

# The $\omega/\lambda 5$ Surrogate Immunoglobulin Light Chain Is Expressed on the Surface of Transitional B Lymphocytes in Murine Bone Marrow

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## Summary

The membrane immunoglobulin heavy chain ( $\mu$ m) plays a feedback role during the pre-B stage of B lymphocyte differentiation. In pre-B cell lines,  $\mu$ m associates with two surrogate light chain proteins. The  $\omega$  chain is disulfide linked to  $\mu$  and was predicted to be the product of the  $\lambda 5$  gene. The  $\iota$  chain is noncovalently associated with  $\mu$ m. We demonstrate that the  $\omega$  protein is indeed the product of the  $\lambda 5$  gene and that  $\mu$ ,  $\omega$ , and  $\iota$  are coassociated in the same complex. Antibodies against the  $\omega/\lambda 5$  protein demonstrate the existence of a subpopulation of "transitional" bone marrow B cells that express  $\mu$ m and  $\omega$  on the cell surface. The majority of these cells also express surface  $\kappa$  light chains, indicating that in B lymphoid ontogeny the  $\lambda 5$  gene is inactivated after the onset of  $\kappa$  light chain expression.

The membrane Ig H chain protein ( $\mu$ m)<sup>1</sup> serves two distinct functions during B cell differentiation. In B cells,  $\mu$ m associates with  $\kappa$  or  $\lambda$  L chains, is transported to the cell surface, and functions as the antigen receptor. In pre-B cells, the  $\mu$ m protein plays a feedback role during differentiation; the synthesis of this protein leads to the shut-off of H chain gene rearrangement, thus mediating allelic exclusion at the H chain locus (1–5). This protein also provides a cue to a pre-B cell permitting it to proceed to the stage of L chain gene rearrangement (6, 7). Pre-B cells have conventionally been described as cells in which  $\mu$  chains are intracellular and are not covalently associated with L chains (8–10). In pre-B cells purified from mouse bone marrow, as well as in a large number of pre-B tumor lines both in the mouse as well as in man,  $\mu$ m is a short-lived intracellular protein that is rapidly degraded (11, 12).

The  $\omega$  protein is a pre-B-specific protein that forms disulfide-linked tetramers with  $\mu$  (13). A second non-disulfide-linked protein,  $\iota$ , is also associated with  $\mu$  in pre-B cells (14). In a subset of murine and human pre-B cell lines (which we refer to as type II pre-B cells, as opposed to type I cells in which  $\mu$ m is intracellular),  $\mu$ m has been demonstrated on the cell surface (15–19). Cell surface  $\mu$ m in these lines is associated with the  $\omega$  and  $\iota$  surrogate Ig L chains (13, 14) or equivalent molecules (20). Based on preliminary radiolabeled sequencing and peptide mapping, we had suggested that the  $\omega$  protein was the product of the pre-B-specific  $\lambda 5$  gene (21, 22). The  $\lambda 5$  gene is the murine homologue of a human

pre-B-specific gene, the 14.1/16.1 gene (23, 24). Indeed, since human pre-B cells also express a protein that is disulfide linked to  $\mu$  (20, 24), this protein was predicted to be the product of the 14.1/16.1 locus and was designated the human  $\omega$  protein (24). Preliminary peptide mapping suggested that the  $\iota$  protein may be the product of the VpreB gene (25). The predicted proteins encoded by the murine  $\lambda 5$  gene and the human 14.1/16.1 gene resemble conventional L chains in their COOH-terminal halves and contain penultimate cysteine residue; much of the NH<sub>2</sub>-terminal portions of these proteins have no homology to Igs. These protein would be predicted to associate with the CH1 domain of  $\mu$  but not with the V domain. The protein encoded by the VpreB gene could conceivably associate with the VH domain. No evidence exists to indicate whether  $\omega$  and  $\iota$  are part of the same complex with  $\mu$ m or form separate complexes with the H chain.

