A Novel Mechanism for B Cell Repertoire Maturation Based on Response by B Cell Precursors to Pre-B Receptor Assembly

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Summary

The expression of different sets of immunoglobulin specificities by fetal and adult B lymphocytes is a long-standing puzzle in immunology. Recently it has become clear that production of immunoglobulin µ heavy chain and subsequent assembly with a surrogate light chain to form the pre-B cell receptor complex is critical for development of B cells. Here we show that instead of promoting pre-B cell progression as in adult bone marrow, this complex inhibits pre-B cell growth in fetal liver. Curiously, we identify a fetal-associated Vµ11 µ heavy chain that allows continued pre-B proliferation in fetal liver. Interestingly, this heavy chain does not associate efficiently with a surrogate light chain, providing a previously unrecognized mechanism for skewing the expression of distinctive Vµ genes toward fetal through early neonatal life.

Immune responses during the neonatal period show significant differences from those in the adult, with striking deficiencies in the ability to respond to certain antigens (1, 2), although the mechanism for this shift is not yet completely understood. Consistent with their restricted ability to respond to antigenic challenge, fetal and neonatal B cell precursors use a restricted set of heavy chain Vµ (lg heavy chain variable region) genes, preferentially from segments proximal to the Ig diversity element (D), such as VµB1X in mice (3, 4). The expansion from a restricted set of genes used by neonatal B cells to the wide variety employed in the adult (3–5) is referred to as repertoire maturation. Although the phenomenon has been appreciated for many years, the molecular and cellular mechanisms that result in this change are not yet understood. Since fetal B cells preferentially express Vµ segments proximal to D, ordered accessibility to recombination has been suggested as a possibility. Yet this cannot be the full explanation, since preferential rearrangement of D-proximal Vµ segments is also observed in B precursor cells in adult bone marrow (6). In addition, productive rearrangements of such genes predominate in fetal cells but become infrequent in adult precursors (7–9). Furthermore, some Vµ genes not proximal to D, such as Vµ11, also show expression biased to B cells generated from the fetal stage through the early neonatal stage (fetally/neonatally) (10, 11). These observations imply that mechanism(s) in addition to rearrangement accessibility, possibly the differential control of B cell development by particular Vµ genes, shape the distinctive repertoires of fetal and adult times.
differential response by fetal pre-B cells to SLC-μ provides a previously unrecognized mechanism for skewing the expression of distinctive V<sub>H</sub> genes toward fetal/neonatal life.

Materials and Methods

**Tg Mice.** A functionally rearranged V<sub>H</sub>11 gene was cloned from a CD5<sup>+</sup> B cell-derived hybridoma cell line (2-2G4) secreting an IgM antibody reacting with bromelain-treated mouse red blood cells (10). The rearranged gene, known to reside on a 4.8-kb EcoRI/EcoRI fragment, was ligated into an IgM antibody reacting with bromelain-treated mouse red blood cells (10). The rearranged gene, known to reside on a 4.8-kb EcoRI/EcoRI fragment, was ligated into the pICEM19H C<sub>μ</sub> plasmid vector. Appropriate V<sub>H</sub> orientation was established using several clones with appropriate restriction enzymes. The 17-kb V<sub>H</sub>11-C<sub>μ</sub> construct was gel isolated and used for microinjection. Litters so generated were screened by PCR amplification for rearranged V<sub>H</sub>11-1<sub>C</sub> constructs. The 4.8-kb founders were mated to C.B-17cr, allowing discrimination of the transgene C<sub>μ</sub> allele (g<sup>h</sup>) from the endogenous C<sub>μ</sub> (g<sup>h</sup>). Two lines, high copy BR 1 and low copy BR 5, were backcrossed more than six times to C.B-17cr and used for analyses. Human (H<sub>u</sub>) μ Tg mice originally generated by M. Nussenzweig (22) and Rag-1<sup>−/−</sup> mice generated by M. Nussenzweig (22) and Rag-1<sup>−/−</sup> mice were provided by M. Weigert (Princeton University, Princeton, NJ).

