Flanked on both sides by 12-base spacer recombination signal sequences, each DH gives the developing B cell access to six reading frames (RFs) of distinctly different germline sequence. In practice, from shark to mouse to human, sequences encoded by RF1 generated by deletion dominate the mature B cell repertoire (7, 8). RF1 is typically enriched for tyrosine and glycine codons. Together, these two neutral amino acids contribute $\frac{1}{4} \times 40\%$ of the amino acids in the CDR-H3 loop (8, 9).

To test the role of germline RF1 sequence in determining CDR-H3 amino acid usage and to test whether a mouse starting with altered germline sequence could recreate a wild-type CDR-H3 repertoire by somatic means, we replaced central codons for RF1-encoded tyrosine and glycine in DFL16.1, the most JH-distal DH, with codons for arginine, histidine, and asparagine and deleted the intervening DH. We found that B lineage cells in the bone marrow of these mice maintained their preference for RF1 through the mature, recirculating IgM$^+$IgD$^+$B cell stage of development. As a result, usage

Tyrosine and glycine constitute 40% of complementarity determining region 3 of the immunoglobulin heavy chain (CDR-H3), the center of the classic antigen-binding site. To assess the role of DH RF1-encoded tyrosine and glycine in regulating CDR-H3 content and potentially influencing B cell function, we created mice limited to a single DH encoding asparagine, histidine, and arginines in RF1. Tyrosine and glycine content in CDR-H3 was halved. Bone marrow and spleen mature B cell and peritoneal cavity B-1 cell numbers were also halved, whereas marginal zone B cell numbers increased. Serum immunoglobulin G subclass levels and antibody titers to T-dependent and T-independent antigens all declined. Thus, violation of the conserved preference for tyrosine and glycine in DH RF1 alters CDR-H3 content and impairs B cell development and antibody production.

Unlike H chain complementarity determining regions 1 and 2, which are entirely encoded by the VH gene segment, CDR-H3 is created de novo by the VDJ rearrangement process (1–3). Imprecision in joining these gene segments permits exonucleolytic loss as well as palindromic (P junction) gain of terminal VH, DH, and JH sequence. The terminal deoxynucleotidyl transferase (TdT) catalyzed insertion of N nucleotides at the sites of joining permits the inclusion of nongermline sequence into CDR-H3 (1, 2). Together, these mechanisms create a CDR-H3 repertoire that ranges from unmodified and intact germline-encoded sequence to rearrangements where extensive nibbling and N addition no longer permit identification of the original D$_H$. The extensive range of diversity available to CDR-H3 has functional consequences because its location at the center of the antigen-binding site, as classically defined, permits this interval to often play a significant role in antigen recognition and binding (4–6).

The online version of this article contains supplemental material.
of arginine, asparagine, and histidine content in mature CDR-H3 loops tripled, whereas tyrosine and glycine content was reduced by one half.

This change in CDR-H3 amino acid content had functional consequences. Homozygous mutant mice exhibited a consistent reduction in total B cell numbers, a redistribution of peripheral B cell subsets, and a decrease in total and antigen-specific antibody levels. These findings confirm the power of the mechanisms used to regulate RF usage (7, 9, 10) and demonstrate a significant role for the D_H gene segment in controlling CDR-H3 amino acid composition. They illustrate the importance of CDR-H3 amino acid content in B cell development and antibody production.

RESULTS
Generation of the \( \Delta D-iD \) mouse
We replaced the central portion of DFL16.1, the most V_H-proximal D_H (11), with the complete inverted coding sequence of DSP2.2 (Fig. 1). This allowed us to replace central RF1 codons for tyrosine and glycine with those for arginine, histidine, and asparagine. We termed this hybrid DFL16.1-inverted DSP2.2 D element \( iD \) for inverted D. DFL16.1 sequence encoding tyrosine and serine at the 5′ and 3′ termini of the D_H gene segment was retained to preserve microhomology between the 3′ end of the D_H and the 5′ end of J_H (9, 10). \( iD \) RF1 generated by inversion (i-RF1) encompasses the original tyrosine enriched sequence of DSP2.2 RF1. \( iD \) RF2 and RF3 by deletion and

Figure 1. Generation of a single D_H-containing IgH locus by use of the Cre-loxP system. (A) Illustration of the D_H locus. Wild-type (WT), the locus after targeted replacement of DSP2.2 by DFL16.1 (iD, and the locus after Cre-mediated deletion leaving the single iD gene juxtaposed to the J_H locus (\( \Delta D-iD \)) are shown. V and C denote the full set of germline variable gene segments and constant region exons, respectively. (B) The sequence of \( iD \). DSP2.2 has been embedded within DFL16.1 in an inverted orientation (in red). The average hydrophobicity is noted in parentheses. (C) Generation of the \( \Delta D-iD \) allele. Southern blot analysis of tail DNA from wild-type (wt/wt), heterozygous iD (iD/wt), and heterozygous D_H locus deleted iD (\( \Delta D-iD \)) mice. (D) The D_H RF1 sequences of the wt and \( \Delta D-iD \) D_H alleles. The mutant D_H locus has lost the neutral, hydrophilic amino acids present at the center of the 13 WT D_H gene segments, but has gained the positively charged amino acids present in iD. The interval corresponding to the region replaced by the inverted DSP2.2 sequence is outlined in yellow.
RF1 and RF3 generated by inversion (i-RF2 and i-RF3) maintain a preference for hydrophobic amino acids, and RF3 and i-RF3 continue to incorporate termination codons. We used gene targeting via homologous recombination in a BALB/c embryonic stem cell line (12) to create an IgH allele (depleted DH locus with a single, mutated DFL16.1 gene segment containing inverted DSP 2.2 sequence [\( \Delta D-iD \)]) limited to this single, modified DH.

