Immunoglobulin γ2b Transgenes Inhibit Heavy Chain Gene Rearrangement, but Cannot Promote B Cell Development

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Summary

Transgenic mice with a γ2b transgene were produced to investigate whether γ2b can replace μ in the development of B lymphocytes. Transgenic γ2b is present on the surface of B cells. Young transgenic mice have a dramatic decrease in B cell numbers, however, older mice have almost normal B cell numbers. Strikingly, all γ2b-expressing B cells in the spleen also express μ. The same is true for mice with a hybrid transgene in which the μ transmembrane and intracytoplasmic sequences replace those of γ2b (γ2b-μmem). The B cell defect is not due to toxicity of γ2b since crosses between γ2b transgenic and μ transgenic mice have normal numbers of B cells. Presence of the γ2b transgene strongly enhances the feedback inhibition of endogenous heavy chain gene rearrangement. Light chain genes are expressed normally, and the early expression of transgenic light chains does not improve B cell maturation. When the endogenous μ locus is inactivated, B cells do not develop at all in γ2b transgenic mice. The data suggest that γ2b cannot replace μ in promoting the developmental maturation of B cells, but that it can cause feedback inhibition of heavy chain gene rearrangement. Thus, the signals for heavy chain feedback and B cell maturation appear to be different.

The development of B lymphocytes is intimately linked to the expression of Ig genes (reviewed in reference 1). Production of membrane-bound IgM is required for B cell maturation, as mice whose μ gene membrane exons have been inactivated by targeted recombination lack B cells (2). Furthermore, membrane-bound IgM is required in the control of the rearrangement of Ig genes by feedback inhibition (reviewed in reference 3). During B cell development feedback inhibition occurs first at the stage of heavy chain gene rearrangement, followed later by the cessation of Ig gene rearrangement due to the shutoff of V(D)J recombinase. Both μ and δ heavy chains can promote feedback inhibition at the stage of heavy chain gene rearrangement and, in combination with light chains, at the stage of V(D)J recombinase shutoff (3, 4). It is clear from work with transgenic mice that the membrane form of μ (μm) is required for both types of feedback inhibition, and that the secreted form (μs) does not mediate feedback (5, 6). The μ and δ proteins have related transmembrane domains and identical short cytoplasmic tails (Lys-Val-Lys) (7). The γ heavy chains have a similar degree of homology to μ and δ in the transmembrane portion, but have a much longer intracytoplasmic domain (7). All three heavy chain classes differ considerably in the CH domains. In light of these structural differences, it was of great interest to compare the effect of γ2b in B cell development to that of μ and δ. Presumably, if γ2b did not have one or all of the effects of μ and δ, sequence differences between μ/δ and γ2b may be implicated in the maturation promoting and/or feedback effects.

We have produced several different transgenic mouse lines with a γ2b gene, presumably representing different chromosomal insertion sites (8), as well as mice carrying a γ2b transgene whose transmembrane and intracytoplasmic portions have been replaced by those of μ (γ2b-μmem). All express the γ2b transgene as shown by tissue-specific γ2b RNA synthesis (8 and not shown). Here we describe the analysis of B cells of these transgenic lines in comparison with B cells from normal littermates and μ transgenic mice.

Materials and Methods

Transgenic Mice. The 343-1 and 348A γ2b transgenic lines have been described (8). The γ2b-μmem transgene, 1241-5-3 (9), was constructed by ligating the V_H through the CH3γ2b region on a 10.6-kb PvuI to KpnI fragment that also includes the majority of pUC13cm^r from pVCM to the μ membrane exons contained on a 2.6-kb KpnI to PvuI fragment from pV_H167μ (5, 8). The transgene was isolated as an 11.4-kb SalI to PvuI fragment by gel electrophoresis and elution from hydroxyapatite (10). The 243-4 μ transgenic line used as a control has been described (5, 11). The μ transgene encodes both the secreted and membrane forms of μ

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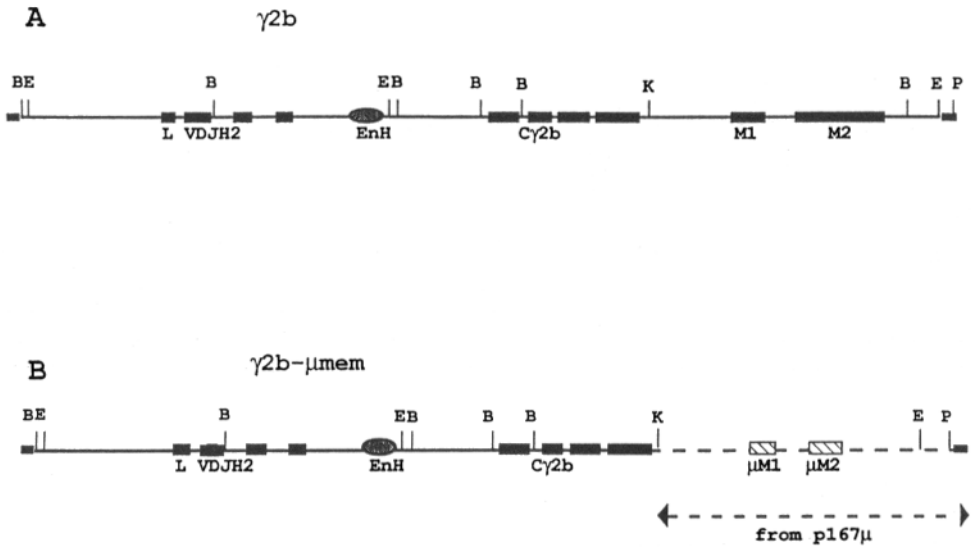

Figure 1. Schematic representation of γ2b (*A*) and γ2b-μmem transgene constructs (*B*). The transgene constructs are drawn approximately to scale. Filled boxes represent exons, the filled oval represents the heavy chain enhancer, and the boxes at the ends indicate pUC13 vector sequence. *B*, BamHI; *E*, EcoRI; *K*, KpnI; *P*, PvuI. (*A*) γ2b transgene. (*B*) γ2b-μmem hybrid transgene. Striped boxes and dotted line indicate region derived from the μ locus.

and contains the VDJ_H region from myeloma MOPC 167 (11). The μdelS transgenic line contains a transgene that is a deletion derivative of the $V_H167\mu$ transgene used to generate the 243-4 μ line. The transgene was derived by deletion of a 1.3-kb BclI fragment containing the secreted terminus of the μ gene. This transgene produces only the membrane form of μ. The μ knockout line (μmt) was obtained from K. Rajewsky (University of Köln, Köln, Germany) via C. Sidman (University of Cincinnati, Cincinnati, OH). This line contains a disruption of the membrane region encoding exons of the μ heavy chain (2). The λ2 transgenic line 1275-4 contains a functionally rearranged λ2 light chain gene from plasmacytoma MOPC 315 and is regulated by the heavy chain enhancer (12).

The founder mice of the γ2b-only mice and the μ and μdelS mice were derived from (SJL × C57BL/6)F_1 and were bred in the first generation with F_1 mice. Later generations (for 5–7 yr) were backcrossed to C57BL/6. The λ2- and γ2b-μmem transgenic mice were produced and propagated in C57BL/6. The 343-1, 348A, 1241-5-3, and 243-4 lines have the following Brinster designations: Tg(Igh)Bri49, Tg(Igh)Bri50, Tg(Igh)Bri128, and Tg(Igh)Bri35, respectively. Their approximate transgene copy numbers are: 8, 22, 14, and 6, respectively.

Flow Cytometry. Flow cytometry (FACS®; Becton Dickinson & Co., Mountain View, CA) was performed essentially as described using the following antibodies (12). Biotinylated rat anti–mouse CD45R (clone RA3-6B2) (PharMingen, San Diego, CA) at 1:100, PE-conjugated monoclonal rat anti–mouse κ (Becton Dickinson & Co.) at 1:50, FITC-conjugated goat anti–mouse γ2b (GAMγ2b; Southern Biotechnology Assoc., Birmingham, AL) at 1:100, PE-conjugated GAMμ (Southern Biotechnology Assoc.) at 1:100, PE-conjugated Streptavidin (Jackson ImmunoResearch Labs., Inc., West Grove, PA) at 1:80, FITC-conjugated or biotinylated mouse anti–mouse IgH-6a (μ^a), clone DS-1 at 1:100 (kindly provided by Dr. Jim Kenny, Frederick Cancer Center, Frederick, MD), and biotinylated mouse anti–mouse IgH6b (μ^b), clone AF6-78 at 1:100 (PharMingen). Samples were analyzed by FACScan® using Consort30, FACScan®, or LYSIS software (Becton Dickinson & Co.). A total of 10^4 lymphocytes as determined by forward and side scatter were analyzed per sample. Where indicated, dead cells were excluded using propidium iodide staining. Just before analysis, 10–20 μl of 1 mg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) was added to each sample. In these cases, the data collection gate was set on live cells and was regated on lymphocytes upon analysis.

