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Deletion of the DQ52 Element Within the Ig Heavy Chain Locus Leads to a Selective Reduction in VDJ Recombination and Altered D Gene Usage

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The process of V(D)J recombination that leads to the assembly of Ig gene segments is tightly controlled during B cell differentiation. Two germline transcripts, one of which (μ0) originates from the promoter region of DQ52, may control the accessibility of the heavy chain locus. Here, we present the analysis of a mouse line in which the DQ52 gene together with its regulatory sequences is deleted by a Cre/loxP-based strategy. In F1 (DQ52−/−) mice, the use of the JH3 and JH4 elements in DJ or VDJ junctions of the DQ52− allele was strongly reduced in both the bone marrow pre-B and spleen cells, while the JH1 and JH2 elements were used with normal frequencies. In addition, IgM B cells of bone marrow and spleen used the DQ52− allele less frequently. On DJ joints of the DQ52− allele, there was 2 times less processing of JH3 ends, which resulted in clearly increased addition of P nucleotides. Although the use of D elements in DJ joints was quite similar, an altered D repertoire was found in VDJ joints of the DQ52− allele. In splenic B cells of the DQ52−/− mouse the amino acid distribution of the CDR3 was skewed, probably to compensate for the altered processing of JH3 ends. Thus, we have shown an interesting selective effect of the DQ52 region on controlling accessibility to 3′ JH elements on the Ig locus, which also seems to influence the processing of DJ joints. We propose a model in which the DQ52 promoter region enhances the induction of secondary DJ rearrangements.


The differentiation of B lymphocytes from committed precursor cells to Ab-secreting plasma cells proceeds through multiple steps that are defined by changes in the gene expression pattern and the assembly of Ig variable gene segments (V, D, and J). These events are strictly regulated in a tissue-, lineage-, and stage-specific fashion (1–4). The assembly of Ig gene segments by a site-specific recombination mechanism, referred to as V(D)J recombination, is initiated at the H chain gene locus, first by rearrangement of a DH to a JH segment on both chromosomes, followed by joining of a VH gene segment to the DJ complex. Assembly of the L chain genes, involving joining of a VL to a JL segment in one of the two light gene families, generally follows productive assembly and expression of the IgH chain (for review, see Ref. 2).

Based on the assumption that the V(D)J recombination machinery is active throughout early lymphocyte development, the availability of gene segments to the recombine in various Ig loci, a concept referred to as accessibility, might be a key factor in controlling the rearrangement process (5, 6). Previous studies have suggested that regulation of rearrangement is affected by cis-acting DNA elements that target the recombine activity to the gene segments. Among the candidate regulatory elements, promoters and enhancers involved in transcriptional control of the IgH locus are of particular interest (5, 6). For example, in mice lacking the IgH intron enhancer, the efficiency of the initial DH to JH rearrangement is only marginally affected, while the VH to DJ joining is practically absent. These data predict the existence of an additional cis-regulatory element(s), different from the IgH intron enhancer, which is particularly important for D to J rearrangement (7, 8).

A candidate element for such a regulatory role has been described by Kottmann et al. (9). This gene element has been mapped in the DQ52 locus, in the sequence flanking the most JH proximal D gene, DQ52. It has been shown to be a complex genetic element that interacts with several DNA binding proteins and is transcriptionally active in B cells, as measured by transient transfection assays (10). Additional experiments have revealed that one of the DNA binding proteins interacting with the DQ52 locus is PAX-5 (T. Tallone et al., manuscript in preparation). Several other features also suggest a special role for the DQ52 locus in the primary assembly of IgH genes.

1) The DQ52 element is preferentially used, together with DFL16.1, in early or primary D-J rearrangements (11–13). Interestingly, because the initial gene rearrangement events are not entirely lineage restricted, inappropriate rearrangement of IgH gene in thymocytes at an intermediate stage of maturation can occur (∼30–50% of peripheral T cells have DH to JH joins). Surprisingly, DQ52 is involved in these inappropriate early rearrangements with unexpectedly high frequency (14–16).

2) DQ52 is the only DH element to be transcribed very early before the onset of the V(D)J recombination (17, 18). These transcripts, called μ0 germline transcripts (19) can be considered one of the earliest indications of B-lineage commitment (18) and appear to originate, in both mouse and human, from a region immediately 5′ to the DQ52 element (17, 20) (T. Tallone et al., manuscript in preparation). Thus, it is possible that the DQ52-JH region of the IgH locus might become preferentially accessible as a consequence of early events underlying commitment to the lymphoid/B cell lineages.

3) The genomic organization of the DQ52...
locus is highly conserved between species (the position, immediately 5′ of the JH segments), and the sequence around the DQ52 element shows high interspecies homology but low homology with other D elements within the same species (21).

To test the role of the DQ52 locus in the regulation of VDJ recombination and B cell development, we have generated mutant mice lacking the DQ52 gene with its regulatory sequences. We demonstrate here that deletion of the DQ52 region results in selective reduction of VDJ recombination, incomplete processing of D-J joints and altered D gene usage.