How does  $\mu$ m mediate its feedback role in pre-B cells? We have previously noted (14) that six polypeptides are associated with the  $\mu$ m-surrogate L chain complex in pre-B lymphocytes, and we have suggested that this complex may be involved in signal transduction, generating an appropriate second messenger or messengers that lead to further differentiation. The recent and more complete demonstration of "CD3-like" and other  $\mu$ -associated polypeptides in B lineage cells (26–29), one of which is the product of the mb-1 gene (30) and another possibly the product of the B29 gene, which shares some homology with mb-1 (31), suggests that some or all of these polypeptides may be involved in a signal transduction step in pre-B lymphocytes.

It is unclear whether in pre-B cells  $\mu$ m delivers a feedback

<sup>1</sup> Abbreviation used in this paper:  $\mu$ m, membrane Ig H chain protein.

signal from an intracellular location or from the cell surface. Indeed, the existence of a pre-B stage in the bone marrow in which  $\mu$ m is expressed on the cell surface remains to be established. We report here that the  $\omega$  surrogate L chain is the product of the  $\lambda$ 5 gene, that  $\mu$ ,  $\omega$ , and  $\iota$  are present in the same complex, and that murine bone marrow, but not spleen, contains a "transitional" B lymphoid subpopulation that expresses  $\mu$  and  $\omega/\lambda$ 5 on the cell surface. The majority of these cells also express surface  $\kappa$  L chains, indicating that at the pre-B to B cell transition, activation and expression of the  $\kappa$  locus precedes the shut-off of  $\lambda$ 5 gene expression, and occurs either immediately before or concomitantly with the acquisition of the ability to transport the  $\mu$ m complex to the cell surface. This result also suggests that models involving the generation of a differentiation signal from intracellular  $\mu$ m merit serious consideration.

## Materials and Methods

**Antibodies and Immunoprecipitation.** A synthetic peptide, AGP-RCSHPALPSKPQF, based on the  $\lambda$ 5 cDNA sequence, was conjugated to BSA using a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminoethyl) carbodiimide; Pierce Chemical Co., Rockford, IL), and antipeptide antibodies were generated in rabbits using protocols described previously (12). IgG was purified on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) following the manufacturer's instructions.

Metabolic labeling, cell surface iodination, immunoprecipitation, and analysis on SDS polyacrylamide gels was performed as described previously (13). The lysis buffer used in all experiments was 0.5% Triton X-100 in 10 mM Tris, pH 7.4, with 3 mM  $MgCl_2$  and 10 mM NaCl. *N*-glycanase (Genzyme, Boston, MA) treatment of immunoprecipitates was performed as follows: immune complexes were dissociated and eluted in 50  $\mu$ l of 1% boiling SDS and incubated for 12 h at 37°C with 3 U of enzyme, before analysis.

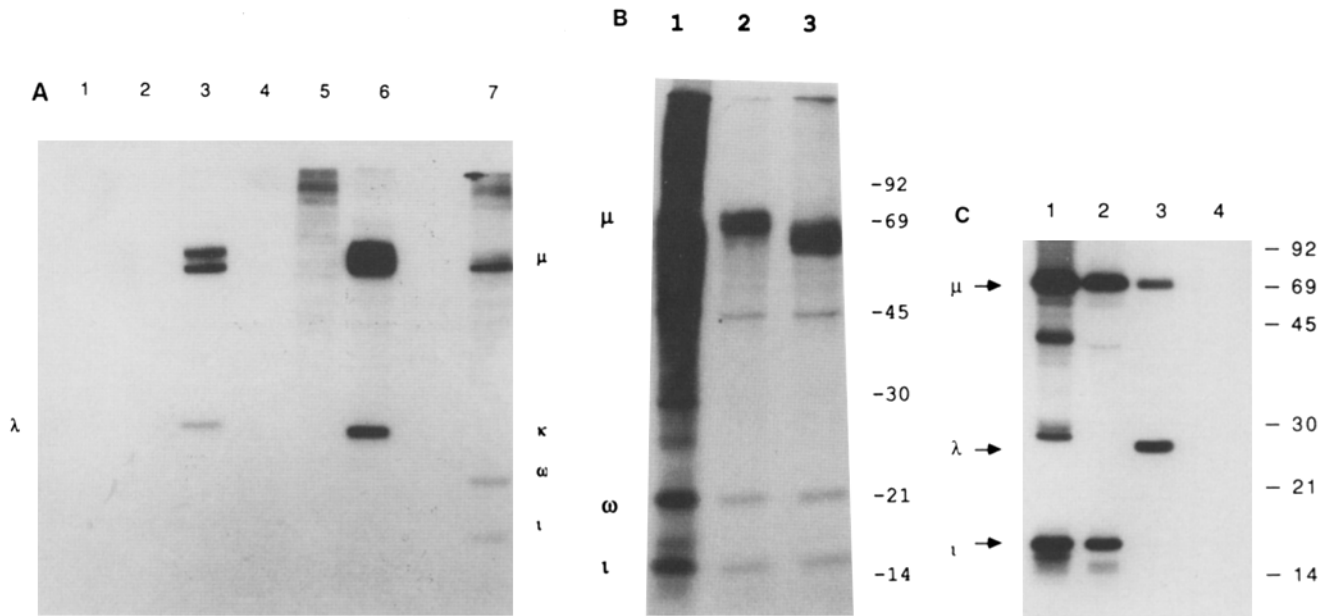
For reimmunoprecipitation studies, a detergent lysate of metabolically labeled NFS 5.3 cells was initially immunoprecipitated using 10  $\mu$ g of anti- $\mu$  and protein A-Sepharose. The precipitate was washed and resuspended in 100  $\mu$ l of 2% (wt/vol) SDS/2%  $\beta$ -mercaptoethanol and boiled for 5 min. The dissociated supernatant was diluted in 2 ml of lysis buffer. One half was reprecipitated with an anti- $\mu$  antibody, and the other with the anti- $\lambda$ 5 antibody.

