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Molecular requirements for the μ-induced light chain gene rearrangement in pre-B cells

Antonio Iglesias, Manfred Kopf, G.Stuart Williams, Bernd Bühler and Georges Köhler

Max-Planck-Institut für Immunbiologie, Freiburg, FRG

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During B cell differentiation rearrangement of immunoglobulin (Ig) genes is partially regulated by the Ig proteins. Rearrangement of heavy (H) chain genes is inhibited, whilst that of light (L) chain genes is induced by the membrane form of the μ H chain. In order to analyse additional structural requirements of μ induced L chain gene rearrangement we transfected wild-type μ and mutant μ constructs lacking functional exons encoding the first or second constant domains into Abelson murine leukemia virus (AMuLV) transformed pre-B cells. All μ chains are expressed on the surface of the pre-B cell and all associate with ω and ι, two proteins forming a surrogate light chain, necessary for μ membrane expression. Nevertheless, only wild-type μ and not the mutant μ proteins promote L gene rearrangement. A heterodimer of proteins with M_r of 33 kd and 36 kd was found associated with wild-type but not with the mutant μ proteins. Continuous presence of μ is required for L chain gene recombination since loss of μ stopped and readdition of μ started L gene rearrangement. We propose that the protein complex composed of μ and the 33 kd/36 kd protein heterodimer is responsible for the activation of the L chain gene locus and its rearrangement.

Key words: AMuLV pre-B cells/B cell differentiation/L gene rearrangement/μ-associated proteins/mutant μ proteins

Introduction

The variable part of immunoglobulin (Ig) heavy (H) and light (L) chain genes is encoded by separated gene segments. Variable (V), diversity (D) and joining (J) segments are brought together during B cell maturation (Tonegawa, 1983). Assembly of the V(D)J gene segments is a regulated process. During B cell development, rearrangement and expression of Ig heavy (H) chain genes precedes the joining and expression of the light (L) chain genes (Alt *et al.*, 1984). Furthermore only one of the two alleles of the Ig genes is ever expressed in B lymphocytes, a phenomenon known as 'allelic exclusion' (Pernis *et al.*, 1965). In both processes expression of the membrane form of μ (μ_m) plays a central role. Transgenic mice expressing rearranged H chain genes often showed incomplete rearrangement of the endogenous H chain genes, thus implying that allelic exclusion is the result of cessation of joining at the second allele, once a functional rearrangement has occurred (Rusconi and Köhler, 1985; Weaver *et al.*, 1985; Iglesias *et al.*, 1987; Nussenzweig *et al.*, 1987). In Abelson murine leukemia virus (AMuLV) transformed pre-B cell lines, L chain genes rearranged only in those cell lines that expressed endogenous or transfected μ_m, strongly suggesting that μ_m also signals initiation of L chain gene joining (Reth *et al.*, 1987). In order to dissect further domains of the μ H chain constant region (in addition to the membrane region) important for the regulation of rearrangement, we constructed variants of a μ gene lacking either the first or the second constant domains and looked for their biological properties. The first constant domain of μ (Cμ1) was a likely candidate to be involved in regulating gene rearrangement for several reasons. First, L chain covalently attaches to Cμ1. Second, BiP, a heat shock-like protein residing in the endoplasmic reticulum also binds to the first constant domain and retains μ until it is replaced by a L chain (Haas and Wabl, 1983; Bole *et al.*, 1986; Hendershot *et al.*, 1987; Munro and Pelham, 1986; Sitia *et al.*, 1990). Third, the products of two pre-B cell specific genes, λ5 and V_{pre-B}, form a surrogate L chain of which λ5 binds covalently to Cμ1 (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987; Kudo *et al.*, 1989). They allow membrane expression of μ in pre-B cells which have not yet rearranged their L chain genes (Pillai and Baltimore, 1987, 1988; Tsubata and Reth, 1990).

The two mutant genes ($\mu\Delta1$ and $\mu\Delta2$) as well as their wild-type counterpart (μ) were transfected into an AMuLV transformed pre-B cell line and their ability to induce L chain gene rearrangement was studied. All μ proteins were expressed on the cell surface in the absence of conventional L chains. All μ proteins were associated with two small proteins corresponding in size to λ5 and V_{pre-B}. Nevertheless only the wild-type but not the mutant μ proteins induced L chain gene rearrangements. Rearrangement correlated with the presence of a heterodimer of two proteins of 33 kd and 36 kd, which was found to be non-covalently associated with μ and, to a lesser extent, with $\mu\Delta2$ but not with $\mu\Delta1$ proteins.

Results

Expression of the μ constructs in the AMuLV-transformed pre-B cell line 33.1.1$^-$

The pre-B cell line 33.1 was obtained by transformation of bone marrow cells of a $\mu + \kappa$ transgenic mouse (Rusconi and Köhler, 1985) with the Abelson murine leukemia virus (AMuLV). 33.1.1$^-$ and 33.1.1$^+$ are sublines of 33.1, selected by cell sorting for loss or retention, respectively, of surface transgene expression, (C.Paige, unpublished results). In subclones of 33.1, different rearrangements at both the H and L gene loci are observed (data for the L gene locus are given in Table I). The H gene alleles of 33.1.1$^+$ show a VDJ and a DJ rearrangement and those of 33.1.1$^-$ contain two VDJ rearrangements that must be non-functional, because no μ chain is detectable in this cell line (Table I). Both pre-B cell sublines have retained the L gene locus in

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Table I. Characteristics of AMuLV-transformed pre-B cell lines

	Surface μ proteins[a]	μ proteins in lysates[b]	Recombinase activity[c]	κ mRNA[d]	κ rearrangements[e]
Sublines					
33.1 (μ,κ)	nd	nd	250	27.8	6/12
33.1/8 (μ,κ)	nd	nd	nd	31.5	3/23
$33.1.1^-$ (−)	1	1	100	0.6	0/42
$33.1.1^+$ (μ,κ)	66	11	1	100.0	0/14
Transfectants of $33.1.1^-$					
μ-1	3	13	160	12.0	7/17
μ-2	1	4	25	0.5	2/40
μ-3	9	12	< 2	12.0[f]	0/32
μ-4	9	14	40	13.1	nd
μ-5	17	7	330	5.0	9/40
μ-6	21	nd	30	2.6	18/20
$\mu\Delta$1-3	17	12	11	< 0.1	0/46
$\mu\Delta$1-4	26	14	1000	12.1	all[g]
$\mu\Delta$1-5	22	12	12	< 0.1	0/34
$\mu\Delta$1-6	60	nd	200	1.5	nd
$\mu\Delta$1-7	26	11	250	1.0	0/44
$\mu\Delta$1-8	8	nd	40	< 0.1	0/28
$\mu\Delta$1-10	40	18	100	0.2	0/72
$\mu\Delta$2-11	9	nd	200	0.1	0/40

