Double recombination of a single immunoglobulin κ-chain allele: Implications for the mechanism of rearrangement

(gene rearrangements/inversion/antibody)

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ABSTRACT DNA fragments containing immunoglobulin κ-chain sequences from two different plasmacytomas (PC 3609 and PC 7043) were found by blot-hybridization studies to be dissociated from germ-line sequences on both the 3' and 5' ends. These fragments were cloned, sequenced, and found to contain the structural features of a product of two recombination events. Each contained a variable (V_κ) gene segment recombined with a joining (J_κ) gene segment followed by the characteristic κ light chain V–J reciprocal structure, a 5' J_κ flanking sequence joined to a 3' V_κ flanking sequence. These segments of DNA represent double recombination products (DRPs) of the same κ-chain allele. The DRP from PC 3609 contains a normal V–J1 recombination, while the DRP from PC 7043 contains an aberrant V–J2 recombination, resulting in a frameshift. The reciprocal structure in the PC 3609 DRP is the result of a V–J2 recombination; the reciprocal structure in the DRP of PC 7043 is the result of a V–J3 recombination and appears to have been derived directly from the productive κ-chain gene recombination in that plasmacytoma. These products demonstrate the capacity of a single κ light chain immunoglobulin allele to undergo multiple V–J recombinations. Furthermore, the presence of a V–J recombination and its reciprocal product in the same cell is inconsistent with a segregating mechanism, such as sister chromatid exchange, but is consistent with an inversion mechanism.

The expression of immunoglobulin genes is dependent on the recombination of separate gene segments. For the κ light chain, this involves joining one of several hundred variable region gene (*V*) segments to one of four functional joining region gene (*J*) segments that lie ≈2.5–3.9 kilobases (kb) upstream of a constant region gene (*C*) segment (1). One approach to analyze the products of immunoglobulin gene recombination has been to clone and sequence these genes from plasmacytomas, which represent a monoclonal expansion of a single, antibody-producing plasma cell (2–5). A number of recombination products have been described for the κ-chain locus. It has been shown that V–J recombination is frequently imprecise, resulting in either functional diversification of antibody specificity or frameshift mutations, which generate aberrant transcripts (4, 5). Aberrant recombinations of immunoglobulin genes help to explain the phenomenon of allelic exclusion in antibody-producing cells; only one allele is functionally expressed. Current concepts of allelic exclusion suggest that once a functional recombination takes place, rearrangement ceases (6). One of the implications of allelic exclusion is that, if an aberrant recombination takes place on one allele, the other allele still has the potential to generate a functional gene recombination. However, the possibility of an aberrantly rearranged allele undergoing a

second, corrective, recombination is typically not considered.

From studies with plasmacytomas and normal B cells, it also was found that many of these cells contain DNA representing the reciprocal product of a V–J recombination (7–10). This DNA is characterized by a back-to-back joining of the highly conserved heptamer and nonamer sequences that flank germ-line V_κ and J_κ segments (8–10). However, in all of the examples examined to date, no direct correlation has been found between the reciprocal product and a V–J joined in the same cell. These observations, along with evidence that some cells lack reciprocal products, led to a proposal that V–J recombination occurs by sister chromatid exchange (7). The key feature of this model is that the joined V–J sequences and their reciprocal products segregate, thus explaining their lack of correlation within the cell. However, immunoglobulin gene recombination on one allele may involve secondary recombinations that could obscure the relationship between recombined κ-chain genes and reciprocal products. Indeed, hybridization studies have revealed fragments of immunoglobulin DNA that are dissociated from the germ-line sequences on both the 3' and 5' ends (7, 11). It has been suggested that these fragments could arise by two V–J recombination events on the same κ allele.

One proposed model that does not predict the segregation of recombination products suggests that immunoglobulin gene segments rearrange by an inversion event (11). Inversion has been demonstrated in one case for an immunoglobulin heavy chain diversity (*D*), gene segment (12) and has been suggested as the mechanism that generated two related products in the myeloma MOPC 41 (13). However, the product of the *D* inversion was clearly irregular, and neither of the related products in the MOPC 41 myeloma contains a *V* gene segment. Recently, an expression vector was constructed that provided a selection method dependent on inversion of κ light chain gene segments (14). It was demonstrated that the transformants that grew in the selective medium contained a V–J product of an inversion event. While this study clearly demonstrated that inversion of κ-chain gene segments can occur, it could not rule out other mechanisms because products of any other mechanism would not have been detected in the selection system.

