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Stimulation of κ Light-Chain Gene Rearrangement by the Immunoglobulin μ Heavy Chain in a Pre-B-Cell Line

ALAN M. SHAPIRO,[1] MARK S. SCHLISSEL,[2,3]† DAVID BALTIMORE,[2,3]‡ AND ANTHONY L. DEFRANCO[1,4]*

Department of Biochemistry, University of California, San Francisco, California 94143-0448[1]; Whitehead Institute, Cambridge, Massachusetts 02142[2]; Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[3]; and G.W. Hooper Foundation and Department of Microbiology and Immunology, University of California, San Francisco, California 94143-0552[4]

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B-lymphocyte development exhibits a characteristic order of immunoglobulin gene rearrangements. Previous work has led to the hypothesis that expression of the immunoglobulin μ heavy chain induces rearrangement activity 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-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, and this correlated with an enhanced frequency of κ immunoglobulin gene rearrangement compared with that in the matched μ^- cell lines. This increased κ gene rearrangement frequency was not due to a general increase in the amount of V(D)J recombinase activity in the μ^+ cells. Consistently, introduction of a functionally rearranged μ gene into one of the μ^- pre-B-cell lines resulted in a fivefold increase in κ gene rearrangements. In three of the four clonally matched pairs with increased κ gene rearrangements, the increase in rearrangement frequency was not accompanied by a significant increase in germ line transcripts from the C_κ locus. However, in the fourth pair, K.40D, we observed an increase in germ line 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 germ line C_κ transcript correlated with the measured frequency of rearranged κ genes. 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.

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 (2, 44). 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 inside the pre-B cell (9). 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 (10, 15, 18, 36, 38, 53). In μ^+ pre-B cells, light-chain genes (κ or λ) undergo rearrangement (1, 31, 39). When light-chain gene rearrangement is successful, the 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 the heavy-chain genes than of the light-chain genes. In this case, it would be statistically more likely for a heavy-chain gene to rearrange first. Alternatively, the order of rearrangement could be regulated such that the V(D)J recombinase is initially targeted to the Ig heavy-chain (IgH) locus and is then directed away from the IgH locus and towards the light-chain loci once the heavy-chain μ protein has been expressed (2, 6, 47). Regulation of V(D)J recombinase targeting might also explain allelic exclusion, the fact that a single B cell has only one functionally rearranged heavy-chain allele.

Currently, the weight of evidence argues for a regulated model of Ig gene rearrangement. For example, cells with two functional IgH rearrangements are frequent in mice heterozygous for a mutation that prevents the μ protein from being expressed in its membrane form (25). Moreover, mice homozygous for this mutation have greatly reduced κ gene rearrangement and a complete arrest of B-cell development (25). These results suggest that the membrane form of the 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 (37, 45, 51, 57). This decrease is observed if the transgene expresses the membrane form of μ but not if it expresses the secretory form (33, 37), again indicating a regulatory role for the membrane form of the 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 (21, 42, 54). 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 the μ chain in this event (6, 19, 27, 48). We have isolated a matched series of μ^- and μ^+ cell lines from the A-MuLV-transformed cell line K.40 (5) and have measured the frequency of κ rearrangements in them. Although some κ rearrangements were seen in the μ^- siblings, μ-chain ex-

* Corresponding author.
† Present address: Department of Medicine and Molecular Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
‡ Present address: Rockefeller University, New York, NY 10021.

pression, 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 targeting of the recombinase to the κ locus.

MATERIALS AND METHODS

Cell lines and tissue culture. The cell line K.40 (5) 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 (26) was obtained from J. M. Bishop (UCSF). WEHI-231 (56) was obtained from Noel Warner (Becton-Dickinson). Cells not under selection were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HyClone, Irving Scientific, or JR Scientific), 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 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 (*Limulus* amoebocyte lysate chromogenic assay; Whittaker Biochemicals). Cells used in experiments were kept in log-phase growth with a density below 7×10^5 cells per ml.

Cytoplasmic immunofluorescence staining of cells. Samples of 10^5 cells were centrifuged onto slides with a Cytospin 2 (Shandon Southern Instruments, Inc.), air dried, fixed in 100% ethanol, washed twice in phosphate-buffered saline (PBS) without divalent cations plus 1% bovine serum albumin plus 0.1% NaN_3 and stained either with 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 of polyvinyl alcohol [Air Products, Inc.] dissolved in 52.2 ml of PBS plus 22.8 ml of glycerol and 1 ml of NaN_3 and filtered through a Whatman no. 1 filter). The numbers of μ^+ cells and κ^+ cells on the entire slide were counted under the fluorescence microscope. In some experiments, the cell recovery of each sample was determined directly by including an internal standard of human B-lymphoblastoid Daudi cells prestained with 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated goat anti-human IgG-IgM (heavy plus light chains) (0.02%) (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 fluorescence-activated cell sorter (FACS) buffer (PBS plus 1% fetal calf serum plus 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 plus 4 μg of propidium iodide per ml.

Isolation of cell lines with endogenously rearranged μ genes. Cells expressing a functionally rearranged endogenous μ gene were isolated by a sib selection approach. First, K.40 cells were subcloned by limiting dilution. The six K.40 subclones (A to 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 immunofluorescence 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 thawed together and grown in tissue culture for a minimal length of time to decrease the chance of changes that might occur following extensive growth in tissue culture (4).

