The basis for the mechanistic bias for deletional over inversional $V(D)J$ recombination

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$V(D)J$ recombination between recognition sites in the genome is characterized by certain biases. At some loci, proximal sites undergo recombination substantially more frequently than distal ones. The joining of D_H/J_H is an example of this. Because the D_H element bears signal sequences on each side, inversion would be expected as often as deletion in D_H/J_H recombination. However, the markedly favored outcome is deletion, entailing utilization of the closer recombination site. One model proposed to explain these biases is the tracking model in which the recombinase tracks from one site to the other. Here, we have directly tested for various types of tracking in $V(D)J$ recombination and have found no indication that it occurs. In addition, we have created D_H-J_H minilocus substrates for analysis of the basis for the preference for deletion. We find that we can reproduce the deletional bias for the system. Moreover, by flipping the orientation of the D segment, we can reverse the bias such that the frequency of inversions can exceed the number of deletions. These results indicate (1) that there is no intrinsic topological preference in this reaction, and (2) that the sequence of the signal and coding ends determines the bias.

[Key Words: Rearrangement; $V(D)J$ recombination; topology; tracking; inversion; deletion]

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Among higher eukaryotes, site-directed recombination has, thus far, only been described in the immune system of vertebrates. Genomic rearrangement systems, such as this, pose particular problems of specification so as to avoid inadvertent rearrangement events. The basis for much of this specification is still uncertain. The assembly of the variable domain exons of the immunoglobulin and T-cell receptor (TCR) molecules occurs by a site-specific recombination reaction called $V(D)J$ recombination (for review, see Lieber 1991; Schatz et al. 1992). The V , D , or J segments have an adjacent signal sequence consisting of a palindromic heptamer and an A/T-rich nonamer separated by either a 12- or 23-bp spacer. A single recombination event is directed by a pair of joining signals, one with a 12- and the other with a 23-bp spacer signal (hereafter designated as 12- and 23-signals).

One of many questions about this reaction concerns how the two signals and the recombination activity assemble. One hypothesis has been that the recombination activity binds to one signal, and collisional interactions of this protein-DNA complex with the other signal generate the ternary complex. A second hypothesis has been that the protein-DNA complex formed at one signal tracks processively along the DNA until it reaches a sec-

ond signal (Yancopoulos et al. 1988). This hypothesis predicts that a signal will recombine with the nearest compatible signal with some preference to distant ones. Although the second hypothesis seems at odds with the notion that $V(D)J$ recombination is a random exon assembly system, work from several laboratories has been cited in favor of some form of tracking. First, the joining at the murine λ locus markedly favors proximal over distal segments (Storb et al. 1989). Second, at the TCR γ locus, the temporal onset of $V\gamma 2$, $V\gamma 3$, and $V\gamma 4$ rearrangement correlates with distance. Third, the utilization of V_H gene segments that are more proximal to D/J_H is 3- to 30-fold greater than the utilization of more distal V_H gene segments during fetal development (Yancopoulos et al. 1988). Finally, the strongest support for the tracking hypothesis (Kurosawa and Tonegawa 1982) lies in the data that it was originally proposed to explain: the well-established fact that deletional $V(D)J$ recombination occurs one to two orders of magnitude more frequently than inversional recombination in D_H/J_H joining at the heavy-chain locus (Meek et al. 1989). Because the D_H element bears signal sequences on each side, inversion would be expected as often as deletion in D_H/J_H recombination (Hesse et al. 1987), but the markedly favored outcome is deletion, entailing the utilization of the closer recombination site. The way a tracking model could be invoked to explain this is that the 12-signal of

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the D_H element that gives rise to deletion is at least 10 bp closer to the 23-signal than the 12-signal that would give rise to inversion. Modified proposals invoking a looser type of tracking, such as jumping followed by local tracking, might account for the few inversions that do occur in $D-J_H$ joining and still allow for randomness of the exon assembly process.

Here, we have studied how signals interact by looking for biases in the outcome of the recombination reaction when multiple possible outcomes exist by using extra-chromosomal plasmid $V(D)J$ recombination substrates. We find that there is no form of tracking between signals. Yet the large bias for deletional over inversional $V(D)J$ recombination can be recapitulated on these substrates. Their analysis indicates that the large bias in favor of deletions is the result of two factors: (1) Sequence differences between the two sides of the D segment can favor usage of the proximal 12-signal, which leads to deletion, and (2) reactions that require two joints to form (inversions) have a lower probability of going to completion than reactions that require only one joint to form.

