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Down-Regulation of *RAG1* and *RAG2* Gene Expression in PreB Cells after Functional Immunoglobulin Heavy Chain Rearrangement

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Summary

Two waves of immunoglobulin gene rearrangements, first of the heavy, then of the light chain gene loci form functional immunoglobulin genes during B cell development. In mouse bone marrow the differential surface expression of B220 (CD45R), c-*kit*, CD25, and surrogate light chain as well as the cell cycle status allows FACS separation of the cells in which these two waves of rearrangements occur. The gene products of two recombination activating genes, *RAG1* and *RAG2* are crucial for this rearrangement process. Here, we show that the expression of the *RAG* genes is twice up- and down-regulated, at the transcriptional level for *RAG1* and *RAG2*, and at the posttranscriptional level for RAG2 protein. Expression levels are high in D → J_H and V_H → DJ_H rearranging proB and preB-I cells, low in preB cells expressing the preB cell receptor on the cell surface, and high again in V_L → J_L rearranging small preB-II cells. In immature B cells expressing on the cell surface *RAG1* and *RAG2* mRNA is down-regulated, whereas RAG2 protein levels are maintained. Down-regulation of *RAG1* and *RAG2* gene expression after productive rearrangement at one heavy chain allele might be part of the mechanisms that prevent further rearrangements at the other allele.

Introduction

During precursor B cell development germline DNA segments of the immunoglobulin gene loci are joined by a site-specific recombination process to form functional genes. The gene products of two recombination activating genes, *RAG1* and *RAG2*, are crucial for this rearrangement process (Schatz et al., 1989; Oettinger et al., 1990; Mombaerts et al., 1992; Shinkai et al., 1992). The immunoglobulin heavy (H) chain locus is transcribed and rearranged before the immunoglobulin light (L) chain loci (Alt et al., 1984, Lennon and Perry 1990). Whenever a functional V_HDJ_H rearrangement has occurred in a precursor (pre) B cell, that cell will express a preB cell receptor on the surface, which is formed by the membrane-bound μH chain in complex with the surrogate L chain (Karasuyama et al., 1994; Rolink et al., 1994, Winkler et al., 1995). The surrogate L chain is composed of two proteins encoded by the preB cell–specific Vpre-B and λ5 genes (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). Less than 1% of all preB cells and later stages of immature and mature B cells express two productively V_HDJ_H-rearranged H chain gene loci (Kitamura and Rajewsky, 1992; Ehlich et al., 1994), an observation termed allelic exclusion of the expression of the H chain locus.

The preB cell receptor is involved in the selection and amplification of preB cells by signaling the proliferative expansion of preB cells that have succeeded in a functional V_HDJ_H rearrangement (Karasuyama et al., 1994; Rolink et al., 1994). The expression of the membrane-bound μH chain also mediates allelic exclusion by turning off V_H to DJ_H rearrangements at the second H chain allele (Kitamura and Rajewsky, 1992; Ehlich et al., 1994). It is not clear at the present time whether surrogate L chain in a preB cell receptor participates in signaling allelic exclusion (Kitamura et al., 1991).

In mouse bone marrow the expression of the tyrosine kinase c-*kit* and the interleukin-2 receptor α chain (CD25) distinguishes c-*kit*$^+$ CD25$^-$ proB and preB-I cells from c-*kit*$^-$ CD25$^+$ preB-II cells (Rolink et al., 1994; Chen et al., 1994; see Rolink et al., 1994, for a comparison of nomenclatures used in preB cell development by different laboratories). PreB-II cells can further be subdivided into large cycling cells and small resting cells. The preB receptor is exclusively expressed on a fraction of large cycling preB-II cells. It is specifically recognized by a monoclonal antibody (MAb) named SL156, which binds to surrogate L when it is associated with μH chains, but not when it is free or associated with a complex of glycoproteins (gp130, gp 35–65) (Karasuyama et al., 1993; Winkler et al., 1995). Expression of the preB receptor defines the boundary between the early proB and preB-I cell stages with immunoglobulin genes in germline or DJ_H configuration, respectively, and large preB-II cells with a productive V_HDJ_H rearrangement (Melchers et al., 1994; Winkler et al., 1995).

Various analyses have suggested that a productive rearrangement at one H chain allele mediates H chain allelic exclusion, i.e., prevents further V_H to DJ_H rearrangements at the other allele (Alt et al., 1984; Weaver et al., 1985). This should occur at the earliest preB cell stage where a productively rearranged H chain locus becomes expressed, i. e., in large cycling preB-II cells expressing the preB receptor. Part of the mechanisms to achieve allelic exclusion could be to turn off *RAG* expression transiently. This has prompted us to analyze the expression of *RAG* genes during early B cell developmental stages.

Results

Separation and Characterization of Different Developmental Stages of B Lineage Cells in Mouse Bone Marrow

Cells of different stages of B cell development were sorted by fluorescence-activated cell sorting (FACS) from bone

marrow of normal young mice into six populations, all of them expressing the pan B marker B220 (CD45R) on the cell surface. Table 1 summarizes the surface markers employed in double and triple color FACS to enrich for the different B lineage subpopulations. Details are given in the Experimental Procedures section. Representative FACS sorter data are shown in Figure 1. The purity of the different sorted cell fractions was between 96%–98% after reanalysis.