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 (8) and pμ (16) as follows. First, the *Sal*I site in the 6.2-kb plasmid pMX1112neo (8) was eliminated by cutting with *Sal*I, filling in with the Klenow fragment of DNA polymerase I, and blunt-end ligation. This pneoA1 plasmid was then cut with *Eco*RI, and *Sal*I linkers were ligated into this site. Then the 12.35-kb *Xho*I-*Sal*I fragment of pμ, containing the μ gene derived from the hybridoma 17.2.25 line (16), was inserted into the engineered *Sal*I site of pneoA1. The resulting plasmid had the 3′ long terminal repeat 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 per ml. Cells in 0.5 ml of PBS were mixed with 15 μg of DNA linearized by *Sal*I and dissolved in 7.5 μl of TE (10 mM Tris, pH 8.0, 1 mM 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 V, 960 μF). After shocking, cells were incubated for 10 min and then diluted into 10 ml of complete RPMI 1640 medium. After growth for 24 h, selection for G418 resistance was initiated. Transfectants were screened for μ expression by immunofluorescence, and isolates with the desired level of μ expression were subcloned by limiting dilution and stored frozen in liquid N_2. These transfectants were thawed together for experiments and grown for a minimal amount of time before analysis to minimize the chance of changes that occur during prolonged tissue culture.

Immunoblotting for μ expression. K.40 siblings and transfectants were lysed in Nonidet P-40 lysis buffer, and soluble proteins were separated on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to a nitrocellulose filter, incubated with 2% bovine serum albumin, and washed as previously described (14). 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, 150 mM NaCl) containing 0.05% Tween 20. The alkaline phosphatase-labeled protein bands were visualized by using 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) and Nitro Blue Tetrazolium (Bio-Rad) as described previously (14).

PCR assay for κ rearrangement frequency. The polymerase chain reaction (PCR) measurement of frequency of rearranged κ alleles in a population was done as described elsewhere with some modifications (48). For preparation of

DNA samples, the cells were centrifuged, washed once by centrifugation in PBS with divalent cations, and split equally into duplicate microcentrifuge tubes (10^6 cells per tube). The cells were pelleted for 15 s in a microcentrifuge and resuspended in 200 μl of PCR lysis buffer (10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 200 μg of gelatin per ml, 0.45% Nonidet P-40, 0.45% Tween 20, and 60 μg of proteinase K per ml). Samples were incubated at 56°C for 1 h and later for 10 min 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 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.0 mM $MgCl_2$; 200 μg of gelatin per ml; 0.2 mM final concentrations (each) of dATP, dGTP, dCTP, and dTTP (Pharmacia); oligonucleotide primers at 0.05 μM; and 1 U of *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 (Perkin-Elmer Cetus) under conditions described previously (48). 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 (32). Gels were blotted to either Zetabind (AMF) or GeneScreen Plus (New England Nuclear) filters according to the manufacturer's instructions. After prehybridization for 12 h, filters were hybridized for 18 h at 42°C in a 50% formamide hybridization solution containing, per ml, 10^6 cpm of a ^{32}P-labeled probe made from the 369-nucleotide (nt) *Rsa*I fragment of pHJ_κ (29). The amount of ^{32}P-labeled probe bound to amplified products was determined by electronic counting with either a Betascope 603 blot analyzer (Betagen) or a PhosphorImager (Molecular Dynamics) machine. The number of rearranged κ genes per 10,000 or 20,000 genomes of the K.40 subclones was determined by comparison with 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 line, 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 (Perkin-Elmer Cetus) was examined by assaying 48 identical κ rearrangement standards together. The measured values were found to be within twofold of each other. PCRs 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 (49). In the assay for D-to-J rearrangements, PCRs 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, PCRs were run with a mixture of three different degenerate primers with homologies to the conserved framework region 3 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 (49).

Measurement of C_κ germ line transcripts. Total RNA was extracted from cells by the acid guanidinium thiocyanate-phenol method (11), but with only a single isopropanol precipitation step. Twenty micrograms of RNA was electrophoresed through a 1.2% agarose gel containing 2 M formaldehyde, buffered with MOPS [3-(*N*-morpholino)-propanesulfonic acid], and transferred to a membrane (GeneScreen; New England Nuclear). The 0.8- and 1.2-kb C_κ germ line transcripts were detected by using a ^{32}P-labeled 350-nt PCR fragment amplified from the 0.8-kb C_κ germ line 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, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 125 μg of denatured salmon sperm DNA per ml, after which the membrane was washed in 0.1× SSC (1× SSC is 150 mM NaCl plus 15 mM Na citrate, pH 7.0)–1% SDS at 65°C. Quantitation of the 0.8- and 1.2-kb C_κ germ line transcripts (scanned as a single unit) was done with the PhosphorImager (Molecular Dynamics). Equivalent amounts of RNA were demonstrated to be present in each lane, by stripping the blots by boiling for 20 min in 0.1% SDS and then reprobing them with a constitutively expressed gene, that for glyceraldehyde-phosphate dehydrogenase (13).

