A Truncated Heavy Chain Protein Relieves the Requirement for Surrogate Light Chains in Early B Cell Development[1]

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Early B cell development depends upon the surface expression of Ig heavy chain protein (μ) in a signaling complex known as the pre-B cell receptor (pre-BCR). In addition to μ, the pre-BCR consists of the surrogate light chains VpreB and λ5 and the transmembrane signal transduction proteins Ig-α and Ig-β. Expression of this complex is associated with changes in surface marker expression, gene transcription, and Ig gene rearrangement. Mutations preventing the expression of either μ or λ5 result in developmental arrest, but the precise roles of the various components of the pre-BCR remain unclear. Using mice transgenic for a surface-expressed, but truncated, form of μ that cannot associate with surrogate light chains, we have studied the role of surrogate light chains in B cell development. We found that expression of the truncated μ transgene resulted in changes in surface marker expression, germline κ locus transcription, and V(D)J recombinase targeting indistinguishable from those induced by intact μ protein. These experiments lead us to conclude that surrogate light chains, while necessary for the assembly of the wild-type pre-BCR, are not directly involved in pre-BCR signaling or otherwise required for early B cell development. ***The Journal of Immunology,*** **1997, 159: 1265–1275.**

Developing lymphocytes assemble their Ag receptor genes through a series of highly regulated site-specific DNA recombination reactions known as V(D)J recombination (1). This process of gene assembly is ordered, with Ig heavy chain gene rearrangement generally preceding Ig light chain gene rearrangement (2). The developing cell monitors this assembly process through the activity of a pre-B cell receptor (pre-BCR)[3] (reviewed in Ref. 3). The pre-BCR consists of three parts: a heavy chain protein (μ), the surrogate light chains (SLCs) λ5 and VpreB, and the signal transduction molecules Ig-α and Ig-β (4, 5).

The pre-BCR is expressed on the surface of pre-B cell lines and primary B cell precursors in the bone marrow (4–9). Ab cross-linking of this complex in transformed pre-B cell lines results in the induction of intracellular calcium flux (10, 11) and of κ light chain gene rearrangement (12). Inability to express this receptor due to transgenic disruption of its components leads to a block in B cell development at the pro- to pre-B cell transition (13–15). Because of its signal transduction capacity and its expression at a critical stage of development, the pre-BCR is thought to be a key regulator of early B cell development.

Studies relying on transgenic and targeted mutant mice have revealed several aspects of normal B cell development that depend upon expression of the pre-BCR. First, the pre-BCR is required for changes in the expression of an array of surface markers that characterize normal B cell development (14–19). Second, transcription of many lineage-specific genes, including unrearranged light chain genes (germline transcripts), SLC genes, and terminal deoxynucleotidyl transferase, is regulated across the pre-B cell transition (18, 20). Finally, signals provided by the pre-BCR affect the regulation of Ig gene rearrangement in developing B cells (17, 21–24).

Regulation of the gene rearrangement program is necessary to ensure that an individual mature B cell expresses only one heavy chain and one light chain (25). This phenomenon, known as allelic exclusion, is critical to the subsequent clonal selection of the B cell repertoire. Feedback regulation of V(D)J recombination by its products, Ig heavy and light chains, is involved in mediating allelic exclusion. Mutant mice unable to assemble the pre-BCR fail to show allelic exclusion of heavy chain gene rearrangement (15, 17, 26). In addition, expression of heavy chain protein increases accessibility of the κ light chain locus to the V(D)J recombinase in vitro (27) and the frequency of V-to-Jκ gene rearrangement in murine bone marrow (21, 24) and in transformed cell lines (12, 28, 29).

Because of its importance as a developmental regulator, attention has focused on the roles of the various components of the pre-BCR. In the absence of surrogate or conventional light chain, Ig heavy chain protein associates with the endoplasmic reticulum (ER) protein BiP and is ultimately degraded (4, 30). Conventional or surrogate light chains when present in the ER compete with BiP for binding to heavy chain, leading to its surface transport. Therefore, it is presumed that one certain function of the SLCs is to serve as chaperones, allowing proper folding of heavy chain protein and transport of pre-BCR to the cell surface. Mice with a transgenic disruption of the λ5 SLC gene, whose B cells are largely blocked at the pre-B stage, gradually accumulate mature peripheral B cells (15). Presumably, a low level of precocious κ light chain gene rearrangement (31) allows κ light chains to functionally replace SLCs in the pre-B receptor; a fraction of B cell progenitors may then proceed through development (32).

Transformed pre-B cell lines transfected with μ expression vectors have been used to study the effect of μ-chain on B cell development. In a virally transformed progenitor B cell line, a full-length μ construct was reported to activate κ gene rearrangement,

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[3] Abbreviations used in this paper: BCR, B cell receptor; SLC, surrogate light chain; ER, endoplasmic reticulum; CS, calf serum; HCD, heavy chain disease; PTPIC, protein tyrosine phosphatase IC; ITAM, immune tyrosine activation motif; LM-PCR, ligation-mediated PCR; RAG-1, recombination activating gene-1.

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whereas transfection of a surface-expressed, truncated version of heavy chain that could not associate with the SLCs failed to do so (12). This truncated heavy chain, lacking V_H and CH1 domains, escaped ER retention and was highly expressed on the cell surface, presumably because it lacks sequences that mediate interaction with BiP (33). The failure of this truncated heavy chain to activate κ gene rearrangement despite its transport to the cell surface implies that the SLCs are involved directly in pre-BCR signaling. In addition to serving a chaperone function, the SLC component of the pre-BCR might be involved in specific interactions with ligands in the microenvironment of developing B cells. It is also interesting to note that SLCs have been detected on the surface of pro-B cells in association with other, as yet unidentified, proteins, leading to the suggestion that they might play a role in development apart from their association with heavy chain (34, 35).

Since the process of transformation interferes with the regulation of signaling pathways in pre-B cell lines (36, 37), we have taken a transgenic approach to define the role of the SLCs in pre-BCR signaling. By studying the effects of a wild-type and a mutant heavy chain transgene on B cell development in several mutant mouse strains, we sought to determine whether SLC expression was required for pre-BCR function or other aspects of early B cell development apart from its role as a heavy chain chaperone.

Materials and Methods

Production and maintenance of transgenic mice

All mouse lines (wild-type BALB/c, National Cancer Institute; RAG-1-deficient (18), human heavy chain transgenic (22); truncated μ transgenic (this study); μ membrane exon knockout (13); and $\lambda 5$ knockout mice (15)) were maintained in microisolator cages in a pathogen-free facility.

Truncated μ transgenic mice were generated by pronuclear injection of an *Eco*RI to *Xho*I fragment of pED234 μ (12) (a gift from M. Reth) into C57/B6 × C3H fertilized ova. Founders were identified by PCR (see below) and bred to the BALB/c background for three or more generations. Transgene expression was stable over the course of several generations and was inherited in a Mendelian fashion.

Genotyping

One centimeter of tail was used to prepare DNA for typing. Tails were incubated overnight at 55°C in 400 μl of sample buffer (10 mM Tris, pH 8.0; 1 mM EDTA; 400 mM NaCl; and 0.5% SDS) with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN). One hundred and fifty microliters of 5.5 M NaCl was added, samples were spun in a microfuge at maximum speed for 10 min, and supernatants were removed to a fresh tube with 500 μl of ethanol. Samples were mixed by vortexing, and DNA was pelleted at maximum speed for 5 min, washed in 400 μl 70% ethanol/H_2O, repelleted, and vacuum-dried for 5 min. Sample DNAs were diluted to 20 ng/μl in PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; 2.0 mM $MgCl_2$; 0.45% Tween-20; and 0.45% Nonidet P-40) and heated to 95°C for 5 min to inactivate residual proteinase K. Two microliters of each DNA was used for PCR.

Below are the sequences of the genotyping PCR primers with the annealing temperatures in parentheses after each set. Basic PCR conditions were 94°C for 1 min; T°C annealing (as noted for each primer, below) for 1 min; 72°C for 2 min, for 28 cycles. The primer sequences were: RAG-1-deficient: RAGL, 5′-CAGTACCAAGCTTCTTGCC; RAGR, 5′-ATCT TGCGCGGGACACTTG (T°C = 56); human μ: HCH2, 5′-TCCAAGC TCATCTGCCAGGCCACGG; HCH3, 5′-ATAGGTGGTCAGGTCTG TGACCAGG (T°C = 60); truncated μ: DJtru, 5′-CTATAGTTATATCT CTGG; JH4, 5′-TCCCTCAAATGAGCCTCCAAAGTCC (T°C = 56); μ membrane knockout: memko5, 5′-CTCTGTAACCACTCACCACC; mem1095, 5′-GGTGCTGTAGAAGAGGCTCA (T°C = 65); and $\lambda 5$ knockout: L5L196, 5′-AGCTCAGCAGAAAGGAGCAGAGCTG; L5IN196, 5′-CTGGCCTTGCAATTGATCG (T°C = 58).

