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Non-conservative homologous recombination in human B lymphocytes is promoted by activation-induced cytidine deaminase and transcription

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ABSTRACT

During secondary immunoglobulin (Ig) diversification in vertebrates, the sequence of the variable region of Ig genes may be altered by templated or non-templated mechanisms. In both cases, cytidine deamination by activation-induced cytidine deaminase (AID) in the transcribed Ig loci leads to DNA lesions, which are repaired by conservative homologous recombination (HR) during Ig gene conversion, or by non-templated mutagenesis during somatic hypermutation. The molecular basis for the differential use of these two pathways in different species is unclear. While experimental ablation of HR in avian cells performing Ig gene conversion may promote a switch to somatic hypermutation, the activity of HR processes in intrinsically hypermutating mammalian cells has not been measured to date. Employing a functional HR assay in human germinal centre like B cell lines, we detect elevated HR activity that can be enhanced by transcription and AID. Products of such recombination events mostly arise through non-conservative HR pathways, while the activity of conservative HR is low to absent. Our results identify non-conservative HR as a novel DNA transaction pathway promoted by AID and suggest that somatic hypermutation in germinal centre B cells may be based on a physiological suppression of conservative HR.

INTRODUCTION

Appropriate repair of DNA damage is essential for the survival of all organisms to prevent unwanted genetic changes, and is thus sustained by an intricate network of DNA repair pathways. DNA lesions encountered by a moving replication fork can be bypassed directly by low fidelity translesion polymerases, often leading to point mutations. Alternatively, the damage may be overcome by pathways based on homologous recombination (HR), using the information of the intact sister chromatid or another homologous DNA region as template (1). Replication fork collapse upon sustained stalling can result in DNA double strand breaks which may also be repaired by HR. Here, an alternative pathway is non-homologous end joining (NHEJ) that religates broken DNA ends without controlling origin and correctness, and may hence be rather error-prone.

HR is usually considered a very precise and error-free DNA repair pathway, as it uses a homologous copy of the damaged gene as template (2). However, HR may also lead to net loss of DNA or other genetic aberrations. Recent models therefore divide HR into conservative HR (including gene conversion) that leads to a copying or exchange of sequence patches without net DNA loss, and non-conservative HR that may lead to deletions, gene fusions or other genetic aberrations (3–6). In higher eukaryotes, the mechanistic basis and enzymatic requirements for the decision between these two pathways are poorly understood to date. Clearly, though, faithful conservative HR is important for genetic stability of

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a cell, as in its absence the processing of DNA damage is performed by error-prone alternative repair pathways such as translesion synthesis, NHEJ or non-conservative HR.

B lymphocytes abundantly introduce lesions into their DNA during immunoglobulin (Ig) diversification. Therefore, DNA repair mechanisms must be tightly regulated in these cells to maintain integrity of the overall genome, but also allow for the required genetic alterations (1). In the case of diversification of the variable region of Ig genes, differential repair of the DNA lesions triggered by activation-induced cytidine deaminase (AID) even leads to different outcomes. During Ig gene conversion in chicken and some mammals, conservative HR with upstream pseudogenes causes templated changes. During somatic hypermutation, different repair pathways contribute to non-templated mutagenesis, including replication over AID-induced uracils, an error-prone variant of mismatch repair and translesion synthesis involving multiple error-prone polymerases and the Rad6 pathway (7–10). Also, removal of the uracils by uracil-*N*-glycosylase (UNG) results in abasic sites, which may subsequently be processed into DNA single strand breaks (9). AID action on both DNA strands may thus lead to staggered double strand breaks that might serve as substrates for HR (11).

An involvement of HR in somatic hypermutation was in fact postulated, based on detection of double strand breaks in hypermutating cells in the G2 phase of the cell cycle when HR is the predominant repair pathway, and on the recruitment of the HR factors Rad51 and Rad52 to these breaks (11,12). The contribution of HR to somatic hypermutation is, however, questionable as the critical HR factors Rad54 and Rad54B are not required for hypermutation (13). Also, in the chicken B cell line DT40 constitutive Ig gene conversion is converted to somatic hypermutation by inhibition of HR in *trans* or in *cis*: the inactivation of critical HR factors (the Rad51 paralogues Xrcc2/3 or Rad51B) or the deletion of the pseudogenes that function as donors for HR lead to a drop in Ig gene conversion and a concomitant rise in somatic hypermutation activity (14–16). It may therefore appear that ablation of HR, rather than its recruitment, is a prerequisite for somatic hypermutation to occur.

To critically evaluate the role of HR in human B lymphocytes, we have applied a functional HR assay to human B cell lines. We report that human germinal center like B lymphoma cells are characterized by high recombination activity, which can be boosted by AID or transcription. Analysis of the recombination products revealed a surprising predominance of non-conservative HR, suggesting a very low activity of conservative HR in hypermutating human B cells. AID induced non-conservative HR is a novel DNA transaction pathway that may contribute to Ig diversification by formation of the previously recognized hybrid V genes, and may also affect lymphomagenesis by the generation of genetic aberrations.

MATERIALS AND METHODS

Vectors

The recombination reporter cassette and the GFP control gene (17) were cloned into the pBC230 vector under control of the CMV-promoter, the Ig enhancers E3 and Ei and a matrix attachment region (MAR) (18,19). To delete the donor GFP gene, the vector was cut with BstBI and religated. The reporter for conservative recombination was generated by cutting the original vector with BstBI and KpnI to delete 94 bp of the donor GFP gene.

A doxycycline-regulable recombination reporter was generated from pRTS-1 (20) by exchange of the Eμ enhancer by the CMVieE enhancer, insertion of a truncated nerve growth factor receptor ($NGFR_t$) gene into the SfiI sites and insertion of the recombination cassette between the AscI and SwaI sites. From this construct, the HA-AID expression vector was generated by exchange of the $NGFR_t$ gene by a HA-AID-IRES-$NGFR_t$ cassette (21) and deletion of the recombination cassette. For inducible Rad51 overexpression, the Rad51 coding region was PCR amplified, fused to an IRES-$NGFR_t$ cassette and ligated in place of the HA-AID-IRES-$NGFR_t$ cassette into the same vector.

