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Corrective Recombination of Mouse Immunoglobulin Kappa Alleles in Abelson Murine Leukemia Virus-Transformed Pre-B Cells

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Previous characterization of mouse immunoglobulin κ gene rearrangement products cloned from murine plasmacytomas has indicated that two recombination events can take place on a single κ allele (R. M. Feddersen and B. G. Van Ness, Proc. Natl. Acad. Sci. USA 82:4792–4797, 1985; M. A. Shapiro and M. Weigert, J. Immunol. 139:3834–3839, 1987). To determine whether multiple recombinations on a single κ allele can contribute to the formation of productive V-J genes through corrective recombinations, we have examined several Abelson murine leukemia virus-transformed pre-B-cell clones which rearrange the κ locus during cell culture. Clonal cell lines which had rearranged one κ allele nonproductively while maintaining the other allele in the germ line configuration were grown, and secondary subclones, which subsequently expressed κ protein, were isolated and examined for further κ rearrangement. A full spectrum of rearrangement patterns was observed in this sequential cloning, including productive and nonproductive recombinations of the germ line allele and secondary recombinations of the nonproductive allele. The results show that corrective V-J recombinations, with displacement of the nonproductive κ gene, occur with a significant frequency (6 of 17 κ-producing subclones). Both deletion and maintenance of the primary (nonfunctional) V-J join, as a reciprocal product, were observed.

The expression of immunoglobulin by B cells is dependent on gene segment rearrangements which are unique to lymphoid development (41). Highly conserved, site-specific sequences flanking variable (V) and joining (J) gene segments of the kappa (κ) light-chain locus direct the complex enzymology required for DNA cutting and ligation (2). In addition to the functional products generated from rearrangement of the κ locus, many defective products have been observed (3, 7, 10, 12, 14, 15, 19, 27, 30, 32, 36, 40, 43, 44). In fact, from both plasmacytomas and normal B cells it has been estimated that more than one-third of all κ rearrangement events are aberrant (11). Collectively, these aberrant rearrangements have suggested that clonal selection may actually mask an otherwise inefficient rearrangement process.

In addition to functional and nonfunctional κ transcription units, Southern blot hybridization analyses, coupled with cloning and sequencing, have identified reciprocal products of V_κ-J_κ recombination (13, 21–23, 37, 39, 42). These by-products of V-J joining events are characterized by the back-to-back fusion of the recombination signal sequences (the conserved heptamers and nonamers) that border the germ line gene segments. The characterization of reciprocal products supports the intrastrand DNA inversion mechanism of V-J recombination. We and others (12, 38) previously characterized additional products of κ locus recombination in mouse plasmacytomas. These novel elements were shown to contain a V_κ-J_κ rearrangement followed by the reciprocal element of another V_κ-J_κ rearrangement. We designated these double recombination products (DRPs). Significantly, it appeared that in one plasmacytoma, a secondary, productive V-J recombination had displaced an earlier, nonproductive V-J element on the same allele, thereby generating a DRP (see Fig. 7). Depending on germ line V gene segment orientation, a second corrective event on a single allele could either delete the previous V-J join or maintain it as a DRP element (12, 23).

The potential for secondary recombination of a single κ allele can be incorporated into a schematic pathway of B-cell development (32). The germ line kappa locus (κ^0) can rearrange productively (κ^+) or aberrantly (κ^-) (Fig. 1). The dashed lines in Fig. 1 indicate that nonproductive V-J rearrangements may be replaced or removed by subsequent productive V-J joins occurring on the same κ allele (12). Obviously, owing to the presence of multiple V and J elements in the genome, committed pre-B cells might be allowed several attempts at generating functional immunoglobulin genes by this scheme.

The DRPs characterized in plasmacytomas represent the end products of rearrangement. Although the presence of DRPs suggested multiple recombination occurring on a single κ allele, several distinct pathways may explain their existence (12). One of these pathways, that of secondary, corrective recombination, was investigated in the work described in this report. We have examined an Abelson murine leukemia virus-transformed bone marrow cell line that spontaneously rearranges κ light-chain gene segments in culture (34). A sequential clonal analysis was used to further investigate the dynamics of κ rearrangement and, in particular, to assess secondary recombination on a single allele. We present evidence directly demonstrating that multiple rearrangements, including corrective recombination, can occur at a single κ locus.

MATERIALS AND METHODS

The Abelson murine leukemia virus-transformed cell line P8 was obtained from Fred Alt, Columbia University, New York, N.Y., and has been previously described (34). Cells were grown in RPMI 1640 medium with 10% fetal bovine serum (Hyclone) and 50 μM β-mercaptoethanol. Lipopolysaccharide (LPS)-induced cultures were treated with 15 μg

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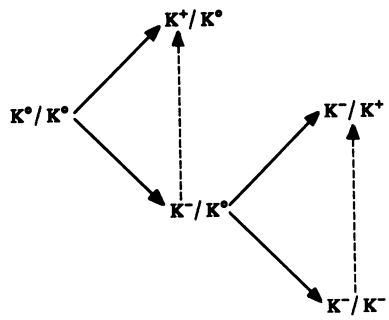

FIG. 1. Schematic pathway for for κ gene rearrangement. The dashed lines indicate that nonproductive rearrangements may be replaced or removed by subsequent productive V-J joins occurring on the same κ allele.