Materials and Methods

DQ52 gene localization

The DNA containing the DQ52 element, the JH cluster, and the intron enhancer was isolated from plasmids pMC184-8 (22) and pSV2gmpVDI-Cα (23). The Xhol-SacI fragment containing the DQ52 element with its regulatory sequences (10) (T. Tallone et al., in preparation) was replaced by a cassette consisting of a neomycin resistance gene and a HSV-tk gene flanked by loxP elements (24). The wild-type sequence was further modified by exchanging a HindIII site in the intron between JH3 and JH4 to an EcoRV site by the ligation of a short oligo-nucleotide linker (Fig. 1B). The modified wild-type sequence was further modified by exchanging a XhoI site in the EcoRV site for a VQ52 (5′-AGGGATCCTTGTGAAGGGATCTACTACTGTGACGATGTTG) called V5.2 primer pairs (Fig. 2D): first pair, DFL16.1/REC/EXT (5′-GCTGGCACAAGTTGCTGCGGCGCAATAAGTGA) and JH3/INT (5′-ACGTTCCTGAGAGCTACGCTAACCAGTACGTTTG), and second pair, DFL16.1/REC/INT (5′-GTTTTCGTCTGAGATATATCAGATCAGCTCAGTACGTTG), and DFL16.1/REC primers are specific to DFL16.1 but have only 2–3 bp mismatches to most members of the DSP2 family (33). PCR products were purified with Quick Spin columns (Qiagen, Chatsworth, CA), and 1/10th of the purified reaction was digested with either EcoRV or HindIII. The digested PCR products were fractionated on a gel and blotted to a filter, which was hybridized with the 5′-labeled oligonucleotide probe JH3 (5′-TCTCCACTTACCTAATACGTACTACGTTG) by standard procedures. To confirm that the PCR products were fully digested with EcoRV, the filters were washed and rehybridized with an oligonucleotide probe, EcoRV/KO (5′-CAGAACTGAAGCTCCTCCAGTCCCTCGAGGAG) that is specific for the introduced EcoRV modification on the targeted allele.

Cloning of the PCR products for sequencing

Bone marrow cell lysates or spleen DNA samples were used to amplify PCR products as described above with either D509 or V558 and 3′ JH4 (5′-AACTTGCAGAGGCTCACCGTACCTCCGGAGGAG) primers. Twenty-five microliters of each PCR was electrophoretically fractionated, bands corresponding to JH3 rearrangements were cut out from the gel, and the DNA was eluted. One microliter of eluted DNA was amplified with either D509 or V558, as in the primary reaction, and JH/KO primers. PCR products were cloned into plasmids, and nucleotide sequencing was performed using the automated DNA Sequencer A.L.F. using an Auto Read Kit supplied by Pharmacia Biotech (Piscataway, NJ).

Generation of Abelson murine leukemia virus (Ab-MuLV)-transformed pro-B cell lines

For generation of Ab-MuLV-transformed cell lines, DQ52+/− mice were crossed onto a RAG−/− background (34). Bone marrow cells were isolated from femurs of DQ52+/−/RAG−/− mice and transformed as previously described (35). The Ab-MuLV-transformed cells obtained were classified by flow cytometry as pro-B because they were surface positive for the B-lineage markers B220 and CD43 (data not shown).

Preparation of a probe of the mutated DQ52 locus and S1 nuclease protection assay

Genomic DNA from the liver of DQ52−/− mice was prepared, and a PCR fragment was amplified with 5′ KO1 and DQ/KO3.1 (5′-TCTTCGACCTTACGAAACTCTAC) primers. A KpnI/BglII subfragment from this PCR product was cloned into a plasmid vector and sequenced. Subsequently, a BglII/BglII fragment 3′ of DQ52 was added to the plasmid. A KpnI/PstI insert (containing the mutated DQ52 locus) was purified and used for the generation of a radiolabeled DNA probe (see Fig. 7A). The denatured plasmid fragment was hybridized with the ST3 oligonucleotide and radio-labeled with Klenow DNA polymerase (Pharmacia). The radiolabeled probe was then purified on a polyacrylamide gel by excision of the appropriate insert (containing the mutated DQ52 locus) was purified and used for the generation of a radiolabeled DNA probe (see Fig. 7A). The denatured plasmid fragment was hybridized with the ST3 oligonucleotide and radio-labeled with Klenow DNA polymerase (Pharmacia). The radiolabeled probe was then purified on a polyacrylamide gel by excision of the appropriate sized fragment. The labeled ssDNA was eluted with the Stratagene Elution Howe, 1993). Bone marrow cell lysates were transfected with the linearized targeting vector as previously described (26) and screened with nested PCR using the primers 5′-GATCTTCGACCTTACGAAACTCTAG and OLNeo5 (5′-GCGATGTTCTTGTGGCC; first round), and 5′KO5 (5′-GAGCAGAGAATGATAGTAGTTGG) and OLNeo2A (5′-CCAGGAACCTCCTGCAATG; second round). Targeted ES cell clones were transfected with 20 μg of supercoiled pMC-CreN, a modified version of the pMC-Cre expression vector (27), and selected with ganciclovir for loss of the selection cassettes by site-specific recombination as previously described (28). The DQ52-deleted ES cell clones were injected into C57BL/6 blastocysts to obtain chimeric mice as described (26). Single-cell suspensions of spleen and bone marrow (BM) were prepared according to standard procedures and analyzed by flow cytometry. Cells were stained with the following mAb: anti-I−α (5′KO5; 29), anti-Iα, AF6-78.25 (30), 5′-TCAACATTCTTGCCCTCTTTAATACGTACGTTG, and 5′-GAGGATCCTTGTGAAGGGATCTACTACTGTGACGATGTTG) by standard procedures. To confirm that the PCR products were fully digested with EcoRV, the filters were washed and rehybridized with an oligo-nucleotide probe, EcoRV/KO (5′-CAGAACTGAAGCTCCTCCAGTCCCTCGAGGAG), that is specific for the introduced EcoRV modification on the targeted allele.

