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A novel regulatory myosin light chain gene distinguishes pre-B cell subsets and is IL-7 inducible

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We describe a novel regulatory myosin light chain gene (termed precursor lymphocyte-specific regulatory light chain or PLRLC) that is expressed specifically in precursor B and T lymphocytes. *PLRLC* is the first example of a regulatory myosin light chain gene which displays specific expression in non-muscle cells. *PLRLC* is expressed in adult bone marrow derived normal and transformed pre-B cells; in the former, *PLRLC* expression levels are induced by the pre-B cell specific growth factor interleukin-7 (IL-7). *PLRLC* is not expressed in either transformed pre-B cells derived from fetal liver or in normal fetal liver pre-B clones grown in the presence of IL-7. Therefore this gene provides the first marker that clearly distinguishes these two pre-B subsets. Finally, several of the different *PLRLC* transcripts potentially encode regulatory myosin light chains with unique structural features. The unique distribution, regulation and structural features of the *PLRLC* gene products suggest an important role for PLRLC during lymphocyte development.

Key words: adult bone marrow/fetal liver/interleukin-7/myosin light chain/pre-lymphocytes

Introduction

Mammalian development depends on the progression of cells along discrete differentiation pathways. The lymphoid lineage has provided useful insights into such differentiation pathways, largely due to the ready availability of cell lines representing progressive stages within this lineage. The differentiation of the B-lymphoid lineage has been divided into three general stages (Alt *et al.*, 1987). B cell progenitors (pro- and pre-B cells) reside in the fetal liver and adult bone marrow; they are characterized by a lack of surface immunoglobulin (sIg) and active recombination of immunoglobulin heavy (IgH) and/or light (IgL) chain gene segments. Productive assembly and expression of IgH and IgL chain genes generate sIg^+ B cells that migrate to peripheral lymphoid organs (spleen and lymph nodes). Subsequent mitogenic or antigenic activation drives B cells to the terminally differentiated, immunoglobulin-secreting plasma cell stage. T-lineage lymphocytes proceed through a differentiation programme in thymus which is in many respects analogous to that of B cells (Marrack and Kappler, 1987).

Progression of both B and T cells through their developmental programmes is mediated by a complex network of regulatory proteins, commonly termed lymphokines or interleukins. Cells that represent various stages of development exhibit distinct interleukin response patterns. The lymphokine interleukin-7 (IL-7) is produced by stromal cells in the bone marrow where it elicits a proliferative but not a differentiation response from pre-B cells; IL-7 does not act upon more mature B-lineage cells (Namen *et al.*, 1988; Lee *et al.*, 1989). In contrast, IL-7 elicits responses from T-lineage cells at different stages, including pre-T cells as well as activated mature T cells (Chazen *et al.*, 1989; Widmer *et al.*, 1990). Accordingly, the expression of the IL-7 receptor gene is restricted to cell lines representing the pre-B stage of B cell development, while it is found in cell lines representing all stages of T cell development (Park *et al.*, 1990; E.M.Oltz and F.W.Alt, unpublished data).

The isolation of genes specific to the pre-B cell stage has yielded molecules that participate in lineage specific developmental processes. For example, the pre-B specific genes $\lambda 5$ and *Vpre-B* together encode a surrogate Ig light chain that associates with Ig μ heavy chain proteins prior to completion of IgL gene assembly (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990). It has been postulated that this pseudo-IgM complex, displayed on the surface of pre-B cells, directs subsequent developmental events. Activation of various lymphokine response pathways also is expected to recruit a subset of developmentally important gene products. Recent studies have demonstrated that exposure of pre-B cells to IL-7 enhances the expression of the pre-B specific surface antigen BP-1 (Sherwood and Weissman, 1990), as well as that of the N-*myc* and c-*myc* genes (Morrow *et al.*, 1992).

We have recently isolated several genes that are expressed specifically in pre-B cells (Yancopoulos *et al.*, 1990). We now report that one of these genes is a novel member of the regulatory myosin light chain (or myosin light chain-2, *mlc-2*) gene family, unique in both nucleotide sequence and expression patterns from previously reported regulatory myosin light chain genes. Our evidence demonstrates that this gene is specifically expressed by precursor lymphocytes; furthermore, its expression distinguishes fetal liver and adult bone marrow derived pre-B cells. Finally, expression of this gene is induced in normal bone marrow pre-B cells upon exposure to IL-7.

Results

Expression of PB.65-related transcripts only in cell lines representing precursor lymphocytes

The PB.65S cDNA clone is a partial copy of an RNA sequence expressed in certain Abelson murine leukemia virus (A-MuLV) transformed pre-B cell lines but not in several lines that represent later stages of the B cell pathway (Yancopoulos *et al.*, 1990). To elucidate more thoroughly PB.65 expression during lymphocyte development, we assayed for PB.65 hybridizing transcripts in RNA derived from a broad panel of lymphoid and non-lymphoid cell lines. The PB.65S probe hybridized to 0.8 and 1.4 kb transcripts which were expressed only in certain pre-B and pre-T lymphocyte cell lines (Figure 1, lanes 1–3 and 6–8). Cell lines that expressed complete Ig (B and plasma cells; Yancopoulos *et al.*, 1990) or T-cell receptors (S33 and EL4; Figure 1, lanes 4 and 5) lacked expression of 0.8 and 1.4 kb PB.65 hybridizing transcripts as did a macrophage cell line (M1; Figure 1, lane 9) and two fibroblast lines (Ltk^- and NIH 3T3; Figure 1, lanes 10 and 11). Under the normal hybridization stringencies used the PB.65S probe also hybridized to a 1.0 kb transcript which was expressed ubiquitously (Figure 1). Under more stringent conditions, hybridization to the 1.0 kb transcript could be selectively eliminated (data not shown). Various analyses demonstrated that this ubiquitous transcript arose from a gene (*mRLC*, see below) related but not identical to that which encoded the 0.8 and 1.4 kb transcripts.