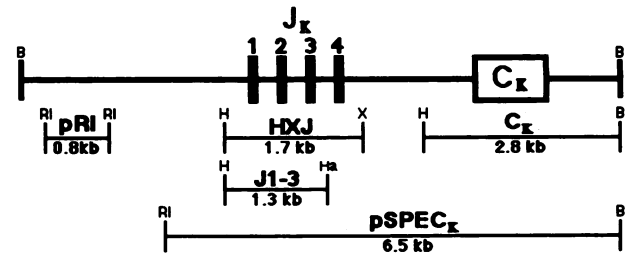

FIG. 2. Mouse kappa locus probes. The top line represents the approximately 12-kb *Bam*HI restriction fragment that contains the κ joining (J)- and constant (C)-region gene segments. Probes isolated from this locus are schematically shown below. Sizes of the probes are given in kilobase pairs, and restriction enzymes used to generate each probe are indicated: B, *Bam*HI; RI, *Eco*RI; H, *Hin*dIII; X, *Xba*I; Ha, *Hae*III. Not drawn to scale.

of LPS (Difco Laboratories) per ml for 15 to 20 h prior to harvest. Selection for surface κ expression was carried out by using goat anti-mouse κ antibody (Southern Biotechnology Associates, Inc.) essentially as described previously (45). Approximately 10^8 cells were panned in each experiment. The adherent cell fraction was cultured for 1 to 2 weeks before further panning or dilution subcloning. Typically, secondary subclones were generated from a population that had been successively panned two or three times prior to dilution subcloning.

Whole-cell protein lysates were produced essentially as previously described (34) and stored at 4°C. The pre-B-cell line 70Z/3 (31) was used as the positive control for LPS induction and κ protein production. Lysates were applied to Nytran (Schleicher & Schuell, Inc.) in a 96-well dot blot apparatus. The membrane was blocked in 5% nonfat dry milk–TBS (100 mM Tris [pH 7.5], 200 mM NaCl) at 37°C with shaking for 2 h, washed 3 times for 10 min each in TBS, and blotted dry on 3MM paper (Whatman, Inc.). A horseradish peroxidase-conjugated goat anti-mouse κ antibody (2 to 5 μg/ml in 1% nonfat dry milk–TBS; Southern Biotechnology) was aliquoted into a 96-well plate (75 μl per well). The membrane was placed face down onto the 96-well plate such that protein dots were in registry with wells containing antibody solution. A sheet of Parafilm, two sheets of blotting paper, and a glass plate were layered onto the membrane and secured by four spring clamps. The sandwich was inverted and incubated at room temperature for 4 h or overnight at 4°C. The membrane was washed once in TBS–0.05% Nonidet P-40 for 1 min and three times in TBS for 15 min each. Two different horseradish peroxidase substrate solutions (3-amino-9-ethylcarbazole and 4-chloro-1-naphthol) were prepared as suggested by the antibody supplier (Southern Biotechnology). A red (3-amino-9-ethylcarbazole) or a blue-black (4-chloro-1-naphthol) deposit was evident after 30 min of treatment. Finally, membranes were rinsed for 2 to 10 h in H_2O.

Kappa surface staining was performed with fluorescein-conjugated rat anti-mouse μ heavy-chain antibody (b7.6 [6]) and rabbit anti-mouse κ light-chain antibody (Cappel Laboratories) as previously described (26). Stained cells were analyzed by using a fluorescent microscope (Nikon). Individual cultures were scored positive (++) if more than 50% of counted cells (>100 counted) showed bright staining. Cultures were labeled negative (−−) if fewer than 5% of them stained.

High-molecular-weight DNA from individual subclone lines was isolated as described previously (4). A high concentration of *Bam*HI (50 U/μl) was used to digest genomic DNA samples as specified by the manufacturer (International Biotechnologies, Inc.). Agarose gels (0.8 to 1.0%) were prepared as described previously (24). Samples (5 to 10 μg) of restricted DNA were electrophoresed at 1.5 to 2 V/cm for 25 to 40 h. Gel-fractionated DNA was capillary transferred to nylon membranes (Nytran; Schleicher & Schnell) in 15× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), baked, prehybridized, and hybridized as suggested by the manufacturer. Hybridization solutions contained 1×10^6 to 2×10^6 cpm of nick-translated probe per ml. The final membrane wash was done in 0.1× SSC–0.5% sodium dodecyl sulfate at 60°C for 15 to 90 min. Autoradiography was performed for 2 to 7 days with X-Omat AR film (Eastman Kodak Co.) and intensifying screens.

Blots for rehybridization were wetted completely in H_2O and washed in three changes (400 ml each) of 0.4 M NaOH at 42°C for 15 min each with constant shaking. Blots were then neutralized in 1 M Tris (pH 7.5)–0.2× SSC–0.1% sodium dodecyl sulfate for 20 min and finally rinsed in 0.2× SSC–0.2% sodium dodecyl sulfate. Prehybridization was then followed by hybridization with a different ^{32}P-labeled probe.