Amino acid utilization in \( \Delta D-iD \) mature B cells reflects dominant use of iD RF1

We sorted bone marrow B lineage cells on the basis of surface expression of CD19, CD43, IgM, BP-1, and IgD (13, 14) (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052217/DC1), isolated total RNA, performed RT-PCR using primers specific for C\( \mu \) and the VH7183 family as representative of the repertoire as a whole, and performed detailed sequence analyses of the expressed repertoire as described previously (12, 14).

We found no evidence of selection during development for use of D\( _H \) RFs that lacked arginine, histidine, and asparagine. In fraction B CD19\(^+\)CD43\(^+\)BP-1\(^-\)IgM\(^-\) progenitor B cells, 74% of the sequences used RF1 and, in fraction F CD19\(^+\)CD43\(^+\)IgM\(^+\)IgD\(^+\) mature, recirculating B cells, 80% used the mutant RF1 (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052217/DC1). Although D\( _H \) inversions were more frequent in \( \Delta D-iD \) B cells than in the wild-type or depleted D\( _H \) locus with single DFL16.1 gene segment (\( \Delta D-DFL \)), their prevalence did not increase with development even though iD i-RF1 recapitulates the normally preferred tyrosine-enriched sequence of DSP2.2 RF1. Among the 326 iD-containing CDR-H3 sequences, four contained iD sequences in i-RF1 and seven used i-RF2 (Tables S1 and S2, available at http://www.jem.org/cgi/content/full/jem.20052217/DC1). The prevalence in fraction B progenitor B cells of inverted sequences was equivalent to fraction F mature, recirculating B cells (4 out of 71 vs. 4 out of 76, respectively) (Fig. S2). Of 225 DFL16.1-containing sequences from the \( \Delta D-DFL \) mice and the 902 wild-type sequences containing an identifiable DH, none contained a DH inversion (\( P < 0.01 \) and \( P < 0.001 \), respectively).

We found no evidence of selection for sequences that had undergone extensive exonucleolytic loss at the termini of the iD gene segment (Fig. 2). The germline contribution of the iD gene segment remained virtually unchanged between fraction B progenitor B cells and fraction F mature, recirculating B cells with 95% of the CDR-H3 intervals containing identifiable iD\( _H \) sequence in each. To control for the effect of eliminating 12 out of the 13 DH gene segments in the DH locus, we compared this pattern of D\( _H \) retention to that previously observed in WT mice (14).
We found no evidence of selection for increased N nucleotide content with development (Fig. 2). We did observe a decrease in the average number of N nucleotides inserted between D and J between fraction C CD19^+ CD43^+ BP-1^IgM^- early pre-B cells and fraction D CD19^+ CD43^- IgM^- IgD^- late pre-B cells. However, this pattern contradicts the expected result if there was selection for N nucleotide-encoded amino acids in response to surrogate light chain-associated selection pressures.

The stability of exonucleolytic loss and N region gain created CDR-H3 repertoires whose average length remained unchanged during development (Fig. 2 and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20052217/DC1). The preservation of iD sequence contributed to a predominance of arginine, asparagine, and histidine at all stages of bone marrow repertoire development examined (Fig. 3 A). Together, these amino acids comprised approximately one third of the amino acids in the CDR-H3 loop, tripling their contribution to the repertoire when compared with controls (P < 0.001). Conversely, the contribution of tyrosine and glycine to the loop was halved (P < 0.001). Persistence of the charged amino acids was associated with enrichment for CDR-H3 loops with

![Figure 3. Amino acid usage and average hydrophobicity profiles for CDR-H3 sequences containing the mutated iD D_{i} gene segment during progressive stages in B cell development. (A) Distribution of individual amino acids in the CDR-H3 loop of sequences from homozygous ΔD-iD, ΔD-DFL, and wild-type (WT) mice as a function of B cell development according to Hardy et al. (reference 13). The distributions are calculated through analysis of 9,322 individual amino acids from 342 unique ΔD-iD CDR-H3 loops, 3,710 amino acids from 242 unique ΔD-DFL CDR-H3 loops, and 17,583 amino acids from 1,074 unique WT CDR-H3 loops. (B) Distribution of average CDR-H3 hydrophobicities in V_{7183}DJCμ transcripts from homozygous ΔD-iD mice in Hardy B-lineage bone marrow fractions B through F (reference 13). The normalized Kyte-Doolittle hydrophobicity scale (reference 49) has been used to calculate average hydrophobicity. Although this scale ranges from −1.3 to +1.7, only the range from −1.0 (charged) to +1.0 (hydrophobic) is shown. Prevalence is reported as the percent of the sequenced population of unique, in-frame, open transcripts from each B lineage fraction. To facilitate visualization of the change in variance of the distribution, the vertical lines mark the preferred range average hydrophobicity previously observed in WT fraction F (reference 14). The number of unique V_{7183}DJCμ sequences analyzed is shown for each developmental B cell subset. Among the ΔD-iD sequences with loops, there are 172 sequences in fractions B, C, and D in the normal and charged range, and 10 sequences with an average hydrophobicity >0.600. There are 158 sequences in fractions E and F in the normal and highly charged range (≤0.700), but none in the highly hydrophobic range (≥0.700) (P < 0.01, χ²). The number of sequences in each fraction is shown. (C) Average hydrophobicity and standard error of mean of CDR-H3 intervals from V_{μ} 7183 DJCμ transcripts from homozygous ΔD-iD, ΔD-DFL, and WT mice in Hardy B-lineage bone marrow fractions B through F.]{http://www.jem.org/cgi/content/full/jem.20052217/DC1}
an average normalized Kyte-Doolittle hydrophobicity value of less than -0.700 (Fig. 3 B and Table S3, available at http://www.jem.org/cgi/content/full/jem.20052217/DC1).