Ribonuclease Protection. Ribonuclease protection assay (RPAs)[1] were performed using the RPA and RPAII kits (Ambion Inc., Austin, TX) according to the manufacturer's instructions. Briefly, 10–30 μg of sample RNA plus yeast tRNA to give a total of 30 μg of RNA was hybridized overnight (>8 h) with 2×10^5 cpm of each probe in a 20-μl reaction volume at 53°C. RNase digestion was carried out at 30°C using a 1:100 dilution of solution R (RNaseA and T1) followed by resolution of protected fragments on 5% denaturing acrylamide/urea gels. The T_7 β-Act plasmid (Ambion Inc.) contains a 250-bp KpnI to XbaI fragment from pAL41 (13) in the USB vector pT_7-1. Restriction with DdeI and in vitro transcription using T_7 RNA polymerase results in a 160-nucleotide (nt) protected fragment in RPA. The CH3MA probe was generated by cloning the 458-bp AccI to MscI fragment containing γ2b CH3 and poly(A) signal for the secreted γ2b message into the SmaI site of Bluescript KS+. Linearization with XbaI and in vivo transcription with T_3 RNA polymerase generates a ~548-nt transcript that protects 404-nt secreted γ2b and 297-nt membrane γ2b fragments in RPA (see Fig. 3). The CH4AH probe was generated by cloning the 726-bp ApaLI to HindIII fragment, including μCH4, the secreted μ terminus, and poly(A) signal into Bluescript KS+. Linearization with BamHI and in vitro transcription using T_3 RNA polymerase protects a 519-nt secreted μ fragment and a 333-nt membrane μ fragment in RPA. The Cκ-2 probe was generated by PCR amplification of a 625-bp fragment containing the last 307 bp of the J-Cκ intron and the first 318 bp of the Cκ exon (essentially the whole coding region). The fragment was cloned into the SmaI site of Bluescript KS+ (Stratagene, La Jolla, CA), and antisense Cκ transcripts are generated by linearization with BamHI and transcription using T_3 RNA polymerase. Processed Cκ mRNAs result in a 318-nt protected fragment.

[1] *Abbreviations used in this paper:* M, membrane; nt, nucleotides; RPA, ribonuclease protection assay.

Results

Splenic B Lymphocytes at Different Ages. The results from two transgenic lines carrying a functionally rearranged γ2b transgene and one line containing a hybrid γ2b-μmem transgene are reported here. Fig. 1 shows a scheme of each transgene construct.

Splenic lymphocytes from littermate or age-matched normal and γ2b transgenic mice were analyzed by FACS® in order to compare their B cell populations. Neonatal mice from the γ2b 343 line have very few B cells. Less then 1% of splenic lymphocytes are either μ or κ positive compared with 11–12% in normal littermates (Table 1). 2-wk-old γ2b-μmem transgenic mice have a similar defect (data not shown). At 4 wk of age, the γ2b transgenic lines have a significantly lower percentage of κ^+ or μ^+ (mIg^+) cells compared with normal mice or with μ transgenic mice. The defect is most severe in the 343 line, which has only 11% μ^+ cells compared with 54% in normal mice (Table 1). The percentage of κ^+ cells matches that of μ, indicating that there are essentially no B cells that express γ2b without also expressing μ. Surprisingly, when the transmembrane and intracytoplasmic portion of μ is substituted for that of γ2b, the resulting γ2b-μmem transgenic mice have the same phenotypes as γ2b transgenic

Table 1. *Flow Cytometric Analysis of Splenic Bone Marrow B Cells*

	Mouse Type	κ	μ	γ2b		No. of spleen cells*
						× 10^6
Spleen of 6-d-old mice	Normal (*n* = 7)	12.5 ± 3.0	11.5 ± 2.1	2.3 ± 1.2		8.5 (3.8–13.2)
	343-1 (*n* = 2) γ2b	0.2 ± 0.3	0.25 ± 0.5	1.0 ± 0.3		8.6 (7.2–10.0)
					B220	
Spleen of 4-wk-old mice	Normal (*n* = 13)	56.1 ± 1.8	54.2 ± 2.9	3.05 ± 1.5	64.3 ± 2.5	51 (39–90)
	343-1 (*n* = 9) γ2b	11.7 ± 2.7	11.4 ± 2.2	14.5 ± 4.3	25.6 ± 1.8	16 (10–27)
	348A (*n* = 2) γ2b	39.8 ± 7.3	35.6 ± 3.8	5.1 ± 3.8	44.4 ± 0.6	25 (23–27)
	1241-5-3 (*n* = 5) γ2b-μmem	16.0 ± 3.8	16.8 ± 3.9	15.7 ± 3.4	44.3 ± 7.2	19 (16–22)
	243-4 (*n* = 4) μ	49.8 ± 2.1	28.8 ± 7.4 (end) 28.8 ± 10.8 (trg)	2.4 ± 0.4	52.6 ± 4.4	42 (31–56)
					$B220^{lo}$	$B220^{high}$
Bone marrow cells of adult mice‡	Normal (*n* = 5)	51.1 ± 3.8	50.5 ± 5.3	10.3 ± 4.2	51.3 ± 8.4	30.3 ± 7.6
	343-1 (*n* = 5) γ2b	18.2 ± 8.7	21.0 ± 10.7	11.1 ± 1.8	47.8 ± 18.3	11.2 ± 7.5

Cells were stained for the indicated markers and analyzed by FASCcan®. A total of 10^4 lymphocytes as defined by forward scatter and side scatter were analyzed per sample. The average percentage of lymphocytes positive for the given marker is indicated at a 90% confidence level as determined by *t* test.
* Average number of nucleated spleen cells. Range of values is in parenthesis.
‡ μ transgenics were analyzed using allotype-specific antibodies. end, endogenous μ^b; trg, transgenic μ^a.