The structure of fragments containing κ-chain gene segments dissociated on both the 3' and 5' ends is of interest because it could provide direct evidence of multiple V–J recombinations of a single κ-chain locus and could provide a link between V–J products and reciprocal products. We have isolated and sequenced cloned DNA fragments from two different plasmacytomas that contain structural features indicating that they were generated from two V_κ–J_κ recombination events. One of these appears to have been generated, in part, as the reciprocal product of the productive V_κ–J_κ

Abbreviations: DRP, double recombination product; *V, J, C,* and *D,* variable, joining, constant, and diversity region genes; kb, kilobase(s); bp, base pair(s).

rearrangement in the same cell. These observations show directly that multiple recombinations can occur on the same κ-chain allele and have important implications concerning the mechanism of immunoglobulin gene rearrangement.

MATERIALS AND METHODS

One plasmacytoma used in this study, PC 7043, expresses κ light chains of the $V_\kappa 21$ group; plasmacytoma PC 3609 expresses a κ light chain from the $V_\kappa 14$ group (15). DNA was isolated as previously described (16). DNA modification enzymes were used in accordance with the manufacturers' guidelines (New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories). Agarose gel electrophoresis and transfer of DNA to nitrocellulose (Schleicher & Schuell) was done as specified by Maniatis *et al.* (17). Hybridization probes pEC$_\kappa$, pR1, and pC$_\kappa$ have been described (7). The 1.7-kb germ-line $J_\kappa 1$–4-containing fragment, HXJ, was produced by *Hin*dIII/*Xba* I digestion of pEC$_\kappa$. The physical maps of all probes are shown in Fig. 1. Hybridization, wash conditions, and autoradiography were as described (7). For cloning, preparative *Bam*HI digests of plasmacytoma DNA were fractionated by electrophoresis through low-melting-temperature agarose, and size-restricted fragments containing the κ-chain gene double recombination product (DRP) were extracted and ligated into λ phage Charon 27 (obtained from F. Blattner). After *in vitro* packaging, the recombinant phage were screened by hybridization to the HXJ probe (18). Fragments of DNA to be sequenced were subcloned into compatible sites or blunt-ended and ligated into *Sma* I sites of phage M13 vectors mp8–11. Nucleotide sequencing was done by the method of Sanger *et al.* (19) using either the universal primer supplied in the New England Biolab M13 kit or synthetic oligonucleotides generated by the phosphoramidite method (20) using a Beckman System 1 DNA Synthesizer.

RESULTS

Evidence for DRPs in Plasmacytomas. Several plasmacytomas have been found that contain fragments of DNA that hybridize to J_κ-containing probes but not to probes containing DNA upstream of the J_κ locus, nor to probes containing C_κ (7). The Southern blots of *Bam*HI-digested DNA from the plasmacytomas PC 3609 and PC 7043 show that each contains two restriction fragments that hybridize to the C_κ probe (Fig. 1). Both κ-chain alleles in PC 3609 have undergone rearrangement [one productively (κ^+) and one nonproductively (κ^-)], whereas in PC 7043 one of the κ-chain alleles remains in the germ-line configuration (κ^0) and the other has rearranged productively (κ^+). PC 3609 and PC 7043 also contain a rearranged fragment that hybridizes to the upstream probe (labeled U), and this presumably represents the reciprocal product of a V_κ–J_κ recombination. In addition, both plasmacytomas contain a fragment that hybridizes to probes containing J_κ sequences but not to probes containing U or C_κ sequences. Since the germ-line DNA both 3' and 5' of J_κ is missing, it appears likely that two recombination events generated these fragments, and we designated them "double recombination products" (DRPs).

