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Interallelic Class Switch Recombination Contributes Significantly to Class Switching in Mouse B Cells

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Except for the expression of IgM and IgD, DNA recombination is constantly needed for the expression of other Ig classes and subclasses. The predominant path of class switch recombination (CSR) is intrachromosomal, and the looping-out and deletion model has been abundantly documented. However, switch regions also occasionally constitute convenient substrates for interchromosomal recombination, since it is noticeably the case in a number of chromosomal translocations causing oncogene deregulation in the course of lymphoma and myeloma. Although asymmetric accessibility of Ig alleles should theoretically limit its occurrence, interallelic CSR was shown to occur at low levels during IgA switching in rabbit, where the definition of allotypes within both V and C regions helped identify interchromosomally derived Ig. Thus, we wished to evaluate precisely interallelic CSR frequency in mouse B cells, by using a system in which only one allele (of b allotype) could express a functional VDJ region, whereas only interallelic CSR could restore expression of an excluded (a allotype) allele. In our study, we show that interchromosomal recombination of VH and Cy or Ca occurs in vivo in B cells at a frequency that makes a significant contribution to physiological class switching: trans-association of VH and Cy genes accounted for 7% of all a mRNA, and this frequency was about twice higher for the γ3 transcripts, despite the much shorter distance between the JH region and the Cy3 gene, thus confirming that this phenomenon corresponded to site-specific switching and not to random recombination between long homologous loci. The Journal of Immunology, 2005, 174: 6176–6183.

Although naive B cells express IgM plus IgD through alternative splicing of a single primary μ/δ H chain gene transcript, BCR-dependent activation in peripheral lymphoid organs turns on the expression of γ, α, or ε IgH chains with new effector functions. Once triggered by BCR ligation, class switch recombination (CSR) is also tuned by the type of T cell help (1). Germline transcription (GT) always precedes CSR to a given constant (C) gene in the same activated cells, and the induction of CSR by specific stimuli correlates directly with their ability to induce or suppress specific GT before CSR occurs (2). Gene-targeting studies demonstrated definitively that transcription from I promoters is a necessary prerequisite to CSR (3–6). Subsequent to GT of IgH C genes, repetitive switch (S) regions are the targets of the B cell-specific activation-induced deaminase, which attacks deoxycytidine residues and initiates DNA breaks on transcribed DNA (7). Due to the activity of other enzymes, including uracyl-DNA-glycosylase, exonuclease-1, and the nonhomologous end-joining machinery, paired S regions (transcribed at the same time on the same chromosome) then recombine and trigger productive expression of a new Ig C gene (8, 9). DNA circles, including the complete looped-out intervening sequences, may readily be detected as by-products in cells that actively undergo CSR (10–12).