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Results

Deletion of the DQ52 locus

To study the in vivo function of the murine DQ52 gene in conjunction with its cis-acting regulatory elements, the DQ52 region was deleted by gene targeting. The targeting vector was designed so that a 240-bp Xhol-SacI fragment containing the D segment as well as all the described flanking sequences with promoter and/or protein binding activities (10) (T. Tallone et al., manuscript in preparation) was replaced by neomycin resistance and HSV-tk cassettes (Fig. 1A). Two loxP sites from the bacteriophage P1 flanked

3 Abbreviations used in this paper: ES, embryonic stem; Ab-MuLV, Abelson murine leukemia virus; RAG, recombine-acti- vating-gene; RSS, recombination signal sequence; BM, bone marrow.
the selection cassettes (24). The loxP sites were added to remove the promoter/enhancer-containing selection cassettes after homologous recombination with the Cre enzyme to avoid any potential compensatory or enhancing activity of these regulatory elements (28). In addition, to carry out a quantitative V(D)J recombination analysis in heterozygous mice, a wild-type HindIII site between JH3 and JH4 was replaced by an EcoRV site in the targeting vector to mark the targeted chromosome (see below). The targeting vector was transfected into ES cells of BALB/c origin (25). After selection, 20 PCR positive clones were obtained and further analyzed by Southern blotting to confirm the proper targeting event and the simultaneous presence of the introduced EcoRV site (Fig. 1B). Of 20 clones, nine contained the EcoRV modification. Two of these ES clones were transiently transfected with Cre, and after negative selection with ganciclovir, all of the surviving clones carried the expected loxP deletion (Fig. 1A) as analyzed by PCR (not shown). By blastocyst injection, chimeric mice were generated that transmitted the mutation into the germline, and the offspring of these mice were analyzed by PCR (not shown). The correct Cre-mediated deletion was confirmed by PCR cloning and sequencing of

FIGURE 1. Gene targeting of the DQ52 element. A, The targeting strategy. Genomic locus: The genomic Ig heavy chain locus comprising the DQ52 and JH1 to JH4 elements and the heavy chain intron enhancer (E\textsubscript{m}) is shown. Targeting vector: The targeting vector with the DQ52 element (Xhol/SacI fragment) replaced by neomycin resistance (neo) and HSV-thymidine kinase (HSV-tk) cassettes, flanked by 2 loxP sites. The HindIII site between JH3 and JH4 is converted to an EcoRV site. Targeted allele: the expected targeted allele after homologous recombination is shown. DQ52-deleted allele: The expected genomic locus after Cre-mediated site-specific recombination between the introduced loxP sites. The locations of the two probes used in the Southern blotting analysis are shown on top of the map. For clarity, not all sites of the indicated restriction enzymes are shown. B, Confirmation of the correct targeting events and presence of the EcoRV site by Southern blotting. DNA from the PCR-positive ES cell clones was digested with either EcoRV or EcoRV+BamHI and hybridized with an external 3' genomic probe or with a NEO probe that hybridizes specifically to the neomycin resistance cassette. Left panel, BALB/c, genomic DNA from BALB/c liver; clone 14 and clone 24, DNA from ES cell clones 14 and 24, respectively. EcoRV, EcoRV-digested DNA. A germline fragment can be detected as a 15.0-kb band. A correct targeting event without (clone 14) or with the simultaneous EcoRV modification (clone 24) can be detected as a 12.8- or 10.0-kb band, respectively (see also A). Middle panel, In EcoRV+BamHI-digested DNA only the correct targeting event with the EcoRV modification can be resolved from the germline band. Right panel, Hybridization with NEO probe demonstrates that the targeting vector is integrated only once and in the correct position.
FIGURE 2. Selective reduction in rearrangement frequency demonstrated by PCR analysis. A, To compare the rearrangement frequency of the mutated with the wild-type IgH allele, PCR was performed with DSF (or with aVH-specific primer) and JH4 primers from the DNA of DQ52\(^{-/-}\) mice. The PCR product was digested with either HindIII or EcoRV. Because the HindIII site between JH3 and JH4 is wild-type allele specific and the introduced EcoRV site is specific for the targeted allele (see Fig. 1A), a direct quantification of rearrangement frequency is possible by comparing the intensities of the undigested (JH1, JH2, and JH3) and the digested (DJH1, DJH2, and DJH3) bands by densitometric analysis. The use of both enzymes in the analysis also allowed both vertical (e.g., JH3 vs DJH3) and horizontal (e.g., JH3 EcoRV vs JH3 HindIII) comparison of the bands. Left panel. The principle is shown for DQ52\(^{-/-}\) mice. The bands were hybridized with the JH3 probe (hybridizing to PCR products from both alleles). PCR from BALB/c DNA was used as a control. Products from this amplification can only be digested by HindIII. Right panel. The filters were rehybridized with the EcoRV probe, which is specific for the targeted allele as it does not hybridize to PCR products from BALB/c DNA. Hybridization with this probe is used to confirm that the digestions are complete, which is absolutely required for a proper analysis. B, DNA from sorted BM B220 low IgM\(^{+}\), total BM, or total spleen cells of DQ52\(^{-/-}\) mice was analyzed by PCR for DJ and VDJ rearrangements by the use of DSF- or VH-specific primers in conjunction with the JH4 primer. Three VH families, VH7183, VH952, and VH588, were selected for the analysis. The PCR products were digested with EcoRV and HindIII and analyzed as described in A. Representative examples after EcoRV digestion of three independent experiments are shown. On the right, the sizes of VH588 rearrangements to each JH are given. All DQ52\(^{-/-}\) mice are on a 100% BALB/c background. C, Results from the densitometric analysis of several experiments are presented as the mean ± SD. Because the absolute values of band intensities were very different, values of the wild-type chromosome were set at 100%, and SD values are only shown for the DQ52\(^{-/-}\) chromosome. The number of experiments and the significance (by paired Student’s t test) are: B220 low IgM\(^{+}\) cells, \(p < 0.01\) (\(n = 3\)); total BM cells, \(p < 0.05\) (\(n = 2\)); and total spleen cells, \(p < 0.01\) (\(n = 5\)). D, Principle of the PCR analysis for the circular excision products (containing signal joints) in DH-JH4 recombination. RSS sequences are shown as triangles. Primers for the PCR are shown as arrows. E, Analysis of JH4 rearrangements by detection of signal joints as depicted in D. Results from sorted BM B220 low IgM\(^{+}\) pre-B cells of one RAG-2\(^{-/-}\) chimeric and two DQ52\(^{-/-}\) F1 mice (labeled a and b) are shown. JH4* shows the undigested PCR fragment, and DJH4* shows the digested PCR fragment. Band intensities after ethidium bromide staining were quantified by densitometric analysis. EcoRV-digested fragments were compared with the corresponding HindIII fragments. The mean reduction of the frequency of DQ52\(^{-/-}\) chromosome was 68.5% for the two F1 mice (\(p < 0.05\), by paired Student’s t test).
DNA from BM cells of homozygous DQ52<sup>−/−</sup> mice. The first experiments were performed with mice derived from two independent ES cell clones (clones 5 and 24), which showed identical phenotypes.

