International Immunology, Vol. 5, No. 12, pp. 1609–1618 © 1993 Oxford University Press

Rearrangement and expression of κ light chain genes can occur without μ heavy chain expression during differentiation of pre-B cells

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Key words: B cell differentiation, Ig gene rearrangement, κL chain protein expression, stromal cell/IL-7 reactive pre-B cells

Abstract

The kinetics of κ light (κL) chain gene rearrangement and expression on mRNA and protein level has been studied with four stromal cell/IL-7 reactive, long-term *in vitro* proliferating pre-B cell lines and clones, two from fetal liver of normal mice and two from fetal liver of EμH – *bcl*-2 transgenic (*bcl*-2-tg) mice. These pre-B cell lines and clones are DJ_H-rearranged on both H chain alleles. Two of the clones harbor H chain rearrangements which do not allow the expression of V_HDJ_H rearranged H chain genes as μH chain proteins. Upon removal of IL-7 from the pre-B cell cultures all four cell lines rearrange $V_H – DJ_H$ and $V_L – J_L$ gene segments, loose the surface expression of c-*kit*, CD43, and surrogate light chain, as well as the capacity to be clonable on stromal cells in the presence of IL-7. Pre-B cells from normal mice die by apoptosis during differentiation, while those from *bcl*-2-tg mice do not. All four lines and clones express comparable levels of mRNA for μH and κL chains with the same time kinetics during 3 days of differentiation. However, only two of the four pre-B cell lines and clones express μH chain protein, whereas all four pre-B cell lines and clones express κL chain protein at comparable levels between 2×10^5 and 1.4×10^6 κL chain molecules per cell. These results suggest that μH chain expression is not mandatory for rearrangement and normal expression of κL chain genes when pre-B cells differentiate to B cells.

Introduction

During B cell differentiation the genes encoding the variable regions of IgH and IgL chains are somatically assembled from V_H, D, and J_H, and V_L and J_L gene segments respectively (1). The various rearrangement events apparently take place in an ordered fashion (2–4), first by rearranging D – J_H segments, followed by $V_H – DJ_H$ at the H chain locus (5). $V_\kappa – J_\kappa$ rearrangements occur later (6,7), while $V_\lambda – J_\lambda$ gene segments are rearranged last (3,8). The ordered rearrangement of H and L chain genes has favored the hypothesis that the expression of μH chain proteins is a prerequisite for κL chain gene rearrangements (2,9).

The μH chains can associate with a surrogate light chain encoded by the precursor B cell specific genes λ_5 (10) and $V_{pre\text{-}B}$ (11) to form an Ig-like complex (12,13). It has been suggested that this Ig-like complex triggers L chain gene rearrangements during B cell differentiation (14–16).

However, the view that the ordered rearrangement of H and L chain genes is regulated by the expression of μH protein was recently challenged by several studies. (i) Epstein – Barr virus-transformed human pre-B cell lines were found to express κ, as well as λ light chains, while H chain genes remained unrearranged (17). (ii) Some Abelson murine leukemia virus (A-MuLV) transformed pre-B cells derived from SCID mice were found to attempt κL chain gene rearrangements in the apparent absence of μH protein (18,19). (iii) Analysis of membrane μH-deficient (μMT) mice (20) revealed that surface deposition of a functional μH chain into the cell membrane was not necessary to allow κL chain gene rearrangements *in vivo* (21). Furthermore, mice with a targeted deletion of the J_H locus show normal frequencies of κL chain gene rearrangements in sorted pre-B cell fractions of the bone marrow, although H chain genes cannot be rearranged in these mutant mice (22,23).

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Transmitting editor: O. Mäkelä

Received 18 August 1993, *accepted* 14 September 1993

Since all *in vitro* data have been obtained using transformed cell lines, we have tried to elucidate the question of a putative regulatory role of μH chains for κL chain rearrangement by comparing the *in vitro* differentiation of normal murine pre-B cells, which either can or cannot express μH chains. This approach allows a detailed analysis of the kinetics of κL chain gene rearrangements and of the expression of κL chain protein in the absence or presence of μH chains.

Pro-B and pre-B I cells of the mouse can be cultured for long periods of time on stromal cells in the presence of IL-7 (24) (pro-B cells, all Ig loci in germline configuration; pre-B I cells, H chain loci DJ_H rearranged; pre-B II cells, at least one H chain locus V_HDJ_H rearranged; for nomenclature see also 25 and 26). These cells retain the capacity to differentiate to sIg^+ B cells upon removal of IL-7 *in vitro* (24). They are mostly DJ_H rearranged on both H chain loci and retain their L chain loci in germline configuration. They are generally $B220^+$, express the pre-B cell specific genes λ_5 and $V_{pre\text{-}B}$, are c-*kit*$^+$, $CD43^+$, and HSA^+, while they may or may not express BP-1 (27–29).

Subcloning of pre-B cell lines sometimes results in pre-B cell clones which are DJ_H rearranged on both alleles in a way, that a subsequent V_H–DJ_H rearrangement cannot lead to a productively rearranged H chain locus capable of expressing μH chain protein. The *in vitro* differentiation of two such clones, CL18 derived from a normal BDF_1 mouse, and BCL-2-11, from a EμH–*bcl*-2 transgenic (*bcl*-2-tg) mouse (30,31), is compared with that of control pre-B cells, which are able to rearrange both their H chain and L chain loci non-productively and productively to generate sIg^- and sIg^+ B cells. The kinetics of κL chain rearrangement and the expression of κL chain protein is measured to answer the question whether or not expression of μH chain protein is necessary for the rearrangement and expression of κL chain genes.

