Brief Definitive Report

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 V_H11 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 V_H 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 V_H (Ig heavy chain variable region) genes, preferentially from segments proximal to the Ig diversity element (D), such as V_H81X 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 V_H 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 V_H 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 V_H genes not proximal to D, such as V_H11, 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 V_H genes, shape the distinctive repertoires of fetal and adult times.

Early B lineage development is critically dependent on expression of Ig μ heavy chain at the pre-B stage, where it associates with a surrogate light chain (SLC), composed of two molecules, λ5 and VpreB (12, 13), which collectively form a complex known as the pre–B cell receptor (BCR). The importance of the pre-BCR in this process is illustrated by the developmental arrest induced upon elimination of any of these components in gene-targeted mice (14–16). Successful pre-BCR assembly induces several hallmark events associated with progression from the pro-B to large pre-B stage in the bone marrow (17), including downregulated expression of genes involved in Ig rearrangement, such as terminal deoxynucleotidyl transferase (TdT) (18, 19) and the recombinase-activating genes (Rag), Rag-1 and Rag-2 (20, 21). These changes coincide with a sharp proliferative expansion in bone marrow, which interestingly occurs at precisely the stage where representation of the fetally biased V_H81X shows a profound decrease (7). This alteration of V_H representation at the stage of μ-dependent proliferation suggests to us that differences in the V region of the μ heavy chain itself may critically influence the growth of B cell precursors during fetal and adult development.

By establishing V_H11 transgenic (Tg) mouse lines expressing two different levels of μ heavy chain, in comparison with other V_H μ Tg lines, we demonstrate in this paper a distinctive in vitro proliferative response by fetal pre–B cells to pre-BCR assembly, with clear in vivo consequences on subsequent B cell development. We propose that this

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differential response by fetal pre–B cells to SLC-μ provides a previously unrecognized mechanism for skewing the expression of distinctive V_H genes toward fetal/neonatal life.

Materials and Methods

Tg Mice. A functionally rearranged V_H11 gene was cloned from a $CD5^+$ 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 λ phage arms and used for generating a library. Positive clones were identified on colony lifts by hybridization with pJ11, and the λ phage was converted into a phagemid vector. The EcoRI 4.8-kb fragment was gel purified, modified by trimming the 3′ end with XhoI, and then ligated into the pICEM19H Cμ plasmid vector. Appropriate V_H orientation was established by mapping several clones with appropriate restriction enzymes. The 17-kb V_H11-Cμ construct was gel isolated and used for microinjection. Litters so generated were screened by PCR amplification for rearranged V_H11-J_H1 (Ig heavy chain joining segment) using DNA made from tail fragments. Tg^+ founders were mated to C.B-17Icr, allowing discrimination of the transgene Cμ allotype (Igh^a) from the endogenous Cμ (Igh^b). Two lines, high copy BR1 and low copy BR5, were backcrossed more than six times to C.B-17 and used for analyses. Human (Hu) μ Tg mice originally generated by M. Nussenzweig (22) and Rag-1^- mice generated by E. Spanapoulou and D. Baltimore (15) were provided by E. Spanopoulou (Mt. Sinai School of Medicine, New York) and maintained in our animal facility. 3H9 Tg animals (23) 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-30F1). Reanalysis 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). $2–5 \times 10^4$ cells ($CD43^+HSA^+B220^+$) 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 10^5–10^6 cells were immunoprecipitated with antibodies to μ (M41), λ5 (LM34-8E), 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 horseradish peroxidase–conjugated M41 rat anti–mouse μ mAb, washed, developed using Supersignal Substrate (Pierce, Rockford, IL), and exposed to Reflection 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 membrane. V_H11-μ immunoprecipitate was loaded in an adjacent lane. Filters were immunoblotted with an antiubiquitin antibody (MAB1510; Chemicon International, Temecula, CA).

Reverse Transcriptase PCR Analysis. cDNA was prepared from total RNA as previously described (18) and then amplified with primers specific for β-actin, TdT, Rag-1, and Rag-2. PCR, blotting, and hybridization were performed as previously described (18).

