

Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced *V-to-DJ* recombination at the *IgH* locus

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The *Pax5* gene coding for the transcription factor BSAP has an essential role in B lymphopoiesis and midbrain development. Here we present a detailed analysis of the B-cell phenotype of *Pax5* mutant mice that revealed a differential dependency of fetal and adult B lymphopoiesis on this transcriptional regulator. B-cell development is arrested in the bone marrow at the early pro-B (pre-BI) cell stage, which is characterized by expression of the early markers *c-kit*, CD43, $\lambda 5$, V_{preB} , and HSA and the absence of the later markers CD25 and BP-1. These pre-BI cells fail to express the BSAP target gene *CD19* and are capable of long-term proliferation in vitro in the presence of stromal cells and IL-7. B-lymphoid progenitors could not be detected in the fetal liver of *Pax5* mutant embryos. However, *Pax5*-deficient fetal liver cells gave rise to the development of pre-BI cells in bone marrow on transplantation into lethally irradiated mice. These data indicate different functions of Pax5 in the distinctive microenvironments of fetal liver and adult bone marrow. As shown by PCR analyses, the pre-BI cells in *Pax5*-deficient bone marrow have undergone D_H -to- J_H rearrangement of the immunoglobulin heavy-chain locus at normal frequency. In contrast, V_H -to- D_HJ_H rearrangements were reduced ~50-fold in *Pax5*-deficient pre-BI cells, suggesting a role for Pax5 in the developmental pathway controlling *V-to-DJ* recombination.

[Key Words: *Pax5* inactivation; early B-cell development; fetal and adult B lymphopoiesis; $V_H D_H J_H$ rearrangement; BSAP target genes]

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B lymphocytes develop from hematopoietic stem cells through an orderly process of differentiation that results in antigen-responsive B cells with individual immunoglobulin surface receptors. This developmental process can be dissected into different stages according to the expression of specific cell surface markers and the sequential rearrangement of immunoglobulin heavy (*IgH*) and light (*IgL*) chain genes (for review, see Rolink and Melchers 1991). In addition, B-cell populations can be distinguished by their growth factor requirements (Hayashi et al. 1990). In the classification scheme of Hardy et al. (1991), sequential stages of mouse B cell development have been divided into seven distinct cell fractions (A–F) based on differential expression of CD43, heat stable antigen (HSA), BP-1, IgM, and IgD (Fig. 1; bottom). Rolink et al. (1994) have instead used the analysis of cell size

and the expression of *c-kit*, CD25, and the surrogate light chains V_{preB} and $\lambda 5$ to fractionate B lymphocytes according to their developmental stage (Fig. 1, top). As shown by these studies, the earliest B-cell progenitors in the bone marrow are large cycling cells expressing CD43, *c-kit*, as well as the surrogate light chain proteins on their surface (Hardy et al. 1991; Karasuyama et al. 1994; Rolink et al. 1994), are in the process of D_H -to- J_H rearrangement of the *IgH* locus (Ehlich et al. 1993; Li et al. 1993; ten Boekel et al. 1995) and can be cloned in vitro on stromal cells in the presence of IL-7 (Rolink et al. 1991b). Completion of a productive V_H -to- D_HJ_H rearrangement results in transient expression of the μ chain in association with the surrogate light chain proteins on the cell surface (Karasuyama et al. 1990, 1994; Tsubata and Reth 1990; Winkler et al. 1995). Signaling through this pre-B cell receptor transiently down-regulates expression of the recombination activating genes RAG-1 and RAG-2 (Grawunder et al. 1995), promotes allelic exclusion at the *IgH* locus (Kitamura and Rajewsky 1992; Löffert et

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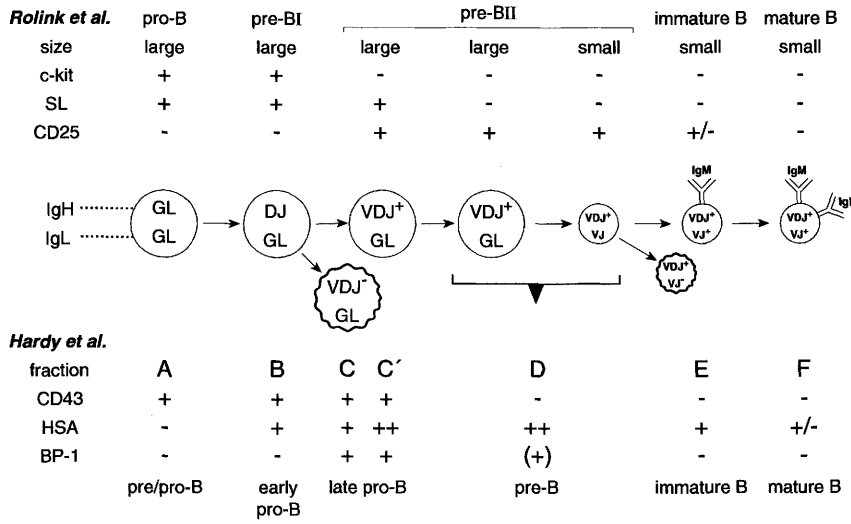


Figure 1. Schematic diagram of murine B-cell development. The different developmental stages of B lymphopoiesis are shown together with their characteristic cell surface markers, which are used for classification according to Rolink et al. (1994) (*top*) or Hardy et al. (1991) (*bottom*). The pan-B cell marker B220 is expressed at all stages. The predominant configuration of the immunoglobulin genes at each developmental stage is indicated, as it was determined by ten Boekel et al. (1995) (Rolink's nomenclature) and by Ehlich et al. (1993, 1994) (Hardy's nomenclature). Large and small circles denote proliferating and resting cells, respectively. Cells destined to die are indicated by wavy outlines. As the correlation between the two classification systems is not straightforward in all aspects, the reader is referred to the original literature for details. (GL) Germ line; (SL) surrogate light chain; (IgH) immunoglobulin heavy chain; (IgL) immunoglobulin light chain; (HSA) heat-stable antigen.

al. 1996), and induces proliferative cell expansion (Karasuyama et al. 1994; Rolink et al. 1994) as well as differentiation to small pre-B cells (Kitamura et al. 1991, 1992). Following successful *IgL* (κ or λ) gene rearrangement, immature B cells emerge that synthesize the IgM form of the B-cell receptor and become subjected to selection by antigen. Subsequent expression of homing receptors enables these cells to populate peripheral lymphoid organs where they participate as mature B cells in immunological reactions (Law and Clark 1994).

Several transcription factors have been identified recently that regulate important aspects of B-cell development (for review, see Busslinger and Urbánek 1995; Hagman and Grosschedl 1994). Among them is the B-cell-specific activator protein (BSAP), which is encoded by the *Pax5* gene (Adams et al. 1992). This transcription factor is expressed at all stages of B lymphopoiesis except in terminally differentiated plasma cells (Barberis et al. 1990), recognizes DNA via its amino-terminal paired domain (Czerny et al. 1993), and uses a carboxy-terminal regulatory module consisting of activating and inhibitory sequences to control gene transcription (Dörfler and Busslinger 1996). BSAP (*Pax5*) has been implicated in the regulation of several B-cell-specific genes primarily based on the identification of BSAP-binding sites in their control regions. Putative BSAP targets are the *I ϵ* promoter (Liao et al. 1994) and 3' enhancers of the *IgH* and *IgL κ* loci (Singh and Birshstein 1993; Neurath et al. 1994; Roque et al. 1996) as well as the genes coding for the cell surface protein CD19 (Kozmik et al. 1992), the tyrosine kinase Blk (Zwollo and Desiderio 1994), the transcription factor XBP-1 (Reimold et al. 1996), and the surrogate light chains $\lambda 5$ and VpreB1 (Okabe et al. 1992).

Targeted inactivation of the *Pax5* gene in the mouse germ line revealed essential functions of this transcription factor in early B-lymphopoiesis and midbrain devel-

opment (Urbánek et al. 1994). *Pax5* mutant mice fail to produce small pre-B, B, and plasma cells and therefore lack any immunoglobulin in their serum owing to a complete arrest of B-cell development at an early stage. The bone marrow of these mice generates, however, large B220⁺ CD43⁺ B lymphocytes characteristic of early B-cell progenitors (Urbánek et al. 1994; see Fig. 1). Here we have determined the precise developmental stage of the differentiation block by extensive analysis of the expression profile of cell surface proteins and the rearrangement status of immunoglobulin genes in *Pax5*-deficient B lymphocytes. These experiments revealed a differential requirement for *Pax5* in fetal and adult B lymphopoiesis. Whereas *Pax5* is essential for progression beyond the early pro-B (pre-BI) cell stage in bone marrow, it is required for differentiation of the earliest B-lineage-committed precursor cells in the fetal liver. The pre-BI cells from *Pax5*-deficient bone marrow could be cultured in vitro on stromal cells in the presence of IL-7 and were shown to lack expression of the BSAP target gene *CD19*. Moreover, these pre-BI cells have efficiently undergone only *D_H-to-J_H* rearrangements at the *IgH* locus which implies a role for *Pax5* in the developmental pathway controlling *V_H-to-D_HJ_H* recombination.