**Selective reduction of the frequency of rearrangements on the DQ52<sup>−/−</sup> allele**

The effect of DQ52 deletion on the rearrangement frequency at the IgH locus was first analyzed. This analysis was performed by PCR amplification of rearranged genes from heterozygous DQ52<sup>−/+</sup> mice with either DH or VH primers in combination with a JH4 primer. The principle and specificity of the method are presented in detail in Fig. 2A. DJ rearrangements were analyzed by amplifying DNA of DQ52<sup>−/+</sup> mice from total spleen and BM cells as well as from BM cells sorted to the pre-B cell fraction (B220<sup>low</sup>IgM<sup>−</sup>). PCR products amplified with DSF- and JH4-specific primers were digested with either EcoRI or HindIII and analyzed by Southern blotting in combination with a densitometric analysis (for details, see Materials and Methods). DSF primer amplifies rearrangements of all other DH genes except DQ52. Rearrangements of VH genes from three VH families, VH7183, VHQ52, and VH558, were amplified with the corresponding V7183, VQ52, and V558 primers and analyzed as described above.

Representative examples of the analysis are shown in Fig. 2B. Results from the densitometric analysis of several experiments are presented as the mean ± SD in Fig. 2C. In all three cell populations a strong reduction of both DJ and VDJ rearrangements to JH3, but not to JH1 or JH2, elements of the DQ52<sup>−/−</sup> allele was found. Reduction in the rearrangement frequency to JH3 was between 50 and 70% for DH, VH7183, and VH558 elements, whereas it was between 15 and 35% for the VHQ52 family members compared with the wild-type allele (Fig. 2C).

The selective reduction of JH3 rearrangements was rather surprising. Therefore, it was important to know whether rearrangements to JH4 were reduced as well. This could not be analyzed in our assay due to the location of the introduced EcoRV site (see Fig. 1A). Rearrangements can also be analyzed by demonstrating the presence of circular excision products in actively rearranging tissues. Based on this fact we designed PCR analysis of excision products that were produced after DH to JH4 rearrangements. Wild-type and DQ52<sup>−/−</sup> alleles could be discriminated in a similar manner as described above (Fig. 2D). PCR primers used in the analysis detected rearrangements of DFL16.1 and most members of the DSP2 family. DNA of DQ52<sup>−/−</sup> mice from sorted BM B220<sup>low</sup>IgM<sup>−</sup> pre-B cells was amplified, and the PCR products were analyzed as described above. DH to JH4 rearrangements were reduced by 61 and 76% in two pre-B cell populations (Fig. 2E). The reduction was even more dramatic (96%) when DQ52<sup>−/−</sup> pre-B cells from complemented RAG-2<sup>−/−</sup> chimeras were analyzed (Fig. 2E).

**Cell number analysis**

The reduced rearrangement frequency on the DQ52<sup>−/−</sup> allele stimulated us to analyze whether it would also result in a reduction of B cell numbers. To analyze this we crossed DQ52<sup>−/−</sup> mice on the BALB/c (IgM<sup>+</sup>) background to wild-type C57BL/6 (IgM<sup>−</sup>) mice and used IgM allele-specific Abs to analyze the F1 offspring soon after birth. There was no difference in the relative amounts of IgM<sup>+</sup>- and IgM<sup>+</sup>positive B cells in control F1 mice, whereas there was a significant reduction in the amount of IgM<sup>+</sup> B cells (DQ52<sup>−/−</sup> B cells) in DQ52<sup>−/−</sup>F1 mice (Fig. 3). The relative number of IgM<sup>+</sup> B cells was reduced to a similar extent at most analyzed time points after birth. The average ratio of IgM<sup>+</sup> B cells to IgM<sup>+</sup>B cells was 38 vs 62% for the BM cells and 43 vs 57% for splenocytes, respectively. A similar reduction was detected among peritoneal B1 cells (CD5<sup>+</sup>, IgM<sup>+</sup>) of 3-wk-old DQ52<sup>−/−</sup>F1 mice (data not shown). Compared with normal BALB/c mice, the reduction of B cell numbers in homozygous DQ52<sup>−/−</sup> mice was not evident. Also, no difference in the proportions of different B cell precursor populations was found in the BM between DQ52<sup>−/−</sup> and normal BALB/c mice (data not shown).

**DH usage is altered in DJ and VDJ rearrangements**

After noticing that rearrangements to JH3 and JH4 were strongly reduced on the DQ52<sup>−/−</sup> allele we decided to clone and sequence VH<sup>558</sup>-JH3 rearrangements to determine whether the reduced frequency of rearrangements was connected to changes in the potential Ab repertoire (altered DH and VH usage). Due to the vast number of VH members in the VH558 family and their broad distribution within the VH<sup>558</sup> locus, only VH558-JH3 rearrangements were sequenced to avoid bias caused by a limited number of VH<sup>558</sup> elements and their strict location in the VH<sup>558</sup> locus. DH rearrangements were isolated from sorted BM B220<sup>low</sup>IgM<sup>−</sup> pre-B cells of homozygous DQ52<sup>−/−</sup> and normal BALB/c mice (for details, see Materials and Methods). VH<sup>558</sup> rearrangements were isolated from sorted pre-B cells of DQ52<sup>−/−</sup> mice and from spleen cells of homozygous mice. D elements were recognized by comparing the sequences obtained to the published germline sequences (38–40). Some D genes have a minor nucleotide change at their 5’ end or even have an identical coding region when compared with the other D gene sequences. Therefore, some D genes could not be discriminated from each other and were grouped as a recognition family (Fig. 4).
D usage in DJ rearrangements was first analyzed (Fig. 4A). In the great majority (63%) of rearrangements of DQ52<sup>−/−</sup> mice, DFL16.1 was used. The other D elements used more than once were DFL16.2 (11%) and DSP2.2 (18%). DJ rearrangements of pre-B cells from BALB/c mice showed a similar preference for DFL16.1 (40%) and DFL16.2 (20%; Fig. 4A). However, a shift toward the use of more 5′ D elements was noticed in DQ52<sup>−/−</sup> rearrangements (e.g., no usage of DSP2.8, but more frequent usage of DFL16.1; see also Fig. 8), although all differences were not significant.