Cell culture

3T3 fibroblasts (American Type Culture Collection, Rockville, MD) were cultured in a 5% CO_2 incubator at 37°C in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 μM β-ME, and antibiotics.

Isolation and purification of primary cells

Cells for RT-PCR, gene rearrangement assays, and broken-end LM-PCR analyses were obtained from various transgenic mouse lines. Mice were killed between the ages of 4 and 8 wk for these analyses. Bone marrow cells were recovered from femurs, tibias, and humeri by flushing bones with 10% calf serum/1× PBS (CS/PBS). The cell suspension was passed through nylon mesh, layered on Ficoll, and centrifuged at 20°C at 400 × *g* for 15 min. Live cells at the buffy coat were aspirated by pipette. These cells were then washed in several volumes of CS/PBS, pelleted, and resuspended in CS/PBS containing biotinylated anti-CD19 mAb (38) for 15 min on ice. Cells were washed once in 2 ml/10^7 cells of CS/PBS and once in an equal volume of 0.5% BSA/PBS, then resuspended in 90 μl/10^7 cells of 0.5% BSA/PBS. Ten microliters of streptavidin-coated paramagnetic beads (Milltenyi Biotech, Sunnyvale, CA) per 10^7 cells were added, and the mixture was incubated at 4°C for 15 min. Cell suspensions were passed through nylon mesh onto a MiniMACS column (Milltenyi Biotech) attached to a magnet at 4°C. Columns were washed twice with 0.5% BSA/PBS, and cells were collected from the flow-through and designated depleted. The column was removed from the magnet, and cells designated enriched were washed free in several milliliters of 0.5% BSA/PBS. The purity of the cell populations was monitored by flow cytometry after staining bound biotinylated anti-CD19 Ab with streptavidin-phycoerythrin. Enriched fractions (3–8 million cells) were >90% CD19 positive.

Flow cytometric analysis of primary cells

Bone marrow cells were harvested from femurs as described above. One million cells per sample were stained in wash buffer (5% CS/PBS/0.01% NaN_3) with 10 μl of Ab (previously titrated) for 15 min on ice. Samples were then washed twice with 1 ml of wash buffer. Detection of biotinylated Abs required a second incubation with diluted avidin-Quantum Red (Sigma Chemical Co.) for 10 min on ice, followed by two 1-ml washes. Samples were then resuspended in 500 μl of wash buffer. Ten to twenty thousand events were collected on live cell gated samples using CellQuest Software and a FACScan (Becton Dickinson, Mountain View, CA). Data analysis was performed using the same program.

Antibodies used in these analyses included monoclonal biotinylated rat anti-mouse CD19 (38); FITC-conjugated monoclonal mouse anti-human μ (G20–127, PharMingen, San Diego, CA); goat anti-mouse μ antiserum, FITC- or PE-conjugated (Southern Biotechnology Associates, Birmingham, AL); monoclonal FITC-conjugated rat anti-mouse CD43 (S7) (16); and monoclonal rat anti-mouse CD22 (PharMingen).

Immunofluorescence analysis of primary cells

Primary cells were isolated as described and placed into ice-cold RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 μM β-ME, and antibiotics. Live cells were purified on a Ficoll gradient and washed in several volumes of cold 1× PBS. Samples were stained on ice for 15 min as described with phycoerythrin-conjugated goat anti-mouse μ antiserum (Southern Biotechnology) or control goat serum diluted in RPMI medium. Cells were washed three times with 1× PBS and fixed by resuspension in 1 ml of 1× PBS/0.5% paraformaldehyde for 30 min on ice. Cells were gently pelleted, washed with 1 ml of 1× PBS, and spotted onto glass slides with coverslips for fluorescence analysis. Cells were viewed and photographed on a Zeiss microscope (New York, NY).

Preparation of RNA and RT-PCR

RNA was prepared from 3 to 5 million cells as described previously (39), and 1 μg of RNA was converted to randomly primed cDNA using Moloney murine leukemia virus RT obtained from Life Technologies (Grand Island, NY) (40). PCR conditions for various transcripts were previously described (41).

Preparation of DNA and PCR analysis of gene rearrangement and double-strand DNA breaks

DNA from several million purified cells was prepared using a Puregene kit (GENTRA Systems, Inc., Research Triangle Park, NC). Samples were normalized for DNA content by limited PCR amplification of a nonrearranging locus (CD14). PCR conditions, primers, and hybridization probes for Ig gene rearrangement and control CD14 assays were described previously (24, 40).

The assay for broken signal ends is diagrammed in Figure 5*A* and was described previously (42). Briefly, 2 μg of DNA was ligated to a double-stranded, blunt-ended linker oligonucleotide using T4 DNA ligase (Boehringer Mannheim). Linker-ligated DNA was then subjected to two rounds of nested PCR using a linker-specific primer and a pair of locus-specific primers. PCR products were analyzed by electrophoresis through agarose gels (FMC, Rockford, ME), blotted onto nylon membranes (ZetaBind, Cuno,

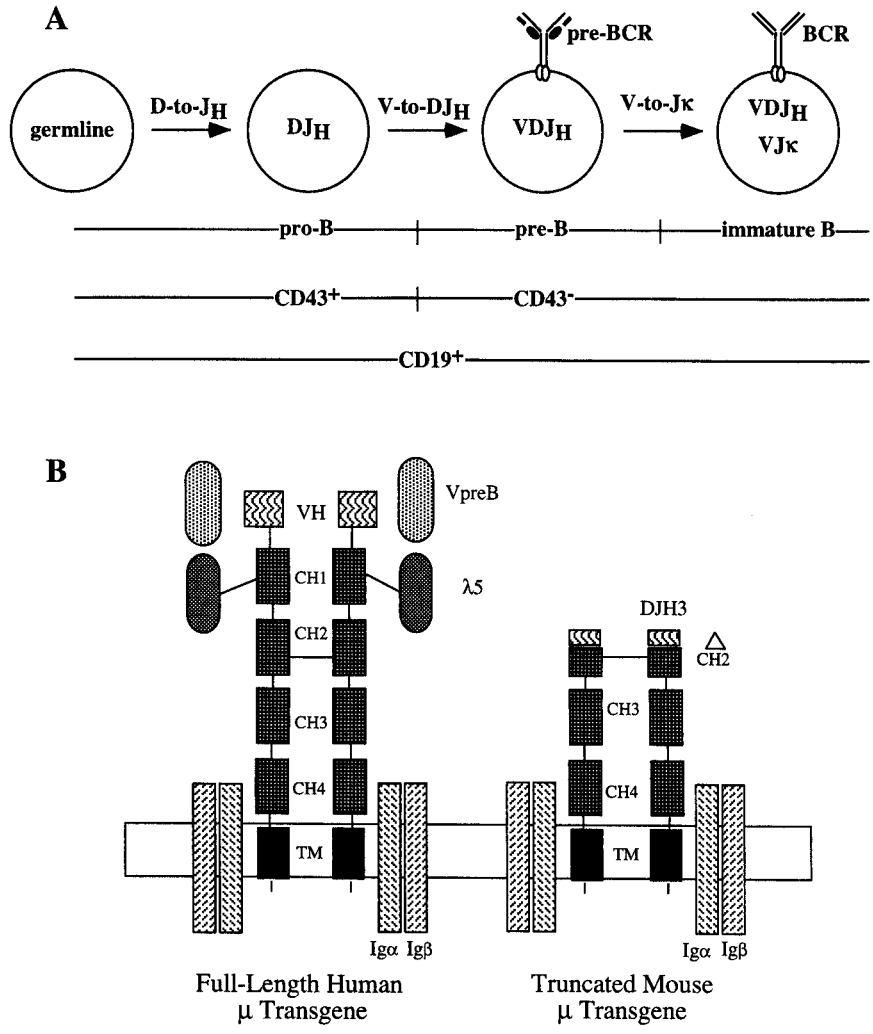

FIGURE 1. Diagrams of the stages of B cell development and proteins encoded by the heavy chain transgenes. *A*, Diagram depicting the order of gene rearrangements and the expression of receptor complexes during B cell development. The proteins anchoring the receptors in the membrane, depicted as paired ovals, are heterodimers of Ig-α and Ig-β. The pattern of expression of surface markers CD43 and CD19 is indicated below the diagram. *B*, The full-length μ (21) and the truncated μ (tμ) (12) transgenes are shown in cartoon form as they would be found in a pre-B cell receptor complex, projecting into the extracellular space. The full-length μ transgene encodes the variable (V_H; stippled box), constant (CH1–4; solid boxes), and transmembrane (TM; black box) domains of heavy chain. The tμ transgene encodes a D_H segment (DSP2) rearranged to the J_H3 segment (stippled box) (62) ligated to the 3′ portion of CH2 and the remainder of the heavy chain coding exons (solid and black boxes). Other components of the pre-B cell receptor are indicated: the SLC proteins VpreB and λ5 are shown as shaded ovals, and the signal transduction components Ig-α and Ig-β (hatched boxes) are indicated by long rectangles crossing the plasma membrane (open box).

