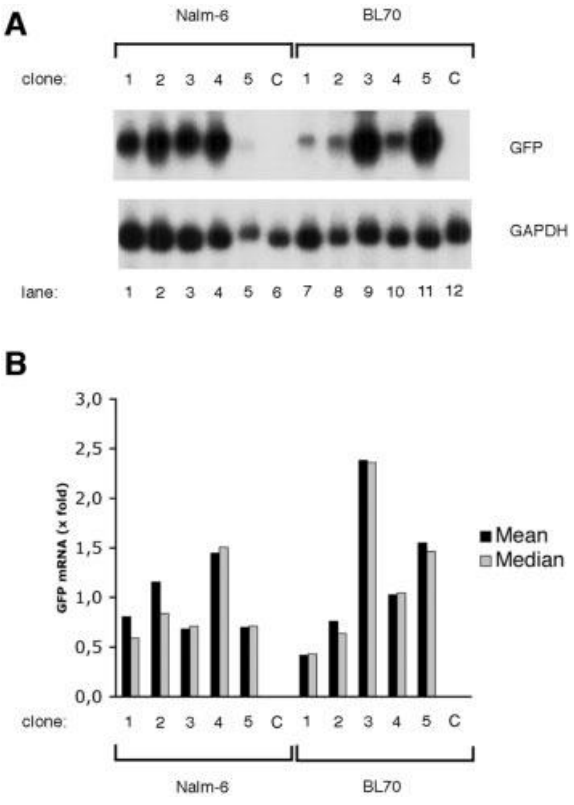

Fig. 7. Comparison of GFP transcript levels among Nalm-6 and BL70 transfectant clones. (A) Northern blot analysis for GFP and GAPDH transcripts in stable transfectant clones (clone numbers 1–5 correspond to Fig. 4). (B) Quantitative real-time PCR for GFP transcripts in the transfectant clones 1–5 from both cell lines Nalm-6 and BL70. The mean and median of four independent real-time PCR results per transfectant clone is shown. Nalm-6 transfectant clones were not thymidine treated. C: Untransfected founder cell line Nalm-6 and BL70, respectively.

AID overexpression has no influence on thymidine-induced mutagenesis in Nalm-6 cells

To address whether AID overexpression interferes with the thymidine-induced mutation mechanism, five AID-overexpressing Nalm-6 transfectants containing the ptk-GFP*stop* transgene were treated with thymidine. Instead of determining the mutation frequency, which may not be sufficient to address small changes in the mutation rate caused by AID overexpression, the actual mutation rate for these five AID-overexpressing clones was assessed. The mutation rates ranged from 1.4×10^{-5} to 6.5×10^{-5} mut/bp/gen and did not differ significantly from the mutation rates in Nalm-6 transfectants lacking transgenic AID expression (Table 1). Exogenous AID expression, even at high expression levels, had therefore no apparent influence on the thymidine-induced reversion rates.

Discussion

We have studied the effect of AID overexpression on the mutability of a GFP reporter transgene in two different human B cell lymphoma cell lines, the pre-B cell line Nalm-6 and the Burkitt lymphoma cell line BL70. AID overexpression induced a mutator phenotype in BL70 cells, as determined by an increased GFP reversion frequency. This observation is in agreement with the current dogma, implying that AID protein overexpression by itself is sufficient to trigger somatic hypermutation in cell lines [9, 10] [33]. Northern blot analysis was performed to determine the endogenous AID transcription level in the cell lines Nalm-6, BL70 and Mutu. Transcription of the endogenous AID locus is very poor in BL70 and completely absent in Nalm-6 cells. The endogenous AID levels in Mutu cells are sufficient to trigger hypermutation on a *GFP* transgene (data not shown), whereas the AID levels in BL70 cells are not sufficient to cause hypermutation. Overexpression of recombinant AID was required to induce hypermutation in BL70 cells.