When VH558 to DJH3 rearrangements in BM B220<sup>low</sup>IgM<sup>−</sup> pre-B cells were analyzed, interesting differences between the wild-type and DQ52<sup>−/−</sup> alleles were noticed (Fig. 4B). D elements of DFL16.1 and DSP2.3 families were not used at all on the DQ52<sup>−/−</sup> allele, while they were used in 27 or 20%, respectively, of VDJ joints of the wild-type allele (both p < 0.05, by Student's t test). In contrast, D elements of the DSP2.1 family were frequently used on the DQ52<sup>−</sup> chromosome (31 vs 0% on the wild-type allele; p < 0.05; Fig. 4B). Thus, on the DQ52<sup>−</sup> allele, the DFL16.1 was not used at all in VDJ joints, although it had been the most frequently used D family in DJ joints (compare Fig. 4, A and B).

Processing of the rearranging gene elements is altered

When the sequences of nonselected DJ rearrangements were analyzed in detail, it was noticed that the average DJ length (nucleotide length from the beginning of the DH coding sequence to the first codon in the JH element) was only slightly longer on the DQ52<sup>−/−</sup> than on the wild-type allele (10.4 vs 9.15 codons; not significant; Fig. 5A). The usages of the three reading frames were similar in DQ52<sup>−/−</sup> and wild-type joints. However, JH3 elements were much more frequently intact, without trimming (47 vs 15%; p < 0.05, by Student's t test), and P nucleotides were added more frequently to JH3 (36 vs 5%; p < 0.05) in DQ52<sup>−/−</sup> than in BALB/c rearrangements (Fig. 5A). Interestingly, a similar difference in the processing of JH3 was detected in VDJ joints isolated from spleen cells of DQ52<sup>−/−</sup> and BALB/c mice despite the strong cellular selection (Fig. 5C).

Altered amino acid composition in the CDR3 region

To understand the selection against the usage of DFL16.1 and DFL16.2 on Igs derived from the DQ52<sup>−</sup> allele, we analyzed the amino acid composition of the CDR3 region obtained from functional VDJ rearrangements of both BALB/c and DQ52<sup>−/−</sup> spleen sequences in Fig. 5) are plotted as the percentage of D usage related to the total number of sequences. Identified DH elements are shown. DH elements that could not be unambiguously identified are marked (*) and are given as D recognition families (DSP2.10* = DSP2.10, DSP2.11; DSP2.1* = DSP2.3, DSP2.4, DSP2.6; DSP2.1* = DSP2.1, DSP2.5; DSP2.1* = DSP2.1, DSP2.4, DSP2.6; DSP2.1* = DSP2.1, DSP2.5; DFL16.1* = DFL16.1 and DFL16.2). A, DH usage in DJ rearrangements of BM B220<sup>low</sup>IgM<sup>−</sup> cells from BALB/c and DQ52<sup>−/−</sup> mice. B, DH usage in V<sub>H</sub>558DJ rearrangements of BM B220<sup>low</sup>IgM<sup>−</sup> cells from DQ52<sup>−/−</sup> mice. C, DH usage in V<sub>H</sub>558DJ rearrangements of spleen cells from BALB/c and DQ52<sup>−/−</sup> mice. All DQ52<sup>−/−</sup> and DQ52<sup>−</sup> mice are on a 100% BALB/c background. Sorted B220<sup>low</sup>IgM<sup>−</sup> cells were determined to be BP-1<sup>+</sup> pre-B cells, and they did not contain any IgD<sup>+</sup> cells (not shown).
FIGURE 5. Processing of JH3 occurs less frequently on the DQ52-/- allele. Sequences of PCR-amplified rearrangements to the JH3 element are shown. All cloned sequences are shown; there were no duplicates. A, DJ rearrangements of BM B220lowIgM2 cells from BALB/c (top) and DQ52-/- (bottom) mice. The reading frames (fr) are shown. The usage of intact JH3 elements (47% -/- vs 15% BALB/c; p < 0.05, by Student’s t test) and the number of P nucleotides (36% -/- vs 5% BALB/c; p < 0.05) were different between DQ52-/- and BALB/c rearrangements. B, VH3DJ rearrangements of BM B220lowIgM- cells from DQ52-/- mice. B220lowIgM- sorted cells were confirmed to be IgD+ by FACS staining. C, VH3DJ rearrangements of spleen cells from BALB/c and DQ52-/- mice. B and C, Productive rearrangements (1) and nonproductive rearrangements (2) are shown. The identified D element (DH) is given on the right. *, DH recognition families (see Fig. 4). P, P nucleotides; N, N nucleotides. All DQ52-/- and DQ52-/- mice are on a 100% BALB/c background.
cells. Amino acid sequences of CDR3 regions (Fig. 6) were translated from the corresponding VDJ sequences shown in Fig. 5C and presented in a color code as in the report by Wilbert et al. (41). It was noticed that the complete usage of the 5' end of JH3 resulted in the incorporation of more hydrophobic amino acids (shown in dark blue) in this region (mean number of hydrophobic amino acids in BALB/c and DQ52−/− sequences, 2.0 and 2.9, respectively). In contrast, the amino acids encoded by the 3' end of the VH gene and the DH element were more charged (red and purple) and polar (green and light blue) in DQ52−/− sequences than in the wild-type ones (Fig. 6). This shift to more charged and polar amino acids seemed to be caused by an altered usage of D elements in the selected sequences of the DQ52− allele. A hydropathicity index was calculated for CDR3 (42) on a scale with Ile, as the most hydrophobic amino acid of 4.5, the neutral amino acid Gly with an index of −0.4, and Arg as the amino acid with the highest charge of −4.5. D elements with a mean hydropathicity index of −1.0 (for reading frame 1), such as DFL16.1 and DFL16.2, that were frequently used in DJ rearrangements (Fig. 4A) were replaced by D elements with a higher negative hydropathicity index, i.e., with a more polar character such as DSP2.1 (index, −1.56) or DSP2.2 (index, −2.18) on the DQ52−/− CDR3s. This was in contrast to BALB/c sequences (Fig. 4C). Both effects, more hydrophobic amino acids in the 3' part and more polar/charged amino acids in the 5' part of the CDR3s of the DQ52−/− mouse, seemed to compensate each other, because a similar average hydropathicity was calculated for the wild-type and DQ52−/− sequences (hydropathicity index of −0.1 for wild-type and −0.38 for DQ52−/− CDR3). Despite larger JH3 segments due to reduced trimming, the CDR3 length was only marginally larger in DQ52−/− sequences than in BALB/c sequences (mean length, 10.8 vs 9.50 aa; not significant). Thus, despite the similar hydropathicity index, the CDR3s of splenic B cells in the DQ52−/− mouse had an altered amino acid composition compared with the wild-type B cells.