Cell culture, recombination assays and western blots

The human B cell lines Raji, BJAB, 721, EREB2-5 and the human T cell line Jurkat were cultured in RPMI-1640 (Invitrogen, Carlsbad, California, USA) containing 10% fetal bovine serum, penicillin, streptomycin, sodium pyruvat and glutamine at 37°C in a humidified 5% CO_2 atmosphere. Three to four days after electroporation, selection was started by addition of 50–400 μg/ml hygromycin (Invitrogen) or 0.8 μg/ml puromycin (Sigma-Aldrich, St.Louis, Missouri, USA), and was typically complete 14 days after transfection. Every 2–4 days, 10^5 living cells were analysed for GFP+ cells on a FACScan (Becton Dickenson, Franklin lakes, NJ, USA). For analysis of transcription effects, aliquots of single cell clones containing the inducible reporter vector were induced in weekly intervals by addition of 1 μg/ml doxycycline, and the number of GFP positive cells was determined by FACS analysis over a period of 2 weeks. The factor of transcription effect was calculated for each subclone by the formula: $[(1/n_b)\sum b_i]/[(1/n_a)\sum a_i]$ (Figure 2D; a, closed circles; b, open circles). *P*-values were derived by Student's *t*-tests. The BJAB subclone inducible for HA-AID expression by application of doxycycline was generated by integration of the HA-AID expression vector into the genome after linearization with AscI and XmnI. This clone was analysed for transcription effect as described above by induction of AID expression and transcription in parallel.

For overexpression of AID in batch cultured BJAB cells, the inducible HA-AID expression vector was transfected in parallel with the reporter vector and cells were cultured in the presence of 1 μg/ml doxycycline. Copy numbers were determined by quantitative PCR in the Light cycler (Roche, Basel, Switzerland) with primers CMV3for: 5′-GCATTATGCCCAGTACATGACC-3′

and CMV4rev: 5′-CGGTTCACTAAACGAGCTCTGC-3′ for the CMV promoter in the vector and hHPRT2: 5′-CTAATGTGATAGACTACTGCTTTG-3′ and hHPRT3: 5′-CCAAACTCAACTTGAACTCTC-3′ for the genomic HPRT locus. Copy numbers were calculated according to the instructions of the manufacturer.

AID protein levels and Rad51 protein levels were determined as described (22) and by anti-Rad51 (Santa Cruz, Santa Cruz, California, USA) and peroxidase conjugated anti-goat IgG (Sigma) antibodies. Anti-actin (Santa Cruz) and peroxidase conjugated anti-mouse IgG (Promega, Madison, Wisconsin, USA) were used for standardization.

Analysis of recombination products

After plasmid rescue from 10^6 transfected cells by Hirt-extraction (17) and retransformation into electrocompetent XL1 blue bacteria (Stratagene, Santa Clara, California, USA), plasmids were prescreened by PacI digestion to excise the recombination cassette and further analysed by other restriction digests and sequencing. GFP+ cells were sorted on a MoFlo cell sorter (Dako Cytomation, Glostrup, Denmark) and expanded. After DNA extraction from GFP+ cells by the QIAamp Kit (Qiagen, Hilden, Germany) or direct lysis with 0.5 µg/µl Proteinase K in 10 mM Tris–HCl pH 7.5 for 2.5 h at 56°C, followed by 10 min at 100°C, Taq PCR was performed with primers GFP1: 5′-AAGAAATGGCTAGCAA AGGAGAAG-3′ in combination with either GFP2: 5′-CTCAGTTGTACAGTTCATCCATG-3′ or Puro5: 5′-ACACATTCCACAGGGTCGAGG-3′. The PCR products were analysed by restriction digestion with Bst1107I and subsequent Southern blot analysis with a full-length GFP probe.

RESULTS

High recombination activity in germinal centre-like B lymphoma cells

To study HR in human B cells, we used an episomally replicating EBV-based vector carrying a CMV promoter-driven direct repeat of two defective GFP genes. The acceptor GFP gene is inactivated by a frameshift insertion and can be reconstituted to a functional GFP by HR with the donor GFP gene, of which the 5′ coding sequence is deleted (Figure 1A) (17). The reporter is able to differentiate between conservative and non-conservative recombination pathways (Figure 1A), each of which may be based on multiple molecular mechanisms [see (3–6) and Discussion section for details].

Upon transfection into human Raji B cells, a time dependent increase in GFP+ cells was observed (Figure 1B) which is clearly dependent on homology-directed repair, as no GFP+ cells were seen with a reporter construct in which the donor GFP is deleted (Figure 1B). After completion of selection for vector-bearing cells (around day 14), the increase in GFP+ cells over time is indicative of ongoing recombination activity.

