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TRANSGENIC EXPRESSION OF VpreB-3 UNDER THE CONTROL OF THE IMMUNOGLOBULIN HEAVY CHAIN ENHANCER AND SV40 PROMOTER

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INDEXING WORDS

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SYNOPSIS

Recently we isolated a novel gene, VpreB-3 gene from the cDNA library of a pre-B cell clone. This gene is selectively expressed in pre-B and bone marrow-derived B cell lines. Its products are associated with the immunoglobulin heavy (IgH) chain in pre-B cell line. In the present study, to address the role of VpreB-3 on B cell development, transgenic mice carrying the VpreB-3 gene under the control of the IgH enhancer and SV40 promoter were produced. The transgenic mice expressed the VpreB-3 gene in bone marrow and spleen at a high level compared with control mice. In the thymus, the expression of the transgene was also detected, although its level was low. Flow cytometry analysis revealed that the frequency of $CD45R^+$ and μ^+ B cells were reduced in the bone marrow of 2 of the 11 transgenic mice and also reduced in the spleen of 1 of the transgenic mice. Furthermore, the results of a stromal cell-dependent B cell culture assay suggested that early B cell development, the differentiation from $CD45R^-$ B progenitor cells to $CD45R^+$ pro-B cells, was delayed in the

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bone marrow cultures of 3 of the 5 transgenic mice compared with the control mice. These results suggested that VpreB-3 products may play some role in early B cell development at the stage of $CD45R^-$ B progenitor cells before the expression of surface μ chains.

INTRODUCTION

B cell development is characterized by the ordered rearrangement of immunoglobulin variable region genes.[1] In the first step, progenitor cells rearrange a D_H to a J_H segment of a heavy (H) chain gene, giving rise to pro-B cells. Subsequently, a V_H gene joins to the D_H-J_H complex. If this results in a functional $V_HD_HJ_H$, then pre-B cells, namely $\mu^+\kappa^-$ B cells, are generated. Finally, after the functional rearrangement of a light (L) chain gene, they develop into surface IgM-bearing ($sIgM^+$) B cells.

It has been reported that during the development of the $\mu^+\kappa^-$ B cells, the membrane-bound μ (μm) chain is associated with the so called surrogate light chain (SL), λ5 [23] and VpreB-1 [14] which are specifically transcribed in pre-B cells, in place of the κ-L chain.[4, 11, 15, 20, 25] Both the VpreB-1 and λ5 genes have homology to the V region and the J_λ-C_λ region, respectively, and have been shown to form an immunoglobulin-like VpreB-1/λ5/μ complex.[8, 26] Analysis of immunoglobulin transgenic mice [16, 18, 27] suggested that the expression of the μm is essential for allelic exclusion, preventing further gene rearrangements in the second IgH locus. Reth et al showed [22] using pre-B cell lines transfected with immunoglobulin gene constructs that the expression of the μm is required for V_κ to J_κ rearrangement of κ light chain gene. Furthermore, analysis of λ5-deficient mice [12] and μm-deficient mice [13] showed that in the absence of these proteins, B cell development is blocked at the pre-B cell stage. These results suggested that the surrogate light chains/μ complex is required in pre-B cells to allow

their maturation into B cells.

We found a novel gene, VpreB-3 (formerly named 8HS20) which has an immunoglobulin domain structure and 36% amino acid sequence homology to VpreB-1.[24] Biochemical analysis revealed that the VpreB-3 proteins associate with μ chains in $\mu^+\kappa^-$ pre-B cells, like the proteins encoded by VpreB-1 and λ5.[19, 24] Northern blot analysis revealed that VpreB-3 is selectively transcribed in pre-B cells and bone marrow-derived B cell lines and it is expressed only at low levels in later stage B cells, such as IgM-producing hybridoma cells, B cell lymphoma and splenocytes.[24] Thus it is thought that the expression of this gene is dependent on the differentiation stage of B cells. In the present study, to examine the function of the VpreB-3, I generated transgenic mice carrying the VpreB-3 gene under the control of the IgH enhancer and the SV40 promoter and examined the effects of the transgene on B cell development.

