

$\lambda 5$, But Not μ , Is Required for B Cell Maturation in a Unique $\gamma 2b$ Transgenic Mouse Line

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Summary

$\gamma 2b$ transgenic mice have a severe B cell defect, apparently caused by strong feedback inhibition of endogenous H-gene rearrangement coupled with an inability of $\gamma 2b$ to provide the survival/maturation functions of μ . A unique $\gamma 2b$ transgenic line, named the C line, was found to permit B cell development. When the C line is crossed with a μ -membrane knockout line, $\gamma 2b^+$ B cells develop in the homozygous knockout. In contrast, a transgenic line representative of all the other $\gamma 2b$ lines is completely B cell deficient when μ -mem is deleted. Strikingly, the C phenotype is dominant in C \times other $\gamma 2b$ transgenic line crosses. There is no evidence for higher $\gamma 2b$ transgene expression or other position effects on the transgene in the C mouse. The sequences of the three $\gamma 2b$ transgene copies in the C line are identical to that of the original transgene. These results have led to the conclusion that in the C line the transgene integration constitutively induces a gene whose expression can replace μ . To more clearly delineate the stage at which the altered phenotype of the C line is expressed, C mice were crossed onto a $\lambda 5$ knockout background. In the absence of $\lambda 5$, the C line produces no B cells. Since it was also found that $\gamma 2b$ can associate with the surrogate light chain (sL; $\lambda 5/V_{pre-B}$), the crosses between C line $\gamma 2b$ mice and $\lambda 5$ knockout mice suggest that $\gamma 2b/sL$ is required for B cell maturation in this mouse line. Thus, $\gamma 2b$ alone is unable to replace μ for pre-B cell survival/maturation; however, in combination with an unknown factor and the sL, $\gamma 2b$ can provide these nurturing functions.

B lymphocyte development is tightly controlled by the ability of the maturing pre-B cells to synthesize first heavy chains and later light chains (1). Failure to correctly rearrange V_{DJ_H} at the early pre-B cell stage, or V_{J_K} or V_{J_L} at the late pre-B cell stage leads to apoptotic death at the respective stages. Normally the functional early heavy chain is μ . Transgenic μ as well as δ have been shown to be able to substitute for endogenously produced μ (for a review see reference 2). These transgenes can replace the maturation function as well as the heavy chain feedback function of endogenous μ . However, $\gamma 2b$ was shown to be unable to substitute for μ (3). Whereas transgenic $\gamma 2b$ causes strong feedback inhibition of endogenous heavy chain gene rearrangement, $\gamma 2b$ cannot promote B cell maturation, leading to an arrest at the stage of large, B220^{lo} pre-B cells and a severe B cell depletion (3, 4). Very young $\gamma 2b$ transgenic mice have hardly any B cells in the spleen; at 4 wk of age $\sim 10\%$ of total spleen lymphocytes are B cells, and only after 16 wk of age do the B cell numbers reach near normal levels (3). All B cells that do develop express endogenous μ . This B cell defect is not due to toxicity of $\gamma 2b$, since B cell development proceeds normally when the $\gamma 2b$ mice are crossed with μ transgenic mice (3). In the absence of endogenous μ , $\gamma 2b$ transgenic mice are absolutely B cell depleted as shown in crosses with μ -membrane (mem) knockout mice (3). Thus, apparently $\gamma 2b$ has the feedback

function of μ , but not the ability to cause survival/maturation of pre-B cells.

These findings were made with four different $\gamma 2b$ transgenic lines in our laboratory and apparently are also true for $\gamma 2b$ transgenic mice produced by others (5, 6), as well as for $\gamma 1$ transgenic mice (7). However, in the course of our studies we observed one unique $\gamma 2b$ transgenic mouse line whose B cell development appeared normal, except that the cells produced essentially no μ . This mouse line is the subject of this report.

Materials and Methods

Transgenic Mice. The 343-1 $\gamma 2b$ transgenic (Tg[Igh]Bri49) and the 243-4 μ transgenic (Tg[Igh]Bri35) lines used as controls have been described (8-10). The 348C line (the C line) is derived from the 348-4-8 (Tg[Igh]Bri50) line which contained multiple integration sites (3, 10). The μ knockout line was obtained from K. Rajewsky (Universität Köln, Köln, Germany) through C. Sidman (University of Cincinnati, Cincinnati, OH) (11). The $\lambda 5$ knockout line was obtained from K. Rajewsky through M. Cooper (University of Alabama, Birmingham, AL).

Flow Cytometry, Southern Blot Analysis, and RNase Protection Assays. These were performed as described in Roth et al. (3).

Cosmid Cloning. High molecular weight DNA was prepared from C $\gamma 2b$ kidney and liver DNA from four transgenic mice. Cosmid inserts were generated by DpnII partial digest giving an

average molecular mass of 35–45 kb. Ligations of digested genomic DNA and BamHI, SmaI double-digested cosmid vector c2RB DNA (12) were performed overnight at 14°C. After packaging, the cosmid library was transduced into *Escherichia coli* strain DH10B (Bethesda Research Laboratories, Gaithersburg, MD). Colony lifts and hybridization was performed essentially as described (13). Two cosmid clones containing transgene sequences were identified. The pOOH3 and pOOH6 cosmid clones were mapped by Southern analysis in comparison to genomic DNA from C γ 2b transgenic mice. Subclones were generated by SmaI digestion of the cosmid clones and ligation of the SmaI fragments into the EcoRV site of BluescriptKS+ (14).

DNA Sequencing. Double strand DNA sequencing was performed using the Sequenase II kit (United States Biochem. Corp., Cleveland, OH). The following primers listed 5' to 3' along the γ 2b transgene were used: T7 promoter (6T7P20) and T3 promoter (T3) were from New England Biolabs Inc. (Beverly, MA); 5' of leader (5L2=AAATACAGCATGCCACACTGTG); VH (5'V γ 2b=GCTGAGCTGATGAGGCCTGGG); VH (VD) γ 2b=TAAAGC-CACACTGACCGCA); VH (JV300=CAGTAGAGACCGAAC; JH2 (3'JH2 γ 2b=CTGAGGAGACTGTGAGAGTGTG); 3' of JH2 (3J2=AGGGACCTAGCGGCA); 5' of CH1 (5bCH1=CCGAGGCCACTGATAAG); CH1 (G2b-221=GATACACTGGTTCTCTCC); 5' of Hinge (5GbH=CCCATACCAGGACAACTTT); 3' of Hinge (3bH-2=AGGAGCTGATGAGGAGAGATGG); 5' of CH2 (5CH2=GTCCT ACC AGA GCTCCACT); CH2 (GbCH2=TATCAAGGATGACTCATG); 3' of CH2 (3CH2=GGCTGAGTGGAAGGCTGTCC); 3' of CH2 (3CGb2=CTGCTGGTGCGGCA); 5' of CH (5CH3Gb=CCACTTCAGCCATGACC); CH3 (HCH3 γ 2b-11=TTCTAAGCTTACAGGGCTAGTCAGAGCTCC); CH3 (G2b-1615=GTTTTTCATATTGAGCTTGCTA); 3' of CH3 (3bCH3=GGATATGGGTGCCAGTGT); 3' of CH3 (3CGb3=ATGGATATGGGTGCC); 5' of M1 (5 γ 2b M1=CAGCATCAACCATGTTGGG); 3' of M1 (3bM1-2=AGCAAAGTACATCCTACTA); 5' of M2 (5GbM2-2=CACAGGATGGAGG-GTC); and 3'UTR (UT-2=GCTGCTCTGCACATGGTCC).