Transcription of S regions from I promoters is under the control of various transcription factors, the expression of which is modulated by cytokines. In addition, most of these promoter elements undergo regulatory interactions with enhancers located at the 3’ end of the IgH locus, which altogether constitute the 3’ IgH regulatory region (13–15). Although physical interactions between I promoters and 3’ enhancers were not demonstrated directly, they were strongly postulated to occur in cis, according to either a linking or a looping model (14, 16). The 3’ cis-regulatory elements were indeed shown to recruit and/or direct histone acetyltransferase activities together with transcription factors and to induce chromatin remodeling of linked genes (17, 18). Regulatory elements may thus stimulate S region transcription and S region accessibility by counteracting a repressive chromatin structure generated by histone deacetylase-recruiting proteins such as LSF. Although it is now demonstrated that such changes in chromatin structure of transcriptionally active Ig genes require interactions among transcription factors, histones, and other cofactors to remodel and displace nucleosomes, it is not clear whether this remodeling occurs on one or both Ig alleles. Because only events occurring on the functionally expressed VDJ allele result in phenotypic changes and are thus positively selected, less is known about the status of the allelically excluded IgH locus. The recently demonstrated asymmetry of Ig alleles with regard to nuclear localization and to early vs late replication has been postulated to favor the occurrence of monoallelic V(D)J rearrangement (19–21). However, this asymmetric accessibility of Ig genes is unlikely to extend to the whole locus and, regarding CSR, it is in fact known for a long time that the silent allele also undergoes intrachromosomal recombination in up to
75% of switched cells (22). In addition, as a process targeted to transcribed S regions under the control of the cytokine milieu, CSR tends to involve the same C gene on both the productive and the nonproductive alleles of a given B cell, and it is difficult to ascertain in such conditions whether switching indeed occurred through intrachromosomal deletion or whether the recombined alleles are the products or reciprocal exchanges (23). It was also shown recently that B cells from heterozygous mice, in which only one Jμ allele was functional, underwent GT of the 3′-SS region at a similar rate on both alleles (24). Finally, all the necessary conditions for interchromosomal exchanges at target S regions are likely present in most stimulated B cells, and such exchanges may have been overlooked and underestimated by comparison to the prevalent cis-recombination model. Trans-recombination within the IgH locus has been previously documented only for the rabbit Eγ locus has been previously documented only for the rabbit Cγ genes and shown to account for 3–7% of rabbit IgA (25, 26). It was demonstrated in this model by characterizing the association of Sp/Sax from both IgH alleles that trans-association of Vμ and Cμ occurred at the DNA level during CSR and not during VDJ rearrangement (26). Because the situation of the multiple rabbit Cγ genes may have been peculiar, we wished to evaluate the frequency of interallelic CSR in mouse B cells by comparison to the canonical model of intrachromosomal deletion. To that goal, we generated a mouse strain in which one IgH allele was rendered nonfunctional by inserting of a frameshifted additional Vκx exon between Jμ4 and Eμ (fr-Vκ mutation). This mutation prevented the production of functional VDJ-Cμ mRNA without deleting any regulatory element of the locus; after cre-mediated deletion of the neo selection marker, the mutation did not introduce any exogenous regulatory element that could have modified the transcriptional status of the locus. In addition, this nonfunctional α allotype locus could not be rescued by secondary rearrangements, as would have been the case for an out-of-frame VμD1Jμ exon, so that fr-Vκfr-Vκ homozygous mice were unable to produce any detectable μ IgH and had no mature B cell. In heterozygous mutant animals with a wild-type allele from the “a” allotype IgH, and α H chains encoded by the mutated a allotype IgH locus.

Materials and Methods

Gene targeting

Plasmid pUC18-Jμ, containing a 6.1-kb EcoRI genomic fragment of the mouse IgH locus, was digested at a unique Nael site, where a cassette including an out-of-frame mouse Vκxexon (fr-Vκ exon) and a tk-promoter-driven neomycin-resistance gene (tk-neo) flanked by loxP sites was inserted (see map on Fig. 1A). The fr-Vκ exon had been designed with a +1 frameshift downstream of its 5′ acceptor splice site and another +1 frameshift upstream of its 3′ donor site. The construct was linearized before transfection by using the unique pUC-derived NdeI site. Cells of the embryonic stem (ES) cell line CK35 were transfected with the linearized vector by electroporation and selected using G418 (400 μg/ml). BamHI restriction and Southern blot analysis with a 3′ probe located outside of the construct (CH1μ probe) identified recombintants (see Fig. 1B). Two ES cell clones showing homologous recombination and fr-Vκ-neo insertion were injected into C57BL/6 blastocysts, and the resulting male chimeras were mated with C57BL/6 females. Germline transmission in heterozygous mutant mice was achieved by coat color, and the presence of the fr-Vκ-neo knockout IgH allele was checked by Southern blots of BamHI-restricted DNA and PCR. Mutant mice with the fr-Vκ-neo insertion were mated with Ella-cre transgenic mice (a kind gift from Dr. H. Westphal, used under a noncommercial research license agreement from DuPont Pharma (Wilmington, DE)) and yielded fr-Vκ mutant mice (see Fig. 1C). The progeny was checked by PCR (Nael PCR (32 cycles at 55°C), as shown in Fig. 1C) for the occurrence of cre-mediated deletion, and this was confirmed by Southern blot, yielding a 10.9-kb BamH1 band for the fr-Vκ-neo knockout and a 9.4-kb BamH1 band for the cre-deleted allele.

