Brief Definitive Report

Circular DNA Resulting from Recombination between V-(D)-J Joining Signals and Switch Repetitive Sequences in Mouse Thymocytes

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Summary

During the course of analyzing circular DNA in mouse thymocytes, novel recombinants were identified with immunoglobulin heavy chain joining gene and switch region probes. These circles represent excision products of recombination between the heptamer-nonamer motif for V-(D)-J joining and a repetitive sequence for class switching. The molecular mechanisms that generate "hybrid circles" are discussed.

Somatic DNA recombination plays a key role in activating and diversifying the Ig and TCR genes during lymphocyte development. For the V-(D)-J type of joining, recombination signal sequences (RSS's) are found adjacent to each germline V, D, or J segment, consisting of a highly conserved heptamer, CACTGTG, and nonamer, GGTTTTTGT, separated by a spacer of constant length (1). Normally, recombination occurs between one RSS containing a 12-bp spacer and a second RSS containing a 23-bp spacer; this is the so-called 12/23-bp spacer rule.

Another type of rearrangement, known as class switch recombination, occurs only in the Ig H chain genes. This recombination is responsible for changing the isotype of Ig H chains, by replacing an upstream set of C gene exons with another downstream set. Switch recombination takes place between a pair of sites, one in the intron between the J_H and $C\mu$ gene, the other in a region upstream from one of the other C genes (2). These recombination sites are variable and lie in the switch (S) regions. The S regions lack conserved recombination signal sequences, such as the heptamer-nonamer motifs or V-(D)-J joining, but are rich in repetitive sequences. Although little is known about the enzymatic machinary, it is generally believed that two distinct "recombinases" mediate V-(D)-J joining and class switch recombination.

To study the mechanisms for V-(D)-J joining and class switch recombination, we and others have previously characterized extrachromosomal circular DNA in lymphocytes. Thymocyte circular DNA contains the excision products derived from the V-(D)-J joining of TCR genes (3, 4). More recently, it was shown that switch-activated B cells contain circular DNA derived from the switch recombination between two distinct S regions (5–8). In general, the characterization of circular DNA in lymphocytes has shown that both V-(D)-J joining and the Ig class switch are accompanied by intramolecular DNA deletion, which results in covalently closed excision products.

During the course of analyzing circular DNA in thymocytes, we noticed that clones positive with Ig J_H region probes could be isolated from the circular DNA library. Previous work on the J_H genes from T cell lines showed that IgH D-to-J joining often occurs in T lineage cells (9). Curiously, we have found that most of the rearranged clones isolated with J_H region probes did not contain the normal signal joint of two fused RSS's, but instead contained structures resulting from recombination between a RSS of D or J segments and a site in the switch repetitive region. In this report, we characterize the unusual circular molecules by restriction enzyme mapping and DNA sequencing.

Materials and Methods

Preparation of Circular DNA. Thymus glands from 3-wk-old BALB/c mice were used for the preparation of thymocyte circular DNA as previously described (3). The circular DNA material was treated with ATP-dependent DNAase of *Micrococcus luteus* (U.S. Biochemical Corp., Cleveland, OH) to eliminate residual chromosomal DNA. A phage library was made with λgtWES.

Flow Cytometric Analysis. For FACS® staining analysis (Becton Dickinson & Co., Mountain View, CA), cells from disrupted thymic tissue were passed through Nytex, and washed three times in HBSS with 2% FCS, 0.1% azide. Cells (10^6) were incubated at 4°C for 1 h with saturating amounts of FITC-conjugated anti-Thy-1.2 mAb (30-H12; Becton Dickinson & Co.) or FITC-conjugated anti-B220 mAb (RA3-6B2; courtesy of N. Glaichenhaus, American Type Culture Collection, Rockville, MD). After washing, cells were analyzed on a FACS® 440 (Becton Dickinson & Co.).

DNA Probes. DNA probes used for screening of the circular DNA clones are as follows: (*a*) $D_{FL16.1}$, 0.8-kb BamHI-BamHI; (*b*) 3′-$D_{SP2.8}$, 340-bp PstI-PstI; (*c*) 5′-J_H, 1.6-kb EcoRI-XbaI;

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(*d*) 3′-D_{Q52}, 335-bp BglII-BglIII; (*e*) J_{H1}, 597-bp BamHI-HindIII; (*f*) J_{H2}, 407-bp HindIII-BamHI; (*g*) J_{H3}, 384-bp BamHI-HindIII; (*h*) 3′-J_H, 682-BP XbaI-EcoRI; (*i*) J_{H1-4} (includes D_{Q52}), 4.0-kb XbaI-XbaI; (*j*) 5′-Sμ, 309-bp EcoRI-XbaI; and (*k*) 3′-Cμ, 1.0-kb HindIII-HindIII. The J_H-positive clones were isolated with probe *i*, the 5′-Sμ-positive clones were isolated with probe *j*.