Results

The γ 2b Transgene of the C Line Permits B Cell Development and Suppresses the Synthesis of μ . In other γ 2b transgenic mice a severe depletion of B cells was seen in spleen and other lymphoid organs (Table 1 343; 3). In contrast, the C line has only slightly fewer B cells than normal mice (Table 1). The proportion of B220⁺ or κ ⁺ cells is essentially the same as in μ transgenic mice, slightly reduced from those in normal mice. However, most of the peripheral B cells in the C mouse produce γ 2b, 89% of B220⁺ cells are γ 2b⁺, and only ~17% express μ . This is in marked contrast to other γ 2b transgenic mice in which every γ 2b⁺ B cell co-expresses μ (Table 1; 3). Thus, whereas normally γ 2b does not permit B cell development and only few pre-B cells mature (those that have managed to rearrange endogenous μ), in the C line B cell development proceeds in the absence of μ expression.

The C γ 2b Bone Marrow Contains Maturing B Cells. Analysis of the bone marrow compartment of C γ 2b transgenic mice was performed in order to further characterize B cell development in these mice (15, 16).

Adult C bone marrow has reduced numbers of lymphoid cells in comparison to normal adult mice. Only 15% of the total nucleated cell population in C bone marrow expresses B220 in comparison to 26% in normal mice (data not shown). However, the distribution and phenotype of cells within the lymphoid compartment of C mice is generally normal (Table 2), although there seems to be a wide variation in the relative percentages of cells of a given phenotype.

Two populations of B220⁺ cells are readily distinguishable within the lymphoid population in normal and C bone marrow. B220^{lo} cells constitute 38–60% of the lymphoid population in normal bone marrow in comparison to 18–46%

Table 1. Flow Cytometric Analysis of Splenic B Cells from 4-wk-old C γ 2b Mice*

Mouse type	κ	μ^{\ddagger}	γ 2b	B220	No. of spleen cells ($\times 10^6$) [§]
Normal <i>n</i> = 13	56.1 \pm 1.8	54.2 \pm 2.9	3.05 \pm 1.5	64.3 \pm 2.5	51 (39-90)
C γ 2b <i>n</i> = 7	44.7 \pm 9.1	8.5 \pm 4.0	43.7 \pm 5.0	48.9 \pm 10.1	37 (18-50)
343 γ 2b	11.7 \pm 2.7	11.4 \pm 2.2	14.5 \pm 4.3	26.6 \pm 1.8	16 (10-27)
243-4 μ <i>n</i> = 4	49.8 \pm 2.1	28.8 \pm 7.4	2.4 \pm 0.4	52.6 \pm 4.4	42 (31-56)
		(end)			
		28.8 \pm 10.8			
		(trg)			

*Spleen cells from 4-wk-old mice were stained for the indicated markers and analyzed by FACScan[®]. A total of 10⁴ lymphocytes as defined by forward and side scatter were analyzed per sample. The average percentage of splenic lymphocytes from 4-wk-old animals positive for the given markers is indicated at a 90% confidence level as determined by *t* test.

[‡] μ transgenics were analyzed using allotype-specific antibodies. *end*, endogenous μ^b ; *trg*, transgenic μ^a .

[§] Average number of nucleated cells in the spleen. () Range of values.

^{||} Data from reference 3.

Table 2. Flow Cytometric Analysis of Bone Marrow Cells from Adult C $\gamma 2b$ Mice*

Mouse type	κ	μ	$\gamma 2b$	B220 ^{lo}	B220 ^{high}
			%		
Normal	45.3	48.8	3.9	60.4	33.6
Normal	52.1	59.0	15.3	38.3	41.0
C $\gamma 2b$	23.8	5.3	28.3	17.9	21.5
C $\gamma 2b$	51.2	3.2	41.9	34.9	34.5
C $\gamma 2b$	55.8	1.7	55.7	46.2	43.8

* Bone marrow from adult 12–16 wk-old mice was stained for the indicated markers and analyzed by FACScan[®]. A total of 10⁴ lymphocytes as defined by forward and side scatter was analyzed per sample. The percentage of lymphocytes positive for the given marker is indicated.

of B220^{lo} cells in the lymphoid population of C bone marrow (Table 2). B220^{hi} cells representing more mature B cells constitute 34–41% of the lymphoid population in the normal bone marrow, and 22–44% in C bone marrow.

Within the κ^+ population in the bone marrow, immature B cells can be distinguished by low B220 expression from more mature cells that are B220^{hi}. FACScan[®] analysis (Becton Dickinson & Co., Mountain View, CA) confirmed that all the B220^{hi} cells also express mIg (data not shown). The B220^{lo} population consists of both mIg⁻, pre-B cells and immature mIg⁺, B cells. The percentage of κ^+ cells is similar in normal and C bone marrow (Table 2). In normal mice, κ^+ cells express μ , whereas in C mice, κ^+ cells are $\gamma 2b^+$ and only few express endogenous μ .

Thus, the C bone marrow contains a relatively normal distribution of B cell subpopulations. In addition, the phenotype of the emerging B cells is identical to the B cells observed in the spleen. There does not appear to be any perturbation in the ability of developing B cells to progress through the developmental pathway. This distinguishes the C mice from other $\gamma 2b$ lines which exhibit a block within the pre-B cell compartment (3).

Crosses to a μ Transgenic Line Reveal that the C Line Is Capable of IgM Surface Expression. C line mice were crossed to a μ transgenic line in order to determine if the C line has an inherent defect in the ability to express membrane IgM. This μ transgenic line exhibits incomplete heavy chain allelic exclusion (3, 9). FACScan[®] analysis reveals that splenic lymphocytes from C \times μ double transgenic mice coexpress $\gamma 2b$ and transgenic μ at the cell surface (not shown). The lack of endogenous μ (μ^b) expression of C mice is maintained in the double transgenics (Table 3).

The results indicate that C mice can express high levels of mIgM at the cell surface. Thus, they do not have an inherent defect preventing expression of mIgM. The C lineage seems to be more efficient in promoting heavy chain allelic

Table 3. Flow Cytometric Analysis of Splenic B Cells from C $\gamma 2b$ Crossed to 243-4 μ Mice*

Mouse type	κ	$\mu^{a\dagger}$	$\mu^{b\dagger}$	$\gamma 2b$	B220
4–6 wk					
Normal	63.3	1.5	53.1	4.4	67.2
243-4 μ	56.0	35.1	41.1	4.6	61.3
	54.6	37.6	36.9	3.5	59.5
	60.4	39.1	42.8	2.7	64.4
C $\gamma 2b$	23.4	3.2	12.2	34.8	31.4
C $\gamma 2b$	30.3	19.4	10.9	33.9	31.4
\times	20.7	12.6	6.9	22.9	28.0
243-4 μ	30.5	21.1	7.0	31.8	33.8
16 wk					
Normal	75.1	2.0	67.5	2.3	82.3
	72.7	3.1	72.1	3.2	78.5
243-4 μ	56.7	34.7	40.6	6.5	62.7
	50.7	35.8	45.6	9.9	68.3
C $\gamma 2b$	32.5	5.6	7.8	32.3	49.5
	60.8	3.9	2.5	63.9	62.9
C $\gamma 2b$	40.3	21.0	6.0	27.1	58.1
\times	38.2	18.8	5.9	30.6	54.6
243-4 μ	41.7	19.7	4.7	33.5	59.1

* Spleen cells from offspring of C $\gamma 2b$ by 243-4 μ crosses were stained for the indicated markers and analyzed by FACScan[®]. A total of 10⁴ lymphocytes as defined by forward and side scatter was 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 .

exclusion and development of transgene expressing B cells than the 243-4 μ lineage.

