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Induction of sterile transcription from the κL chain gene locus in V(D)J recombinase-deficient progenitor B cells

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Abstract

B cell development in RAG-2-deficient (RAG-2T) mice is impeded at an early stage, due to the inability of these animals to rearrange their endogenous Ig gene loci. Expression of an *Eμ-bcl-2* transgene in these mice did not change this phenotype. However, stromal cell/IL-7-reactive B cell progenitors (pro-B cells) were found in fetal liver and bone marrow of RAG-2T and RAG-2T/*Eμ-bcl-2* transgenic mice in numbers comparable to normal mice. Like cells from normal mice they are c-kit$^+$, surrogate L chain$^+$ and CD25$^-$, and can proliferate *in vitro* for long periods of time. Upon IL-7 deprivation, they can be induced to differentiate into c-kit$^-$, surrogate L chain$^-$ and CD25$^+$ cells that are no longer clonable on stromal cells and IL-7. Furthermore, sterile transcription from the κL chain gene loci is induced. The latter was also observed with pro-B cells directly isolated *ex vivo* from the bone marrow of RAG-2-deficient animals. The results suggest that progenitor B cell differentiation can occur in cells from V(D)J recombinase-deficient mice to the stage where κL chain gene rearrangements would normally be initiated. It further indicates that some molecular programs of early B cell differentiation can take place in the absence of Ig gene rearrangements.

Introduction

The variable regions of B and T cell antigen receptors are somatically assembled from V, D and J gene segments that are separated in the germline (1). Early B cell differentiation is characterized by ordered rearrangements of first D to J_H then V_H to DJ_H gene segments on the Ig heavy (H) chain locus, and finally of V_κ to J_κ or V_λ to J_λ segments in the Ig light (L) chain loci (2,3). It has been suggested that progression along the pathway of B cell differentiation is guided by the surface-expression of proteins from productively rearranged Ig gene loci, i.e. by DJ_HC_μ proteins (D_μ proteins), μH chains, κ and λ L chains (4,5). Furthermore, it was proposed that one of the ways by which productive rearrangements of the IgH chain gene locus could be monitored by precursor B cells would be to form pre-B cell receptors composed of D_μ proteins or μH chains in association with surrogate L chain (5), encoded by the pre-B cell specific genes λ_5 (6) and $V_{pre\text{-}B}$ (7).

This view of B cell differentiation was supported by the analysis of mice carrying a targeted (T) deletion of the exon encoding the transmembrane portion of μH chain (μMT mice) (8), of the λ_5 gene (λ_5T mice) (9) or a targeted deletion of the entire J_H gene cluster of the IgH gene locus (J_HT mice) (10,11). All these mutant mice are impeded during early B cell development at the transition from DJ_H-rearranged pre-B-I to V_HDJ_H-rearranged pre-B-II cells (12) (for nomenclature, see 13).

An inductive role of μH chain expression for early pre-B cell differentiation, especially in the control of L chain gene rearrangements, has been implied from experiments with A-MuLV-transformed pre-B cell lines (14–17). Furthermore, it has been suggested that μH chain expression *in vivo* enhances the frequency of κL chain gene rearrangements by activating sterile transcription from the L chain gene loci (18).

However, an inductive role of μH chains for the latter process has been put into question by the analysis of κL chain gene rearrangements in pre-B cell subsets of μMT and J_HT mice (11), as well as by the demonstration that κL chain protein can be expressed after *in vitro* differentiation of pre-B cell clones, which are unable to express μH chain protein (19).

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Mice in which the *RAG-2* gene has been disrupted by targeted mutation (RAG-2T mice) cannot rearrange their Ig and TCR gene loci and are impeded in early T and B lymphopoiesis (20). In order to assess the influence of Ig gene rearrangements on the overall capacity of early B lineage cells to differentiate to more mature cellular stages, stromal cell/IL-7-dependent cell lines (21) were established from bone marrow and fetal liver of RAG-2T mice, as well as of RAG-2T mice expressing an *Eμ-bcl-2* transgene (22). Expression of the *bcl-2* transgene facilitates this analysis, since it suppresses apoptosis, which occurs during the *in vitro* differentiation of cells from normal mice (23).

Methods

Mice

(C57BL/6×DBA/2)F_1 (BDF_1) mice, which were used for the isolation of wild-type cells, were purchased from BRL (Füllinsdorf, Switzerland). A breeding pair of RAG-2T mice was kindly provided by Dr F. W. Alt (The Children's Hospital, Boston, MA) and breeding of the RAG-2T strain was continued in the specific pathogen-free facility of the Basel Institute for Immunology. *Eμ-bcl-2* transgenic (tg) mice were originally obtained by Dr A. Strasser (The Walter and Elisa Hall Institute for Immunology, Melbourne, Australia) and were bred to the RAG-2T background at the animal facilities of the Basel Institute for Immunology. Mice were tested for the presence of the *bcl-2* transgene and the neomycin resistance gene being inserted into the RAG-2 coding region by means of PCR on tail DNA.

Screening of offspring for the bcl-2 transgene and the neo insertion by analytic PCR on tail DNA

Genomic DNA was extracted from 0.2–0.5 cm tail DNA derived from 3-week-old offspring. Tails were incubated for 8–14 h at 56°C in 500 μl lysis buffer (50 mM Tris–HCl, pH 8.2, 5 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.5 mg/ml proteinase K). Debris was removed by centrifugation for 30 s at 12,000 r.p.m. at room temperature. Proteins in the supernatant were precipitated by adding 200 μl saturated NaCl and incubating for 20 min on ice, and were removed from the solution by centrifugation at 12,000 r.p.m. for 15 min at 4°C. DNA in the supernatant was precipitated by adding 1 ml ethanol and was obtained by centrifugation at 12,000 r.p.m., 15 min at 4°C. DNA pellets were washed once in −20°C cold 70% ethanol, air-dried and dissolved in 100 μl 10 mM Tris–HCl, pH 8.3; 1 μl was used for subsequent PCR amplification. Forward and reverse primers for the detection of the bcl-2 transgene and the RAG-2/neo-locus were as follows: bcl-2 tg: forward: 5′-TCACACCACAGAAGTAAGTAAGGTTCC-3′, reverse: 5′-GGTCATGTGTGTGGAGAGCGTCA-3′, RAG-2/neo locus: forward: 5′-GCAACATGTTATCCAGTAGCCGGT-3′, reverse: 5′-TTGGGAGGACACTCACTTGCCAGT-3′, reverse: 5′-GTATGCAGCCGCCGCATTGCATCA-3′. By using a mixture of reverse primers for the latter reaction, the wild-type and RAG-2T alleles could be analyzed in the same reaction, allowing identification of heterozygous and homozygous wild-type or RAG-2T genotypes.

Conditions for PCR were: 1 cycle at 94°C (40 s), 32 cycles at 94°C (20 s), 60°C (25 s) and 72°C (90 s).