The pRI and $pSPEC_\kappa$ probes (Fig. 2) have been previously described (12). The constant-region probe (C_κ) and the two joining-region probes (HXJ and J1-3) were isolated from the $pSPEC_\kappa$ clone. DNA probes were radioactively labeled by using a nick translation kit (Bethesda Research Laboratories, Inc.) and [α-^{32}P]dCTP (Du Pont, NEN Research Products/Amersham Corp.). Specific activities above 1×10^8 cpm/μg were routinely obtained.

RESULTS

Analysis of primary subclones of the P8 cell line. The initial cell line, designated P8, has previously been shown to produce intracellular μ heavy chain and rearrange the κ locus during cell culture (34). The P8 cell line was grown for 1 to 2 months prior to limiting-dilution subcloning (one cell per five wells). Single-cell clones were subsequently expanded to generate whole-cell lysates for protein assays and genomic DNA for assessing gene rearrangement. Cell cultures were routinely induced with LPS to increase κ protein expression and detection in the assays that were used (see Materials and Methods).

Southern blot hybridization of *Bam*HI-digested genomic DNA from each of the 27 primary subclones was performed with the C_κ probe (Fig. 2). A single 12-kilobase (kb) fragment was found in 11 of the primary subclones, indicating that no

TABLE 1. P8 primary subclones

Clone	Size (kb) of *Bam*HI C_κ fragment		Protein assay[a]	Probable genotype[b]
	I	II		
P8	12	12	−	κ^0/κ^0
P8.B	8.5	7.5	+	κ^+/κ^-
P8.F			−	κ^d/κ^d
P8.G	12	9.0	−	κ^0/κ^-
P8.4	12	4.5	−	κ^0/κ^-
P8.13		7.5	+	κ^d/κ^+
P8.28	12	10	−	κ^0/κ^-
P8.34	9.7	6.0	−	κ^-/κ^-
P8.61	12	8.5	−	κ^0/κ^-

[a] Solid-phase dot blot assay for kappa protein.
[b] κ^0, Germ line (unrearranged) allele; κ^+, productively rearranged allele; κ^-, nonproductively rearranged allele; κ^d, deleted allele.

κ rearrangement had occurred in these subclones. None of these cells expressed κ protein. Analysis of the other subclones demonstrated that nine had rearranged one κ allele, four had rearranged both κ alleles, two had deleted both κ alleles, and one contained a single rearranged κ allele. Although no predominant pattern of primary V-J rearrangement was observed in the P8 cell line, analysis of other Abelson murine leukemia virus pre-B-cell lines (P. Brodeur, personal communication), along with fetal and neonatal murine B-cell hybridomas (20), has suggested preferential V_κ gene usage. By grouping identical P8 clones, a total of eight different primary subclones were generated (Table 1). The Southern blot and protein dot blot analyses of the eight unique primary subclones are shown in Fig. 3. Based on the protein assays, six of these subclones failed to produce detectable κ protein. These results are consistent with those previously reported with the P8 cell line (34).

Sequential subcloning to assess corrective recombination. To determine whether multiple recombinations on a single κ allele can contribute to the formation of productive V-J genes, we isolated secondary subclones and analyzed them as described above. Figure 4 schematically shows how primary subclones containing one germ line (κ^0) allele and the other allele nonproductively rearranged (κ^-) were examined for corrective recombination. These clones were grown in culture and panned (see Materials and Methods) to enrich for cells subsequently producing κ protein (Fig. 4B). By repeating the Southern blot analysis on secondary subclone DNA, the frequency of secondary, corrective rearrangement versus rearrangement of the germ line allele was determined. The possible genotypes of secondary subclones are shown in Fig. 4C.

The defect of the κ^- allele in the primary subclone could include a wide variety of abnormalities involving any one or more elements of the κ gene structure. Because our goal was to assess the potential for secondary recombinations, we did not investigate the exact nature of each aberrant defect. Kappa protein production thus became the crucial assay to assign the status of each rearranged allele. Relevant clones were assayed by surface staining to confirm the dot blot immunodetection assay.

Secondary subclones of the P8.4 cell line. Primary subclone P8.4 (Fig. 3; Table 1) was further investigated by the scheme shown in Fig. 4 because it was determined to be κ^0/κ^- (i.e., one germ line allele and one nonproductively rearranged). Southern blot and protein analyses were performed on 42 independent subclones derived from the panning selection of