**Immunofluorescence of Live Cells.** NFS 5.3, AJ9, and WEHI 231 cells (see references 12 and 13 for sources of cell lines) were stained with pre-immune and immune (anti- $\lambda$ 5) rabbit IgG and fluorescent goat anti-rabbit IgG using standard procedures. In brief, 2  $\mu$ g IgG was used in the first step and a 1/20 dilution of FITC-labeled (Fab)<sub>2</sub> fraction of goat anti-rabbit IgG (Caltag) was used as the second-step reagent. BALB/c mouse bone marrow cells were obtained by flushing femurs and tibiae followed by Tris/ $NH_4Cl$  lysis of erythrocytes. Spleen cells were obtained using standard procedures. Samples were simultaneously stained using anti- $\lambda$ 5 (with a FITC-labeled second antibody as described above) and PE-labeled goat anti- $\mu$  (Caltag), used at a 1/10 dilution. An anti- $\kappa$  mAb (PE conjugated; Becton Dickinson & Co., Mountain View, CA) was used to stain NFS 5.3 (negative control), WEHI 231, and bone marrow cells. Samples were analyzed on a FACScan analyzer (Becton Dickinson & Co.). Typically, 5,000–10,000 gated cells were counted for single-color analysis and 50,000–100,000 for two-color analysis.

## Results

**$\mu$ ,  $\omega$ , and  $\iota$  Are Coassociated in Pre-B Cell Lines.** We have previously reported that  $\mu$  in pre-B lymphocytes is associated with a disulfide-linked surrogate L chain,  $\omega$  (originally described as an 18-kD protein; we have subsequently revised our estimation of its size to 20 kD), and a smaller non-disulfide-linked 16-kD chain that we refer to as  $\iota$ . Preliminary radiolabeled sequencing and peptide maps suggested that  $\omega$  was the product of the  $\lambda$ 5 gene and that  $\iota$  was the product of the vPreB gene. Since these chains were identified by their association with  $\mu$ , it could be argued that  $\mu$ 2 $\omega$ 2 complexes constitute a separate species from  $\mu$  complexes. To confirm that the  $\omega$  chain is the product of the  $\lambda$ 5 gene and to obtain evidence regarding the coassociation of  $\mu$ ,  $\omega$ , and  $\iota$ , we raised an antibody against a synthetic peptide based upon a portion of the predicted coding sequence of exon 2 of the  $\lambda$ 5 gene. Immunoprecipitation of metabolically labeled pre-B cells with anti- $\lambda$ 5 and anti- $\mu$  revealed that these antisera recognize identical complexes. On analysis of immunoprecipitates on non-reducing/reducing two-dimensional gels (data not shown), identical off-diagonal species ( $\sim$ 74 and 20 kD corresponding to  $\mu$  and  $\omega$ , respectively) were observed with both anti- $\mu$  and anti- $\lambda$ 5, essentially replicating our previously published observations with anti- $\mu$  immunoprecipitations (18). The anti- $\lambda$ 5 antibody did not crossreact with  $\kappa$  or  $\lambda$  L chains. Metabolically labeled lysates from two B cell lines AJ9 ( $\mu$ ,  $\lambda$ ), and WEHI 231 ( $\mu$ ,  $\kappa$ ) were immunoprecipitated in parallel with a lysate from a type II pre-B cell line, NFS 5.3, with anti- $\lambda$ 5 and control antibodies. As seen in Fig. 1 A, anti- $\lambda$ 5 antibodies bring down  $\mu$ ,  $\omega$ , and  $\iota$  from NFS 5.3 (lane 7) but do not associate specifically with any proteins in the two B cell lines (lanes 2 and 5). The specificity of this antibody for  $\omega$  and the absence of cross reactivity with  $\kappa$  and  $\lambda$  L chains was further confirmed (as described below) by cell surface staining of AJ9, WEHI 231, and mouse spleen cells, all of which were surface  $\lambda$ 5 negative.