Crosses between $\gamma 2b$ Transgenic Lines Reveal Dominance of the C Phenotype. The C $\gamma 2b$ line can efficiently promote B cell development and produce peripheral B cells that do not express endogenous μ . In contrast, B cell development in the 343-1 and other $\gamma 2b$ transgenic lines is dependent upon production of a functional μ chain (3). In order to determine whether C $\gamma 2b$ transgene expression could functionally substitute for μ and rescue B cell development in the 343 lineage, C $\gamma 2b$ mice were crossed with 343 $\gamma 2b$ mice.

Analysis of 4–8-wk-old offspring revealed that the C \times 343 double transgenic mice are virtually identical to C-only transgenic mice (Fig. 1). Splenic lymphocytes in the C and C \times 343 mice express $\gamma 2b$, but not μ , on the surface. In contrast, all splenic lymphocytes in 343-only mice express

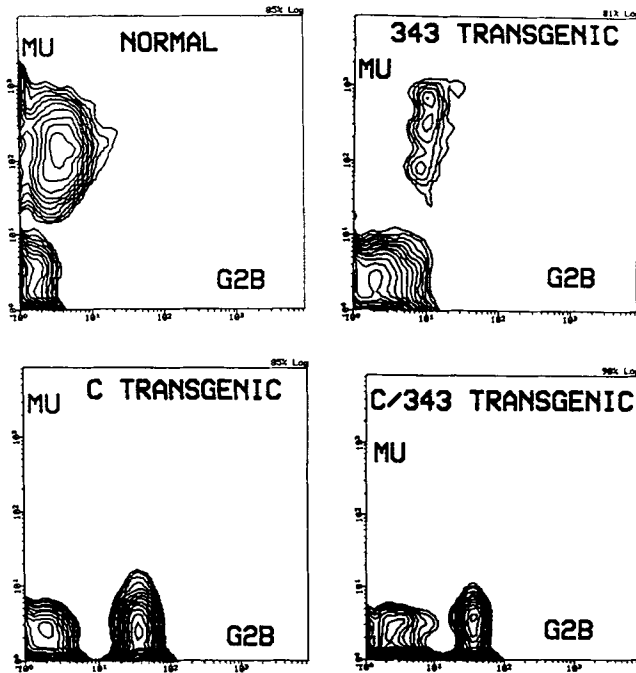


Figure 1. FACS[®] analysis of splenic lymphocytes from adult (C × 343 γ 2b) F₁ mice. Contour plots depict expression on splenic lymphocytes of μ -PE staining on the y-axis and γ 2b-FITC staining on the x-axis for representative offspring from a C × 343 γ 2b cross. The genotype is indicated within each plot.

μ on the cell surface. In addition, the presence of the C transgene in C × 343 double transgenic mice rescues the B cell defect characteristic of 343 γ 2b mice (not shown). 46–50% of splenic lymphocytes in C and C × 343 mice in this experiment expressed B220. Only 33% of splenic lymphocytes in 343 mice expressed B220. In addition, bone marrow from C × 343 double transgenic mice exhibits populations of B220^{lo}, γ 2b⁺ and B220^{hi}, γ 2b⁺ cells similar to those observed in C mice, but absent in 343 mice (data not shown).

Thus, the presence of the C γ 2b transgene in 343 transgenic mice can act in a dominant manner to relieve the requirement for endogenous μ production. The presence of the 343 γ 2b transgene copies does not interfere with the generation of mature mIg⁺ B cells when an alternative, functional heavy chain is present.

Expression of γ 2b mRNA. Analysis of C × 343 double transgenic mice indicates that cell surface expression patterns of B220 and mIg characteristic of lymphocyte populations in C spleen and bone marrow are dominant (see above). In addition, we wanted to characterize γ 2b transcript levels at an early developmental time point as the C and 343 lines exhibit distinct expression levels at this stage. The 343 line expresses high levels of γ 2b in day 12 fetal liver, whereas the C line does not exhibit appreciable γ 2b transcript levels until day 14 (14).

C × 343 double transgenic embryos express high levels of γ 2b mRNA in fetal liver at day 12 of development (Fig. 2). The RNA expression pattern is identical to that of 343-

only transgenic embryos: secreted γ 2b mRNA levels exceed membrane γ 2b mRNA levels which in turn exceed endogenous μ transcript levels. As in previous analyses, C-only embryos express secreted γ 2b transcripts at barely detectable levels at day 12, and membrane γ 2b is undetectable at this time. Both γ 2b transgenic lines, as well as their double transgenic offspring express levels of μ mRNAs similar to normal mice. We presume this to represent mainly sterile μ transcripts, but this has not been further investigated.

Thus, whereas C × 343 double transgenic mice show an identical cell surface expression pattern to C lineage mice, embryos exhibit the high γ 2b transcript levels characteristic of 343 lineage mice. To determine if in the bone marrow the expression of the C transgene was favored, bone marrow mRNA from normal, conventional γ 2b (343), and C line γ 2b transgenic mice was analyzed (Fig. 2, B and C). Results very similar to the fetal liver were obtained. The conventional γ 2b mice produce slightly more γ 2b mRNA of both the membrane and the secreted forms than the C line mice (compare the γ 2b bands with the actin bands). In both lines again, the secreted form is predominant. These data imply that the dominant C phenotype does not result from higher levels of expression of the transgene. Furthermore, the 343 × C crosses show that the high levels of γ 2b expression in the 343-1 line are not toxic to B cells.

The C Line Is Capable of Promoting B Cell Development in the Absence of a Functional Endogenous μ Locus. To definitively determine whether C γ 2b is fully functional in promoting B cell development, the C γ 2b line was crossed with mice carrying a homozygous deletion of the μ membrane exons (11). The ability of C γ 2b transgenic offspring lacking any developmentally functional μ gene (C γ 2b, μ mt^{-/-}) to produce B cells was examined by FACS[®] analysis of splenocytes (Fig. 3). Mice of the (C γ 2b, μ mt^{-/-}) genotype produce γ 2b⁺-only B cells as efficiently as C γ 2b littermates carrying one wild-type μ locus (C γ 2b, μ mt^{+/-}). Previously, we performed similar crosses between a μ knockout and a μ transgenic line (3). In the absence of a functional endogenous μ locus, μ transgenic mice (μ +, μ mt^{-/-}) retain the ability to produce a normal composition of splenic lymphocytes (3). Thus, both μ and C γ 2b transgenes are capable of restoring B cell development in homozygous μ knockout mice, unequivocally demonstrating the ability of these transgenes to generate all the required signals for proper completion of the B cell developmental pathway. This is in striking contrast to other γ 2b transgenic lines which have a complete inhibition of B cell development in a μ mt^{-/-} background (3).

