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Early expression of Ig μ chain from a transgene significantly reduces the duration of the pro-B stage but does not affect the small pre-B stage

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Abstract

During B cell development, V–J rearrangements at the Ig heavy μ chain (IgH μ chain) locus occur in early cycling precursors (pro-B stage). Subsequently, rearrangements at the Ig light (IgL) chain locus occur in late resting precursors (small pre-B stage). To study the effects of μ chain expression on the rate of B cell development, purified hematopoietic stem cells (HSC) bearing a μ chain transgene or wild-type HSC were transferred into immunodeficient *RAG-2*$^{-/-}$ mice and B cell development was followed over time. In addition, cycling B cell precursors were pulse-labeled by the injection of BrdU into transgenic and wild-type mice, and the production of BrdU-labeled κ$^+$ and λ$^+$ B cells was followed over time. These experiments suggested that early expression of the μ chain from the transgene significantly shortened the duration of the pro-B stage and immediately drove the precursors to differentiate into small pre-B cells. By contrast, the presence of the transgene did not affect the small pre-B stage, where IgL rearrangements occur. Thus, κ and λ rearrangements occurred only after the arrest of cell cycling as previously shown in wild-type mice, even when the μ chain is artificially expressed earlier in B cell development.

Introduction

Based on the state of cell cycling and the expression of the $V_{pre\text{-}B}$/λ5 surrogate light (SL) chain and IgH μ chain, CD45R$^+$ (B220$^+$) B cell precursors can be subdivided into four stages: pro-B [cycling, SL$^+$cytoplasmic-μ$^-$(c-μ$^-$)], large pre-B (SL$^+$c-μ$^+$ or SL$^-$c-μ$^+$), small pre-B (resting, SL$^-$c-μ$^+$) and newly-generated immature B cells [resting, surface-IgM$^+$ (sIgM$^+$)] (1–4). Rearrangements at the IgL loci (the Igκ and Igλ loci) occur mostly in the small pre-B cell stage (5–7). Thus, B cell precursors can be more simply subdivided into two stages: early cycling precursors and late resting precursors, which undergo rearrangements at the IgL locus.

A defect in the expression of the μ chain arrests B cell development at the pro-B stage (8,9), as seen in *RAG-2*$^{-/-}$ mice, which are unable to undergo V(D)J recombination (10,11). This block can be released in *RAG-2*$^{-/-}$ mice bearing a μ chain transgene, and pro-B cells can differentiate into large pre-B cells and then into small pre-B cells (12–14). The presence of the μ chain is thus essential for the differentiation of early cycling precursors into late resting precursors.

While it is clear that μ chain expression suppresses another V_H to D_HJ_H rearrangement (15–17), it has been debated whether the expression of the μ chain activates $V_κ$–$J_κ$ rearrangements (18). Transfection of a μ chain gene into an Abelson murine leukemia virus (A-MuLV)-transformed pre-B cell line, p17-27, induced $V_κ$–$J_κ$ rearrangements, supporting a role for the μ chain in the activation of $V_κ$–$J_κ$ rearrangements (19–21). J_H-deficient mice, however, can initiate low levels of $V_κ$–$J_κ$ rearrangements (22). Hence, $V_κ$–$J_κ$ rearrangements can be initiated without the μ chain, whereas it remains unclear whether or not the expression of the μ chain gives rise to signals for enhancing IgL rearrangements.

While previous studies have shown that the μ chain is

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essential for the generation of small pre-B cells, the present study addresses the effect of the μ chain on the rate of B cell differentiation. For this purpose, we analyzed B cell development with time *in vivo* comparing μ chain transgenic and non-transgenic mice. The transgene expressed the μ chain very early during B cell development causing the inhibition of endogenous V_H to D_HJ_H rearrangements (data not shown). The results indicated that early expression of the μ chain from the transgene significantly accelerated B cell development in the early cycling precursor stage but did not affect the late resting precursor stage.

Methods

Mice

Breeding stocks of *RAG-2*$^{-/-}$ mice (Ly5.1$^-$Ly5.2$^+$) were kindly provided by Drs C. Reeder (NCI, Frederick, MD) and F. Alt (The Children's Hospital, Boston, MA). Mice carrying the Sp6 μ chain transgene (23) were provided by Dr Antonio Iglesias (Max-Planck-Institute for Immunology, Freiburg, Germany) and back-crossed twice with C57BL/6 mice to obtain mice heterozygous for the transgene and non-transgenic littermates. The presence of the transgene was verified by staining the peripheral blood with an anti-idiotype antibody (24). Mice were maintained at the animal facility at the Basel Institute under specific pathogen-free conditions.

Antibodies for flow cytometry

The following primary antibodies were used in this study: phycoerythrin (PE)-coupled ACK-4 [anti-c-*kit* (25); PharMingen, San Francisco, CA], FITC-, PE- or allophycocyanin-coupled RA3-6B2 [anti-B220/CD45R (26); PharMingen], FITC- or biotin-conjugated 104-2.1 [anti-Ly5.1 (27)], FITC- or biotin-conjugated A20-1.7 [anti-Ly5.2 (27)], biotin-conjugated goat anti-κ antibody (Southern Biotechnology, Birmingham, AL), biotin-conjugated LO-MM (anti-mouse IgM; Caltag, San Francisco, CA), biotin-conjugated R26-46 [anti-mouse λ_1 and λ_2 light chain (28), PharMingen], PE-conjugated 217-170 [anti-mouse IgD^b (29); PharMingen]. Second step reagents included streptavidin–TriColor (Caltag) or streptavidin–Red613 (Gibco/BRL, Gaithersburg, MD).

Injection of hematopoetic stem cells (HSC) into RAG-2$^{-/-}$ *mice*

Bone marrow cells were taken from 5- to 7-week-old transgenic and non-transgenic littermates. The CD45R$^-$c-*kit*^{high} population was purified by sorting and 10^4 purified cells from μ chain transgenic or non-transgenic littermates were injected i.v. into 4 Gy irradiated *RAG-2*$^{-/-}$ mice.