[a]Surface expression of μ proteins was determined by flow cytometry and is expressed relative to the value found in $33.1.1^-$.
[b]μ proteins in lysates were measured by radio immunoassay (RIA) and is expressed relative to the background value found in $33.1.1^-$.
[c]The recombinase activity is measured with the transient assay of Hesse *et al.* (1987) and is described in Materials and methods. The values are given relative to the number obtained in $33.1.1^-$ (7/3800 = 100%).
[d]The κ mRNA levels are given relative to the value of $33.1.1^+$. All values are corrected for loading errors with the values obtained with the ribosomal probe S12 (Figure 3).
[e]κ rearrangements are expressed as the fraction of κ alleles found in a joined configuration of the total κ alleles detected in the Southern blot analysis. nd, not determined.
[f]In this transfectant κ mRNA of a smaller size (~1 kb) than normal (1.2 kb) is found (see Figure 3).
[g]The same two κ rearrangements were found in all subclones of this transfectant.

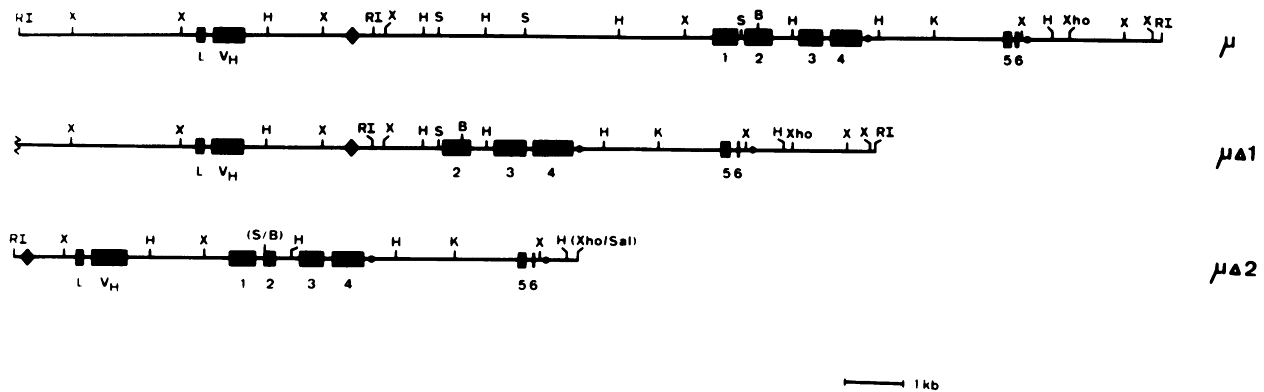

Fig. 1. Restriction maps of the μ constructs used in the transfection experiments. Coding regions are shown as black rectangles and intervening sequences as a line. The constant region exons of μ are numbered 1−6. L indicates the leader exon and V_H the variable region exon of the μ gene from hybridoma Sp6 (TNP specificity, Köhler *et al.*, 1982). (◆) denotes the heavy chain enhancer; (●) indicates polyadenylation sites. A vertical wavy line in $\mu\Delta$1 indicates a small 0.6 kb deletion introduced here to abrogate the *Eco*RI site. (RI) *Eco*RI; (X) *Xba*I; (H) *Hin*dIII; (S) *Sac*I; (B) *Bam*HI; (K) *Kpn*I; (Xho) *Xho*I. Fused restriction sites that are lost by joining are shown in parentheses.

a germline configuration (see below). Furthermore, Southern blot analysis of DNA from these subclones revealed complete loss of the transgenes in $33.1.1^-$ (not shown). For these reasons $33.1.1^-$ was chosen as recipient in gene transfer experiments, aimed to study the role of the wild-type and mutant μ proteins in the process of L chain gene rearrangement.

The wild-type μ and the mutant μ gene constructs $\mu\Delta$1 and $\mu\Delta$2 (Figure 1) were transfected into a $33.1.1^-$ and several transfectants were analysed for expression of the corresponding proteins. Of many transfectants obtained with the construct $\mu\Delta$2 only the transfectant $\mu\Delta$2-11 is included in this analysis as the only one expressing the transfected proteins at levels comparable to those in μ or $\mu\Delta$1 transfectants (see Table I). Apparently, deletion of the second constant exon results in generally lower levels of μ protein expression both in pre-B and also in myeloma cells (not shown). Flow cytometric analysis of the transfected cell lines was performed using a biotinylated monoclonal antibody specific for the third domain of murine μ chain (1M41). The

staining intensity of all transfectants is summarized in Table I, expressed as the mean fluorescence intensity relative to the fluorescence in the negative parental line 33.1.1$^-$. Although surface expression for a defined construct can vary, all three different μ proteins are expressed on the surface of the transfected cells with an overlapping range of intensities (Table I and Figure 2, upper panel). On the average, surface staining of $\mu\Delta1$ transfectants was ~2-fold higher than that of wild-type μ transfectants although comparable amounts of proteins were found intracellularly (Table I). Staining of μ transfectants with a monoclonal antibody specific for murine κ L chains revealed a small proportion of cells positive for κ, seen as small shoulders in the FACS histograms of the μ transfectants but absent in the staining profile of the parental cell line 33.1.1$^-$ (Figure 2, lower panel). Similar staining profiles for cell surface κ were not observed in $\mu\Delta1$ or $\mu\Delta2$ transfectants (data not shown). The results shown in Figure 2 demonstrate that the majority of the wild-type μ and all mutant μ proteins are expressed on the surface of the transfectants in the absence of L chains and suggest ongoing L gene rearrangement and expression in a fraction of the wild-type μ, but not in $\mu\Delta1$ and $\mu\Delta2$ transfectants.

Recombining capacity of the transfectants

As all transfectants went through manipulations including electroporation-mediated gene transfer and drug selection over several weeks, we included an independent control to check for retention of recombining potential. Using the joining substrate pJH201 (Hesse *et al.*, 1987), the V gene recombining capacity of a given cell line can be rapidly assessed in a transient transfection assay. Testing our cell lines with this assay allowed us to distinguish between transfectants devoid of recombinase activity and transfectants that retained an intact recombining machinery and yet did not show L gene rearrangement. The line 33.1.1$^+$ had 1% and the line μ-3 had <2% of the recombining capacity of 33.1.1$^-$. They are essentially unable to recombine the exogenous substrate (Table I). The other cells lines all have recombinational activities within a factor of 3 of the parental line. Some transfectants ($\mu\Delta1$-3 and $\mu\Delta1$-5) show a ten-fold reduced and one ($\mu\Delta1$-4) a ten-fold increased activity (Table I). Whether these deviations reflect real differences of the activity of the recombination machinery is not clear. Interestingly however, the $\mu\Delta1$-4 transfectant was the only line which had spontaneously rearranged both endogenous κ alleles before transfection (see below).