When two pre-B cell lines, NFS 5.3 (type II) and 54.3 (type I), were metabolically labeled and immunoprecipitated in parallel with anti- $\lambda$ 5 and anti- $\mu$  antibodies, both antisera specifically precipitated  $\mu$ ,  $\omega$ , and  $\iota$ , indicating that these three chains are associated with one another in the same complex. In Fig. 1 B, an analysis of an immunoprecipitation experiment using NFS 5.3 cells is presented demonstrating that these three chains are brought down both by anti- $\lambda$ 5 (lane 1) and anti- $\mu$  (lane 2) antibodies (see also Fig. 1 A, lane 7). A portion of the anti- $\mu$  immunoprecipitate was treated with *N*-glycanase and analyzed in parallel (lane 3), confirming that the  $\mu$  H chain has N-linked carbohydrate side chains, while  $\omega$  and  $\iota$  are not *N*-glycosylated. We have previously shown (13, 14) that surface iodination of NFS 5.3 cells followed by anti- $\mu$  immunoprecipitation reveals the presence of cell surface  $\mu$ ,  $\omega$ , and  $\iota$ ;  $\omega$ , in contrast to  $\mu$  and  $\iota$ , is poorly iodinated (14), but can be seen as a species disulfide linked to  $\mu$  (13). Recent transfection experiments (32, 33) have confirmed the association of the  $\mu$ ,  $\lambda$ 5 and vPreB gene products and also indicate that the  $\lambda$ 5 gene product is poorly iodinated (32),



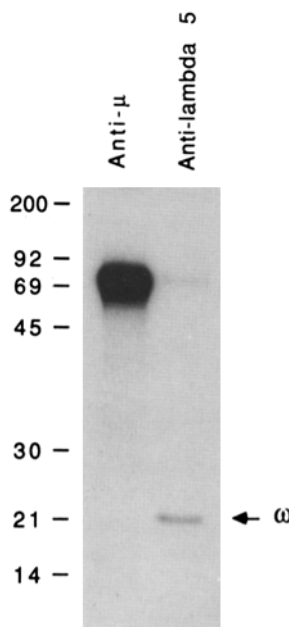
**Figure 1.**  $\mu$ ,  $\omega$ , and  $\iota$  are associated with one another in the same complex. (A) Lysates from metabolically labeled B cell lines AJ9 (lanes 1–3) and WEHI 231 (lanes 4–6) were immunoprecipitated using pre-immune serum (lanes 1 and 4), the anti- $\lambda 5$  antiserum (lanes 2 and 5), and anti- $\mu$  (lanes 4 and 6). Lane 7 contains an anti- $\lambda 5$  immunoprecipitate derived in parallel from metabolically labeled NFS5.3 cells. Samples were analyzed on a 12.5% SDS-polyacrylamide gel. (B) A lysate from metabolically labeled NFS 5.3 cells was immunoprecipitated separately with anti- $\mu$  (lane 2) and anti- $\lambda 5$  (lane 1) antibodies. A portion of the anti- $\mu$  immunoprecipitate was treated with *N*-glycanase (lane 3). Samples were analyzed on a 12.5% SDS-polyacrylamide gel. The position of migration of molecular mass markers (in kD; Rainbow markers; Amersham Corp., Arlington Heights, IL) are depicted adjacent to lane 3. (C) Lysates from cell surface iodinated NFS 5.3 (type II pre-B) and AJ9 (B, expressing  $\mu$  and  $\lambda$ ) were immunoprecipitated with anti- $\mu$  (lanes 1 and 3) and anti- $\lambda 5$  (lanes 2 and 4) antibodies. Samples were analyzed on a 12.5% SDS-polyacrylamide gel.

