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V(D)J Recombination: Signal and Coding Joint Resolution Are Uncoupled and Depend on Parallel Synapsis of the Sites

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V(D)J recombination in lymphoid cells is a site-specific process in which the activity of the recombinase enzyme is targeted to signal sequences flanking the coding elements of antigen receptor genes. The order of the steps in this reaction and their mechanistic interdependence are important to the understanding of how the reaction fails and thereby contributes to genomic instability in lymphoid cells. The products of the normal reaction are recombinant joints linking the coding sequences of the receptor genes and, reciprocally, the signal ends. Extrachromosomal substrate molecules were modified to inhibit the physical synapsis of the recombination signals. In this way, it has been possible to assess how inhibiting the formation of one joint affects the resolution efficiency of the other. Our results indicate that signal joint and coding joint formation are resolved independently in that they can be uncoupled from each other. We also find that signal synapsis is critical for the generation of recombinant products, which greatly restricts the degree of potential single-site cutting that might otherwise occur in the genome. Finally, inversion substrates manifest synaptic inhibition at much longer distances than do deletion substrates, suggesting that a parallel rather than an antiparallel alignment of the signals is required during synapsis. These observations are important for understanding the interaction of V(D)J signals with the recombinase. Moreover, the role of signal synapsis in regulating recombinase activity has significant implications for genome stability regarding the frequency of recombinase-mediated chromosomal translocations.

The exons encoding the immunoglobulin and T-cell receptor variable region domains are assembled during lymphocyte ontogeny by V(D)J recombination (reviewed in references 24 and 34). This site-specific reaction is directed by a pair of joining signals recognized by components of the recombination activity. The consensus sequence of each signal consists of a palindromic heptamer and an A/T-rich nonamer. These two elements are separated by a 12-base spacer at one signal and a 23-base spacer at the other. The recombination crossover point for each signal is at the end of the heptamer on the side distal from the nonamer.

During recombination, coding ends and signal ends are generated by recombinase-mediated cleavage at the crossover sites. The two coding ends are joined to form what is termed a coding joint, and the two signal ends are joined to form a signal joint. Modification of the coding ends occurs by terminal deoxynucleotide transferase-associated nucleotide addition (7, 19, 29), by P-element addition (18, 29), and by nucleolytic chewback of the exposed ends prior to joint resolution (reviewed in references 21 and 24). These modifications are important to the diversification of the antigen-binding repertoire.

Elucidating the order of the steps in this reaction and their mechanistic interdependence is important to our understanding of how the reaction fails and thereby contributes to genomic instability in lymphoid cells. Failed V(D)J recombination reactions ending in chromosomal translocations are detectable in the lymphoid tissues of approximately 50% of the human population (1, 28). Moreover, at least 20% of all childhood malignancies appear to involve translocations derived from site-directed lymphoid recombinations (25). Determining the degree to which steps of the V(D)J recombination reaction are tightly coupled is essential for understanding the extent of V(D)J recombinase-mediated genomic instability.

For those loci that undergo recombination by inversion, the necessity for both coding joint and signal joint resolution is obvious: the failure of either joint to form results in a chromosomal break. In addition, the detection of large circular products containing signal joints from loci rearranging by deletion (31) raises the possibility of an obligate coupling of coding joint and signal joint resolution.

In contrast, three lines of work relate to the possibility that signal joints and coding joints are uncoupled. First, it has been known for some time that there is independent processing of coding and signal ends. End modifications commonly observed at coding joints are infrequently detected at signal joints (20, 27). However, this differential processing of the two sets of recombinant ends in no way precludes the mechanistic interdependence in their resolution. Second, data that may be considered relevant to the uncoupling of signal joint and coding joint formation are obtained from the disruption of V(D)J recombination by the murine severe combined immunodeficiency (*scid*) defect. In mice expressing the *scid* mutation, signal joint formation occurs in the absence of normal coding joint formation (4, 26, 33). However, it is important to note that this uncoupling may be part of the *scid* genetic defect. Third, free signal ends have been detected in normal thymic T cells, whereas unresolved coding ends were not detected (33). It is unclear whether this observation indicates differential survival of free coding and signal ends; uncoupling of the formation of these two products; or mechanistic coupling, but with different resolution half-times. Hence, the issue of interdependence of coding joint and signal joint resolution has not been tested.