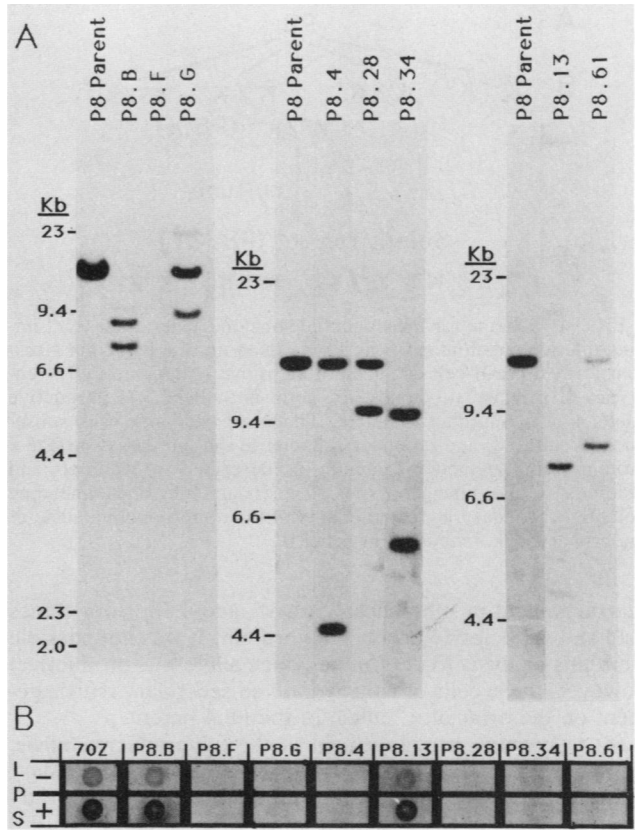

FIG. 3. P8 primary subclone analysis. (A) Genomic Southern blot analysis of *Bam*HI-digested DNA isolated from the P8 parent and eight different primary subclone cell cultures. The blots (three separate) were hybridized with the $pSPEC_\kappa$ probe (P8 parent, P8.B, P8.F, and P8.G blots) or the C_κ probe (the other two blots). The migration of lambda DNA and *Hin*dIII markers is indicated at the left of each panel. (B) Solid-phase dot blot analysis of cell culture lysates for the detection of κ protein (see Materials and Methods). Lysates from LPS-induced cultures are presented in the lower windows. 70Z represents the positive control, and because both κ alleles of P8.F have been deleted, it represents a negative control.

the P8.4 primary clone. The P8.4 clone (Fig. 5A, lane 2) displays one germ line allele (12 kb) and one rearranged allele (4.5 kb). From the use of J region probes (Fig. 2) and further restriction analysis, we determined that the recombination in the P8.4 clone was to J3 (data not shown). DNA from a multiclonal population of P8.4, obtained by selecting cells expressing surface κ (Fig. 5A, lane 4), exhibited a variety of bands distinct from the parent and primary subclone (Fig. 5A, compare lanes 1 through 4). DNA from nonselected P8.4 cells maintained in long-term culture (Fig. 5A, lane 3) did not display a similar pattern. Neither the P8 parent population nor the clonal P8.4 primary subclone produced κ protein, as assessed by the protein dot blot (Fig. 5B). However, many of the secondary subclones expressed κ protein and displayed altered patterns of κ gene rearrangement (Fig. 5A and B, lanes 5 through 21). In these and all other experiments, protein lysates were prepared from the same batch of cells from which genomic DNA was isolated (see Materials and Methods).

(i) Rearrangement of the germ line allele. Subclones 4.2, 4.4, and 4.6 rearranged the κ germ line allele and, based on the solid-phase protein assay, did so productively. Both κ alleles of the P8.4 primary clone appear to have undergone

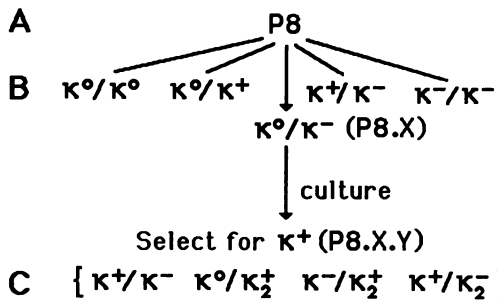

FIG. 4. Scheme for P8 sequential subclone generation. (A) Limiting-dilution subcloning of the P8 Abelson murine leukemia virus-transformed pre-B-cell line generating primary subclones. (B) Genotypes of primary subclones: κ^0, germ line allele; κ^+, productive allele; κ^-, nonproductive allele. The κ^0/κ^- subclone was subsequently cultured, and secondary subclones that produced surface κ protein were selected by panning as described in Materials and Methods. (C) Possible genotypes of secondary subclones, including potential secondary κ_2 recombinants of the nonproductive allele of the primary κ^0/κ^- subclone in panel B.

rearrangement in subclones 4.5, 4.15, and 4.18. Both clones 4.15 and 4.18 clearly produced κ protein. It was not possible from this analysis to determine which allele was productive; however, these cells obviously inolved secondary rearrangement on the original κ^- allele of the P8.4 parent.

(ii) Corrective rearrangement of the nonproductive allele. Subclones 4.7, 4.9, 4.11, and 4.12 demonstrated secondary rearrangements of the original 4.5-kb κ allele without any rearrangement of the germ line allele (Fig. 5A, lanes 11, 13, 15, and 16). Each of these subclones produced κ protein as detected by the dot blot and surface-staining assays. The dot blot assay for the 4.9 lysate was not conclusive, but the κ surface-staining assay revealed more than 70% of a fresh 4.9 culture to be κ positive. These results indicate that secondary, corrective rearrangements of the κ^- allele in the P8.4 cell line result in a κ^+ allele. Additional hybridization analysis of the secondary subclones confirmed that only J4 was retained in the rearranged κ^+ allele (see Fig. 6).

