The EMBO Journal vol.12 no.5 pp.1827–1834, 1993

# A novel gene product associated with μ chains in immature B cells

**Takuji Shirasawa[1,5], Kazuo Ohnishi[1], Shinji Hagiwara[1,2], Kazuhiro Shigemoto[1], Yutaka Takebe[3], Klaus Rajewsky[4] and Toshitada Takemori[1,6]**

[1]Department of Immunology, NIH, Tokyo, [2]Research and Development Division, Sandoz Pharmaceuticals, Tokyo, [3]Center for AIDS Research, NIH, Japan and [4]Institute for Genetics, University of Cologne, Cologne, Germany

[5]Present address: Department of Molecular Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

[6]Corresponding author

Communicated by K.Rajewsky

**A previously unreported B cell specific gene, which we have named 8HS-20, was isolated from the cDNA library of a pre-B cell clone by subtraction and differential hybridization. This gene is selectively expressed as a 0.75 kb transcript in pre-B and bone marrow-derived B cell lines; a transcript of the same size is also found in bone marrow and, albeit at low levels, in spleen. The deduced amino acid sequence of the 8HS-20 cDNA displayed homology to a B cell specific gene, VpreB-1, and to members of the immunoglobulin supergene family including Vλ, Vκ, $V_H$, TCRVα, Vβ and CD8. Biochemical analysis using purified antiserum against 8HS-20 oligopeptides indicates that the gene encodes proteins with mol. wts of 13.5, 14, 15.5 and 16 kDa, which associate with μ chains in pre-B cell lines, and that these molecules are expressed concomitantly with VpreB-1 and λ5 gene products in the same cell lines.**

*Key words:* B cell maturation/immunoglobulin supergene family/μ chain complex/μ chain expression/surrogate light chains

## Introduction

The IgM antigen receptor on B cells is composed of antigen-binding Igμ heavy (H) and light (L) chains non-covalently associated with disulfide-linked heterodimers of the proteins designated IgMα and Igβ (Hermanson *et al.*, 1988; Hombach *et al.*, 1988, 1990; Sakaguchi *et al.*, 1988; Campbell and Cambier, 1990; Stappert and Reth, 1990; Reth *et al.*, 1991). However, in B cell development, the expression of membrane μ chain precedes that of L chains; in pre-B cells, in which the genes encoding L chains have not yet been rearranged, μ chains are expressed on the membrane in association with 'surrogate' L chains, molecules of 21 and 15.5 kDa (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987; Pillai and Baltimore, 1987; Kerr *et al.*, 1989; Takemori *et al.*, 1990; Cherayil and Pillai, 1991; Nishimoto *et al.*, 1991). The 21 and 15.5 kDa proteins are encoded by the λ5 and VpreB-1 genes, respectively, and are selectively transcribed in pre-B cells (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). These molecules appear to play a crucial role in μ chain transport and expression (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990).

In addition to the λ5 and VpreB-1 gene products, the μ chains of some pre-B cell lines also appear to be associated with a 14 kDa molecule (Takemori *et al.*, 1990). This 14 kDa protein had previously been observed to associate with intracellular μ chains shortly after their synthesis, whereas the λ5 and VpreB-1 gene products appear to form a complex with a significant amount of μ chain later on. However, the origin and nature of the 14 kDa molecule was not clear. In the present report we describe a novel murine B cell specific gene, designated 8HS-20, which is selectively expressed in pre-B and bone marrow-derived B cell lines. This gene displays homology to L chain V region domain (VL) and VpreB and encodes a 14 kDa protein which associates with intracellular μ chains in pre-B cells.

## Results

### *8HS-20 gene is expressed in pre-B cell lines*

The 8HS-20 cDNA clone was isolated from the cDNA library of a pre-B cell clone 496-138 by subtraction and differential hybridization (see Materials and methods). As shown in Figure 1A, 8HS-20 is selectively expressed as a 0.75 kb transcript in the $\mu^-$ pre-B cell line 46-6 (Takemori *et al.*, 1990) and the bone marrow-derived B cell line CYG34 (Nishikawa *et al.*, 1988) as well as in the $\mu m^+$ pre-B cell lines Ig6.3 (Takemori *et al.*, 1990) and 70Z/3 (Kincade *et al.*, 1981; data not shown). A message of the same size is expressed, albeit at a low level, in splenocytes and in the hybridoma cell line B1-8 which secretes IgM antibodies with specificity for the hapten NP (Reth *et al.*, 1978). The level of the message was more or less the same in splenocytes irrespective of whether the splenic B cells were stimulated with bacterial lipopolysaccharide (LPS), suggesting that B cell activation by LPS did not influence the expression of the 8HS-20 gene. In contrast, the message was undetectable in the B cell lymphoma WEHI231 (Boyd and Schrader, 1981), in the plasmacytoma P3U-1 and in non-B-lymphoid lines including the erythroid lineage line T3-C12 (Ikawa *et al.*, 1976), the macrophage line 5RM (kindly provided by Dr Soejima) and the T cell line EL-4 (T.Shirasawa and T.Takemori, data not shown). In tissues, 0.75 kb 8HS-20 transcripts were exclusively found in bone marrow and, at low level, in spleen (Figure 1B). A 0.9 kb transcript hybridizing with the 8HS-20 cDNA probe was detectable in lung on longer exposure (Figure 1B, middle panel), although a cDNA clone corresponding to this longer mRNA was not isolated from the pre-B cell cDNA library.

Southern blot analysis showed that a single 12 kb *Eco*RI fragment hybridized with the 8HS-20 cDNA probe under stringent washing conditions in mouse liver and 46-6 genomic DNA (Figure 1C, right panel), indicating that