DNA Sequencing and Other DNA Methods. Nucleotide sequences were determined by the chain-termination method with dideoxynucleotides (10). DNA clones were analyzed by standard procedures.

Results and Discussion

The mouse thymocyte circular DNA library was screened with a 4.0-kb XbaI-XbaI probe containing D_{Q52} and the entire J_H region. With this probe, >200 J_H-positive clones were isolated from the library of 350,000 recombinant phage. The frequency of J_H-positive clones was comparable with that previously observed for screenings with TCR J_α probes (3). This indicates that J_H excision events are not rare in thymocytes from 3-wk-old mice. 38 of these clones were single plaque purified and analyzed further with several J_H region probes. The hybridization data showed that 31 of these clones represent excision products in which both D_{Q52} and the J_H segments remain in the germline configuration. Seven other J_H-positive clones contained rearrangement in the 5′ region. They were positive with J_{H1-4} and 3′J_H probes, but negative with the 5′J_H probe. The library was also screened with the 5′-Sμ probe, a 309-bp EcoRI-XbaI fragment. 23 5′-Sμ-positive clones were single plaque purified and analyzed further with various IgH probes. These hybridization experiments showed that most of the Sμ-positive clones also contain J_{H1-4}, and 3′-J_H sequences. Some of thse clones were further analyzed by restriction mapping. They include clones ICD116, ICD137, ICD144, ICD162, ICD171, ICD172, and ICD165.

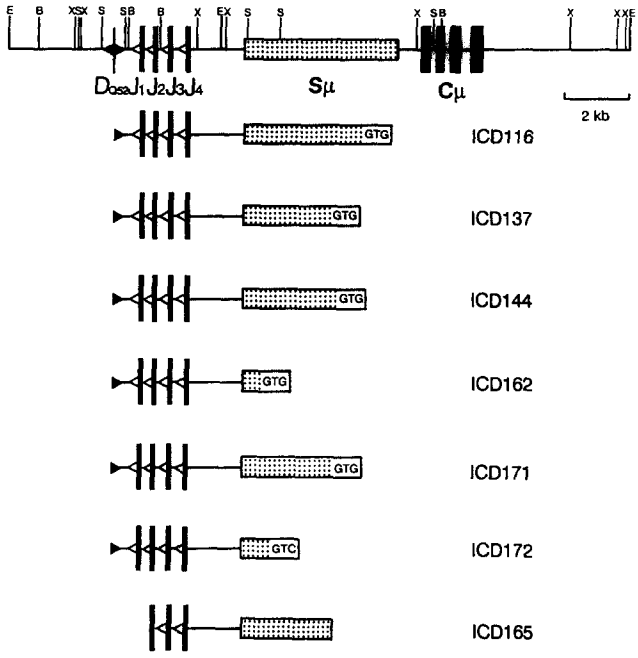

Figure 1. Circular DNA molecules resulting from recombination between RSS and switch sequences. Seven clones were analyzed by restriction enzyme mapping. By comparing the maps of recombinants with those of the germline regions, recombination sites were predicted. DNA regions excised into the circles are shown. In the map, switch (Sμ) regions are stippled. Coding sequences (D_{Q52}, J_H's, and Cμ exons) are indicated by vertical bars. Triangles represent recombination signal sequences (RSS's) for V-(D)-J joining. Restriction sites are shown: EcoRI (E), BamHI (B), XbaI (X), and SacI (S).

