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

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 January 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the study interesting, well executed and appropriate for the journal. They bring up some specific issues as detailed below that you should be able to address. Given these comments I'd like to invite you to submit a revised manuscript, taking the raised issues into account. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

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In this manuscript, the authors place presumed transcription terminators at three different places within the IgH locus (one upstream of S-mu and the others up/downstream of S-g3) in order to gain information on the importance of S-region transcription on IgH class-switching.

The finding that placing the terminator upstream (but not downstream) of Sg3 substantially inhibits switching to IgG3 is clearly established, interesting and is a discovery suitable for publication in EMBO J. It is a very nice finding and provides the best evidence to date that transcription through Sg3 is necessary for switching and the result is important for our understanding of the regulation of CSR in general. Ideally, it would have been accompanied by a negative control using insertion of a multiply point-mutated terminator (which does not lead to transcription termination) but this is not a control that I would specifically request: the control with the downstream inverted terminator suffices for this purpose.

One request I do make, however, is that the authors should be much more cautious in comparing/contrasting the effects of inserting the terminator 5' of S-g3 with that of inserting it 5'-of S-mu. The two situations are drastically different. The development of splenic B cells depends entirely on the production of IgM H-chains, but with no similar requirement for IgG3 expression. Therefore, there could well have been very strong selection for all sorts of, for example, epigenetic changes to allow the overcoming of any inhibitory effects on IgM expression of the terminator insertion upstream of S-mu. [Indeed, a comparison of the B cell profiles in bone marrow and spleen would be consistent with this.] The same selection would not apply to either of the insertions flanking S-g3, since there is no equivalent selection for IgG3 expression during B cell development.

The ideal way for the authors to address this criticism would be by comparing switching on the knock-in allele in heterozygous mice carrying one wild-type and one knock-in allele: productive IgM expression could be provided by the WT allele with no skewing effects of selection operating on the knock-in allele. I appreciate that such an experiment might be technically challenging - though maybe it would be possible to develop a PCR-based assay to selectively detect switching on the knock-in allele if the WT allele is derived from a different IgH allotype?

An alternative title might be 'Sense transcription through the S region is essential for Ig class-switch recombination'.

There might be some sense in transferring the actual RT-PCR data in Figs 3 and 7 to supplementary data but incorporating the RT-PCR quantitation histograms within Figs 2 and 6 respectively. Figure 5 can be left as is, since it supports the main finding of the paper.

In conclusion, I consider the manuscript highly suitable for EMBO J (the IgG3 CSR finding is striking and interesting) but would ask either that the authors are either more circumspect in their discussion of the IgM/IgG3 CSR comparison (removing for example the last sentence of the Abstract) or, ideally, that they perform the requested switching experiment with heterozygous mice if this is readily feasible in a reasonable time frame.

Referee #2 (Remarks to the Author):

This paper is the first to directly test the hypothesis that anti-sense transcription is equivalent to sense transcription through the mammalian Ig class switch regions. The authors use transcription poly-A sites to trigger transcription termination either before the switch region (sense direction) or after the switch region for the anti-sense direction. They find that blockage of anti-sense transcription has no effect at Sg3, whereas blockage of sense transcription markedly decreases Ig class switch recombination.

Though other studies have nicely touched on this issue (Zarrin and Alt's inverted S regions in PNAS several years ago), the studies here provide a different and extremely valuable set of findings that are quite important for considering models of Ig class switch mechanism. The use transcription termination to manipulate the direction of transcription through S regions, as done here by Khamlichi et al, provides the clearest answer to the importance of the direction of transcription for Ig switching. This is a beautiful study.
Minor

1. P. 5, line 4. The authors could cite the review by Yu and Lieber DNA Repair 2003 (last Figure) for a diagram of what might arise for this case.