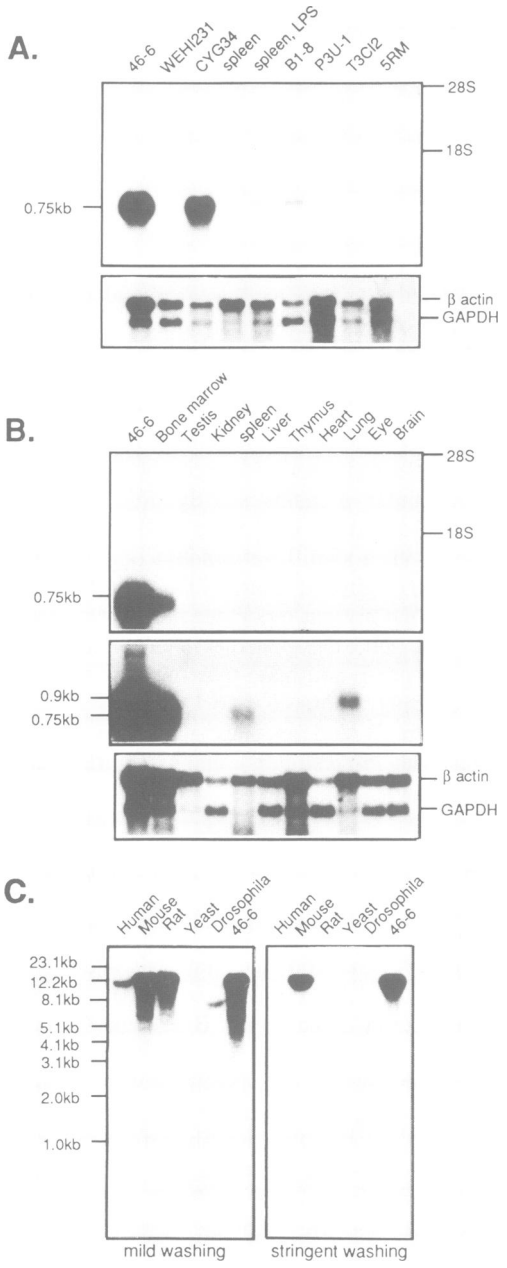



**Fig. 1.** (**A**) Northern blot analysis of 46-6, WEHI231, CYG34, splenocytes, splenocytes cultured in the presence of LPS (Gibco, San Francisco) at a final concentration of 20 μg/ml for 24 h, B1-8, P3U-1, the erythroid cell line T3-Cl2 and the macrophage cell line 5RM, probed with 8HS-20 cDNA. (**B**) Northern blot analysis of the expression of 8HS-20 in 46-6, and bone marrow, testis, kidney, spleen, liver, thymus, heart, lung, eye and brain of BALB/c mice. The same filter probed with 8HS-20 was exposed for 20 h (top panel) and 70 h (middle panel) to Kodak XAR-5 film. Thereafter, the filter was re-probed with β-actin and GAPDH cDNAs (lower panel). (**C**) Southern blot analysis of human placenta, mouse kidney, rat kidney, *S.cerevisiae, Drosophila* and 46-6 probed with 8HS-20 cDNA. Each filter was washed under mild (left) and stringent (right) conditions as described in Materials and methods.

8HS-20 is a single copy gene and that the gene is not rearranged in somatic cells. Southern blot analysis carried out under mild washing conditions indicated that genes cross-hybridizing with 8HS-20 were detectable in genomic DNA from human, rat and *Drosophila* (Figure 1C, left panel) as well as from rabbit and chicken (data not shown).

### *The structure of the 8HS-20 gene*

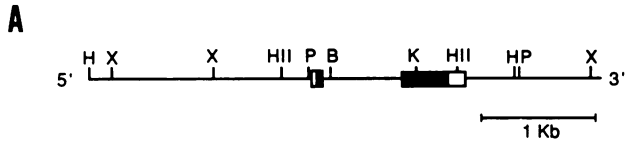
The 8HS-20 cDNA clone consists of 583 nucleotides containing a single open reading frame of 123 amino acids, which would encode a putative polypeptide with a predicted molecular mass of 13 kDa (11 kDa without leader peptide). This open reading frame was flanked by 30 bp of 5′ untranslated region (UT) and 181 bp of 3′ UT containing three tandem repeats of a polyadenylation signal (Figure 2B). Using this cDNA as a probe three clones, 8HS-20g2, 8HS-20g13 and 8HS-20g27, were isolated from the genomic library. The restriction maps of a 3.3 kb *Xba*I and a 3.6 kb *Hin*dIII fragment of 8HS-20g2 were determined (Figure 2A) and the 2630 nucleotides of the *Xba*I−*Hin*dIII subfragment containing the 8HS-20 coding sequence were sequenced (Figure 2B). Comparison of the genomic sequence with the cDNA sequence of 8HS-20 revealed that the gene consists of two exons separated by a 732 bp intron (Figure 2A and B). The first exon encodes a stretch of 19 amino acids which is hydrophobic and has a leucine-rich region, typical of an IgV region signal sequence. An open reading frame encoding 104 amino acids defines the second exon, which is limited at the 5′ end by the splicing signal CAG/T at nucleotide position 1671 (Figure 2B, underlined). In addition, another splicing signal, CAG/G, is present at nucleotide 1719, accompanied by six pyrimidine-rich nucleotides upstream of the consensus sequence. This additional splicing signal obeys the canonical rules (Breathnach and Chambon, 1981). No recombination signal heptamer or nonamer sequences were found at the 3′ end of the 8HS-20 gene.

The 5′ region of the 8HS-20 gene contains a TATA sequence which is absent from the promoter region of VpreB genes (Okabe *et al.*, 1992a). The conserved octanucleotide ATTTGCAT, located ~100 bp 5′ to the initiator ATG of all Vλ and Vκ genes (Falkner and Zachau, 1984; Parslow *et al.*, 1984), is located at position 785, 97 bp 5′ to the ATG initiation codon. There are sequence homologies to other *cis*-acting regulatory elements including a consensus motif indispensable for the promoter activity of the VpreB-1 gene (Okabe *et al.*, 1992b; data not shown). The nucleotide sequence around the ATG initiator codon is compatible with Kozak's consensus sequence (ACCATGG) including C at position −2, C at position −1, and G at position +4 (Kozak, 1983).

### *The 8HS-20 gene displays homology with members of the immunoglobulin supergene family*

A homology search for the 8HS-20 cDNA nucleotide sequence in the GenBank and EMBO databases revealed that 8HS-20 represents a previously unreported gene. The 8HS-20 cDNA sequence encoding the longest open reading frame has 53−54% homology with VpreB-1 and the human Vλ, pag-1, which may belong to Vλ subgroup V (Hughes-Jones *et al.*, 1990) (T.Takemori, data not shown). In addition, at positions 1864−1967 (see Figure 2) the 8HS-20 cDNA has 59−64% homology with the framework region (FR)III of murine Vλ1 (Bernard *et al.*, 1978) and murine Vκ, 106−10E, which belongs to the Vκ21 subgroup (Reininger *et al.*, 1990) as well as human Vλ, pag-1, and Vκ, EV15, which is closely related to the subgroup 1 Vκ gene, HK102 (Stavnezer *et al.*, 1985).