Cell culture

Splenocytes from 6- to 8-wk-old mice were activated in vitro in RPMI 1640 medium supplemented with 10% FCS and 20 μg/ml LPS from Salmonella typhi marium (Sigma-Aldrich) at a density of 10⁷ cells/ml. At day 3, aliquots of cells were removed to prepare RNA.

Lymphocytes from Peyer’s patches were isolated, and RNA was prepared.

Allotype-specific ELISA

Sera from wHfr-Vκ heterozygous mutant mice, BALB/c (wHfr-Wκ), and C57BL/6 (wHfr-Wκ) mice were analyzed for the presence of “a” allotype IgA (IgAa) by ELISA. All IgAa evaluations were performed in duplicate. ELISAs were performed in polycarbonate 96-multiwell plates (Maxisorb; Nunc), coated overnight at 4°C (100 μl/well) with 0.5 mg/ml monoclonal for IgAa capture Abs (BD Pharmingen) diluted in 0.05 M sodium bicarbonate buffer (2 μg/ml). After blocking and washing steps, 50 μl of sera (first diluted to 1/50) or isotypic standard IgAa (BD Pharmingen) were added. After washing, 100 μl/well alkaline phosphatase-conjugated goat antiserum specific for mouse IgA class (Southern Biotechnology Associates) diluted in 0.1% Tween 20PBS at 2 μg/ml was added and adsorbed during 1.5 h at 37°C. After washing, alkaline phosphatase activity was assayed on 1 mg/ml alkaline phosphatase substrate (Sigma-Aldrich) and blocked with addition of 3 M NaOH; optic density was measured at 405 nm in a Spectra-trac photometer (Packard).

Flow cytometry analysis

Single-cell suspensions from peripheral blood or lymphoid tissues were washed in PBS/5% FCS and stained (5 × 10⁶ cells/assay) with various Abs as follows: anti-B220 conjugated with SpectralRed; anti-c-Kit, anti-CD43 (BD Biosciences), and anti-CD25 (Beckman Coulter) conjugated with PE; and anti-IgM conjugated with FITC (Jackson ImmunoResearch Laboratories).

For the analysis of IgM allotypes, splenocytes were labeled using PE-conjugated anti-IgM and FITC-conjugated anti-IgM* (Southern Biotechnology Associates) before LPS cell stimulation. Data were acquired on 1.5 × 10⁶ viable cells using a Coulter XL apparatus (Beckman Coulter).

Confocal microscopy

For the analysis of IgAa-producing cells, splenocytes of Peyer’s patch cells were fixed with paraformaldehyde and cold methanol and then simultaneously labeled with FITC-conjugated anti-IgA and biotin-conjugated anti-IgA (Southern Biotechnology Associates). After three washes, followed by incubation with cyanin 5-conjugated streptavidin (Southern Biotechnology Associates), and another three washes, slides were analyzed using a LSM510 confocal microscope (Zeiss). To determine the percentage of a allotype expression in fr-Vκ+ mice, >100 IgA-secreting cells were counted. The assay was carried out on five independent heterozygous mice and live control a/a and b/b mice.

Transcript analysis

For RT-PCR, total bone marrow RNA was used as template. For VDJ mature transcript analysis, primers were Vρ1183 and Cmr (Cμ reverse, complementary to the Cμ CH1 exon) (32 cycles at 55°C). For fr-Vκ transcript analysis, the primers used were 5′fr-Vκ (a forward primer internal to the knockin Vκ exons and Cmr) (32 cycles at 55°C).

Sequences of primers were as follows: 5′ Nael, 5′TCAGGTTAAGA ATGGCCTCTCC-3′; 3′ Nael, 5′TTCAAAGGTCTGTGAGATCCC-3′; Vρ1183, 5′CGTACCAAGAASAMCMCTTGCWGAAAATGASC-3′; CH1μ (5′GATTCCTGGACTCGACCTGGTTGTTAATGACGTCGTCCTTTGGTCTCT-3′; CHω, 5′GGCAC GTTCTCAAGGTCGTCGTCG-3′; Cmr, 5′GGAACATTGGGAGGACT GACT-3′; and 5′fr-Vκ, 5′CTGTAGGCTGGAGGCGGCTACGG-3′ (with degenerate positions coded as follows: S = C or G, M = A or C, W = A or T).