Table 1 also summarizes our knowledge of the expression of other surface and intracellular markers in these B lineage subpopulations, determined in previous experiments (Rolink et al., 1994), and the status of rearrangements in the immunoglobulin H and L chain gene loci previously determined in single cell polymerase chain reaction (PCR) analyses (Ten Boekel et al., 1995).

Differential forward scatter analysis has been used to sort the B220$^+$ CD25$^+$ cells into large cycling and small resting cells. Previous experiments (Rolink et al., 1994; Winkler et al., 1995) have shown that approximately one-third of all large CD25$^+$ cells express the preB cell receptor, while the rest of the large and the small B220$^+$ CD25$^+$ cells do not express surrogate L chain. In the large cycling CD25$^+$ preB cells, the κL chain loci were found to be in germline configuration, while the small CD25$^+$ preB cells have the L chain loci V_LJ_L rearranged to the same extend as sIg$^+$ immature and mature B cells (Ten Boekel et al., 1995). We therefore concluded that small resting CD25$^+$ preB cells represent the later stage of development (Rolink et al., 1994).

We analyzed RNA prepared from the different fractions semiquantitatively for the expression of genes known to be expressed in the B cell lineage by reverse transcription-PCR (RT-PCR). As shown in Table 1, only the earliest c-*kit*$^+$ fraction of proB and preB-I cells expressed TdT, whereas RNA for λ5 and VpreB (data not shown) was detected also in the large cycling preB-II cells. Sterile transcripts of the κL chain locus became detectable in the fraction of large CD25$^+$ cells and were maximally expressed in small CD25$^+$ preB cells. The results confirm earlier analyses from our laboratory (Rolink et al., 1994) and suggest that TdT, involved in N-region insertions found in H, but not in L chain V(D)J joints, is turned off as soon as the H chain loci have been functionally rearranged and before L chain loci are rearranged. Surrogate L chain expression continues in large preB-II cells to ensure the expression of the preB receptor and is turned off before L chain gene rearrangements are induced. Transcription of the κL chain locus, as germline transcripts, precedes rearrangements, in agreement with earlier findings (Schlissel and Baltimore, 1989).

Since large B220$^+$ SL156$^+$ cells did not express sterile κL chain transcripts, while the large B220$^+$ CD25$^+$ cells did, we conclude in agreement with earlier findings (Winkler et al., 1995) that the preB receptor-positive fraction represents the intermediate stage between the c-*kit*$^+$ proB/preB-I and the large CD25$^+$ preB-II cells.

RAG Gene Expression Is Turned Down in PreB Cells Expressing the PreB Cell Receptor on the Cell Surface

RNA levels for *RAG1* and *RAG2* were analyzed by a semiquantitative RT-PCR assay (Figure 2). RT-PCR products of RNA expressed from a housekeeping gene, encoding hypoxanthine-guanine phosphoribosyl transferase (HPRT), served as controls for the quantity of RNA in the preparation. *RAG1* or *RAG2* was coamplified in the same tube with HPRT. We found a bimodal RNA expression of *RAG* genes in the pre-B cell fractions. Whereas the c-*kit* expressing proB and pre-BI cells and the small CD25$^+$ preB-II cells expressed high levels of both *RAG1* and *RAG2* mRNA, cells expressing the pre-B cell receptor on the surface were found to express barely detectable levels.

The RT-PCR products of serial dilutions of the cDNA reaction were blotted and hybridized with radiolabeled probes and the hybridization signal was quantified by means of a PhosphorImager. At constant levels of expression of HPRT, *RAG1* and *RAG2* mRNA levels were at least

Table 1. Development Stages of B Cell Differentiation Sorted for Analysis of *RAG* Expression

Developmental stage	proB and preB-I	preB-II			Immature B	Mature B
Cell surface marker used for sorting of B220$^+$ cells	c-*kit*$^+$	SL156$^+$	Large CD25$^+$	Small CD25$^+$	IgM$^+$ IgD$^-$	IgM$^+$ IgD$^+$
Cell cycle status[a]						
Percent of cells in S, G2, or M	25–30	60–70	60–70	<5	<5	<5
Rearrangement status[b]						
IgH	DJ	VDJ	VDJ	VDJ	VDJ	VDJ
IgL	GL	GL	GL	$V_\kappa J_\kappa$	$V_\kappa J_\kappa$	$V_\kappa J_\kappa$
				$V_\lambda J_\lambda$	$V_\lambda J_\lambda$	$V_\lambda J_\lambda$
Gene expression[c]						
TdT	+++	–	–	–	–	–
λ5	+++	++	+	–	–	–
Sterile κ light chain	–	–	++	+++	++	+

[a] Cell cycle data were determined by 2–3 independent experiments.
[b] Data were summarized from a study by ten Boekel et al. (1995).
[c] mRNA expression levels for TdT; λ5, and a 0.8 kb sterile transcript from the κL chain locus were determined by semiquantitative RT-PCR. Pluses indicate relative expresison levels: three pluses, strong; two pluses, intermediate; one plus, weak. Minus indicates expresison levels below detection limits in this assay.

