Regulation of B Lymphocyte Development by the Truncated Immunoglobulin Heavy Chain Protein Dμ

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Summary

The development of B lymphocytes from progenitor cells is dependent on the expression of a pre–B cell–specific receptor made up by a μ heavy chain associated with the surrogate light chains, immunoglobulin (Ig)α, and Igβ. A variant pre–B cell receptor can be formed in which the μ heavy chain is exchanged for a truncated μ chain denoted Dμ. To investigate the role of this receptor in the development of B cells, we have generated transgenic mice that express the Dμ protein in cells of the B lineage. Analysis of these mice reveal that Dμ expression leads to a partial block in B cell development at the early pre–B cell stage, probably by inhibiting V_H to D_HJ_H rearrangement. Furthermore, we provide evidence that Dμ induces V_L to J_L rearrangements.

During B cell differentiation, the genes encoding the heavy and light chains of the immunoglobulin molecules are assembled from germline gene segments in an ordered fashion (1, 2). Initially, a D_H segment is joined to a J_H segment in the heavy chain locus on both chromosomes. Subsequently, a V_H gene segment is rearranged to the D_HJ_H complex. If this renders a functional rearrangement, a μ heavy chain is expressed on the cell surface, together with the surrogate light chains encoded by the genes λ5 and Vpre-B (3–5). This complex, denoted the pre–B cell receptor (pBCR),[1] has been shown to be of vital importance for maturation of B lymphocytes. Thus, in mice deficient in this receptor, B cell differentiation is arrested at an early stage (6–9). Moreover, the pBCR gives a signal to the cell to stop further rearrangements on the heavy chain locus (10–13) and to upregulate rearrangement of the light chain gene segments (14, 15). Due to an inexact joining mechanism, the D_H can be rearranged to the J_H in three possible reading frames (RFs). A majority of the D_H segments carry their own promoter and an ATG translational initiation codon. When the D_H is rearranged to a J_H in RF2, according to the nomenclature of Ichihara et al. (16), this D_HJ_H complex can be translated into a truncated μ chain, denoted Dμ (17). A well-documented underrepresentation of RF2 in $V_HD_HJ_H$ as well as D_HJ_H joints (18–20) has been suggested to be mediated by this protein expressed on the cell surface (19). The mechanism by which this occurs is, however, unknown. It has been postulated that the Dμ protein in association with the surrogate light chains (Dμ pBCR) possess signalling properties similar to those given by the pBCR, including signals mediating allelic exclusion (21–24). If so, cells expressing the Dμ protein on the cell surface would be arrested at an early developmental stage due to the absence of a complete μ heavy chain.

To investigate the effect of Dμ expression on B cell differentiation, we generated mice transgenic for the Dμ protein under control of its endogenous promoter (Dμ-endo) or, alternatively, under control of the pre–B cell and B cell–specific mb-1 promoter (Dμ–mb-1; references 25–27).

Materials and Methods

Transgenic Constructs. The Dμ transgenic constructs were created by PCR amplification of D_HJ_H rearrangements, using DNA from large pre–B cells as template. The primers (Fig. 1 *A*, *a* and *b*) hybridize to sequences 0.42 kb 5′ of the D_H segment and 0.62 kb 3′ of J_H4, respectively. The PCR products were sequenced, and a fragment consisting of DFL16.1 joined to J_H4 in RF2 was cut with NotI and EcoRI, and cloned into pBluescript. A second construct in which the endogenous promoter was replaced with the mb-1 promoter, was generated by PCR amplification using primers c and b (Fig. 1 *A*), and the fragment was cut with BamHI/EcoRI and cloned into pBluescript containing the mb-1 promoter. The 0.3-kb fragment containing the mb-1 promoter was isolated by PCR according to the published sequence (27). Next, the plasmids were cut with Xba1 and Xho1, and ligated with a 9.8-kb XbaI–XhoI fragment from the plasmid p21-H22 (10), provided by Dr. T. Leandersson (University of Lund, Lund, Sweden), containing the complete membrane heavy chain

[1]*Abbreviations used in this paper:* HSA, heat stable antigen; pBCR, pre–B cell receptor; RF, reading frame.