Antibodies and FACS staining

Two-color FACS analyses were carried out as described in (21). Biotinylated mAb specific for c-kit, the IL-7R (both a kind gift of Dr S. I. Nishikawa), CD19, CD40 (Rolink, unpublished) CD25, CD23 and CD43 (PharMingen, San Diego, CA) were revealed by streptavidin–phycoerythrin (PE) (Southern Biotechnology Associates, Birmingham, AL). Three-color FACS analyses were carried out using FITC-labeled anti-B220 (CD45R) antibody RA3-6B2 (PharMingen), PE-labeled anti-c-kit antibody and biotinylated anti-NK1.1 (PharMingen, San Diego, CA) or anti-CD19 antibodies, which were revealed by streptavidin–TriColor (Caltag, San Diego, CA).

Isolation and cell culture of stromal cell/IL-7-dependent B cell progenitors

Stromal cell/IL-7-dependent cell lines were derived from fetal liver and bone marrow of wild-type and mutant mice as described (21). They were grown at 37°C, 10% CO_2 on a semiconfluent layer of γ-irradiated (2000 rad) ST-2 stromal cells (24) in IMDM medium containing 1×non-essential amino acids (Gibco/BRL, Basel, Switzerland), 1×penicillin/streptomycin (Gibco/BRL), 5 μg/ml porcine insulin (Sigma, St Louis, MO), 5×10^{-5} M β-mercaptoethanol, 0.03% Primatone RL (Quest International, Naarden, The Netherlands), 1% FCS (Gibco/BRL) and 1% conditioned medium from a J558 myeloma cell line transfected with an IL-7 expression vector (a kind gift of Dr T. H. Winkler, Basel Institute for Immunology), containing 5×10^4–10^5 U/ml recombinant IL-7. Cells grown under these conditions were considered as undifferentiated (day 0) cells.

Differentiation was achieved by washing cells from IL-7 cultures three times in medium without IL-7 and culturing them in the absence of IL-7 either with or without γ-irradiated (2000 rad) ST-2 stromal cells for 1, 2 or 3 days. Cells were harvested by centrifugation, washed once in PBS and assayed directly or (for molecular analyses) were shock-frozen in liquid nitrogen and stored at −80°C.

Short-term culture of FACS-sorted cells

$B220^+$/$c\text{-}kit^+$ cells from bone marrow were isolated by FACS as described (25). Between 5×10^4 and 10^5 cells/well were set up in 200 μl medium in a 96-well plate without IL-7 and stromal cells. Cells at the day of sorting were considered as undifferentiated cells, those that have been cultured for various days at 37°C, 10% CO_2 were considered as differentiated cells (e.g. after day 2 of culture). Cell viability was monitored by counting cells in a Neubauer hemocytometer using PBS, 0.2% Trypan blue.

Limiting dilution analysis

Undifferentiated cells and cells after various time points of differentiation were harvested and the number of viable cells was determined by Trypan blue exclusion. The cells washed once in medium containing IL-7 and serial 2-fold dilutions of the cell suspensions were seeded into 96-well plates covered with a semiconfluent layer of γ-irradiated ST-2 cells. Cells were cultured at 37°C, 10% CO_2 in medium containing IL-7 and growth of colonies was assayed with an inverted microscope after 7 days of culture. Results of limiting dilution analyses were graphically analyzed using semi-logarithmic

representation of the data. According to Poisson's distribution the concentration at which 37% of the wells score negative for growth of pre-B cell colonies contained on average one clonable cell per well.

Forward scatter, side scatter and DNA content analysis of pre-B cells

Cells were incubated in PBS containing 25 μg/ml propidium iodide (PI) (Sigma) for 20 min on ice and were analyzed for forward/side scatter on a FACScan (Becton Dickinson, Mountain View, CA) equipped with Lysys software. Viable cells could be discriminated from apoptotic cells (appearing at very low forward scatter values) by their ability to exclude PI and therefore stain negative for fluorescence detectable in channel 2 or 3 (FL2 or FL3). By this procedure, small resting lymphocytes (also appearing at small forward scatter) could unequivocally be discriminated from apoptotic cells.

For DNA content analysis, 5×10^4–5×10^5 cells were resuspended in 200 μl ice-cold PBS, were mixed with 2 ml ice-cold 70% ethanol, 30% PBS and were incubated for 30 min on ice. Cells were harvested by centrifugation, resuspended in 800 μl PBS, to which 100 μl RNase A (1 mg/ml) and 100 μl PI (400 μg/ml) were added, and the cells were incubated for 30 min at 37°C.

DNA content was analyzed by measuring FL3 and, excluding doublets, by also measuring FL3 width.

Northern blot analysis

Total RNA was extracted from undifferentiated cells and cells that were cultured in the absence of IL-7 by acid guanidinium thiocyanate extraction (26). Aliquots of 15 μg of total RNA per lane were analyzed by Northern blotting for the expression B cell related mRNAs as described (23). The same blots were hybridized to the indicated probes (β-actin served as a control for RNA loading), which were derived from the following constructs: RAG-1, a mixture of 410, 950 and 1300 bp fragments obtained by *Hin*dIII digestion of M6-BSK+, containing the entire RAG-1 cDNA; RAG-2, a 2100 bp fragment obtained by *Not*I digestion of MR2-BSK+, containing the mouse RAG-2 cDNA (both plasmids were kind gifts of Dr D. Schatz, Yale University). Constant μH chain probe, a 536 bp *Pst*I–*Pst*I fragment cloned into M13mp8, comprising the third C_μ exon up to the 5′ part of the fourth C_μ exon. Constant κ probe: a 330 bp *Hpa*I–*Ava*II fragment, comprising 90% of the constant κ exon. β-actin probe, a 522 bp fragment (from position 220 to 742 of GenBank sequence MUSACTBR). λ_5, a 444 bp fragment of the λ_5 cDNA spanning exons 1–3 from position 364 of exon 1 (GenBank code MUSIGRL51) to position 354 of exon 3 (GenBank code MUSIGRL53). $V_{pre\text{-}B}$, a 447 bp fragment of the $V_{pre\text{-}B}$ cDNA from position 133 to 580 of GenBank code MUSVPREB1. TdT, a 541 bp fragment from position 150 to 691 of GenBank code MUSTDTR. The fragments of C_κ, β-actin, λ_5, $V_{pre\text{-}B}$ and TdT were all cloned into M13mp19, isolated as single-strand DNA and labeled by primer extension.

RT-PCR assay for mRNA expression in FACS enriched cells

Total RNA for cDNA synthesis was prepared using RNAzol B (Biotecx, Houston, TX) according to the manufacturer's recommendations. RNA was reverse-transcribed using a Superscript II cDNA synthesis kit (Gibco Life Technologies, Gaithersburg, MD) as recommended by the manufacturer. Serial 5-fold dilutions of the various cDNA samples were subjected to PCR using primer pairs for the indicated genes (see below). Aliquots of the PCR-amplified DNA were fractionated by agarose gel electrophoresis and transferred to nylon membranes for 1 h using 0.4 M NaOH. Blots were prehybridized (1 h) and hybridized (4 h) at 65°C in 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA with the same probes also used for Northern blots. Radioactivity on the blots was revealed by X-ray autoradiography and was quantitated by means of a Phosphorimager equipped with Image-Quant™ Software (Molecular Dynamics, Mountain View, CA).