Results

B-cell development is arrested at the early pro-B (pre-BI) cell stage in bone marrow of Pax5-deficient mice

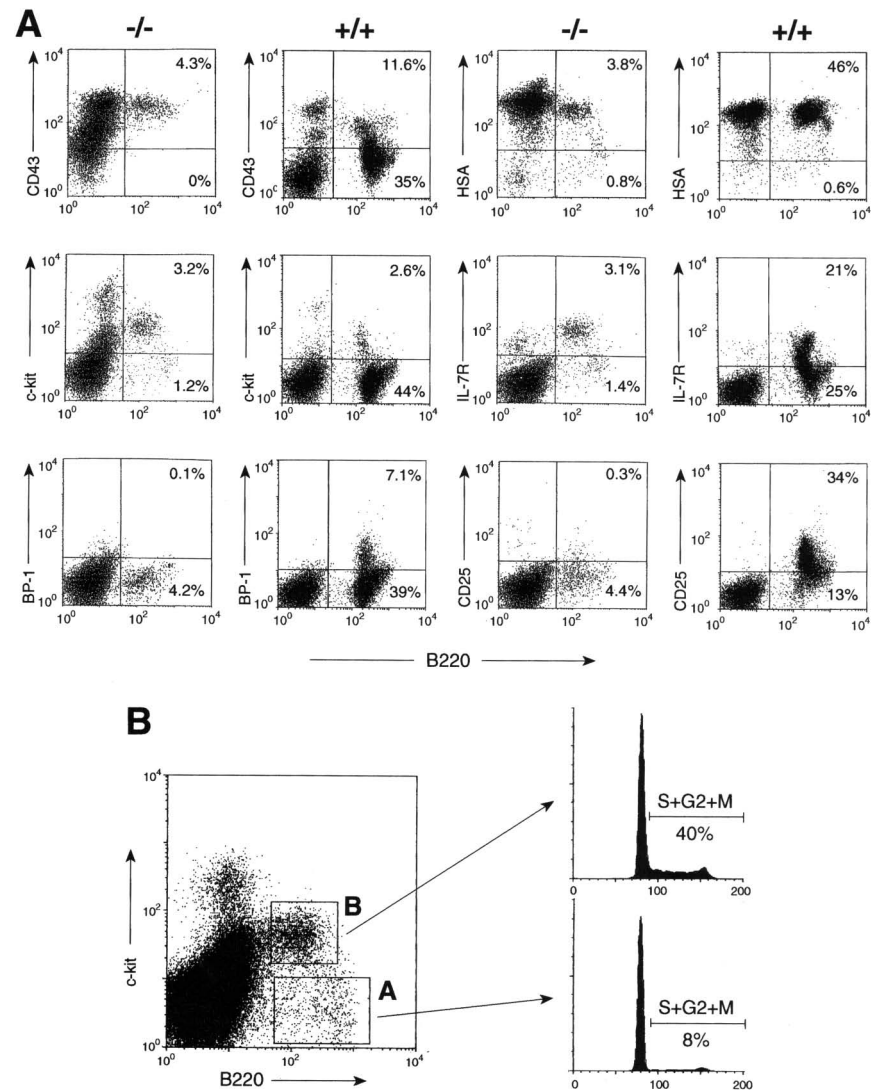
Our previous phenotypic analysis of *Pax5* mutant mice demonstrated that the absence of *Pax5* arrests B-cell development in the bone marrow at an early stage corresponding to large B220⁺CD43⁺ B lymphocytes (Urbánek et al. 1994). As indicated in Figure 1, these early B lymphocytes can be subdivided into different developmental

stages according to the expression of the surface markers HSA, BP-1, *c-kit*, and CD25 (IL-2R α). To further characterize the Pax5-dependent differentiation block, we have used antibodies recognizing these stage-specific proteins for flow cytometric analysis of bone marrow cells from Pax5 mutant and control wild-type mice. Because the majority of Pax5 mutant mice die within 3 weeks after birth (Urbánek et al. 1994), we have analyzed the B-cell compartment (defined by expression of the pan-B-cell marker B220) only up to the age of 2 weeks when these mice are still largely free of disease symptoms. As illustrated in Figure 2A (and data not shown), the majority of the B220⁺CD43⁺ B lymphocytes in Pax5-deficient bone marrow express *c-kit*, HSA, IL-7R, and λ 5 (3–3.8% of all cells). The same cells, however, lack CD25, BP-1, and markers of late B-cell differentiation (IgM, IgD, CD21, CD23, CD40) on their cell surface in contrast to wild-type bone marrow cells. This expression profile defines the Pax5-deficient B lymphocytes as early pro-B cells of fraction B in Hardy's nomenclature (Hardy et al. 1991;

Fig. 1), and as pro/pre-BI cells in the classification scheme of Rolink et al. (1994) (Fig. 1). A small subset of Pax5-deficient B220⁺CD43⁺ cells (~1%; Fig. 2A) expresses neither of the early markers *c-kit*, HSA, and IL-7R and is therefore likely to correspond to Hardy's fraction A (see Fig. 1), which even includes non-B-lymphoid cells such as progenitors of natural killer cells (Rolink et al. 1996).

The proliferation of Pax5-deficient B lymphocytes was next studied by separating bone marrow cells of Pax5 mutant mice by fluorescence-activated cell sorting into B220⁺*c-kit*⁺ and B220⁺*c-kit*⁻ cells. Nuclei were prepared from both cell fractions, and their DNA content was analyzed by flow cytometry (Fig. 2B). Only relatively few B220⁺*c-kit*⁻ cells (8%) in fraction A proved to be in the cell cycle. In contrast, 40% of all B220⁺*c-kit*⁺ cells in fraction B were in the S, G₂, or M phase of the cell cycle, which compares favorably with 45% measured for the B220⁺*c-kit*⁺ cell population in wild-type bone marrow (Rolink et al. 1994). We conclude, therefore, that Pax5 is

Figure 2. B-cell development is blocked at the pre-BI cell stage in bone marrow of Pax5 mutant mice. (A) Flow cytometric analysis. Bone marrow cells from Pax5 mutant (-/-) and wild-type (+/+) mice at the age of 2 weeks were analyzed by flow cytometry using a FITC-conjugated anti-B220/CD45R antibody (RA3-6B2) in combination with either a biotinylated anti-CD43 (S7), anti-HSA (CD24; M1/69), anti-*c-kit* (ACK4), anti-IL-7R (A7R34), anti-CD25 (IL-2R α ; 7D4) or anti-BP-1 (6C3) antibody. Biotin-conjugated antibodies were revealed by incubation with PE-coupled streptavidin. The percentages of B220⁺ cells in each quadrant are indicated. (B) Cell cycle analysis. Bone marrow cells from a 2-week-old Pax5 mutant (-/-) mouse were stained with FITC-conjugated anti-B220 (CD45R) and biotinylated anti-*c-kit* antibodies (visualized by PE-conjugated streptavidin) and then separated by fluorescence-activated cell sorting into the two fractions indicated at left. Nuclei of the sorted cell populations were prepared, stained with ethidium bromide, and analyzed for their DNA content (shown at right). The percentage of cells in the S, G₂, and M phases of the cell cycle is indicated.



not required for proliferation of early pro-B (pre-BI) cells. However, it is essential for progression of B-cell development beyond this early stage in bone marrow.

Reduced V_H -to- D_HJ_H recombination at the IgH locus in Pax5-deficient pre-BI cells

The status of immunoglobulin gene rearrangement was next studied in B lymphocytes isolated from bone marrow of Pax5-deficient mice. For this purpose, individual B220⁺c-kit⁺ cells were first sorted and then analyzed by a recently developed and subsequently modified PCR assay that allows amplification of germ-line and rearranged gene segments from the immunoglobulin loci of a single cell in two steps (Ehlich et al. 1994; ten Boekel et al. 1995). In the first PCR reaction, the IgH and IgL_κ loci were amplified simultaneously with a mixture of 11 5' primers homologous to V_H , $V_κ$, D_H and upstream J_H1 sequences in combination with two 3' primers located downstream of the J_H4 and $J_κ5$ segments. In the second round of amplification, the products of the first PCR were analyzed in separate reactions with primer combi-

nations that were specific for each rearrangement event (Ehlich et al. 1994; ten Boekel et al. 1995). As summarized in Figure 3, PCR products were obtained in this manner from 54 B220⁺c-kit⁺ cells of Pax5-deficient bone marrow. These amplified DNA fragments were characterized according to their size (ten Boekel et al. 1995), and 27 of them were cloned at random followed by DNA sequencing (Fig. 3B). This analysis indicated that 9 alleles of the IgH locus were still in germ-line configuration, whereas 65 alleles underwent D_HJ_H rearrangement (Fig. 3A). D_H -to- J_H joining is usually imprecise at the recombination breakpoint because of deletion of D_H and J_H sequences as well as insertion of template-independent nucleotides (N and P) (Desiderio et al. 1984; Lafaille et al. 1989). The D_HJ_H joints in Pax5-deficient B lymphocytes were normal in both of these aspects, as shown by DNA sequence analysis (Fig. 3B). Importantly, neither $V_H D_H J_H$ nor $V_κ J_κ$ rearrangements could be detected in our sample of Pax5-deficient pre-BI cells (Fig. 3A). This finding was not an artifact of the V_H -specific primers used, as they were able to amplify efficiently $V_H D_H J_H$ -rearranged alleles from individual pre-BII cells of wild-type mice

rearrangements			alleles
germline			9
$D_H J_H$			65
$V_H D_H J_H$			0
$V_κ J_κ$			0