The DRPs were cloned as *Bam*HI restriction fragments into the phage λ cloning vector Charon 27. Restriction fragments from each clone were subcloned into M13 (mp8–11) for sequencing by the Sanger dideoxy chain-termination method (ref. 19; see Fig. 2). Fig. 2 illustrates the structural features determined from the nucleotide sequence shown in Fig. 3. Each DRP clone shows an extended open reading frame which, when translated, contains the invariant amino acids characteristic of mouse V_κ regions (21). Comparison with the available protein sequence data indicates that the V_κ in the PC 3609 DRP is identical to the productive
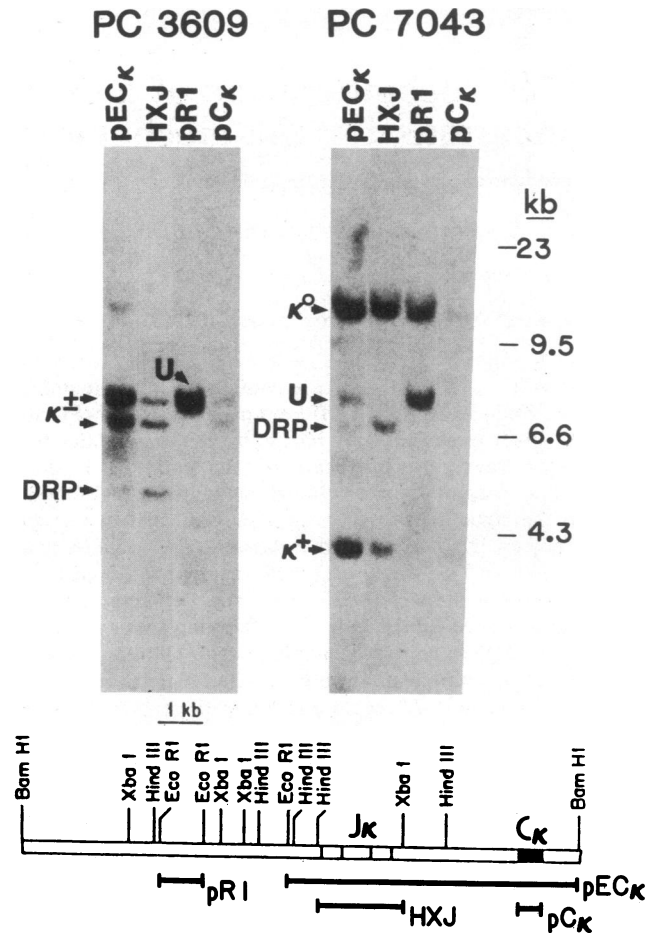

FIG. 1. (*Upper*) Southern blot hybridization of *Bam*HI-digested DNA from the plasmacytomas PC 3609 and PC 7043, probed as indicated. (*Lower*) The source of each probe. The hybridizing fragments are labeled as follows: κ^0, germ line; κ^+, productive rearrangement; κ^-, nonproductive rearrangement; U, rearranged, upstream (reciprocal); and DRP. It has not yet been determined which of the two C_κ-containing fragments in the PC 3609 is productive and which is nonproductive.

V_κ of PC 2367 (22), which differs from the prototype $V_\kappa 8$ family (21) by one amino acid in the first framework region (amino acids 1–23). The V_κ in the PC 7043 DRP is identical in the first framework region to the productive V_κ in J604 (23), which has been designated as a member of the $V_\kappa 19$ family (21). Comparisons with germ-line sequences of the κ-chain locus (24) show that the V gene segment in the DRP from PC 3609 is joined to $J1$, maintaining a proper open reading frame. In the PC 7043 DRP, the V gene segment is joined to $J2$ such that there is a shift in the normal J reading frame. This type of imprecise recombination has been previously observed (4, 5).

Both DRP clones also contain the highly conserved heptamer–nonamer sequences that flank germ-line J_κ and V_κ segments (25, 26), joined back-to-back (see Figs. 2 and 3). The DRP from PC 3609 contains the 23-base-pair (bp) spaced heptamer–nonamer sequences derived from the sequence 5' of $J2$, while the DRP from PC 7043 contains the 24-bp spaced heptamer–nonamer derived from the sequence 5' of $J3$. The 12-bp spaced heptamer–nonamer joined to each J_κ flanking sequence is characteristic of the sequences 3' of all V_κ genes (26). Thus, in addition to a V_κ–J_κ recombination, each DRP contains the sequence characteristic of a reciprocal product of a V_κ–J_κ recombination.