Methods

Mice

Female (C57 BL/6 ×DBA/2)F_1 (BDF_1) mice of different age were obtained from the Institut für Medizinisch Forschung AG (Füllingsdorf, Switzerland). *bcl*-2-tg mice (30,31) were bred at our own animal facilities from breeding pairs originally obtained from Dr A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). BDF_1 embryos from time-pregnant C57 BL/6 females and *bcl*-2-tg embryos from time-pregnant heterozygous *bcl*-2-tg females were provided by the breeding facilities at the Basel Institute for Immunology. The appearance of vaginal plugs was counted as day 0 of gestation.

Cell lines

The stromal cell line PA-6 (32) was kindly given to us by Dr H. Kodama (University of Ohu, Japan). Pre-B cell clones CL 18 (24) and PAL-1 (27) were established from day 14 fetal liver of normal BDF_1 embryos as previously described (24). Pre-B cell line BCL-2-5 and pre-B cell clone BCL-2-11 were obtained from fetal liver of 16 day old *bcl*-2-tg embryos by limiting dilution as described (24). Cell-line 5-7 is a stromal cell independent, IL-7 dependent subline of a normal pre-B cell clone 5 (24), which had become sIg^+ (95% μ^+, κ^+), and served as a positive control for Western blots developed with μH and κL chain specific antibodies.

mAbs and flow cytometric analysis

The mAbs 187.1 (rat anti-mouse κL chain) (33), ACK-4 (rat anti-mouse c-*kit*) (27), and S7 (rat anti-mouse CD43) (29) were purified from hybridoma culture supernatants using Protein G–sepharose columns (Pharmacia, Uppsala, Sweden), and were biotinylated according to standard protocols. Biotinylated mAbs LM34 (rat anti-mouse λ_5) (34) and VP245 (rat anti-mouse $V_{pre\text{-}B}$) (34) were a kind gift of Dr H. Karasuyama, Basel Institute for Immunology. Biotinylated mAbs 6C3 (rat anti-mouse BP-1), M1/69 (rat anti-mouse HSA), and 7D4 (rat anti-mouse IL-2Rp55, CD25), and FITC-labeled mAbs C2 (rat anti-mouse CD71), RA3-6B2 (rat anti-mouse B220, CD45R), and R6-60.2 (rat anti-mouse IgM) were obtained from Pharmingen (San Diego, CA). Polyclonal horseradish peroxidase-labeled rat anti-mouse κL chain and rat anti-mouse IgM antibodies for Western blotting were obtained from Southern Biological Associates (Birmingham, AL). Surface staining of cells and FACS analysis was carried out as previously described (24). Stainings using biotinylated antibodies were developed with streptavidin–FITC (Amersham, Buckinghamshire, UK), except for the λ_5 and $V_{pre\text{-}B}$ specific antibodies LM34 and VP245, which were developed with streptavidin–phycoerythrin (PE) (Southern Biotechnology Associates). Viable cells were either gated by exclusion of propidium iodide (in the case of FITC stainings) or by forward and side scatter (in the case of PE stainings). FACS analysis was carried out by means of a FACScan (Becton Dickinson, Mountain View, CA) equipped with an argon laser tuned to 488 nm. Data were acquired and analyzed using the Lysis software package (Becton Dickinson).

Cell cultures and limiting dilution analysis

Culture of pre-B cells and PA-6 stromal cells was carried out as described (35). Briefly, pre-B cells were cultured on a semiconfluent layer of γ-irradiated PA-6 stromal cells in medium containing 100–200 U/ml IL-7. To obtain differentiated pre-B cells, cells were washed three times in medium without IL-7 (to remove any IL-7 contamination) and were cultured on a semiconfluent layer of γ-irradiated PA-6 stromal cells in medium without IL-7. Differentiated cells were harvested after 1, 2, and 3 days of culture.

Limiting dilution analysis was carried out in 96-well flat-bottom plates containing an irradiated PA-6 stromal cell layer (10^4 cells/well). Pre-B cell suspensions were diluted by serial 2-fold dilutions in medium containing IL-7. Cultures were scored after 6 days of culture with an inverted microscope for growth of pre-B cell colonies.

Northern blot analysis

Total cellular RNA was extracted from differentiating pre-B cells with acid guanidinium thiocyanate–phenol–chloroform (36) and analyzed by Northern blotting exactly as described (35).

Western blot analysis

Differentiated cells from normal pre-B cell clones CL18 and PAL 1 were enriched for viable cells by centrifugation using Ficoll-Paque (Pharmacia). Between 5×10^6 and 2×10^7 living cells were resuspended in 100 μl lysis buffer (2% NP40, 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, 2 mM NaN_3, 2 mM phenylmethyl sulfonyl fluoride) and were allowed to lyse for 20 min on ice. Nuclei were separated from cytoplasmic lysates

by centrifugation at 12 000 r.p.m , 4°C, 15 min. Supernatants were diluted in lysis buffer and appropriate dilutions were used to fractionate cytoplasmic proteins under reducing conditions on a 15% protein gel (37). Fractionated proteins were electroblotted onto nitrocellulose filters (BioRad, Richmond, CA) in 192 mM glycine, 25 mM Tris base, 20% methanol for 4 – 6 h at room temperature with constant stirring (38). Immunodetection of *x*L chains and *μ*H chains was carried out using horseradish peroxidase-labeled antibodies specific for either mouse *x*L chains or mouse IgM and an ECL Western blotting kit (Amersham) according to the manufacturers protocol. Chemiluminescence on the blots was detected using X-OMAT AR X-ray films (Eastman Kodak, Rochester, NY) with exposure times varying between 10 and 60 min.

Molecular analysis of preB cells

DJ_H rearrangements of the heavy chain locus can be amplified and detected using a polymerase chain reaction (PCR) primed by oligonucleotides, which bind 5′ of each D segment and 3′ of the J_{H4} gene segment. In germline configuration the primers will be located too far apart to be amplified by a PCR, in case of a V_HDJ_H rearrangement, the primer binding site 5′ of D would be deleted. The primers binding 5′ of D and 3′ of J_{H4} have been described by Gu *et al.* (39), and can detect all DJ_H joints except those formed by DFL16.2 and DQ52 gene segments.