PB.65 is derived from a novel regulatory myosin light chain gene

Additional PB.65 hybridizing cDNA sequences were isolated by screening pre-B cell cDNA libraries. Most isolated cDNAs represented transcripts derived from the same gene as the PB.65 sequence, as determined by nucleotide sequence comparisons with the PB.65S clone and high stringency Northern analyses (data not shown). Nucleotide sequence analyses of these cDNA clones demonstrated that they derived from a gene highly related to known myosin regulatory light chain genes (Figure 2A and see below); thus, we have referred to this novel gene as precursor lymphocyte-specific regulatory light chain gene (*PLRLC*). Based on nucleotide sequence analyses, we further subdivided the *PLRLC* cDNAs into a least six distinct types of transcripts all of which derived from a single *PLRLC* gene through differential promoter utilization and/or differential RNA processing (Figures 2 and 6; described in detail below). Additional isolated cDNA sequences derived from the ubiquitously expressed 1.0 kb transcript and had >95% homology to a ubiquitously expressed, rat derived regulatory myosin light chain (*RLC*) gene (Taubman *et al.*, 1987); therefore this sequence is referred to as mouse *RLC* (*mRLC*). This assignment was further supported by Northern analyses that demonstrated ubiquitous expression of the *mRLC* transcript in cell lines and tissues (see below).

PLRLC is expressed in bone marrow but not fetal liver pre-B cell lines

Expression of *PLRLC* in B lineage cells was observed only in cell lines that represent the pre-B stage of development. However, only a subset of pre-B lines had a high level of expression, indicating a further restriction. To elucidate further the potential significance of the latter result, we

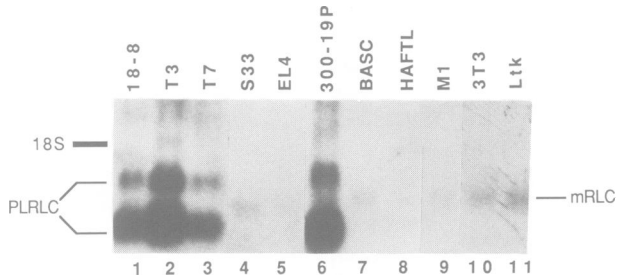

Fig. 1. Expression of PLRLC in cell lines. Approximately 10 μg of total RNA from the indicated sources was fractionated on a 1% formaldehyde gel and blotted onto Zeta Probe nylon membrane (BioRad). The filters were hybridized with the PB.65S cDNA probe (consisting of the final 14 bp of *PLRLC-B* non-myosin sequences, fused to nucleotides 265–427 and 619–695 of *PLRLC-A*). The cell lines surveyed include pre-B cells: 18-8tk^- (lane 1), 300-19P (lane 6) (Alt *et al.*, 1984); BASC6-C2 (lane 7), HAFTL-1 (lane 8) (Holmes *et al.*, 1986); pre-T cells: T3 (lane 2) and T7 (lane 3) (Ferrier *et al.*, 1990); mature T cells: S33 (lane 4, a previously undescribed α/β TCR positive line, data not shown) and EL4 (lane 5) (Shevach *et al.*, 1972); fibroblasts NIH-3T3 (lane 10) and Ltk^- (lane 11); and the macrophage line M1 (lane 9) (Sachs, 1978). Lanes 1–5 and 6–11 are from separate blots.

assayed for *PLRLC* sequences in RNA preparations from a broad panel of established pre-B cell lines; these lines had various degrees of Ig gene rearrangements and represented several distinct murine strains. All of 29 A-MuLV transformants derived from adult bone marrow expressed relatively high levels of the 0.8 and 1.4 kb *PLRLC* transcripts (Figure 3). In contrast, substantial levels of *PLRLC* transcripts were not observed in any of 46 A-MuLV transformants derived from fetal liver (Figure 3). As a control, we found that transcripts of the *PLRLC* homologue, *mRLC*, occurred at similar abundance in all of these lines (Figure 3). The Balb sarcoma virus transformed, bone marrow derived pre-B cell line (BASC6-C2; Holmes *et al.*, 1986) did not express substantial levels of *PLRLC* (Figure 1, lane 7); the significance of this apparent exception to the expression patterns established with A-MuLV transformants is unknown.

We considered the possibility that differential expression of *PLRLC* in cell lines arose from variations in establishment protocols or during long-term passage in culture. To address this issue, we established additional A-MuLV transformed pre-B cell lines from murine fetal livers and maternally derived murine bone marrow. None of the 36 newly generated fetal liver lines expressed substantial levels of *PLRLC* transcripts (Figure 3), whereas all ten bone marrow lines derived from the maternal donor and other mice expressed high levels (Figure 3). Therefore high levels of *PLRLC* transcripts are generally found in A-MuLV transformants derived from bone marrow but not in those derived from fetal liver. Finally, these differences in *PLRLC* expression are probably mediated at the transcriptional level because the *PLRLC* gene was found to be hypomethylated in several bone marrow derived transformants and hypermethylated in several fetal liver derived transformants (data not shown).

PLRLC expression in normal cells parallels that in transformed cell lines

To assess whether the restricted pattern of *PLRLC* gene expression observed in cell lines also occurs *in vivo*, total