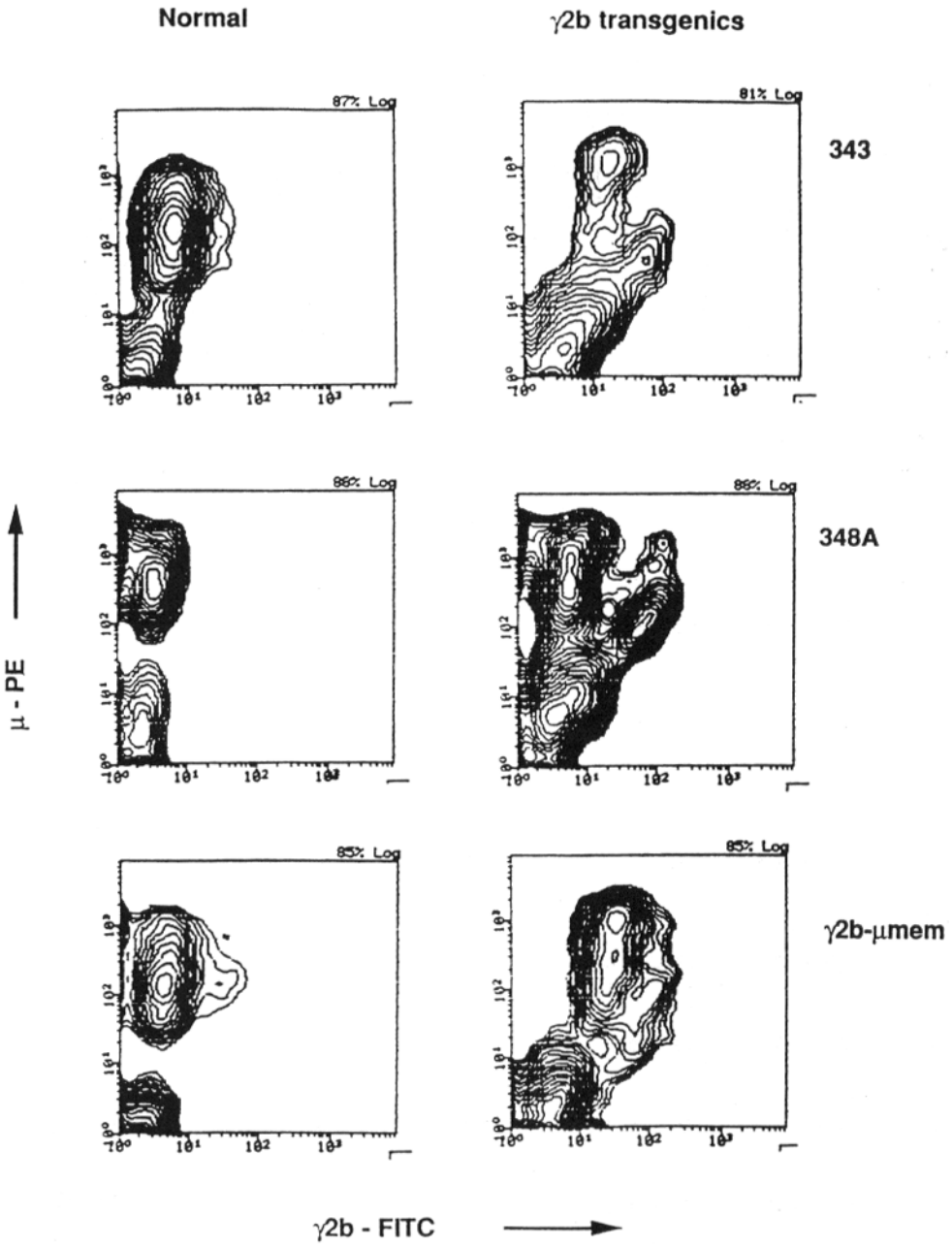

Figure 2. Two-color FACS® analysis of splenic lymphocytes from adult γ2b mice. μ-PE staining on the *y*-axis and γ2b-FITC staining on the *x*-axis are shown for representative 343 γ2b, 348A γ2b, and γ2b-μmem transgenic mice, each in comparison with a normal littermate.

mice (Table 1). In contrast, in μ transgenic mice, the percentage of κ^+ cells is greater than endogenous μ, indicating that ~21% B cells express transgenic μ without endogenous μ (Table 1) (5, 14, 15).

By 16 wk, B cells in the γ2b mice have increased to near normal numbers (not shown). But, unlike in μ and δ transgenic mice (5, 6, 16), B cells of the γ2b mice always coexpress γ2b and endogenous μ (Fig. 2). Again, the γ2b-μmem transgenic mice have the same phenotype as γ2b transgenics (Fig. 2). In the 343 γ2b and γ2b-μmem lines virtually all B cells coexpress μ and γ2b. In the 348A line, a large proportion of μ^+ B cells are $\gamma2b^{lo}$ or $\gamma2b^-$. It is not known whether in these cells the transgene has been inactivated or whether the endogenous μ H chain preferentially associates with the L chain that happens to be produced by these cells.

Thus, splenic B cells from γ2b mice coexpress μ on all $\gamma2b^+$ cells. The replacement of the γ2b transmembrane and intracytoplasmic regions with those of μ is not sufficient to permit the development of γ2b-only B cells. In addition, γ2b and γ2b-μmem mice are defective in the formation of B cells, as revealed by the low B cell numbers in young mice. This defect is overcome with age and the severity of the defect varies among transgenic lines. Preliminary analysis of two additional γ2b transgenic lines agrees with these findings (not shown).

The γ2b Transgene Is Expressed Early and at High Levels during Fetal Development. Analysis of lymphoid bone marrow cultures and Abelson murine leukemia virus–transformed bone marrow pre-B cells indicated that the 343 mice express γ2b in the pre-B compartment (17). This implies that the trans-

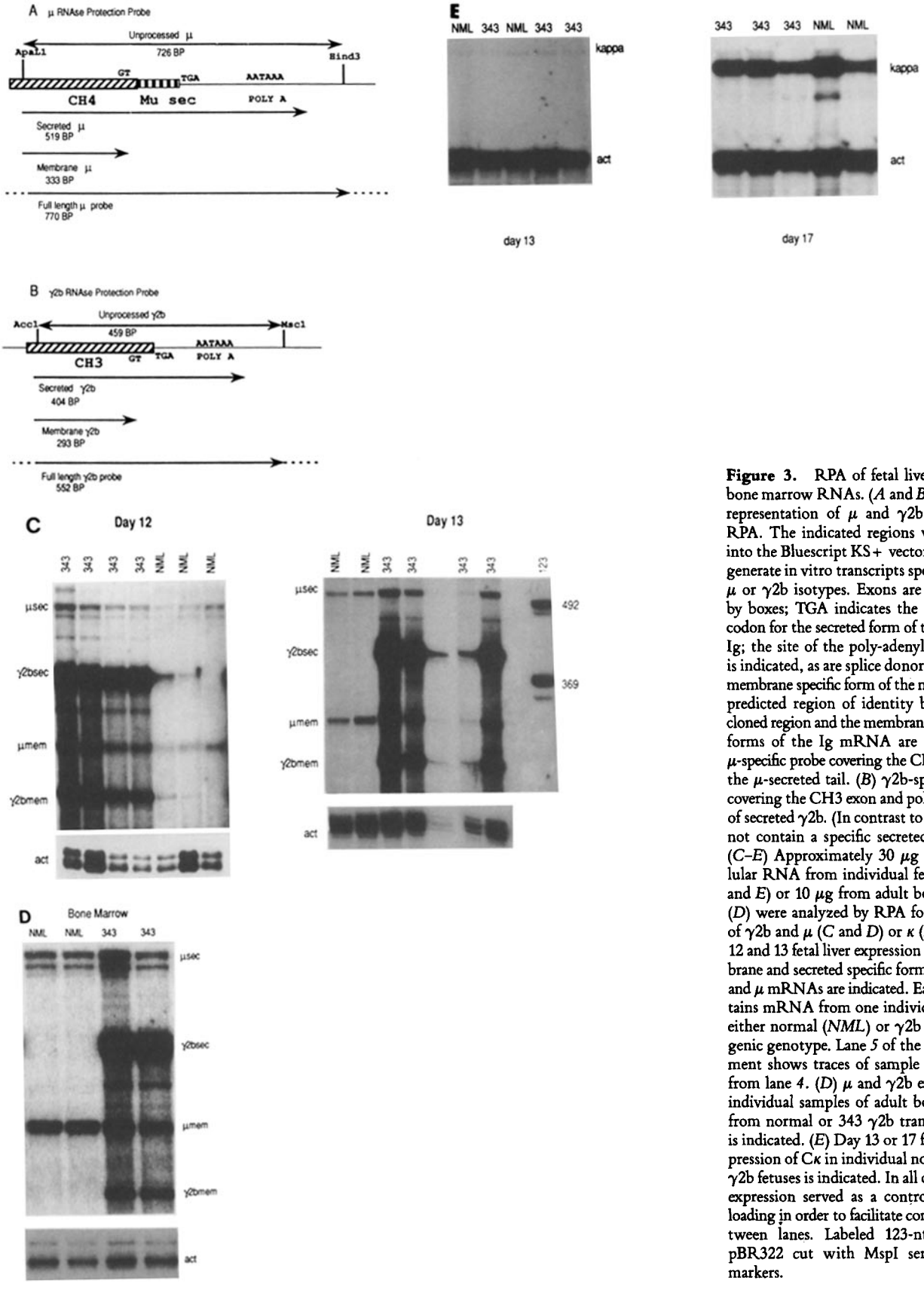

Figure 3. RPA of fetal liver and adult bone marrow RNAs. (*A* and *B*) Schematic representation of μ and γ2b probes for RPA. The indicated regions were cloned into the Bluescript KS+ vector in order to generate in vitro transcripts specific for the μ or γ2b isotypes. Exons are represented by boxes; TGA indicates the termination codon for the secreted form of the indicated Ig; the site of the poly-adenylation signal is indicated, as are splice donor sites for the membrane specific form of the mRNA. The predicted region of identity between the cloned region and the membrane vs. secreted forms of the Ig mRNA are shown. (*A*) μ-specific probe covering the CH4 exon and the μ-secreted tail. (*B*) γ2b-specific probe covering the CH3 exon and poly(A) region of secreted γ2b. (In contrast to μ, γ2b does not contain a specific secreted terminus.) (*C–E*) Approximately 30 μg of total cellular RNA from individual fetal livers (*C* and *E*) or 10 μg from adult bone marrow (*D*) were analyzed by RPA for expression of γ2b and μ (*C* and *D*) or κ (*E*). (*C*) Day 12 and 13 fetal liver expression of the membrane and secreted specific forms of the γ2b and μ mRNAs are indicated. Each lane contains mRNA from one individual fetus of either normal (*NML*) or γ2b (*343*) transgenic genotype. Lane *5* of the 13-d experiment shows traces of sample carried over from lane *4*. (*D*) μ and γ2b expression in individual samples of adult bone marrow from normal or 343 γ2b transgenic mice is indicated. (*E*) Day 13 or 17 fetal liver expression of Cκ in individual normal or 343 γ2b fetuses is indicated. In all cases β-actin expression served as a control for RNA loading in order to facilitate comparison between lanes. Labeled 123-nt ladder or pBR322 cut with MspI served as size markers.

