

Processing of Switch Transcripts Is Required for Targeting of Antibody Class Switch Recombination

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Summary

Antibody class switching is mediated by somatic recombination between switch regions of the immunoglobulin heavy chain gene locus. Targeting of recombination to particular switch regions is strictly regulated by cytokines through the induction of switch transcripts starting 5' of the repetitive switch regions. However, switch transcription as such is not sufficient to target switch recombination. This has been shown in mutant mice, in which the I-exon and its promoter upstream of the switch region were replaced with heterologous promoters. Here we show that, in the murine germline targeted replacement of the endogenous $\gamma 1$ promoter, I-exon, and I-exon splice donor site by heterologous promoter and splice donor sites directs switch recombination in activated B lymphocytes constitutively to the $\gamma 1$ switch region. In contrast, switch recombination to IgG1 is inhibited in mutant mice, in which the replacement does not include the heterologous splice donor site. Our data unequivocally demonstrate that targeting of switch recombination to IgG1 in vivo requires processing of the I $\gamma 1$ switch transcripts. Either the processing machinery or the processed transcripts are involved in class switch recombination.

Key words: class switch • recombination • splicing • switch transcript • B lymphocytes

B cells can exchange, by immunoglobulin class switch recombination, the isotype of the antibody they express during an immune response. Upon activation, gene segments of a particular constant region (C_H) are fused to the variable region exons of the immunoglobulin heavy-chain (IgH) gene. This DNA recombination occurs between switch (s) regions located upstream of each constant region gene (for review see references 1–3).

Switch recombination is directed to particular Ig classes by cytokines (for review see reference 4) which induce transcripts of individual unrearranged switch regions, the switch transcripts, before recombination (5–8). A common switch recombinase machinery may then recognize the committed heavy-chain gene segments. Targeting of class switch recombination becomes independent of cytokines if the promoter of a particular unrearranged IgH gene segment is replaced by a constitutively active promoter (7, 8).

Switch transcription initiates from the I_H -exon, located upstream of the switch regions, and proceeds through the switch region and the C_H exons. The switch region and the C_H introns are spliced out of the switch transcript pre-mRNA.

Little is known about the mechanism by which these switch transcripts control class switch recombination. Neither the process of switch transcription alone nor the production of a stable transcript is sufficient to direct switch recombination (7–10). Switch transcripts seem not to encode for polypeptides involved in recombination.

It has been suggested that in addition to transcription, the processing of switch transcripts might be necessary for class switch recombination (7, 8). In mice that lack a 114-bp fragment spanning the 3' part of the I $\gamma 1$ -exon and the 5' part of the $s\gamma 1$ region, including the splice donor site, class switch to IgG1 was abolished, whereas the switch transcription, driven by the human metallothionein (hMT) promoter, was not affected. This result led to speculations on the relevance of the splice donor site. However, an alternative explanation for this finding could be the presence of a recombination control element within the 114-bp fragment.

To clarify the relevance of the endogenous I $\gamma 1$ -exon splice donor site for switch recombination, we describe here the targeted replacement of the entire endogenous I $\gamma 1$ -exon by an artificial I-exon and an adenoviral (Ad) splice donor site. We show unambiguously that processing

of switch transcripts, or the processed switch transcripts themselves, are required for switch recombination.

Materials and Methods

Mice. All mice used in this study were bred and kept in the animal facility of the Institute of Genetics (University of Cologne, Germany). CD1 female mice for preparation of morulae were obtained from Charles River (Sulzfeld, Germany). All animal experiments were performed in accordance with institutional and state guidelines.

Construction of the Ad-hMT Gene-targeting Vector. We amplified the Ad splice donor site as an XhoI fragment (sequence data are available from EMBL/GenBank/DDBJ under accession number X02996, positions 6062 to 6241) from the plasmid pH3E-5 (a gift from Dr. K. Hilger-Eversheim, Institute of Genetics), containing the Ad type V-HindIII E insert cloned into the HindIII site of pBR322. The splice donor fragment contained 28 nucleotides of the first exon of the tripartite leader from the Ad-major-late region (11), plus 151 nucleotides of the first intervening sequence (12). The Ad-hMT vector has been constructed by cloning the XhoI fragment of the Ad splice donor site into the SalI site of the hMT targeting vector directly downstream of the neomycin resistance gene (*neo*)-hMT promoter cassette, which includes a bacterial sequence replacing the I γ 1-exon. The hMT vector has been described in detail (7). The Ad-hMT construct consisted of an 8-kb homologous sequence to the 5' γ 1 region, the replacement cassette containing *neo* in reverse orientation, the hMT promoter, a bacterial sequence, and the Ad splice donor site, and 0.8-kb homology to the γ 1 region. A thymidine kinase gene (*TK*) for negative selection was introduced at the 5' end of the construct.

