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EVIDENCE THAT THE INTERVENING SEQUENCE WAS EXCISED AS A LINEAR MOLECULE DURING D_β-J_β REARRANGEMENT IN T-CELL RECEPTOR β CHAIN GENE LOCI

Teruo Ino*+, Masami Hirano+ and Yoshikazu Kurosawa*

*Institute for Comprehensive Medical Science, and +Department of Internal Medicine, Fujita-Gakuen Health University, School of Medicine, Toyoake, Aichi, Japan 470-11

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SUMMARY : In a previous paper (Proc. Natl. Acad. Sci. USA 84: 4264, 1987) we reported an unusual DNA rearrangement in T-cell receptor β chain gene loci in cells from a patient with human T-cell leukemia. A $D_{\beta1}$-$J_{\beta2.3}$ junction was found on one chromosome, while the other chromosome kept the germline configuration. Although the DNA fragment located between the $D_{\beta1}$ and $J_{\beta2.3}$ loci should have disappeared from the cells, it was found on chromosome 6 as an inserted segment. We have now determined the nucleotide sequences bordering both sides of the inserted segment. The signal sequence for D_β-J_β rearrangement at the 5' side of $J_{\beta1.2}$ gene seems to have been used for the insertion. The 3' end of the inserted segment corresponded to the edge of the signal heptamer at the 5' side of $J_{\beta2.3}$ which was used for the initial $D_{\beta1}$-$J_{\beta2.3}$ joining. This indicates that, during D_β-J_β rearrangement, the intervening sequence was excised as a linear molecule.

Variable regions of T-cell antigen receptors (TCR) are encoded by two or three split genes : V, (D) and J (for reviews see refs 1, 2). During T-cell ontogeny, V-(D)-J joinings occur to form complete V genes. In the vicinities of germline V, D and J genes, there are characteristic heptamers, such as CACAGTG or CACTGTG, and nonamers, like ACAAAAACC or GGTTTTTGT. These oligomers are separated by regular spacers, one 12 nucleotides long and another 23 nucleotides long. The rearrangements seem to occur as follows: The putative recombinase for V-(D)-J rearrangements recognizes two sets of the signal heptamer and nonamer, one containing a 12-nucleotide spacer and the other a 23-nucleotide spacer. An endonuclease first cuts the side of the signal heptamer. A ligase joins both heptamers, resulting in a head-to-head structure referred to as a "reciprocal joint"(3). The edges of the coding regions neighboring the heptamers are processed by an exonuclease and ligated to form a "coding joint"(3). Apparently, immunoglobulin (Ig) and TCR genes both utilize the same recombination mechanism(4, 5). Recently, Fujimoto and Yamagishi(6), and Okazaki et al. (7) identified extrachromosomal circular DNA containing the above head-to-head structure derived from TCR α-chain and β-chain gene loci in mouse thymocytes. The circular DNA seems

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to represent excision products of V_α-J_α, V_β-D_β and D_β-J_β joinings. However, there was no evidence as to whether both reactions (ie. cutting by an endonuclease and joining by a ligase) are coupled during the recombination process, or whether an initial linear product generated by an endonuclease becomes a circular DNA later. In this report we present evidence that the intevening sequence was excised as a linear molecule during D_β-J_β rearrangement in T-cell receptor β chain gene loci.

MATERIALS AND METHODS

A T-cell tumor analyzed in this study and clone 1-4E11 were described in a previous paper (8). Clone GC1-4 was isolated from Maniatis' human genomic library (9) according to Benton-Davis' plaque hybridization method (10). Clone 3-7E6 was isolated from the leukemia cell DNA by the ordinary cloning procedure as described by Sakano et al. (11). The nucleotide sequence was determined by the dideoxy method (12). Southern hybridization was carried out by the same method as described (13).

