Deletions of Immunoglobulin Cκ Region Charactherized by the Circular Excision Products in Mouse Splenocytes

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Summary

We have identified circular DNAs containing the κ light chain constant region (Cκ), as well as the excision products of Vκ-Jκ and Vκ-Jκ joining in adult mouse splenocytes. Analysis of Cκ-positive circular DNA clones revealed two recombination sites (intron recombining sequence [IRS]1 and -2) within the germline Jκ-Cκ intron region and the recombining sequence (RS) located downstream of the Cκ exon. While RS contains a conserved heptamer and nonamer separated by a 23-bp spacer on the 5′ side, IRS1 sequence is an isolated heptamer without an obvious nonamer, and IRS2 contains a variant heptamer, CACAAAA. Since IRS1 and IRS2 recombined with both RS (23-bp spacer signal) and Vκ (12-bp spacer signal) with significant frequency, intron recombination sites seem to have dual recombination signals. These findings provide direct evidence that Cκ deletion preceding λ gene rearrangement can occur by looping out and excision. Increased accessibility of inefficient recombinational loci within the intron may enable recombinase to accept wide signal sequence variation.

Materials and Methods

Construction of Circular DNA Clone Library. Spleen cells were obtained from five 7-wk-old female BALB/c mice. Circular DNAs were prepared from 2 × 10⁸ cells, and a phage library was prepared by cloning the BamHI fragments of circular DNA into the coliphage Charon27 X vector as previously described (12, 18). Phage titers per microgram of vector DNA were 10⁵ for self ligation and 3 × 10⁶ for the recombinants.

DNA Hybridization. Plaque hybridization and Southern blot
hybridization were performed according to the methods of Maniatis et al. (21). All DNA probes were used as purified inserts. Probes were as follows: a 2.1-kb HindIII C fragment of IgWES IgH701-C (22) for C, a 1.7-kb HindIII-Xbal fragment (23) for J, an 8.6-kb EcoRI fragment of Ig25λ (24) for J, a 280-bp Hpal-AvalI fragment of a Igκ cDNA clone (gift from Dr. T. Honjo, Kyoto University) for Cκ, a 0.9-kb ApaLI fragment of pKDE5 clone (this study) for RS, a 199-bp ApaLI-HindIII fragment of pKDE5 clone for IRS1, a 498-bp SspI fragment of pKDE4 clone (this study) for IRS2, and 11- and 5.2-kb BamHI fragments of rat mitochondrial DNA (25) for mouse mitochondrial DNA.

DNA Sequencing. BamHI-digested circular DNA clones were recloned into a pHSG399 plasmid. Nucleotide sequences were determined by the dideoxy chain termination method (26) using appropriate specific primers synthesized based on the RS 650-bp database, MUSIGKRS: GACACTGCTCTTTACCAGT (20-mer; 245-264) for RS; and the Jx-Cκ, 7,466-bp database base, MUSIGKC2: GAAGCCACAGACAGCACACAC (21-mer; 1184-107) for Jx; AACAATTAAACAGTTAGAC (21-mer; 1164) for Jx; CACAAGTTACCCAAACAGAAC (21-mer; 1820) for Jx; CTACCATGAAAACCTGTGTC (20-mer; 2136) for Jx; AGTCTGTCACATCTCTGTTCT (21-mer; 3255) for IRS1; AACAACTTAACAAGGTTAGAC (21-mer; 3235) for IRS1; and CACAAGTTACCCAAACAGAAC (21-mer; 3255) for IRS1). Nucleotide sequences of Cκ compared are X24(27), 17-1A (28), V139 (29), 61B8 (30), AN02K and AN11K (31), Q52 (32), k2 (33), VL38C (34), Vκ (35), V-L7 and V-L6 (36), V-D3 (37), f173 and V-L8 (38), V1C (39), and V24 (40).