Generation and Screening of Homologous Recombinant Embryonic Stem (ES) Cell Clones. The NotI linearized Ad-hMT vector was transfected into 129/Ola derived E14-1 ES cells (13). ES cells were cultured on primary embryonic fibroblasts and manipulated as described in Torres and Kühn (14). Homologous recombinant ES clones were identified by Southern blot analysis. Genomic DNA was digested with EcoRI and hybridized with the external 9-kb SspI-EcoRI fragment of the γ 1 switch region (15). Homologous recombinant clones were confirmed by digestion with KpnI hybridizing with a 1,550-bp XhoI-EcoRV neomycin probe from pFRT2neo (9).

Generation of Targeted Ad-hMT Mice. A correctly targeted ES cell clone was aggregated with CD1 morulae prepared from 6-wk-old superovulated mice and implanted into foster mothers as described previously (14). Male chimeric mice were crossed to female C57BL/6 mice to obtain heterozygous mutant mice. Offspring were analyzed for transmission of targeted and wild-type IgH alleles by PCR using as the 5' primer 5'-TAGTCCCTGCCTTTGCTCTG-3' (primer 1) and, for the Ad-hMT allele, the 3' primer 5'-ATCGCCTTCTTGACGAGTTC-3' (primer 2), and, for the wild-type allele, the 3' primer 5'-GCCTCGTCAGAAAGATTGGT-3' (primer 3). Homozygous mice were generated by intercross.

Analysis of IgG1 Serum Titers and Cytometric Analysis of LPS-stimulated Splenic B Cells. Serum concentrations of IgG1^a and total IgG1 of unimmunized mice were determined by allotype-specific ELISA as described (13). The frequency of IgG1 class switch was assayed by flow cytometric analysis of LPS- (Sigma Chemical Co., Steinheim, Germany) activated splenic B cells *in vitro*. Resting B cells were obtained by depletion of CD43⁺ cells from spleen cells prepared from 8–12-wk-old mice using anti-CD43 microbeads and high-gradient magnetic cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The efficiency of depletion was controlled by flow cytometric analysis with anti-CD43 antibodies (Miltenyi Biotec). B cells were cultured in RPMI, 10% FCS, 50 μ M β -mercaptoethanol, 2 mM glutamin, and 10 U/ml penicillin-streptomycin in a concentration of 10⁶ cells/ml and stimulated on day 0 either with LPS alone (40 μ g/ml) or with LPS and recombinant murine IL-4 (5 ng/ml; Genzyme Corp., Cambridge, MA). Cells were harvested on day 5, fixed in formaldehyde (2%; Merck, Darmstadt, Germany), permeabilized by saponin (0.5%, Sigma Chemical Co.), (16), and stained for cytoplasmic IgG1 with digoxigenized monoclonal rat anti-mouse IgG1 (0.5 μ g/ml; gift from Miltenyi Biotec) and antidigoxigenin (Boehringer Mannheim, Mannheim, Germany) conjugated to Cy5 (Amersham, Braunschweig, Germany). Cytometric analysis was performed using a FACScan[®] (Becton Dickinson, Mountain View, CA).

Results

Generation of Ad-hMT Targeted Mice. For the genetic modification of the IgG1 locus, we constructed an Ad-hMT targeting vector (Fig. 1 A). It contained a heterologous