RESULTS AND DISCUSSION

In a previous paper (8) we reported an unusual DNA rearrangement in TCR β chain gene loci residing on chromosome 7 in cells from a patient with human T-cell leukemia. In these cells, a $D_{\beta1}$-$J_{\beta2.3}$ junction was found on one chromosome, while the other chromosome kept the germline configuration. Although the DNA fragment located between $D_{\beta1}$ and $J_{\beta2.3}$ loci should have disappeared from the cells, it was found on chromosome 6 as an inserted segment. If the excision product during $D_{\beta1}$-$J_{\beta2.3}$ joining had taken on a circular form and its insertion into chromosome 6 occurred without deletion of the DNA, the inserted fragment should have contained the head-to-head structure as described in Introduction. Such a DNA region could have been identified by Southern hybridization with the 3'-$D_{\beta1}$ probe shown in Fig.1 as a rearranged band in EcoRI-digested DNA. Since V_β-D_β joining usually occurs after D_β-J_β formation and the 3'-$D_{\beta1}$ region should be deleted during normal $D_{\beta1}$-$J_{\beta1}$ or $D_{\beta1}$-$J_{\beta2}$ rearrangement, the rearranged bands detectable by 3'-$D_{\beta1}$ probe would have been the products of insertion by the above process. Based on these principles we searched for examples of insertion of reciprocal-joint-containing (RJC) fragments into other chromosome loci in more than 40 T-cell leukemia cells. Using four DNA probes : the C_β probe, 5'-$D_{\beta1}$ probe and $J_{\beta2}$ probe described in a previous paper (8), and 5'-$D_{\beta2}$ probe located upstream of $D_{\beta2}$ gene, we were able to determine the types of DNA rearrangement by Southern hybridization. Among them, we found unusual DNA rearrangements (ie, other than D_β-J_β or V_β-D_β) of TCR β-chain genes in three leukemia cells: two were obtained from patients with adult T cell leukemia and another was from chronic T cell leukemia (unpublished). However, the 3'-$D_{\beta1}$ probe detected no examples of

