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Molecular Characterization of Extrachromosomal Circular DNAs from Differentiating Embryonic Stem Cells

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ABSTRACT. **Embryonic E14 stem cells were differentiated to parietal yolksac-like flat cells in vitro in the absence of added feeders and LIF (Leukemia Inhibitory Factor). We cloned circular DNAs from the differentiating E14 cells. Out of 9 DNA inserts with the unique sequence, one clone showed a chromosomal rearrangement which could have occurred between a pair of short inverted repeats. Recombination mechanism is discussed in view of two other circularization events of the flanking sequences between short inverted repeats shown in differentiated P19 embryonal carcinoma cells.**

Mammalian cells contain a heterogeneous population of circular DNAs that are composed entirely of chromosomal sequences. They include chromosomal excision products of antigen receptor V(D)J recombination and immunoglobulin class switch recombination (5, 7, 11, 21, 23, 29, 34), extrachromosomal DNAs containing interspersed repetitive sequences (6, 14, 17, 26) or tandem repeats (2, 15, 16, 18) and more or less uncharacterized small polydisperse circular (spc) DNAs (36, 37, 39).

Chromosomal excision products are generated by intrachromosomal recombination mediated by V(D)J signal sequences in lymphoid cells. The canonical signal sequences of V(D)J recombination can be classified as one of patchy short inverted repeats. The specificity of V(D)J recombination requires the correct recognition of such inverted short homology regions by the RAG1 and RAG2 proteins (4, 33). Yet, we have obtained no evidence for circular DNA fragments indicating a V(D)J-like recombination in non-lymphoid cells.

Immunoglobulin class switch recombination generates extrachromosomal circular DNAs by joining the two switch (S) regions located upstream of each constant region. The S region consists of tandem repetition of short unit sequences composed of the primordial pentamer sequences (C/G)TG(A/G)G which show homology to some of the general recombinogenic sequences such as χ and mini-satellite sequences (1, 27). However, switch recombination sites tend to share little homology (11, 12, 21, 34). Thus S-S recombination seems to be mediated through a mechanism different from typical homologous recombination.

Both short (SINE) and long (LINE) interspersed repetitive sequences are known as retroposons, as expected for a reverse transcript (30). Although extrachromosomal retroposon-containing circular DNAs fit the idea of putative intermediates or by-products of RNA-mediated transposition, they also match the excision products generated by homologous recombination between flanking direct repeats (24). Alphoid satellite DNA and Sau3A family DNA enriched in spc DNA are explained as the result of homologous recombination events within the long array of tandem repeats (16, 24).

Extrachromosomal circular DNAs emerged in the course of lymphocyte development as reviewed by Lewis (19) and Harriman *et al.* (8) and also less frequently in the process of early development of mouse embryo and in vitro differentiation of embryonal carcinoma (EC) cells (37). Extrachromosomal circular DNAs isolated from lymphocytes have been well characterized as the excision products of DNA rearrangement occurring during differentiation. However, spc DNAs emerging during differentiation of non-lymphoid cells are less well characterized. In a previous investigation (13), we performed molecular characterization of spc DNAs from EC cells induced to differentiate *in vitro*. Although repetitive sequences were enriched in these spc DNA fragments, we could assign two breakpoints of circular DNA with unique sequences from the germline sequence. In this study, we characterized spc DNAs emerging in the process of differentiation of embryonic stem cells, and identified the germline sequence flanking the junction of circle formation. Taken together they suggest a circular DNA formation, facilitated by patchy short inverted repeats flanking short direct repeats. This

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suggests a simple cut- and paste-type model for spc DNA formation.

MATERIALS AND METHODS

λpCF2 vector construction. The strategy of vector construction was based on Miki *et al.* (22). A *Sal*I/*Eco*RI fragment within the multicloning site (MCS) of pHSG399 was replaced by an oligonucleotide linker harboring two *Bsa*I sites convertible to *Eco*RI and *Hin*dIII adaptors. The phage vector Charon27 was cleaved sequentially by *Eco*RI and *Hin*dIII. The modified pHSG399 was cleaved by *Bsa*I (New England Biolabs. Inc.) and ligated to the two *Eco*RI and *Hin*dIII arms of Charon 27. In this phage λ-plasmid composite vector λpCF2, the original *Eco*RI and *Hin*dIII cloning sites of Charon 27 were removed by repair ligation and thus *Not*I and *Sac*II-linkered pHSG399 was cloned into Charon 27 as shown in Fig. 1a. A pCF2 plasmid can be obtained by *Not*I digestion of λpCF2 and ligation followed by transformation of bacterial cells (Fig. 1b).