2. If the authors are going to cite cell-free RNA/DNA hybrid data, such as Mizuta et al., 2003, then to be fair, they should also cite other cell-free data that was published earlier for RNA/DNA hybrids at various switch regions (Daniels, GA et al., Nucl. Acids Res. 1995 Dec 25;23(24):5006-11. PMID: 8559658 and also the reference to the 1994 Reaban & Griffin paper in JBC).

Referee #3 (Remarks to the Author):

Both sense and antisense transcription have been described for the IgH locus. Previous results indicate clearly that sense transcription is important for class switch recombination (CSR). This paper examines whether antisense transcription also contributes. This question is important because both DNA strands are targeted by AID, and yet the substrate for AID is single-stranded DNA, and yet sense transcripts can only form R-loops with the bottom strand, which leaves the top strand single-stranded. Mechanisms for generating ss DNA on the bottom strand have been proposed and are appropriately discussed in the introduction in this paper.

This paper describes the effect of inserting bidirectional transcription terminators at three sites in the IgH locus, asking their effect on CSR and germline transcripts. One site is upstream of Sµ (Su), and this one has little effect on CSR and germline (Iu-Cu) transcripts, although developing B cells are reduced about 2-fold. The second one is inserted upstream of Sg3. This insertion has a marked effect on CSR to IgG3 specifically, actually ablat ing it. This mutant also reduces overall transcript levels for the germline (GL) g3 RNA. The third mouse has the termination site inserted 3' to Sg3, and this has no effect on CSR or overall GL transcript levels.

The 2nd mouse, with the poor CSR to IgG3, has reduced sense and antisense transcripts upstream and downstream of Sg3, although the authors conclude differently (see below). The 3rd mouse shows reduced antisense transcripts upstream of Sg3, suggesting that the terminator, which is located downstream of Sg3, prevents antisense transcription through Sg3. And yet CSR is normal. Thus, the third mouse provides the best evidence supporting their hypothesis that antisense transcripts are not involved in promoting CSR. However, this mouse does have antisense transcripts downstream of Sg3, and they don't test how far they extend into Sg3, so one wonders if they actually extend into Sg3, which could contribute to CSR.

Figs 3E and 5E show sense and antisense transcripts but the description of the data do not seem to match the results, although it could be that I don't know what lanes are being referred to. The lanes need to be numbered. Also what is the difference between "hexamers" and cDNA? Are they both cDNA but one used oligo dT and the other random hexamer primers? There is no information in the legend or the methods that I could find. I will have to assume the hexamer samples are some sort of control since they are the same in both WT and mutant. I will assume this for my conclusions below.

In describing Fig 5E, they say that the sense transcripts downstream of Sg3 are barely detectable in the mutant mice with the termination site located upstream of Sg3. If I am right about which lanes to compare, the transcripts in WT are also barely detectable and even fainter in the mutant. I don't think this confirms a "strong termination of sense transcription" - as the authors write. On the other hand, the downstream antisense transcripts are more convincingly reduced in the mutant, but the authors write that antisense transcription was not hindered by the mutation! From these data in Fig 5E, the authors conclusion that only sense transcription at Sg3 is important for CSR does not seem justified, as the fold reduction of antisense transcription seems to be as great as the reduction of sense transcription shown in Fig 5E, so how can one decide which is important? One cannot compare the absolute amounts of transcripts since the primers are different and so the efficiency of PCR can differ.

But then when they finally discuss the third mouse (Fig 7D), the data are more convincing viz a viz their conclusion, since in this mouse antisense transcripts are reduced in the regions both upstream
(especially) and downstream of Sg3, and yet CSR is not reduced. Also, sense transcripts are not affected.

I think these results are potentially important, but the presentation needs to be improved. Also, I think it is important to determine in the third mouse whether antisense transcripts through Sg3 are indeed reduced. I don’t think it is sufficient to simply look upstream of Sg3 and conclude they did not go through Sg3. The locations of the primers for sense and antisense transcripts should be clearly marked on Fig 1B.