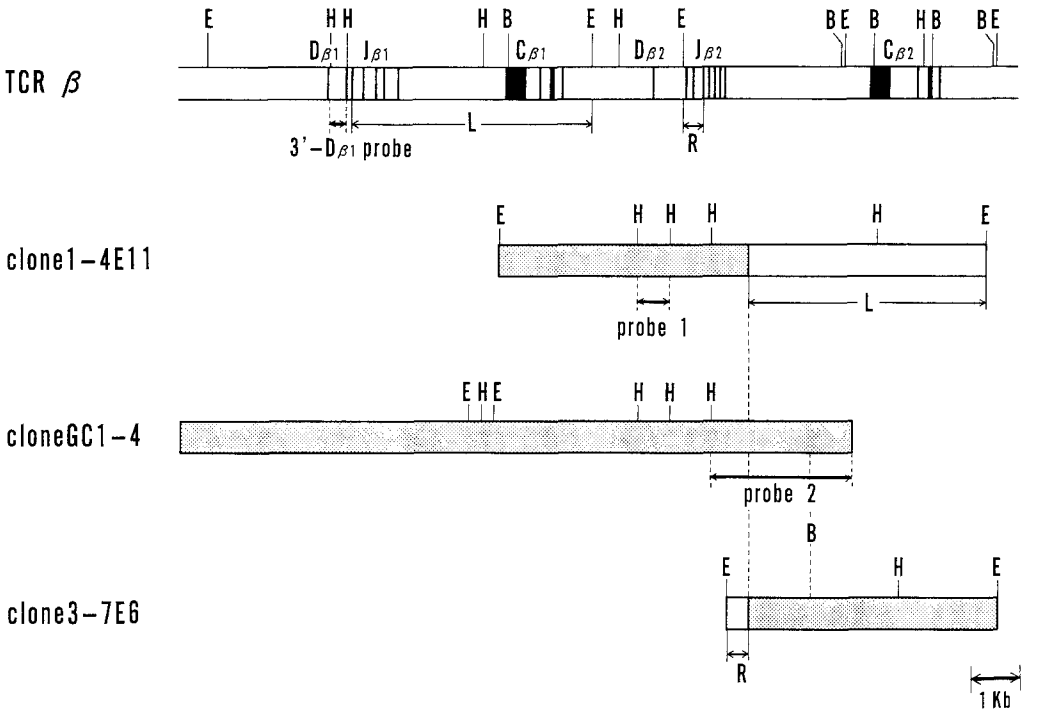

Fig.1. Restriction maps of human TCR β chain gene loci and the clones analyzed in this study. The DNA segment from $J_{\beta1.2}$ to the 5'-flanking region of $J_{\beta2.3}$ was inserted into chromosome 6 in a human T-cell leukemia cell. Restriction map of TCR β chain gene loci was from ref.14. Equivalent portions of clone 1-4E11 and clone 3-7E6 were aligned with those of germline clone GC1-4. L in TCR and clone 1-4E11 and R in TCR and clone 3-7E6 indicate the equivalent regions. Abbreviations : E, EcoRI; H, HindIII; B, BglII.

rearranged bands. As shown in Fig.2 (a), even the T-cell leukemia cell DNA described in a previous paper (8) gave only the germline band, indicating that the 3'-$D_{\beta1}$ region which should have existed on the excised fragment was deleted after insertion.

In order to characterize the inserted region and the mechanism of insertion in the T-cell leukemia cells preciously reported (8), we obtained two clones: one is from Maniatis' human genomic library and the other from the T cell leukemia cell DNA which is the same as used in a previous paper (8). Using probe 1 shown in Fig.1, a germline clone GC1-4, containing the inserted region was isolated. Figure 2(b,c) shows Southern hybridization of BglII-digested DNA from placenta and the T-cell leukemia cell. Although probe 2 identified several faint bands other than 11 kb major band in placenta DNA, two distinct rearranged bands were detected at 14 kb and 6 kb in the the leukemic cell DNA (Fig. 2(c)). Since the 6 kb band was also detected as a rearranged band by C_β probe (Fig. 2(b)), this band should contain the 3' edge of the inserted fragment. The 14 kb band is derived from its 5' edge. Thus, probe 2 identified both ends of the inserted region. Probe 2 detected only one rearranged band at 6 kb in the

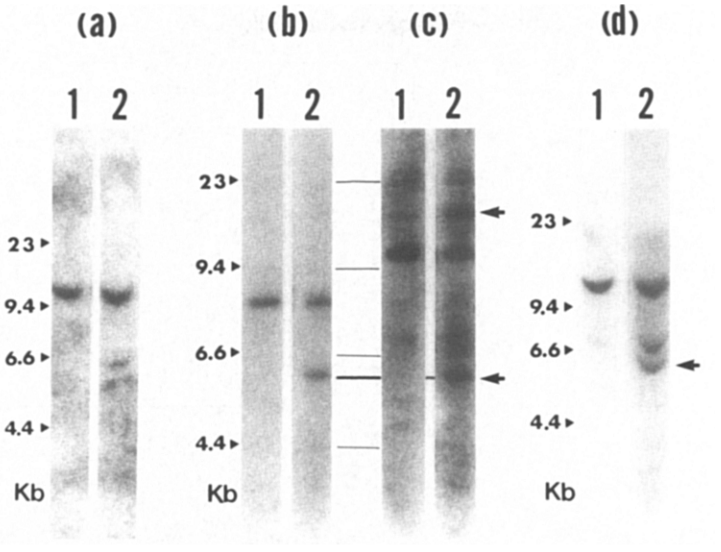

Fig.2. Southern hybridization of placenta DNA and the DNA of the leukemia cells. Restriction enzymes : (a, d) EcoRI ; (b, c) BglII. Probes : (a) 3'-$D_{\beta 1}$ probe; (b) C_{β} probe; (c, d) probe 2. DNAs : 1, placenta ; 2, leukemia cell. The rearranged bands are indicated by thick arrows.

