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Terminal Deoxynucleotidyl Transferase Expression During Neonatal Life Alters D<sub>H</sub> Reading Frame Usage and Ig-Receptor-Dependent Selection of V Regions

Aaron J. Marshall,* Noelle Doyen,† Laurent A. Bentolila,† Christopher J. Paige,* and Gillian E. Wu*‡

During neonatal life, Ig diversity is limited in many respects. The absence of terminal deoxynucleotidyl transferase (TdT) expression with the consequent lack of nontemplated addition during the neonatal period, coupled with the predominant usage of a single D<sub>H</sub> reading frame (RF), leads to severe limitations of diversity in the CDR3 region of Ig heavy (H) chains. The neonatal Ig H chain repertoire is also characterized by restricted V<sub>H</sub> usage, with predominant expression of certain V<sub>H</sub> segments, such as V<sub>H</sub>81x, that are rarely evident during adult life. In this report, we examine the effect of enforced TdT expression on the neonatal repertoire of V<sub>H</sub>81xDJ<sub>H</sub> rearrangements. We find that TdT synthesis abrogates D<sub>H</sub> RF bias during the fetal/neonatal period through a Ig-receptor-independent mechanism. These findings suggest that D<sub>H</sub> RF bias during neonatal life is determined largely by homology-directed joining. We also find that TdT synthesis alters the selection of productively rearranged V<sub>H</sub>81xDJ<sub>H</sub> alleles in the neonatal spleen through a Ig-receptor-dependent mechanism. Analysis of predicted CDR3 amino acid sequences indicates that positive selection of V<sub>H</sub>81x-encoded H chains is correlated with the presence of a consensus sequence immediately adjacent to the V<sub>H</sub><sub>5</sub> segment. These data support the hypothesis that the CDR3 region is critical in determining the ability of V<sub>H</sub>81x-encoded H chains to form functional receptors that support positive selection of B lymphocytes. Together, our results demonstrate that TdT can indirectly influence the Ig repertoire by influencing both receptor-dependent and receptor-independent selection processes.


The ability of the immune system to respond to the vast array of potential pathogens encountered during the life of an organism depends on the diverse repertoire of Ag receptors expressed by B and T lymphocytes. Ag-receptor diversity is created in large part by the process of V(D)J recombination (1), in which separate DNA elements are imprecisely joined to form the variable domains of Ag-receptor genes. The enzymatic machinery underlying V(D)J recombination is being elucidated. Key molecules in this process are the recombination-activating genes RAG-1 and RAG-2, as well as other molecules not solely specific for V(D)J recombination, including the DNA-dependent protein kinase (DNA-PK) complex and XRCC4 (2, 3). Terminal deoxynucleotidyl transferase (TdT) is an auxiliary enzyme that is not essential for recombination but is responsible for the majority of nontemplated (N) nucleotide additions between the joining DNA segments (4–6). Generation of functional Ig heavy (H) and light (L) chain genes through V(D)J recombination occurs in a stepwise process during B cell development in the primary lymphoid organs (7).

A large body of evidence has accumulated indicating that the preimmune repertoire of Ig receptors is nonrandom in terms of both gene segment usage and characteristics of the DNA junctions (8–15). Bias in the recombination process and/or cellular selection through Ig receptors are thought to account for the nonrandom nature of the Ig repertoire; however, the details of these selection mechanisms and the relative impact of each type of selection mechanism on the Ig repertoire have not been determined. The Ig H chain repertoire displays two interesting nonrandom characteristics that have been particularly well studied: 1) unequal usage of D<sub>H</sub> reading frames (RF), and 2) overusage of the V<sub>H</sub>81x gene segment. D<sub>H</sub> elements can be read in three different frames (designated RF1, RF2, and RF3), and in both forward and inverted orientations depending on how they join with the J<sub>H</sub> element. However, examination of DJ<sub>H</sub> and VDJ<sub>H</sub> rearrangements made in vivo showed that most joins use RF1 in the forward orientation (12, 14, 16). Two nonexclusive hypotheses have been proposed to explain this bias in RF usage. Because DJ<sub>H</sub> rearrangements in RF2 can give rise to expression of truncated μ protein (DP; Ref. 17), one hypothesis is that cells making DJ<sub>H</sub> rearrangements in RF2 are counterselected through a D<sub>μ</sub> protein-surrogate L chain (SLC)-receptor complex (18–21). Together with the fact that DJ<sub>H</sub> rearrangements in RF3 frequently encode stop codons, this cellular selection mechanism could explain the predominant usage of RF1 in productive VDJ<sub>H</sub> rearrangements. The second hypothesis suggests that the presence of short sequence homologies in the 3′ end of D<sub>H</sub> elements and the 5′ end of J<sub>H</sub> elements directs the choice of recombination sites such that RF1 predominates (14, 16, 22). Because the