In contrast to BL70 cells, AID overexpression in the human pre-B cell line Nalm-6 failed to induce a detectable mutator phenotype. We tested a whole panel of independent AID-overexpressing Nalm-6 cotransfectants, to exclude that genomic integration site effects interfered with the mutability of the ptk-GFP*stop* transgene; none of them showed an increased mutation rate. The pAID-ngfr transfectant clones generated in this study expressed HA-AID at various protein levels, probably due to different copy numbers and/or genomic integration sites. This was independently observed for BL70 and Nalm-6 transfectants. However, irrespective of the HA-AID protein expression level, Nalm-6 cotransfectants did not hypermutate the GFP reporter transgene. In contrast, all analyzed BL70 AID-overexpressing cotransfectants hypermutated the GFP reporter gene, even at varying HA-AID protein levels. To exclude that AID-induced mutations had affected the function of the *AID* transgene, as observed by Martin and Scharff [13], the *AID* transgene from one pAID-ngfr + ptk-GFP*stop* Nalm-6 cotransfectant clone (highest AID expression) was PCR amplified and sequenced. Sequence analysis confirmed that the AID-coding region of the transgene had not acquired any mutations, and thus, the AID protein should be still functional (data not shown). Taken together, this indicated that AID overexpression failed to induce a detectable mutator phenotype on the *GFP* transgene in the cell line Nalm-6, irrespective of the genomic integration site and the AID protein levels.

The Nalm-6 cell line has one rearranged Ig heavy-chain locus; the second Ig heavy-chain locus and the light-chain loci alleles are still in germ-line configuration [34]. We intended to sequence the endogenous heavy-chain locus of AID-overexpressing Nalm-6 transfectant clones. However, the endogenous μ heavy-chain protein was hardly detectable by cytoplasmic anti-Ig heavy-chain immunostaining, presumably due to weak Ig heavy-chain transcription (data not shown). Sequencing of the endogenous μ chain locus was therefore dismissed, because mutation rates correlate with the transcription levels during somatic hypermutation [20, 32, 35, 36]. Weak transcription of the *GFP* transgene in Nalm-6 cells could also have been a reason for the failure to detect a mutator phenotype in AID-expressing Nalm-6 cells. However, Nalm-6 and BL70 transfectants expressed the *GFP* transgene at comparable levels.

In comparison to all other published AID-overexpressing cell line systems, the cell line Nalm-6 behaves absolutely exceptionally, and the molecular cause for its failure to respond to AID remains completely elusive. It cannot be excluded that events downstream of the direct AID function (cytidine deamination) are impaired in Nalm-6 cells; however, RT-PCR for *trans* factors that have been suggested to play a role in the somatic hypermutation mechanism showed no obvious difference between Nalm-6 and BL70 cells.

The current model implies that AID deaminates cytidine residues directly in single-stranded DNA templates [11, 28]. According to this model, nuclear translocation of the AID protein would be a prerequisite for its function. However, immunostaining of transfectant cells for the HA-tagged recombinant AID protein revealed a predominant localization in the cytoplasm (data not shown). This observation confirmed data presented by several groups [37–40] who did not see any evidence of nuclear localization for a functional GFP-AID (or RFP-AID) fusion protein. The immunostaining in

BL70 and Nalm-6 transfectants did not show any obvious difference with regard to the localization of intracellular HA-tagged AID protein (data not shown). Nevertheless, we cannot exclude that different amounts of recombinant AID protein actually translocate into the nucleus of BL70 and Nalm-6 cells. The hnAID protein contains a bipartite nuclear localization signal sequence [38], which is frequently mutated in Hyper-IgM syndrome 2 patients [41]. Nuclear localization as the active site of AID function is also suggested by its homology with APOBEC-1. APOBEC-1 protein is localized in the cytoplasm and in the nucleus, with its physiological active site in the nucleus, where it edits the apolipoprotein B mRNA [42]. It has been proposed that the nuclear translocation of APOBEC-1 is achieved by a chaperon-guided mechanism [43, 44].

It is unlikely that a B cell-specific factor is involved in the putative translocation of AID into the nucleus, because AID-induced hypermutation also occurs in fibroblasts. On the other hand, AID overexpression may lead to the non-physiological situation where the active (or better regulated) transport of AID is no longer needed for nuclear translocation. However, in Nalm-6 cells, this non-physiological nuclear translocation may even be actively suppressed. Okazaki et al. [14] suggested that B cells may have a protective system to prevent excessive activity of AID. It is unclear at what level this putative protective system may work. If the nuclear transport (active or passive) of recombinant AID protein is impaired in Nalm-6 cells, it will be interesting to overexpress an AID protein containing a strong nuclear localization sequence, thus forcing it into the nucleus.

