Sense transcription through the S region is essential for immunoglobulin class switch recombination

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Class switch recombination (CSR) occurs between highly repetitive sequences called switch (S) regions and is initiated by activation-induced cytidine deaminase (AID). CSR is preceded by a bidirectional transcription of S regions but the relative importance of sense and antisense transcription for CSR *in vivo* is unknown. We generated three mouse lines in which we attempted a premature termination of transcriptional elongation by inserting bidirectional transcription terminators upstream of S_μ, upstream of S_{γ3} or downstream of S_{γ3} sequences. The data show, at least for S_{γ3}, that sense transcriptional elongation across S region is absolutely required for CSR whereas its antisense counterpart is largely dispensable, strongly suggesting that sense transcription is sufficient for AID targeting to both DNA strands.

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Introduction

The generation of primary repertoire of immunoglobulin (Ig) and T-cell receptor requires a developmentally regulated and lineage-specific recombination between variable (V), diversity (D) and joining (J) segments through V(D)J recombination. The process is regulated by accessibility control elements including transcriptional promoters and enhancers (Hesslein and Schatz, 2001; Krangel, 2003; Cobb *et al*, 2006). The E_μ enhancer, located between J_H segments and C_μ exons,

has a critical role in germ-line (GL) transcription of D_H and J_H segments and in V(D)J recombination. This enhancer regulates the production of a set of sense and antisense transcripts: I_μ transcripts that initiate at the I_μ promoter associated with E_μ enhancer, μ0 transcripts that initiate at a promoter upstream of DQ52, the most distal D_H segment, and potentially D_H and J_H antisense transcripts (Perlot *et al*, 2005, 2008; Afshar *et al*, 2006; Bolland *et al*, 2007).

Upon antigen challenge, mature B cells undergo two other diversification processes: somatic hypermutation (SHM) that introduces point mutations at the V-region exons and class switch recombination (CSR), which further diversifies antibody constant (C) domains. Both processes require transcription of the target sequences and the single-stranded DNA-specific cytidine deaminase, activation-induced cytidine deaminase (AID). However, how does AID specifically target some sequences (e.g., V regions) while sparing adjacent sequences (e.g., C regions) remains largely unknown (Di Noia and Neuberger, 2007; Peled *et al*, 2008; Maul and Gearhart, 2010).

CSR occurs between highly repetitive S sequences located upstream of all the C genes except C_δ and involves double-strand breaks at the recombining S sequences (Stavnezer *et al*, 2008). Targeted deletion of the whole S sequences severely impairs CSR, demonstrating their critical importance for efficient CSR (Shinkura *et al*, 2003; Khamlichi *et al*, 2004). Transcription of S sequences is derived from GL promoters called I promoters upstream of S sequences. The transcripts run through a non-coding I exon and intronic S sequences and undergo polyadenylation downstream of the C exons. Splicing enables fusion of I exon to the C region yielding sterile transcripts (Chaudhuri and Alt, 2004). GL transcription is regulated in a major part by the 3' regulatory region (3' RR) located downstream of the IgH locus (Khamlichi *et al*, 2000; Manis *et al*, 2002). The transcriptional orientation of S sequences is important for CSR *in vivo* as inversion of S_{γ1} decreases CSR to IgG1 (Shinkura *et al*, 2003). There is evidence that AID associates with the chromatin of the target S sequences in a GL transcription-dependent manner through a direct interaction with the elongating transcription machinery (Nambu *et al*, 2003). In addition, a high density of RNA polymerase II (Pol II) molecules was detected at S regions indicating a stalling of Pol II molecules (Rajagopal *et al*, 2009; Wang *et al*, 2009).

During transcriptional elongation, GL transcripts form RNA–DNA hybrids with the template strand exposing the single-stranded, non-template strand. The main function of GL transcripts is thought to promote accessibility of S sequences through co-transcriptional generation of R loops and possibly other structures that provide the substrates for AID (reviewed in Chaudhuri and Alt (2004)). In this context, it was found that AID-initiated mutations were detectable at some 150 bp downstream of I promoters, rise sharply at S sequences before falling off at their downstream boundaries, and are undetectable at C

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regions (reviewed in Di Noia and Neuberger (2007)). However, there is clear evidence that both DNA strands are targeted by AID *in vivo* (Rada *et al*, 2004; Xue *et al*, 2006). Therefore, AID must somehow gain access to the presumably protected cytosines of the C-rich template strand.

How does AID achieve this function is presently unclear. Besides the hypothesis that transcription bubbles may be sufficient for AID attack, there are several non-mutually exclusive possibilities: (1) intracellular RNase H may degrade the RNA of the DNA/RNA duplex resulting in a collapsed structure in which misaligned repeats are exposed as single-stranded regions on both strands (Yu *et al*, 2003), (2) transient supercoiling of DNA upstream of elongating Pol II molecules may have a role for AID targeting (Shen and Storb, 2004), (3) other factors such as the single-stranded DNA binding protein RPA may target AID to non-R loop-forming sequences (Chaudhuri *et al*, 2004) and (4) splicing of the GL transcripts may be involved in the collapse or destabilization of the DNA/RNA duplex (reviewed in Yu and Lieber (2003)). In this context, previous work showed that mutation of the donor splice site of $\text{I}\gamma 1$ exon inhibited CSR to IgG1 despite normal GL transcription (Hein *et al*, 1998), (5) the antisense transcripts at S regions (Julius *et al*, 1988; Apel *et al*, 1992; Morrison *et al*, 1998; Perlot *et al*, 2008) may somehow contribute to AID targeting.