I. Bergqvist and U.-C. Tornberg contributed equally to this work.

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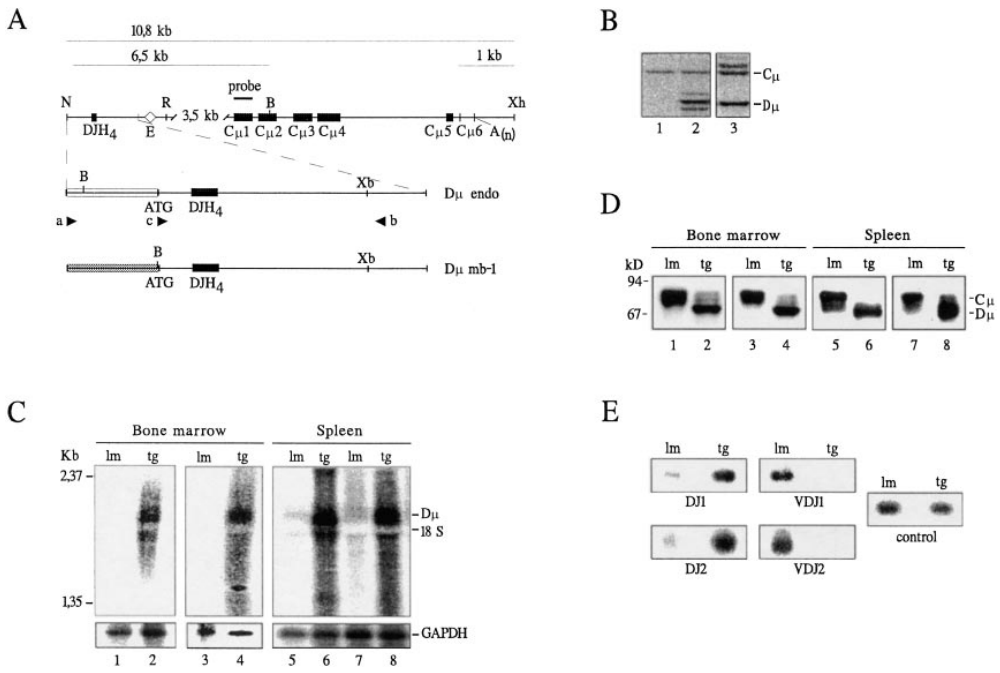

Figure 1. (*A*) Schematic outline of the transgenic constructs used. The Dμ-endo construct included a 0.3-kb fragment containing the endogenous promoter (*open box*), a DFLJH4 rearrangement in RF2, the IgH enhancer (*E*), and the complete Cμ sequence. *Arrowheads*, the location of PCR primers used to generate the constructs. The probe used for Southern blot analysis is depicted in the figure together with the size of the BamHI fragment detected in transgenic mice (6.5 kb). The construct used to create Dμ–mb-1 transgenic mice was derived from the Dμ-endo construct by replacing the region upstream of the ATG with a 0.3-kb fragment containing the mb-1 promoter (*shaded box*). *B*, BamHI; N, NotI; *R*, EcoRI; *Xb*, XbaI; *Xh*, XhoI. (*B*) Southern blot analysis of genomic tail DNA digested with BamHI and hybridized with Cμ exon 1 as a probe. The 6.5-kb Dμ transgenic band and the ~10-kb band representing the endogenous Cμ locus are indicated. Lane *1*, C57/BL6; lane *2*, Dμ-endo, founder 13; lane *3*, Dμ–mb-1, founder 23. The transgenic band in lane *3* is 0.3 kb shorter than in lane *2*, due to the insertion of a BamHI site 3′of the mb1 promoter in the construct. (*C*) Northern blot analysis of total RNA from bone marrow and spleen cells hybridized with a probe complementary to the D_HJ_H joint used in the constructs. Blots from transgenic (*tg*) mice and littermate controls (*lm*) are shown. Lanes *1*, *2*, *5*, and *6*: Dμ–mb-1 line 26. Lanes *3*, *4*, *7*, and *8*: Dμ-endo line 11. The 1.9-kb Dμ transcript and the 18 S ribosomal RNA are indicated in the figure. GAPDH was used as a control for quantification. (*D*) Expression of the Dμ protein in transgenic mice. Proteins extracted from bone marrow or spleen cells were analyzed by Western blot. A band of ~69 kD was detected in transgenic mice using an anti-IgM antibody. Blots representing transgenic (*tg*) mice and littermate controls (*lm*) are shown. Dμ–mb-1, lanes *1*, *2*, *5*, and *6*; Dμ-endo, lanes *3*, *4*, *7*, and *8*. (*E*) $V_HD_HJ_H$ and D_HJ_H rearrangements in Dμ-endo transgenic mice. $B220^+CD43^+$ pre–B cells were isolated by cell sorting from transgenic mice (*tg*) and from littermate controls (*lm*). Semiquantitative PCR (28) was performed using a primer hybridizing to a sequence downstream of JH2, together with primers complementary to either recombination sequences 5′of all D regions or to members of the J558 family. As the target sequence for the JH primer is not included in the transgenic construct, there is no amplification of the transgene itself. Limited PCR amplification of a nonrearranging locus (λ5) was used to normalize the DNA content in the reactions. PCR products were hybridized with a probe complementary to the JH1 and JH2 exons, or to the λ5 gene. The upper and lower panels show rearrangements to the JH1 and the JH2 gene segments, respectively.