To isolate genomic DNA, 5×10^5 cells were washed with PBS, lysed by boiling (5 min) in 500 μl PBS, and treated with proteinase K (Boehringer, Mannheim, Germany, 0.2 mg/ml) at 55°C for 2 h. The preparations were boiled again to inactivate proteinase K, extracted once with phenol:chloroform:isoamylalcohol (25:24:1), and once with chloroform:isoamylalcohol (24:1). DNA was precipitated with 1 volume of isopropanol and 0.1 volume of 3 M Na – acetate, pH 5.2, at −20°C for 1 h. After centrifugation at 12 000 r p.m. for 15 min at 4°C, the pellet was washed with 70% ethanol, air dried, and dissolved in 500 μl 10 mM Tris – HCl, pH 8.3. Aliquots of 5 μl of this preparation were subsequently used for PCR amplification.

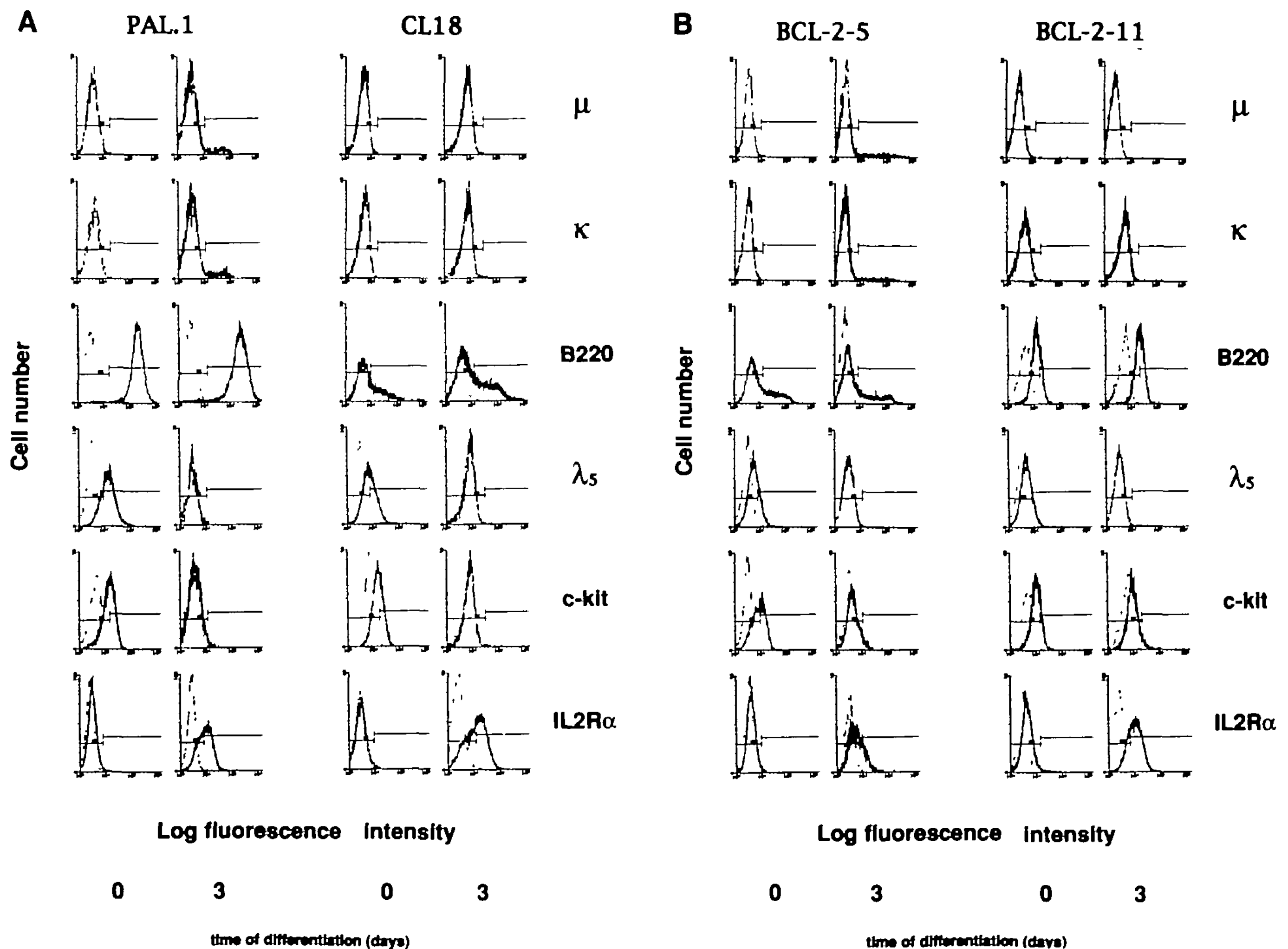

Fig. 1. *Expression of a set of B cell differentiation markers (black histograms) on long-term proliferating pre-B cell clones PAL.1 (left) and CL18 (right) from normal BDF₁ mice (a) and of pre-B cell line BCL-2-5 (left) and clone BCL-2-11 (right) from bcl-2-tg mice (b) before and after 3 days of differentiation in vitro. All stainings were carried out with either directly FITC-labeled antibodies (μ, B220) or biotinylated antibodies, which were developed by means of streptavidin – FITC (x, c-kit, Il-2Rα) or streptavidin – PE ($λ_5$). Histograms of control stainings (secondary reagent alone) are displayed in light grey color.*

PCR reactions were performed according to the enzyme manufacturer's protocol (Perkin-Elmer Cetus, Norwalk, CT). The amplification protocol was 20, 94°C; 2 min at 72°C; 35 cycles on a Perkin-Elmer Cetus Thermal Cycler. The PCR products were finally separated and visualized on a 1.5% agarose gel containing 5 μg/ml ethidium bromide.