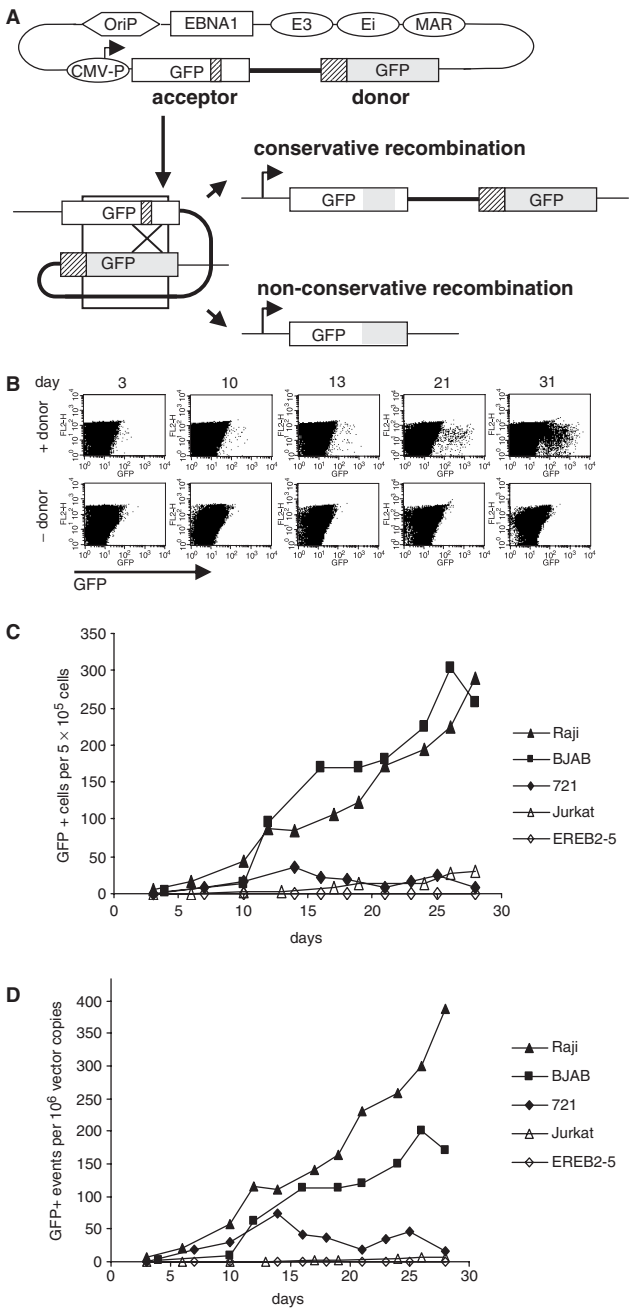

Figure 1. Differences in HR activity in human B cells. (**A**) The GFP-based recombination cassette and the two potential recombination products. The acceptor GFP contains a frameshift insertion, and the donor GFP is inactivated by an N-terminal deletion (striped boxes). CMV-P, CMV promoter; Ig enhancer elements: E3, 3′ enhancer; Ei, intron enhancer; MAR, matrix attachment region. Epstein-Barr-virus elements: replication origin OriP and the EBV nuclear antigen (EBNA) 1 gene. (**B**) Detection of GFP+ cells over time in Raji cells transfected with the recombination reporter (upper plots) or a reporter lacking the donor GFP (lower plots). (**C**) Frequencies of GFP+ cells over time in the Burkitt lymphoma line Raji, the Burkitt-like line BJAB, the two human lymphoblastoid cell lines 721 and EREB2-5 and the T cell line Jurkat. B cells were transfected with the recombination reporter containing Ig enhancers and Jurkat with a construct lacking the enhancers. Selection for vector bearing cells was completed around day 14. Data are representative of ≥5 (Raji, BJAB, 721) or 2 (Jurkat, EREB2-5) experiments. (**D**) Frequencies of GFP restoring events per vector copy over time in the respective cell lines.

To investigate whether this recombination activity is a general feature of human B cells, we determined recombination activities in the Burkitt lymphoma line Raji, the Burkitt-like line BJAB, the two human lymphoblastoid cell lines 721 and EREB2-5 and the Jurkat T cell line. We used the batch approach to avoid experimental skewing in fluctuation analysis due to potential clonal variabilities in B cells (23). High HR activities were observed in Raji and BJAB, 721 displayed intermediate activity, and barely any recombination was detectable in EREB2-5 and Jurkat. A representative sample set is displayed in Figure 1 without (C) or with (D) normalization of HR activities to episome copy numbers. A broader application of this assay was limited by low transfection rates of most B cell lines and by rapid episome loss or silencing in others.

For Raji, the most active cell line in recombination, the amount of GFP+ cells divided by the average copy number of the reporter in the cells was used to calculate recombination rates. Multiple independent experiments in the course of this study yielded recombination rates of 1×10^{-5} to 5×10^{-5} per gene copy and cell division. This is clearly above the spontaneous HR rates observed for other human cells using the same or similar recombination cassettes (10^{-6} to 10^{-7}) (17,24,25).

We conclude that high recombination activity is not a general feature of human B cells, but is rather due to activities that vary between different cell lines or cellular differentiation stages. As recombination activating gene (Rag) expression can be excluded as the major cause for recombination activity in these cells (26), we concentrated on examining the influence of processes that are characteristic for germinal centre B cells.

Effect of transcription on recombination

Spontaneous recombination, as measured in our assay system, may be due to several reasons, including a high frequency of breaks introduced into the DNA during transcription or replication. To gain insight into the causes of the high recombination activity in human B cells, we investigated the influence of transcription on recombination using a doxycycline-regulable episomal vector system (Figure 2A) (20). In this vector, a bidirectional regulable promoter drives coupled expression of the recombination cassette and the $NGFR_t$ gene. Because of variability in the quality of expression regulation in this system (20), vector-containing Raji and BJAB subclones were generated.

As GFP expression in this system requires transcription of the reporter as well, three culture aliquots of the subclones were induced for transcription at different time points, followed by determination of GFP+ cells over 2 weeks of continuous induction. We analysed 34 Raji and 35 BJAB subclones showing high inducibility and reasonable stability of $NGFR_t$ expression, which represents consistent reporter expression. As exemplarily shown in Figure 2B and C, the analysis revealed a complex picture for transcription influence. Some clones show a rapid increase in GFP+ cells upon transcription induction, while in others a small continuous increase in the number of GFP+ cells was seen, likely representative of