DH JH junctions			type		
DH	P,N	JH	DH	JH	RF
TCT ATG ATG GCT AC	G TGA	TAC TAT GCT ATG GAC TAC TGG	Dsp2.9	J4	II *
TC TAC TAT GAT AAC GAC	CCT AC	C TAT GCT ATG GAC TAC TGG	Dsp2.2	J4	I
T CTA CTA TGA TTA CGA C	GC	TAC TAT GCT ATG GAC TAC TGG	Dsp2.2	J4	III
T CTA CTA TGG TAA CTA	AGG	GCT TAC TGG	Dsp2.1/2.5	J3	III
TC TAC TAT GG	G AAA AGG G	AC TAT GCT ATG GAC TAC TGG	Dsp2	J4	I
T CTA CTA TGA TTG CGA C	GC	TAC TAT GCT ATG GAC TAC TGG	Dsp2.2	J4	III
C CTA CTA TAG TAA C	CC	TAC TGG	Dsp2.X	J3	III
CCT AGT ATG GTA ACT AC	G TC	G TTT GCT TAC TGG	Dsp2.8	J3	II
TC TAC TAT AGG AAC GAC	TAT	TAC TAT GCT ATG GAC TAC TGG	Dsp2.10	J4	I
T TTA TTA CTA CGG TAG TAG C	CC G	TT GCT TAC TGG	Dfl16.1	J3	III
CC TAC TAT AGG TAC GAC	GGG GC	G CCT GGT TTG CTT TAC TGG	Dsp2.11	J3	I
CC TAC TAT	TT	G CCT GGT TTG CTT TAC TGG	Dsp2	J3	I
TTT ATT ACT ACG GTA GTA GCT AC	C C	AC TAT GCT ATG GAC TAC TGG	Dfl16.1	J4	II
TT TAT TAC TAC GGT AGT AGC	CCC GGG GC	C TAT GCT ATG GAC TAC TGG	Dfl16.1	J4	I
TTT ATT ACT ACG GTA GTA	TCC CG	C TGG TTT GCT TAC TGG	Dfl16.1	J3	II
C CTA CTA CTA TAG TAA C	CC	TAC TAT GCT ATG GAC TAC TGG	Dsp2.X	J4	III
TC TAC TAT GGT AAC TAC	GTA TAT	TAC TAT GCT ATG GAC TAC TGG	Dsp2.1/2.5	J4	I
T CTA TGA TGG TTA CTA C	TT	TAC TAT GCT ATG GAC TAC TGG	Dsp2.9	J4	III
TCT ACT ATG G	AG G	TG GAC TAC TGG	Dsp2	J4	II
T CTA CTA TGA TTA CGA C	GA GGG GGG A	CC TGG TTT GCT TAC TGG	Dsp2.2	J3	III
CC TAC TAT AGT AAC TA	G CAT	TAC TAT GCT ATG GAC TAC TGG	Dsp2.X	J4	I
C CTA GTA TGG T	T	C TTT GAC TAC TGG	Dsp2.8	J2	III
TCT ACT ATG ATT ACG AC	G GG	C TTT GAC TAC TGG	Dsp2.2	J2	II
TC TAC TAT GGT AAC TAC	TTT	TAC TGG TAC TTC GAT GTC TGG	Dsp2.1/2.5	J1	I
CCT ACT ATA GGT ACG AC	G ACG	GCT ATG GAC TAC TGG	Dsp2.11	J4	II
CCT ACT ATA GT	C	GCT ATG GAC TAC TGG	Dsp2.X	J4	II
TCT ACT ATG ATT ACG AC	G GGA CGG TAT GT	T ATG GAC TAC TGG	Dsp2.2	J4	II

Figure 3. Status of immunoglobulin gene rearrangement in bone marrow pre-BI cells of Pax5-deficient mice. (A) Summary of the PCR analysis of immunoglobulin gene rearrangements in single cells. B220⁺c-kit⁺ cells from bone marrow of 2-week-old Pax5 (-/-) mice were stained and sorted as described in Fig. 2B. In total, 88 individual cells were subjected to PCR analysis according to Ehlich et al. (1994) with the modifications described by ten Boekel et al. (1995). Both IgH alleles were detected in 20 cases (23%), a single PCR band was observed in 34 samples (38.5%) and no amplification was obtained in 34 cases (38.5%). Numbers refer to the occurrence of the different immunoglobulin gene rearrangements. (B) Junctional sequences of the D_HJ_H -rearranged IgH genes in Pax5-deficient B220⁺c-kit⁺ cells. DNA fragments amplified from 27 D_HJ_H -rearranged alleles were cloned and sequenced. The type of J_H and D_H segments used as well as the D_H reading frame (RF; conventionally standardized relative to J_H) were determined as described (Ichihara et al. 1989; Chang et al. 1992). Template-independent nucleotides (N), which are inserted by terminal deoxynucleotidyl transferase (Alt and Baltimore 1982; Desiderio et al. 1984), and short palindromic sequences (P), which are complementary to the ends of the recombining gene segments (Lafaille et al. 1989), are listed. N sequence insertion generated a stop codon in reading frame II of one D_HJ_H -rearranged allele (indicated by asterisk), therefore preventing translation of the D $μ$ protein.

(data not shown). The failure to detect $V_H D_H J_H$ rearrangements in Pax5-deficient pre-BI cells is therefore significant and contrasts with results obtained in previous single-cell PCR analyses of wild-type bone marrow cells. Ehlich et al. (1994) found that the early pro-B cells in Hardy's fraction B contain 24% (9/38) of all analyzed *IgH* alleles in the $V_H D_H J_H$ -rearranged configuration. Likewise, ten Boekel et al. (1995) observed $V_H D_H J_H$ joining in 13% (4/30) of the *IgH* alleles analyzed from B220⁺*c-kit*⁺ pre-BI cells. Based on these two studies, we would have expected to identify 10–18 $V_H D_H J_H$ rearrangements among the 74 *IgH* alleles analyzed from Pax5-deficient pre-BI cells. The absence of any $V_H D_H J_H$ -rearranged allele strongly suggests that the incidence of V_H -to- $D_H J_H$ rearrangement is at least 10-fold reduced in the absence of Pax5.

To verify the significance of this PCR result, we have sorted B220⁺*c-kit*⁺ cells from the bone marrow of wild-type and Pax5 mutant mice and used a pool of 10,000 cells rather than individual B lymphocytes as starting material for PCR amplification by the two-step protocol described above. As expected, *IgH* gene sequences could be amplified readily in germ-line and $D_H J_H$ -rearranged configurations from both wild-type and three Pax5 mutant mice (Fig. 4A). In contrast, the pre-BI cells of only one of the three mutant mice gave rise to an amplification product with $V_H J_558$ -specific primers. Therefore, $V_H D_H J_H$ rearrangements involving members of the prominent $V_H J_558$ gene family were present in Pax5 mutant mice just at the detection limit of our PCR assay (Fig. 4A). Similar results were also obtained by PCR amplification with primers specific for the $V_H 7183$ and $V_H Q52$ gene families (data not shown). As 70–80% of all $V_H D_H J_H$ rearrangements normally occur within these three V_H gene families (Yancopoulos et al. 1988; Malynn et al. 1990), we conclude that $V_H D_H J_H$ rearrangements are rare in pre-BI cells of Pax5 mutant mice.

The frequency of $V_H D_H J_H$ rearrangements in Pax5-deficient B lymphocytes was next estimated by a quantitative PCR assay. For this experiment we have sorted CD43⁺B220⁺ cells, as this population contains both *c-kit*⁺ and *c-kit*⁻ cells and therefore includes all B lymphocytes of Pax5-deficient bone marrow (Fig. 2A). Increasing numbers of B220⁺CD43⁺ cells from Pax5 mutant and wild-type mice were analyzed by 30 cycles of PCR amplification with $V_H J_558$ -specific primers. As shown in Figure 4B, only few $V_H D_H J_H$ rearrangements could be amplified from 10,000 B220⁺CD43⁺ cells of Pax5 mutant mice in marked contrast with the situation observed with wild-type littermates. The amplified $V_H D_H J_H$ fragments were quantitated by normalization to a PCR product from the $C_H 1$ region of the *IgH* locus that was used as a control for the number of genomes analyzed. As indicated by this quantitation, the incidence of $V_H D_H J_H$ rearrangements in B220⁺CD43⁺ cells was 50- to 70-fold reduced in Pax5 mutant mice compared with wild-type animals. The combined results of all PCR experiments demonstrate therefore that the absence of Pax5 results in a low frequency of V_H -to- $D_H J_H$ rearrangements at the pre-BI cell stage.

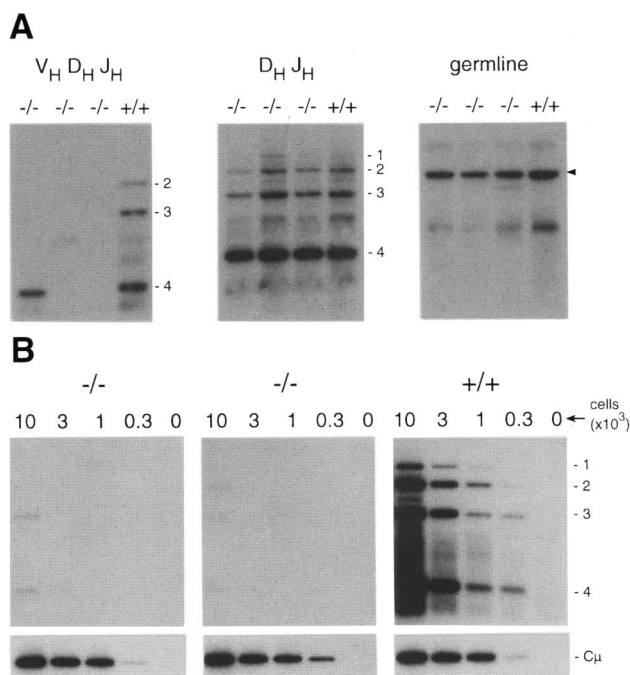


Figure 4. Reduced V -to- D J recombination at the *IgH* locus in pre-BI cells of Pax5-deficient mice. (A) PCR analysis of pre-BI cells. B220⁺*c-kit*⁺ cells were sorted from the bone marrow of three Pax5 mutant mice (-/-) and one wild-type littermate (+/+) at the age of 2 weeks. Ten thousand of these pre-BI cells were subjected to the same two-step PCR amplification protocol that was used for the analysis of *IgH* gene rearrangements in single cells (Fig. 3; Materials and Methods). PCR amplification products were detected by Southern blot hybridization with a 120-bp probe containing DNA sequences 3' of the $J_H 4$ segment. $V_H D_H J_H$ rearrangements involving members of the prominent $V_H J_558$ gene family were amplified with a $V_H J_558$ -specific primer pair (Ehlich et al. 1994). Numbers at right refer to the J_H segments used for rearrangement; the arrow points to the position of the amplified germ-line fragment. Cloning and sequencing of the only $V_H D_H J_H$ fragment amplified from Pax5 (-/-) pre-BI cells indicated that it consisted of just one unique sequence, therefore corresponding to a single V_H -to- $D_H J_H$ rearrangement event. (B) Quantitation of $V_H D_H J_H$ rearrangements in B220⁺CD43⁺ cells. Increasing numbers (300 to 10,000) of B220⁺CD43⁺ cells, which were sorted from the bone marrow of two mutant mice (-/-) and one wild-type littermate (+/+), were analyzed by 30 cycles of PCR amplification with a $V_H J_558$ -specific primer pair, and the four different $V_H D_H J_H$ fragments were detected by Southern blot analysis (see Materials and Methods). The autoradiographs shown were exposed for the same time period. A DNA segment from the $C_H 1$ region of the *IgH* locus was amplified in parallel to control for the number of genomes analyzed. Quantitation of the $V_H D_H J_H$ signals by PhosphorImager analysis and normalization to the $C_H 1$ signal indicated a 50- to 70-fold lower incidence of $V_H D_H J_H$ rearrangements in the Pax5 mutant cells compared with the wild-type cells.