Transfection Assays. DNA constructs were introduced into the ret 02/1 cell line by electroporation as previously described (19). The SP6μ plasmid contains a *neo*^{r} gene derived from pSV2-neo in addition to the heavy chain gene (25). For transfections with transgenic constructs (V_H11, $V_H11/V\kappa9$, V_H3H9 [23], and V_H81X [26]), cells were cotransfected with pSV2-neo to allow selection with G418. A rearranged Vκ9Jκ2Cκ light chain gene, present on an 8.4-kb BamHI fragment, was cloned from the 2-2G4 hybridoma (10). BamHI-digested DNA of this size was ligated into λ phage arms, the library was screened with nick-translated pECk probe, and a positive clone was ligated into pBluescript II KS (Stratagene, La Jolla, 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_H segments on pre-BCR assembly. Expression of several μ heavy chains with different V_H (V_HDJ_H) 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_H81X or V_H11 μ chains do not show significant TdT or Rag-1 downregulation when compared to the parental line (Fig. 1). Since V_H81X μ chains often do not associate well with λ5 (27), it was reasonable to ask whether pre-BCR assembly was inefficient in V_H11 transfectants.

To assess the extent of pre-BCR assembly with V_H11 μ, we performed immunoprecipitation experiments. SDS-PAGE analysis revealed that the V_H11 μ protein expressed in transfected cell lines exists as two forms, a predominant species with an M_r 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

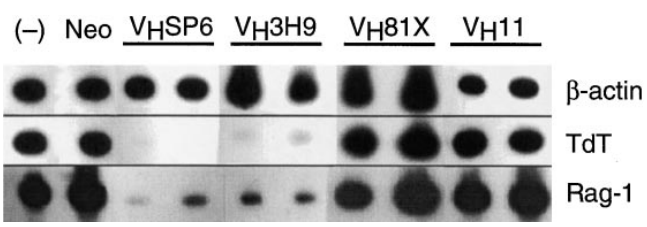

Figure 1. Effect of μ heavy chains with different V regions on downregulation of TdT and Rag-1 message levels as revealed by reverse transcriptase PCR. RNA prepared from the ret02/1 cell line parent (−), from a clone transfected with neo alone (*neo*), and from two clones each generated by transfection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

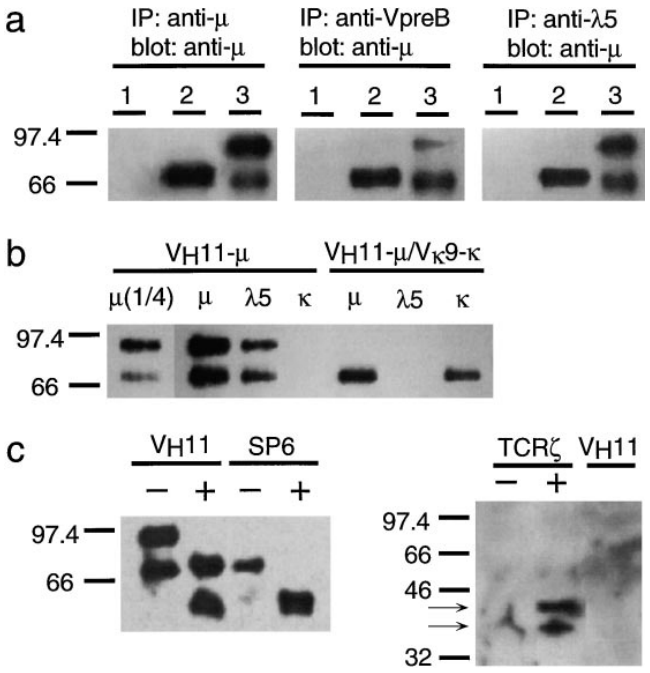

Figure 2. (*a*) SDS-PAGE analysis of pre-BCR components reveals incomplete assembly of V_H11-μ with SLC. Cell lysates from nontransfected (lane *1*), V_HSP6-μ–transfected (lane *2*), and V_H11-μ–transfected (lane *3*) ret02/1 cells were immunoprecipitated and then blotted as shown. Densitometry indicates that the intensity of the upper to lower band in the V_H11-μ–transfectant is 5:1 with anti-μ, 0.1:1 with VpreB, and 2:1 with anti–λ5. Numbers on the left indicate the M_r of standards (in kD). (*b*) Vκ9 eliminates the slowly migrating V_H11-μ species. Immunoprecipitation of V_H11 and V_H11/Vκ9 transfectants with antibodies as indicated, then blotted with anti-μ. The left-most lane shows a short (1/4 time) exposure for the V_H11 transfectant anti-μ immunoprecipitate. (*c*) Deglycosylation (*left panel*) does not eliminate the aberrant V_H11-μ species. Control (−) and treated (+) μ immunoprecipitates from V_H11 and SP6 transfectants. Antiubiquitin immunoblotting (*right panel*) does not detect V_H11-μ heavy chain. As a positive control, TCR-ζ ubiquitination was induced by incubation of thymocytes with pervanidate (+ lane).

