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Germ line transcription in mice bearing *neo*^{r} gene downstream of Iγ3 exon in the Ig heavy chain locus

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Abstract

Class switch recombination (CSR) is preceded by germ line transcription that initiates from promoters upstream of switch (S) sequences and terminates downstream of associated constant genes. Previous work showed that germ line transcripts and their processing are required for CSR and that germ line transcription is regulated in a major part by a regulatory region located downstream of the Ig heavy chain locus. This long-range, polarized effect can be disturbed by inserting an expressed neomycine resistance (*neo*^{r}) gene. To contribute to a better understanding of the mechanism of such a long-distance regulation, we generated knock-in mice in which a *neo*^{r} gene was inserted downstream of Iγ3 exon leaving intact all the necessary elements for germ line transcription and splicing. We show that the expressed *neo*^{r} gene interferes with transcription initiation from Iγ3, and that it impairs but does not block S recombination to Cγ3. Moreover, we show for the first time that the *neo*^{r} gene provides through chimeric *neo*^{r}-Cγ3 transcripts the necessary elements for splicing of germ line transcripts by activating two novel cryptic splice sites, one in the coding region of the intronless *neo*^{r} gene and the other in the Iγ3-Cγ3 intron.

Introduction

In the mouse, the constant region (C) genes are organized in the following order: 5′-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3′. Upon antigen challenge, populations of mature B cells expressing IgM and/or IgD can undergo a class switch recombination (CSR) that specifically affects C genes through a deletional process whereby a downstream C gene is brought to proximity of a rearranged VDJ gene, enabling expression of one of the downstream isotypes (IgG, IgE or IgA) (1).

CSR occurs between highly repetitive switch (S) sequences located upstream of all the C genes except Cδ. This process is controlled by a series of signals in which the B cell antigen receptor, cytokines and B cell–T cell interactions play a critical role. Activation and targeting of CSR can be mimicked *in vitro* by a combination of certain mitogens and cytokines to induce or suppress germ line transcription of specific C genes (1).

CSR is often directed to the same S sequences on both homologous chromosomes (2–4) and is preceded by a biallelic germ line transcription directed by I germ line promoters located upstream of S sequences (5). The transcripts run through the I exon and the S sequences and undergo polyadenylation downstream of the C exons. Splicing enables fusion of the I exon to the C and excision of the intervening sequences yielding sterile, non-coding transcripts (6).

Previous work suggested that germ line transcription was necessary for the accessibility of S sequences to putative recombinases affecting cleavage (6). Several studies also showed that germ line transcripts form RNA–DNA hybrids with the template strand (7–11), whereas the single-stranded, non-template strand form long and stable R loops *in vivo* which may serve as substrates for activation-induced cytidine deaminase (12). The latter was shown to associate with the chromatin of the target S sequences in a germ line transcription-dependent manner, maybe through a direct interaction with the transcription machinery (13).

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Different knockout experiments highlighted the importance of the germ line transcription for efficient CSR to the target genes (14–18). However, although necessary, germ line transcription is not sufficient and processing of germ line transcripts is required for efficient CSR (16, 19–22), but the role of the splicing machinery in CSR remains unclear.

Cis-regulatory elements located upstream of the germ line promoters or downstream of the Ig heavy chain (IgH) locus control CSR by regulating germ line transcription from the target I promoters (1, 23–27). The Ig heavy chain 3′ regulatory region (3′RR) which contains four DNase I hypersensitive sites (5′-hs3a, hs1-2, hs3b, hs4-3′) (25) was suggested to function as a long-range control region that regulates various germ line promoters following appropriate stimulation (23, 24). Mutant mice were generated in which hs3a, hs1-2 or hs3b/hs4 were replaced with an LPS-inducible neomycine resistance (*neo*^{r}) gene. The mutant B cells were severely impaired in their ability to switch to most isotypes due to altered germ line transcription of the corresponding C genes spread over ~100 kb (23, 24). However, the removal of the *neo*^{r} cassette had no remarkable effect on CSR for individual deletions of hs3a or hs1-2 (24), whereas the joint deletion of hs3b/hs4 affected CSR to most isotypes but still less severely than with the replacement mutation (26). Additional insights into the mechanisms by which the 3′RR modulates germ line transcription came from the analyses of mutant mice in which the expressed selectable marker was inserted within the IgH C locus upstream of the 3′RR (18, 21, 28, 29). For example, Cε or the Iγ2b exon was replaced with a *neo*^{r} gene. Analysis of mutant B cells showed that germ line transcription and CSR to upstream C genes were impaired (with the exception of *Cγ1*) but the downstream C genes were not affected (29).

Based on the above studies, it has been proposed that the inhibition of CSR resulted from a '*neo* effect' that may reflect a potential competition of the *neo*^{r} promoter for control elements in the 3′RR, and that CSR could be regulated by the relative ability of various I promoters to compete for activities displayed by the 3′RR (23, 24). In addition, *neo*^{r} gene insertion into the IgH C locus seems to disturb the polarized long-range effect of the 3′RR on germ line promoters as long as they are located upstream, but not downstream, of the *neo*^{r} insertion site (29).