I also think it would be much clearer if the presentation could be condensed. It seems it would be much better to present the third mouse first and then the second and first. The first mouse is not important and does not really test the hypothesis, although the results could be mentioned. Since the terminator inserted upstream of Su did not block transcription very much, results with this mutant are not conclusive. Also, it is logical to insert the terminator downstream of Sg3 if one wants to inhibit antisense transcription through Sg3, so it makes sense to present this mouse first. The current order of presentation makes one read through all the negative results first, expecting something interesting due to the abstract and introduction, and one gets disappointed and annoyed. It is already known that sense transcription through Sg3 is required for CSR so the results from insertion of a terminator upstream of Sg3 are not novel.

The discussion is too long and does not really discuss previous findings of anti-sense transcription and their own results clearly. I suggest that things that could be cut are all the stuff on the third page of the discussion, and more. Fig 8 adds nothing and is not even discussed.

I think the title should reflect the authors’ conclusions - that antisense transcription across Sg3 does not appear to regulate CSR. The title is too vague, as it stands. They should be able to make this conclusion stronger if they test more completely the structure of the antisense transcripts in their third mutant.

1st Revision - authors’ response
02 February 2011

Reply to Referee #1:

Remark 1:
"...but would ask either that the authors are either more circumspect in their discussion of the IgM/IgG3 CSR comparison (removing for example the last sentence of the Abstract) or, ideally, that they perform the requested switching experiment with heterozygous mice if this is readily feasible in a reasonable time frame."

Reply:
We totally agree with the Referee with regard to IgM H-chain-mediated selection versus the lack of such selection for IgG3-expressing B cells. Throughout the work on pAp-mu mouse line, we have been aware of the complex problems related to selection mechanisms. That is one of the reasons why we have been very cautious in not drawing definitive conclusions from the pAp-mu mutation in a wild type background (see below), although we acknowledge that we did not directly address the issue, raised by the Referee, in the present work. With regard to the interesting experiment suggested by the Referee, it requires heterozygous mice with appropriate genetic background (e.g. mutant 129/wild type Black6), which are unfortunately not available right now. Additionally, the use of specific allotype-based primers, for PCR or DC-PCR on genomic DNA, which we have performed for another study, was not, in our hands, that conclusive. Thus, as the Referee himself acknowledges, this experiment “is not easily feasible in a reasonable time frame”, but more importantly, it does not question the core message of our study.

More specifically, although we cannot formally exclude the involvement of IgM-based selection mechanisms in late B cell development, we think that they cannot solely account for the decrease seen in pAp-mu mouse line. Indeed, by investigating, as we did, the effect of the mutation in RAG2-deficient background, one can analyse mu0 and Imu germ-line transcription while getting rid of the selection mechanisms that operate on IgM H-chain-expressing B cells. Although the results were clear-cut, we have been very cautious in our statements. Thus, in the Results section (p. 7), we wrote: “…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 pApµ mutation was brought into a Rag2−/−-deficient background.” In the Discussion section (p. 15 and 17), we went on to say: “While this indicates that 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.” (p. 15).

In summary, we think that we have been circumspect enough to not draw firm conclusions from the pApµ model (in the presence of selection mechanisms). In contrast, the conclusion drawn from the pApµ/RAG2-deficient model (a partial inhibition of transcription elongation by the pAp cassette) is fully justified, although it cannot be transposed to germ-line transcription during CSR. In order to clarify the issue further, we did the following:

- We added a sentence in the Discussion section (p.15, at the end of the first paragraph) in order to tone down our statement and to draw attention to the difference between the two situations.
- We removed the last sentence of the abstract in order to meet the alternative request made by the Referee.

Remark 2:
“There might be some sense in transferring the actual RT-PCR data in Figs 3 and 7 to supplementary data but incorporating the RT-PCR quantitation histograms within Figs 2 and 6 respectively. Figure 5 can be left as is, since it supports the main finding of the paper.”