Results

Circular DNA Clones Containing Cκ Gene Loci. We purified circular DNAs from spleen cells of 7-wk-old BALB/c mice and cloned the fragments after BamHI digestion into the Charon 27 phage vector (cloning capacity up to 9.2 kb). BamHI sites, which are located a short distance downstream of Cκ, and upstream of RS (Fig. 1), were expected to be useful for cloning presumptive excision products of Cκ gene rearrangements (2, 4).

We screened circular DNA clones by plaque hybridization with probes of Cκ DNA. Out of 1.2 × 10⁶ phage, we obtained 136 Cκ-positive clones (Table 1). The same set of filters was screened with probes of Cμ (Cμ) and Jκ (Jκ and Jκ). 14 Cκ-positive clones represented the excision products of class switch recombination ongoing in unstimulated spleen cells (18) and 953 Jκ-positive clones represented those of Vκ-Jκ rearrangements performed in the bone marrow (11, 12). Approximately 60% of the phage clones contained mitochondrial DNA.

To characterize the Cκ-positive clones, we selected four clones at random and compared the restriction fragments (Fig. 1). Four clones contained inserts of 5.7 kb, a size similar to the BamHI fragment expected in the excision product of RS

Table 1. Plaque Hybridization of Circular DNA Clones

| No. of clones screened | IRS2 + Cκ + RS+ hybridized with probes: | IRS2 - Cκ + RS+ hybridized with probes: |
|-----------------------|----------------------------------------|----------------------------------------|
|                       | IRS1+ IRS1-                           | IRS1+ IRS1-                           |
|                       | Jκ+ Jκ-                               | Jκ+ Jκ-                               |
|                       |                                        | Cκ+ RS+                               |
|                       |                                        | mt+                                    |
| 1.2 × 10⁶             | 14 850 103                             | 74 8                                  |
| 200                   | ND ND ND                              | ND ND ND                              |

The number of circular DNA clones in the phage libraries characterized by appropriate probes is shown. A set of phage plaque filters was hybridized successively with different probes. Mitochondrial DNA probe-positive (mt+) clone is also included.
DNA rearrangement with the J\_C\_ intron. Comparison of the C\_ positive clones with the germline RS locus and J\_C\_ intron suggested rearrangements between RS and the J\_C\_ intron (pKDE4-8) and between V\_ and J\_ (pKDE1-3). Upon closer inspection of the restriction map, a 5.3-kb (pKDE4) insert was shown to contain RS recombined with a site in the J\_C\_ intron, which is more proximal to C\_ than that of the 5.7-kb insert. Clone pKDE4 may represent a new recombination site in J\_C\_ intron.

Identification of Recombination Signals of RS/J\_C\_ Rearrangements. A synthetic primer downstream of IRS1 sequence (4) reacted with clone pKDE5 but not with pKDE4. Therefore, breakpoints of clones pKDE4 and -5 were sequenced using synthetic primers upstream of RS sequence (650 bp, MUSIGKRS, GenBank; reference 4) and downstream of IRS1 sequence (2), respectively (Fig. 2). Clone pKDE5 contained a precisely fused signal joint of RS with the IRS1 within the J\_C\_ intron. Clone pKDE4, however, showed a novel sequence fused with the upstream sequence of RS. By comparison with the published germline J\_C\_ sequence (7,466 bp, MUSIGKC2, GenBank), this novel sequence can be assigned downstream from position 3601. At the junction with RS, a variant signal heptamer, CACAAAA, without an obvious signal nonamer was observed. This is the second signal nonamer within the J\_C\_ intron, and is therefore termed as the second intron recombinating sequence (IRS2), which is distinguished from the known IRS (IRS1). Identity of these two IRS's with the usual intron recombinating points rearranged with V\_ found in myelomas (41) is not clear. To measure the frequency of these two types of recombinants, we prepared two probes, 5'RS (0.9-kb ApaLI fragment) and 3'IRS1 (199-bp ApaLI-HindIII fragment), from clone pKDE5, and a 3'IRS2 probe (498-bp SspI fragment) from clone pKDE4, respectively (Fig. 1). We obtained 82 clones positive for both the GS and RS probes. Of these 82 clones, 74 clones were double positive for IRS1 and IRS2 probes and eight were single positive for IRS2 (Table 1). The RS sequence, therefore, recombines with either IRS1 or IRS2 in the J\_C\_ intron and deletes the C\_ exon from the chromosome.