Long-term proliferation potential of Pax5-deficient pre-BI cells in vitro

The growth factor requirement of B-lymphoid cells provides yet a third criterion to define their developmental

stage. For instance, pre-BI cells from the bone marrow of wild-type mice can be cultured *in vitro* on stromal cells in the presence of IL-7 (Rolink et al. 1991b). The long-term proliferation capacity of these cells is known to be critically dependent on the expression of *c-kit* and IL-7R (Rolink et al. 1991a; Sudo et al. 1993), both of which are expressed on *Pax5*-deficient B lymphocytes (Fig. 2A). To examine the *in vitro* proliferation potential of these cells, we have used fluorescence-activated cell sorting to seed individual B220⁺*c-kit*⁺ cells from the bone marrow of *Pax5* mutant mice into single wells containing stromal ST2 cells and IL-7 medium. Under these conditions, one out of five cells grew into a colony that could be further propagated as a cell line in culture (data not shown). The same cloning frequency (1/5) was determined previously for B220⁺*c-kit*⁺ cells isolated from the bone marrow of wild-type mice (Rolink et al. 1993). Therefore, the absence of *Pax5* does not affect the clonability of bone marrow pre-BI cells.

The cell surface phenotype of *in vitro* propagated pre-BI cells lacking *Pax5* was next compared, by flow cytometric analysis, with that of wild-type pre-BI cells. As shown in Figure 5, both cell populations expressed similar levels of B220, *c-kit*, CD43, IL-7R, and VpreB, but differed in the synthesis of the BP-1 and CD19 proteins, which were absent from *Pax5*-deficient pre-BI cells. These results combined with the *in vivo* data of Figure 2A indicate that, at this level of analysis, the *Pax5*-defi-

cient pre-BI cells have apparently not altered their phenotype on *in vitro* culturing.

B-cell-specific gene expression in Pax5-deficient pre-BI cells

BSAP (*Pax5*) is only one of several transcription factors that have essential roles in early B-cell development (for review, see Busslinger and Urbánek 1995). The availability of homogeneous pre-BI cell populations differing only by the presence or absence of *Pax5* made it possible to investigate whether BSAP regulates the expression of other known transcription factors. As shown by the RNase protection experiment in Figure 6, the expression of the transcription factors PU.1 (Scott et al. 1994), Ikaros (Georgopoulos et al. 1994), EBF (Lin and Grosschedl 1995), E2A (Bain et al. 1994; Zhuang et al. 1994), Sox-4 (Schilham et al. 1996), Oct-1 (Sturm et al. 1988), Oct-2 (Corcoran et al. 1993), and OBF-1 (Kim et al. 1996; Schubart et al. 1996) was unaffected at the mRNA level by the absence of *Pax5*. Similarly, the *Pax5* mutation did neither influence transcription of the recombination activating genes *RAG-1* (Schatz et al. 1989) and *RAG-2* (Oettinger et al. 1990) nor expression of the germ-line transcript *I μ* which initiates within the *IgH* enhancer region (Lennon and Perry 1985). These latter observations are in agreement with our finding that *Pax5*-deficient pre-BI

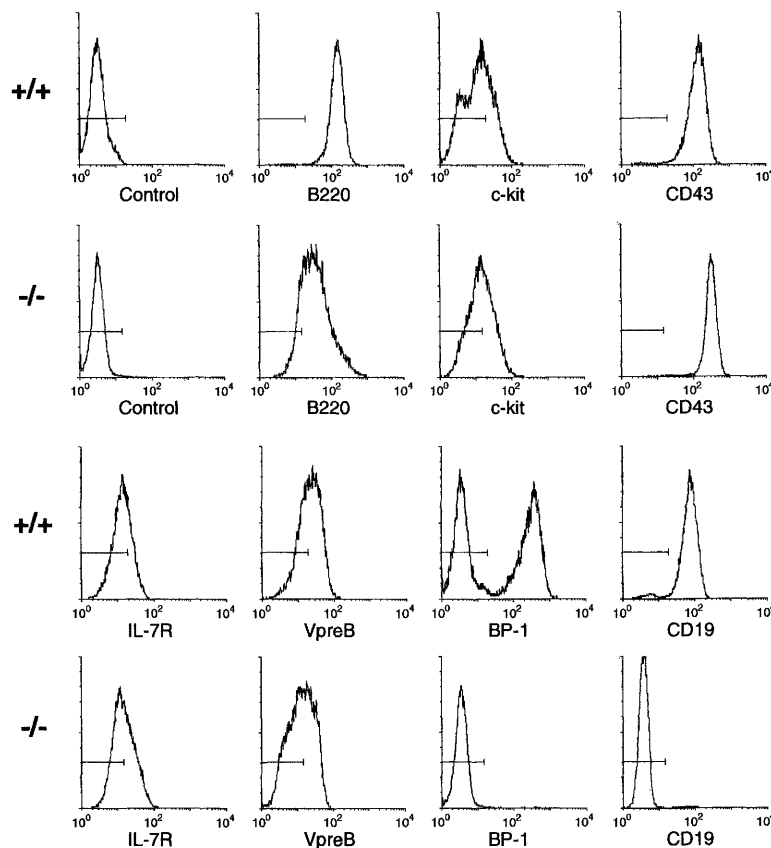


Figure 5. Cell surface phenotype of *in vitro* cultured pre-BI cells from bone marrow of wild-type and *Pax5* mutant mice. Bone marrow cells from wild-type (+/+) and *Pax5* mutant (-/-) mice at the age of 2 weeks were plated under limiting dilution conditions on ST2 cells in the presence of IL-7. For each genotype, several pre-BI cell colonies were pooled 10 days later. After 24 days in culture, the growing pre-BI cell populations were subjected to flow cytometric analysis by staining with the indicated antibodies as described in the legend to Fig. 2A.

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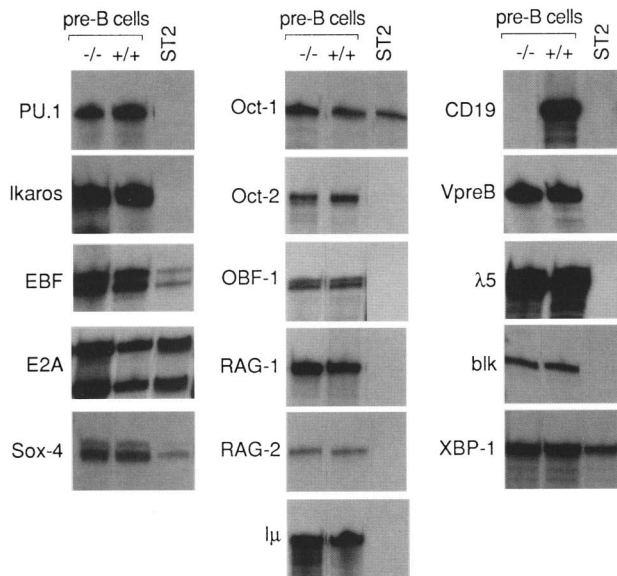


Figure 6. Gene expression in Pax5-deficient pre-BI cells. Pre-B cells isolated from bone marrow of wild-type (+/+) and Pax5 mutant (-/-) mice were propagated in vitro on stromal ST2 cells in the presence of IL-7. Total RNA (10 µg) prepared from the two pre-B cell populations and ST2 cells was analyzed by RNase protection assay for the presence of the gene transcripts indicated at left. For the generation of different riboprobes see Materials and Methods. Only the relevant parts of the autoradiograph containing the RNase-protected fragments are shown.

cells are capable of D_H/J_H recombination at the *IgH* locus (Fig. 3).

The B-cell-specific genes coding for CD19 (Kozmik et al. 1992), Blk (Zwollo and Desiderio 1994), XBP-1 (Reimold et al. 1996), $\lambda 5$, and VpreB1 (Okabe et al. 1992) have been suggested to be direct targets for regulation by the transcription factor BSAP (Pax5). However, of all these genes, only transcription of *CD19* was reduced in the absence of Pax5 (Fig. 6). Interestingly, not even basal-level expression of the *CD19* gene could be detected in Pax5-deficient pre-BI cells in agreement with the observation that the CD19 protein was absent from the surface of these cells (Fig. 5). These data therefore provide genetic evidence that *CD19* expression is critically dependent on BSAP in contrast with the other genes tested.