Immunofluorescence staining, analysis and cell sorting

For phenotypic analysis, single cell suspensions from bone marrow were stained with mAb as indicated in the figure legends. Cells ($1–3\times10^6$) were incubated with purified mAb at 5–10 μg/ml in PBS/5% FCS for 15–30 min on ice and washed once with PBS/5% FCS. Flow cytofluorometric analysis was performed on a FACscan (Becton Dickinson, Mountain View, CA). Three-color staining of anti-Ly5.1/anti-Ly5.2/anti-CD45R, anti-Ly5.2/anti-CD45R/anti-κ and Ly5.2/anti-CD45R/anti-λ was used to quantitate donor-type CD45R$^+$, s-κ$^+$ and s-λ$^+$ B cells respectively. Cells present in the lymphocyte gate, as defined by light scatter (30), were analyzed to examine κ$^+$ and λ$^+$ B cells. Fluorescence data are presented as logarithmic histograms or dot plots, using Lysys software (Becton Dickinson). Sorted cell populations were reanalyzed for their purity and were found to be >98% pure.

PCR analysis to quantitate recombination

From the bone marrow of recipient mice, Ly5.1$^+$Ly5.2$^-$B220$^+$ donor-type B-lineage cells were sorted and stored as 5 μl aliquots containing 1000 cells in 0.5 ml microtubes at –70°C. The protocol of Ehlich *et al.* (22) was followed for PCR analysis of DNA rearrangements. Amplified samples were run on a 1.5% agarose gel, followed by Southern blotting. The filter was hybridized, following a previously described protocol (22,31).

BrdU labeling in vivo and detection of BrdU$^+$ B cells in the bone marrow by flow cytometry

5-Bromo-2-deoxyuridine (Sigma, St Louis, MO) was dissolved in PBS at 1 mg/ml. Mice were injected i.p. with 200 μg BrdU. Mice were killed at various time points and the bone marrow cells were isolated. Sorter purified cells (CD45R^{low}sIgM$^-$, B220^{low}sIgM$^+$sIgD$^-$κ$^+$, CD45R^{low}sIgM$^+$sIgD$^-$λ$^+$) were treated as described previously (32). Cells were stained with FITC-labeled anti-BrdU antibody (Becton Dickinson) and underwent cytofluorometric analysis. Unlabeled cells stained with anti-BrdU mAb and labeled cells lacking anti-BrdU mAb staining were used as negative controls.

Results

Transferred bone marrow stem cells reconstituted B cell compartments in RAG-2$^{-/-}$ *recipient mice*

HSC purified from μ chain transgenic and non-transgenic littermates were transferred into *RAG-2*$^{-/-}$ mice. After the engraftment (time zero), B cell development from injected HSC was followed over time. The CD45R(B220)$^-$c-*kit*^{high} fraction (1.4% among nucleated cells in the bone marrow of μ chain transgenic and non-transgenic littermates; Fig. 1A, R2) was sorted to purify HSC. The c-*kit*^{high} fraction has been reported to contain HSC, while the c-*kit*^{low} fraction contains committed hematopoietic precursors (25,33,34). In addition, μ chain expression has been shown to down-regulate the expression of c-*kit* (35). As such, the CD45R$^-$ c-*kit*^{high} fraction did not contain committed B cell precursors.

A total of 10^4 sorted cells from μ chain transgenic or non-transgenic littermates were injected i.v. into γ-irradiated (4 Gy) Ly5.1$^-$Ly5.2$^+$ *RAG-2*$^{-/-}$ mice. Figure 1(B) shows the flow cytometric analysis of B cell development in the bone marrow at 23.5 days after the injection of HSC derived from non-transgenic mice. Ly5.1$^+$Ly5.2$^-$ donor-type cells can be distinguished from Ly5.1$^-$Ly5.2$^+$ host-type cells by the absence of the Ly5.2 congenic marker (the R3 gate shown in Fig. 1B, d). The (e) and (f) dot plots in Fig. 1(B) display cells present in both the gate for lymphocytes (R1) and the gate for donor-type cells (R3). The percentage of surface κ$^+$ B cells among the donor-type CD45R$^+$ cells was calculated to be 21.1% based on the following calculation: 7.4%(κ$^+$CD45R$^+$ cells)/35%(total CD45R$^+$