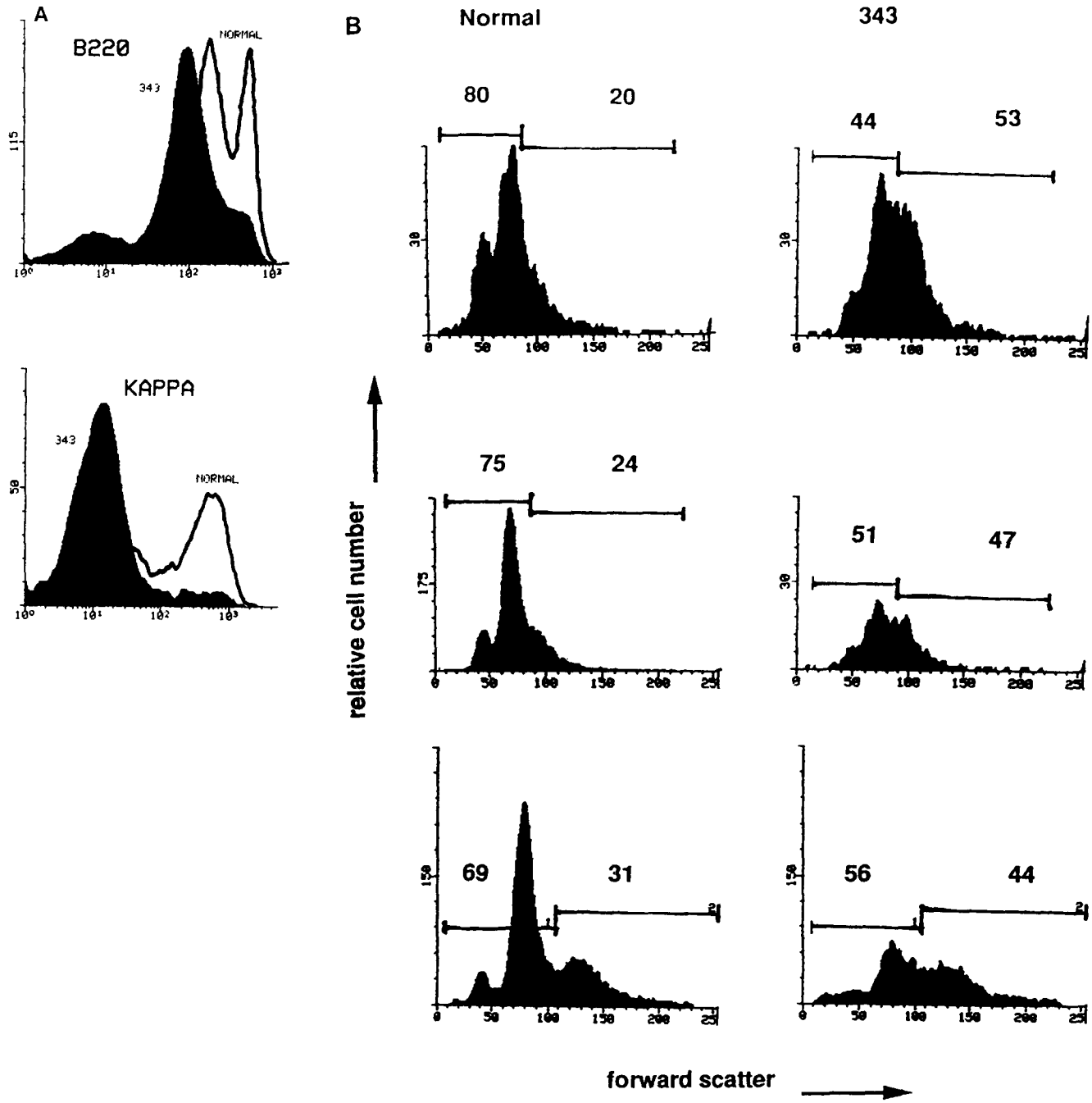

Figure 4. FACS® analysis of bone marrow from 343 γ2b transgenic and normal mice. (*A*) Anti B220 (*top*) and anti κ (*bottom*) staining. The *x*-axis indicates the fluorescence intensity of staining for the indicated marker using PE-coupled antibodies. The *y*-axis indicates the number of bone marrow lymphocytes positive for each level of staining with the indicated marker. *Blackened area*, 343 γ2b transgenic; *outlined area*, normal mouse. (*B*) $B220^{lo}$ bone marrow lymphocytes of 343 γ2b mice. $B220^{lo}$, mIg^- bone marrow cells reanalyzed for cell size by forward scatter are indicated by histograms. The percentage of large, less mature cells is indicated in the right-hand region of each panel. The percentage of smaller, more mature cells is indicated in the left-hand region. Three individual adult 343 γ2b mice are shown each in comparison with a normal littermate.

gene is expressed appropriately to mediate allelic exclusion, namely around the time of the first μ expression when developing B cells are beginning to rearrange their endogenous H chain genes (18, 19, 20). In light of the observed lack of allellic exclusion in mature cells described above, we sought to define more precisely the stage at which the γ2b gene is first expressed. We used an RPA for analysis of fetal liver (Fig. 3), because B cell development occurs in a synchronous wave in this organ (20). Individual fetal livers at days 12, 13, 15, and 17 of gestation were analyzed. Expression of γ2b is readily detectable and several-fold higher than that of μ in day 12 fetal liver from 343 γ2b transgenic mice (Fig. 3

C). Both membrane and secreted γ2b mRNAs are produced, although the level of secreted mRNA exceeds that of membrane mRNA (Fig. 3 C). This prevalence of secreted γ2b mRNA is in agreement with data that show an excess of secreted over membrane γ2b mRNA in lymphoma and myeloma lines (21, 22). The level of γ2b expression increases through day 17 (days 15 and 17 not shown).

The data indicate that the γ2b transgene is expressed at an appropriate developmental point to mediate allelic exclusion, namely during the time of endogenous H gene rearrangement. The levels of membrane γ2b are approximately equivalent to or higher than membrane μ levels at every time point. In light of these findings, the lack of H gene allelic exclusion seen in the peripheral B cells of γ2b transgenic mice does not appear to be due to insufficient or delayed expression of membrane γ2b.

The Bone Marrow Compartment Reveals a Block in B Cell Development in γ2b Transgenic Mice. To assess the stage at which B cell development is inhibited in γ2b transgenic mice, cells of the B cell lineage were analyzed in the bone marrow. FACS® analysis of bone marrow from normal and 343 γ2b mice shows two B220 populations (Fig. 4 *A* and Table 1). In normal mice, the $B220^{lo}$ population comprises 51% of the lymphoid compartment in bone marrow and the $B220^{hi}$ population comprises 30% (Table 1). However, in the bone marrow of 343 γ2b transgenic mice the $B220^{hi}$ population is decreased to only 11% of the lymphoid cells (Table 1). In addition, in normal mice, 50% of the bone marrow lymphoid cells express IgM on the cell membrane (mIgM) while only 18% of 343 bone marrow lymphoid cells are mIg positive as indicated by κ staining (Fig. 4 *A* and Table 1). As in the spleen, all 343 bone marrow cells that are γ2b positive are also μ positive (data not shown). While few cells in 343 bone marrow express mIg at the cell surface, mRNA for membrane γ2b readily can be detected (Fig. 3 *D*).