**Cell Staining and Culture.** Single cell suspensions of bone marrow (from 3-mo-old animals) or fetal liver (from animals at day 16 of gestation) were stained, analyzed, and sorted as previously described (17, 18) with anti-CD45R (B220) (allophycocyanin-6B2), anti-CD43 (fluorescein-S7), and anti-CD24/HSA (phycoerythrin-M1). Single cell suspensions of bone marrow (from 3-mo-old animals) or fetal liver (from animals at day 16 of gestation) were stained, analyzed, and sorted as previously described (17, 18) with anti-CD45R (B220) (allophycocyanin-6B2), anti-CD43 (fluorescein-S7), and anti-CD24/HSA (phycoerythrin-30F1). R analysis of sorted fractions consistently showed purities >95%. The FLST2 stromal line-dependent proliferation assay was performed as previously described (15, 17), except that cultures were supplemented with 100 U/ml of recombinant human IL-7 (gift of S. Gillis, Immunex Corp., Seattle, WA). 2–5 × 10<sup>5</sup> cells (CD4<sup>+</sup>/HSA<sup>+</sup>/B220<sup>−</sup>) were sorted per 1 ml well. Cells were harvested after 4 d, stained as above, and analyzed by flow cytometry.

**Immunoprecipitation Analysis.** Digitonin lysates prepared from 10<sup>7</sup>–10<sup>8</sup> cells were immunoprecipitated with antibodies to μ (M 41), λ<sub>5</sub> (LM 34-8E), VpreB (VP245), or κ (187, 1), and then proteins were resolved by SDS-10% PAGE as previously described (19). After electrotransfer to PVDF membranes (DuPont-NEN, Boston, MA), filters were blocked with milk protein, probed with horse-radish peroxidase-conjugated M41 rat anti–mouse μ mAb, washed, developed using Supersignal Substrate (Pierce, Rockford, IL), and exposed to R effection film (DuPont-NEN). In some experiments, μ immunoprecipitates were first deglycosylated by treatment with anhydrous trifluoromethanesulfonic acid according to the manufacturer’s protocol (“GlycoFree”; Oxford GlycoSystems, Bedford, MA) before electrophoresis. Freshly isolated thymocytes were stimulated for 5 min at 37°C with 0.1 mM pervanadate to induce ubiquitination of TCR-ζ (24). The TCR complex was immunoprecipitated from lysates of treated or untreated thymocytes using anti-TCR-β mAb, resolved by SDS-11% PAGE, and transferred to PVDF membranes. V<sub>H</sub>11-μ immunoprecipitate was loaded in an adjacent lane. Filters were immunoblotted with an antibi ubiquitin antibody (MAB1510; Chemicon International, Temecula, CA).

**Results and Discussion**

Transfection of μ heavy chain into pro-B cell lines results in the downregulation of TdT expression (19), providing a model system for investigating the effect of different V<sub>H</sub>11 segments on pre-BCR assembly. Expression of several μ heavy chains with different VH(V<sub>H</sub>D<sub>H</sub>) regions, such as SP6 and 3H9, in the pro-B cell line ret02/1 results in diminished message levels of TdT as well as Rag-1 (Fig. 1). These changes mimic those normally seen in the differentiation of pro-B cells to the early pre-B cell stage in vivo (18–21). However, not all μ heavy chains are equally efficient in this assay: transfectants with V<sub>H</sub>81X or V<sub>H</sub>11 μ chains do not show significant TdT or Rag-1 downregulation when compared to the parental line (Fig. 1). Since V<sub>H</sub>81X μ chains often do not associate well with λ<sub>5</sub> (27), it was reasonable to ask whether pre-BCR assembly was inefficient in V<sub>H</sub>11 transfectants.

To assess the extent of pre-BCR assembly with V<sub>H</sub>11 μ, we performed immunoprecipitation experiments SDS-PAGE analysis revealed that the V<sub>H</sub>11 μ protein expressed in transfected cell lines exists as two forms, a predominant species with an M<sub>W</sub> 20 kD greater than other μ heavy chains tested, and a minor species with near normal mobility (Fig. 2 A). These μ species exhibited different extents of association with SLC: although the conventionally size μ pairs with λ5

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of μ heavy chains with different V regions on downregulation of TdT and Rag-1 message levels as revealed by reverse transcription PCR. RNA prepared from the ret02/1 cell line parent (−), from a clone transfected with neo alone (neo), and from two clones each generated by transfection with the four different V<sub>H</sub>-μ constructs indicated. β-actin levels were used to compare efficiency of RNA/cDNA preparation.