Cosmid Cloning Reveals that the C Line Contains Three Intact, Wild-type Copies of the γ 2b Transgene. The characteristics of the C γ 2b mice described so far indicate that whereas the C line has a dominant ability to promote B cell development, this cannot be explained by a dominant (earlier or higher) expression of γ 2b in the C line compared with other γ 2b transgenic lines. This implies that the C γ 2b line possesses some characteristic fundamentally different from other γ 2b lines.

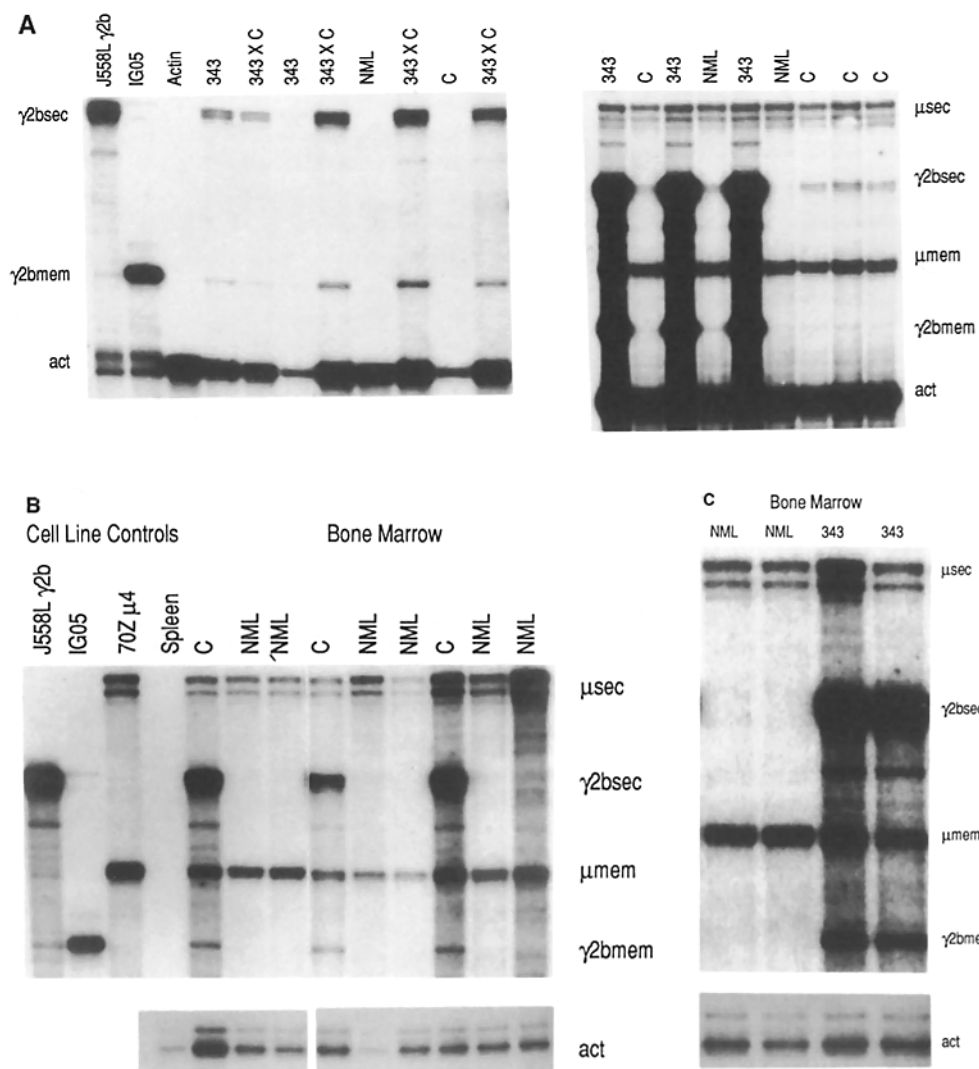


Figure 2. RNase protection assay RPA analysis. (A) Day 12 fetal liver from C \times 343 embryos. Approximately 30 μ g of total cellular RNA from individual fetal livers was analyzed by RPA for expression of γ 2b and β -actin; the membrane and secreted specific forms of the γ 2b are indicated; each lane contains RNA from an individual fetal liver with the indicated genotype. The samples in the right panel were also analyzed for μ expression. The J558L- γ 2b lane represents control RNA from a myeloma line transfected with the linearized γ 2b transgene and expresses predominantly secreted γ 2b (10). Ig05 is transfected with an altered γ 2b gene that produces predominantly membrane γ 2b (44). The actin lane (*act*) contains fetal liver RNA hybridized to only the β -actin probe. (B) Bone marrow from normal mice and C- γ 2b transgenic mice, and, as controls, cell lines as indicated: J558L- γ 2b and Ig05 are as in A; 70Z- μ 4 is a transfectant of the 70Z/3 late pre-B cell line with the VH167 μ transgene; it expresses equal levels of membrane and secreted μ . (C) Bone marrow from normal and conventional γ 2b (343) transgenic mice.

It was possible that a transgene copy in the C line could have sustained a mutation changing the structure and function of the transgene product to become functionally like μ . In the course of the analysis of other independent transgenic lines we have found evidence for point mutations and short deletions that likely had occurred before transgene integration (17, 18).

Thus, the transgenes of the C line were cloned in order to determine if the sequences had been altered. From the initial characterization of the C line we knew that no major deletions or structural alterations had occurred during transgene integration (14). An integration site map was developed to facilitate identification of each of the three transgene copies. The integration site analysis confirmed the copy number to be precisely three in the C γ 2b lineage, organized as a consecutive array with each copy in the same orientation (14). The map revealed that the three transgene copies differ at the extreme 5' ends and that two of the copies have incurred small deletions of polylinker sequence. These deletions are

minor and do not extend into regulatory or coding regions of the transgene. There is no evidence for deletion of any transgene sequence at the 3' end of any of the copies.

Two overlapping cosmid clones (pOOH3 and pOOH6) containing the entire C transgene array, as well as pieces of flanking DNA from either side of the locus, were isolated (Fig. 4). Their integrity was confirmed by Southern analysis compared with genomic DNA from C transgenic mice (not shown). The pOOH6 cosmid contains all three transgene copies and \sim 3 kb of 5' flanking DNA and that of the pOOH3 cosmid contains \sim 15 kb of 3' flanking DNA, the 3' transgene copy and part of the middle transgene copy extending to a BamHI site in the CH1 domain of the constant region (Fig. 4).