Sequencing

The PCR primers used for amplification of the DJ_H rearranged genes contain sites for restriction enzymes *EcoRI* and *XbaI*. After restriction enzyme digestion of the PCR products, the digestion mixture was separated on a 1.5% UltraPure Agarose gel (BRL, Bethesda, MD) and bands of interest were cut out. The DNA was isolated with the NaI glassbeads method (Geneclean II kit, Bio 101 Inc., La Jolla, CA), ligated into the M13mp19 vector (New England Biolabs, Beverly, MA) and sequenced (Sequenase, United States Biochemicals, Cleveland, OH).

Results

Phenotype of pre-B cells from normal and bcl-2-tg mice before and after 'in vitro' differentiation

Long-term *in vitro* proliferating pre-B cell clones CL18 and PAL.1 orginating from day 14 fetal liver of BDF_1 mice, as well as pre-B cell line *bcl*-2-5 and clone *bcl*-2-11 from day 16 fetal liver of *bcl*-2-tg mice all divide every 20 – 24 h at 37°C on stromal cells in the presence of IL-7. All four cells lines and clones express various levels of B220, and are positive for c-*kit*, CD43, CD71 (*transferrin receptor*), *and the surrogate light chain encoded by* λ_5 and $V_{pre\text{-}B}$ (Fig. 1 and data not shown). Furthermore, undifferentiated pre-B cells do not express sIg, evident from an inability to be stained for μH and xL chain. They are only weakly positive or completely negative for the IL-2Rα chain p55 (Fig. 1).

Upon differentiation in the absence of IL-7, pre-B cell clone PAL.1 and pre-B cell line BCL-2-5 gave rise to 7.0 and 5 6% respectively (μ,x)-sIg$^+$ B cells among living cells within 3 days (Fig. 1). The pre-B cell clones CL18 and BCL-2-11, however, did not express detectable levels of Ig on the cell surface after differentiation (Fig. 1). Surface expression of c-*kit*, surrogate light chain, CD43, and CD71 was down-regulated on all four pre-B cell lines and clones, while B220 expression remained unchanged and IL-2Rα chain expression was upregulated (Fig. 1 and data not shown). Within 3 days of *in vitro* differentiation >99% of viable cells of all four pre-B cell lines and clones lost the capacity to grow on stromal cells in the presence of IL-7 (Fig. 2). We conclude that the kinetics of differentiation, as measured by the changes in surface marker expression and by changes in the cloning frequencies of differentiated cells on stromal cells in the presence of IL-7, is comparable in all pre-B cell lines and clones independent of the expression of surface Ig.

DJ_H rearrangements of pre-B cell clones CL18 and BCL-2-11

PCR amplification of DJ_H rearrangements (see Methods) revealed that PAL.1 and BCL-2-11 are clonal, with two different DJ_H rearrangements on the two heavy chain alleles (Table 1). The BCL-2-5 pre-B cell line had DJ_H rearrangements to all four J_H segments (Table 1), indicating that BCL-2-5 had continued to rearrange D to J_H *in vitro* (29). Only one DJ_H rearrangement could be detected by a DJ_H PCR for pre-B cell clone CL18, even when including D_{Q52} and $D_{FL16.2}$ specific primers in the PCR (Table 1), while in Southern blots with CL18 two different DJ_H rearrangements were identified (24). Since transfer of CL18 pre-B cells into SCID mice did not result in the generation of measurable levels of serum IgM, it can be assumed that both chromosomes are non-functional for H chain expression (24).

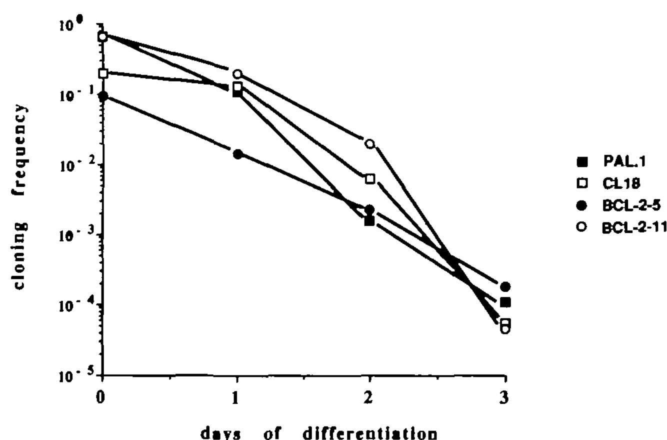

Fig. 2. Cloning frequency of long-term proliferating pre-B cell lines and clones on stromal cells in the presence of IL-7 after various times of *in vitro* differentiation in the absence of IL-7. Frequencies of pre-B cells with the capacity to express μH chain protein (PAL.1 and BCL-2-5) are represented by closed symbols, open symbols denote pre-B cell clones CL18 and BCL-2-11, which are not able to express μH chains upon differentiation. The data of one of three experiments was selected for this figure.

Table 1. DJ_H rearrangements of long-term *in vitro* differentiating pre-B cell lines and clones

Line or clone	H chain locus	Reading frame[a]	Rearrangements
PAL.1	1. allele: DJ_{H2}	one	functional
PAL.1	2. allele: DJ_{H4}	three	non-functional
CL18	1. allele: DJ_{H2}	three	non-functional
CL18	2. allele: ?	?	apparently non-functional
BCL-2-5	DJ_{H1},DJ_{H2},DJ_{H3},DJ_{H4}		functional and non-functional
BCL-2-11	1. allele: DJ_{H1}	three	non-functional
BCL-2-11	2. allele: DJ_{H2}	one	non-functional

[a]For definition of reading frames see Kaartinen and Mäkelä (40).