Results

Substrates with multiple 12-signals: Tracking from the 23- to the 12-signal

We were interested in whether the $V(D)J$ recombination activity shows any evidence of scanning along the DNA from signal to signal. First, we chose to examine if there was any preference for the 23-signal to recombine with the nearest 12-signal. To test this, a substrate was constructed with a single 23-signal and three 12-signals, $p3 \times 12$ (Fig. 1). On this substrate, the 23-signal interacts with any one of the three 12-signals, resulting in deletional recombination. The coding joint is retained on the plasmid, and the signal joint is deleted. This substrate was used to transfect a murine pre-B-lymphoid cell line (1-8) that we have shown previously to actively rearrange $V(D)J$ recombination substrates (Hesse et al. 1987; Lieber et al. 1987). Restriction analysis of the recombination products recovered from the cells shows that each of the three 12-signals is used in roughly one-third of the products (Fig. 1). In this experiment, and all others reported in this study, the interpretation of signal usage based on restriction analysis was confirmed by sequencing several representative recombinant products. We infer that there is no evidence of preferential usage of any signal based on proximity relative to the 23-signal. Hence, there is no evidence of a recombination bias owing to a tracking mechanism from the 23-signal in either direction around the circular minichromosome substrate.

Substrates with multiple 23-signals: Tracking from the 12- to the 23-signal

Although tracking from the 23- to the 12-signal is not apparent in the above results, the possibility existed that

Figure 1. Multiple 12-signal substrate: $p3 \times 12$. (A) The structure of the multiple 12-spacer signal substrate $p3 \times 12$. The triangles represent $V(D)J$ recombination heptamer-spacer-nonamer signals: ($\blacktriangle$) The 23-spacer signal (23-signal); ($\triangle$) 12-signal. A deletional recombinant will result when the 23-signal recombines with any of the three 12-signals (1,2,3). (STOP) The prokaryotic transcription terminator; (cat) the chloramphenicol acetyltransferase gene, which confers chloramphenicol resistance in *E. coli*; (P) the lactose operon promoter; (amp) the β -lactamase gene, which confers ampicillin resistance in *E. coli*; (ColE1) the prokaryotic origin of replication; (PyT) the polyomavirus gene for large T antigen; (Py ori) the polyoma origin of replication; (6.2-kb stuffer) the 6262-bp *Clal*-*Clal* fragment from bacteriophage λ that has been inserted to increase the distance between the 12- and 23-signals. Restriction endonuclease sites: (B) *Bam*HI; (C) *Clal*; (E) *Eco*RI; (H) *Hind*III; and (S) *Sal*I. (B) The distribution of 12-signal usage in $p3 \times 12$. $p3 \times 12$ was transfected into 1-8 cells, and the recombinant products were recovered 48 hr after transfection. 12-Signal usage in the recombinant products was determined by restriction analysis. (events) The number of recombinant substrates analyzed and distributed according to which 12-signal is used in the recombination reaction.

tracking might occur from the 12- to the 23-signal. This would not be revealed in the analysis with the $p3 \times 12$ substrate. To test this, a plasmid, $p3 \times 23$, was constructed with one 12- and three 23-signals (Fig. 2). Analysis of recombinants of this substrate also showed no evidence that the nearest 23-signal was favored for recombination with the 12-signal. The results show that each of the three 23-signals engages in recombination in approximately one-third of the events. Therefore, we infer that tracking does not occur from the 12- to the nearest 23-signal in either direction around the circular minichromosome substrate.

Figure 2. Multiple 23-signal substrate: p3x23. The orientation of the recombination signals in p3x23 is shown. p3x23 was transfected into 1-8 cells, and the recombinant products were recovered 48 hr after transfection. A deletional recombinant will result when the 12-signal recombines with any of the three 23-signals {1,2,3}. The recombinant products were analyzed by restriction analysis to determine which 23-signal was used. The distance between the 12-signal and the 23-signal triplet is 6.5 kb. The remainder of the plasmid outside of this recombination zone is identical to that in p3x12 (see Fig. 1A). (events) The number of recombinant substrates analyzed and distributed according to which 23-signal was used in the recombination reaction.