Although highly charged CDR-H3 loops were retained in the mature AD-iD B cell repertoire, highly hydrophobic sequences followed the normal pattern of loss during development (P < 0.01) (Fig. 4 B and Table S4, available at http://www.jem.org/cgi/content/full/jem.20052217/DC1)(12,14). The selective loss of these highly hydrophobic intervals in AD-iD shifted the average hydrophobicity of the CDR-H3 repertoire firmly into the charged range (Fig. 4 C).

Use of VH7183 and JH gene segments is minimally affected by the change in CDR-H3 sequence

The global effect of the shift in CDR-H3 hydrophobicity could have been ameliorated by a shift in VH or JH utilization, but we found no evidence that this had occurred either. As a population, V<sub>H</sub>7183 usage in AD-iD B lineage cells proved highly similar to controls (Fig. 4). J<sub>H</sub> utilization in AD-iD during development differed from WT in that J<sub>H</sub>4 usage was diminished and J<sub>H</sub>1 usage was enhanced; however, this usage pattern matched that previously observed in AD-DFL (12, 14).

Forced use of a D<sub>H</sub> containing charged sequence impairs B cell production

When compared with WT littermate controls, AD-iD mice exhibited a consistent reduction in total B cell numbers. The absolute numbers of CD19<sup>+</sup> cells in the bone marrow, spleen, and peritoneal cavity of homozygous AD-iD BALB/c mice (Table I) were two thirds of that observed in WT littermate controls (P = 0.004, P < 0.0001, and P = 0.001, respectively). The ratio of Igκ- to Igλ-bearing cells in AD-iD B cells was similar to WT littermate controls (unpublished data).

Although total B cell numbers were decreased, some individual B cell subsets exhibited normal numbers and others demonstrated increases (Table I). The average numbers of CD19<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>BP-1<sup>-</sup>-fraction B cells, primarily pro-B cells, were higher than littermate controls (P = 0.05). The numbers of CD19<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>BP-1<sup>+</sup>-fraction C cells, primarily early pre-B cells, were essentially unchanged. When compared with wild-type littermate controls, the CD19<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>BP-1<sup>-</sup>-fraction D late pre-B cell and CD19<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>BP-1<sup>-</sup>-fraction E immature B cell subpopulations exhibited a 30% decrease in cell numbers (P = 0.008 and P = 0.05, respectively). This pattern of a progressive decrease in cell numbers with development in bone marrow was similar to that previously observed in AD-DFL mice (Fig. 2) (12).

In the spleen, the absolute numbers of cells in the transitional T1 (CD19<sup>+</sup>AA4.1<sup>+</sup>IgM<sup>hi</sup>CD23<sup>-</sup>) and T2 (CD19<sup>+</sup>AA4.1<sup>+</sup>IgM<sup>hi</sup>CD23<sup>-</sup>) subsets were similar to that in both WT and AD-DFL (Table I, and Fig. 5) (12, 14). However, when compared with controls, the absolute numbers of T3 (CD19<sup>+</sup>AA4.1<sup>+</sup>IgM<sup>lo</sup>CD23<sup>-</sup> and mature follicular (M) (CD19<sup>+</sup>CD23<sup>+</sup>CD21<sup>-</sup>) AD-iD B cells were reduced by one half (P = 0.002, P < 0.0001, and P < 0.0001, respectively).

Figure 4. VH 7183 and JH gene segment use during B cell development. VH 7183 and JH use is reported as the percent of the sequenced population of unique, in-frame, open transcripts for Hardy fractions B (left) through F (right) according to the scheme of Hardy et al. (reference 13). Both VH and JH segments are arranged in germline order. The number of sequences analyzed is shown. (top) VH 7183 and JH use in homozygous AD-iD mice (AD-iD), (middle) VH 7183 and JH use in homozygous AD-DFL mice (AD-DFL), (bottom) VH 7183 and JH use in wild-type, homozygous IgM<sup>-</sup> mice (WT). Significant differences between WT and AD-iD are labeled with asterisks (*, P < 0.01, ***, P < 0.001 etc.; χ²).
whereas T3 and mature B cell numbers were indistinguishable from WT in ΔD-DFL. In marked contrast with the decrease in the absolute numbers of mature follicular cells, the absolute numbers of CD19+CD23hiCD21hi marginal zone (MZ) B cells were increased by one third (P = 0.03) in ΔD-iD.