Taken together, with the exception of lines 33.1.1$^+$ and μ-3, all lines show recombinational activity. We therefore conclude that the lines analysed did not change their overall recombining potential after wild-type or mutant μ transfection.

Northern blot analysis of the transfectants

On-going rearrangement at the κ locus of μ transfectants might result in detectable amounts of mature κ mRNA. Northern analysis of total mRNA of the different transfectants revealed varying amounts of κ transcripts, detected with a probe specific for the constant exon of the κ gene (Figure 3). In contrast to the parental line 33.1.1$^-$, most of the μ transfectants (Figure 3, μ-1 to μ-6) expressed high amounts of κ mRNA of a size identical to the mature transgenic κ transcripts (Figure 3 and Table I). As an exception, transfectant μ-2 contains very low levels of κ mRNA. This

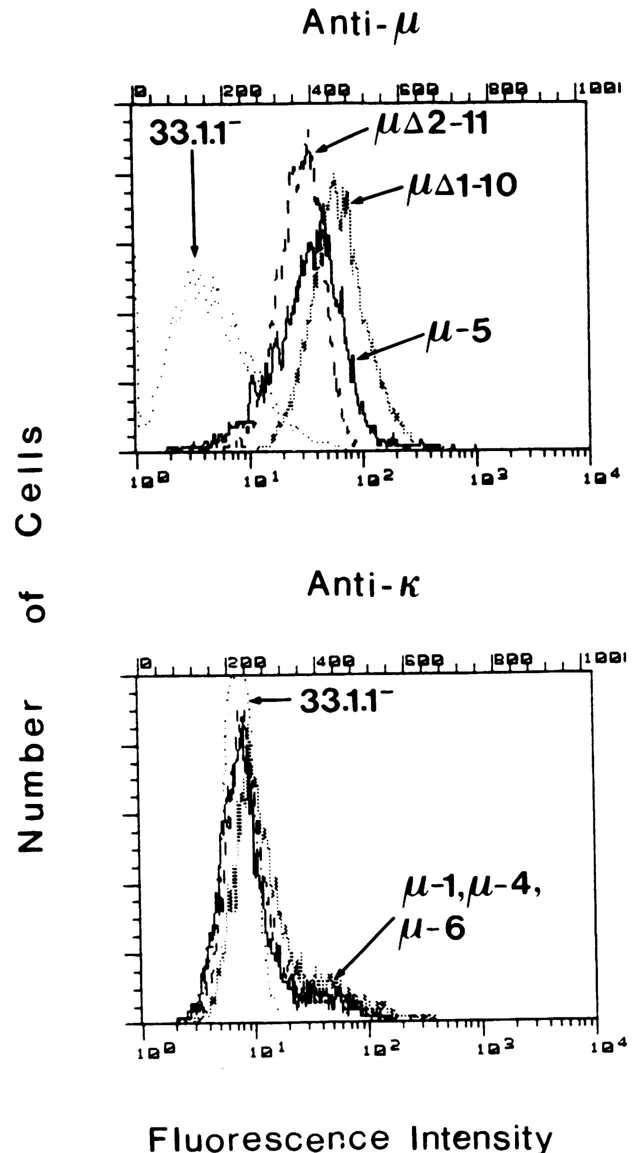

Fig. 2. Flow cytometric analysis of parental 33.1.1$^-$ and transfected cells. The staining histograms for the indicated cell lines with the anti-μ mAb 1M41 or the anti-κ mAb 187.1 are overlayed with the corresponding profile of 33.1.1$^-$.

transfectant expressed also the smallest amounts of μ protein and has the lowest frequency of κ rearrangements (Table I). Likewise, transfectant μ-3 contains considerable amounts of κ transcripts that are of smaller size (~1 kb) than normal (1.2 kb), and therefore may correspond to immature Cκ transcripts (Nelson *et al.*, 1985; Leclercq *et al.*, 1989). κ transcripts are also present in $\mu\Delta1$-4, consistent with the κ rearrangements detected in this transfectant (see below). On the average, μ transfectants show a ten-fold increase in the κ mRNA levels of correct size as compared with the parental line 33.1.1$^-$ (Table I). In contrast, no increase in the κ mRNA levels is detected in mutant μ transfectants as compared with 33.1.1$^-$. RNA transcripts for $\lambda5$, $V_{pre\text{-}B}$ and mb-1, the gene encoding the μ-associated protein IgMα (Sakaguchi *et al.*, 1988; Hombach *et al.*, 1990) are also present in all cell lines at roughly equivalent amounts (Figure 3). Only the line 33.1.1$^+$ gave consistently ~4-fold lower values for $\lambda5$ and $V_{pre\text{-}B}$ mRNA when scanned on independent gels.

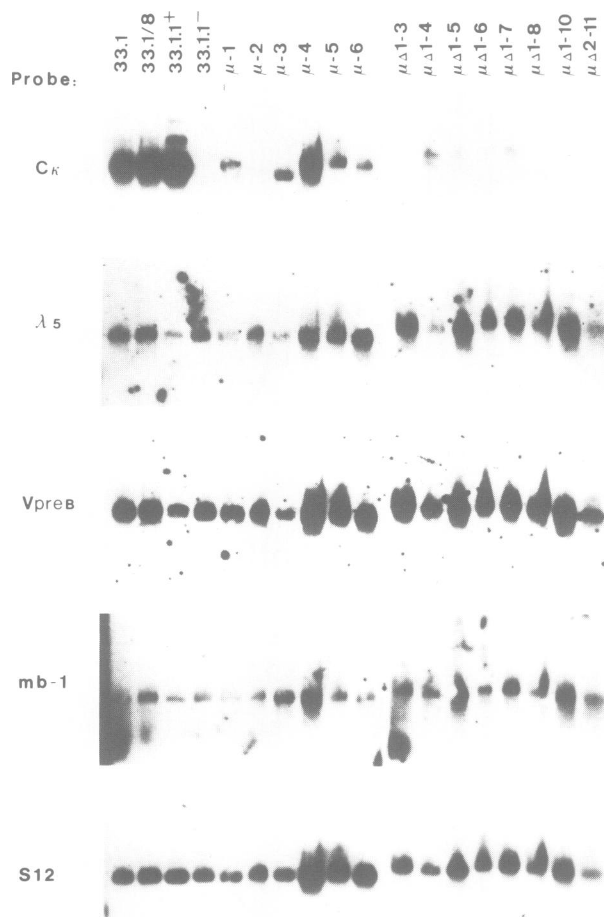

Fig. 3. Northern blot analysis of RNA from pre-B cell lines and transfectants. 15 μg of total RNA of the cell lines indicated were used in the experiment. The same blot was used in subsequent hybridizations after stripping off the preceding probe. The probes used are indicated left and described in the text and in Materials and methods.