Inc., Meriden, CT), and hybridized with T4 DNA kinase-labeled oligonucleotide probes specific for each locus.

Results

Association with surrogate light chains is not required for the pre-BCR to alter the expression of surface markers on B cell progenitors

To assess the role of SLCs in pre-BCR signaling, we compared the effects of two different heavy chain transgenes on B cell development (Fig. 1*A*). The first transgene encodes a full-length human heavy chain protein (μ) whose expression is directed by the B-lineage-specific V_H promoter and heavy chain intronic enhancer (Fig. 1*B*). This transgene has been shown to properly regulate B cell development in previous studies and can be readily distinguished from endogenous murine heavy chain with specific Abs (18, 22). The second transgene, also controlled by the V_H promoter and the heavy chain intronic enhancer, encodes a functionally rearranged DJ segment ligated to a truncated μ constant exon 2 followed by constant exons 3, 4, and the transmembrane exons (12) (Fig. 1*B*). Although lacking the V_H, CH1, and half of the CH2 domains of μ and unable to associate with either of the SLCs, this truncated μ protein (tμ) is expressed at the cell surface in transfected cell lines (12, 43). Upon cross-linking using μ-specific antisera, tμ can initiate intracellular signals (12, 43).

Figure 2*A* shows expression in the bone marrow of the full-length μ and tμ proteins after breeding these transgenes into a RAG-1-deficient background. In RAG-1-deficient mice, B cell development is arrested at the CD43$^+$ pro-B cell stage (Fig. 1*A*) (16, 44, 45). These B cells are unable to initiate gene rearrangement and, therefore, cannot express endogenous heavy chain protein. B-lineage cells were identified with a mAb to the lineage-specific marker CD19. Transgene surface expression was detected with either a monoclonal anti-human μ Ab (for full-length μ) or an anti-murine μ antiserum (for tμ). Bone marrow B cells from RAG1-deficient mice stain with the anti-CD19 Ab but lack μ expression. Full-length μ protein is undetectable on the cell surface of RAG-1-deficient/μ transgenic cells as previously reported (18, 19). The tμ protein, however, is expressed in a majority of RAG1-deficient B cell precursors at a level similar to native μ on the surface of mature, wild-type B cells (Fig. 2*A*, compare *upper right* and *lower right panels*).

Pre-BCR signaling results in changes in cell surface markers (Fig. 1*A*). It was shown previously that pre-BCR expression can promote the maturation of RAG-deficient B cell progenitors from the pro-B cell (CD43$^+$) to the pre-B cell (CD43$^-$) surface phenotype (16, 18, 19). The *upper panel* in Figure 2*B* shows wild-type bone marrow B cells (CD19 positive) divided into CD43$^+$ pro-B cells (*upper right quadrant*) and the CD43$^-$ pre- and mature B cells (*upper left quadrant*). Cells from RAG-1-deficient mice accumulate at the CD43$^+$ pro-B cell stage. Expression of either μ or tμ proteins promotes the development of RAG-deficient B cells to the CD43$^-$ stage of development (Fig. 2*B*).

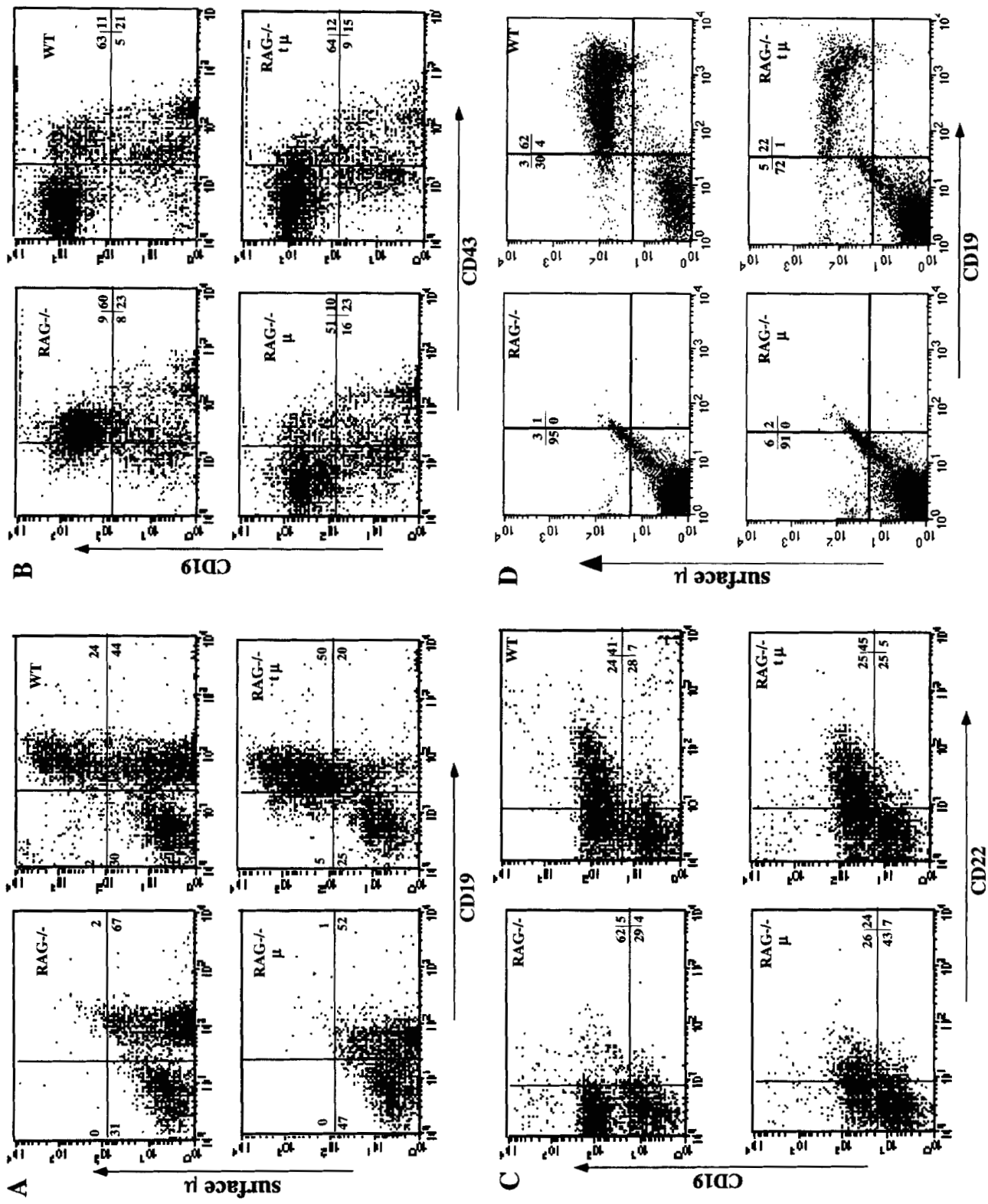

FIGURE 2. Flow cytometric analysis of the effects of heavy chain transgenes on bone marrow B cells from RAG-1-deficient mice. *A*, Detection of surface μ expression on developing bone marrow B cells. Single cell suspensions of whole bone marrow isolated from 4- to 6-wk-old wild-type (WT; BALB/c), RAG-1-deficient ($RAG^{-/-}$), and RAG-1-deficient/heavy chain transgenic mice ($RAG^{-/-}$, μ or tμ) were stained with Abs to the B-lineage-specific marker CD19 and with mAb or antisera specific for μ (see *Materials and Methods*). Data were gated on lymphocytes based on their characteristic forward and side light scatters. The percentages of scatter-gated cells in each quadrant are indicated. *B*, Expression of the developmentally regulated surface marker CD43. Bone marrow cells from the mice described above were stained with Abs to CD19 and CD43. Early B cell precursors fall in the *upper right*, $CD43^+$ quadrant, while more mature cells are found in the *upper left*, $CD43^-$ quadrant (16). Percentages of scatter-gated cells in each quadrant are indicated. *C*, Expression of the B-lineage-specific protein CD22. Cells described above were also stained with Abs specific for CD19 and CD22, a protein involved in signaling via surface Ig (48). The percentages of scatter-gated cells are shown in each quadrant. *D*, Expression of surface μ chain on splenic B cells in RAG-1-deficient and transgenic mice. Splenocytes were harvested from mice of the indicated genotypes and stained with Abs specific for CD19 and μ chain (murine or human). Data were gated on lymphocytes based on their characteristic forward and side light scatters.