The lack of somatic hypermutation in AID-overexpressing Nalm-6 transfectants is also reminiscent of the observations made by Faili et al. [45] in the human cell line BL2. The authors observed that B cell receptor engagement in combination with additional coreceptor cross-linking was required to trigger somatic hypermutation in the endogenous Ig heavy-chain locus of BL2 cells. However, AID protein is already expressed in BL2 in the absence of receptor engagement, without inducing mutations in the Ig loci. These results suggested that AID function is governed by a yet undefined mechanism. Indeed, data from Chaudhuri et al. [18] support the view that AID may be part of a bigger protein complex. We envision that under physiological conditions, AID function is tightly regulated with regard to its deaminating activity and its substrate specificity, potentially by other *trans* factors. This putative "AIDosome" complex is required to prevent the potentially harmful mutagenic effect of AID seen under non-physiological (overexpression) conditions.

In addition, our results show that the GFP-based reversion assay can be used to study the effect of potentially mutagenic drugs in cell lines. Thymidine treatment induced a profound mutator phenotype in Nalm-6 cells. In some experiments, up to 90% of all ptk-GFP*stop* Nalm-6 transfectant clones showed significant frequencies of GFP revertants after 3 days of thymidine treatment (data not shown). In individual Nalm-6 transfectant clones, mutation rates up to 7.9×10^{-5} mut/bp/gen were measured. Yet, this thymidine-induced mutator phenotype is not due to an induction of endogenous AID expression. Thymidine-treated Nalm-6 cells did not express endogenous AID. It will require further studies to understand the molecular mechanism by which thymidine treatment induces such a profound mutator phenotype in the Nalm-6 cell line. Finally, the data presented here demonstrate that the GFP-based mutation assay can be used to study protein- and chemical-induced mutator phenotypes.

Materials and methods

Plasmid construction

For plasmid pAID-ngfr, an HA-tag was fused in frame 5′ to the human *AID* coding region. The HA-tagged hnAID was cloned into the Eco RI site of pExp, bringing the *AID* gene under the transcriptional control of the bos promoter. The IRES-NGFR cassette was excised as an Xho I fragment from plasmid pMC35a and cloned into a Sal I site 3′ adjacent to the HA-tagged hnAID. Plasmid ptk-GFP*stop* is identical to the previously published plasmid pE [23].

Primary analysis

For transfection, Nalm-6 and BL70 cells were washed once with PBS, then electroporated (250 V, 850 μF) either with ptk-GFP*stop* alone or with ptk-GFP*stop* + pAID-ngfr. Independent, stably transfected clones (24) were transferred into 24-well plates containing 2 ml fresh selection medium. As soon as the transfectant clones in the 24-well plates exhausted the medium, the frequency of revertant cells per 10^5 viable cells was analyzed by flow cytometry.

Expansion analysis

To determine the mutation rates in various Nalm-6 transfectant clones, 5×10^6 cells were stained using the cell tracker dye PKH-26 (Sigma, St. Louis, MO), according to the recommendation of the manufacturer. For each transfectant clone, two separate cultures were established, one containing 1 mM thymidine and 0.1 mM cytidine, the other without thymidine and cytidine. Each day (every 24 h), an aliquot was removed from the cultures, and the number of fluorescent and non-fluorescent cells in 1×10^6 viable cells was analyzed by flow cytometry. The cell division rate was assessed by the linear decrease of the PKH-26 intensity, also measured by flow cytometry [32]. The mutation rate was determined by applying the curve fit function from program CA-Cricket Graph III 5.2 (Computer Associates International, Islandia, NY).

Preparation of mRNA samples and Northern blot analysis

Northern blot analysis was performed as recommended in [46]. Total RNA was extracted from expanding cultures with TRIZOL Reagent (Invitrogen), and 15 mg of total RNA was loaded per lane. Fragments from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and the AID gene were radiolabeled using a random-primer labeling kit (Boehringer Mannheim) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech).