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3 Abbreviations used in this paper: TdT, terminal deoxynucleotidyl transferase; H, heavy; L, light; SLC, surrogate L chain; N, nontemplated; P, palindromic; RF, reading frame; Ig, transgenic; pre-BCR, pre-B cell receptor; P/NP, productive to nonproductive ratio; μmT, μ membrane exon-targeted.

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influence of sequence homologies on recombination site choice is most readily apparent in the absence of TdT (22), this mechanism might be expected to play a greater role in determining D<sub>H</sub> RF usage during fetal life, where TdT is absent, than adult life, where TdT is present (23).

The V<sub>H</sub> segment called V<sub>H</sub>81x has been shown to be highly overused in B cell precursors, but rarely used in mature B cells. V<sub>H</sub>81x is used in 20–40% of VDJ<sub>H</sub> rearrangements isolated from early B cell precursors, but in only 4–5% of rearrangements from mature, peripheral B cells (10, 24). During adult B cell development, this decline in usage of V<sub>H</sub>81x is accompanied by a progressive decrease in the ratio of productive to nonproductive V<sub>H</sub>81xDJ<sub>H</sub> rearrangements (25–28), suggesting that V<sub>H</sub>81x-encoded H chains are removed through cellular selection mechanisms. Consistent with this hypothesis, V<sub>H</sub>81x-encoded H chains often fail to associate with SLC proteins to form the pre-B cell receptor (pre-BCR) (29, 30), which provides signals essential for B cell differentiation (31, 32). Interestingly, this selective disfavoring of productive V<sub>H</sub>81xDJ<sub>H</sub> rearrangements does not appear to operate during fetal/neonatal B cell differentiation, where a high productive to nonproductive (P/NP) ratio of V<sub>H</sub>81xDJ<sub>H</sub> rearrangements is still observed in mature B cell populations (27, 33, 34).

Here we report studies designed to assess the impact of TdT synthesis on molecular and cellular selection mechanisms operating during the Ig repertoire development at the endogenous IgH locus. We use TdT-transgenic (tg) mice (35) to determine the effect of enforced TdT synthesis on the neonatal repertoire of V<sub>H</sub>81xDJ<sub>H</sub> rearrangements. We provide evidence that the presence of N addition in the neonate interferes with both molecular selection processes influencing D<sub>H</sub> RF usage and cellular selection processes influencing the P/NP ratio of V<sub>H</sub>81xDJ<sub>H</sub> rearrangements is still observed in mature B cell populations (27, 33, 34).

We isolated VDJ gene segments from neonatal spleens of the TdT mice and from fetal livers of the mT mice, which were maintained as a homozygous strain, and from mT bone marrow show a significantly different pattern of D<sub>H</sub> RF usage, with greater than 80% RF1 usage, confirming previous findings. How- ever, the V<sub>H</sub>81xDJ<sub>H</sub>4 junctional sequences revealed the expected presence of N addition in the Tg samples and near absence of N addition in the non-tg samples (Table I and Fig. 1). While the frequency of N addition in the TdT-tg neonatal spleens is similar to that observed in adult tissues, the average length of the additions is approximately twofold lower (Table I).