L chain gene rearrangement

To investigate directly the potential of the μ constructs in inducing L gene rearrangement, we made subclones of individual transfectants and subjected their genomic DNA to Southern analysis. The DNAs were digested with the restriction enzymes *Hin*dIII or *Xba*I and, after blotting, hybridized with probes covering the κ J region and the κ intron region (not shown). All the wild-type μ transfectants analysed in this way showed rearrangements at the L gene locus (Table I). The exception of μ-3 is explained by the loss of its recombining capacity (see above). In contrast, no κ rearrangements are found in similar analysis of subclones of μΔ1 and μΔ2 transfectants with the exception of μΔ1-4. This transfectant contains appreciable amounts of κ mRNA (see Figure 3 and Table I) and displays κ proteins on the cell surface of only a few cells (not shown). Southern analysis of DNA from 21 subclones of this transfectant revealed two identical κ rearrangements in all subclones, indicating that two non-productive rearrangement events had occurred before transfection. Weak additional signals indicate on-going κ replacement rearrangements, which explain the few surface κ positive cells in this line (data not shown). Omitting the line without recombinase activity (μ-3) we find 36 rearranged κ alleles of a total of 117 alleles analysed in subclones of wild-type μ transfectants. No rearranged κ allele could be detected in 224 alleles screened in subclones of μΔ1 and in 40 alleles analysed in μΔ2 subclones. From these data we conclude that only the wild-type μ protein but not the truncated forms of it have the capacity to efficiently induce L chain gene rearrangement.

Characterization of the proteins associated with the transfected μ proteins

We asked next for possible differences in the proteins associated with the wild-type or mutant μ proteins in the transfectants. Indeed, different μ associated protein complexes are found in wild-type μ versus μΔ1 and μΔ2 transfectants. Figure 4 shows a typical SDS−polyacrylamide gel of the μ and associated proteins after immune precipitation of metabolically ([^{14}C]leucine) labelled lysates of different cell lines with rabbit antibodies specific for murine IgM (similar results are obtained with an anti-μ monoclonal antibody, not shown). No μ proteins were precipitated from a 33.1.1$^-$ lysate. In 33.1.1$^+$, only the transgenic μ and κ proteins are precipitated. In wild-type μΔ1 and μΔ2 transfectants, two small proteins are co-precipitated whose sizes (21 kd and 17 kd) correspond to the expected size of the gene products of λ5 and V_{pre-B}. Two additional proteins of M_r 33 kd and 36 kd are also co-precipitated with μ and to a lesser extent, with μΔ2, but not with μΔ1 (Figure 4, digitonin). In none of the μΔ1 transfectants were the μΔ1 chains found associated with the 33 kd/36 kd proteins. As a control the μΔ1-10 line was supertransfected with the wild-type μ gene. Anti-μ precipitation then revealed the two μ chains and the 33 kd/36 kd proteins (not shown). These latter two proteins are only co-precipitable with wild-type μ from digitonin lysates whereas the putative λ5 and V_{pre-B} products are easily detectable in immune precipitates of both digitonin or NP-40 lysates (Figure 4). For the μΔ2 transfectant, scanning analysis of the lanes of the gel in Figure 4 revealed a three- to four-fold reduction in the amount of the 33 kd/36 kd proteins associated with the μΔ2 chain, as compared with a similar amount of the wild-type μ chain. Association with λ5/V_{pre-B} is not affected by the deletions. The complex of proteins associated with wild-type μ in 33.1.1$^-$ transfectants are also demonstrated in an independent AMuLV-transformed pre-B cell line, transfected with the same μ construct (IIB4 in Figure 4), and in four other independent AMuLV pre-B cell lines derived from μ transgenic mice which express the transgenic μ protein (not shown). We conclude therefore that the observed complex of proteins linked to μ is not a peculiarity of the 33.1.1$^-$ line but reflects a more general feature of AMuLV-transformed pre-B cells which express μ.

Next we asked, whether the 33 kd/36 kd proteins are associated with μ on the cell surface of transfectants. Figure 5 shows an SDS−polyacrylamide gel of iodinated surface proteins of the different cell lines, after digitonin lysis and immunoprecipitation with rabbit anti-mouse IgM antibodies. A protein of 33 kd is co-precipitated with wild-type surface μ only in the μ-6 transfectant (indicated by an arrow in Figure 5). This result might indicate that only the smaller, 33 kd component is associated with μ on the cell surface. Alternatively, the result could simply reflect poor iodination of the larger, 36 kd protein, due to steric non-accessibility or low tyrosine content.

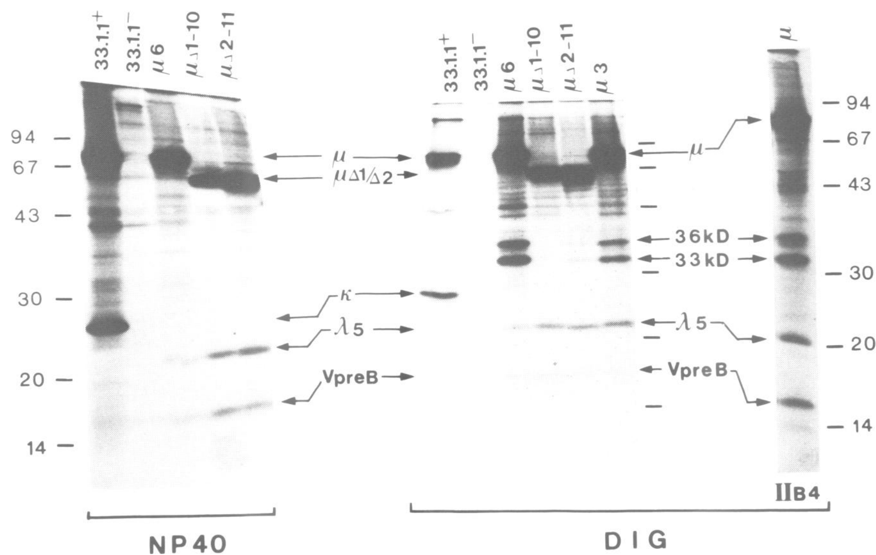

Fig. 4. Analysis of μ associated proteins in transfected lines. The indicated cell lines are metabolically labelled with [^{14}C]leucine, lysed either with NP40 or digitonin (DIG) and the lysates are immuno-precipitated with rabbit anti-IgM antibodies. The precipitates are run on reducing 12% SDS−polyacrylamide gels. The right-most slot shows the proteins immuno-precipitated from the line IIB4, also expressing the μ construct (Figure 1). The positions of the proteins μ, μΔ1, μΔ2, 36 kd, 33 kd and putative λ5 and V_{pre-B} gene products are indicated with arrows. The position and size of mol. wt markers is indicated with horizontal bars left and right. Upon scanning of the proteins in the digitonin gel for transfectants μ-6, μΔ1-10 and μΔ2-11, the peak areas corresponding to μ proteins, 36 kd and 33 kd proteins, and putative ω and ι were measured. The resulting 33 kd : μ and 36 kd : μ ratios are 0.17 and 0.18 for μ-6 and 0.04 and 0.03 for μΔ2-11. The ratios of ω/ι : μ are comparable in all transfectants.