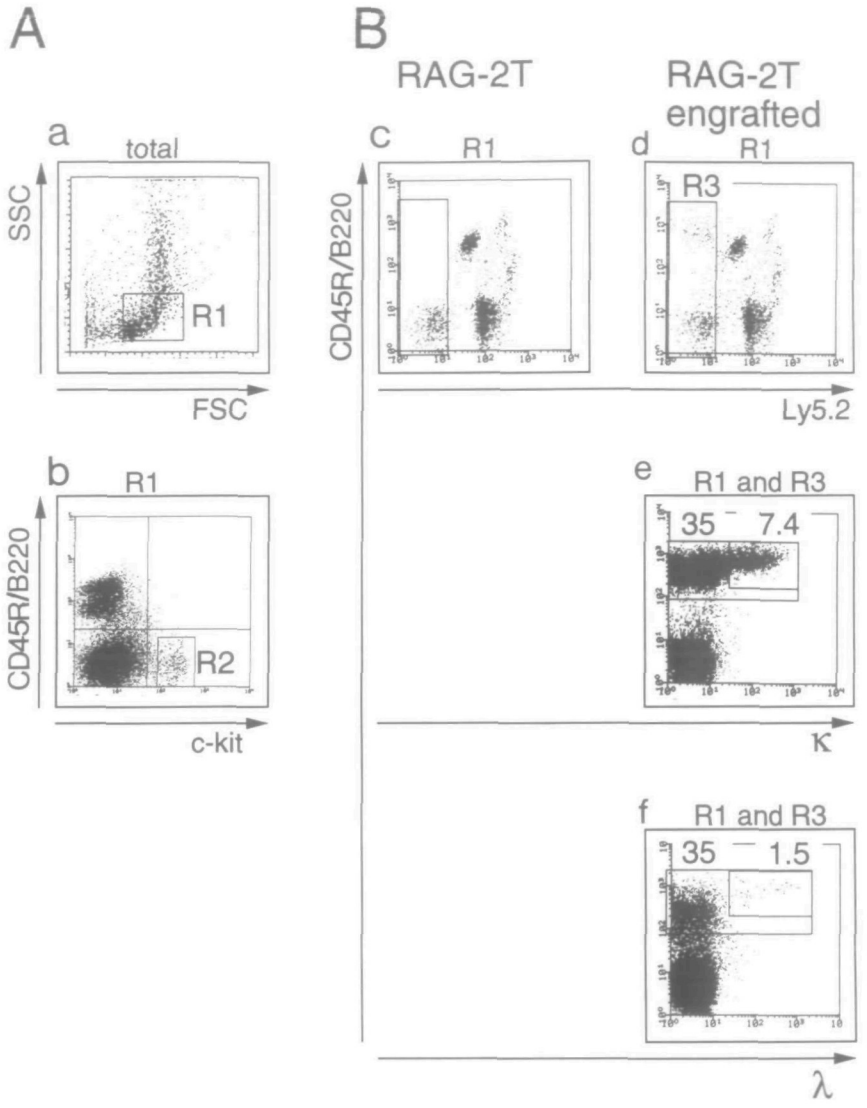

Fig. 1. Purification of HSC and analysis of bone marrow of a recipient mouse engrafted with the purified HSC. (A) Purification of HSC. Bone marrow cells were stained by two-color flow cytometry to measure the expression of CD45R (B220) and c-*kit* The R1 gate was defined in a dot plot of forward scatter (FSC) versus side scatter (SSC) (a). Cells presented in the R1 gate are displayed in the (b) dot plot and the R2 gate was defined as c-kit^{high}CD45R^{-}. The cells in both the R1 and R2 gates represented the HSC fraction, and the sorted cells were injected into 4-Gy-irradiated Ly5 2^{+} *RAG-*$2^{-/-}$ mice. (B) Analysis of cells in a reconstituted mouse Bone marrow cells were analyzed by three-color flow cytofluorometry to measure the expression of Ly5.2 versus CD45R versus κ, and Ly5 2 versus CD45R versus λ, in an unreconstituted *RAG-*$2^{-/-}$ (RAG-2T) mouse (d) and a reconstituted *RAG-*$2^{-/-}$ mouse (d–f) Cells present in the gate, which is indicated on each dot plot, are displayed (c–f) Donor-type cells (Ly5.1^{+}Ly5 2^{-}) in the reconstituted mouse were identified by the absence of the recipient marker, Ly5.2 (R3 gate in d) and cells in both the R1 and R3 gates are displayed in the (e) and (f) dot plots in (B). The percentage of CD45R^{+} B-lineage cells among donor-type cells is shown by numbers on the left in (e) and (f), and numbers on the right in these indicate the percentage of CD45R$^{+}\kappa^{+}$ (e) and CD45R$^{+}\lambda^{+}$ cells (f) among the donor-type cells

cells)×100%. Similarly, the percentage of surface λ^{+} B cells among the donor-type CD45R^{+} cells was calculated to be 4.3% based on the following calculation: 1.5%(λ^{+}CD45R^{+} cells)/35%(total CD45R^{+} cells)×100%.

The early expression of transgenic μ chain advanced the generation of sIgM^{+} B cells

Figure 2(A) represents the flow cytometric analysis of bone marrow cells from μ chain transgenic and non-transgenic littermates. Figure 2(B) shows an identical analysis to that discussed in Fig. 1(B), i.e. flow cytometric analyses of bone marrow cells from reconstituted mice sacrificed at the indicated days postgrafting. The percentage of small pre-B cells was slightly smaller in a μ chain transgenic mouse than in a non-transgenic littermate (64 versus 70%, Fig. 2A). By contrast, the histograms in Fig. 2(B) show that transferred *transgenic* HSC (HSC derived from μ chain transgenic mice) consistently yielded higher percentages of small pre-B cells than transferred *non-transgenic* HSC. The dot plots in Fig. 2(B) show that *transgenic* HSC produced κ^{+} B cells by