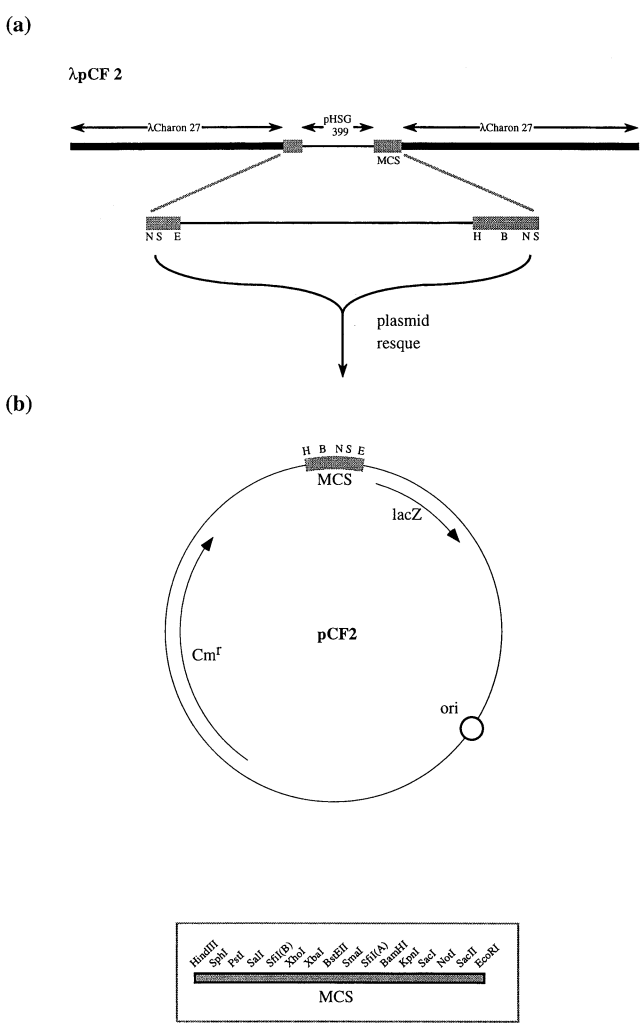


Fig. 1. Structure of the phage-plasmid composite vector λpCF2 (a) and the rescued plasmid pCF2 with several restriction sites of the multicloning site (MCS) (b). H, *Hin*dIII; B, *Bam*HI; N, *Not*I; S, *Sac*II; E, *Eco*RI; ori, replication origin of plasmid pHSG399.

Cell preparation. E14 embryonic stem cells from 129/Ola mouse blastocysts were kindly provided by Dr. M. Hooper (32), and were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 20% fetal bovine serum (BioSerum), 10^{-4} M β-mercaptoethanol, and 10^3 U/ml LIF (ESGRO). Stock cultures were passaged every 3 days by plating 5×10^6 cells with 3×10^6 mitomycin C-treated NHL7 feeder cells (25) in a 90 mm dish, and the culture medium was changed every day. Differentiation of ES cells was induced by removing the feeder cells and LIF from the culture, and cells were harvested on day 5 when approximately one-third of the cells had shown differentiated morphology.

Construction of a circular DNA clone library. Extrachromosomal circular DNA molecules were prepared from approximately 5×10^6 E14 cells induced to differentiate and purified by use of ATP-dependent exonuclease to remove contaminating linear DNA, as described previously, except for *Xho*I treatment (38). They were digested with *Bgl*II, ligated to calf intestine phosphatase (CIP)-treated *Bam*HI arms of the λpCF2 phage vector and packaged *in vitro*. Recombinant phage formed clear plaques on *E. coli* LE392. Recombinant phage titers per microgram of vector were 3×10^5.