As shown in Fig. 3, the V_κ flanking sequence joined to the $J3$ flanking sequence in the PC 7043 DRP is identical to the

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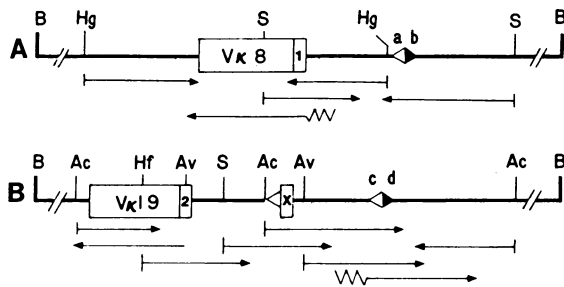*Proc. Natl. Acad. Sci. USA 82 (1985)* 4795

FIG. 2. Sequencing strategy and schematic representation of the DRPs cloned from PC 3609 (*A*) and PC 7043 (*B*). Only the relevant portion of each clone is shown. Arrows indicate the extent of sequencing of individual M13 subclones. Each subclone was sequenced at least two times. Jagged lines represent sequences primed with a synthetic oligonucleotide (20-mer). The V_κ groups are designated; the numbered boxes represent J_κ gene segments; X represents the nonfunctional *J*-like sequence between *J2* and *J3*. The open and closed triangles labeled "a" and "b" represent the heptamer–nonamer sequences that flank *J2* and a V_κ gene segment (unknown group), respectively. The triangles labeled "c" and "d" represent heptamer–nonamer sequences that flank *J3* and a $V_\kappa 21$ gene segment. For sequence details, see Fig. 3. B, *Bam*HI; H, *Hind*III; Hg, *Hgi*AI; S, *Sau*3A; Ac, *Acc* I; Hf, *Hinf*I; Av, *Ava* II.

flanking sequence of a germ-line $V_\kappa 21$ gene that is closely related to or the same as subgroup D $V_\kappa 21$ genes (27). This is particularly noteworthy because the productive rearrangement in PC 7043 involves a subgroup D $V_\kappa 21$ join to *J3* (28). Sorting out the recombination products in PC 3609 is more complex because both κ-chain alleles are rearranged. Although the source of the V_κ flanking sequence in the DRP from PC 3609 is currently not known, it is possible that the DRP is related to one of the V_κ–J_κ recombinations in the same cell line, since there is a V_κ–*J2* recombination in PC 3609 (ref. 28; unpublished data) that could have generated the DRP reciprocal structure.

DISCUSSION

A DRP Could Be Generated in Two Ways. Several hybridization studies have provided data suggesting that multiple recombinations may occur on a single allele of a κ light chain

gene (7, 10, 11). We have cloned and sequenced products of κ chain gene recombination resulting from two *V–J* recombination events on the same allele. Each contains a V_κ gene segment joined to a J_κ gene segment, followed by the sequences characteristic of a reciprocal product of V_κ–J_κ recombination (Fig. 3). A DRP could be generated in either of two ways (Fig. 4). A recombination of a *V* gene segment to *J2*, *J3*, or *J4* can generate a reciprocal product containing one or more complete J_κ segments. These reciprocal segments could still serve as substrates for additional V_κ–J_κ recombination events (Fig. 4A). It seems likely that *J*-containing reciprocal products may be located in much closer proximity to *V* gene segments as a result of the reciprocal exchange. This close proximity may significantly increase the probability of a second *V–J* recombination. It is possible that recombination of *J*-containing reciprocals could continue until a *V–J1* recombination occurred; the reciprocal of this event would no longer contain viable *J* segments. This idea would explain why all previously reported reciprocal products in plasmacytomas contain the sequence that flanks *J1* (8–10). This also would explain why reciprocal products in cloned Abelson murine leukemia virus-transformed PD cell lines apparently rearrange in subsequent subclones (11).

The $V_\kappa 8$–*J1* join in the PC 3609 DRP maintains a proper reading frame (Fig. 3). In addition, the signal peptide and splice junctions appear to be functional, and the highly conserved octanucleotide of $\approx$100 bp 5′ of all V_κ signal peptide-encoding elements (29) is present (data not shown). Therefore, it appears that the *V–J* of the DRP in PC 3609 would have been functional if it were the primary rearrangement on that κ allele, although we cannot be sure it would exclude further recombinations. It seems more likely that the DRP in PC 3609 was the result of a secondary V_κ–J_κ rearrangement of a reciprocal product containing the *J1* gene segment, as in Fig. 4A.

