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Role of immunoglobulin [subscript mu] heavy chain in the regulation of [subscript kappa] light chain gene rearrangement

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Role of Immunoglobulin μ Heavy Chain in the Regulation of κ Light Chain Gene Rearrangement

by

Alan Murray Shapiro

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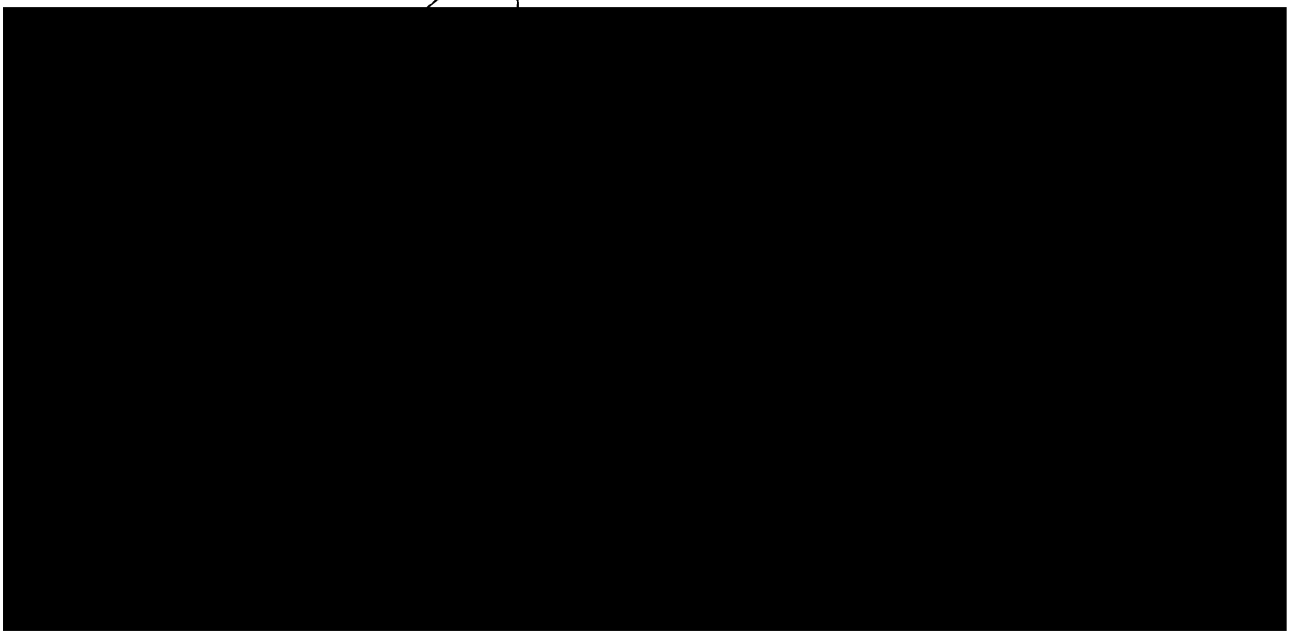

This thesis is dedicated to my friends

who have stood by me throughout my graduate training.

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The work described in chapter one was a collaborative effort with Mark Schlissel both when he was a fellow in the lab of David Baltimore at the Whitehead Institute and as a assistant professor at John Hopkins University School of Medicine. Mark Schlissel determined the IgH genotype (Table 1) and measured the κ rearrangement frequency (Figure 4) of the K.40 B-lineage pairs . In addition, Mark Schlissel also confirmed by PCR that the transcripts detected by Northern hybridization in Figure 6 were Cκ germline transcripts.

Further acknowledgments for advice and support are at the end of chapter one.

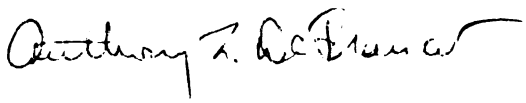
Role of Immunoglobulin Heavy Chain μ in the Regulation of Light Chain κ Rearrangement

Alan Murray Shapiro

ABSTRACT

B lymphocyte development exhibits a characteristic order of immunoglobulin (Ig) gene rearrangements. Sucessful Ig rearrangements may not only mark the maturational stages but may be responsible for inducing progression through B cell development. Previous work has led to the hypothesis that expression of Ig μ heavy chain induces rearrangement at the κ light chain locus. To examine this issue in more detail, we isolated five matched pairs of μ^- and endogenously rearranged μ^+ cell lines from the Abelson murine leukemia virus (A-MuLV) transformed pro-B cell line K.40. In four of the five μ^+ cell lines, substantial expression of μ protein on the cell surface was observed by immunoflourescence and this correlated with an enhanced frequency of κ immunoglobulin gene rearrangement compared to the matched μ^- cell lines. The increased κ rearrangement frequency, measured by quantitative PCR, was not due to a general increase in V(D)J recombinase activity in the μ^+ cells which was determined by introducing an exogenous V(D)J recombination substrate into cells by transient transfection. Consistently, introduction of a functionally rearranged μ gene by electroporation into one of the μ^- pre-B cell lines resulted in a 5-fold increase in κ gene rearrangements. In three of the four clonally matched pairs with increased κ rearrangements, the increase in κ gene rearrangement frequency was not accompanied by a significant increase in germline transcripts from the C_κ locus, as detected by northern hybridization. However, in the fourth pair, K.40D, we observed an increase in germline transcription of the κ locus after expression of μ protein encoded by either an endogenously rearranged or a transfected functional heavy chain allele. In these cells, the

amount of the germline C_κ transcript correlated with the frequency of rearranged κ genes measured. These results support a regulated model of B cell development in which μ protein expression in some way targets the V(D)J recombinase to the κ gene locus.



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INTRODUCTION

This thesis is concerned with the temporal control of immunoglobulin (Ig) rearrangement, especially with the role that μ heavy chain plays in the regulation of κ light chain rearrangement. To introduce my work, I will briefly describe the structure and assembly of immunoglobulins and then give an overview of what is known about B cell development and the approaches used to study it. The remainder of my introduction will lay both the theoretical and experimental basis of my project.

Antibody Molecules

Antibody molecules have as their basic structural unit two identical heavy chains and two identical light chains joined together via disulfide bonds to form a heterotetramer. Antibodies can be produced in either membrane-bound or secreted forms. The heavy and light chains of antibodies each have one variable domain and one or more constant domains. The variable domains from one heavy chain and from one light chain combine to form an antigen binding pocket. The variable domains are encoded by several gene segments which are rearranged during B cell development to generate the large number of specificities observed in the antibody repertoire. The heavy chain is generated by bringing together three classes of segments: variable (V_H), diversity (D_H) and joining (J_H). These recombinations generate a variable exon, which is linked to the constant region (Cμ) exons by RNA splicing. The light chain is generated by bringing together two classes of segments: variable (V_κ or V_λ) and joining (J_κ or J_λ), adjacent to the corresponding constant region (C_κ or C_λ). When rearrangements of the heavy chain gene and light chain gene have been completed, the newly constructed genes direct the synthesis of the heavy and light chains that combine to form an immunoglobulin molecule.

B Cell Development

B lymphocytes are derived from hematopoietic stem cells which are located in the liver during fetal development and later are present in the bone marrow. These stem cells give rise to multipotential cells that can generate a subset of the hematopoietic lineages. Under appropriate conditions, the multipotential stem cells differentiate into cells committed to the B lymphocyte lineage. The growth dependence of B cell precursors for either contact with stromal cells and/or for molecules derived from them changes during development as will be discussed in more detail below. Along with these changes, developing B cells exhibit an ordered rearrangement of their Ig genes and differential expression of a number of cell surface molecules. These changing properties have been used to follow B lymphocyte development and to characterize developmental intermediates.

The earliest cells observed to be committed to the B lymphocyte lineage exhibit joining of the heavy chain gene segments D_H to J_H, the expression on the cell surface of the B220 form of CD45, the expression of a number of genes that are important for B cell development, and the additional requirement of interleukin 7 (IL-7) for growth. Cells containing one or two DJ_H rearrangements are referred to as pro-B cells or committed stem cells. These pro-B cells express recombinase activating genes RAG-1 and RAG-2 (Oettinger *et al.*, 1990; Schatz *et al.*, 1989; Schatz *et al.*, 1992), which play essential roles in V(D)J recombination events. Pro-B cells typically exhibit ongoing rearrangement of the heavy chain locus (V to DJ_H rearrangements and some D-region replacement) and very little or no light chain rearrangement. In fetal liver cell culture, completion of D_H to J_H rearrangement on both heavy chain alleles coincides with the expression of B220 (Cumano and Paige, 1992). Ig-α, the *mb-1* gene product, is expressed just prior to or concomitantly with D_H to J_H rearrangement (Palacios and Samaridis, 1992). Ig-α forms

a heterodimer with Ig-β (B29 gene product) and this heterodimer forms a complex with membrane IgM, the formation of which is required for surface expression of IgM in B cells (Reth, 1992). As will be discussed below, Ig-α and Ig-β may play important roles in events occurring in the pre-B cell, once Ig μ heavy chain is expressed. Pro-B cells also express the alternative or surrogate light chains ω (λ5 gene product) and ι ($V_{pre\text{-}B}$ gene product) (Palacios and Samaridis, 1992). Another property of pro-B cells is their growth requirement. The multipotential cells that give rise to B lymphocyte precursors require stromal cell contacts and stromal cell derived factors other than IL-7 for growth and differentiation. A further characteristic of pro-B cells is that their growth becomes dependent on stromal cell derived IL-7 in addition to stromal cell contact (Era *et al.*, 1991; Hayashi *et al.*, 1990; Takeda *et al.*, 1989). Thus, commitment to the B cell lineage involves acquisition of a wide range of B lineage-specific properties.

The next step in B cell development involves rearrangement of a V_H gene segment to the DJ_H site. An in-frame rearrangement of this type results in the production of a functional heavy chain and the resulting cell is now referred to as a pre-B cell. The transition from pro- to pre- B cell triggered by μ heavy chain expression also is accompanied by the shutdown of expression of both CD43 (Hardy *et al.*, 1989), a cell surface adhesion molecule, and c-kit (F. Melchers, personal communication). Loss of expression of CD43 and other cell-surface molecules in pre-B cells may result in decreased interaction with the stromal cells. This is also accompanied by the ability to grow in the presence of IL-7 alone, without the need for other stromal cell factors or contacts (Era *et al.*, 1991; Hayashi *et al.*, 1990; Lee *et al.*, 1989). Subsequent heavy chain rearrangement is generally not seen in pre-B cells, whereas light chain rearrangements now begin. Heavy chain μ is found primarily inside the pre-B cell and is associated with the products of the λ5 and $V_{pre\text{-}B}$ genes (Hollis *et al.*, 1989; Karasuyama *et al.*, 1990; Pillai and Baltimore, 1988; Tsubata and Reth, 1990). As will be discussed

later, during the pre-B cell stage it appears that this $\mu/\lambda5/V_{pre\text{-}B}$ complex also can be expressed on the cell surface (Cherayil and Pillai, 1991; Misener *et al.*, 1991; Nishimoto *et al.*, 1991; Takemori *et al.*, 1990). In non-transformed B cell precursors this complex is present only transiently, since shortly after μ is expressed in pre-B cells, the levels of both λ5 and $V_{pre\text{-}B}$ mRNA and protein are greatly reduced (R. Hardy; F. Melchers; presented at Taos meeting on B cell development, 1993). Thus, in the developing B cell the expression of functional μ heavy chain leads to several significant changes in its properties.

The pre-B cell developmental stage is often divided into 2 sub-stages consisting of large, cycling pre-B cells and small, non-cycling, pre-B cells (Kincade, 1981). It is thought that the large pre-B cells are those cells that have recently rearranged a functional μ chain and have started or are ready to start rearrangement of light chain genes. Small pre-B cells are thought to result from a cessation of growth by the large pre-B cells and to be the immediate precursors of the surface IgM^+ B lymphocytes. In these cells, light chain rearrangement is either ongoing or completed with a functional light chain rearranged but not yet detectably expressed (Henderson *et al.*, 1992). Light chain rearrangement in pre-B cells generally occurs at the κ locus prior to the λ locus (Korsmeyer *et al.*, 1981; Lewis *et al.*, 1982). If no functional κ chain is produced from rearrangement at the two κ alleles, then the constant region of κ is often deleted by the joining of a recombination recognition sequence 3' to C_κ to a heptamer within the J_κ-C_κ intron (Durdik *et al.*, 1984; Siminovitch *et al.*, 1985). Next, rearrangement occurs at the λ locus. Rearrangement at the λ locus does not require previous κ gene rearrangement, since mice with the κ intronic enhancer deleted by homologous recombination exhibit no κ gene rearrangement but still have λ gene rearrangement (K. Rajewsky, presented at Taos meeting on B cell development, 1993). When light chain rearrangement, either at the κ or λ locus, produces a functional molecule, this light chain can be expressed along

with heavy chain to produce a functional IgM molecule. The cell expressing membrane IgM, now called a B cell, leaves the bone marrow and migrates into the periphery. To summarize, large cycling pre-B cells give rise to small non-cycling pre-B cells and then, after successful light chain rearrangement, to B cells.

Approaches to the Study of B Cell Development

From the late 1970's to early 1980's, it was observed that μ heavy chain was produced prior to κ light chain in cells derived from fetal liver and adult bone marrow (Levitt and Cooper, 1980; Siden *et al.*, 1981). This was also seen in Abelson murine leukemia virus (A-MuLV)-transformed B lymphocyte precursor cell lines (Alt *et al.*, 1981). In these systems, in transgenic mice (to be discussed later) and in other transformed B cell precursors, evidence accumulated for regulated temporal control of μ heavy chain and κ light chain rearrangement. Each of these systems for studying B lymphocyte development has its advantages and disadvantages. In fetal liver, B lymphocyte development occurs synchronously and events can be timed following fertilization of the embryo. In contrast, B cell development in the adult bone marrow is asynchronous and therefore only a very small number of cells at a particular stage can be isolated. B lymphocyte precursors from both fetal liver and bone marrow have been cultured on stromal cell lines to try to reproduce the environment in which they normally develop (Whitlock *et al.*, 1985). Given the appropriate conditions, growth and differentiation of B cell precursors does occur in these cultures and this has led to examination of the requirements for these events. One disadvantage of this approach is that B cell precursors cultured on stromal cells are a polyclonal mix of cells at varying stages of B lymphocyte development. Biochemical studies and molecular studies of un-transformed bone marrow cells and fetal liver cells were not possible for many years because there were no cell-surface markers that could be used to enrich for a homogenous

population and because of the diversity of Ig gene rearrangements. With the recent advent of single cell and general PCR techniques as well as markers that delineate different stages of B lymphocyte development, such as CD43, molecular studies on these cells are now practical.

A second approach that has been used to study B cell development has been to transform B lymphocyte precursors with A-MuLV (Alt *et al.*, 1981). B lymphocyte precursors from both fetal liver cells and adult bone marrow can be transformed by A-MuLV and most such lines are apparently 'locked' at the stage of development in which they were transformed. This generates homogenous clonal populations of cells and is particularly useful for characterization of Ig gene rearrangements. In some A-MuLV lines such as 300-19P (Reth *et al.*, 1985) and K.40 (Beck-Engeser *et al.*, 1987), the cells will progress through B cell development to the stage in which they produce both heavy and light chain (Reth *et al.*, 1985). Of course, it should be remembered that A-MuLV lines express a highly active *v-abl* oncogene, which may perturb the developmental effects that are being studied. Nevertheless, these cell lines have provided considerable information about properties of B lymphocyte development.