In both normal and 343 γ2b bone marrow, all $B220^{hi}$ cells are also mIg^+, while the $B220^{lo}$ population consists of both mIg^- and mIg^+ populations representing pre-B and B cells, respectively. In addition, the $B220^{lo}$, mIg^- population contains both large and small pre-B cells that can be distinguished based on forward light scatter. In normal mice, 69–80% of the $B220^{lo}$ pre-B cells are the small more mature type, while only 44–56% of 343 γ2b transgenic $B220^{lo}$ pre-B cells are of this type (Fig. 4 *B*).

The data suggest that the paucity of B cells observed in the spleens of younger γ2b mice may result from an inefficiency in generating B cells. This defect manifests itself in the bone marrow as a decrease in the $B220^{hi}$ population. It appears that the block in B cell development may occur within the $B220^{lo}$ pre-B cell compartment, which is skewed to contain a greater proportion of larger, less mature cells. The data imply that γ2b transgenic mice are not able to generate small pre-B (and subsequently B cells) as efficiently as normal mice. All the $B220^{hi}$ cells that emerge in γ2b transgenic bone marrow express μ, thus implying that μ may be required for this step of B cell development and that γ2b cannot function as a substitute.

γ2b Expression Is Not Toxic to B Cells, but It Inhibits Heavy Chain Gene Rearrangement. The high levels of expression of γ2b in the 343 fetal liver and bone marrow and the dramatic decrease in the population of more mature $B220^{hi}$ cells in γ2b transgenic mice raised the possibility that the γ2b transgenic heavy chain is toxic to developing B cells. Alternatively, γ2b may not be able to provide certain physiological signals that are essential for development. To distinguish between these alternatives, a functional μ transgene (from mouse line 243-4) was crossed into the 343 γ2b transgenic background.

Similar to previous findings, 7-wk-old 343 γ2b mice exhibit a dramatic decrease in splenic B cells (Table 2). The 243-4 μ transgenic mice, on the other hand, have almost normal numbers of B cells. Crossing the γ2b transgenic mice with the μ transgenic mice leads to a reversal of the B cell defect characteristic of γ2b transgenic mice. There is a considerable increase in the population of $B220^+$ and κ^+ cells in γ2b × μ double transgenic mice at both 7 and 16 wk in comparison with the 343 γ2b only littermates (Table 2). These findings suggest that the γ2b transgene product itself is not inhibitory to B cell development in the presence of a functional μ chain.

The μ transgenic mice show little feedback inhibition of endogenous μ genes. These μ transgenic mice have few transgenic μ–only cells, but endogenous μ–only and endogenous μ plus transgenic μ–positive B cells represent the majority of B cells in the spleen of the μ transgenic mice (Fig. 5 *A*). There is a striking decrease in the percentage of cells expressing endogenous μ in the double transgenic mice in comparison with transgenic μ-only mice (Fig. 5 *A*), showing that γ2b strongly inhibits endogenous H gene rearrangement. However, all the γ2b expressing cells also coexpress transgenic μ (Fig. 5 *B*).

Thus, the γ2b protein does not act in a dominant manner to disrupt B cell development. The exclusion of endogenous μ gene expression in $\gamma2b^+/\mu^+$ double transgenic B cells reveals a pronounced rearrangement feedback effect by the γ2b transgene. The feedback presumably occurs through inhibition of heavy chain gene rearrangement and does not impede the expression of the μ transgene.

The feedback effect by γ2b is further demonstrated in hybridomas from γ2b transgenic mice (Table 3). Only 18% of hybridomas from normal mice have either a germline heavy chain or a DJ rearrangement on one of the heavy chain alleles. However, 59% of γ2b transgenic hybridomas have an unrearranged or incompletely rearranged heavy chain allele.

Expression of a Transgenic Light Chain Does Not Improve B Cell Development in γ2b Transgenic Mice. The data suggest a functional inadequacy of γ2b compared with μ during B cell development. One characteristic attributed to heavy chain expression is the induction of light chain gene rearrangement (23). To test the possibility that the defect in B cell development characteristic of γ2b transgene expression is due to an inability to induce light chain gene expression and rearrangement, the 343 γ2b line was crossed to the 1275-4 λ2 transgenic line. This transgene causes partial feedback on both

Table 2. *Flow Cytometric Analysis of Splenic B Cells from 343-1 γ2b Crossed to 243-4 μ Mice*

	Mouse Type	κ	μ^{a*}	μ^{b*}	γ2b	B220
7 wk‡	243-4	47	25	31	2	48
	μ	51	43	47	2	57
		51	27	43	2	52
		50	41	33	3	53
	343-1	13	5	16	13	25
	γ2b	16	4	18	16	28
		17	8	17	17	24
	343-1 γ2b	40	39	11	17	41
	X	28	28	12	27	31
	243-4 μ	49	47	8	15	N.D.
16 wk	Normal	61	2	60	5	72
		68	1	66	5	71
	243-4	61	29	44	5	71
	μ	66	39	52	5	72
	343-1	30	6	31	31	57
	γ2b	28	6	32	21	54
	343-1 γ2b	53	53	10	49	70
	X	49	41	16	41	56
	243-4 μ	56	53	11	47	62

Spleen cells from offspring of 343-1 γ2b × 243-4 μ crosses were stained for the indicated markers and analyzed by FACScan®. A total of 10^4 lymphocytes as defined by forward scatter and side scatter were analyzed per sample. The percentage of splenic lymphocytes from each animal positive for the given marker is indicated.

* μ expression was analyzed using allotype-specific antibodies. μ^b, endogenous μ; μ^a, transgenic μ.

‡ No mice without a transgene were present in this litter.

H and L chain gene rearrangement, presumably because it is expressed in early pre-B cells due to the presence of the heavy chain enhancer (12).

Table 4 summarizes the analysis of (343 γ2b × 1275-4 λ2) offspring from three independent experiments. As before, 343 γ2b transgenic spleen cells express μ and κ as well as γ2b. The percentage of λ^+ cells in 343 transgenic spleen is 5%, equivalent to that in normal mice. Similar to previous results (12), the λ2 transgenic mice express λ on 33% of splenic lymphocytes and κ on 26%. In double transgenic γ2b × λ2 offspring the percentage of λ expression is increased compared with γ2b-only mice. Many cells coexpress κ and λ (not shown). Compared to γ2b transgenic mice the numbers of Ig^+ B cells do not increase. Again, all the B cells that emerge in the spleen express μ, and most also express γ2b. Thus, the presence of a functionally rearranged light chain expressed early in B cell development does not result in the repair of the B cell depletion or the production of B cells expressing the γ2b transgene alone.

Induction of κ Transcription during Fetal Development Is Normal. To further analyze a possible effect of the γ2b transgene on light chain expression, we determined the levels of κ mRNA in fetal livers of γ2b mice in comparison with normal littermates. Using RPA analysis we examined Cκ transcripts at days 13, 15, and 17 of gestation. The probe used in these studies detects but does not distinguish between rearranged and germline κ transcripts (see Materials and Methods). At day 13, κ transcripts are barely detectable in both normal and 343 γ2b transgenic fetal liver (Fig. 3 *E*). By days 15 (not shown) and 17 (Fig. 3 *E*), the levels of κ expression have substantially increased. However, no differences are seen in the relative levels of κ expression between normal and γ2b transgenic mice at any time.

The findings indicate that normal mice do not initiate κ transcription earlier nor to a higher level at any of the time points analyzed in comparison to γ2b transgenic mice. Since a correlation exists between the rate of transcription at the κ locus and the rearrangement of κ genes (24), no significant differences in κ gene rearrangement are likely to be occuring normal vs. γ2b mice. Thus, the defect in B cell development characteristic of γ2b transgenic mice does not seem to result from a failure to induce light chain production.