In the latter context, the expression pattern of antisense transcripts appears to mirror that of their sense counterparts: they are synthesized before CSR, they are produced constitutively at $\text{S}\mu$ region, and they are induced at the same time and in the same stimulation conditions as the sense transcripts at downstream S regions (Perlot *et al*, 2008). Several hypotheses have been put forward to account for the function of antisense transcripts during CSR including promotion of Pol II stalling, stabilization of secondary structures upon potential collision of transcriptional complexes moving in opposite directions, and contribution to the modulation of chromatin topology beyond simple transcriptional opening (Roa *et al*, 2008; Teng and Papavasiliou, 2009; Maul and Gearhart, 2010).

In this study, we provide evidence that antisense switch transcripts are largely dispensable for CSR *in vivo*. We also report the striking finding that elongation past premature polyadenylation (PolyA) and pause sites is more efficient in μ locus than in $\gamma 3$ locus.

Results

Insertion of premature transcriptional termination sites in μ and $\gamma 3$ regions

The transcriptional terminator cassette (hereafter called pAp cassette) was designed so that it provides polyA sites in both sense and antisense orientations. In the sense orientation, the pAp cassette was made up of a synthetic polyA site and pause site (Levitt *et al*, 1989) followed by SV40 late polyA site and its own pause site (Carswell and Alwine, 1989). In the antisense orientation, the cassette consists of SV40 early polyA and pause sites followed by a synthetic polyA site. The cassette thus provides two polyA sites in the sense orientation and three polyA sites in the antisense orientation (Supplementary Figure S1A). The pAp cassette was inserted at 168 bp downstream of $\text{I}\mu$ exon (the knock-in will be referred to as pAp μ), 251 bp downstream of $\text{I}\gamma 3$ exon (here-

after pAp γ) or ~ 500 bp downstream of $\text{S}\gamma 3$ core repeats (hereafter $\text{S}\gamma 3$ -pAp) (Supplementary Figure S1B–G). All analyses were carried out on homozygous mice. In order to rule out the introduction of any mutation in the pAp cassette upon the integration events and the deletion of the selectable marker at the three insertion sites, the cassette was PCR-amplified from genomic DNA of the corresponding splenic B cells and sequenced, no mutation was found (Supplementary Figure S1A and data not shown).

Analysis of early B-cell development in pAp μ mice

Preliminary observations showed somewhat surprisingly no sign of immunodeficiency in pAp μ mice, which would be expected if V(D)J recombination and transcription were completely shut down. To check at which developmental stage the mutation exerts its effect, we first analysed B-cell populations in the bone marrow by flow cytometry. A ~ 2.5 -fold decrease was seen for the $\text{B}220^+$ population in pAp μ mice ($\sim 22\%$) compared with WT controls ($\sim 59\%$) (Supplementary Figure S2A). Double positive $\text{B}220^+ \text{IgM}^+$ population was decreased by half in pAp μ bone marrows ($\sim 6\%$) compared with WT controls ($\sim 13\%$) (Supplementary Figure S2B). A slight accumulation was found for mutant pro-B cells ($\text{B}220^+ \text{IgM}^- \text{CD}43^{\text{high}}$) ($\sim 30\%$ for pAp μ and $\sim 20\%$ for WT) and a decrease in pre-B cell population ($\text{B}220^+ \text{IgM}^- \text{CD}43^{\text{low}}$) ($\sim 70\%$ for pAp μ and $\sim 80\%$ for WT) (Supplementary Figure S2C). The results show that insertion of the pAp cassette in μ intron slightly impairs, but does not block early B-cell development.

Normal V(D)J recombination in pAp μ mice

In order to further characterize the step at which the impairment occurs, we analysed V(D)J recombination at the IgH locus. Genomic DNA was prepared from sorted pro-B cells ($\text{B}220^+ \text{IgM}^- \text{CD}43^{\text{high}}$) and pre-B cells ($\text{B}220^+ \text{IgM}^- \text{CD}43^{\text{low}}$) and was subjected to PCR using a set of specific primers. No obvious decrease was detected in D_H to J_H rearrangement in pAp μ pro-B cells compared with WT controls. Likewise, V_H to $\text{D}_\text{H}\text{J}_\text{H}$ rearrangement was normal be it for the proximal $\text{V}_\text{H}7183$ or the distal $\text{V}_\text{H}558$ gene families (Supplementary Figure S3 and data not shown). $\text{V}\kappa\text{J}\kappa$ was also normal (Supplementary Figure S3). Thus, the mutation has no apparent effect on V(D)J recombination in pAp μ mice.

Decreased GL transcription in the bone marrow of pAp μ mice

The lack of effect of the pAp μ insertion on V(D)J recombination led us to investigate whether the decrease in B-cell population correlates with a decrease in V(D)J transcription. To this end, total RNA from magnetically sorted $\text{CD}19^+$ bone marrow B cells was subjected to RT-PCR using a degenerate primer specific for the largest $\text{V}_\text{H}558$ gene family and a primer specific for the $\text{C}\mu 1$ exon. $\text{VDJ}\text{C}\mu$ transcript levels were clearly decreased in pAp μ mice compared with WT controls (Figure 1A), indicating a partial inhibition of transcriptional elongation, though we cannot exclude the involvement of checkpoints in the bone marrow.

In order to analyse the extent of the transcriptional read-through independently of selection mechanisms that operate against the B cells that fail to express a functional (pre-) B-cell receptor, the pAp μ mutation was brought into an $\text{Rag}2^{-/-}$ deficient background. Total RNA was prepared from bone