In the peritoneal cavity, the absolute numbers of B1a cells (CD19+CD5+Mac-1+) in ΔD-iD mice were one half (P < 0.0001) that of WT, and the B1b population (CD19+CD5−Mac-1+) was reduced by 25% (P = 0.06) (Table I and Fig. 5). In contrast with the reduction in mature B cell numbers in the bone marrow and spleen, the numbers of peritoneal cavity B2 cells (CD19+CD5−Mac-1−) were slightly increased (P = 0.27) when compared with controls.

Humoral immune responses are impaired

We measured serum immunoglobulin levels in homozygous ΔD-iD and WT littermates at 8 wk of age. The geometric mean concentration of all four IgG subclasses in the sera of ΔD-iD mice was significantly less than that of WT (P = 0.02, P = 0.0004, P = 0.003, and P = 0.0002, respectively) (Fig. 6 A). The serum concentration of IgM and IgA was comparable to WT (P = 0.17 and P = 0.51, respectively).

Immune responses to both T-dependent and T-independent antigens were impaired. In WT BALB/c mice, intravenous challenge with DEX elicits a T-independent response that is dominated by λ light chain–bearing antibodies that express a diverse range of antigen-binding sites with heterogeneous CDR–H3 sequences (15, 16). The IgM and the Igλ anti-DEX responses in homozygous ΔD-iD BALB/c mice (Fig. 6 B) were significantly lower than those in WT (P < 0.01). In BALB/c, the primary response to the nitrophenylacetyl hapten of [4-hydroxy-3-nitrophenyl] acetyl–γ globulin (NP19-CGG) requires T cell help and contains a large fraction of IgG1 antibody (17). After intraperitoneal challenge, the anti-NP IgG response in ΔD-iD mice was threefold diminished when compared with WT littermates (P < 0.03) (Fig. 6 C). In BALB/c, immunization with purified tetanus toxoid (TT) elicits a T-dependent response that is dominated by κ light chain–bearing antibodies (18). After oral immunization with a recombinant strain of Salmonella that expresses the Tox C fragment of TT (19), the IgM anti-TT response in ΔD-iD mice proved equivalent to WT littermate controls, but the IgG response was approximately fourfold diminished (P = 0.0004) (Fig. 6 D). ELISA analysis using anti-κ and anti-λ reagents documented a preference for κ L chain–bearing antibodies in both the ΔD-iD and WT anti-TT response (unpublished data).

**DISCUSSION**

Replacement of Dλi, RF1-encoded tyrosine and glycine with codons for arginine, histidine, and asparagine resulted in extensive substitution of the latter positively charged amino acids for the former neutral ones in the CDR–H3 repertoire. These findings document the power of the mechanisms used to regulate Dλi RF choice (7, 9, 10). Maintenance of the terminal coding sequence of DFL16.1 (Fig. 1) preserved the ability to undergo microhomology-directed DλiRF1→JHi recombination (9, 10). iD RF2 was placed in-frame with the upstream DFL16.1 ATG translation start site to permit Dμ protein production, which can invoke mechanisms of allelic exclusion (7, 9). The inclusion of termination codons in iD RF3 limited its usage. Rearrangement by deletion remained

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**Table I. Cell numbers in bone marrow and spleen of normal and mutant mice**

| Bone marrow | Total cells x 10⁶ | CD19+ x 10⁶ | B x 10⁶ | C x 10⁶ | D x 10⁶ | E x 10⁶ | F x 10⁶ |
|-------------|------------------|-------------|--------|--------|--------|--------|--------|
| WT          | 9                | 12.9 (0.3)  | 3.0 (0.2) | 2.6 (0.3) | 2.1 (0.3) | 19.0 (1.4) | 7.1 (0.4) | 3.9 (0.5) |
| ΔD-iD       | 12               | 11.6 (0.4)  | 2.1 (0.2) | 4.3 (0.6)e | 2.1 (0.3) | 13.6 (1.2)f | 4.9 (0.5)b | 1.9 (0.3)d |
| Spleen      | Total cells x 10⁶ | CD19+ x 10⁶ | B1a x 10⁶ | B1b x 10⁶ | B2 x 10⁶ | M x 10⁵ | M x 10⁵ |
| WT          | 8                | 53.9 (3.3) | 22.5 (1.0) | 1.1 (0.1) | 1.3 (0.1) | 2.2 (0.1) | 1.4 (0.1) | 14.5 (1.4) |
| ΔD-iD       | 10               | 42.3 (1.7)e | 14.3 (0.6)f | 1.2 (0.1) | 1.2 (0.0) | 1.2 (0.1)e | 1.9 (0.2)b | 7.8 (0.5)f |

| Peritoneal cavity | Total cells x 10⁶ | CD19+ x 10⁶ | B1a x 10⁶ | B1b x 10⁶ | B2 x 10⁶ |
|-------------------|------------------|-------------|--------|--------|--------|
| WT                | 10               | 31.0 (3.2)  | 11.5 (0.7) | 4.2 (0.4) | 1.4 (0.2) | 2.4 (0.3) |
| ΔD-iD             | 10               | 23.7 (1.4)h | 7.8 (0.6) | 1.9 (0.6) | 1.9 (0.1) | 2.8 (0.3) |