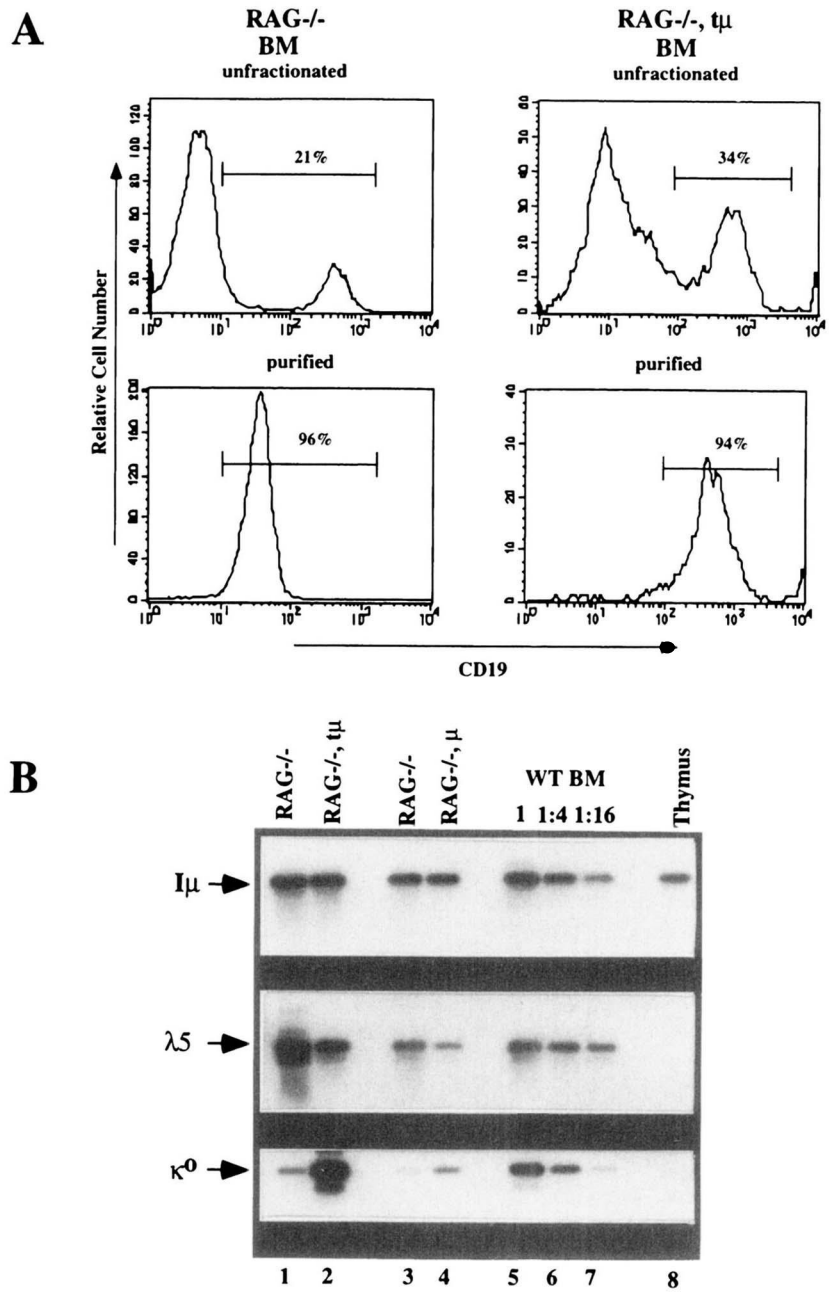

FIGURE 3. Influence of heavy chain transgenes on the expression of developmentally regulated transcripts in purified bone marrow B cells from RAG-1-deficient mice. *A*, Purification of cells used for RT-PCR analysis. Cells from the bone marrow of several 4- to 6-wk-old mice of the indicated genotypes were collected, and B cell precursors were purified by positive selection based on the expression of CD19 (see *Materials and Methods*). *Upper panels* show the anti-CD19 Ab staining of unfractionated pools of bone marrow cells, with the percentage of $CD19^+$ B cell precursors shown above the bar. *Lower panels* show the anti-CD19 staining of the positively selected cells. The reduction in the level of anti-CD19 staining after selection is due to the interference by the magnetic particles used in the selection with detection of the bound anti-CD19 Ab. Staining with other B cell-specific Abs and forward vs side scatter characteristics confirm the identity of the purified populations (not shown). Bone marrow cells from wild-type mice and from RAG-1-deficient, full-length μ transgenic mice were purified similarly (data not shown). *B*, RT-PCR analysis of B cell-specific transcripts. Equivalent amounts of total RNA purified from the cells described in *A* were reverse transcribed and analyzed by PCR with primers specific for the indicated transcripts (see *Materials and Methods*) (18). Controls included PCR analysis of serial dilutions of wild-type (WT) $CD19^+$ bone marrow B cell cDNA (a series of fourfold dilutions into RNase-free water; *lanes 5–7*), cDNA prepared from thymocyte RNA (>90% $Thy1^+$ T cells; *lane 8*), and a mock cDNA reaction (no RNA; data not shown). Products were separated by gel electrophoresis, blotted to nylon filters, hybridized with radioactive probes specific for each transcript, and revealed by autoradiography. Iμ indicates the lymphocyte-specific transcript used to confirm the integrity and equivalence of each cDNA sample. λ5 indicates the λ5 SLC transcript, and κ^o indicates the germline κ locus transcript.

In addition, both μ and tμ regulate the surface expression of other B-lineage markers such as CD2 (data not shown), MHC class II (data not shown), and CD22 (Fig. 2*C*). CD22 is a B-lineage-specific Ig superfamily member involved in the modulation of signals through IgM (46, 47). CD22 remains largely uncharacterized in early murine B cell development, but human CD22 is thought to be expressed only in the cytoplasm until cells reach the $sIgD^+$, mature B cell stage (48). We consistently observe induction of surface expression of CD22 at the pre-B stage in both mutant and wild-type mice (Fig. 2*C* and data not shown).

The full-length and truncated μ transgenes differ in their abilities to promote the colonization of the spleen with RAG-deficient B cells (Fig. 2*D*). RAG-deficient mice lack splenic B cells. Expression of the tμ, but not the μ, transgene results in the appearance of significant numbers of B cells in the spleens of RAG-deficient mice.