MATERIALS AND METHODS

Production of VpreB-3 transgenic mice

To strongly express the transgene to B cells, I constructed the expression vector as shown in Fig. 1. Briefly, a 0.5 kb SalI - XhoI fragment of SV40 polyadenylation (polyA) signal sequence was inserted into the SalI site of pBlue Script SKII$^-$ (Strategene, CA). To the 5-side of the polyA signal sequence, a 1.9 kb Pst I fragment of the genomic VpreB-3 DNA and 2 kb Hind III fragment including mouse IgH enhancer[2] and SV40 promoter[3, 6, 21] were inserted. Then, the 4.4 kb SacI - XbaI fragment of the expression vector was microinjected into fertilized eggs of C57BL/6 mice, and the viable eggs were implanted into oviducts of pseudopregnant ICR female mice.

To determine the presence or absence of the transgene, 10 ng of genomic DNA from a tail biopsy was introduced to DNA dot blot analysis, using the 240 bp fragment of the

SV40 poly A additional signal sequence as the probe. The DNA dot blot analysis showed that several lines of transgenic mice were produced. The effects of the transgene on B cell development were examined using offspring from line No. 41 and 118 of those mice.

Northern blot analysis

Total RNAs were extracted from various organs of the transgenic and littermate mice using the guanidine isothiocyanate method.[5] Ten μg of total RNA was electrophoresed in 1.2 M formaldehyde-1.2% agarose gel and transferred to a nitrocellulose membrane. Hybridization was carried out under stringent conditions with a ^{32}P-labeled VpreB-3 cDNA probe. Filters were stripped and rehybridized with mouse β actin cDNA probe.

RNase protection assay

The RNase protection assay was carried out, as described previously.[17] To prepare the RNA probe, the 369 bp EcoRI-BamHI fragment of the VpreB-3 transgenic vector (Fig. 1) was subcloned into the pBlue Script SK II$^-$ vector and this plasmid was linearized with EcoRI, and then transcribed with T3 polymerase (Promega, Madison) in transcription buffer containing [α-^{32}P] CTP. Next, 10 μg of total RNA was hybridized with the ^{32}P-labeled RNA probe overnight at 65 ℃ in 80% formamide hybridization buffer followed by digestion with RNase A and T1 (Sigma, St. Louis, MO) at 37 ℃ for 1 h. The protected fragments were separated on 6% polyacrylamide-urea denaturing gel by electrophoresis and were exposed to X-ray film.

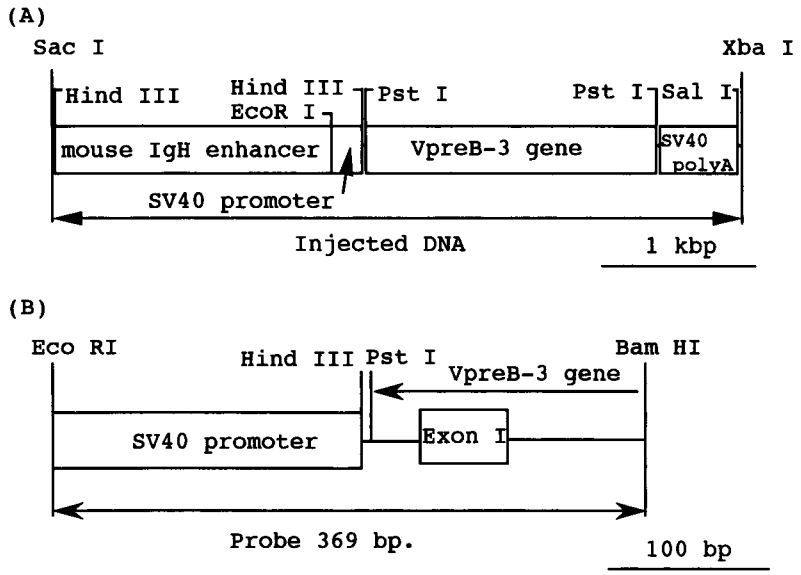

Fig. 1. Construction of the expression vector and the **probe for RNase protection assay.**
A. The VpreB-3 expression vector.
B. The probe for RNase protection assay.