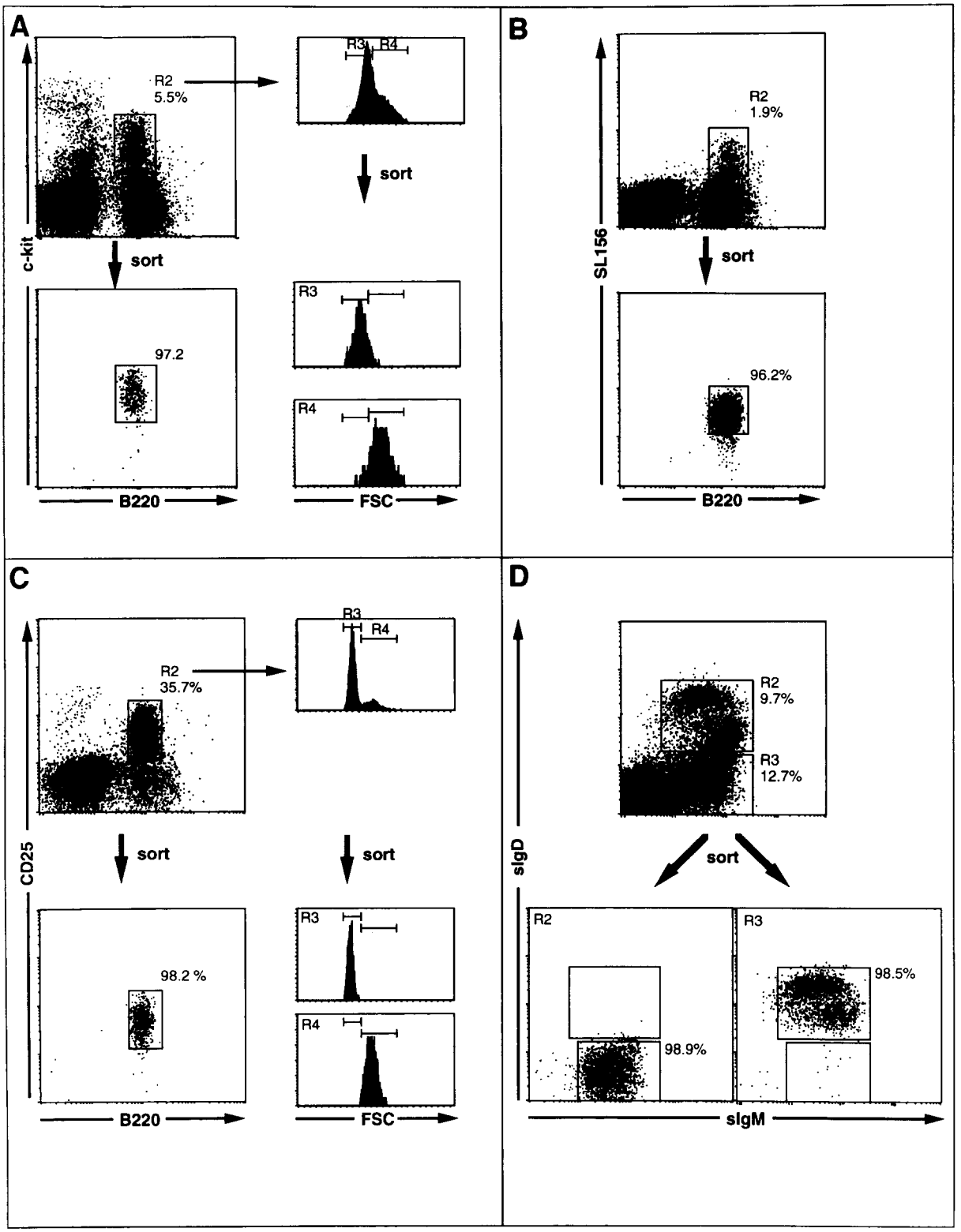

Figure 1. Flow Cytometric Analysis and Cell Sorting of Developmental Stages of B Lymphoid Cells in Bone Marrow from BDF1 Mice

Cells falling in the extended lymphoid gate are displayed. The percentages of cells within the rectangular sorter gates are shown in the figure. For A–C, bone marrow cells were depleted of sIg$^+$ cells before analysis and cell sorting.

(A) Double staining with anti-B220 and anti-c-*kit* antibodies and sorting of c-*kit*$^+$ proB/preB-I cells. For the analysis of RAG2 protein, double-positive cells were further fractionated according to forward light scatter characteristics (see Figure 4 for analysis of DNA contents).

(B) Double staining with anti-B220 and SL156 and sorting of preB cell receptor-positive cells.

(C) Double staining with anti-B220 and anti-CD25 and sorting of large and small CD25$^+$ preB-II cells.