A computer search of the NBRF protein sequence database revealed that the deduced amino acid sequence of 8HS-20 between position +1 and +103 displayed 38% homology with human Vλ, pag-1, 37% homology with murine VpreB-1




B

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   1  CTAGACCCAGACTATACCAGGGGTAGTGTATTAGTCAGGAGTCTTTAGAGTCACAAAACT
  61  TATGGAATGTCTCTCTATATTGAGGGAATTTATTGGAACGACTTACAGTCTGTAGTCCAA
 121  CTAACCCAACAATGGTCAGCTGTGGATGGGAAGTCCATGGACCTGGTAGTTGCTCAGTCC
 181  CACTAGGGTAGCTATTTCAGCTGGCCTTCTGTATAAGCTGGAAGCCTGAAGTAGGCTCCA
 241  ACAGATGTGCTGGCCAGTAAGTGCAAGCAGGTGAAGAGAGTGAGCCTTCCTTCTTCCAAT
 301  GTCCGTATGGAGGCCTTCCAGCCAGAAGGTGTGGCCCAGATTAAAGATGTGTACCACCAT
 361  GCCTGGATCTGGGACTTGCTTGTCCCAGGCTGACGCAGAGGTCTCCTTGCCTCAGTCTCC
 421  TGGGATTAACAGCGTGTACTACCTTGCCTGGGCCTAAGCTCTCCATGCCACTATGGCCTC
 481  AAGATCTCTGAGCCAAATCCAGGTCAGAAATGTGTGTCTTCCAGCCACAAGATCTGAATC
 541  ACAGGTGTGCCCTCCAATTCTGGATCATAGTTCATTTCAGATATAGTCAGTTGACCACCA
 601  GAAATAGTCATTACAGGTAGATATACACTAGGAAAGGGTATGAGAGGCTGTCTGGGGAAT
 661  GCCAACTTCCTGCAAATGGATGTGAAGGGCTCTGATCTCCTGCTGGCTGAGATGCCAGGA
 721  CATTTGGAAGATAGGGCCTGACCCTGGCCCCAGACACATGTGTCGTCCTTCAGTCAGTGG
 781  GCTGATTTGCATGCTCTATTGTGGTCCAGAGACTTCCCCATCCCTGTACTACAAGCATCC
                  1 ----> cDNA
 841  GGAGCACAGTACTGCAGAACTCTAGGTCGGCCTTTCTGCTTCTGGCCATGGCCTGCCCTG
                  ↑                                M A C P G  5

 901  GTTGCCTGCCTCTGCTCCTGATAGGAACCTTTGTGGCAGGTACGGCACATCCAGGAACGA
        C L P L L L I G T F V A V                              18

 961  GGTGGGGGTTGGCACCAGCTCAGGGAAACTCATGCAAAGAAGTGAATCAACAGGGTAAGG
1021  GGGGTGGATCCTAGGAGAGGCATTGGGGTAGAGCAGAGAGCCGGCTGTAGGCAACTCTGT
1081  CCAGGACTAGGGGAAAGCCACTTTCCGTGTGGACCTTAGCTTCCCAATTTGTGGGTCTGC
1141  TTAGACTGTGGTCCTTCAGTCGTCTCTGAGGAGAGGGCAACTTCTTGCCTTTGTTTCTCA
1201  GAGAGGAGGCTAATAAAGATGGGCAGCCCCCTGCTCCACAGATCCTTTAAGGCAATCCTC
1261  TGTGGCCAGCAGTTTGAAAGTCAAGGATGACAGTTAAGCATTCTGAGAGGTCTGGAGGGC
1321  AGTTACTTTGTAGCTGAGGTTGAGTTATTAGATTGAGGGGTAAGGGTAGAGAGAAGGAGG
1381  GTAGTAGGTACTAGGGGGACCTAGAAGGTCACACTTGAACACTAGGCTCTCAACTGAACA
1441  TGGATTCTTGGAGACAGGACAGTAATGCTAATTTAGGGGTCACTTATTCACAAGCCCCCA
1501  TTCACAGAGGAAACCGGAACGTAGATGGAGCAAACTATGTCTCACAGAACCATCCAGGGC
1561  ACTAGGCTCTGAAAAATATTTGGGAGTGGGAATCTTTGCAGTCTGATAAAATATTTGTAG
1621  CAAGGTAGGAAGGAGCCCAGGCTAGCCCTTAGTGGGTCTGTGTCTTTTTCAGTCTTTCAG
                                                        F Q  20

1681  CCAACTCTGACCCAGCCTGACGCGTTCTCAGTCTTCCCAGGCCAAGACGCTCATCTCAGC
      P T L T Q P D A F S V F P G Q D A H L S  40

1741  TGCACCATCAATTCCCAGCATGCCACCGCTGGAGATATTGGGGTGTCGTGGTACCAGCAG
      C T I N S Q H A T A G D I G V S W Y Q Q  60

1801  CAGCCAGGAAGTGCTCCCCATCTTCTCTACTATTACGCAGAAGAGGAACACTATCGGCCC
      Q P G S A P H L L Y Y Y A E E E H Y R P  80

1861  GCTGACATCCCCGACAGATTCTCAGCTACCGTGGATGCAGCCCACAACGCCTGTATCCTC
      A D I P D R F S A T V D A A H N A C I L 100

1921  ACCATCAGCCCCGTGCTCCCGGAGGATGATGCCGATTACTTCTGTTCCATCGCCCACACC
      T I S P V L P E D D A D Y F C S I A H T 120

1981  TTTGAACCCTAGGGCTGGGTTGAACATCTTCTGTCTCCTAGGTCTGCCCCCTGACCTTGG
      F E P *  (123)

2041  GCCTCCTCTTTTTCTCTTAAGTCTTGCTTTCTTTCCTTCCTCTGTGAAATGAGTTAATAA
2101  CACTCGACGGGTCAACCACTGTCCTGAGAACTGTGAGATCCTGTGTAAACAAATAAATAA
2161  AATAAATGTCAAATTGGAGGATGTGGAGGTTGGGATTTTGATGATTGTTGTCTAAAGAGG
2221  AAACACCTGAGACCCTTAGGGAATGTGGCCCAGCTTTGTCCCTATCCCTTAGGGGTTTAG
2281  CTTCCCTCCTGGCCTTCCAGTGACCTGAAGGTAGTGAAACAGGAACCAACACTTGCCACC
2341  CTCCCCCGAACCCCCGCCCCCAAGTCCTGCATGGCTGATTGTGGTCCAACGCTCATGGTT
2401  CTTGGGTGGCTGGTCCCCATGGAGTTCATGCTGATGGGGAGGGGAGGGGCAGCCCTGCCT
2461  CTTTTCTGAAGTCTCCGGGCAGGTTCCCAAAGATCTTGATGGTTAGAGGCAGGTGTTCTC
2521  AGAAGGGCCGTGAGGGGCAGAAATGAGGATATTGAAAGCAGGTAAGAGATTATGCAGCAT
2581  CCTTCCCTGCTCAGAAACAGTGTGTTCTACTGATTCCCTGAGGGAAGCTT  (2630)
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**Fig. 2.** Restriction map, genomic sequence and deduced amino acid sequence of 8HS-20. (**A**) Structure and restriction map of the 8HS-20 gene. Exons and coding regions are represented by open and solid boxes, respectively. Restriction enzymes: H, *Hin*dIII; X, *Xba*I; HII, *Hin*cII; P, *Pst*I; B, *Bam*HI; K, *Kpn*I. (**B**) Nucleotide sequence of 8HS-20 genomic DNA (*Xba*I−*Hin*dIII fragment) with deduced amino acid sequence of the open reading frame. Numbering of nucleotides and amino acids is shown on the left and right, respectively. The first nucleotide of the 8HS-20-2 cDNA is at position 858 in the genomic sequence and is indicated by an arrow. The 8HS-20-2 cDNA consists of 583 nucleotides corresponding to the positions of 858−939 and 1673−2173 in the genomic sequence. Numbering of amino acid residues starts with 888 as the first position of the leader and proceeds to 1678 as the first position of the putative mature protein. The polyadenylation signal is underlined. The splicing signals GT and AG are double underlined. The asterisk points to the termination codon TAG. CCAATT and TATA sequences in the putative promoter region are boxed.