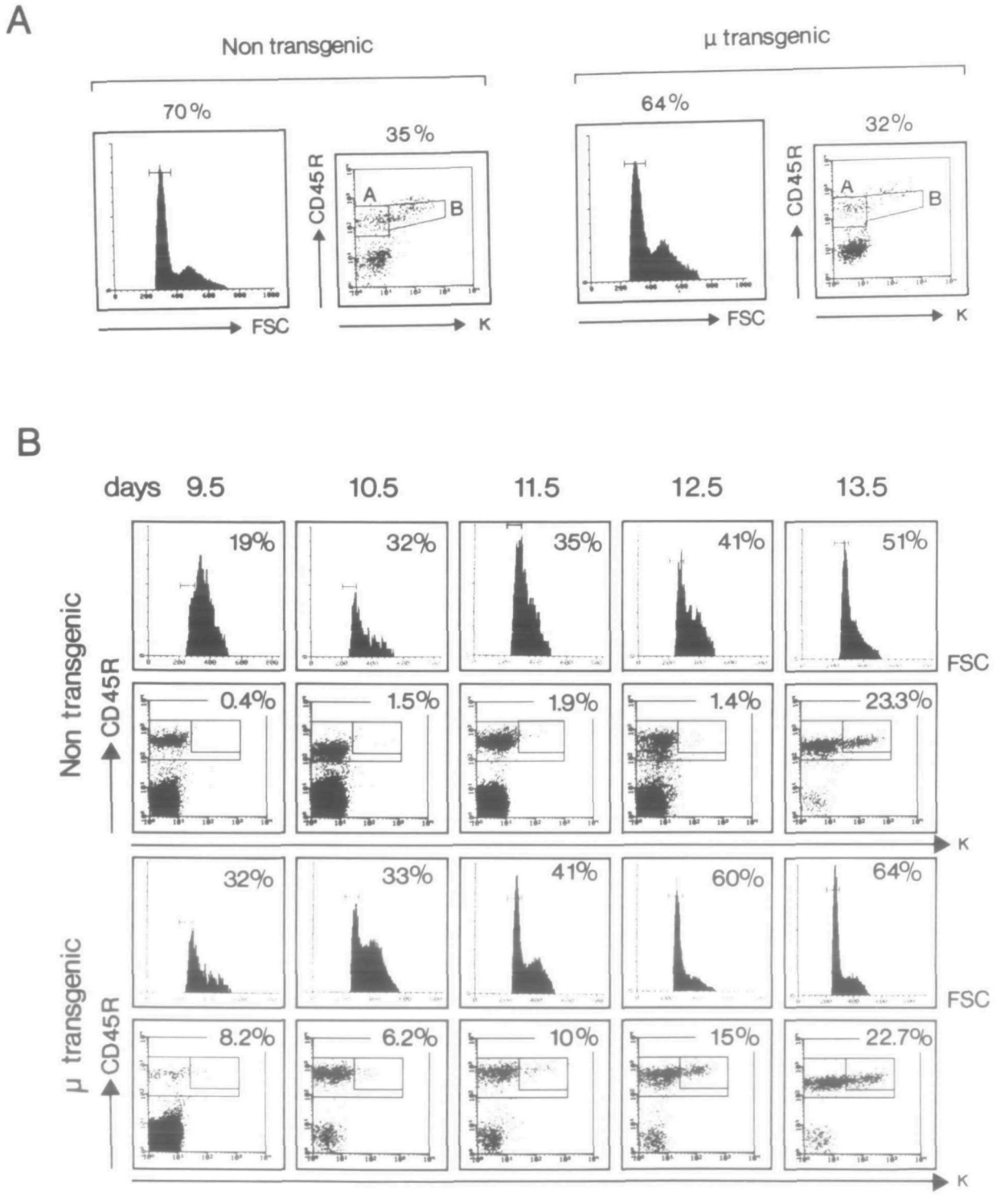

Fig. 2. HSC bearing transgenic μ chain gave rise to small pre-B cells and κ^+ B cells earlier than *non-transgenic* HSC in the reconstituted mice. (A) Flow cytometric analysis of B cell precursors in the bone marrow of μ chain transgenic and non-transgenic littermates. Histograms show the forward scatter (FSC) of $CD45R^{low}sIgM^-$ B cell precursors as defined by the A gate in the dot plots. The numbers in the histograms show the percentage of small pre-B cells among the $CD45R^{low}sIgM^-$ B cell precursors. Dot plots display the whole population of mononuclear cells. The numbers in the dot plots show the percentage of newly-generated $CD45R^{low}\kappa^+$ B cells (B gate) among the whole $CD45R^{low}$ cell population (A + B gates). $CD45R^{high}$ cells were not counted, because they were recirculated from the periphery (30). (B) Reconstituted *RAG-2*$^{-/-}$ mice were sacrificed on the indicated days postgrafting and the bone marrow cells were analyzed as shown in Fig. 1(B). The histograms show the forward scatter (FSC) of the Ly5 $2^-CD45R^+sIgM^-$ fraction (donor-type B cell precursors) in the first and third rows of Fig. 1(B) The numbers represent the percentage of small pre-B cells among the donor-type B cell precursors. The dot plots show the expression of κ versus CD45R (B220) of $Ly5.2^-$ donor-type cells. Numbers given in the dot plots indicate the percentage of κ^+ B cells among the donor-type $CD45R^+$ cells.

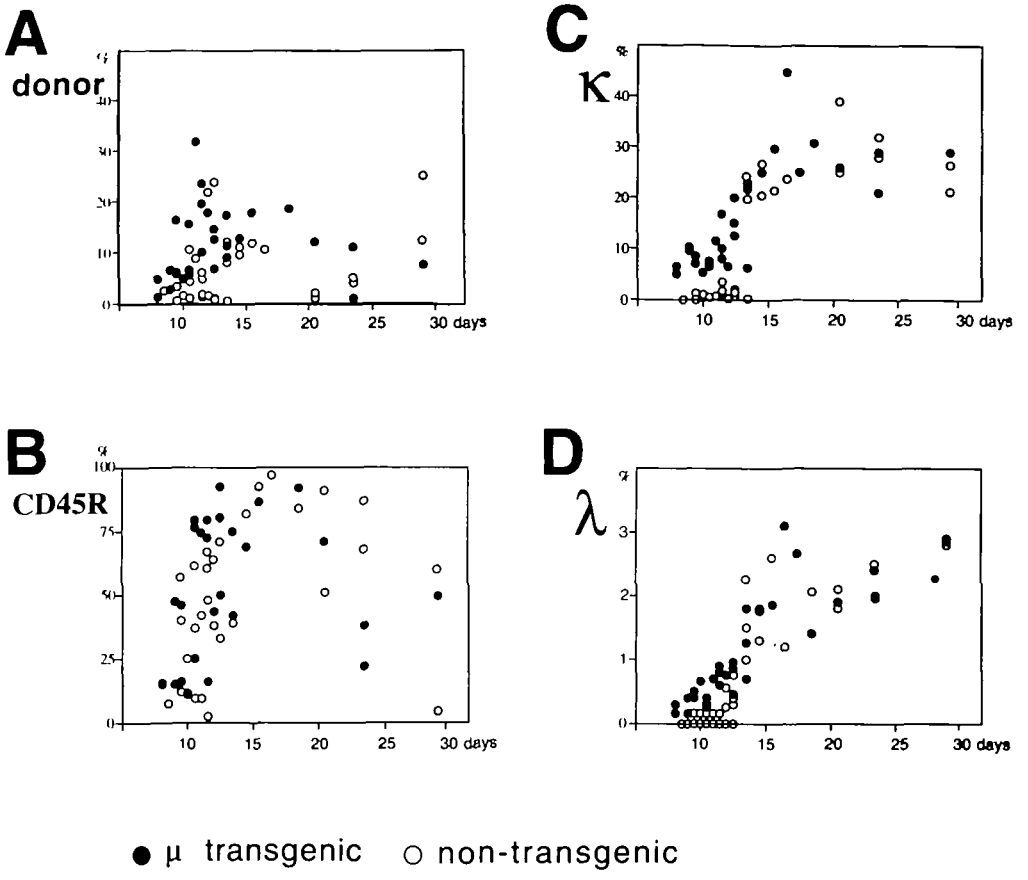

Fig. 3. B cell development from transferred HSC in recipient mice. (A–D) Each circle represents the result for an individual reconstituted mouse. The reconstitution of donor-type cells is compared between recipient mice engrafted with HSC from the μ chain transgenic mice (closed circles) and those engrafted with HSC from non-transgenic littermates (open circles). The percentage of donor-type cells among all nucleated bone marrow cells (A), the percentage of $CD45R^+$ cells among the donor-type cells (B), and the percentage of κ^+ B cells (C) and λ^+ B cells (D) among the donor-type $CD45R^+$ cells are displayed. Time zero represents the day of engraftment.