Genetic manipulation of B cell development in the whole animal has been made possible through the generation of transgenic and mice with targeted gene disruptions. Using transgenic mice, the effect of premature expression or overexpression of a gene can be studied. This type of system is useful for studying dominant effects such as allelic exclusion which will be discussed below. Since premature or over-expression of a gene is a non-physiological condition, transgenic mice are not as useful for the study of whether a gene is required for a developmental process. The best way to study the absolute requirement for a gene, is to eliminate its expression, so that the effects of its absence can be observed. As will be described below, this has been done for the

membrane form of μ heavy chain (Kitamura *et al.*, 1991) and for the λ5 gene (Kitamura *et al.*, 1992). Of course, careful analysis of gene disruption phenotypes are needed to determine when a gene acts during development since the accumulated effects on a process are observed. Thus, the observed phenotype could reflect a defect at an earlier stage. In addition, if either no effect or a small effect is observed in a gene disruption study, this does not indicate the gene plays no role in the studied process. Such a result could indicate that there are either alternative processes and/or genes that can assume the function of the disrupted gene. Nonetheless transgenic and gene disruption studies in mice permit genetic manipulation of B cell development in a complex whole animal system and thus provide an increasingly important strategy for dissection of this pathway.

Regulation of Ig Gene Rearrangements

It has been generally observed that heavy chain rearrangements are completed and the resulting IgH gene is expressed prior to light chain gene rearrangement and expression (Alt *et al.*, 1987; Rolink and Melchers, 1991). Typically, this order of Ig rearrangements has been observed in developing fetal liver cells and in A-MuLV-transformed B cell precursors (Alt *et al.*, 1981; Maki *et al.*, 1980; Perry *et al.*, 1981). It should be noted, however, that light chain κ rearrangement and expression has been seen in the absence of heavy chain rearrangement in exceptional cases (Blackwell *et al.*, 1989; Hendrickson *et al.*, 1990; Kitamura and Rajewsky, 1992; Kubagawa *et al.*, 1989).

Is the typical temporal order of Ig gene rearrangements the result of stochastic processes or is it brought about by some form of regulation? The temporal order could result from the heavy chain loci having a higher frequency of rearrangement than the light chain loci. If this were the case, it would be statistically more likely for a heavy chain gene to rearrange successfully first. Alternatively, regulatory events could target the

V(D)J recombinase to the heavy chain locus initially and to the light chain loci after expression of a functionally rearranged μ gene product. The weight of evidence currently favors the regulated model.

One of the strongest arguments for the regulated model is the absolute requirement for expression of the membrane form of heavy chain μ (μ_m) for the production of mature B lymphocytes. Recently, Kitamura et al. used homologous recombination to generate a mouse that contained a homozygous disruption of the transmembrane exon of μ heavy chain, such that μ_m could not be produced (Kitamura *et al.*, 1991). These mice were totally deficient in the generation of mature B lymphocytes indicating a requirement for μ_m protein expression for B cell development. It was also observed that these mice had a 20-fold reduction in κ gene rearrangement in the bone marrow as compared to normal mice (Kitamura and Rajewsky, 1992). This work argued that μ_m is not necessary for the induction of κ gene rearrangement, but its presence does lead to more efficient rearrangement of the κ gene. The experiments presented in this thesis provide evidence for μ stimulating an increase in κ rearrangement over the low level that occurs in its absence. Interestingly, mice heterozygous for the μ transmembrane domain disruption generated a significant number of B cells with two functional IgH rearrangements (Kitamura and Rajewsky, 1992). This is in striking contrast to normal B cells which rarely have more than one functional IgH rearrangement, a phenomenon termed allelic exclusion. Presumably the defective allele is unable to effect the shut down of further IgH rearrangement, which is responsible for allelic exclusion. Conversely, mice expressing functionally rearranged μ transgenes exhibit decreased rearrangement of endogenous μ heavy chain genes (Rusconi and Köhler, 1985; Storb, 1987; Weaver *et al.*, 1985). These results indicate that μ_m is needed for the completion of B lymphocyte development and, moreover, that it acts as a positive regulator of κ gene rearrangement and as a negative regulator of subsequent μ rearrangements

Molecular studies on B cell precursors from A-MuLV-transformed cell lines also support the regulated model of B cell development. For example, a regulatory role for μ heavy chain was suggested by Reth et al. when they found that μ heavy chain expression was required for the rearrangement of the κ locus in the Abelson transformed pro-B cell line, 300-19P. Light chain κ gene rearrangement was observed only after expression of the membrane form of μ protein; the secretory form of μ heavy chain was ineffective (Reth *et al.*, 1987). This result suggested that μ heavy chain may act by a signaling mechanism to initiate κ gene rearrangement. This effect has not been uniformly seen however. For example, Blackwell et al. observed that transfecting a functional μ chain into an A-MuLV SCID pre-B cell line did not significantly increase the low level of κ gene rearrangements already seen in these μ^- cells (Blackwell *et al.*, 1989). In the last two years, an effect of μ protein on κ gene rearrangements has been reported in two additional publicatons (Iglesias *et al.*, 1991; Tsubata *et al.*, 1992). Iglesias et al. showed that both the first and second constant domain of μ ($C_\mu1$ and $C_\mu2$) are needed for the stimulation of κ gene rearrangement (Iglesias *et al.*, 1991). Similarly, Tsubata et al. showed that a truncated μ chain lacking both the variable and $C_\mu1$ domain did not stimulate κ gene rearrangement (Tsubata *et al.*, 1992). Interestingly, crosslinking of the latter truncated μ molecule with anti-μ antibody did stimulate κ gene rearrangement. One attractive hypothesis suggested by these observations is that binding of proteins, such as alternative light chains encoded by the λ5 and $V_{pre\text{-}B}$ genes, to these μ domains may activate the signaling function of μ and stimulate κ gene rearrangement. Taken together, these studies with A-MuLV-transformed B cell precursors support the role of μ heavy chain in regulating light chain κ rearrangement and suggest that this regulation may occur via a signaling mechanism.

In addition to full length μ protein, a truncated protein called Dμ can be produced from the IgH locus, and this Dμ protein has been shown to play a negative regulatory role during B cell development. Although D_H to J_H joining can be in any one of three reading frames, in mature antibodies there is a strong skewing to reading frame 1 (RF1) (Ichihara *et al.*, 1989; Kaartinen and Mäkelä, 1985). In the third reading frame (RF3), most D-J rearrangements usually result in translational stops accounting for the under representation of this reading frame in secreted antibodies. In the second reading frame (RF2), there are no translational stops, and yet antibodies utillizing this reading frame are uncommon. Interestingly, in this reading frame, many of the D_H elements have both transcriptional and translation start sites, that when joined to J_H-Cμ, permit the expression of a truncated form of μ heavy chain called Dμ protein. Like μ heavy chain, Dμ protein can be expressed as either a membrane or a secreted protein due to alternative splicing. Dμ protein can be expressed on the surface of pro-B cells in a complex with the λ5 and $V_{pre\text{-}B}$ gene products (Tsubata *et al.*, 1991). The membrane form of Dμ has been shown to be necessary for the reading frame bias, since mice heterozygous for the disruption of the membrane exon of μ do not show a negative selection against RF2 in the allele containing the disruption (Gu *et al.*, 1991). It has been proposed that membrane bound Dμ gives a negative signal which somehow selects against the cell expressing it. At this point, it is unclear whether Dμ's negative regulation of B cell development is due to the shutdown of heavy chain or light chain rearrangements, premature stimulation of light chain rearrangement, or some other process. The fact that the membrane form of Dμ is responsible again suggests some sort of signaling mechanism is involved.

A Signaling Model for B Cell Development

If μ expression regulates Ig gene rearrangements by a signaling mechanism, how could this signaling be generated? As described above, a truncated μ was able to induce

κ rearrangement upon crosslinking with anti-IgM antibodies in pre-B cells (Tsubata *et al.*, 1992). This is reminiscent of the commonly used method for inducing mIgM signaling in mature B cells. In mature B-lymphocytes, cell surface expression of Ig heavy chain requires association with κ or λ light chains and with the Ig-α and Ig-β accessory proteins. Crosslinking of this membrane Ig (mIg) complex by antigen generates a signal that includes protein tyrosine phosphorylation and phosphoinositide breakdown (DeFranco, 1992). Interestingly, μ chain in pre-B cells can form a mIg-like structure by complexing with at least two polypeptides, originally called ι (iota, 18kd) and ω (omega, 22 kd) (Pillai and Baltimore, 1987). The genes for ω and ι, λ5 and $V_{pre\text{-}B}$ respectively, were isolated as pre-B cell specific genes (Kudo and Melchers, 1987; Sakaguchi and Melchers, 1986). The λ5 gene product shows homology to light chain constant regions and the $V_{pre\text{-}B}$ gene product has homology to light chain variable regions (Venkitaraman, 1992). The ability of the λ5 and $V_{pre\text{-}B}$ gene products to form a complex with μ was confirmed by expressing these genes together with μ heavy chain in either an Ig negative myeloma or a fibroblast cell line (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990). These observations have led many investigators to refer to the λ5 and $V_{pre\text{-}B}$ gene products collectively as alternative or surrogate light chains, to denote the hypothesis that they take the place of light chains in their complex with μ heavy chain.

The importance of λ5 in B cell development was demonstrated by disruption of the gene in mice. In these mice, there was a large reduction in the rate of B lymphocyte production. Thus, although the λ5 alternative light chain may not be absolutely required for development of B cells, it plays an important role. It is attractive to think that the λ5 gene product may do this by participating in the signaling function of μ in pre-B cells. In any case, the ability of μ to associate with alternative light chains in pre-B cells and to form a mIg like structure suggests a possible signalling role for μ in these cells.

Signaling by IgM in mature B cells appears to go through the disulfide linked Ig-α and Ig-β heterodimer that is associated with IgM (Hombach *et al.*, 1990). This heterodimer is also necessary for surface IgM expression (Matsuuchi *et al.*, 1992; Venkitaraman *et al.*, 1991). Both Ig-α and Ig-β share a structural motif in common with the cytoplasmic region of CD3-ζ chain (Reth, 1989). The CD3-ζ chain intracellular domain can activate signaling in T-cells when crosslinked as part of a CD8/CD3-ζ chimera (Irving and Weiss, 1991). Signaling can also be activated by a Tac/CD3-ε chimera which has a very similar structural motif, although the pattern of protein tyrosine phosphorylation was different from that of Tac/CD3-ζ (Letourneur and Klausner, 1992). The cytoplasmic tails of Ig-α and Ig-β can associate in an affinity column with distinct cytoplasmic effectors (PI-3 kinase (Ig-α and Ig-β), Lyn & Fyn (Ig-α) and unidentified proteins pp40 & pp42(Ig-β)) suggesting that these effectors mediate signaling by the mIgM/Ig-α/Ig-β complex in mature B cells (Clark *et al.*, 1992). Indeed, motifs from Ig-α and Ig-β can activate signaling in B cells in the context of chimeric proteins (Law and DeFranco, unpublished observations). In pre-B cells, the μ-alternative light chain complex associates with Ig-α and Ig-β (Nakamura *et al.*, 1992). Thus, it is attractive to postulate that Ig-α and Ig-β may also mediate signaling through the μ-alternative light chain complex in a similar fashion as in mature B cells. Thus signaling may be responsible for informing the cell that μ protein is being expressed and the subsequent steps may properly proceed.

If the complex of μ and alternative light chains is responsible for generating a signal that indicates the presence of μ in the pre-B cell, the question arises as to the nature of this signal and how it is generated. Does the μ-alternative light chain complex need to be expressed on the cell-surface in pre-B cells to generate this signal? One reason for thinking that cell-surface expression might be important for signaling is that the cytoplasmic components of signaling tend to be located at the plasma membrane and

therefore cell-surface expression might be needed to engage these components. At this point, there is no experimental evidence indicating whether or not the μ-alternative light chain complex needs to get to the surface to mediate its putative effects.

One way to assess the significance of the surface expression of the μ-alternative light chain complex is to observe if it occurs during the pre-B cell stage in normal B lymphocyte development. Most studies looking at the surface expression of μ have been done in pre-B cell lines. Most of these, however, express only a small amount of μ on their surface. In contrast, a minority of μ^+ transformed pre-B cell lines express a significant amount of μ on the surface of the cell (Gordon *et al.*, 1981; Hendershot and Levitt, 1984; Paige *et al.*, 1981; Tsubata and Reth, 1990). In two studies looking at normal B lymphocyte development in human fetal bone marrow (15-24 week gestation) (Nishimoto *et al.*, 1991) and murine bone marrow (Cherayil and Pillai, 1991), different conclusions were reached about the significance of surface expression of the μ-alternative light chain complex. Nishimoto et al. observed about 25% of pre-B cells express low levels of μ-alternative light chain on their cell surface and concluded that they represented a distinct stage in B cell development. On the other hand, Cherayil and Pillai examined the expression of λ5, μ, and κ in murine bone marrow cells using fluorescenated antibodies and two-color flow cytometry (Cherayil and Pillai, 1991). They found about 5-10% of the B-lineage cells (pro-B, pre-B, and B cells) co-expressed high levels of μ and λ5 in the bone marrow. But since the same fraction of cells co-expressed κ and λ5, they assumed that these cells were not pre-B cells but rather were "transitional" B cells that had begun to express rearranged κ chain but had not shut down λ5 expression yet. Therefore, they concluded it was unlikely that surface μ positive pre-B cells were a "physiological" intermediate during B lymphocyte development. It is possible, however, that a population of pre-B cells that express a small to moderate amount of μ on the cell surface was overlooked. At this point it is possible that μ-

alternative light chain is expressed on the cell surface of pre-B cells during B cell development and that it may be important for B cell development. Further analysis of this issue will be necessary.

In those pre-B cells lines that express the μ-alternative light chain complex on the surface, crosslinking of this complex by either anti-μ or anti-λ5 antibodies causes an increase in cytoplasmic free calcium (Justement *et al.*, 1990; Misener *et al.*, 1991; Takemori *et al.*, 1990). Increases in intracellular free calcium were also observed in pre-B cells treated with monoclonal antibodies directed against the Ig-α molecule, which, along with Ig-β, form a complex with μ and alternative light chains. Curiously, these monoclonal antibodies either gave a small or negligible response when used to crosslink Ig-α in B lymphoma lines (Nomura *et al.*, 1991) suggesting some structural or functional difference between the mIgM/Ig-α/Ig-β complex and its pre-B cell equivalent. In any case, the complex of μ/λ5/$V_{pre\text{-}B}$/Ig-α/Ig-β does exhibit signaling ability in pre-B cells. In experiments using anti-μ antibodies, an increase of internal calcium was observed but without a measurable increase in phosphoinositide breakdown (Takemori *et al.*, 1990). In B cell lines, the calcium response is a more sensitive measure of membrane IgM engagement than phosphoinositide breakdown (Blum *et al.*, submitted, 1993). Therefore, it is possible that anti-μ treatment of pre-B cells induces small increases in phosphoinositide breakdown that are undetectable by the methods used, but are sufficient to mediate a rise in intracellular free calcium. Again, these results provide further support for the signaling ability of the μ-alternative light chain complex.

