Direct evidence for intrastrand DNA inversion of κ immunoglobulin gene segments in two murine plasmacytomasRodney Feddersen^{1,2} and Brian Van Ness^{1*}¹Institute of Human Genetics and Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455 and ²Department of Biochemistry, The University of Iowa, Iowa City, IA 52242, USA

Received August 7, 1989; Revised and Accepted November 2, 1989

ABSTRACT

The products of kappa immunoglobulin gene recombination have been characterized in two murine plasmacytomas to examine the relationship between V-J products and reciprocal elements. By cloning, sequencing, hybridization, and application of the polymerase chain reaction, we have established the direct relationship of the kappa recombination products in these cells. The results provide stronger support for the intrastrand mechanism of kappa gene recombination as well as demonstrating a role for secondary, corrective recombinations.

INTRODUCTION

The process of kappa (κ) immunoglobulin gene rearrangement results in both functional and non-functional joining of variable (V) and joining (J) gene segments (1–3). By-products of kappa V-J recombination have also been characterized (4–7). These products are characterized by the back-to-back fusion of the heptamer and nonamer sequences that flank germline V and J segments, and have demonstrated that recombination involves a reciprocal exchange of DNA (3–5). Interestingly, the vast majority of V-J reciprocal products observed contain a V flanking sequence joined to the sequence flanking J1, despite the fact that V segment joining is distributed among all four J segments of the kappa locus. The observation that V-J1 reciprocal products (VJ1RP) did not always correspond to the V-J usage in a given B cell, as well as the frequent loss of reciprocal products, were the basis for the proposal of unequal sister chromatid exchange (uSCE) as the mechanism for V-J joining (8). Segregation of V-J and reciprocal products into different daughter cells served to explain the lack of direct reciprocity of V-J and VJ1RPs found in a given cell.

More recently other products of kappa gene recombination have been reported (4,9). The double recombination product (DRP) is characterized by a V-J join followed by the reciprocal fusion of the flanking sequences of another V-J join (see Figure 5). These products demonstrated that more than one recombination could occur at a single allele. We previously reported the detailed characterization of a DRP from a murine plasmacytoma that contained a productive V κ 21D join to J3 (4). Significantly, the reciprocal element of the DRP in this cell contained the sequences which flanked the germline V κ 21D and J3 gene segments. This observation refuted uSCE, and supported models which proposed intrastrand DNA inversion (10). In addition, the characterization of the DRP suggested that because secondary recombinations could occur on a single allele, the relationships of V-J products and their reciprocal products may be obscured. Moreover, it has been suggested that murine V κ gene segments may lie in either transcriptional orientation relative to the J κ locus (10,11) as has been demonstrated for the human kappa locus (12,13). As proposed by models

Figure 1. Schematic representation of the various mouse immunoglobulin κ probes employed for hybridization analysis. All probes have been previously described (4). Restriction enzymes used to isolate probes from the germline (12 Kb; κ°) locus are as follows: B=Bam HI, RI=Eco RI, H3=Hind III, Hf=Hinf I and X=Xba I.

of DNA inversion, some reciprocal products would be deleted, depending on the germline orientation of the rearranging V_κ gene segment.

Although it is now generally accepted that kappa recombination can occur by intrastrand inversion, supporting evidence is limited to a single example in a plasmacytoma (4) and artificial constructs characterized under selective conditions (14–16). Conscious of multiple V-J recombination per allele, we sought to characterize the relationships of the products of kappa recombination in other plasmacytomas. Specifically, the prediction of V-J and reciprocal products generated by a single intrastrand DNA inversion was assessed. In addition to cloning, sequencing and hybridization studies, the polymerase chain reaction (PCR) was employed to establish the relationship of the products of kappa gene segment rearrangement.