(Kudo and Melchers, 1987), 35% homology with human V$\varkappa$, EV15, 34% homology with murine V$\varkappa$, 106−10E, 27% homology with murine V$\lambda$1 and 21−27% homology with products of the murine immunoglobulin supergene family, namely $V_H$ and TcRV$\alpha$, V$\beta$ and CD8 (Figure 3). Although 8HS-20 demonstrated amino acid homology to VpreB-1 and VL region segments, including V$\lambda$1, they also exhibit some structural differences: VpreB-1 and 8HS-20 contain four and three extra amino acids, respectively, within the region corresponding to the CDR2 of V$\lambda$1 and two extra

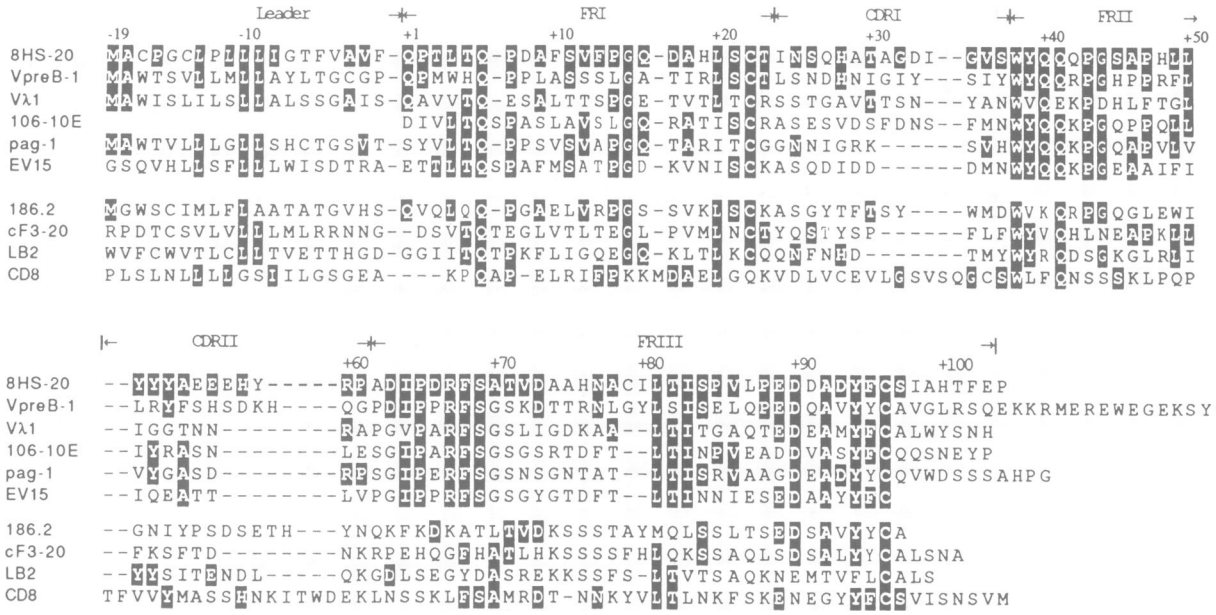


**Fig. 3.** Alignment of the deduced amino acid sequence of 8HS-20 with the sequences of murine VpreB-1 (Kudo and Melchers, 1987), Vλ1 (Bernard *et al.*, 1978), V$\varkappa$, 106−10E (Reininger *et al.*, 1990), human Vλ, pag-1 (Hughes-Jones *et al.*, 1990), human V$\varkappa$, EVK15 (Stavnezer *et al.*, 1985), murine $V_H$, V186.2 (Bothwell *et al.*, 1981), TCRV$\alpha$, cF3-20 (Chou *et al.*, 1986), TCRV$\beta$, LB2 (Patten *et al.*, 1984) and CD8 (Nakauchi *et al.*, 1985). 106−10E was cloned from a hybridoma producing an IgM antibody specific for bromeaine-treated erythrocytes, pag-1 from a hybridoma producing IgG1 with specificity for erythrocyte membrane antigen, and 186.2 from an IgM-producing hybridoma with specificity for the hapten, NP. EVK15 was cloned from Epstein−Barr virus transformed cells of a $\varkappa$-deficient patient. cF3-20 was cloned from an alloreactive cytotoxic T cell clone and LB2 from a helper T cell line which recognizes chicken erythrocytes. Residues homologous between 8HS-20 and any of the other sequences are in black boxes. The numbering of positions follows that given for 8HS-20 in Figure 2. Leader, CDRI and II and FRI, II and III indicate the locations of the leader, the complementarity determining regions (CDRs) and framework regions (FRs) found in the variable regions of Vλ1.