Absence of B lymphopoiesis in the fetal liver of Pax5 mutant embryos

During embryogenesis, B-cell development occurs predominantly in the fetal liver, which is highly enriched in B220⁺c-kit⁺ B lymphocytes. These cells can be cloned from wild-type embryos at a high frequency in the presence of stromal cells and IL-7 (Rolink et al. 1993). Consistent with this notion, we could readily establish pre-B-cell lines from fetal liver of wild-type (+/+) and heterozygous mutant (+/-) embryos at day 17 (Fig. 7A). Much to our surprise however, we failed to clone any pre-B cells from the fetal liver of nine embryos lacking Pax5

(-/-) (Fig. 7A). This finding suggested strongly that the fetal liver is unable to support the development of early B-lymphoid progenitors in the absence of Pax5. This conclusion was confirmed by flow cytometric analysis of liver cells from day-17 fetuses (Fig. 7B). B lymphocytes identified by the expression of the B-cell markers B220 and CD19 constitute only a small subset (3–4%) of all cells at this developmental age both in wild-type and heterozygous mutant embryos. Most of these B lymphocytes correspond, however, to progenitor cells as defined by the expression of *c-kit* on their cell surface. This progenitor cell population was entirely absent in the fetal liver of Pax5 mutant embryos (Fig. 7B). To corroborate this result at the molecular level, we have analyzed, by RNase protection assay, the expression of the B-cell-specific genes *B29*, $\lambda 5$, *VpreB1*, and *EBF* in fetal liver of wild-type and mutant embryos (Fig. 8A). All of these genes are transcribed in a Pax5-independent manner at early stages of B-cell development as shown by their expression in Pax5-deficient pre-BI cells of bone marrow (Fig. 6; unpubl. data). However, these genes were expressed in the fetal liver of homozygous mutant embryos

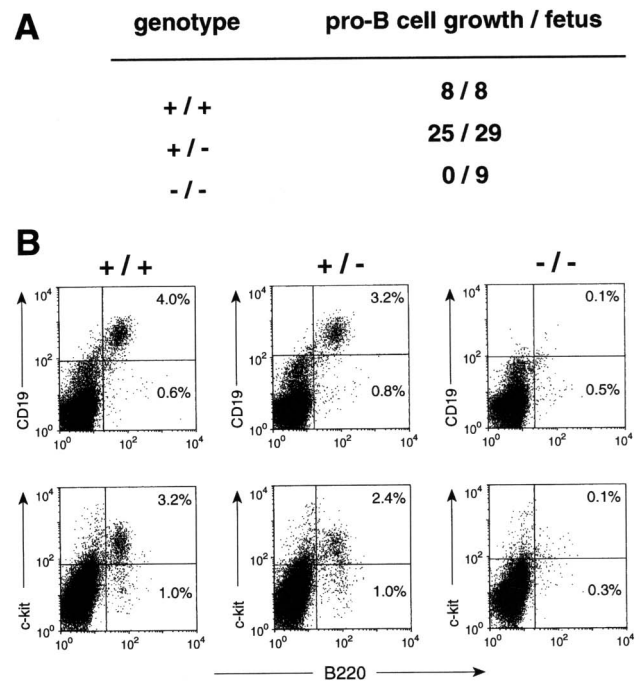


Figure 7. Absence of pro-B cells in the fetal liver of Pax5 mutant embryos. (A) Summary of the in vitro cloning experiments. Fetal liver cells from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mutant embryos at day 17 were plated under limiting dilution conditions on ST2 cells in the presence of IL-7. Individual colonies were pooled, and their growth was assessed after 3 weeks of in vitro culture. (B) Flow cytometric analysis. Fetal liver cells from day-17 embryos of the indicated genotypes were analyzed by flow cytometry using FITC-conjugated anti-B220/CD45R (RA3-6B2) and biotinylated anti-CD19 (1D3) or anti-c-kit (ACK4) antibodies (revealed by incubation with PE-coupled streptavidin). The percentages of B220⁺ cells are indicated in each quadrant.

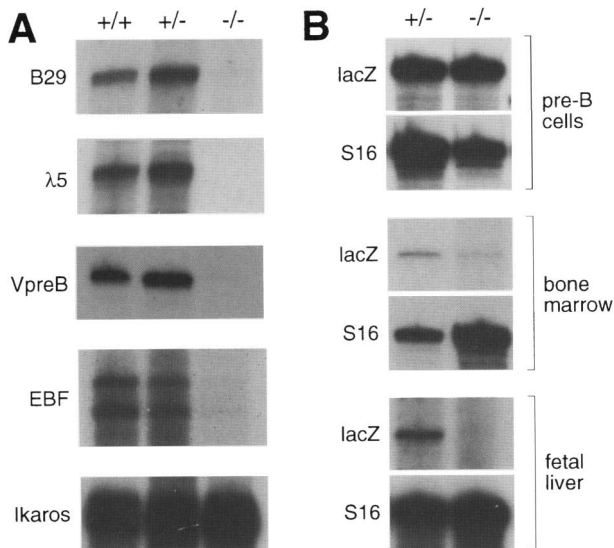


Figure 8. Dramatic reduction of B-cell-specific gene expression in fetal liver of *Pax5* mutant embryos. (A) Expression of B-cell-specific genes in fetal liver cells. Total RNA was isolated from fetal liver of day-18.5 embryos of all three genotypes, and 20 μ g of each RNA preparation was analyzed by RNase protection assay with riboprobes specific for the gene transcripts indicated at left. Only the relevant parts of the autoradiographs containing the RNase-protected signals are shown. (B) *lacZ* expression in *Pax5* mutant B lymphocytes. *LacZ* mRNA transcribed from the targeted *Pax5* locus (Urbánek et al. 1994) was detected by RNase protection assay in total RNA isolated from in vitro cultured bone marrow pre-B cells, from bone marrow of 16/19-day-old mice and from fetal liver of day-18.5 embryos of heterozygous (+/-) and homozygous (-/-) mutant genotype. Transcripts coding for the small ribosomal protein S16 were co-mapped as internal reference RNA.

only at a very low level, if at all, compared with wild-type and heterozygous embryos (Fig. 8A). In contrast, the *Ikaros* gene was equally well transcribed in the fetal liver of all three genotypes consistent with its broader expression pattern within the hematopoietic system (Georgopoulos et al. 1994). In summary, the combined results of three experimental approaches (in vitro cloning, flow cytometry, and expression analysis) demonstrate that mutation of the *Pax5* gene arrests B lymphopoiesis in the fetal liver at an even earlier developmental stage than in bone marrow.

The two distinct phenotypes could in principle result from a difference in the developmental onset of *Pax5* expression during fetal and adult B lymphopoiesis. To address this question, we have taken advantage of the fact that the targeted *Pax5* allele carries an *Escherichia coli lacZ* gene under the transcriptional control of the endogenous locus (Urbánek et al. 1994). RNase protection analysis of *lacZ* mRNA was therefore used to monitor *Pax5* expression in bone marrow, fetal liver, and in vitro cultured pre-BI cells of heterozygous and homozygous mutant mice. As shown in Figure 8B, the *lacZ* gene

was transcribed in both pre-B-cell lines to a similar level. In agreement with this observation, *lacZ* transcripts were also detected in bone marrow, where the disparity in transcript levels reflects the difference in B-lymphocyte numbers between heterozygous and homozygous mutant mice (Urbánek et al. 1994). We conclude therefore that the *Pax5* gene is transcribed even before the developmental stage at which B-cell differentiation is aborted in the bone marrow of *Pax5*-deficient mice. In contrast, no *Pax5* transcripts could be detected in the fetal liver of homozygous mutant embryos (Fig. 8B), therefore indicating that *Pax5* is essential for fetal B-lymphopoiesis right from the onset of its expression.

B-cell-autonomous effect of the Pax5 mutation

Early lymphocyte development is dependent strictly on the interaction with appropriate stromal cells in the primary lymphoid organs (Rolink and Melchers 1991). Interestingly, stromal cells of the thymus express *Pax-1*, another member of the *Pax* family, and mutation of this gene in *undulated* mice interferes with stromal cell differentiation, therefore resulting in abnormal T-cell development (Wallin et al. 1996). In analogy to this situation, it is also conceivable that a primary site of *Pax5* function could be the stromal cells in bone marrow and that the block of B-cell development is merely the secondary consequence of abnormal stromal cell differentiation in *Pax5* mutant mice. To distinguish between such an indirect mode of action and a cell-autonomous effect of the *Pax5* mutation, we have reconstituted lethally irradiated wild-type mice by transplantation of bone marrow cells from *Pax5*-deficient donor mice. Three months after cell transfer, the reconstituted bone marrow of these mice contained only B220⁺CD43⁺ B lymphocytes, as shown by flow cytometric analysis (Fig. 9A). The majority of these cells also expressed *c-kit* and IL-7R on their surface, which further identifies them as pre-BI cells. The absence of CD19 expression on these B lymphocytes demonstrated efficient reconstitution of the bone marrow by *Pax5* mutant donor cells. As illustrated by the absence of the late differentiation marker IgM and CD40, B-cell development was, however, still blocked completely in all B-lymphoid progenies of the transferred *Pax5*-deficient stem cell that was now embedded in wild-type tissue. We conclude, therefore, that the effect of the *Pax5* mutation is B-cell autonomous.