It should be noted that these model studies used crosslinking by anti-IgM antibody to activate the signaling properties of μ in pre-B cell lines. Transfection of a μ expression vector into the 300-19P17-27 ($\underline{VDJ}^-/\underline{VDJ}^-$) cell line resulted in an increase in κ rearrangement frequency but there was no further increase upon crosslinking with anti–μ

(Tsubata *et al.*, 1992). This experiment indicates that the μ-alternative light chain complex may generate whatever signal is needed to stimulate κ rearrangement without a requirement for exogenous crosslinking. It could be that exogenous crosslinking of μ-alternative light chain complex generates the same type of signals, possibly at an amplified level, that μ generates in the pre-B cell even in the absence of crosslinking.

Additional Regulation of V(D)J Recombination

In addition to the importance of intrinsic events occurring during B cell development such as the expression of μ_m protein, extrinsic events are also likely to play a role in regulating V(D)J recombination. Stromal cell contact or derived factors clearly play a role in the differentiation of B lymphocyte precursors (Rolink and Melchers, 1991). Interestingly, during development of B lineage cells, the growth and differentiation requirements change. The multipotential cells that give rise to B lymphocyte precursors are dependent on stromal cell contacts and derived factors, with kit-ligand being a major component of these interactions (Billips *et al.*, 1992). Precursors committed to the B cell lineage require IL-7 in addition to stromal cell signals in order to grow and progress to the pre-B cell stage. After a functional μ heavy chain is produced, the pre-B cells lose their dependence on stromal cell signals and become dependent on IL-7 alone (Hayashi *et al.*, 1990) . At a late stage in B cell development, following light chain rearrangement but possibly prior to its expression, the pre-B cells can no longer proliferate in reponse to IL-7 stimulation (Henderson *et al.*, 1992). It appears that it is the expression of μ heavy chain and/or κ light chain that acts to change the growth and differentiation requirements of the B cell precursors in cell culture (Era *et al.*, 1991; Reichman-Fried *et al.*, 1990). It is not known whether there are any external factors that directly influence κ rearrangement, but IL-7 is required for the growth of the pre-B cells prior to κ gene rearrangement. Although it has been hypothesized that the μ-alternative

light chain complex serves as a receptor for an external factor, the current weight of evidence is that the mere presence of this complex inside or on the surface of the cell is sufficient to mediate its regulatory effect (Iglesias *et al.*, 1991; Reth *et al.*, 1987; Tsubata *et al.*, 1992). Thus, it appears that the external factors (stromal cell contacts and IL-7) are permissive for B lymphocyte development, but it is the actual immunoglobulin chains that signal the transition to subsequent developmental stages with their unique growth and differentiation requirements.

Accessibility Model of Regulated Ig Rearrangement

Rearrangements of Ig heavy and light chain genes and of the various T cell receptor genetic loci are all thought to be mediated by a single enzymatic entity, referred to as V(D)J recombinase (Schatz *et al.*, 1992). V(D)J recombination in both T- and B-lymphocytes correlates with RAG-1 and RAG-2 expression (Oettinger *et al.*, 1990; Schatz *et al.*, 1989), and interference with either RAG-1 or RAG-2 function by targeted gene knockout completely eliminates V(D)J recombination (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). Moreover, when a DNA construct containing the recombination signal sequences from either immunoglobulin or T-cell antigen receptor genes are introduced by transfection into B cell or T cell lines with ongoing V(D)J recombination, significant rearrangement occurs in either cell type (Blackwell and Alt, 1984; Lieber *et al.*, 1987; Yancopoulos *et al.*, 1986). So how does V(D)J recombinase select the proper gene to rearrange at the proper time and in the proper lineage? For instance, if V(D)J rearrangements occurred in a random fashion, then rearrangements of T cell receptor genes should occur in B lymphocytes. This has generally not been observed (Yancopoulos *et al.*, 1986; Calman and Peterlin; 1986) although there is some indirect evidence that such rearrangement may occur in human B-cell tumors (Pelicci *et al.*, 1985). These observations suggested that the chromosomal contexts of the antigen

receptor genes are important for determining where V(D)J recombination occurs in a given situation.

In order to explain these observations, Alt and colleagues have proposed that the rearrangement of immunoglobulin and T-cell receptor genes are regulated by modulating the accessibility of these genes to the recombination machinery (Yancopolous and Alt, 1985). In general terms, this accessibility model states that the consecutive rearrangements observed in developing B lineage cells (D_H to J_H, V_H to DJ_H, V_L to J_L) are the result of sequential opening of each region to the recombination machinery. This sequential opening of the various loci is accompanied by production of sterile germline transcripts, and the occurrence of these transcripts correlates well with V(D)J recombination of the loci in question (Lennon and Perry, 1990; Schatz *et al.*, 1992; Schlissel *et al.*, 1991). It is unknown whether the germline transcripts are directly involved in stimulating V(D)J recombination or are simply evidence of opening-up the region, making it accessible to protein complexes such as V(D)J recombinase and RNA polymerase. Moreover, what an opened or accessible region reflects in molecular terms is unknown. Nevertheless, the accessibility model and variations of it are currently favored by many investigators in the field.

How can the accessibility model explain the temporal control of IgH and IgL gene rearrangement? The most straightforward hypothesis would be that the heavy chain loci become accessible to V(D)J recombinase first and the recombinase generates a functional heavy chain gene. Next, the unrearranged heavy chain loci V_H genes become inaccessible, and the light chain loci become accessible (first κ then λ) to V(D)J recombinase. The recombinase then generates a functional light chain and V(D)J recombinase expression is shut down. Other possibilities can be imagined. Instead of accessibility of IgH occurring before acessibility of IgL, both IgH and IgL genetic regions

could become accessible at the same time and additional factors could be responsible for directing the V(D)J recombinase, first to the heavy chain loci and later to the light chain loci.

A central issue for regulated models for the temporal control of heavy and light chain gene rearrangement is how expression of a functional heavy chain leads to a change in V(D)J recombination. One hypothesis is that some product, either message or protein, of the newly produced heavy chain gene somehow sends a signal to the recombination machinery to start rearranging light chain κ. As discussed above, there is some evidence for the hypothesis that this μ protein forms a complex with alternate light chains and Ig-α/Ig-β, and that this complex generates receptor signaling reactions. It is attractive to think that such a signal could regulate either Ig gene accessibility or Ig gene recombination targeting factor(s).

The Mechanism of Temporal Control of Ig Gene Rearrangement

The targeting of the V(D)J recombinase could be mediated by enhancer-binding proteins. Evidence for a link between transcription and rearrangement comes from experiments using transgenic reporter constructs. Using transgenic constructs containing T-cell recombination elements, V_β , D_β, and J_β linked to the μ heavy chain constant region, either with or without the μ heavy chain enhancer, Ferrier et al. found that rearrangement only occurred when the μ heavy chain enhancer was present in the construct (Ferrier *et al.*, 1990). Rearrangements of D_β to J_β occur in these constructs in both B- and T- cells, both of which exhibit enhancer activity of Eμ. In contrast, V_β to DJ_β rearrangements in the construct were only observed in T-cells, where the construct's unrearranged V_β gene was transcribed, and not in B-cells where the V_β gene was not

transcribed. Similarly, Engler et al. observed that stably transfected V(D)J recombination reporter constructs missing an enhancer element had a reduced efficiency of recombination (Engler *et al.*, 1991). These results suggest that cis-acting transcription elements may regulate V(D)J recombination.

In addition to experiments using reporter constructs, there is correlative evidence that suggests that transcription factors may regulate κ gene rearrangement. As described above, transcription of germline C_κ and V_κ gene segments correlates temporally with κ gene rearrangement (Lennon and Perry, 1990; Schlissel *et al.*, 1991; Van Ness *et al.*, 1981). One example of this involves the treatment of A-MuLV pro-B cells with lipopolysaccharide (LPS) which increases both C_κ transcription and κ gene rearrangement frequency (Schlissel and Baltimore, 1989). LPS is known to activate the transcription factor NF-κB, which binds to an enhancer sequence in the intron between J_κ and C_κ. One possibility is that NF-κB has a dual role in both the enhancement of transcription and in the stimulation of rearrangement at the κ locus (Schatz *et al.*, 1992). It should possible to test this hypothesis by using NF-κB expression vectors to cause over-production of this enhancer binding protein. A similar experiment has been done in the pre-T cell line 2017. Over-expression of μ enhancer binding protein E47 following transfection with an expression vector resulted in a marked increase in D_H to J_H rearrangement and induction of germline transcription originating in the J_H-C_μ intron (Schlissel *et al.*, 1991).

Other experiments have found that the correlation between V(D)J recombination and transcription is not absolute. For example, a V_κ-J_κ transgenic reporter construct could rearrange in T-cells in the absence of detectable transcription from the construct (Goodhardt *et al.*, 1989). It is not clear whether the kappa enhancer was responsible for stimulating rearrangement of this introduced gene. Similarly a transiently transfected

minichromosome without an enhancer element exhibited efficient V(D)J recombination when introduced into a pre-B cell line (Hsieh *et al.*, 1992). The behavior of these constructs argues against a requirement for transcription per se to activate rearrangement.

If transcription is not required for V(D)J recombination, then how are these processes related? In addition to transcription, DNase I hypersensitivity and hypomethylation have been observed in the regions in which V(D)J recombination is occurring (Mather and Perry, 1983; Persiani and Selsing, 1989; Yancopoulos *et al.*, 1986). Both hypomethylation and increased DNase I sensitivity are thought to be markers of increased accessibility of the region in which they are found (Razin and Riggs, 1980; Weisbrod *et al.*, 1980). Since it has been suggested that CpG methylation plays a role in inactivation of genetic regions (Ceder, 1988; Doerfler *et al.*, 1990; Selker, 1990), Hsieh and Lieber examined the role of CpG methylation in regulating V(D)J recombination on a minichromosome recombination substrate (Hsieh and Lieber, 1992). CpG methylation was observed to inhibit V(D)J recombination on a replicating minichromosome but not on a minichromosome that does not replicate. Curiously, CpG methylation inhibited transcription in both cases. From these observations, it was suggested that CpG methylation did not cause inaccessiblity to V(D)J recombinase until the minichromosome was replicated. From this and previous results, Hsieh and Lieber argued that it is unlikely that transcription potentiates V(D)J recombination. It is more likely that activation of both transcription and V(D)J recombination reflect the increased accessibility of a CpG hypomethylated region (Hsieh and Lieber, 1992).

The experiments examining the relationship between transcription and the activation of V(D)J recombination suggest at least two possibilities for how the two processes are related. Transcription could be directly involved in making an immunoglobulin genetic region more accessible to V(D)J recombinase. Although V(D)J

recombination has been observed in the absence of transcription, it is still possible that a low level of transcription could be causally involved, perhaps by physically altering the region, thereby promoting V(D)J recombination. Alternatively, increased transcription and V(D)J recombination at a given locus could be the result of the 'opening-up' of the region by enhancer binding proteins or by some other process that activates both transcription and V(D)J recombination.

In the work reported in this thesis, I have examined the extent and nature of regulation of κ gene rearrangement by μ heavy chain in clonally matched pairs of μ^- and μ^+ cell lines and in clones of a μ^- cell line transfected with a μ heavy chain expression vector. The frequency of κ gene rearrangement in these cell lines has been determined and the presence of μ heavy chain has been found to increase this parameter. There is no corresponding increase in the amount of V(D)J recombinase present in these cells, indicating that it is the targeting of the recombinase that is being regulated. Finally, as accessibility to the recombinase is often correlated with transcription of unrearranged Ig gene segments, the effect of μ heavy chain expression on germline Cκ transcription was also analyzed. Under some circumstances, μ heavy chain expression did correlate with increased germline Cκ transcription. Thus, increased κ gene rearrangement may, in part, involve increased accessibility. In addition, there may be increased targeting of recombinase to the κ locus by a mechanism that does not involve accessibility.

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STIMULATION OF κ LIGHT CHAIN REARRANGEMENT BY IMMUNOGLOBULIN μ HEAVY CHAIN IN A PRE-B CELL LINE

INTRODUCTION:

B-lymphocytes develop from hematopoietic stem cells in an ordered progression, characterized by the rearrangement and subsequent expression of the heavy and light chain immunoglobulin (Ig) genes (Alt et al., 1987; Rolink and Melchers, 1991). Typically, the μ heavy chain gene rearranges first in B cell precursors with D_H to J_H recombination followed by V_H to DJ_H recombination. The μ gene product is found primarily intracellularly in the pre-B cell (Levitt and Cooper, 1980; Siden et al., 1981). In some normal pre-B cells and in some pre-B lines, however, a significant amount of μ protein is expressed on the cell surface (Gordon et al., 1981; Paige et al., 1981; Hendershot and Levitt, 1984; Tsubata and Reth, 1990; Cherayil and Pillai, 1991; Nishimoto et al., 1991). In μ^+ pre-B cells, light chain genes (κ or λ) undergo rearrangement (Maki et al., 1980; Alt et al., 1981; Perry et al., 1981) When light chain gene rearrangement is successful, light chain combines with μ to form membrane IgM, which is expressed on the surface of the B cell.

Ordered rearrangement of Ig genes could be due to a higher frequency of rearrangement of heavy chain genes than light chain genes. In this case, it would be statistically more likely for a heavy chain gene to rearrange first. According to this view, the observation that a single B cell has only one functionally rearranged heavy chain allele (allelic exclusion) would be a consequence of an inefficient rearrangement process that infrequently generates in-frame gene rearrangements (Coleclough, 1983). Alternatively, the order of rearrangement could be regulated such that the V(D)J recombinase is initially targeted to the heavy chain locus and is then directed away from the IgH locus and towards the light chain loci once heavy chain μ protein has been expressed (Alt et al., 1987; Blackwell and Alt, 1989; Schatz et al., 1992). Currently, the weight of evidence argues for the latter explanation. For example, cells with two

functional IgH rearrangements are frequent in mice heterozygous for a mutation that prevents μ protein from being expressed in its membrane form (Kitamura and Rajewsky, 1992). Moreover, mice homozygous for this mutation have greatly reduced κ gene rearrangement and a complete arrest of B cell development (Kitamura and Rajewsky, 1992). These results suggest that the membrane form of μ heavy chain plays a critical regulatory role in B cell development. A similar conclusion has been reached from experiments with mice expressing a functionally rearranged μ transgene, which exhibit decreased rearrangement of the endogenous heavy chain gene (Rusconi and Köhler, 1985; Weaver et al., 1985; Storb, 1987; Nussenzweig et al., 1987). This decrease is observed if the transgene expresses the membrane form of μ but not if it expresses the secretory form (Nussenzweig et al., 1987 ; Manz et al., 1988) again indicating a regulatory role for the membrane form of μ heavy chain.

Evidence for the regulated model of B cell development also comes from experiments with Abelson murine leukemia virus (A-MuLV) transformed μ^- pro-B and μ^+ pre-B cell lines (Reth et al., 1987; Iglesias et al., 1991; Tsubata et al., 1992). κ gene rearrangement was examined in cells that expressed μ either following rearrangement of their endogenous IgH genes or following transfection with a functionally rearranged μ gene. Expression of μ by either means resulted in κ gene rearrangements. In contrast, other studies have observed κ gene rearrangements in the absence of μ chain expression and have questioned the role of μ chain in this event (Blackwell and Alt, 1989; Kubagawa et al., 1989; Schlissel and Baltimore, 1989; Hendrickson et al., 1990). We have isolated a matched series of μ^- and μ^+ cell lines from the A-MuLV-transformed cell line K.40 (Beck-Engeser et al., 1987) and have measured the frequency of κ rearrangements in them. Although some κ rearrangements were seen in the μ^- siblings, μ chain expression, from either an endogenously rearranged allele or a transfected allele, consistently led to an increased frequency of κ rearrangements. This increase was not

accompanied by an increase in V(D)J recombination activity for an exogenous substrate and therefore seems to reflect increased targetting of the recombinase to the κ locus.