Test of topological preference for deletion or inversion

Given that tracking does not appear to operate in $V(D)J$ recombination, we sought other explanations for some of the observed recombination biases in the genome. The most striking example of such a bias is in D_H/I_H recombination. Here, there is a very large bias in choice of rearrangement options, even though the distance difference between the two options is small. To study this issue, we chose a generalized substrate design that recapitulates D_H/I_H joining at the immunoglobulin heavy-chain locus (Fig. 3). These substrates give rise to deletional or inversional recombinant products, depending on which one of the two 12-signals is used in the recombination reaction. We were interested in examining any biases favoring either deletional or inversional recombination intrinsic to such an arrangement of signals. To remove any signal usage bias resulting from differences in signal quality, we constructed a substrate with the same consensus 12-signal positioned at both ends of the idealized D_i element cassette (Fig. 4). Two versions of this DJ substrate were constructed (pD_i-J_i and pJ_i-D_i) that differed only in the orientation of the idealized D_i element cassette. Analysis of recombination products of substrate pD_i-J_i showed a frequency of 0.95 deletions for every inversion (Fig. 4A). Recombinants from pJ_i-D_i had a frequency of 2.4 deletions for every inversion (Fig. 4B). The two 12-signals in D_i are identical in sequence; therefore, the small difference in deletion/inversion frequency between these two substrates may result from differences in the coding end sequences attached to the 12-signals. In a separate study, we have observed small differences in recombination frequency between substrates that differed only at their coding end sequences (R. Gerstein and M.R. Lieber, unpubl.). Regardless of this

disparity between the two orientations of the idealized D_i element, the deletion/inversion ratios here and in earlier studies (Hesse et al. 1987) clearly indicate that there are no significant topological biases in this reaction. Therefore, the large preference for deletions in D_H/I_H joining must have its basis in the sequences surrounding the recombination sites.

The basis for a deletional recombination bias in D/I_H joining

Could the predominance of deletional recombination between D_H and I_H elements observed at the immunoglobulin heavy-chain locus be explained by a difference in recombination strength between the two sides of the D element? For example, the sequence of the signal in the deletional orientation might resemble the consensus signal sequence more closely than the signal in the inversional orientation and, thus, give rise to a predominance

A Physiologic Orientation

B Reverse Orientation

Figure 3. Schematic representation of the recombinant products of a generalized DJ substrate. (A) D element in physiologic orientation. A deletion will result when the 23-signal recombines with the 12-signal labeled 2. Completion of the recombination reaction requires the ligation of the D -coding end to the J -coding end, forming the coding joint c_2 . An inversion will result when the 23-signal recombines with the 12-signal labeled 1. The intervening DNA is inverted, and two ligations occur forming two joints: the coding joint c_1 and the signal joint s_1 . (B) D element in reverse orientation. Deletion requires the formation of one coding joint, c_2 . Inversion requires the formation of one coding joint, c_1 , and one signal joint, s_1 .

of deletional recombination events. To test this hypothesis, a substrate, $pD_{SP}-J_i$, was constructed containing the murine *D* element $D_{SP2.2}$ in its physiological orientation (Fig. 5A). The deletion/inversion ratio for this substrate was 2.6. This ratio is much smaller than the observed ratio for endogenous D_H/J_H joining. Nevertheless, this

substrate does exhibit some bias for deletions, and we wondered whether the bias would reverse when we reversed the orientation of the $D_{SP2.2}$ segment (Fig. 5B). The reverse orientation substrate, pJ_i-D_{SP} , yielded a deletion/inversion ratio of 0.43. Hence, the deletion/inversion bias followed the orientation of the $D_{SP2.2}$ segment

very closely. Like the data from the idealized DJ substrate, pD_i-J_i , these data indicated that there is no intrinsic topological bias. Moreover, the $pD_{SP}-J_i$ and pJ_i-D_{SP} data indicate that when there is a bias in recombination owing to a difference in recombination potential of the two sides of the D segment, the bias follows the orientation of the sequence.

Although the above substrates yielded useful information about DJ joining, they did not recapitulate the large bias in DJ joining observed in the genome. We considered that the quality of the signal at the J segment might influence the deletion/inversion ratio; that is, a real J_H 23-signal might raise the deletion/inversion ratio. Indeed, this was the case. Substrate $pD_{SP}-J_{H3}$ is exactly like $pD_{SP}-J_i$, except that the J_{H3} segment is positioned in place of J_i (Fig. 5C). The deletion/inversion ratio for this substrate is 28. Hence, the J segment markedly influences the deletion/inversion ratio; moreover, it recapitulates the bias at the endogenous locus.