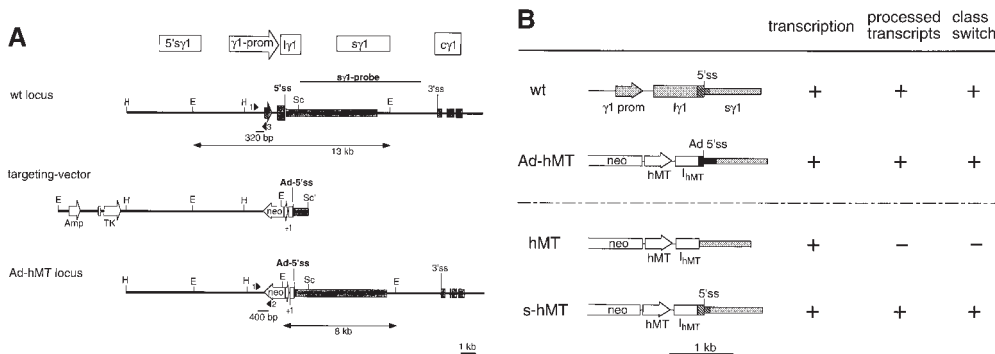


Figure 1. Targeting of the murine γ 1 wild-type locus using a modified hMT vector with an inserted Ad splice donor site (*Ad-5'ss*). (A) Genomic organization of the murine IgG1 wild-type locus, the targeting vector, and the targeted Ad-hMT allele. The γ 1 probe used for Southern screening of homologous recombinant ES cell clones is represented as a black bar. Filled triangles indicate the primers used for screening of chimeric offspring. H, HindIII; E, EcoRI; Sc, ScaI; Amp, ampicillin resistance gene; TK, herpes simplex virus thymidine kinase gene; *neo*, neomycin resistance gene. (B) Detail of the γ 1 wild-type and Ad-hMT targeted loci. Sequences introduced by targeting vectors are shown in white: *neo* gene (*neo*), hMT promoter (*hMT*), artificial I γ 1 exon (*I γ 1*); or in black: Ad splice donor site (*Ad*). The 114-bp fragment of wild-type sequence is represented as a hatched box. The phenotype of the mice is indicated with respect to transcription (*transcription*), splicing of the transcripts (*processed transcripts*), and class switch to IgG1 (*class switch*).

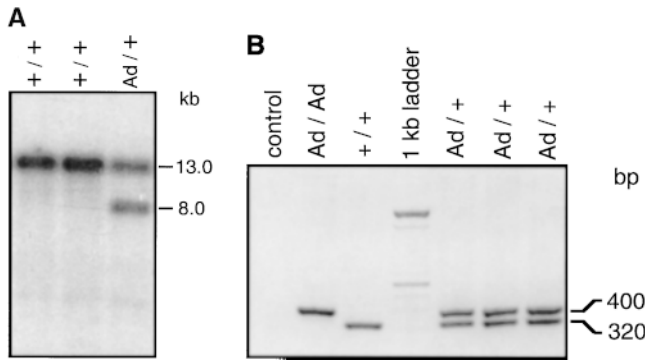


Figure 2. (A) Analysis of homologous recombinant ES cell clones by Southern blot using an $\gamma 1$ probe. EcoRI digestion of genomic DNA yielded fragment sizes of 13.0 and 8.0 kb of the wild-type (+) and Ad-hMT (Ad) alleles, respectively. (B) Analysis of heterozygous and homozygous targeted mice and C57BL/6 wild-type littermates. Agarose gel electrophoresis of PCR products of tail DNA amplified with primers 1, 2, and 3.

splice donor site inserted into the SalI site directly 3' of the neo-hMT promoter cassette of the targeting vector, which we originally used to generate the hMT mutant mice as described previously (7). We chose an 179-bp fragment from Ad type V, which contains all *cis* elements needed for efficient splicing, and a 9-bp splice donor site (12). No coding sequence is present in this fragment. Both the sequence of the Ad splice donor site (GGG GTAGT) as well as the sequence of the endogenous $\gamma 1$ splice donor site (GCG GTAAGT) are 78% homologous to the consensus sequence (17). The resulting Ad-hMT construct (Fig. 1 A) was targeted into wild-type E14-1 ES cells derived from 129/Ola mice (IgH^a). Homologous integration was confirmed by Southern hybridization (Fig. 2 A, and data not shown). In the mutant Ad-hMT IgH locus the endogenous 1.7-kb 5' $\gamma 1$ region containing the $\gamma 1$ promoter and the I $\gamma 1$ -exon is replaced by an inversely oriented neo, the hMT promoter, a bacterial sequence replacing the I-exon and the Ad splice donor site. The correct insertion of the Ad splice donor site in the targeted ES cell clone was verified by sequencing. Thus, Ad-hMT mice and hMT mice differ only in the presence of the Ad splice donor site (Fig. 1 B).

We generated nine chimeric mice by aggregation of one ES cell clone with morulae prepared from female mice of the CD1 outbred strain. Heterozygous targeted mice were obtained by crossing the chimeras with C57BL/6 (IgH^b). Heterozygous and homozygous mutant mice were identified by PCR of genomic DNA from tail biopsies using primers 1, 2, and 3 (Fig. 2 B). The effect of the inserted splice donor site on IgG1 class switching was analyzed in mice heterozygous and homozygous for the mutation.