Immunoprecipitation

Metabolic labeling of cells and immunoprecipitations were performed as described previously.[24] Briefly, cells were labeled with [^{35}S] Cys (0.5 mCi, ICN 51002) for 3 h at 37 ℃ and lysed with ice-cold PBS lysis buffer [10 mM phosphate buffer (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 5 mM EDTA] containing 15% glycerol and 10 mM iodoacetamide, supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF), 50 μg/ml leupeptin, 100 μg/ml pepstatin, and 20 μg/ml aprotinin before use. The cell lysates were pre-cleared with preimmune rabbit immunoglobulin coupled to Sepharose 4B and incubated with purified anti-VpreB-3 antiserum [24] immobilized on Sepharose 4B at 4 ℃ for 4 h. After washing the Sepharose beads with

PBS lysis buffer, the samples were analyzed on SDS-PAGE (15%).

Bone marrow cell culture

Bone marrow cells were isolated from the littermate control and the transgenic mice and resuspended with RPMI 1640 medium containing 5% fetal calf serum, 50 mg/L of streptomycin, $5X10^4$ units/L of penicillin, and $5X10^{-5}$ M of β-mercaptoethanol. The cells were then cultured on the PA6 stromal cells in a 25 cm^2 flask for 14 days as previously described[7] and then 20 U/ml of recombinant IL-7 (kindly provided by Dr. Tetsuo Sudo, Toray Research Institute, Yokohama, Japan) was added to the culture medium. The culture medium was exchanged every 3 days.

Cell surface staining

The bone marrow cells, spleen cells and the cultured bone marrow cells from the littermate control and the transgenic mice were used for the cell surface staining. The cells were incubated with FITC-conjugated monoclonal anti-μ (#331.12; Pharmingen, San Diego, CA) and biotin-conjugated monoclonal anti-CD45R (#RA3-6B2; Pharmingen) for 60 min on ice. The cells were then further incubated with Phycoprobe PE-streptavidin (Biomeda, Foster city, CA) for 30 min on ice. The stained cells were analyzed by FACScan (Becton- Dickinson, Mountain View, CA). Dead cells were excluded by propidium iodide staining.

RESULTS

The expression of VpreB-3 mRNA in the transgenic mice

First, I evaluated the expression of VpreB-3 mRNA in the bone marrow and spleen by Northern blot analysis (Fig. 2). The expression of VpreB-3 in the bone marrow and spleen of control mice was at low levels compared with the

murine $\mu^{+}\kappa^{-}$ pre-B cell line 46-6. However, the transgenic mice strongly expressed VpreB-3 mRNA in both the bone marrow and spleen and the expression levels were comparable with the pre-B cell line 46-6.

Next, I confirmed the expression of the VpreB-3 transgene by RNase protection assay. From the results in Fig. 3, it was shown that transgenic mice expressed VpreB-3 transgene in the spleen and thymus, which is transcribed from SV40 promoter. The expression of the transgene was also detected in the bone marrow (data not shown). The thymus of the transgenic mice expressed the VpreB-3 transgene at low levels compared with the spleen, while the expression of VpreB-3 was not detected in the thymus of the control mice as previously reported.[24] Furthermore, although I examined the expression of the transgene in the liver and kidney, the expression of the transgene was not detectable in either organs (data not shown).

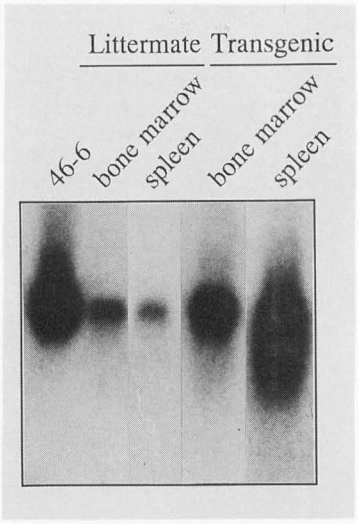

Fig. 2. Northern blot analysis of VpreB-3.
Ten μg of total RNA prepared from each sample was subjected to agarose gel electrophoresis followed by transfer to a nitrocellulose filter and hybridization with the ^{32}P-labeled Vpre-B3 cDNA probe.