The RNA PCR assay was performed on randomly primed cDNA as described previously (49). Briefly, 3 μg of guanidinium-purified total RNA was reverse transcribed in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). A fraction of first-strand cDNA was used as a template in the PCR assay with primers κ^0, C_κ (49), and O-PR1 (34). The κ^0 and O-PR1 primers anneal to sequences 5′ to $J_\kappa1$ which are deleted upon gene rearrangement, so these assays detect transcripts only 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 plasmid (22). 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 by a slightly modified version of the protocol of Lieber et al. (30). Cells were incubated with circular pBlueRec DNA (0.3 to 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 10 ml of complete RPMI 1640 growth medium and grown for 48 h, during which time the cell level was kept under 7×10^5 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 minipreparations (46) including phenol-chloroform extraction but omitting RNase treatment. Recovered DNA was resuspended in 25 μl of TE. DNA samples were digested with the methylation-requiring enzyme *Dpn*I for 8 h to cleave unreplicated DNA that may not have entered the lymphoid cells. The DNA samples were introduced into the XL-1-blue (Stratagene) *Escherichia coli* cells by electropo-

ration using the Bio-Rad Gene Pulser protocol. Two microliters 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, and 200 Ω. The cells were resuspended in 1 ml of a growth medium made of 2% Bacto Tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose and incubated at 37°C for 1 h with shaking at 225 rpm. The electroporated bacteria were then plated on Luria broth agar plates containing 80 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml, 150 μM isopropyl-β-D-thiogalactopyranoside, 100 μg of ampicillin per ml, and 10 μg of tetracycline per ml and scored for the LacZ phenotype by blue-white color.

Statistical methods. The Student *t* test was used to generate a *P* value to determine the statistical significance of a difference between two sets of samples.

RESULTS

Isolation and characterization of matched μ^- and μ^+ pre-B-cell lines. To examine the role that the 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 a lower but detectable rate of light-chain gene rearrangement (5). K.40 cells have a nonfunctional rearrangement (*VDJ*$^-$) of one IgH allele and a partial rearrangement (*DJ*) of the other allele (5). 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 fluoresceinated 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 μ^- K.40 subclone were isolated (Fig. 1). By 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 a full-length μ chain, cell lysates were examined by immunoblotting with anti-μ antibody (Fig. 2). In cell lines expressing full-length μ, immunoblotting usually reveals two adjacent μ protein bands which differ in their degree of glycosylation (50). All of 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, with the exception of K.40F.2, all of the K.40 μ^+ subclones expressed μ on the cell surface at reasonably high levels (Fig. 3A to C). These findings were unexpected, as most pro-B-cell lines express the μ protein primarily intracellularly and express only low levels on the cell surface. A few other examples of this phenotype have been reported previously (15, 18, 38, 53).

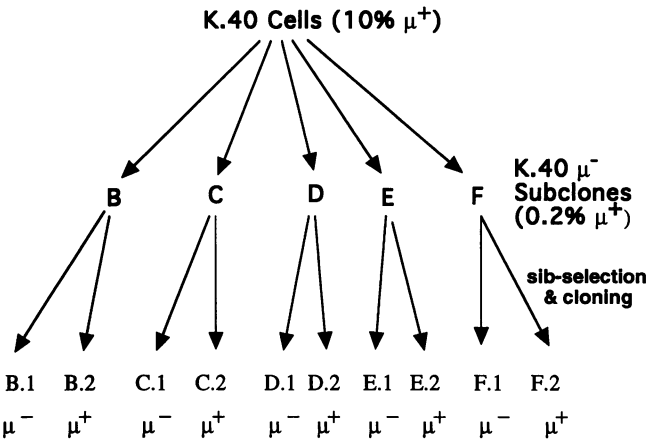


FIG. 1. Generation of clonally matched B-lineage cell pairs. Starting from a population of the pro-B-cell line K.40 (*VDJ*$^-$/*DJ*) in which 10% of the cells expressed the μ chain, as assessed by immunofluorescence, six μ^- subclones were isolated by limiting dilution cloning. These six subclones each 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.

The immunoblotting experiment (Fig. 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) (17, 43). Interestingly, three of the μ^+ derivatives of K.40 (D.2, E.2, and F.2) continued to express the Dμ protein. PCR analysis of genomic μ-chain alleles of K.40E.2 and K.40F.2 demonstrated that these Dμ$^+$ K.40 derivatives retained their DJ alleles (data not shown). These results suggest that full-length μ protein in these cells was generated by a V-region replacement event on the *VDJ*$^-$ chromosome, leaving the Dμ-expressing DJ_H allele on the other chromosome intact. In contrast, flow cytometry suggested that K.40D.2 cells were not clonal (Fig. 3B) and still contained about 20% μ^- (and presumably Dμ$^+$) cells in the population. Several subclones of K.40D.2 were isolated, and these cell lines expressed μ on 100% of the cells and did not express Dμ. The K.40B.2 and C.2 cells also expressed the full-length

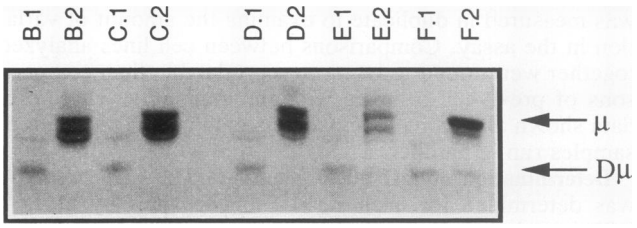


FIG. 2. Heavy-chain μ and Dμ expression in B-lineage pairs. Proteins from detergent-soluble lysates of K.40 subclones were resolved by SDS-polyacrylamide gel electrophoresis, and the expression of μ and Dμ proteins was assessed by immunoblotting with alkaline phosphatase-conjugated anti-mouse IgM heavy chain-specific antibody. Colorimetric detection was used to visualize alkaline phosphatase-stained bands. The positions of the μ and Dμ proteins are indicated by arrows.