(D) Triple staining with anti-B220, anti-IgM, and anti-IgD and sorting for IgM$^+$IgD$^-$ immature B cells and IgM$^+$IgD$^+$ mature B cells. B220$^+$ B cells were gated.

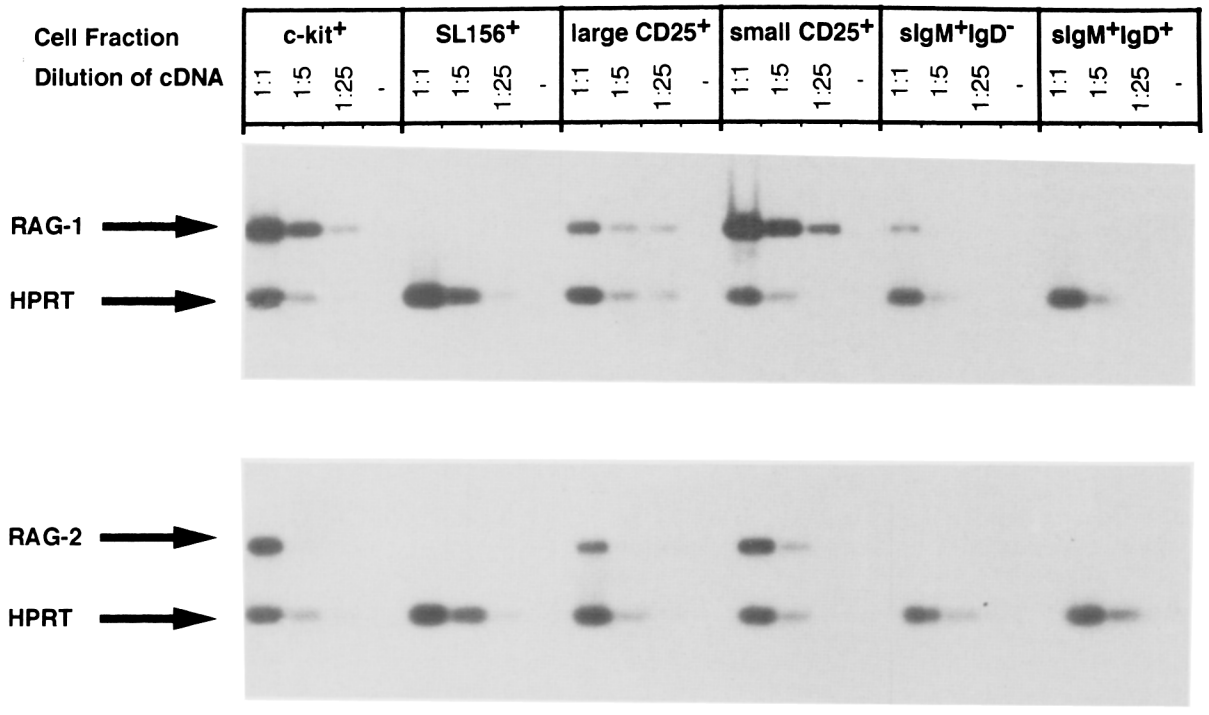

Figure 2. Expression of *RAG1* and *RAG2* Genes at Different Stages of B Lineage Development

B lineage cells of different developmental stages from bone marrow were sorted by FACS after dual or triple labeling, $B220^+$ $CD25^+$ cells were further subfractionated into large and small cells according to forward scatter characteristics. Equilibrated dilutions of cDNA transcribed from RNA of sorted cells were subjected to PCR amplification of individual genes with coamplification of the HPRT gene in the same reactions. After electrophoresis and Southern blotting, the PCR products were detected by hybridization with specific probes.