These phenotypes illustrate the usefulness of *neo* effect approach in gaining some insights into the mechanisms underlying long-range interactions in complex loci. However in the studies where the 3′RR was left intact, the structure of the elements known to be required for accurate germ line transcription and processing, namely the I promoter, the canonical splice sites or the C gene was altered (14–22, 28, 29). Therefore, it was interesting to study the *neo* effect by leaving intact all these elements. To this end, we generated knock-in mice bearing the *neo*^{r} gene in a sense orientation downstream of the Iγ3 exon leaving intact all the necessary elements for proper germ line transcription of *γ3* gene. We asked if and how *neo*^{r} gene insertion would alter transcription

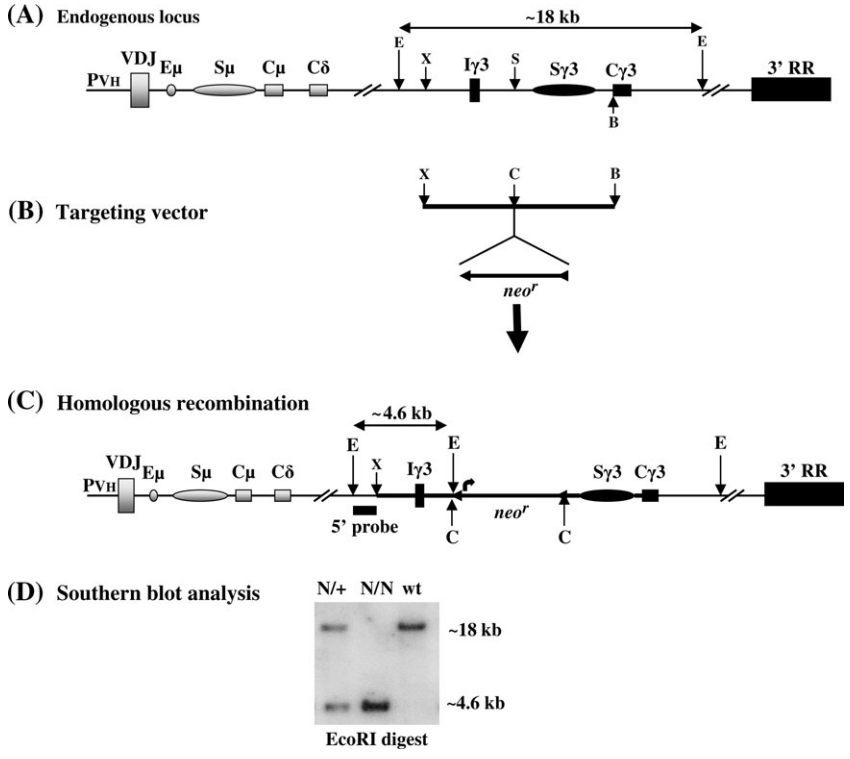

Fig. 1. Insertion of *neo*^{r} cassette downstream of Iγ3 exon. (A) Structure of the targeted locus: scheme of a wt allele showing a rearranged IgH locus and highlighting the γ3 gene as well as the 3′RR (not to scale). (B) Structure of the targeting vector in which a *neo*^{r} cassette flanked by two loxP sites (filled triangles) was inserted into the *Sph*I site (S) between Iγ3 and Sγ3. The *Sph*I site was modified into a *Cla*I site (C) in the targeting vector. (C) Structure of the targeted allele. The *neo*^{r} cassette was inserted in the sense orientation (indicated by the arrow) ~250 bp downstream of Iγ3 exon. The location of the 5′ probe (1-kb *Eco*RI–*Xho*I fragment) is indicated. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; S, *Sph*I; X, *Xho*I. (D) Southern blot analysis of wt and mutant mice. Genomic DNA was extracted from the mice tails and digested with *Eco*RI. The 5′ probe distinguishes the wt band (~18 kb) from the *neo*^{r} recombinant band (~4.6 kb).

initiation from Iγ3 promoter and from the upstream Iμ promoter and how this would affect long-range interaction between the 3′RR and Iγ3.

Methods

Targeting vector and mice

A γ3 targeting construct was generated using a plasmid containing an ~8-kb *Xho*I–*Bam*HI fragment spanning Iγ3 and Sγ3. An *Sph*I site 250 bp downstream of the Iγ3 donor splice site was replaced by a *Cla*I site. A *neo^r* cassette in which the selectable marker is under the control of the thymidine kinase (tk) promoter was then introduced as a 1.3-kb *Cla*I fragment in the sense orientation. An HSV tk gene was inserted in *Not*I site for negative selection. ES cells (cell line CK35) were transfected by electroporation, and selected using G418 (400 μg ml^{-1}) and ganciclovir (2 μM). Recombinant clones were identified by Southern blot analysis with an external probe (1.0-kb *Eco*RI–*Xho*I as a 5′ probe) after an *Eco*RI digest. An internal probe (1.9-kb *Hin*dIII–*Bam*HI at the end of the 3′ arm) was also used after an *Eco*RI + *Bam*HI digest. Two ES clones showing homologous recombination were injected into C57Bl/6 blastocysts, the male chimeras were then mated with C57Bl/6 females. Germ line transmission of the mutation was checked by Southern blot by using the same digests and probes.

Spleen cell cultures

Splenocytes from 6- to 8-week-old mice were activated *in vitro* in RPMI 1640 medium supplemented with 10% FCS, 50 μM of 2 β-mercaptoethanol and 20 μg ml^{-1} of LPS (*Salmonella typhimurium*; Sigma, St Louis, MO, USA) at a density of 10^6 cells ml^{-1}. At days 0, 2, 4 and 5, aliquots of cells were removed for RNA preparation.

Flow cytometry analysis

At day 5 of LPS stimulation, splenocytes (5×10^5 cells per assay) were labeled by using spectral red-conjugated anti-B220 and PE-conjugated anti-IgM, anti-IgG3 or anti-IgG2b (Southern Biotechnology). Data were obtained on 1.5×10^4 viable cells by using a Coulter XL apparatus (Beckman Coulter, Fullerton, CA, USA).

ELISA assays

Sera or supernatants from spleen cell cultures (harvested after 5 days of stimulation) were analyzed for the presence of IgM, IgG3 and IgG2b by ELISA as described (26). For statistical analysis, we used Student's *t*-test; *P* values of less than 0.017 were considered to be statistically significant.