Association with surrogate light chains is not required for pre-BCR-induced alterations in transcription

To determine whether μ protein can affect the regulation of transcription independent of its association with surrogate light chains, we purified bone marrow B cells from RAG-1-deficient mice with or without full-length μ and tμ transgenes using biotinylated anti-CD19 Ab and streptavidin-coated paramagnetic beads. FACS analysis showed that >90% of the purified cells were from the B lineage (Fig. 3*A*). We purified RNA from each population of cells and analyzed the expression of developmentally regulated transcripts by RT-PCR (Fig. 3*B*). Amplification of Iμ, a noncoding RNA transcribed from the heavy chain locus at similar levels throughout B cell development (49), showed that there were similar amounts of amplifiable material in each sample. We showed previously that bone marrow B cells expressing a full-length μ

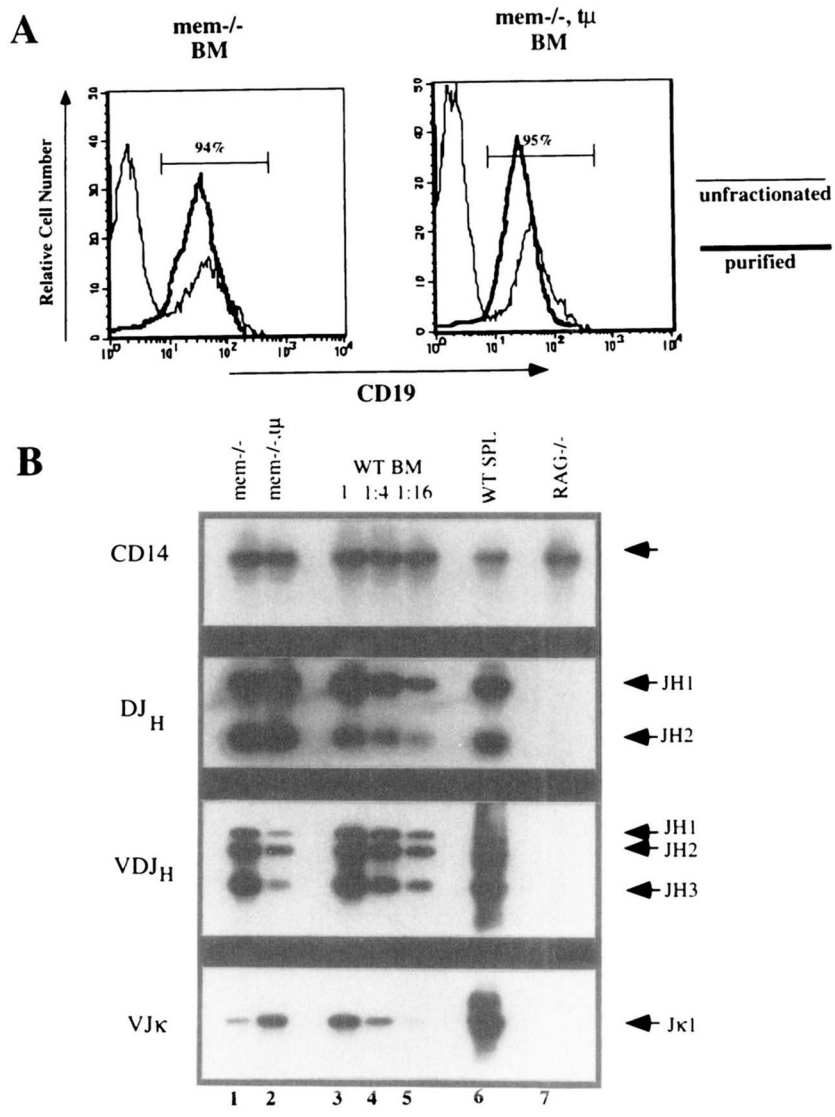

FIGURE 4. Influence of the tμ transgene on Ig gene rearrangements in B cell precursors from mice with a targeted disruption of the μ transmembrane exon. *A*, Purity of cells used in rearrangement assays. Cells from the bone marrow of several 4- to 6-wk-old μ membrane exon knockout mice ($mem^{-/-}$) with or without the tμ transgene were collected, and B cell precursors were purified by positive selection based on the expression of CD19 (see *Materials and Methods*). Thin lines show the CD19 staining of unfractionated pools of bone marrow cells, and heavy lines show the CD19 staining of the positively selected cells. The bars indicate the purity of the positively selected cells. Wild-type (WT) and RAG-1-deficient ($RAG^{-/-}$) bone marrow B cells and WT splenic B cells were purified similarly (data not shown). *B*, Analysis of gene rearrangements in B cell precursors. DNA was purified from the cells described in *A*, and equivalent amounts were used in PCR reactions designed to quantify gene rearrangement (24). Controls included DNA from purified wild-type (WT) bone marrow B cells (a series of four-fold dilutions into $RAG^{-/-}$ DNA (*lanes 3–5*), wild-type (WT) splenic B cells (*lane 6*), and RAG-1-deficient bone marrow cells (*lane 7*)) and a reaction lacking template DNA (not shown). Products were separated by gel electrophoresis, blotted to nylon filters, hybridized with radioactive probes specific for each locus, and revealed by autoradiography. Amplification of CD14, a nonrearranging locus, was used to confirm the equivalence of DNA in each sample (*top panel*). Rearrangements of D and J_H gene segments are shown in the *second panel*, complete heavy chain V_H-to-DJ_H rearrangements are shown in the *third panel*, and Vκ-to-Jκ1 rearrangements are shown in the *bottom panel*.

transgene increase germline transcription at the κ light chain locus while decreasing transcription of the surrogate light chain gene λ5 (18, 24). We found that the tμ transgene caused an increase in germline κ transcription and a decrease in λ5 transcription similar to those caused by the full-length μ transgene (Fig. 3*B*, *lanes 1–4*).

Pre-BCR regulates gene rearrangement independent of its association with surrogate chains

Another outcome of pre-BCR signaling is the regulation of gene rearrangement at the heavy and light chain Ig loci. As noted above, mutant mouse lines unable to express pre-BCR fail to allelically exclude rearrangement of their heavy chain loci (17, 26). Signals from the pre-BCR also increase the efficiency of κ light chain gene rearrangement (24). To determine whether these developmental functions can occur when SLCs are not part of the pre-BCR, the tμ transgene was bred to the recombination-competent, μ membrane exon knockout mouse line (13). These mice have a targeted disruption of the membrane exon of μ; they can rearrange their Ig loci, but cannot express μ on the cell surface. They show an early arrest of B cell development and failure of heavy chain allelic exclusion (13, 17).

Bone marrow B cells were purified from μ membrane exon knockout ($mem^{-/-}$) mice with and without the tμ transgene as described above. Reanalysis showed the purified fractions to consist of >90% $CD19^+$ cells (Fig. 4*A*). DNA was purified from these cells and the products of completed Ig gene rearrangement were analyzed by PCR (Fig. 4) (40, 50). The B cell samples contained equivalent amounts of amplifiable DNA, as shown by the control amplification of a nonrearranging locus (CD14). Each population also had similar levels of D_H to J_H gene rearrangement (Fig. 4*B*, compare *lanes 1* and *2*). We found a striking difference, however, in the frequency of V_H-to-DJ_H rearranged alleles. Cells from $mem^{-/-}$ + tμ transgenic mice had a significant reduction in completed V_H-to-DJ_H rearrangements compared with cells from the $mem^{-/-}$ mice. This leads us to conclude that the tμ transgene signals the allelic exclusion of the endogenous heavy chain loci in a manner similar to that of the wild-type heavy chain (21–24, 51).

We found the opposite effect on gene rearrangement at the κ light chain locus. Cells from $mem^{-/-}$ mice showed a modest amount of completed Vκ-to-Jκ rearrangement, while the $mem^{-/-}$ + tμ transgenic B cells showed a significant increase in κ gene rearrangements (Fig. 4*B*, *lanes 1* and *2*). In a fashion similar to full-length μ (24), the tμ transgene increased the frequency of