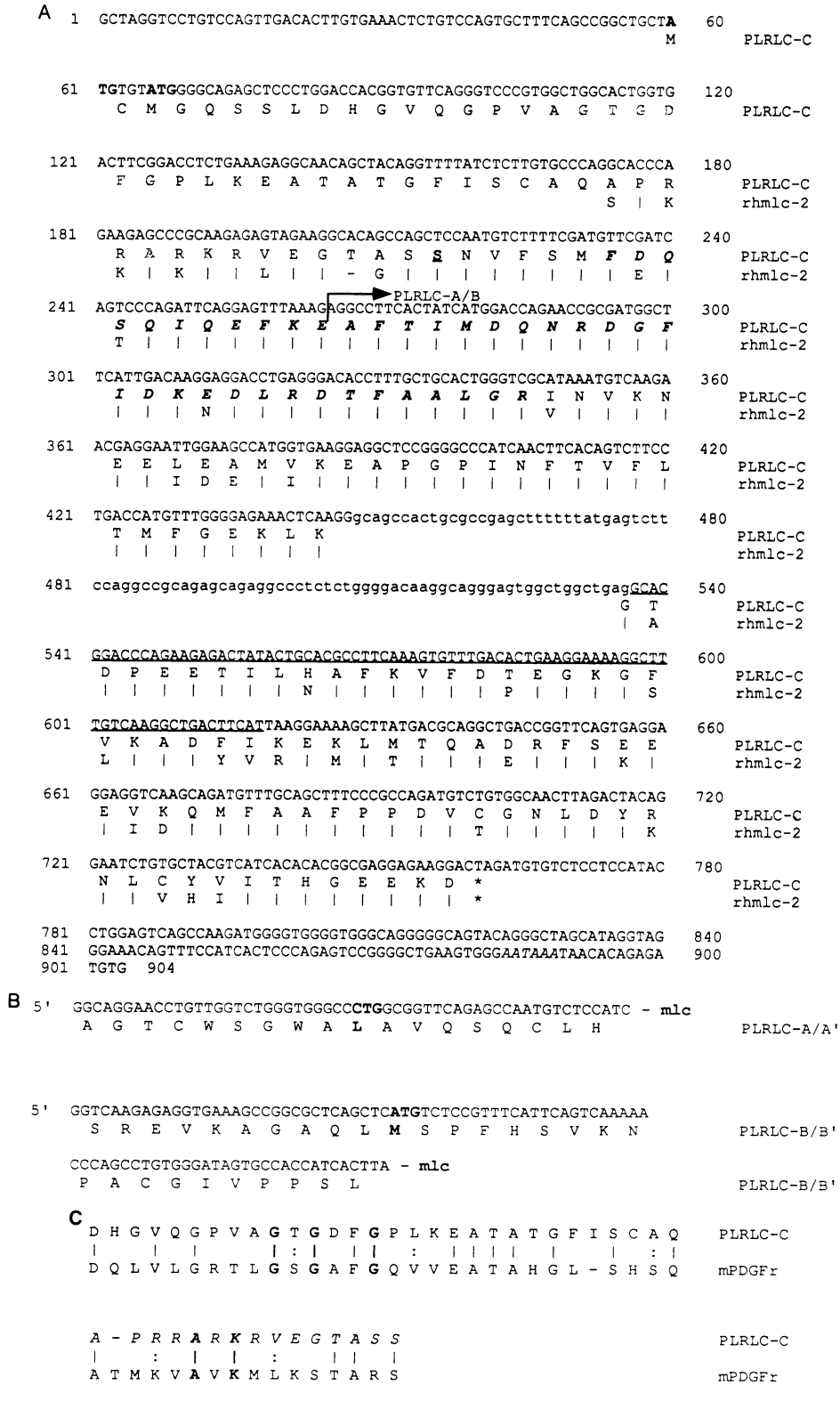

Fig. 2. Nucleotide sequence of *PLRLC* cDNAs. (**A**) Nucleotide sequence of the *PLRLC-C* cDNA and comparison of its predicted amino acid sequence with its closest reported homologue, rat cardiac myosin light chain 2 (Hendersen *et al.*, 1988). Vertical lines indicate amino acid identities. In-frame methionine codons are shown in bold print, * indicates termination codons and italicized nucleotides indicate polyadenylation sequences. Amino acid residues presented in outlined script compose the nucleotide binding domain while those important for the EF-hand are shown in bold italics. The serine residue targeted by mlc-2 protein kinases is underlined in bold print. Lower case letters are used for the intron 4/5 sequences found in this cDNA and the nucleotides representing exon 5 (deleted in some cDNAs) are underlined. The coding sequences homologous to *mlc-2* genes present in cDNA classes *PLRLC-A* and *-B* begin precisely at the exon 2/3 junction which is indicated by an arrow. (**B**) Non-myosin 5′ sequences for the *PLRLC-A* and *-B* cDNA classes and their predicted translations. Possible in-frame initiation codons are in bold print. (**C**) A comparison of nucleotide binding domain regions of the *PLRLC-C* cDNA and murine PDGFr (Yarden *et al.*, 1986). The non-myosin 5′ portion of *PLRLC-C* is shown in normal print and the regulatory myosin light chain portion is italicized. Residues critical in forming the NBD are shown in bold print.

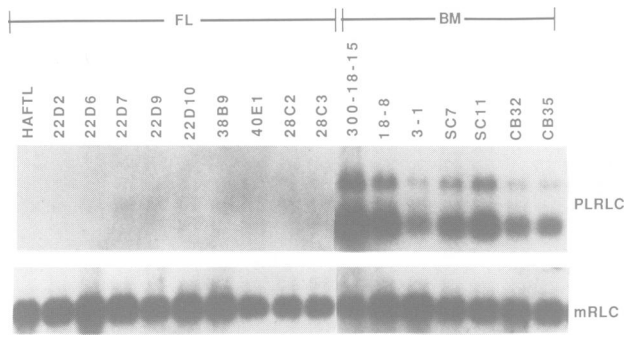

Fig. 3. *PLRLC* gene expression is restricted to bone marrow derived pre-B cell lines. Northern blots containing 10 μg of total RNA from previously described cell lines (Alt *et al.*, 1984) were sequentially hybridized with PB.65S cDNA and *mRLC* probes. FL, fetal liver derived Abelson lines (except HAFTL-1 which was transformed with the Harvey sarcoma virus): BM, bone marrow derived Abelson cell lines. Numerous additional Abelson pre-B cell lines were analysed for *PLRLC* expression. All bone marrow derived cell lines were positive for *PLRLC* transcripts, while only the *mRLC* transcript was detected in fetal liver lines. Lines not shown included: bone marrow, 300-18, 300-19P and 18-8tk$^-$ (Figure 1), 1848 and 3-1 (Alt *et al.*, 1984); CB31, CB35, CB36, CB37, SC2, 3, 11, 12 and 24 (Malynn *et al.*, 1988), BM1C1 (maternally derived analogue to the FLO series below, see text) 500-1, 7, 17, 22, 43, 45, 56, 82 and 83 (Ma *et al.* 1991); fetal liver, FLO-1 to -36 (newly established FL lines, see text).