constant region. The plasmids containing the final constructs were digested with NotI and XhoI, the inserts were gel purified and injected into fertilized oocytes of F1(C57BL/6 × CBA) mice. The injected zygotes were transplanted into oviducts of pseudopregnant female mice. Tail DNA from offspring was digested with BamHI, and a 0.3-kb probe comprising the Cμ1 exon was used for Southern blot, detecting a band of 6.2/6.5 kb in transgenic mice (Fig. 1 *B*).

PCR Amplifications and Southern Blot Analysis. PCR amplifications performed to isolate the gene fragments used for the transgenic vectors were carried out over 30 cycles (1 min at 95°C, 1 min at 55°C, and 2 min at 72°C) in a programmable thermal controller (PTC-100; MJ Research Inc., Watertown, MA), using DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland) with the supplied buffer. The following primers were used for PCR amplification: (*a*) 5′-CGCGCGGCCGCTCAAAGCACAATGCCTGG-3′, (*b*) 5′-GGAATTCCTTCTAATATTCCATACACATA-3′, and (*c*) 5′-AAGGATCCATGACAACTGAAACTCAACC-3′.

Quantification of IgH chain rearrangements were performed according to Costa et al. (28). In brief, DNA was prepared by lysing 4–16 × 10^4 $CD43^+$, $B220^+$, IgM^- cells in 200 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 60 μg/ml proteinase K), incubating them at 55°C for 2 h, and then precipitating the DNA with isopropanol. The DNA was dissolved in water, and used for PCR at a concentration of ~5–20 ng/reaction. PCR amplifications were carried out for 30 cycles (30 s at 95°C, 2 min at 55°C, and 1 min and 45 s at 72°C) in a programmable thermal controller (PTC-100; MJ Research Inc.), using DynaZyme II DNA polymerase (Finnzymes Oy) with the supplied buffer. Limited PCR amplification of a nonrearranging locus (λ5) was used to normalize the DNA content in the reactions. The following primers, at a concentration of 100 ng/reaction, were used for PCR amplification: JH: 5′-GGCTCCCAATGACCCTTTCTG, DH: 5′-GTCAAGGGATCTACTACTGTG, VJ558: 5′-TCCTCCAGCACAGCCTACATG, 5′λ5: 5′-CAAGTCTGACCCCTTGGTCACTC, 3′λ5: 5′-TGTGAGGCATCCACTGGTCAGATA. One-tenth of the 50 μl PCR reaction was run on a 1.7% agarose gel, blotted onto Zeta-Probe GT blotting membranes (BioRad, Hercules, CA) and hybridized with a 510-bp probe spanning the JH1 and JH2 exons (for detection of D_HJ_H and $V_HD_HJ_H$ rearrangements) or a 690-bp probe (29; provided by Dr. L. Mårtensson, University of Lund, Lund, Sweden) for detection of λ5. Intensity of the bands was determined using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA).

Northern Blot Analysis. Total RNA from spleen and BM was

Table 1. *Number of IgM$^+$ B Cells and of Total Nucleated Cells*

Transgenic construct	Founder	TG/LM*	Newborn liver: Nucleated cells × 10^6	Newborn liver: IgM$^+$ cells × 10^4	*n*	3-wk-old spleen: Nucleated cells × 10^6	3-wk-old spleen: IgM$^+$ cells × 10^6	*n*	Adult spleen: Nucleated cells × 10^6	Adult spleen: IgM$^+$ cells × 10^6	*n*
Dμ-endo	11	TG	12.5 (± 3.9)†	3.5 (± 0.6)	3	18.2 (± 6.3)	2.9 (± 2.6)	4	37.0 (± 4.2)	18.2 (± 1.9)	2
		LM	16.4 (± 2.4)	51.5 (± 9.8)	4	36.2 (± 6.2)	13.7 (± 4.1)	5	79.0 (± 19.8)	42.8 (± 7.1)	2
Dμ–mb-1	26	TG	7.1 (± 1.4)	7.4 (± 2.2)	5	ND	ND		ND	ND	–
		LM	8.2 (± 2.3)	22.3 (± 8.9)	5	ND	ND		ND	ND	–