Northern blots

Total cellular RNA preparation and northern blotting were carried out as described (26). Probes used for hybridization were the following: for Cμ transcripts, a 1.2-kb *Xba*I–*Hin*dIII genomic fragment containing the murine Cμ1 to Cμ3 region; for γ transcripts, a 1.7-kb *Bam*HI–*Sph*I genomic fragment containing the Cγ3 region and cross-hybridizing with all γ constant transcripts; for mb-1 transcripts, a 0.5-kb *Pvu*II mb-1 cDNA fragment and for *neo^r* transcripts, a 1.0-kb version of the cassette devoid of the tk promoter.

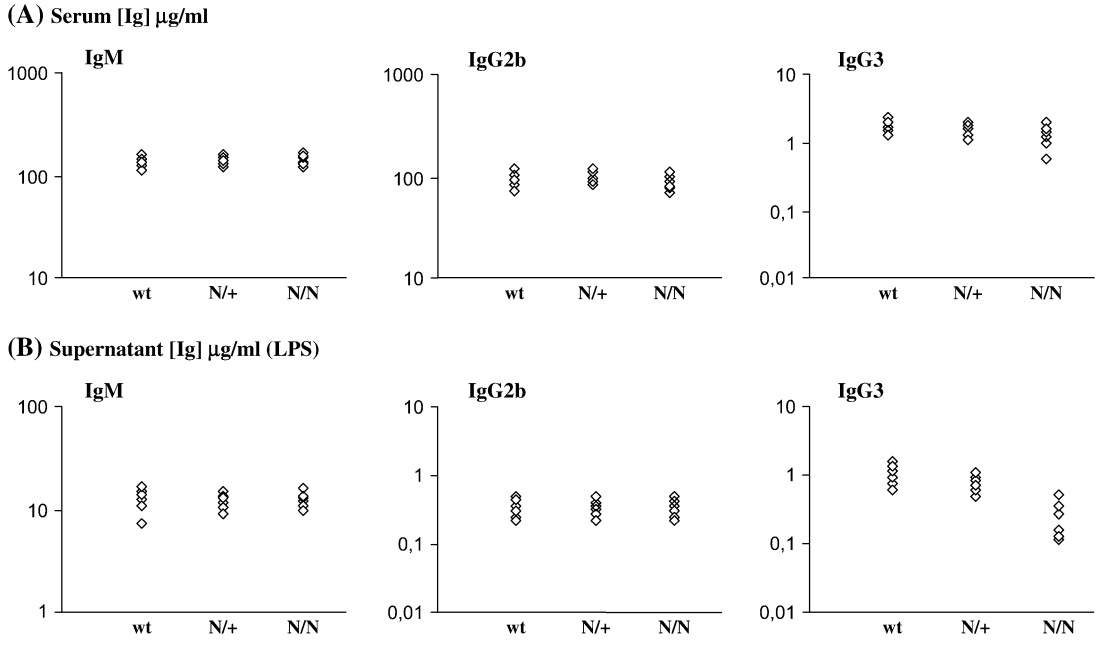

Fig. 2. Analysis of Ig production in the sera and culture supernatants. (A) Ig production in unimmunized mice. Analysis of IgM, IgG3 and IgG2b secretion in 6-week-old mice was done by ELISA. Six mice from each genotype were analyzed. (B) ELISA analysis of IgM, IgG3 and IgG2b secretion after LPS stimulation. In three separate experiments, splenocytes from six littermates (two of each genotype) were analyzed for LPS-induced IgM, IgG3 and IgG2b secretion. The ELISA has been performed twice. For IgM: 12.5 ± 2.2 μg ml^{-1} (+/+), 11.90 ± 1.91 μg ml^{-1} (N/+), 12.50 ± 1.78 μg ml^{-1} (N/N). For IgG2b: 0.55 ± 0.21 μg ml^{-1} (+/+), 0.59 ± 0.19 μg ml^{-1} (N/+), 0.62 ± 0.17 μg ml^{-1} (N/N). For IgG3: 1.14 ± 0.25 μg ml^{-1} (+/+), 1.05 ± 0.27 μg ml^{-1} (N/+), 0.53 ± 0.32 μg ml^{-1} (N/N).

Oligonucleotides and reverse transcription–PCR analysis of germ line transcription

NeoATG primer: 5′ ATGGGATCGGCCATTGAACAA 3′. Primers Imf, Cmr, Ig3f, Cg3r, Ig2bf, Cg2br, Acti-4 and Acti-5, the reverse transcription (RT)–PCR conditions and the expected sizes of the PCR products have been described (26, 30).

Neo-Cγ3 cloning and sequencing

The two PCR products (using NeoATG and Cg3 primers) were eluted, cloned in Topo I vector (Invitrogen, Gröningen, The Netherlands) and transfected in TG1 bacterial strain. Sequences were determined on both strands by using M13 universal and internal primers.

Results

Generation of mice carrying a neo^r cassette downstream of Iγ3 exon

A targeting vector was designed in order to insert the tk-*neo^r* cassette downstream of Iγ3 but without altering the structure of Iγ3 or that of the splice donor site and its surrounding sequences. To this end, we inserted the cassette between Iγ3 and Sγ3, in an *Sph*I site located 250 bp downstream of the canonical splice donor site (Fig. 1A). Among 360 resistant clones, four clones showed a recombinant band with the expected size (~4.6 kb, with *Eco*RI digest) by using an external 5′ probe. Two recombinant clones were injected into blastocysts and both allowed germ line transmission. Heterozygous (N/+) and homozygous (N/N) mice were analyzed by Southern blot using the same digest and 5′ probe (Fig. 1B). The use of an internal probe confirmed the absence of random integration (not shown).