As the 33 kd and 36 kd μ-associated proteins are similar in size to the two recently described surface IgM-associated glycoproteins, IgMα and Igβ (Hombach *et al.*, 1990), we decided to directly compare both sets of proteins. To this end we mixed an aliquot of the affinity-purified antigen receptor from the myeloma variant J558Lμm3 (Hombach *et al.*, 1988, a kind gift of J.Wienands), containing IgMα and Igβ, with an immuno-precipitate of a metabolically labelled lysate of the μ-6 transfectant (as in Figure 4) and run the mixture in a 2-D SDS−polyacrylamide gel. As for IgMα and Igβ, the 33 kd and 36 kd proteins described here are non-covalently attached to μ but covalently bound to each other. However, comparison of the silver-stained IgMα and Igβ proteins (shown in Figure 6A) with the radioactively labelled 33 kd and 36 kd proteins (Figure 6B) clearly demonstrates different migration properties for the two pairs of proteins. IgMα and Igβ are larger in size than the 33 kd/36 kd proteins, respectively, and their mobility properties in the second, reducing dimension of our 2-D SDS−polyacrylamide gel correspond well to their reported sizes of 34 kd (IgMα) and 39 kd (Igβ) (Hombach *et al.*, 1990). Additionally, both the 33 kd and the 36 kd proteins have a discrete size, in contrast to the broad pattern of modified forms typically displayed by Igβ (Hombach *et al.*, 1990; and Figure 6). Therefore, we conclude that the 33 kd/36 kd proteins studied here are different from the described IgMα and Igβ proteins.

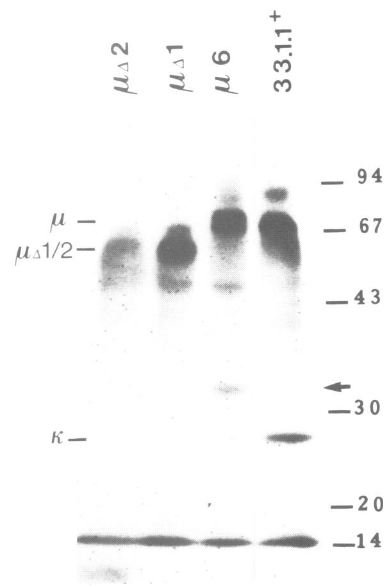

Fig. 5. SDS−PAGE analysis of surface iodinated cell lines. The indicated cell lines were surface labelled with ^{125}I, lysed with digitonin and the lysates were immuno-precipitated with rabbit anti-IgM antibodies. The immuno-precipitates were run in 12% SDS−PAGE and the gel was dried and autoradiographed. μ and κ proteins are indicated. The position of the 33 kd protein is indicated by an arrow.

Expression of μ and κ does not stop κ rearrangement

According to current models (Ritchie *et al.*, 1984) a pre-B cell that expresses surface H and L chains should no longer rearrange the κ locus. This is indeed the case in the 33.1.1$^+$ line (Table I). In contrast, the pre-B cell lines 33.1 and 33.1/8 expressed the transgenic μ and κ proteins on the surface and continue rearranging their endogenous L chain loci (Table I). Thus we were interested in the nature of the proteins associated with μ in these cell lines. In Figure 7, the proteins co-precipitated with μ in metabolically radiolabelled lysates of 33.1, 33.1/8, 33.1.1$^+$ and of the μ-6 transfectant are shown. The transgenic μ and κ proteins are precipitated in all three cell lines but, in addition, the complex of λ5/V_{pre-B} and 33 kd/36 kd proteins are also precipitated with μ in cell lines 33.1 and 33.1/8. Quantification of the scanning curves of the lanes in the gel of Figure

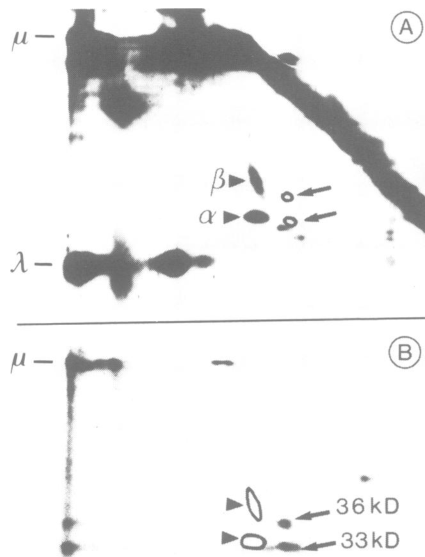

Fig. 6. Co-electrophoresis of the 33 kd/36 kd proteins with the IgM-associated proteins IgMα/Igβ.μ-6 cells were metabolically labelled with [^{14}C]leucine and digitonin lysates were immuno-precipitated using rabbit anti-IgM antibodies. The immuno-precipitate was mixed with NP-affinity-purified IgM proteins from a digitonin lysate of the surface IgM positive myeloma variant J558Lμm3 (Hombach et al., 1988) and run first under non-reducing conditions in an 8% SDS−polyacrylamide gel and then in the second dimension in a 10% reducing SDS−PAGE. The 2-D gel was first silver-stained to make IgMα and Igβ visible (A). After drying, the gel was exposed to reveal the radioactively labelled 33 kd and 36 kd proteins (B). The position of the 33 kd/36 kd proteins and of IgMα/Igβ are indicated by circles in A and B, respectively.