RESULTS:

Isolation and Characterization of Matched μ^- and μ^+ Pre-B Cell Lines

To examine the role that Ig heavy chain plays in regulating κ light chain gene rearrangement during B lymphocyte development, we chose the K.40 pro-B cell line which exhibits a high rate of heavy chain gene rearrangement and significant light chain gene rearrangement (Beck-Engeser et al., 1987). K.40 cells have a non-functional rearrangement ($\underline{VDJ}^-$) of one IgH allele and a partial rearrangement ($\underline{DJ}$) of the other allele (Beck-Engeser et al., 1987). First, five μ^- subclones of K.40 were isolated by limiting dilution cloning. These K.40 subclones were chosen because, like the parental cells, they were mostly μ^- but did have a significant number of μ^+ cells in the population (~0.2%). The μ^+ cells in these populations are likely to be the result of gene rearrangements that brought a V_H gene to one of the two alleles after the cloning event. In addition, the presence of such cells is indicative of V(D)J recombinase activity being present. To isolate a μ^+ derivative of each K.40 subclone, sib-selection based on cytoplasmic staining with fluorescenated anti-μ antibody was done to enrich for μ^+ cells. The cell populations enriched for μ^+ cells were then subjected to limiting dilution cloning and μ^- and μ^+ pairs of cloned siblings for each pair were isolated (Figure 1). Using this procedure, five matched pairs of μ^- pro-B and μ^+ pre-B lines with functional rearrangements of endogenous IgH loci were obtained.

To confirm that all of the μ^+ K.40 subclones did, in fact, express full length μ chain, cell lysates were examined by immunoblotting with anti-μ antibody (Figure 2). In cell lines

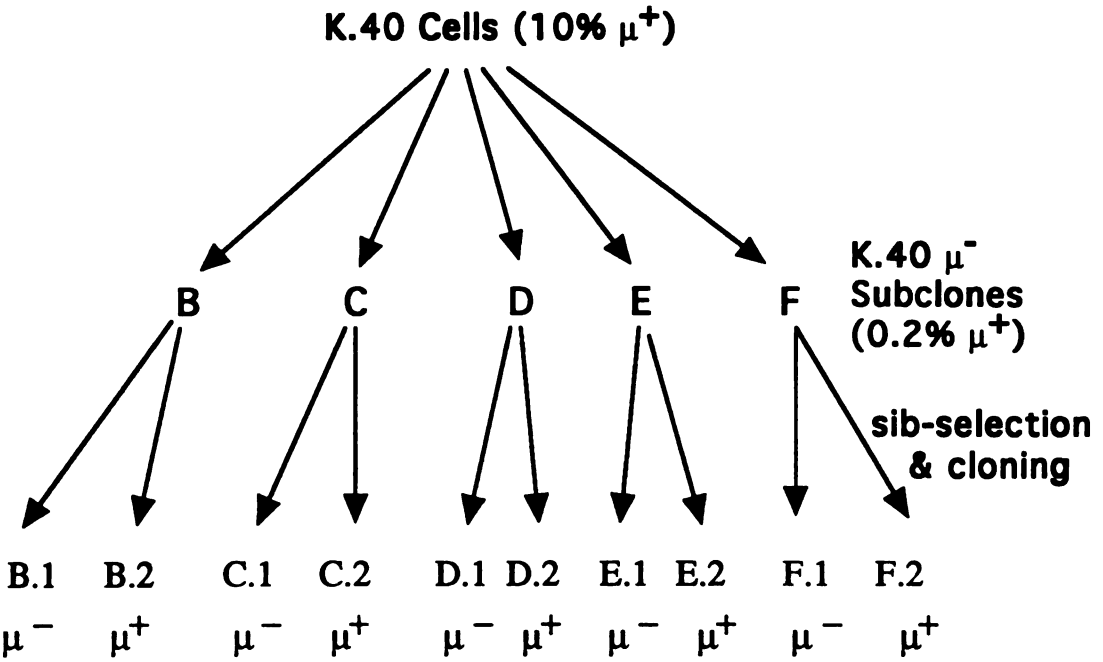


Figure 1: Generation of clonally matched B-lineage cell pairs

Starting from a population of the pro-B cell line K.40 (VDJ $^-$ / DJ) in which 10% of the cells expressed μ-chain, as assessed by immunofluorescence, six μ^- subclones were isolated by limiting dilution cloning. These six subclones each exhibited ongoing rearrangement of the IgH locus resulting in approximately 0.2% μ^+ cells in the populations soon after recloning. Sister clones that were μ^- or μ^+ were isolated from five of the six K.40 subclones by sib-selection. For each clonally matched pair, the μ^- member was referred to as X.1 and the μ^+ member was referred to as X.2.

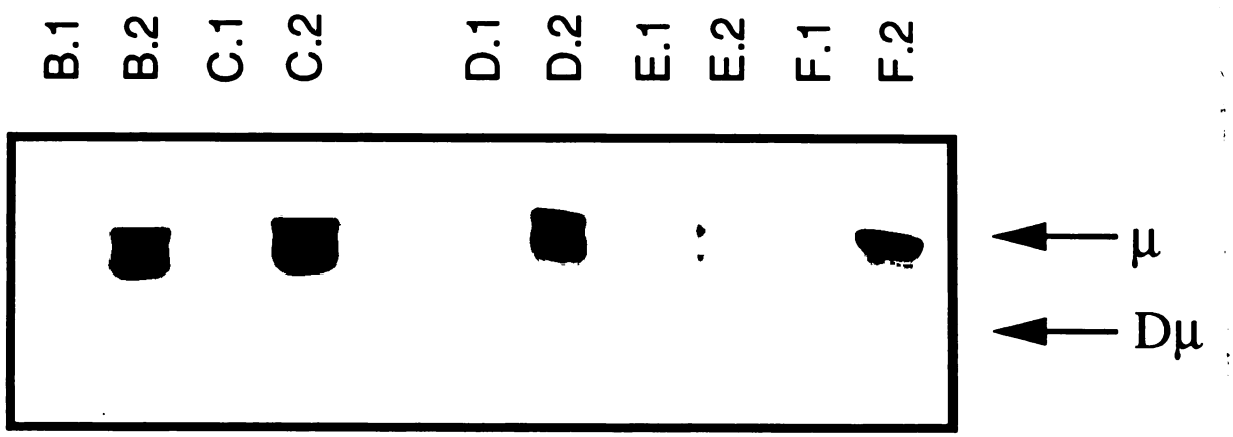


Figure 2: Heavy chain μ and Dμ expression in B-lineage pairs

Protein from detergent-soluble lysates of K.40 subclones were resolved by SDS-PAGE, and the expression of μ and Dμ protein were assessed by immunoblotting with alkaline phosphatase-conjugated anti-mouse IgM heavy chain-specific antibody. Colorimetric detection was used to visualize alkaline phosphatase stained bands. The positions of μ and Dμ proteins are indicated by arrows.

expressing full length μ, immunoblotting usually reveals two adjacent μ protein bands which differ in their degree of glycosylation (Sidman et al., 1981). All the μ^+ cells isolated by sib-selection except K.40F.2 expressed both μ chain bands. K.40F.2 expressed only the lower molecular weight band indicating less extensive glycosylation, which could reflect a change in intracellular localization. Staining of cell surface heavy chain μ on K.40 subclones with fluoresceinated anti-μ antibody followed by flow cytometery showed that all of the K.40 μ^+ subclones expressed μ on the cell surface at reasonably high levels with the exception of K.40F.2 (figs. 3A-C) . These findings were unexpected as most pro-B cell lines express μ protein primarily intracellularly and do not express substantial levels on the cell surface. A few other examples of this phenotype have been reported (Gordon et al., 1981; Paige et al., 1981; Hendershot and Levitt, 1984; Tsubata and Reth, 1990).

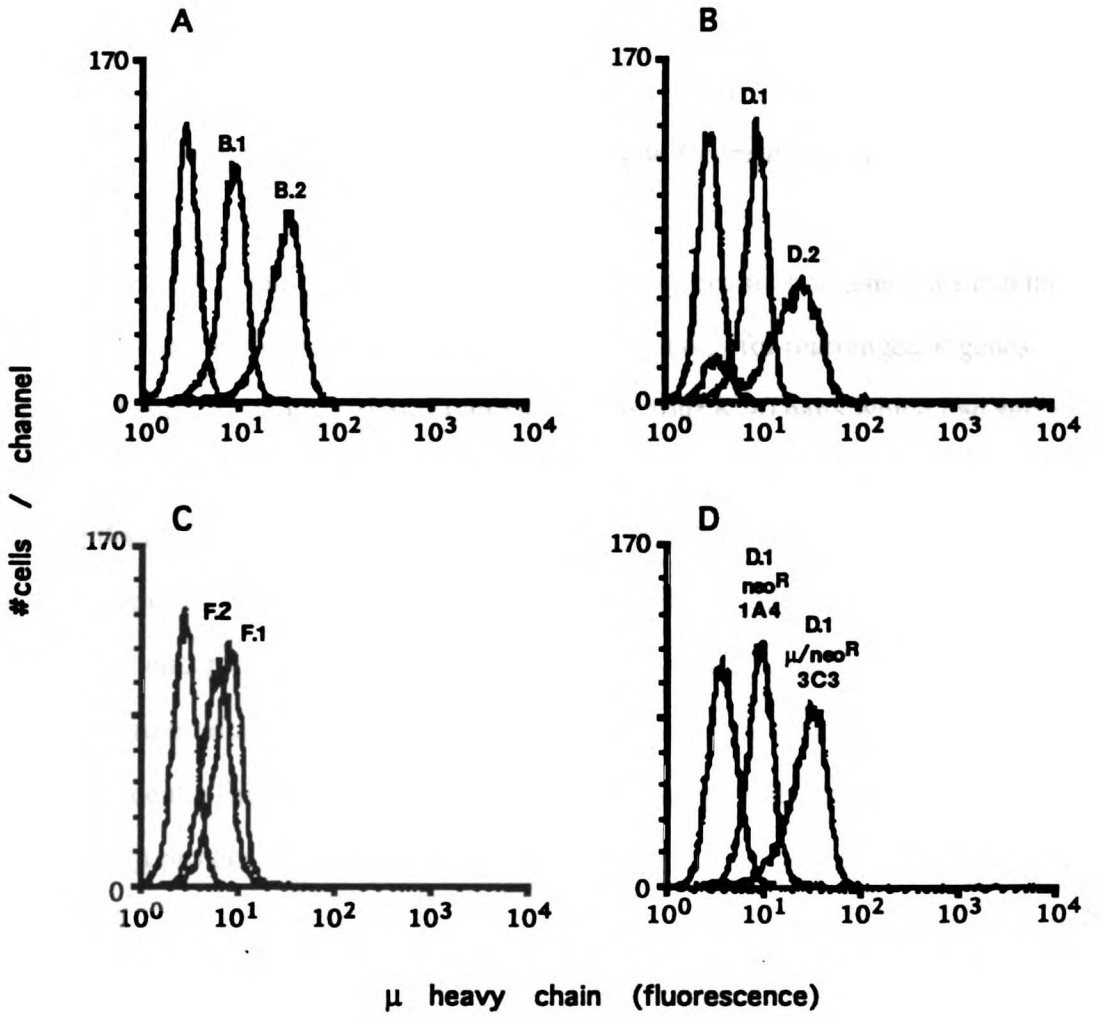
The immunoblotting experiment (Figure 2) demonstrates that all five of the μ^- subclones expressed a shorter version of μ protein. This truncated μ protein is probably the Dμ protein that is synthesized from most DJ alleles in which D and J regions are linked in one particular reading frame (reading frame 2) (Reth and Alt, 1984; Gu et al., 1991). Interestingly, three of the μ^+ derivatives of K.40 (D.2, E.2 & F.2) continued to express Dμ protein . This suggested that full length μ protein in these cells was generated by a V region replacement event on the $\underline{VDJ}^-$ chromosome, leaving the Dμ–expressing DJ_H allele on the other chromosome intact. This possibility was supported by polymerase chain reaction (PCR) analysis of genomic μ chain alleles of each sibling. These analyses demonstrated that the three $D\mu^+$ K.40 derivatives still retained their DJ alleles (data not shown). In the case of K.40E.2 and K.40F.2, this explanation is probably correct. In contrast, K.40D.2 cells expressed a low level of Dμ protein, and moreover, only 80% of them expressed μ on the cell surface (Figure 3B). These observations suggest that the original K.40D.2 cell line was not clonal and contained both cells that expressed only full

Figure 3: Flow cytometric analysis of K.40 siblings and K.40D transfectants for surface heavy chain μ expression

Intact cells were directly stained with fluorescein-conjugated goat anti-mouse μ heavy chain antibody and examined by flow cytometry. The curve to the far left of each histogram represents the autofluorescence profile of unstained cells.

A. Surface heavy chain μ expression of K.40B.1 & K.40B.2 cells. Results for K.40C.1, K.40C.2, K.40E.1 and K.40E.2 were comparable to K.40B.1 and K.40B.2 except that K.40E.2 had a slightly higher surface heavy chain μ expression. **B.** Surface μ chain expression of K.40D.1 & K.40 D.2 cells. About 20% of K.40D.2 cells were surface μ^-. Several μ^+ subclones derived from K.40D.2 were all 100% surface μ^+ suggesting that the K.40D.2 cells were not clonal as originally isolated. **C.** Surface μ chain expression in K.40F.1 & K.40 F.2 cells.

D. Surface μ chain expression in K.40D.1neo^R subclone 1A4 and in K.40D.1 μ/neo^R subclone 3C3. Results for the K.40D.1neo^R subclones 1A5 & 1B2 were comparable to 1A4 and for the K.40D.1 μ/neo^R subclones 3C1, 3C2, & 3D5 were comparable to 3C3.


A
170
0
B.1
B.2
10^0 10^1 10^2 10^3 10^4
B
170
0
D.1
D.2
10^0 10^1 10^2 10^3 10^4
C
170
0
F.2
F.1
10^0 10^1 10^2 10^3 10^4
D
170
0
D.1
neoR
1A4
D.1
μ/neoR
3C3
10^0 10^1 10^2 10^3 10^4
#cells / channel
μ heavy chain (fluorescence)

length μ and other cells that expressed Dμ, probably without full length μ. This interpretation was confirmed by re-cloning these cells, as several subclones of K.40D.2 were obtained that expressed μ on 100% of the cells and did not express Dμ. The K.40B.2 and C.2 cells also expressed full length μ chain but not Dμ protein. These cell lines presumably resulted from V_H to DJ_H rearrangement, resulting in loss of the ability to produce Dμ protein. PCR analysis of μ chain alleles of the K.40B.2 and K.40C.2 siblings supported this conclusion (Table 1).