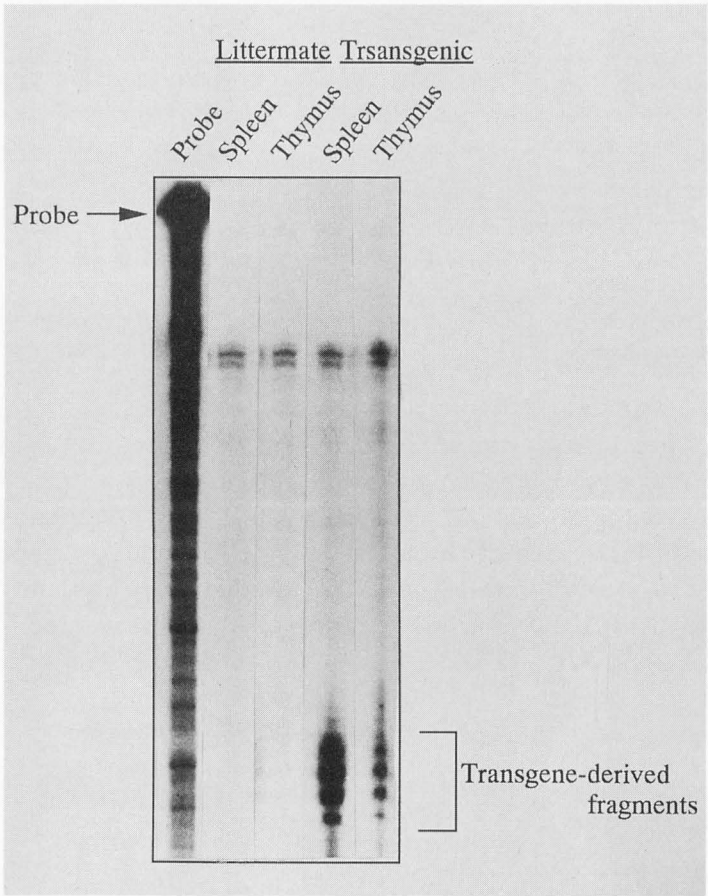

Fig. 3. RNase protection assay of VpreB-3 transgene.
Total RNA was hybridized with the RNA probe, then digested with RNase A and T1. The protected fragments were separated on 6% polyacrylamide-urea gel. The protection bands of the transgene which is transcribed from SV40 promoter were expected to be 154, 153 and 147 bp.[3] Therefore, it is considered that the lowest band in the figure may result from the secondary structure of the transgene-derived protected fragments. The 81 bp protection band derived from the endogenous VpreB-3 was detected in the spleen of both the control and transgenic mice (data not shown).

VpreB-3 gene products in the transgenic mice

The properties of the VpreB-3 gene product were studied by immunoprecipitation using a rabbit antiserum raised against the C-terminal peptide of VpreB-3. As shown in Fig. 4, the antiserum precipitated molecules from the cell

lysate of the $\mu^+\kappa^-$ pre-B cell line Ig6.3, migrating with apparent molecular weights (Mw) of 13 - 14 kDa and 15.5 - 16 kDa. Moreover, analysis using two-dimensional (2D) gel electrophoresis indicated that the VpreB-3 gene encodes molecules with apparent Mw of 14 and 16 kDa in the PI range 4.1 - 4.2 and with apparent Mw of 15.5 and 13 - 13.5 kDa in the PI range 4.7 - 4.8. The results of Western blot analysis indicated that these molecules were the products of the VpreB-3 gene (data not shown). However, these molecules were not detectable in the $\mu^+\kappa^+$ B cell lymphoma WEHI 231.

The expression of these molecules, which are considered to be the VpreB-3 gene products, were compared in the littermate control mice and the VpreB-3 transgenic mice. As shown in Fig. 4, the VpreB-3 transgenic mice strongly express the VpreB-3 gene products in the bone marrow and spleen compared with control mice. The expression levels were comparable with that of $\mu^+\kappa^-$ pre-B cell line Ig6.3, as expected from the results of the Northern blot analysis.