9.5 days postgrafting, while *non-transgenic* HSC produced essentially no κ^+ B cells until 12.5 days postgrafting.

Figure 3 summarizes the time course of B cell development from transferred HSC in recipient mice and each dot represents an individual recipient mouse. The percentages of donor-type cells among nucleated bone marrow cells (Fig. 3A), $CD45R^+$ ($B220^+$) cells among donor-type cells (Fig. 3B), and κ^+ B cells (Fig. 3C) and λ^+ B cells (Fig. 3D) among donor-type $CD45R^+$ cells were compared between host mice transferred with *non-transgenic* HSC (open circles) and those grafted with *transgenic* HSC (closed circles). The two groups of recipients produced donor-type $CD45R^+$ cells at the same time, 8–10 days postgrafting (Fig. 3B). Although *transgenic* and *non-transgenic* HSC differentiated into $CD45R^+$ cells at the same time postgrafting, *transgenic* HSC differentiated into $sIgM^+$ B cells earlier than *non-transgenic* HSC. In fact, *transgenic* HSC yielded substantial numbers of κ^+ B cells as early as day 8 and the percentage of κ^+ B cells among donor-type $CD45R^+$ cells increased around day 11 (Fig. 3C). In marked contrast, in the mice reconstituted with *non-transgenic* HSC, κ^+ B cells were virtually undetectable until 12.5 days postgrafting and the number of κ^+ B cells rose rapidly at 13 days postgrafting.

Consistent with the generation of κ^+ B cells, *transgenic* HSC yielded substantial numbers of λ^+ B cells 9–13 days postgrafting, while *non-transgenic* HSC were not capable of generating λ^+ B cells during this period (Fig. 3D).

The early expression of the μ chain from the transgene advanced the onset of κ rearrangements

In order to assess V_κ–J_κ rearrangements among donor-type precursors, the relative numbers of V_κ–J_κ joints in donor-type $CD45R^+$ cells were measured by PCR (36) at various times postgrafting. The assay for V_κ–J_κ joints was restricted to rearrangements involving the $J_\kappa 1$ segment, which is one of four functional J_κ segments and is used most frequently in V_κ–J_κ rearrangements (37). In this analysis, the number of V_κ–J_κ rearrangements in sorted splenic κ^+ B cells was used as the standard (100%) as described previously (22). Figure 4 shows a representative Southern blot analysis of PCR amplified fragments after a 28 cycle amplification. Recombined V_κ–J_κ fragments were detectable earlier in the postgrafting period in donor-type $CD45R^+$ cells from *transgenic* HSC than in cells from *non-transgenic* HSC. This study indicates that the presence of transgenic μ chain indeed advanced the onset of V_κ–J_κ rearrangements.

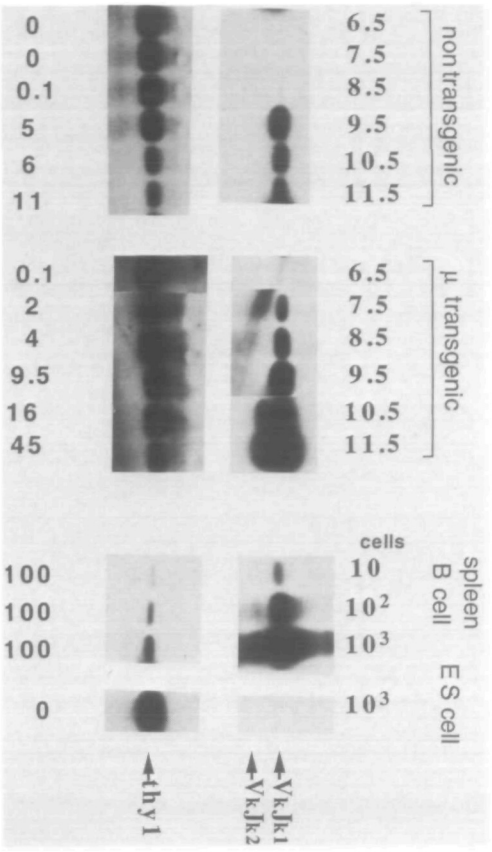

Fig. 4. PCR analyses of κ rearrangements reveal that μ chain expression from the transgene advances the onset of κ rearrangements during B cell development in the reconstituted mice. On the indicated days after engraftment, donor-type CD45R$^+$ cells were isolated from recipient mice engrafted with wild-type HSC and from recipient mice engrafted with HSC carrying the μ chain transgene Chromosomal DNA from 10^3 cells was amplified, blotted and hybridized to analyze V_κ–J_κ joints and the *Thy-1.2* gene (internal control). The arrows show the sizes of the expected amplified products. Data from the indicated numbers of sIgM$^+$ splenic B cells (positive control) and ES cells (negative control) are also presented The intensities of V_κ–J_κ1 joints are quantitated on appropriate autoradiographic exposures. Under each lane, the extent of κ rearrangements is shown as a percentage of that in C57BL/6 splenic B cells. The data were corrected for variations in cell input based on the *Thy-1.2* gene amplification product.

The early expression of transgenic μ chain did not activate IgL rearrangements in early cycling B cell precursors

The engraftment of HSC revealed that early expression of the μ chain from the transgene caused the early onset of κ rearrangements. In order to investigate whether or not early expression of μ chain from the transgene activated κ rearrangements in the early cycling precursor stage, we injected a single dose of BrdU i.p. into μ chain transgenic and non-transgenic mice to pulse-label cycling cells. In normal B cell development (Fig. 5A), early cycling B cell precursors (pro-B and large pre-B cells) are expected to incorporate BrdU, while late resting B cell precursors (small pre-B cells), which undergo IgL rearrangements, are not expected to incorporate BrdU in the absence of cell division. The appearance of κ$^+$ B cells at 8 days post-engraftment suggests that CD45$^+$ cells are capable of expressing sIgM from a very early developmental time. Thus, once early precursors acquire in-frame V_κ–J_κ or V_λ–J_λ rearrangements, they would rapidly express κ or λ on the cell surface. As such, the rates of output of BrdU-labeled κ$^+$ and λ$^+$ B cells should reflect the rates of V_κ–J_κ and V_λ–J_λ rearrangements respectively.