(iii) Additional secondary subclones. A number of other secondary subclones of the P8.4 primary clone displayed more than two C_κ hybridizing fragments (Fig. 5A, lanes 19 and 20). In addition, several secondary subclones contained multiple bands of varying intensities (data not shown). These subclones could have been the result of inefficient cloning or continuing rearrangements during the early propagation of the cell line used for analysis. These polyclonal cultures were not informative in our analysis. Similarly, some secondary subclones (Fig. 5A, lanes 5, 7, 12, 14, and 21) exhibited the P8.4 primary clone pattern of C_κ hybridizing fragments (12 and 4.5 kb), suggesting that the panning procedure as applied, was not 100% efficient for separating κ positive and negative cells.

P8.4 primary and secondary cell line clonality. To determine the presence of other κ locus rearrangement products, the blot shown in Fig. 5A was alkali washed and rehybridized with the pRI probe (Fig. 2). This probe will hybridize to the germline (κ^0) *Bam*HI fragment and, because it is derived from sequence upstream of the J_κ cluster, will detect reciprocal products of V_κ-J_κ recombination. In addition to the 12-kb germ line fragment, the pRI probe detected a unique fragment of 25 kb in the P8.4 primary subclone (Fig. 5C). This is probably the reciprocal product of a V_κ-J_κ recombi-

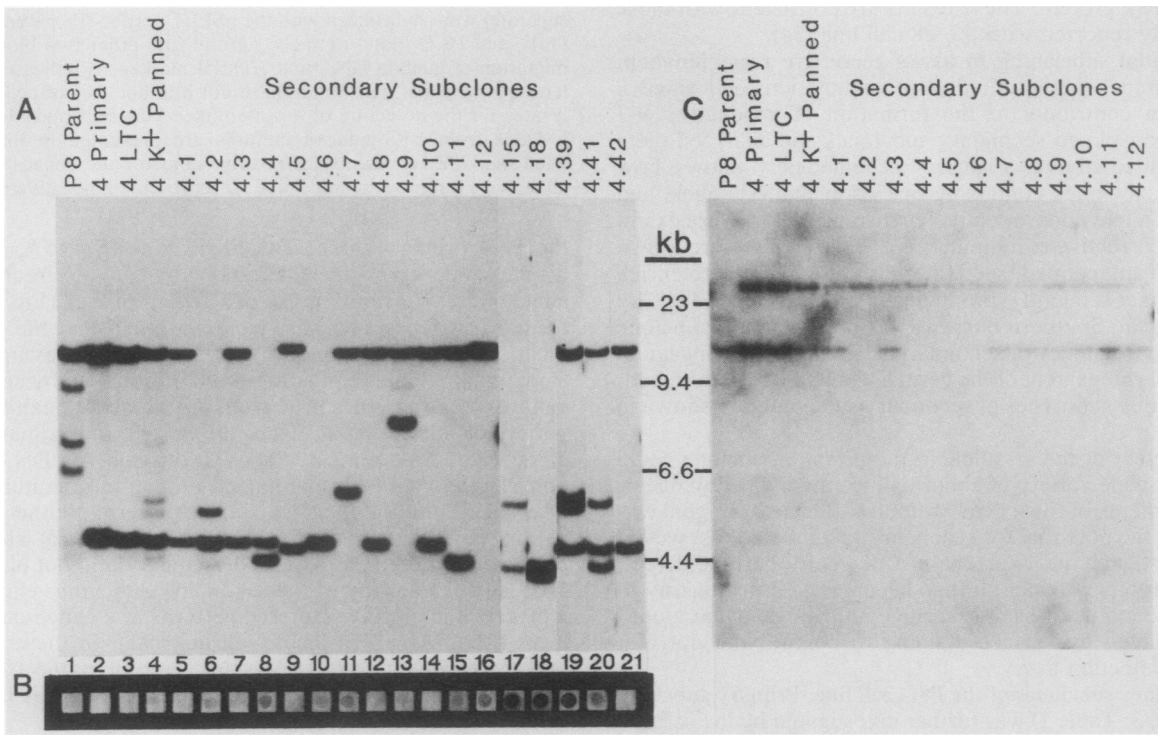

FIG. 5. P8.4 secondary subclone analysis. (A) Southern blot analysis of *Bam*HI-digested genomic DNA isolated from P8 and P8.4 subclone cell lines, hybridized with the C_κ probe. (B) Solid-phase protein assay of corresponding cell lysates. (C) Alkali-washed blot from panel A, rehybridized with the pRI probe. The migration positions of *Hin*dIII-digested lambda DNA fragments are indicated in kilobase pairs between panels A and C.