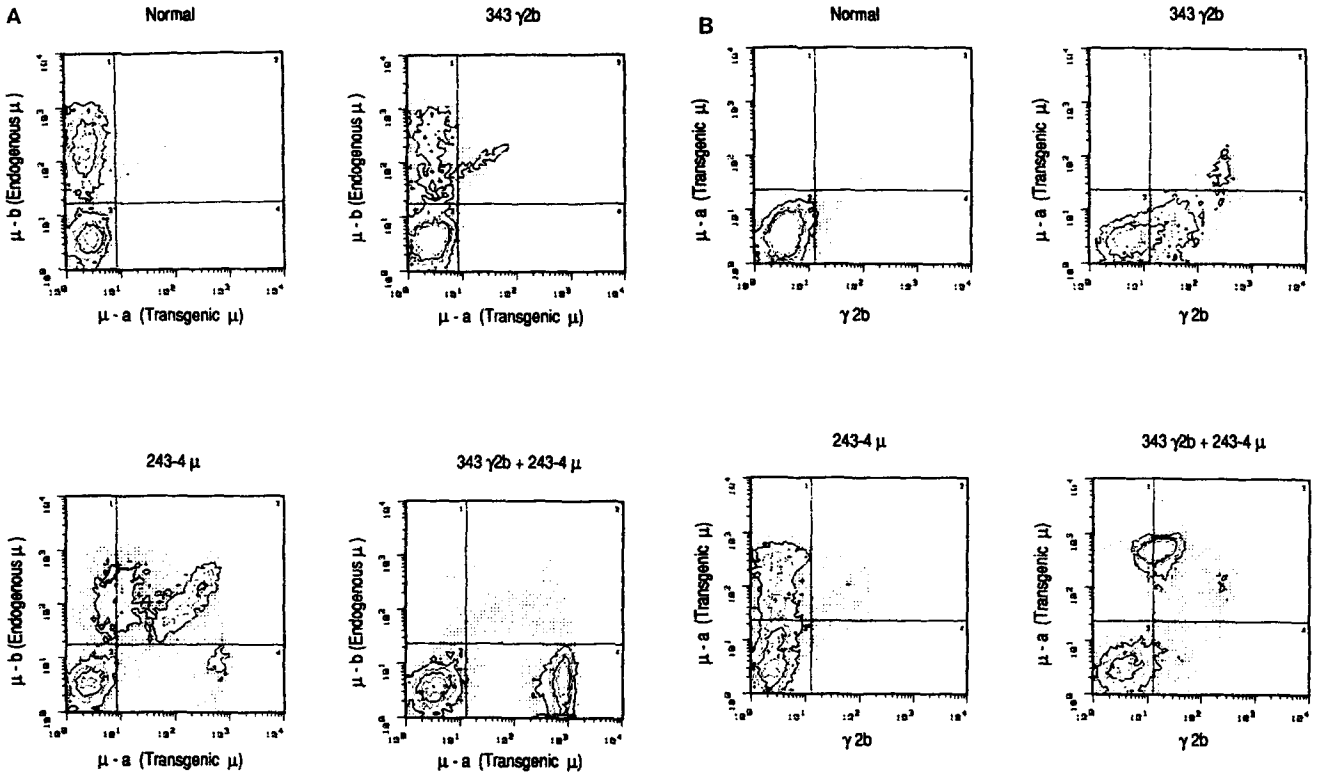

Figure 5. 343 γ2b × 243-4 μ transgenic mice. (*A*) Endogenous μ and transgenic μ staining of splenic lymphocytes: endogenous μ (μ^b-PE) on the *y*-axis and transgenic μ (μ^a-FITC) on the *x*-axis. The genotypes are indicated. (*B*) Transgenic μ and γ2b staining on splenic lymphocytes. Transgenic μ expression is shown on the *y*-axis and transgenic γ2b-FITC expression on the *x*-axis. The mice were 16 wk old.

No Mature B Cells Develop in γ2b Transgenic Mice in the Absence of a Functional Endogenous μ Locus. The production of membrane-bound H chain is an absolute requirement for B cell development (2). To test the hypothesis that γ2b cannot promote B cell development beyond the pre-B cell stage, 343 γ2b transgenic mice were crossed to μ knockout mice. Spleen and bone marrow from the offspring of these crosses were analyzed at 8 wk of age for expression of B220 and mIg (Fig. 6).

Nontransgenic mice heterozygous for the disruption of the μ membrane domain express B220 and κ on 47% of splenic lymphocytes (Tγ2b−, μm+/−; Fig. 6 *A*). In comparison, only a background staining of ~1% positive cells is seen in homozygous μ knockout mice (Tγ2b−, μm−/−). Homozygous μ knockout mice also expressing the γ2b transgene (Tγ2b+, μm−/−) do not produce κ-expressing B cells and are phenotypically similar to nontransgenic μ knockout mice (Tγ2b−, μm−/−). Thus, the presence of the γ2b transgene does not allow the rescue of B cells in the absence of membrane-bound μ. Heterozygous μ knockout, γ2b transgenic mice, however, are able to produce splenic B cells (Tγ2b+, μm+/−; Fig. 6 *A*).

As expected, the bone marrow of the γ2b −/−; μm −/− mice contains only $B220^{lo}$ cells (Fig. 6 *B*), but also in γ2b transgenic mice, regardless of the μ genotype, very few $B220^{hi}$ cells are seen in the bone marrow (Fig. 6 *B*). This is consistent with the failure to produce B cells in the presence of the γ2b transgene. These cells are lower in numbers than in the analysis shown in Table 1, presumably because the mice in Fig. 6 *B* are younger (see Discussion).

As a control, we performed a similar analysis using a μ transgenic line, μdelS. This transgenic line expresses only the membrane form of μ, and exhibits efficient B cell development and feedback inhibition of endogenous μ expression (Fig. 6 *C*, Kim, J. Y. and P. Roth, unpublished results). In the absence of a functional endogenous μ locus, μdelS transgenic mice (μdelS+; μm−/−) retain the ability to produce a normal composition of splenic lymphocytes (Fig. 6 *C*). Thus, the peripheral B cell population can be reconstituted with a single type of heavy chain molecules.

Discussion

From the analysis of these γ2b and γ2b-μmem mice, we conclude that the γ2b heavy chain cannot functionally substitute for μ in all aspects of B cell development. While γ2b or γ2b-μmem are able to mediate feedback inhibition of endogenous H gene rearrangement, they do not promote maturation or survival of B cells.

B Cell Development Is Blocked at an Early, Pre-B Cell Stage in γ2b Transgenic Mice. The bone marrow of γ2b transgenic mice shows a marked decrease in the $B220^{hi}$ population.

Table 3. *IgH Rearrangement and Ig Secretion in γ2b Transgenic and Normal Hybridomas*

H genes*	γ2b transgenic hybridomas		
	No.‡	Percent	Secreted Ig§
G	6(9)‖	22	6 γ2b, 3 none
DJ/DJ	0		
VDJ	8	30	6 μ and γ2b, 1 γ2b, 1 μ
VDJ/G	1	4	1 μ and γ2b
VDJ/DJ	9	33	8 μ and γ2b, 1 γ2b
VDJ/VDJ	3	11	3 μ and γ2b
	Normal mouse hybridomas		
	No.	Percent	Secreted Ig
G	0		
DJ/DJ	0		
VDJ	18	82	8 μ, 10 γ2b
VDJ/G	1	4	1 γ2b
VDJ/DJ	3	14	2 μ, 1 γ2b
VDJ/VDJ	0		

Hybridomas were generated from splenic LPS blasts of 4-wk-old mice. Culture supernatants were screened in an ELISA for the presence of secreted Ig. DNA was analyzed by Southern blots for IgH rearrangement.
* Configuration of detectable H genes. G, germline; DJ, D to J rearrangement; VDJ, V to D to J rearrangement. In cases where only a single allele is indicated, the other allele could either have been lost or gives an indistinguishable pattern on Southern blots.
‡ Number of hybridomas containing the indicated gene configurations.
§ Secreted Ig detected in hybridoma culture supernatant by isotype-specific ELISA.
‖ Three hybridomas that did not secrete Ig were excluded from the calculation of percentages since it is not known if they may have arisen from pre-B cells.

This population contains both recirculating and newly produced B cells that express mIg (25, 26). The $B220^{hi}$ cells that are present in γ2b transgenic bone marrow exhibit an identical phenotype as peripheral splenic B cells from the same mice. All the $\gamma2b^+$ cells also coexpress endogenous μ at the cell surface. This is an important observation because it indicates that γ2b-only B cells are not produced.

In contrast to the defect in the $B220^{hi}$ population, the percentage of cells in the immature $B220^{lo}$ population appears relatively normal, however, the distribution of cell sizes within this pre-B cell compartment is skewed towards larger, less mature cells in γ2b transgenic bone marrow (Table 1 and Fig. 4 *B*). Large, $B220^{lo}$ early preB cells have begun to rearrange D to J_H genes at the H loci; these cells transit to the next preB stage and become small, $B220^{lo}$, noncycling cells with rearranged VDJ_H genes and germline κ genes (26–28). In the normal development pathway, the transition from large to small $B220^{lo}$ pre B cells is concomitant with the decrease of CD43 and the acquisition of cytoplasmic μ expression (26, 27). This latter population is decreased in γ2b transgenic mice similar to mice with homozygous deletion of the λ5 gene or the μ membrane gene segment (2, 29, 30). The implication is that γ2b transgenic mice are not able to generate small, late stage, pre-B cells as efficiently as normal mice, and subsequently exhibit a depletion of later stages of $B220^{hi}$ bone marrow and splenic B cells.