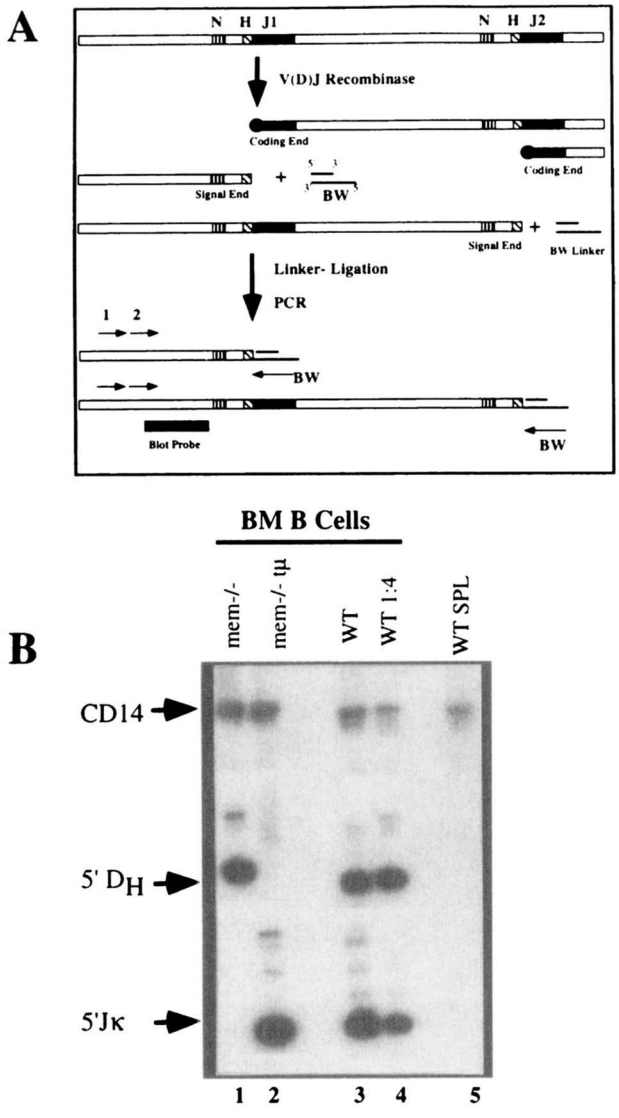

FIGURE 5. The influence of the tμ transgene on the abundance of recombination signal sequence breaks at Ig loci in mem$^{-/-}$ mice. *A*, Diagram of the LM-PCR assay for detection of signal ends in genomic DNA. The diagram shows two joining gene segments (black bars labeled J1 and J2) with their flanking recombination signal sequences (labeled N for nonamer and H for heptamer). Cleavage by the V(D)J recombinase yields broken coding and signal ends. The BW linker oligonucleotide is shown as an asymmetric pair of lines. PCR primers are indicated by smaller black arrows, labeled 1 and 2. After linker ligation of BW to the blunt ends in genomic DNA, primary PCR using linker primer BW and locus-specific primer 1 is followed by another PCR with the same linker-specific primer and a nested locus-specific primer (primer 2). Amplified signal ends are detected by hybridization with a radiolabeled locus-specific DNA probe (Blot Probe). Different sets of primers and probes are used to detect signal ends at DH and Jκ loci. *B*, LM-PCR assays of bone marrow DNA from μ membrane exon knockout B cell precursors with and without the tμ transgene. Double-stranded signal end breaks at Ig loci were analyzed using the assay shown in *A* and material from cells purified as described in Figure 4*A*. The amplification of CD14 (*top panel*) was included as a control for the equivalence of purified DNA samples. The spleen sample (*lane 5*) is included as a negative control, since broken signal ends are below the limits of detection in mature B cells. The *middle panel* shows the double-strand breaks upstream of DH gene segments indicative of V_H-to-DJ_H rearrangement (42). The *bottom panel* shows double-strand breaks upstream of the Jκ1 gene segment indicative of Vκ-to-Jκ gene rearrangement. Note that the range of this particular assay is quite narrow in that double-strand breaks in a wild-type bone marrow sample (WT) diluted 1/16 are undetectable (not shown).

endogenous V-to-Jκ rearrangements despite its negative effect on endogenous heavy chain gene rearrangement.

Assessing the abundance of VDJ_H- or Vjκ-rearranged alleles is an imperfect measure of the targeting of the V(D)J recombinase, since developing B cells undergo selection based on the expression of heavy and light chains. Proliferation or death of cells with particular gene rearrangements might give rise to misleading results. We recently reported a more precise way of measuring the targeting of the recombinase (42). The V(D)J recombinase generates double-stranded DNA breaks at the ends of rearranging gene segments. Two pairs of ends are created in this process: coding ends and signal ends (Fig. 5*A*) (52, 53). Using a ligation-mediated PCR assay (diagrammed in Fig. 5*A*), we showed previously that detection of signal ends indicates active recombination at a particular locus (42, 54). Since this assay detects intermediates in recombination rather than its products, which may be subject to selection, it more accurately reflects the amount of active recombination at a particular locus.

Using this assay on DNA isolated from the purified mem$^{-/-}$ and mem$^{-/-}$ + tμ bone marrow B cells described above, the abundance of breaks upstream of D_H gene segments and Jκ gene segments was analyzed (Fig. 5*B*). We observed a significant 5′-of-D_H broken-end signal in B cells from mem$^{-/-}$ mice that was completely suppressed in B cells from mem$^{-/-}$ + tμ mice (compare *lanes 1* and *2*). Breaks upstream of D_H gene segments indicate loci undergoing V-to-DJ_H rearrangement (42). Conversely, the presence of the tμ transgene increased the frequency of detectable breaks upstream of Jκ (indicating active V-to-Jκ gene rearrangement) in this same pair of DNA samples. These results confirm the apparent ability of the tμ transgene to signal heavy chain allelic exclusion and increase the efficiency of Vκ-to-Jκ gene rearrangement by targeting recombinase activity in the absence of SLC involvement.

We conclude from these experiments that by the criteria of surface marker changes, regulation of transcription, and retargeting of the V(D)J recombinase, a pre-BCR containing tμ is functional in the absence of the ability to form a conventional, SLC-containing pre-BCR complex.

Does λ5 have a role in development independent of its association with heavy chain protein?

These data lead to two hypotheses concerning the role of SLCs in early B cell development. First, the SLCs may simply be chaperone proteins that allow μ to assemble into a pre-BCR complex where it is competent to send developmental signals. Alternatively or in addition, the SLCs might have functions in development that are not related directly to their association with heavy chain in the pre-BCR but are essential for the development of B cells.

To search for a function for the λ5 SLC apart from its direct association with the pre-BCR, mice bearing the tμ transgene were bred to the λ5 knockout mouse line (15). Mice with a targeted disruption of λ5 display an incomplete block in B cell development at the pro- to pre-B cell transition and failure of heavy chain allelic exclusion. Both of these phenotypes have been attributed to the inability of λ5 knockout mice to form a pre-BCR complex (26). If λ5 serves only as a chaperone for heavy chain folding in the pre-BCR, cells with tμ should progress through development as assessed by surface markers, and the transgene should restore heavy chain allelic exclusion while inducing κ gene rearrangement. If λ5 has an additional role, developmental arrest might persist regardless of expression of the tμ transgene.

In agreement with previous reports (15) we found that λ5-deficient bone marrow B cells are predominantly CD43$^+$ (Fig. 6*A*). Expression of the tμ transgene efficiently promotes the transition