RT-PCR

cDNA was obtained by a standard procedure using Superscript II (Gibco/BRL) and subjected to 45 cycles in a thermal cycler, with each cycle consisting of 45 s at 94°C, 30 s at 63°C, and 30 s at 72°C. The following primer combinations were used: hnAID sense 5′-GGACACCACTATGGACAGCC-TCTTG-3′, antisense 5′-GGAAGTTGCTATCAAAGTCCTAAAG-TACG-3′; DNA polymerase eta sense 5′-CTGTGCCCTTAC-CCGCTATGATG-3′, antisense 5′-GAATGATTCCAGAGACGTG-GTTGC-3′; DNA polymerase iota sense 5′-TGTGTGCT-TCTGCAACCTTAAAGCAC-3′, antisense 5′-TGGAAGCTGCTT-GAAGACTTCTTGG-3′; DNA polymerase zeta sense 5′-CAG-CATGTTGCAGTCATCCTC-3′, antisense 5′-AGTGAACATCCTT-GACTCGATG-3′; UDG sense 5′-ACTCCCCGCTGAGGTGCTTC-3′, antisense 5′-CTGGGGTGCCTGAGGAAAGC-3′.

Quantitative RNA analysis

Total RNA was extracted from expanding cultures with TRIZOL Reagent (Invitrogen), and 1 μg of total RNA per culture was reverse-transcribed into cDNA by a standard procedure using Superscript II (Gibco/BRL). For relative quantification by real-time PCR, each cDNA was analyzed in a LightCycler with software version 3.5, using a FastStart DNA Master SYBR Green I kit (Roche Diagnostics). For each primer pair, a standard curve was developed. Annealing temperatures and elongation times were optimized for product generation and exclusion of artifacts. The primer sequences were as follows: GFP sense 5′-AGGAGGACGGCAACATCCTG-3′, antisense 5′-TCGTTGGGGTCTTTGCTCAG-3′; CD19 sense 5′-CTCCTTCTCCAACGCTGAGT-3′, antisense 5′-TGGAAGTGT-CACTGGCATGT-3′.

Western blot analysis

For immunoblot analysis, cell lysates were prepared by homogenizing PBS-washed cells (3×10^6 cells per lysate) in 2× Laemmli buffer (120 mM Tris-HCl pH 6.8, 4.0% SDS, 20% glycerol, 100 mM DTT, 0.01% bromophenol blue). No cell line-specific differences in protein yields between BL70 and Nalm-6 lysates were detectable. Proteins (30 μg/lane) were separated by electrophoresis in 12% SDS-acrylamide gels and transferred to a nitrocellulose membrane. After blocking with 5% skim milk in PBS, the membrane was incubated overnight at 4°C with either primary rat anti-HA antibody (Roche Applied Science) or primary rabbit anti-GAPDH antibody (Abcam). After washing, the membrane was incubated at room temperature for 3 h with the horseradish peroxidase-conjugated anti-rat (Santa Cruz Biotechnology) or anti-rabbit secondary antibody (Promega), respectively. Finally, the result was visualized with an ECL kit (Amersham Pharmacia Biotech).

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References

1 **McKean, D., Huppi, K., Bell, M., Staudt, L., Gerhard, W. and Weigert, M.,** Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. USA* 1984. **81:** 3180–3184.

2 **Rajewsky, K.,** Clonal selection and learning in the antibody system. *Nature* 1996. **381:** 751–758.

3 **Muramatsu, M., Sankaranand, V. S., Anant, S., Sugai, M., Kinoshita, K., Davidson, N. O. and Honjo, T.,** Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J. Biol. Chem.* 1999. **274:** 18470–18476.

4 **Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. and Honjo, T.,** Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000. **102:** 553–563.

5 **Liu, Y. J., Malisan, F., de Bouteiller, O., Guret, C., Lebecque, S., Banchereau, J., Mills, F. C., Max, E. E. and Martinez-Valdez, H.,** Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. *Immunity* 1996. **4:** 241–250.

6 **Jacob, J., Kelsoe, G., Rajewsky, K. and Weiss, U.,** Intraclonal generation of antibody mutants in germinal centres. *Nature* 1991. **354:** 389–392.

7 **Berek, C., Berger, A. and Apel, M.,** Maturation of the immune response in germinal centers. *Cell* 1991. **67:** 1121–1129.

8 **Oppezzo, P., Vuillier, F., Vasconcelos, Y., Dumas, G., Magnac, C., Payelle-Brogard, B., Pritsch, O. and Dighiero, G.,** Chronic lymphocytic leukemia B cells expressing AID display dissociation between class switch recombination and somatic hypermutation. *Blood* 2003. **101:** 4029–4032.