The reverse orientation substrate, $pJ_{H3}-D_{SP}$, had a lower deletion/inversion ratio, as expected (Fig. 5D). However, it is interesting that the deletion/inversion ratio did not fully invert upon reversal of the D_{SP} segment in this case.

The analysis was broadened to include a second D segment, $D_{FL16.1}$. The deletion/inversion ratio for this substrate was high regardless of whether it recombined with

J_i or with J_{H3} (Fig. 6A or C), thus recapitulating the rearrangement of this segment in the genome. Interestingly, in neither of the corresponding reverse orientations (pJ_i-D_{FL} and $pJ_{H3}-D_{FL}$) did the deletion/inversion ratio fully invert (Fig. 6B or D). For $pJ_{H3}-D_{FL}$, the deletion/inversion ratio remained almost the same.

Discussion

Tracking mechanisms for $V(D)J$ recombination

Our results rule out tracking mechanisms for the interaction of the 12- and 23-signals in $V(D)J$ recombination. The tracking hypothesis for $V(D)J$ recombination has been suggested as a possible explanation for some of the unexplained biases in rearrangement order and frequency at the endogenous loci. For example, the joining biases at the immunoglobulin λ and TCR γ loci correlate with their segment order and distance. Our data show no evidence of preferential recombination of the 12-signal with the first 23-signal encountered, regardless of which direction tracking might proceed around the plasmid.

Our data also indicate that there is no tracking from the 23-signal toward multiple 12-signals. Tracking has been suggested in this direction to explain the bias that J_H segments have for recombining with D_H segments by deletion much more frequently than by inversion (Kuro-

Figure 6. Deletion and inversion with $D_{FL16.1}$, the consensus 23-signal, and J_{H3} . The orientation of the recombination signals is shown above the deletion and inversion data for that substrate. The recombination substrates $pD_{FL}-J_i$, pJ_i-D_{FL} , $pD_{FL}-J_{H3}$, and $pJ_{H3}-D_{FL}$ were transfected into 1–8 cells, and the recombinant products were recovered 48 hr after transfection. (A) $pD_{FL}-J_i$: The consensus 23-signal with the $D_{FL16.1}$ cassette in physiological orientation. (B) pJ_i-D_{FL} : The consensus 23-signal with $D_{FL16.1}$ in reverse orientation. $pD_{FL}-J_i$ and pJ_i-D_{FL} are identical to each other except for the orientation of the $D_{FL16.1}$ cassette. (C) $pD_{FL}-J_{H3}$: The J_{H3} element with $D_{FL16.1}$ in physiological orientation. (D) $pJ_{H3}-D_{FL}$: The J_{H3} element with $D_{FL16.1}$ in reverse orientation. $pD_{FL}-J_{H3}$ and $pJ_{H3}-D_{FL}$ are identical to each other except for the orientation of the $D_{FL16.1}$ cassette. In both substrates, 300 bp separates the $D_{FL16.1}$ cassette and the single 23-signal. The remainder of both plasmids outside of this recombination zone is identical to that in $p3 \times 12$ (see Fig. 1A). In substrates with the $D_{FL16.1}$ cassette in physiological orientation ($pD_{FL}-J_i$ and $pD_{FL}-J_{H3}$), a deletion will result when the 12-signal labeled 1 is used in recombination with the 23-signal. In both of these substrates an inversion will result when the 12-signal labeled 2 is used. With the $D_{FL16.1}$ cassette in reverse orientation (pJ_i-D_{FL} and $pJ_{H3}-D_{FL}$), deletions result when the 12-signal labeled 2 is used and inversions result when the 12-signal labeled 1 is used. (events) The number of recombinant substrates analyzed and distributed according to whether

the recombination reaction resulted in a deletion (del) or inversion (inv). (normalized) The number of events after correction for differences in plating efficiency between deletional and inversional recombinants (see Materials and methods). The 95% confidence intervals for the del/inv ratios are as follows: $pD_{FL}-J_i$, 18.01–20.21; pJ_i-D_{FL} , 0.30–0.43; $pD_{FL}-J_{H3}$, 15.94–18.34; $pJ_{H3}-D_{FL}$, 11.50–17.06.

sawa and Tonegawa 1982). These studies not only rule out strict tracking mechanisms but also variations of such a mechanism in which there is jumping followed by local tracking. Because the serial sets of signals are adjacent to one another, there is little opportunity for randomization by jumping between signals; hence, jumping followed by local tracking would have been apparent, if it occurred. Upon finding that no form of tracking occurred, we did a further analysis of what factors might operate in the D_H to J_H joining process to bias the reaction pathway.