DRPs also could arise by an alternative route. If one assumes that more than one *V–J* recombination can occur on a single κ-chain allele, then it is also possible that an aberrant recombination can be corrected by a second V_κ recombination to J_κ gene segments 3′ of the first V_κ–J_κ (Fig. 4B). The DRP from PC 7043 could have been generated by this pathway. The *V–J* recombination in the PC 7043 DRP contains an aberrant recombination between the $V_\kappa 19$ and *J2*
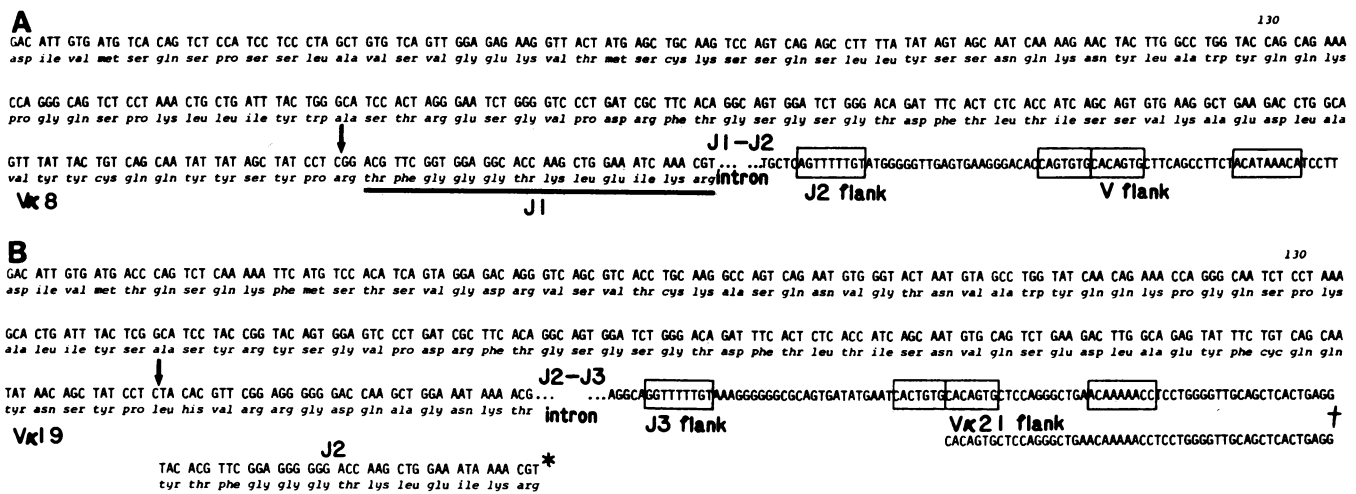

FIG. 3. Nucleotide sequence of the DRPs from PC 3609 (*A*) and PC 7043 (*B*). Translation of the nucleotide sequence into amino acid sequence is also shown where appropriate. Each DRP contains a V_κ gene segment joined to a J_κ gene segment (*J1* in *A*, *J2* in *B*), followed by the back-to-back joining of sequences that flank a J_κ gene segment (*J2* in *A*, *J3* in *B*) and a V_κ gene segment. The characteristic heptamer–nonamer sequences are boxed. The V_κ flanking sequence in the PC 7043 DRP is shown to be identical to the sequence (†) that flanks a $V_\kappa 21$ germ-line gene sequence. The *V–J* recombination site, designated with an arrow, maintains the normal J_κ reading frame in *A* but results in a frameshift in *B*. The proper $J_\kappa 2$ reading frame (∗) is presented to demonstrate this shift (∗, from ref. 24; †, from ref. 27).
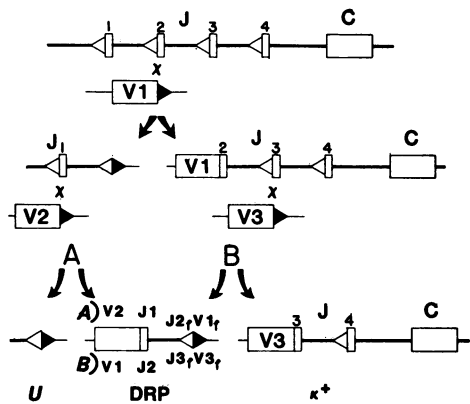
FIG. 4. DRPs can be generated in two ways. The first recombination is shown to involve a *V1* to *J2* join, generating a reciprocal product containing *J1*, followed by the back-to-back joining of the *J2–V1* flanking (f) sequences. In DRP designated *A*, reciprocal products containing *J* gene segments can undergo further *V–J* recombination. In this case *V2* is shown joining to *J1*, resulting in a *J1–V2* reciprocal product and a DRP containing the *V2–J1* gene segment, followed by the reciprocal portion of the first *V1–J2* recombination. In DRP designated *B*, if the *V1–J2* recombination is aberrant, then a second recombination could join a *V3* to *J3* (κ⁺, if productive). The reciprocal of this recombination would be a DRP containing the first aberrant *V1–J2* join, followed by the sequences that flank *J3* and *V3*.