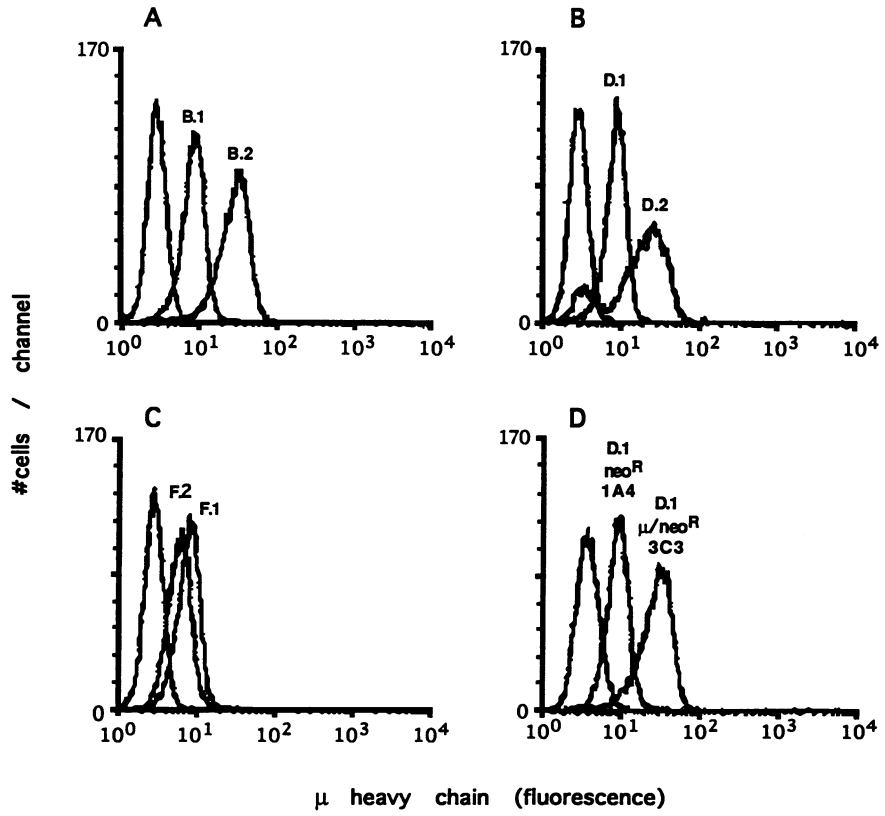


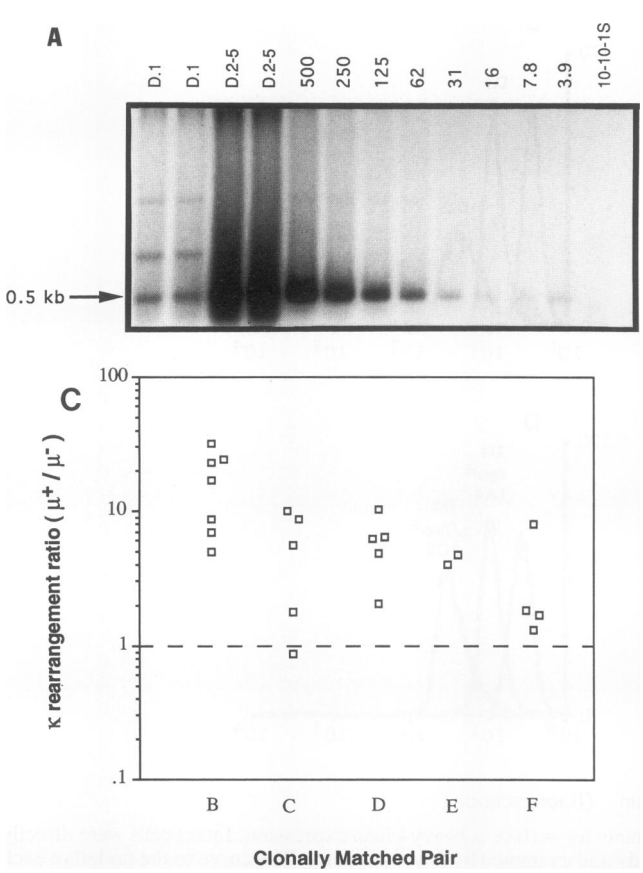
FIG. 3. Flow cytometric analysis of K.40 siblings and K.40D transfectants for surface μ heavy-chain expression. Intact cells were directly stained with fluorescein-conjugated goat anti-mouse μ heavy chain antibody and examined by flow cytometry. The curve to the far left of each histogram represents the autofluorescence profile of unstained cells. (A) Surface μ heavy-chain expression of K.40B.1 and K.40B.2 cells. Results for K.40C.1, K.40C.2, K.40E.1, and K.40E.2 were comparable to those for K.40B.1 and K.40B.2 except that K.40E.2 had a slightly higher surface μ heavy-chain expression. (B) Surface μ-chain expression of K.40D.1 and 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 and K.40F.2 cells. (D) Surface μ-chain expression in K.40D.1 Neo^R subclone 1A4 and in K.40D.1 μ Neo^R subclone 3C3. Results for the K.40D.1 Neo^R subclones 1A5 and 1B2 were comparable to those for 1A4, and results for the K.40D.1 μ Neo^R subclones 3C1, 3C2, and 3D5 were comparable to those for 3C3.