Deletional vs. inversional $V(D)/J$ recombination

What is the basis for the large values of the deletion/inversion ratio for the endogenous heavy-chain D to J joining? Our substrate reconstructions recapitulate this bias with deletion/inversion ratios of 28 and 17 for the $D_{SP2.2}$ and $D_{FL16.1}$ joining with J_{H3} , respectively. Factors that could determine such ratios are (1) topological bias of the reaction for deletion over inversion (i.e., the ability of the recombinase to sense whether the 12- and 23-signals are in direct or inverted orientation relative to each other), and (2) effects from the sequence of the coding and signal ends.

We have examined the first possibility extensively, an intrinsic topological bias for deletion versus inversion. Our data clearly indicate that there is none. First, $pD_{SP}-J_i$ and pJ_i-D_{SP} show that there can be a perfect reversal of the deletion/inversion ratio based entirely on the flipping of the D segment. This indicates that the sequences of the coding and signal ends involved in the reaction fully account for the deletion/inversion ratio. Second, the deletion/inversion ratio for substrates with identical competing signals, as in pD_i-J_i , is close to one. Finally, if there were a topological bias in this system, it might be expected to have its basis in the torsional strain associated with the synopsis of the 12- and 23-signals. We have done a study of the dependence of the deletion/inversion ratio based on the distance between the D and J segments over the range from 330 to several thousand base pairs, and we find that the deletion/inversion ratio remains constant (G.H. Gauss and M.R. Lieber, unpubl.). Hence, there is no intrinsic topological bias for deletion over inversion in $V(D)/J$ recombination.

The sequence of the recombining ends is the only factor that can be involved in determining the deletion/inversion ratio. The sequence of the recombinant ends, however, can have two different and discernible effects on the deletion/inversion ratio. First, the sequences, especially the signal ends, may affect how efficiently the recombination activity binds and cuts. Second, the coding and signal ends also may influence how efficiently the coding and signal joints form. For example, if we assume that only the first effect was operative, namely that after binding and cutting at the 23-signal of the J element and at the stronger 12-signal of the D element, all cut ends are resolved with 100% efficiency, then the deletion/inversion ratio for the reverse orientation should be the reciprocal of the deletion/inversion ratio

for the physiologic orientation. This is definitely not the case. Consider the substrate pairs $pD_{SP}-J_{H3}$, $pJ_{H3}-D_{SP}$ and $pD_{FL}-J_{H3}$, $pJ_{H3}-D_{FL}$. In these cases, the deletion/inversion ratio remains high despite reversal of the D segment (cf. Fig. 5C with D; Fig. 6C with D). This can only be explained if the efficiency of coding and signal joint formation is not always 100%. We suggest that the sequence at the signal and coding ends can have an effect on the efficiency of forming the signal and coding joints. Some sequences may resolve more efficiently than others. Because inversional recombination requires two joints to form, whereas deletional recombination requires only one, and because the probability of forming each joint can be less than one, the probability of completing an inversion event can never be greater than that for completing a deletion event for the same two recombination sites. Simply stated, it is harder to achieve completion of two joints than one; hence, deletion is favored.

More explicitly, the deletion/inversion ratios reflect two factors: (1) the bias between the two signals flanking D (i.e., the strength with which they bind the recombinase and are cut), and (2) the efficiency with which the one-joint product (deletion) forms relative to the two-joint product (inversion). If all joints (signal and coding joints for inversion, coding joints for deletion) form with high efficiency, then the second factor (joint resolution) does not have any effect and only the first factor (signal strength) has an influence. This means that if signal 2 in Figure 3 is optimal for binding and cutting and signal 1 is not, then the deletion/inversion ratio will have a value larger than unity in the physiologic orientation and less than unity in the reverse orientation. Furthermore, the ratios for the two orientations will be reciprocal. If they are not reciprocal, then not all joints are being formed with 100% efficiency; this raises the deletion/inversion ratio because inversion requires two joints to form, not just one.