Mutant Ad-hMT Mice Produce Normal Titers of IgG1 In Vivo. To analyze IgG1 class switching in vivo we measured the IgG1 titer in IgH^{Ad-hMT/b} mice by an allotype-specific ELISA. Since the C57BL/6 IgH allotype produces IgG1 of the b haplotype, analysis of the IgG1^a titer directly determines the IgG1 level produced from the 129/Ola derived

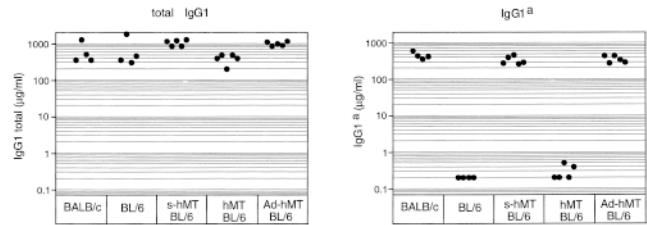


Figure 3. In vivo analysis of IgG1 production in heterozygous targeted F₁ and wild-type control mice. Immunoglobulin levels of IgG1^a and of total IgG1 (IgG1^a and IgG1^b) were determined in sera of 8–12-wk-old mice by ELISA. The detection limit was 0.2 $\mu\text{g/ml}$.

targeted Ad-hMT alleles (IgH^a). In IgH^{Ad-hMT/b} mice, serum IgG1^a concentrations were $\sim 1,000$ -fold above the titers of IgH^{hMT/b} mice, which show a constitutive transcription of the IgG1^a locus but lack the 114-bp endogenous fragment (Fig. 3). Titers comparable to IgH^{Ad-hMT/b} mice (mean, 365 $\mu\text{g/ml}$) were detected in sera of IgH^{s-hMT/b} mice, which have retained the 114 bp of endogenous sequence (mean, 340 $\mu\text{g/ml}$) and in BALB/c mice (IgH^{a/a}, mean, 453 $\mu\text{g/ml}$). IgH^{hMT/b} mice produce very low levels of serum IgG1^a (mean 0.4 $\mu\text{g/ml}$), whereas in C57BL/6 wild-type mice (IgH^{b/b}) serum IgG1^a was not detectable (< 0.2 $\mu\text{g/ml}$). All animals had comparable titers of total IgG1 (IgG1^a plus IgG1^b).

IgG1 Switch Frequency of Activated B Cells in Ad-hMT Mice. For the analysis of IgG1 class switching of B lymphocytes in vitro, we isolated resting B cells from homozygous Ad-hMT mice (IgH^{Ad-hMT/Ad-hMT}) and wild-type littermate controls by depletion of CD43⁺ cells from spleen. 99% pure CD43⁻, resting B cells were stimulated with LPS or with LPS and IL-4. On day 5, activated B cells were stained intracellularly for expression of IgG1 (Fig. 4). As expected, B cells from wild-type mice switched to expression of IgG1 only in the presence of LPS and IL-4. Due to

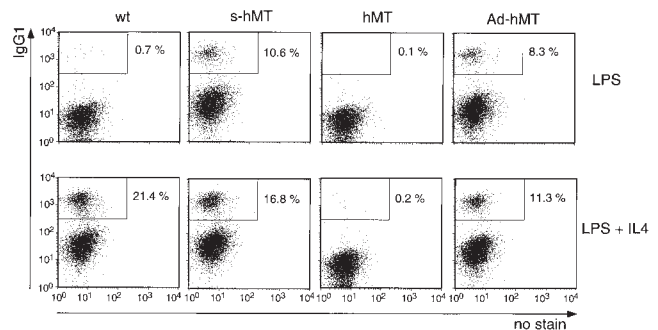


Figure 4. Flow cytometric analysis of LPS-stimulated splenic B cells of pooled homozygous targeted mice and their wild-type littermates. By depletion of CD43⁺ spleen cells using the MACS system, resting B cells (CD43⁻) were isolated and cultured with LPS or with LPS + IL-4. After 5 d cells were fixed and stained intracellularly for expression of IgG1. Due to constitutive activity of the hMT promoter, class switch to IgG1 is independent of IL-4 in B cells from targeted mice. A representative example of two independent experiments is shown. (Note that in all cultures with IgG1 switched B cells, there is a basal level staining of all B cells for IgG1, probably due to Fc- γ R bound IgG1 on the surface.)

constitutive activity of the hMT promoter in the targeted mice, recombination to $\gamma 1$ is independent of IL-4. Thus, differences between the various targeted alleles became evident in B cells stimulated only with LPS. IgH^{Ad-hMT/Ad-hMT} B cells, stimulated with LPS, switched to IgG1 at frequencies comparable to IgH^{s-hMT/s-hMT} B cells (8.3% vs. 10.6%), whereas IgH^{hMT/hMT} B cells did not switch to IgG1 at all (<0.1%). In the presence of LPS plus IL-4, the frequency of switched cells from IgH^{Ad-hMT/Ad-hMT} mice and IgH^{s-hMT/s-hMT} mice was enhanced by a factor of 1.4 and 1.6, respectively, as compared to the cultures with LPS alone. This effect is probably due to the stronger proliferative induction of stimulated B cells by IL-4.