**Materials and Methods**

**Mice and DNA samples**

TdT-tg mice were generated as described (35) and were maintained by brother-sister matings at the Ontario Cancer Institute Animal Facility. As the generation of TdT-tg mice proved to be difficult, these data are based on a single TdT-tg line. Individual spleens were harvested at 1–2 days after birth, and DNA was prepared and typed for the presence or absence of the transgene by PCR, as described (35). Examination of the B lineage populations in neonatal and adult TdT-tg spleens by flow cytometry revealed no significant differences from controls (data not shown). DNA from two tg or three non-tg pups were pooled. We obtained µ membrane exon-targeted (µmT) mice (32) from the laboratory of Dr. Klaus Rajewsky (Institute for Genetics, Cologne, Germany) through Dr. Len Schultz (Jackson Laboratories, Bar Harbor, MN). Timed matings of homozygous µmT mice were conducted and fetal livers were harvested at day 16 of gestation (morning after mating = day 0). DNA was prepared from pooled livers from a single pregnancy. DNA was also prepared from bone marrow cells obtained from an adult (8-wk-old) µmT mouse.

We isolated VDJ gene segments from neonatal spleens of the TdT mice and from fetal livers of the µmT mice although it would have been as informative to keep the perinatal tissue the same. Because of breeding problems we were not able to obtain timed pregnancies for the TdT mice, whereas the µmT mice, which were maintained as a homozygous strain, proved to be relatively simple to obtain. However, because the information extracted from the perinatal µmT V<sub>H</sub>81x rearrangements depends only on the absence of N addition (determined by using perinatal tissue) and the absence of receptor-mediated selection (determined by the µmT mutation), we were satisfied that the information should not be influenced by which perinatal tissue was used.

**Amplification and sequencing of V<sub>H</sub>81xDJ<sub>H</sub> rearrangements**

V<sub>H</sub>81xDJ<sub>H</sub> rearrangements were amplified from the indicated DNA samples as described (34). Briefly, total VDJ<sub>H</sub> rearrangements were amplified for 30 cycles with a degenerate V<sub>H</sub> primer (V<sub>H</sub> all) and a J<sub>H</sub>4 primer, diluted 1/500 and then reamplified for 25 cycles with a V<sub>H</sub>81x-specific primer and an internal J<sub>H</sub>4 primer (J<sub>H</sub>4IN). The amplified V<sub>H</sub>81xDJ<sub>H</sub> rearrangements were then cloned using the TA cloning kit (Invitrogen, San Diego, CA), according to the manufacturer’s protocols. Randomly picked clones were then sequenced using the T7 sequencing kit (Pharmacia, Piscataway, NJ) in conjunction with a sequencing primer specific for the 3' end of V<sub>H</sub>81x (5'-GCAATACCAAGAAGACC-3').

**Results**

**Level of N addition in TdT-tg neonatal spleen**

To determine the effect of transgenic expression of TdT on the neonatal repertoire, we examined VDJ<sub>H</sub> rearrangements in TdT-tg and control neonatal spleen. Spleens were harvested from TdT-tg and non-tg littermates at 1–2 days after birth, and genomic DNA was extracted. VDJ<sub>H</sub> rearrangements using the V<sub>H</sub>81x gene segment and the J<sub>H</sub>4 gene segment were amplified from these DNA samples using PCR. The V<sub>H</sub>81x gene segment was chosen for these studies because its genomic sequence is known (10), allowing the accurate identification of N regions, and because productively rearranged V<sub>H</sub>81xDJ<sub>H</sub> alleles are subject to interesting selection processes (25, 26, 28, 33, 34, 36), which we speculated might be influenced by TdT expression. The amplified rearrangements were cloned into a plasmid vector, and randomly selected clones were sequenced (Fig. 1).

Examination of the V<sub>H</sub>81xDJ<sub>H</sub>4 junctional sequences revealed the expected presence of N addition in the Tg samples and near absence of N addition in the non-tg samples (Table I and Fig. 1). While the frequency of N addition in the TdT-tg neonatal spleens is similar to that observed in adult tissues, the average length of the additions is approximately twofold lower (Table I).