We have analyzed coding joint and signal joint resolution on plasmid substrates under circumstances in which one of

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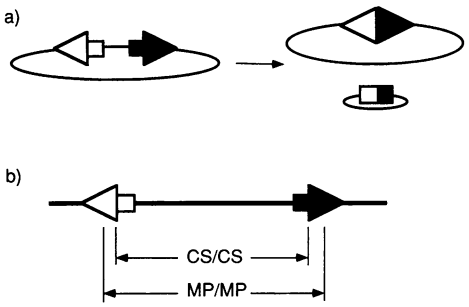
the two joint resolution reactions was markedly constrained. Disruption of normal joint resolution was achieved by reducing the distance between recombination signals. The data presented here demonstrate that coding joint and signal joint formation are separable events. In addition, restricting signal-signal interaction results in a dramatic inhibition of recombination. Therefore, while no obligate coupling of coding joint and signal joint resolution is evident, it appears that signal synapsis is required for the initiation of the recombination reaction. Finally, we propose that signal synapsis occurs only when the recombination signals are in parallel alignment. The role of V(D)J signal synapsis in mediating the activity of the recombinase is discussed within the context of genome stability and chromosomal translocations.

MATERIALS AND METHODS

Plasmid construction. The basic structures of plasmids used as extrachromosomal V(D)J recombination substrates (Fig. 1 to 3) have been described previously (11, 26). The inversion substrate pI279 was constructed by modification of pJH288 (26), in which the 12-spacer recombination signal segment was replaced with the paired oligonucleotides ml124 and ml125 and the 23-spacer signal was replaced by the paired oligonucleotides ml126 and ml127 (see below for sequences). The orientation of the signals is as shown in Fig. 3. pI89 was derived from pI279 by replacing the 227-bp *Cla*I segment containing the *oop* transcription terminator of bacteriophage λ with the paired oligonucleotides ks1 and ks2 (see below), containing a rho-independent transcription terminator (6) and *Cla*I-compatible ends. An additional inversion substrate, pI105, was constructed by reversing the orientation of the 23-spacer signal of pJH289 (23) and replacement of the oop terminator with the ks1-ks2 terminator as described above. The orientations of both recombination signals in pI105 are opposite those in pI89 and pI279.

pC329, which is the same as pJH290 described previously (26), is a deletion substrate which retains the coding joint in recombined molecules. Derivatives of pC329 with reduced intersignal distances were prepared as follows. pC139 has the 227-bp *Cla*I segment containing the *oop* terminator of pC329 replaced with ks1 and ks2 as described above. pC102 has the oop terminator excised without replacement. pC81 was derived by partial *Sal*I-*Bam*HI digestion of pC329 followed by treatment with mung bean nuclease and ligation, yielding a clone with a complete 12-spacer signal flanked by intact *Sal*I and *Bam*HI sites. A 23-spacer signal was inserted at the *Bam*HI site in the proper orientation for a coding joint substrate (see Fig. 2).

pS247 is a deletion substrate which retains the signal joint on recombined molecules (Fig. 1). It was constructed from pI279 by replacing the 12-spacer signal with the oligonucleotide pair hj1-hj2 (see below). pS57 was derived from pS247 by replacing the *oop* terminator with the ks1-ks2 terminator as described above. A series of plasmid recombination substrates in which the intersignal distances were increased in 2-bp increments was derived from pS57. These plasmids were constructed by cleaving at unique restriction sites located between the recombination signals, filling in the overhangs by DNA polymerase I (large fragment; Boehringer Mannheim Biochemicals), and religation. The substrates with their respective filled restriction site(s) are as follows: pS59 (*Cla*I), pS61a (*Sal*I), pS61b (*Bam*HI), pS63 (*Sal*I and *Cla*I), pS65 (*Sal*I and *Bam*HI), and pS67 (*Sal*I, *Cla*I, and *Bam*HI). Klenow filling of the *Bam*HI site generates an additional *Cla*I site; pS67 was cut with *Cla*I and filled to produce an additional substrate, pS69. The actual distances are listed in Table 1.



Substrate	Intersignal Distance (bp) MP/MP	CS/CS	Recombination (R)
pS247	280	247	2.1 (9,167/427,614)
pS57	90	57	0.97 (3,228/331,720)
pS40	73	40	< 0.001 (0/100,320)

FIG. 1. Effect of intersignal distance reduction on signal joint substrates. V(D)J recombination signals are shown in the orientation for substrates retaining recombinant signal joints. The open triangle represents the 12-spacer signal consisting of a consensus heptamer, a 12-bp spacer segment, and consensus nonamer. The closed triangle represents the 23-spacer signal consisting of a consensus heptamer, a 23-bp spacer segment, and consensus nonamer. Recombination crossover sites are located immediately adjacent to the heptamer of each signal (the base of each triangle). (a) Recombination reaction products of signal joint substrates are depicted. The deleted intersignal fragment containing the coding joint is shown as a closed circle, although this structure has not been detected in physical assays of transfection harvests. (b) The distances between signal midpoints (MP/MP) and recombination crossover sites (CS/CS) are given for each substrate. Recombination for each substrate was determined by assessing replicated (*DpnI*^{r}) substrate molecules by the genetic detection assay (see Materials and Methods). Recombination values (*R*) are percentages of replicated substrate molecules that have recombined. Values in parentheses are the total number of recombinants divided by the total number of replicated substrate molecules assessed from four transfections of each substrate. For a description of plasmid structure, including antibiotic resistance markers, see Hesse et al. (11) and Lieber et al. (26).

pS40 was constructed from pS247 by replacing the *oop* terminator with a 36-bp insert of the annealed oligonucleotides ks3 and ks4. The ks3-ks4 insert contains a rho-independent transcription terminator and has one *Sal*I-compatible end and one *Bam*HI-compatible end.