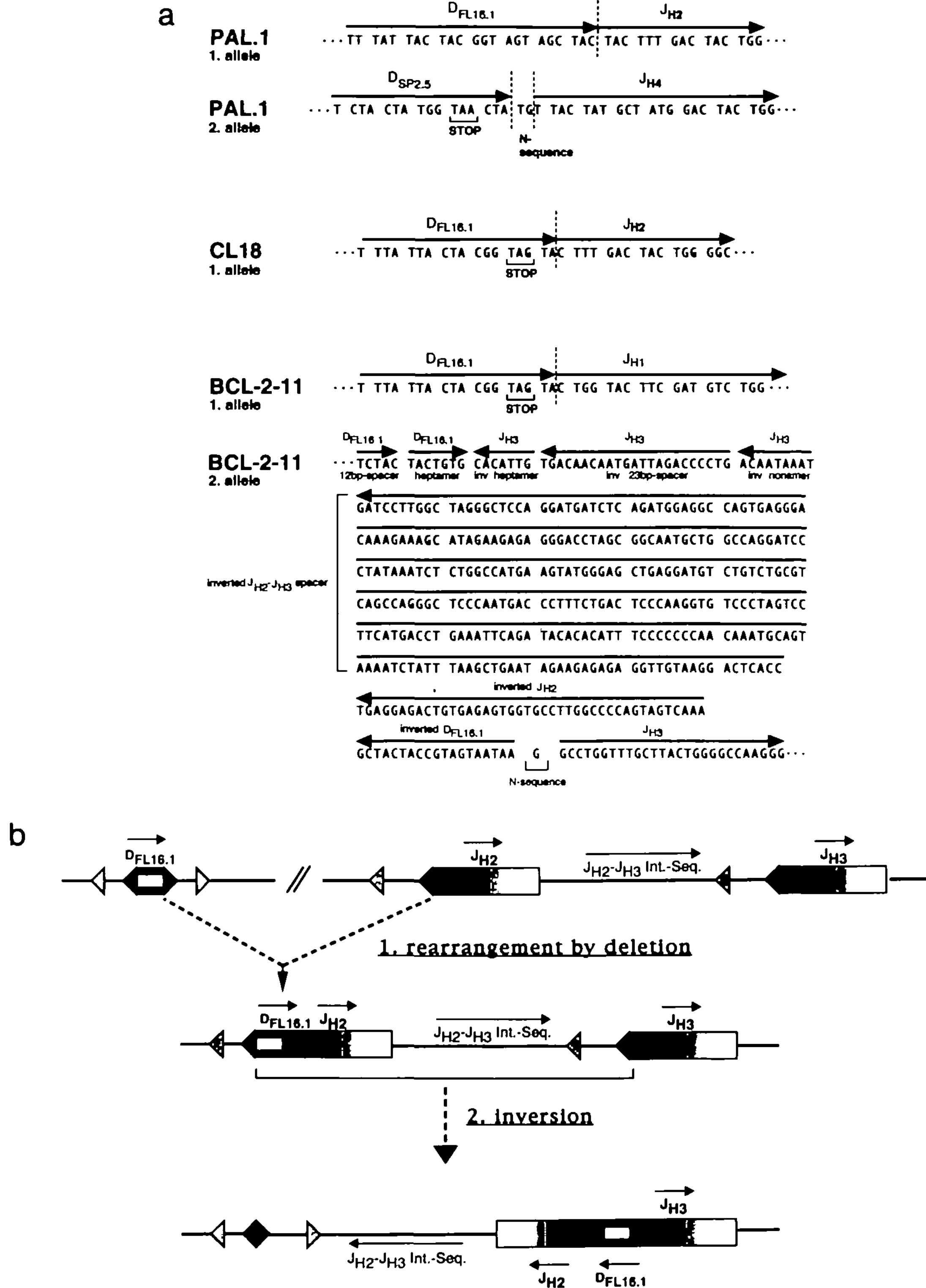

Fig. 3. (a) Nucleotide sequences of cloned DJ_H joints from pre-B cell clones PAL.1, CL18, and BCL-2-11. In the case of normal DJ_H joints, codons imposed by the fixed rfs of J_H gene segments are emphasized by spaced typing. Stop codons are indicated by STOP. Nucleotide sequences originating from different D and J_H gene segments as well as recombination signal sequences (RSS) are marked. Their transcriptional orientation is indicated by arrows. Although all pre-B cells are fetal liver-derived, PAL.1 contains two nucleotides of N-sequence, which correlates with the finding that PAL.1 is a rare clone expressing detectable levels of mRNA specific for terminal deoxynucleotidyl transferase (data not shown). (b) Schematic representation of the rearrangement events, which might have occured on the second H chain allele of pre-B cell clone BCL-2-11. First a normal deletional DJ_H rearrangement between $D_{FL16.1}$ and J_{H2} had taken place. This was apparently followed by a VDJ recombinase-mediated, inversional rearrangement using the RSS 5′ of J_{H3} (containing a 23 bp spacer) and the RSS 5′ of $D_{FL16.1}$ (containing a 12 bp spacer).