In our assay, as in the genome, we only recover completed reaction products. Incomplete joints are left as linear DNA molecules and may be analogous to chromosomal breaks in the genome (Roth et al. 1992). Such ends may be destroyed rapidly or may be rescued by alternative end-joining processes that are unrelated to $V(D)/J$ recombination. Alternative end-resolution processes are often associated with a significant degree of exonucleolytic "chew-back." Large deletions that may represent such rescued coding ends are not uncommon at the endogenous antigen receptor loci (Alt et al. 1981; Nottenburg et al. 1987).

The D_H/J_H joining step has been the subject of intense interest not only because of its skewed bias toward deletions but also because it is commonly involved in chromosomal translocations (Cleary and Sklar 1985; Bakhshi et al. 1987; Tsujimoto et al. 1988; Tycko and Sklar 1990). Recent evidence indicates that the incidence of chromosomal translocations involving the D_H/J_H joining step is dramatically higher than suspected previously. More than 50% of routine human tonsil specimens contain evidence of the bcl-2 chromosomal translocation involving this locus (Limpens et al. 1991). The extent to

which recombinant ends fail to go to resolution in our experimental recapitulations of D_H/J_H joining (reflected in the nonreciprocal deletion/inversion ratios for many of the orientation pairs) may explain the common presence of translocations generated from the endogenous D_H/J_H joining step.

The present study recapitulates the *in vivo* deletion/inversion ratio. It rules out intrinsic topological biases and clearly indicates that the sequences of the signal and coding ends are the dominant factor determining the deletion/inversion ratio. On the basis of a separate study, we know that coding end sequences, like signal sequences (Hesse et al. 1989), can affect the efficiency of both the signal and coding joining halves of the reaction (R. Gerstein and M.R. Lieber, unpubl.). The effects of signal and coding end sequence on the reaction appear to be general to $V(D)J$ recombination and are not specific to D_H and J_H joining.

Materials and methods

Plasmids

All plasmids used in this study are derivatives of pJH298, which has been described previously (Lieber et al. 1988). p3×12 (Fig. 1) is identical to pJH298, except for three modifications. First, the orientation of the *Bam*HI–*Bam*HI fragment containing the 23-signal was reversed. Second, a 101-bp synthetic oligonucleotide containing three 12-signals was ligated into the *Sal*I site. Third, to increase the distance between the 12- and 23-signals, the 6262-bp *Clal*–*Clal* fragment from bacteriophage λ (nucleotides 46,430–4,198) was ligated into the *Clal* site nearest the 23-signal of pJH298. p3×23 (Fig. 2) is identical to p3×12, except for two modifications. First, the 101-bp *Sal*I–*Sal*I fragment containing the triple 12-signal was removed and a 39-bp synthetic oligonucleotide containing a single 12-signal was inserted. Second, the *Bam*HI–*Bam*HI fragment containing the 23-signal was removed and a 147-bp synthetic oligonucleotide containing three 23-signals was ligated into the regenerated *Bam*HI site. The D/J model substrates used in this study all have the same general structure: a D -like gene segment and a J -like gene segment. The D element consists of two 12-signals placed in heptamer-to-heptamer orientation and separated by 17–23 bp of “coding sequence.” The J element consists of a single 23-signal and 15 bp of coding sequence. The D element in the idealized D/J model substrate, pD_i-J_i , is a 79-bp oligonucleotide with two consensus 12-signals separated by 17 bp (Fig. 4A). The J_i element has a consensus 23-signal. The remainder of pD_i-J_i outside of this recombination zone is identical to pJH298. pJ_i-D_i (Fig. 4B) is identical to pD_i-J_i , except the orientation of the double 12-signal oligonucleotide has been reversed. $pD_{SP}-J_i$ (Fig. 5A) is identical to pD_i-J_i , except the D_i element has been replaced by an 84-bp synthetic oligonucleotide with an equivalent sequence to the murine immunoglobulin $D_{SP2.2}$ segment. The orientation of the $D_{SP2.2}$ segment relative to the consensus 23-signal on $pD_{SP}-J_i$ is the same as the germ line orientation of $D_{SP2.2}$ relative to the J_H elements at the murine immunoglobulin heavy-chain locus (physiologic orientation). pJ_i-D_{SP} (Fig. 5B) is identical to $pD_{SP}-J_i$, except the orientation of the $D_{SP2.2}$ segment has been reversed (the $D_{SP2.2}$ segment is in the reverse orientation in pJ_i-D_{SP}). $pD_{SP}-J_{H3}$ and $pJ_{H3}-D_{SP}$ (Fig. 5C,D) are identical to $pD_{SP}-J_i$ and pJ_i-D_{SP} , respectively, except that the consensus 23-signal has been replaced with a 65-bp synthetic oligonucleotide with equivalent sequence to the murine J_{H3} gene segment.