Total RNA was treated with DNase I (Invitrogen Life Technologies) according to the supplier’s instructions. Treated RNA (1 μg) was reverse transcribed by addition of reverse transcriptase (Invitrogen Life Technologies) and PCR-amplified. For Ig α-chain transcripts, amplification was performed for 32 cycles; hybridization was at 57°C, and the primers were Vρ1183 and CHar. For γ-chain transcripts, amplification was performed for 32 cycles; hybridization was at 61°C, and the primers used were Vρ1183 and CHγ1rev. Amplified products were cloned into the TOPO 1 vector (Invitrogen Life Technologies) and sequenced or analyzed by restriction.
Allotype assignment of cloned PCR fragments

A PCR was performed on each TOPO 1 vector using the same primers as those used to amplify α or γ3 transcripts. PCR products were further digested by SstI for α transcripts and PstI for γ3 transcripts to determine allotypes.

To confirm allotypes, cloned fragments were sequenced from both ends using the M13Reverse and M13(-20) forward primers and an automated sequencer (Applied Biosystems).

Results

Insertion of a mutated Vk exon between Jμ4 and Eμ and generation of mutant animals

To create a nonfunctional IgH allele without deleting any regulatory element, and in the absence of any inserted non-Ig sequence, we designed an IgH mutation by which the locus was rendered nonfunctional through the insertion of an out-of-frame supplementary fr-Vκ exon. To that goal, mouse ES cell clones were generated by the insertion between Jμ4 and Eμ of a cassette, including a frameshift-mutated and promoterless Vk exon and a floxed neo gene (Fig. 1A). After injection into blastocysts, germline transmission of the mutation, and in vivo deletion of the neo gene, a stable mouse line carrying the fr-Vκ insertion was obtained (Fig. 1, B and C). Homozygous animals were derived and bred with C57BL/6 mice to yield heterozygous animals carrying a wild-type C57BL/6 IgH locus from the b allotype, together with a mutant allele from the 129-Ola background, i.e., from the a allotype.

B cell development in homozygous mutant fr-Vκfr-Vκ animals

Pathological studies of tissues from homozygous animals and their littermates were conducted. Spleens of reduced size devoid of any germinal centers and the absence of Peyer’s patches were noticed in fr-Vκfr-Vκ homozygous animals. When bone marrow from fr-Vκfr-Vκ mice was checked by flow cytometry, double staining with anti-B220 and anti-μ Abs showed the lack of surface IgM+ B cells (Fig. 2A). B cells were also absent from bone marrow and spleen (Fig. 2, B and C). Bone marrow featured an expanded B220+, CD43+, CD25−, surface IgM+ pro-B compartment, whereas the B220+, CD43−, CD25+ pre-B cell compartment was absent (Fig. 2A and data not shown). Accordingly, a complete serum Ig deficiency appeared in the fr-Vκfr-Vκ homozygous strain (data not shown).

Nonfunctional VDJ mature transcripts including the supplementary fr-Vκ exon are detectable in both homozygous and heterozygous mice

Although normal-size VDJ-Cμ transcripts were undetectable by RT-PCR in bone marrow cells from homozygous mutant animals, abnormal, enlarged transcripts including the additional Vk exon (VDJ-Vκ-Cμ transcripts) were detected in bone marrow from both homozygous and heterozygous mice (Fig. 3A). Although the knockin Vk exon carried frameshifts at both its 5’ and 3’ splice site, neither in-frame nor out-of-frame VDJ junctions allowed in-frame translation of Cμ, so that there was no way of synthesis of any H chain with a functional Cμ region (in agreement with the B-less phenotype of homozygous animals).