MATERIALS AND METHODS

Plasmacytoma DNA was kindly provided by M. Weigert (Institute for Cancer Research, Fox Chase, PA). Restriction digests were performed as recommended by the manufacturer (Bethesda Research Labs or New England Biolabs). Blot hybridization analyses were as previously described (4). Blots used for re-hybridization were wet completely in water and washed in three changes of 0.4M NaOH at 42°C for 15 min each, then neutralized in 1M Tris pH 7.5/0.2×SSC/0.1%SDS for 20 min. The membrane was rinsed in 0.2×SSC/0.2% SDS. No residual signal was detected following a two week exposure of the alkaline washed blot. All probes used have been described elsewhere (4) and were radioactively labelled using a nick translation kit (BRL) and α - 32 P-dCTP (New England Nuclear) to specific activities greater than 1×10^8 cpm/ μ g.

Genomic cloning was as previously described (4). Briefly, size selected Bam HI libraries were constructed from DNA isolated from a low melting agarose gel and screened with an appropriate kappa locus probe. The lambda cloning vector EMBL 3 was used and packaged with the Gigapak bacteriophage system (Vector Cloning Systems).

Figure 2. Products of α gene recombination in PC 613.

A) Multi-probe blot hybridization analysis of Bam HI digested genomic liver and PC 613 DNA. B) Simplified restriction maps and sequencing strategies of cloned α recombination products. For the 9 kb V-J1 clone a J1 specific oligo (bold 'o') was used as a sequencing primer. Other symbols on sequencing arrows indicate deletion subclones. The joined open and filled triangles shown on the 15 kb VJIRP clone represent heptamer/nonamer sequences flanking germline J and V gene segments, respectively. C) Blot hybridization analysis of Bam HI digested liver DNA, probed with a 3 kb Bam HI/Pst I fragment isolated from the 5' region of the V-J clone (vertically striped box).

DNA sequencing was as previously described (4). The J1 specific oligonucleotide used for double stranded sequencing was employed as instructed by the Sequenase Version 2 kit (United States Biochemical).

Polymerase chain reactions were performed as recommended by the manufacturer of the kit components (Cetus). Each 100 μ l reaction mix contained 20 picomoles of the two

Figure 3. DNA sequence of PC 613 κ recombination products. A) Sequence of the productive V κ 9-J1 clone. The V κ 9 invariant amino acid residues are indicated with bold numbers above. Splice sequences are indicated with arrows. The 18 bp sequence synthesized for PCR use is indicated (Oligo 18). B) Sequence of the VJ1RP clone. Sequence begins at the Hind III site 80 bp 5' of J1 (ref.20). Heptamer/nonamer sequences associated with J1 and V gene segments are indicated. The 17 bp sequence synthesized for PCR use is indicated (Oligo 17).

specific oligonucleotides (shown in Fig. 3). The oligonucleotides were synthesized by the Microchemical Facility of the Institute of Human Genetics (U. of Minnesota). Reaction conditions, using 1 mM MgCl₂ in the reaction buffer, were as follows: 1 min at 95°C, 1.5 min at 40°C, and 0.75 min at 72°C for 30 cycles, with an additional 1 sec/cycle automatic extension. One-tenth of each reaction was electrophoresed in a 3% agarose gel in a Tris-Borate buffer containing ethidium bromide. Gels were briefly destained by soaking in water and photographed employing UV illumination and an MR-2 Polaroid camera.

RESULTS

Characterization of the kappa rearrangement products of PC 613

Initial characterization of the products of kappa rearrangement was accomplished by blot hybridization of the DNA with a variety of probes (Fig. 1). As shown in Figure 2, blot hybridization analysis of plasmacytoma (PC) 613 demonstrates rearrangement of a single kappa allele. Previous studies identified a single 5.3 kb primary κ transcript that indicates the V recombination was to J1 (17). Amino terminal sequencing of the kappa protein suggested that the V region is a member of the V κ 9 subgroup (1,18). The genomic DNA isolated from PC 613 contained a VJ1RP, as identified by its characteristic hybridization