TABLE 2. Kappa status in P8.4 and P8.G secondary subclones

Cell line and subclone	Size (kb) of C_κ *Bam*HI fragment		Other kappa locus products[a]	Solid-phase protein assay	Immunoglobulin surface staining[b]		Kappa genotype[c]	
	I	II			μ	κ	I	II
P8 parent	12	12		−	<10%	<10%	κ^0	κ^0
P8.4 primary subclone	12	4.5	25-kb U′	−	−−	−−	κ^0	κ^-
Secondary subclones								
4.1[d]	12	4.5	25-kb U′	−	ND	ND	κ^0	κ^-
4.2[d]	5.0	4.5	25-kb U′	+	ND	ND	κ^+	κ^-
4.4[d]	4.3	4.5	25-kb U′	+	ND	ND	κ^+	κ^-
4.5[d]	13	4.4	25-kb U′	−	ND	ND	κ^-	κ_2^-
4.6	4.5	4.5	25-kb U′	+	ND	ND	κ^+	κ^-
4.7[d]	12	6.0	25-kb U′, 8.0-kb DRP	+	++	++	κ^0	κ_2^+
4.9	12	8.5	25-kb U′	−	++	++	κ^0	κ_2^+
4.11	12	4.3	25-kb U′	+	++	++	κ^0	κ_2^+
4.12	12	12.5	25-kb U′	+	++	++	κ^0	κ_2^+
4.15	5.5	4.2	25-kb U′	+	ND	ND	$\kappa^\pm$	$\kappa_2^\pm$
4.18	4.2	4.2	25-kb U′	+	ND	ND	$\kappa^\pm$	$\kappa_2^\pm$
4.29	6.0	4.5	25-kb U′	+	ND	ND	κ^+	κ^-
4.30	5.5	4.5	25-kb U′	+	++	++	κ^+	κ^-
4.31	4.4	4.5	25-kb U′	−	ND	ND	κ^-	κ^-
4.39[d]	Polyclonal	Polyclonal	25-kb U′	±	ND	ND	$\kappa^\pm$	$\kappa^\pm$
4.42	3.5	4.5	25-kb U′	−	ND	ND	κ^-	κ^-
P8.G primary subclone	12	9.0	22-kb U′	−	−−	−−	κ^0	κ^-
Secondary subclones								
G.15[d]	8	9.0	22-kb U′	+	++	++	κ^+	κ^-
G.56[d]	Polyclonal	9.0	22-kb U′	+	ND	ND	κ^+	κ^-

[a] U′, Upstream reciprocal; DRP, double recombination product.
[b] μ heavy-chain and κ light-chain surface staining is positive (++) if more than 50% of cells stained and negative (−−) if less than 5% of cells stained; ND, not determined.
[c] κ^0, Germ line; κ^+, productively rearranged; κ^-, nonproductively rearranged; κ_2, secondary rearrangement; $\kappa^{+/-}$, cannot distinguish κ^+ from κ^- allele.
[d] Representative clone of two or more individual isolates.

nation, since it failed to hybridize with the C_κ probe. It is noteworthy that all 42 secondary subclones that were isolated from the P8.4 panning selection exhibited this unique 25-kb fragment. This established the clonal relationship of the P8.4 cell line with the secondary subclones derived from it.

A complete listing of the sequential subclone characterizations of P8.4 is provided in Table 2. Review of the 42 secondary subclones of P8.4 showed that several displayed identical genotypes and phenotypes, suggesting that identical clones had been isolated. The 42 original P8.4 subclones are therefore condensed to 16 unique subclones in this tabulation.

Reciprocal products of secondary κ recombination. In our previous analysis of κ-expressing plasmacytomas, we characterized a fragment which contained a nonfunctional V_κ-J_κ join followed by the back-to-back fusion of the signal sequences which flank germ line V_κ and J_κ segments (12). This product was probably the result of a corrective V-J rearrangement which generated a DRP containing a previous nonfunctional V_κ-J_κ followed by the reciprocal product of the secondary, corrective V_κ-J_κ. A DRP has a characteristic hybridization pattern: it hybridizes to a J_κ-containing probe, but fails to be detected by the C_κ and pRI probes. A multiprobe analysis of DNA from the P8.4 primary and secondary subclones 4.7 and 4.9 is shown in Fig. 6. As shown above, the 4.7 and 4.9 hybridization patterns included the 6- and 8.5-kb *Bam*HI fragments, respectively, that hybridized to the C_κ probe (Fig. 6A). When these lanes were probed with the J1-3 probe (Fig. 2), the P8.4 primary subclone displayed an identical pattern to that seen with C_κ, whereas the rearranged C_κ bands in the secondary subclones

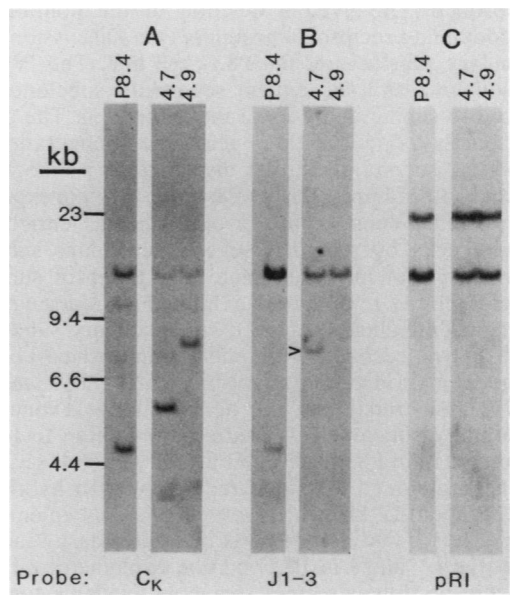

FIG. 6. DRP detection. Southern blot analysis of *Bam*HI-digested DNA from primary subclone P8.4 and secondary subclones 4.7 and 4.9, hybridized with the C_κ probe (A), the J1-3 probe (B), and the pRI probe (C). The open arrowhead in panel B identifies the DRP fragment in subclone 4.7. Phage λ and *Hin*dIII markers are indicated at the left.