Figure 1 Analysis of GL transcription in bone marrow of pApμ mice. (A) CD19⁺ cells were sorted from bone marrows of WT and pApμ mice and RT-PCR was performed on total RNA. The PCR was performed on five-fold serial dilutions starting with the 1/5 dilution. A degenerate primer that binds the VHJ558 gene family and Cμr primer were used to amplify the VDJCμ sequence. The actin transcript was used for normalization of single-stranded DNA input. (B) Scheme of the structure of the DQ52-Cμ domain indicating the initiation sites of μ0 and Iμ transcripts (arrows). The localization of the pAp cassette upstream of Sμ, the endogenous polyA site 3' of Cμ and the primers used as well as the spliced forms of μ0 and Iμ transcripts are shown. (C) CD19⁺ cells were sorted from bone marrows of Rag2^{-/-} and pApμ/Rag2^{-/-} mice and RT-PCR was performed on total RNA. The PCR was performed on five-fold serial dilutions. Contamination by genomic DNA was excluded by using a minus RT control for each reaction. The λ5 surrogate light chain transcript was used as input control. (D) Transcript levels were quantified on 1/5, 1/25 and 1/125 dilutions for μ0 exon and Iμ + Cμr, and on 1/25 and 1/125 dilutions for μ0F + Cμr and Iμ exon. For GL transcription, μ0 and Iμ GL transcript levels from Rag2^{-/-} controls were set up as 100% (*n* = 2). A full-colour version of this figure is available at *The EMBO Journal* Online.

marrow purified CD19⁺ B cells and subjected to RT-PCR using primers that are specific for μ0 or Iμ exons (primers 3'DQD1-Fw/DDJH1-Rev and IμFw/IμRev, respectively) or for the spliced forms of μ0 and Iμ transcripts (μ0-Fw/Cμr and Iμ-Fw/Cμr) (Figure 1B). While initiation of μ0 and Iμ exon transcripts in pApμ/Rag2^{-/-} mice was comparable to that of Rag2^{-/-} controls (Figure 1C and D), a mild decrease (~1.5-fold) was detected for both μ0 and Iμ spliced transcripts (reflecting a bypass of the pAp cassette) in pApμ/Rag2^{-/-} mice versus Rag2^{-/-} controls (Figure 1C and D). The data indicate that the pApμ insertion has no effect on the initiation step of μ0 and Iμ GL transcription and that it

impairs but clearly does not totally block transcriptional elongation through Sμ region.

Analysis of splenic B cells in non-immunized pApμ mice

The decrease seen in B220⁺IgM⁺ population in pApμ bone marrow may lead to an alteration of B-cell homeostasis in the periphery. Therefore, we sought to know if the mutation would affect the accumulation of the mutant B cells in the spleen. To this end, we resorted to flow cytometry by using an anti-B220, anti-IgM and anti-IgD antibodies. We found that the mutant B cells replenished normally the spleen at levels comparable to WT controls. In fact, we consistently found a

Figure 2 Cell surface Ig expression on stimulated pApμ splenic B cells. (A) Splenic B cells from WT, pApμ and AID^{-/-} mice were cultured for 4.5 days and stained with anti-B220 and anti-IgG1 for anti-CD40 + IL4-stimulation, with anti-B220 and anti-IgG3 for LPS + anti-IgD-dextran-stimulation or with anti-B220 and anti-IgA for LPS + TGF-β + B-LYS-stimulation. (B) The percentages of switched splenic B cells are indicated on the top right of the quadrant. The data shown are representative of four independent experiments. A full-colour version of this figure is available at *The EMBO Journal* Online.

slight but reproducible ($n = 3$) increase in B220⁺IgM⁺ and B220⁺IgD⁺ populations in pApμ spleens compared with WT controls (Supplementary Figure S4A and B). Altogether, the data show that replenishment of the mutant spleens occurs normally with a slight accumulation of IgM- and/or IgD-expressing populations, thus excluding any major alteration of late B-cell homeostasis in pApμ mice.

Decreased CSR in activated pApμ B cells

We then asked if and how the pApμ mutation would affect CSR to downstream isotypes. To this end, we resorted to *ex vivo* activation of purified splenic B cells from WT, pApμ and AID^{-/-} mice. B cells were cultured in the presence of anti-CD40 + IL4 which induce switching to IgG1, of LPS + anti-IgD-dextran which induce switching to IgG3 and IgG2b or of LPS + TGF-β + B-LYS which induce switching to IgA. Surface expression was monitored by flow cytometry at day 4.5 using an anti-B220 antibody and anti-IgG1, anti-IgG3 or anti-IgA antibodies. Surface expression was decreased ~1.3–2.3-fold for all isotypes tested but was clearly not extinguished in pApμ activated B cells (Figure 2A and B). Thus, insertion of

the pAp cassette upstream of Sμ results in a generalized but mild defect in CSR to downstream isotypes.

The decreased CSR at the genomic level correlates with a decreased μ GL transcription in pApμ mice

In order to check that the decrease in CSR seen by FACS occurs at the genomic level, we resorted to DC-PCR (Chu *et al*, 1992). At day 4.5 post-stimulation, genomic DNA was prepared from WT, pApμ and AID^{-/-} splenic B cells that have been induced to switch to IgG1 or IgG3 and subjected to DC-PCR. CSR to Cγ1 and Cγ3 was clearly decreased in stimulated splenic mutant B cells (Figure 3A), demonstrating that the impairment of CSR in pApμ mice occurs indeed at the genomic level.

To analyse GL transcription, total RNA was prepared at day 2 from activated WT and pApμ B cells, was reverse transcribed and amplified in semi-quantitative conditions using isotype-specific GL transcript primers (Figure 3B). We will hereafter use the term GL transcripts meaning the ‘classical’ spliced GL transcripts derived from I promoters. Iγ3–Cγ3 and Iγ2b–Cγ2b GL transcript levels were comparable in WT and