Figure 4. Polymerase chain reaction showing the relationship of the α recombination products in PC 613. A) Schematic representation of the V-J clone and the location of Oligo 18 (Sequence shown in Fig. 3A). B) Schematic representation of the VJ1RP and the location of Oligo 17 (Sequence shown in Fig. 3B). C) Predicted germline V_x9 sequence showing expected relationship of Oligo 18 and Oligo 17. The predicted PCR reaction product is shown (393 bp) with its unique Kpn I site indicated. D) Analysis of the PCR product generated from Oligo 17/Oligo 18 primed synthesis with mouse liver DNA. A 3% agarose gel was used to resolve PCR products. Control lane contains the 500 bp standard provided in the Cetus amplification kit. The PCR/Kpn I lane contains PCR product (same as in lane 2) digested with restriction enzyme Kpn I.

pattern (Fig. 2A). This product was detected as a 15 kb Bam HI fragment with the pEck and pR1 probes, but not the J850 probe. This hybridization pattern is consistent with the reciprocal product of a V-J1 event. However, a relationship remained to be established between the V flanking sequence present in the VJ1RP and the germline V α 9 segment that was productively joined in PC 613.

The Bam HI fragments containing the productive V-J product as well as the VJ1RP were each cloned into the EMBL 3 phage vector (see Methods). Simplified restriction maps and DNA sequencing strategies are shown in Figure 2B, with the relevant portion of each sequence presented in Figure 3. The functional product contains a V α 9 gene segment joined in-frame to J1, and includes a signal sequence with 5' promoter elements. The VJ1RP displays the nonamer/heptamer flanking J1 fused to another nonamer/heptamer. The 12 bp spacer of the second recombination signal sequence is representative of a germline V α associated element (11).

Assuming a single DNA inversion event resulted in the generation of the two products in PC 613, a germline organization was predicted based on the lengths of known segments of the cloned DNAs. As illustrated in Figure 2B, a germline V α 9 containing Bam HI fragment should be comprised of the 8.5 kb portion of the VJ1RP clone and 3.8 kb of the α + clone. Thus the germline V α 9 segment rearranged in PC 613 was predicted to lie on a 12 kb Bam HI fragment. To investigate this possibility, a 3 kb Bam HI/Pst I fragment was isolated from the 5' region of the α + clone and used as a probe against Bam HI digested DNA from mouse liver. Indeed, a single hybridization signal at 12 kb was detected (Fig. 2C). Because this result may be ambiguous, the relationship of the PC 613 kappa products was further investigated by application of the polymerase chain reaction. Oligonucleotides were synthesized based on the sequence of the two cloned products in PC 613. The location of the oligonucleotides are shown in Fig. 3, and schematically in Fig. 4A and B. By application of these primers in a PCR reaction with germline (liver) DNA, the detection of an amplified product would demonstrate the linkage of the V flanking sequence in the VJ1RP and the V α 9 coding sequence. The predicted product defined by these two oligo primers was 393 bp (303 bp from the oligo 18 primed V α 9 portion and 90 bp from the oligo 17 primed V flank of the VJ1RP, Fig. 4C). As shown in Fig. 4D a single 393 bp product was obtained from the PCR reaction. Confirmation of the PCR product identity was obtained by digestion with Kpn I which was predicted to cleave the product into fragments of 121 bp and 272 bp (Fig. 4D, lane 4). These results clearly established that a single DNA inversion event resulted in the production of the V α 9-J1 product, with retention of the reciprocal product in the same cell.

Characterization of the kappa rearrangement products of PC 3609

In contrast to the rearrangement of a single allele in PC 613, both kappa alleles were rearranged in PC 3609 (Fig. 5). Blot hybridization analysis of PC 3609 identified four products of kappa gene segment recombination, in agreement with a previous report (4). Two Bam HI fragments, 7.0 and 7.6 kb, were detected with the J1-4 and C α probes but not the pR1 probe. This hybridization pattern suggested these were the two V-J recombination products. Two reciprocal products were also detected. The 7.7 kb reciprocal product was a VJ1RP based on its hybridization with the pR1 probe only and restriction map (Fig. 5A&B). The fourth kappa product (5 kb), detected with only the J1-4 probe, has been previously cloned and identified as a DRP (4). DNA sequencing of the DRP showed that it contained a V α 8-J1, followed by the reciprocal element of a V-J2 recombination (see Fig. 5B). Protein analysis of the kappa light chain from PC 3609