Serum antibodies and in vitro Ig production by LPS-activated splenocytes

In order to analyze the sera, N/N mice were bred, the progeny was bled at week 6 and the serum content in IgM, IgG3 and IgG2b was analyzed by ELISA. No difference could be detected between the mutant N/+ or N/N mice and the wild-type (wt) controls with regard to IgM, IgG3 or IgG2b (Fig. 2A).

To check if IgG3 production *in vitro* would be affected by *neo^r* insertion, we used LPS that induces splenic B cells to

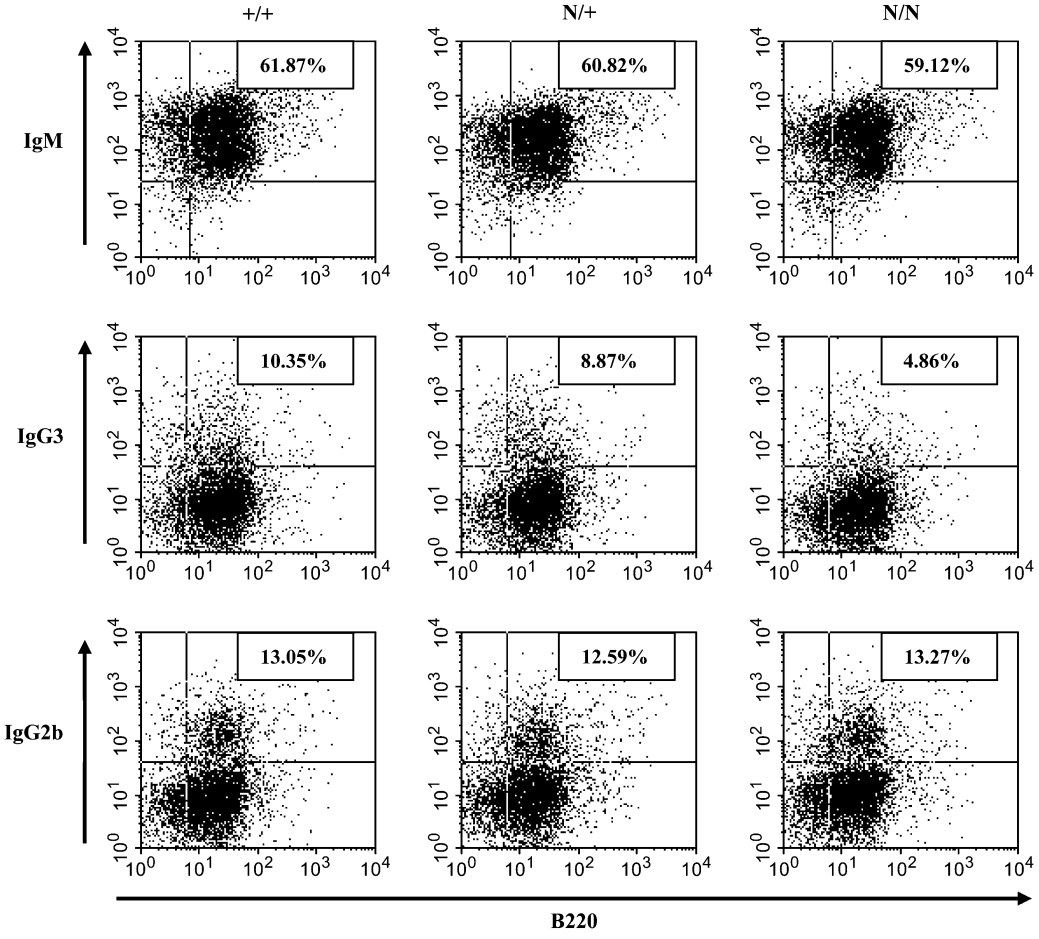

Fig. 3. Cell-surface Ig expression on stimulated splenocytes. Splenocytes from +/+, N/+ or N/N mice were cultured for 5 days with LPS (to induce switching to IgG3 and IgG2b) and stained with anti-B220 and anti-IgM, anti-IgG3 or anti-IgG2b. The percentages of switched splenic B cells among the B220+ populations are indicated on the top right of the quadrant. The data shown are representative of three independent experiments. For IgM: 67.4 ± 6.0% (+/+), 65.06 ± 5.91% (N/+), 64.00 ± 6.02% (N/N). For IgG2b: 14.40 ± 1.83% (+/+), 13.90 ± 1.63% (N/+), 14.58 ± 1.50% (N/N). For IgG3: 9.96 ± 1.71% (+/+), 7.57 ± 1.57% (N/+), 4.53 ± 0.61% (N/N).

switch to IgG3 and IgG2b. Since *γ2b* gene is located far downstream of the insertion site, it was used as an internal control for a potential alteration of Ig production by LPS-stimulated splenic B cells.

Total splenocytes from littermates were stimulated with LPS for 5 days and supernatants were analyzed by ELISA. Whereas no difference was detected for IgM and IgG2b production between +/+, N/+ and N/N mice, a statistically significant impairment was found for IgG3 production by N/N splenocytes. IgG3 levels were roughly half those from wt ($P = 0.0003$) or N/+ splenocytes ($P = 0.0008$) (Fig. 2B). These results indicate that the *neo* effect specifically alters IgG3 production with no detectable effect on IgM or IgG2b production.

Cell-surface Ig expression in mutant mice

We also checked potential alteration of CSR in the mutant mice by studying cell-surface expression of IgM, IgG3 and IgG2b isotypes on activated splenic B cells. Splenocytes from +/+, N/+ or N/N mutant mice were activated with LPS for 5 days to induce switching to IgG3 and IgG2b. Cell-surface expression of IgM, IgG3 and IgG2b was then monitored among the B220+ populations. Whereas no difference could be detected between +/+ and mutant N/+ and N/N splenic B cells for IgM and IgG2b surface expression, a slight defect on IgG3 cell-surface expression was found on mutant N/+ splenocytes (7.57 ± 1.57% instead of 9.96 ± 1.71% in wt) and a more severe decrease in the case of N/N splenocytes (4.53 ± 0.61%) (Fig. 3). These data further show that *neo* effect specifically targets the IgG3 isotype.