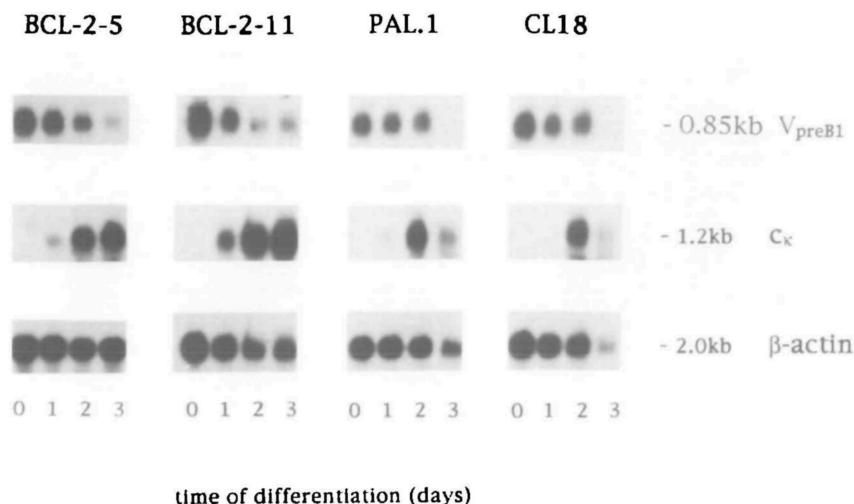

Fig. 4. Northern blot analysis of all four pre-B cell lines and clones at different times after induction of differentiation in the absence of IL-7. All lanes were loaded with 15 μg total RNA. The same blot was hybridized successively with the indicated probes. All autoradiographies were obtained by overnight exposure at – 70°C. μH chain specific mRNA was also detectable with all four pre-B cell clones and lines upon differentiation (data not shown).

However, the primary structure of the DJ_H joint of the second chromosome needs to be detemined.

Sequences for DJ_H rearrangements of all pre-B cell clones are given in Fig. 3(a). They show that BCL-2-11 has both heavy chain alleles rearranged in a way which does not allow the expression of a functional heavy chain protein. On one allele a DJ_{H1} rearrangement has occured in reading frame (rf) III (40). In this rf the D segment contains a TAG stop codon. $D_{FL16\,1}$ used in this rearrangement is the most 5′ located D segment, so that a secondary (and thus potentially functional) DJ_H rearrangement can no longer be made on this allele. The second allele contains an inversion of a region spanning the DJ_{H2} plus the adjacent $J_{H2}-J_{H3}$ intervening sequence, which should have occured after a normal $D_{FL16\,1}-J_{H2}$ rearrangement in rfI (Fig. 3b). This region apparently was inverted using the conserved RS sequences 5′ of J_{H3} and 5′ of $D_{FL16\,1}$, evident in the perfectly head to head fused signal joints 5′ of the inverted sequence (Fig. 3a). Again, no additional D segment is left to allow a subsequent functional DJ_H joining. Thus we conclude, that pre-B cell clone BCL-2-11 has two DJ_H-rearranged H chain alleles, which do not allow expression of a μH chain protein upon V_HDJ_H rearrangement.

The DJ_{H2} rearrangement of pre-B cell clone CL18 also uses the most 5′-located $D_{FL16\,1}$ in rfIII, resulting in a stop codon within the D segment (Fig. 3a). In this case the second allele has not yet been identified. It is expected that the second allele is in a configuration which does also not allow the expression of μH chains after V_HDJ_H rearrangement.

Kinetics of κ chain mRNA expression

The extent of κL chain gene expression as a consequence of $V_\kappa-J_\kappa$ gene rearrangement in differentiating pre-B cells was first assessed by measuring the amount of mRNA coding for κL chain protein relative to mRNA for β-actin. In Northern blot analysis a mature $V_\kappa J_\kappa C_\kappa$ mRNA is 1.2 kb in size and can thus be discriminated from a sterile transcript of C_κ alone, which is detectable at a size of 0.8 kb (41). The kinetics of induction of κL chain mRNA expression upon differentiation is nearly identical in pre-B cells with functional DJ_H rearrangements (BCL-2-5, PAL.1) and those with non-functional DJ_H rearrangements (BCL-2-11, CL18) (Fig. 4). The apparent decrease in the amount of κL chain mRNA in normal pre-B cells at day 3 of differentiation is probably due to extensive apoptosis which normal pre-B cells undergo upon differentiation and which affects the quality of the isolated RNA. This is also evident from the decrease of mRNA for β-actin, although to a lesser extent. mRNA for the pre-B cell specific gene $V_{pre\text{-}B}$ gradually decreased with time of differentiation in all four pre-B cell lines and clones. This indicates that the pre-B cells were indeed differentiating to more mature cells. These results suggest that the frequency of κL chain gene rearrangements and their subsequent expression as mature κL chain mRNA at different time points of *in vitro* differentiation in pre-B cells can occur in the absence or presence of μH chain protein.

Expression of κL chain protein

The level of κL chain protein expression in pre-B cells differentiating for 3 days to B cells was determined by Western blotting. In order to enable a quantitative analysis of the amount of κ protein produced by differentiated cells, a κ protein standard (MOPC 41) was included and serial 2-fold dilutions of cytoplasmic proteins from cells of the same genetic background were carried out on the same blots. Furthermore, each blot included cytoplasmic protein preparations of a sIg^+ cell line 5-7 (as a positive control) and of undifferentiated pre-B cells In Fig. 5 the results of one representative experiment out of three independent experiments are shown.

Undifferentiated pre-B cells do not produce detectable levels of either μH or κL chain (Fig. 5a, lanes B and J; and Fig. 5b, lanes B and H). μH chain protein is also not detectable in BCL-2-11 and CL18 cells differentiated for 3 days (Fig. 5a, lanes C–H; and Fig. 5b, lanes C–G respectively), in contrast to BCL-2-5 and Pal.1 cells differentiated for 3 days, in which μH chain protein is detectable.