By comparing restriction enzyme cleavage maps, the recombination sites on the clones were predicted. In Fig. 1, DNA regions excised into the circular DNA are shown underneath the germline map of the J_H-Cμ region. Clones were charac-

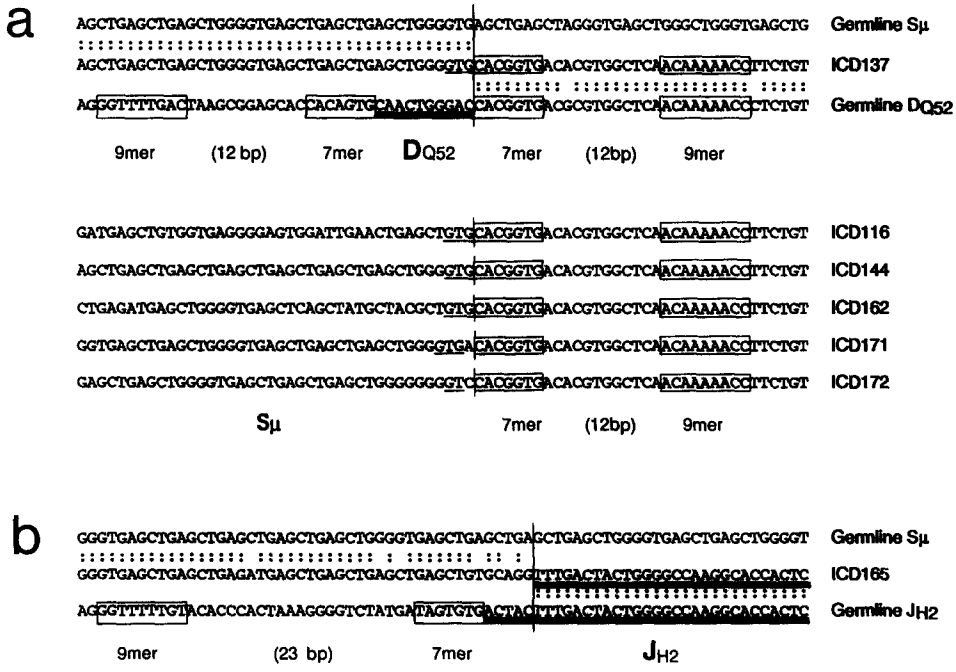

Figure 2. Nucleotide sequences of seven clones of hybrid circles. Clones ICD116, ICD137, ICD144, ICD162, ICD171, ICD172, and ICD165 were analyzed. Sequences around the breakpoint were compared with their corresponding germline sequences. Precise recombination sites (indicated by vertical lines) were determined by comparing the sequences of recombinant clones with those of germline counterparts. For the clone ICD165, the best fitted Sμ consensus sequence (MUSIGCD09) was used as the germline sequence. Heptamers and nonamers of the RSS's are boxed. D_H- and J_H-coding sequences are bold underlined. The S_μ trinucleotide sequence GTG, found at the breakpoint, is underlined. Colons between two sequences indentical residues.

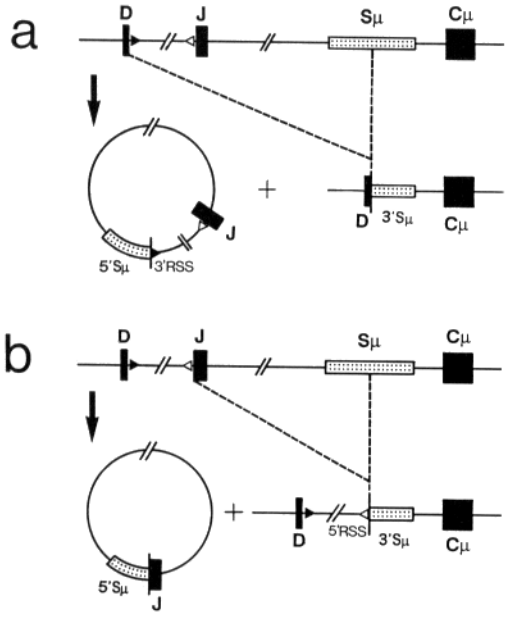

Figure 3. Schematic diagrams of hybrid circle formation. Two types of circles are generated between the RSS and Sμ sequences, depending upon relative orientations of RSS to S_μ sequence. In pathway *a*, the 3'-RSS of the D_H recombines with S_μ sequence, and both are retained on the circle, while the D_H-coding sequence remains on the chromosome. In pathway *b*, the 5'-RSS of J_H recombines with Sμ, but remains on the chromosome. The J_H-coding sequence, recombined with Sμ sequence, is retained on the circle. Coding sequences for D, J, and Cμ exons are shown as filled bars. Switch regions are stippled. Triangles represent RSS's.