**TdT expression in the neonate alters D<sub>H</sub> RF usage through an Ig-receptor-independent mechanism**

In VDJ<sub>H</sub> rearrangements isolated from both neonatal and adult mouse tissues, D<sub>H</sub> elements are most often found to be joined to the J<sub>H</sub> element such that they would be read in the RF designated RF1 (12, 14, 16). We compared D<sub>H</sub> RF usage in V<sub>H</sub>81xDJ<sub>H</sub> rearrangements isolated from TdT-tg and non-tg neonatal spleen (Fig. 1). Rearrangements isolated from neonatal spleen show greater than 80% RF1 usage, confirming previous findings. However, the V<sub>H</sub>81xDJ<sub>H</sub>4 rearrangements from TdT-tg neonatal spleen show a significantly different pattern of D<sub>H</sub> RF usage, with <30% using RF1 (Fig. 1). This result suggests that the presence of TdT interferes with the process(es) which establish biased D<sub>H</sub> RF usage during neonatal life.

Although the effect on RF usage is apparent among nonproductive rearrangements (Fig. 1), it is still possible that TdT is influencing selection at the level of the D<sub>H</sub> receptor expressed from DJ<sub>H</sub> rearrangements in RF2 (17–21). To distinguish Ig-receptor-independent effects from receptor-dependent effects we compared D<sub>H</sub> RF usage in V<sub>H</sub>81xDJ<sub>H</sub>4 rearrangements isolated from µmT fetal liver or bone marrow cells (Figs. 2 and 3). These populations provide a sample of rearrangements generated in the absence or presence of TdT, respectively, that have not been influenced by Ig-receptor-mediated selection processes. It was found that >80% of the rearrangements from µmT fetuses use RF1 (Fig. 3), suggesting that establishment of D<sub>H</sub> RF bias during fetal/neonatal life is not dependent on receptor-mediated selection processes. In contrast, rearrangements isolated from µmT bone marrow show a
more random pattern of D\(\text{H}\) RF usage (Fig. 3), as shown previously (18). These data indicate that TdT activity results in the alteration of D\(\text{H}\) RF usage by an Ig-receptor-independent mechanism. Neonates with TdT activity have an altered selection of productive V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements that occurs through an Ig-receptor-dependent mechanism.

Selection of productive V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements differs in fetal and adult B cell progenitors in that fetal cells positively select these rearrangements while adult cells negatively select these rearrangements (27, 33, 34). Because TdT represents one gene known to be differentially expressed in fetal and adult B cell progenitors (23), we hypothesized that TdT may be a critical factor determining this differential selection of productive V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements. If this were the case, we would expect the P/NP of V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements in TdT-tg neonatal spleen to be different from in non-tg littermates. Indeed, when we compared the P/NP of V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements from non-tg neonatal spleens to a high P/NP (2.4), as observed previously (33, 36). However, V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements from TdT-tg neonatal spleens exhibit a significantly lower P/NP (0.32), indicating a disruption of the positive selection for productive rearrangements. Thus, synthesis of TdT does appear to be a critical factor in determining the selection of functional V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements.

We next examined whether the effect of TdT synthesis on selection of productive V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements is due to an Ig-receptor-dependent or Ig-receptor-independent mechanism.

**FIGURE 1.** V\(\text{H}\) 81xDJ\(\text{H}\) junctional sequences isolated from neonatal spleens of TdT-tg or control mice. DNA was prepared from neonatal spleens harvested from 1- to 2-day-old TdT-tg mice or littermate controls. V\(\text{H}\) 81xDJ\(\text{H}\) junctional sequences were amplified by PCR and sequenced. Identical sequences isolated from the same sample are only listed once. Sequences are divided into productive and nonproductive using the criteria that productive rearrangements must have the V\(\text{H}\) and J\(\text{H}\) elements in frame and no stop codons in the CDR3. Nucleotides printed in bold type represent potential palindromic (P) additions (43). Underlined sequences could have come from either the D\(\text{H}\) or the J\(\text{H}\) element and therefore represent homology overlaps.