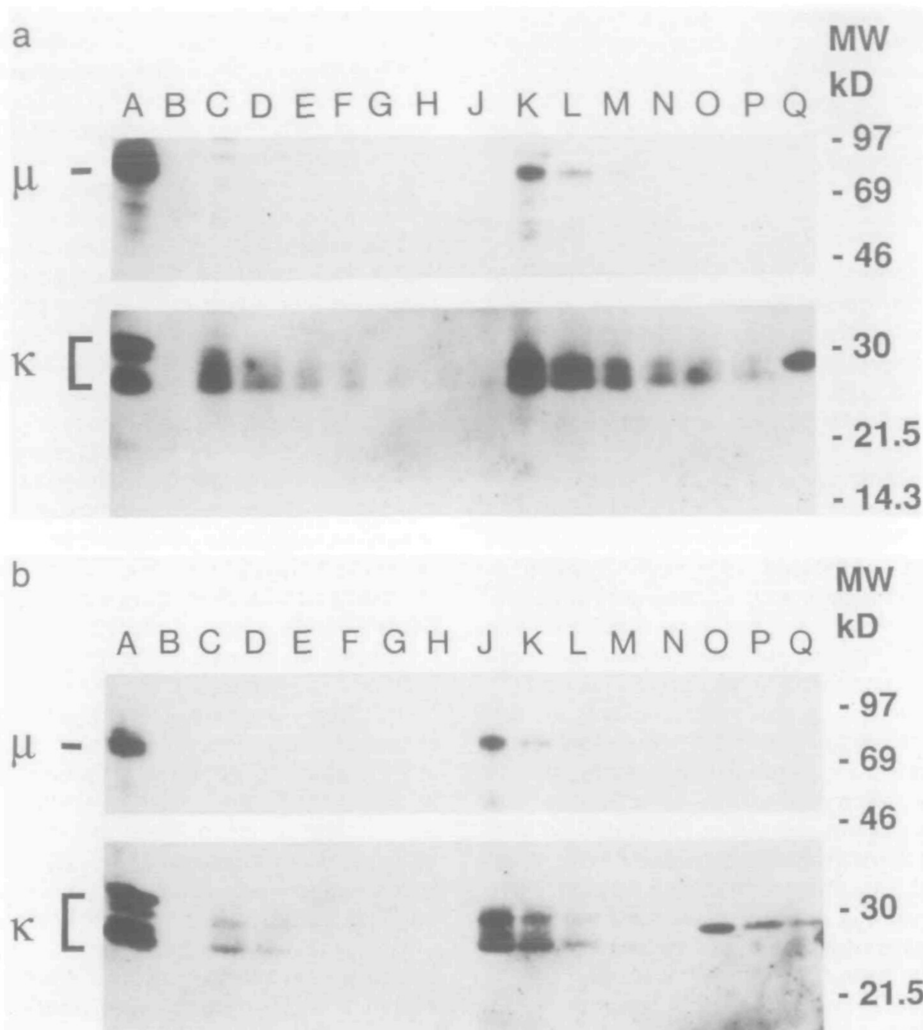

Fig. 5. (a) Western blot analysis of undifferentiated and differentiated cells of pre-B cell clone BCL-2-11 (lanes B – H) and pre-B cell line BCL-2-5 (lanes K – P) The blot was developed with antibodies specific for IgM (top) and κL chain protein (bottom) as indicated. Lane A contains proteins extracted from the sIg$^+$ cell line 5-7 as a positive control (2 × 10^5 cells). Lane Q contains 30 ng of purified MOPC41 κL chain protein as standard. Lane B: 6 × 10^6 undifferentiated BCL-2-11 pre-B cells; lanes C – H: serial 2-fold dilutions of proteins extracted from differentiated BCL-2-11 cells, beginning with 6 × 10^6 cells in lane C. Lane J: 6 × 10^6 undifferentiated BCL-2-5 pre-B cells, lanes K – P: serial 2-fold dilutions of proteins extracted from differentiated BCL-2-5 cells, beginning with 6 × 10^6 cells in lane K. (b) Western blot analysis of undifferentiated and differentiated cells of pre-B cell clones CL18 (lanes B – G) and PAL.1 (lanes H – N). Lane A: same as in Fig. 5(a). Lanes O – Q contain serial 2-fold dilutions of purified MOPC41 κL chain protein standard, beginning with 30 ng in lane O. Lane B: 2 × 10^6 undifferentiated CL18 pre-B cells; lanes C – G: serial 2-fold dilutions of proteins extracted from differentiated CL18 cells, beginning with 2 × 10^6 cells in lane C. Lane H: 2 × 10^6 undifferentiated PAL.1 cells; lanes J – N: serial 2-fold dilutions of proteins extracted from differentiated PAL.1 cells, beginning with 2 × 10^6 cells in lane J. Since pre-B cells derived from normal BDF_1 mice undergo extensive apoptosis upon differentiation, living cells were enriched by Ficoll centrifugation prior to protein extraction.

However, κL chain protein can be detected in differentiated cells from all four pre-B cell lines and clones. It appears that κL chain protein is always slightly more abundant in differentiated cells from normal mice than in those from *bcl*-2-tg mice. This could be due to a reduced rate of biosynthesis or an increased turnover rate of κL chain proteins in bcl-2-tg cells. Normal, as well as bcl-2-tg cells, which are unable to express μH chain protein, express ~3 to 4 fold lower levels of κL chain protein when compared with cells which also express μH chains. This could either be a consequence of an increased stability of κL chains complexed by μH chains or may just reflect an increased amount of κL chain protein which is retained by μH chain protein in the form of Ig complexes on the cell membrane and in the cytoplasm. We conclude that in normal differentiating pre-B cells κ light chain protein can be expressed without μH chain protein.

The average number of κL chain molecules in a differentiated cell can be calculated by a comparison of the intensity of the κL chain protein bands relative to the MOPC41 standard. In Fig. 5(a and b), ~7 × 10^6 BCL-2-11 cells, 2 × 10^6 BCL-2-5 cells, 3 × 10^6 CL18 cells, and 1 × 10^6 PAL.1 cells contain ~30 ng of κL chain protein. (These estimations are also in agreement with the additional Western blots performed with different preparations of cytoplasmic proteins not shown in Figure 5.)