Figure 3 Analysis of CSR and GL transcription in activated pApμ splenic B cells. (A) Genomic DNA from anti-CD40 + IL4- and LPS + anti-IgD-dextran-stimulated WT, pApμ and AID^{-/-} B cells was subjected to DC-PCR to detect Sμ-Sγ1 and Sμ-Sγ3 switching, respectively. Three-fold dilutions of circularized DNAs were subjected to PCR. Samples were normalized using nAchRe SphI DC-PCR. (B) RT-PCR was performed on WT and pApμ LPS + anti-IgD-dextran-activated B cell RNAs (day 2) for IμF-IμR, IμF-Cμr, IμF-pA1, Iγ3-Cγ3 and Iγ2b-Cγ2b pre-switch transcripts, and from anti-CD40 + IL4-activated WT and pApμ B cell RNAs for pre-switch Iγ1-Cγ1. Single-stranded cDNAs or five-fold dilutions thereof were subjected to PCR using appropriate primers. (C) RT-PCR was performed on WT, pApμ and AID^{-/-} GL transcripts from LPS + anti-IgD-dextran-activated pApμ B cell RNAs (day 4.5) for IμF-Cμr, IμF-pA1, IμF-Cγ3 and IμF-Cγ2b post-switch transcripts, and from anti-CD40 + IL4-activated WT, pApμ and AID^{-/-} RNA for IμF-Cγ1 post-switch transcripts. PCR conditions were as in (B). (D) Quantification of pre-switch and post-switch transcript levels in pApμ mice. The levels from WT controls were set up as 100%. Standard deviations ($n = 3$) are shown. (E) Strand-specific RT-PCR for Sμ was performed on splenic B cells activated with LPS + anti-IgD-dextran. The relative position of the primers used is shown in the upper scheme. The thick arrows indicate the primers used for strand-specific reverse transcription. In the lower panel, the random hexamers, sense or antisense RT primers used with (+) or without (-) reverse transcriptase are indicated. Sense-specific PCR (upper) and antisense-specific (lower) PCR upstream or downstream of the pAp insertion site are depicted ($n = 2$). A full-colour version of this figure is available at *The EMBO Journal* Online.

pAp μ B cells activated with LPS + anti-IgD-dextran. The same pattern was found for I γ 1–C γ 1 GL transcripts in anti-CD40 + IL4-activated B cells (Figure 3B and D). With regard to μ transcripts, I μ exon transcripts (reflecting initiation) were equally abundant in WT and pAp μ B cells. Additionally, we used I μ F and pA1 primers that would detect mutant transcripts that end up at the pAp site and probably some μ pre-mRNAs that reach C μ polyA site. Transcripts in the form of I μ –pA1 (485 bp) were clearly present in pAp μ splenic B cells in both stimulation conditions (Figure 3B and data not shown). In contrast, spliced I μ –C μ GL transcripts were decreased ($\sim$ 3-fold) in activated pAp μ B cells compared with WT controls (Figure 3B and D) regardless of the stimulation condition (data not shown).

We also analysed post-switch GL transcripts in the form of I μ –C x (C x being any switched isotype), which are a good marker of the efficiency of CSR. Given the location of the pAp μ insertion site, one might expect that the vast majority of CSR events will preserve the pAp cassette in the newly generated hybrid S μ –S x regions. Thus, the defect in CSR can be directly correlated with the effect of the mutation. Total RNA at day 4.5 post-stimulation was prepared from WT, pAp μ and AID-deficient mice and amplified with I μ –C x primers (x being μ , γ 1, γ 3, γ 2b) or I μ –pA1 primers. Post-switch transcripts corresponding to all isotypes tested were readily detectable, but were clearly decreased (3–4-fold decrease depending on the isotype) in pAp μ mice (Figure 3C and D). Again, unspliced I μ –pA1 transcripts were detected in pAp μ mice but not in control mice as expected (Figure 3C).

The identification of antisense transcripts in primary B cells (Perlot *et al*, 2008) led us to investigate whether the decreased CSR in pAp μ mice correlates with a decrease in sense or antisense transcription. To this end, we used a strand-specific RT–PCR (Bolland *et al*, 2007; Perlot *et al*, 2008) to analyse sense and antisense transcription upstream and downstream of the pAp insertion site. As shown in Figure 3E, unprocessed sense and antisense transcripts were detected both upstream and downstream of the insertion site in WT and pAp μ mice.

Altogether, the results demonstrate that the pAp μ mutation has no effect on downstream pre-switch GL transcripts and that a substantial fraction of μ and post-switch GL transcripts can elongate past the pAp site and undergo splicing. Thus, the premature pAp site impairs but does not completely stop elongation of μ GL transcripts or post-switch GL transcripts. The data also show that there is a correlation between the defect of CSR at the genomic level and the decrease of μ GL transcript levels. In contrast, we could not conclude about the relative importance of sense versus antisense transcription regarding the impairment of CSR.

Complete and specific abrogation of CSR in pAp γ mice

In order to analyse the effect of pAp γ mutation on CSR, we resorted to *ex vivo* activation of pAp γ splenic B cells. Surface expression of IgG1 was comparable in WT and pAp γ B cells when activated with anti-CD40 + IL4 (Figure 4A). In contrast, while surface expression of IgG3 was readily detectable in WT B cells activated with LPS + anti-IgD-dextran, that of their pAp γ counterparts was completely absent (Figure 4B). The same conclusion could also be drawn from serum and culture supernatant analyses by ELISA. IgG2b titres of pAp γ sera or supernatants from

LPS + anti-IgD-dextran stimulation were comparable to those of WT controls. In contrast, only background levels of IgG3 were detected in the sera or culture supernatants from pAp γ mice (Figure 4C and D).

The results show that insertion of the pAp cassette upstream of S γ 3 sequence leads to a complete and specific extinction of IgG3 production *in vivo* and *ex vivo* and that the block is B-cell autonomous and cannot be ascribed to some inhibitory signals *in vivo*.

The absence of CSR to IgG3 in pAp γ mice correlates with a shutdown of sense transcription

In order to demonstrate that the shutdown of CSR to C γ 3 occurs at the genomic level, genomic DNA was prepared from WT, pAp γ and AID $^{-/-}$ splenic B cells induced to switch to IgG1 or IgG3 and subjected to DC-PCR. While CSR to IgG1 was comparable between WT and pAp γ splenic B cells induced with anti-CD40 + IL4, the data clearly showed a total absence of CSR to IgG3 in pAp γ B cells activated with LPS + anti-IgD-dextran (Figure 5A). Thus, abrogation of CSR to IgG3 occurs at the genomic level.