**FIGURE 2.** V\(\text{H}\) 81xDJ\(\text{H}\) junctional sequences isolated from fetal liver or bone marrow of μT\(\text{M}\) mice. Sequences were generated and presented as in Fig. 1. When deletion of the V\(\text{H}\) or J\(\text{H}\) element is more than the sequences shown in the figure, the number of nucleotides deleted is shown in place of the V\(\text{H}\) or J\(\text{H}\) sequence.

**Table I.** Junctional diversity of V\(\text{H}\) 81xDJ\(\text{H}\) rearrangements isolated from various mice and tissues

<table>
<thead>
<tr>
<th>Sample</th>
<th>N Addition V-D</th>
<th>N Addition D-J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control NS</td>
<td>8.3</td>
<td>2.5</td>
</tr>
<tr>
<td>TdT-tg NS</td>
<td>60.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Adult spleen</td>
<td>86.4</td>
<td>4.6</td>
</tr>
<tr>
<td>μT(\text{M}) FL</td>
<td>11.5</td>
<td>1.3</td>
</tr>
<tr>
<td>μT(\text{M}) BM</td>
<td>92.9</td>
<td>4.8</td>
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<tr>
<th>Sample</th>
<th>N Addition V-D</th>
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<tr>
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<tr>
<td>μT(\text{M}) FL</td>
<td>11.5</td>
<td>1.3</td>
</tr>
<tr>
<td>μT(\text{M}) BM</td>
<td>92.9</td>
<td>4.8</td>
</tr>
</tbody>
</table>

μT\(\text{M}\) Day 16 Fetal Liver

<table>
<thead>
<tr>
<th>Sample</th>
<th>N Addition V-D</th>
<th>N Addition D-J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control NS</td>
<td>8.3</td>
<td>2.5</td>
</tr>
<tr>
<td>TdT-tg NS</td>
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<tr>
<td>μT(\text{M}) BM</td>
<td>92.9</td>
<td>4.8</td>
</tr>
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</table>
more frequently give rise to functional receptors. Therefore, we mined by the CDR3 sequence in such a way that N-less sequences of rearrangements using each of the three D H RFs was determined for the ability of V H 81x to generate functional receptors is determined from only the nonproductive rearrangements to minimize bias due to cellular selection based on functional H chains; however, a similar pattern can be observed among productive rearrangements (see data in Figs. 1 and 2).

thus examined V H 81xDJ H rearrangements isolated from μMT fetal liver or bone marrow to determine the P/NP generated in the absence or presence of TdT without the influence of Ig-receptor-mediated selection (Fig. 4). It was found that the rearrangements from μMT fetal liver and bone marrow both had a P/NP close to that which would be expected from random joining (0.3–0.5), indicating that the presence of TdT activity does not significantly alter the P/NP ratio of V H 81xDJ H structures in the absence of Ig-receptor-mediated selection. Rearrangements from μMT fetal liver had a significantly lower P/NP than in non-tg neonatal spleen (Fig. 4), indicating that the high P/NP in normal neonates is dependent on Ig-receptor-mediated selection. In contrast, the P/NP observed in TdT-tg neonatal spleen was similar to that in μMT fetal liver, indicating that the Ig-receptor-mediated selection occurring in the neonatal spleen is largely abrogated by TdT activity. Together, these results suggest that TdT activity results in an alteration the P/NP ratio of V H 81xDJ H structures by influencing an Ig-receptor-dependent selection process.

Role of the CDR3 sequence in Ig-receptor-dependent selection of productive V H 81xDJ H rearrangements