Table 2. C\_\(~+\)RS\_ Circular DNA Clones Analyzed

| Clones   | Size (kb) | V\_ subfamily | J\_ | Frame |
|----------|-----------|---------------|-----|-------|
| pKDE 1   | 8.1       | V\_4,5 (X24; 100) | J\_2 | -     |
| pKDE 2   | 4.5       | V\_8 (17-1A; 99.4) | J\_5 | (-)   |
| pKDE 3   | 6.5       | V\_8 (V139; 100) | J\_5 | -     |
| pKDE 29  | 5.2       | V\_8 (6188; 97.3) | J\_5 | -     |
| pKDE 30  | 4.3       | V\_4,5 (AN02K; 100) | J\_5 | -     |
| pKDE 31  | 7.2       | V\_x1 (Q52; 97.4) | J\_5 | -     |
| pKDE 32  | 5.3       | V\_x12,13 (k2; 84.5) | J\_x2 | +     |
| pKDE 33  | 8.0       | V\_9 (V\_unc; 81.9) | J\_x2 | -     |
| pKDE 34  | 4.4       | V\_x23 (AN11K; 86.4) | J\_x4 | -     |
| pKDE 35  | 7.2       | V\_x1 (Q52; 99.2) | J\_x5 | -     |
| pKDE 36  | 6.6       | V\_28 (V\_ser; 81.2) (IRS) | J\_x5 | -     |

Most homologous V\_x and percent homology are shown in parentheses. +, in frame; (+), in frame with nonsense codon; -, out of frame. Clone pKDE36 has a signal joint (S) of V\_xIRS.

Figure 2. Nucleotide sequences at the recombination sites of circular DNA clones. The recombinant sequences are compared with their corresponding germline sequences of RS (MUSIGKRS) and IRS1. Conflicts between MUSIGKJC2 data base and the IRS1 germline sequence are as follows: bases 3175 (G to C), 3176 (C to G), and 3201 (deletion). The signal sequences are underlined and the coding joints are shown by arrows.
Nucleotide sequencing revealed the $V_\epsilon$ sequence rearranged with $J_\epsilon$. In Table 2, the most homologous $V_\epsilon$ sequence and the percent homology are summarized for each clone. Most sequences of at least 100-bp nucleotides are assigned to a known $V_\epsilon$ subfamily (43, 44), based on the criterion of 80% homology threshold. Only one translational reading frame (pKDE32) out of 10 coding joints was in phase and free of nonsense codons. Coding joint of pKDE2 was inframe but included a premature termination codon.

Clone pKDE36 did not contain the $J_\epsilon$ region but reacted with the IRS1 probe. Nucleotide sequencing was performed with a specific nucleotide primer downstream of IRS1 (Fig. 2). This clone contained the precise head-to-head fusion of two heptamers in a signal joint resulting from $V_\epsilon$ subfamily recombining with the sequence downstream of IRS1. There is no precedent for such a $V_\epsilon$/IRS signal joint.