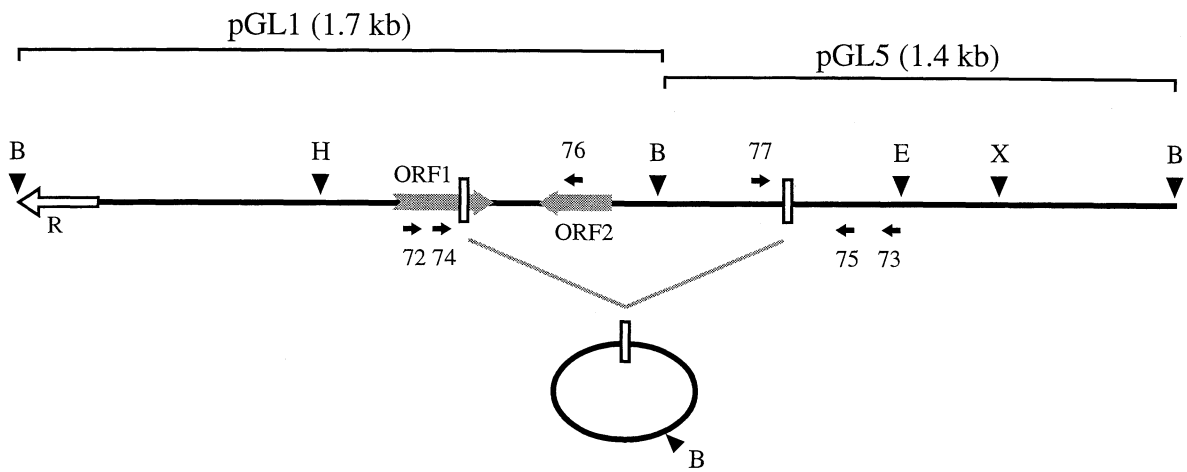
DNA hybridization. Southern hybridizations were performed according to the methods of Maniatis *et al.* (20). Genomic DNA was prepared from approximately 1×10^8 uninduced E14 cells. In brief, cells were digested with lysis buffer (100 mM Tris·HCl [pH 8.5], 5 mM EDTA, 0.2% SDS, 200 mM SDS and 100 μg/ml proteinase K) overnight at 56°C, fol-

Table I. CHARACTERIZATION OF CLONED E14 CIRCULAR DNA FRAGMENTS.

Fragments		genomic bands[a)]
DNA clone	size (kb)	
ES 8	4.1	4.1
1	3.2	r
6	2.7	2.7
7	2.5	r
2	1.6	r
11	1.4	1.4
3	1.2	1.2
14	1.1	1.1
5	0.9	r
4	0.8	1.4, 1.7
10	0.75	0.75
13	0.6	0.6
12	0.6	r
9	0.5	0.5

[a)] Every Southern banding pattern of *Bgl*II-digested genomic DNA was either unique bands shown by kb or repetitive smear bands (r).

(a)



(b)

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pES4   TTGCCTTTAGTGAAGTAATTTATTAAAACTATCCATTTCAAGGTTCAAAAGATGGTTGTCATGT
pGL1   AG-A-A-C-CAA---A-TCAA---G--T-A----------------------------------
pGL5   ------------------------------CCACTT-AAC-A-TGCAGAA--AACATGT-
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Fig. 2. Structure of the complete circular DNA cloned in pES4. (a) A 0.8 kb-sequence of pES4 circular DNA fragment is aligned with the sequences of their germline counterparts, pGL1 and pGL5. Two rectangles indicate a short homology at the breakpoints on the circular DNA clone. Orientations of PCR primers are shown by arrows starting from each numbered sps primer sequence, the open reading frames are shown by shaded arrows and the R sequence of LINE is by an open arrow. H, *Hin*dIII; B, *Bgl*II; E, *Eco*RI; X, *Xba*I. (b) Nucleotide sequences surrounding breakpoints of circular DNA fragments. The recombinant structure is compared with the corresponding germline sequences. Nucleotides which are homologous to circular DNA fragments are indicated by a dash. A 4-bp short homology at cross-over junctions is shown by rectangles.

lowed by ethanol precipitation and then dissolved in 200 μl distilled water. DNA probes used for Southern hybridization were the circular DNA fragments shown in Table I. All post-hybridization washes were at high stringency (0.5 × SSC, 0.1% SDS, 68°C).

DNA amplification. A genomic DNA equivalent to 5×10^5 cells (1 μl) after being induced to differentiate was subjected to two rounds of PCR using pairs of nested primers. They were sps 72/73 for the first round and sps 74/75 for the second round or a pair of sps 76/77 primers. The primers were designed from the pGL1 and pGL5 nucleotide sequences of DDBJ/EMBL/GenBank databases accession no. D86124: sps 72, GTGAAACCTTACACTTGGGA; sps 73, TCCTGCAC AACAGTCTGTAA; sps 74, TCTCCAAGTCACTGTCTTT G; sps 75, GATACACAGACATGATGGCA; sps 76, AGGT GAAAGCCCTGCATTGT; sps 77, GGTCTTGGAGATGA AAACCA (Fig. 2a). Amplification was performed with Taq polymerase as follows: an initial 4 min incubation at 94°C, 30 cycles at 94°C for 1 min, 50°C for 2 min and 72°C for 3 min with a final 5 min elongation step at 72°C. PCR fragments were cloned into MCS of pCF2.