* *TG*, transgenic; *LM*, littermates.
† Numbers given within parenthesis represent standard deviation.

isolated using Ultraspec RNA isolation system (Biotecx, Houston, TX). The RNA was electrophoresed in a 1.2% agarose/formaldehyde gel, transferred to Zeta-Probe GT blotting membranes (BioRad), and hybridized according to the manufacturer's recommendations. A 0.8-kb fragment from the transgene construct, spanning the D_HJ_H complex, was used as a probe to detect Dμ transcripts. κ transcripts were detected using a 0.4-kb fragment comprising the 3′ portion of the Cκ gene, and a 0.9-kb probe containing part of the mouse mb-1 gene (25; provided by Dr. Michael Reth, Institute for Biology III, Freiburg, Germany) was used to determine the amount of B cell–derived RNA in the samples.

Western Blot Analysis. Proteins were prepared by lysing 3–10 × 10^6 cells from bone marrow, or from spleen, in 100 μl sample buffer (135 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 10% 2-mercaptoethanol, and bromophenolblue indicator [BFB]) and applying the suspension to a 5–15% SDS-PAGE gradient gel. Fractionated proteins were electroblotted onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) using a Trans-Blot cell (BioRad). Immunodetection of the Dμ chain was carried out using a horseradish peroxidase–labeled anti-IgM antibody (Southern Biotechnology Associates, Birmingham, AL) and an ECL Western blotting kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer's protocol.

Flow Cytometry Analysis and Cell Sorting. Bone marrow cells were flushed out of the femurs with HBSS. Spleen cells were obtained by homogenization of the organ in the same medium. Cells were collected by centrifugation, resuspended in FACS medium (3% fetal calf serum and 0.1% sodium azide in PBS), counted, and 10^6 cells/25 μl were incubated with antibodies. The antibodies used were: biotin-coupled anti-B220 RA3.6.B2 (30), FITC-labeled anti-IgM (Southern Biotechnology Associates), FITC-labeled anti-CD43 (PharMingen, San Diego, CA), biotin-coupled anti–heat stable antigen (HSA) (PharMingen), and streptavidin PE–labeled anti–BP-1 (PharMingen). PE– and Cychrome–conjugated streptavidin were obtained from PharMingen. Stained cells were analyzed on a FACSCalibur® (Becton Dickinson, Mountain View, CA). For cell sorting, bone marrow cells were stained with the same reagents and separated on a FACStar Plus® (Becton Dickinson).