With a molecular weight of 22.5 kDa for κL chains and assum-

ing that ~50% of all differentiated cells may express κL chains from a productively rearranged κL chain locus, it follows that one differentiated CL18 and one PAL.1 cell contains $\sim 4.8 \times 10^5$ and 1.4×10^6 κL chain molecules respectively. For BCL-2-11 and BCL-2-5 the numbers are 2×10^5 and 7.1×10^5 κL chain molecules per cell respectively.

Discussion

Mammalian B cell differentiation is characterized by somatic DNA rearrangements of Ig genes located on three different chromosomes. There is a substantial body of evidence indicating that Ig genes are rearranged in an ordered fashion (see Introduction). However, it is still controversial as to what controls the sequential rearrangement of first heavy, and later κ and λ light chain genes.

The protein products of rearranged Ig genes have been implied in the regulation of an ordered progression of H and L chain gene rearrangements, especially since it has been shown that κL chain gene rearrangement can be induced in a H chain negative (null) A-MuLV transformed pre-B cell line by expressing a membrane bound μH protein (15). However, it has to be considered that the A-MuLV cell line used for this study was derived from a cell line previously shown to be prone to differentiation *in vitro* (7). No clear correlation between μH chain protein expression and κL chain gene rearrangement could be seen, since many sublines retained the κ locus in germline configuration, although μH chain proteins were expressed at high levels. Furthermore, analysis of A-MuLV transformed pre-B cell lines derived from SCID mice revealed that some of these cells attempted κL chain gene rearrangements in the absence of μH chain protein. More importantly, cells with silent κL chain loci could never be induced to attempt κL chain gene rearrangement after transfection with a membrane μH chain expression vector (18,19).

The results presented in this study are not consistent with a model suggesting that H chain protein is necessary for κL chain gene rearrangement since differentiation and κL chain gene rearrangements appear to occur to the same extent in pre-B cells which can, and in pre-B cells which cannot, express μH chain protein. Our findings support the conclusion reached earlier with human transformed cell lines, that κL chain rearrangements can occur in the absence of μH chain expression. They are also in agreement with recently published data, showing that the frequency of κL chain gene rearrangement in sorted pro-B and pre-B cell fractions of bone marrow is not affected by the presence or absence of H chain protein or H chain gene rearrangement (22,23).

We find that *in vitro* differentiation of pre-B cells measured by changes in surface marker expression and loss of clonability is also independent of H chain protein expression or surface deposition. These changes upon *in vitro* differentiation are also observed with pre-B cell lines and clones from SCID mice, RAG-2 deficient mice (unpublished observation), and from λ_5T mice (42).

In vivo, the Ig-like complex of μH chain with a surrogate L chain apparently exerts its role in selecting large $B220^+$, c-kit^+, $CD43^+$ pre-B cells, with a productive H chain gene rearrangement, into the pool of small $B220^+$, c-kit^-, $CD43^-$ pre-B II cells in the bone marrow, since B cell differentiation is blocked at that particular stage in λ_5T and μMT mice (21,42,43). The μH chain – surrogate L chain complex mediated positive selection and expansion of productively rearranged pre-B II cells in the bone marrow does apparently not occur in our pre-B cell culture system, probably because an important environmental factor for this selection is missing *in vitro*.

A mutation in the λ_5 gene impairs the formation of a pre-B II compartment in bone marrow, which is present in normal mice and comprises $\sim 50-70 \times 10^6$ cells in a young (4 – 6 week old) animal (26). However, λ_5T mice slowly fill up the pool of peripheral B cells from a pre-B I compartment [$\sim 2 \times 10^6$ cells in a normal 4 – 6 week old animal (26)]. This has led to the hypothesis that immature B cells in normal mice are not only generated from pre-B II cells, but also may be directly generated from the much smaller pre-B I compartment (22) If this is true, it can be expected that immature B cells exist, which harbor non-productively rearranged H chain loci and thus are not able to express μH chains, but nevertheless express κ or λL chain protein. Although these cells should be functionally inactive (since they cannot display Ig on their surface), they could be the target of a malignant transformation, which, in the end, may lead to Bence – Jones protein producing plasmacytomas. To test this hypothesis, an analysis of the configurations of H chain loci in such plasmacytomas is under way.

The independence of κL chain gene rearrangements from H chain protein expression in normal pre-B cells supports a model of an ordered Ig gene rearrangement that might mainly be regulated by the sequential accessibility of first H chain, and later κ and λ chain gene loci for the enzymes of the VDJ – recombinase complex (44 – 46). Thus, rearrangements of H and L chain gene segments are carried out in a stochastic way as proposed by Coleclough and colleagues (47), initially with a higher probability of H chain over L chain gene rearrangements. It remains further to be elucidated whether intrinsic preferences exist for $V_H - DJ_H$ over $V_\kappa - J_\kappa$ rearrangements, as they appear to exist for $V_\kappa - J_\kappa$ over $V_\lambda - J_\lambda$ rearrangements (48). The model further implies that once a hematopoietic stem cell becomes commited to the B cell lineage, a molecular program of differentiation is initiated that can take place independent of external stimuli and can lead to the generation of immature sIg^+ B cells in the absence of proliferation.

Acknowledgements

We like to thank Drs Klaus Karjalainen and Shunichi Takeda for critically reading the manuscript. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd, Basel, Switzerland.

Abbreviations

A-MuLV	Abelson murine leukemia virus
PCR	polymerase chain reaction
PE	phycoerythrin
rf	reading frame
tg	transgenic
RSS	recombinase signal sequence
μMT	μH-deficient mice

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