The frequency of these two types of $C_\epsilon^+R_\epsilon^-$ recombinants was measured using a $J_\epsilon$ probe (1.7-kb HindIII-BglIII fragment). Of 54 $C_\epsilon^+R_\epsilon^-$ clones, 48 clones were positive for $J_\epsilon$ probe, but six clones were negative (Table 1). Out of six $C_\epsilon^+R_\epsilon^-J_\epsilon^-$ clones, four clones were IRS1$^+$IR$S2^-$ and two clones were IRS1$^-$IR$S2^+$. Since no obvious signal sequence was found in the flank between IRS1 and IRS2, IRS2 signal sequence is likely to be used to generate the signal joint with $V_\epsilon$ in these two $J_\epsilon^-IRS1^-IRS2^+C_\epsilon^-R_\epsilon^-$ clones. These rearranged $C_\epsilon^-R_\epsilon^-$ fragments may represent the final

| Table 3. $C_\epsilon^-R_\epsilon^+$ Circular DNA Clones Analyzed |
|------------------------------------------------------------|
| Clones | Size (kb) | $V_\epsilon$ subfamily used in SJ |
|---------|-----------|---------------------------------|
| pKDE 11 | 6.6       | $V_\epsilon$23 (V-L7; 100) |
| pKDE 12 | 6.5       | $V_\epsilon$21 (V21B; 100)   |
| pKDE 13 | 5.2       | $V_\epsilon$9 (F173; 96.8)   |
| pKDE 14 | 6.6       | $V_\epsilon$23 (V-L7; 100)   |
| pKDE 15 | 6.2       | $V_\epsilon$7-1 (V-L6; 89.3) |
| pKDE 16 | 4.4       | $V_\epsilon$4,5 (V-L8; 92.8) |
| pKDE 17 | 5.1       | $V_\epsilon$9 (F173; 86.3)   |
| pKDE 18 | 5.2       | $V_\epsilon$1 (V1C; 77.4)    |
| pKDE 19 | 7.5       | $V_\epsilon$4,5 (V-L8; 90.4) |
| pKDE 20 | 7.5       | $V_\epsilon$4,5 (V-L8; 90.4) |
| pKDE 21 | 6.0       | $V_\epsilon$1 (V1C; 99.6)    |
| pKDE 22 | 4.8       | $V_\epsilon$7-1 (V-L6; 80.9) |
| pKDE 23 | 6.2       | $V_\epsilon$24 (V24; 71.2)   |
| pKDE 24 | 10.0      | $V_\epsilon$4,5 (V-L8; 88.5) |
| pKDE 25 | 6.5       | $V_\epsilon$21 (V21B; 100)   |
| pKDE 26 | 4.8       | $V_\epsilon$28 (Vser; 82.9)  |
| pKDE 27 | 5.0       | $V_\epsilon$28 (Vser; 82.7)  |
| pKDE 28 | 5.3       | $V_\epsilon$12,13 (F2; 78.0) |

Most homologous $V_\epsilon$ and percent homology are shown in parentheses.
The pattern of $\kappa$ usage in the excision products of $\kappa$ was compared with a tentative genetic map of the $\kappa$ locus (43, 44). The distributions of used $\kappa$ are shown in Fig. 3 along with the summarized data of $\kappa$ usage in the excision products of $\kappa$ rearrangements (11, 12). The $\kappa$ usage in the terminal phase of $\kappa$ rearrangements were dispersed throughout the locus (Fig. 3, A and B), in contrast to the biased usage of $\lambda$-distal $\kappa$ in primary rearrangement and of $\lambda$-proximal $\kappa$ in secondary deletional events (Fig. 3, C and D). Since approximately half of the $\kappa$ gene segments lie in the opposite polarity on the chromosome relative to the $J_k$ segments (46), primary inversion events may engage distal $\kappa$ gene segments while preserving proximal genes for secondary rearrangements by deletion. However, at the terminal phase of $\kappa$ rearrangements after multiple rounds of inversion and deletion, the original chromosomal position of $\kappa$ may have been shuffled. Usage of dispersed $\kappa$ in the early and late rearrangements of genes has been shown by others (47, 48) and is in contrast to the biased usage of $\lambda$-proximal $\kappa$ in VDJ rearrangements (1).