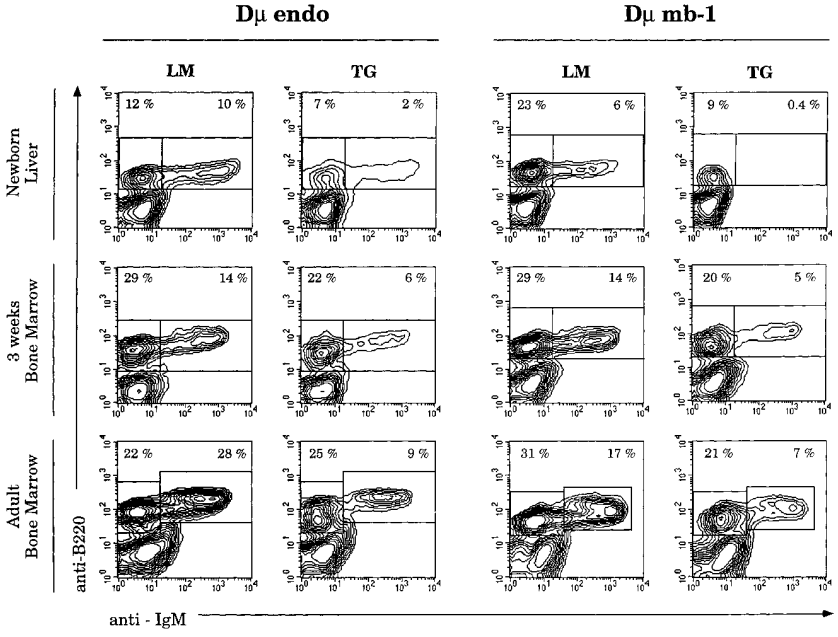

Figure 2. Flow cytometric analysis of newborn liver and bone marrow cells from Dμ-endo transgenic mice, Dμ-mb-1 transgenic mice, and littermate controls. Cells were stained with anti-B220-PE and anti-IgM-FITC and analyzed on FACScan®. The lymphocyte population was gated according to standard forward- and side-scatter values. The numbers above the framed areas represent the percentage of B220$^+$IgM$^-$ and B220$^+$IgM$^+$ cells out of the total number of gated lymphocytes.

Results and Discussion

Dμ Expression Leads to Arrest in V_H to D_HJ_H Rearrangements. Five founder transgenic mouse lines were established expressing Dμ under the control of the endogenous D_H promoter, and four lines were established with Dμ expression controlled by the mb-1 promoter (25–27; Fig. 1 *A*). Each of the founder mice were crossed to C57BL/6 mice and were analyzed for integration of the transgene by genomic Southern blots using a probe hybridizing to the Cμ1 exon (Fig. 1 *B*). Transcription of transgenic Dμ in splenic and bone marrow cells from transgenic mice was demonstrated by Northern blot analysis (Fig. 1 *C*). Western blot analysis of cell lysates was used to confirm the expression of transgenic Dμ protein. Thus, Dμ protein was readily detected in bone marrow cells and in spleen cells from transgenic, but not from littermate mice (Fig. 1 *D*). To directly test if the expression of Dμ protein would affect the $V_HD_HJ_H$ rearrangement process, the relative amount of complete $V_HD_HJ_H$ and of incomplete D_HJ_H rearrangements was estimated in $B220^+CD43^+$ early B cell progenitors using a semiquantitative PCR assay (28). As illustrated in Fig. 1 *E*, the relative amount of complete $V_HD_HJ_H$ rearrangements was found to be severely reduced in the transgenic mice compared to littermate controls. In contrast, DJ_H rearrangements were more abundant in the transgenic mice. Together, these data provide evidence for that expression of the Dμ protein mediates inhibition of V_H to DJ_H rearrangements.

Partial B Cell Depletion in Dμ Transgenic Mice. To study the effect of the transgenically expressed Dμ protein on the B cell compartment, newborn liver, bone marrow, and spleen cells from two transgenic lines were analyzed by flow cytometry. Analysis of B lymphocytes from newborn mice revealed an ~3- and 15-fold reduction of IgM positive cells in the liver (Table 1, Fig. 2). In 3-wk-old mice, B cell numbers were reduced about fourfold in transgenic mice compared to littermate controls (Table 1). In adult spleen, the number of total B cells was approximately twofold lower in the transgenic mice (Table 1), whereas the T cells numbers were apparently unchanged (data not shown). At all time points analyzed, no significant difference in the proportion of $CD5^+$ and $CD5^-$ B cells in the peritoneum was observed, indicating that the generation of B-1 cells and of conventional B cells were similarly affected by the transgenic Dμ expression (data not shown).

Impairment of B Cell Differentiation in Dμ Transgenic Mice Occurs at the Pro/Pre B Cell Stage. To identify the stage at which the B cell development was affected by the transgenic expression of Dμ, bone marrow cells from adult mice were analyzed by flow cytometry. The number of immature and mature B cells ($B220^+IgM^+$) was diminished approximately two- to threefold in transgenic mice, whereas the $B220^+IgM^-$ population, including most B cell progenitors, was similar or only slightly reduced compared to littermate controls (Fig. 2).

The stages of B cell differentiation have been subdivided into fractions (A–F) on the basis of expression of the cell surface markers B220, CD43, HSA, BP-1, and IgM (31). Analysis of bone marrow cells using these markers revealed that the $B220^+CD43^+$ early progenitors were slightly increased in transgenic mice, whereas $B220^+CD43^-$ cells were fourfold reduced compared to littermate controls (Fig. 3). These results suggested that the observed block in B cell development induced by Dμ expression occurs before the transition of late pro–B cells to the pre–B cell stage.