aTotal nucleated cells that excluded trypan blue. In the bone marrow, values shown are cell counts per femur (average cellularity of two femurs collected from each experimental animal) of paired 8-wk-old homozygous ΔD-iD (ΔD-iD) or homozygous wild-type (WT) littermate progeny of heterozygous ΔD-iD/WT BALB/c mice. The standard error of the mean is shown in parentheses. The number of cells in fractions B (CD19+CD43+HSA-BP-1−), C (CD19+CD43+HSA-BP-1−), D (CD19+CD43−IgM+IgD−), E (CD19−CD43−IgM−IgD−), and F (CD19−CD43−IgM−IgD−) was determined from the relative proportion of total cells. Cell counts per spleen of 8-wk-old homozygous ΔD-iD or wt littermate progeny of WT BALB/c mice. In the spleen, transitional T1 (CD19+AA4.1+slgM+CD23−), T2 (CD19+AA4.1+slgM+CD23−), and T3 (CD19+AA4.1+slgM+CD23−) splenic B cell subsets were described as described by Allman et al. (reference 46). Marginal zone (MZ, CD19+CD21+CD23−) and mature (M, CD19+CD21+CD23+) B cell subsets were determined as described by Oliver et al. (reference 47). In the peritoneal cavity, values shown are average cell counts per single peritoneal lavage of 8-wk-old homozygous ΔD-iD or WT littermates. The number of B1a (CD19+CD5+), B1b (CD19+Mac-1+CD5−), and B2 (CD19+Mac-1−CD5−) B cell subsets were determined as described by Oliver et al. (reference 47). In the peritoneal cavity, values shown are average cell counts per single peritoneal lavage of 8-wk-old homozygous ΔD-iD or WT littermates. The number of B1a (CD19+CD5+), B1b (CD19+Mac-1+CD5−), and B2 (CD19+Mac-1−CD5−) B cell subsets were determined as described by Oliver et al. (reference 47).

*P ≤ 0.05.

*P ≤ 0.001.

*P ≤ 0.0005.

*P ≤ 0.0001 versus BALB/c wild-type littermates.
favored over inversion. Together, these mechanisms maintained the preference for use of RF1 in spite of the change in the coding sequence of the mutant D_H. However, D-D fusion is a very rare event, and none of the 902 WT sequences with identifiable D_H contained a recognizable D-D fusion using our threshold of D_H identification. This suggests that D-D fusion would be unlikely to contribute “ameliorated” sequences at a higher rate than that observed for iD inversion.

Because sequences with WT characteristics generated by iD inversion or by a combination of terminal sequence loss and N addition were detected in early B cell progenitors, selection for these alternative sequences could have recreated a “WT” CDR-H3 repertoire in mature B cells. We had previously documented selection during development for a specific range of CDR-H3 lengths, amino acid content, and average hydrophobicity in both WT (14) and ΔD-DFL mice (12), thus this seemed a likely outcome in ΔD-iD. However, ΔD-iD RF1-encoded arginine, histidine, and asparagine CDR-H3 remained overrepresented at all stages of bone marrow B cell development examined, including the mature, recirculating CD19^+IgM^+IgD^+ pool. Somatic selection did not shepherd the repertoire toward the WT range, even though the end result was a reduction in mature B cell numbers and antibody production. These data suggest that the somatic mechanisms normally used to select the repertoire can be subordinate to evolutionary conservation of germline sequence in regulating CDR-H3 content even though B cell function may suffer.

Early B cell development in the ΔD-iD mice followed a pattern similar to that observed in ΔD-DFL mice, which are limited to a single D_H of normal, tyrosine- and glycine-enriched sequence (12). This pattern included an accumulation of fraction B pro-B cells, normalization of the number of fraction C early pre-B cells, and a reduction in fraction D late pre-B and fraction E immature B cell numbers. The decreased efficiency with which developing ΔD-iD B cells transit through the bone marrow appears to reflect the loss of D_H locus sequence rather than the change in the sequence of the remaining D_H.

A potential mechanism that could have affected early repertoire development is incomplete access or altered use of the full germline array of V_H and J_H gene segments. We chose V_H7183 as the representative V_H family because all of the family members encoded by the IgH allele have been defined previously (20), key patterns of individual V_H7183 gene segment utilization are well established (20–22), it accounts for a manageable 10% of the active repertoire (23), and V_H7183 gene segments have been shown to contribute to both self- and nonself-reactivities (24, 25).

With a few limited exceptions, V_H7183 gene segment usage appeared minimally affected by the central coding sequence of D_H or by the global alteration of CDR-H3 loop amino acid content.

Compared with WT, J_H4 usage was depressed and the use of J_H1 was enhanced. This pattern matched that previously observed among ΔD-DFL sequences, suggesting that the
alteration in JH usage was independent of the change in the coding sequence of the single, remaining D\H{52} (12). JH\H{4} usage has been previously shown to be diminished in VDJ rearrangements from mice lacking DQ52 together with cis-regulatory sequence immediately upstream of this JH-proximal gene segment (26). Together, these data suggest that the alteration in JH usage is the result of the deletion of the remainder of the DH locus, perhaps, as previously suggested (26), as a result of a loss of the ability to engage in secondary D-J rearrangements.

Efficient transition from fraction B, primarily pro–B cells, to fraction C, early pre–B cells, is associated with successful creation of a functional H chain (13). Accumulation of pro–B cells has been observed in the context of a deletion of DFL16.1 through JH\H{1}, inclusive, in C57BL/6 mice (27) and in the context of the \Delta D-DFL deletion in BALB/c (12).