μ chain but not the Dμ protein. These cell lines presumably resulted from V_H-to-DJ_H rearrangement, resulting in a 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 (data not shown).

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 by a quantitative PCR assay for rearranged κ genes (48) (Fig. 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. Alternatively, the higher rate of κ gene rearrangement could be due to a higher level of V(D)J recombinase in the μ^+ cells. To examine this possibility, the recombinase activity of each sibling was tested by introduction of an artificial recombinase substrate (22). 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 (20). In the C and F pairs, the level of recombinase activity was quite similar in the μ^- and μ^+ matched pair of cell lines (Table 1). In the E pair, the μ^- sibling had a fourfold-higher level of recombinase compared with that of 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 compared with that in the μ^- sibling. 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 with that in their μ^- siblings. In K.40C.2 and K.40E.2 cells, however, 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 four of the five K.40 μ^+ cells.

Effects of transfected μ heavy chain on κ gene rearrange-



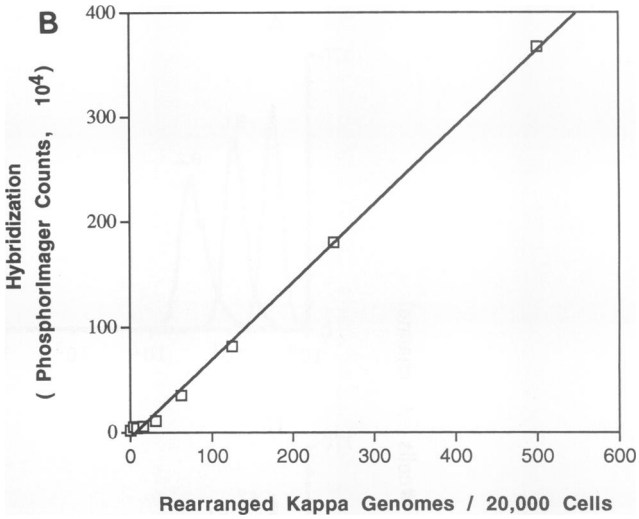


FIG. 4. Increased κ rearrangement frequency in μ+ siblings of K.40 matched pairs. The frequency of κ rearrangement for each K.40-derived sibling was measured by a quantitative PCR assay. (A and B) Method of measuring κ rearrangement frequency by PCR. Lysates of K.40D siblings containing 20,000 cell equivalents along with a dilution series of κ rearrangement standards were run on the thermocycler under conditions described previously (48). The K.40D siblings were run in duplicate on the thermocycler, while the κ rearrangement standards were run in singlet. The PCR products were subjected to electrophoresis through a 1.2% agarose gel and blotted onto a filter. The rearranged κ gene PCR product was detected by hybridization with a labeled 369-nt DNA fragment that spans $J_κ1$ and most of the $J_κ1$-$J_κ2$ spacer. (A) Southern blot of the amplified PCR products of K.40D.1, K.40D.2-5, and the κ rearrangement standards. The amount of amplified product was determined by scanning with the PhosphorImager and measuring the amount of signal (PhosphorImager counts) within the amplified 536-nt band. The PhosphorImager counts were 378,000 and 447,000 for D.1 and 3,670,000 and 3,330,000 for D.2-5. The κ rearrangement standards consisted of WEHI-231 B-lymphoma DNA, which has a functionally rearranged κ allele, titrated into a total of 20,000 cell equivalents of murine T-lymphoma 10-10-1S DNA, which has no κ gene rearrangements. (B) Standard curve derived from κ rearrangement standards which has a r^2 value of 0.998. (C) Graph of the κ rearrangement ratio of the μ+ to the μ− member of each clonally matched pair. Each datum point shown is the ratio of the number of κ rearrangements in the μ+ member of the clonally matched pair to the number in the μ− member. Each datum 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 points, the same sets of DNA samples were analyzed in two separate PCR assays and the ratios were 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 represents a ratio of one, i.e., equal κ rearrangement frequency in both μ− and μ+ cell lines.

ment. To see whether 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 (21, 42, 54). 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 under the microscope. G418-resistant transfectants that were uniformly μ+ and produced an amount of μ roughly equal to that of the μ+ sibling K.40D.2 were subcloned and subjected to further analysis. 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. Four of the K.40D.1 μ Neo^R subclones were tested and found to express comparable amounts of μ protein on the cell surface, as assessed by flow cytometery (Fig. 3D). 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 compared with the level in the Neo^R-only transfectants (Fig. 5). Analysis of variance between the κ gene rearrangement frequency of the K.40D.1 μ Neo^R and 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, since subclones with various levels of κ rearrangement expressed equivalent amounts of μ protein on the cell surface.