Effects of Endogenous μ Heavy Chain on κ Gene Rearrangement

The effect of endogenously rearranged μ heavy chain on κ gene rearrangement frequency was measured using a quantitative PCR assay for rearranged κ genes (Schlissel and Baltimore, 1989) (Figure 4). In the four K.40 pairs which had surface μ expression, the frequency of κ gene rearrangement was significantly greater in the μ+ sibling than in the μ– sibling (4-fold to 17-fold). In the fifth pair (K.40F), which lacked surface μ expression, there was little or no increase in the number of κ gene rearrangements present in the μ+ population. Thus, rearrangement of the endogenous IgH locus to produce an in-frame μ gene was correlated with increased κ gene rearrangement. This correlation was particularly strong for those μ^+ K.40 derivatives that expressed μ protein on the cell surface.

The most straightforward interpretation of these results is that expression of μ protein induces κ gene rearrangement and that K.40F.2 is unusual in some regard. An alternative explanation for the increased κ gene rearrangement in the μ^+ siblings is that the selection of cells expressing μ heavy chain also selected for cells that displayed increased κ gene rearrangement for some other reason. For example, in enriching for μ^+ cells through sib-selection, it is possible that cells with a higher level of recombinase activity were selected

Cell Line	Mu allele 1	Mu allele 2
K.40B.1	DJ_3	VDJ_4
K.40B.2	VDJ_3	VDJ_4
K.40C.1	DJ_3	VDJ_4
K.40C.2	VDJ_3	VDJ_4
K.40D.1	DJ_3	VDJ_4
K.40D.2	DJ_3	VDJ_4
K.40E.1	DJ_3	VDJ_4
K.40E.2	DJ_3	VDJ_4
K.40F.1	DJ_3	VDJ_4
K.40F.2	DJ_3	VDJ_4

Table 1: IgH genotype of K.40 B-lineage pairs

Using two separate PCR reactions, one for D_H to J_H rearrangements and the other for V_H to DJ_H rearrangement, the IgH genotype was determined for each K.40 B-lineage pair.

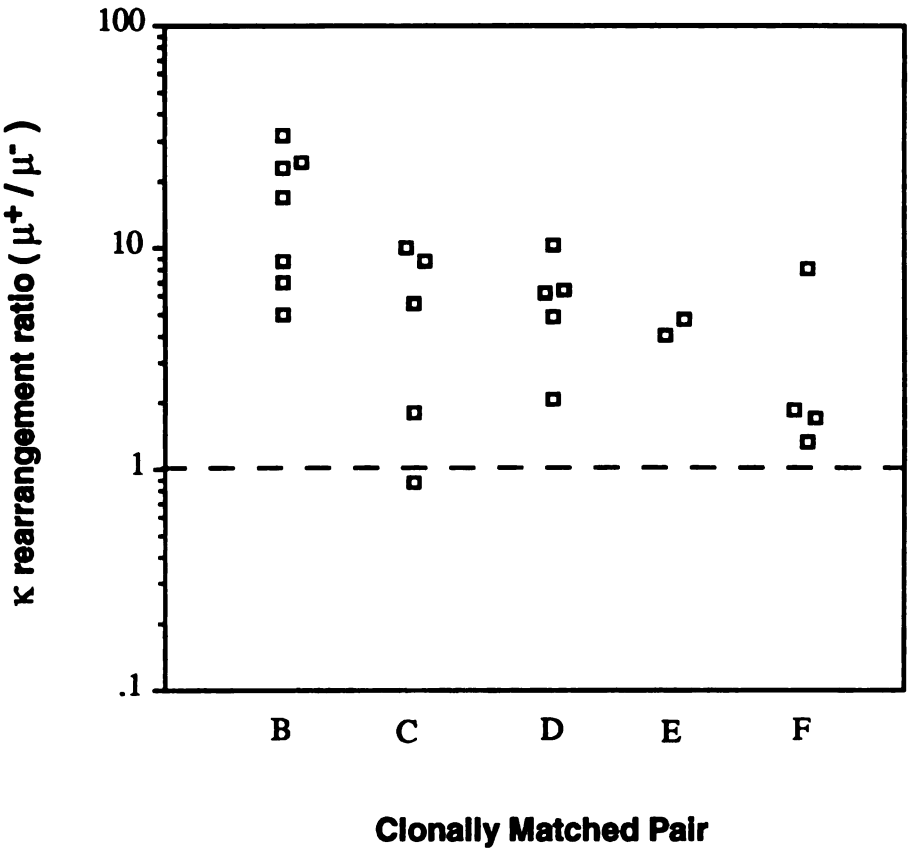


Figure 4: Increased κ rearrangement frequency in μ^+ siblings of K.40 matched pairs.

The frequency of κ rearrangement for each K.40 derived sibling was measured by using a quantitative PCR assay. Each data point shown is the ratio of the number of κ rearrangement in the μ^+ member of the clonally matched pair to the number in the μ^- member. Each data point represents an analysis of a distinct pair of DNA samples in a single PCR assay, except for two of the K.40D points and both of the K.40E points on the graph. For those K.40D and K.40E points, the same sets of DNA samples were analyzed in two separate PCR assays, the ratios averaged and plotted as a single point. For the K.40D points, the ratios were within 50% of the average and the K.40E ratios were within 15% of the average. The dotted horizontal line in the figure represents a ratio of one, i.e., equal κ rearrangement frequency in both μ^- and μ^+cell lines.

(Alt et al., 1992). Such cells, if they exist, would be expected to rearrange the μ gene at a high frequency, and thus be over-represented in the μ^+ pool. In this case, the higher rate of κ gene rearrangement could be due to a higher level of V(D)J recombinase rather than a regulatory effect of μ heavy chain on the choice of target by the recombinase. To examine this possibility, recombinase activity of each sibling was tested by introduction of an artificial recombinase substrate. This substrate consisted of a bacterial plasmid that contained an insert with consensus V(D)J recombination signal sequences in the middle of its lacZ gene and a polyoma origin of replication (Kallenbach et al., 1990). V(D)J recombination removes the insertion in lacZ and generates an active lacZ gene 1/3 of the time. Substrates of this type are rearranged by V(D)J recombinase without regard to the nature of the endogenous antigen receptor genes that are targeted by the recombinase in those cells (Hesse et al., 1987). This assay was used to test relative V(D)J recombinase levels in the K.40 sibling clones (Table 2). In the C & F pairs, the level of recombinase activity was quite similar in the μ^- and μ^+ matched pair of cell lines. In the E pair, the μ^- sibling had a 4-fold higher level of recombinase as compared to the μ^+ sibling. In the D pair, there was a small increase in recombinase activity and in the B pair there was a significantly higher level of recombinase in the μ^+ sibling as compared to the μ^- sibling. Clearly, there was no systematic increase in recombinase level in the μ^+ subclones. It is possible that the increased V(D)J recombinase level in the K.40B.2 cells, and to a lesser extent in the K.40D.2 cells, was responsible, in part, for the increased number of κ gene rearrangements in these cells, compared to their μ^- siblings. However, in K.40C.2 and K.40E.2 cells, κ gene rearrangement frequency was increased with the same or lower V(D)J recombinase activity. Thus, it seems unlikely that greater VDJ recombinase activity can account for the increased κ rearrangement observed in 4 of the 5 K.40 μ^+ cells.

Recombination Frequency for Paired Sister Clones

Cell lines	μ^-	μ^+	n
K.40B	0.21% (±.10)	0.93% (±.24)	4
K.40C	2.0% (±.69)	2.0% (±.46)	8
K.40D	1.8% (1.6-1.9%)	3.8% (3.7-3.9%)	2
K.40E	1.8% (1.6-2.1%)	0.48% (.47-.49%)	2
K.40F	0.99% (.90-1.1%)	1.6% (1.4-1.8%)	2

Table 2: Recombinase activity in clonally matched B-lineage pairs

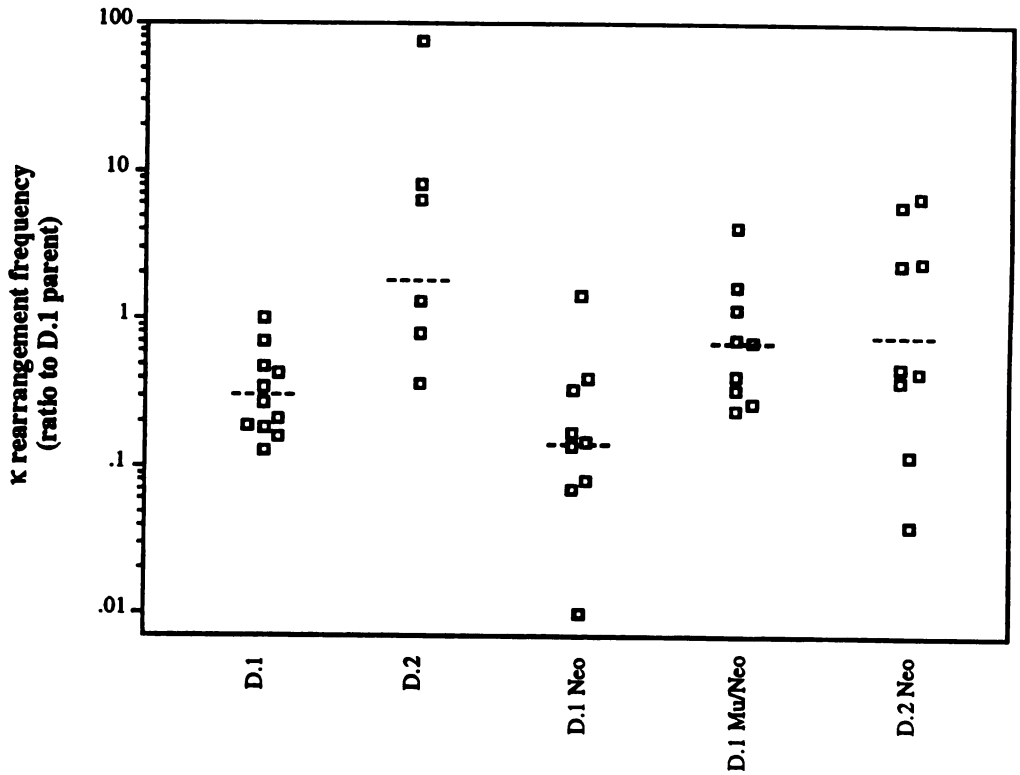
Recombinase activity was determined by transient transfection of K.40 sister clones with a V(D)J recombinase reporter plasmid, pBlueRec. Forty-eight hours after transfection, the cells were lysed and the extra-chromosomal DNA was isolated. The frequency of V(D)J recombination of these molecules was determined by transforming bacteria and then scoring for lacZ phenotype on X-gal plates. The recombination frequency ((# blue colonies x 3)/ # total [blue + white] colonies) x 100% was determined at least in duplicate for the siblings and transfectants. For samples assayed more than two times, the standard error of the mean was used to estimate the error. For samples assayed in duplicate, the range of recombination frequencies obtained is given.

Effects of Transfected μ Heavy Chain on κ Gene Rearrangement

These results are consistent with the hypothesis that μ heavy chain expression resulted in the increase in κ gene rearrangement in the clonally matched pairs. Alternatively, more complicated explanations can be envisioned. It is possible that these events are correlated but not causally related. For example, perhaps a developmental transition has to occur as a prelude to IgH rearrangement in these cells, then this transition might also lead to a higher frequency of κ gene rearrangement. To see if μ is directly responsible for causing increased κ gene rearrangements, we introduced a functionally rearranged μ gene into K.40D.1 cells by transfection and assessed its effect on κ gene rearrangement (Reth et al., 1987; Iglesias et al., 1991; Tsubata et al., 1992). For this purpose, the plasmid pμneoA1 was constructed. This plasmid contains a functionally rearranged μ gene and a neomycin resistance gene. This plasmid or a control plasmid containing just the neo^R gene was transfected into K.40D.1 cells and clones were examined for μ expression by immunofluorescence of permeabilized cells. G418 resistant transfectants that produced an amount of μ roughly equal to that of the μ^+ sibling K.40D.2 were subcloned and subjected to further analysis. As was seen with K.40D.2, the K.40D.1 μ/neo^R cells expressed a considerable amount of μ protein on the cell surface as assessed by flow cytometery (Figure 3D). Preliminary results suggested that a threshold level of μ expression was necessary to see an effect on κ gene rearrangement (data not shown), so low-expressing transfectants were not included in the analysis. Nine independent subclones from each transfection were obtained and analyzed by PCR for the number of κ gene rearrangements in the population. Although there was considerable clone-to-clone variation, we observed an average 4.5-fold increase in κ gene rearrangements in the μ/neo^R transfectants as compared to the neo^R only transfectants (Figure 5). Analysis of variance between the κ gene rearrangement frequency of the K.40D.1 μ/neo^R and

Figure 5: Increased κ rearrangement frequency in μ^+ transfectants as compared to μ^- transfectants

The frequency of immunoglobulin κ gene rearrangement for each K.40 transfectant and subclone was measured and compared to the number of κ gene rearrangements of K.40D.1 cells, as in figure 4. The points in the columns labeled K.40D.1 and K.40D.2 represent independent subclones isolated from the original K.40D.1 and K.40D.2 parents. K.40D.1 neo^R, K.40D.1 μ/neo^R, and K.40D.2 neo^R samples are all neo^R or μ^+ neo^R subclones derived from transfection of K.40D.1 or K.40D.2 with the pneoA1 plasmid or the linked pμneoA1 plasmid. These subclones were isolated by G418 selection and recloning of transfectants expressing the desired genes. The data for this figure were collected from four separate PCR assays, and samples from K.40D.1 and K.40D.2-5 were analyzed in each assay to test for variability between assays. The ratio of the frequency of κ rearrangement of K.40D.2-5 to K.40D.1 was consistent for the four PCR assays (7.8 ± .55). The K.40D.1 and K.40D.2 subclones were analyzed in two different PCR assays in which related subclones were assayed together. About half of the K.40D.1neo^R, K.40D.1 μ/neo^R, and K.40D.2 neo^R subclones were analyzed in one PCR assay and the remainder were analyzed in a second assay. As the standard curve of the two transfectant PCR assays were quite similar, comparisons could be made directly between samples analyzed in the two assays. To control for variability between these two assays, one subclone each of the K.40D.1neo^R, K.40D.1 μ/neo^R, and K.40D.2 neo^R sets were run in both PCR assays . The κ rearrangement values generated in the two assays for these subclones were well within two-fold.


κ rearrangement frequency
(ratio to D.1 parent)
100
10
1
.1
.01
D.1
D.2
D.1 Neo
D.1 Mu/Neo
D.2 Neo

K.40D.1 neo^R subclones revealed that this difference is statistically significant ($p<0.02$). It should be noted that the variability of κ gene rearrangement in the K.40D.1 μ/neo^R subclones could not be explained by differences in the amount of surface expression of μ heavy chain.

We also measured the frequency of κ gene rearrangement in the non-transfected lines K.40D.1 (μ^-) and K.40D.2 (μ^+) along with a number of subclones isolated from each of these cell lines. The average frequency of κ gene rearrangements in K.40D.2 and its subclones was found to be 6.1-fold greater than that in K.40D.1 and its subclones ($p<0.005$) (Figure 5). Finally, we transfected K.40D.2 with the neomycin resistance gene and measured the frequency of κ gene rearrangement in these transfectants. The average frequency in K.40D.2 neo^R subclones was found to be 5.1-fold greater than that of K.40D.1 neo^R subclones ($p<0.05$). Curiously, the average κ gene rearrangement frequency of K.40D.1 neo^R and K.40D.2 neo^R subclones were about 2-fold lower than their non-transfected subcloned counterparts. It may be that either the details of the isolation of these cells, the expression of neo^R gene, or the growth in G418 were in some way responsible for the reduced frequency of κ gene rearrangement in the transfected subclones.