Figure 5. Products of κ gene recombination in PC 3609. A) Multi-probe blot hybridization analysis of Bam HI digested genomic DNA. Kappa locus probes (see Fig. 1) used to hybridize each lane are identified at the top. B) Restriction maps and sequencing strategies of cloned κ recombination products. Symbols are as described in Fig. 2. Restriction sites: Bam=Bam HI, Xba=Xba I, Hd3=Hind III, Hph=Hph I, Hc2=Hinc II, Hgi=Hgi AI, Pvu2=Pvu II, Hae3=Hae III, Kpn=Kpn I, Bg2= Bgl II and Pst=Pst I.

Figure 6. DNA sequence of the V-J1 portion of the 7 kb V-J (χ^-) clone from PC 3609. Numbered amino acids indicate V χ 8 invariant residues. Codons # 93, 94, and 95 (boxed) indicate differences with V χ 8 family proteins. The site of V/J1 recombination is indicated by the upward arrow. For comparison, the normal J1 reading frame is shown.

demonstrated a functional V χ 14 rearrangement (1,18), although J usage was not determined. Northern analysis of nuclear mRNA identified primary transcripts of 5.3 and 5.1 kb (17), suggesting both V-J1 and V-J2 transcription.

The four products of kappa rearrangement in PC 3609 were each cloned into phage vectors (see Methods). Restriction maps and areas sequenced are shown in Fig. 5B. From the sequence of the 7.0 kb clone (Fig. 6) a frameshift mutation was identified at a V χ 8-J1 recombination site, thus identifying this product as non-functional (χ^-). Interestingly, the V gene segment used in this recombination is very similar, but not identical, to the V χ 8 found in the DRP. The other V-J clone (7.6 kb) was not sequenced, but based on restriction mapping and protein data, it must contain the functional V χ 14-J2 recombination product (χ^+).

In order to investigate the relationship that PC 3609 clones may share with each other, a dual probe blot hybridization analysis was conducted. Based on the cloned fragments of kappa recombination, predictions of the germline V χ organization were deduced. Figure 7 illustrates the application of this analysis. The V-J1 found in the DRP fragment and the VJ1RP could be the products of a single DNA inversion event. By combining the 1.37

Figure 7. Relationship of DRP and VJ1RP κ recombination products from PC 3609. Schematic representation of the A) DRP, B) VJ1RP and C) predicted germline V κ 8 locus. Probes used for dual blot hybridization analysis are shown as solid and hatched boxes. D–E) Blot hybridization of liver DNA digested with restriction enzymes indicated and probed with the DNA fragments indicated above. Two quantities of Bam HI digested DNA, 5 μ g [Bam (5)] and 10 μ g [Bam (10)], were analyzed.

Figure 8. Relationship of DRP and productive V κ 14-J2 recombination products from PC 3609. Schematic representation of the A) DRP, B) V κ 14-J2 product and C) predicted germline V κ 14 locus. Probes used for dual hybridization analysis are shown as vertical lined and stippled boxes. D-E) Blot hybridization of liver DNA digested with restriction enzymes indicated and probed with DNA fragments indicated above. Digests are as indicated in Fig. 7.

Figure 9. Proposed pathway for κ gene recombination by DNA intrastrand inversion. A) PC 613. Retention of the reciprocal product of V-J recombination requires that the germline $V_{\kappa 9}$ gene segment be in the opposite transcriptional orientation relative to the J-C locus. A single inversion event retains all products on the same chromosome. Open and closed triangles represent heptamer/nonamer sequences flanking germline J and V segments, respectively. B) PC 3609. The primary recombination produces a non-functional $V_{\kappa 8}$ -J1 with retention of the VJ1RP. Secondary recombination of $V_{\kappa 14}$ to J2 produces the functional κ gene, with retention of its reciprocal as a DRP.

kb 5' portion (Bam HI site to J1) of the DRP clone with the 1.08 kb 3' portion (heptamer to Bam HI site) of the VJ1RP clone, a 2.5 kb Bam HI germline fragment was predicted (Fig. 7C). Probes isolated from each of the two clones should then detect the same 2.45 kb Bam HI fragment in liver (germline) DNA. As shown in Fig. 7D and E, this was in fact the case. In addition, the same probes detected common fragments obtained from other restriction enzyme digests of liver DNA. The faint bands detected with each probe likely represent V_{κ} family members that share common flanking regions. This result strongly suggests that the coding portion of the $V_{\kappa 8}$ in the DRP clone and the V 3' flanking region in the VJ1RP clone were generated from a single DNA inversion with retention of the reciprocal product in PC 3609.