**A weak aberrant μ transcript was detected in the targeted allele**

The reduction of rearrangements to JH3 and JH4, but not to JH1 or JH2, was an unexpected finding that indirectly suggested that the phenotype might be due to a partial effect of the deletion to the

![FIGURE 6](http://www.jimmunol.org/ Downloaded from)

The amino acid composition of the CDR3 reveals differences between IgH chains derived from DQ52− and wild-type alleles. The amino acid sequences of CDR3 regions of functional VDJH3 rearrangements in the spleen of BALB/c and DQ52−/− mice are shown (corresponding to sequences in Fig. 5C). Amino acids are given in a color code (41). Amino acids coded by DH segments are underlined. #: H, Y, Mean number of amino acids His and Tyr; #: S, T, N, mean number of Ser, Thr, and Asn; #: chrg. AA, mean number of charged amino acids in the CDR3; #: hyph. AA, mean number of hydrophobic amino acids encoded by the JH3 region plus P nucleotides. On the right, the CDR3 length (between C of V_H and Y of JH3) for each sequence and the mean CDR3 length are shown.
regulation of VDJ recombination. Therefore, it was analyzed whether the \( \mu^0 \) transcript that originates from the DQ52 promoter (10) (T. Tallone et al., manuscript in preparation) was completely absent in homozygous DQ52\(^{-/-} \) mice. cDNA from BM cells of BALB/c and DQ52\(^{-/-} \) mice was amplified by PCR with a sense primer that hybridized 3' of the DQ52 deletion and an antisense C\(_m\) primer as described previously (43). Surprisingly, a \( \mu^0 \) transcript could also be amplified with these primers from DQ52\(^{-/-} \) BM cells (data not shown), although the correct deletion of the DQ52 locus including its promoter region was confirmed by cloning and sequencing. Additional experiments were performed to map start sites and quantify the aberrant \( \mu^0 \) transcript from the targeted allele. For this purpose, Abelson transformed pro-B cell lines were generated from BM cells of mice that were heterozygous for the DQ52 deletion in the RAG-2\(^{-/-} \) background. Expression of \( \mu^0 \) transcripts was analyzed simultaneously from both alleles by S1 nuclease protection assay. A radiolabeled probe for the assay was generated by primer extension from a plasmid containing the DQ52 deletion cloned from the targeted allele (see Materials and Methods and Fig. 7A). Thus, the probe was specific for the detection of all potential transcripts originating from the targeted locus upstream of the DQ52 deletion. However, transcripts arising from the wild-type allele also generated S1 nuclease-resistant fragments of about 160 bp in length (Fig. 7, A and B) that served as an internal control for the normal level of \( \mu^0 \) transcript. Transcripts of the wild-type allele started from several initiation sites, as typical for TATA-less promoters. Interestingly, only two minor transcripts (A and B) initiating around the remaining loxP site were detected on the targeted allele, but their intensities were only 10 and 5%, respectively, that of the wild-type \( \mu^0 \) transcript (Fig. 7B). Thus, despite the complete deletion of the natural start sites of the \( \mu^0 \) transcript, two very weak aberrant sterile transcripts starting from the inserted modifications of the DQ52 locus could be detected on the DQ52\(^{-/-} \) allele.

**Discussion**

We have demonstrated in this work that deletion of the DQ52 element with its surrounding regulatory elements causes a selective reduction of rearrangements to JH3 and JH4. The reduction can be seen both at the level of primary DJ rearrangements in the BM pre-B cell population as well as at the level of VDJ rearrangements in the periphery. Therefore, we think that the reduction is due either to an altered accessibility of the locus or to a lowered efficiency of the recombinatorial machinery. It also appears that the reduced frequency of rearrangements seen at the level of VDJ joining is due to the lower number of DJ target elements rather than a separate mechanism operating on the accessibility of V\(_{\mu}\) gene segments. We have also demonstrated that there is a lower number of B cells with Ig receptors derived from the DQ52\(^{-/-} \) allele than from the wild-type heavy chain allele in F1 mice. The reduction in the number of B cells is probably caused by the selective impairment of DJ rearrangements, but selection at the level of the IgH chain in pre-B cells is probably an additional mechanism (see below).