Also evident from the data in Figure 5 is that there was considerable variation in κ gene rearrangement frequency among like transfectants and subclones. These variations could be partly due to the randomness of when rearrangements occur in culture. When a κ gene rearrangement occurs soon after the cloning event, it will contribute a larger number of rearranged κ genes to the population than will a κ gene rearrangement event happening later. This aspect of the methodology may explain why the K.40D.1 subclones had lower κ gene rearrangement frequencies than the parental cell line - perhaps by chance the first κ rearrangement in the parental cell line occurred relatively

soon after cloning. Of course, the higher the rate of rearrangement, the greater the probability that a κ gene rearrangement will occur early following subcloning, so a general correlation is expected between the rate of rearrangement and the number of rearranged κ genes, the measured parameter. In addition, some of the variation observed may reflect true differences in κ rearrangement rate among like subclones or transfectants. This phenomenon has been reported by others (Alt et al., 1992), although we did not see a general loss of V(D)J recombinase activity in the various derivatives of K.40D.1 (see below). For these reasons, we believe that averaging of results from multiple subclones, as we have done here, is currently the most reliable way of assessing κ gene rearrangement rate.

To determine if expression of an exogenous μ heavy chain protein influenced recombination activity, various K.40D derivatives were assayed for V(D)J recombinase activity using the transiently transfected artificial substrate (Table 3). The recombinase activity of these transfectants were comparable to each other. No correlation was found between the frequency of κ gene rearrangement and the level of recombinase in the transfected cells. Moreover, we did not observe a systematic loss in V(D)J recombinase assay upon transfection or subcloning of these cells. Taken together, these data strongly support the conclusion that μ heavy chain expression is responsible for promoting κ gene rearrangement. Moreover, this regulation does not require increased V(D)J recombinase activity as measured with an introduced recombinase substrate.

Effects of μ Heavy Chain on Germline Cκ Transcription

To address the mechanism of how heavy chain μ increases the frequency of κ gene rearrangements, the effect of μ on the transcription of the unrearranged C_κ gene was examined. Two germline transcripts of 0.8 kilobase (kb) and 1.2kb (the latter also

Type	Cell Line	Average κ Rearrangement Frequency (Rearr. / 20,000 genomes)[a]	Recombination Activity (%)[b]
K.40D siblings	D.1 Parent	36	1.8 (1.6-1.9)
	D.2-5	280	3.8 (3.7-3.9)
K.40D.1 transfected	D.1 neo 1A3	2.3	0.52 (.45-.58)
with the neo^R gene	D.1 neo 1A4	0.32	1.5 (±.12)
	D.1 neo 1A5	6.1	1.5 (±.31)
	D.1 neo 1B1	51	2.5 (1.9-3.0)
	D.1 neo 1B2	14	1.6 (1.3-1.8)
K.40D.1 transfected	D.1 μ/neo 3C1	59	2.4 (1.9-2.9)
with μ and neo^R genes	D.1 μ/neo 3C2	41	4.4 (4.0-4.7)
	D.1 μ/neo 3C3	150	1.6 (1.4-1.7)
	D.1 μ/neo 3D5	8.4	1.3 (1.2-1.3)
	D.1 μ/neo 5A4	9.7	1.7 (±.46)
K.40D.2 transfected	D.2 neo 7A6	1.3	1.3 (.75-1.8)
with neo^R gene	D.2 neo 7B4	240	1.8 (1.7-1.9)

Table 2: Comparison of the frequency of κ rearrangement and recombinase activity in K.40D derivatives

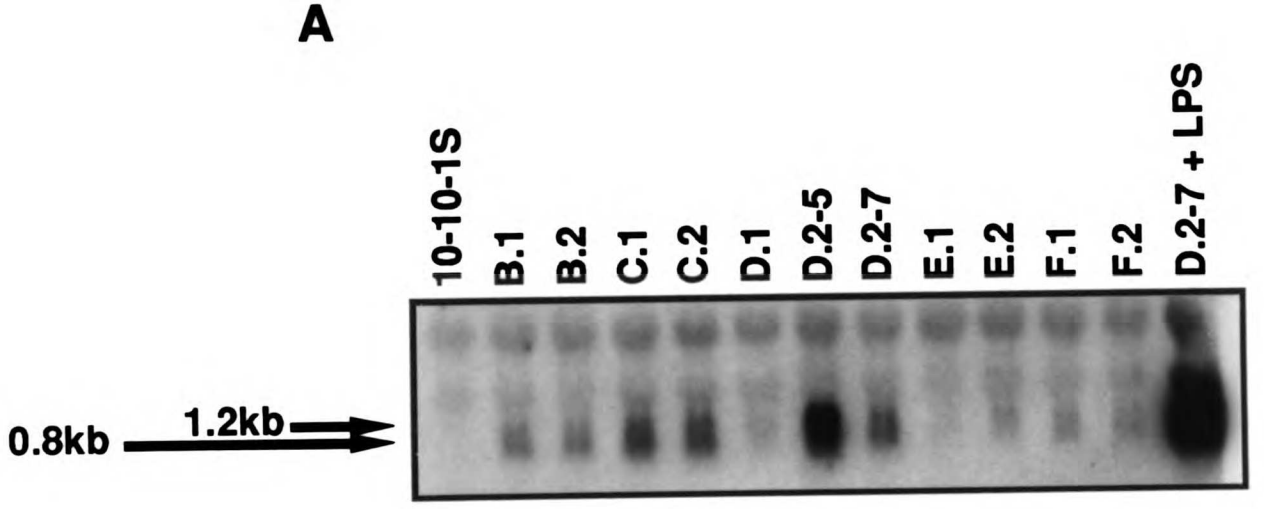
[a] The frequency of κ rearrangement for each K.40D derivative was measured by using a quantitative PCR assay. [b] The recombination activity was determined by transient transfection of the K.40D derivatives with a V(D)J recombination reporter plasmid, pBlueRec, as described in the legend of Table 1. For samples assayed more than twice, the standard error of the mean was used to estimate the error. For samples assayed in duplicate, the range of measured recombination frequencies is given.

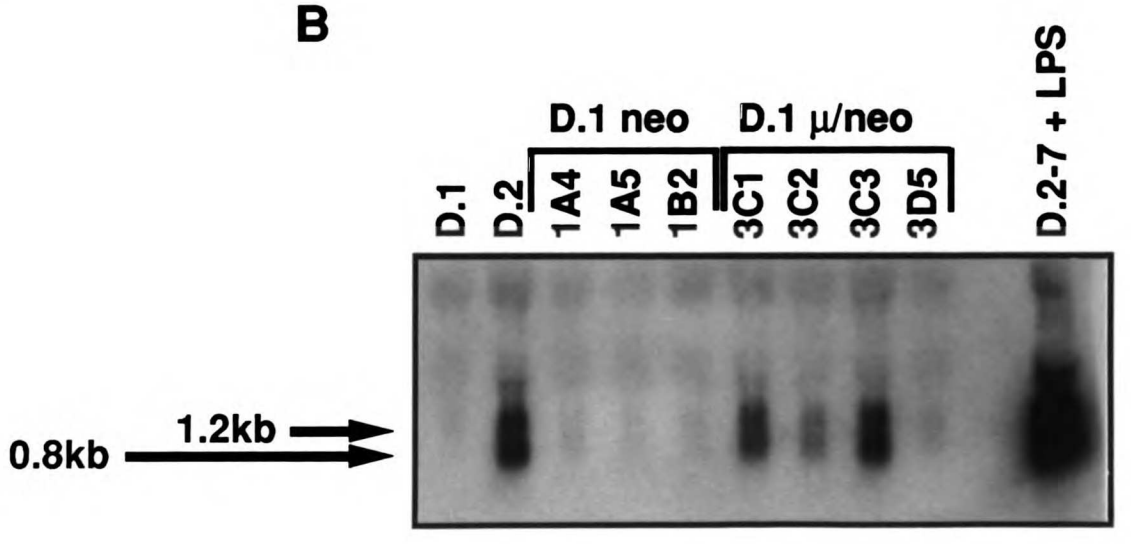
referred to as 1.1kb (Leclercq et al., 1989)) from the C_κ locus have been described (Martin and Van Ness, 1990). It has been previously observed that increases in germline transcription correlate with increased κ gene rearrangement frequency (Schlissel and Baltimore, 1989). Whether these germline transcripts actually promote κ gene rearrangement or are merely reflective of the increased accessibility of the κ locus to protein complexes such as RNA polymerase or V(D)J recombinase is not known (Alt et al., 1992; Schatz et al., 1992). RNA from each of the μ^- and μ^+ clonally matched siblings was isolated and subjected to Northern analysis using a κ_0 probe that detects germline C_κ transcripts (Figure 6A). For four out of the five K.40 pairs , the level of germline C_κ transcription was quite similar for both the μ^- and μ^+ sibling. Only in the case of the K.40D pair was there a considerable increase (~5-fold) in the amount of C_κ germline transcription in the μ^+ sibling as compared to the μ^- sibling. Very similar results were obtained using a quantitative RNA PCR assay for C_κ germline transcription (Schlissel and Baltimore, 1989) (data not shown). Moreover, these PCR experiments confirmed that the 1.2kb transcript is a germline C_κ transcript rather than a rearranged κ germline transcript. Thus, with the exception of the K.40D pair, there was no correlation between increases in C_κ germline transcription and the increased κ gene rearrangement frequency in the K.40 matched pairs. Since K.40D had been used for the transfection studies, κ germline transcription was examined in the some of the K.40D.1 neo^R only and μ/neo^R transfectants to see if the increase in germline C_κ transcription in K.40D.2 was due to μ chain expression (Figure 6B). All of the neo^R only transfectants had a very low level of C_κ germline transcription like the parental K.40D.1 cells. In contrast, the K.40D.1 μ/neo^R transfectants exhibited increased C_κ germline transcription. Increased C_κ germline transcription in the transfectants was confirmed by the quantitative RNA PCR assay for germline C_κ transcription (data not shown). Moreover, there was a linearrelationship between the level of germline transcription and the frequency of κ gene rearrangement observed in K.40D.2 and K.40D.1 μ/neo^R transfectants (Figure 6C). In

Figure 6: Expression of κ germline transcripts in K.40 siblings and K.40D derivatives

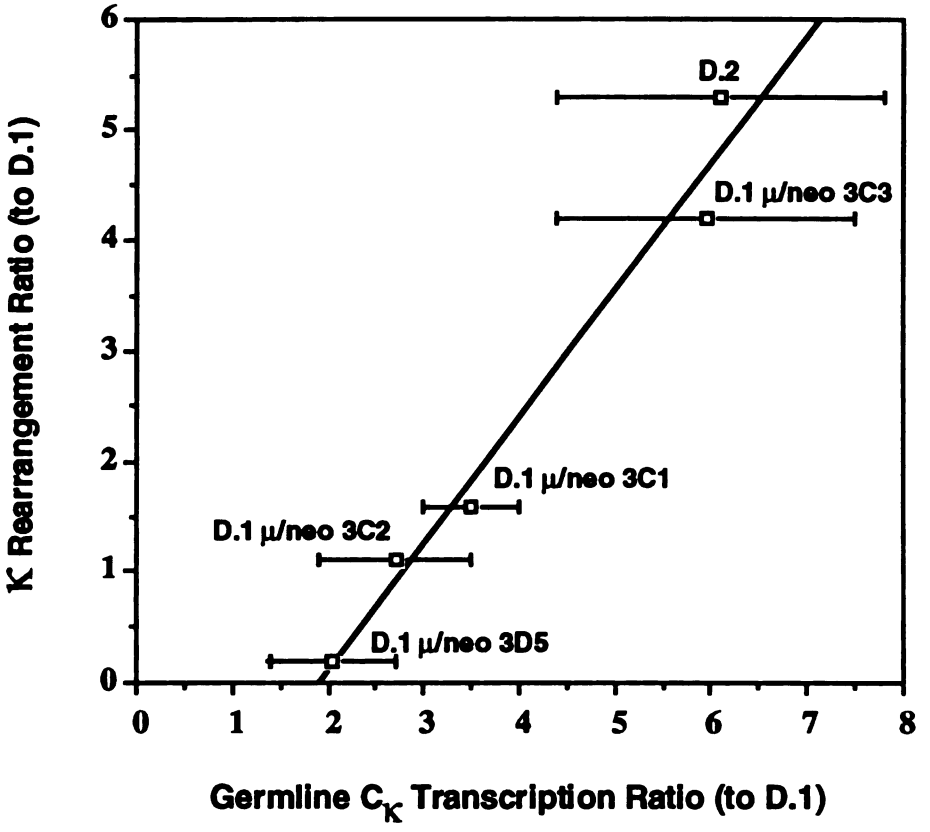
20 μg of total RNA was resolved on a 1% agarose-formaldehyde gel and expression of both a 0.8kb and 1.2kb C_K germline transcripts were detected by hybridization with a labeled 350 nt PCR fragment amplified from a Cκ germline transcript cDNA. RNA loading for each lane was examined by comparing the upper ribosomal RNA bands and by re-probing with GAPDH (data not shown) and was found to be approximately equivalent. RNA from LPS treated (1 μg/ml, 24 hours) K.40D.2-7 was used as a positive control for the germline C_K transcripts.

A. Northern blot of K.40 siblings. K.40D.2-5 and K.40D.2-7 are subclones of K.40D.2. 10-10-1S, a murine thymoma line, was used as a negative control. **B.** Northern blot of K.40D.1, K.40D.2 and K.40D.1 neo^R & μ/neo^Rtransfectants. **C.** Correlation of amount of C_K germline transcription versus κ gene rearrangement frequency in K.40D.2 and K.40D.1 μ/neo^R transfectants. The graph is the average of values derived from two separate Northern blots of C_K germline transcripts. The r^2 value for the first Northern was 0.98, for the second Northern was 0.92 and for the average of the two was 0.97.


A
10-10-1S
B.1
B.2
C.1
C.2
D.1
D.2-5
D.2-7
E.1
E.2
F.1
F.2
D.2-7 + LPS
0.8kb
1.2kb


B
D.1 neo
D.1 μ/neo
D.1
D.2
1A4
1A5
1B2
3C1
3C2
3C3
3D5
D.2-7 + LPS
0.8kb
1.2kb

C


K Rearrangement Ratio (to D.1)
Germline C_K Transcription Ratio (to D.1)
D.2
D.1 μ/neo 3C3
D.1 μ/neo 3C1
D.1 μ/neo 3C2
D.1 μ/neo 3D5
0
1
2
3
4
5
6
7
8

contrast, the K.40D.1 neo^R (μ^-) clones failed to exhibit a correlation between these two parameters. Thus, for K.40D.1 there was a direct correlation between the amount of C_κ germline transcription induced by μ chain expression and the frequency of κ gene rearrangements. K.40D.1 was unique in this regard.