RT-PCR assays were carried out using the following primer-pairs: forward primer for 0.8 kb κ^0 transcript, 5′-CAGTGAGGAGGGTTTTTGTACAGCCAGACAG-3′; forward primer for 1.1 kb κ^0 transcript, 5′-CATGGAAGTGAAATGGCTGTAGCCTAATG-3′; reverse primer for both κ^0 transcripts, 5′-CTCATTCCTGTTGAAGCTCTTGACAATGGG-3′; forward, reverse primers for β-actin, 5′-GTGGGAATTCGTCAGAAGGACTCCTATGTG-3′, 5′-GAAGTCTAGAGCAACATAGCACAGCTTCTC-3′; forward, reverse primers for λ_5, 5′-GGTAGAATTCGCTCACCAACACACTAC-3′, 5′-GAGATCTAGACTGCAAGTG AGGCTAGAG-3′; forward, reverse primers for $V_{pre\text{-}B}$, 5′-CAGGTCTAGAGCC TGGCCTGGACGTCTG-3′, 5′-GTCTGAATTCCTCCAGAGCCTAAGATCCC-3′; forward, reverse primers for TdT, 5′-GATTTCTAGACTTGGTCCTCTTCATTTTGG-3′, 5′-CAAGGAATTCCCTCTGTGTCTTTCATGCTG-3′; forward, reverse primers for RAG-1, 5′-TCTTGAATTCTCTCATGGTCAAGTGTCCCG-3′, 5′-CAGTTCTAGAGGACACATTCTTCAGTGGGG-3′. All PCRs were carried out with 1 cycle at 94°C for 40 s, followed by 26–29 cycles at 94°C for 20 s, 55°C for 25 s and 72°C for 90 s, depending on the abundance of the transcripts.

Results

Analysis of early B lineage cells in mice with a disrupted RAG-2 gene

Bone marrow and fetal liver of RAG-2T and RAG-2T/*Eμ-bcl-2* tg mice were found to contain normal numbers of $B220^+$ pro-B cells with the surface phenotype of DJ_H-rearranged pre-B-I cells, expressing the proto-oncogene *c-kit* (Fig. 1A) (13,23) Expression of an *Eμ-bcl-2* transgene, which is functionally active in $B220^+$/c-kit^+ cells of RAG-2T/*Eμ-bcl-2* tg mice (Fig. 1B), did neither promote B cell differentiation in the bone marrow, nor the generation of B lineage cells in the peripheral lymphoid organs expressing mature markers, like CD21 or CD23 (Fig. 1a). This appears to be in contrast to a study by Strasser *et al.* (27), in which C.B-17-SCID mice expressing a *Eμ-bcl-2* transgene were found to accumulate surface IgM^- B lineage cells with mature markers (CD21, CD23) in the peripheral lymphoid organs. Although SCID mice also exhibit a defect in V(D)J recombinase activity, it has to be kept in mind that lymphocytes of these mice, unlike those from RAG-2T mice, are able to initiate and to perform (although at reduced frequencies) rearrangements of Ig and TCR gene loci (28,29) (see below).

Limiting dilution experiments revealed that the frequencies of stromal cell/IL-7-reactive B cell precursor in fetal liver and