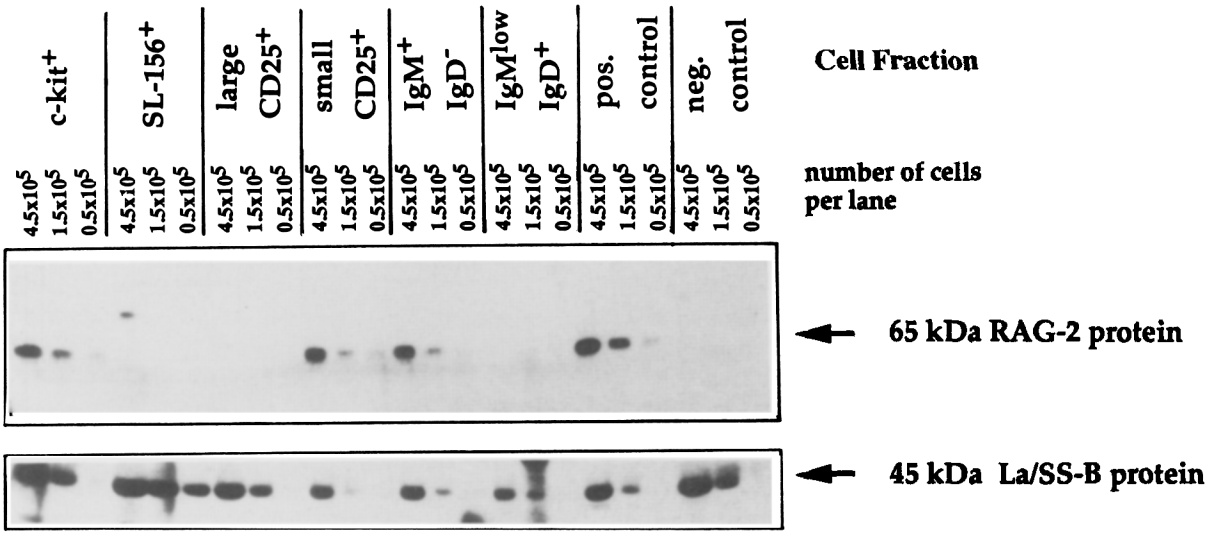

Figure 3. RAG2 Protein Expression in Fractionated Cells of Early B Cell Development

Protein extracts from FACS-sorted cells from each fraction were analyzed by immunoblotting with affinity-purified polyclonal rabbit anti-RAG2 antibodies. The stromal cell/IL-7-dependent preB cell line BCL-2-5 expressing high levels of RAG2 protein after two days of in vitro culture in the absence of IL-7 (Rolink et al., 1993) served as a positive control, extracts from X63 myeloma cells were used as negative controls. Specificity of the antibodies was further verified by Western blot analysis of in vitro transcribed/translated RAG2 protein as well as analyzing extracts from RAG2T versus wild-type preB cell lines (data not shown). Protein loading was controlled by reprobing the blots for SS-B (La) protein with a human SLE serum.

30- to 70-fold lower in the SL156$^+$ fraction than in the c-*kit*$^+$ fraction.

In the fraction of the large CD25$^+$ cells, which includes preB cell receptor-positive cells (Winkler et al., 1995) *RAG* gene expression was approximately 5- to 10-fold lower than in the small CD25$^+$ fraction. Low levels of expression were found in sIgM$^+$ immature B cells, while in mature B cells *RAG* expression was below detection levels.

RAG2 Protein Levels in Cells at Different Developmental Stages of B Cell Differentiation

RAG2 protein contents in total cell lysates from the sorted B cell populations were analyzed by Western blotting (Figure 3). Interesting differences could be seen between mRNA expression and RAG2 protein content in the different B lineage subpopulations. In c-*kit*$^+$ pro- and preB-I cells, as well as in small preB-II cells, high levels of mRNA expression coincided with high RAG2 protein content. In the preB receptor-positive cells both mRNA and protein was below detection limits. However, although *RAG1* and *RAG2* mRNA were already detectable in the large CD25$^+$ preB-II cells, RAG2 protein remained undetectable in this cell fraction. On the other hand, the sIgM$^+$ immature B cells contained RAG2 protein levels as high as the small CD25$^+$ preB-II cells, while *RAG2* mRNA expression appeared to be considerably lower in immature B cells.

These results either suggest that *RAG2* mRNA and RAG2 protein have different turnover rates at different precursor B cell stages, or that in large preB receptor-negative preB-II cells *RAG2* mRNA expression is regained but protein has not yet been made. Conversely, *RAG2* mRNA expression is being turned off in immature B cells, while previously made RAG2 protein remains intact in these cells.

RAG2 Protein Levels Fluctuate with the Cell Cycle in Early c-*kit*$^+$ ProB and PreB-I Cells

It has been shown that RAG2 protein accumulates in the G1 phase within the cell cycle (Lin and Desiderio, 1994). This prompted us to assay for RAG2 protein content in different phases of the cell cycle of normal proB and preB-I cells isolated ex vivo (Figure 4). Since the majority of all c-*kit*$^+$ proB and preB-I cells are in cell cycle, we sorted them by differential forward scatter analysis into small and large cells, the majority of which we found to be in the G1, respectively, the S/G2/M phases of the cell cycle. (Figure 4A). Whereas small and large cells expressed similarly high levels of *RAG1* and *RAG2* mRNA (Figure 4B), RAG2 protein was detectable only in the small cells (Figure 4C). Thus, the regulated accumulation of RAG2 protein in G1 phase of the cell cycle can also be seen in normal proB and preB-I cells of bone marrow within one developmentally homogeneous cell population.

Discussion

The bimodal up- and downmodulation of *RAG* gene expression during B cell development is impressive in its extent. Expression is up in proB and preB-I cells, down in large preB receptor-positive preB-II cells, begins to come up again in large preB receptor-negative preB-II cells, is fully up again in small preB-II cells, is downregulated to a large extent in immature B cells, and appears to be off in mature B cells.