Individual transgene copies were subcloned and each subclone containing a specific region of the C transgene array was subjected to sequence analysis. The entire coding region sequence of each transgene copy was determined, as well as splice donor and splice acceptor sequences at the borders of

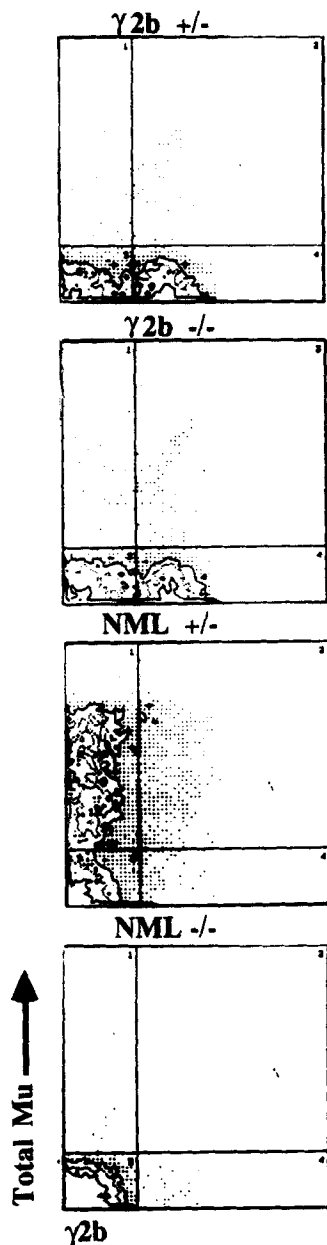


Figure 3. FACS[®] analysis of spleen from C $\gamma 2b$ transgenic mice \times μ knockout mice. Plots depict expression on splenic lymphocytes of μ -PE on the y-axis and $\gamma 2b$ -FITC on the x-axis. (+/-) Wild-type or disrupted, respectively, μ locus in $\gamma 2b$ transgenic or normal littermates (NML).

each exon, and its respective introns and compared to the published sequence for the BALB/c $\gamma 2b$ locus and the original transgene (not shown) (10, 19).

Several nucleotide substitutions, deletions, and additions were identified in the variable region of all three transgene copies (not shown, but these sequence data are available from EMBL/GenBank/DDBJ under accession number M21294). These nucleotide changes result in alteration of the leader and V region sequence predicted by Tsang et al. (10). All the nucleotide changes were found to be present in the $\gamma 2b$ plasmid used to create these lines. In addition, sequence analysis of a portion of the variable region DNA from the 343-1 $\gamma 2b$ line confirmed the presence of these mutations. Thus, it seems that all the nucleotide changes observed by sequence analysis of the C transgene copies are accountable as errors in the published sequences.

The cosmid analysis conclusively demonstrates that the C $\gamma 2b$ lineage contains three intact and wild-type $\gamma 2b$ transgene copies. Thus the structure of the $\gamma 2b$ transgene product expressed from the C locus cannot be responsible for the unique phenotype that characterizes the C $\gamma 2b$ lineage. This suggests that the integration site may influence the generation of the C-specific phenotype.

Crossing the C Line $\gamma 2b$ Transgene into a $\lambda 5$ Knockout Background Eliminates B Cell Development. Given that the C line can promote B cell development independent of μ , it appeared possible that the $\gamma 2b$ transgene was integrated near and activated a locus which normally was expressed after signaling via the pre-B cell receptor μ /surrogate light chain (sL)¹. Possible candidate genes could be kinases or other molecules involved in the signaling cascade, perhaps acting independently of the requirement for a functional pre-B cell receptor. To evaluate if a pre-B cell receptor was required for B cell development in the C line, we crossed the $\gamma 2b$ transgene of the C line (and as a control of the 343-1 $\gamma 2b$ line [3]) with mice whose $\lambda 5$ locus was inactivated by homologous recombination (20).

The results clearly show that no B cells develop in the C transgenic line when $\lambda 5$ is not expressed (Fig. 5). The B cell

¹ Abbreviation used in this paper: sL, surrogate light chain.

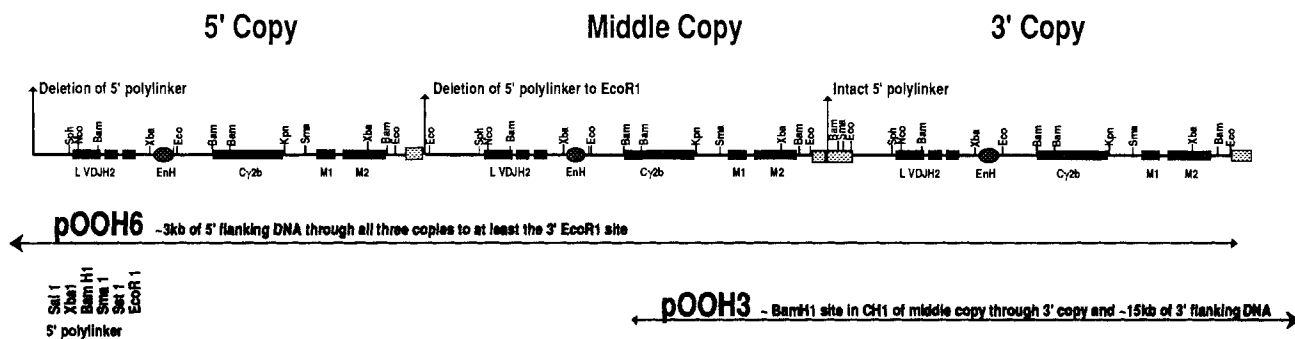


Figure 4. Schematic representation of transgene orientation and 5' end configuration in the C $\gamma 2b$ line. The three transgenes in the C line are in the same orientation and each contains the indicated unique 5' end structure. (Solid boxes) Exons; (filled ovals) the heavy chain enhancer present in each copy; and (stippled boxes) the pUC13 vector sequence remaining on the ends of the copies. The region contained within each isolated cosmid clone is indicated below. (Sph) SphI; (Nco) NcoI; (Bam) BamHI; (Xba) XbaI; (Eco) EcoRI; (Kpn) KpnI; and (Sma) SmaI.

profiles of homozygous $\lambda 5$ knockout mice with a $\gamma 2b$ transgene are the same regardless of the presence of the C- or the 343-1 $\gamma 2b$ transgenes. Both show essentially no B cells. This suggests that $\gamma 2b$ in combination with the sL ($\lambda 5/Vpre-B$) is required for B cell development in the C mouse.

Normally early pre-B cells do not express $\gamma 2b$. The ability of this heavy chain to associate with $\lambda 5$ and Vpre-B was assessed by transfecting the transgene into the pre-B cell line 70Z/3 that expresses the sL. Immunoprecipitation of $\gamma 2b$

from ^{35}S -methionine-labeled cells reveals coprecipitation of two proteins with the expected mobility of the sL $\lambda 5$ and Vpre-B (not shown). In addition, LPS stimulation of these cells results in replacement of the sL by conventional light chain once κ expression is induced (Roth, P. E., and A. DeFranco, unpublished results). Thus $\gamma 2b$ heavy chains can physically associate with sL to form a pre-B cell receptor and expression of this receptor is required for complete B cell development in the C line of $\gamma 2b$ transgenic mice.