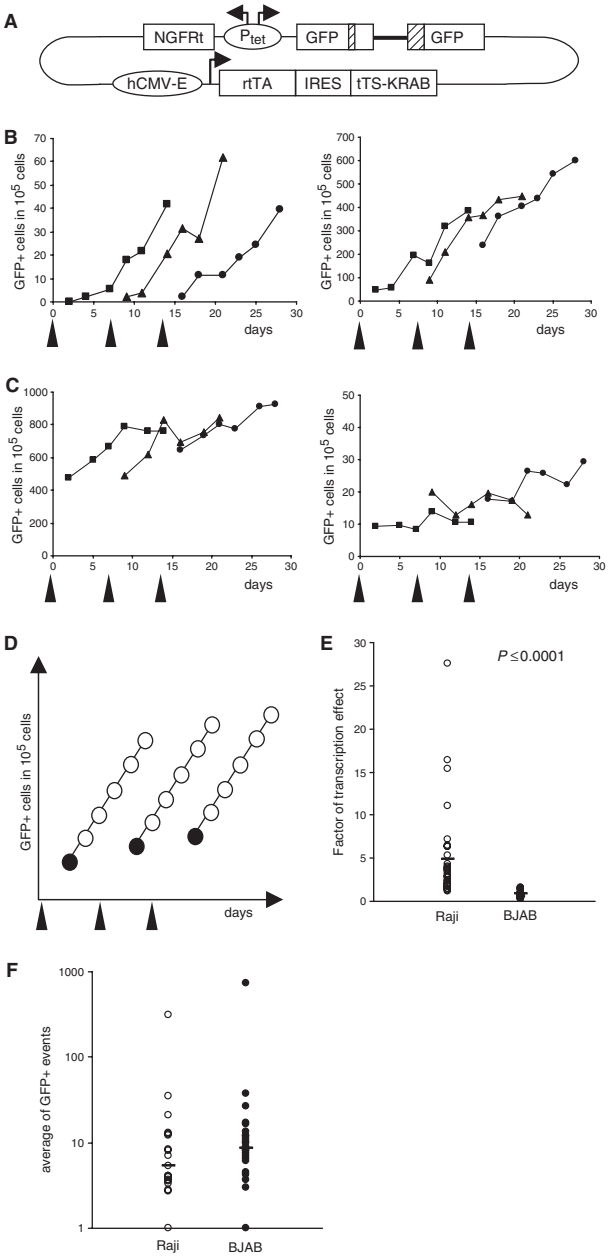

Figure 2. Transcription promotes recombination in Raji but not in BJAB cells. (**A**) Schematic structure of the vector used for the analysis of transcription effects on recombination. The bidirectional doxycycline-inducible promoter driving expression of the recombination cassette and $NGFR_t$ and elements for doxycycline dependent regulation are indicated. The vector system does not contain Ig enhancer elements. Frequencies of GFP+ cells after doxycycline addition in four representative out of 34 Raji (**B**) and 35 BJAB (**C**) subclones containing the inducible reporter. Arrowheads and different symbols indicate doxycycline addition to aliquots of each subclone. (**D**) Schematic illustration for calculation of the factor of transcription effect: the average number of GFP+ cells generated after transcription induction (average of all values represented by open circles) was divided by the average number of GFP+ cells detected at the beginning of transcription induction (average of all values represented by closed circles). (**E**) Factors of transcription effect for all Raji and BJAB subclones analysed with the inducible reporter. Each dot represents one clone. (**F**) Average numbers of GFP+ cells for all subclones of Raji and BJAB were calculated using the whole data set (i.e. average of all data represented by open and closed circles in D). Each dot represents one clone. The difference between the data sets is not statistically significant ($P > 0.1$).

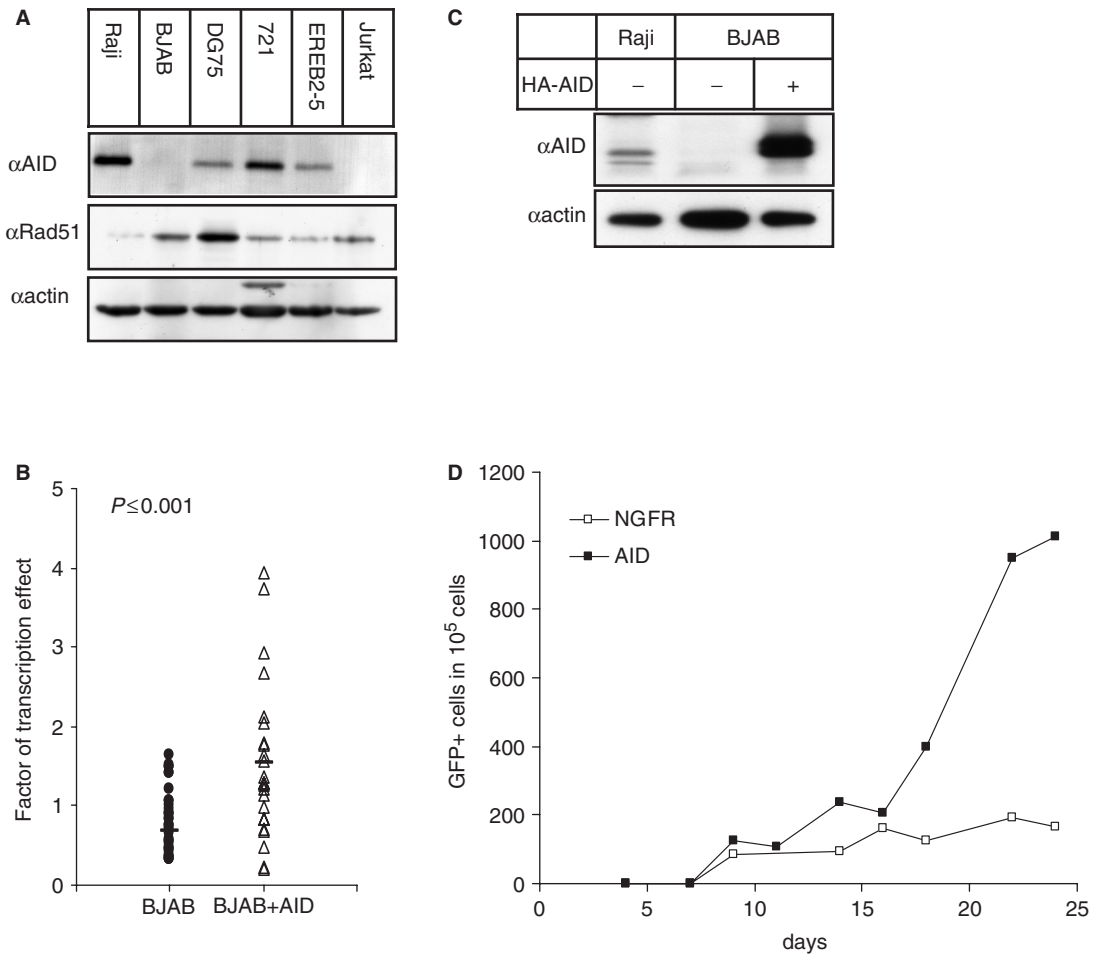

Figure 3. Effect of AID on recombination activity. (**A**) AID and Rad51 protein levels in the cell lines studied. (**B**) Factor of transcription effect of BJAB subclones without and with overexpression of HA-AID. (**C**) Comparison of endogenous and HA-tagged AID protein expression levels in Raji and BJAB cells transfected with the HA-AID- or $NGFR_t$-expression vector. A minute lower migrating band seen in some experiments for endogenous AID likely represents a degradation product. (**D**) Recombination events in BJAB cells transfected with the reporter and the HA-AID- or $NGFR_t$-expression vector. Completion of selection of cells bearing both the recombination reporter and the overexpression vector was achieved around day 16.