Our measurements of *RAG* mRNA and RAG2 protein expression levels are limited on one side by the relative purity of the subpopulations of cells and, on the other hand, by the relative stability of mRNA and protein that, in principle, could be different at different stages of B cell development. If we consider that our FACS-purified preB cell populations were at best 96%–98% pure, it is, however, all the more impressive that the large SL156$^+$ preB-II cells express *RAG1* and *RAG2* mRNA and RAG2 protein at barely detectable levels. It might well be that *RAG* expression is completely switched off in these cells and that the low level of mRNA detected in these cells is due to a contamination from, most likely, c-*kit*$^+$ precursors expressing high levels of *RAG* genes. Furthermore, we show that the down-regulation of *RAG1* and *RAG2* mRNA expression in the SL156$^+$ population is not merely correlated with their cell cycle status, since c-*kit*$^+$ proB and preB-I cells enriched for cells in S/G2 or M showed high levels of *RAG1* and *RAG2* mRNA.

Large preB receptor-negative preB-II cells were found to express *RAG* mRNA, whereas RAG2 protein was below detection limits. As others have described that RAG2 protein, but not RNA levels, are decreased in cycling precursor lymphocytes that were enriched for cells in S, G2/M (Lin and Desiderio, 1994), this obviously applies to the large CD25$^+$ fraction. These cells have, according to their developmental stage, regained *RAG2* mRNA expression, but still display undetectable RAG2 protein levels, since 60%–70% of these cells are in S, G2/M (Table 1).

Immature resting B cells showed very low levels of *RAG* mRNA, in agreement with earlier results by Li et al. (1993). RAG2 protein content, however, was found to be as high as in small preB-II cells. This result might be expected if the RAG2 protein produced at high levels in the small CD25$^+$ preB-II cells exerts a long half-life. The continued expression of RAG2 protein is compatible with recent findings that immature B cells may still continue to rearrange their L chain loci even when an immunoglobulin molecule is expressed on the surface (Harada and Yamagishi, 1991; Radic et al., 1993; Rolink et al., 1993; Tiegs et al., 1993).

In a previous study, Li et al. (1993) have separated early stages of B cell development by means of differential expression of CD43, HSA, and BP-1 on B220$^+$ cells (Hardy's fractions A–F, Hardy et al., 1991). *RAG* mRNA expression was found to be high at all proB and preB cell stages (B–D). The great majority of the cells in fraction C, however, have recently been found to be composed of nonproductively VDJ rearranged preB cells (Ehlich et al., 1994). These data indirectly indicate that the cells need a productive V_HDJ_H to shut down *RAG* expression. The prediction would therefore be that the C′ fraction (the CD43$^+$ fraction of cells that has probably undergone productive V_HDJ_H rearrangement and is cycling; Hardy et al., 1991) should be negative or very low for *RAG* expression.