In an attempt to establish closer links between the defect of CSR at the genomic level and the potential impairment of sense or antisense transcription, we analysed GL transcripts from WT or pAp γ splenic B cells activated with LPS + anti-IgD-dextran. The levels of I γ 2b–C γ 2b GL transcripts were comparable in WT and mutant pAp γ B cells (Figure 5B and D). In contrast, a drastic decrease was found for spliced I γ 3–C γ 3 transcripts in pAp γ B cells despite normal transcription initiation (I γ 3F–I γ 3R primers) (Figure 5B and D). The transcripts in the form of I γ 3–pA1 (570 bp) were readily detected (Figure 5B) further demonstrating that I γ 3 promoter is in an open configuration, and that most detectable transcripts that originate from I γ 3 fail to bypass the pAp site. Hence, the low level of GL transcription across S γ 3 is clearly not sufficient for detectable CSR to IgG3 in pAp γ mice.

We also analysed post-switch transcripts at day 4.5 post-stimulation. I μ –C γ 2b transcripts were equally abundant in WT and mutant B cells (Figure 5C and D). As expected, I μ F–pA1 transcripts were not detected in pAp γ B cells (Figure 5C). More importantly, I μ –C γ 3 post-switch transcripts were barely detectable in pAp γ splenic B cells (Figure 5C and D) further confirming the lack of CSR to IgG3 in pAp γ B cells.

These data demonstrate that transcription initiation from I γ 3 promoter is intact in pAp γ mice and that the vast majority of γ 3 GL transcripts fail to efficiently elongate past the pAp site. Thus, the data strongly suggest that failure to efficiently transcribe S γ 3 region in the sense orientation is likely to account for the specific lack of CSR to IgG3 in pAp γ mice.

In order to prove this, we used strand-specific RT–PCR to analyse sense and antisense transcription. Sense transcripts were readily detected upstream of the insertion site in both WT and pAp γ mice (Figure 5E, see also Figure 8). In contrast, sense transcripts downstream of the insertion site were barely detectable in pAp γ mice compared with WT mice, further confirming the strong termination of sense transcription by the pAp cassette. Interestingly, antisense transcripts were readily detected downstream of the insertion site in both WT and pAp γ mice, indicating that antisense transcription of S γ 3 occurred and was not hindered by the mutation. In contrast, only a faint signal was detected for antisense transcripts upstream of the insertion site in pAp γ mice,

Figure 4 Cell surface Ig expression on stimulated pAp B cells. Splenic B cells from WT, pApγ and AID^{-/-} mice were cultured for 4.5 days and stained with anti-B220 and anti-IgG1 for anti-CD40 + IL4-stimulation (**A**) or with anti-B220 and anti-IgG3 for LPS + anti-IgD-dextran-stimulation (**B**). (**C**) ELISA analysis of IgG3 and IgG2b secretion at 4.5 days post-stimulation. Splenic B cells from six littermates of WT or pApγ mice were analysed for LPS + anti-IgD-dextran-induced production of IgG3 and IgG2b ($n = 2$). (**D**) Serum analysis of IgG3 and IgG2b from 8-week-old WT or pApγ mice was done by ELISA. Six mice from each genotype were analysed ($n = 2$). A full-colour version of this figure is available at *The EMBO Journal* Online.

suggesting that few Pol II molecules have managed to elongate past the pAp cassette. We conclude that the abrogation of CSR to IgG3 in pApγ mice correlates with the lack of sense transcription of *Sγ3* and that antisense transcription of *Sγ3* in the absence of its sense transcription is not sufficient for CSR to IgG3.

Normal CSR to IgG3 in *Sγ3*-pAp mice

In order to provide further functional support to the notion that it is the sense transcription of *Sγ3* that counts for efficient CSR to IgG3, we generated a third mouse line in which the pAp cassette was inserted downstream of *Sγ3*. We reasoned that because of the bidirectional function of the pAp cassette, *Sγ3* would be transcribed in the sense orientation while its antisense transcription should be blocked. Therefore, should CSR to IgG3 occur, the correlation with sense transcription would be more firmly grounded.

In order to analyse the effect of *Sγ3*-pAp mutation on CSR, we first resorted to *ex vivo* activation of splenic B cells. No obvious difference could be detected between WT and *Sγ3*-pAp activated B cells regarding surface expression of IgG1 and, somewhat surprisingly, of IgG3 (Figure 6), indicating normal CSR to IgG3. Thus, in stark contrast to the situation in pApγ mice, insertion of the pAp cassette downstream of *Sγ3* does not affect CSR to IgG3.

Normal CSR to IgG3 in the absence of *Sγ3* antisense transcription in *Sγ3*-pAp mice

We confirmed this result at the genomic level by using DC-PCR. No obvious difference could be seen between *Sγ3*-pAp and WT control regarding $Sμ > Sγ3$ switching (Figure 7A). To check that no AID-induced mutation occurred at the pAp cassette, we again amplified the cassette from genomic DNA of activated B cells and definitively excluded this possibility (data not shown).

The *Iγ3*-*Cγ3* and *Iγ2b*-*Cγ2b* pre-switch transcripts were equally abundant in WT and *Sγ3*-pAp activated B cells (Figure 7B). Conversely, *Iμ*-*Cγ3* and *Iμ*-*Cγ2b* post-switch transcript levels were comparable in WT and mutant B cells further confirming normal CSR to IgG3 and IgG2b in *Sγ3*-pAp mice (Figure 7C).