recombination independently of transcription induction. To evaluate these data for the effect of transcription on recombination activity, the clones were classified by a factor that describes the ratio of the average number of GFP+ cells generated by doyxcycline induced transcription, divided by the average number of GFP+ cells detected directly after transcription induction, taking into account the data from all three induction aliquots (Figure 2D). Accordingly, the factor should clearly exceed a value of 1 for a transcription effect. The classification revealed a strong promoting effect of transcription on HR activity in Raji subclones, with a mean value of 4.8. In contrast, in BJAB subclones recombination activity is not considerably promoted by transcription, as deduced from a mean value of 0.8 (Figure 2E). Consistent with the previous batch experiments, Raji and BJAB subclones show similar average levels of GFP+ cells with variability among the clones (Figure 2F). Taken together, one can conclude that in Raji cells HR activity is highly stimulated by transcription.

Promotion of HR by AID

The Raji Burkitt lymphoma cell line is characterized by constitutive hypermutation activity, as determined by a hypermutation reporter in an identical vector context (18). The effect of transcription on recombination seen in these cells may be caused by AID, which is also active on transcribed genes. Indeed, one obvious difference between BJAB and Raji is their AID protein level (Figure 3A).

In order to assess whether AID affects HR activity, we generated BJAB cells inducible for HA-AID expression and performed the experiment to assess transcription effects as described above (Figure 3B). Indeed, upon overexpression of AID in BJAB cells, the mean value of the factor of transcription effect was significantly increased from 0.8 to 1.5 (Figure 3B), indicating a promoting effect of AID in concert with transcription on total HR activity.

To further characterize the effect of AID on HR activity, we cotransfected the recombination reporter and an

AID overexpression vector into BJAB cells. Following selection for cotransfected cells (day 16), a significant increase in recombinant frequencies was clearly detectable upon AID overexpression (Figure 3C and D). Taken together, the results reveal that both transcription and AID have a highly promoting effect on induction of recombination activity.

Preferential use of non-conservative recombination pathways

Generation of GFP+ cells in our assay is indicative of homology-directed recombination between the two GFP genes, which can lead to two possible recombination products (17). In one product, the recombination cassette is retained while in the other product, the two GFP genes are fused and the spacer sequence is deleted (Figure 1A). These products refer to particular pathways of recombination, termed conservative and non-conservative, respectively (3,27–30).

To assess the overall recombination events on the reporter, we first extracted episomes from Raji cells harbouring the reporter for 35 days and transformed them into *Escherichia coli*. In total, 243 plasmids were rescued and the precise conformation of the reporter cassette was studied by restriction analysis. Total 237 reporter plasmids showed the restriction pattern of the originally transfected plasmid and only six displayed evidence for recombination. Thus, excessive breakage and ligation of the reporter does not occur in the course of the experiment.

One of the six altered plasmids (R) showed a repair event where the two GFP genes are fused (Figure 4A), restoring the functionality of the GFP gene as demonstrated in Figure 4B. In our reporter system, this kind of recombination product may arise either from rare gene conversion with a crossover event, or alternatively from pathways that are grouped as non-conservative HR and are defined by such net loss of DNA.

In the five other plasmids, deletion events disrupting the GFP tandem repeat were detected (Figure 4A and B). Sequence analysis of the junctions of these deletions revealed that NHEJ has been used for repair. Thus, breaks occurring in our reporter plasmid are repaired both by HR and NHEJ at a relative frequency consistent with previous observations (30).

We then decided to look closer at the structure of the resultant HR products by approaches that can differentiate between conservative HR and non-conservative pathways. GFP-positive cells were sorted at an episome copy number of 1–2 per cell, and the status of the recombination reporter was analysed by the PCR strategies shown in Figure 4C. As expected, amplification of the acceptor GFP gene with two internal primers, followed by cleavage with Bst1107I (which is specific for repaired GFP), clearly detected homology-dependent repair events in all cell lines investigated (Figure 4D). In contrast, the PCR reaction on the acceptor GFP using a 3′ primer located between the two GFPs amplified some residual unrepaired copies of the reporter, but failed to detect repair events in these cell lines (Figure 4D). This finding implies that in fact most of the HR events result from non-conservative recombination pathways which delete the internal sequence. This notion was further confirmed by restriction analysis of recovered episomes from sorted GFP+ cells. Most of them had deleted the internal part of the recombination cassette, leading to a productive GFP gene (data not shown). Control experiments confirmed that recombined episomes that have retained or deleted the internal sequence lead to equal levels of GFP expression in the cells (Figure 4F). Chromosomally integrated reporters also showed a clear preference for non-conservative HR in the cell lines investigated (Figure 4E), indicating that the preference for this deletion-associated HR pathway is not due to the episomal nature of the reporter.

To study the ratio of non-conservative and conservative HR in these cells in more detail, we additionally applied a reporter specific for conservative recombination (Figure 5A). This specificity can be achieved by locating the donor GFP gene upstream of the acceptor gene or by inactivation of the donor GFP gene by deletion of both the 5′- and the 3′-end of the coding sequence. Due to these modifications, non-conservative recombination cannot restore a functional GFP gene. We chose the latter approach to ensure better comparability of the two reporters.