Preliminary analysis of hybridomas obtained from \Delta D-DFL BALB/c mice has shown that the nonfunctional \Delta D-DFL allele is frequently found in a germline, unrearranged state (12). Inefficiency in the initiation, progression, or completion of D\H{}→J rearrangement as a result of D H locus deletion may be the cause of the relative increase in the number of fraction B cells as well as in the alteration of JH usage.

Progression from fraction C, early pre–B cells, to fraction D, late pre–B cells, requires successful assembly of a pre–B cell receptor (28). Potential mechanisms for the decrease in the absolute number of cells in fraction D include an impaired ability to associate with surrogate light chain, as well as altered reactivity of the pre-BCR, including auto-reactivity. Passage to fraction E, which contains immature B cells, then requires both in-frame light chain rearrangement and successful association of the rearranged L chain with its H chain.

The geometric mean concentrations of anti-DEX IgM and Ig\L{} antibody titers 7 d after immunization were 10 μg/ml [4–23] vs. 79 μg/ml [43–143], and 11 μg/ml [2–63] vs. 83 μg/ml [51–134], respectively; P < 0.01. Titors in preimmune sera were <1 μg/ml; not depicted. (C) The primary T-dependent IgG response to NP19-CGG in homozygous \Delta D-iD mice is diminished when compared with WT littermates. **, P < 0.01; ***, P < 0.001. (D) TT-specific antibody responses in \Delta D-iD mice. 24 homozygous \Delta D-iD and 24 WT littermate controls were each orally immunized with 250 μl of 5 × 10^8 rSalmonella-ToxC. 4 wk later, plasma samples were collected and subjected to TT-specific ELISA. The data are shown as the reciprocal log2 titer. For \Delta D-iD the mean IgM and IgG anti-TT titers were 9.1 ± 0.2 and 15.1 ± 0.5, respectively; whereas, for WT IgG the mean titer were 9.3.1 ± 0.2 and 17.5 ± 0.3 (P = 0.64 and P = 0.0004, respectively).
partner (13). Fraction E cells may lose surface IgM expression during receptor editing or may be released from the bone marrow to undergo maturation in the periphery, which is associated with surface coexpression of IgD. Potential mechanisms for the decrease in the absolute numbers of cells in fraction E include failure to undergo proper L chain rearrangement, leading to (a) a block in the progression from D to E; (b) a more rapid progression from E to the transitional cell population in the periphery as a result of enhanced success of H-L partnering; or, conversely, (c) enhanced receptor editing (29) as the result of functional failure of H-L partnering, causing inflation in the numbers of cells identified as fraction D. This latter scenario seems less likely because fraction D numbers were also depressed. Formal testing of these hypotheses will require kinetic evaluation of developing B cell populations in both ΔD-DFL and ΔD-iD mice (30).

Although the initial pattern of B lineage cell production in ΔD-iD proved similar to that observed in ΔD-DFL, a striking divergence in B cell numbers was observed among the splenic follicular and MZ populations, and among recirculating B cells in the bone marrow. The decrease in numbers in the ΔD-iD T3 transitional population paralleled the reduction in mature, follicular B cells. It has been hypothesized that the antibody repertoires expressed by mature B cells in the follicles and MZ of the spleen are shaped by selection for antigen specificity (31), including negative selection of B cells bearing autoreactive antibodies (32) and positive selection in both the recirculating mature B cell pool and the MZ (31, 33–35). Highly charged CDR-H3 sequences, especially long arginine-containing CDR-H3, are thought to be more likely to generate self-reactive antibodies (36, 37); and preliminary studies indicate that IgG immunoglobulin CDR-H3 repertoires in the spleen is a current focus of investigation.

In the peritoneal cavity, the numbers of D-iD T3 transitional population paralleled the reduction in mature, follicular B cells. It has been hypothesized that the antibody repertoires expressed by mature B cells in the follicles and MZ of the spleen are shaped by selection for antigen specificity (31), including negative selection of B cells bearing autoreactive antibodies (32) and positive selection in both the recirculating mature B cell pool and the MZ (31, 33–35). Highly charged CDR-H3 sequences, especially long arginine-containing CDR-H3, are thought to be more likely to generate self-reactive antibodies (36, 37); and preliminary studies indicate that IgG anti-DNA antibodies are more common in the sera of young ΔD-iD mice than WT (unpublished data). It is possible that the increase in MZ cell numbers and the decrease in follicular and recirculating mature B cell numbers reflect a redirection of ΔD-iD B cells as a consequence of altered patterns of autoreactivity. Analysis of the extent and quality of self-reactivity among ΔD-iD B cells is being actively pursued in our laboratory.

Although it remains unclear whether the T3 subset consists of precursors to the mature, follicular compartment, these data would suggest that the cells in this compartment have suffered the same selective block as that experienced by splenic mature B cells. The sequence composition of immunoglobulin CDR-H3 repertoires in the spleen is a current focus of investigation.

In the peritoneal cavity, the numbers of ΔD-iD B1a and B1b cells are reduced. This effect is most pronounced for the B1a population, which is enriched for sequences derived from perinatal progenitors that tend to be more heavily influenced by germline sequence as the result of diminished N addition (13). The ΔD-iD B2 population, which is derived from the same pool of conventional B cells that circulate through the blood and into the spleen and bone marrow (13), achieved slightly higher absolute numbers than WT. It is unclear whether this represents a compensatory increase in numbers as a result of an increase in available physiologic space or whether the altered repertoire facilitates homing into the peritoneal cavity. Kinetic studies to address this question are currently ongoing in our laboratory.