Stage-specific alterations in the steady-state levels of

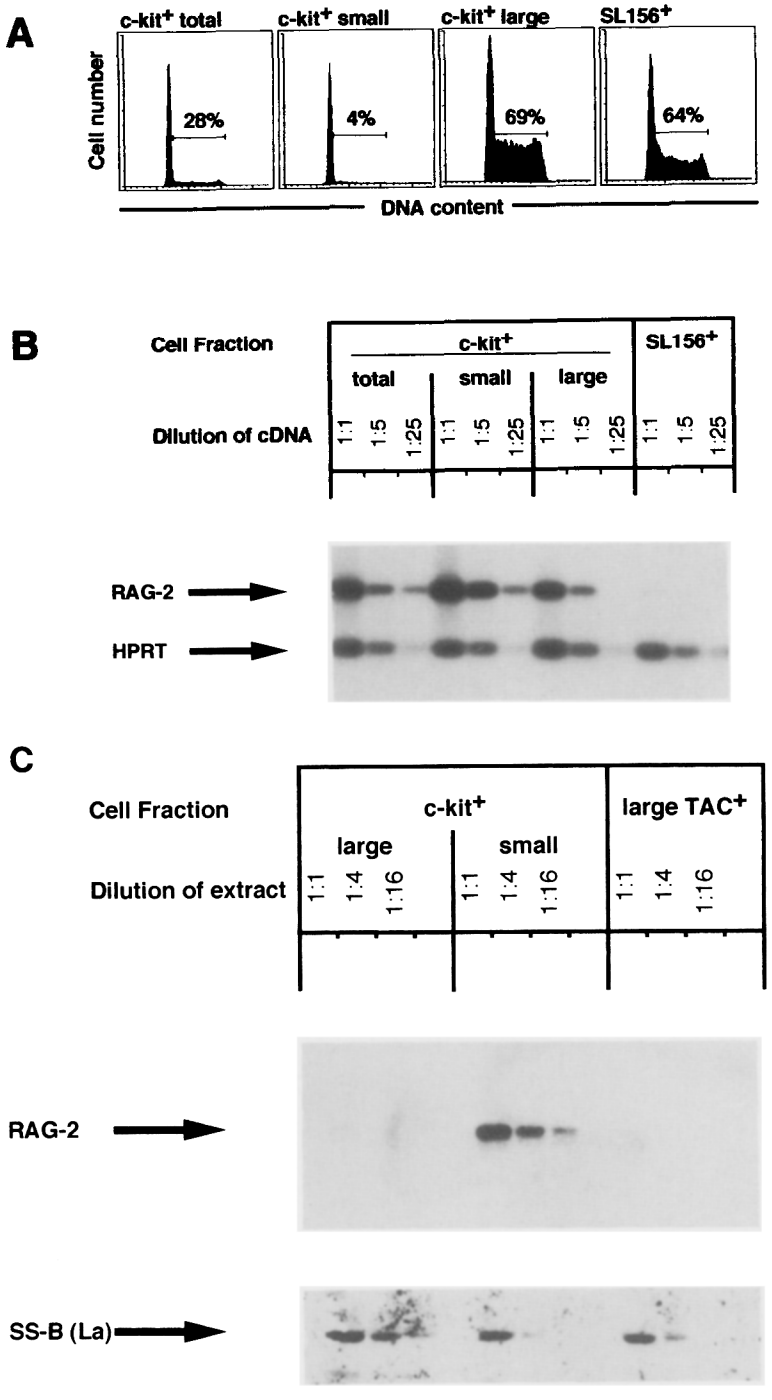

Figure 4. Cell Cycle–Dependent Regulation of RAG2 Protein but Not of *RAG2* mRNA Accumulation in Early B Cell Progenitors Ex Vivo

(A) DNA content of *c-kit*$^+$ and SL156$^+$ preB cells after sorting. The percentage of cells in S, G2, or M is denoted in the histogram display. *c-kit*$^+$ preB-I cells were subdivided into large and small cells according to forward scatter characteristics.

(B) Expression of *RAG2* mRNA analyzed in the sorted fractions by reverse transcription PCR.

(C) Assay for RAG2 protein in sorted fractions. Cell extracts were analyzed for RAG2 protein and the SS-B (LA) protein by immunoblotting with a RAG2-specific antiserum or with a serum from a patient with SLE as described in Figure 3.

RAG1 mRNA during thymocyte development with low levels of *RAG1* mRNA in late CD4$^-$ and CD8$^-$ cells have been described (Wilson et al., 1994). This developmental stage is likely to coincide with the expression of a newly rearranged T cell receptor β chain, which is displayed on the surface with the pre-T α chain (Saint-Ruf et al., 1994). The inverse correlation of *RAG* gene expression with the expression of the pre-T cell receptor should be examined.

Our results show that the expression of *RAG1* and *RAG2* is tighly regulated during B cell development at the RNA level and that, in addition, RAG2 protein accumulation is regulated across the cell cycle. The data suggest that the presence of *RAG* mRNA and RAG2 protein coincides with immunoglobulin gene rearrangements, VDJ rearrangements at the H chain gene locus in *c-kit*$^+$ pro and preB-I cells and VJ rearrangements at the L chain gene loci in small CD25$^+$ preB-II cells. These two waves of *RAG* gene expression are separated by a proliferative expansion phase of cells with a functional IgH gene rearrangement where the *RAG1* and *RAG2* RNA is down-regulated, and

RAG2 protein becomes undetectable. During the expansion phase, when recombination is transiently shut off, accessibility of the immunoglobulin gene segments might be controlled in a way that allows L chain gene rearrangement and prevents further $V_H \rightarrow DJ_H$ rearrangements (Alt et al., 1992). The down-regulation of the transcription of *RAG* genes after productive immunoglobulin H chain rearrangement and during the proliferative expansion of preB-II cells might be part of the mechanisms that prevent further rearrangements at the other H chain allele until this second allele has become inaccessible for the recombination machinery.

Experimental Procedures

Mice

Female 3- to 5-week-old (C57Bl/6J × DBA/2J)F1 mice were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland). RAG2T mice (Shinkai et al., 1992) were bred at our own animal facilities from breeding pairs originally obtained from Dr. F. Alt, Howard Hughes Medical Institute, Harvard Medical School, Boston.

Antibodies

The rat MAbs SL156 was established from fusions of X63–Ag8.653 cells with spleen cells from rats immunized with purified mλ5Vpre-B complex as described (Karasuyama et al., 1993; Winkler et al., 1995), purified, and conjugated with biotin by standard methods. The rat MAb ACK-4 (anti-mouse c-*kit*; Ogawa et al., 1991), provided by Dr. S. Nishikawa, and the rat anti-mouse IgD MAb NIM-R9 (Parkhouse et al., 1992), provided by Dr. R. Parkhouse, were conjugated with biotin using standard procedures. The rat MAb M41 specific for mouse IgM (Leptin et al., 1984) was conjugated with fluorescein isothiocyanate or biotin using standard procedures. The fluorescein isothiocyanate– and APC-conjugated MAb RA-6B2 (anti-CD45R, B220) and biotin-conjugated 7D4 (anti-CD25, TAC) rat MAbs were purchased from Pharmingen (San Diego, California).

For the generation of polyclonal anti-RAG2 antibodies, amino acids 70–516 of murine RAG2 were expressed in bacteria as a fusion protein with a mutant pseudomonas exotoxin (Bruggemann et al., 1991). Rabbits were immunized with the partially purified fusion protein in Freund's complete adjuvant and boosted with the immunogen in Freund's incomplete adjuvant. Polyclonal antiserum was affinity purified against the RAG2 polypeptide, expressed as a fusion protein with the maltose binding protein (pMAL, New England Biolabs). The generation and purification of the antibody will be described in detail elsewhere (T. M. J. L. and D. G. S., unpublished data).