We also measured the frequency of κ gene rearrangement in the nontransfected 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 ($P < 0.005$) (Fig. 5). Finally, we transfected K.40D.2 with the neomycin resis-

TABLE 1. Recombinase activity in clonally matched B-lineage pairs[a]

Cell line	Recombination frequency [% (SEM or range)] for paired sister clones		n
	μ^-	μ^+	
K.40B	0.21 (0.10)	0.93 (0.24)	4
K.40C	2.0 (0.69)	2.0 (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 (0.47–0.49)	2
K.40F	0.99 (0.90–1.1)	1.6 (1.4–1.8)	2

[a] 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 extrachromosomal DNA was isolated. The frequency of V(D)J recombination of these molecules was determined by transforming bacteria and then scoring for the LacZ phenotype on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. The recombination frequency {(number of blue colonies × 3)/[total number (blue + white) of colonies] × 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.

tance 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 frequencies of K.40D.1 Neo^R and K.40D.2 Neo^R subclones were about twofold lower than those of their nontransfected 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 was in some way responsible for the reduced frequency of κ gene rearrangement in the transfected subclones.

Also evident from the data in Fig. 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

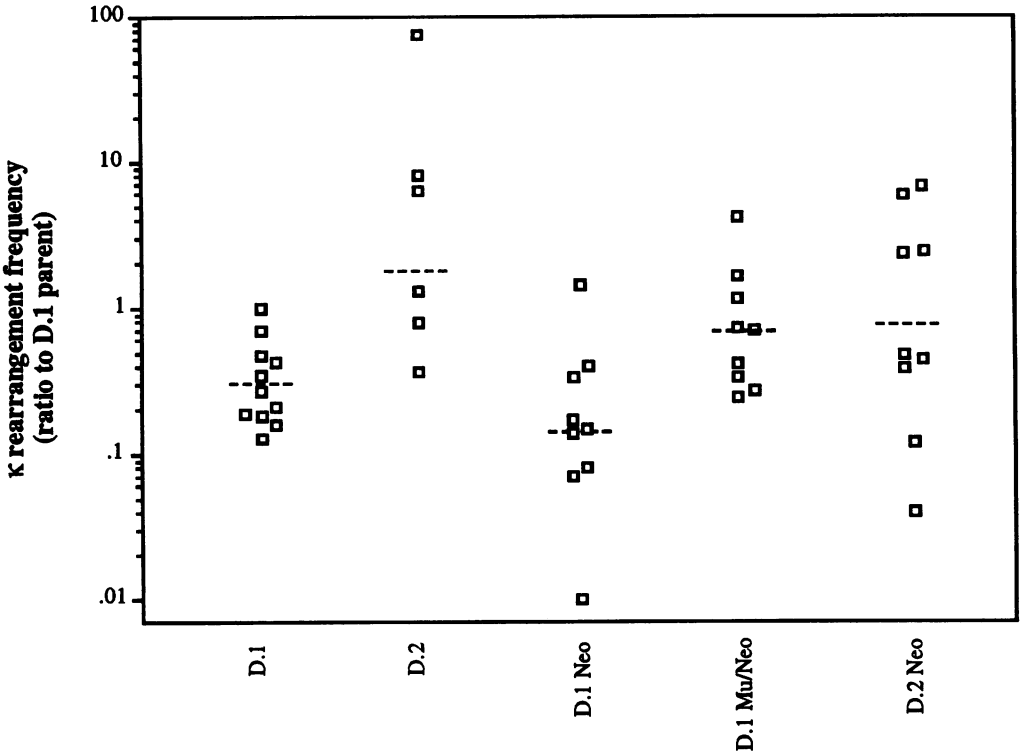


FIG. 5. Increased κ rearrangement frequency in μ^+ transfectants compared with that in μ^- transfectants. The frequency of Ig κ gene rearrangement for each K.40 transfectant and subclone was measured and compared with the number of κ gene rearrangements of K.40D.1 cells, as in Fig. 4. The points in the columns for 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 dashed lines represent the median values for the data in each column. 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 that of K.40D.1 was consistent for the four PCR assays (7.8 ± 0.55). 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.1 Neo^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 curves 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.1 Neo^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 twofold.

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

Type	Cell line	Avg κ rearrangement frequency (no. of rearrangements/ 20,000 genomes)[a]	Recombination activity [% (SEM or range)][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 with the Neo^R gene	D.1 Neo 1A3	2.3	0.52 (0.45–0.58)
	D.1 Neo 1A4	0.32	1.5 (0.12)
	D.1 Neo 1A5	6.1	1.5 (0.31)
	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 with the μ and Neo^R genes	D.1 μ Neo 3C1	59	2.4 (1.9–2.9)
	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 (0.46)
K.40D.2 transfected with the Neo^R gene	D.2 Neo 7A6	1.3	1.3 (0.75–1.8)
	D.2 Neo 7B4	240	1.8 (1.7–1.9)

[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 Table 1, footnote *a*. 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.