gene segments. If this rearrangement had occurred first at the *J* locus, it likely would have resulted in a nonproductive transcript. A second $V_\kappa 21$–*J3* rearrangement on the same allele could have corrected the aberrant *V–J*, generating a DRP containing the reciprocal product of the productive recombination. The possibility that multiple recombinations can serve to correct aberrant rearrangements on the same allele is not typically addressed in models of allelic exclusion. If this conclusion is correct, then the rate of aberrant recombinations of the κ-chain locus may have been underestimated (28), and, until a functional light chain is produced, a single κ-chain allele is not excluded from further recombinations. However, current views of allelic exclusion predict that replacement of a productive rearrangement by a second recombination is rare or nonexistent.

Implications of the DRP from PC 7043 for the Mechanism of Rearrangement. The relationship of the DRP reciprocal structure and the productive V_κ–J_κ join in PC 7043 also has important implications in considering a mechanism of rearrangement. The reciprocal portion of the PC 7043 DRP contains a characteristic V_κ flanking sequence, which is homologous to the sequence that flanks the germ-line $V_\kappa 21$ gene used in the productively rearranged κ⁺ allele of the same cell. If the mechanism of immunoglobulin gene rearrangement involves sister chromatid exchange, the reciprocal product of a V_κ–J_κ recombination would be expected to segregate into a different cell. Thus, the relationship we observed between the productive *V–J* and the DRP in PC 7043 does not support a segregating model. While it is formally possible that a subgroup D $V_\kappa 21$ reciprocal product segregated into a cell, which then also rearranged subgroup D $V_\kappa 21$, we think this is unlikely. For this reason, and because one of the κ alleles in PC 7043 is in the germ-line configuration (κ⁰), we assume that all of the recombination products were generated on the same κ allele.

The *V–J* recombination in an inversion event is not that different from sister chromatid exchange; both involve reciprocal exchanges between double-stranded DNA. However, the key difference is that the products of an inversion would not segregate. To explain why some cells lack reciprocal products, it has been suggested that some V_κ gene

segments lie in the same transcriptional orientation as the J_κ gene segments (in which case an inversion would delete the reciprocal product of a V_κ–J_κ join), while others lie in the opposite orientation (in which case an inversion would retain a reciprocal product) (11). At present there are no data on the orientation of V_κ genes relative to the J_κ gene segments. However, it may be relevant that recently two V_γ gene segments from a T-cell line, which share the conserved flanking sequence homology of immunoglobulin genes, were determined to be in a head-to-head (transcriptionally opposite) orientation (30).

Fig. 5 demonstrates how each of the products of PC 7043 κ-chain gene recombination could have been generated by a series of inversion events. Initially, the $V_\kappa 21$ gene is drawn in the same transcriptional orientation as the J_κ gene segments, while the $V_\kappa 19$ gene is drawn in the opposite orientation. By inversion, any recombination of a $V_\kappa 21$ to a J_κ gene segment would result in a deletion of the reciprocal product. However, if the first event were the recombination of a $V_\kappa 19$ gene, which in the germ-line DNA lies 5' of the $V_\kappa 21$ gene family (31), the reciprocal product would be retained, and all *V* genes 3' of the $V_\kappa 19$ would end up in an orientation opposite to that found in the germ line. If the first, $V_\kappa 19$ recombination were aberrant, a second inversion of a V_κ to another 3' J_κ could take place. In Fig. 5 a second recombination is shown involving a $V_\kappa 21$ inversion to *J3*. Since the $V_\kappa 21$ gene family was put in the opposite orientation by the first event, the reciprocal product would now be retained. A DRP would be generated that contains an aberrant $V_\kappa 19$–*J2* rearrangement, followed by the reciprocal back-to-back structure containing
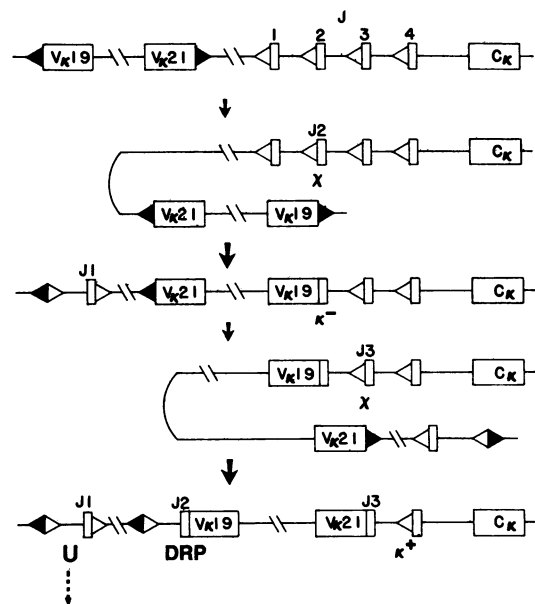