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and VpreB, this is not true of the other species. Only about half of the larger \( \mu \) species is complexed with \( \lambda 5 \), and even less (~5%) is associated with VpreB (Fig. 2 a). Since both \( \lambda 5 \) and VpreB are required for pre-BCR function, these data demonstrate that almost none of the larger \( \mu \) protein is assembled into complete pre-BCR complexes. Moreover, because the larger \( \mu \) form makes up 80% of \( V_\mu 11-\mu \), the assembled pre-BCR is decreased by fivefold in these transfectants.

Significantly, the aberrant molecular form of \( V_\mu 11-\mu \) was not detected in cells cotransfected with \( V_k9 \) light chain (Fig. 2 b), the light chain found frequently in mature \( V_\mu 11-\mu \) cells (10, 11). This suggests that the higher \( M_r \) \( \mu \) species represents a posttranslationally modified form, possibly targeted for degradation due to incomplete association with SLC, since a light chain capable of assembling with \( V_\mu 11-\mu \) eliminates the aberrant band. Deglycosylation of \( V_\mu 11-\mu \) (Fig. 2 c, left) did not collapse the double band. Furthermore, the aberrant species was not detected in an antiubiquitin immunoblot (Fig. 2 c, right). The nature of modification responsible for the slowly migrating \( \mu \) species is still under study, and failure to completely assemble with SLC does not always generate it, since \( V_\mu 81X \) chains that failed to associate with \( \lambda 5 \) showed conventional size \( \mu \) (27). Nonetheless, our data suggest that the downregulation of TdT and Rag gene expression in \( \mu \) heavy chain transfectants depends upon efficient assembly of a complete pre-BCR and therefore suggests that both \( V_\mu 81X-\mu \) and \( V_\mu 11-\mu \) are inefficient in mediating these changes.

To test whether normal nontransfected pre-B cells developing in vivo also show a comparable dependence on particular \( V_\mu 11-\mu \), we examined two lines of \( V_\mu 11-\mu \) transgenic mice representing low (BR5) and high (BR1) copy number, comparing them with several other \( \mu \) transgenic mouse lines (26). None of these data from the cell line transfection experiments. In addition, a human Tg (Hu \mu), previously shown to promote B cell development in mice (15, 22), also induced downregulated Rag-2 expression. Thus the extent of bone marrow pre-B cell progression in Ig transgenic mice, as monitored by changes in gene expression that we measured, appears to be dependent on \( V_\mu \)-mediated pre-BCR assembly, with \( V_\mu 11 \) being particularly ineffective.

Unexpectedly, the high copy number BR1 \( V_\mu 11-\mu \) Tg mice showed Rag-2 downregulation, different from the transfection data (Fig. 3 a); however, analysis of the \( \mu \) protein in these two lines provides a potential explanation. Although the novel and conventional \( \mu \) species are both generated in developing B lineage cells in these Tg mice, the conventionally sized \( \mu \) predominates in pre-B cells from the high copy number BR1 mouse, whereas BR5 pre-B
cells showed predominance of the aberrant size $\mu$ (Fig. 3 b), similar to the transfectants. Importantly, while BR1 pre-B cells express more total $\mu$ protein than do pre-B cells from non-Tg mice, the $\mu$ levels in the BR5 line are closer to those in wild-type mice. Thus, we consider that the BR5 line, which expresses physiological levels of $\mu$ and fails to promote R ag-2 downmodulation, provides a realistic picture of V\text{H}11 function in vivo. Moreover, the downregulation of R ag-2 in the BR1 line likely reflects superphysiologic $\mu$ expression that is presumably able to compensate for the inefficient assembly of V\text{H}11 with SLC.

To further pursue how the efficiency of pre-BCR assembly influences pre-B and subsequent B cell development, we next tested the growth response of fetal and adult pre-B cells to $\mu$ expression in stromal cell culture (Fig. 4), since a proliferative burst is another characteristic associated with early pre-B cell progression in the bone marrow. As Fig. 4 a shows, analysis of short term cultures of pre-B cells sorted from bone marrow of competent (H\text{u} \mu, or BR1 $\mu$) and incompetent (BR5 $\mu$) adult Tg mice (on a R ag-1$^+$ background) revealed that Tg expression had relatively little effect on cell growth, with any enhancement in proliferation largely balanced by differentiation and exit from cell cycle. Strikingly, however, analysis of the comparable pre-B fraction isolated from fetal liver of the same transgenic mouse lines revealed that H\text{u} $\mu$ or BR1 $\mu$ expression arrested cell growth. In contrast, the BR5 V\text{H}11 line showed little inhibition of fetal liver B-lineage proliferation, and instead allowed continued pre-B cell growth.