Oligonucleotides. The following oligonucleotides were used in construction of the plasmid recombination substrates and as hybridization probes: hj1, 5′-TCGAGGTTTTTGT TCCAGTCTGTAGCACTGTGTCGACAT-3′; hj2, 5′-CGAT GTCGACACAGTGCTACAGACTGGAACAAAAACC-3′; ks1, 5′-CGATCGCCAGCCCGCCTAATAGGCGGGCTTT TTTTTG-3′; ks2, 5′-CGCAAAAAAAAGCCCGCCTATTA GGCGGGCTGGCGAT-3′; ks3, 5′-TCGACTCAGCCCGCC TAATGAGCGGGCTTTTTTTTG-3′; ks4, 5′-GATCCAAA AAAAAGCCCGCTCATTAGGCGGGCTGAG-3′; ks9, 5′-G TGCTACAGACTGGAAC-3′; ks10, 5′-TACTCCACTGTC TGGCT-3′; ks12, 5′-TGCAGGTCGACACAGTG-3′; ml124, 5′-TCGACACAGTGGTACAGACTGGAACAAAAACC-3′; ml125, 5′-TCGAGGTTTTTGTTCCAGTCTGTACCACTG TG-3′; ml126, 5′-GATCCACAGTGATTCATATCACTGCG CCCCCGTTACAAAAACCA-3′; and ml127, 5′-GATCTG

GTTTTTGTAACGGGGGCGCAGTGATATGAATCACT GTG-3'.

Cell culture and transfection. The Abelson murine leukemia virus-transformed pre-B-cell line 204-1-8 (30) was used for all transfections. Cell cultures were maintained in RPMI 1640 (Irvine Scientific) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah), 50 μM β-mercaptoethanol, 2 mM L-glutamine, and 100 U of penicillin-streptomycin (Irvine Scientific) per ml. Cells were grown to a density of 1×10^6 to 2×10^6/ml prior to transfection. For each transfection, approximately 3×10^6 cells were osmotically transfected with 0.15 μg of purified substrate DNA as described previously (11). After incubation in closed flasks at 37°C for approximately 48 h, plasmid DNA was recovered in a modification of the rapid alkaline procedure of Birnboim and Doly (3) as described previously (11).

Genetic detection of recombinants. Recombined substrates molecules of substrates containing either the *oop* or rho-independent transcription terminator were detected by using the V(D)J recombination assay as described previously (11). Briefly, *Escherichia coli* DH10B transformed with input or recombined substrate molecules acquires ampicillin resistance (Amp^r). Recombinants also confer chloramphenicol resistance (Cam^r) on transformed bacteria as a result of inversion or deletion of the transcriptional terminator that blocks expression of the chloramphenicol acetyltransferase gene on the input substrate. Replication of transfected plasmids was used as an indicator of entry into lymphoid cells and was assessed by *Dpn*I digestion of recovered substrate molecules. Recombination was quantified as the percentage of $DpnI^r$ Amp^r colonies that are also Cam^r (Amp^r Cam^r/Amp^r). This value was obtained by growth of transformed bacteria on LB-ampicillin agar (100 μg of ampicillin per ml) and LB-ampicillin-chloramphenicol agar (100 μg of ampicillin and 11 μg of chloramphenicol per ml). Plasmid was recovered from selected colonies by the rapid alkaline procedure of Birnboim and Doly (3) for verification of recombinants by restriction digest analysis and/or sequencing.

Recombinant plasmids were distinguished from input by changes in restriction digest patterns. Digestion at restriction sites flanking the recombination signals was coupled with a second endonuclease cutting between the signals on unrecombined plasmid. Recombinants of deletion substrates were identified by loss of the second restriction site as well as changes in the expected restriction pattern of the first endonuclease. Recombinants of signal joint and inversion substrates with precise signal joints were identified by the formation of an additional *Apa*LI restriction site between the *lac* promoter and the chloramphenicol acetyltransferase gene of the substrate. Inversion recombinants were also identified by changes in the position of a restriction sites located near either the 12-spacer or 23-spacer signal. Additional analysis of selected recombinants was done by sequencing of miniprep DNA. All sequencing was carried out on double-stranded substrates by using Sequenase version 2.0 (U.S. Biochemical) according to the manufacturer's protocol.