Gene expression in stimulated mutant splenocytes

We then asked if the LPS-inducible expression of *neo^r*, *μ* and *γ3* genes would mirror the profiles found by ELISA and FACS. To this end, total RNA from unstimulated or LPS-stimulated splenocytes was hybridized with a *neo* probe, a Cμ probe and a Cγ3 probe that cross-hybridizes with all γ transcripts. Abundant *neo* transcripts could readily be detected in the mutant mice but not in the wt control upon LPS stimulation, clearly indicating that *neo^r* gene is induced by LPS. The same pattern holds true for *μ* gene expression which was vigorously induced by LPS in all the samples. For γ gene expression, although an LPS inducibility was obvious, we could not draw a definitive conclusion about the specific alteration of *γ3* gene expression since the probe recognized all γ transcripts (not shown).

Germ line transcription in stimulated mutant splenocytes

To gain insight into the *neo* effect at the germ line transcriptional level, we resorted to RT–PCR in semi-quantitative conditions using a set of specific primers for germ line

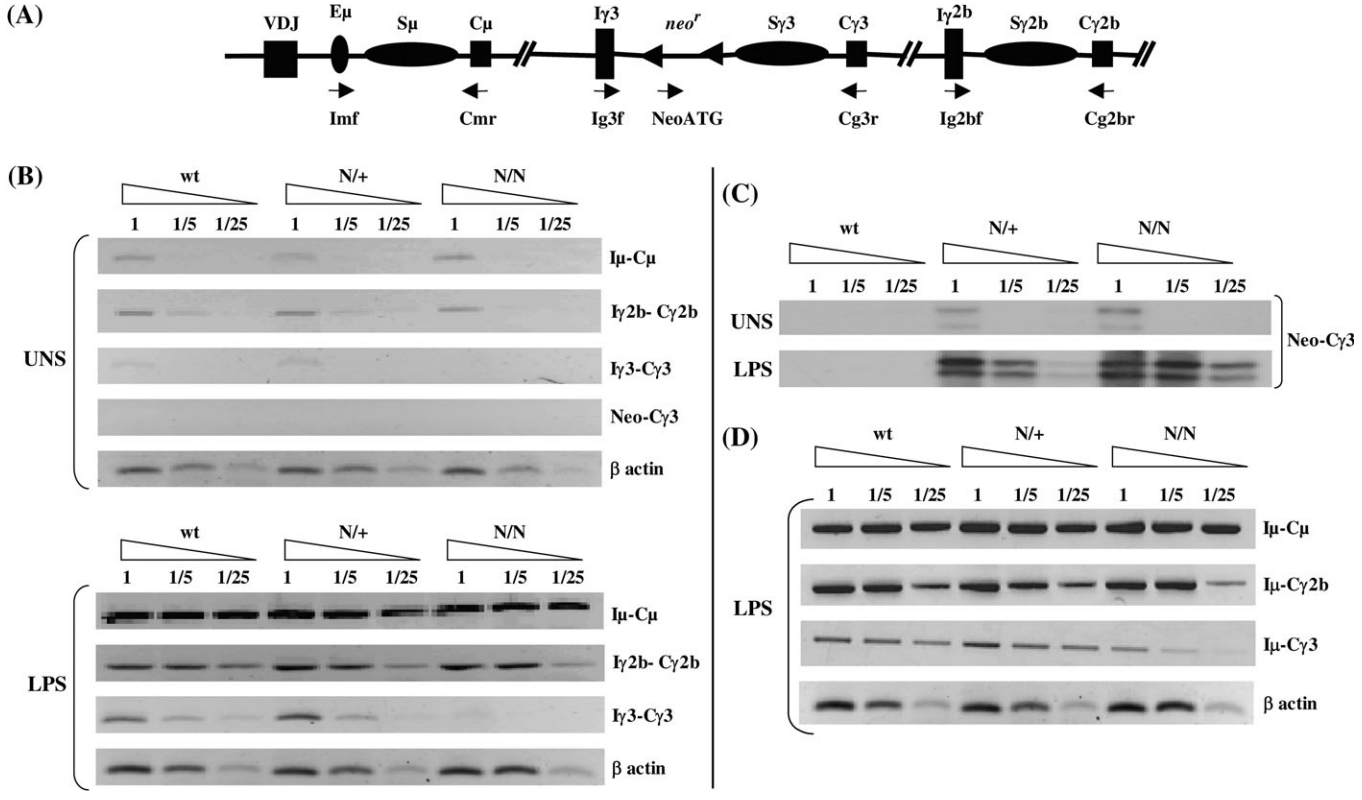

Fig. 4. Analysis of germ line transcription. (A) Relative localization of the oligonucleotides used in PCR. (B) RT–PCR was performed on wt, N/+ or N/N germ line transcripts from unstimulated (UNS) and from LPS-activated splenocytes RNA (day 2) for Iμ-Cμ, Iγ2b-Cγ2b, Iγ3-Cγ3 or for Neo-Cγ3 germ line transcripts, respectively. Single-stranded cDNAs or dilutions thereof (1/5 and 1/25) were subjected to PCR using appropriate primers. (C) To detect Neo-Cγ3 chimeric transcripts, RT–PCR was performed as in (B) using NeoATG and Cg3r primers, followed by a Southern blot and hybridization with a radiolabeled *neo* probe. (D) Analysis of hybrid germ line transcripts was performed as in (B) except that the RNA was prepared from splenocytes 4 days post-stimulation. β-Actin transcripts were used as controls.

promoters and C exons that enable a clear distinction between the different isotypes.