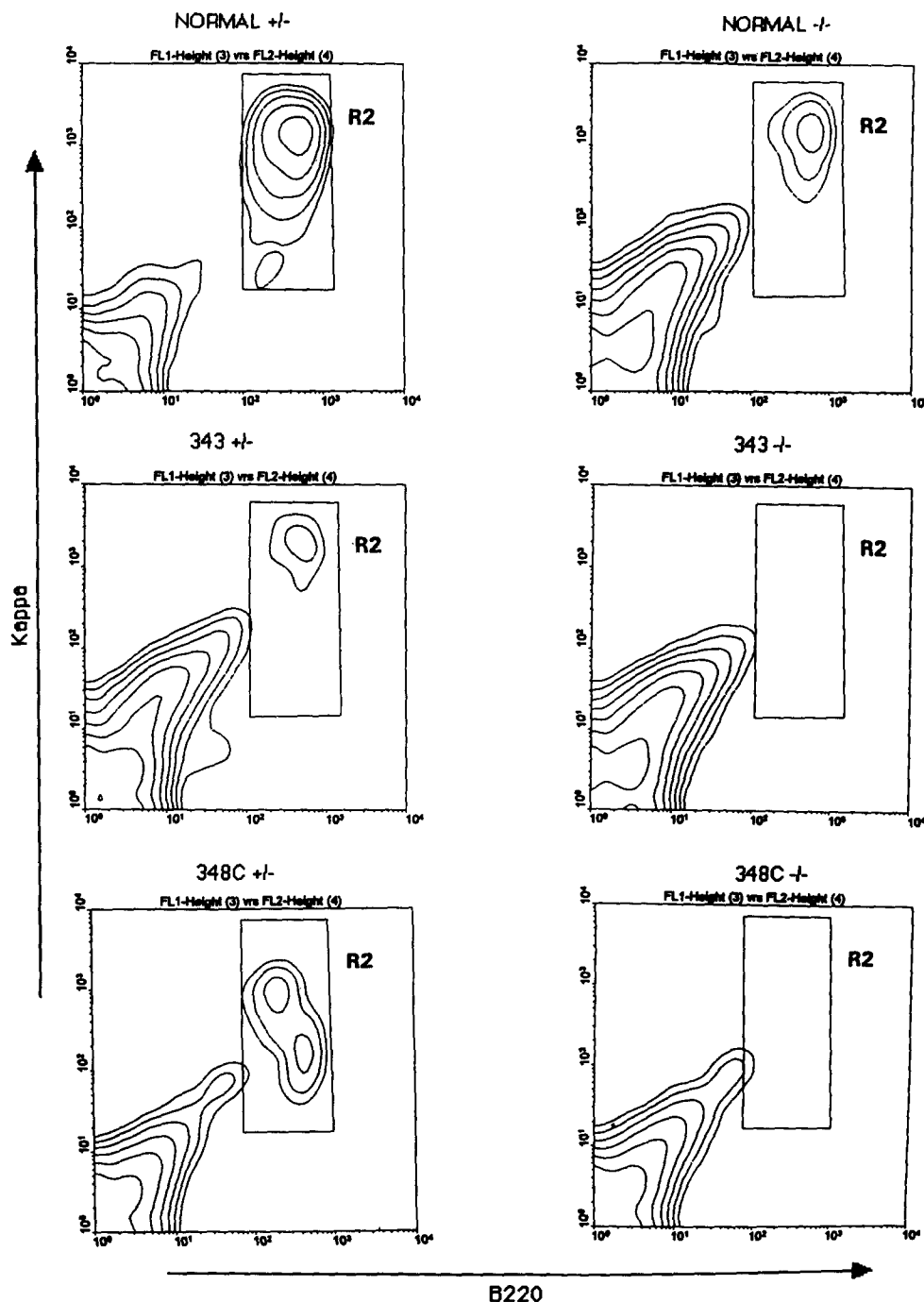


Figure 5. FACS[®] analysis of spleen from C $\gamma 2b$ transgenic \times $\lambda 5$ knockout mice. Splens from 4-wk-old heterozygous (+/-) and homozygous (-/-) mice were stained with PE-conjugated anti- κ antibody and FITC-conjugated anti-B220 antibody. The percentages of cells in the R2 window were: (NORMAL -/-) 2.9%; (NORMAL +/-) 33.1%; (343 -/-) 0.6%; (343 +/-) 1.8%; (348C -/-) 1.4%; and (348C +/-) 10.8%. The R2 gatings for the 348C mice differ slightly from the others as they were done in a separate experiment.

TRANSGENIC $\gamma 2b$ IN B CELL DEVELOPMENT

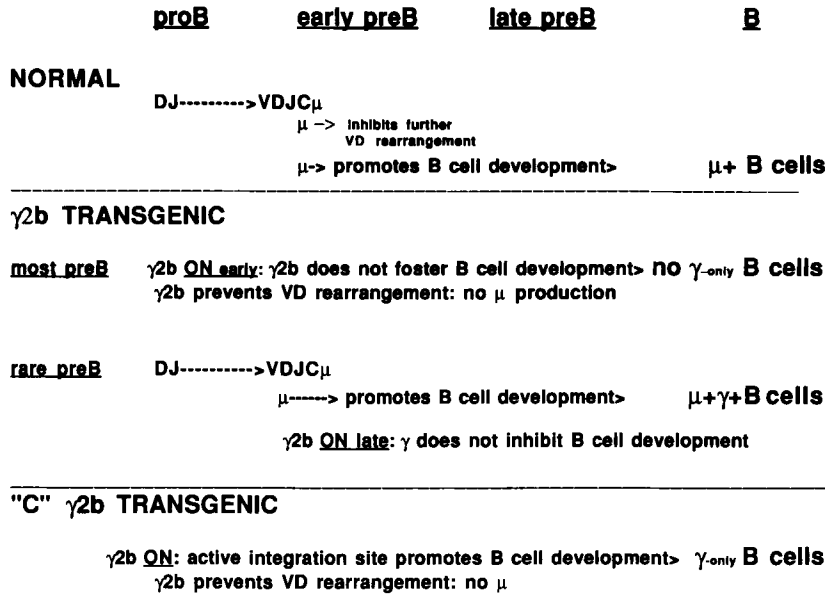


Figure 6. Model for B cell development in two types of $\gamma 2b$ transgenic mice. The model outlines the inhibition of Ig gene rearrangement mediated by $\gamma 2b$ during the pro-B through early pre-B cell stages. The activation of an endogenous gene due to transgene integration in the C mouse results in the maturation or survival of pre-B cells which can then continue through the complete developmental pathway to give rise to mature splenic lymphocytes expressing only $\gamma 2b$ heavy chains. In other $\gamma 2b$ transgenic mice only rare pre-B cells correctly rearrange an endogenous VDJ μ gene before feedback inhibition by the $\gamma 2b$ transgene.

Discussion

This paper reports the analysis of a unique $\gamma 2b$ transgenic mouse that has essentially normal B cell development, in contrast to all other $\gamma 2b$ transgenic mice (3-6) (Fig. 6). The C line is, however, similar to other $\gamma 2b$ transgenic lines in that the $\gamma 2b$ transgene is expressed in fetal liver, bone marrow, and the spleen, and it causes feedback inhibition of endogenous heavy chain gene rearrangement.

$\gamma 2b$ Transgenes Promote Allelic Exclusion of Endogenous Heavy Chains. All $\gamma 2b$ transgenic mice analyzed by us exhibit strong allelic exclusion of endogenous heavy chains (3; this paper). This seems also to be the case for $\gamma 2b$ transgenic mice reported by others (5, 6). The findings with the C mouse support this conclusion: most B cells express $\gamma 2b$ only, and in crosses with a μ transgenic mouse, the expression of endogenous μ chains is suppressed much more strongly than in the μ transgenic mice without the $\gamma 2b$ transgene (Table 3). Thus, the ability for heavy chain gene feedback is shared by μ , δ (21), and $\gamma 2b$. These three isotypes are closely related in their transmembrane domain and it is possible that the feedback requires that domain. Replacing the transmembrane domain of a $\gamma 2b$ transgene with that of μ still permits the feedback inhibition, but does not restore the B cell maturation defect of $\gamma 2b$ mice (3).