The cell lines were transfected with either reporter, selected for vector-containing cells and the number of GFP+ cells was determined at the same time point. One of the results summarized in Table 1 and Figure 5C is shown in Figure 5B. In agreement with the results shown in Figure 4, all tested cell lines predominately used non-conservative recombination pathways. There are interesting differences in the proportion of conservative recombination, though. While activity of this pathway was barely detectable in Raji, BJAB and 721 cells, the Burkitt lymphoma cell line DG75 showed a substantial percentage of conservative recombination, consistent with its suitability for gene targeting, a process requiring conservative recombination (31). The clear bias to non-conservative HR in human B lymphoma cell lines is thus not due to technical constraints of the assay, but rather caused by a relatively low activity of conservative HR pathways in these B cells.

Potential reasons for the defective conservative HR in Raji and BJAB cells include impaired damage recognition, damage signalling or repair factor recruitment. Both cell types formed γH2AX/53bp1-foci spontaneously (data not shown), indicating that recognition of DNA breaks is intact. Interestingly, however, protein levels of the critical HR factor Rad51 were very low in these cells (Figure 3A). While this feature may contribute to the HR defect, it is not the sole responsible factor, as Rad51 re-expression did not rescue conservative HR capacity (Figure 5D and E). Thus, the repair defect observed may be caused by impairment in more than one critical player, or an upstream component that regulates their expression or function.

DISCUSSION

In the present study, we show that germinal centre like B lymphoma cell lines display high recombination activity that could be linked to transcription in the actively

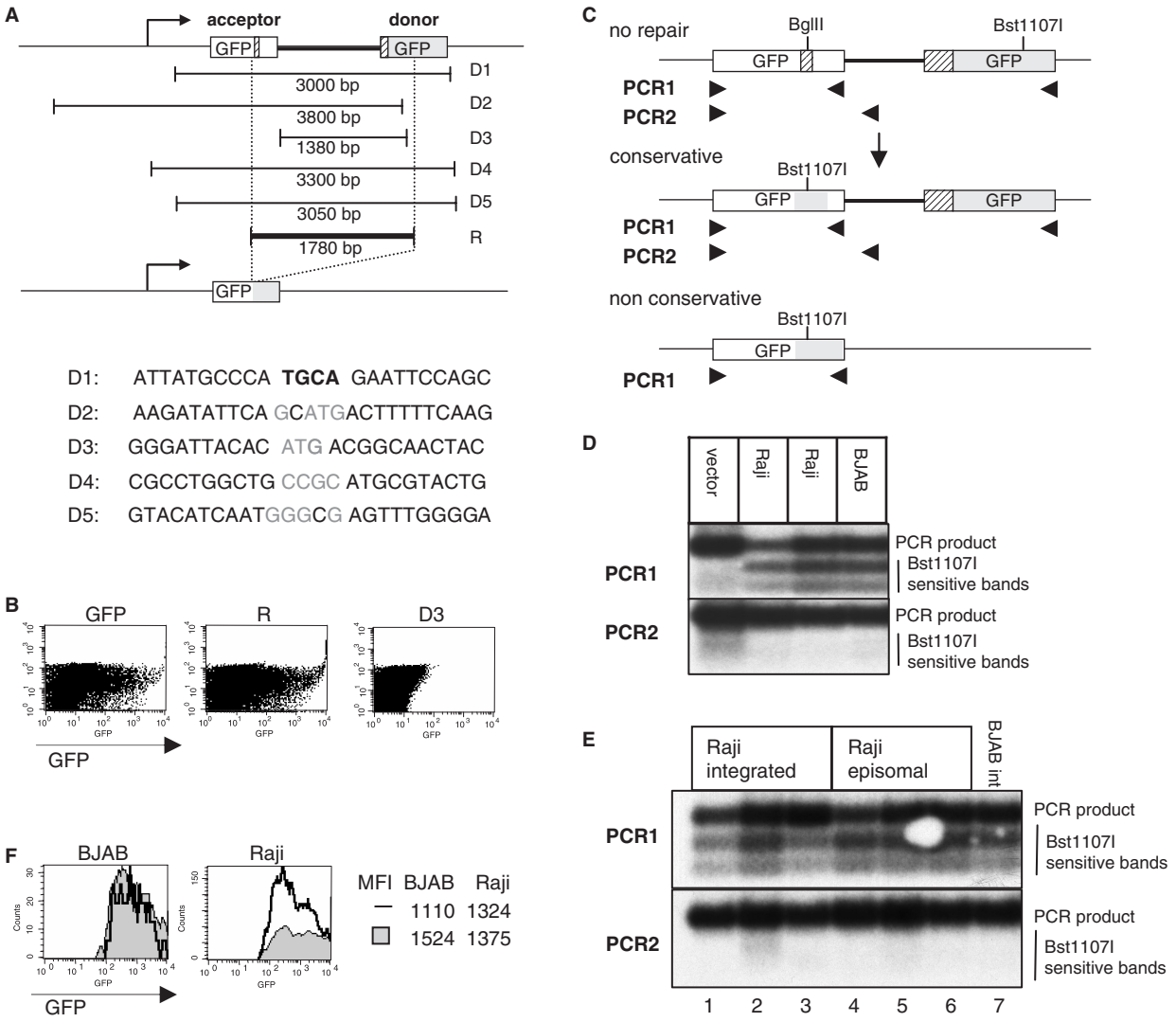

Figure 4. Preferential non-conservative recombination in human B cells. (**A**) Structural and sequence analysis of recovered reporter plasmids from Raji cells (after 35 days of culture) showing a GFP restoring recombination event (R; thick bar) or destructive deletions (D1-5; thin bars). Gaps in the sequence information display junctions with micro-homologies (grey) or insertions (bold). (**B**) Analysis for GFP expression capacity of two recovered reporter plasmids (R and D3) by retransfection into Raji cells. GFP indicates the control with a vector containing the functional GFP gene. (**C**) Schematic view of the PCR/restriction approach for analysis of recombination products. Primers for the respective PCRs (arrowheads) and the Bst1107I restriction site used for discrimination of repaired GFP genes are shown. (**D**) PCR/restriction/Southern blot analysis of DNA from sorted GFP+ cells from Raji and BJAB cells. Two independent experiments are shown for Raji. The transfected vector was used as a negative control. Full-length GFP was used as probe. (**E**) PCR/restriction/Southern blot analysis of chromosomally integrated and episomal reporters in Raji and BJAB cells analysed as in D. (**F**) Comparison of the GFP fluorescence intensity of the two possible recombination products (see C) in BJAB and Raji cells. Black line: non-conservative recombination product; grey: conservative recombination product. Corresponding mean fluorescence intensities (MFI) are listed.