Following 4 h of pulse BrdU labeling, BrdU was incorporated by 31 and 29% of CD45R$^+$sIgM$^-$ B cell precursors from wild-type and μ chain transgenic mice respectively. Figure 5(C) shows the percentages of BrdU-labeled κ$^+$ and λ$^+$ B cells among newly-generated B cells in the bone marrow (30). In both groups of mice, the kinetics of BrdU-labeled κ$^+$ B cell output as well as λ$^+$ B cell output were very similar. Following the injection of BrdU, substantial fractions of BrdU-labeled κ$^+$ and λ$^+$ B cells appeared at 12 and 36 h respectively, and the percentages of BrdU-labeled κ$^+$ and λ$^+$ B cells reached maximum levels by 36 and 72 h respectively. These observations indicate that μ chain transgenic as well as wild-type mice underwent κ and λ rearrangements with the same kinetics after the arrest of cell cycling. The fraction of BrdU$^+$ cells in μ chain transgenic mice was consistently lower than that in non-transgenic littermates. The number and/or rate of cell division in early B cell precursors in μ chain transgenic mice were likely smaller than in non-transgenic mice, causing lower incorporation of BrdU on a single cell basis.

Discussion

The expression of the μ chain immediately drives early cycling B precursors to differentiate into the late resting precursors.

We performed the following two experiments to study the kinetics of B cell development comparing wild-type and μ chain transgenic mice: transfer experiments where B cell development from transferred HSC was followed over time and BrdU-labeling experiments where B cell development after the transition from the early cycling to the late resting precursor stages was followed over time. The transfer experiments showed that early expression of the μ chain from the transgene significantly reduced the duration of B cell development from HSC to immature B cells. The BrdU-labeling experiments showed that the presence of transgenic μ chain did not affect the length of the late resting precursor stage. These observations suggest that early expression of the μ chain from the transgene reduces the length of the early cycling precursor stage.

This conclusion is consistent with previous studies that measured the fraction of B cell precursors at different stages in μ chain transgenic mice (2,35,38). These studies showed that the presence of a μ transgene reduced the proportion of precursor B cells at an early stage. It should be noted that T cell development apparently differs in this regard from B cell development. TCR αβ thymocytes undergo V–J rearrangements in the double-negative (DN, CD4$^-$CD8$^-$) stage. In contrast to a striking decrease in the number of CD45R$^+$sIgM$^-$ B cell precursors in μκ transgenic mice (38), the number of DN thymocytes was not reduced in TCR αβ transgenic mice (39). Hence, DN thymocytes in TCR αβ transgenic mice may need the same maturation time as required in wild-type mice to differentiate into the next developmental stage by initiating