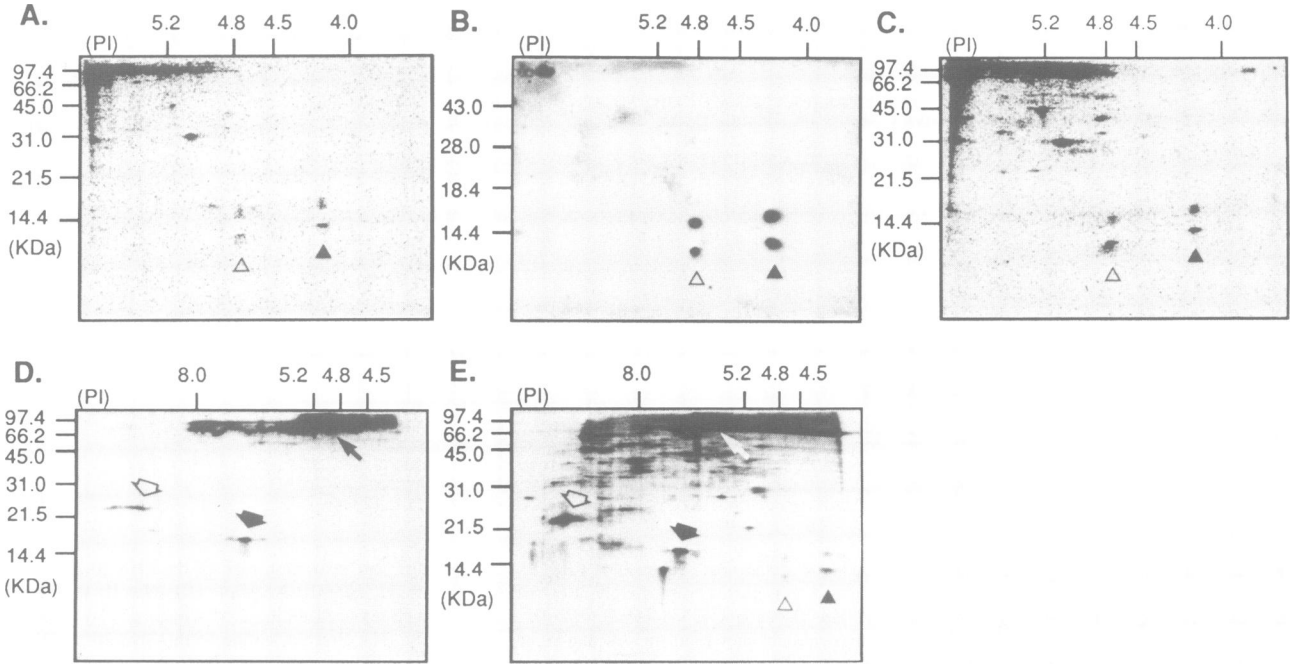


**Fig. 4.** Assembly of the 8HS-20 gene product with $\mu$ chains in a pre-B cell line Ig6.3. (**A**, **C** and **E**) were metabolically labelled with [$^{35}$S]cysteine. The labelled cell lysates of Ig6.3 were immunoprecipitated with purified anti-8HS-20 antiserum raised against $Cys^{96}$−$Phe^{104}$ peptide (A) or purified goat anti-$\mu$ antiserum (C and E). A murine fibroblast cell line, $Ltk^-$, which was transfected with $\mu$, λ5 and VpreB-1 genes (Karasuyama *et al.*, 1990), was labelled with [$^{35}$S]cysteine. The culture supernatant of the $Ltk^-$ transfectant was immunoprecipitated with goat anti-$\mu$ antiserum (**D**). The immunoprecipitated proteins were resolved by IEF (A and C) or NEPHGE (D and E) in one dimension, followed by SDS−PAGE under reducing conditions. In Western blot analysis (**B**), Ig6.3 proteins immunoprecipitated with anti-8HS-20 antiserum were separated by IEF/SDS−PAGE, blotted onto an Immobilon membrane and incubated with anti-8HS-20 antiserum followed by $^{125}$I-labelled protein A. Open and solid arrowheads show the 8HS-20 molecules. Open and solid arrows show λ5 and VpreB-1 products, respectively. The white arrow indicates $\mu$ chain.

amino acids in the region corresponding to the Vλ1 FRIII. Glutamine and serine are conserved at positions 69 and 70 within the FRIII in VpreB-1, Vλ1 and other VL region segments, whereas these amino acids are replaced by alanine and threonine in 8HS-20. VpreB-1 also contains an additional 19 amino acids at the C-terminus (Kudo and Melchers, 1987) compared with 8HS-20 and Vλ1. Cysteines are found at amino acid positions 22 and 96 and a tryptophan at position 38 in the 8HS-20 deduced protein sequence. These residues could be engaged in intrachain disulfide-bond formation, a characteristic structural feature of Ig domains. In addition to these cysteine residues, 8HS-20 has an extra cysteine residue at position 79, possibly forming interchain disulfide-links with cysteine residues on other molecules such as the immunoglobulin heavy chain (see below).

### *The 8HS-20 gene product associates with μ chains in pre-B cell lines*

The properties of the 8HS-20 gene product were characterized by two-dimensional (2D) gel electrophoresis using a rabbit antiserum raised against the C-terminal peptide of 8HS-20 (see Materials and methods). As shown in Figure 4A, the antiserum precipitated molecules from the cell lysate of the cell line Ig6.3 migrating with apparent mol. wts of 14 and 16 kDa in the pI range 4.1−4.2 (solid arrowheads) and with apparent mol. wts of 15.5 and 13−13.5 kDa in the pI range 4.7−4.8 (open arrowheads). The results of Western blot analysis, shown in Figure 4B, indicated that these molecules were the products of the 8HS-20 gene. We tentatively designated the molecules migrating at pH 4.1−4.2 8HS-20α and those at pI 4.7−4.8 8HS-20β (see Discussion). Molecules with the same properties were also detectable in Ig6.3 by immunoprecipitation using anti-μ antibodies, which indicates that the 8HS-20 gene products are associated with μ chains (Figure 4C). These molecules appear to be expressed in bone marrow cells also, as shown by immunoprecipitation using one-dimensional SDS−PAGE (S.Hagiwara, unpublished). Preliminary experiments suggested that the 16 and 15.5 kDa molecules are generated from the 14 and 13.5 kDa molecules, respectively, by glycosylation, since peptide *N*-glycosidase F (PNGase F) treatment of the precipitates with anti-8HS-20 antiserum resulted in the loss of the 16 and 15.5 kDa spots but not the 14 and 13−13.5 kDa ones on a 2D gel (K.Ohnishi, unpublished).

The λ5 protein migrated with an apparent mol. wt of 21−22 kDa and at fairly basic pH, and VpreB-1 with an apparent mol. wt of 15.5 kDa and pH 6.8 on 2D non-equilibrium pH gradient electrophoresis/SDS−PAGE (NEPHGE/SDS−PAGE, Figure 4D, solid and open arrows). When a lysate of the Ig6.3 line was precipitated with anti-μ antibodies and the precipitate electrophoresed under the same conditions, spots appeared in the position of 8HS-20 as well as in those of λ5 and VpreB-1 (Figure 4, solid and open arrows). This indicates that μ chains in Ig6.3 are associated with the λ5, VpreB-1 and 8HS-20 gene products. Northern blot or immunoprecipitation analysis using anti-μ antibodies suggested that the λ5, VpreB-1 and 8HS-20 genes or their products are concomitantly expressed also in other cell lines, including the $\mu^-$ pre-B cell lines 46-6 and SCID7 (Ogawa *et al.*, 1989), the $\mu^+$ pre-B cell line 70Z/3 and the $\mu^+\kappa^+$ B cell line CYG34 (data not shown).

When the anti-μ immunoprecipitates of Ig6.3 were run on 2D diagonal electrophoresis, molecules of 22, 16 and 14 kDa running either at the diagonal or an off-diagonal position were identified (Figure 5A). The 22 kDa molecule on this gel is considered to be the λ5 gene product. The Western blot analysis shown in Figure 5B revealed that the 14 kDa molecule migrating at either the diagonal or an off-diagonal position and the 16 and 13.5 kDa molecules on the diagonal were revealed by anti-8HS-20 antiserum (Figure 5B). These results suggest that all these molecular species are encoded by the 8HS-20 gene but that only the 14 kDa molecule is able to assemble with μ chains via interchain disulfide-linkage. Anti-μ antibodies co-precipitated the 16 kDa molecule at the off-diagonal position on 2D electrophoresis (Figure 5A). Since this molecule does not react with anti-8HS-20 antibodies, it may not be encoded by the 8HS-20 gene.