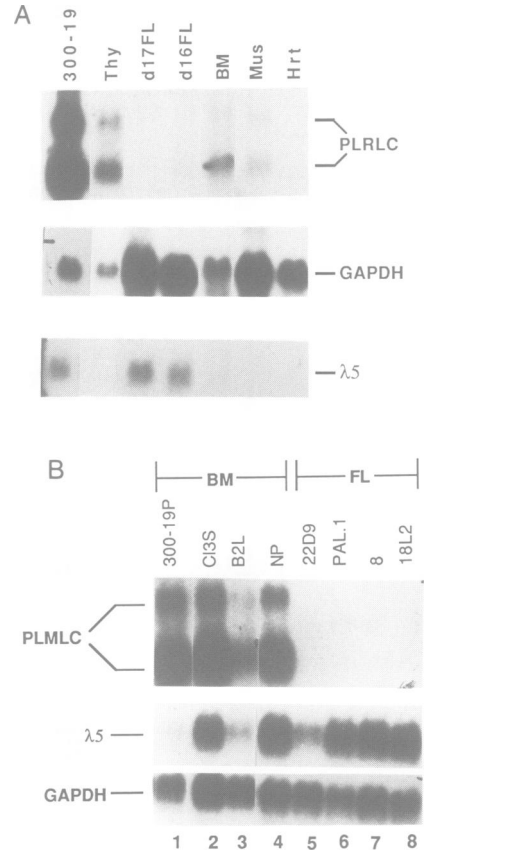

Fig. 4. Expression of PLRLC in normal cells. (**A**) Total RNA was extracted from indicated murine tissues and analysed by Northern blotting procedures [with the exception of the FL samples which were first selected for poly(A) containing RNA]. The blot was sequentially hybridized with probes for *PLRLC* (GR1132, nucleotides 562–853, Figure 2A), *GAPDH* (control for RNA levels) and the pre-B specific λ*5* gene (control for pre-B cell derived RNA). Lanes: Thy, thymus; FL, fetal liver; BM, bone marrow; Mus, skeletal muscle; Hrt, heart. (**B**) Northern blots were prepared from 10 μg total RNA derived from either bone marrow (lanes 2–4) or fetal liver (lanes 6–8) pre-B cell lines that were cloned from respective tissues on stromal cells, in the continual presence of IL-7 (Rolink *et al.*, 1991). Bone marrow (300-19P) and fetal liver (22D9) Abelson lines were included as controls. The blot was sequentially probed with the *PLRLC-A*, λ*5* and *GAPDH* cDNAs. Two additional fetal liver pre-B cell lines (C18 and F13-5, not shown) also lacked detectable levels of *PLRLC* expression.

RNA from murine tissue samples was assayed by Northern blotting for hybridization to a probe that displays minimal cross-hydridization to the highly expressed *mRLC* transcript (Figure 4A). To standardize the amount of total RNA added to each lane, we stripped the blot and assayed for hybridization to a glyceraldehyde phosphate dehydrogenase (*GAPDH*) probe and, to estimate the relative amount of pre-B cell derived RNA in each tissue sample, we further assayed the blot for hybridization to a pre-B specific λ*5* cDNA probe (Figure 4A). Specific *PLRLC* transcripts were observed solely in primary lymphoid organs (i.e. those that harbour lymphocyte precursors) and in skeletal muscle; highest levels were observed in thymus RNA (Figure 4A). Peripheral lymphoid tissues that contain only mature lymphocytes (i.e. spleen and lymph nodes) did not display detectable levels of *PLRLC* transcripts (Yancopoulos *et al.*, 1990; data not shown). Comparisons of *PLRLC* transcript levels in RNA from bone marrow and fetal liver revealed dramatic differences. Densitometric analyses, normalized for *GAPDH* signal levels, revealed that the relative abundance of *PLRLC* transcripts was >50-fold greater in bone marrow RNA than in fetal liver RNA. Furthermore, assuming that all of the *PLRLC* expression in bone marrow derives from pre-B cells, normalization of λ*5* expression levels indicates that *PLRLC* expression is at least 100-fold greater in bone marrow pre-B cells than in those of fetal liver.

To confirm the basis of the dramatic differential in *PLRLC* expression observed in bone marrow versus fetal liver, we assayed for expression of *PLRLC* transcripts in a series of normal fetal liver or bone marrow derived pre-B cell clones that were propagated on stromal cells in the presence of the IL-7 pre-B growth factor. Previous phenotypic characterization of such lines has shown them to represent normal, early pre-B cells with their IgH loci in either germline or a DJ_H rearranged configuration (Rolink *et al.*, 1991). Strikingly, all of three independent pre-B clones derived from bone marrow expressed levels of *PLRLC* transcripts comparable to those found in bone marrow derived A-MuLV transformants, whereas none of the five fetal liver derived normal pre-B clones expressed detectable *PLRLC* transcripts (Figure 4B). As a control, expression of the λ*5* gene was detected in all clones examined (Figure 4B). Therefore the expression patterns of the *PLRLC* gene observed in transformed cell lines directly parallels those of their normal cellular counterparts.

PLRLC transcripts are induced in bone marrow pre-B cells by IL-7

Both the fetal liver and bone marrow derived pre-B clones were propagated in the presence of IL-7 (Figure 4B); the lack of *PLRLC* expression in the former indicated that expression of this gene is not part of a response programme to this pre-B growth factor in these cells. To confirm whether or not this was also the case in bone marrow derived pre-B cells, we assayed whether or not expression of *PLRLC* transcripts in marrow derived pre-B cell cultures was

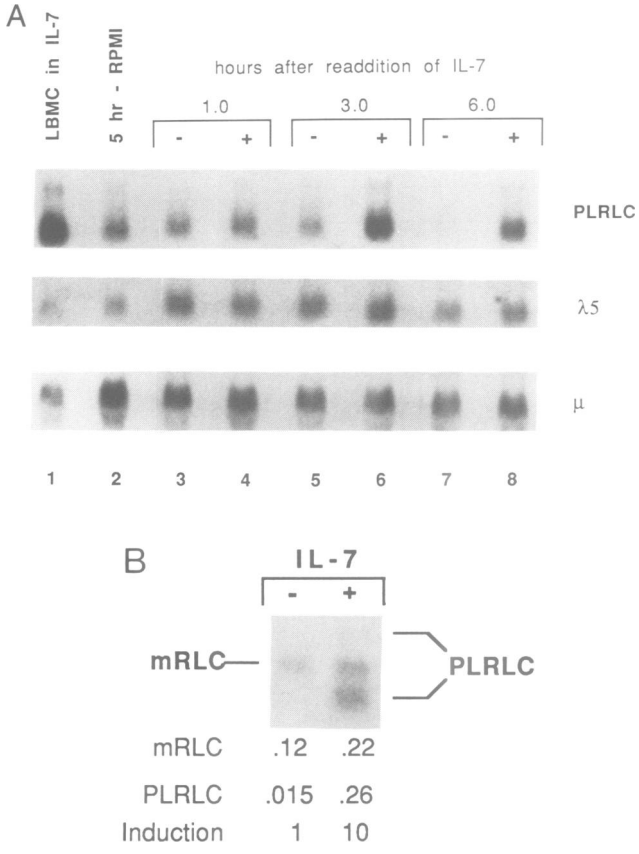

Fig. 5. IL-7 induction of *PLRLC* specific transcripts. (**A**) Regulation of *PLRLC* transcripts in long-term lymphoid bone marrow cultures. LBMC derived pre-B cells were propagated in the presence of IL-7 for 2 weeks; the resulting cells were removed from IL-7 (lane 1) and propagated in RPMI for 5 h (lane 2). Half of these cells were then replated in IL-7 supplemented media and samples were removed from the parallel cultures at the indicated time points (no IL-7, lanes 3, 5 and 7; with IL-7, lanes 4, 6 and 8). The blot was sequentially probed for expression of *PLRLC* (PB.65S), λ5 and IgH Cμ. (**B**) Northern blot analysis of RNA from purified $sIg^-/B220^+$ bone marrow pre-B cells propagated for 14 h in the presence (+) or absence (−) of IL-7. *PLRLC* expression was examined by hybridization with the PB.65S cDNA probe. Controls for RNA levels were provided by cross-hybridization of the probe to the *mRLC* transcript. The values for signal levels that were obtained by densitometry and used to calculate the relative induction of *PLRLC* are shown at the bottom.