Horseradish peroxidase (HRPO)–labeled polyclonal goat anti-rabbit IgG and HRPO-labeled goat anti-human IgG were purchased from Southern Biotechnical Associates, (Birmingham, Alabama).

A human serum from a patient with systemic lupus erythematosus (SLE) containing high levels of anti-SS-B (La) autoantibodies was a gift from Dr. J. R. Kalden, Institut. für Immunologie und Rheumatologie, Erlangen, Federal Republic of Germany.

FACS Staining and Sorting

Preparation of bone marrow cells from femurs, staining, and cell sorting was done as described (Rolink et al., 1994; Winkler et al., 1995). For the separation of the different preB cell stages, bone marrow cells were depleted of sIgM$^+$ cells using magnetic beads prior to staining as described (Winkler et al., 1995). Sorted cells were reanalysed after the sort and were between 96%–98% pure.

For the analysis of DNA content in the sorted fractions, the cells were fixed in 70% ethanol in phosphate-buffered saline for 30 min on ice, pelleted, and digested with RNase A (0.1 mg/ml) for 30 min at 37°C and than stained with propidiumiodine (40 μg/ml). The cells were analyzed on a FACScan instrument (Becton Dickinson, Mountain View, California) with gating on fluorescence signal area versus signal width to exclude doublets.

RT-PCR

Total RNA for cDNA synthesis was prepared using RNAzol B (Biotecx Laboratories, Incorporated, Houston, Texas) according to the recommendations of the manufacturers. RNA was reverse-transcribed using Superscript II reverse transcriptase (GIBCO Life Technologies, Gaithersburg, Maryland), 1 mM dNTPs, 1 μg random hexameric oligoncleotides, and the supplied buffer. RT–PCR assays were carried out using the following primer pairs: *RAG1*, 5′-TGCAGACATTCTAGCACTCTGG-3′, 5′-ACATC TGCCTTCACGTCGAT-3′; *RAG2*, 5′-CTTCTCTAGAGATTCCTGCTACCTCCCACC3′, 5′-TGTGGAATTCACTGCTGGGGTACCCAGGGG-3′; HPRT, 5′-GCTGGTGAAAAGGACCTCT-3′, 5′-CACAGGACTAGAACACCTGC-3′. TdT, 5′-GATTTCTAGACTTGGTCCTCTTCATTTTGG-3′, 5′-CAAGGAATTCCCTC TGTGTCTTTCAT GCTG3′; λ5, 5′-GAGATCTAGACTGCAAGTGAGGCTAGAG-3′, 5′-CTTGGGCTGACCTAGGATTG-3′; 0.8 kb sterile κL chain, 5′-CAGTGAGGAGGGTTTTTGTACAGCCAGACAG-3′, 5′-CTCATTCCTGTTGAAGCTCTTGACAATGGG-3′. All PCRs were carried out with 1 cycle at 94°C for 40 s, followed by 30 cycles at 94°C for 20 s, at 55°C for 15 s, and at 72°C for 60 s. The following probes were used for PCR–Southern blot analysis: *RAG1*, a 950 bp fragment obtained by HindIII digestion of M6-BSK(+), containing the 5′ region of *RAG1* cDNA; *RAG2*, a 2100 bp fragment obtained by NotI digestion of MR2-BSK(+), containing the mouse *RAG2* cDNA (Oettinger et al., 1990). The probes specific for the HPRT, TdT, λ5, and Cκ genes were generated by cloning PCR fragments into Bluescript.

The RT–PCR of the sorted fractions was repeated with samples from three different sorts of all B cell subpopulations and the results were comparable.

In two experiments, the hybridization signals were quantitated by means of a PhosphorImager equipped with ImageQuant software (Molecular Dynamics, Mountain View, California).

Immunoblotting

Total cellular protein was extracted from cells by lysing cells for 30 min at 4°C in 0.5% deoxycholate, 1% NP-40, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 50mM Tris–HCl (pH 8.0) containing 2.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A. Cellular debris was removed by centrifugation at 12,000 × g at 4°C for 15 min (Lin and Desiderio, 1993). Samples were diluted in reducing SDS–PAGE sample buffer and boiled for 5 min before electrophoresis on 12% SDS–polyacrylamide gels. Western blotting was carried out as previously described (Grawunder et al., 1993). Blots were incubated with the affinity-purified polyclonal rabbit anti-RAG2 antibody (see above) as primary reagent. Specific signals were revealed with HRPO-labeled polyclonal goat anti-rabbit IgG and an ECL-detection kit (Amersham International, Bucks, England) as recommended by the manufacturer. For reprobing the filter, the HRPO on the filters was inactivated with phosphate-buffered saline, 10 mM NaN_3, washed extensively with phosphate-buffered saline–Tween, and incubated with a 1:5,000 dilution of the human autoimmune serum as a control for protein loading.

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