In order to analyse the contribution of sense and antisense transcription to the normal CSR to IgG3 in *Sγ3*-pAp mice, we used the strand-specific RT-PCR assay. Sense transcripts were readily detected upstream and downstream of the insertion site in both WT and *Sγ3*-pAp mice. Likewise, antisense transcripts were readily detected downstream of the insertion site in both WT and *Sγ3*-pAp mice. More importantly, antisense transcripts were undetectable upstream of the insertion site in *Sγ3*-pAp mice compared with WT control (Figure 7D, see also Figure 8). We conclude that in *Sγ3*-pAp mice, normal

Figure 5 Analysis of CSR and GL transcription in activated pApy splenocytes. **(A)** Genomic DNA from anti-CD40 + IL4- and LPS + anti-IgD-dextran-stimulated WT, pApy, and AID^{-/-} B cells was subjected to DC-PCR (S_μ-S_{γ1} and S_μ-S_{γ3}, respectively). Three-fold dilutions of circularized DNAs were subjected to PCR. Samples were normalized using nAchRe SphI DC-PCR. **(B)** RT-PCR was performed on WT and pApy GL transcripts from LPS + anti-IgD-dextran-activated pApy B cells' RNA (day 2) for Iy3F-Iy3R, Iy3F-pA1, Iy3-Cy3 or Iy2b-Cy2b pre-switch transcripts. Single-stranded cDNAs or five-fold dilutions thereof were subjected to PCR using appropriate primers. **(C)** RT-PCR was performed on WT and pApy GL transcripts from LPS + anti-IgD-dextran-activated B cells' RNA (day 4.5) for IμF-pA1, IμF-Cγ3 and IμF-Cγ2b post-switch transcripts. PCR conditions were as in **(B)**. **(D)** Quantification of pre-switch and post-switch transcript levels in pApy mice. The levels from WT controls were set up as 100%. Standard deviations (*n* = 3) are shown. **(E)** Strand-specific RT-PCR for Sγ3 was performed on pApy splenic B cells activated with LPS + anti-IgD-dextran. The relative position of the primers used is shown in the upper scheme. The thick arrows indicate the primers used for strand-specific reverse transcription. In the lower panel, the random hexamers, sense or antisense RT primers used with (+) or without (-) reverse transcriptase are indicated. Sense-specific PCR (upper) and antisense-specific (lower) PCR upstream or downstream of the pAp insertion site are depicted (*n* = 2). A full-colour version of this figure is available at *The EMBO Journal* Online.

CSR to IgG3 correlates with sense transcription of $S\gamma 3$. These results, together with the data from pAp γ mice, show that antisense transcription of $S\gamma 3$ does not significantly contribute to normal CSR to IgG3.

Discussion

Insertion of a transcriptional terminator upstream of $\gamma 3$ efficiently blocks $\gamma 3$ GL transcription elongation and results in a total and specific abrogation of CSR to IgG3, whereas μ GL transcriptional elongation is only mildly affected in

similar conditions, leading to a moderate decrease of CSR to downstream isotypes. We conclude that transcription elongation past the pAp cassette at μ and $\gamma 3$ loci is differentially regulated *in vivo*, although we cannot presently exclude the remote possibility that we may be dealing with highly selected mature B-cell clones in pAp μ mice but not in pAp γ mice.

Analysis of early B-cell development in pA μ mice revealed a decrease in B220⁺ population that was not due to a failure to rearrange the variable gene segments but to a partial inhibition of μ transcription. While this indicates that

Figure 6 Cell surface Ig expression on stimulated Sγ3-pAp splenic B cells. Splenic B cells from WT, Sγ3-pAp and AID^{-/-} mice were cultured for 4.5 days and stained with anti-B220 and anti-IgG1 for anti-CD40 + IL4-stimulation (**A**) or with anti-B220 and anti-IgG3 for LPS + anti-IgD-dextran-stimulation (**B**) ($n=3$). A full-colour version of this figure is available at *The EMBO Journal* Online.

the pAp cassette does not completely block elongation at μ locus, we cannot exclude the implication of selection mechanisms (von Boehmer and Melchers, 2010) so that only clones that have successfully expressed a functional pre-BCR and BCR would survive. However, the fact that the blockade at $\mu 0$ and $I\mu$ elongation step is not complete in Rag2^{-/-} background, while initiation is normal, clearly shows that the ability to bypass the pAp cassette is inherent to μ transcription elongation.

Despite the structural similarities between μ and $\gamma 3$ transcription units, there are some important differences. In particular, $E\mu$ enhancer acts both as an enhancer and as a GL promoter at μ locus, whereas $I\gamma 3$ GL promoter has no known enhancer function. Additionally, while $S\mu$ is transcribed throughout the B-cell development, $S\gamma 3$ is selectively transcribed after B-cell activation. Another difference stems from the fact that $E\mu/I\mu$ -derived transcription is constitutive (Li *et al*, 1994) whereas $I\gamma 3$ -derived transcription is inducible.

Taking into account the fact that μ and $\gamma 3$ introns are of relatively the same size, the distance of the pAp cassette from the splice sites of μ and $\gamma 3$ sense transcripts, is unlikely to account for the difference in the phenotypes of pAp μ and pAp γ mice. Interestingly, while sense μ transcripts are readily detected downstream of the pAp cassette in pAp μ mice, their $\gamma 3$ counterparts are barely detectable in pAp γ mice, suggesting that $\gamma 3$ transcripts downstream of the cleavage site(s) (3' of the polyA sites of the pAp cassette) undergo extensive degradation before reaching $S\gamma 3$ (where the generated DNA/RNA duplex may possibly contribute to stabilize them) and the $C\gamma 3$ -1 acceptor splice site. Strikingly, when the pAp cassette is moved downstream of the $S\gamma 3$ (in Sγ3-pAp mice), the transcripts downstream of the pAp cassette appear to be just as abundant as their WT counterparts.