We found no obvious alteration in Iμ-Cμ and Iγ2b-Cγ2b transcripts in LPS-stimulated mutant splenocytes. In contrast, transcripts in the form of Iγ3-Cγ3 were hardly detectable in the N/N LPS-stimulated mutant splenocytes (Fig. 4B). Attempts to detect chimeric transcripts in the form of Iγ3-Neo in homozygous LPS-stimulated mutant splenocytes failed (not shown), suggesting that, within the sensitivity limits of our PCR assay, germ line transcription from Iγ3 promoter was impaired.

Interestingly, we detected chimeric Neo-Cγ3 transcripts (see below) in the N/+ and the N/N LPS-stimulated mutant splenocytes but not in the wt control. To our surprise, two weak fragments were consistently amplified in LPS-stimulated splenocytes by using the NeoATG and Cg3r primers. The two fragments were readily detectable by Southern blot using

(A)

***neo^r* sequence**

TGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGG
CTGACCGCTTCCTCGTGCTTTACG**GT**ATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTC
TGAGGGGATCGGCAATAAAAAGACAGAATAAAACGCACGGGTGTTAGGTCGTTTGTTCGGATCAGCTTCCGATCATATTCAAT
AACCCTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTCGGTCT

Cγ3 sequence

ctgtctgataacctcactcatcctcctatcttgc**agCTACAACAACAGCCCCATCTGTCTATCCCTTGGTCCCTGGCTGCAGT**
GACACATCTGGATCCTCGGTGACACTGGGATGCCTTGTCAAAGGCTACTTCCCTGAG

Neo-Cγ3 junction (0.8 kb)

CGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGC
TGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGC
TTCCTCGTGCTTTACG**CTACAACAACAGCCCCATCTGTCTATCCCTTGGTCCCTGGCTGCAGTGACACATCTGGATCCTCGGT**
GACACTGGGATGCCTTGTCAAAGGCTACTTCCCTGAG

(B)