modulated by the presence or absence of IL-7. For these experiments, non-adherent cells were removed from stromal layers of established lymphoid bone marrow cultures (LBMCs) that were propagated in the presence of IL-7 for 2 weeks. These cells exhibited high steady-state levels of *PLRLC* transcripts (Figure 5A, lane 1). Removal of IL-7 from the growth medium of these cells for 5 h resulted in a reduction of *PLRLC* transcript levels (Figure 5A, lane 2). These cultures then were divided and maintained in parallel with or without IL-7. Samples were then removed from the parallel cultures after an additional 1, 3 and 6 h and assayed for *PLRLC* expression. Cells maintained in the absence of IL-7 displayed a progressive decrease in the levels of *PLRLC* transcripts, whereas those maintained in IL-7 retained the original levels of *PLRLC* expression. In contrast, expression of the IgH Cμ and λ5 genes (which are expressed by pre-B cells) was not significantly altered by the presence or absence of IL-7 (Figure 5A). These data clearly demonstrate IL-7 dependent modulation of *PLRLC* transcripts in these cultured pre-B cells.

To confirm further this observation, we tested the ability of IL-7 to regulate *PLRLC* expression in pre-B cells directly after their isolation from bone marrow by a differential panning procedure (Lee *et al.*, 1989). Cells from this purified population were maintained for 14 h with or without IL-7 and then assayed for *PLRLC* RNA expression as described above. With the short treatment periods employed, possible selection for a population of pre-B cells that express higher levels of *PLRLC* transcripts was minimized. Comparisons of *mRLC* with *PLRLC* transcript levels by densitometry revealed an ~10-fold induction of *PLRLC* expression in IL-7 treated cells (Figure 5B). In addition, S1 nuclease analyses revealed that all of the different *PLRLC* transcript types were similarly induced (data not shown). Together, the findings described above indicate that *PLRLC* expression is part of an IL-7 responsive pathway in bone marrow pre-B cells, but IL-7 does not confer high level steady-state expression on fetal liver pre-B cells.

PLRLC transcripts may encode novel myosin light chains

We have employed a combination of cDNA cloning, S1 nuclease mapping and genomic cloning analyses to determine the structure and derivation of a series of *PLRLC* transcripts (described in the legend to Figure 6). Previously described regulatory myosin light chain genes contain seven exons; ORFs of transcripts derived from these genes initiate at an ATG codon which spans the exon 1/2 junction and terminates in the 5′ portion of exon 7 (see *mlc-2* transcript in Figure 6A) (Nudel *et al.*, 1984). Diversity of myosin complex gene transcripts, primarily due to alternate promoter utilization and differential transcript processing, is not uncommon; yet the proteins encoded by these different transcripts are structurally similar (Periasamy *et al.*, 1984; Lenz *et al.*, 1989). The transcripts that arise from the *PLRLC* gene display a similar diversity, but unlike the situation with previously defined regulatory myosin light chains some of the alternative *PLRLC* transcripts have the potential to encode markedly different proteins.

One *PLRLC* transcript (*PLRLC-D* in Figure 6A) appears to have an overall structure similar to those of previously identified regulatory myosin light chains. S1 nuclease protection analyses indicate that while this is the only *PLRLC* transcript detectable in skeletal muscle, it is one of the least abundant transcripts in pre-B cells (data not shown). The more abundant *PLRLC* transcripts (*PLRLC-A*, *-A′*, *-B*, *-B′* and *-C* in Figure 6A) contain novel (i.e. non-myosin related) 5′ sequences fused to either exon 2 or 3 (Figures 2A and 2B) and are found exclusively in precursor lymphocytes. Some of these transcripts are also unusual as compared to previously defined regulatory myosin light chain gene transcripts in that they lack exon 5 (see *PLRLC-A′* and *-B′* in Figure 6A). Comparative analyses of *PLRLC* cDNA and genomic clones revealed that the non-myosin coding sequences are derived from regions upstream of the respective exons to which they are fused (Figure 6B). Notably, the non-myosin N-terminus encoded by the *PLRLC-C* transcript displayed a significant homology to the nucleotide binding domain of the platelet derived growth factor receptor (Figure 2C) (Yarden *et al.*, 1986) and therefore could potentially encode a functionally novel