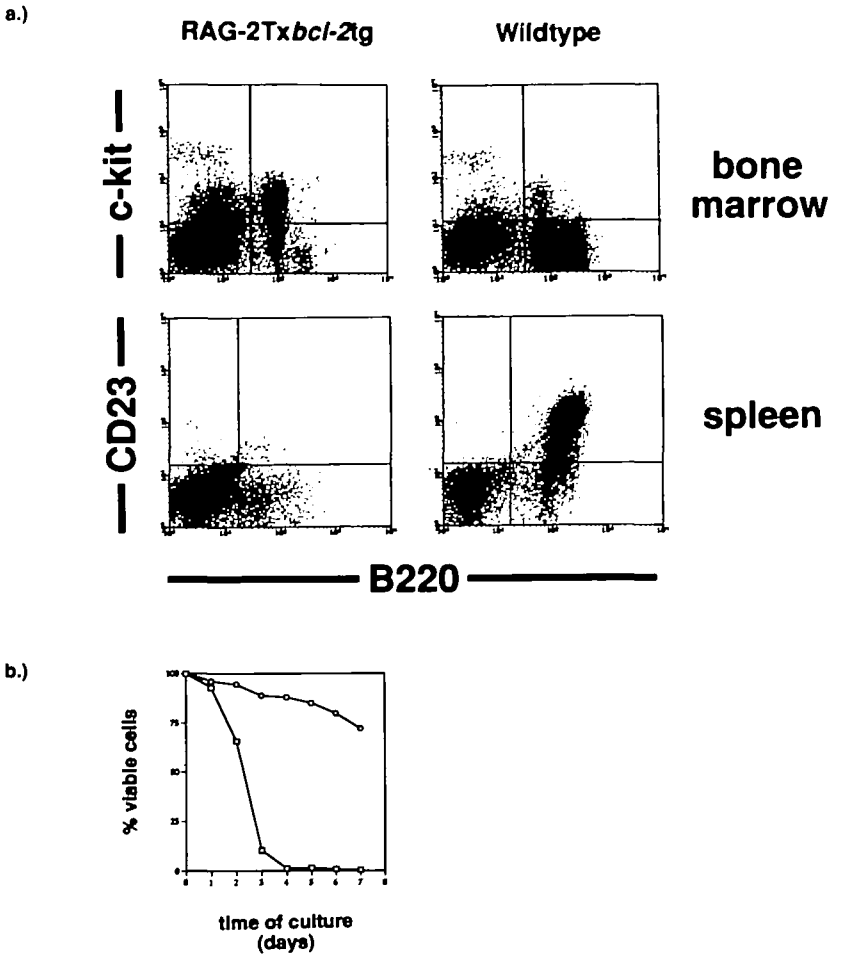


Fig. 1. Expression of an *EμH-bcl-2* transgene in RAG-2-deficient mice does not lead to accumulation of differentiated B lineage cells in bone marrow or spleen. (A) FACS analysis was carried out with cell suspensions prepared from bone marrow and spleen of three or four age-matched (4 weeks old) RAG-2T and RAG-2T/*EμH-bcl-2* tg mice, as well as normal BDF_1 mice. Comparison of the percentages of $B220^+$ cells in bone marrow and spleen of RAG-2T and RAG-2T/*EμH-bcl-2* tg mice revealed no significant differences (13–18% for bone marrow and 10–14% for spleen in individual mice of both genetic backgrounds) as compared with wild-type mice exhibiting 40–50% $B220^+$ cells in bone marrow and 58–66% $B220^+$ cells in spleen. The total cellularity of bone marrow and spleen in individual RAG-2T and RAG-2T/*EμH-bcl-2* tg mice was comparable (data not shown). Two-color FACS analysis using anti-B220 mAb and a variety of B lineage related mAb in RAG-2T and RAG-2T/*EμH-bcl-2* tg mice has revealed that the $B220^+$ cells found in bone marrow and spleen expressed early, pre-B cell associated markers, like c-kit, CD43 or the IL-7R, but no FACS-detectable levels of markers found on more mature B lineage cells, like CD25, CD40, CD21 or CD23. As an example, B220/c-kit (representing an early B cell differentiation marker) and B220/CD23 (as a representative for the late B cell differentiation markers) double-fluorescence stainings are given for bone marrow and spleen respectively of RAG-2T/*EμH-bcl-2* tg as well as control mice (similar stainings were obtained with RAG-2T mice, not shown). (B) Functionality of the *bcl-2* transgene in B cell progenitors of RAG-2T/*EμH-bcl-2* tg mice was assayed by culturing FACS-purified $B220^+$/c-kit^+ cells. While 75% of input cells could be recovered after 7 days of tissue culture from RAG-2T/*EμH-bcl-2* tg mice (open circles), $B220^+$/c-kit^+ cells from RAG-2T mice (open squares) were rapidly lost from cultures containing no exogenous IL-7 and <1% viable cells could be recovered after 4 days of culture. These results indicate that the *bcl-2* transgene is active in $B220^+$/c-kit^+ B cell progenitors of RAG-2T/*EμH-bcl-2* tg mice and can prevent apoptosis during *ex vivo* culture in the absence of growth factors.

bone marrow of RAG-2T and RAG-2T/*Eμ-bcl-2* tg mice are comparable to those of normal mice.

Several long-term proliferating, stromal cell/IL-7-reactive cell lines were established from day 16 fetal liver and from bone marrow of RAG-2T and RAG-2T/*Eμ-bcl-2* tg mice. All cell lines exhibited proliferation kinetics which were indistinguishable from each other and from fetal liver or bone-marrow derived pre-B-I cells of normal BDF_1 and of *Eμ-bcl-2* tg mice (data not shown).

These cell lines were analyzed by FACS for the expression of B lineage related surface markers (Fig. 2). The results of these analyses demonstrate that mutant and normal cell lines expressed B220, c-kit, surrogate light chain (encoded by λ_5 and $V_{pre\text{-}B}$) (Fig. 2), as well as CD43, CD44, CD71 (the transferrin receptor) and heat stable antigen (data not shown). In contrast, they did not express CD25 (IL-2Rα chain) (Fig. 2) or surface IgM (sIgM), CD23 (FcεRII) and CD40 (data not shown). Since the surface phenotype of mutant and normal cell lines appears indistinguishable, we conclude that a pre-B-I like stage of B lineage differentiation can be reached

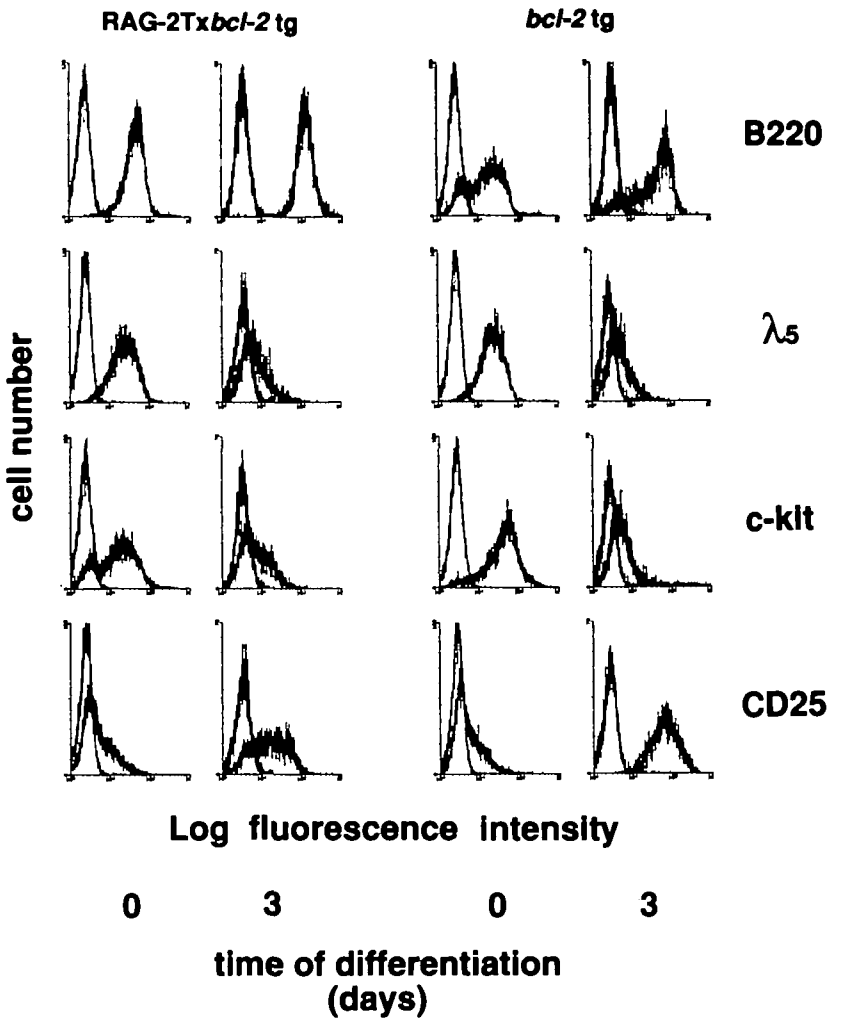


Fig. 2. Stromal cell/IL-7-dependent B cell progenitors from RAG-deficient mice undergo the same phenotypic changes upon IL-7 withdrawal that are characteristic for the *in vitro* differentiation of stromal cell/IL-7-reactive pre-B cells from normal mice. Cell lines from wild-type, RAG-2-deficient and RAG-2-deficient/*Eμ-bcl-2* tg mice were analyzed by FACS for the expression of B220, surrogate L chain, c-kit and CD25 (as shown), as well as CD43, HSA and IL-7R, CD40, CD21 or CD23 (not shown). Removal of IL-7 from the cultures resulted in the down-modulation of pre-B cell specific markers [surrogate L chain, c-kit (right panel) and CD43 (not shown)] and up-regulation of CD25 expression (lower right) for RAG-2-deficient/*Eμ-bcl-2* tg (left), as well as wild-type *Eμ-bcl-2* tg cells (right). This process occurred in the absence of proliferation and without significant loss of cells during 3 days of culture.

without any rearrangements in the Ig gene loci, a conclusion already reached earlier by the analysis of the RAG-2T mutation (20).

Stromal cell/IL-7-dependent progenitor B cell lines from RAG-2T mice can differentiate in vitro

Whenever IL-7 is removed from cultures of continuously proliferating pre-B-I cells of normal mice *in vitro*, they cease to proliferate and differentiate into $sIgM^+$ as well as $sIgM^-$ immature B cells within 2–3 days (21). Differentiation is further accompanied by a loss of the capacity to proliferate on stromal cells in the presence of IL-7, by the induction of apoptosis, and by the disappearance and concomitant appearance of a set of B lineage specific differentiation markers (21).