To further investigate the nature of the early block in fetal B lymphopoiesis, we have also used fetal liver cells of *Pax5* mutant embryos to reconstitute the hematopoietic system of lethally irradiated wild-type mice (Fig. 9B). Interestingly, the bone marrow of these reconstituted mice was able to generate pre-BI cells expressing CD43, *c-kit*, and IL-7R quite in contrast with the fetal liver of the donor embryos. The B-cell phenotype is therefore identical in reconstituted mice regardless of whether *Pax5*-deficient fetal liver or bone marrow cells were used for transplantation. These data indicate that different functions of *Pax5* are required to support early B-cell

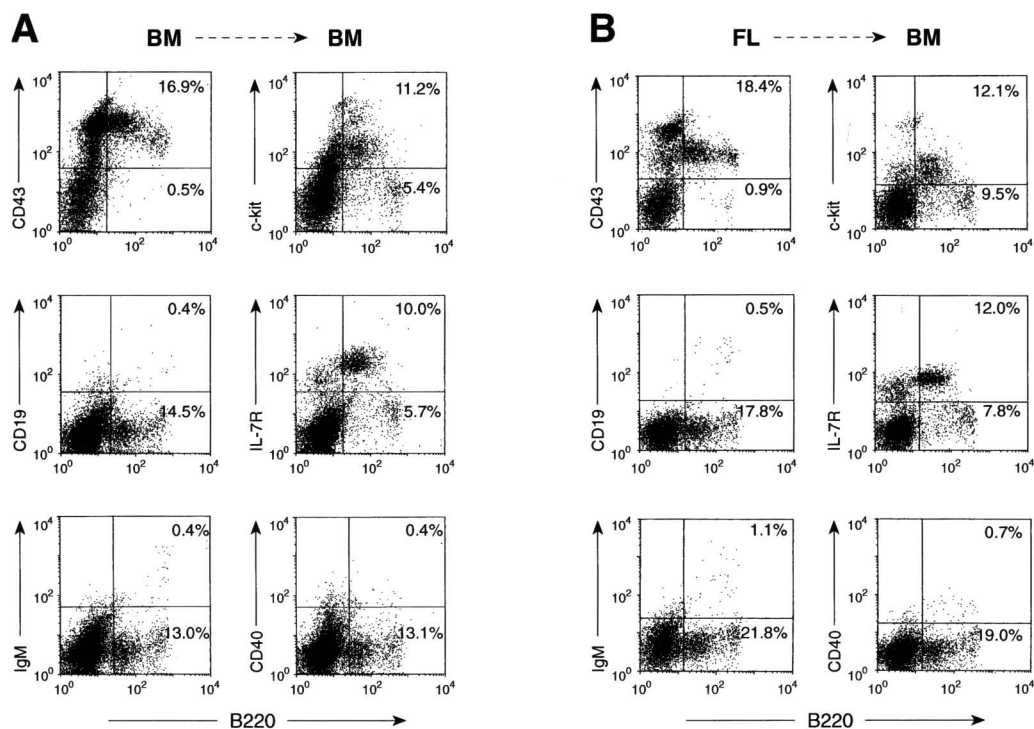


Figure 9. Cell autonomous effect of the *Pax5* mutation in B-cell development. (A) Reconstitution of the hematopoietic system with bone marrow of *Pax5*-deficient mice. The hematopoietic system of lethally irradiated wild-type mice was reconstituted by transplantation of bone marrow (BM) cells from 16-day-old homozygous *Pax5* mutant mice (see Materials and Methods). (B) Reconstitution with *Pax5*-deficient fetal liver cells. Lethally irradiated wild-type mice were rescued by injection of fetal liver (FL) cells from day-13.5 embryos lacking *Pax5*. In both cases, the B-cell compartment in the bone marrow of reconstituted mice was analyzed 3 months after transplantation by flow cytometry as described in the legend to Fig. 2A and in Materials and Methods.

development in the distinctive microenvironments of fetal liver and adult bone marrow.

Discussion

Differential dependency of fetal and adult pro-B-cell development on Pax5

The developmental control genes of the *Pax* family are associated with mouse mutants and human disease syndromes and code for transcription factors with important functions in embryonic pattern formation (for review, see Stuart et al. 1994). One member of this gene family, *Pax5*, has an essential role not only in the morphogenesis of the developing midbrain, but also in early B lymphopoiesis (Urbánek et al. 1994). Here we have demonstrated that B-cell development is arrested at the early pro-B (pre-BI) cell stage in the bone marrow of *Pax5*-deficient mice. The B lymphocytes of these mice were identified as pre-BI cells on the basis of their expression of *c-kit*, IL-7R, CD43, HSA, $\lambda 5$, and VpreB as well as by the absence of CD25, BP-1, and later differentiation markers on the cell surface (see Fig. 1). Moreover, these *Pax5*-deficient pre-BI cells could be cultured in vitro in the presence of stromal cells and IL-7 with the same efficiency as the corresponding cells from wild-type bone marrow. Pre-BI cells could, however, not be detected in

the fetal liver of *Pax5* mutant embryos as shown by the lack of in vitro clonable B lymphocytes and by an almost complete absence of B-cell-specific gene expression. *Pax5* is therefore required at two different developmental stages in fetal and adult B lymphopoiesis (Fig. 10). In adult bone marrow, *Pax5* is essential for progression of B-cell development beyond the early pre-BI cell stage, whereas in the fetal liver it is required already for differentiation of the earliest B-lineage-committed precursor cells. Bone marrow transplantation experiments furthermore demonstrated that the developmental block in adult B-lymphopoiesis results from a direct, cell autonomous effect of the *Pax5* mutation on B-cell differentiation instead of being the indirect consequence of an interference with stromal cell differentiation. Interestingly, *Pax5*-deficient fetal liver cells could give rise to the development of pre-BI cells in reconstituted bone marrow, contrary to the situation encountered in the embryo. This apparent discrepancy may indicate a critical function of *Pax5* for stromal cell development in the fetal liver, although there is to date no evidence for *Pax5* expression in these cells. Therefore, we consider it more likely that the earliest B-cell progenitors depend for development or survival in the fetal liver microenvironment on their own expression of BSAP (*Pax5*) target genes that are not essential for pro-B cell development in

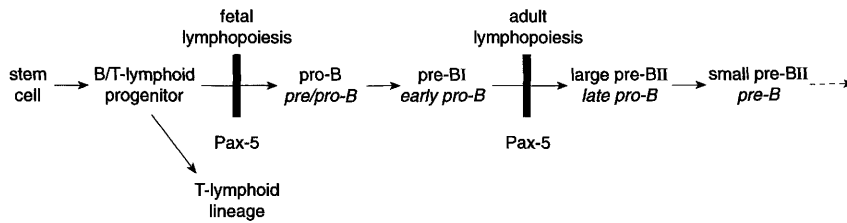


Figure 10. Differential dependency of pro-B cell development on *Pax5* in fetal and adult B lymphopoiesis. A schematic diagram of early B-cell differentiation is shown together with the developmental block observed in fetal and adult B lymphopoiesis of *Pax5* mutant mice. The different developmental stages are referred to in the nomenclature of Rolink et al. (1994) (*top* line) and Hardy et al. (1991) (*bottom* line in italics).

adult bone marrow. Consistent with this notion, *Pax5* expression is indispensable right from its onset in fetal B lymphopoiesis, whereas its expression in pre-BI cells of the bone marrow does not appear to fulfill a critical function during early pro-B cell development.

Differences between fetal and adult B lymphopoiesis have been observed previously. The enzyme terminal deoxynucleotidyl transferase (TdT), which is responsible for N sequence insertions in the D_H/J_H and $V_H/D_H/J_H$ joints of the *IgH* gene, is not expressed in lymphocytes of the fetal liver in contrast to bone marrow (Desiderio et al. 1984; Li et al. 1993). Similarly, the B-lymphoid-specific myosin light chain gene *PLRLC* is transcribed only in pre-B cells of the bone marrow, but not in fetal liver cells (Oltz et al. 1992; Li et al. 1993). The hematopoietic progenitor cells of fetal liver and bone marrow also differ in their capacity to regenerate the two different B-cell subsets consisting of the conventional CD5⁻ B lymphocytes and the CD5-expressing B-1 cells (for review, see Hardy and Hayakawa 1994; Kantor and Herzenberg 1993). Fetal liver cells can efficiently reconstitute both B-cell populations in transplantation experiments, whereas bone marrow cells of adult donor mice predominantly give rise to conventional B lymphocytes (Kantor et al. 1992; Hardy and Hayakawa 1994). These phenotypic differences are most probably not related to any *Pax5* function as both B-cell populations are equally dependent on this transcription factor for their development (Urbánek et al. 1994). To our knowledge, however, *Pax5* provides the first example of a transcription factor with distinct roles in fetal and adult B lymphopoiesis.

The *Pax5* gene is expressed in the B-lymphoid lineage up to the mature B-cell stage and consequently has also been implicated in the regulation of different aspects of late B-cell differentiation (for review, see Busslinger and Urbánek 1995). For instance, *Pax5* is considered to be involved in the proliferation control of mature B lymphocytes, as antisense oligonucleotide inhibition of BSAP synthesis prevented splenic B-cells from entering the cell cycle on mitogenic stimulation (Wakatsuki et al. 1994). This hypothesis has recently gained support by the discovery that *Pax5* is recruited as an oncogene by t(9:14)(p13;q32) translocations in a subset of non-Hodgkin's lymphomas (Busslinger et al. 1996). This type of chromosomal translocation inserts the E_{μ} enhancer of the *IgH* locus upstream of two *Pax5* promoters, which may interfere with the down-regulation of *Pax5* expression during plasma cell differentiation (Busslinger et al. 1996). In this regard it is, however, important to note

that the early pre-BI cells of both wild-type and *Pax5*-deficient mice proliferate equally well in vivo and in vitro. Therefore, the proliferation of B lymphocytes does not require *Pax5* expression early in the lineage, but may become dependent on this transcription factor at later stages of B-cell differentiation.

Targeted gene inactivation in the mouse germ line was also used to elucidate the function of other transcription factors in the genetic control of B-cell differentiation. PU.1, a member of the Ets transcription factor family, appears to act high up in the transcriptional hierarchy, as disruption of its gene prevents the generation of progenitors for both the lymphoid and myeloid lineages (Scott et al. 1994). Deletion of a subset of the zinc finger proteins encoded by the *Ikaros* locus results in complete failure of B- and T-cell development (Georgopoulos et al. 1994). Once committed to the B-lymphoid lineage, cells become dependent on the function of the *EBF* and *E2A* genes in addition to *Pax5*. Mice lacking the early B-cell factor EBF (Lin and Grosschedl 1995) or the helix-loop-helix transcription factors E12 and E47 encoded by the *E2A* gene (Bain et al. 1994; Zhuang et al. 1994) are able to generate only a small number of CD43⁺B220⁺ cells in the bone marrow. In both cases, these early pro-B cells express a very limited set of B-cell-specific genes, contain the *IgH* locus still in germ-line configuration and do not transcribe the *Pax5* gene (Bain et al. 1994; Lin and Grosschedl 1995). B-cell development appears therefore to be blocked at an earlier stage in the bone marrow of *EBF* and *E2A* mutant mice compared with animals lacking *Pax5*. However, a different situation prevails in fetal liver where *Pax5* is already required for full commitment to the B-lymphoid lineage. Progression of B-cell development to the small pre-B-cell stage is furthermore dependent on the function of the transcription factor Sox-4 (Schilham et al. 1996), whereas Oct-2, OBF-1, and the p50 subunit of NF- κ B have critical roles in the activation of mature B-cells to undergo plasma cell differentiation (Corcoran et al. 1993; Sha et al. 1995; Kim et al. 1996; Schubart et al. 1996). Importantly, the expression of none of these regulatory genes was affected in *Pax5*-deficient pre-BI cells. Therefore, *Pax5* does not appear to exert its effect on B-cell development by controlling the expression of other known transcription factors.