corroborating our previous observation. To confirm that the anti- $\lambda 5$  antibody recognizes the  $\mu$ ,  $\omega$ , and  $\iota$  complex on the cell surface, surface iodination of NFS 5.3, a type II pre-B cell line, and AJ9, a B cell line, was performed, followed by immunoprecipitation with anti- $\lambda 5$  and anti- $\mu$ . Anti- $\mu$  and anti- $\lambda 5$  both reveal, as predicted, the presence of  $\mu$  and  $\iota$  (Fig. 1 C, lanes 1 and 2) in NFS 5.3 cells. In AJ9, although anti- $\mu$  precipitated  $\mu$  and  $\lambda$  as expected (lane 3), anti- $\lambda 5$  antibodies did not react with any species in these cells (lane 4). We have previously commented (14) on the presence of up to six polypeptides associated with the  $\mu$ m-surrogate L chain complex. The associated proteins seen in Fig. 1 B, lane 1, and C, lanes 1 and 2, are presumed to be products of the “CD3-like” complex that may be involved in the  $\mu$ m-dependent signal transduction process.

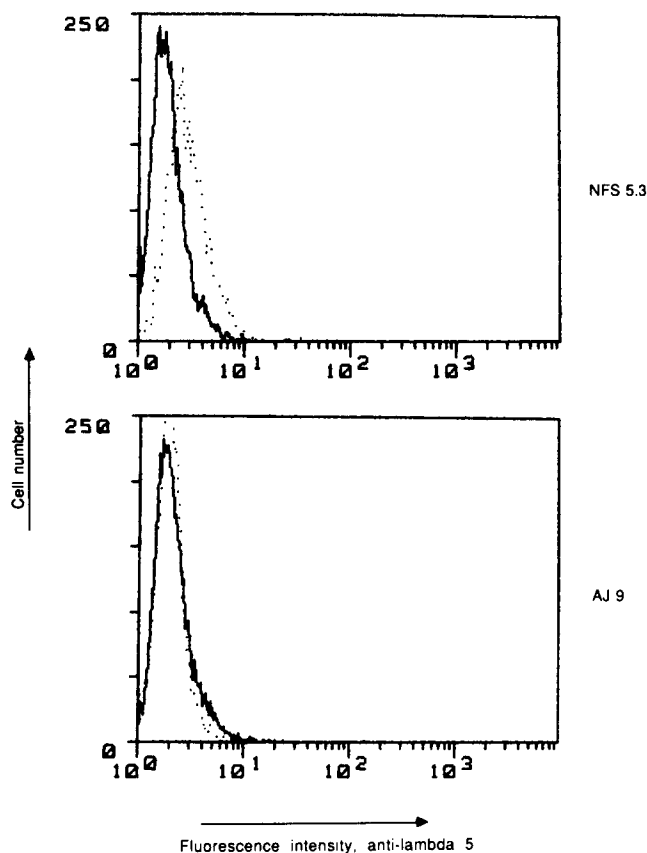
We performed studies with metabolically labeled pre-B cells involving dissociation of anti- $\mu$ -derived immunoprecipitates followed by reprecipitation with anti- $\mu$  and anti- $\lambda 5$ ; an anti- $\mu$  antibody reprecipitated the  $\mu$  protein (Fig. 2, lane 1), and the anti- $\lambda 5$  antibody reprecipitated a 20-kD protein (Fig. 2, lane 2).