Thus, within the framework of current models of co-transcriptional processing (Moore and Proudfoot, 2009;

Richard and Manley, 2009), the failure of Pol II molecules to efficiently transcribe Sγ3 in pAp γ mice stems, at least in part, from a failure to protect the 3' RNA transcripts from degradation before splicing. In the case of Sγ3-pAp mice, the insertion site lies at some 2 kb from $C\gamma 3$ -1 acceptor splice site and downstream of the core Sγ3 where the majority of R loops form (Huang *et al*, 2006), and where Pol II molecules stall (Wang *et al*, 2009). Hence, one possibility is that the high density of stalled Pol II at Sγ3 (and perhaps the stable DNA/RNA duplex structures) protects the transcripts against cleavage, enabling Pol II molecules to rapidly reach the $C\gamma 3$ -1 acceptor splice site. Thus, in this scenario, the density of Pol II arises as a critical determinant at the basis of the phenotypes of the three mutant mouse lines, and more specifically in the differential ability to ignore premature termination sites. Whether Pol II molecules recruit or lack specific factors at the three pAp sites is presently unknown. Whatever the case, these factors need not be recruited in a developmentally regulated manner as described for instance for membrane versus secreted forms of μ transcripts (Takagaki *et al*, 1996), as the differential ability to bypass the pAp sites is manifest in the very activated splenic B cells of the corresponding mouse lines.

Previous studies involved enhancers in the stimulation of elongation past premature or endogenous termination sites (Yankulov *et al*, 1994; Krumm *et al*, 1995; Blau *et al*, 1996; Brown *et al*, 1998; Sawado *et al*, 2003). Thus it is plausible that $E\mu$ enhancer is involved in the elongation of μ transcripts, which may confer to $S\mu$ some features that would be missing in downstream S regions, for instance, by acting as a powerful entry site for Pol II as a high density of Pol II molecules was detected at $E\mu/I\mu$ region (Rajagopal *et al*, 2009; Wang *et al*, 2009; Pavri *et al*, 2010). In addition, different sets of transcripts run through $S\mu$ region throughout B-cell development before, and after, the onset of V(D)J

Figure 7 Analysis of CSR and GL transcription in activated $S\gamma 3$ -pAp splenic B cells. **(A)** Genomic DNA from anti-CD40 + IL4- and LPS + anti-IgD-dextran-stimulated WT and $S\gamma 3$ -pAp B cells was subjected to DC-PCR ($S\mu \rightarrow S\gamma 1$ and $S\mu \rightarrow S\gamma 3$, respectively). Three-fold dilutions of circularized DNAs were subjected to PCR. Samples were normalized using nAchRe SphI DC-PCR. **(B)** RT-PCR was performed on WT and $S\gamma 3$ -pAp GL transcripts from LPS + anti-IgD-dextran-activated B cells' RNA (day 2) for $I\gamma 3$ - $C\gamma 3$ and $I\gamma 2b$ - $C\gamma 2b$ pre-switch transcripts. Single-stranded cDNAs or five-fold dilutions thereof were subjected to PCR using appropriate primers. **(C)** RT-PCR was performed on WT or $S\gamma 3$ -pAp GL transcripts from LPS + anti-IgD-dextran-activated B cells' RNA (day 4.5) for $I\mu F$ - $C\mu R$, $I\mu F$ - $C\gamma 3$ and $I\mu F$ - $C\gamma 2b$ post-switch transcripts. PCR conditions were as in **(B)**. **(D)** Strand-specific RT-PCR for $S\gamma 3$ was performed on $S\gamma 3$ -pAp splenic B cells activated with LPS + anti-IgD-dextran. The relative position of the primers used is shown in the upper scheme. The thick arrows indicate the primers used for strand-specific reverse transcription. In the lower panel, the random hexamers, sense or antisense RT primers used with (+) or without (-) reverse transcriptase are indicated. Sense-specific PCR (upper) and antisense-specific (lower) PCR upstream or downstream of the pAp insertion site are depicted ($n = 2$). A full-colour version of this figure is available at *The EMBO Journal Online*.

rearrangement. Such continuous transcription may imprint on $S\mu$ chromatin some structural and/or epigenetic marks that distinguish it from downstream S regions. Moreover, after CSR, post-switch transcripts, though decreased, are readily detectable in pAp mice irrespective of the acceptor switch site or the stimulation condition, suggesting that $E\mu$ enhancer, which remains intact in any CSR event, continues to activate transcription elongation of the hybrid $S\mu/Sx$ sequences.

In stark contrast to pAp μ , the only set of Pol II molecules that reach the pAp cassette in pAp γ mice, before CSR, are those that initiate transcription from $I\gamma 3$ promoter. Therefore, the strong inhibition of transcriptional read-through in pAp γ mice could, at least in part, be due to a weaker GL promoter. Taking into account the fact that the 3'RR controls $I\gamma 3$ -derived GL transcription (Khamlichi *et al*, 2000; Manis *et al*, 2002), one might speculate that the 3'RR acts at the elongation step downstream of the pAp γ site. An effect of a locus control

region on elongation has been reported in the β -globin locus (Sawado *et al*, 2003). We propose that $E\mu$ enhancer and the 3'RR may facilitate the transition of Pol II molecules from stalling to efficient elongation at $S\mu$ and downstream S regions, respectively.

By using an inducible switch substrate in a B lymphoma line, it was suggested that there exists a linear correlation between the transcription level of $S\alpha$ and the efficiency of switching to IgA (Lee *et al*, 2001). Our results support this notion and extend it to $S\mu$ and $S\gamma 3$ *in vivo*. More specifically, we provide evidence that, at least for $S\gamma 3$, there is a correlation between the level of sense transcription and the efficiency of CSR to IgG3.