We conclude that processing of the I $\gamma 1$ switch transcripts is required for class switching to IgG1 in vivo and in vitro.

Discussion

Recent studies have suggested that in addition to transcription, processing of switch transcripts may be required to induce recombination of the corresponding switch region (7, 8). We have shown previously that a 114-bp fragment of I $\gamma 1$ wild-type sequence is necessary to target switch recombination to IgG1 (Fig. 1 B; 7). Whether the 114-bp fragment is required as a splice donor site, a recombination control element, or a transcriptional enhancer has not been clear so far.

In this study, we demonstrate unequivocally that it is the splice donor element which is required to target switch recombination to the $\gamma 1$ region in vivo. In mutant Ad-hMT mice, which contained an Ad splice donor site replacing the 114-bp fragment, serum concentrations of IgG1 were at the same level as in wild-type mice. In contrast, serum concentrations of IgG1 were reduced about three orders of magnitude in hMT mice (Fig. 1 B) lacking the I $\gamma 1$ splice donor site.

At first glance, our results contradict experiments published recently by Harriman et al. (8), who replaced the I α promoter by the phosphoglycerate kinase promoter and the entire sequence of the I α -exon by a human hypoxanthine phosphoribosyltransferase (hHPRT) minigene. Despite the deletion of the endogenous I α splice donor site, mutant mice produce normal titers of IgA in the serum. α switch recombination in these mice might be due to splicing within the intron of the hHPRT minigene 5' of the α switch region, as suggested by the authors. However, the data also do not exclude splicing of primary transcripts between the splice donor site of the hHPRT minigene and the splice acceptor of the first C α -exon, resulting in a situation comparable to B cells from Ad-hMT mice or wild-type B cells. In another study, deletion of the entire I ϵ -exon without replacement by splice signals nearly but not completely abolishes class switch recombination to Ige (10). Again, the residual frequency of switch recombination to Ige could be explained by alternative splicing between cryptic splice sites and C ϵ , which may occur according to the data shown by the authors.

Our data clearly show that processing of switch transcripts is required for targeting of switch recombination in vivo. This argues against the original idea that switch transcription as such may simply improve accessibility of switch regions for the action of a switch recombinase (5, 18, and for review see reference 4).

A different picture arises from experiments using extrachromosomal or integrated switch recombination substrates in vitro (19–23). In all systems recombination between switch regions can be detected without active transcription. Some switch plasmids undergo recombination at high frequencies only if they are transcribed (24, 25). A requirement for splicing of those plasmid transcripts has not been described so far. However, in our own experiments, we have observed that the switch recombination frequency could be increased more than twofold on transcribed extrachromosomal switch substrates, transfected into activated, primary B cells if the plasmid transcripts are spliced (Petry, K., G. Siebenkotten, R. Christine, and A. Radbruch, manuscript submitted for publication). This suggests that a basic recombination frequency of extrachromosomal switch substrates may be due to recombination machines other than the switch recombinases, but for switch recombination, transcription and splicing of transcripts may be required.

Our study does not answer the question of how switch transcripts are involved in the process of switch recombination. An attractive idea is that the spliced switch transcripts or the spliced intronic switch regions would interact with the recombination machinery. Either in *trans*, as a component of the switch recombinase, or in *cis*, rehybridizing with the DNA template, thus stabilizing an RNA–DNA triple helix which then could be recognized by the recombination machinery. This idea is supported by findings that transcription of G-rich regions, present in switch regions or in mitochondrial DNA of yeast, can generate stable RNA–DNA hybrids in vitro (23, 26, 27). Proteins with RNA- as well as DNA-binding domains have been shown recently to be involved in the recombination of switch substrates (28).

An alternative hypothesis would be that components of the spliceosome are required for the process of recombination. Nucleophosmin and nucleolin, two of the identified components of the recently described switch region transfer activity (28) have been shown to be also involved in the processing of ribosomal RNA in the nucleolus (29, 30), which is dependent on the presence of small nucleolar ribonucleoproteins (snoRNPs) (31).

The link between splicing and recombination has been documented recently also in other transcription-based recombination systems such as the integration of yeast intron RNA into DNA via reverse splicing and the involvement of genes in double strand formation during the meiotic recombination and in RNA splicing in yeast (32, 33). The common basic chemistry involved in splicing of RNA and in recombination of DNA supports the upcoming idea that splicing may have more potential than only to process RNA.

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