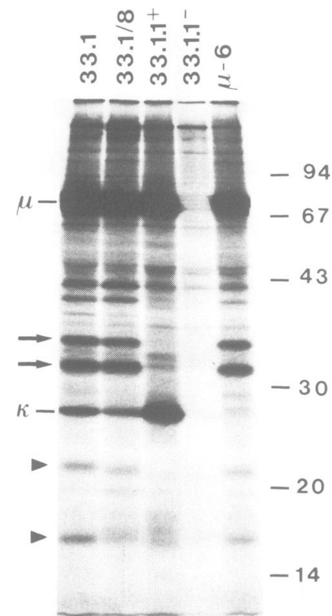

Fig. 7. Analysis of μ associated proteins in pre-B cell lines. Metabolic labelling, lysis, immuno-precipitation and gel electrophoresis for the indicated lines are as in Figure 5. The immuno-precipitated μ and κ proteins as well as the 33 kd/36 kd (arrows) and $\lambda5/V_{pre\text{-}B}$ (arrowheads) are indicated. Size and position of mol. wt markers are shown right. Measurement of the peak areas obtained by scanning the lanes corresponding to 33.1, 33.1/8 and 33.1.1$^+$ gave a κ : μ ratio of 0.41 for 33.1.1$^+$, 0.059 for 33.1 and 0.058 for 33.1/8.

7 demonstrated an eight-fold reduced ratio of κ versus μ in the cell lines 33.1 and 33.1/8 as compared with the κ:μ ratio in the cell line 33.1.1$^+$, indicating that an excess of transgenic μ over transgenic κ is produced in the rearranging pre-B cell lines 33.1 and 33.1/8. As the rabbit anti-IgM antiserum is also able to precipitate free κ proteins we can rule out the possibility that large amounts of κ are produced but poorly associated to μ in these cell lines. The amounts of μ are similar in the three cell lines, thus pointing to an up-regulation of κ production in the 33.1.1$^+$ line. In fact, a 3- to 4-fold increased steady-state level of κ transcripts in 33.1.1$^+$ versus 33.1 and 33.1/8 is found in the Northern experiment shown in Figure 3.

In summary, the results suggest that AMuLV-transformed pre-B cell lines expressing μ proteins are able to proceed to rearrangement of the L gene locus as long as the μ proteins are in excess over κ and associated with the 33 kd/36 kd heterodimer and possibly the ω and ι surrogate L chains.

Discussion

The rearrangement of Ig genes is a temporally ordered and regulated process in developing pre-B cells. The actual products of this process, the Ig proteins, themselves play a central role in the regulation of the recombining events. The first antibody chain to be produced during B cell development, the μ H chain, exerts a double effect. It inhibits further rearrangement at the H gene locus (Rusconi and Köhler, 1985; Weaver et al., 1985) and induces rearrangement of L genes (Reth et al., 1987). For both functions, the membrane form of μ (μ_m) is required (Nussenzweig et al., 1987 and 1988; Manz et al., 1988; Reth et al., 1987). In the present paper we further define the protein elements required in the process of μ-induced L gene rearrangement.

The continued expression of excess μ promotes κ rearrangement

Our data confirm and extend former results (Reth et al., 1987) demonstrating that μ proteins efficiently induce L chain gene rearrangement and show that deletions of the first or second constant domains of μ abrogate this capacity.

By loss of the μ and κ transgenes, we obtained the cell line 33.1.1$^-$ which retained the capacity to rearrange an exogenous recombination substrate, but failed to recombine its κ locus. Upon transfection and expression of a wild-type (μ) or mutant (μΔ1, μΔ2) μ genes, κ rearrangements were detected in all 33.1.1$^-$ wild-type μ transfectants (5 out of 5, omitting the recombinase negative line μ-3, Table I), whereas no κ rearrangements were induced by the mutant μ proteins (Table I). μ independent rearrangement of the κ locus was observed once (μΔ1-4) in 29 lines studied (21 subclones of 33.1.1$^-$ plus the 8 mutant μ transfectants, Table I). Thus, in our study, spontaneous κ rearrangement is ~30-times less frequent than the μ-induced one (Table I). This is also indicated by the low κ mRNA levels detected in 33.1.1$^-$ and the mutant μ transfectants (Figure 2 and Table I). Spontaneous, μ-independent κ rearrangement has been documented in other pre-B cell lines (Schlissel and Baltimore, 1989), in murine AMuLV-transformed pre-B cell lines derived from immuno-deficient SCID mice (Blackwell

et al., 1989a) and in Epstein–Barr virus transformed human pre-B cell lines (Kubagawa *et al.*, 1989). They were detected at low levels using a sensitive PCR technique (Schlissel and Baltimore, 1989) and were rare events in the SCID study. Since μ and not κ proteins are easily detected in normal pre-B cells, spontaneous κ rearrangement may not be a major pathway in B cell development. The lines 33.1 and 33.1/8 produce both transgenic μ and κ proteins (Figure 7) and, at the same time, show rearrangements of the endogenous κ loci. This appears to contradict the proposed model (Ritchie *et al.*, 1984), according to which rearrangement at the Ig loci ceases once a pre-B cell expresses complete IgM molecules. However, close analysis of the Ig proteins produced in 33.1 and 33.1/8 revealed an excess of μ over κ proteins. This contrasts with the equimolar ratio of μ:κ found in the non-rearranging line 33.1.1$^+$ (Figure 7). This line shows a 100-fold decrease in recombinase activity and a four-fold reduction in the levels of λ5 and $V_{pre\text{-}B}$ mRNA, as compared with 33.1.1$^-$ (Figure 3). Also, ω and ι proteins are not found associated with μ in this cell line (Figures 4 and 7). In fact, the overall characteristics of the line 33.1.1$^+$ correspond better to the phenotype of early B cells than to that of pre-B cells. One could hypothesize that κ-rearranging activity persists in 33.1 and 33.1/8 because free μ chains are present in these cell lines. Binding of all μ proteins to L chains, as is the case in 33.1.1$^+$, would lead to down-regulation of recombinase activity and to reduced synthesis of the pre-B cell specific proteins λ5 and $V_{pre\text{-}B}$ as the cells proceed to the next differentiation stage.

Overall, we find in wild-type μ 33.1.1$^-$ transfectants a similar high frequency of κ gene rearrangement to that observed in the parental 33.1 and in the 33.1/8 lines (Table I). We conclude that only continued expression of (excess) μ signals κ rearrangment.