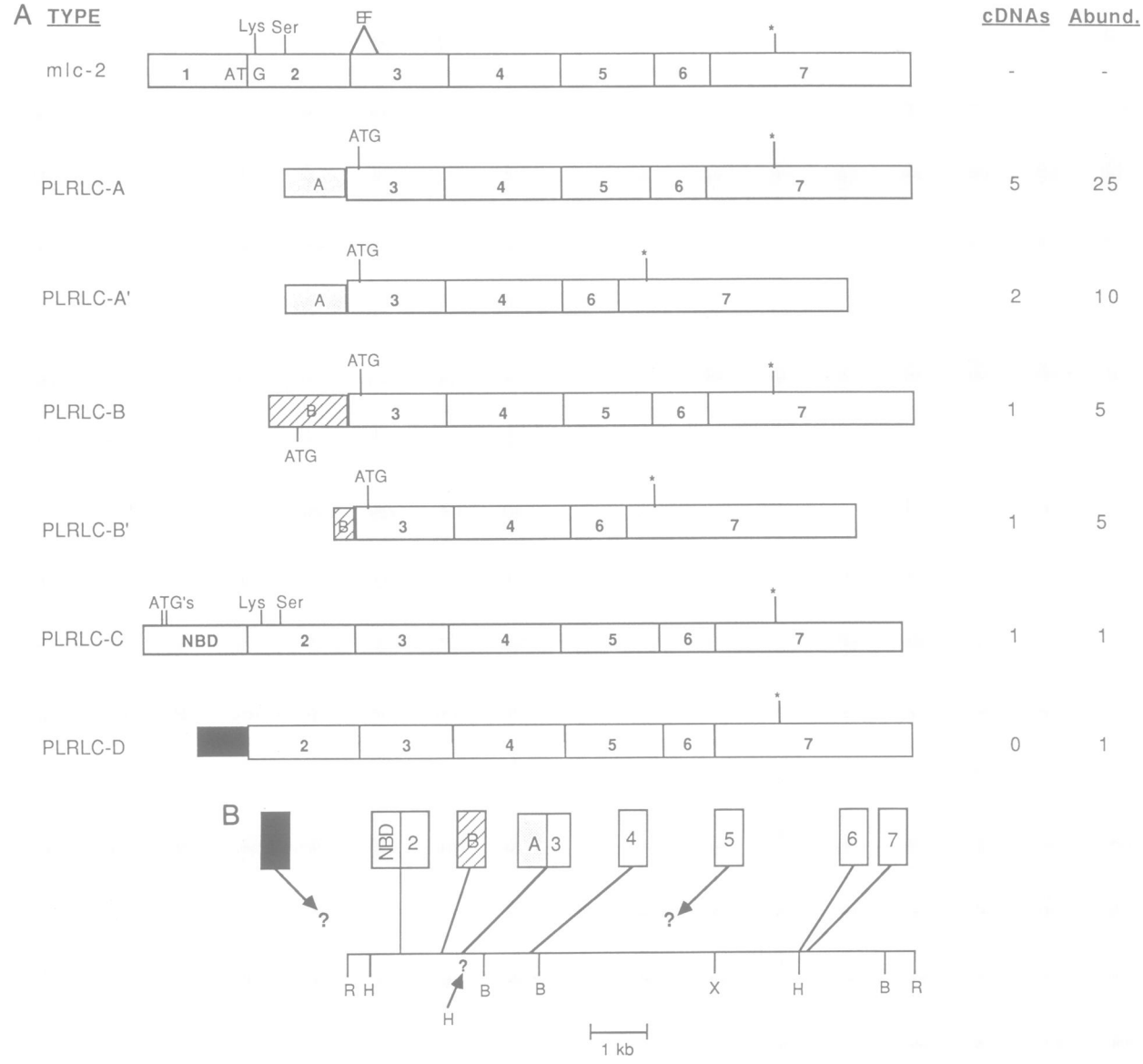

Fig. 6. The *PLRLC* gene gives rise to multiple transcripts. (**A**) Schematic representations of *PLRLC* transcripts and corresponding cDNAs; primary nucleotide sequence data have been deposited in the EMBL Databank under the accession numbers X65979 (*PLRLC-A*), X65980 (*PLRLC-B*) and X65981 (*PLRLC-C*). All *PLRLC* cDNA structures are compared with those reported for other *mlc-2* cDNAs, exemplified by rat skeletal muscle *mlc-2* (Nudel *et al.*, 1984). *Mlc-2*: the structure of rat skeletal muscle *mlc-2* transcript; the *mlc-2* ORF initiates at an ATG that spans the exon 1/2 junction and terminates in exon 7. Numbered boxes denote the typical exonic structure of the reported *mlc-2* genes and highlighted structural features include Ser which represents the mlc-2 kinase target, EF which represents the Ca^{2+} binding EF-hand (present but not shown in all PLRLC transcript types); * represents the termination codon. *PLRLC-A*: this sequence corresponds to exons 3−7 of reported *mlc-2* genes preceded by a unique 5′ (non-myosin) sequence that in various cDNA isolates varied in length from 45−71 bp (box A). The first in-frame AUG codon of *PMLC-A* transcripts was in the 'exon 3' sequences of the myosin ORF; although an in-frame CUG codon, occasionally utilized for translational initiation (Bernards and delaMonte, 1990), was present in the unique 5′ sequence (Figure 2B). *PLRLC-A'*: this set of sequences contained an ORF identical to that of *PLRLC-A* except that it lacks the 81 bp homologue to the sequence encoded by exon 5 of *mlc-2* genes. *PLRLC-B*: this sequence contained abbreviated *mlc-2* ORFs identical to those observed for the *PLRLC-A* transcripts, but had a distinct 89 bp 5′ (non-mlc2) sequence (denoted by box B). The *PLRLC-B* cDNA sequence contained an ORF that included an in-frame AUG codon within the novel 5′ sequence and could encode a 20 amino acid non-myosin N-terminus appended to the downstream *mlc-2* homologous sequences (Figure 2B). *PLRLC-B'*: this cDNA was identical to *PLRLC-B* except that it lacked exon 5 coding sequences. *PLRLC-C*: this sequence contained a complete *mlc-2* ORF (including exon 2) but was interrupted by an 86 bp sequence found between exons 4 and 5 of the *mlc-2* genes; S1 nuclease assays with probes spanning this region of the *PLRLC-C* clone confirmed that the predominant form of the *PLRLC-C* transcript in cell lines and tissues lacked this intronic sequence (see Materials and methods). The *PLRLC-C* sequence contained a 180 bp sequence 5′ to exon 2 which contains two in-frame AUG codons and that potentially encodes a 35 amino acid, non-myosin, N-terminus (Figure 2A); the highlighted NBD represents the nucleotide binding domain completed by the highlighted Lys residue which is potentially encoded by this sequence (Figure 2C). *PLRLC-D*: as cDNA sequences representing this transcript were not isolated, its structure was inferred from S1 nuclease protection analyses (see Materials and methods) to include exon 2 of the classical *mlc-2* message and exclude the novel 5′ sequences of the *PLRLC-C* transcript. Columns at the right of the figure indicate the number of each cDNA type isolated (10 in total) and the approximate relative abundance of each transcript in the pre-B cell line 300-19P (as determined by S1 nuclease assays, see Materials and methods). (**B**) Partial restriction map and structure of a *PLRLC* genomic clone as derived from mapping and nucleotide sequence data (not shown). Non-myosin 5′ sequences of the *PLRLC-A* and *-A'* cDNAs, represented by box A, are present in the intron separating exons 2 and 3 and are contiguous with exon 3. Non-myosin 5′ sequences for the *PLRLC-B* and *-B'* cDNAs, denoted by box B, are also present in intron 2/3 but are non-contiguous (~450 bp 5′). Enzyme sites shown: R, *Eco*RI; X, *Xho*I; B, *Bam*HI; and H, *Hin*dIII. The exact locations of exons 5 and the putative *PLRLC-D* 5′ exon as well as a final *Hin*dIII site have not been identified (all denoted by a ?).

regulatory myosin light chain related product (see Discussion). All *PLRLC* transcripts that have been characterized in this study apparently correspond to the smaller set of transcripts identified by Northern blotting (ca. 800 bp); thus additional *PLRLC* transcripts must exist.