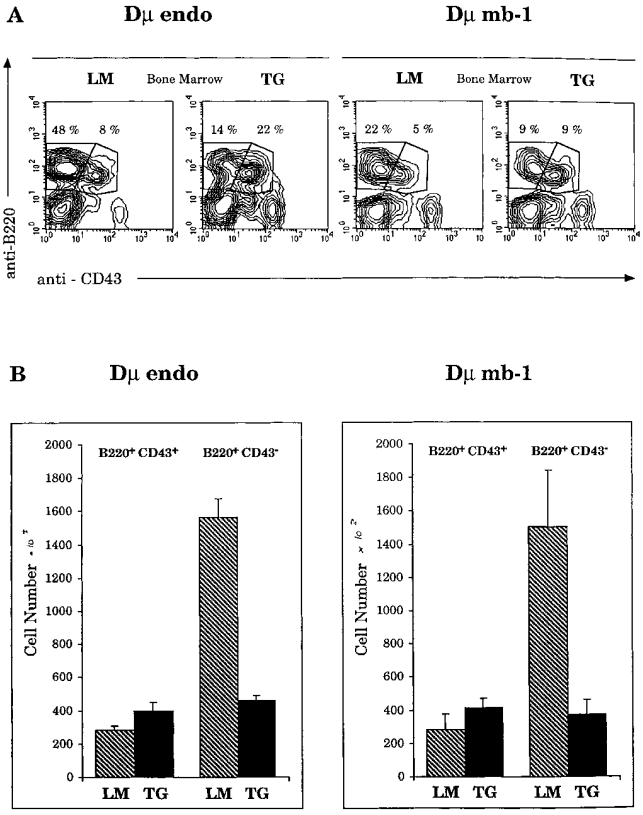

Figure 3. Flow cytometric analysis of bone marrow cells from 3-wk-old Dμ-endo transgenic mice, Dμ–mb-1 transgenic mice, and littermate controls. (*A*) FACS® profiles of cells stained with anti-CD43 and anti-B220 and analyzed on FACScan®. The lymphocyte population was gated according to standard forward- and side-scatter values. The numbers above the framed areas represent the percentage of $B220^+CD43^-$ and $B220^+CD43^+$ cells out of the total number of gated lymphocytes. (*B*) Numbers of $B220^+CD43^-$ and $B220^+CD43^+$ cells per femur displayed as histogram plots.

To further dissect at what point in early B cell differentiation Dμ expression exerts its effect, BP-1, CD43, and HSA expression was used to subdivide B cell progenitor cell populations (31). As shown in Fig. 4, the $CD43^{int}BP1^+$ cell population (late pro–B cells) was slightly increased in transgenic mice compared to littermate controls. However, although the number of $CD43^{int}BP1^+$ cells with low expression of HSA was almost twofold higher in the transgenic versus wild-type mice, the number of $CD43^{int}BP1^+$ cells with high level expression of HSA was similar. Thus,