While we have shown that the productive $\kappa$-$J_k$ rearrangements occur in approximately one out of every three rearrangements (12), only 1 out of 10 $\kappa$-$J_k$ coding sequences excised by the RS recombination showed a productive $\kappa$ gene rearrangement (Table 2). Upon closer inspection of rearrangement status, all $\kappa$'s rearranged with the other $J_k$'s (which can serve as a substrate for further rearrangement) showed one productive rearrangement of four coding joints. This agrees with previous data showing a lack of feedback inhibition by functional $\kappa$ gene rearrangement (12). These results suggest that premature B cells that have generated both intermediate and dead-end products in stochastic mechanisms may switch to $\lambda$ gene rearrangements by RS recombination.

Recognition sequences that mediate site-specific recombination flank all Ig gene segments involved in V(D)J gene rearrangement. A complete recognition sequence contains a highly conserved palindromic heptamer (head), which is contiguous to the end of the coding sequence, and a characteristic nonamer (tail), separated from the heptamer by a non-conserved spacer region of either 12 or 23 bp. Joining appears to occur only between elements flanked, respectively, by recognition sequences having spacers of 12 and 23 bp (12/23 joining rule) (8, 9). In Fig. 4, we summarize the recognition sequences involved in the RS recombination. While the RS sequence contains complete recognition signals, IRS1 and IRS2

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Comparison of signal sequences. Consensus V and J sequences (8); V21C and J1 (23); IgH Dsp2 (49); $\delta$Re's and $\psi$Jc (17); $\kappa$ internal heptamer (6, 7); and other sequences from this paper. More or less essential nucleotides in the consensus sequences are underlined (51). The bases found in a consensus sequence are marked by an asterisk.

Adult mouse splenocytes, we identified circular DNA excised by the recombination of RS DNA and obtained 82 Cc $\delta$S clones and 67 Cc $\delta$S clones, representing RS/$J_k$-$C_k$ and RS/$\kappa$ rearrangement, respectively. From the same library, we also obtained an equivalent amount of 103 J1 $\kappa$ clones that represent the excision products of $\kappa$-$J_k$M rearrangements. The coincidence in frequency of excision products by RS recombination and $\kappa$-$J_k$ recombination is consistent with the notion of a regulatory role for $C_k$ deletion in the initiation of $\lambda$ gene rearrangement.

According to the studies on $\lambda$ and $\kappa$ gene rearrangements in pre-B cell differentiation (45), RS rearrangement occurs mainly toward the end of the $\kappa$-recombinating phase of pre-B cell development, before the initiation of $\lambda$ recombination. Therefore, the rearrangement status of $\kappa$ and $J_k$ found in the excision products of $C_c$-deleting recombination may represent the terminal phase of $\kappa$ rearrangements before $\kappa$ to $\lambda$ isotype switching. We found that the $J_k$ segment located at the $3'$ end of the $J_k$ cluster is most frequently used (Table 2), in contrast to the biased usage of $J_k$ at the most $5'$ side observed in the excision products of primary $\kappa$-$J_k$ rearrangement (12). This suggests that $J_k$ is the last $J_k$ used after the multiple rounds of $\kappa$-$J_k$ recombinations.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Multiple recombination pathways resulting in the inactivation and deletion of $C_c$ exon. Arrow shows the direction of donor to acceptor signal in deletional recombination by normal or pseudonormal joining. Inversional recombinations are also possible for $\kappa$'s in the opposite polarity. Recombination signal sequences and the polarity are shown as a filled triangle for a 23-bp spacer signal, an open triangle with the rectangle for a 12-bp spacer signal, a single open triangle for an isolated heptamer, and rectangles for $V$, $J$, $C$ exons.
sequences seem to be isolated heptamers without obvious nonamer sequences. Other isolated recognition heptamers with physiological roles are found in the 3'-internal region of most Vn genes used for Vn gene replacements (6, 7). IRS1 recombined with either a 23-bp spacer signal (RS) or a 12-bp spacer signal (Vn), forming head-to-head fused heptamers. The palindromic nature of the heptamer would suggest the possibility of either direct or inverted signal alignment in an intermediate structure. However, signal heptamers of IRS may have been polarized as head to tail, thereby preventing Ck inversion mediated by IRS/RS rearrangements. Alternatively, the IRS heptamer may be provided by a binary spacer signal. Weak nonamer signal motifs are recognizable at either 12- or 23-bp distance downstream of the signal heptamer (Fig. 4). Other binary spacer signals containing an additional heptamer have been implied in the IgH D gene fragment (49, 50).