Pro-B cell lines of RAG-2T (not shown) and RAG-2T/*Eμ-bcl-2* tg mice underwent the same changes of surface marker expression upon removal of IL-7 from the cultures, but as expected, do not generate $sIgM^+$ cells (Fig. 2). This indicates that differentiation to more mature cellular stages in these mutant cells is not blocked at the transition from pre-B-I to pre-B-II cells *per se*. In line with these results were analyses of the frequencies of cells clonable on stromal cells in the presence of IL-7, which decreased from 1 in 1–5 to ~1 in 10^4 during 3 days of *in vitro* differentiation, with both cells from RAG-deficient and from normal mice (Fig. 3). Finally, IL-7 deprivation in cultures of RAG-2T pro-B cells lead to growth arrest and the induction of apoptosis. As expected, the latter was inhibited in *bcl-2* tg cells. After 3 days of IL-7 deprivation, surviving cells became small, resting lymphocytes as evid-

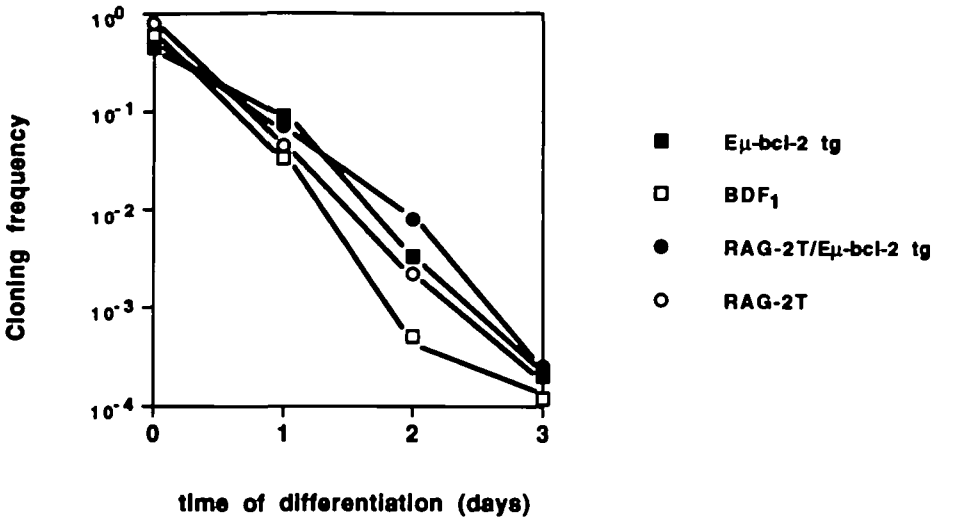
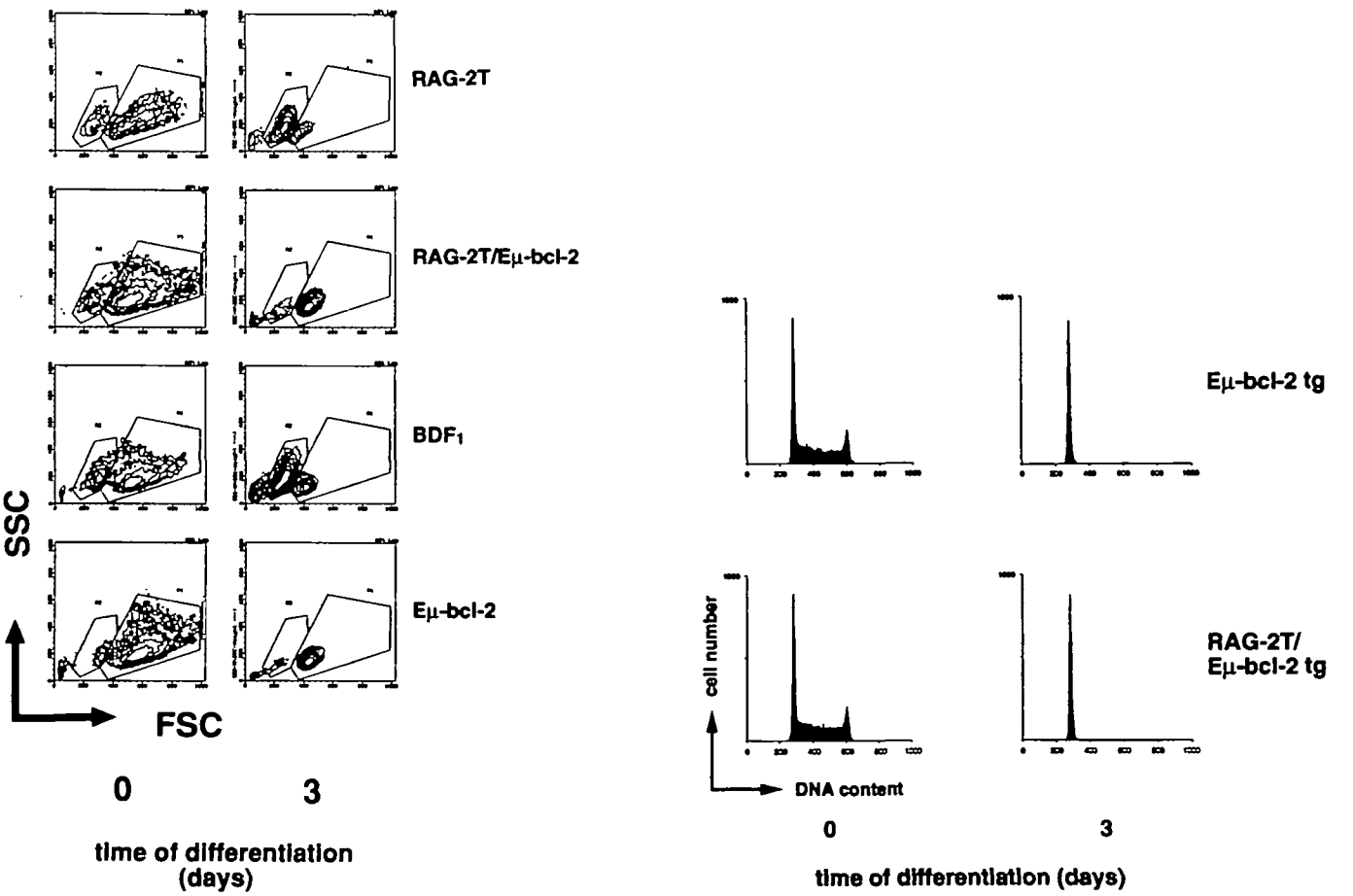


Fig. 3. After removal of IL-7, cell lines from V(D)J recombinase deficient loose their capacity to grow on stromal cells and IL-7. Stromal cell/IL-7-dependent cell lines from RAG-2-deficient (circles) and wild-type (squares) mice, either expressing a bcl-2 transgene (closed symbols) or not (open symbols), were cultured for 1, 2 and 3 days in the absence of IL-7. The frequency of cells reclonable on stromal cells and IL-7 after these time-points was analyzed by limiting dilution analysis and is displayed as a semi-logarithmic plot. Like wild-type cells, all RAG-2-deficient cell lines tested gradually lost their capacity to respond to stromal cells and IL-7 during culture without IL-7. One representative experiment out of two is shown.



enced by decreased forward/side scatter values (Fig. 4a), with >98.5% of the cells entering G_0/G_1 of the cell-cycle (Fig. 4b).