B Cell Development Is Similar in C $\gamma 2b$ Transgenic Mice to μ Transgenic Mice. B cells expressing mIg accumulate somewhat more slowly in C transgenic mice than in normal mice. This is similar to the reduction seen in the 243-4 μ transgenic line examined in comparison to the C line (Table 1). Other studies (22-25) have also shown that Ig transgenic mice exhibit a reduction in the size of their B cell population, although the severity of the depletion varies. In addition to the variability in B cell populations between different transgenic lines, there is variability in the level of feedback regulation resulting in allelic exclusion in different transgenic lines.

The C line exhibits stronger allelic exclusion than the 243-4 μ line (Table 3). However other μ transgenic lines have been shown to exhibit a similar degree of allelic exclusion as the C line (26, 27).

A large proportion of $\gamma 2b/\mu$ double-producing B cells is seen in the spleen of μ - x $\gamma 2b$ -transgenic crosses. This suggests that selection against cells which produce two heavy chains is not automatic. (It may of course play a role in antigen-specific tolerance). Furthermore, feedback inhibition of H-gene rearrangement by $\gamma 2b$ heavy chains clearly is very significant (Table 3). Taken together, these results further support the existence of heavy chain feedback, rather than selection, as the major mechanism for heavy chain allelic exclusion (28).

Why is the C Mouse Able to Promote B Cell Development? The findings presented here show that the C line differs from all other $\gamma 2b$ transgenic lines (3-6) in its permissiveness of B cell development. Most B cells in the C line express $\gamma 2b$ only, the C line transgene can rescue the B cell defect of another $\gamma 2b$ transgenic line, and C line B cells develop in the absence of μ expression in a μ knockout background.

What is the molecular basis for the unique phenotype in the C $\gamma 2b$ transgenic line? Below we summarize the mechanisms by which the $\gamma 2b$ transgene in the C line could affect B cell development. An important basis for our conclusions is the dominance of the C phenotype in crosses with another $\gamma 2b$ transgenic line.

(a) It is unlikely that the C effect is due to inactivation of an endogenous gene, since the effect is dominant. The transgene has been carried as a hemizygous integration for many years, thus a normal locus would always be present. The C phenotype is also independent of the sex of the parent transmitting the transgene, indicating that the normal locus cannot be inactivated by parental imprinting.

(b) The integration of the $\gamma 2b$ transgene in the C line does not qualitatively nor quantitatively change the expression of

$\gamma 2b$ compared to other $\gamma 2b$ transgenic lines in a way that can explain the phenotypic differences. Since the C phenotype is dominant, a position effect would have to express itself in a positive way, such as earlier or stronger expression or an increase in the membrane form of the $\gamma 2b$ mRNA. Comparing $\gamma 2b$ expression in transgenic lines shows that the expression of $\gamma 2b$ is somewhat weaker and slightly delayed in the C line (Fig. 3). Furthermore, the ratio of membrane to secreted $\gamma 2b$ mRNA is shifted in favor of the secreted form in both lines of $\gamma 2b$ transgenic mice (Fig. 3). Finally, in crosses between the C and 343-1 $\gamma 2b$ transgenic lines, the mRNA profile of the double transgenic is identical to that of the conventional $\gamma 2b$ line (343), whereas B cell development proceeds identically to the C line.

Furthermore, different $\gamma 2b$ transgenics express different levels of $\gamma 2b$ (3). The expression in the C mouse seems to be between the two extremes, the 343 and 348A mice, in level and timing. The 343 mice have the strongest feedback effect and the lowest number of B cells in young mice, whereas the 348A mice have the least feedback and the highest number of B cells. Nevertheless, both 343 and 348A express μ on all B cells, in marked contrast to the C line. It appears thus very unlikely that the expression pattern of the $\gamma 2b$ transgene itself is responsible for the B cell developmental effect seen in the C line.

(c) A third possibility seemed that the transgene in the C line might be altered in a way that would allow it to mimic μ during B cell development. However, as shown, the three transgene copies in the C line are identical to those in the other three $\gamma 2b$ transgenic lines produced in our laboratory (3, 10).

(d) This leaves the possibility that the transgene integration site itself mediates the phenotype observed in the C line. Transgene expression is regulated by the strong heavy chain intronic enhancer. Essentially all transgenic mice with transgenes regulated by this enhancer express the transgene regardless of chromosomal integration site (2). It is possible that an endogenous locus is coordinately activated with the $\gamma 2b$ transgene due to the insertion of the heavy chain enhancer. This is analogous to B cell neoplasias associated with translocation or mutation events resulting in alterations in expression patterns of normal cellular genes (29).

Since the original founder mouse was a (C57BL/6 \times SJL) F_2 , the possibility existed that the transgene in the C mouse was integrated in a SJL-derived chromosome retained despite the backcrossing to C57BL/6. The difference to the conventional transgenics could have been due to maintenance of this SJL component in the C mouse. However, crossing of the conventional $\gamma 2b$ mouse lines, 343 and 348A, with SJL did not result in a C phenotype (Kurtz, B., and U. Storb, unpublished observations).

One of the functions of μ appears to be the upregulation of light chain gene rearrangement in pre-B cells, although a basal level of light chain rearrangement does not require μ (20, 30–32). We previously investigated the possibility that $\gamma 2b$ was unable to upregulate light chains. That analysis indicated that the B cell defect observed in $\gamma 2b$ transgenic mice was not alleviated when the 343-1 $\gamma 2b$ line was crossed with

a λ transgenic line (3). The λ transgene in that analysis is regulated by the heavy chain enhancer and should therefore be expressed early enough to provide high levels of light chains (33). Furthermore, in fetal liver and bone marrow of the 343-1 $\gamma 2b$ line, the levels of endogenous κ mRNA appear to be normal (3). In the C line too, there does not appear to be any change in the timing or level of expression of endogenous κ (14). Additionally, we have localized the integration site of the transgene to chromosome 13 (Kurtz, B., A. Fernald, M. LeBeau, and U. Storb, unpublished results), thus it cannot directly affect either κ (chromosome 6) or λ (chromosome 16) light chain genes. Taken together, these results make it unlikely that the phenotype in the C line results from transgene insertion activating either of the endogenous light chain loci.