*The Anti- $\lambda 5$  Antibody Specifically Stains a Type II Pre-B Cell Line but Not B Cell Lines Expressing Conventional  $\kappa$  or  $\lambda$  L Chains.* In human pre-B cell lines, anti- $\lambda$  L chain antibodies have been reported to crossreact with a  $\mu$ -associated  $\omega$ -like protein (20). To further confirm the specificity of the anti- $\lambda 5$  antibody, we used this antibody to stain a type II pre-B cell

line as well as B cell lines that express  $\kappa$  and  $\lambda$  L chains. The anti- $\lambda 5$  antibody specifically stained the surface (Fig. 3, top) of NFS 5.3 cells. This antibody did not stain AJ9, a B cell line on whose surface a conventional  $\lambda$  L chain is associated



**Figure 2.** Dissociation and reimmunoprecipitation of anti- $\lambda 5$  immune complexes. NFS 5.3 cells were metabolically labeled with [ $^{35}$ S]methionine, lysed, and immunoprecipitated with an anti- $\lambda 5$  antibody. The immune complexes were dissociated and after dilution, reprecipitated with anti- $\mu$  and anti- $\lambda 5$  antibodies. Samples were analyzed on a 12.5% SDS-polyacrylamide gel. The position of migration of molecular mass markers (in kD; Rainbow markers; Amersham Corp.) is indicated. The position of the  $\omega$  band is highlighted by an arrow.



**Figure 3.** Anti- $\lambda 5$  antibodies stain type II pre-B cells but B cells. A type II pre-B cell line, NFS 5.3, and B cell line, AJ9, were separately stained with anti- $\lambda 5$  IgG and nonimmune IgG as the first antibody and FITC-labeled anti-rabbit IgG as the second-step reagent. Solid lines represent pre-immune fluorescence and dotted lines indicate fluorescence with anti- $\lambda 5$ .

with  $\mu$ m (Figure 3, *bottom*) and also did not stain WEHI 231 cells, which express high levels of surface  $\mu$  and  $\kappa$  (data not shown). No surface staining of mouse spleen cells was observed with this antibody (see below), confirming the absence of crossreactivity of this antibody to conventional L chains.

*Mouse Bone Marrow Contains a Population of Cells Expressing Surface  $\mu$ ,  $\omega/\lambda 5$ , and  $\kappa$ .* Pre-B cell lines expressing rearranged H chain genes in both mouse and man can be categorized by their inability to express  $\mu$ m on the cell surface (type I pre-B cells) or their ability to do so (type II pre-B cells). Since the majority of pre-B cell lines are of type I and since studies on bone marrow pre-B cells have confirmed the existence of largely type I cells (intracellular  $\mu$ , no conventional L chains), pre-B cells are widely considered to be cells that do not express  $\mu$  on the cell surface. A particular problem has been the absence of reagents that could distinguish between a B stage surface  $\mu$ m complex ( $\mu$ m and conventional  $\kappa$  or  $\lambda$  L chains) and a pre-B stage surface  $\mu$ m complex ( $\mu$ m and surrogate L chains). We performed two-color double immunofluorescence studies on murine bone marrow cells using antibodies against  $\lambda 5$  and  $\mu$ . As seen in Fig. 4, *top*, two subpopulations expressing surface  $\mu$ m were identified. Approxi-



**Figure 4.** Two-color immunofluorescence reveals a  $\kappa/\omega$  double-positive cell population in murine bone marrow. (*Top*) Bone marrow cells were simultaneously stained with rabbit anti- $\lambda 5$  and FITC-conjugated goat anti-rabbit IgG to stain  $\omega$ - and PE-conjugated goat anti- $\mu$  to stain  $\mu$ m. Two-color FACS analysis was performed with anti- $\mu$ -stained cells revealed along the vertical axis and anti- $\lambda 5$  staining along the horizontal axis. Single-positive  $\mu$ -only cells are seen in quadrant 1, double-positive cells in quadrant 2, and double-negative cells in quadrant 3. No cells were detected that stained only with the anti- $\lambda 5$  antibody (quadrant 4). (*Bottom*) Two-color FACS analysis of bone marrow cells stained with anti- $\kappa$  and anti- $\lambda 5$  reveals that all cells that stain with anti- $\lambda 5$  also express surface  $\kappa$ .

mately 0.5% of total marrow cells (0.2–0.5% in three different experiments; between 5 and 10% of B lineage cells, which include pro-B, pre-B, and B cells) constituted a  $\mu/\omega$  double-positive population that we initially presumed to represent type II pre-B cells. No single-positive  $\omega$ -only cells were detected. Approximately 3% of total marrow cells ( $\sim 30$ –40% of total B lineage cells) were single-positive  $\mu$ -only cells and represent B cells (which are known to have transcriptionally inactivated the  $\lambda 5$  gene).