terized also by DNA sequencing (Fig. 2). For each clone, three sequences were compared in the vicinity of the breakpoint; they were germline Sμ, germline D_H or J_H, and the breakpoint region of the clones. By comparing these sequences, the precise recombination site was identified. In six of the seven clones sequenced, the 3'-RSS of D_{Q52} was joined with the Sμ repetitive sequence (Fig. 2 *a*), although recombination sites in the Sμ region were all different and scattered (Fig. 1). In Fig. 3 *a*, the formation of circular DNA using the 3'-RSS of D and Sμ sequence is schematically shown. As described above, we have isolated many unrearranged J_H clones in the circular DNA library. They were most likely derived from larger circles that were excised with the RSS of D segments located further upstream from the 5' EcoRI site in the J_H region.

Unlike the clones described above, ICD165 was negative with the D_{Q52} probe, although it was positive with the J_{H2}-spanning and 5'-Sμ probes, and probes between these two. For this clone, the recombination sites were predicted to lie in the Sμ region and at J_{H2}. In Fig. 2 *b*, the recombinant sequence is compared with germline J_H and Sμ sequences. At the breakpoint, there is a precise match of J_{H2} coding sequence, which is joined to a typical Sμ repetitive sequence. The mapping predicted that the recombination site lies in the middle of the overall Sμ region (Fig. 1). In ICD165, the RSS is not retained at the recombination breakpoint, but the J_H coding sequence is. Therefore, the clone ICD165 is analogous to the excision product of so-called pseudonormal recombination (3, 11), in which rearranged coding sequence is retained on the circular DNA, and the signal joint is formed on the chromosome (Fig. 3 *b*).

In this report, we have characterized novel circular DNAs that represent excision products of recombination between the RSS and a switch repetitive sequence (Fig. 3). These "hybrid circles" were discovered during the course of analyzing thymocyte circular DNA. Since the FACS® analysis demonstrated that the thymocyte sample contained <0.3% B220-positive cells, it is unlikely that these Ig circular DNAs were isolated from B lineage cells. In addition to the unusual recombination reported here, D-to-J joining of IgH is rather common in T cells. Why the Ig genes are rearranged in T cells is an unresolved question. However, it is assumed that a common recombinase is responsible for both Ig and TCR gene rearrangements, and that the Ig J_H region is activated for recombination at early stages of T cell development. It is somewhat curious that Ig Sμ region is involved in the rearrangement in T lineage cells. More puzzling here is why the RSS can join with the Sμ sequence. To account for the origin of the Ig "hybrid circles", one possibility is that some basic components are shared by the V-(D)-J and class switch recombinases. Another possibility is that the GTG in the switch repetitive sequence was recognized by the V-(D)-J recombinase, as if it were part of an RSS heptamer. As shown in Fig. 2 *a*, the trinucleotide GTG was found in the Sμ sequence at the breakpoints in most of the clones. In the heptamer, the trinucleotide adjacent to the recombination site is essential (12, 13), and appears to serve by itself as a joining signal, at least in the V_H gene replacement (14, 15).

In the Ig H chain genes, the J_H-Sμ region often serves as a target for aberrant DNA rearrangement. It has been postulated that the V-(D)-J or switch recombinase is responsible for aberrant rearrangements in some lymphoid tumors (16). The "hybrid circles" described in this report appear to be formed, at least in part, by the action of a V-(D)-J recombinase, but in a manner that is aberrant with respect to the 12/23-bp spacer rule. We have analyzed T cell lines and T cell hybridomas for the Sμ rearrangement on the chromosome. Among 24 samples analyzed, rearrangement was found in one pre-T cell line, KKA (17), which is Thy-1^+ and $CD3^-$. The Sμ region was not rearranged in the other T cell samples tested, most of which represent mature stages of development (our unpublished observation). It is possible that the hybrid circle formation may be limited to certain stages of T cell maturation in the thymus. Deregulation of the recombination machinery may occur in dying T cells, leading to the formation of hybrid circles. Further studies with separated thymocyte populations will elucidate the biological significance of hybrid circle formation in thymocytes. It is of interest to study whether this type of recombination also occurs in B cells. However, if it occurs during B cell ontogeny, such cells would be either blocked in their ability to form functional VDJ structures or blocked in their ability to undergo class switch at later stages. In any case, the discovery of hybrid circles is of intrinsic interest for the understanding of the recombination mechanisms of antigen receptor genes.

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