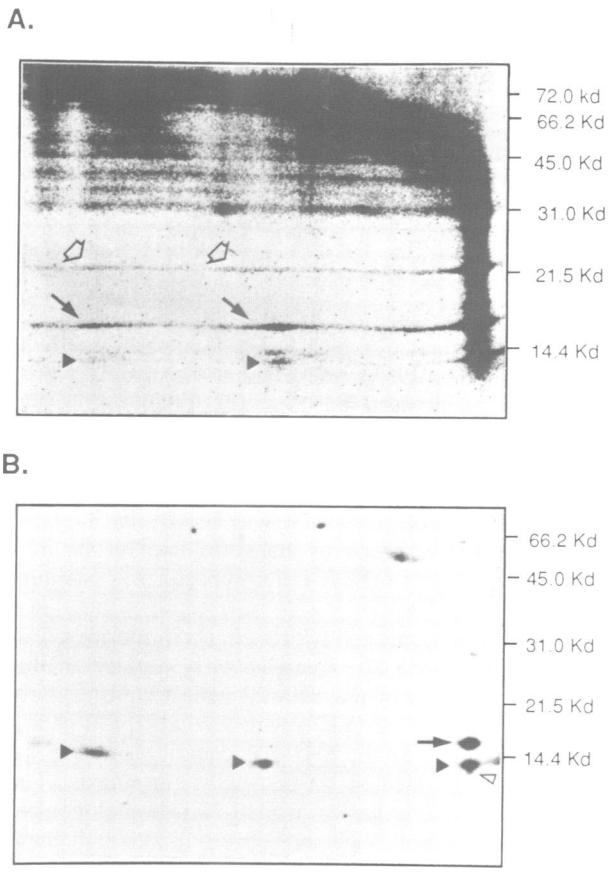


**Fig. 5.** Analysis of 8HS-20 gene products and their association with μ chains in Ig6.3 on diagonal gels. (**A**) Ig6.3 was labelled with [$^{35}$S]cysteine, lysed and immunoprecipitated with goat anti-μ antibodies. The precipitates were electrophoresed under non-reducing conditions on a 6% SDS−polyacrylamide gel in the first dimension and under reducing conditions on a 15% SDS−polyacrylamide gel in the second dimension. (**B**) Ig6.3 was lysed and immunoprecipitated with anti-μ antibodies. The precipitates were resolved on diagonal gels as in (A), blotted onto an Immobilon membrane and incubated with anti-8HS-20 antiserum followed by $^{125}$I-labelled protein A. The closed arrowhead indicates the 14 kDa molecule, the open arrowhead the 13.5 kDa molecule, the solid arrow the 16 kDa molecule and the open arrow the 21 kDa molecule.

## Discussion

The expression of the membrane form of the μ chains is a critical step in B cell development already at the pre-B cell stage, i.e. before L chain expression (Iglesias *et al.*, 1987;

Nussenzweig *et al.*, 1987; Reth *et al.*, 1987; Era *et al.*, 1991; Kitamura *et al.*, 1991). At this developmental stage the μ chains form a complex with pre-B specific 'surrogate' L chains, and two proteins serving as surrogate L chains have been characterized in the past in molecular terms, namely λ5 and VpreB-1 (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). The λ5 and VpreB-1 proteins appear to be critical for μ chain transport and surface expression (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990). Together with other associated molecules (Hermanson *et al.*, 1988; Hombach *et al.*, 1988; 1990; Sakaguchi *et al.*, 1988; Campbell and Cambier, 1990; Stappert and Reth, 1990; Reth *et al.*, 1991), the μ chain-surrogate L chain complex may be involved in the control of the proliferation of pre-B cells as well as H chain allelic exclusion and onset of L chain gene rearrangement (Iglesias *et al.*, 1987; Nussenzweig *et al.*, 1987; Reth *et al.*, 1987; Era *et al.*, 1991; Kitamura *et al.*, 1991). It was shown that targeted disruption of the λ5 gene in the germ line results in a significant depletion of the B cell compartment (Kitamura *et al.*, 1992).

A third protein associated with μ chains in murine pre-B cells, designated 8HS-20, is molecularly defined in this paper. The structure of the 8HS-20 gene is most closely related to V region gene (VL) segments and VpreB genes, but in contrast to these the 8HS-20 genomic gene consists of two exons separated by an unusually long intron. The deduced amino acid sequence of 8HS-20 is homologous to that of VLs including Vλ1 and VpreB-1, although these proteins exhibit some structural differences among each other; VpreB-1 and 8HS-20 contain extra amino acids within the regions corresponding to CDRII and FRIII of Vλ1. VpreB-1 carries a long C-terminal portion which is not observed in 8HS-20 and VL. Like the λ5 and VpreB-1 genes, the 8HS-20 gene is exclusively expressed in pre-B cell lines and also in $\mu^+\kappa^+$B cell lines derived from bone marrow which co-express the μ chain-surrogate L chain complex (Takemori *et al.*, 1990). These cell lines were observed to co-express the λ5 and VpreB-1 genes or their products, suggesting that the λ5, VpreB-1 and 8HS-20 genes are coordinately regulated during early B cell maturation. The λ5 and VpreB-1 genes are turned off at a later stage of B cell maturation and are not expressed in the cells present in peripheral lymphoid organs (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). In contrast, the 8HS-20 gene is expressed in an IgM-producing hybridoma cell line and in splenocytes, albeit at low levels, suggesting that the expression of the 8HS-20 gene is not regulated in a strictly stage-specific manner.

In the human, three proteins with apparent mol. wts of 16, 18 and 22 kDa were identified as associating with μ chains in pre-B cell lines (Kerr *et al.*, 1989). The 18 and 22 kDa molecules are encoded by the human VpreB and 14.1 genes, respectively (Bauer *et al.*, 1988; Hollis *et al.*, 1989). Since the deduced amino acid sequence of 14.1 revealed that its C-terminal shares homology with Jλ and Cλ and its N-terminal half with the λ5 gene product, it was suggested that the 14.1 and λ5 are human and mouse homologues (Hollis *et al.*, 1989). Regarding the additional 16 kDa molecule, serologically λ-like, it seems that neither the 14.1 gene nor one of the genes which have been isolated as homologues of 14.1 encodes this molecule (Chang *et al.*, 1986; Schiff *et al.*, 1989; Bossy *et al.*, 1991). Since a 8HS-20-homologous sequence appears to be present in human genomic DNA as detected by low stringency hybridization, it is tempting to speculate that the 16 kDa molecule might be encoded by a human homologue of the 8HS-20 gene.

We demonstrate that four molecules migrating on SDS gels with apparent mol. wts of 14 and 16 kDa (pI 4.1–4.2; 8HS-20β) and 15.5 and 13–13.5 kDa (pI 4.7–4.8; 8HS-20α) were 8HS-20 gene products. In a preliminary experiment, with the B cell line WEHI231 transfected with 8HS-20 cDNA, anti-8HS-20 antiserum immunoprecipitated 8HS-20β but not 8HS-20α (K.Ohnishi, unpublished), suggesting that the 8HS-20α observed in pre-B cells might be generated by post-translational modification, perhaps peculiar to the stage of B cell maturation, or by alternative RNA processing. Consistent with this, genomic and cDNA sequences of 8HS-20 show that an additional splicing site is found within exon 2. Although isolated 8HS-20 cDNA clones have so far been observed to contain a single open reading frame of 123 amino acids, another form of 8HS-20 mRNA could be generated by the choice of an additional splicing site to which the leader is joined (see Figure 2). This mRNA might be translated as a molecule containing 111 amino acids with a predicted molecular mass of 12 kDa (10 kDa without leader peptide). Further experiments are needed to settle this point.