The observations on the effect of TdT activity on receptor-dependent selection of productive V H 81xDJ H rearrangements suggest that ability of V H 81x to generate functional receptors is determined by the CDR3 sequence in such a way that N-less sequences more frequently give rise to functional receptors. Therefore, we examined the productive V H 81xDJ H rearrangements positively selected in the neonatal spleen to determine whether they exhibit any restrictions in CDR3 amino acid sequence that could be attributed to receptor-mediated selection (Fig. 5). We observed a clear conservation in the four amino acids immediately adjacent to the V H 81x gene segment (positions 95–98 of the H chain; Ref. 37). All (100%) of the sequences contained a histidine residue at the first position adjacent to the V H 81x segment (position 95). The amino acids at positions 96–98 also show striking conservation, with hydrophobic amino acids being nearly absent and glycine, tyrosine, asparagine, and serine comprising approximately 80% of the amino acids. Notably, positions 96 and 98 consist mainly of glycine, tyrosine, and serine, while position 97 differs in that 41% of sequences contains asparagine residues with the remainder mainly containing tyrosine or serine. By tabulating the most frequent amino acids at positions 95–98, a degenerate consensus sequence can be derived (Fig. 5). Over half (58.8%) of the sequences from non-tg neonatal spleen conform to this consensus sequence (marked with an asterisk in Figure 5) with most of the remaining sequences containing only a single nonconsensus amino acid. As expected, this consensus sequence is present at a lower frequency (16.7%) in TdT-tg neonatal spleen (Table II).

To determine the extent to which the sequence conservation identified is dependent on receptor-mediated selection, we determined five samples with which the consensus sequence occurs among productive V H 81xDJ H sequences from μMT fetal liver or bone marrow (Fig. 6A). As another measure of conformity to the consensus sequence, the percent of amino acids deviating from the consensus sequence defined in Fig. 5 was also determined for the various samples (Fig. 6B). None of the sequences from μMT bone marrow contained the consensus CDR3 sequence, suggesting that generation of this sequence is rare in the presence of TdT. In contrast, the consensus sequence was present among the sequences from μMT fetal liver; however, the frequency of rearrangements containing the consensus appears lower than that in the normal neonatal spleen (33.3% vs 58.8% of rearrangements; Fig. 6A), and the percent of amino acids deviating from the consensus is significantly higher (39% vs 15%; Fig. 6B). In addition, only 66.7% of the productive sequences from μMT fetal liver encode histidine at position 95 in contrast with 100% in the normal neonatal spleen.

Table II. Selection for a consensus CDR3 sequence among productive V H 81xDJ H rearrangements

<table>
<thead>
<tr>
<th>Samplea</th>
<th>Frequency of Sequences with Consensus CDR3</th>
<th>Percent of Amino Acids Deviating From Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control NS</td>
<td>10/17 (58.8%)</td>
<td>10/68 (14.7%)</td>
</tr>
<tr>
<td>TdT-tg NS</td>
<td>1/6 (16.7%)</td>
<td>11/24 (45.8%)</td>
</tr>
<tr>
<td>μMT FL</td>
<td>3/9 (33.3%)</td>
<td>14/36 (39.9%)</td>
</tr>
<tr>
<td>μMT BM</td>
<td>0/4 (0.0%)</td>
<td>10/16 (62.5%)</td>
</tr>
<tr>
<td>Normal BM pre-Bb</td>
<td>0/7 (0.0%)</td>
<td>15/28 (53.6%)</td>
</tr>
<tr>
<td>Normal Adult Spleend</td>
<td>2/5 (40%)</td>
<td>6/20 (30%)</td>
</tr>
</tbody>
</table>

a NS, neonatal spleen; FL, fetal liver; BM, bone marrow.
b Frequency of productively rearranged sequences conforming to the degenerate amino acid sequence defined in Fig. 5.
c The percent of amino acids that deviate from the degenerate consensus defined in Fig. 5.
d Spleen sequences taken from Refs. 26–29.
Thus, while the consensus sequence is more frequently generated in the absence of TdT activity than in the presence of TdT activity, there also appears to be a receptor-mediated selection for this sequence in the neonatal spleen.

Compilation of published V<sub>H</sub>81xD<sub>J</sub>4 sequences from adult bone marrow pre-B cells or adult spleen (25–28, 33, 34) provides further evidence for receptor-mediated selection of the consensus sequence. Productive V<sub>H</sub>81xD<sub>J</sub>4 sequences derived from normal bone marrow pre-B cell populations confirm that the consensus sequence is rarely generated in the presence of TdT (0/8 sequences). However, 40% (2/5) of the productive sequences from adult spleen have the consensus sequence, despite the rare occurrence of these rearrangements in the bone marrow. The overall percent of amino acid deviations from consensus is also lower in the adult spleen sequences than in the bone marrow pre-B cell sequences (30% vs 56.3%). Together with our results, these data indicate that receptor-mediated selection results in a skewing of CDR3 sequences immediately adjacent to the V<sub>H</sub> segment toward a hydrophilic consensus sequence.