Pax5 and the B-cell-specific control of V(D)J recombination

The somatic assembly of immunoglobulin heavy- and

light-chain genes from different coding segments is achieved in a highly regulated and temporally ordered fashion by the site-specific $V(D)J$ recombination system (for review, see Lewis 1994). Joining of the D_H and J_H segments is initiated at the IgH locus soon after B-cell lineage commitment, and this process is completed on both IgH alleles at the early pro-B (pre-BI) cell stage (Ehlich et al. 1993; Li et al. 1993). At this time in B-cell development, V_H -to- D_HJ_H recombination is activated and then continues to operate until the late pro-B-cell stage (large pre-BII) (Ehlich et al. 1993; Li et al. 1993) when surface expression of a functionally rearranged μ heavy-chain protein signals allelic exclusion and therefore prevents further rearrangement at the IgH locus (Kitamura and Rajewsky 1992). Using different PCR assays, we have demonstrated that the majority of the pre-BI cells in Pax5-deficient mice contain the IgH locus in the D_HJ_H -rearranged configuration. The frequency of V_H - D_HJ_H rearrangements is, however, reduced 50-fold in Pax5-deficient pre-BI cells compared with wild-type cells. These results therefore suggest an involvement of Pax5 in the pathway controlling V_H -to- D_HJ_H recombination.

The chromatin accessibility of the recombination substrates appears to be a key factor in the control of the $V(D)J$ rearrangement process as indicated by the observation that the transcription of germ-line immunoglobulin gene segments usually precedes their DNA rearrangement (Yancopoulos and Alt 1985; Schlissel and Baltimore 1989; Schlissel et al. 1991b). Transcriptional activation may therefore target immunoglobulin genes for $V(D)J$ recombination. In agreement with this hypothesis, $V(D)J$ recombination is impaired by germ-line deletion of the intronic enhancers of the IgH locus (Chen et al. 1993; Serwe and Sablitzky 1993) and $IgL\kappa$ gene (Takeda et al. 1993) or by the lack of the $E\mu$ enhancer on transgenic recombination substrates (Ferrier et al. 1990). As both intronic enhancers do not contain any high-affinity binding sites for BSAP (Barberis et al. 1990), it is unlikely that Pax5 mediates its effect on $V(D)J$ recombination by direct interaction with these regulatory regions. Alternatively, Pax5 may control V_H -to- D_HJ_H rearrangements by activating germ-line V_H gene transcription. The promoter activity of V_H and V_κ genes is known to critically depend on a regulatory sequence that is bound by the transcription factors Oct-1 and Oct-2 (Falkner and Zachau 1984; Mason et al. 1985). However, both of these transcriptional regulators as well as their B-cell-specific coactivator OBF-1 (Kim et al. 1996; Schubart et al. 1996) are equally well expressed in the presence or absence of Pax5. To date we therefore do not have any evidence for either a direct or indirect role of Pax5 in transcriptional regulation underlying the $V_HD_HJ_H$ rearrangement process.

It has been known for some time that D_HJ_H rearrangements at the IgH locus promiscuously occur also in cells of the T-lymphoid lineage (Born et al. 1988; Cory et al. 1980; Kurosawa et al. 1981). In contrast, V_H -to- D_HJ_H rearrangements of the IgH gene take place only in B lymphocytes and therefore constitute a more stringently

regulated step of the $V(D)J$ recombination process. The ubiquitously expressed transcription factors of the $E2A$ gene have been implicated in the control of D_HJ_H rearrangements both by gain- and loss-of-function experiments. Ectopic expression of the E47 protein resulted in the activation of D_HJ_H , but not of $V_HD_HJ_H$ rearrangements in a pre-T-cell line (Schlissel et al. 1991a). Moreover, the pro-B-cells of E2A-deficient mice neither express the recombination activating gene RAG-1 nor do they undergo D_H -to- J_H rearrangements at the IgH locus (Bain et al. 1994). Here we have shown that loss of the B-cell-specific transcription factor BSAP (Pax5) affects the B-lymphoid-restricted V_H -to- D_HJ_H joining step of IgH assembly. In this context it is of interest to note that the B-cell phenotype of Pax5-deficient mice differs at least in two aspects from that of mice with targeted disruption of genes involved in the expression of the pre-B-cell receptor. First, based on the expression of cell surface markers, B-cell development appears to be arrested at an earlier stage in Pax5 mutant mice (early pro-B cells; fraction B) than in mice lacking RAG-1, $Ig\beta$, $\lambda 5$ ($\lambda 5T$), the joining region (J_HT) or the membrane exon (μMT) of the IgH locus, all of which are blocked at the late pro-B-cell stage (fraction C) (Ehlich et al. 1993; Spanopoulou et al. 1994; Gong and Nussenzweig 1996). Second, expression of a rearranged μ heavy-chain transgene in Pax5 mutant mice did not allow B lymphocytes to advance in B-cell development (C. Thévenin and M. Busslinger, unpubl.) in contrast with the situation observed with RAG-deficient mice where complementation with a μ transgene facilitates progression to the small pre-B cell stage (Rolink et al. 1994; Spanopoulou et al. 1994; Young et al. 1994). The lack of complementation therefore indicates that the failure to undergo V_H -to- D_HJ_H rearrangements is unlikely to be the reason for the developmental block observed in the bone marrow of Pax5 mutant mice.

Regulation of B-cell-specific gene expression by BSAP (Pax5)

Insight into the regulatory function of BSAP critically depends on the identification of genes that are controlled by this transcription factor. The in vitro clonability of Pax5-deficient pre-BI cells now provides a useful tool to search for such target genes. Comparative expression analysis of over 30 known B-cell-specific genes in wild-type and Pax5-deficient pre-BI cells resulted in the identification of three genes, $CD19$, $mb-1(Ig\alpha)$ and $N-myc$, which are down-regulated in the absence of BSAP at this early stage of B-cell development. Whereas the levels of $N-myc$ and $mb-1$ transcripts were reduced consistently (S. Nutt, unpubl.), expression of the $CD19$ gene was entirely lost in Pax5-deficient pre-BI cells (Fig. 6). In accord with this finding, we have identified previously a high-affinity BSAP-binding site in the -30 region of the $CD19$ gene, which is fully occupied in vivo in B cells and is therefore likely to mediate BSAP-dependent transcriptional initiation (Kozmik et al. 1992). Moreover, $CD19$ expression can be induced readily in an estrogen-dependent manner in Pax5-deficient pre-BI cells expressing a

BSAP-estrogen receptor fusion protein (S. Nutt, unpubl.). Together, these data unequivocally identify *CD19* as a genuine BSAP target gene. The expression of *blk*, *XBP-1*, $\lambda 5$, and *VpreB1* was, however, unaffected by the absence of BSAP, which strongly argues against a critical role of this transcription factor in the regulation of these genes in apparent contradiction to published data (Okabe et al. 1992; Zwollo and Desiderio 1994; Reimold et al. 1996).

Could the lack of *CD19* expression be responsible for the early arrest of B-cell development in *Pax5*-deficient mice? The *CD19* protein is known to associate with surface immunoglobulin receptors where it acts as a co-stimulatory molecule to lower the threshold for antigen-dependent signaling (Carter and Fearon 1992). In agreement with this function, the processes of B-cell activation, selection and maturation are impaired severely in mice lacking *CD19* (Engel et al. 1995; Rickert et al. 1995). However, B-lymphoid development up to the mature B-cell stage was unperturbed in the bone marrow of these mice, demonstrating that *CD19* cannot be one of the critical target genes responsible for the differentiation block in *Pax5* mutant mice. Therefore, we will have to face the challenge to systematically search for such target genes, which should be facilitated greatly by the availability of *Pax5*-deficient pre-BI cell lines.

Materials and methods

Mice

All analyses including the bone marrow transplantation experiments were performed with wild-type and *Pax5* mutant mice that were bred on the hybrid background C57BL/6 \times 129/Sv (Urbánek et al. 1994). The *Pax5* genotype was determined by Southern blot analysis (Urbánek et al. 1994) or by PCR assay using the following oligonucleotides: 5'-ACAGTCCCCTTACCCCTTCCCCCAAATGAA-3' (primer 1); 5'-GTCCCTCCTAACAAAGTCTCCCTATTCCAC-3' (primer 2); and 5'-GTGAGCGAGTAACAACCCGTCGGATTCTCC-3' (primer 3). A 693-bp PCR product was amplified from the wild-type *Pax5* allele with primer pair 1/2 and a 783-bp DNA fragment from the mutant *lacZ* allele with the pair 1/3.