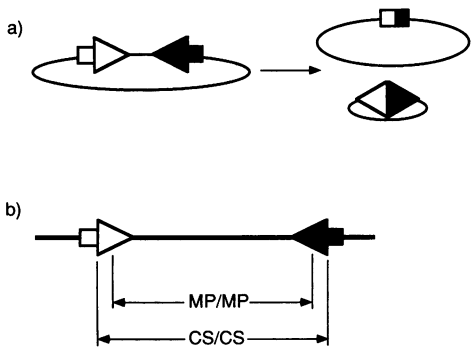
Physical detection of recombinants. Recombined substrate molecules were also detected by a combination of restriction endonuclease digestion and hybridization of colony lifts with specific oligonucleotide probes. Plasmid DNA was recovered from transfected 204-1-8 cultures as described above. A portion of each harvest was restriction digested with *Dpn*I and a combination of *Dpn*I plus *Bam*HI. Parallel mock reactions were included. *E. coli* DH10B electrocompetent cells were transformed with digested or mock-digested plasmid and plated onto LB-ampicillin agar as described above.

Recombinant colonies were confirmed by lift hybridization with oligonucleotide probes specific for the 12-spacer signal (ks9), the 23-spacer signal (ks10), and the 23-spacer signal heptamer and flanking coding-end region, including the *Bam*HI site (ks12). Colonies were picked and spot plated onto LB-ampicillin agar in a 10-by-10 grid array along with colonies containing known recombined and input plasmids as negative and positive controls, respectively. Plates were incubated at 37°C for at least 12 h. Colonies were directly lifted onto dry nitrocellulose filters (BA85; Schleicher & Schuell, Keene, N.H.) for 2 min. Filters were transferred sequentially, bacteria side up, to blotting papers saturated with 0.5 N NaOH–1 M Tris-HCl (pH 7.4) and 0.5 M Tris-HCl (pH 7.4)–1.25 M NaCl for 5 min each. After drying for 30 min, filters were baked at 80°C for 2 h and then stored at −20°C or used immediately for hybridization.

Prior to hybridization, filters were washed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) three times for 15 min at room temperature and once for 90 min at 65°C. Filters were prehybridized in 6× SSC–5× Denhardt's solution–0.05% sodium pyrophosphate ($Na_4P_2O_7$)–0.5% SDS–40 μg of sheared, denatured salmon sperm DNA per ml at 45°C for at least 1 h. Hybridization with kinase-treated oligonucleotides was carried out at 45°C in 6× SSC–1× Denhardt's solution–0.05% $Na_4P_2O_7$–40 μg of sheared, denatured salmon sperm DNA per ml for at least 12 h. Oligonucleotides were end labeled to high specific activity (approximately 5×10^5 dpm/ng) with [γ-^{32}P]ATP and polynucleotide kinase (U.S. Biochemical) according to the manufacturer's specifications. Approximately 500 pg of labeled oligonucleotide was added per ml of hybridization solution. Filters were washed three times for 15 min at room temperature and once for 30 min at 5°C above the hybridization temperature in 6× SSC–0.05% $Na_4P_2O_7$. Filters were dried briefly at room temperature and exposed on film (Kodak XAR) with intensifying screens at −80°C for 24 to 48 h. For reprobing, filters were stripped by being washed twice for 15 min in a solution of 0.1× SSPE–0.1% SDS heated to boiling and once for 5 min in the same solution at room temperature. Filters were exposed on film, as described above, for 3 days to assess the efficacy of signal removal before rehybridization.

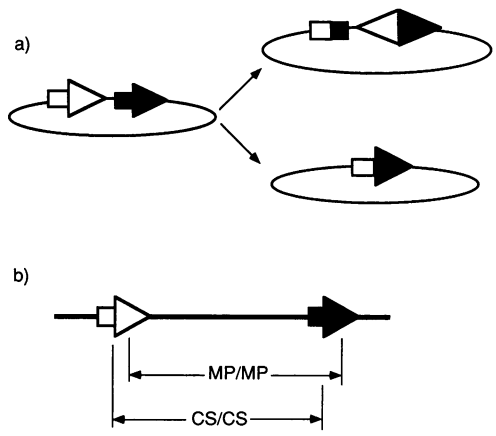
RESULTS

In the recombination reaction, two sets of recombinant ends are generated by cleavage of the DNA at the two signal sequences. Recombinant signal joints and coding joints result from the ligation of each set of ends. In deletional V(D)J recombination, the resolution of one joint requires the formation of a closed circle. To examine the possible interdependence of signal joint and coding joint resolution, plasmid recombination substrates were modified such that coding joint and signal joint formation were mechanistically uncoupled. To do this, we employed a strategy that utilized the rapid increase in free energy requirements for short segments of DNA to bend. Reducing the distance between recombination signals imposes a physical limitation on the ability of the intervening DNA to bend sufficiently for both sets of recombinant ends to form covalently closed circles. The joint resulting from ligation of the closely spaced ends is inhibited without imposing similar constraints on the reciprocally generated joint.