Furthermore, consistent with in vitro analysis, the frequency of pre-B cells in liver of newborn R ag-1$^+$ Tg mice was significantly reduced (relative to nontransgenic mice) in Tg animals with SLC-associating V\text{H} genes, such as H\text{u} $\mu$, but showed near normal levels in BR5 Tg mice (Fig. 4 b).

Importantly, when analyzed on a R ag-1$^+$ background, the majority of BR5 B220$^+$ B-lineage cells in spleen of neonatal (1 wk) BR5 mice were surface V\text{H}11 Tg$^+$, without endogenous $\mu$ surface expression (Fig. 4 c). In contrast, V\text{H}11 $\mu$ did not promote efficient B cell development in adult BR5 mice, since B cells in these mice comprised predominantly cells bearing exclusively endogenous $\mu$. As with the R ag-2 downregulation analysis, the BR1 line appeared normal, showing decreased pre-B cell development early in ontogeny (Fig. 4 b) and predominance of Tg$^+$ B cells in the adult (Fig. 4 d), likely due to superphysiologic V\text{H}11 $\mu$ levels. In summary, cells in fetal liver expressing SLC-nonassociating V\text{H} regions show a growth advantage compared to cells with SLC-associating V\text{H} segments. In contrast, the reverse is true in adult bone marrow where successful pre-BCR assembly is important for B lineage progression.

Our analysis suggests that a bias in V\text{H} representation can occur after successful V\text{H}-D\text{J}\text{H} rearrangement due to interaction (or lack thereof) with SLC, and this assembly-mediated response differs between fetal and adult pre-B cells. We suggest that skewed representation of B cells expressing certain V\text{H} $\mu$, such as V\text{H}81X or V\text{H}11, generated during fetal time can result from active inhibition of clonal expansion of other B cells bearing SLC-associating $\mu$. This model predicts a significant (and different) change in VDJ representation at the late pre-B cell stage during both fetal and adult B cell development. Most V\text{H}81X and V\text{H}11 productive heavy chain sequences reveal a fetal/neonatal origin reflected by a low level of CDR3 diversity (28, 29), due to low levels of TdT during the fetal stage, which results in little addition of extra nucleotides at heavy chain V-D and D-J junctions (18, 30). This has led some to suggest that the representation of certain V\text{H} genes (such as V\text{H}81X and V\text{H}11) is biased by a requirement for nucleotide homology
at the junctions (28, 31), such that TdT-mediated addition in the adult would result in a preponderance of nonproductive joints. However, recent analysis of \( \text{V}_H^{81X} \) sequences generated from adult bone marrow pre-B cell cultures has revealed considerable CDR3 diversity in productive rearrangements (9), demonstrating that these can occur in the presence of TdT. Thus, the difference in TdT levels with ontogeny cannot account for the skewed repertoire difference.

As animals mature from the fetal to the adult stage, the potential to generate a more diverse \( \text{V}_H \) repertoire increases due to TdT expression. This increasing heterogeneity of Ig heavy chain, important for generating more diversity in the adult, may require a more intricate mechanism for selection of appropriate \( \text{V}_H \) genes, such as screening \( \text{V}_H \) structures for an ability to pair with light chains. If this is the case, then SLC may provide a template for an “average” light chain structure. This process would be less important during fetal B lymphopoiesis where TdT is absent, resulting in expression of a more restricted set of \( \text{V}_H \) regions. Thus a dependence of the pro-B to pre-B transition on efficient pre-BCR assembly in adult mouse bone marrow could be viewed as an elaboration on a simpler mode of B cell development represented in fetal liver.

Whether B cell development with these SLC-nonassociating \( \text{V}_H \) genes is completely SLC-independent remains to be determined, since SLC is expressed at the highest levels on the surface of B lineage cells at a developmental stage before heavy chain expression (32), where it could conceivably provide a growth signal independent of \( \mu \). Regardless of a possible role for SLC in the pro-B receptor, it appears that the pre-BCR phase of fetal B lymphopoiesis is quite distinctive compared to that in adult bone marrow, allowing selective expansion of cells with certain \( \text{V}_H \) genes. We speculate that over evolution, useful \( \text{V}_H \) regions have been selected into the germline repertoire and that the distinctive response of fetal pre-B cells to SLC association provides a mechanism for their preferential expression. Determining why fetal B cells should express this primordial repertoire remains an interesting subject for future studies.

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