hypermutating cell line Raji, and was greatly enhanced by AID. In contrast to Ig gene conversion in chicken B cells, HR in human B cells is strongly biased to non-conservative HR that may lead to deletions, gene fusions or translocations rather than to copying or exchange of short sequence patches. The low activity of conservative HR in these cells may explain why AID induced DNA lesions are processed by somatic hypermutation rather than Ig gene conversion. We also anticipate that AID-induced non-conservative DNA recombination may contribute to Ig diversification as well as to lymphomagenesis.

It is noteworthy that some spontaneous HR, as indicated by GFP+ cells, was detectable in all cell lines investigated, irrespective of AID expression levels or hypermutation capacity. This is not surprising, as the assay employed here was originally developed to investigate spontaneous mitotic HR presumably arising due to replication-associated DNA breaks (17,24). This form of HR may constitute most of the activity observed in the AID^{low} BJAB cells as well as in the non-hypermutating but AID expressing 721 and EREB2-5 lines. It is thus likely that the enhanced HR activity in hypermutating B cells is stimulated by replication-associated breaks as well as by transcription and AID. In agreement with ligation-mediated PCR experiments, DNA breaks leading to HR may hence occur without AID but will be increased upon

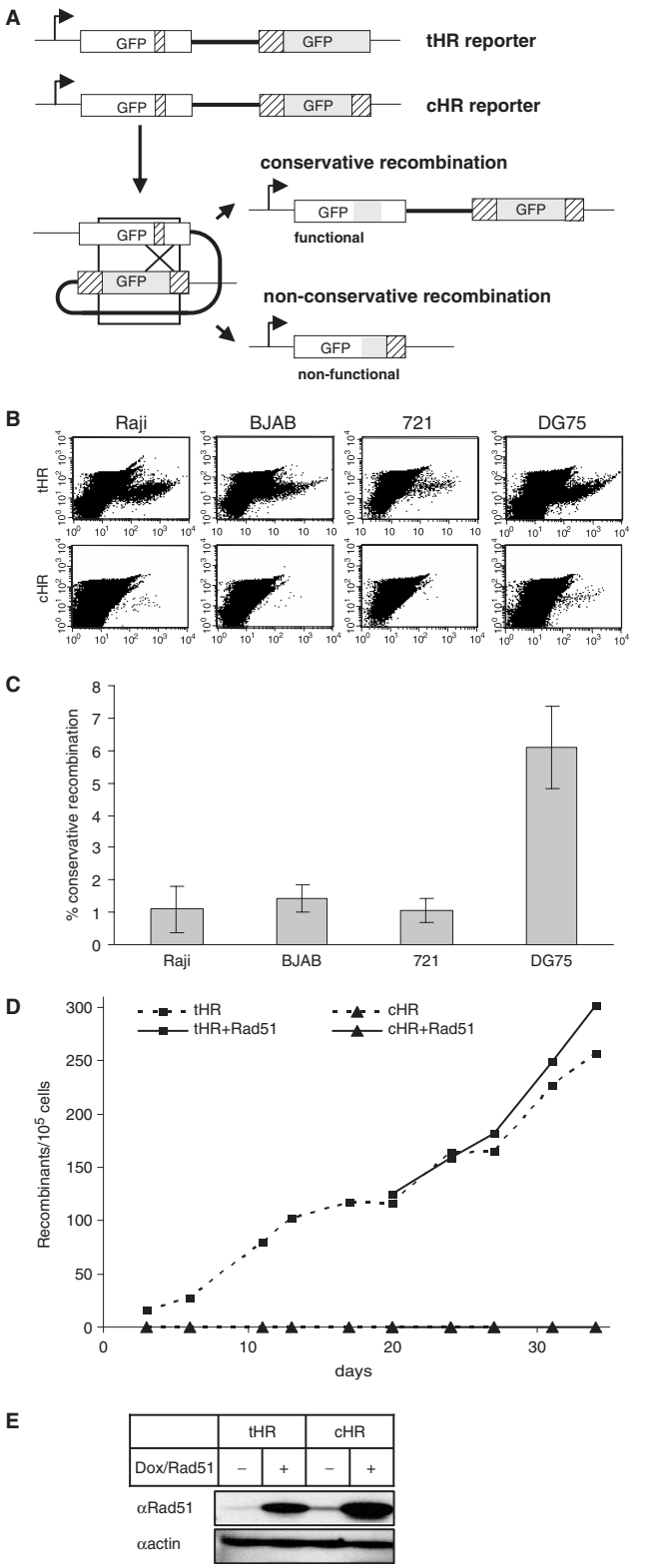

Figure 5. Differences in the proportion of conservative recombination in human B cells. (**A**) Schematic structure of the previously used reporter for all recombination pathways (tHR) and the reporter specific for conservative recombination (cHR). (**B**) Flow cytometry dot plots for frequencies of GFP+ cells in the Burkitt and Burkitt like lymphoma cell lines Raji, BJAB and DG75 and the lymphoblastoid cell line 721 for the reporter detecting all recombination events (tHR, upper row) and the reporter specific for conservative recombination (cHR, bottom row). (**C**) Percentage of conservative recombination in the cell lines analysed in B. Absolute numbers are listed in Table 1. (**D**) Rad51 overexpression does not rescue conservative HR in Raji cells. A clone carrying the inducible Rad51 overexpression vector was transfected with the tHR and cHR reporters, and upon completion of selection (day 20), Rad51 was induced in one aliquot of the culture by doxycycline addition. (**E**) Successful Rad51 induction in the analyses shown in (D) was assessed by western blot.