Reply:
We would prefer to show the RT-PCR and strand-specific RT-PCR data together, as they conversely provide a more solid ground to the findings of each figure.

Remark 3:
“An alternative title might be ‘Sense transcription through the S region is essential for Ig class-switch recombination’.”

Reply:
The title has been changed. We adopted the one proposed by the Referee.

Reply to Referee #2:

Remark 1:
“P. 5, line 4. The authors could cite the review by “Yu and Lieber” DNA Repair 2003 (last Figure) for a diagram of what might arise for this case.”

Reply:
We have added the reference (see Introduction (p. 5) and References (p. 36)).

Remark 2:
“If the authors are going to cite cell-free RNA/DNA hybrid data, such as “Mizuta et al., 2003”, then to be fair, they should also cite other cell-free data that was published earlier for RNA/DNA hybrids at various switch regions (Daniels, GA et al., Nucl. Acids Res. 1995 Dec> 25;23(24):5006-11. PMID: 8559658 and also the reference to the 1994 Reaban & Griffin paper in JBC).”

Reply:
The two references have been added (see Discussion (p. 18) and References (p. 29 & 33).

Reply to Referee #3:

Remark 1:
“The 2nd mouse, with the poor CSR to IgG3, has reduced sense and antisense transcripts upstream and downstream of Sg3, although the authors conclude differently (see below).”

Reply:
What the data show (Fig. 5) and what we wrote throughout the corresponding Results and Discussion sections is that the 2nd mouse line (pAp-g) has 1) normal sense transcripts upstream of the pAp cassette but reduced sense transcripts downstream of the cassette, and 2) normal antisense
transcripts across Sg3, but reduced transcripts upstream of the cassette. Therefore, our conclusions, which are clearly summarized in Fig. 8, are quite different from what the Referee attributes to us.

Remark 2:
"... the third mouse provides the best evidence supporting their hypothesis that antisense transcripts are not involved in promoting CSR. However, this mouse does have antisense transcripts downstream of Sg3, and they don't test how far they extend into Sg3, so one wonders if they actually extend into Sg3, which could contribute to CSR."

Reply:
What we show (see Fig. 7 and also Fig. 8, which is helpful in this regard) and discuss, is that, in the third mouse line (Sg3-pAp), antisense transcripts are normal downstream of the pAp cassette, but are undetectable upstream of the cassette. Therefore, the question of how far the antisense transcripts extend into Sg3 does not seem to be pertinent.

Remark 3:
"Figs 3E and 5E show sense and antisense transcripts but the description of the data do not seem to match the results, although it could be that I don't know what lanes are being referred to. The lanes need to be numbered. Also what is the difference between "hexamers" and cDNA? Are they both cDNA but one used oligo dT and the other random hexamer primers? There is no information in the legend or the methods that I could find. I will have to assume the hexamer samples are some sort of control since they are the same in both WT and mutant. I will assume this for my conclusions below."

Reply:
1) we added a couple of sentences in the materials and methods section to outline the salient features of the technique (p. 21), 2) we added a sentence to further clarify the corresponding figure legends (p. 24, 25 & 26), 3) we provide a scheme to show the relative position of the primers used in each strand-specific RT-PCR (see also remark 5), and 4) we cited another paper which provides additional technical details. In this way, we think that any misunderstanding should be avoided.

Remark 4:
"In describing Fig 5E, they say that the sense transcripts downstream of Sg3 are barely detectable in the mutant mice with the termination site located upstream of Sg3...On the other hand, the downstream antisense transcripts are more convincingly reduced in the mutant, but the authors write that antisense transcription was not hindered by the mutation! From these data in Fig 5E, the authors conclusion that only sense transcription at Sg3 is important for CSR does not seem justified."