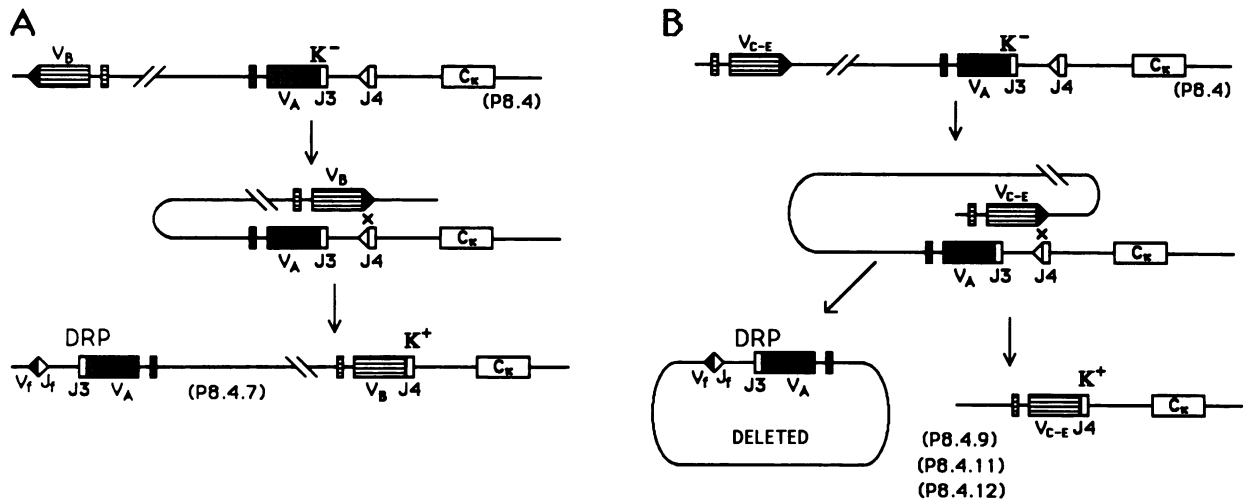

FIG. 7. Corrective recombinations in P8.4. The triangles flanking V and J gene segments represent the recombination signal sequences (conserved heptamer and nonamer). The site of corrective recombination is indicated by X. (A) The nonproductive V_A-J3 rearrangement is replaced by a productive V_B-J4 join. A DRP fragment is generated as a by-product of this corrective recombination. This pathway of rearrangement represents the P8.4 and P8.4.7 subclones. (B) Corrective recombination as characterized in the P8.4.9, P8.4.11, and P8.4.12 subclones. Displacement of the nonproductive V_A-J3 gene by the $V_{C\text{-}E}$-J4 rearrangement results in loss of the reciprocal product. The fate of $V_{B\text{-}E}$-J4 intervening DNA in panels A and B is dependent on the transcriptional orientation of the V gene segment as previously proposed (14, 23).

failed to hybridize (Fig. 6B). Secondary subclones 4.11 and 4.12 displayed a similar pattern of hybridization (results not shown). This confirmed that although the original primary P8.4 clone contained a rearrangement to J3, the secondary subclones contain κ^+ rearrangements of V_κ to J4. A unique band of 8 kb was detected by the J1-3 probe in the 4.7 subclone (Fig. 6B). This fragment failed to hybridize to the pRI probe (Fig. 6C) or the C_κ probe (Fig. 6A): the expected hybridization characteristics of a DRP. No such product was detected in the 4.9, 4.11, or 4.12 subclones, suggesting that despite two recombinations on the same allele, the second event probably involved a deletion of the nonfunctional V_κ-J_κ-containing reciprocal products (see Discussion).

Secondary subclones of the P8.G cell line. The P8.G primary subclone and subsequent secondary subclones were subjected to similar analyses to those for P8.4. The original P8.G clone had one κ allele in germ line configuration (κ^0), whereas the second allele was displayed as a 9-kb *Bam*HI fragment (Tables 1 and 2). Like P8.4, no κ protein expression was detected. When a culture of P8.G was enriched for κ-positive cells by panning, several secondary subclones expressing κ protein were isolated. A group of subclones represented by G.15 and G.56 exhibited κ^+ rearrangements of the germ line allele (Table 2). No secondary subclone of P8.G displayed secondary recombination of the 9-kb allele. Subsequent hybridization analysis with the J1-3 and HXJ probes demonstrated that the nonproductive event in the P8.G primary subclone involved recombination to J4 (data not shown). Like P8.4, the P8.G primary and all secondary subclones contained a unique reciprocal (pRI-hybridizing) fragment (about 22 kb) which served as a convenient clonal marker. Thus, failure to demonstrate secondary rearrangement of the κ^- allele in P8.G can be explained by the fact there were no downstream J segments available for a corrective event to occur on this allele.