9 **Martin, A., Bardwell, P. D., Woo, C. J., Fan, M., Shulman, M. J. and Scharff, M. D.,** Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* 2002. **415:** 802–806.

10 **Yoshikawa, K., Okazaki, I. M., Eto, T., Kinoshita, K., Muramatsu, M., Nagaoka, H. and Honjo, T.,** AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. *Science* 2002. **296:** 2033–2036.

11 **Petersen-Mahrt, S. K., Harris, R. S. and Neuberger, M. S.,** AID mutates E. col suggesting a DNA deamination mechanism for antibody diversification. *Nature* 2002. **418:** 99–103.

12 **Betz, A. G., Milstein, C., Gonzalez-Fernandez, A., Pannell, R., Larson, T. and Neuberger, M. S.,** Elements regulating somatic hypermutation of an immunoglobulin kappa gene: critical role for the intron enhancer/matrix attachment region. *Cell* 1994. **77:** 239–248.

13 **Martin, A. and Scharff, M. D.,** Somatic hypermutation of the AID transgene in B and non-B cells. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 12304–12308.

14 **Okazaki, I. M., Hiai, H., Kakazu, N., Yamada, S., Muramatsu, M., Kinoshita, K. and Honjo, T.,** Constitutive expression of AID leads to tumorigenesis. *J. Exp. Med.* 2003. **197:** 1173–1181.

15 **Nakamuta, M., Oka, K., Krushkal, J., Kobayashi, K., Yamamoto, M., Li, W. H. and Chan, L.,** Alternative mRNA splicing and differential promoter utilization determine tissue-specific expression of the apolipoprotein B mRNA-editing protein (Apobec1) gene in mice. Structure and evolution of Apobec1 and related nucleoside/nucleotide deaminases. *J. Biol. Chem.* 1995. **270:** 13042–13056.

16 **Lau, P. P., Zhu, H. J., Baldini, A., Charnsangavej, C. and Chan, L.,** Dimeric structure of a human apolipoprotein B mRNA editing protein and cloning and chromosomal localization of its gene. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 8522–8526.

17 **Bransteitter, R., Pham, P., Scharff, M. D. and Goodman, M. F.,** Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 4102–4107.

18 **Chaudhuri, J., Tian, M., Khuong, C., Chua, K., Pinaud, E. and Alt, F. W.,** Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* 2003. **422:** 726–730.

19 **Dickerson, S. K., Market, E., Besmer, E. and Papavasiliou, F. N.,** AID mediates hypermutation by deaminating single stranded DNA. *J. Exp. Med.* 2003. **197:** 1291–1296.

20 **Ramiro, A. R., Stavropoulos, P., Jankovic, M. and Nussenzweig, M. C.,** Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. *Nat. Immunol.* 2003. **4:** 452–456.

21 **Sale, J. E., Calandrini, D. M., Takata, M., Takeda, S. and Neuberger, M. S.,** Ablation of XRCC2/3 transforms immunoglobulin V gene conversion into somatic hypermutation. *Nature* 2001. **412:** 921–926.

22 **Bachl, J. and Wabl, M.,** An immunoglobulin mutator that targets G.C base pairs. *Proc. Natl. Acad. Sci. USA* 1996. **93:** 851–855.

23 **Bachl, J. and Olsson, C.,** Hypermutation targets a green fluorescent protein-encoding transgene in the presence of immunoglobulin enhancers. *Eur. J. Immunol.* 1999. **29:** 1383–1389.

24 **Rogozin, I. B. and Kolchanov, N. A.,** Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochim. Biophys. Acta* 1992. **1171:** 11–18.

25 **Mizushima, S. and Nagata, S.,** pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 1990. **18:** 5322.

26 **Cohen, J. H., Revillard, J. P., Magaud, J. P., Lenoir, G., Vuillaume, M., Manel, A. M., Vincent, C. and Bryon, P. A.,** B cell maturation stages of Burkitt's lymphoma cell lines according to Epstein-Barr virus status and type of chromosome translocation. *J. Natl. Cancer Inst.* 1987. **78:** 235–242.

27 **Hurwitz, R., Hozier, J., LeBien, T., Minowada, J., Gajl-Peczalska, K., Kubonishi, I. and Kersey, J.,** Characterization of a leukemic cell line of the pre-B phenotype. *Int. J. Cancer* 1979. **23:** 174–180.