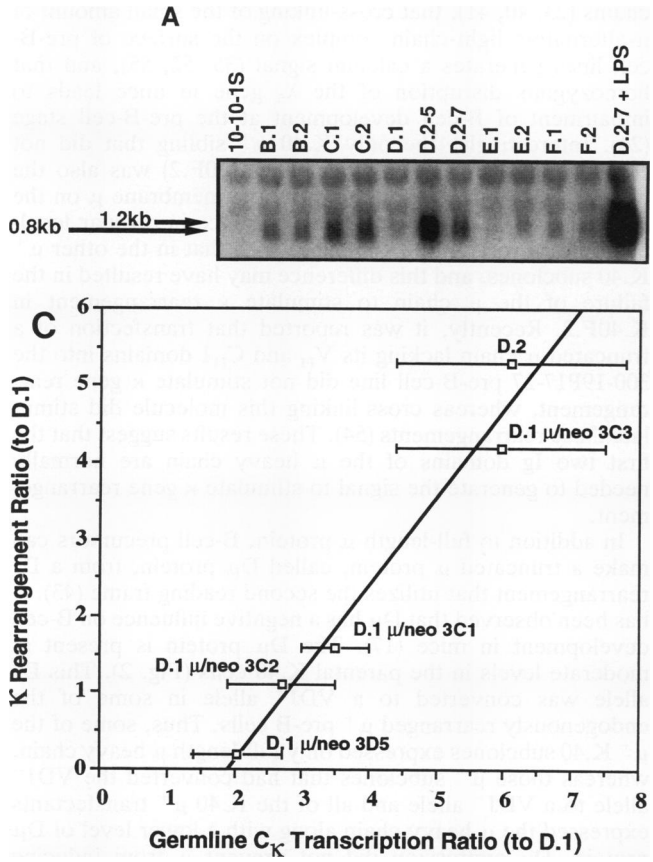
cell line: perhaps by chance the first κ rearrangement in the parental cell line occurred relatively soon after cloning. Of course, the higher the rate of rearrangement is, the greater is the probability that a κ gene rearrangement will occur early following subcloning, so a general correlation is expected 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 (4), 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 the κ gene rearrangement rate.

Various K.40D derivatives were assayed for V(D)J recombinase activity with the transiently transfected artificial substrate (Table 2). The recombinase activities 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 activity 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 the μ heavy chain on germ line C_κ transcription. To address the mechanism of how the μ heavy chain increases the frequency of κ gene rearrangements, the effect of μ on the transcription of the unrearranged C_κ gene was examined. The C_κ locus has been observed to generate germ line transcripts of 0.8 and 1.2 kb (the latter is also referred to as 1.1 kb [28]) (34). It has previously been observed that increases in germ line transcription correlate with increased κ gene rearrangement frequency (48). Whether these germ line 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 (3, 47). RNA from each of the μ^- and μ^+ clonally matched siblings was isolated and subjected to Northern (RNA) analysis using a κ_0 probe that detects germ line C_κ transcripts (Fig. 6A). For the K.40F pair, which had no increase in κ gene rearrangements, the level of germ line C_κ transcription was comparable for both the μ^- and μ^+ siblings. For three of the four K.40 pairs with increased κ gene rearrangements, the level of germ line C_κ transcription was also quite similar for both the μ^- and μ^+ siblings. Only in the case of the K.40D pair was there a considerable increase (approximately fivefold) in the amount of C_κ germ line transcription in the μ^+ sibling compared with that in the μ^- sibling. Very similar results were obtained with a quantitative RNA PCR assay for C_κ germ line transcription (48) (data not shown). Moreover, these PCR experiments confirmed that the 1.2-kb transcript is a germ line C_κ transcript rather than a rearranged κ transcript. Thus, with the exception of the K.40D pair, there was no correlation between increases in C_κ germ line transcription and the increased κ gene rearrangement frequency in the K.40 matched pairs. Next, κ germ line transcription was examined in the some of the K.40D.1 Neo^R-only and μ Neo^R transfectants to see whether the increase in germ line C_κ transcription in K.40D.2 was due to μ chain expression (Fig. 6B). All of the Neo^R-only transfectants had a very low level of C_κ germ line transcription like the parental K.40D.1 cells. In contrast, the K.40D.1 μ Neo^R transfectants exhibited increased C_κ germ line transcription. Increased C_κ germ line transcription in the transfectants was confirmed by the quantitative RNA PCR assay for germ line C_κ transcription (data not shown). Interestingly, there was a linear relationship between the level of germ line transcription and the frequency of κ gene rearrangement observed in K.40D.2 and K.40D.1 μ Neo^R transfectants (Fig. 6C). In 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 germ line 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. (42), Iglesias et al. (21), and Tsubata et al. (54) had demonstrated that μ heavy-chain