EcoRI-digested DNA of the leukemia cells (Fig. 2(d)). Judging from the restriction map of the inserted region, the size of rearranged band corresponding to the 5' edge is fortuitously the same as the germline band. The 6 kb-EcoRI fragment identified with probe 2 contains the 3' edge of the inserted fragment. We cloned this fragment and named it clone 3-7E6. We determined the nucleotide sequences of the boundaries of the inserted segment and the germline sequences at the inserted region. The nucleotide sequence of the inserted segment's 5'-boundary is compared with germline sequences of the inserted region and TCR gene loci (14) in Fig.3(a).

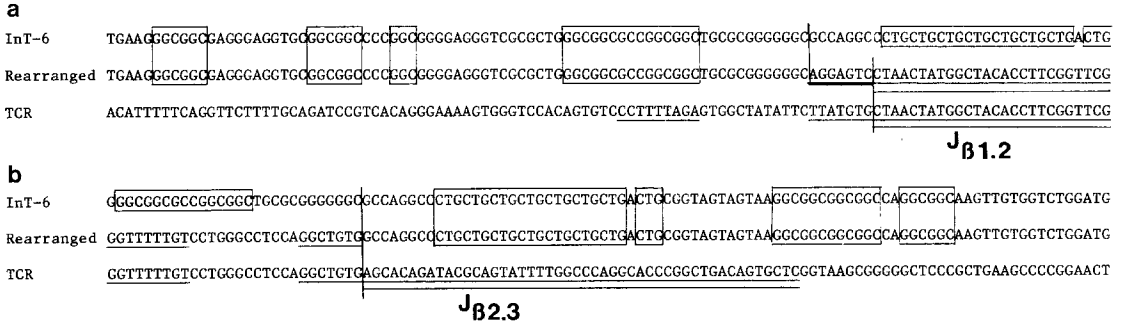

Fig.3. Comparison of the nucleotide sequences around the boundaries of the inserted fragment. a. (upper) Germline sequence of InT-6 region. (middle) The sequence around the 5'-boundary of the inserted segment. (lower) Germline sequence of TCR β chain gene (14). b. (upper) Germline sequence of InT-6 region. (middle) The sequence around 3'-boundary of the inserted segment. (lower) Germline sequence of TCR β chain gene loci. Vertical lines indicate boundaries of the inserted segment. Signal nonamer and heptamer sequences are underlined by thin lines. J_{β} genes are underlined by two lines. "N" segment is underlined by a thick line. Repeating sequences of GGC and CTG are boxed.

The breaking point in TCR gene loci is the edge of the signal heptamer at the 5' side of $J_{\beta1.2}$ gene. Although there is no typical signal sequence in the germline sequence of the inserted region, it is likely that the recombination machinery for V-(D)-J joining was utilized for the insertion for the following reasons: The boundary in T-cell receptor β chain gene loci corresponds exactly to the edge of the signal heptamer. The extranucleotide AGGAGTC, so-called N segment (15), was inserted into the boundary. Several reports (16-19) show that translocation involving Ig or TCR gene loci seems to be mediated by the recombinase, but they could not find a typical signal sequence in the recombination partner. We refer to the inserted region of TCR β chain gene-containing fragment in chromosome 6 as InT-6. In InT-6 region, GC-rich sequence and repetitive sequences of GGC and CTG can be seen (Fig.3).