μ acts by activating the κ locus, resulting in rapid κ rearrangement

Transcriptional activity of V gene segments is supposed to precede Ig gene rearrangement (Blackwell *et al.*, 1986; Schlissel and Baltimore, 1989). Germline transcripts of unrearranged Ig V gene segments have in fact been described both *in vitro* (Blackwell *et al.*, 1989b) and *in vivo* (Yancopoulos and Alt, 1985). Exposure of pre-B cells to bacterial lipopolysaccharide (LPS), a polyclonal mitogen, activates transcription at the κ locus, detectable as germline $C\kappa$ transcripts (Nelson *et al.*, 1985; Leclercq *et al.*, 1989; Blackwell *et al.*, 1989a), and eventually results in κ gene rearrangement (Schlissel and Baltimore, 1989). The small κ transcripts present in transfectant μ-3 hybridize with a $J\kappa$-1 probe (not shown) and correspond in size to what is expected for germline $J\kappa$-$C\kappa$ transcripts (~1 kb, Leclercq *et al.*, 1989). Nonetheless, this transfectant is unable to bring about L gene rearrangement, due to the lack of recombinase activity (Table I). In this transfectant, the complex of μ and associated proteins is indistinguishable from that found in κ rearranging μ transfectants (Figure 4). It is therefore tempting to speculate that transfectant μ-3 is 'frozen' at the stage immediately prior to κ rearrangement.

Such a model implies that κ rearrangement is a rapid event, since no germline $C\kappa$ transcripts were detectable with mature κ mRNA in μ transfectants (Figure 3), in spite of the fact that not all subclones have κ rearrangements and that on-going κ rearrangements can be detected (Table I, not shown).

What signals L gene rearrangement?

Taken together, our results suggest that the frequency of κ gene rearrangements in pre-B cells is enhanced by the formation of a protein complex including μ, a heterodimer of proteins of 33 kd and 36 kd in size and possibly ω and ι. Both in murine and human pre-B cells, the complex of the two small proteins (ω, M_r 22 kd and ι, M_r 17 kd) encoded by the λ5 and $V_{pre\text{-}B}$ genes has been shown to serve as surrogate L chains for binding and surface expression of μ (Kerr *et al.*, 1989; Tsubata and Reth, 1990). We found ω and ι to be associated with all our μ proteins (Figure 4), thus indicating that their binding is not affected by deletion of the first or second μ constant domain. It has been suggested that ω binds covalently to μ via the cysteine residue present in $C\mu 1$, whereas ι associates with μ through non-covalent interaction with the V_H region (Kudo *et al.*, 1989). In addition, ω and ι are shown to be able to bind to each other in the absence of μ (Misener *et al.*, 1990). Indeed we observed association of ω/ι with the mutant protein $\mu\Delta 1$, thus suggesting that the interaction of ι with the V_H region is sufficient to form the complex $\mu-\omega-\iota$. Thus, the role of ω and ι in the rearrangement process is not directly addressed in our experiments, as they do not differentially bind to the mutant μ proteins.

The results shown in Table I, and Figures 4 and 7 indicate a correlation between the capacity to induce L gene rearrangement and the ability to associate and form a complex with the 33 kd/36 kd protein heterodimer. A similar heterodimeric pair of μ-associated proteins with M_r of 34 kd and 39 kd, termed IgMα and Igβ have been shown to be required for surface expression of IgM and signalling in mature B cells (Hombach *et al.*, 1990; Campbell and Cambier, 1990). Although similar in size, the two μ-associated protein heterodimers are not identical: (i) The 33 kd/36 kd proteins are found associated with wild-type μ but not with a mutant μ protein that lacks the first constant domain and are only weakly associated with a mutant μ lacking the second constant domain. These findings map the binding site(s) for the 33 kd/36 kd heterodimer within or around the first constant domain of μ, whereas the μ-binding site for IgMα/Igβ has been localized to the last, membrane-proximal, constant domain and the transmembrane portion of μ (Hombach *et al.*, 1990; Williams *et al.*, 1990). This interpretation is underlined by the observation that we were unable to precipitate our heterodimer from the 33.1.1$^+$ line, whose increased κ chain production possibly results in blocking of the $C\mu 1$ domain (Figure 7). (ii) We have directly shown that the size of our 33 kd/36 kd proteins is different from the size of the IgMα/Igβ proteins (Figure 6). Furthermore, the typical broad pattern of modified Igβ proteins is not found for either of the 33 kd or 36 kd proteins (Figure 6), and in addition, the 33 kd/36 kd proteins are not found associated with IgM in the myeloma variant J558Lμm3 (Figure 6A). (iii) As all cell lines analysed display surface μ proteins, (Figures 2 and 5 and Table I), we assume that all μ proteins are associated with IgMα/Igβ, independently of the mutations. However, the 33 kd/36 kd heterodimer is easily co-precipitated with μ proteins in digitonin lysates of the lines 33.1 and 33.1/8 and of wild-type μ and even $\mu\Delta 2$ transfectants, but is not detectable in immuno-precipitates of the line 33.1.1$^+$ and in $\mu\Delta 1$ transfectants (Figures 4 and 7). The fact that we do not detect IgMα/Igβ in our immuno-precipitates could be explained by the labelling and immuno-precipitation procedures used in our

experiments. In fact, a 50-fold higher specific labelling than used in our experiments, followed by a specific NP-affinity enrichment of the IgM complex is needed to visualize IgMα/Igβ (see Materials and methods; Hombach *et al.*, 1988; Wienands *et al.*, 1990).

While these findings altogether suggest that the 33 kd/36 kd heterodimer is composed of one or two proteins unrelated to IgMα/Igβ, we cannot presently rule out the possibility that they result from pre-B cell specific modifications of IgMα and/or Igβ. Such differentially modified forms of IgMα/Igβ could serve different functions in pre-B and mature B cells. Furthermore, to explain the apparent mapping of our heterodimer to the Cμ1 domain, one could envisage that excess free μ chains might aggregate *via* their Cμ1 domains and thereby stabilize the 33 kd/36 kd association at the C-terminal end. Along this line, such aggregates could be the triggering event for L chain gene rearrangement.

It is known that Ig crosslinking leads to activation of B cells (for review see, Reth, 1991). It has been proposed that ligands in bone marrow stromal cells serve to crosslink the μ−ω−ι surface receptor and thereby induce κ gene rearrangement (Melchers *et al.*, 1989). However, AMuLV-transformed pre-B cells undergo κ gene rearrangement in the absence of bone marrow-derived stromal cells and thus, binding of surface μ to putative ligands on stromal cells is not required. What then triggers this process in AMuLV pre-B cells? It has been shown that cross-linking of surface μ *via* anti-IgM antibodies in pre-B cells results in Ca^{2+} influx (Takemori *et al.*, 1990). In the wild-type μ transfected AMuLV pre-B cells, μ receptor crosslinking could be mediated by a putative ligand on the same cell. Alternatively, crosslinking could represent an intrinsic property of the surface μ complex. We speculate that the 33 kd/36 kd heterodimer is needed for crosslinking of μ on the cell surface. This would generate intracellular signals directed to activate the κ locus for transcription and recombination. The purification and molecular characterization of the 33 kd and 36 kd proteins will further help to elucidate the mechanism(s) underlying μ-dependent L chain gene rearrangement in pre-B cells.