A Model for B Cell Development in γ2b Transgenic Mice. We propose a model for B cell development in γ2b transgenic mice in which the majority of B cell precursors developing in the bone marrow express high levels of γ2b early in the developmental pathway (Fig. 7). In developing pre-B cells the γ2b transgene product causes cessation of further H gene rearrangement. This effect is similar to that of endogenous μ chains and transgenic μ or δ chains. After this point, the function of γ2b diverges from μ in this model. Large, $B220^{lo}$ pre-B cells seem to require a signal generated pri-

Table 4. *FLow Cytometric Analysis of Splenic B Cells from 343-1 γ2b Crossed to 1275-4 λ2 Mice*

Mouse type	κ	λ	μ	γ2b	B220
Normal (n = 9)	45.6 ± 5.9	4.8 ± 3.0	48.0 ± 6.0	8.7 ± 5.4	47.1 ± 9.4
343-1 γ2b (n = 8)	17.4 ± 7.1	4.9 ± 1.7	20.8 ± 7.4	11.9 ± 2.4	29.0 ± 2.1
1275-4 λ2 (n = 8)	25.6 ± 6.8	33.0 ± 7.4	33.1 ± 6.4	7.7 ± 1.6	30.7 ± 10.0
343-1 γ2b X 1275-4 λ2 (n = 11)	10.8 ± 5.4	16.0 ± 4.6	20.5 ± 4.0	14.7 ± 2.3	29.7 ± 1.0

Spleen cells from offspring of 343-1 γ2b × 1275-4 λ2 crosses were stained for the indicated markers and analyzed by FACScan®. The data are an average of three experiments. A total of 10^4 lymphocytes as defined by forward scatter and side scatter were analyzed per sample in one experiment. In the other two experiments, propidium iodide was used to eliminate dead cells. The analysis was then regated on lymphocytes. The average percentage of splenic lymphocytes positive for the given marker is indicated at a 90% confidence interval as determined by t test.

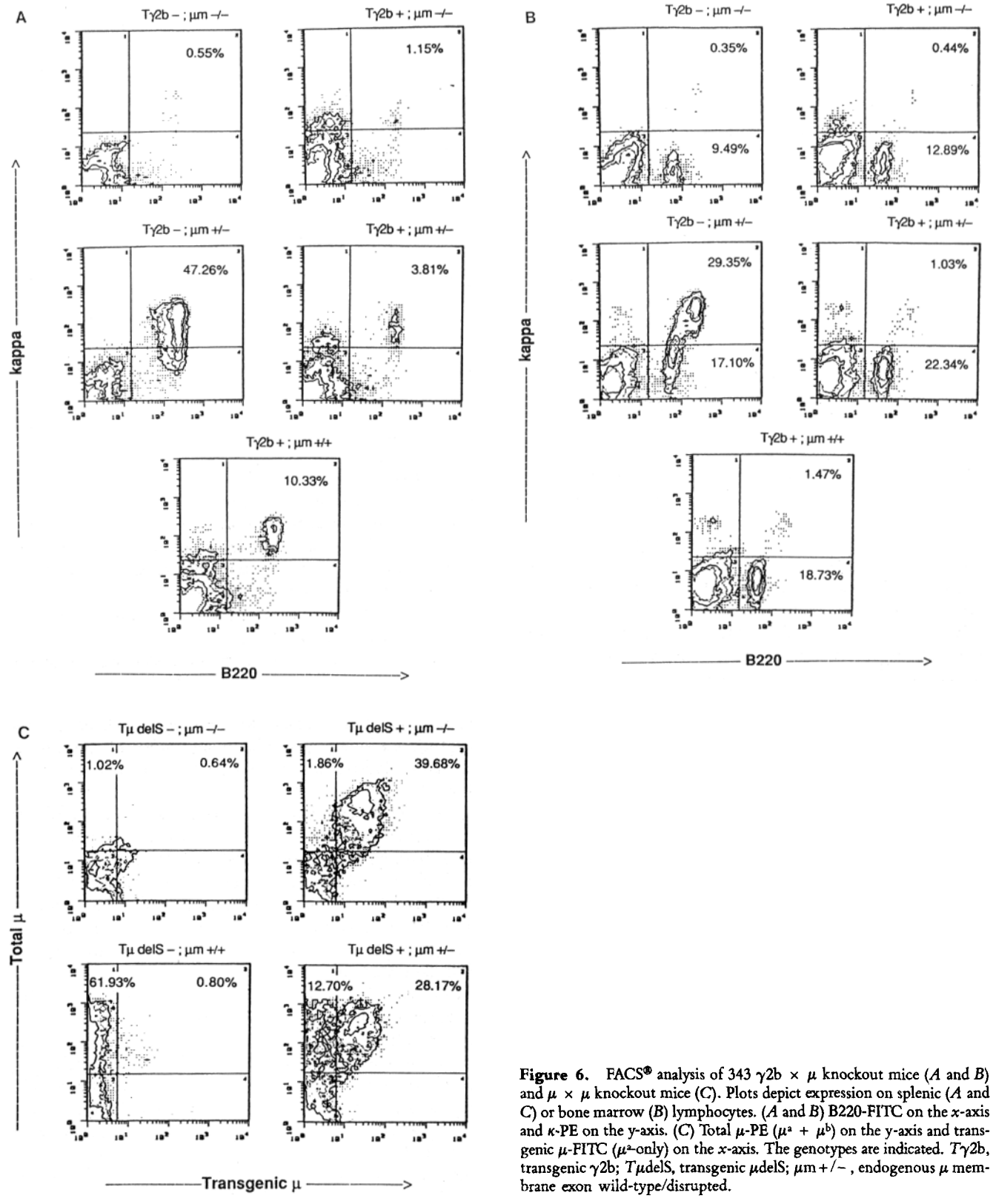

Figure 6. FACS® analysis of 343 γ2b × μ knockout mice (*A* and *B*) and μ × μ knockout mice (*C*). Plots depict expression on splenic (*A* and *C*) or bone marrow (*B*) lymphocytes. (*A* and *B*) B220-FITC on the *x*-axis and κ-PE on the y-axis. (*C*) Total μ-PE ($\mu^a + \mu^b$) on the y-axis and transgenic μ-FITC (μ^a-only) on the *x*-axis. The genotypes are indicated. *T*γ2b, transgenic γ2b; *T*μdelS, transgenic μdelS; μm +/−, endogenous μ membrane exon wild-type/disrupted.

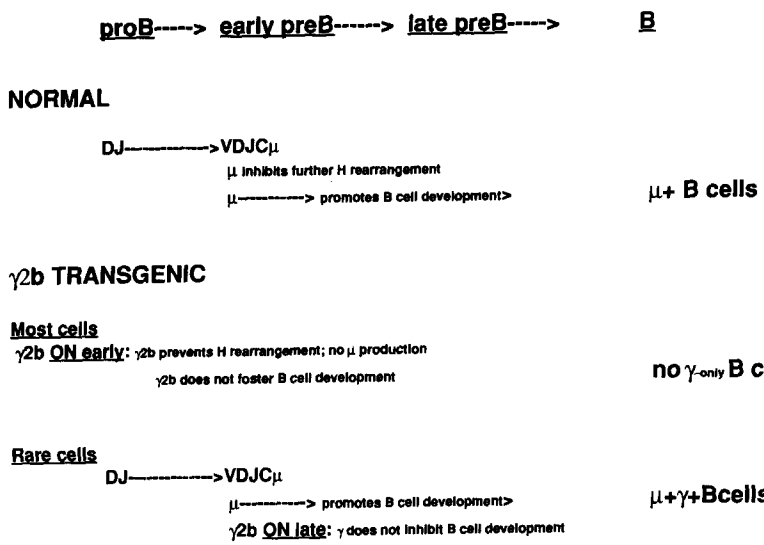

Figure 7. Model of B cell development in γ2b transgenic mice. The model outlines the inhibition of Ig gene rearrangement mediated by γ2b during the pro-B through early pre-B cell stages. B cell development arrests before the late pre-B cell stage resulting in a general B cell defect in γ2b transgenic mice. Rare cells arise that rearrange endogenous heavy chain genes, and these cells can progress through the complete developmental pathway to give rise to mature splenic lymphocytes.

marily via the pre-B cell receptor in order to progress to become small $B220^{lo}$ late pre-B cells (2, 29, 30). Expression of membrane μ is apparently required for this transition. The failure of γ2b to generate this survival or differentiation signal, in combination with the reduction of rearrangement at the endogenous H gene loci, results in the B cell defect characteristic of γ2b transgenic mice.