According to Hesse et al. (51), the first three bases (CAC) closest to the head in the consensus heptamer sequence are strictly required, and the middle base, A, is less stringently specified, while the sixth and seventh positions (AA) of the nonamer may be necessary for efficient recombination. In this context, IRS1 and IRS2 contain similar signal sequence motifs. Less recombination efficiency of IRS2 with the RS compared with the IRS1/RS recombinations is possibly due to the variant heptamer (CACAAAA) (Fig. 4). A deletional rearrangement analogous to the IRS/RS recombination is the variant heptamer have been implied in the IgH D gene fragment (49, 50).

While the SRec1 sequence contained a complete recombination signal, SRec2 and -3 sequences seem to be less efficient and may be necessary for efficient recombination. In this context, IRS1 and IRS2 contain similar signal sequence motifs. Less recombination efficiency of IRS2 with the RS compared with the IRS1/RS recombinations is possibly due to the variant heptamer (CACAAAA) (Fig. 4). A deletional rearrangement analogous to the IRS/RS recombination is the joining of TCR δ gene deleting elements (δRec2) with ψ1α flanking the Ca gene, leading to Ca exon deletion (17, 52). While the δRec1 sequence contained a complete recombination signal, δRec2 and -3 sequences seem to be less efficient due to the lack of the necessary requirements.

Although the presence of dual recognition signals requires modification of the 12/23-bp joining rule, all recombinations analyzed in splenocytes contained standard recombination products (coding joints and signal joints) with no unusual products; neither hybrid joints nor open-and-shut joints (53) were observed. The prevalence of nonstandard recombination for extrachromosomal substrates may not accurately reflect rearrangement in the endogenous context.

There are several pathways to delete or inactivate the κ genes before the initiation of λ gene rearrangement as summarized in Fig. 5. The RS/IRS recombination results in a deletion of the Ca region, whereas the RS/Vn recombination deletes the entire Jκ-Ca region. These recombination results in the elimination of the κ enhancer elements located on both sides of Ca (54). Thus, Ca deletion could prevent the useless expression of nonfunctional κ genes in λ chain-producing cells and ensure λ gene use. Selsing et al. (55) have proposed that the RS recombinants could encode a trans-acting factor that signals the activation of the λ genes for recombination. We provided direct evidence of Ca excision products generated by the standard recombination in both pathways of RS/IRS and RS/Vκ joining. We also provided evidence for standard IRS-Vκ recombination products mediated by either inversive or pseudornormal joining depending on the orientation of Vκ. Recently, a novel two-step rearrangement pathway, in which Jκ/IRS1 rearrangement forms a signal joint capable of further Vκ recombination, has been shown to occur with significant frequency in the Abelson MuLV-transformed cell line (42). These standard rearrangements of IRS with Jκ or Vκ effectively exclude functional expression from that allele. Thus, IRS plays a key role for both recombination pathways resulting in Ca deletion and the cessation of nonfunctional κ gene expression. An unusual recombination event for the Vκ-IRS hybrid joint and pseudornormal Jκ-IRS joining has been found in myelomas (56) and plasmacytomas (57), respectively. Although the Jκ-Ca intron is transcriptionally active after multiple Vκ-Jκ rearrangements, the destractive recombinational activity of IRS loci remains limited, perhaps due to the weak recombination signal motifs. Activation of the IRS loci for recombination resulting in Ca deletion may occur as part of the developmental switch that leads to λ chain rearrangement.

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