FIG. 5. A series of inversion events could generate all of the recombination products seen in PC 7043. A $V_\kappa 19$ gene segment is shown in germ line to be in the opposite transcriptional orientation as the J_κ gene segments, while the $V_\kappa 21$ gene segment is in the same orientation as the J_κ segments. Recombination by inversion of the $V_\kappa 19$ gene to *J2* would result in the retention of the reciprocal product and reorientation of the $V_\kappa 21$ gene. If the $V_\kappa 19$–*J2* product were aberrant, a second inversion could join another *V* (in this case a $V_\kappa 21$) productively to a remaining *J* (in this case *J3*). The reciprocal product of this inversion (a DRP) would also be retained. Since the first reciprocal product still contains a germ-line *J1*, it likely will undergo further *V–J* recombination to generate a *J1* reciprocal (indicated by dotted line). The rearranged *J1* reciprocal (*U*), a DRP containing an aberrant $V_\kappa 19$–*J2* recombination followed by a *J3*–$V_\kappa 21$ reciprocal product, and a productive $V_\kappa 21$–*J3* recombination (κ⁺) are present in PC 7043.

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the sequences flanking the *J3* gene segment and the $V_\kappa 21$ gene segment. The DRP of PC 7043 fits this description (see Fig. 3). In addition, the first recombination would have generated a reciprocal product that would have sequence upstream of the J_κ locus and would be detectable with the upstream (pR1) probe. The Southern blot in Fig. 1 shows that in addition to the κ^+, κ^0, and DRP fragments, a rearranged upstream fragment is detected (labeled *U* in Fig. 1). As would be predicted from the idea that reciprocal fragments appear to undergo further recombination if they contain *J* gene segments (Fig. 4*A*), the rearranged upstream fragment in PC 7043 contains only *J1* flanking sequences (not shown) and, therefore, is unlikely to be directly related to the $V_\kappa 19$–*J2* gene rearrangement. In the scheme proposed in Fig. 5, the $V_\kappa 19$ gene is shown to result in retention of the reciprocal fragment. This is in apparent contradiction to previous data, which suggested that the $V_\kappa 19$ rearrangement in the MPC-11 myeloma deleted the $V_\kappa 21$ gene family on one allele (31). Since secondary rearrangements could have deleted the $V_\kappa 21$ gene segments in MPC-11, the orientations proposed in Fig. 5 are not necessarily inconsistent.

Based on our observations, the apparent lack of relationship between κ light chain *V–J* recombination products and the reciprocal products previously characterized could be explained by secondary *V–J* rearrangements on the same allele. The DRPs of PC 3609 and PC 7043 demonstrate the capacity of the κ-chain allele to undergo multiple *V–J* recombinations. In addition, the DRP of PC 7043 was most likely derived from a *V–J* recombination in the same cell, an observation that would not be consistent with a segregating mechanism. Some additional plasmacytomas we have surveyed contain fragments with hybridization properties predicted of DRPs. It will be important to determine if other DRPs are derived from *V–J* recombinations in the same cell. Many plasmacytomas lack DRPs, presumably because secondary recombination events have occurred that delete them. While a series of inversion events can account for the recombination products observed in PC 7043, this single example does not necessarily rule out other possible mechanisms, such as secondary deletion–reinsertions of reciprocal products. A clearer understanding of the mechanism of immunoglobulin gene rearrangements awaits additional examples and, ultimately, a complete study of the enzymology involved.

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