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 V   A   D   R   Y   Q   D   I   A   L   A   T   R   D   I   A   E   E   L   G   G
GTG GCG GAC CGC TAT CAG GAC ATA GCG TTG GCT ACC CGT GAT ATT GCT GAA GAG CTT GGC GGC
 E   W   A   D   R   F   L   V   L   Y   A   T   T   T   A   P   S   V   Y   P   L
GAA TGG GCT GAC CGC TTC CTC GTG CTT TAC Gct aca aca aca gcc cca tct gtc tat ccc ttg
 V   P   G   C   S   D   T   S   G   S   S   V   T   L   G   C   L   V   K   G   Y
gtc cct ggc tgc agt gac aca tct gga tcc tcg gtg aca ctg gga tgc ctt gtc aaa ggc tac
 F   P   E
ttc cct gag
```

(C)

***neo^r* Sγ3 sequence**

ACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA
GGGGATCGGCAATAAAAAGACAGAATAAAACGCACGGGTGTTAGGTCGTTTGTTCGGATCAGCTTCCGATCATATTCAATAAC
CCTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTCGGTCTGAAGAGGAGTTTACGTCCAGCCAAGCTAACTTGGC
GCCCCCACTAGTCCCATCGATGGctgaggagctcagggaactatgaaacagctgagaggaactgaggcacctaaaaatttaag
gtaccaagttgagcagccacaggagagcataggggacc

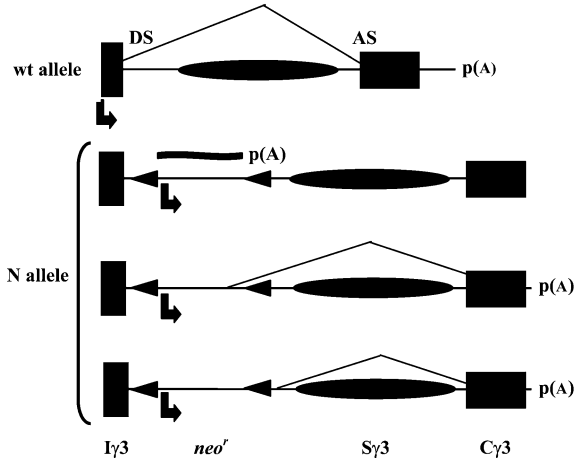
Cγ3 sequence

ctgtctgataacctcactcatcctcctatcttgc**agCTACAACAACAGCCCCATCTGTCTATCCCTTGGTCCCTGGCTGCAGT**
GACACATCTGGATCCTCGGTGACACTGGGATGCCTTGTCAAAGGCTACTTCCCTGAG

Neo-Cγ3 junction (1.1 kb)

ACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA
GGGGATCGGCAATAAAAAGACAGAATAAAACGCACGGGTGTTAGGTCGTTTGTTCGGATCAGCTTCCGATCATATTCAATAAC
CCTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTCGGTCTGAAGAGGAGTTTACGTCCAGCCAAGCTAACTTGGC
GCCCCCACTAGTCCCATCGATGGctgaggagctcagggaactatgaaacagctgagaggaactgaggcacctaaaaatttaag
CTACAACAACAGCCCCATCTGTCTATCCCTTGGTCCCTGGCTGCAGTGACACATCTGGATCCTCGGTGACACTGGGATGCCTT
GTCAAAGGCTACTTCCCTGAG

(D)



neo-coding sequence as a probe (Fig. 4C), though only in the undiluted sample from unstimulated splenocytes, which may be due to the basal activity of the *neo^r* gene promoter or to some pre-activated splenocytes. Quantification of the two signals using an Instant imager following hybridization with a *neo* probe revealed an equivalent abundance of the two transcript species (not shown).

Conversely, post-switch hybrid transcripts in the form of Iμ-Cx (x denoting any C gene after CSR) (31) were reduced in the case of Iμ-Cγ3 but not in the case of Iμ-Cγ2b in LPS-stimulated N/N mutant splenocytes (Fig. 4D) confirming the profiles seen by FACS and ELISA of supernatants from *in vitro* LPS-stimulated splenocytes (Figs 2 and 3).

Altogether, these results show that insertion of an expressed *neo^r* gene downstream of Iγ3 exon impairs transcription initiation from Iγ3 promoter, but not from Iμ located far upstream or from Iγ2b downstream of *γ3* gene. Instead, tk promoter is used to initiate germ line transcription which runs through the *neo^r* coding sequence, and at least in part through the Sγ3 and Cγ3 sequences.

neo^r insertion activates two cryptic splice sites

Previous work showed that although necessary, germ line transcription is not sufficient and processing of germ line transcripts is required to achieve normal levels of CSR (16, 19–21). In our mice, *neo^r* gene promoter was able to substitute for Iγ3 promoter. In addition, we consistently detected two cDNA species by RT–PCR using NeoATG and Cg3r primers. Therefore, in order to delineate the splice junctions between the *neo^r* and Cγ3 transcripts, we cloned and sequenced the two fragments from three independent PCRs. Surprisingly, analysis of the junction sequence in the shorter fragment (~0.8 kb) showed that a cryptic splice site had been activated within the coding sequence of the intronless *neo^r* gene, whereas in the larger fragment (~1.1 kb) another cryptic splice site, located in the intron sequence between *neo^r* insertion site and Sγ3, had been used. In both cases, the donor sites were normally spliced to the canonical Cγ3 splice acceptor site (Fig. 5A and C).

These results strengthen the idea that processing of germ line transcripts is a critical parameter in the molecular mechanisms leading from germ line transcription to CSR.

Discussion

Besides its use as a selectable marker, *neo^r* gene has been used as a tool to unravel the mechanisms underlying long-range interactions in complex loci. Of note, other selectable markers were also used (17, 21), but for the sake of coherence, we will keep on using the term *neo* effect as long as it is probably the promoter that counts in this effect and not the coding sequence of the selectable marker. It is noteworthy that, at least within the IgH locus, the *neo* effect does not seem to depend on the *neo^r* transcription orientation or on the widely used phosphoglycerate kinase (21, 23, 24, 28, 29) or tk (26, 32, this study) promoters driving *neo^r* gene expression.

The *neo* effect was shown to alter the effect of the 3′RR on I promoters (except maybe for Iγ1) located upstream but not downstream of *neo^r* insertion site (21, 29), indicating that the *neo^r* gene somehow disturbs the interaction between the 3′RR and I promoters at a distance that may exceed 100 kb. However, the replacement mutations described in these studies (21, 29) altered the structure of critical elements for germ line transcription (I promoter, I or C exons). This does not affect the interpretation of the phenotypes analyzed as far as intact upstream or downstream promoters (relative to *neo^r* insertion site) are concerned. For the replaced sequences, it remains uncertain whether the phenotype obtained results from the alteration of these elements themselves, from *neo* effect or from both. Therefore, it was interesting to analyze the *neo* effect by leaving intact all the necessary elements for germ line transcription and CSR. To this end, we inserted a *neo^r* cassette in the sense orientation between the Iγ3 exon and Sγ3 sequence. Both Iγ3 and Iγ2b promoters are LPS-responsive, thus germ line transcription of and CSR to *γ2b* gene (located downstream of *neo^r* insertion site) can be used as internal controls in LPS-mediated CSR to IgG3 and IgG2b.

We found that the LPS-inducible *neo^r* gene specifically impairs germ line transcription from the upstream Iγ3 promoter but not from the downstream Iγ2b promoter in agreement with previous findings (29). In addition, *neo^r* insertion in the IgH C locus has no effect on the upstream μ gene expression. It would be interesting to check if *neo^r* interference is confined to a chromatin domain (under the influence of the 3′RR) whose 5′ boundary lies within the large matrix attachment region upstream of the γ3 locus (33).