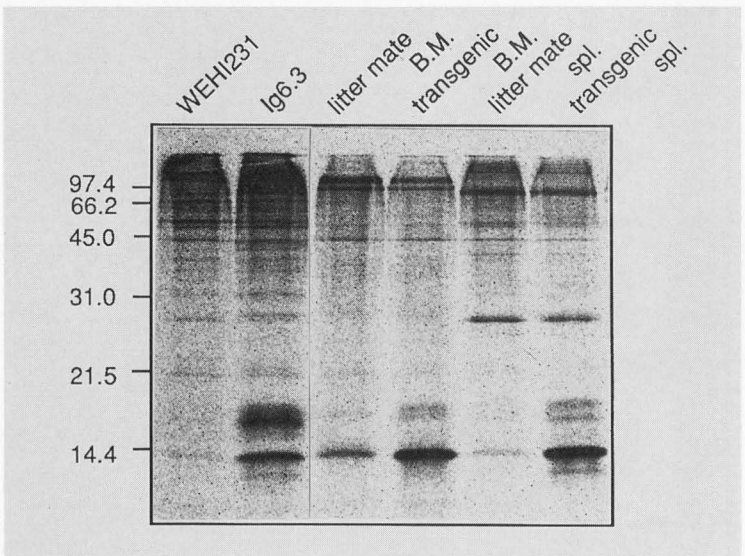

Fig. 4. Analysis of VpreB-3 gene products.
Cells were labeled with [^{35}S] cysteine, lysed and immunoprecipitated with anti-VpreB-3 antibody. The precipitates were electrophoresed under reducing conditions on a 15% SDS-polyacrylamide gel. The left shows apparent molecular weights (kDa).
B.M. : bone marrow, spl. : spleen.

B cell frequency in the VpreB-3 transgenic mice

B cells in bone marrow and spleen were analyzed by flow cytometry using anti-μ and anti-CD45R antibodies. Cells from normal littermates served as controls. As shown in Table I, the frequency of B cells was reduced to half in the bone marrow of one of the six 5 - 6 week old transgenic mice and also in the bone marrow and spleen of only one of the five 8 - 12 week old mice, but in the rest of the transgenic mice B cells were present at an equal frequency as in the control mice. Although cells from transgenic mice over 16 weeks old were also examined, the frequency of B cells was not changed. The frequency of κ chain expressing B cells in the spleen appeared to be also unchanged (data not shown).

The peritoneum is populated in part by a distinct subset of B cells characterized by the expression of the Mac I surface antigen. The origin of these Ly1 B cells is thought to be different from that of the conventional B cells and Ly1 B cells have the ability to self-renew. Although the frequency of the Ly1 B cells in the peritoneum of the transgenic mice was examined, the frequency appeared to be also equal to that of the control mice (data not shown).

The frequency of T cells in the thymus was also determined using anti-CD4 and anti-CD8 antibodies. However, the frequency of the $CD4^+$ and $CD8^+$ cells in the thymus of the transgenic mice appeared to be unchanged from that of control mice.

Effects of the VpreB-3 transgene on B cell development in the stromal cell-dependent bone marrow cell culture

The effects of the VpreB-3 transgene on in vitro B cell development using stromal cell were studied. Bone marrow cells from the VpreB-3 transgenic mice and littermate control mice were cultured on the PA6 stromal cell layer

Table I. The frequency of B cells in bone marrow and spleen from littermate and transgenic mice.
Cells from littermate control (L) and transgenic mice (T) were stained with anti-CD45R and anti-μ antibodies. The stained cells were analyzed by FACScan. The total number of bone marrow cells or spleen cells from the littermate control and transgenic mice was almost equal.

			Positive cells (%)	
age (weeks)	L / T	Tissue	CD45R$^+$	μ^+
5 - 6	L	Bone marrow	11.4	3.1
	T	Bone marrow	7.3	1.6
8 - 12	L	Bone marrow	7.3	1.9
	T	Bone marrow	3.3	0.8
	L	Spleen	35.0	30.9
	T	Spleen	13.3	13.5

Table II. Effects of the VpreB-3 transgene on early B cell development in the bone marrow cell culture.
The total cell numbers for the control and transgenic mice were 1.5 X 10^7, 8 X 10^6 cells, respectively at 7 days after adding IL-7. The total number of cells were almost equal at 14 days after adding IL-7.

	Positive cells (%)		
	- IL-7 (14 days)	IL-7 (20 U/ml)	
	Pre-culture	7 days	14 days
Littermate			
CD45R$^+$	2.95	26.7	34.8
CD45R$^+$ μ^+	0.07	0.00	1.73
Transgenic			
CD45R$^+$	2.34	3.47	25.6
CD45R$^+$ μ^+	0.00	0.00	1.18

for 14 days without IL-7 (i.e., preculture). $CD45R^+$, μ^- B cells and $CD45R^+$, μ^+ B cells in bone marrow cells were depleted during the preculture period and then 20 U/ml of IL-7 was added to the culture medium. The frequency of $CD45R^+$ and μ^+ B cells was determined by using flow cytometry. Five independent experiments were performed. It was shown that early B cell development was delayed in the bone marrow cultures from 3 of the 5 transgenic mice compared with control mice. The appearance of $CD45R^+$ B cells was delayed in the cultures from 3 transgenic mice. A representative result of the retardation of B cell development is shown in Table II. While 26.7% of the cells had become $CD45R^+$ B cells in the control culture by day 7, only 3.47% of the cells had become $CD45R^+$ B cells in the cultures from the transgenic mice. However, by day 14, the difference had become slight. The frequency of $CD45R^+$ B cells was 34.8% and 25.6% in the control culture and in the culture from transgenic mice, respectively.