IgM expression in heterozygous fr-Vκa/+ animals

Although the mutated fr-Vκ was expected to result in premature termination of IgH transcripts whatever the reading frame of the VDJ exon, we verified that heterozygous a/b allotype animals with a mutant IgHb allele possessed B cells with only b allotype membrane IgM and no expression of IgMα (whereas control wild-type a/b C57BL/6 × 129 mice simultaneously carried cells that expressed either IgMα or IgMβ allotype) (Fig. 3B). In heterozygous mutant animals, the null expression of the IgMα allotype also indicated that the blocking effect of the fr-Vκ mutation was not rescued by gene conversion between Cμ genes, random interallelic homologous recombination, nor by trans-splicing between mature or germline Cμ transcripts originating from both alleles.

High-frequency interallelic recombination during α and γ3 isotype switching

To examine interallelic recombination during switching to the α and γ3 isotypes in heterozygous fr-Vκ+/ B cells, we amplified H chain mRNAs from Peyer’s patches and LPS-stimulated splenocytes by RT-PCR and studied individual cDNA clones for the association of functional VμH genes with either the Ca or Cγ3 H chain exons. The quantitation of interallelic recombination was then conducted by DNA digestion to determine for each cDNA clone its C region allotype. VμH-Cα or VγH-Cγ3 cDNA clones could readily be isolated, the C regions of which originated from either the a or the b allotype (Fig. 4). Mouse Ca genes display a single nucleotide polymorphism, which generates a SstI restriction site on CμH1α for only the b allotype (Fig. 5A). We found by digestion of

FIGURE 1. Gene targeting. A, Map of the IgH DQ52/HICμ region in its germline configuration and after frameshift Vk-neo knockin followed with cre-lox recombination; probes for Southern blotting are shown (restriction sites as follows: B, BamHI; N, Nael; Bg, BgII; the arrow indicates the transcriptional orientation of neo). B, Southern blot of knockin ES cells carrying the Vk-neo insertion. The CH1 μ 3’ probe detects a 9.0-kb BamHI wt band and a 10.9-kb band from the targeted locus. C, PCR analysis of mice with germline transmission of the knockin Vk gene.
120 clones (Fig. 5B) obtained from two mice that 7.0 ± 2.0% of VDJ-Cγ sequences were from the α allotype and were thus transcribed from an interchromosomally recombined locus (Fig. 4). Single-nucleotide polymorphism sequencing confirmed allotype assignment beyond the unique SstI site for all α allotype clones.

Accordingly, digestion of the mixed-PCR products from a VH7183/CH3 amplification carried on polyclonal fr-VH/CH3 B cells, detected not only the main band corresponding to the wild-type cis-recombined α allotype locus (307-bp fragment) but also a significant proportion of a band with the size of the α allotype (510-bp) fragment (Fig. 5C).

The murine Cγ3 gene displays a polymorphism generating an additional PstI restriction site on CH1 from the α allotype (Fig. 6A). Restriction of α allotype PCR-amplified clones by PstI yielded two fragments of 330 and 117 bp due to the presence of a single PstI site (Fig. 6B). By contrast, the additional α allotype-specific PstI site yielded additional fragments of 195 and 128 bp (due to the cleavage of the α allotype-specific 330-bp fragment) (Fig. 6B). Digestion of 120 independent clones (Fig. 6B) obtained from two mice revealed that 17.95 ± 1.15% of VDJ-Cγ3 transcripts were from the α allotype and thus witnessed interallelic recombination (Fig. 4). This percentage was checked by sequencing all α allotype clones. Accordingly, digesting the mixed VH7183/CH3 PCR product amplified from polyclonal heterozygous fr-VH/CH3 B cells confirmed the predominance of the α allotype restriction pattern together with additional fragments fitting with the expected α allotype pattern (Fig. 6C).

**FIGURE 2.** B cell development in mutant animals. Freshly isolated bone marrow (A and B) and spleen (C) cells were isolated and stained with fluorescently labeled Abs. Representative results from wt/wt and fr-VH/CH3 animals are shown.

**FIGURE 3.** A, Analysis of Cγ3 gene transcription. Total RNA was isolated from wt/wt, fr-VH/CH3, and fr-VH/CH3 bone marrow cells. RT-PCR assessed the presence or absence of VDJ mature transcripts and VH transcripts. Amplification was performed in semiquantitative conditions by using serial cDNA dilutions as templates. B, IgM but not IgMa expression in mutant heterozygous animals. Freshly isolated spleen cells were stained with fluorescently labeled Abs. Representative data from wt/wt α and wt/α animals are shown.