A preference for tyrosine and glycine in the CDR-H3 loop is common to jawed vertebrates (8). Tyrosine is 10-fold overrepresented in CDR-H3 repertoires when compared with protein sequence in general, and tyrosine and glycine typically provide approximately 4 out of every 10 amino acids in the CDR-H3 loop (38). Raaphorst et al. (39) have proposed that use of highly charged sequence in CDR-H3 might reduce H chain stability. By extension, charged sequence could also have adversely affected binding to surrogate light chain or L chain. Persistence of highly charged sequences through the mature B cell stage in the ΔD-iD mice described in this paper suggests that structural stability is unlikely to be the primary force driving the preservation of tyrosine and glycine in CDR-H3.

Another hypothesis to explain preference for tyrosine and glycine in CDR-H3 is that repertoires enriched for these amino acids might facilitate achievement of optimal humoral immune responses to antigen (8, 40, 41). Although homozygous ΔD-iD mice expressed normal serum concentrations of IgM and IgA, serum levels of all four IgG subclasses were reduced, indicating that the ability of homozygous ΔD-iD B cells to respond properly to a broad range of antigens might have been compromised. We tested this hypothesis by challenging the mice with DEX, a T cell–independent antigen, and with NP19-CGG (17, 42) and rSalmonella-Tox C (19, 43), both T cell–dependent antigens. After immunization, IgM titers to DEX and IgG titers to NP and TT were significantly lower than controls. Tyrosine and glycine in CDR-H3 may be required for production of efficient antigen-binding sites. Correlations between the change in CDR-H3 amino acid content to the antigen specificity or affinity of their host immunoglobulin will require in vitro production of representative antibodies followed by analysis of their structures and antigen-binding characteristics.

Our studies indicate that the sequence of D is RFI establishes CDR-H3 amino acid usage. Somatic selection was not sufficient to recreate a "primary" repertoire enriched for tyrosine and glycine, even though B cell and antibody production was impaired. We conclude that there are limits to the power of somatic selection of the expressed repertoire that require prior selection on a genomic or evolutionary scale. Thus, it might be said that the D locus of “diversity” gene segments can also be considered a locus of “delimiting” elements, wherein the D gene segment serves not only to diversify but also to constrain the composition of CDR-H3 into a range more likely to yield optimal immune function.

MATERIALS AND METHODS

Generation of targeted ES cells and the ΔD-iD mouse. The R1-2 charon phage containing the BALB/c DFL16.1 locus was a gift from Y. Kurosawa (Fujita Health University, Toyoake, Japan) (7). An 800–base pair BglII fragment containing DFL16.1 was modified by PCR-based site-directed
mutagenesis. Nost and KpnI cloning sites were inserted 50 base pairs downstream of the 3' recombination signal sequence and the central portion of DEL1.1 was replaced by inverted DSP2.2 sequence. Creation of the ID mutant was confirmed by DNA sequencing. Additional germline sequence was added 5' and 3' of the modified IgHl fragment, creating a targeting construct with a 4.2-kb 5' long arm and a 0.7-kb 3' short arm. A $laxP-neo-loxP$ cassette was inserted into the Nost–KpnI cloning sites and an HSV-TK selection cassette was inserted into the tip of the long arm.

ESDJQ2-KO, derived from WT BALB/c-1 ES cells of the IgH haplotype, had been targeted to insert a $laxP$ site in lieu of a 240-base pair XhoI–SacI fragment containing the $DQ52$ gene and a putative 5' cis-regulatory element (26). Using standard protocols (44), this cell line was transfected with Ascl-linearized $ID$ targeting vector ($p$HWS84). One ES clone was identified by Southern blot analysis and DNA sequencing to contain the $ID$ gene in cis with the $DQ52$-deleted mutation and was injected into C57BL/6J blastocysts. Two $ID$ chimeric males were bred to yield BALB/cJ $iD$ mice, and two WT sibling pairs (8 wk old) were immunized intravenously with 100 $\mu$g of Dextran B-1355S in saline (a gift from T.I. Novobrantseva, Center for Blood Research, Boston, MA) precipitated in potassium aluminum sulfate (alum) in saline. Mice were bled from tail veins weekly.

Quantitative anti-DEX and anti-NP ELISA assays were performed using DEX- and NP-BSA (gifts from J.F. Kearney) or unimmunized 8-wk-old animals as controls (Southern Biotechnology Associates, Inc.).

To compare TT-specific antibody responses between $\Delta D$-iD and WT littermates, a recombinant Salmonella typhimurium BRD 847 (aroA, arsD) expressing the Tox C fragment of TT (rSalmonella-Tox C) was used (19). Cohorts of 24 mice were given a primary oral dose of 5 $\times$ 10$^6$ rSalmonella-Tox C at day 0. Plasma samples were collected at day 28 for the analysis of TT-specific IgG antibodies by ELISA. Figs. S1–S4 give representative FACS profiles for phenotypic differentiation of B lineage, bone marrow, spleen, and peritoneal cavity cells in homozygous $\Delta D$-iD and WT mice.