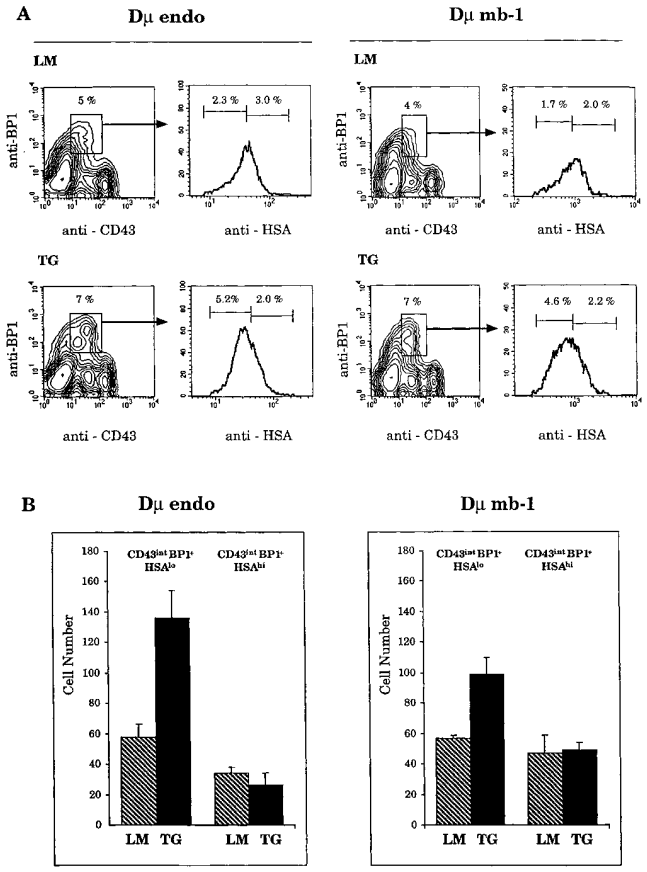

Figure 4. Flow cytometric analysis of bone marrow cells from 3-wk-old Dμ-endo transgenic mice, Dμ–mb-1 transgenic mice, and littermate controls. (*A*) FACS® profiles of cells stained with anti-CD43, anti-BP-1, and anti-HSA, and analyzed on FACScan®. The lymphocyte population was gated according to standard forward and side-scatter values. Cells expressing BP-1 and intermediate levels of CD43 (indicated as a framed population) were analyzed for HSA expression and are displayed as a separate histogram. (*B*) Numbers of $CD43^+$, $BP\text{-}1^+$, HSA^{high} and $CD43^+$, $BP\text{-}1^+$, HSA^{low} cells per femur are displayed as histogram plots.

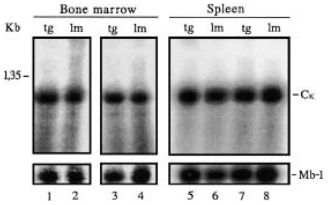

Figure 5. Northern blot analysis of total RNA from bone marrow and spleen cells hybridized with a probe complementary to the Cκ. Blots from transgenic (*tg*) mice and wild-type littermates (*lm*) are shown. Lanes *1*, *2*, *5*, and *6*: Dμ-mb-1 line 26. Lanes *3*, *4*, *7*, and *8*: Dμ-endo line 11. The κ transcript is indicated in the figure. mb-1 was used as a control for quantification.

the partial block in B cell development induced in the Dμ transgenic mice occurs at the transition of the fraction C to the fraction C′ in the nomenclature of Hardy et al. (31), i.e., at the developmental stage where μ chain expression on the cell surface is required for further differentiation.

Our results provide support for the hypothesis that the Dμ pBCR can mediate a block in B cell development, probably by inhibiting further V_H to D_HJ_H rearrangement (32, 33) similar to the pBCR (10–13). This mechanism appears, however, to allow leakage of cells that can complete $V_HD_HJ_H$ rearrangement in the presence of Dμ expression. This is not surprising in view of the observed production of endogenous rearrangements in transgenic mice expressing a IgH chain (10–13). It is predicted from the proposed action of Dμ expression that most or all of these mature B cells should contain only one complete $V_HD_HJ_H$ rearrangement. Experiments addressing this issue are presently ongoing.

Dμ Induces Light Chain Rearrangements. In addition to inhibiting V_H to D_HJ_H rearrangements, Dμ expression has been suggested to mediate induction of V_L to J_L rearrangements (21, 32). If so, progenitor B cells of the Dμ transgenic mice would be expected to rearrange the light chain locus despite the arrest in B cell development and the possible impairment of V_H to D_HJ_H rearrangements. To test this hypothesis, we analyzed the levels of expression of κ chain messenger RNA in bone marrow cells. The levels of κ transcripts were found to be similar in transgenic mice compared to littermate controls (Fig. 5). Since in transgenic mice there is a three- to four-fold reduction in the B cell progenitors that normally produce L chain transcripts, these results suggest that Dμ expression induces V_L to J_L in progenitors that normally would contain the light chain loci in germline configuration. It appears, thus, that Dμ can replace the complete μH chain in terms of mediating induction of V_L to J_L rearrangements.

We conclude from these results that the Dμ pBCR can mediate a block in B cell development, probably by inhibiting V_H to D_HJ_H rearrangements, as well as inducing V_L to J_L rearrangements. In contrast, Dμ cannot substitute for the requirement of μH chain expression for pre–B cell transition. These observations are in agreement with previous reports demonstrating that Dμ counterselection is mediated through the transmembrane domain of the membrane Dμ protein (33) and is dependent on the expression of Igβ (23) and Syk (22). It has been suggested that the inability of Dμ to mediate pre–B cell transition would be due to a failure to pair with L chains (24). This explanation seems unlikely, however, because counterselection of the Dμ protein-encoding RF2 appears to occur before the stage of L chain expression (34). An alternative explanation may be that the Dμ pBCR and the pBCR generate qualitatively different signals (12). Further analysis of the Dμ transgenic mice will be able to directly assess this possibility.

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