The sequences of the λ5 and VpreB-1 genes show a strong homology to those of the Cλ and Vλ genes, respectively. λ5 contains a preterminal cysteine residue capable of forming a disulfide linkage with μ chain, similar to conventional light chains. It is suggested that the λ5 and VpreB gene products form an L-chain-like complex in the absence of μ chains, probably by association between the N-terminal end of λ5 and the C-terminal portion of the VpreB-1 gene product (Kudo *et al.*, 1989). When μ chains are synthesized, the λ5 and VpreB-1 complex binds μ chains where VpreB-1 most likely binds the $V_H$ domain and λ5 could bind the $C_H1$ domain, probably through a disulfide linkage. Accordingly, the μm chain in pre-B cells form a complex composed of λ5 and VpreB-1 and display an IgM-like structure. In addition, these proteins bind intracellular Dμ chain which lacks the $V_H$ domain (Tsubata *et al.*, 1991), suggesting that λ5 may play a crucial role in the assembly of the μ–λ5–VpreB-1 complex.

Unlike VpreB-1, the 8HS-20 product lacks a long C-terminal portion responsible for the non-covalent association with the λ5 protein (Kudo *et al.*, 1987). Within our limited analysis by immunoprecipitation resolved on a one-dimensional SDS gel, we could not observe 8HS-20 antiserum to coprecipitate molecules with mol. wts close to those of the λ5 and VpreB-1 gene products, in spite of the fact that λ5 and VpreB-1 molecules appear to be synthesized coordinately in the same cells (S.Hagiwara, unpublished). This supports the idea that 8HS-20 binds μ chain independently and not in association with λ5 and VpreB-1. We have previously observed that a 14 kDa molecule, which is now considered to be a product of the 8HS-20 gene, associates with μ chains shortly after their synthesis, whereas μ chain assembly with the λ5 and VpreB-1 complex becomes dominant later on (Takemori *et al.*, 1990; K.Ohnishi, in preparation). When Ig6.3 was labelled with $Na^{125}I$ and the lysates were immunoprecipitated with anti-μ antiserum followed by the resolution of the immunoprecipitates on 2D

isoelectric focusing (IEF)/SDS−PAGE, spots did not appear in the position of 8HS-20 (K.Ohnishi, in preparation). This suggests that the 8HS-20 gene product is not expressed in association with μ chains on the cell surface. From these results we speculate that 8HS-20 may contribute to μ chain transport in pre-B cells at an initial phase, prior to λ5 and VpreB-1. Further analysis will be needed to ascertain whether the 8HS-20 gene indeed plays a critical role in normal B cell development.

## Materials and methods

### *Cell lines*

The immature B cell clone 46-6 was obtained by transforming BALB/c bone marrow cells with a temperature-sensitive (ts) mutant of A-MuLV at 35°C (Takemori *et al.*, 1987). Clones Ig6.3 and Ig6.11, the former expressing μm and the latter μ and κ chains, were established from 46-6 cells that had been cultured at a non-permissive temperature. The cell line 70Z/3 is a chemically induced pre-B cell lymphoma (Kincade *et al.*, 1981). B cell clones SCID7 and CYG34 were established from BALB/c bone marrow cells by culturing the cells on stroma cells derived from bone marrow. These were kindly given by Dr Nishikawa (1988). SCID7 is in the stage of $\mu^-$ pre-B cells. CYG34 was initially a $\mu^+$ pre-B cell but all the cells became $\mu^+\kappa^+$ during cultivation in the presence of feeder cells. WEHI231 is a B cell lymphoma (Boyd and Schrader, 1981) and B1-8 is a hybridoma cell line which produces antibody (μ/λ1) with specificity for the hapten NP (Reth *et al.*, 1978). T3-Cl2 is an erythroid lineage cell line and was kindly given to us by Dr Ikawa (Ikawa *et al.*, 1976). The macrophage cell line 5RM was derived from spleen cells transformed with SV40, which was kindly provided by Dr Soejima. All cell lines were cultured at 37°C except 46-6 which was cultured at 35.5°C.

### *Isolation of 8HS-20 cDNA*

The 8HS-20 cDNA clone was isolated from the cDNA library of the pre-B cell clone 496-138 cultured at 39.5°C for 2 days, using a subtracted probe prepared from 496-138. 496-138 was transformed with a ts mutant of A-MuLV and matured to express surface IgM when cultured at 39.5°C for 4 days (Takemori *et al.*, 1987). Under these conditions, the cells co-expressed the μ-surrogate L chain complex. The cDNA library of 496-138 was constructed by the method of Okayama and Berg (1982). The library was amplified once and plasmid DNA was prepared to transform competent DH5 for the subsequent subtractive screening. To prepare the subtracted cDNA probe, $^{32}$P-labelled first strand cDNA of 496-138 cultured at 39.5°C was hybridized with a 20-fold excess of poly(A)$^+$ RNA of 496-138 cultured at 35.5°C in 20 μl of hybridization solution (0.5 M sodium phosphate, 0.1% SDS, 5 mM EDTA and 500 μg/ml herring sperm DNA) at 68°C for 28 h (Crot = 154). After the first round of hybridization, single-stranded probes were separated by hydroxylapatite (HAT) column chromatography (Bio-Rad; Hedrick *et al.*, 1984). The subtracted first strand cDNA probes were re-hybridized under the same conditions as the first round of subtractive hybridization with a mixture of poly(A)$^+$ RNA derived from 496-138 cultured at 35.5°C and another pre-B cell line, 5-49, cultured at 39.5°C and at 35.5°C. 5-49 was established from bone marrow cells transformed with wild-type A-MuLV (Takemori *et al.*, 1987). Colonies ($1 \times 10^5$) were screened by a standard hybridization technique (Sambrook *et al.*, 1989). 150 positive colonies were isolated with subtracted cDNA probe and re-screened by differential hybridization. Briefly, positive colonies were transferred to four nitrocellulose filters and each of the replicated filters was hybridized with the first strand cDNA probe of 496-138 cultured at 35.5°C, 496-138 cultured at 39.5°C, 5-49 cultured at 35.5°C or 5-49 cultured at 39.5°C. Twenty-five clones hybridized to the cDNA probe of 496-138 cultured at 39.5°C but not to that of 5-49 cultured at 39.5°C. One of these clones, 8HS-20, was found to be specific for immature B cells by Northern blot analysis and was analysed further. It was observed that the level of 8HS-20 message was significantly higher in 496-138 cells cultured at 39.5°C than in cells cultured at 35.5°C. In order to isolate the full length cDNA clone, a cDNA library was prepared from mRNA of 46-6. Double-stranded cDNA was synthesized, attached to an *Eco*RI linker and ligated to λZap vector (Stratagene, CA). The 0.5 kb *Bam*HI cDNA insert of 8HS-20 was used as a probe to screen $6 \times 10^5$ recombinants and eight out of 60 positive clones were analysed further. The two largest cDNA inserts, 8HS-20-2 (0.6 kb) and 8HS-20-13 (0.6 kb), were mapped with restriction endonucleases and 8HS-20-2 was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase (United States Biochemicals).