We conclude that the characteristic changes observed during the *in vitro* differentiation of stromal cell/IL-7-dependent pre-B-I cells from normal mice are also observed when pro-B cell lines from RAG-2T and RAG-2T/*Eμ-bcl-2* tg mice differentiate *in vitro*. Hence, Ig gene rearrangements appear not to be required for this form of differentiation.

Differentiation of RAG-2T pro-B cell lines *in vitro* was furthermore analyzed by changes in the transcription of B cell lineage related genes. Northern blot analyses of total RNA extracted from undifferentiated and differentiated pre-B-I cells from normal and *Eμ-bcl-2* tg mice revealed that transcription of both the RAG-1 and RAG-2 genes was induced within 1–2 days of differentiation (23). The same was observed with cells from RAG-2T and RAG-2T/*Eμ-bcl-2* tg mice (Fig. 5). RAG-2T cells were found to transcribe a 2.4 kb instead of a 2.1 kb RAG-2 mRNA, due to the insertion of the neomycin resistance gene. This indicates that the neomycin insertion does not alter the transcriptional control of the RAG-2 gene (20).

Induction of sterile transcription from the κL chain gene loci in cells from RAG-2T mice

Finally, transcription of unrearranged Ig gene loci (sterile transcription) was monitored. Studies in many laboratories have accumulated experimental evidence suggesting that rearrangements of Ig genes are preceded by, and may, in fact, be activated by sterile transcription of the corresponding locus (30–32). It has been proposed that sterile transcription might, in fact, be mandatory for Ig gene rearrangements in a process that 'opens' the chromatin structure of the rearranging locus (2,33).

Results in Fig. 5 show that undifferentiated RAG-2T pro-B and pre-B-I cells from normal mice transcribed a sterile 3.0 kb mRNA from the μH chain locus. This transcript (Ic_μ) represents a sterile message starting in the intron between J_H4 and the first exon of C_μ (34,35). Such a transcript has previously been shown to be expressed in early B lineage cells of both normal as well as RAG-deficient mice (36–38). Upon removal of IL-7 from cultures of RAG-2T cells, the 3.0 kb Ic_μ message was either retained or even slightly increased in quantity for all normal and mutant cell lines (Fig. 5). A 2.7 kb mRNA expressed from a V_HDJ_H-rearranged H chain locus (39–41) became detectable only in cells from normal mice and from *bcl-2* tg mice after 2–3 days of differentiation (Fig. 5) but, as expected, not in differentiated cells from RAG-2T mice which keep their Ig gene loci in germline configuration.

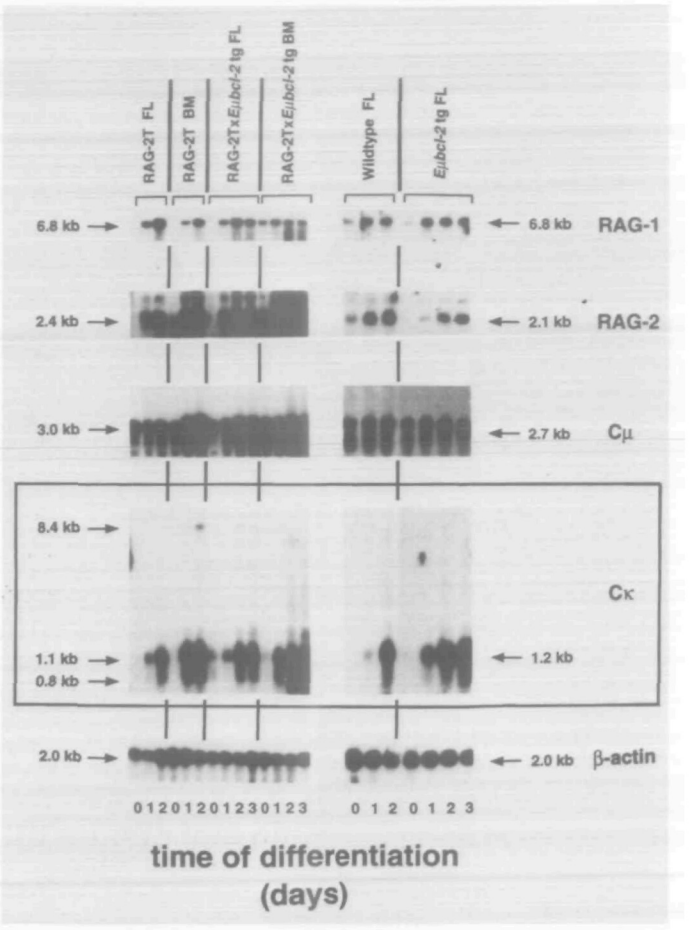
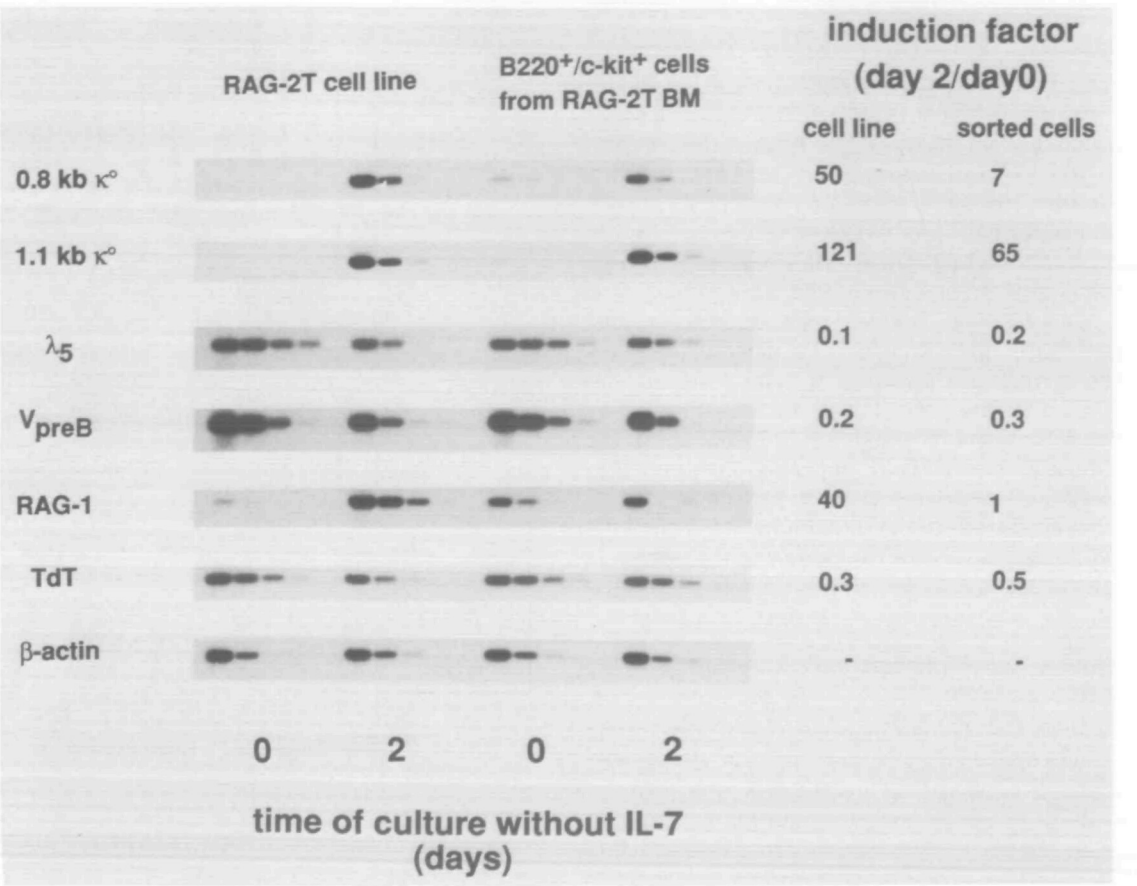