Substrate	Intersignal Distance (bp) MP/MP	CS/CS	Recombination (R)
pC329	296	329	1.0 (2,230/226,020)
pC139	106	139	0.2 (590/258,400)
pC102	69	102	0.2 (535/250,030)
pC81	48	81	0.01 (54/514,880)

FIG. 2. Effect of intersignal distance reduction on coding joint substrates. (a) V(D)J recombination signals are shown in the orientation for substrates retaining recombinant coding joints; symbols and abbreviations are as described in the legend to Fig. 1. Recombination reaction products of coding joint substrates containing coding joints (upper) and signal joints (lower) are shown. (b) Intersignal distances are given for coding joint substrates as described for Fig. 1. Recombination values (R) are percentages of replicated substrate molecules that have recombined as determined by the physical detection assay (see Materials and Methods). Values in parentheses are the total number of recombinants divided by the total number of replicated (*Dpn*I^r) substrate molecules assessed from four transfections of each substrate.



Substrate	Intersignal Distance (bp) MP/MP	CS/CS	Recombination (R)
pI279	284	279	0.8 (313/38,600)
pI105*	100	105	0.03 (76/254,468)
pI89	94	89	0.002 (3/134,420)

* - recombination signals are in reverse orientation of that shown.

FIG. 3. Effect of intersignal distance reduction on inversion substrates. V(D)J recombination signals are shown in the orientation for substrates undergoing recombination by inversion; symbols and abbreviations are as described in the legend to Fig. 1. (a) Recombination products are shown for both inversion (upper) and hybrid (lower) reactions. (b) Intersignal distance are given as described for Fig. 1. Recombination values (R) are percentages of replicated substrate molecules that have recombined as determined by the physical detection assay (see Materials and Methods). Values in parentheses are the total number of recombinants divided by the total number of replicated (*Dpn*I^r) substrate molecules assessed from four transfections of each substrate.

Intersignal distances for our substrates were selected to correlate with the rapid increase in the free energy of bending as described by Wang and Giaever (39) and the inhibition of cyclization of short fragments of DNA described by Shore et al. (36). On the basis of these studies, the efficiency of cyclization resulting in covalently closed circles is greatly reduced at lengths of less than 237 bp. The smallest DNA circles reported in the literature are 99 bp and form only in the presence of one of the strongest DNA-bending proteins, HU (13); attempts to form smaller circles under the same circumstances have been unsuccessful. To assess the effects of bending constraints on our recombination substrates, we constructed recombination substrates with intersignal distances ranging from 40 to 329 bp, as measured from cut site to cut site.

Three distinct types of substrates were used in this analysis. One substrate series was constrained for coding joint formation but not signal joint formation (Fig. 1). Another substrate series was constrained for signal joints but not coding joints (Fig. 2). In the third series, inversional recombination was tested, in which both joints were constrained (Fig. 3). The intersignal distance can be expressed as the distance between the midpoints of each signal or as the distance between the recombination crossover sites. Both are given for each substrate in the figures. Throughout most of this analysis, we refer to the latter value, as it represents the site of a defined activity of the recombinase, the cleavage of the substrate, and the length of the constrained segment. The midpoint-to-midpoint values may more accurately reflect the positioning of components of the recombinase on the signals and their interaction and therefore provide a more equitable basis for comparing different signal orientations.

Signal joint formation is independent of coding joint formation. The effect of inhibiting coding joint formation on the resolution of signal joints was examined by using substrates on which signal joints are retained on recombinant plasmids and on which the joining of coding ends is constrained (Fig. 1). Reducing the distance between the signals (Fig. 1) down to 57 bp (pS57) did not significantly affect the efficiency of signal joint formation (recombination value [R] = 0.97; Fig. 1). Completion of the coding joint in this case requires the formation of a 57-bp circle. Because the circle of deleted DNA would be more than 40 bp smaller than the smallest described DNA circle (2, 13), we conclude that resolution of the coding joint is likely to be very inefficient, if not absent entirely. Any obligatory coupling of signal joint formation to coding joint formation should have greatly reduced the efficiency of signal joint resolution for this substrate. It did not, indicating that signal joint resolution is independent of coding joint resolution.

Further reduction of the intersignal distance by 17 bp down to 40 bp (pS40; Fig. 1) shows a striking inhibition of both coding joint and signal joint formation ($R < 0.001$). Results obtained previously with a substrate of similar design and having an intersignal distance of 16 bp showed severely inhibited but detectable recombination (23). The recombination of a substrate much shorter than pS40 indicates that prevention of simultaneous binding of both sites

by the recombinase proteins is not the basis for the reduction in reaction efficiency. We instead suggest that signal synapsis is required for efficient initiation of recombination.