The Requirement for μ May be Circumvented in the C $\gamma 2b$ Transgenic Line by Intersection of Signaling Pathways. Since all other possibilities appear unlikely, we postulate that a protein, which is not a light chain, is produced in the C line, and that expression of this protein during B cell development can overcome the block normally seen in $\gamma 2b$ transgenic mice. This protein would presumably be produced by a gene activated by the heavy chain enhancer in the transgene. Since μ is not required for B cell development in the C line, presumably a signal must be generated that circumvents μ . The signals generated by μ apparently operate through the pre-B cell receptor in combination with the sL, $\lambda 5$ and Vpre-B (16, 20). Thus, circumventing the signal could occur at several levels, namely, the pre-B cell receptor itself, a ligand for the receptor, or a downstream event within the signaling cascade of the pre-B cell receptor. However, a downstream event could not act autonomously, since the crosses with the $\lambda 5$ knockout mice indicate that signaling through a pre-B cell receptor is required for B cell development in the C line. Unless $\lambda 5$ has an as yet unknown function, that is independent of the pre-B cell receptor, the lack of B cells in the $\lambda 5$ knockout mice that carry the C $\gamma 2b$ transgene suggests that $\gamma 2b$ has a signaling role in B cell development in the C line and that $\gamma 2b$ functions in association with $\lambda 5$. Consistent with this is the finding that the $\gamma 2b$ heavy chain expressed in these transgenic mice can associate with the $\lambda 5$ and Vpre-B chains when transfected into a sL positive cell line.

Why, if a sL complex is required for B cell development in the C line, do other $\gamma 2b$ transgenes not promote development? Apparently, a $\gamma 2b$ /sL receptor is not sufficient to drive B cell development (Fig. 7). An additional function is required and the C $\gamma 2b$ line is unique among $\gamma 2b$ transgenic lines in being able to provide this signal. The postulated gene activated in the C line is unlikely to participate in an event upstream to the expression of $\gamma 2b$ /sL since B cell development arrests at the same (3, 20) or slightly later (Kurtz, B., and U. Storb, unpublished observations) pre-B cell stage in conventional $\gamma 2b$ transgenic lines compared to the $\lambda 5$ knockout mice. Thus the function of the unknown gene in the C line is either coincident with the $\gamma 2b$ /sL signal, or is a downstream event induced by the $\gamma 2b$ /sL signal.

A complex signal may be generated by the μ /sL, but only a partial signal by the $\gamma 2b$ /sL. Indeed, μ , in association with

A model of B-cell development in the C line

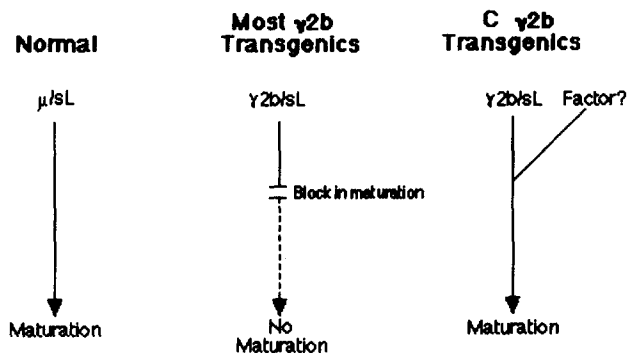


Figure 7. Scheme of signaling via different pre-B cell receptors. An unknown factor makes a $\gamma 2b/sL$ receptor signal functionally equivalent to a μ/sL receptor signal.

conventional light chain, has been shown to mediate a multitude of signals upon activation of the B cell antigen receptor (for a review see reference 34). In the C line, a protein may be recruited which in combination with the $\gamma 2b/sL$ receptor, results in signals indistinguishable from those generated by μ/sL . Since double transgenic mice with the C $\gamma 2b$ and μ transgenes have normal B cell development and express μ , if such a protein is present, it may not interfere with pre-B cell receptor signaling via μ/sL .

Another possibility for the postulated gene product activated in the C line may be a ligand for the $\gamma 2b/sL$ receptor. This could be either a molecule present in other cells in the bone marrow or a protein induced within the pre-B cell after receptor assembly. Other cells interacting with pre-B cells that could express the $\gamma 2b/sL$ receptor ligand could be stromal or T cells. However, the effect is unlikely to be due to stromal cells since the C phenotype can be adoptively transferred to irradiated mice (Roth, P. E., and U. Storb, unpublished results). The bone marrow recipients produce $\gamma 2b$ -only B cells and it is not likely that stromal cells have been preserved in the transferred cells.

T cells, however, could express the postulated gene activated in the C line. T cells are known to transcribe heavy chain transgenes under the control of the heavy chain intronic enhancer (2). However, T cells are apparently not required for normal B cell development since introduction of transgenic heavy and light chain genes into recombination activating gene (RAG) knockout mice, which lack T cells, is sufficient to allow B cell development (35, 36). Expression of different factors can influence pre-B cell growth and differentiation, thus it remains a formal possibility that a gene functioning in T cells could be activated in the C line to promote B cell development.

An additional possibility is that the postulated gene is expressed within pre-B cells in a post pre-B cell receptor function. As discussed, it seems likely that the $\gamma 2b/sL$ receptor

provides only a subset of the signals generated by a μ/sL receptor. Thus, activation of a gene that is normally a downstream target of pre-B cell receptor signaling could compensate for the missing signal. It is also possible that the activated gene does not normally function during B cell development, but can substitute for the missing signal when ectopically expressed. Indeed, functional redundancy has been demonstrated in TCR signaling. For example, increased expression of either the T cell-specific isoform of $p59^{\text{fyn}}$, (fyn^T), or the alternative isoform prevalent in brain, (fyn^B), results in improved responsiveness to TCR stimulation demonstrating that fyn^B can substitute for fyn^T (37).

Finally, another potential function of the postulated gene activation event in the C line could be to promote cell survival. Genes such as *Bcl-2*, can promote cell survival and block induction of apoptosis (38–40). In addition, *Bcl-2* and its partner, *Bcl-x*, do not reside on chromosome 13 (the site of the C transgene integration), but on chromosome 1 (41) and 2 (Thompson, C., personal communication), respectively, making it unlikely that these genes are specifically involved in mediating B cell development in the C line. It remains possible that as yet unknown genes can mediate pre-B cell survival. Perhaps one of the functions of signaling through the pre-B cell receptor is to induce cell survival or progression through the cell cycle to expand developing pre-B cells with functional heavy chain gene rearrangements. Developing pre-B cells have a finite life span and in the absence of appropriate signals it is likely that these cells apoptose in the bone marrow (42, 43). A failure of $\gamma 2b$ to promote cell survival is consistent with the severe B cell defect observed in most $\gamma 2b$ transgenic mice (3). Thus, activation of a gene involved in cell survival or cell cycle progression could mediate the phenotype characteristic of the C line. It is important to note however, that signaling through the $\gamma 2b/sL$ receptor is required either to activate this gene or in addition to activation of this gene to render the cells susceptible to its activity.

In summary, we present the characterization of a unique $\gamma 2b$ transgenic line. Unlike all other $\gamma 2b$ transgenic lines, the C line produces B cells expressing only $\gamma 2b$ heavy chains. We have shown that this phenotype is mediated neither by a unique expression pattern of the C transgene, nor by an alteration of the transgene itself. We postulate instead that $\gamma 2b$, in combination with the sL and an unknown factor, can replace μ in B cell development (Fig. 7). The precise mechanism promoting B cell development in this unique line has not been fully elucidated. We offer several possibilities for functions that could be activated in the C line. Most of these possibilities can be tested experimentally. These studies reiterate the complexity of requirements for proper B cell development. The production of a functional heavy chain mediates progression through the developmental pathway, but it is clear from these studies that multiple signals are required to complete this pathway. It is hoped that this unique line will lead to new insights into the signals necessary for B cell development.

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