AID activity (11,12,32,33). While it appears likely that such breaks are due to the DNA damage inflicted by AID-mediated cytidine deamination, other modes of AID interference with DNA repair cannot be excluded at present (34). According to the DNA-deamination model, though, diverse pathways of mutagenesis are activated by AID-induced uracils and abasic sites (9) before they are processed to DNA breaks that may give rise to HR (11). Thus, intermediates that cause mutagenesis may eventually lead to recombination, in striking accordance with the situation encountered during AID-induced class switch recombination (35,36).

The total HR activity measured here reflects not only the amount of breaks but also the extent to which the breaks are repaired by HR rather than by other repair pathways. This depends on multiple factors and may vary among different cell types and differentiation stages. One must consider that in general, cells possess perfectly error-free repair pathways for most DNA lesions (i.e. also for typical AID induced lesions such as uracils and abasic sites), and might thus be capable of preventing both mutagenesis and recombination caused by AID. From studies of spontaneous mitotic recombination, it is known that the conversion of DNA lesions into HR intermediates during replication is substantially enhanced in cells deficient for damage sensors such as p53 and ATM (17,37,38), and both factors are low in germinal center cells (39,40). Also, the lymphoma cell lines we used are mutated in the p53 gene (41). Such damage sensors or other repair factors may in fact have a higher impact on the final HR activity than the AID levels in the respective cell.

The most striking finding of the present study is the strong predominance of recombination pathways that

Table 1. GFP+ cells generated by non-conservative and conservative recombination in B cell lines

Cell line	GFP+ cells in 10^6 living cells		Conservative recombination (%)
	tHR reporter	cHR reporter	
Raji	1953	31	1,5
	3063	19	0,6
BJAB	1062	15	1,4
	1922	16	0,8
721	833	9	1,1
	188	1	0,5
DG75	1954	102	5,2
	955	67	7,0

Results of two independent experiments are shown for each cell line. Cells were analysed 14 days and 24 days after transfection for the first and second experiment, respectively.

lead to a fusion of donor and acceptor GFP genes and deletion of the intervening sequence. Formally, such recombination events may be explained in the classical HR models by crossovers (3,5). Although we cannot exclude crossover events because the reciprocal products are not maintained in the experimental setting, they can not explain the predominance of these recombination products in the present study. Crossovers are suppressed during mitotic recombination and are also only a rare side effect of conservative HR during meiosis (42). Rather, non-conservative recombination pathways such as single-strand annealing or break-induced replication, which also lead to these products, are generally considered responsible for their predominance (3,6,27–30). Here, the crucial mechanistical difference to conservative HR is that after invasion of the damaged DNA strand into the homologous gene, subsequent DNA synthesis and ligation does not involve the return of the invading strand to the originally damaged DNA heteroduplex (3,5). Hence, fusion of the donor and acceptor genes and deletion or exchange of associated gene sequences occurs. With the reporter system used, we may not differentiate between the different subpathways of non-conservative HR. While break-induced replication requires only one DNA break, single strand annealing is based on two breaks, one within each homologous copy. In case of AID-induced DNA lesions, which generally occur in a 1kb region downstream of the promotor, the latter scenario seems less likely. Irrespective of the exact mechanism, though, non-conservative recombination may lead to genetic aberrations, as depending on the location of the homologous gene copies, deletion of intervening sequences (in tandem arrays), gross rearrangements (in inverted repeats) or chromosomal translocations (in interchromosomal HR) may occur.

This non-conservative HR has been observed in different experimental systems before, in particular in cells that show defects in critical HR factors (27,28). Hence, it may be used in the B cell lines in this study due to a low activity of factors promoting conservative HR (such as gene conversion). Such an interpretation is in accordance with the observation that inhibition of such critical HR factors leads to hypermutation in DT40 cells. Also, it is notable that in the virtual absence of conservative HR, we could observe all three alternative pathways—translesion synthesis (i.e. hypermutation), NHEJ and non-conservative HR—in hypermutating cells.

We have seen this bias to non-conservative HR in case of AID-induced and spontaneous HR in B cells and even some non-B cell lines (data not shown), so it may reflect a low activity of conservative HR in many human cells. On the other hand, our system detects substantial conservative HR in human lymphoid DG75 cells that show efficient gene targeting (31). While physiological mechanisms regulating any differential use of HR pathways have not been described to date, defects in conservative HR can be observed in some tumor cells (43). We also note, though, that differential activity of HR factors in murine-activated versus germinal centre B cells has been suggested before (44). In this context, it is interesting that, reminiscent of the situation in normal germinal centre cells, Rad51 expression is much lower in Raji and BJAB than in DG75 cells that are able to perform conservative HR. However, this appears not the sole responsible factor for the phenomenon, as Rad51 reexpression in Raji did not rescue conservative HR. Among other candidates, defective ATM damage signalling due to impaired p53 function in the cells deserves attention, as this is again reminiscent of the situation in normal germinal centre cells (39). It will thus be very interesting to investigate which mechanisms B lymphocytes may use for differential HR pathway choice, as well as to investigate potential implications for B cell lymphoma.

In sum, we show that HR and hypermutation do coexist in human B cells and are both promoted by AID, but neither process is a prerequisite for the other one to occur. We surmise, though, that AID does not induce substantial Ig gene conversion in hypermutating human B cells because of a low activity of the underlying repair pathway, conservative HR. Rather, the products of AID-induced non-conservative recombination on the endogenous Ig loci in human B cells would be hybrid V genes, i.e. functional HR-generated fusion products of V genes, which have previously been observed as a byproduct of hypermutation (45). Considering the potential mistargeting of AID activity to non-Ig genes, we also anticipate that AID-induced non-conservative HR may contribute to genetic rearrangements and chromosomal translocations of other genes during lymphomagenesis. While experimental approaches to assess these questions are difficult for the endogenous Ig loci (46), the GFP reporter system presented here provides a feasible tool to tackle these issues and can also be transferred to the *in vivo* situation to study the regulation of HR activity and pathway choice in mammalian B cells.

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