To determine whether some or all of the cells expressing surface  $\mu$  and  $\omega/\lambda 5$  are actually type II cells, or if they represented a transitional B cell population that had not yet switched off  $\lambda 5$  expression, we performed two-color analyses using antibodies against  $\lambda 5$  and  $\kappa$ . As seen in Fig. 4, *bottom*, the majority of the surface  $\lambda 5$  population also expressed surface  $\kappa$  L chains and represents a “transitional” B cell population.

The number of double-positive  $\kappa/\lambda 5$  cells was identical in two separate determinations to the number of double-positive  $\mu/\lambda 5$  cells. On two-color analysis for  $\kappa$ - and  $\lambda 5$ -positive cells, even when a large number of a gated population of surface  $\lambda 5$ -positive cells was studied, no single-positive  $\lambda 5$  cells were detected. This suggests that the transitional B cell population in addition to expressing  $\omega/\lambda 5$  may be restricted to the conventional  $\kappa$  isotype. Although it is possible that a small proportion of these cells may represent "true" type II cells that do not yet express  $\kappa$  L chains, it is clear that the overwhelming majority of the surface  $\lambda 5$  cells simultaneously express both conventional and surrogate L chains.

Double immunofluorescence and two-color analysis was performed on mouse spleen cells using anti- $\mu$  and anti- $\lambda 5$  antibodies and also using anti- $\kappa$  and anti- $\lambda 5$  antibodies. Approximately 30% of splenic cells were surface  $\mu$  positive and  $\sim 30\%$  were surface  $\kappa$  positive. No spleen cells were stained with the anti- $\lambda 5$  antibody. Transitional B cells probably represent an early stage of ontogeny that is restricted to the marrow in adult mice. It is clear that the anti- $\lambda 5$  antibody shows no crossreactivity whatsoever to conventional  $\kappa$  or  $\lambda$  L chains, which are relatively well represented in the spleen.

## Discussion

Three interesting molecular and cellular events that characterize the pre-B to B cell transition are the surface expression of  $\mu$ m, the transcriptional activation of the  $\kappa$  locus, and the shut off of  $\lambda 5$  gene expression. The temporal order of

these events is an important issue in B cell ontogeny. In this study, we have confirmed that the  $\omega$  surrogate L chain is the product of the  $\lambda 5$  gene and that in pre-B cell lines  $\mu$ ,  $\omega$ , and  $\iota$  are indeed associated with each other in the same complex. Our demonstration of a bone marrow population of "transitional" B cells expressing both surface  $\omega/\lambda 5$  and conventional  $\kappa$  L chains indicates that the activation of the  $\kappa$  locus precedes the inactivation of  $\lambda 5$  gene expression. We have previously noted that in type II pre-B cell lines,  $\mu$ m is transported to the cell surface in association with surrogate L chains and that the expression of a  $\kappa$  L chain is not an absolute requirement for the surface expression of  $\mu$ . Although this evidence from cell lines suggests that the first of the above-mentioned triad of events at this cellular transition is the surface transport of  $\mu$ m, our data on murine bone marrow surface  $\omega$  cells do not provide convincing evidence for the existence of a significant proportion of type II pre-B cells. The "physiological" relevance of type II pre-B cell lines has been always open to question. Both NFS 5.3 and 70Z/3 have rearranged  $\kappa$  loci even though they do not normally express NF $\kappa$ B or  $\kappa$  transcripts. They were both derived from B lymphomas, which may have originally represented transitional B cells. We do not exclude the existence of a "physiological" population of type II cells but must assume that such a stage must either be extremely transient or that levels of surface  $\mu$  and  $\omega$  are extremely low (in which case, signalling through such a receptor may be of questionable significance). Attempts are in progress to see if a bone marrow type II population can be convincingly defined.

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