Sequencing. Circular DNA and genomic DNA cloned into λpCF2 were rescued as plasmid clones by *Not*I digestion. Nucleotide sequences were determined by the dideoxy chain termination method using the universal M13 primer M4 or reverse primer RV.

RESULTS

Development of an efficient cloning system for spc DNA. DNA cloning techniques have been of great value in characterizing the molecular structure of eukaryotic extrachromosomal circular DNAs, as shown in the identification of excision products of antigen receptor V(D)J recombination and immunoglobulin class switch recombination (8, 19). However, spc DNAs emerge much less frequently in the process of differentiation of embryonic stem cells than in lymphoid cells.

Thus, most of the spc DNA clones isolated are mitochondrial DNA fragments. The conventional circle DNA cloning procedures using phage vectors include (I) counter selection of mitochondrial DNA fragments and (II) recloning into plasmid vectors. Therefore, it is very difficult to get a reasonable number of clones of very low frequent spc DNA using the usual procedures. In the present study, we developed a new efficient DNA cloning system: purification of circular DNA using ATP-dependent exonuclease to remove contaminating linear DNA (38), digestion with *Bgl*II and cloning into the *Bam*HI site of the phage λ-plasmid composite vector, λpCF2 which is capable of plasmid rescue as constructed in this study (Fig. 1). In this system, a single mitochondrial *Bgl*II fragment of 15 kb is not packageable into the λpCF2 vector (cloning capacity up to 9.0 kb). After isolation of phage clones of interest, pCF2 plasmid with spc DNA inserts can be obtained by *Not*I digestion and ligation followed by transformation of bacterial cells.

Spc DNA clones isolated from differentiating E14 embryonic stem cells. When embryonic E14 stem cells are cultured in the absence of added feeders and LIF, they multiply rapidly for several days and differentiate to parietal yolksac-like flat cells. In culture for 5 days without feeder cells and LIF, 20 to 30% of E14 cells differentiated. Approximately 3×10^7 cells were harvested and the one-sixth was subjected to circular DNA preparation as described except for the *Xho*I digestion (38). A circular DNA library was made by cloning *Bgl*II-digested circular DNA into the *Bam*HI site of the λpCF2 composite vector. We selected 14 clear plaque-forming units at random and isolated DNA inserts of various length (Table I). These DNA inserts were used as hybridization probes for Southern blots of undifferentiated E14 DNA that had been digested with *Bgl*II. Out of 14 DNA inserts of 1.6 kb in average length, 5 contained repetitive sequences mostly showing a hybridization smear. Only one clone, ES4, showed two discrete bands of 1.7 kb and 1.4 kb which differed from the probe (0.8 kb). This suggests that the circularization joint is located within the 0.8 kb fragment of ES4. For the rest of the clones, the probe DNA was identical with the germline DNA in size and possibly representing a partial sequence of the larger spc DNAs.

Breakpoint on ES4 circular DNA fragment. To determine the joint for circle formation, we prepared a genomic library of E14 DNA which was digested with *Bgl*II and cloned into the *Bam*HI site of the λpCF2 vector, and screened for germline sequence fragments homologous to the rearranged fragment of the ES4 circular DNA clone. We recovered two germline clones; GL1 and GL5, which correspond to genomic Southern bands in size. We sequenced the entire region of ES4 and both ends of the germline clones, GL1 and GL5

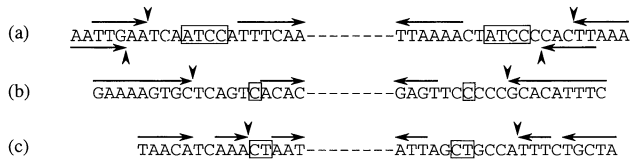

Fig. 3. A pair of short inverted repeats on the germline sequence flanking the circular DNA fragments isolated from the differentiated embryonic stem cell line E14 (a) and embryonal carcinoma cell line P19 (b and c). The sequence (a) is derived from pGL1, pGL5 and pES4; (b) from RP9, pR8 and pR2; (c) from pR24, pR23 and pR19 (13). The sequence within the circular DNA fragment is shown by dashes and short homologies at cross-over junctions are boxed. Inverted signal repeats of interest are marked by a pair of convergent arrows and the presumable cutting sites at the signal border by arrow heads.