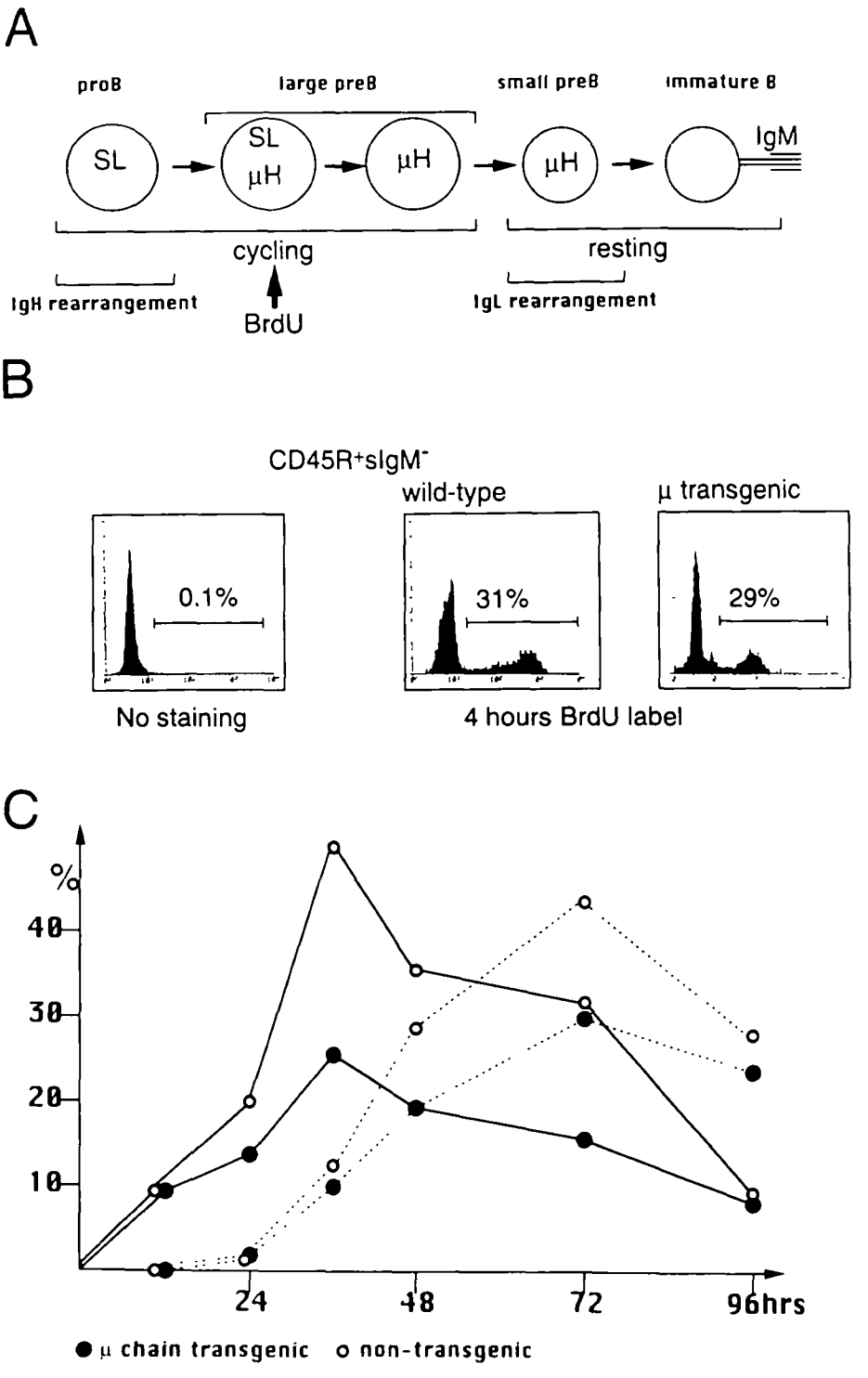

Fig. 5. *In vivo* pulse BrdU labeling reveals that early expression of μ chain from the transgene does not reduce the duration of the small pre-B cell stage (A) Strategy for BrdU-labeling experiments. CD45R$^+$ B lineage cells in the bone marrow were subdivided into four stages based on the state of cell cycling and expression of the surrogate light chain (SL), μ chain and IgL chain Precursor cells at the pro-B and large pre-B cell stages would be expected to incorporate BrdU. These cells should differentiate into resting small pre-B cells, which undergo IgL rearrangements without incorporating BrdU. The appearance of BrdU-labeled B cells is thus dependent on the kinetics of productive IgL rearrangements. (B) The histograms show the staining by anti-BrdU antibody The histogram on the left represents a negative control where unlabeled CD45R^{low}sIgM$^+$ bone marrow cells were stained with anti-BrdU antibody. The histogram on the right shows anti-BrdU staining of the CD45R^{low}sIgM$^-$ bone marrow cells derived from mice pulse-labeled with BrdU for 4 h. The BrdU-labeled fraction is delineated by a horizontal bar. (C) The appearance of BrdU-labeled κ$^+$ B cells (solid line) and BrdU-labeled λ$^+$ B cells (dotted line) in the μ chain transgenic mice (closed circles) and their non-transgenic littermates (open circles) after a single injection of BrdU. At the indicated time after the injection of BrdU, newly-generated κ$^+$ and λ$^+$ B cells were isolated from the bone marrow and purified by cell sorting. Sorted cells were stained with anti-BrdU antibody. The percentage of cells which were BrdU-labeled is displayed.

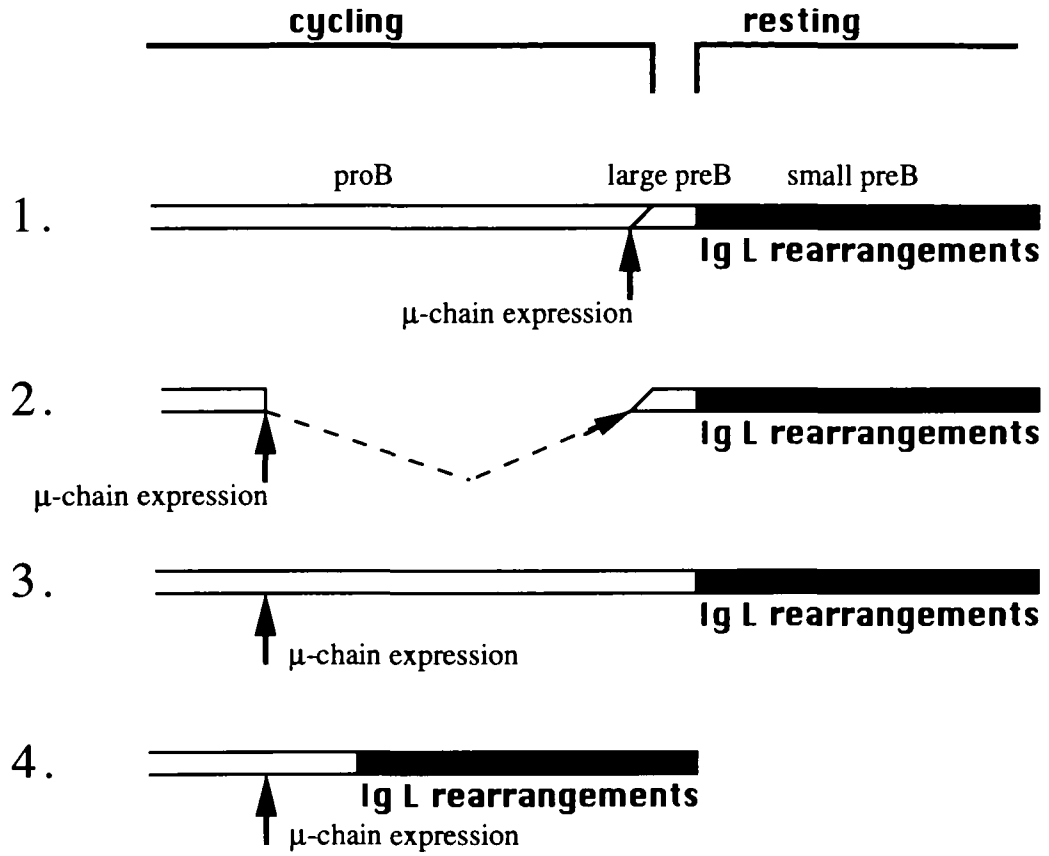

Fig. 6. Early expression of the μ chain from the transgene significantly reduced the duration of the pro-B cell stage but did not affect the small pre-B cell stage B cell development in wild-type mice (first model) can be subdivided into two stages the early cycling precursor stage (pro-B) and the late resting precursor stage (small pre-B) The stage where κ and λ rearrangements occur is shown by closed bars. In wild-type mice, these rearrangements occur exclusively in precursors in the resting phase (first model). The present experiment revealed that, as shown in the second model, early expression of the μ chain from the transgene immediately drove early cycling precursors into the late resting stage (broken line), but did not affect the late resting precursor stage. The present experiments invalidated the third and fourth models The third model predicts that HSC need a fixed maturation time to differentiate into the late resting stage, where IgL rearrangements occur. The fourth model predicts that μ chain expression directly activates the IgL loci for V–J rearrangements, while the precursors are still proliferating.

the expression of other factors, in addition to the expression of the transgenic TCR.