While we could not conclude on the relative importance of sense and antisense transcription of $S\mu$ for the targeting and efficiency of CSR, the data from pAp γ and $S\gamma 3$ -pAp mice confer the crucial role to the sense transcription of $S\gamma 3$ in CSR to IgG3. These data exclude a major role for the putative

Figure 8 Graphical summary of the effects of pAp insertions on CSR. The schemes represent μ and $\gamma 3$ transcription units. pAp cassette is indicated by a red circle. The endogenous polyA sites (pA) are shown. The thick arrow shows normal transcription. The thin arrow indicates decreased transcript levels. The dotted arrows mean that the termination sites have not been determined. The dotted lines indicate that the transcriptional start sites have not been identified. A full-colour version of this figure is available at *The EMBO Journal Online*.

collision of opposite transcription machineries in the efficiency of CSR. They cannot be explained by a double-stranded RNA made up of sense and antisense transcripts at the polyA sites that might mask these sites (reviewed in Faghihi and Wahlestedt (2009)). Rather, the recruitment of the critical factors for CSR to IgG3 seems to be primarily conditioned by the efficient transcription of $S\gamma 3$ sequence in the sense orientation.

It thus appears that sense transcription is sufficient for AID activity on both DNA strands of transcribed $S\gamma 3$, indicating that a double-stranded RNA structure is not mandatory for AID targeting and action. How does AID attack the presumably protected template strand is presently unknown and several mechanisms have been proposed (see Introduction). It is also unclear whether AID targets both strands at the same time *in vivo* or whether it attacks the non-template strand first. From the pAp γ model (in which CSR to IgG3 is inhibited despite antisense transcription of $S\gamma 3$), it would appear that mutation of the non-template strand is the limiting step suggesting a stepwise process, and indicating that sense transcription-mediated generation of structures such as R loops is a crucial factor for the efficiency of CSR *in vivo*. This would confer a major role to the G content of the sense transcript as suggested previously (Reaban *et al*, 1994; Daniels and Lieber, 1995; Mizuta *et al*, 2003; Shinkura *et al*, 2003; Yu *et al*, 2003).

Yet, it is still puzzling how AID targets the exposed arm of the loop while it is recruited to the transcribed strand. Recent studies indicate that PKA is specifically recruited to S regions to promote localized phosphorylation of AID and binding of RPA (Vuong *et al*, 2009), and that the stalling factor Spt5 associated with RNAP II facilitates AID recruitment to its targets (Pavri *et al*, 2010). Whether these associations occur during antisense transcription is presently unclear. Nonetheless, it could be that during sense transcription, stalled RNAP II, Spt5 and PKA recruit increased number of AID molecules and allow sufficient time for its access to the exposed strand. Alternatively, the move of AID from the transcribed strand to the exposed strand may be an active

and regulated process that requires specific cofactors and/or post-translational modifications of AID.

It has been noted (Roa *et al*, 2008) that if antisense transcription had a major role, one would expect a mutation pattern downstream of the antisense start site similar to that of the sense start site, which is clearly not the case *in vivo* (Xue *et al*, 2006) although more complex scenarios may be envisaged. Thus, our results provide a basis for the prediction that transcription-associated high rate of mutation correlates with sense transcription during SHM.

Materials and methods

GL transcription in bone marrow

B cells from bone marrow were sorted by using CD19-magnetic microbeads and LS columns (Miltenyi Biotec) and total RNA was reverse transcribed (Invitrogen) and subjected to PCR. Quantification was as described (Haddad *et al*, 2010).

Spleen cell cultures

After removal of red blood cells, a single-cell suspension from spleens of 6–8-week-old mice was obtained. *Ex vivo* stimulation was as described (Boboila *et al*, 2010; Zhang *et al*, 2010). Negatively sorted B cells were activated with 25 $\mu\text{g}/\text{ml}$ of LPS (Sigma) and anti-IgD-dextran (Fina BioSolutions) at 3 ng/ml or with 0.5 $\mu\text{g}/\text{ml}$ of anti-CD40 (eBiosciences) and 20 ng/ml of IL4 (eBiosciences) or with 25 $\mu\text{g}/\text{ml}$ of LPS, 3 ng/ml of anti-IgD-dextran, 10 ng/ml of IL4, 2 ng/ml of TGF- β (R&D systems), 5 ng/ml of IL5 (R&D systems) and 5 ng/ml of B-LYS (R&D systems). At days 2 and 4.5, aliquots of cells were removed for RNA preparation.

Flow cytometry analysis of CSR

At day 4.5, activated B cells were stained with anti-B220-APC and anti-IgG1-FITC, with anti-B220-APC and anti-IgG3-FITC or with anti-B220-APC and anti-IgA-FITC (Biolegend). Data were obtained on 3×10^4 viable cells by using a Coulter XL apparatus (Beckman Coulter, Fullerton, CA).

ELISA on serum and culture supernatants

ELISA was performed as described (Pinaud *et al*, 2001; Khamlichi *et al*, 2004).

GL transcription in stimulated splenic B cells

For GL transcription, the primers, the RT-PCR conditions and the expected sizes of the PCR products have been described (Oruc *et al*, 2007; Haddad *et al*, 2010). pA1, GATGGAGAGCGTATGTTAGTAC. Additional primers used in this assay are listed in the Supplementary data. For unprocessed sense and antisense transcripts, primers downstream of pAp μ and RT-PCR conditions were as described (Perlot *et al*, 2008). Briefly, reverse transcription is performed with strand-specific primers. The latter generate sense- and antisense-specific cDNAs, respectively, which are then used as templates for two different PCRs that control each other: sense-specific and antisense-specific PCR leading to overlapping products (Bolland *et al*, 2007; Perlot *et al*, 2008). Additional controls include reverse transcription with random hexamers (which do not allow distinction between sense and antisense transcripts), and controls for genomic DNA contamination.

DC-PCR

Negatively sorted B cells were activated as described above. At day 4.5, live cells were sorted with dead cell removal kit (Miltenyi) as per manufacturer's instructions. Genomic DNA was purified and subjected to DC-PCR essentially as described (Chu *et al*, 1992). The primers used are listed in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal Online* (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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