Fig. 5. Nucleotide sequence of Neo-Cγ3 hybrid cDNAs and localization of cryptic splice sites. (A) The short PCR fragment (846 bp) amplified with NeoATG and Cg3r primers was cloned and sequenced on both strands. Top: *neo^r* sequence derived from the amplified fragment is shown in capital letters, the stop codon and the two polyadenylation sites are underlined and the cryptic splice donor site in the coding region is shown in bold and underlined. Middle: Cγ3 sequences are shown in bold capital letters and the Iγ3-Cγ3 intron sequences in lower case letters and the splice acceptor site is shown in bold and underlined. Bottom: the junction between *neo^r* and Cγ3 sequences. (B) The use of a cryptic splice donor site within the coding sequence of *neo^r* gene normally spliced to the Cγ3 acceptor site leads to a Neo-Cγ3 open reading frame. *neo^r* nucleotide sequence is shown in capital letters and Cγ3 sequence is shown in lower case letters. neo^r amino acid sequence is shown in capital italic letters and Cγ3 amino acids are shown in bold capital letters. (C) The large PCR fragment (1145 bp) amplified with NeoATG and Cg3r primers was cloned and sequenced on both strands. Top: *neo^r* sequence is shown in capital letters, the stop codon and the two polyadenylation sites are underlined, the Iγ3-Cγ3 intron sequence downstream of the cassette is shown in lower case letters and the cryptic splice donor site is in bold and underlined. Middle: Cγ3 sequences are shown in bold capital letters and the Iγ3-Cγ3 intron sequences in lower case letters and the splice acceptor site is shown in bold and underlined. Bottom: the junction between *neo^r* and Cγ3 sequences. (D) Schemes of the transcript species in the wt and mutant γ3 loci (not to scale). In the wt locus (top), Iγ3 transcript exon is spliced to Cγ3 exons using canonical donor (DS) and acceptor (AS) splice sites. In the mutant locus, three transcript species were detected: the major normal *neo^r* transcripts using the *neo^r* polyadenylation sites p(A) (second line) and two minor *neo^r*-Cγ3 hybrid transcripts were detected when transcription could proceed through Sγ3 and Cγ3 (third and fourth lines). In the hybrid transcripts, two novel cryptic splice donor sites were activated, equally used and normally spliced to the canonical Cγ3 acceptor site. The arrows indicate the transcription orientation of the *neo^r* gene.

Previous work showed that germ line transcription *per se* is not sufficient for CSR and processing of germ line transcripts is a critical parameter in CSR (19–22, 30) but the mechanism remains unclear. Our results support these findings and show that in the absence of the canonical Iγ3 splice donor site, and within the sensitivity limits of our RT–PCR assay, two other cryptic splice sites were activated, and were equally used for splicing to the normal Cγ3 acceptor site. Cryptic splice sites are not detectably used in wt pre-mRNA, but are activated as a result of a mutation occurring most often at the canonical splice site. Surprisingly, one of the donor sites was activated within the *neo^r* coding sequence leading to a Neo-Cγ3 fusion protein. Whether the latter protein plays a role in any aspect of CSR to Cγ3 is presently unclear. The second cryptic site was located between *neo^r* gene and Sγ3 sequences, and therefore led to no fusion protein. The two Neo-Cγ3 hybrid transcripts occurred on the same allele since the corresponding cDNAs could be amplified in the heterozygous B cells. The activation of a cryptic splice site within the coding region of *neo^r* gene could explain the phenotype seen in mice in which most of the Iγ2b exon (including the splice donor site) was replaced by a *neo^r* gene leading to normal CSR to Cγ2b (18). However, we cannot exclude the possibility that the activation of the cryptic splice site within *neo^r* in our mice depends on the insertion site of the cassette, obviously on its transcription orientation and its distance from endogenous splice sites.

We found that *neo^r* gene could substitute for Iγ3 promoter for germ line transcription initiation and provided (or activated) splice sites, however, CSR to Cγ3 was still lower in LPS-activated mutant N/N splenic B cells than in their N/+ or +/+ counterparts. Although we cannot rule out other possibilities, one likely explanation is that most of the transcription process ends at the polyadenylation sites of the *neo^r* gene leading to normal *neo^r* transcripts, while some transcription runs through the whole *neo^r* gene and proceeds through the Sγ3 and Cγ3 sequences leading to hybrid Neo-Cγ3 transcripts. It could be that only the latter ones have bearing on CSR to Cγ3 since they will alone enable the formation of R loops and not normal *neo^r* transcripts that contain no Sγ3 sequences. This leaves open the question whether transcribed *neo^r* sequence itself forms an R loop. Alternatively, one might speculate that transcribed *neo^r* sequence somehow impairs CSR to Cγ3 through an alteration of Sγ3 chromatin or by generating a secondary (or higher order) structure that may impair access of CSR machinery to Sγ3 sequences. Although we cannot formally exclude this possibility, we think it unlikely, because we should then expect a more drastic decrease in CSR to Cγ3 due to the cumulative effect of both the decreased germ line transcription of Sγ3 sequences (most of transcription stops at *neo^r* polyadenylation sites) and the limited access of CSR machinery to Sγ3 sequences by the expressed *neo^r* gene. Clearly, this topic requires further investigations.

Within the IgH locus, *neo* effect seems to be associated with the LPS inducibility of the *neo^r* gene (24, 26, 32, 34, this study), a feature that is lost when the *neo^r* gene is inserted outside the IgH locus (24). Therefore, the LPS inducibility could have a predictive value in that it may enable delimitation of domains under the influence of Eμ (32) or of the 3′RR (21, 28, 29). It may thus be used to delineate the boundaries of the 3′RR activity required for CSR. Indeed, mutant mice bearing *neo^r* gene downstream of hs4 displayed no obvious alteration of CSR (34) strongly suggesting that the activity that enhances transcription from upstream germ line promoters is mainly effected by (a combination of) elements contained within the ~34 kb spanning hs3a, hs1-2, hs3b and hs4.

How the long-range interaction between the 3′RR and the I promoters takes place and how an LPS-inducible *neo^r* gene interferes with this interaction are presently unclear. Whether *neo* effect is exerted through alteration of local chromatin structure or through promoter 'competition' with specific upstream I promoters (29) awaits further analyses. In any case, we think it unlikely that competition or higher affinity of *neo^r* promoter to transcription factors recruited under the influence of the 3′RR can explain the available data. If it were the case, one might expect an alteration of germ line transcription from downstream as well as from upstream I promoters relative to *neo^r* insertion site (with the seemingly exception of Iγ1). Further, rather than through a strict looping model (35), the effect of the 3′RR on upstream I promoters may result from the propagation of a signal from the 3′RR (35, 36) that is somehow stopped by the expressed *neo^r* gene leading to an 'insulation' of upstream but not of downstream I promoters, except maybe for Iγ1 promoter which has additional regulatory sequences (37–40) that may enable a relative 3′RR-independent germ line transcription.

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Abbreviations

C	constant region
CSR	class switch recombination
IgH	Ig heavy chain
neo^r	neomycine resistance
3′RR	Ig heavy chain 3′ regulatory region
RT	reverse transcription
S	switch
tk	thymidine kinase
wt	wild type

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