Previous characterization of the reciprocal portion of the DRP in PC 3609 suggested that it may have been generated from a DNA inversion event that produced the functional $V_{\kappa 14}$ -J2 product (4 and see Fig. 8). By adjoining the 5' (2.5 kb) and 3' (3.45 kb) portions of the two clones, a 6.0 kb Bam HI germline fragment was predicted (Fig. 8C). Probes isolated from the 5' portion of the $V_{\kappa 14}$ clone and from the 3' region of the V flank in the DRP were hybridized to various restriction digests of genomic liver DNA. Common fragments were detected in all corresponding lanes (compare Fig. 8D and E), suggesting that the DRP and V-J2 products of PC 3609 are related by a single recombination event. Again, minor bands probably reflect cross hybridization with additional family members. These results also strongly suggest that the $V_{\kappa 14}$ -J2 recombination took place by a DNA strand inversion which served to displace the previous non-functional $V_{\kappa 8}$ -J1 product.

DISCUSSION

While it is generally accepted that kappa recombination occurs by DNA intrastrand inversion with concomitant retention or deletion of the intervening DNA (11), direct evidence from naturally occurring products has been presented for only one plasmacytoma (4). By analyzing the relationship of products from two additional plasmacytomas we have found evidence that now provides stronger support for intrastrand DNA inversion. Analysis of the products of kappa recombination examined in this study strongly suggest that V-J products and related

reciprocal products were retained in the same cell. PC 613 was chosen for analysis based upon the simplicity of kappa recombination that it exhibited. A single DNA inversion event can be invoked to generate both the functional V κ 9-J1 product and the VJ1RP (Fig. 9A). By application of the polymerase chain reaction we could demonstrate germline linkage of the V flanking region present in the VJ1RP and the V coding region present at the productive V-J gene. Apparently the regulatory process involved in allelic exclusion prevented further recombinations. The intrastrand inversion model also predicts that the retention or deletion of reciprocal products is dependent on the orientation of the germline V segment, prior to its recombination. Accordingly, the germline V κ 9 gene segment must lie in the opposite transcriptional orientation relative to the J-C locus to account for retention of the reciprocal product.

The rearrangement product relationships are more complex in PC 3609 since both kappa alleles have undergone recombination, and there are two reciprocal products. As previously noted, the V κ 8-J1 portion of the DRP clone has an octamer sequence mutation: a C residue at the first position instead of the consensus A residue (4). This suggests that an otherwise functional V-J join may have been unable to efficiently express mRNA (19). As shown in Fig. 9B, this non-functional allele could then be replaced by a secondary recombination on the same allele, similar to the process proposed in another plasmacytoma (4). This corrective recombination can explain the production of the two related products observed in PC 3609: the productive V κ 14-J2 and a DRP containing the initial V κ 8-J1 linked to the reciprocal product of the replacing V κ 14-J2. Because the fragments cloned and characterized in this study represent the end products of a multi-step process, the pathways proposed can only be speculated. However, the inter-relationships of V-J products and reciprocals demonstrated in this report clearly support the intrastrand mechanism of recombination and suggest a significant role for secondary, corrective pathways on a single kappa allele. Using Abelson-MuLV transformed pre-B cells we have recently demonstrated that corrective recombination does contribute significantly to expression of the mouse kappa repertoire (21).

ACKNOWLEDGEMENTS

We thank M. Weigert for providing plasmacytoma DNA. This work was supported by Grant GM 37687 from the National Institutes of Health.

*To whom correspondence should be addressed

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