Three previously described gene deletions, namely IgH enhancer (E\(_{\mu}\)) (8, 44), PAX-5 (45), and IL-7R (46), have a selective negative effect on VDJ recombination at the IgH locus. Deletion of any of these genes causes a strong reduction in V to DJ joining, although DJ joining is practically unaffected. However, the effect of the E\(_{\mu}\) deletion on VDJ recombination is far milder than that of the TCR\( \beta \) enhancer (E\( \beta \)). Deletion of E\( \beta \) practically inactivates both D to J and V to DJ joining at the TCR\( \beta \) locus (47, 48), suggesting that additional cis-acting elements have to be involved in the activation of D to J recombination at the IgH locus (8).

Therefore, deletion of the DQ52 locus was a logical concept to test this hypothesis. This is, in fact, the first gene deletion (except for the Rag gene deletions) that significantly inhibits DJ rearrangements at the IgH locus, although the effect is selective to JH3 and JH4. One possible explanation is that there are several elements controlling the activation of DJ recombination, and we have deleted only one of them (see model discussed below). The other alternative is that the DQ52 locus is the key element, but it has been only partially inactivated by this particular deletion. All known start sites of the \( \mu^0 \) transcript are in the deleted region, and therefore, \( \mu^0 \) transcription should have been eliminated in the DQ52\(^{-/-} \) allele. However, a weak \( \mu^0 \)-like sterile transcript was detected in the targeted allele. This transcript started in two positions around the loxP site retained in the locus after successful Cre-mediated deletion of DQ52. It remains obscure whether this weak (only 1/10th of the wild type) transcriptional activity was able to reverse the effect of the targeted deletion. A novel mutant mouse line in which the rest of the D gene locus, except for DFL 16.1, has been removed seems to shed additional light on this question. Preliminary analysis of rearrangements in these mutant mice shows that the targeted deletion of the DH locus has a very similar, although stronger, selective effect on the usage of JH elements in rearrangements as the deletion of DQ52 (H. W. Schroeder, Jr., personal communication). This suggests that transcription from the cryptic sites does not dramatically alter the effect of the DQ52 deletion. Formally, we cannot absolutely exclude that insertion of the EcoRV linker between JH3 and JH4 may also influence DJ rearrangement. We think this is very unlikely, because we have inserted only a few-base pair-long linker to exchange HindIII to EcoRV without deleting DNA. In addition, this region is not highly conserved (49). Allele-specific changes in the close proximity of the wild-type HindIII site can be found in several mouse strains. Previously, germline sterile transcripts, potentially involved in the regulation of rearrangement, have been eliminated at TCR or Ig loci by gene targeting in three mouse models. Deletion of TCR D\( \delta \)1 and Jk K1-K11 promoters leads to the significant reduction of rearrangement at D\( \delta \)1-J\( \beta \)1 and Jk loci, respectively (50, 51). In contrast, deletion of the T early \( \alpha \) element selectively down-regulates TCR \( \alpha \) rearrangements to the most 5' \( \alpha \) genes (52). These data together with DQ52 deletion indicate that the accessibility of each rearranging locus is individually controlled, most likely by multiple gene elements.

Our finding that processing of JH3 elements was altered in DQ52-deficient mice is rather surprising, but the present progress in the knowledge of VDJ recombination might offer a proper explanation. The VDJ recombination process is initiated by targeting of the Rag-1 and Rag-2 proteins to the conserved recombination signal sequences (RSSs). Thereafter, double-strand cleavage of DNA occurs precisely at the junction between an RSS and the adjacent coding sequence (for review, see Ref. 53, 54). The coding ends are then processed and ligated by additional activities of several factors involved in general DNA double-stranded break repair to form a coding end (for review, see Ref. 55, 56). Gene-targeting experiments have confirmed that transcriptional enhancers play an important role in the regulation of VDJ recombination at TCR and Ig loci (6, 57). It has been generally postulated that these enhancers control for recombinatorial accessibility. However, Hempel et al. have demonstrated that deletion of the TCR\( \beta \) enhancer reduces the accessibility of the locus for recombinase far less than further processing of the coding ends (57). In addition, it has been shown that enhancers can selectively regulate the rearranging elements. By using a human TCR\( \delta \) minilocus system it was demonstrated that inactivation of the E\( \delta \) enhancer selectively reduces formation
of double-stranded breaks 5′ of Jβ1 but not 3′ of Dδ3 (58). We have now demonstrated that deletion of the DQ52 locus causes a selective alteration in the processing of JH3, but not DH, elements in the DJ rearrangements of unselected BM preB cells. This suggests that a cis-acting sequence flanking DQ52 plays a similar role in the IgH locus to the one played by Eβ and Eδ enhancers in the