DISCUSSION

Although the availability of multiple V and J gene segments of the κ locus creates the potential for immense antibody diversity, the results of the recombination process appear to be quite error prone (11). Defects in rearrangement are evident at all levels of expression, including pseudo-V genes and splice site usage and frameshift mutations resulting from aberrant V-J joining (see references 14 and 15 and references therein). However, nonproductive rearrangement does not necessarily exclude a κ allele from additional recombination. When germ line V and downstream J gene segments are used, secondary rearrangements might occur. Indeed, the characterization of double recombination products (DRPs) in mouse plasmacytomas (12, 38) strongly suggested that two V_κ-J_κ rearrangements occurred on the same allele. In at least one case, the DRP appeared to have been generated by the displacement of a nonfunctional V_κ-J_κ by a secondary V_κ-J_κ rearrangement. By sequential cloning of a cell line which rearranges κ gene segments during cell culture, we have now directly demonstrated that secondary recombinations can occur on a single allele and that these can serve to correct previous aberrant V-J-containing κ alleles.

The sequential events of corrective recombination that were observed through subcloning of the P8.4 cell line are schematically represented in Fig. 7. In total, 10 of 17 secondary subclones which expressed κ protein exhibited secondary rearrangements of a single allele. Significantly, of these 10, 4 unique κ^+ subclones (4.7, 4.9, 4.11, and 4.12) demonstrated secondary rearrangement of the original κ^- allele while maintaining the other allele in germ line configuration. For several subclones it was not possible to determine whether a corrective allele or a rearranged germ line allele was responsible for κ protein production (4.15 and 4.18 [Table 2]). This suggests that at least 25% of the κ-producing clones characterized had become productive by secondary, corrective V_κ-J_κ recombination.

It is formally possible that gene conversion or somatic mutation could result in changes of restriction fragment size and kappa protein production while maintaining the primary V-J rearrangement. Using J-specific probes (J1-3, HXJ, and others), we have observed V-J3 displacement by subsequent

V-J4 rearrangement in subclones 4.7 and 4.9 (Fig. 6) and 4.11 and 4.12 (data not shown). Obviously, cloning and sequencing of the nonproductive primary V-J together with the secondary productive V-J elements would undoubtedly confirm these observations. It should be noted that no known selective pressure was imposed in the isolation of cells displaying corrective recombination. Our results from random subcloning indicate that secondary rearrangement occurs with a significant frequency in the P8 cell line. We cannot accurately compare this frequency with the in vivo frequency of the immune system, although the results presented here directly demonstrate its potential.

Since the nonproductive allele was transcribed in all primary subclones analyzed (data not shown), further rearrangement may be allowed by an open conformation and accessibility of the locus to the recombinase (8, 46). It is conceivable that cells involving a nonproductive V-J1 rearrangement may display higher frequencies of corrective recombination owing to the availability of three downstream J segments. None of the initial isolates allowed us to address this possibility. In contrast, nonproductive V-J4 rearrangement would force κ rearrangement to the other allele, as was observed with the P8.G set of subclones.

Secondary rearrangements of the κ allele display some analogous features with rearrangements involving heavy-chain gene segments. Secondary V_H rearrangements that replace previous V_H to D-J_H joins have been described (16, 17, 33). Presumably, the deletion of germ line D_H gene segments upon V_H-to-DJ_H joining (1) would prevent the use of downstream J_H gene segments and corrective rearrangement as described in this report. Secondary D-J_H recombination appears, instead, to be a means by which more distal D_H and V_H gene segments are brought into the J_H-C_H region (35). This may also be the effect of initial nonproductive $V_κ$-$J_κ$ events with secondary, productive $V_κ$-$J_κ$ rearrangements generating a more complete expression of the available κ repertoire. In addition, secondary V_H gene conversion of a previously assembled V_HDJ_H gene exon has been reported (18).

The various T-cell-receptor (TCR) loci also display multiple rearrangement events. Reciprocal products of TCR $V_β$-D-$J_β$ gene segment recombination have been detected in the circular DNA fraction isolated from mouse thymocyte nuclei (28). Similarly, TCR α and δ gene segment DRPs have been characterized (29). The interspersed organization of the TCR α and δ loci has suggested the unique ability of primary nonproductive δ rearrangements to potentiate eventual α expression through secondary rearrangement of the same allele (5). Furthermore, it has been reported that apparently productive TCR $V_α$-$J_α$ rearrangements can be replaced by secondary rearrangements on the same allele (25). Recently, secondary κ rearrangements have also been implicated to explain idiotypic heterogeneity of light-chain variant tumor cells (9).

In this study we present direct evidence for sequential rearrangements of a single κ allele which can serve to correct previous aberrant events and generate a DRP element. We previously noted DRPs in about 5% of murine plasmacytomas surveyed (12). Initially, this suggested that the DRP was a novel yet minor product of κ recombination. Although the detection of only one DRP in four P8.4 corrective subclones certainly does not represent a comprehensive analysis, it does confirm that multiple V-J recombination of a single κ allele may occur more frequently than is revealed by simple hybridization analysis. In essence, the loss of DRP elements via deletional DNA inversion (Fig. 7B) has undoubtedly disguised secondary V-J recombination. Thus, through the identification of intermediate pre-B cells, this study has demonstrated that rearranged $κ^-$ alleles, as substrates for further recombination, can become $κ^+$ by corrective recombination and that this can occur with a significant frequency.

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