Fig. 5. Sterile transcription from the κL chain loci is induced upon *in vitro* differentiation of B cell progenitors from RAG-2-deficient mice. Total RNA was extracted from undifferentiated cells and cells that have been cultured in the absence of IL-7 for 1, 2 and 3 days by acid guanidinium thiocyanate extraction (26). Aliquots of 15 μg of total RNA per lane were analyzed by Northern blotting for the expression B cell related mRNAs. The same blots were hybridized to the indicated probes. A β-actin probe was used as control for RNA loading.

These results show, in agreement with previous studies by others (42,43), that sterile transcription of the H chain gene loci can occur without rearrangement. This prompted an analysis of the transcriptional activity of the κL chain gene loci in B cell progenitors from RAG-2T mice before and after induction of *in vitro* differentiation. It revealed that sterile transcription from germline κL chain loci was very low, if

Fig. 4. Stromal cell/IL-7-dependent RAG-2-deficient cell lines become small resting lymphocytes upon IL-7 withdrawal. (A) Forward/side scatter analysis of both RAG-2-deficient and wild-type cells that were either grown on stromal cells and IL-7 (left panel) or were cultured in the absence of IL-7 for 3 days (right panel) show that viable cells (in gate R1) become small lymphocytes as evidenced by a decrease in forward/side scatter values. In the case of non-bcl-2 tg cell lines the majority (80–90%) of the cells become apoptotic during 3 days of culture in the absence of IL-7 and accumulate as PI-stainable cells with low forward scatter values in gate R2 (left-handed small gate). The capability to develop into small lymphocytes is not affected by the RAG-2T mutation. (B) DNA content analysis of RAG-2T/*Eμ-bcl-2* tg and wild-type-*Eμ-bcl-2* tg cells before (left) and after 3 days of culture without IL-7 (right). Cells growing on IL-7 and stromal cells usually contained 62–65% in $S/G_2/M$ phases of the cell cycle, while the small lymphocytes after 3 days of IL-7 deprivation were resting, since >98.5% were in G_0/G_1. No difference was detectable between RAG-2 deficient and wild-type cells.



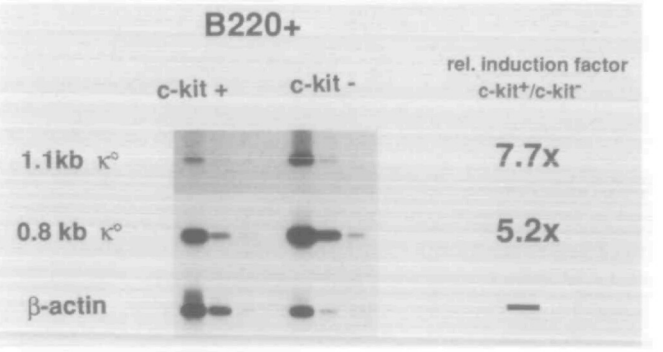


Fig. 6. Induction of sterile transcription from the κL chain gene loci in B cell progenitors from bone marrow of RAG-deficient mice. (A) B220$^+$/c-kit$^+$ cells were isolated by FACS from the bone marrow of 4-week-old RAG-2T mice. Total RNA was extracted from 5×10^4 cells as well as from 5×10^4 cells that were cultured for 2 days in the absence of IL-7 (in this particular experiment 52% of input cells had still been viable after 2 days of culture). cDNA was prepared from these RNA samples and from 1 μg total RNA extracted from a stromal cell/IL-7-reactive RAG-2T cell line (derived from bone marrow) before and after 2 days of IL-7 withdrawal. Serial 5-fold dilutions of the various cDNA samples were subjected to PCR using primer pairs for the indicated genes. Aliquots of the PCR-amplified DNA was fractionated by agarose gel electrophoresis and transferred to nylon membranes. Blots were hybridized to the corresponding genes. Radioactivity on the blots was visualized by X-ray autoradiography and was quantitated by means of a Phosphorimager equipped with Image-Quant™ Software. Ratios of radioactivity detected in differentiated (2 days) versus undifferentiated cells are given for every RT-PCR as relative induction values (on the right). (B) B220$^+$/c-kit$^+$ and B220$^+$/c-kit$^-$ cells were isolated by FACS from bone marrows of 4- to 6-week-old RAG-2T as well as RAG-2T/*Eμ-bcl-2* tg mice. Analysis and quantitation of sterile κL chain transcription was analyzed by RT-PCR/Southern-blotting. The experiment was performed three times on individual samples isolated from RAG-2T and RAG-2T/*Eμ-bcl-2* tg mice. The mean induction factor of sterile κL chain transcription in c-kit$^+$ versus c-kit$^-$ cells was 2.1 ± 0.3 for RAG-2T and 6.5 ± 0.8 for RAG-2T/*Eμ-bcl-2* tg mice. A representative experiment for cell populations isolated from RAG-2T/*Eμ-bcl-2* tg is given.

detectable at all, in undifferentiated (i.e. proliferating) cells from RAG-T and RAG-2T/*Eμ-bcl-2* tg mice. The same was found for cells from normal mice (Fig. 5). However, κL chain transcripts were found to be induced in differentiated cells from normal and RAG-2T cells after 1–2 days of IL-7 deprivation. Whereas differentiating cells from control mice only transcribed a 1.2 kb mRNA originating from V_κ–J_κ-rearranged L chain loci, differentiating pro-B cells from RAG-2T mice induced sterile transcription from germline κL chain loci characterized by mRNA species of 0.8, 1.1 and 8.4 kb (35,37,44) (Fig. 5).