Discussion

It is well established that TdT plays an important role in generation of the Ig repertoire by giving rise to nontemplate-encoded nucleotides at the junctions between joining V<sub>H</sub>, D<sub>H</sub> and J<sub>H</sub> segments (4–6). The differential regulation of N addition during fetal and adult B cell development thus provided compelling evidence for a specific molecular mechanism to “program” different Ig repertoire during fetal and adult life (12, 16). Here we demonstrate that this differential TdT expression has far reaching implications for Ig H chain repertoire generation, which go beyond the direct effect of catalyzing N nucleotide addition. With the caveat that the data emerged from a single mouse line, our results indicate that TdT activity affects the selection process at at least two stages: at the stage of V(D)J joining and at the stage of receptor-mediated selection.

Regulation of N addition during fetal and adult life

Our study uses mice expressing TdT under the control of the N-myc promoter and the E<sub>m</sub>e enhancer (35), which has been shown to give high level expression in lymphoid cells throughout their development (38). However, the overall level of N addition in the TdT<sup>tg</sup> neonatal spleen (Table I) and TdT-tg fetal liver (35) is significantly less than observed in normal adult spleen. Receptor-mediated selection against N-region-containing sequences cannot account for this difference because it is apparent among both productive and nonproductive rearrangements (Fig. 1). Thus, this

**FIGURE 5.** Analysis of CDR3 amino acid sequences in productive V<sub>H</sub>81xD<sub>J</sub>4 rearrangements. Predicted CDR3 amino acid sequences were determined from the DNA sequences in Fig. 1 using the DNA Strider program. Position 1 of the defined consensus sequence is derived partially from the 3' end of the V<sub>H</sub> segment, while positions 2 and 3 are generally encoded by the D<sub>H</sub> segments, and position 4 is encoded by either the D<sub>H</sub> or J<sub>H</sub> element, depending on the CDR3 length. Sequences which fall within the degenerate consensus at all four positions are marked with an asterisk.

Nonconsensus amino acids are displayed in bold. Amino acid positions are numbered according to Ref. 37.
lower level on N addition appears to result from lower TdT activity. It remains to be determined whether this is simply due to lower expression of TdT in the Ig B cell progenitors or to differential posttranscriptional regulation of TdT activity in fetal vs adult B cell progenitors.

During fetal B cell development, D_H RF bias is established through a receptor-independent TdT-sensitive mechanism

During B cell development in the adult bone marrow, it has been shown that D_H RF bias, and specifically suppression of RF2, is dependent on signaling through the D_{H} protein/SLC complex (18–21). In contrast, our data indicates that, during fetal B cell development, D_H RF bias is established independently of expression of \( \mu \)-related proteins on the cell surface. Instead, it appears that during the fetal/neonatal period D_H RF bias is established at the level of V(D)J recombination by a mechanism that requires the absence of TdT. These results provide supporting evidence for the homology-directed joining model as the mechanism determining biased RF usage during fetal/neonatal life (14, 16, 22). Homology-directed joining has been shown to be essential for the establishment of “canonical” junctional sequences in the invariant \( \gamma^\delta \) TCR generated during fetal life, and generation of such receptors is largely abrogated in the presence of TdT (39). Thus, D_H RF bias appears to be a second clear example of the influence of homology-directed recombination in generation of the restricted fetal repertoire. Given the existence of the D_{H}-receptor-dependent mechanism for maintaining biased RF usage, it is surprising that this mechanism does not appear to maintain biased RF usage when homology-directed joining is disrupted in TdT-tg neonates. This could indicate that the D_{H}-receptor-dependent mechanism is only operative during adult B cell differentiation; however, additional experiments will be required to clarify this issue. To determine whether the finding that premature TdT expression interferes with normal neonatal D_H RF bias may be generalizable to other H chains, we reexamined our data of TdT-tg fetal liver sequences reported in (35). Of 16 informative V_{H}DJ_{H} sequences, eight had N additions, and eight did not. Of those without N, seven are in RF1 (88%); of those with N, three are in RF1 (38%). Thus, it may be a general finding that N addition changes the bias for RF1 usage.