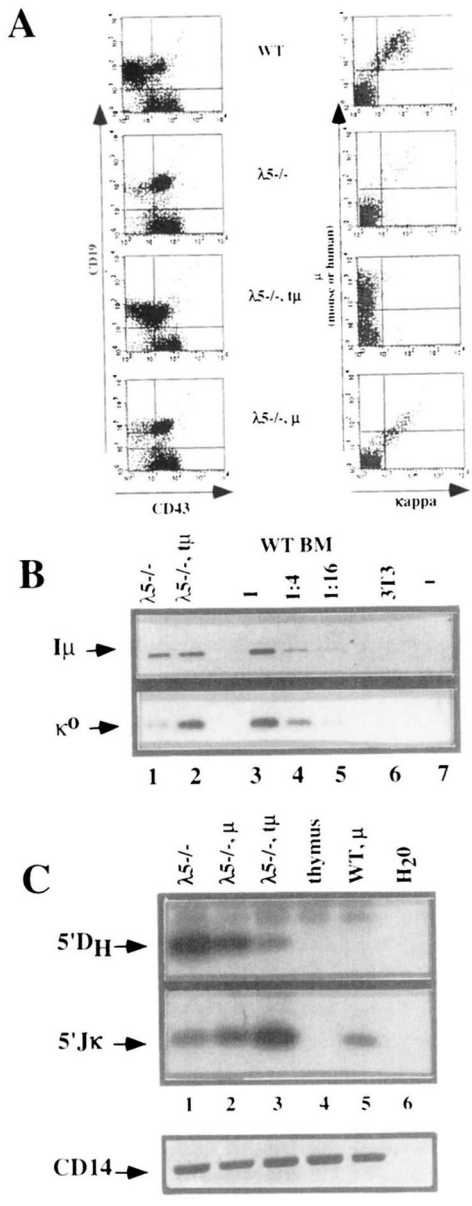

FIGURE 6. The effects of μ or tμ on surface marker expression, transcription, and V(D)J recombination in λ5-deficient mice. *A*, Flow cytometric analysis of cells purified from the bone marrow of 3- to 4-wk-old wild-type (WT), $\lambda5^{-/-}$, $\lambda5^{-/-}$ tμ transgenic, and $\lambda5^{-/-}$ μ transgenic mice using Abs specific for CD19, CD43, murine or human Ig μ, and Ig κ. The data are gated on the forward and side scatter characteristic of lymphoid cells. *B*, RT-PCR analysis of B cell-specific transcripts. Equivalent amounts of RNA isolated from $\lambda5^{-/-}$, $\lambda5^{-/-}$, tμ transgenic, and wild-type purified bone marrow B cells were reverse transcribed and analyzed by PCR using primers specific for each indicated RNA (see *Materials and Methods*) (18). Controls included amplification of a series of fourfold dilutions of wild-type cDNA into RNase-free water (*lanes 3–5*) and NIH-3T3 fibroblast cDNA (*lane 6*; as a nonlymphocyte, negative control), and a mock PCR reaction with no cDNA template (*lane 7*; showing that PCR samples are free of contamination). Products were separated by gel electrophoresis. The Iμ transcript is lymphocyte specific and was used to confirm the equivalence of each lymphocyte cDNA sample (*upper panel*; negative image of an ethidium-stained gel). The expression of the germline transcript (κ^0) is shown in the *bottom panel*, detected by blot hybridization and revealed by autoradiography. *C*, Analysis of double-strand DNA breaks in B cell precursors. DNA was purified from bone marrow cells with the indicated genotypes, and equivalent amounts were subjected to linker ligation and PCR analyses designed to measure double-strand breaks at D_H and Jκ1 recombination signal sequences (as shown in Fig. 5*A*) (42). Control reactions included no template (H_2O; *lane 6*), DNA purified from wild-type thymocytes (*lane 4*), and DNA purified from wild-type μ transgenic bone marrow (*lane 5*). PCR products were separated by gel electrophoresis, blotted to nylon filters, hybridized with radioactive probes specific for each locus, and revealed by autoradiography. The negative image of an ethidium-stained agarose gel of the products of PCR amplification of CD14, a nonrearranging locus, confirms the integrity and equivalence of DNA in each sample (*bottom panel*).

of these λ5-deficient cells from $CD43^+$ to $CD43^-$ (Fig. 6*A*) and activates the surface expression of CD22 (data not shown). In contrast, expression of the full-length μ transgene fails to promote this developmental progression (Fig. 6*A*).

We purified bone marrow cells from 3- to 4-wk-old $\lambda5^{-/-}$, $\lambda5^{-/-}$ + μ, and $\lambda5^{-/-}$ + tμ mice as described for the other mutant mouse lines and tested RNA and DNA isolated from these cells for marker transcripts and V(D)J recombination-associated DNA breaks. It is important to note that there was a significant percentage (12%) of $CD43^-$ cells in the purified population from $\lambda5^{-/-}$ mice. This $CD43^-$ population was somewhat larger in the $\lambda5^{-/-}$ + μ transgenic mice (18%). These $CD43^-$ cells probably represent the "leaky" nature of the developmental arrest in the $\lambda5^{-/-}$ mutant and the ability of precociously rearranged light chain to rescue μ^+ cells that cannot assemble the pre-BCR (32). These cells also contribute to the background of pre-B cell markers in the following analyses.

Figure 6*B* shows the RT-PCR analysis of the germline κ transcript. Equivalent amounts of amplifiable material were present as assessed by amplification of a control transcript from the mouse MHC class I (H2) locus (data not shown). Amplification of the B cell-specific Iμ transcript (*upper panels, lanes 1* and *2*) shows the equivalence of the lymphocyte samples. In the *lower panel* is shown low level, detectable germline κ transcription in purified cells from λ5-deficient mice. Levels of this transcript increased in cells with the tμ transgene (compare *lanes 1* and *2*).

DNA purified from these cells was analyzed by LM-PCR (as diagrammed in Fig. 5*A*) for DNA breaks associated with V-to-DJ_H and V-to-Jκ gene rearrangement. As shown in Figure 6*C*, $\lambda5^{-/-}$ and $\lambda5^{-/-}$ + μ transgenic bone marrow B cells had frequent DNA breaks 5′ of D_H gene segments that were significantly diminished in DNA from $\lambda5^{-/-}$ + tμ cells (compare *lanes 1–3, top panel*). This pattern is indicative of the allelic exclusion of heavy chain rearrangement mediated by tμ, but not by μ, in the absence of λ5. Similarly, analysis of the same DNA samples showed a striking induction of Jκ-associated broken DNA by the tμ transgene (Fig. 6*C*, *middle panel*, *lanes 1–3*). PCR analysis of DJ_H, VDJ_H, and VJκ rearrangements showed corresponding, although less striking, effects of the tμ transgene in the $\lambda5^{-/-}$ background (data not shown).

These effects of tμ are very similar to those of a full-length μ transgene on a wild-type background, as we reported previously (23). This emphasizes the need for SLCs in the transport of full-length heavy chain to the cell surface. The tμ transgene, because its expression is SLC independent, can reach the cell surface and drive pre-B cell differentiation.

How might tμ signal in the absence of SLCs?

Since the surrogate light chains are a consistent feature of an otherwise highly variable pre-BCR repertoire, it is possible that the pre-BCR signals through interaction of the surrogate chains with a ligand in the developing microenvironment. The ability of the

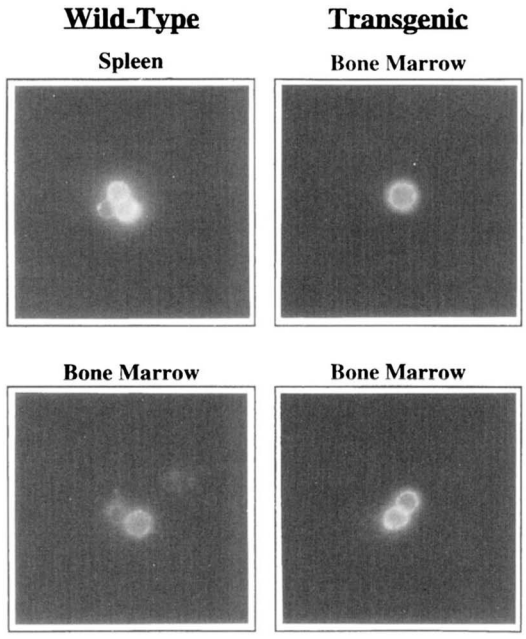

FIGURE 7. Comparison of the distribution of full-length μ and tμ on primary cells. Cells from wild-type mouse bone marrow and spleen (Wild-Type) as well as mem$^{-/-}$ + tμ transgenic mouse bone marrow (Transgenic) were stained with antiserum (phycoerythrin-conjugated goat anti-mouse μ) that detects endogenous and truncated μ and were analyzed by fluorescence microscopy. Representative μ^+ cells from each tissue and genotype are shown. Faintly visible cells are μ negative and have a level of staining equivalent to that obtained using a negative control antiserum (data not shown).

SLC-independent, truncated μ protein to drive pre-B cell development raises the possibility that surface μ can signal in the absence of ligand. However, by analogy with the previous analysis of another form of mutant heavy chain, the V_H-less human heavy chain disease (HCD) protein (55, 56), it was possible that tμ signaled in the absence of SLCs because it spontaneously aggregated at the cell surface (55). To determine whether tμ protein was normally distributed or aggregated on the cell surface, we performed immunofluorescence analysis, comparing bone marrow B cells from mem$^{-/-}$ + tμ transgenic mice to B cells from wild-type bone marrow and spleen (Fig. 7). Staining for surface μ expression showed that the distribution of μ on wild-type B cells and that of tμ on transgenic B cells are indistinguishable. Unlike the HCD protein, tμ did not appear to abnormally aggregate on the surface of developing B cells.

These experiments show that a truncated form of μ heavy chain that cannot associate with the SLCs can nonetheless promote differentiation of pro-B cells into pre-B cells as assessed by changes in surface marker expression, transcription of developmentally regulated genes, and targeting of V(D)J recombination. In addition, the expression of $\lambda 5$ is not required for signaling by the pre-BCR or for other regulated events before the pre-B cell stage of development.

Discussion

We have found that μ protein functions within the pre-BCR independent of its ability to associate with the SLCs VpreB and $\lambda 5$. Both μ and tμ transgenic pre-B cells show characteristic alterations in surface marker expression, decreased $\lambda 5$ transcription, increased germline κ transcription, decreased V-to-DJ_H gene rearrangement, and increased V-to-Jκ gene rearrangement. Since our results are derived from highly purified B cell populations, variation in the fraction of B cells in the marrow of these various mouse mutants cannot account for our observations. μ protein, via its assembly into a pre-BCR but independent of its association with SLCs, directly and actively regulates B cell development. These observations help to define the role of SLCs in B cell development.