(Fig. 2a). As shown in Fig. 2b, these 3 sequences were compared and the two breakpoints for circular DNA formation were identified on the chromosomal sequence. At the junction of the two breakpoints there exists a 4-bp homologous sequence. That is, the chromosomal sequence that forms the circular DNA of ES4 is flanked by a short direct repeat, ATCC. In the vicinity of the breakpoints, there are separated, patchy, short inverted repeats: TTGAA and TTAAA; AATTG and CACTT; TTTCAA and TTAAAA (Fig. 3a).

Genome structure of a complete ES4 circular DNA. To determine whether circular DNAs are generated as the result of chromosomal deletion, we amplified the DNA sequences surrounding the breakpoint of circular DNA fragments by the polymerase chain reaction (PCR) method with the use of appropriate primer pairs (Fig. 2a). Using a pair of the sps primers 72 and 73, we obtained a 1.3 kb PCR fragment from E14 cells induced to differentiate. A complete 1286 bp sequence of this fragment showed the precise joint of the terminal *Bgl*II cloning sites of the ES4 circular DNA fragment. Therefore, we constructed a linear genome sequence of 1310 bp including the complete ES4 circular DNA sequence as registered in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number D86124 (Fig. 2a). This sequence harbored two stretches of open reading frames (ORF1, at least 87 residues; ORF2, 70 residues) of opposite orientation. The ORF1 showed 73% homology in 80 amino acids overlap including physicochemically similar residues to a continuous open reading frame (at least 174 residues) located in the complementary strand 522 bp upstream of the yeast α-type proteasomal gene, PRE5 (9). Since functional domains for proteasomal subunits seem to be conserved in the eukaryotes, the ORF1 may represent the unknown mouse homologue related to the α family of proteasomal subunits. At 1.0 kb upstream from the breakpoint of circular DNA formation, there is a 5′ half of an R repetitive sequence (0.2 kb). Even