Ig-receptor-dependent positive selection of V_{H}\,81x-encoded H chains is dependent on the CDR3 sequences

Several studies have indicated a striking difference in positive selection of V_{H}\,81x-encoded H chains by fetal and adult B cell progenitors (25, 27, 34). We previously put forward two hypotheses to explain this difference (34): 1) fetal and adult B cell progenitors have different requirements for Ig-receptor-mediated selection, or 2) selection of V_{H}\,81x-encoded H chains is dependent on the V_{H}-DJ_{H} junctional sequence (CDR3 region) in such a way that the absence of N addition during fetal life (23) more frequently generates V_{H}\,81x-encoded H chains which have the structure required for positive selection of B cell progenitors. A recent study (40) using an in vitro assay to assess the impact of various transgenic H chains on fetal vs adult B cell development drew the conclusion that fetal and adult B cell precursors differ in their H chain selection requirements, consistent with the first hypothesis. However, the present data provide strong in vivo evidence in support of the second hypothesis, showing that positive selection of V_{H}\,81x-encoded H chains during fetal life is virtually abrogated by transgenic expression of TdT. And, as discussed above, homology-directed joining is likely to be the mechanism responsible for those CDR3 regions that are productive. Thus, in the case of V_{H}\,81x, it appears that the fetal vs adult difference in TdT synthesis is sufficient to determine the fetal vs adult difference in selection of H chains.

Recent studies examining the ability of various \( \mu \) H chains to form a pre-BCR complex (29, 30, 41) provide a probable mechanism for the CDR3-dependent positive selection of cells bearing V_{H}\,81x-encoded H chains. The initial repertoire of H chains generated in the bone marrow was found to contain both H chains capable of forming a pre-BCR complex and H chains incapable of forming this complex, in approximately equal proportions (29). However, the large majority of H chains isolated from later stages of pre-B cell development are capable of forming a pre-BCR, indicative of a positive selection based on pre-BCR formation (29). Several V_{H}\,81x-encoded H chains were tested for the ability to form a pre-BCR complex, and it was found that all N-region-containing V_{H}\,81x-encoded H chains failed to form a complex with SLC (29, 30). To date, the only two V_{H}\,81x-encoded H chains (42) found to form a pre-BCR complex are N-less B cells isolated from fetal cells. Importantly, a transgene encoding a V_{H}\,81x H chain that has no N addition and that can form a pre-BCR (H.-M. Jack, unpublished observation) was shown to support B cell maturation (42), while a transgene encoding a V_{H}\,81x H chain that cannot form a pre-BCR failed to support B cell differentiation (30, 41). Thus, it appears plausible that the differential positive selection of V_{H}\,81x-encoded H chains with simple vs complex CDR3 regions is due to differential association with SLC.

Implications for pre-BCR formation and selection of the neonatal Ig repertoire

Interestingly, the capacity of different V_{H}\,81x-encoded H chains to bind SLC appears to correlate with the ability to bind conventional Ig L chains (41). This indicates that pre-BCR formation may serve as a broad screen for “functionality” of H chains in terms of their ability to fold correctly to form Ig-like receptors (30, 41). In light of this view, it is interesting that the “functionality” of H chains encoded by V_{H}\,81x appears to be extremely sensitive to variations in the CDR3 region. We speculate that H chain proteins encoded by V_{H}\,81x may be inherently unstable and only form a “functional” Ig fold when joined to sequences encoded by simple, conserved DJ_{H} joins that stabilize the Ig domain. This instability could be due to one or more of the unusual amino acid substitutions found in V_{H}\,81x (10). Thus, the V_{H}\,81x segment may be structurally specialized for selective expression in the TdT-negative repertoire generated early in life. It remains to be determined whether other such structurally specialized V_{H} segments exist in the mouse or human.

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