The nucleotide sequence of the 3'-boundary of the inserted segment is also compared with germline sequences of the inserted region and TCR gene loci in Fig.3(b). The 3' end of the inserted segment corresponded to the edge of the signal heptamer at the 5' side of $J_{\beta2.3}$ which was used for the initial $D_{\beta1}$-$J_{\beta2.3}$ joining. As shown by Southern hybridization with the 3'-$D_{\beta1}$ probe (Fig.2a), the 3'-$D_{\beta1}$ region which should have existed on the excised fragment was deleted, but there were no deleted nucleotides at the 3' end. In a previous paper (8), we argued the possibility that the excised fragment took on a circular form. However, since it appears that the head-to-head structure is not re-cut in the middle after formation of circular DNA, we propose that excision-insertion occurred according to the following process (Fig.4): During $D_{\beta1}$-$J_{\beta2.3}$ joining the intervening sequence between $D_{\beta1}$ and $J_{\beta2.3}$ was excised as a linear molecule. An endonuclease in the recombination machinery first cut both the signal heptamer at the 5' side of $J_{\beta1.2}$ and the specific loci in chromosome 6 at ~ GGGGGC↓GCCAGG ~ / ~ CCCCCC↓CGGTCC ~. After insertion of N segment, possibly by terminal transferase (15), ~GGGGGC / ~CCCCCG in chromosome 6 and the edge of $J_{\beta1.2}$ gene was ligated. The 3' end of the excised fragment and the remaining end of GCCAGG~ / CGGTCC~ in chromosome 6 were connected. Thus, the 3' $D_{\beta1}$ region on the excised fragment was deleted.

The major arguments supporting the formation of circular DNA during V-(D)-J joining are based on the presence in thymocytes of circular DNA containing head-to-head structure (6, 7). However, instead of head-to-head structure, Okazaki et al.(7) reported the presence of several extra nucleotides between the heptamers in the presumably reciprocal joints. The observed circular DNAs might be ligated products of excised linear molecules. In certain cases, where the polarity of the relevant genes embedded in the chromosome is opposite, inversion occurs (20). For such

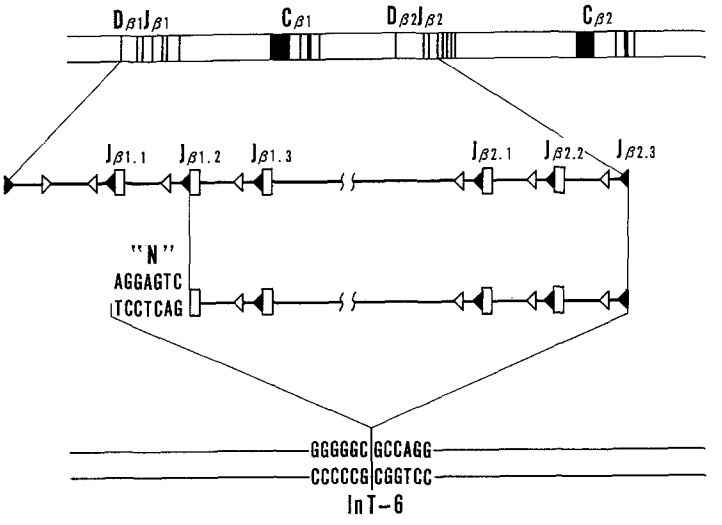

Fig.4. Scheme of excision and insertion. During $D_{\beta 1}$-$J_{\beta 2.3}$ joining, the DNA fragment between $D_{\beta 1}$ and $J_{\beta 2.3}$ genes was excised as a linear molecule. A putative recombinase recognizes the signal heptamer upstream of $J_{\beta 1.2}$ on the excised fragment and the sequence in InT-6 region and cuts both fragments. After insertion of "N" segment, both ends are ligated. Remaining ends of both fragments are ligated. The signal heptamers and nonamers are indicated by closed and open triangles, respectively.

inversion, coupled reactions of cutting and ligation seem to be essential to maintain chromosome integrity. Even so, the initial product(s) of an excised or inverted fragment can be in linear form. This paper reports the first example where both the coding joint of D-J joining and its excised product were identified in one cell. Previous papers (6, 7) described products from heterogeneous cell populations. The isolation and characterization of recombination machinery will help lead to the precise mechanism of recombination.

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