PLRLC is a novel, conserved mlc-2 gene

The *mlc-2* related coding sequences of *PLRLC* cDNAs were most related to those of rat cardiac *mlc-2* (Hendersen *et al.*, 1988); 75% identical at a nucleotide and 79% identical at an amino acid level (87% with conserved changes, Figure 2A). To determine whether the *PLRLC* gene has a conserved and unique counterpart in other species, genomic DNAs from several species were digested with various restriction enzymes and assayed for hybridization to *PLRLC* probes. Under normal stringency, a *PLRLC* cDNA probe hybridized strongly to a limited set of restriction fragments (Figure 7); all of the fragments in mouse genomic DNA could be accounted for by analyses of murine genomic clones that contained the *PLRLC* gene (Figure 6B). Thus, the *PLRLC* probe distinguished *PLRLC* from other murine *mlc-2* genes. Specific hybridization of the *PLRLC* probe to rat and human DNA also was detected under normal stringencies (Figure 7). Preliminary analyses of a rat *PLRLC* (*rPLRLC*) genomic clone showed that all restriction fragments observed in Southern analyses of rat genomic DNA arose from sites within a single *PLRLC* gene. Limited sequence analysis of exon 2 of *rPLRLC*, confirmed that it was unique from previously reported rat *mlc-2* coding sequences (data not shown) but highly related to the *mPLRLC* gene. Thus, the *PLRLC* gene is a unique, evolutionarily conserved member of the *mlc-2* gene family.

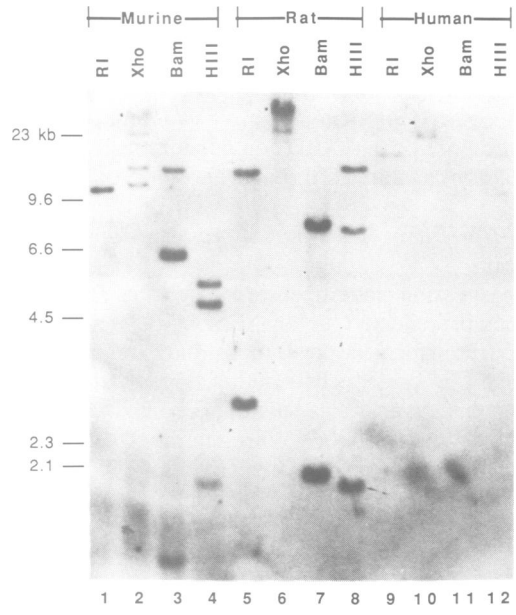

Fig. 7. Evolutionary conservation of the *PLRLC* gene. Genomic DNAs from murine (Abelson line BM1C1, lanes 1–4), rat (liver tissue, lanes 5–8) and human (HeLa cells, lanes 9–12) sources were digested with the indicated enzymes (RI, *Eco*RI; Xho, *Xho*I; Bam, *Bam*HI; and HIII, *Hin*dIII) and subjected to Southern analysis. The blot was hybridized with the *PLRLC-A* cDNA probe under normal stringency. Migration of *Hin*dIII digested λ DNA size markers is indicated.

Discussion

The PLRLC gene is a novel mlc-2 gene

Proteins of the vertebrate myosin complex are encoded by highly conserved, multigene families (reviewed in Harrington and Rodgers, 1984); previously defined family members are either ubiquitously expressed or specifically expressed in certain muscle subtypes (Taubmann *et al.*, 1987). *PLRLC* is the first reported example of a regulatory myosin light chain gene which displays highly restricted expression in non-muscle cells. Its restricted expression to subclasses of precursor lymphocytes and its regulation by a pre-B cell specific growth factor suggest a critical and highly specific role in lymphocyte development.

Myosin light chain-2 proteins represent one component of the myosin complex, which is normally composed of two heavy chains (MHCs), two structural light chains (mlc-1 or 3) and two regulatory light chains (mlc-2; Harrington and Rodgers, 1984). The myosin complex plays a major role in contractile processes of both muscle and non-muscle cells, where it acts in an energy transductory capacity converting ATP hydrolysis into mechanical work. The myosin complex also interacts with actin, a protein which is of major importance both in regulating the inherent ATPase activity of MHCs and in mediating contractile events. In addition to its role in muscle contraction, the actino–myosin complex has been implicated in cytoskeletal redistribution (Pasternak *et al.*, 1989), cell motility, vesicle transport (Pollard and Weihing, 1974) and capping of cell surface receptors (Bourguignon and Bourguignon, 1984; Pasternak *et al.*, 1989). Present evidence suggests that a major function of regulatory light chains is to modulate the ATPase activity of MHCs. In turn, the modulatory ability of the regulatory light chains depends on their phosphorylation status (Fechheimer and Cebra, 1982). The regulatory myosin light chains also possess a conserved Ca^{2+} binding site (EF-hand) (Reinach *et al.*, 1986) although possible roles for Ca^{2+} binding in mammalian *mlc-2* genes remain undefined.

The *PLRLC* gene produces multiple transcripts that have the potential to encode a number of distinct proteins. The *PLRLC-D* transcript type was detected at low levels in skeletal muscle (by S1 nuclease analyses; data not shown); no other transcript types were detected in this tissue. It is conceivable that this transcript may encode a protein structurally similar to those encoded by previously described *mlc-2* genes. All of the remaining characterized *PLRLC* transcripts contain sequences capable of encoding the Ca^{2+} binding EF-hand motifs as well as other mlc-2 structural features including an extended α-helical domain. Several of the transcripts also contain sequences (present in exon 2) that encode a serine residue which is normally targeted by mlc-2 specific protein kinases (Ikebe *et al.*, 1988); conformational changes resulting from phosphorylation of this serine residue allow mlc-2 proteins to positively regulate MHC ATPase activity.

The coding capacity and potential function of the novel 5′ sequences within most of the *PLRLC* transcripts remains to be determined. However, the PLRLC-C transcript encodes a nucleotide binding domain (NBD) at its N-terminus. Thus, putative *PLRLC-C* encoded proteins would include a novel arrangement of three regulatory motifs (NBD, mlc-2 kinase target and EF-hand) within a stretch of 100 amino acids. The putative *PLRLC-C* nucleotide binding domain is composed of sequences derived from both myosin and non-myosin portions of this transcript (Figure 2A and C). The

NBD present in *PLRLC-C* is analogous in structure to those found in a large class of protein kinases (including PDGF receptor and c-src), although the *PLRLC-C* protein would not retain other domains highly conserved in protein kinases and necessary for catalytic activities (Hanks *et al.*, 1988). The presence of this NBD could theoretically endow novel regulatory capabilities through allosteric effects.