Interpreting the activity of tμ during B cell development

The tμ transgene expresses a mutant version of the normal μ protein. The mutant has the useful property of incorporation into a pre-BCR-like complex without recruiting SLCs to the complex (12). One must consider the possibility, however, that tμ, because of its truncation or level of expression, may not act as a valid model for pre-BCR signaling. We offer the following observations in support of the validity of using tμ to study pre-BCR signaling.

The level of surface expression of the tμ pre-BCR far exceeds that of the wild-type pre-BCR (Fig. 2*A*) (18, 19). We presume that this is due to SLCs being limiting in amount, as wild-type pre-B cells have a large amount of cytoplasmic μ protein that is not assembled into a pre-BCR. It is possible that activation of the tμ pre-BCR occurs as a consequence of this elevation in surface expression and not of its physiologic activity. We think that this is unlikely, however, since although elevated, the amount of surface tμ is similar to the amount of surface μ on mature B cells that do not signal constitutively. In addition, our previous studies and those of others have shown that tμ must be cross-linked by Ab to activate calcium flux (43) or κ light chain gene rearrangement (12) in transformed cell lines, again arguing that it does not spontaneously signal.

It is possible that the tμ pre-BCR is structured abnormally and spontaneously aggregates on the cell surface, thereby activating signal transduction. This suggestion stems from observations made during studies of a human HCD protein that is a V_H-less version of μ that escapes retention in the ER (33, 55). The HCD protein, when expressed as a transgene in mice, is transported to the cell surface, where it appears in an aggregated form (57). These workers proposed that this aggregation signals developmental progression. Immunofluorescence analyses of tμ transgenic B cells showed that tμ is distributed over the pre-B cell surface in a pattern identical with that of μ on the surface of newly generated, wild-type bone marrow B cells and dissimilar to that of HCD in transgenic mice (Fig. 7) (57).

These considerations lead us to conclude that we can infer the properties of components of the wild-type pre-BCR by observing the effects of the tμ transgene on B cell development.

How does the pre-BCR signal?

On mature B cells, sIgM in a complex with Ig-α and Ig-β serves as the BCR. Interaction of this receptor with Ag, in conjunction with other regulatory signals, results in the clonal expansion and further differentiation of Ag-selected B cells. In a similar fashion, expression of the pre-BCR is required for the differentiation of pre-B cells (reviewed in Ref. 58). It is uncertain, however, what triggers pre-BCR signaling. One hypothesis is that a ligand exists in the microenvironment of the developing pre-B cell that serves to activate the pre-BCR by surface cross-linking. Given the clonal variation in the variable portion of heavy chain protein and the constancy of the SLCs, it was reasonable to consider the possibility that a putative pre-BCR ligand might interact with the SLC component of the pre-BCR. The current experiments make this possibility much less likely, however, since the tμ transgene can replace full-length μ in generating the pre-BCR signal without recruiting SLCs to the complex. It remains possible that the pre-BCR is activated by a ligand that interacts with one of the constant domains

of the μ chain. The ability of several Fc receptors to recognize the constant domain of various soluble Igs provides precedent for this type of recognition (59).

It is also possible, however, that the pre-BCR does not require a ligand but is constitutively active as consequence of the balance of kinases and phosphatases in pre-B cells. BCR signaling involves the phosphorylation of specific tyrosines in conserved domains of Ig-α and Ig-β (ITAMs) (4). The phosphorylation state of these residues depends on the balance of specific phosphatase and kinase activities within the cell. Developing B cells from mice that lack one such phosphatase, PTP1C, show a greatly increased sensitivity to deletion through interaction with self antigen (56). This same phosphatase, when localized to the cell membrane via its interaction with the transmembrane protein CD22, has been shown to diminish signaling through μ (46). We found that CD22, while undetectable before pre-BCR expression, is clearly expressed thereafter (Fig. 2*C*). We propose that nascent pre-B cells might have a great propensity for μ signaling since they lack CD22. In the absence of CD22, PTP1C would fail to localize to the membrane, and the activity of kinases associated with the pre-BCR would be unopposed. After pre-BCR signaling, elevated CD22 expression would localize PTP1C to the cell membrane and increase the threshold for receptor activation to that observed on mature cells that require exogenous Ag to activate the receptor. In contrast to primary cells, transformed pre-B cells transfected with tμ might fail to signal constitutively because of a higher signaling threshold related to their transformed state.

Since both proteins lack V_H domains, the tμ protein does somewhat resemble the Dμ protein, derived from a subset of endogenous D-to-J rearrangements, that is expressed with Ig-α and Ig-β on some pro-B cells (60, 61). It seems paradoxical at first that expression of Dμ protein leads to the deletion of the Dμ-expressing cell and not the developmental progression and bone marrow emigration observed with expression of tμ (Fig. 2*D*). We propose that Dμ and tμ proteins signal similarly in developing B cells, but due to their distinct structures, signaling generates different outcomes. Dμ associates with the SLCs, but cannot associate with conventional light chains (61). If Dμ signaling, like the pre-BCR signal from μ or tμ (this work and Refs. 18 and 23), results in cessation of heavy chain gene rearrangement and diminished expression of λ5, it will prevent further attempts at V-to-DJ rearrangement and decrease its own expression at the cell surface. Since Dμ-expressing cells cannot assemble a complete heavy chain, and Dμ cannot associate with a conventional light chain, these cells suffer a fate similar to that of other cells that fail to make productive heavy chain gene rearrangements: death by apoptosis. Signals from SLC-independent tμ pre-BCR complex, in contrast, down-regulate SLC expression, but this has no effect on the surface expression of the complex, which can continue to signal and promote B cell development.

Is there a role for λ5 apart from the pre-BCR?

Various groups have reported that the VpreB and λ5 genes are transcribed at the earliest stages of B cell development. Surprisingly, SLCs can be detected on the surface of B cell progenitors that lack heavy chain protein (7–9, 11, 34). Several as yet unidentified proteins coprecipitate with SLCs from these cells. It was proposed that these proteins might act as surrogate heavy chains, forming a surrogate heavy/surrogate light chain signaling complex on pro-B cells. Consistent with this possibility, a recent report showed that developing B cells incapable of expressing Ig-β-arrested development at a stage before V-to-DJ rearrangement (14). It is possible that the surrogate heavy/light complex requires Ig-β to signal a critical checkpoint in early B cell progenitors.

The present studies together with others (15, 26, 31) allow us to conclude that λ5 does not have a role in regulating surface marker changes, transcription, or gene rearrangement apart from its chaperone function for heavy chain. Developing B cells deficient in λ5 progress to the pro-B cell stage in a similar fashion as B cells with a deletion of the membrane exons of μ chain (Figs. 4 and 6). In addition, they undergo the pro-to-pre-B cell transition in identical fashion upon expression of tμ protein (Figs. 4–6). Therefore, the function of SLCs associated with putative surrogate heavy chains remains undetermined.

Surrogate light chains as chaperones

To assure heavy chain allelic exclusion, a developing B cell must monitor its success in assembling a functional heavy chain gene. In the absence of a light chain, however, heavy chain protein is misfolded in the endoplasmic reticulum and ultimately degraded there (4, 30). SLCs, first discovered by subtractive cDNA cloning as transformed pre-B cell-specific transcripts (60), can associate with heavy chain protein in pre-B cells and promote their transport to the cell surface in association with Ig-α and Ig-β (9). Multiple lines of evidence have shown that the pre-BCR is essential for heavy chain allelic exclusion. We have presented experiments that show that SLCs are not required for signaling by this complex and suggest that their role might be limited to that of a chaperone, allowing heavy chain to assemble into the pre-BCR. Similar observations were made recently with HCD gene transgenic mice, in which the HCD protein mediated both allelic exclusion of endogenous heavy chain expression and alteration in CD43 expression in developing B cells (57, 61).

Previous experiments using transfected cell lines had shown that the tμ gene could be transported to the cell surface without SLCs (12). In those experiments, however, the mutant pre-BCR required exogenous cross-linking with anti-μ Ab to affect light chain gene rearrangement while signaling by full-length heavy chain was constitutive. We found that in transgenic mice, signaling by tμ is constitutive and results in heavy chain allelic exclusion, activation of light chain gene rearrangement, and alterations in surface marker and gene expression. The difference between these sets of results might be due to the effects of cell transformation on pre-BCR signaling or to the absence of potential ligands provided by the microenvironment of developing B cells. Our observations lead us to suggest that the major role of SLCs in primary pre-B cells is to promote the assembly of the pre-BCR, apart from which it is dispensable.

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