DISCUSSION

μ chains are expressed in association with "surrogate" L chains encoded by the $\lambda 5$ and VpreB-1 genes in pre-B cells before the expression of L chain.[4, 11, 14, 15, 20, 23, 25] In addition to the $\lambda 5$ and VpreB-1, the proteins encoded by the VpreB-3 gene also associate with the μ chains in the $\mu^+\kappa^-$ pre-B cells.[24]

In the present studies, from the results of the Northern blot analysis, RNase protection assay and immunoprecipitation assay (see Fig. 2, 3 and 4), it has been clearly demonstrated that the transgenic mice in which the expression of VpreB-3 was enhanced, were successfully produced. Flow cytometry analysis of the transgenic mice revealed that the number of $CD45R^+$ and μ^+ B cells were reduced in 2 of the 11 transgenic mice (see Table I). The reduction was observed in the both offspring

from line No. 41 and 118 of transgenic mice. Thus, it appears that the VpreB-3 transgene has the ability to inhibit B cell generation. However, the other molecules could mostly compensate for the inhibition of B cell generation caused by the expression of the transgene in vivo. This suggestion is supported by the results of in vitro bone marrow cultures using the stromal cell line PA6 (see Table II).

The observation, the VpreB-3 gene is selectively transcribed in $\mu^+\kappa^-$ pre-B cells but not in $\mu^+\kappa^+$ B cells,[24] led to the hypothesis that the down regulation of VpreB-3 expression might be a trigger for the expression of μ-κ complex on the cell surface. However, flow cytometry analysis revealed that the number of κ chain expressing B cells in the spleen of the transgenic mice in which the expression of VpreB-3 was enhanced was unchanged from that of normal mice. On the other hand, the results of in vitro bone marrow culture experiments revealed that the frequency of $CD45R^+$ B cells on day 7 after adding IL-7 drastically decreased in the culture from bone marrow cells of the transgenic mice. The decrease was observed in the culture from bone marrow cells of the both offspring from line No. 41 and 118 of transgenic mice and it was notable in the culture from bone marrow cells of mice from line No. 41 compared with No. 118 (data not shown). These results suggest that the expression of the transgene has the ability to induce the retardation of the differentiation from $CD45R^-$ B progenitor cells to surface μ^- $CD45R^+$ pro-B cells and / or inhibit the proliferation of $CD45R^+$ pro-B cells. However, the latter does not seem likely, because $CD45R^+$ B cells were in almost equal numbers on day 14 after adding IL-7, in both the control and the transgenic mice. We (unpublished data) and Karasuyamara et al [9] found that λ5, VpreB-1 and VpreB-3 genes are expressed in not only μ^+ κ^- pre-B cells but also in surface μ^- pro-B cells. Furthermore, the expression of the λ5 was also detected in $CD45R^-$ B

progenitor cells.[10] These results suggest that these molecules function in the μ^- or CD45R$^-$ B progenitor cells of the normal mice. Therefore, the present report helps to clarify the role of VpreB-3 in the B progenitor cells of the normal mice.

The VpreB-3 gene encodes molecules with distinct properties, in terms of Mw and PI value.[24] We suggested that these molecules are generated by alternative RNA processing and glycosilation (S. Hagiwara, in preparation). Ohnishi et al have reported that VpreB-3 products associate with μ chains shortly after their synthesis, but are not expressed in association with μ chains on the cell surface.[19] From these results we speculate that VpreB-3 may contribute to μ chains transport in $\mu^+\kappa^-$ pre-B cells. However, the present finding suggest that VpreB-3 has the ability to function in the B progenitor cells before they express μm. Further detailed analysis using those cells isolated from normal and transgenic mice or VpreB-3 deficient mice is needed to ascertain the role of the VpreB-3.

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