### *Isolation of genomic 8HS-20 gene*

A BALB/c liver genomic library was obtained from the Japanese Cancer Research Resources Bank (L1016), which was a *Sau*3AI partial genomic library constructed in the Charon 28 vector. Plaques ($1 \times 10^6$) were screened with a cDNA probe, 0.6 kb *Eco*RI fragment of 8HS-20-2. Three independent clones, 8HS-20g2, 8HS-20g13 and 8HS-20g27, were plaque purified and mapped by restriction enzymes. These clones covered from 5.5 kb upstream to 9.5 kb downstream of the genomic locus of 8HS-20. 2.5 kb *Xba*I−*Hin*dIII fragment of 8HS-20g2 which covers the two exons of 8HS-20 were recloned into Bluescript plasmid. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977).

### *Northern blot analysis*

Total RNAs were extracted from brain, eye, lung, heart, thymus, liver, spleen, kidneys, testis and bone marrow of BALB/c mice using the guanidine/CsCl method (Chirgwin *et al.*, 1979). Ten micrograms of total RNA was electrophoresed in 1.2 M formaldehyde−1.2% agarose gel and transferred to nitrocellulose (NC) membrane. The blot was hybridized with the 0.6 kb *Eco*RI insert of cDNA clone 8HS-20-2. The washed filter was exposed for 20 or 70 h to XAR-5 film (Kodak) at −70°C with an intensifying screen. Filters were stripped and rehybridized with a mixture of mouse β-actin and rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probes.

### *Southern blot analysis*

High molecular weight DNAs were extracted from the pre-B cell line 46-6, mouse kidney, rat kidney, human placenta and total body of *Drosophila melanogaster*. DNAs of rabbit, chicken and *Saccharomyces cerevisiae* were purchased from Clontech, USA. Five micrograms of high molecular weight DNA was digested with *Eco*RI, fractionated on a 0.8% agarose gel and transferred to NC membranes. Hybridization was in 4 × SSC, 5 × Denhardt's, 0.5% SDS, 50 mM Tris (pH 7.4), 10 mM EDTA and 10% dextran sulfate at 65°C. The washing conditions were 2 × SSC and 0.5% SDS at 50°C for mild washing and 0.1 × SSC and 0.5% SDS at 65°C for stringent washing. The blot was hybridized with the 8HS-20-2 cDNA probe. The filter was exposed to XAR-5 film (Kodak) at −70°C with an intensifying screen.

### *Preparation of 8HS-20 antiserum*

Anti-8HS-20 antisera were obtained from rabbits hyperimmunized with synthetic 8HS-20 oligopeptides corresponding to amino acid residues Cys$^{96}$−Phe$^{104}$ which were conjugated to keyhole limpet haemocyanin. Antiserum was purified by affinity column chromatography using BSA-coupled 8HS-20 oligopeptide.

### *Immunoprecipitation and gel electrophoresis*

Ig6.3 and a murine fibroblast cell line, Ltk$^-$, which was transfected with genomic μ gene and VpreB-1 and λ5 cDNAs (Karasuyama *et al.*, 1990) were metabolically labelled with [$^{35}$S]cysteine (0.4 mCi/3 × $10^7$) at 37°C for 3 h. Ig6.3 cells ($5 \times 10^7$) were lysed with ice-cold PBS lysis buffer [1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS, 10 mM phosphate buffer pH 7.4; 100 mM NaCl, 5 mM EDTA (Kunopka *et al.*, 1984)] 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 or normal goat immunoglobulin (GIg) coupled to Sepharose 4B and incubated with purified G anti-μ antiserum or anti-8HS-20 antiserum immobilized on Sepharose 4B at 4°C for 4 h as previously described (Takemori *et al.*, 1990). In the case of the Ltk$^-$ transfectant, 100 μl of $^{35}$S-labelled culture supernatant was used for immunoprecipitation. For Western blot analysis, non-labelled Ig6.3 cells ($5 \times 10^8$) were lysed, precleared with normal GIg, and incubated with anti-8HS-20 antiserum. The Sepharose beads were washed with PBS lysis buffer and proteins were recoverd with 100 μl 8 M urea containing chick gizzard proteins (Hirai and Hirabayashi, 1983). The samples were analysed on IEF/SDS−PAGE and NEPHGE/SDS−PAGE according to the method of O'Farrell (1975) and O'Farrell *et al.* (1977). A pH gradient was formed with a 1:3 mixture of Biolyte 3-10 and Biolyte 5-7 for IEF and Pharmalyte 3-10 for NEPHGE. Isoelectric points were determined by directly measuring the pH of focusing gel pieces and by measuring focused spots of chick gizzard proteins after Coomassie Blue staining. Autoradiography was done by exposure of a dried gel or an Immobilon membrane to XAR-5 film with

an intensifying screen and to an imaging plate (Fuji-Film Co. Ltd, Tokyo) followed by analysis in a BAS2000 Bio-image analyser (Fuji-Film Co. Ltd). 2D diagonal gel electrophoresis was performed according to Goding and Harris (1981). Cell lysates immunoprecipitated with anti-$\mu$ antiserum from non-labelled or metabolically labelled Ig6.3 cells were electrophoresed under non-reducing conditions on a 15% SDS–polyacrylamide gel. The proteins in the gel were then reduced in the presence of 10% 2-mercaptoethanol, 0.1% SDS and 100 mM Tris, pH 6.8, for 2–3 h. Subsequently, the gel was placed on top of a 12% polyacrylamide–SDS gel and run in the second dimension.

## Acknowledgements

We are grateful to Drs U.Krawinkel and Y.Matsuura for technical advice, to Drs Y.Ikawa, T.Soejima, Y.Matsuura, S.-I.Nishikawa and H.Karasuyama for reagents, to Drs H.Kimoto and M.Taniguchi for discussion, to Dr R.Torres for critically reading the paper, and to Ms H.Murayama and S.Sasaki for typing. This work was supported by a Grant-in-Aid for Special Project Research of the Ministry of Education in Japan to T.T. and in the initial phase by a Special Coordination Fund of the Ministry of Science and Technology in Japan to T.T. and the Deutsche Forschungsgemeinschaft through SFB 73 to K.R.

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*Received on July 24, 1992; revised on January 19, 1993*

## Note added in proof

The nucleotide sequence data reported here have been deposited in the GenBank/EMBL data library under the accession number D13208.