**Immunizations.** To determine basal levels of immunoglobulin isotypes in immunized 8–wk-old $\Delta D$-iD and WT littermates, class-specific unlabeled and alkaline-phosphatase (AP)–labeled antibodies were used (Southern Biotechnology Associates, Inc.).

For the DEX response, homozygous $\Delta D$-iD and WT littermates (9–12 wk) were immunized intravenously with 100 $\mu$g of Dextran B-1355S in saline (a gift from J.F. Kearney) and bled 7 d later. For the NP-CGG response (45), homozygous $\Delta D$-iD and WT littermates (16 wk) were immunized intraperitoneally with 10 $\mu$g of NP$_3$-CGG (gift from T.I. Novobrantseva, Center for Blood Research, Boston, MA) precipitated in potassium aluminum sulfate (alum) in saline. Mice were bled from tail veins weekly.

Quantitative anti-DEX and anti-NP ELISA assays were performed using DEX- and NP-BSA (gifts from J.F. Kearney) or unimmunized 8-wk-old animals as controls (Southern Biotechnology Associates, Inc.).

To compare TT-specific antibody responses between $\Delta D$-iD and WT littermate controls, a recombinant Salmonella typhimurium BRD 847 (aroA, arsD) expressing the Tox C fragment of TT (rSalmonella-Tox C) was used (19). Cohorts of 24 mice were given a primary oral dose of 5 $\times$ 10$^6$ rSalmonella-Tox C at day 0. Plasma samples were collected at day 28 for the analysis of TT-specific IgG antibodies by ELISA. Figs. S1–S4 give representative FACS profiles for phenotypic differentiation of B lineage, bone marrow, spleen, and peritoneal cavity cells in homozygous $\Delta D$-iD and WT mice.

**RNA, RT-PCR, cloning, and sequencing.** Total RNA was prepared from 1–2 $\times$ 10$^6$ cells of each individual Hardy fraction, sorted directly into RLT lysing buffer, using the QIAGEN RNAeasy mini-kit. 30% of the total RNA preparation was used to synthesize first-strand cDNA that was primed with primer CPr1 (5'-GACAGGGGCTCTCGG-3') using AMV reverse transcriptase (Roche Molecular Biochemicals) at 42°C for 1 h. 15% of the cDNA was used to amplify V(D)Jc joint junctions using the QIAGEN Tag PCR Core Kit and the manufacturer's recommended protocol under the following conditions: 95°C denaturation for 2 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final 72°C extension for 10 min. The reaction buffer contained 100 mM Tris–HCl, pH 8.8, 15 mM MgCl$_2$, and 750 mM KCl. Primers used were AF303 (5'-GGGGGCTCCAG-GAGTCTGGGGGA-3'), specific for framework 1 of the $\mu$H1783 family (20), and the C$$\mu$$ exon 1 primer CPr2 (5'-CAGGATCGAGGGGGAAGACATT-GG-3'). PCR products were cloned (TOPO-TA Cloning Kit; Invitrogen) and sequenced using the primer CPr2 and Big Dye chemistry (ABI 377; Applied Biosystems).

**Sequence analysis of CDR-H3.** Gene segments were assigned according to published germline sequences for the IgH gene segments as listed in the ImMunoGeneTics database (http://imgt.cines.fr:8104). The CDR3 of the
immunoglobulin heavy chain was defined to include those residues located between the conserved cysteine (C92) of FR3 and the conserved tryptophan (W103) of FR4. Average hydrophobicity of CDR-H3 was calculated as described previously (13). The sequences reported in this paper have been placed in the GenBank database (accession nos. AY205614–AY205988 and DQ226217–DQ226509).

Statistical analysis. Population means (flow cytometry, sequence data, and ELISA) were analyzed using an unpaired, two-tailed Student’s t test for populations that were normally distributed and with the nonparametric Mann-Whitney for those that were not. Titters against antigen are reported as the geometric mean and 95% confidence intervals. Frequencies within a population (sequence data) were analyzed using a two-tailed Fisher’s exact test. The overall frequencies of amino acids within groups of sequences were studied using a χ² test with 19 degrees of freedom. For frequencies of individual amino acids, a χ² test was performed. Analysis was performed with JMP IN version 5.1 (SAS Institute, Inc.). Means are accompanied by the standard error of the mean.

Online supplemental material. Figs. S1 and S4–S6 illustrate representative flow cytometric gates and analyses of bone marrow, spleen, and peritoneal B-lineage cells from homozygous ∆D-iD and WT littermates. Fig. S2 documents D₄H RF usage during B cell development. Fig. S3 presents the average length of CDR-H3 in VᵢL/VH183/D.createElement transcripts from homozygous ∆D-iD, ∆D-DFL, and WT mice in bone marrow B-lineage subsets. Table S1 lists the nucleotide sequences of CDR-H3 from homozygous ∆D-iD mice that contain an inverted D₄H gene segment. Table S2 presents the predicted amino acid sequences of CDR-H3 that contain an inverted D₄H gene segment. Table S3 presents the predicted amino acid sequences of CDR-H3 whose average Kyte-Doolittle hydrophobicity is less than −0.700. Table S4 presents the Predicted amino acid sequences of CDR-H3 with average Kyte-Doolittle hydrophobicity greater than +0.700. Also included in the supplemental material is an Excel spreadsheet containing the deconstructed nucleotide sequences of the CDR-H3 from homozygous ∆D-iD and littermate control bone marrow B-lineage cells. All online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052217/DC1.

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