FIGURE 7. Mapping of additional weak transcriptional start sites in the targeted DQ52 locus by S1 nuclease protection. A. Method for the simultaneous detection of transcripts arising from the wild-type and the targeted DQ52 locus in a DQ52−/− RAG-2−/− pro-B cell line. The wild-type allele with the DQ52 gene, heptamer (H), nonamer (N), and the DQ52 protein binding boxes A, B, and C are shown. In the targeted allele the XhoI/SalI fragment was replaced by a loxP site. The ST3 oligo-nucleotide was used to generate the S1 probe from a plasmid containing the targeted locus (wavy line). The S1 probe can be used to map 5′ transcriptional start sites from the targeted locus and simultaneously can detect fragments of about 160 bp derived from the wild-type locus. The μ0 transcripts from the wild-type locus are known to originate from the DQ52 boxes A and B (10) (T. Tallone et al., manuscript in preparation). Results from B are also indicated as solid lines; protected fragments are from the wild-type allele (~160 nucleotides (nt)), and the longer protected fragments are from the targeted allele (A, about 240 nt; B, about 175 nt). B, S1 protection analysis of RNA prepared from a DQ52−/− RAG-2−/− pro-B cell line. Samples: lanes 1 and 2, cytoplasmic RNA of the RAG-2−/− wild-type pro-B cell line 63–12; GATC, sequencing reaction with ST3 primer, the sequence of the loxP site is indicated; lanes 3–6, total RNA (3, 4) and cytoplasmic RNA (5, 6) of a DQ52−/− RAG-2−/− pro-B cell line; lanes 7 and 8, transfer RNA; lane 9, S1 probe. On the right, an interpretation of the found transcripts is indicated: the 160-nt protected fragment that arises from the wild-type allele and two minor transcripts (A and B) that arise from the DQ52− allele.
TCR loci, when one assumes that the action of DQ52 is Eμ dependent, as shown previously (10). The analysis of D usage in the sequences from DJ and VDJ rearrangements revealed several interesting observations that merit discussion. First, D usage in VDJ rearrangements from both spleen cells and BM B220low IgM^2 pre-B cells was very similar, but markedly different from the primary DJ rearrangements. This suggests that both BM pre-B cells and peripheral B cells are similarly ligand selected. However, it has been demonstrated by Gu et al. that pre-B cells express a broad random repertoire of V_H genes, whereas in mature peripheral B cells only some sets of V_H genes are dominantly expressed and, therefore, are ligand selected (59). This obvious discrepancy might be explained by the fact that we have amplified VDJ rearrangements primarily from the small pre-B cell subpopulation (fraction D) that is expanded in response to the pre-B cell receptor, composed of a complete heavy chain and surrogate light chains. This expansion may already involve selection for optimal CDR3s. Alternatively, our results may indicate that only optimal CDR3s pair with the surrogate light chains. This idea is further supported by the finding that DFL16.1 and DFL16.2 gene elements were not used in the VDJ rearrangements from the DQ52^- allele of pre-B cells or DQ52^-/^- spleen cells, although they were frequently detected in the corresponding wild-type populations. This was not due to the lack of DFL16 rearrangements, because both DFL16.1 and DFL16.2 genes were very frequently used in the primary DJ joints. Therefore, it is possible that DQ52^-/^- cells expressing a combination of VH558-DFL16.1/2-JH3 do not form a proper CDR3 that can interact with a putative ligand or pair with the surrogate light chains. Altered processing in DQ52^-/^- cells frequently leads to the complete use of JH3 and addition of P nucleotides. This can potentially result in binding sites that have longer CDR3s than the wild-type ones and have a stretch of hydrophobic amino acids at the carboxyl-terminal end of CDR3. Because the size of the CDR3 region is tightly controlled (60) and DFL16.1 is 6 bp longer than most other D elements, DQ52^-/^- B cells expressing an IgH chain with DFL16.1 on the cell surface could be counterselected. Counterselection against DFL16.2 is more difficult to explain, because it has the same germ-line length as all the members of the DSP family. The amino acid analysis of functional rearrangements from BALB/c and DQ52^-/^- mice (given in percentages for DFL16.1, DFL16.2, and DSP2.8 below the map on top).

FIGURE 8. Model showing that the DQ52 region triggers secondary rearrangements. The D-J cluster of the wild-type allele (left) and that of the DQ52^- allele (right) are shown schematically. The positions of the mapped D elements are indicated. On the wild-type allele, DQ52 is frequently used in primary rearrangements to JH1 or JH2 (our example). The cis-regulatory region of DQ52 together with the heavy chain enhancer (Eμ) controls the accessibility of the 3' JH3 and JH4 elements. Quite often the first rearranged D element is replaced by a secondary D-J rearrangement (here, DFL16.1 to JH3). On the DQ52^- allele DQ52 is not available; therefore, 5' D elements are used for primary rearrangements (here, DSP2.2). This D element does not contain the cis-regulatory region, which controls accessibility to JH3 and JH4; therefore, secondary rearrangements occur less frequently. If they occur on this allele, they must involve more 5' D elements, i.e., cause a shift to usage of D elements positioned further 5' (in our example, DFL16.1). This shift was detected in our analysis and is indicated by the frequencies of D usage found in DJ joints of BALB/c and DQ52^-/^- mice (given in percentages for DFL16.1, DFL16.2, and DSP2.8 below the map on top).
In consequence, the higher number of polar/charged amino acids in the 5’ part of the CDR3 seems to balance the longer stretch of hydrophobic amino acids in the 3’ part, resulting in an overall similar mean hydrophaticity index in selected DQ52 9/10 and wild-type CDR3s. This is in good agreement with the strict control of the hydrophaticity of the CDR3 as presented by Schroeder et al. (61). Despite the similar mean hydrophaticity, the amino acid composition of the CDR3 of the DQ52 9/10 allele is altered, which most likely influences the ligand recognition or association with the surrogate light chain of the resulting IgH chain.

To explain our results we propose the following model. JH1 and JH2 genes are accessible for rearrangement in the first wave of recombination simultaneously with DQ52. Therefore, DQ52 elements frequently rearrange to JH1 and JH2 when other DH elements are not yet accessible, and DQ52 is found in early primary rearrangements (11–13); thereafter, other elements (VH, DH and JH) become accessible. However, the accessibility of JH3 and JH4 genes is enhanced by the DQ52 element already rearranged to JH1 (Fig. 8). Thus, the DQ52 promoter region, perhaps in concert with the heavy chain intron enhancer, could act as a trigger for secondary rearrangements, involving other Ds rearranging to further 3’ JH elements, i.e., primarily JH3 and JH4. In DQ52 9/10 B cells the DQ52 gene is not available, and other DH elements substitute it for the primary rearrangements. As a consequence, secondary rearrangements are not initiated to the same extent as in wild-type B cells, as reflected by a lower number of rearrangements to 3’ JH elements (Fig. 8). Assuming that DJ joints of JH3 and JH4 are frequently the result of secondary rearrangements (as indirectly indicated in the human and mouse by comparing fetal and adult JH usages (59–61)), the DQ52 9/10 allele should use more 5’ positioned D elements compared with the wild-type allele. This further 3’ rearrangements (11–13); thereafter, other elements (VH, DH and JH) become accessible. Therefore, the D segment (DQ52) nearest to the J region.

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