**FIGURE 4.** Production of IgAa serum Igs by fr-VH+ mice

As expected, IgAa serum Igs were undetectable in control C57BL/6 mice (wt/wt) but were present at high levels in (wt/wt) BALB/c mice (792.8 ± 115.7 μg/ml). A lower, but significant, level of a allotype serum IgA was also detectable in fr-VH+ mice (148.3 ± 99 μg/ml) (Fig. 7).
Confocal microscopy study of IgAa plasmocytes from Peyer’s patches

Confocal microscopic analysis clearly demonstrated the presence of some IgAa-producing plasmocytes in fr-V/H9260/H11001 mice, whereas controls showed their absence in C57BL/6 mice (wtb/wtb) and the uniform IgAa staining of all IgA-secreting plasmocytes from BALB/c mice (wta/wta) (Fig. 8). The percentage of cells bearing IgAa in fr-V/H9260/H11001 was 5.9%, i.e., fell in agreement with the percentage of "a" allotype transcripts obtained in the RT-PCR study.

Discussion

The vast majority of class-switched IgH chains are derived from V<sub>H</sub> and CH genes joined through cis-recombination. This deleterious pathway of gene rearrangement involves intrachromosomal recombination between S regions located on the same chromosome and is accompanied by the looping-out of intervening DNA.

FIGURE 4. Percentages of cis- and trans-chromosomal recombination during isotype switching to Ca and Cy3. Data are expressed as the mean numbers (±SEM) of clones corresponding to the a or b allotype; independent cDNA clones were obtained from two different wt<sub>b</sub>/fr-V<sub>a</sub> mice and were analyzed by restriction and sequencing.

FIGURE 5. Ca region polymorphism. Total RNA was isolated from Peyer’s patches. A, Location of V<sub>H</sub>/CH<sub>a</sub> RT-PCR amplified fragment. Changes in the nucleotide sequence of the a vs b allotypes are indicated in bold. The S<sub>r</sub>I restriction sequence used for allotype determination is underlined. B, S<sub>r</sub>I-digested V<sub>H</sub>/CH<sub>a</sub> PCR products from different clones were resolved on 2% ethidium bromide-stained agarose gel. C, Total V<sub>H</sub>/CH<sub>a</sub> PCR products from wt<sub>b</sub>/fr-V<sub>a</sub>, BALB/c (wt<sub>a</sub>/wt<sub>a</sub>), and C57BL/6 (wt<sub>a</sub>/wt<sub>b</sub>) were digested by S<sub>r</sub>I and resolved on 2% ethidium bromide-stained agarose gel. The location of the a allotype-specific band in amplified products from heterozygous cells is indicated by an arrow.

FIGURE 6. Cy3 region polymorphism. Total RNA was isolated from LPS-stimulated splenocytes. A, Location of V<sub>H</sub>/CH<sub>endrev</sub> RT-PCR-amplified fragment. The P<sub>s</sub>I restriction sequence used for allotype assignment is underlined. B, P<sub>s</sub>I-digested V<sub>H</sub>/CH<sub>endrev</sub> PCR products from different clones were resolved on 2% ethidium bromide-stained agarose gel. C, Total V<sub>H</sub>/CH<sub>endrev</sub> PCR products from wt<sub>b</sub>/fr-V<sub>a</sub>, BALB/c (wt<sub>a</sub>/wt<sub>a</sub>), and C57BL/6 (wt<sub>a</sub>/wt<sub>b</sub>) were digested by P<sub>s</sub>I and resolved on 2% ethidium bromide-stained agarose gel. The location of the a allotype-specific band in amplified products from heterozygous cells is indicated by an arrow.