Coding joint formation is independent of signal joint formation and is constrained by signal-signal interaction. Having established the uncoupling of signal joint formation from coding joint formation, we assessed the reciprocal situation: uncoupling coding joint resolution from signal joint formation. A panel of recombination substrates was constructed whereby coding joints are retained on recombined plasmid molecules and the formation of signal joints is increasingly constrained by reduction of the intersignal distance (Fig. 2).

These coding joint substrates also show sensitivity to recombination signal proximity. Reduction of the intersignal distance to 102 bp (pC102) results in a fivefold decrease in coding joint resolution (R = 0.2; Fig. 2). Although signal joint formation is constrained in this configuration, the effect on coding joint formation is not dramatic, demonstrating that coding joint formation can be uncoupled from signal joint formation.

Reduction of the intersignal distance by another 21 bp on another coding joint substrate to 81 bp (pC81) results in a 100-fold decrease in coding joint formation (R = 0.01; Fig. 2). This disruption of coding joint resolution is consistent with our proposal that synapsis occurs prior to the relief of strain at one or both of the recombination crossover sites.

Recombination of inversion substrates is the most sensitive to intersignal DNA bending. Inversion substrates (Fig. 3) are the third topological orientation of sites analyzed. We find that recombination is inhibited approximately 25-fold at 105 bp (pI105) and 400-fold at 89 bp (pI89) compared with the control substrate, pI279 (Fig. 3).

An alternative recombination product obtained from inversion substrates is the hybrid joint described by Lewis et al. (23), in which the coding end of one signal and the signal end of the other signal are joined with the intervening DNA deleted (Fig. 3). Because deletional recombination occurs in both signal joint and coding joint substrates with comparable intersignal distances (compare pC102 and pS57, respectively, with pI105 and pI89), it could be argued that the decline in inversional recombination seen in pI105 and pI89 might not be due to constraint of signal synapsis but rather might be due to a particular difficulty associated with forming two joints in a constrained space rather than one joint. To test this possibility, we examined the frequency of hybrid joint formation. Hybrid joint formation on inversion substrates occurs by deletional recombination and hence requires only one joint to form. Although hybrid joints occur among pI89 recombinants, they are detected at greatly reduced frequencies compared with pI279. Hybrids occurred in 0.0025% of recovered pI89 substrate molecules, compared with 0.4% of recovered pI279, a 160-fold reduction. This magnitude of decrease is not significantly different from the 400-fold decrease observed for inversional V(D)J recombination of these two substrates. Therefore, constraint of synapsis does appear to be the basis for the sensitivity of inversion to short intersignal distance. The implications of these findings as they relate to the configuration of synapsing signals are discussed below.

Synapsis is not affected by rotational phasing of the signals. In assessing the recombination frequencies of substrates with different intersignal distances, it is important to note that the orientation of the signals relative to one another may introduce an additional constraint to signal synapsis. If signal synapsis requires a particular stereospecific alignment for V(D)J recombination to occur, phasing of the signals should be evident on substrates restricted in their ability to rotate their recombination signals to the appropriate orientation for synapsis. To test this possibility, a panel of signal joint substrates, derived from pS57, was constructed with the intersignal distance increased from 57 bp to 69 bp in 2-bp increments (Table 1). By utilizing substrates having intersignal distances approaching the flexibility threshold for inhibiting signal interaction, we were able to assess limitations of the DNA to twist into the necessary orientation for synapsis apart from the effects of restricted bending.

TABLE 1. Evidence that rotational phasing of the recombination signals about the DNA axis does not affect the efficiency of the V(D)J recombinase to form signal joints

Substrate	Intersignal distance (bp)[a]		Recombination (R)[b]
	MP/MP	CS/CS	
pS57	90	57	0.4 (2,816/662,832)
pS59	92	59	0.4 (2,624/610,848)
pS61a	94	61	0.7 (3,023/456,480)
pS61b	94	61	0.7 (5,910/856,584)
pS63	96	63	0.8 (6,453/760,320)
pS65	98	65	1.1 (4,231/375,984)
pS67	100	67	0.6 (3,993/664,272)
pS69	102	69	0.7 (5,155/747,360)
pS247	318	285	1.0 (6,059/618,624)

[a] Intersignal distances are given from the midpoint of the 12-spacer signal to the midpoint of the 23-spacer signal (MP/MP) and from the recombination crossover site of the 12-spacer signal to the recombination crossover site of the 23-spacer signal (CS/CS; see Fig. 1).