PLRLC probably has a novel pre-lymphocyte specific function

The expression pattern observed for the *PLRLC* gene is without precedent. Our analyses of both normal and A-MuLV transformed pre-B cells indicate that *PLRLC* is expressed in pre-B cells of adult bone marrow but not in those of fetal liver; future studies of precursor T lymphocytes may reveal a restriction to discrete pre-T cell classes. The *PLRLC* gene also belongs to a small set of identified genes that are regulated by IL-7. One such gene (*BP-1*) encodes a pre-B cell specific transformation associated surface protein that has endopeptidase activity and is postulated to play a role in the IL-7 responsivity of pre-B cells (Sherwood and Weissman, 1990; Wu *et al.*, 1991). The *BP-1* protein is also found on the surface of intestinal and kidney epithelial cells. The N-*myc* nuclear oncogene is rapidly induced by IL-7 treatment of normal pre-B cells (Morrow *et al.*, 1992), perhaps reflecting an important role in mediating the pre-B cell proliferative effects of this lymphokine. N-*myc* is expressed in a variety of cell types but also is pre-B specific within the B cell lineage. However, in contrast to *PLRLC*, expression of the *BP-1* and N-*myc* genes occurs in both bone marrow and fetal liver derived A-MuLV transformants.

The unique restriction of *PLRLC* expression to precursor lymphocyte subclasses and its regulation by IL-7 should provide insights into its function. Recent studies have indicated that fetal and adult pre-B cells are distinct, differing in their abilities to generate a mature $CD5^+$ B cell population (Hardy and Hayakawa, 1991). The presence or absence of *PLRLC* expression probably confers unique phenotypic properties on a given pre-B cell type which may involve forms of cytoskeletal reorganization required for particular pre-B cell capabilities. Such differential capabilities could be conceivably required for cell migration in the structurally distinct differentiation microenvironments of the bone marrow versus the fetal liver, or differences in abilities to perform important pre-B cell functions such as the capping of surface receptors.

Materials and methods

RNA and DNA preparation, blotting analyses and probes

DNA and RNA were prepared, electrophoresed, blotted and probed as described previously (Kohl *et al.*, 1983, 1984). The following probes were used for Northern and/or Southern blotting analyses: λ5, a 700 bp full length cDNA fragment isolated from the cDNA library described below; *GAPDH*, a 1.3 kb *Pst*I fragment from a rat *GAPDH* cDNA (Fort *et al.*, 1985); mRLC, a full length murine cDNA derived from an A-MuLV transformed pre-B cell library (see below); Cμ, a 900 bp *Pst*I fragment from pABM-11 (Bothwell *et al.*, 1981).

S1 nuclease analyses described in the text were performed as described by Kohl *et al.* (1986) except that riboprobes prepared by *in vitro* transcriptions from either the T3 or T7 promoters in pBS were employed in hybridizations (80% formamide, 60°C). Analyses of *PLRLC* transcript ratios were performed with a combination of two probes: (i) 65F4-5′ which originates at bp 1 of the *PLRLC-C* cDNA and terminates at the internal *Hin*dIII site (bp 628) and (ii) the *PLRLC-A* cDNA. The 65F4-5′ probe was also employed to confirm the lack of *PLRLC-C* intron 4/5 sequences in pre-lymphocyte RNA.

Isolation and characterization of PLRLC cDNAs

A pre-B cell cDNA library (described in Yancopoulos *et al.*, 1990) was synthesized from pooled poly(A)$^+$ RNA of three pre-B cell lines (38B9, 22D6P and 300-19P) in the Lambda ZAP phage vector (Stratagene). 2.5×10^5 plaque forming units were plated onto each of four 243 mm^2 plates. Nitrocellulose lifts from each plate (Benton and Davis, 1977) were probed with the PB.65S cDNA and washed under high stringency (0.2 × SSC, 0.1% SDS; 68°C, three times for 20 min). Positive clones were purified and cDNA inserts were isolated by following standard lambda ZAP protocols (Stratagene). The isolated cDNAs and subclones were sequenced directly from the corresponding pBS plasmids by the dideoxy chain termination method according to manufacturer's protocol (US Biolabs).

Cell culture and IL-7 induction studies

Femoral bone marrow cells from 8 week-old Balb/c mice (Jackson Laboratories) were separated on the basis of surface marker expression as described (Lee *et al.*, 1989). The purified $sIg^-/B220^+$ precursor B cells were cultured for 14 h in RPMI-1640 supplemented with 5% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 50 μg/ml penicillin and streptomycin, with or without the addition of 500 U/ml of recombinant murine IL-7 (Namen *et al.*, 1988).

Femoral bone marrow cells from 5–8 week-old C57Bl/6 mice (Jackson Laboratories) were suspended at 10^6 cells/ml of tissue culture media (as above) in 75 cm^2 Falcon T-flasks (Becton-Dickinson). Cells were maintained for at least 4 weeks by replacing half of the media weekly thereby avoiding removal of non-adherent cells (Whitlock and Witte, 1982). IL-7 (500 U/ml) was added to the LBMC and the cells were passaged for an additional 2 weeks. The resultant non-adherent pre-lymphocyte cell population was washed three times with media to remove IL-7, grown in IL-7-free media for 5 h, split and then IL-7 (500 U/ml) was reintroduced into one of the cultures. Aliquots of cells for RNA extraction were removed from both cultures at 1, 3 and 6 h subsequent to reintroduction of IL-7.

The BM1C1 and FLO series of A-MuLV transformed pre-B cell lines were established as previously described (Rosenberg and Baltimore, 1976). The pre-B phenotype of the lines was confirmed by Northern blot analyses for pre-B cell specific genes (data not shown).

The bone marrow and fetal liver derived pre-B cell lines (Figure 4B) were established as described previously (Rolink *et al.*, 1991). The lines CL35, B2L and NP were established from the bone marrow of adult C57BL/6, 2 week-old $(C57BL/6 \times DBA/2)F_1$ and 3 week-old Balb/c mice, respectively. The lines PAL.1 and PAL.8 were derived from day 14 fetal liver, while 18L2 was derived from day 18 fetal liver of $C(57BL/6 \times DBA/2)F_1$ mice. All lines express the pan-B cell specific marker B220 as well as the pre-B cell specific markers λ5 and VpreB. All lines have DJ rearranged IgH loci and are germline in their configurations of κ and λ IgL chain loci.

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