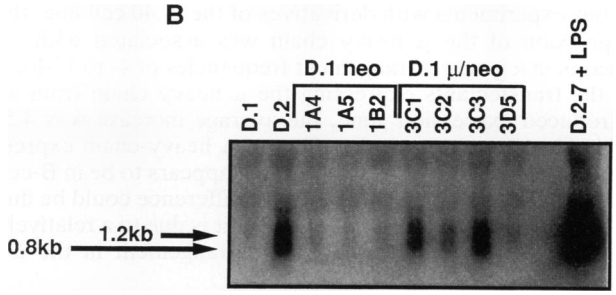


FIG. 6. Expression of κ germ line transcripts in K.40 siblings and K.40D derivatives. Twenty micrograms of total RNA was resolved on a 1% agarose-formaldehyde gel, and expression of both 0.8- and 1.2-kb C_κ germ line transcripts was detected by hybridization with a labeled 350-nt PCR fragment amplified from a C_κ germ line transcript cDNA. RNA loading for each lane was examined by comparing the upper rRNA bands and by reprobing with glyceraldehyde-phosphate dehydrogenase (data not shown) and was found to be approximately equivalent. RNA from lipopolysaccharide-treated (1 μg/ml, 24 h) K.40D.2-7 was used as a positive control for the germ line C_κ 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 and μ Neo^R transfectants. (C) Correlation of the amount of C_κ germ line transcription versus κ gene rearrangement frequency in K.40D.2 and K.40D.1 μ Neo^R transfectants. The graph plots the averages of values derived from two separate Northern blots of C_κ germ line transcripts. The r^2 value was 0.98 for the first Northern blot, 0.92 for the second Northern blot, and 0.97 for the average of the two.

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 differentiating 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 with 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, one of the μ^- K.40 derivatives was transfected with a functionally rearranged μ gene. On average, the nine μ^+ transfectants examined had a four- to fivefold increase in the number of κ gene rearrangements compared with the number in control transfectants. These results provide additional evidence that μ heavy-chain expression is an important regulatory event leading to increased κ gene rearrangement.

In contrast to the results of Reth et al. (42) and Iglesias et al. (21), 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 immunofluorescence 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 (48), in A-MuLV-transformed pro-B-cell lines derived from mice defective at the *scid* locus (7, 19), and in Epstein-Barr virus-transformed human B-cell progenitors (27). In each example, these B-cell precursors lack a functional μ heavy chain. In the 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 blots (immunoblots) or Northern blots were used to detect κ-chain protein or mRNA expression (21, 42). 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 a 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 with that from normal mice, supporting the hypothesis that the membrane form of the μ heavy chain stimulates an otherwise low level of κ gene rearrangement in developing B lymphocytes (25).

In our experiments with derivatives of the K.40 cell line, the expression of the μ heavy chain was associated with increases in κ gene rearrangement frequencies of 4- to 17-fold. In the transfectants expressing the μ heavy chain from an introduced expression gene, the average increase was 4.5-fold. Thus, on average, the effect of μ heavy-chain expression was somewhat less in K40 than it appears to be in B-cell precursors in vivo. This quantitative difference could be due to many things. One possibility is that it is due to a relatively high background rate of κ gene rearrangement in the μ^- K.40 cell lines.

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 (2). 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 (25) 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 redirection 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 (47). The supposition behind this approach is that if the transcription machinery can access a region, so can the 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 of four clonally matched pairs, the increase in κ rearrangement frequency in the μ^+ siblings was not accompanied by a significant change in germ line C_κ transcription. Thus, for the K.40B, C, and E clonally matched pairs, the stimulation of κ gene rearrangement by μ was apparently not due to changes in the accessibility of the κ locus. In the fourth pair, K.40D, μ expression did consistently lead to an increase in transcription of germ line 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 germ line 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 (42), creation of μ-transgenic mice (37, 51), and gene disruption of the membrane form of μ (25) 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 (12), it has been suggested that μ mediates its effects on V(D)J recombinase targeting through a receptor signaling mechanism (21, 25, 42, 54). A number of observations support this hypothesis, including the findings that μ can form complexes with alternative light chains (23, 40, 41), 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 (35, 52, 55), and that homozygous disruption of the λ_5 gene in mice leads to impairment of B-cell development at the pre-B-cell stage (24). 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 with that in the other μ^+ K.40 subclones, and this difference may have resulted in the failure of the μ chain to stimulate κ rearrangement in K.40F.2. Recently, it was reported that transfection of a truncated μ chain lacking its V_H and C_H1 domains into the 300-19P17-27 pre-B-cell line did not stimulate κ gene rearrangement, whereas cross-linking this molecule did stimulate these rearrangements (54). These results suggest that the first two Ig domains of the μ heavy chain 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 (43). It has been observed that Dμ has a negative influence on B-cell development in mice (17). The Dμ protein is present at moderate levels in the parental K.40 cells (Fig. 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 the μ heavy chain along with a lower level 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 (17). 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 an in-frame μ chain by V-region replacement. As a consequence, K.40E.2 and K.40F.2 also retained Dμ expression following μ heavy-chain 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 us an opportunity to study the regulation of κ gene rearrangement in a variety of situations. Our results suggest that the μ heavy chain, 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. (4). They have found that prolonged culture of V(D)J recombinase-expressing cell lines can lead to a loss of the recombinase activity over time. The experiments 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 K.40D.2 that were analyzed did not show systematic loss of V(D)J recombinase activity (Table 2).

Whether C_κ germ line 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 germ line 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 a whole, we suggest that there are multiple steps in the regulation of κ gene 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 germ line transcription, and an additional mechanism for activating κ gene rearrangement possibly involving targeting of the recombinase to the κ locus.

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