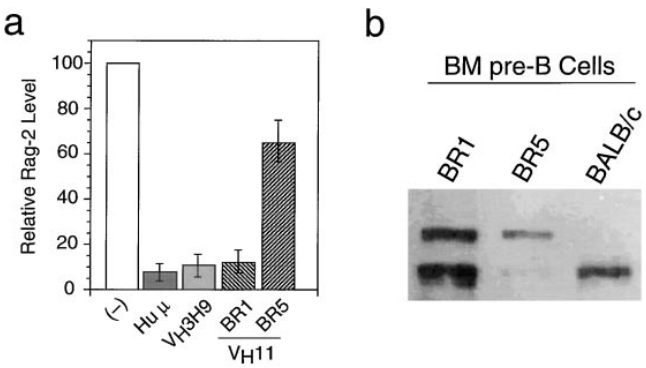

Figure 3. Analysis of gene expression and μ chain protein in pre-B cells. (*a*) RNA prepared from $B220^+CD43^+HSA^+$ cell fraction sorted from bone marrow of Rag-1^-μ Tg mice. All values expressed relative to the maximum level seen with RNA from comparable fraction in Rag-1^- mice (lane −). (*b*) Pre-B cells ($B220^+CD43^+HSA^{hi}$; Fr. C′; reference 17) sorted from BR1 and BR5 Rag-1^- bone marrow and from BALB/c bone marrow were immunoprecipitated and blotted with anti-μ. The relative intensities (with BALB/c set to 1) are 1.2, 1.5 (*upper*, *lower*) for BR1 and 0.5, 0.1 for BR5.

and VpreB, this is not true of the other species. Only about half of the larger μ species is complexed with λ5, and even less (~5%) is associated with VpreB (Fig. 2 *a*). Since both λ5 and VpreB are required for pre-BCR function, these data demonstrate that almost none of the larger μ protein is assembled into complete pre-BCR complexes. Moreover, because the larger μ form makes up 80% of V_H11-μ, the assembled pre-BCR is decreased by fivefold in these transfectants.

Significantly, the aberrant molecular form of V_H11-μ was not detected in cells cotransfected with Vκ9 light chain (Fig. 2 *b*), the light chain found frequently in mature V_H11^+ B cells (10, 11). This suggests that the higher M_r μ 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_H11-μ eliminates the aberrant band. Deglycosylation of V_H11-μ (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 μ species is still under study, and failure to completely assemble with SLC does not always generate it, since V_H81X chains that failed to associate with λ5 showed conventional size μ (27). Nonetheless, our data suggest that the downregulation of TdT and Rag gene expression in μ heavy chain transfectants depends on efficient assembly of a complete pre-BCR and therefore suggests that both V_H81X-μ and V_H11-μ are inefficient in mediating these changes.

To test whether normal nontransformed pre–B cells developing in vivo also show a comparable dependence on particular V_H μ, we examined two lines of V_H11 μ transgenic mice representing low (BR5) and high (BR1) copy number, comparing them with several other μ transgenic mouse lines. To eliminate any effect by endogenous μ expression and to restrict our analysis to the pre–B cell stage, we used Rag-1^- mice bearing Ig Tgs. As Fig. 3 *a* shows, a differential ability to downregulate Rag-2 in adult bone marrow was clearly evident when comparing low copy number BR5 μ with 3H9 μ Tg mice, in agreement with data from the cell line transfection experiments. In addition, a human μ Tg (Hu μ), 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_H-mediated pre-BCR assembly, with V_H11 being particularly ineffective.

Unexpectedly, the high copy number BR1 V_H11 μ Tg mice showed Rag-2 downregulation, different from the transfection data (Fig. 3 *a*); however, analysis of the μ protein in these two lines provides a potential explanation. Although the novel and conventional μ species are both generated in developing B lineage cells in these Tg mice, the conventionally sized μ predominates in pre-B cells from the high copy number BR1 mouse, whereas BR5 pre–B