[b] Determined by the genetic detection assay (see Materials and Methods) from four parallel transfections. Figures in parentheses are counts of (Amp^r Cam^r/Amp^r *Dpn*I^r) colonies used to calculate the recombination frequency for each substrate.

There is little, if any, effect attributable to signal phasing (Table 1). The synapsis of recombination signals is not inhibited in these substrates, indicating that twisting of the signals occurs freely about the DNA helix, nor is the ability of the recombinase to recognize and cleave the signal sequence compromised by twisting; use of *Apa*LI, a restriction enzyme that cuts at precisely formed signal joints, showed that nearly all of these signal joints were precise (data not shown). Therefore, cleavage and ligation occurred precisely at the junction of the signal heptamers and coding sequences, with no increase in nucleotide loss or addition.

DISCUSSION

This study demonstrates that the mechanism of V(D)J rearrangement is sensitive to bending of the DNA between the recombination cut sites. Hence, cleavage and release of coding and signal DNA ends prior to synapsis with another signal must be at least 100-fold less frequent than cuts made after synapsis. Otherwise, we would see these events as an irrepressible level of recombination, insensitive to intersignal distance.

The results reported here indicate that signal joint formation can occur efficiently under conditions in which coding joint formation is expected to be highly constrained. In previously reported studies of lymphoid cells from *scid* mice, signal joint formation usually occurs without coding joint formation (4, 26). However, among pre-B-cell lines from normal mice, there has been no information on this point. The importance of the current study is the demonstration that there is no obligate coupling of signal joint forma-

tion to coding joint formation in normal cells. Moreover, it can now be concluded that the uncoupling that is observed in the murine *scid* defect is not part of the mutant phenotype.

Is coding joint formation dependent on signal joint formation? The substrate pC102 forms coding joints at only fivefold-lower frequency than do substrates with intersignal distances of 329 and 6,000 bp (pC329 in Fig. 2 and pJH290A in reference 26, respectively). Concomitant signal joint formation would require the formation of a circle of 102 bp with only 35 bp between the nonamer ends of the two signals. Such a circle, albeit possible, would form very inefficiently, as judged from analyses of DNA bending (14, 35, 36, 39). We have attempted to detect the predicted circles or free-ended linear DNA molecules generated by recombination of the pC102 substrate but have not been successful in detecting either with use of a variety of physical methods, including hybridization analysis of transfected cell harvests and amplification of the deleted segment by polymerase chain reaction with specific primers. It may be that the stability of such molecules is much lower than that of the plasmid carrying the coding joint reaction product. At this time, we conclude that if 102-bp circles form, they do so with much lower efficiency than in the reciprocal coding joints. Therefore, coding joint formation must be occurring independently of signal joint formation.

Inversion substrates can undergo V(D)J recombination to yield the standard products, coding and signal joints, or the alternative product, hybrid joints (23). The formation of hybrid joints allows us to address the question of whether they result from a different synaptic orientation from the standard products. The data here provide insight on this issue. The observation that hybrid joints are reduced to nearly the same extent as are coding and signal joints in the inversion substrate analysis indicates that the same geometric constraints apply to the generation of all of the V(D)J recombination reaction products. Hence, hybrid joints arise not because of a distinct synaptic orientation of the recombination signals but rather as a distinct resolution from a common reaction intermediate.

Cooperative interaction of DNA-binding proteins is often required for initiating or suppressing activity of genetic loci (5). These interactions may be stereospecifically restricted, as indicated by reports in which the activity of the protein complex is suppressed by half-turn rotations of the binding sites relative to each other (8, 9, 12, 17, 37). However, not all such interactions require stereospecific alignment; for example, the DNA-binding monomers of the DeoR repressor form a flexible dimer linkage that is unaffected by changing the spacing between binding sites (16). Similarly, the V(D)J recombinase does not appear to be sensitive to twisting, suggesting that protein flexibility may be a feature of this system as well. The absence of signal phasing also indicates that the DNA-binding components of the recombinase, although specific for the sequence of the recombination signal, are not restricted to recognizing a particular exposed face of the signal DNA on the nucleosome.

Our results show that recombination by inversion is susceptible to strain at a longer distance than is deletional recombination. Whereas signal joint formation is largely unaffected at the intersignal distance of 57 bp (pS57) and coding joint formation is only 5-fold reduced at an intersignal distance of 102 bp (pC102), inversional recombination is 25-fold reduced at 105 bp and 400-fold reduced at 89 bp (pI105 and pI89, respectively). Indeed, this effect is even more pronounced when these substrates are compared on the basis of their midpoint-to-midpoint distances. We propose that the more marked inhibition of inversion reflects the requirement for a particular alignment of recombination signals at synapsis. When the recombination sites are depicted in a parallel or an antiparallel alignment during synapsis, the data favor a parallel alignment of sites as an explanation for inversional recombination manifesting strain at a longer distance than does either deletional reaction (Fig. 4). An antiparallel alignment of sites during synapsis would have given results opposite those that we obtained; inversion would have shown constraint at shorter, not longer, intersignal distances than did deletion substrates.