DISCUSSION:

Previous work by Reth et. al. (Reth et al., 1987), Iglesias et. al. (Iglesias et al., 1991) and Tsubata et. al. (Tsubata et al., 1992) had demonstrated that μ heavy chain expression in A-MuLV-transformed B cell lines leads to an increase in both κ light chain expression and κ gene rearrangement. In the analysis described in this paper, we examined five distinct clonally matched pairs of μ^- and μ^+ cell lines derived from K.40. Since each pair was cultured together until they were subcloned, the only expected difference between siblings is the presence of endogenously rearranged functional μ heavy chain plus any differentiative events associated with the gene rearrangement and/or expression of the μ heavy chain. Four of the μ chain expressing K.40 subclones, all of which expressed μ on the surface, were found to have an increased number of κ gene rearrangements in the population compared to their μ^- subclones. The fifth μ^+ subclone was distinctive in that it had much lower or no surface μ expression and had little to no increase in κ gene rearrangements. The increase in κ gene rearrangement frequency observed in the four surface μ^+ siblings could not be ascribed to a systematic increase in the activity of V(D)J recombinase, as measured with an exogenous recombination substrate. To further test whether μ expression leads to increased κ gene rearrangement, the μ^- K.40 derivatives were transfected with a functionally rearranged μ gene. On average, the nine μ^+ transfectants examined had a four- to five-fold increase in the number of κ gene rearrangements compared to control transfectants. These results

provide additional evidence that μ heavy chain expression is a pivotal regulatory event leading to increased κ gene rearrangement.

In contrast to the results of Reth et. al. (Reth et al., 1987) and Iglesias et. al. (Iglesias et al., 1991), detectable levels of κ gene rearrangement were observed in our μ^- K.40 subclones. These κ rearrangements appeared to have occurred in the predominant μ^- cells since double immunofluorescent staining of permeabilized cells with anti-μ and anti-κ antibodies showed the presence of cells expressing κ and not μ in the μ^- K.40 subclones (data not shown). These observations agree with the previous report of κ gene rearrangements in A-MuLV-transformed pro-B cell lines (Schlissel and Baltimore, 1989), in A-MuLV-transformed SCID pro-B cell lines (Blackwell et al., 1989; Hendrickson et al., 1990), and in Epstein-Barr virus-transformed human B cell progenitors (Kubagawa et al., 1989). In each example, these B cell precursors lack a functional μ heavy chain. In previous experiments that had failed to detect κ gene rearrangement or κ chain expression in μ^- pro-B cell lines, Southern blots were used to detect κ gene rearrangements and either Western dot-blot or Northern blots were used to detect κ chain protein or mRNA expression (Reth et al., 1987; Iglesias et al., 1991). These methods appear to be less sensitive than the quantitative PCR assay for κ gene rearrangement used in our experiments. Thus, the previous failure to detect κ gene rearrangement or expression in μ^- pro-B cells may have been due to lack of sensitivity rather than a complete absence of κ gene rearrangement in B cell precursors lacking μ heavy chain expression.

A low level of κ gene rearrangement in the absence of full-length μ expression could be a general feature of B lymphocyte development. Mice homozygous for a mutation interrupting the μ membrane exon fail to make functional μ_m protein but still exhibit some κ gene rearrangement in their bone marrow cells, as detected by quantitative

PCR. In this case, the level of κ gene rearrangement in the bone marrow cells was reduced about 20-fold compared to that from normal mice, supporting the hypothesis that the membrane form of μ heavy chain stimulates an otherwise low level of κ gene rearrangement in developing B lymphocytes (Kitamura and Rajewsky, 1992).

The observed order of Ig gene rearrangements is commonly thought to reflect ordered accessibility of IgH and Ig κ loci to V(D)J recombinase (Alt et al., 1987). The low level of κ gene rearrangement observed in A-MuLV-transformed μ^- pro-B cell lines and in bone marrow cells from IgH mutant mice (Kitamura and Rajewsky, 1992) suggests, however, that both the μ heavy chain locus (V_H to DJ_H rearrangement) and the κ light chain locus (V_κ to J_κ rearrangement) become accessible to the V(D)J recombinase system at an early stage of B-cell development. The order of Ig gene rearrangements might be the result of greater accessibility first at the IgH locus and then later at the Ig κ locus. Alternatively, accessibility may be a necessary first step, but directing factors may be required to target the recombinase machinery to each locus sequentially. Production of a functional μ heavy chain could cause the re-direction of the recombinase machinery to the κ light chain locus. One approach to examining the accessibility of a genomic region is to measure the production of mRNA transcripts from promoters in the region (Schatz et al., 1992). The supposition behind this approach is that if the transcription machinery can access a region so can the other proteins involved in VDJ recombination. This logic assumes that it is only the accessibility of the region that is limiting and not the protein factors needed for transcription. In three out of four clonally matched pairs, the increase in κ rearrangement frequency in the μ^+ siblings was <u>not</u> accompanied by a significant change in germline C_κ transcription. Thus, for the K.40B, C & E clonally matched pairs, the stimulation of κ gene rearrangement by μ was apparently not due to change in accessibility of the κ locus. In the fourth pair, K.40D, μ expression did consistently lead to an increase in transcription of germline κ genes. Thus,

it could be that part or all of the mechanism by which μ increased κ gene rearrangement in K.40D cells was via an increase in κ gene accessibility. Indeed, there was a strong correlation between the frequency of κ gene rearrangement and the level of germline C_κ transcription in the K.40D μ^+ siblings and μ^+ transfectants. Taken together, these results suggest that there are at least two ways by which μ protein expression can regulate κ gene rearrangement. One form of control would be by increasing the accessibility of the κ locus to protein factors which are already present and another would involve activating factors that direct the recombinase machinery to the κ locus.

Experiments utilizing transfection in A-MuLV-transformed B cell lines (Reth et al., 1987), creation of μ-transgenic mice (Storb, 1987; Nussenzweig et al., 1987), and gene disruption of the membrane form of μ (Kitamura and Rajewsky, 1992) all argue that it is the membrane form of μ that is involved in the stimulation of κ gene rearrangement and/or the cessation of IgH gene rearrangement. As membrane IgM in B cells is capable of inducing protein tyrosine phosphorylation and phosphoinositide breakdown (DeFranco, 1992) it has been suggested that μ mediates its effects on V(D)J recombinase targeting through a receptor signaling mechanism (Reth et al., 1987; Iglesias et al., 1991; Kitamura and Rajewsky, 1992; Tsubata et al., 1992). The finding that μ can form complexes with alternative light chains (Pillai and Baltimore, 1988; Karasuyama et al., 1990; Reth, 1991), that cross-linking of the small amount of μ-alternative light chain complex on the surface of pre-B cell lines generates a calcium signal (Takemori et al., 1990; Misener et al., 1991; Tsutsumi et al., 1992) and that homozygous disruption of the λ_5 in mice leads to impairment of B cell development at the pre-B cell stage (Kitamura et al., 1992) supports this hypothesis. Interestingly, the only K.40 μ^+ sibling that did not exhibit enhanced κ rearrangement (K.40F.2) was also the only μ^+ sibling that did not express its membrane μ on the cell surface. Thus, there was a difference in cellular localization of μ in K.40F.2 compared to the other μ^+ K.40 subclones and this difference

may have resulted in the failure of μ chain to stimulate κ rearrangement in K.40F.2. Recently, it was reported that transfection of a truncated μ chain lacking its variable and the C_H1 region into the 300-19P17-27 pre-B cell line did not stimulate κ gene rearrangement; whereas crosslinking this molecule did stimulate these rearrangements (Tsubata et al., 1992). These results suggest that the first two Ig domains of heavy chain μ are normally needed to generate the signal to stimulate κ gene rearrangement.

In addition to full-length μ protein, B cell precursors can make a truncated μ protein called Dμ protein from a DJ rearrangement that utilizes the second reading frame (Reth and Alt, 1984). It has been observed that Dμ has a negative influence on B cell development in mice (Gu et al., 1991). The Dμ protein is present at moderate levels in the parental K.40 cells (Figure 2). This DJ allele was converted to a VDJ^+ allele in some of the endogenously rearranged μ^+ pre-B cells. Thus, some of the μ^+ K.40 subclones expressed only full-length μ heavy chain; whereas, those μ^+ subclones that had converted the VDJ^- allele to a VDJ^+ allele and all of the K.40 μ^+ transfectants expressed μ heavy chain along with a lower amount of Dμ protein. Dμ expression did not prevent μ from inducing increased κ gene rearrangement in the K.40 derivatives analyzed. One possibility is that κ gene recombination observed in the μ^- K.40 subclones was in some way induced by Dμ expression, *i.e.*, that Dμ was acting like full-length μ. This hypothesis cannot, however, explain the other examples of the κ gene rearrangements observed in the absence of μ, cited above. In any case, the presence of Dμ apparently did not interfere with the ability of μ to stimulate κ gene rearrangement in the experiments reported here. This is in contrast to the observed negative influence of Dμ on B cell development in mice (Gu et al., 1991). The stage at which Dμ acts to block B cell development is not yet known.

The K.40 siblings and transfectants generated in this study displayed surprising individuality. For example, two or three of the siblings, K.40B.2 and K.40C.2, and probably K.40D.2, generated a functional μ heavy chain by conventional V to DJ gene rearrangement. Two others, K.40E.2 and K.40F.2, generated a functional μ chain by V region replacement. As a consequence, K.40E.2 and K.40F.2 also retained Dμ expression following heavy chain μ rearrangement. The presence of Dμ did not appear to be have a major impact in the increased κ gene rearrangements observed in μ^+ pre-B cells. K.40F.2 was distinct from all of the other μ^+ siblings and transfectants in that it did not express substantial levels of μ protein on the cell surface. Having this range of generated cell lines with a unique set of properties provided an opportunity to study the regulation of κ gene rearrangement in a variety of situations. Our results suggest that heavy chain μ, at least when expressed on the cell surface, plays a pivotal role in the increase in κ gene rearrangement observed in pre-B cells.

Considerable differences were observed in the amount of V(D)J recombinase of the different K.40 subclones. This phenomenon has also been observed by Alt et al. (Alt et al., 1992). They have found that prolonged culture of V(D)J recombinase expressing cell lines can lead to loss of the recombinase activity over time. The experiment reported here were all done with subclones that were grown in culture for only short periods of time following isolation to minimize this problem. Indeed, subsequent subclones and transfectants of K.40D.1 and D.2 that were analyzed did not show systematic loss of V(D)J recombinase activity (Table 2).

Whether C_κ germline transcription, possibly as a measure of 'accessibility', correlates with increases in κ gene rearrangement is less clear cut. Three of the μ^+ siblings had increased κ gene rearrangements without increased C_κ germline transcription. In contrast, K.40D.2 had increased C_κ transcription. Interestingly,

K.40D.1 μ^+ transfectants showed a strong correlation between C_κ transcription and increased κ rearrangement frequency. Drawing on our results as whole, we suggest that there are multiple steps in the regulation of κ rearrangement. Although their relationship to one another remains to be defined, these events include the expression of a functional μ heavy chain, increases in C_κ germline transcription, and an additional mechanism for activating κ gene rearrangement possibly involving targeting of the recombinase to the κ locus.

METHODS:

Cell lines and tissue culture

The cell line K.40 (Beck-Engeser et al., 1987) was obtained from Matthias Wabl (University of California, San Francisco (UCSF)). 10-10-1S, a $CD4^+$ murine thymoma line, was obtained from Dan Littman (UCSF) and Daudi (Klein et al., 1968) was obtained from J.M. Bishop (UCSF). WEHI-231 (Warner et al., 1979) was obtained from Dr. Noel Warner (Becton-Dickinson). Cells not under selection were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Irving Scientific, or JR Scientific), 2mM glutamine, 1mM sodium pyruvate, and 50μM 2-mercaptoethanol at 37°C in an atmosphere containing 5% CO_2. For selection of cells expressing a transfected neo^R gene, G418 (Gibco) was added to the media at a concentration of 1.8 mg/ml (dry powder) and adjusted to pH 7.4 with NaOH. Both the regular and G418 containing-media contained less than 0.1 endotoxin unit / ml (LAL chromogenic assay, Whittaker Biochemicals). Cells used in experiments were kept in log-phase growth with a density below 7×10^5 cells/ml.

Cytoplasmic immunofluorescent staining of cells

Samples of 10^5 cells were centrifuged onto slides using a cytospin 2 (Shandon Southern Instruments, Inc.) air-dried, fixed in 100% ethanol, washed twice in phosphate buffered saline (PBS) without divalent cations + 1% BSA + 0.1% NaN_3 and stained with either rhodamine-conjugated goat anti-mouse μ antibodies (Fisher Biotech) at 100 μg/ml or with fluorescein-conjugated goat anti-mouse κ antibodies (Fisher Biotech) also at 100 μg/ml or with both. After staining, the cells were washed in situ twice, as before, and then mounted with elvanol (14.3 g. polyvinyl alcohol (Air Products, Inc.) dissolved in 52.2 mls PBS plus 22.8 ml glycerol and 1 ml NaN_3, and filtered through Whatman filter #1). The number of μ^+ cells and κ^+ cells on the entire slide were counted under the fluorescent microscope. In some experiments, the cell recovery of each sample was determined directly by including an internal standard of human B lymphoblastoid Daudi cells pre-stained with 7-Amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated goat anti-human IgG+IgM (H+L) (.02%) [Jackson Immunoresearch Labs, Inc.]. Recovery in different experiments varied between 38% and 100% with 60% recovery being typical.

Cell Surface Immunofluorescence

Cell surface μ expression was determined by flow cytometry using a FACScan (Becton Dickinson). Cells were washed in FACS buffer (PBS + 1% FCS + 0.1% NaN_3), stained with fluorescein-conjugated goat anti-murine μ chain (Fisher Biotech), washed again in FACS buffer, and resuspended in FACS buffer + 4 μg/ml propidium iodide.

Isolation of cell lines with endogenously rearranged μ-genes

Cells expressing a functionally rearranged endogenous μ gene were isolated using a sib-selection approach. First K.40 cells were subcloned by limiting dilution. The six K.40 subclones (A-F) had on average 0.2% μ^+ cells. The predominantly μ^- cells were plated into 40 wells of a 96 well plate at a concentration calculated to yield 2 wells that

were enriched for μ^+ cells. After expansion for approximately 10 days, a sample from each well was taken and analyzed for intracellular μ-expression by immunofluorescent staining. The well containing the enriched sample was subjected to another round of sib-selection. With the exception of the K.40A subclone, screening 40 wells was sufficient to find an enriched well. When the frequency of μ^+ cells exceeded 5%, then the enriched K.40 cells were subcloned by limiting dilution and both μ^+ and μ^- sibling clones were obtained. The cloned K.40 derivatives were screened for Mycoplasma contamination (Mycotect Assay, GIBCO) and found to be Mycoplasma-free. These derivatives and additional subclones and transfectants were stored frozen in liquid N_2. Cells used for experiments were grown in tissue culture for minimal length of time to decrease the chance of changes that might occur following extensive growth in tissue culture (Alt et al., 1992).

Introduction of a functional μ gene into K.40-derived cells

DNA-mediated gene transfer into K.40 cells was achieved by electroporation with a plasmid containing a functionally rearranged μ gene and a neo^R gene. The linked plasmid, pμneoA1 was constructed from pMX1112neo (Brown and Scott, 1987) and pμ (Grosschedl et al., 1984) as follows: First, the Sal1 site in the 6.2 kilobase (kb) plasmid pMX1112neo (Brown and Scott, 1987) was eliminated by cutting with Sal1, filling in with the Klenow fragment of DNA polymerase I, and blunt-end ligation. This pneoA1 plasmid was then cut with EcoR1, and Sal1 linkers were ligated into this site. Then the 12.35 kb Xho1-Sal1 fragment of pμ, containing the μ gene derived from the hybridoma 17.2.25 (Grosschedl et al., 1984), was inserted into the engineered Sal1 site of pneoA1. The resulting plasmid had the 3' long terminal repeat (LTR) of the neo^R plasmid adjacent to the V region of the μ gene.