Materials and methods

Cell lines

The line 33.1 was obtained by AMuLV transformation of bone marrow cells from the Sp6 transgenic mouse (Rusconi and Köhler, 1985). 33.1.1$^+$, 33.1.1$^-$ and 33.1/8 are sublines of 33.1. IIB4 is an AMuLV-transformed pre-B cell line derived from Balb/c bone marrow. Both cell lines have been established by C.Paige, Toronto. All sub-clonings were done by limiting dilutions (0.3 cells/well).

DNA constructs

The gene construct for wild-type μ (μ in Figure 1) was made by subcloning two *Eco*RI fragments encompassing the entire genomic VDJ-Cμ region and regulatory flanking sequences from a λ phage clone into the expression vector pSV2gpt (Mulligan and Berg, 1981). To make μΔ1 a 4.5 kb deletion using the two internal *Sac*I fragments was introduced in construct μ. μΔ2 was derived from plasmid p2.11, (a kind gift of A.Traunecker), where the heavy chain enhancer is placed 5′ of the VDJ region and the large intron is reduced by deleting the internal *Xba*I fragments. Digestion of p2.11 with *Sac*I and *Bam*HI, followed by blunt end generation and religation resulted in truncation of the 5′ half of the second constant μ exon including the splice acceptor site. The constructs μΔ1 and μΔ2 direct the production of transcripts and μ proteins devoid of the first or second constant domain, as determined serologically and in Northern experiments (not shown).

Gene transfer experiments

Transfection of the μ constructs into pre-B cells was done by the method of electroporation (Potter *et al.*, 1984). Transfectants were obtained by selection with mycophenolic acid (1.2 μg/ml) in the case of μ and μΔ1 or G418 (1.8 mg/ml) in the case of μΔ2 and are routinely kept in DMEM supplemented with 10% fetal calf serum and the indicated concentrations of selective drugs. Transfection of the recombination substrate pJH201 (kindly provided by Dr K.Mizuuichi) into pre-B cells, recovery of the plasmid fraction after 48 h and bacterial transformation were done as described (Hesse *et al.*, 1987), except that electroporation of competent bacteria was also used as an alternative method to increase the frequency of bacterial transformant colonies (for details see B.Bühler, Diploma Work). Rearrangement of the joining substrate during the transient transfection results in activation of the previously silent bacterial gene chloramphenicol acetyl transferase. The frequency of the joining events which occurred in individual pre-B cell transfectants is assessed by comparing the frequency of ampicillin resistant with that of ampicillin and chloramphenicol double resistant bacterial colonies obtained. Only double resistant colonies containing plasmids with faithful rearrangements, as determined by analysis with the restriction enzyme *Hgi*AI (Hesse *et al.*, 1987), are taken into the account shown in Table I.

Flow cytometric analysis

Cells were stained either with the biotinylated mAb 1M41, specific for the third constant domain of μ (Leptin *et al.*, 1984), followed by Streptavidin−phycoerythrin or with the FITC-conjugated mAb 187.1, specific for murine κ chains (Yelton *et al.*, 1981). Stained cells were analysed in a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA).

Southern and Northern blot analysis

Southern blot analyses were essentially performed as described by Iglesias *et al.* (1987), except that alkaline transfers onto ZetaProbe membranes (BioRad) were done. Routinely 15 μg of DNA of each cell line were used in the analysis. Hybridizations were done at 65°C. All the probes used consist of agarose gel-purified fragments and are ^{32}P-labelled with the random priming method (Feinberg and Vogelstein, 1984). The probes used consisted of a *Hind*III−*Bgl*II and a *Xba*I−*Hind*III DNA fragments encompassing the entire Jκ cluster and Jκ-Cκ intron sequences, respectively (Max *et al.*, 1981). The probe for λ5 consisted of a 0.7 kb *Bam*HI fragment of the cDNA clone pZ183.1 and the probe for $V_{pre\text{-}B}$ was a genomic 3.5 kb *Bam*HI−*Xba*I fragment derived from the plasmid 7pB12-2 (both kindly provided by Dr F.Melchers). The probe for mb-1 was the 1 kb of cDNA insert described in Sakaguchi *et al.* (1988). S12 is a 0.5 kb cDNA fragment of the gene that encodes the mouse ribosomal protein S12 (Ayane *et al.*, 1989). Northern blot analysis were essentially done as described (Maniatis *et al.*, 1982). 15 μg each of total RNA from each cell line analysed were electrophoresed in glyoxal gels. After blotting, hybridization and exposure, the radioactive probe was stripped off and the filter was used in a subsequent hybridization with another probe. Scanning of the signals obtained was done with an Ultroscan XL Enhanced Laser Densitometer (LKB).

Cell labeling, immuno-precipitation, SDS−PAGE, surface iodinations and Western blot analysis

For metabolic labelling, $1-5 \times 10^7$ viable cells were first cultured in leucine-free DMEM medium (Gibco) containing 10% dialysed fetal calf serum for 30 min and then 10 μCi [^{14}C]leucine were added. After incubation for 4 h, cells were washed in PBS and lysed on ice for 30 min either in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA; 1 mM PMSF) or in digitonin lysis buffer (1% digitonin, 20 mM triethanolamine, 10 mM Tris, pH 7.5, 150 mM NaCl; 1 mM EDTA; 1 mM PMSF) followed by centrifugation for 20 min at 24 000 *g*. After pre-clearing of the lysates with 10 μg of normal rabbit serum for 1 h at room temperature, and incubation with protein G−Sepharose for another 1 h and centrifugation, the supernatant was incubated with rabbit anti-murine IgM antibodies for 1−2 h at room temperature, followed by a 1 h incubation with protein G−Sepharose and centrifugation. Pellets were washed three times with lysis buffer, resuspended in sample buffer and boiled. One-dimensional SDS−PAGE was performed in 12% polyacrylamide gels. For two-dimensional SDS−PAGE, protein samples were heated for 15 min at 65°C in non-reducing sample buffer and run in 8% tube gels. Thereafter the gel was reduced in 5% β-mercaptoethanol, 0.1% SDS, 100 mM Tris, pH 6.8 and bromophenol blue at 65°C for 15 min. The tube gel was then fixed with 1% agarose on top of 10% acrylamide gels and electrophoresed.

For surface iodinations, $2-5 \times 10^7$ cells were surface labelled with 0.5 mCi ^{125}I (Amersham Buchler, Braunschweig, FRG), by the lactoperoxide method as described (Haustein, 1975). Labelled cells were washed and digitonin lysis and immuno-precipitations were performed as above.

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