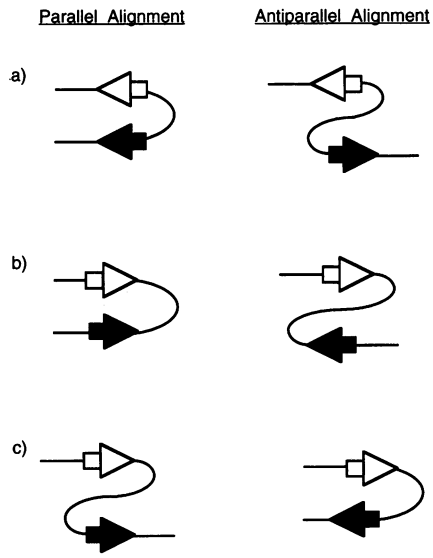


FIG. 4. Parallel versus antiparallel alignment of recombination signals. V(D)J recombination signals are represented for each of the three substrate types: (a) signal joint substrates, (b) coding joint substrates, and (c) inversion substrates. Symbols are as described in the legend to Fig. 1. The conformation of signals and intervening DNA is depicted in both parallel and antiparallel synaptic alignments.

We have considered the possibility that the inhibition of recombination is due to steric interference preventing simultaneous binding of both sites by the recombinase protein assembly. If so, the degree to which the proteins exceed the boundaries of each signal must be great enough to cause interference yet small enough to account for recombination by deletion substrates. For example, the nonamer ends of pC102 are 35 bp (11.9 nm) apart and show little indication of steric effects. In addition, severely limited, but detectable, recombination (200-fold reduced) was reported by Lewis and Hesse (22), using a signal joint substrate in which the heptamers were only 16 bp apart. If one considers protein-protein interference resulting from a highly asymmetric positioning of the recombinase proteins on the signals, inversion substrates should show an equal or intermediate degree of inhibition between the two types of deletion substrates. This is not the case; inversions are more sensitive than either deletional reactions. Hence, steric interference due to simultaneous protein binding at the two sites is unlikely to explain the inefficient recombination of inversion substrates.

It is important to note that the constraint imposed by signal proximity does not play a role in genomic recombination of lymphoid genes. The normally recombining segments are often separated by many kilobases of intervening DNA

and not subject to the constraints described in this study. Although signals can be imagined to synaptically align in either the parallel or antiparallel configuration, our data demonstrate that only the parallel alignment of signals results in a functional interaction.

The studies described here provide a mechanistic explanation for the stability of the T-cell receptor D segments at the T-cell receptor beta and delta loci. These segments are unique in the immune system because they are flanked by a 12-spacer signal on one side and a 23-spacer signal on the other. These signals are separated by a D-segment coding sequence between 8 and 16 bp long in human and mouse cells. If there were no synaptic requirement, then these D segments might be expected to delete out by using the 12-spacer and 23-spacer signals on each side as often as they join with other segments. Our studies indicate that synapsis is required and that this process helps to ensure the stability of the D minigenes until they recombine with a J segment.

It is not yet clear how the V(D)J recombination mechanism goes awry in the events preceding chromosomal translocations. One possible explanation is that the recombinase commonly makes cuts at single heptamer-nonamer sites. The finding of open and shut events, in which the recombinase has cut at the end of one signal and then closed the site (10, 22, 23), is consistent with this possibility. It is important to know the frequency of opening followed by release of the ends. For a variety of reasons, it has not been possible to determine this frequency yet. However, the data here set some limits on how frequently this might occur: greater than 99% of recombination events require a two-signal interaction (Fig. 1 to 3). This markedly reduces the potential cutting in the genome by the V(D)J recombinase. Clearly, other factors also contribute to genome stability. For example, the collision frequency of signals may be influenced by factors such as chromatin structure, signal proximity (i.e., interchromosomal versus intrachromosomal recombination), and the efficiency of recombinase binding to nonconsensus and cryptic recombination sequences. With regard to chromatin structure, CpG methylation may account for 100- to 1,000-fold protection against V(D)J recombination (15). Nevertheless, the exposed portion of the genome may still be large. Hence, while there may be over 10^5 adventitious heptamers in the genome, the requirement for binding and synapsis of two such sites to generate recombinant free ends provides an important barrier to chromosomal translocations.

Ultimately, these issues will be reexamined when the V(D)J recombination reaction is reconstituted in a cell-free system and after crystallographic information is available on how the DNA is bound to the recombination proteins. However, it is only by determining the reaction characteristics in the cell that we can be certain that cell-free reconstitutions are faithfully recapitulating the endogenous reaction.

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