28 **Rada, C., Williams, G. T., Nilsen, H., Barnes, D. E., Lindahl, T. and Neuberger, M. S.,** Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in UNG-deficient mice. *Curr. Biol.* 2002. **12:** 1748–1755.

29 **Zeng, X., Winter, D. B., Kasmer, C., Kraemer, K. H., Lehmann, A. R. and Gearhart, P. J.,** DNA polymerase eta is an A-T mutator in somatic hypermutation of immunoglobulin variable genes. *Nat. Immunol.* 2001. **2:** 537–541.

30 **Diaz, M., Velez, J., Singh, M., Cerny, J. and Flajnik, M. F.,** Mutational pattern of the nurse shark antigen receptor gene (NAR) is similar to that of mammalian Ig genes and to spontaneous mutations in evolution: the translesion synthesis model of somatic hypermutation. *Int. Immunol.* 1999. **11:** 825–833.

31 **Faili, A., Aoufouchi, S., Flatter, E., Gueranger, Q., Reynaud, C. A. and Weill, J. C.,** Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase iota. *Nature* 2002. **419:** 944–947.

32 **Bachl, J., Carlson, C., Gray-Schopfer, V., Dessing, M. and Olsson, C.,** Increased transcription levels induce higher mutation rates in a hypermutating cell line. *J. Immunol.* 2001. **166:** 5051–5057.

33 **Wang, C. L., Harper, R. A. and Wabl, M.,** Genome-wide somatic hypermutation. *Proc. Natl. Acad. Sci. USA* 2004. **101:** 7352–7356.

34 **Drexler, H.,** The leukemia-lymphoma cell line, 1st edn. Academic Press, London 2001

35 **Peters, A. and Storb, U.,** Somatic hypermutation of immunoglobulin genes is linked to transcription initiation. *Immunity* 1996. **4:** 57–65.

36 **Fukita, Y., Jacobs, H. and Rajewsky, K.,** Somatic hypermutation in the heavy chain locus correlates with transcription. *Immunity* 1998. **9:** 105–114.

37 **Rada, C., Jarvis, J. M. and Milstein, C.,** AID-GFP chimeric protein increases hypermutation of Ig genes with no evidence of nuclear localization. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 7003–7008.

38 **Ito, S., Nagaoka, H., Shinkura, R., Begum, N., Muramatsu, M., Nakata, M. and Honjo, T.,** Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. *Proc. Natl. Acad. Sci. USA* 2004. **101:** 1975–1980.

39 **Brar, S., Watson, M. and Diaz, M.,** Activation-induced cytosine deaminase, AID, is actively exported out of the nucleus but retained by the induction of DNA breaks. *J. Biol. Chem.* 2004. **279:** 26395–26401.

40 **McBride, K. M., Barreto, V., Ramiro, A. R., Stavropoulos, P. and Nussenzweig, M. C.,** Somatic hypermutation is limited by CRM1-dependent nuclear export of activation-induced deaminase. *J. Exp. Med.* 2004. **199:** 1235–1244.

41 **Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A. et al.,** Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 2000. **102:** 565–575.

42 **Lau, P. P., Xiong, W. J., Zhu, H. J., Chen, S. H. and Chan, L.,** Apolipoprotein B mRNA editing is an intranuclear event that occurs posttranscriptionally coincident with splicing and polyadenylation. *J. Biol. Chem.* 1991. **266:** 20550–20554.

43 **Yang, Y., Sowden, M. P. and Smith, H. C.,** Intracellular trafficking determinants in APOBEC-1, the catalytic subunit for cytidine to uridine editing of apolipoprotein B mRNA. *Exp. Cell. Res.* 2001. **267:** 153–164.

44 **Blanc, V., Kennedy, S. and Davidson, N. O.,** A novel nuclear localization signal in the auxiliary domain of apobec-1 complementation factor regulates nucleocytoplasmic import and shuttling. *J. Biol. Chem.* 2003. **278:** 41198–41204.

45 **Faili, A., Aoufouchi, S., Gueranger, Q., Zober, C., Leon, A., Bertocci, B., Weill, J. C. and Reynaud, C. A.,** AID-dependent somatic hypermutation occurs as a DNA single-strand event in the BL2 cell line. *Nat. Immunol.* 2002. **3:** 815–821.

46 **Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K.,** Current protocols in molecular biology.Wiley, New York 1995