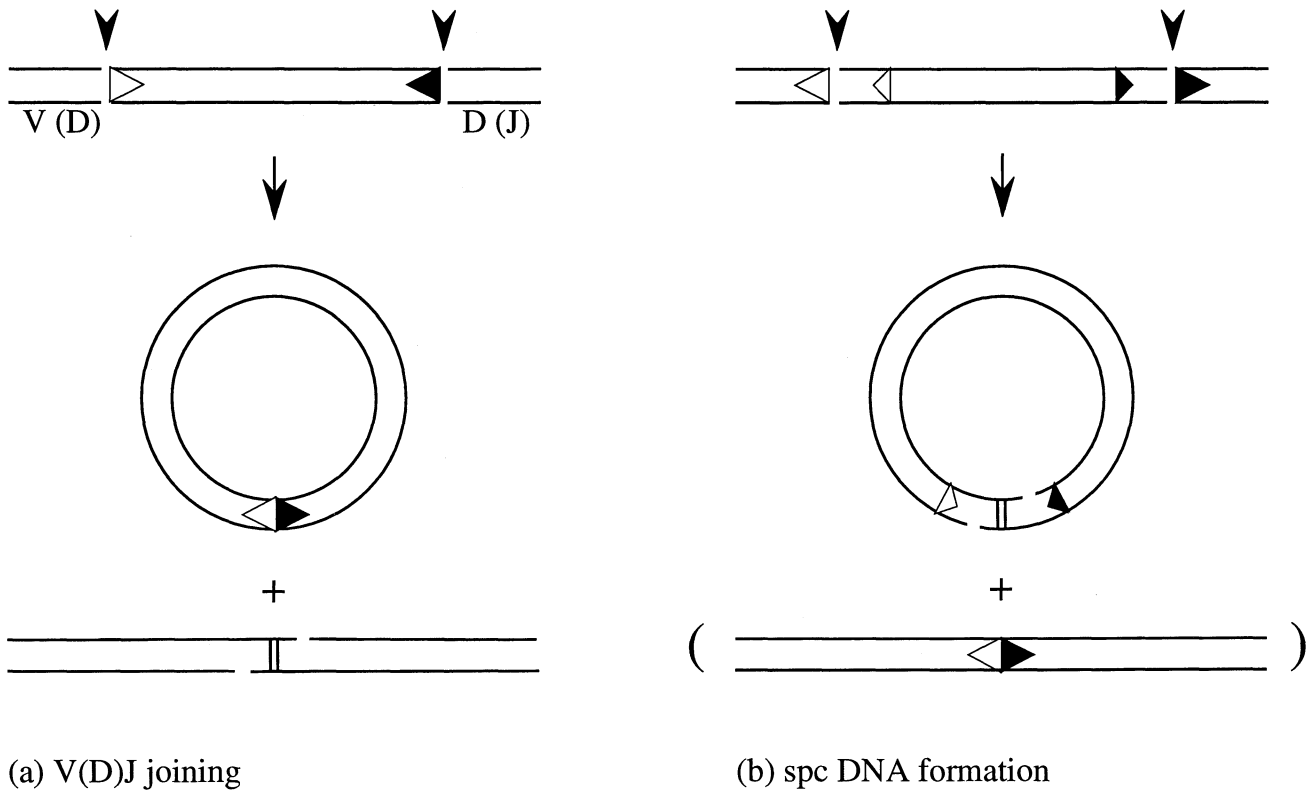

Fig. 4. Two recombination models of V(D)J joining (a) and spc DNA formation (b) mediated by a pair of short inverted repeats (open and closed triangle). The cleavage site is shown by arrow heads. Limited base loss occurring as a consequence of endonucleolytic or exonucleolytic activity is shown by gaps and positions of short homology are denoted by vertical bars. Putative chromosomal structure is parenthesized.

after two rounds of PCR, using pairs of the nested sps primers 72/73 and 74/75, no rearranged band of 379 bp expected from the germline sequence was amplified. Another PCR amplification using the pair of sps primers 76 and 77 failed to show any evidence of generation of extrachromosomal circular DNAs.

DISCUSSION

Large size circular DNA complexes of more than 3 kb emerge during the compaction process of mouse embryos and during the differentiation of embryonal carcinoma cells induced with retinoic acid (37). However, the complete ES4 circular DNA sequence analyzed in this study is only 0.8 kb long, thus ES4 DNA may not represent circular DNAs specific for the early development of mouse embryos.

The presence of extrachromosomal circular DNAs composed of chromosomal sequences does not necessarily mean a reciprocal recombination product of any rearranging chromosomal DNA as verified in V(D)J recombination and class switch recombination in the immune system as reviewed by Lewis (19) and Harriman *et al.* (8). Extrachromosomal fragments may also be generated by other mechanisms, such as regional DNA amplification (31). We have obtained no evidence so far for chromosomal deletion or amplification responsible for spc DNA formation. Spc DNAs found in non-lymphoid cells may be a very small fraction of the total cellular DNA and thus hard to detect even by the PCR method.

Nascent fragments derived from a chromosome may be linear or circularized with the two ends occasionally modified by base deletion or addition. We tried to find a signal sequence for circularization of spc DNA from three sequence data including two circular DNA clones isolated from retinoic acid-treated EC cells (13). It is known that the canonical signal sequences of V(D)J recombination can be regarded as patchy inverted repeats consisting of heptamers and nonamers separated by spacers, and the first three to four residues of the signal heptamer are conserved in the cryptic joining signals (19). Nevertheless, examples of cryptic signal joints are involved in the characteristic precise connection between two signal elements (3, 10, 28). Therefore, we searched for a pair of separated short inverted repeats of at least 5 bp showing more than 80% homology in the flanking region of a rearranging locus. Although the

available data were limited, signal-like inverted repeats were found within a 5 bp-distance outside of recombination sites (Fig. 3). Homologous regions at recombination sites were very short, 1 to 4 bases long. This is consistent with the short junctional homologies of 1.5 bases (0 to 4 bases) observed for end-to-end ligation between unrelated DNA segments in mammalian cells (35).

Although the reciprocal recombination product of a precise signal joint on the chromosome was not confirmed, we propose a simple model for spc DNA formation by the cleavage at the exact edges of joining signals, an exonucleolytic trimming of the fragment disconnected from their signals, and the joining of trimmed ends facilitated by limited sequence homologies as shown in Fig. 4. According to mechanistic and structural criteria, this is a pseudo-normal V(D)J joining, exploiting the degenerate signal sequence (19) and a short homology utilized for class switch recombination (8, 11, 12). Many cryptic joining targets combined with a degenerate signal sequence and a short homology may be scattered in the genome. However, actual recognition of cryptic sites by putative recombinase followed by spc DNA formation may be regulated by the local configuration of the chromosome. Although the biological significance of spc DNA formation remains unclear, spc DNAs appear to emerge in accord with the general rule utilized in V(D)J and class switch recombination, representing a localized flexibility of eukaryotic genomes.

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