Confocal microscopy study of IgAa plasmocytes from Peyer’s patches

Confocal microscopic analysis clearly demonstrated the presence of some IgAa-producing plasmocytes in fr-V<sub>a</sub>/+ mice, whereas controls showed their absence in C57BL/6 mice (wt<sub>a</sub>/ wt<sub>b</sub>) and the uniform IgAa staining of all IgA-secreting plasmocytes from BALB/c mice (wt<sub>a</sub>/wt<sub>a</sub>) (Fig. 8). The percentage of cells bearing IgAa in fr-V<sub>a</sub>/+ was 5.9%, i.e., fell in agreement with the percentage of "a" allotype transcripts obtained in the RT-PCR study.

Discussion

The vast majority of class-switched IgH chains are derived from V<sub>H</sub> and CH genes joined through cis-recombination. This deleterious pathway of gene rearrangement involves intrachromosomal recombination between S regions located on the same chromosome and is accompanied by the looping-out of intervening DNA.
Reciprocal exchange between sister chromatids was also postulated to account for CSR events, then giving rise to one daughter cell carrying a class-switched expressed IgH locus and the other with an unswitched expressed C\textsubscript{\mu} gene, followed with a partial duplication of the C region, but such events are in fact very rare, if any (27). A third model that may account for the expression of non-IgM isotypes is trans-splicing. Many examples of trans-splicing are found among protozoans and lower invertebrates, and it has been shown that leader RNAs from nematodes and trypanosomes can be trans-spliced in mammalian cells (28, 29). Trans-splicing between mature VDJ-C\textsubscript{\mu} and germline I\textsubscript{\alpha}-C\textsubscript{\alpha} transcripts was thus suggested to account for some observations of simultaneous multiple isotype expression of Ig in individual B lymphocytes (30, 31). Although trans-splicing was also suggested for the processing of some rabbit \textalpha\textsubscript{\mu} transcripts, sequencing CSR breakpoints in these cases demonstrated that trans-association of V\textsubscript{\mu}H and C\textsubscript{\alpha} in fact occurred through trans-chromosomal recombination (26). Finally, although extensively looked for, trans-splicing was never demonstrated for Ig transcripts and probably has little role in B cells, if any. In this study, we report about an allelically excluded IgH locus yielding nonfunctional frameshifted transcripts but in which the C\textsubscript{\mu} gene was left intact and could normally undergo GT. The null expression of a allotype IgM in a/b fr-V\textalpha+/+ heterozygous mice with an inactivated IgH\textalpha locus also shows in this case that

**FIGURE 7.** ELISA analysis of IgA\textalpha serum levels in 8-wk-old mice. Data from C57BL/6 (wt\textsuperscript{b}/wt\textsuperscript{b}), wt\textsuperscript{b}/fr-V\textalpha\textsuperscript{a}, and BALB/c (wt\textsuperscript{a}/wt\textsuperscript{a}) mice are shown. Mean Ig levels from all experiments (±SEM) are indicated in italics.

**FIGURE 8.** A, Confocal microscopy study of IgA\textalpha plasmocytes from Peyer’s patches. Peyer’s patches were isolated from wt\textsuperscript{b}/fr-V\textalpha, BALB/c (wt\textsuperscript{a}/wt\textsuperscript{a}), and C57BL/6 (wt\textsuperscript{b}/wt\textsuperscript{b}), and double-stained with antisera specific for all mouse IgA or for only IgA\textalpha. B, Percentage of IgA\textalpha plasmocytes in wt\textsuperscript{b}/fr-V\textalpha, BALB/c (wt\textsuperscript{a}/wt\textsuperscript{a}), and C57BL/6 (wt\textsuperscript{b}/wt\textsuperscript{b}).
trans-splicing between mature, functional Cα and germline Igα-Cα transcripts originating from each allele does not occur.

A fourth CSR model involves interallelic recombination and was documented only for rabbit α genes, thanks to the existence of allotypes in both H chain V and C domains (25, 26). However, the situation of the extensively duplicated rabbit allotypes in both H chain V and C domains (25, 26). However, the situation of the extensively duplicated rabbit α genes (up to 13 copies) may be singular, and their disposition as an array of genes at the end of the locus may increase the likelihood of recombination. Interestingly, alternative trans-CSR could account for the re-expression of an otherwise silent or allelically excluded allele. To study such events in the mouse, where there are no allelotype makers of V regions that can be used with serologic methods, we generated mice with heterozygous a/b IgH C genes but in which the disrupted a allele locus was engineered so as to be allelically excluded. To avoid any modification of chromatin accessibility within this artificially excluded locus, as might have resulted from cis-linked within the productive IgH locus.