Induction of sterile κL chain gene transcription in RAG-2T B cell progenitors isolated ex vivo

To analyze whether the observed induction of sterile transcription from germline κL chain gene loci in RAG-2T pro-B cell lines after IL-7 deprivation is also found upon *in vitro* culture of pro-B cells directly isolated from the bone marrow of RAG-2T mice, $B220^+$/c-kit^+ cells were enriched from RAG-2T bone marrow by FACS. Comparison of germline κL chain transcription levels in undifferentiated cells and cells after 2 days of tissue culture, as assayed by RT-PCR, has demonstrated that sterile transcription from the κL chain gene loci was equally induced in directly *ex vivo* sorted, as well as stromal cell/IL-7-dependent pro-B cell lines from RAG-2T mice (Fig. 6a).

Likewise, the expression levels of pre-B cell specific mRNAs (λ_5, $V_{pre\text{-}B}$ and TdT) were down-modulated in a similar way in *ex vivo* sorted and in long-term proliferating cell lines from RAG-2-deficient animals. These results indicate that early $B220^+$/c-kit^+ pro-B cells found in the bone marrow of RAG-2T mice are not blocked in differentiation *per se*, but are rather subjected to selective processes that require the expression of Ig proteins.

Elevated levels of sterile κL chain transcripts in c-kit⁻ B lineage cells in the bone marrow of RAG-2T mice

A similar differentiation appears to occur also *in vivo* since small numbers of $B220^{low}$/c-kit^- cells were detectable in bone marrow of RAG-2T and RAG-2T×*Eμ-bcl-2* tg mice (Fig. 1a). They may represent the progeny of c-kit^+ pro-B cells. Quantitation of sterile κL chain transcription in FACS-sorted $B220^{low}$/c-kit^+ versus $B220^{low}$/c-kit^- cells from RAG-2-deficient mice by RT-PCR revealed that sterile κL chain transcription was 2- to 6-fold increased in c-kit^- versus c-kit^+ pro-B cells, as measured in three independent sorting experiments (Fig. 6b). These results suggest that the up-regulation of sterile κL chain gene transcription that was observed upon the *in vitro* differentiation of c-kit^+ RAG-2T pro-B cells to c-kit^- cells can also be detected, although to a lesser extent, in c-kit^+ versus c-kit^- B lineage cells in the bone marrow of RAG-2T mice.

Collectively, these results indicate that the induction of sterile transcription from κL chain gene loci in pre-B cells occurs in the context of a cellular program of differentiation that may be influenced by cell to cell contacts and by cytokines. It further appears that RAG-2T pro-B cells can reach a maturation stage, at which κL chain gene rearrangements would normally be initiated. These results also show that neither H chain gene rearrangement nor IgH chain expression are required for the activation of sterile transcription of the κL chain gene loci.

Discussion

Our findings are not easily reconciled with a variety of studies, which use A-MuLV-transformed pre-B cell lines from normal mice, showing that μH chain expression or triggering of a μH chain–surrogate L chain pre-B cell receptor complex signals rearrangements of the κL chain gene loci (14–17), although other studies have come to the conclusion that κL chain gene rearrangements can occur in the apparent absence of μH chain expression in A-MuLV-transformed pre-B cell lines from C.B-17 SCID mice (30,45,46). Transformation of B lineage precursor cells by A-MuLV appears to arrest development at early pre-B cell stages. Those cells generally retain transcriptionally inactive, germline κL chain alleles (47,48). It has been suggested that an active NF-κB–RelB complex cannot be formed in A-MuLV-transformed cells, due to an increased stability of the inhibitory IκBα subunit, which finally interferes with the activation of κL chain transcription via the NF-κB–RelB-dependent κL chain enhancer (48). Inactivation of v-*abl* protein function (47,48) allows pre-B cells to activate the κL chain gene loci by forming an active NF-κB–RelB complex, which may well be part of the normal cellular program of differentiation, which we see initiated in stromal cell/IL-7-dependent cell lines upon the removal off IL-7. Lack of occupancy of the IL-7 receptor signals this cellular program and it is possible that μH chain expression in A-MuLV-transformed pre-B cells is also involved in this signaling.

Differentiation of pro-B and pre-B-I cells caused by the removal of IL-7 *in vitro* occurs without cell division (25). In bone marrow of normal mice, however, pre-B-I cells undergoing a productive V_HDJ_H rearrangements are expanded by proliferation in order to generate a 5- to 10-fold larger pre-B-II cell compartment (49,50). The observed *in vivo* block of early B lymphopoiesis in RAG-deficient, μMT, λ_5T and J_HT mice suggests that a surface bound μH chain–surrogate L chain pre-B cell receptor is required to signal this expansion, which at the same time rescues cells from being actively removed from the pool of differentiating B cell precursors.

In agreement with this view of early B cell development, the introduction of tg μH chains into RAG-1T or RAG-2T mice leads to the re-establishment of a normally sized pre-B-II cell compartment, in which κL chain transcription is active (42,43).

Although the κL chain transcription activity has not been measured in single pre-B-II cells from RAG-1T or RAG-2T with or without a tg μH chain, we would expect from our Northern blot analysis that these cells have comparable κL chain transcription activity.

While we find that an *Eμ-bcl-2* transgene does not promote B cell differentiation in RAG-2T mice, Strasser *et al.* (27) have found large numbers $sIgM^-$ B cells in SCID mice expressing the same transgene. In order to offer a possible explanation for this apparent discrepancy, it is important to note that SCID mice are not completely rearrangement deficient. The findings by Strasser *et al.* are not controlled for this leakiness. In fact, Strasser *et al.* detected increased frequencies of VDJ-rearranged H chain alleles in $B220^+$ bone marrow cells of *bcl-2* tg versus non-tg SCID mice. This finding might indicate

that *bcl-2* can, *in vivo*, rescue VDJ-rearranged precursor B cells but not pro-B cells with the H chain in the germline configuration.

In normal non *bcl-2* tg mice, productively VDJ-rearranged pre-B-II are selected and expanded by proliferation. The expression of the pre-B cell receptor consisting of the μH chain–surrogate L chain is absolutely required for this selection and expansion (13,49,50). In light of this, it would be interesting to know whether the pre-B-II cells rescued in SCID *bcl-2* tg mice are also selected for in-frame VDJ rearrangement.

In conclusion, we propose the hypothesis that at least two molecular programs of differentiation might exist in B lineage cells. One program is characterized by changes in surface marker expression and growth requirements, as well as the control of accessibility and sterile transcription of germline Ig gene loci. The other program affects Ig gene rearrangements. Analysis of differentiating pro-B cells from RAG-2T mice should allow us to define additional molecular processes which lead to changes in surface marker expression and growth requirements, induction of sterile transcription of Ig L chain gene loci, as well as Ig gene rearrangements. It should then be possible to test our hypothesis of two molecular programs of early B cell differentiation by targeted disruption of the expression of the genes controlling these molecular processes during B cell development.

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Abbreviations

PE	phycoerythrin
PI	propidium iodide
tg	transgenic

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