Antibodies and flow cytometric analysis

The following monoclonal antibodies were purified from hybridoma cell supernatants on protein G-sepharose columns (Pharmacia, Uppsala, Sweden) and conjugated with Sulfo-NHS-Biotin (Pierce Chemical Company) as recommended by the suppliers: anti-*CD19* mAb (1D3; Krop et al. 1996), anti-*c-kit* mAb (ACK4; Ogawa et al. 1991), anti-IL-7R mAb (A7R34; Sudo et al. 1993), anti-*VpreB* mAb (VP245; Karasuyama et al. 1993), and anti- μ H chain mAb (M41; Leptin et al. 1984). Anti-B220/CD45R mAb (RA3-6B2) was obtained as fluorescein isothiocyanate (FITC)-conjugated antibody from PharMingen (San Diego, CA), whereas all other monoclonal antibodies were purchased from the same company in biotinylated form: anti-CD25/TAC mAb (7D4), anti-CD43 (S7), anti-BP-1 (6C3), and anti-HSA/CD24 (M1/69). Phycoerythrin (PE)-conjugated streptavidin was obtained from Southern Biotechnology Associates Inc. (Birmingham, Alabama).

Single-cell suspensions were prepared from bone marrow and

fetal liver, stained with different antibody combinations and then analyzed by a FACScan flow cytometer (Becton-Dickinson) as described previously (Urbánek et al. 1994).

Fluorescence-activated cell sorting and cell cycle analysis

Bone marrow cells of 2-week-old *Pax5* mutant mice were stained with anti-B220/CD45R (RA3-6B2) and anti-*c-kit* (ACK4) antibodies and then separated into B220⁺*c-kit*⁺ and B220⁺*c-kit*⁻ cell fractions by fluorescence-activated cell sorting using a FACStar^{plus} flow cytometer. Reanalysis of the sorted cells indicated a purity of $\geq 90\%$ for each cell population. Cell cycle analysis was performed by fixing the sorted cells followed by ethidium bromide staining of isolated nuclei and FACScan analysis of their DNA content as described previously (Rolink et al. 1994).

Transplantation of bone marrow and fetal liver cells

Wild-type female mice (C57BL/6 \times 129/Sv) at the age of 2 months were lethally γ -irradiated with 950 rads (9.5 Gy) and then provided with 2×10^6 to 7×10^6 bone marrow cells from 16-day-old homozygous *Pax5* mutant mice (C57BL/6 \times 129/Sv) by injection into the tail vein. In a second experimental series, wild-type recipient mice of the same age and strain background were lethally irradiated and subsequently received 3×10^6 to 5×10^6 fetal liver cells prepared from day-13.5 embryos (C57BL/6 \times 129/Sv) lacking *Pax5*. The B-cell compartment of the recipient mice was analyzed by flow cytometry 3 months after cell transfer.

PCR analysis of IgH gene rearrangements

Single-cell PCR analyses (Fig. 3) were performed with individual B220⁺*c-kit*⁺ cells that were sorted from bone marrow of two-week-old *Pax5* mutant mice into single wells of a 96-well plate by a FACStar^{plus} flow cytometer. The *IgH* and *IgL κ* gene loci of individual cells were amplified exactly according to the two-step PCR protocol of ten Boekel et al. (1995), which is based on the method of Ehlich et al. (1994). Only the first (Gu et al. 1991) of the two $D_{H}FL16/D_{H}SP2$ -specific 5' primers listed in Table 1 of ten Boekel et al. (1995) was used for PCR amplification of $D_{H}J_{H}$ -rearranged alleles which resulted in DNA fragments of the following sizes: 1880 bp (J_{H1}), 1560 bp (J_{H2}), 1180 bp (J_{H3}), and 610 bp (J_{H4}). The products of the second PCR reaction were cloned directly into the dT-tailed vector pCRII (Invitrogen) and then verified by DNA sequencing.

The same two-step PCR protocol was also employed for the amplification of rearranged *IgH* alleles from 10,000 B220⁺*c-kit*⁺ cells that were sorted from the bone marrow of either wild-type or *Pax5*-deficient mice. Only 25 cycles of amplification were used for each of the two PCR reactions, and the amplified DNA fragments were analyzed by Southern blot hybridization with a 120-bp J_{H4} -specific DNA probe. In this assay, $V_{H}D_{H}J_{H}$ recombination generated DNA fragments of the following sizes: 1720 bp (J_{H1}), 1410 bp (J_{H2}), 990 bp (J_{H3}) and 460 bp (J_{H4}). A 1500-bp PCR product was indicative of the *IgH* germ-line configuration.

For quantitation of $V_{H}D_{H}J_{H}$ recombination, the rearranged alleles of the $V_{H}558$ gene family were amplified by 30 PCR cycles with a $V_{H}558$ -specific primer pair (Ehlich et al. 1994) from increasing numbers of B220⁺CD43⁺ cells that were sorted from the bone marrow of mutant and wild-type mice. As control for the number of B220⁺CD43⁺ cells analyzed, a 2050-bp fragment was amplified from the C_{μ} region of the *IgH* locus with primers described previously (Rolink et al. 1993).

Establishment of pre-B- cell lines

Cell suspensions from bone marrow and fetal liver of wild-type and *Pax5* mutant mice were plated at limiting dilutions on a semi-confluent layer of stromal cells in the presence of IL-7 medium exactly as described (Rolink et al. 1991b, 1993). ST2 cells (Ogawa et al. 1988) that were γ -irradiated with ~1100 rads (11 Gy) in a Gammacell 40 machine were used as stromal cells. The IL-7-containing medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with 2% heat-inactivated fetal calf serum, 0.03% (wt/vol) primatone RL (Quest International, Naarden, The Netherlands), 50 μ M 2-mercaptoethanol, 1 mM glutamine, and 1% conditioned supernatant of rIL-7-producing J558L cells (Rolink et al. 1993). After 1 week of in vitro culture, several pre-B-cell colonies were pooled and further propagated as a cell line.

Riboprobes and RNase protection assay

The following oligonucleotide pairs were used for PCR amplification of the indicated riboprobes: mPU.1, 5'-GCGGAATTCGCGACATGAAGGACAGCATCT-3' and 5'-GCGAAGCTTGCTGAAGTGGTAGGCTT-3'; mIkaros, 5'-GCGGAATTCGCTGCGCAAGACTCCACAGATA-3' and 5'-GCGAAGCTTTGCTCGCCACTCGTGCTGAC-3'; mEBF, 5'-GCGGAATTCACAATAACTCCAAGCACGGG-3' and 5'-GCGAAGCTTGGCATGAGGAGTTATCAACTC-3'; mE2A, 5'-GCGGAATTCCTCAACTACGATGCAGGTCT-3' and 5'-GCGAAGCTTGAGGTCTCTGTGAGAGGTCA-3'; mSox-4, 5'-GCGAAGCTTTTATGGTGTGGTCGCGAGATCG-3' and 5'-GCGAAGCTTGTGCCCCGACTTCACCTTCTT-3'; mOct-1, 5'-GCGGAATTCCTCAGTGAAGAGTCGGGAGA-3' and 5'-GCGAAGCTTATCTGTATGGGCTGAGACAGG-3'; mOct-2, 5'-GCGGAATTCCTGCACATGGAGAAGGAAGTG-3' and 5'-GCGAAGCTTCAGACTGCTAGAAAGCTTGGA-3'; mOBF-1, 5'-GCGGAATTCACCTCCACCCTGCAGTACCA-3' and 5'-GCGAAGCTTGCCTTCCACAGAGAGAGTGTGG-3'; and mVpreB1, 5'-GCGGAATTCCTCCAGGTTCTGCTGAGATA-3' and 5'-GCGAAGCTTGCAGCTTTTCTCCTTCCCACTC-3'; m λ 5, 5'-GCGGAATTCCTCAGCAGAAAGGAGCAGAGCT-3' and 5'-GCGAAGCTTACACACTAAGGTGGCCTTGT-3'; mRAG-1, 5'-GCGGAATTCGGAACCTCTCCACCAGTT-3' and 5'-GCGAAGCTTTCAGCCAGTGATGTTTCAGGAC-3'; mRAG-2, 5'-GCGGAATTCGGACTCCACTCCCTTTGAAGA-3' and 5'-GCGAAGCTTATCCATCGACTGGGCATGTAC-3'; mI μ , 5'-GCGGAATTCCTGGGAATGTATGGTTGG-3' and 5'-GCGAAGCTTATGGCCACATGCAGATCTCTG-3'; mB29, 5'-GCGGAATTCCTGTGGCAGGAACTTCTAGT-3' and 5'-GCGAAGCTTCTCTGTCGAAGAGTCACTATG-3'; mXBP-1, 5'-GCGGAATTCACCATGGTGCCTAGTGTCC-3' and 5'-GCGAAGCTTCTGAGGAGTTGTGTGAGCT-3'.

cDNA transcribed from poly(A)⁺ RNA of the murine pre-B-cell line 70Z/3 was used as template for PCR amplification of PU.1, EBF, Oct-1, OBF-1, XBP-1, and I μ sequences with the primers indicated above. E2A, Oct-2, VpreB1, λ 5, RAG-1, RAG-2, and B29 sequences were amplified from the respective cDNA clone (Kudo and Melchers 1987; Kudo et al. 1987; Hermanson et al. 1988; Schatz et al. 1989; Oettinger et al. 1990; Walker et al. 1990; Wirth et al. 1991). All amplified cDNA fragments were cloned in the antisense orientation into the *Hind*III and *Eco*RI sites of pSP64. The S16 and lacZ riboprobes have been described previously (Urbánek et al. 1994). A 260-bp *Bam*HI-*Eco*RI fragment of plasmid pZipNeo-mCD19 (Krop et al. 1996) and a 150-bp *Apa*I-*Hind*III fragment of the *blk* cDNA clone 102 (Dymecki

et al. 1990) were inserted into the polylinker of pSP64 to obtain the CD19 and *blk* riboprobes, respectively. All subclones were verified by DNA sequencing.

Total RNA was prepared from mouse tissues and pre-B- cell lines using the Trizol reagent (GIBCO-BRL), and 10–20 μ g of each RNA preparation were analyzed by RNase protection assay according to Vitelli et al. (1988) except that a hybridization temperature of 60°C was used.

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