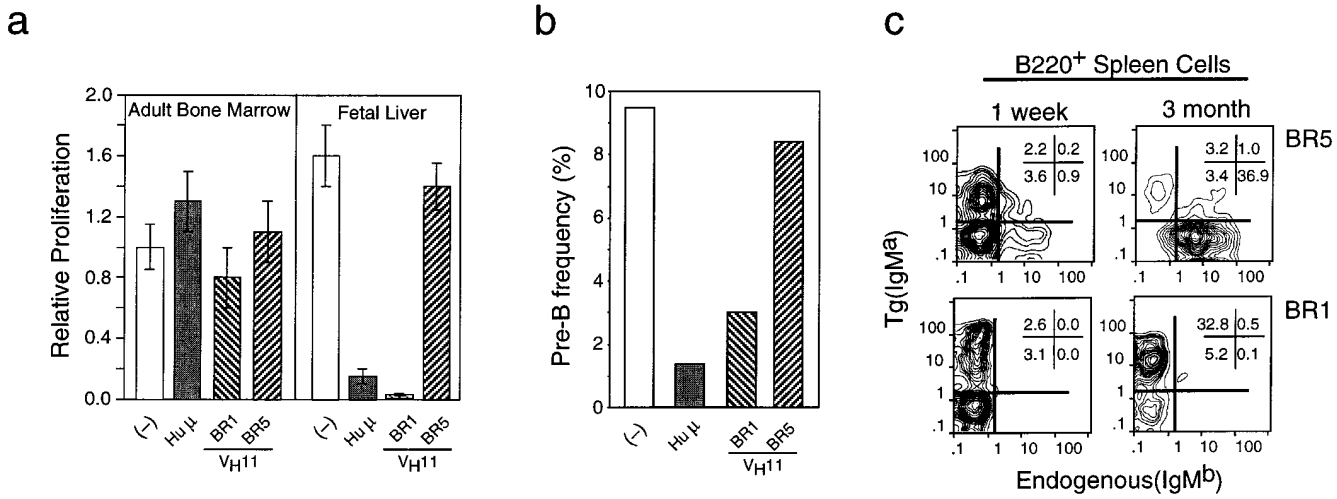

Figure 4. Different consequences of μ heavy chain expression during fetal and adult B cell development. (*a*) Growth effect of various μ transgenes (Rag-1$^-$ background) after 4-d stromal cell–dependent cultures of B220$^+$CD43$^+$HSA$^+$ cells sorted from bone marrow and fetal liver. Live cell number recovered after culture of Rag-1$^-$ bone marrow (average sixfold increase) is set to 1.0. (*b*) Frequency of pre-B cells in liver of newborn Rag-1$^-$ mice bearing indicated μ transgenes compared to nontransgenic mice (−) as determined by flow cytometry. (*c*) Flow cytometry analysis of B220$^+$ cell in spleen of BR5 and BR1 mice at 1 wk and 3 mo. Numbers show percentage of live cells in a lymphocyte size gate for indicated quadrants. Representative data from three analyses.

cells showed predominance of the aberrant size μ (Fig. 3 *b*), similar to the transfectants. Importantly, while BR1 pre-B cells express more total μ protein than do pre-B cells from non-Tg mice, the μ 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 μ and fails to promote Rag-2 downmodulation, provides a realistic picture of V_H11 function in vivo. Moreover, the downregulation of Rag-2 in the BR1 line likely reflects super-physiologic μ expression that is presumably able to compensate for the inefficient assembly of V_H11 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 μ 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 (Hu μ or BR1 μ) and incompetent (BR5 μ) adult Tg mice (on a Rag-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 Hu μ or BR1 μ expression arrested cell growth. In contrast, the BR5 V_H11 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 Rag-1$^-$ Tg mice was significantly reduced (relative to nontransgenic mice) in Tg animals with SLC-associating V_H genes, such as Hu μ, but showed near normal levels in BR5 Tg mice (Fig. 4 *b*). Importantly, when analyzed on a Rag-1$^+$ background, the majority of BR5 B220$^+$ B-lineage cells in spleen of neonatal (1 wk) BR5 mice were surface V_H11 Tg$^+$, without endogenous μ surface expression (Fig. 4 *c*). In contrast, V_H11 μ did not promote efficient B cell development in adult BR5 mice, since B cells in these mice comprised predominantly cells bearing exclusively endogenous μ. As with the Rag-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 *c*), likely due to superphysiologic V_H11 μ levels. In summary, cells in fetal liver expressing SLC-nonassociating V_H regions show a growth advantage compared to cells with SLC-associating V_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_H representation can occur after successful V_H-DJ_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_H μ, such as V_H81X or V_H11, generated during fetal time can result from active inhibition of clonal expansion of other B cells bearing SLC-associating μ. 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_H81X and V_H11 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_H genes (such as V_H81X and V_H11) 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 V_H81X 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 V_H μ 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 V_H μ, such as screening 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 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 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 μ. 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 V_H genes. We speculate that over evolution, useful 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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