Figure 6 shows four models of the time course of B cell development. Osmond estimated that the developmental periods of the $CD45R^{-}TdT^{+}$pro-B, $CD45R^{+}$pro-B, large pre-B and small pre-B cell stages averaged 20, 18, 6.5 and 33 h respectively in wild-type mice (Fig. 6, first model) (1). Early precursors (pro-B) are mostly cycling and late precursors (small pre-B) are in resting phase. Closed bars in Fig. 6 represent the developmental stage during which B cell precursors undergo V–J rearrangements for IgL chain. These rearrangements are normally initiated after the arrest of the cell cycle (5,7). As shown by a dashed arrow in the second model of Fig. 6, the present data suggest that, when the μ chain was artificially expressed by a transgene at a very early developmental time, the precursors omitted subsequent maturation steps in the early cycling precursor stage and immediately differentiated into the late resting precursor stage. Subsequently, the μ chain transgenic mice underwent κ and λ rearrangements after the arrest of cell cycling with the same kinetics as wild-type mice.

The present experiments invalidate the other two models of B cell development (Fig. 6, third and fourth models). In the third model, although very early B cell precursors express transgenic μ chain, they need the same length of maturation time as wild-type precursors to differentiate into immature B cells. The third model seems to apply to thymocyte development in TCR αβ transgenic mice as discussed before. In the fourth model, early expression of transgenic μ chain immediately activates the κ and/or λ locus for V–J rearrangements while the precursor cells are cycling.

The arrest of cell cycling is an obligatory step in the induction of IgL rearrangements

In normal B cell development the arrest of cell cycling appeared to correlate closely with the initiation of IgL rearrangements (6,7,40), but causality was not determined. The present results showed that, despite the significant reduction in the duration of the preceding pro-B stage, μ chain transgenic mice initiated κ and λ rearrangements only after the arrest of cell cycling. This observation implies that

the arrest of cell cycling is an obligatory step for κ and λ rearrangements.

The induction of IgL rearrangements after the arrest of cell cycling is reminiscent of the action of pro-B cell clones established by Rolink *et al.* (41). The pro-B cell clones contain D_HJ_H/D_HJ_H at the IgH locus and germline configurations at the IgL loci; the clones divide in a self-renewing manner in the presence of a feeder cell line, pA6 plus IL-7 (41). With the removal of either pA6 or IL-7, the cells stop cycling, and subsequently initiate κ rearrangements. We found that even the arrest of cell cycling by the addition of hydroxyurea induced IgL rearrangements (unpublished data) These observations imply that the arrest of cell cycling causes IgL rearrangements both *in vitro* and *in vivo*.

Although J_H-deficient mice cannot generate late resting B cell precursors, these mice can initiate low levels of V_κ–J_κ rearrangements (8,22). This observation is not inconsistent with the notion that κ rearrangements are initiated upon the arrest of the cell cycle We speculate that early B cell precursors from J_H-deficient mice undergo IgL rearrangements only when these cells stop cell cycling before cell death

Expression of the μ chain transduces signals to inhibit V_H to D_HJ_H rearrangements, but presumably does not directly activate the IgL genes for V–J recombination

We speculate that the expression of the μ chain does not directly activate the IgL genes for V–J recombination, based on the following observations. There are some pro-B clones that contain abortive IgH rearrangements on both alleles, but still undergo V_κ–J_κ rearrangements with the same kinetics as normal pro-B cell clones (42). These data indicate that the expression of the μ chain does not facilitate IgL rearrangements during the *in vitro* differentiation of pro-B clones. The very efficient induction of κ rearrangement in these pro-B cell clones is more representative of B cell development *in vivo* than the slower induction of κ rearrangement observed in A-MuLV transformed pre-B cell lines (19–21). Given that the arrest of cell cycling apparently triggers IgL rearrangements *in vivo* as well as *in vitro*, the expression of the μ chain may not transduce signals to activate the IgL loci for V–J rearrangements *in vivo*.

The rate of output of $BrdU^+\kappa^+$ and $BrdU^+\lambda^+$ cells reflects the kinetics of κ and λ rearrangements more precisely than assessment of the number of V_κ–J_κ joints by PCR

In order to analyze the kinetics of IgL rearrangements, previous studies measured the number of V_κ–J_κ joints in sorter-purified precursors, using PCR (7,22,43). Although these studies clearly showed the correlation between the expression of surface markers and the state of V(D)J rearrangements, we did not employ this method to assess the effect of μ chain expression on IgL rearrangements for the following three reasons. First, surface markers expressed on late resting B cell precursors may differ between μ chain transgenic and non-transgenic mice, because the length of the pro-B cell stage in μ chain transgenic mice is significantly reduced. While the expression of CD25 and c-*kit* on B cell precursors is regulated by expression of the μ chain (2,35,44), the expression pattern of other surface markers appears to correlate with the length of time after HSC begin differentiating into B-lineage cells. In fact, each subfraction of B cell precursors, which was purified by cell sorter following Hardy's method (6), contains precursors at various developmental stages with respect to the status of V–J rearrangements (43). Second, our previous study showed that sequential V_κ–J_κ rearrangements on one chromosome occur up to three times on average during B cell development, until in-frame V_κ–J_κ rearrangements occur (45,46). The total number of V_κ–J_κ joints measured by PCR does not reflect the presence of sequential V_κ–J_κ rearrangements, while the flow cytometric analysis of $BrdU^+\kappa^+$ B cells can accurately measure the number of in-frame V_κ–J_κ joints. Third, the production rates of small pre-B and B cells have been shown to be 1.7×10^7 and 1.6×10^7 cells/day respectively, and the turnover of small pre-B cells is 3%/h (1). These observations indicate that virtually all late resting precursors in wild-type mice initiate IgL rearrangements and that most of these precursors acquire in-frame κ rearrangements within 33 h. Such a rapid increase in the number of V_κ–J_κ joints could not be accurately measured by PCR.

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Abbreviations

A-MuLV	Abelson murine leukemia virus
DN	double negative
HSC	hematopoetic stem cell
PE	phycoerythrin

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