FIGURE 9. Models for CSR. The major CSR pathway results in the linkage of the VDJ region to one of the C gene (Cs) located downstream of Cµ on the cis-recombination of Sµ/Sx. This model implies a looping-out and deletion, which often happens on both the productive and the silent allele. The alternative CSR pathway consists in the trans-association of Sµ/Sx and may also be associated with the looping-out and deletion of the intervening sequence that includes Cµ and other intervening CH genes on both alleles.

VDJ genes with Cγ3 and Cα C genes from the α allotype. The occurrence of interchromosomal CSR was confirmed at the protein level by the presence of IgA+ serum IgGs detected by ELISA in heterozygous mutant animals and of IgA+ plasmocytes observed by confocal microscopy.

This model demonstrates the frequent occurrence in murine B cells of interchromosomal CSR events that restore expression of the allelically excluded IgH locus for the production of class-switched Abs. In our study, we showed that interallelic recombination of V and Cγ or Cα occurs in vivo in normal B cells at frequencies that make a significant contribution to physiological class switching, because 6–7% of α mRNA originated from such events. This frequency is thus similar to what was reported previously for rabbit α genes (26). In addition, our study indicated that trans-association of Vδ and Cγ3 occurred in 17% of total γ3 mRNA tested, i.e., at a frequency that was higher for the Cγ3 gene than for Cα despite the shorter distance between the Jδ5 region and Cγ3.

Checking trans-CSR for genes located at both ends of the IgH C gene cluster and observing that it does not increase with the distance between Cµ and its switching partner indicates that trans-CSR does not merely rely on homologous recombination. Although the frequency of interallelic homologous recombination should increase with the distance and would be expected as very low for genes lying within a <300-kb (~0.3 cM) interval, interallelic CSR rather appears as a targeted process involving S regions and the CSR machinery (Fig. 9).

The occurrence of such events was not evaluated previously in the mouse between endogenous alleles, but a previous study in VDJ-Cµ transgenic mice also reported interchromosomally derived IgH chains associating transgenic VDJ segments with an endogenous Cγ3 gene (32). In the pathology, interchromosomal recombination between S regions and oncogenes have also been reported in multiple instances (33).

In general, interchromosomal recombination in somatic cells is rare. The high frequency of interallelic recombination in the IgH locus might reflect the frequent occurrence of ssDNA or dsDNA breaks on both alleles during isotype switching, exposing them simultaneously to three possibilities as follows: short internal S-region deletions, cis-CSR, and trans-CSR (26). Interestingly, although dsDNA breaks also occur on both alleles during VDJ recombination, interallelic recombination was not found at this stage (23, 26, 34). It has been shown for VDJ recombination that DNA cleavage is followed by the formation of a limited number (usually one or two) of nuclear foci containing histone γH2AX and proteins involved in chromosome repair (35). It is tempting to speculate that, in the case of VDJ recombination, DNA breaks would rather be repaired independently on each allele, whereas in the case of CSR, a single nuclear location may attract the transcribed S regions from both alleles and concentrate the activities of either activation-induced deaminase and the CSR machinery and/or of repair enzymes, thereby allowing the resolution of double-strand breaks through either cis-CSR or trans-CSR (Fig. 9).

The frequent occurrence of trans-recombination during isotype switching is also reminiscent of the high incidence of murine and human B lineage malignancies (multiple myeloma, Burkitt’s lymphoma, large B cell lymphoma, plasmacytoma, and so on) that arise from various chromosomal translocations linking a S region from the IgH locus to oncogenes such as c-myc, bcl-2, bcl-3, bcl-6, or c-maf, and so on (33, 36). Thus, it confirms the likely ability of the CSR machinery to recruit in a single nuclear specialized compartment various transcribed genes, and not only those which are cis-linked within the productive IgH locus.
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References