Reply:
What the data show in Fig. 5E and what we actually say in describing this figure (and again what we summarize in Fig. 8), reads as follows (p. 12 & 13): “Sense transcripts were readily detected upstream of the insertion site in both WT and pAp-g mice (Figure 5E, see also Figure 8). In contrast, sense transcripts downstream of the insertion site were barely detectable in pAp-g mice compared to 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-g mice, indicating that antisense transcription of Sg3 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-g mice suggesting that few Pol II molecules have managed to elongate past the pAp cassette. We conclude that the abrogation of CSR to IgG3 in pAp-g mice correlates with the lack of sense transcription of Sg3 and that antisense transcription of Sg3 in the absence of its sense transcription is not sufficient for CSR to IgG3.”

Thus, our conclusion, based on the second mouse model, is quite cautious. One of the reasons relates to the nature of the strand-specific RT-PCR assay that the Referee rightly reports. However, in the next paragraph, we highlighted the fact that our conclusions are based on both the second and third models, though the third model is more conclusive with regard to the essential role of sense transcripts (as the Referee acknowledges, see below). Thus, we wrote (p. 13): “In order to provide further functional support to the notion that it is the sense transcription of Sg3 that counts for efficient CSR to IgG3, we generated a third mouse line in which the pAp cassette was inserted downstream of Sg3. We reasoned that because of the bidirectional function of the pAp cassette, Sg3 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.” And we concluded the corresponding Result section (p. 14) by writing: “We conclude
that in Sg3-pAp mice, normal CSR to IgG3 correlates with sense transcription of Sg3. These results, together with the data from pAp-g mice, show that anti-sense transcription of Sg3 does not significantly contribute to normal CSR to IgG3.

Remark 5:
“But then when they finally discuss the third mouse (Fig 7D), the data are more convincing viz a viz their conclusion, since in this mouse antisense transcripts are reduced in the regions both upstream (especially) and downstream of Sg3, and yet CSR is not reduced.”

“Also, I think it is important to determine in the third mouse whether antisense transcripts through Sg3 are indeed reduced. I don’t think it is sufficient to simply look upstream of Sg3 and conclude they did not go through Sg3. The locations of the primers for sense and antisense transcripts should be clearly marked on Fig 1B.”

Reply:
1) The data (see Fig. 7, summarized in Fig. 8) show that unprocessed antisense transcripts are undetectable upstream of the pAp cassette. Therefore, the distinction between upstream and downstream of Sg3 need not be addressed, 2) we have added the location of the primers on the top of each strand-specific RT-PCR figure.

Remark 6:
“... The first mouse is not important and does not really test the hypothesis, although the results could be mentioned. Since the terminator inserted upstream of Su did not block transcription very much, results with this mutant are not conclusive. ...” I suggest that things that could be cut are all the stuff on the third page of the discussion, and more. Fig 8 adds nothing and is not even discussed.”

Reply:
We could not predict the phenotype of Smu mice before getting the data, so the issue of whether this mouse model is important or not is debatable, but it does not change the substance of our finding: i.e. that the pAp cassette does not completely block sense and antisense transcription in this mouse line. But, we felt that we could not show the whole data on pAp-Smu mouse line and then simply ignore them in the Discussion section. We have cautiously proposed that IgH enhancers may play a role in elongation and provided some evidence from the available data and from the literature. Thus, one reads: “...it is plausible that Eµ enhancer is involved in the elongation of µ transcripts” (p. 17), or “one might speculate that the 3’RR acts at the elongation step downstream of the pAp-g site” (p. 17), or “We propose that Eµ enhancer and the 3’RR may facilitate the transition of Pol II molecules from stalling to efficient elongation at Sµ and downstream S regions respectively” (p. 17). Thus, we think that we have been circumspect enough in our discussion of pAp-Smu model. As for figure 8, we believe it is quite helpful since it summarizes our main findings that are discussed throughout the Discussion section, and therefore does not need to be discussed by itself.

Remark 7:
“I think the title should reflect the authors’ conclusions - that antisense transcription across Sg3 does not appear to regulate CSR. The title is too vague, as it stands.”

Reply:
The title has been changed.