In rare pre-B cells of γ2b transgenic mice, endogenous H gene rearrangement proceeds to completion. This could be due to lower levels or later timing of expression of the γ2b gene in a small number of cells, or to early rearrangement events proceeding before γ2b heavy chain signals stop H gene rearrangement. If the endogenous rearrangement is in frame, a functional μ heavy chain will be produced in the developing cell. This cell will then receive the required survival or differentiation signals via its endogenous μ chain and will proceed through further development. Such cells must be very rare, since it takes ≥16 wk to achieve normal B cell levels in the spleen when the mice have a wild-type μ locus (Tables 1 and 2).

The frequency with which this rare event of endogenous H gene rearrangement occurs seems to vary among transgenic lines. The 343 γ2b line shows the most severe depletion of B cells, while the 348A γ2b line exhibits only a modest depletion in B cell number. Presumably the variation in the B cell defect results from variation in levels and timing of transgene expression during B cell development due to integration site effects. Thus, compared with the other lines, the 343 line may express the γ2b transgene earlier (and more strongly), causing a greatly delayed accumulation of μ^+ cells. Conversely, the 348A line, which shows very low numbers of $\gamma2b^+$ splenic B cells, may express γ2b at too low a level in many pre-B cells to cause suppression of endogenous H gene rearrangement.

Possible toxicity of the γ2b transgene has been shown to be unlikely by crossing a functional μ transgene into γ2b transgenic mice (Table 2). This leads to a reversal of the B cell defect, indicating that γ2b expression does not curtail B cell development when a μ chain is present.

The data argue that the failure to produce γ-only mature B cells in γ2b transgenic mice does not result from a failure of γ2b to inhibit feedback of endogenous H gene rearrangement. On the contrary, a striking decrease in endogenous μ expression occurs in γ2b × μ double transgenic mice compared with μ transgenics. Analysis of spleen B cell hybridomas further confirms that γ2b promotes strong feedback inhibition of H gene rearrangement.

As conclusive test for the proposed model of B cell development in γ2b transgenic mice, the 343 γ2b line was crossed with the μmem knockout line. If our model is correct, in the absence of a functional μ chain, no peripheral B cells should be produced in 343 γ2b mice homozygous for the μmem disruption. Indeed, this prediction is borne out in these crosses (Fig. 6).

Thus, the data are consistent with the proposed model that γ2b cannot replace μ in promoting the completion of the B cell developmental pathway. While γ2b appears to mediate feedback on endogenous H gene rearrangement, no B cells emerge either in the bone marrow or in the spleen that express only γ2b heavy chains. There is an absolute requirement for expression of μ in order for pre-B cells to complete their development.

Why Is γ2b Unable to Provide a Maturation Signal? One function attributed to μ chain expression is the induction of L chain gene rearrangement (23, 31). An inability of γ2b to induce L gene rearrangement would be compatible with the proposed model. B cells could develop to the pre-B cell stage and would fail to progress past this stage in the absence of a light chain. Evidence to support such a scenario comes from studies with *scid* mice, which are defective in rearrangement of Ig and TCR genes (32). They exhibit a block in B cell development at a similar stage as μmem knockout mice (26). In the presence of a functional μ transgene, *scid* mice develop normal pre-B cell populations, but very few B cells, presumably due to the inability to readily rearrange light chain genes (33–35).

If γ2b simply fails to induce light chain gene rearrange-

ment, supplying a functionally rearranged light chain transgene expressed in early pre-B cells should relieve the block in B cell development and allow the production of γ2b-only producing B cells at increased frequency in the periphery. However, λ2 × γ2b double transgenic mice do not exhibit an increase in the efficiency of B cell production (Table 4). In addition, all the B cells that populate the spleen express endogenous μ, similar to γ2b-only transgenic mice.

Thus, the block in B cell development is not due to lack of light chain gene rearrangement. This conclusion is strengthened by the fact that no differences in fetal liver κ expression were observed between γ2b transgenic mice and normal littermates.

Others have recently obtained evidence that μ is not required for the induction of light chain gene rearrangement in pre-B cells (2, 29, 30, 36). However, in the presence of μ, L gene rearrangement seems to be upregulated (37). It is intriguing to speculate that γ2b may substitute for μ in the upregulation of light chain gene rearrangement. Perhaps the signals for cessation of heavy chain gene rearrangement, which γ can deliver and upregulation of light chain gene rearrangement, are the same.

The signals for heavy chain gene rearrangement feedback and B cell maturation apparently are different. The γ2b molecule can only give the first Ig gene rearrangement feedback signal that stops heavy chain gene rearrangement, but not the B cell maturation signal, which μ and δ can deliver. Since γ2b appears capable of delivering a signal to stop H gene rearrangement, but not a signal for B cell maturation, either different regions of the heavy chains are involved in the different signals, or a given region of μ can perform both functions, whereas the analogous region of γ2b can perform only one function.

Structural differences between γ2b proteins and μ proteins must be responsible for their functional differences. The most obvious structural difference between μ and γ2b is the cytoplasmic tail (7). However, the fact that the γ2b-μmem transgenic mice exhibit an identical phenotype as the γ2b transgenic mice argues that the μ transmembrane and cytoplasmic tail regions are not sufficient to confer complete functional competence onto a hybrid γ2b-μ gene. It is possible that in the context of the γ2b-μmem hybrid gene the μ transmembrane and cytoplasmic domains may not fold into an appropriate three-dimensional structure. Alternatively, the transmembrane portion may be involved in the H gene feedback only and the membrane domain of most heavy chain isotypes may be able to deliver this signal. The μ and γ proteins differ no more in this protein domain than do μ and δ (7), which both can give a feedback as well as a maturation signal (4). Notably, a tyr-ser doublet within the transmembrane portion, which has been shown to be essential for signal transduction and antigen presentation via mIgM (38), is present in γ2b at an identical position (7).

Before the production of light chain, the heavy chain is expressed in a pre-B cell receptor complex with the surrogate light chains λ5 and VpreB that is also capable of signal transduction (39–42). The importance of this receptor is inferred by the block in early B cell development observed in either μmem or λ5 knockout mice (2, 29). However, λ5 seems not to be required for feedback inhibition of heavy chain gene rearrangement in the few B cell that develop in λ5 knockout mice (29). In analogy, γ2b may not require functional association with λ5 to cause H gene feedback. It is not known whether γ2b can combine with the surrogate light chains. The association between λ5 and μ heavy chains seems to occur through the CH1 domain of μ, as deletions of this domain result in loss of association between μ and these polypeptides (43). Based on the conservation of BiP binding and light chain binding to γ2b via associations within the CH1 domain, it seems reasonable to expect that λ5, which is structurally similar to a light chain, may also associate with γ2b (44–47). However, even if γ2b can bind λ5, a correct signal for B cell maturation may not be created.

mIg is expressed in the context of a cell surface receptor in which the H and L chain polypeptides are associated with Ig-α and Ig-β (48). It is known that all heavy chain isotypes can associate with Ig-α and Ig-β (48–50). This receptor complex participates in signal transduction events resulting from engagement of mIg (51). In addition, multiple protein tyrosine kinases have been found to associate with mIg and to be activated after crosslinking of the mIg receptor (52–54). Many other target substrates have been identified downstream from the mIgM receptor (55). It is likely that certain downstream signaling events may differ between μ- and γ-containing Ig receptors.

The precise signal transduction mechanisms regulating the normal progression of B cell development are beginning to be elucidated. While a number of proteins associated with the pre-B and B cell receptor have been identified, many have not. It appears that γ2b is unable to associate with or efficiently transduce signals through all the pathways in which μ can participate. Further analysis of the γ2b transgenic mice should help to delineate the specific pathways downstream from heavy chain expression that are required to complete B cell development.

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