For electroporation, the cells were washed in PBS and resuspended in PBS at 10^7 cells / ml. Cells in 0.5 ml PBS were mixed with 15 μg of DNA linearized by Sal1 and dissolved in 7.5 μl TE (10mM Tris, pH 8.0, 1mM EDTA) in a 0.4 cm cuvette (Bio-Rad). The cells were chilled on ice for 10 min prior to electroporation with the Gene Pulser apparatus (Bio-Rad) (200 volts, 960 μF). After shocking, cells were incubated for 10 min and then diluted into 10 mls of complete RPMI 1640 medium. After growth for 24 hours, selection for G418 resistance was initiated. Transfectants were screened for μ expression by immunofluorescence, and isolates with desired level of μ expression were subcloned by limiting dilution.

Immunoblotting for μ expression

K.40 siblings and transfectants were lysed in NP-40 lysis buffer and soluble proteins were separated on an 8% sodium dodecyl sulphate (SDS)-polyacrylamide gel, transferred to a nitrocellulose filter, incubated with 2% bovine serum albumin and washed as previously described (Gold et al., 1990). The filter was then probed with an alkaline phosphatase-conjugated goat anti-mouse μ antibody (Jackson Immunoresearch) at 200 ng/ml in Tris-buffered saline (10 mM Tris-HCl, pH 8, 150mM NaCl) containing 0.05% Tween 20. The alkaline phosphatase labeled protein bands were visualized by using 5-bromo-4-chloro-3-indoyl phosphate (Bio-Rad) and nitro blue tetrazolium (Bio-Rad) as described previously (Gold et al., 1990).

PCR assay for κ rearrangement frequency

The PCR measurement of frequency of rearranged κ alleles in a population was done as described with some modifications (Schlissel and Baltimore, 1989). For preparation of DNA samples, the cells were centrifuged, washed once by centrifugation in PBS with divalent cations, and split equally into duplicate microfuge tubes (2×10^6 cells/tube). The cells were pelleted for 15 sec in a microfuge and resuspended in 200 μl

PCR lysis buffer (10 mM Tris pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl, 200 μg/ml gelatin, 0.45% NP-40, 0.45% Tween-20 and 60 μg/ml Proteinase K). Samples were incubated at 56°C for one hour and later 10 minutes at 95°C. Samples were stored at -20°C until analysis.

The PCR was done with either 10,000 or 20,000 cell equivalents of DNA in 2 or 4 μl of the cell lysate. Reactions were run in a buffer containing 10mM Tris-HCl pH 8.3, 50mM KCl, 2.0mM $MgCl_2$, 200 μg/ ml gelatin, 0.2mM final concentration of each dATP, dGTP, dCTP, and dTTP (Pharmacia), oligonucleotide primers at 0.05 μM, and 1 unit Taq polymerase (AmpliTaq, Perkin-Elmer Cetus)] in a total volume of 50 μl. Each sample in a particular PCR assay had the same amount of cell lysate, and each was run in duplicate on the thermocycler (Cetus-Perkin Elmer) using conditions described previously (Schlissel and Baltimore, 1989). Series of the standards were run along with lysis buffer and reaction buffer negative controls. Aliquots of PCR products were electrophoresed through a 1.2% agarose (SeaKem) gel in Tris-borate-EDTA buffer (Maniatis et al., 1982). Gels were blotted to either Zetabind (AMF) or Gene Screen Plus (New England Nuclear) filters according to manufacturers' instructions. After pre-hybridization for 12 hours, filters were hybridized for 18 hours at 42°C in a 50% formamide hybridization solution containing 10^6 cpm/ml of a ^{32}P labeled probe made from the 369nt Rsa I fragment of pHJ_κ (Lewis et al., 1982). The amount of ^{32}P labeled probe bound to amplified products was determined by electronic counting with either a Betascope 603 Blot Analyser (Betagen) or by a PhosphorImager (Molecular Dynamics) machine. The number of rearranged κ genes per 10,000 or 20,000 genomes of the K.40 subclones were determined by comparison to standard curve data run in parallel. Standards for quantitation were made by titrating DNA from the WEHI-231 B lymphoma cell line, which has a rearranged κ allele, into salmon sperm DNA. Alternatively, in some experiments, the B lymphoma DNA was added to DNA from a murine T

lymphoma, 10-10-1S, in which the κ gene was not rearranged, as this may be a closer approximation of the DNA from the K.40-derived subclones.

Accuracy and reproducibility of measurements were assessed in several ways. The well to well variability of the 48 wells in the thermocycler (Cetus-Perkin Elmer) was examined by assaying 48 identical κ rearrangement standards together. The measured values were found to be within two-fold of each other. PCR reactions of all samples were run in duplicate and the amount of product in each PCR sample was measured in duplicate to examine the amount of variation in the assay. Comparisons between cell lines analyzed together were found to be more reproducible than comparisons of pre-B cell samples with the standards. Therefore, data shown all involve comparisons made between different samples run in parallel.

Determination of IgH allele genotype

The IgH genotype was determined for each K.40 B-lineage pair by using a PCR-based approach as described previously (Schlissel et al., 1991). In the assay for D to J rearrangements, PCR reactions were run with D region degenerate primers that were homologous to all of the Dfl16 and Dsp2 D gene families and unique primers 3' to J_H3 or J_H4. To assess V to DJ rearrangements, PCR reactions were run with a mixture of three different degenerate primers with homologies to the conserved framework region 3 (FR3) sequences of three V_H gene families (V_H7183, V_H558, and V_HQ52) plus the J_H4 primer. The amplified PCR products were detected by Southern blotting and hybridization with the appropriate IgH probe as described previously (Schlissel et al., 1991).

Measurement of C_K Germline Transcripts

Total RNA was extracted from cells using the acid guanidinium thiocyanate-phenol method (Chomczynski and Sacchi, 1987), but with only a single isopropanol precipitation step. 20 μg of RNA was electrophoresed through a 1.2% agarose gel containing 2M formaldehyde, buffered with MOPS (3-(N-morpholino)-propane-sulfonic acid) and transferred to a membrane (Genescreen, New England Nuclear). The 0.8 kb and 1.2 kb C_κ germline transcripts were detected using a ^{32}P-labeled 350 nt PCR fragment amplified from the 0.8 kb Cκ germline transcript cDNA as a probe. The probe was labeled by the random-oligonucleotide-priming method (Boehringer Mannheim Biochemicals). Hybridization was achieved in a buffer containing 50% formamide, 1M NaCl, 1% SDS, 10% dextran sulfate, and 125 μg/ml denatured salmon sperm DNA, after which the membrane was washed in 0.1 X SSC (1 X SSC: 150mM NaCl, 15mM Na citrate, pH 7.0) and 1% SDS at 65°C. Quantitation of the 0.8kb and 1.2kb C_κ germline transcripts (scanned as a single unit) was done with the Phospoimager (Molecular Dynamics). Equivalent amounts of RNA were demonstrated to be present in each lane, by stripping the blots by boiling for 20 minutes in 0.1% SDS and then re-probing them with a constitutively expressed gene, glyceraldehyde-phosphate dehyrogenase (GAPDH) (Fort et al., 1985).

RNA PCR assay (RT-PCR) was preformed on randomly primed cDNA as described previously (Schlissel et al., 1991). Briefly, 3 μg of guanidinium-purified total RNA was reverse-transcribed in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (BRL). A fraction of 1st strand cDNA was used as a template in PCR assay with primers κ^0, C_κ (Schlissel et al., 1991) and O-PR1 (Martin and Van Ness, 1990). The κ^0 and O-PR1 primers anneal to sequences 5' to $J_\kappa 1$ which are deleted upon gene rearrangement so these assays only detect transcripts from unrearranged κ genes.

Measurement of V(D)J recombinase activity

The V(D)J recombinase activity of the K.40-derived siblings and transfectants was measured by transiently introducing into cells a plasmid, pBlueRec, containing an artificial recombinase substrate and measuring the frequency of rearranged genes after recovery of the plasmids (Kallenbach et al., 1990) Recombination of the consensus VDJ recombination signal sequences deletes an insert in the lacZ gene and restores a functional β-galactosidase coding region about one-third of the time.

Duplicate samples of K.40-derived cells each were transiently transfected with pBlueRec using a slightly modified version of the protocol of Lieber et al. (Lieber et al., 1987). Cells were incubated with circular pBlueRec DNA (0.3-0.5 μg/ml) under hypotonic conditions for 35 min. The cell were washed with 5 ml of unsupplemented RPMI 1640 instead of 0.5 ml. At the end of the procedure, the cells were resuspended in 10ml complete RPMI 1640 growth medium and grown for 48 h, during which time the cell concentration was kept under 7 x 10^5 by adding fresh media as needed. After 48 h, the cells were pelleted and washed twice in PBS with divalent cations. Plasmid DNA was recovered by the alkaline lysis procedure for bacterial minipreps (Sambrook et al., 1989) including phenol / chloroform extraction but omitting RNase treatment. Recovered DNA was resuspended in 25 μl TE. DNA samples were digested with the methylation-requiring enzyme Dpn I for 8 hours to cleave un-replicated DNA that may not have entered the lymphoid cells. The DNA samples were introduced into the XL-1-blue (Stratagene) E. coli cells by electroporation using the Bio-Rad Gene Pulser protocol. 2 μl of each DNA sample was mixed with 40 μl of electrocompetent XL-1-blue E. coli cells and incubated at 0° C for 30 s. Next, the cells with DNA were placed in a cuvette and subjected to electroporation by a Bio-Rad Gene Pulser set at 25 μF, 2.5 kV & 200Ω. The cells were resuspended in 1 ml of a growth medium made up of 2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose and incubated at 37° C for 1 h shaking at 225 rpm. The electroporated bacteria

were then plated on Luria Broth (LB) agar plates containing 80μg/ml 5-Bromo-4-chloro-3-indoyl-β-D-galactoside, 150μM isopropyl-β-D-thiogalactopyranoside, 100μg/ml ampicillin, and 10μg/ml tetracycline, and scored for lacZ phenotype by blue-white color.

Statistical Methods

The Student's t-test was used to generate a P-value to determine the statistical significance of a difference between two sets of samples.

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CONCLUDING REMARKS

In my work, I have examined the role that heavy chain μ plays in the control of light chain κ rearrangement. In these studies, the presence of μ heavy chain protein on the cell surface correlated with an increased frequency of κ light chain gene rearrangement in K.40 A-MuLV transformed pro-B cells. This effect could not be explained by an increase in V(D)J recombination activity and therefore seems to involve targeting of the V(D)J recombinase to the κ locus. Curiously, two different mechanisms for this targeting seemed to be operating in different K.40 derivatives. In the μ^+ derivatives of one of the siblings (K.40D.1), a linear relationship was seen between the amount of germline Cκ transcription and the frequency of κ gene rearrangement. This suggests that germline transcription or some related parameter such as accessibility was limiting for κ rearrangement in these cells and that μ may have stimulated rearrangement by increasing this parameter. In contrast, derivatives of other K.40 subclones (K.40B.1, K.40C.1 & K.40E.1) exhibited higher levels of Cκ germline transcription in the μ^- siblings suggesting that this parameter was not limiting in these K.40 derivatives. Nonetheless, expression of μ in these K.40 derivatives induced an increase in κ gene rearrangement without an increase in C_κ germline transcription. This observation suggests that μ can increase κ gene rearrangement by a distinct mechanism, perhaps not involving accessibility but rather targetting of the V(D)J recombinase by the expression of factors that direct the recombinase to the κ locus. Thus there may be multiple processes involved with the regulation of κ gene rearrangement.

In the future, there are two different directions that can be taken to extend this line of investigation. The first is to elucidate the mechanism by which μ regulates κ gene rearrangement in cell lines and the second is to set up a system to study the regulation of κ gene rearrangement in normal cells. For the former question it would be possible to

expand on the work of Tsubata *et al.*, and analyze signaling in cell lines using truncated μ constructs that require crosslinking by antibody to stimulate an increase in κ rearrangement frequency (Tsubata *et al.*, 1992). Changes in various signalling pathways (cAMP, Ca^{2+}, PIP_2 breakdown and protein tyrosine phosphorylation) could be examined after crosslinking of the truncated μ molecule. In addition, mutations of the μ transmembrane region that inactivate signaling in mature B cells could be introduced into these truncated μ constructs to help assess μ's contribution to signalling. Chimeras containing the cytoplasmic motif from either Ig-α or Ig-β could be used in the place of the truncated μ chain to examine the role of Ig-α or Ig-β in the stimulation of κ rearrangement by μ heavy chain. To avoid some of the variation inherent in the κ rearrangement assay used for this thesis, a quantitative PCR assay that measures the excision products that occur durring the rearrangement of the κ gene could be developed. The excision assay would only measure ongoing rearrangements at the κ locus and thereby avoid the founder effect which skews the results of the PCR assay utilized in this work. Also, AMuLV-transformed cell lines could be derived from transgenic mice that have a homozygous disruption of the transmembrane exon of the μ gene. This would eliminate the background from endogenous μ rearrangements, since the endogenous μ chains would all be in the secretory form and could not stimulate κ rearrangement (Reth *et al.*, 1987). Another interesting experiment would be to examine the role of the newly described tyrosine kinase BPK (B-cell Progenitor Kinase) also known as ATK (agammaglobulinemia tyrosine kinase) in the regulation of κ rearrangement in pre-B cells (Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). The gene encoding this tyrosine kinase is the gene defective in X-linked aggamaglobulinemia a disease in which B cell development is disrupted. One approach to studying the role of BPK in the regulation of κ rearrangement would be to introduce truncated μ constructs into AMu-LV transformed pro-B cells that have the activity of their BPK gene eliminated by genetic disruption or by the use of a dominant suppressor gene and examine the stimulation of κ rearrangement in these cells.

To extend the study of the regulation of κ gene rearrangement to normal cells, I suggest creating transgenic mice that have the membrane bound truncated μ chain described above as their only μ chain. These mice could be generated by making transgenic mice that expresses the truncated μ construct and breeding them with mice that have a homozygous disruption of the membrane exon of the μ gene. Theoretically these mice should not produce normal B cells but only pre-B cells that contain the truncated μ chain. If surface expression of μ chain occurs during the pre-B cell stage and is necessary for the progression through development, one would expect these mice to have a significant amount of pre-B cells that have the truncated μ chain on their cell surface. It would be of interest to determine whether these pre-B cells grown in stromal cell culture could increase their frequency of κ gene rearrangement upon stimulation with anti-μ antibodies and if so what kind of signal is generated in these cells. A similar experiment could also be done *in vivo* by injecting anti-μ antibodies into mice expressing the truncated μ heavy chain and then harvesting bone marrow cells various times later to look at κ gene rearrangement. If such treatment results in increased κ gene rearrangement in these mice, this would provide direct evidence for the signalling role of μ as compared to the results of Kitamura *et al.* which show a requirement for the transmembrane region of μ but do not address the mechanism of this requirement (Kitamura and Rajewsky, 1992; Kitamura *et al.*, 1991).

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