$pD_{FL}-J_i$ and pJ_i-D_{FL} (Fig. 6A,B) are analogous substrates to $pD_{SP}-J_i$ and pJ_i-D_{SP} , except the $D_{SP2.2}$ gene segment has been replaced with an 89-bp synthetic oligonucleotide with an equivalent sequence to the murine $D_{FL16.1}$. $pD_{FL}-J_{H3}$ and $pJ_{H3}-D_{FL}$ (Fig. 6C,D) are identical to $pD_{FL}-J_i$ and pJ_i-D_{FL} , respectively, except the consensus 23-signal has been replaced with the J_{H3} gene segment.

The sequences of the oligonucleotides used in the construction of our substrates are listed below. To facilitate cloning, these oligonucleotides and their complementary sequences were synthesized (Applied Biosystems 380A synthesizer), and then annealed leaving *Sal*I-compatible (12-signals) or *Bam*HI-compatible (23-signals) 5' overhangs. The heptamer and nonamer of each signal are indicated in boldface. The coding end sequences of the D and J elements are underlined.

Triple 12-signal, p3×12: 5'-TCGACTGCACAGTGCTACAGACTGG-
GACTGGAACAAAAACAGGCACAGTGCTACAGACTGG-
AACAAAAACAGGCACAGTGCTACAGACTGGAACAAA-
AACACTGCAG-3'.

Single 23-signal, p3×12: 5'-GATCCCCGAGGGTTTTGTGTA-
CAGCCAGACAGTGGAGTACTACCACTGTGG-3'.

Triple 23-signal, p3×23: 5'-GATCCCCGAGGGTTTTGTGTA-
CAGCCAGACAGTGGAGTACTACCACTGTGGGATCCCC-
GAGGGTTTTGTACAGCCAGACAGTGGAGTACTACCA-
CTGTGGGATCCCCGAGGGTTTTGTACAGCCAGACAGT-
GGAGTACTACCACTGTGG-3'.

Single 12-signal, p3×23: 5'-TCGACCACAGTGCTACAGA-
CTGGAACAAAAACCTGCAG-3'.

D_i element, pD_i-J_i orientation: 5'-TCGACTGTTTTGTTC-
CAGTCTGTAGCACTGTGTTAACTAGATCTGAATTCAC-
AGTGCTACAGACTGGAACAAAAACAG-3'.

D_{SP} element, physiologic orientation: 5'-TCGACGATTTT-
TGTCAAGGGATCTACTACTGTGTCTACTATGATTACG-
ACCACAGTGATATATCCAGCAACAAAAACCTGCAG-3'.

D_{FL} element, physiologic orientation: 5'-TCGACGCTTTT-
TGTGAAGGGATCTACTACTGTGTTTATTACTACGGTA-
GTAGCTACCACAGTGCTATATCCATCAGCAAAAAACCT-
GCAG-3'.

J_{H3} element: 5'-GATCCATTTATTGTGTCAGGGGTCTAAT-
CATTGTTGTCAATGTGCCTGGTTTGCTTACTCTCG-
GG-3'.

J_i element: 5'-GATCCCCGAGGGTTTTGTACAGCCAGA-
CAGTGGAGTACTACCACTGTGGGATCCCCGGGGATC-
AGCTTG-3'.

$V(D)J$ recombination assay

The $V(D)J$ recombination assay has been described previously (Hesse et al. 1987) but is summarized briefly below. Plasmid substrates are transfected into the Abelson murine leukemia virus-transformed murine pre-B-lymphoid cell line 1-8. While resident in the cells, a fraction of the substrate population undergoes $V(D)J$ recombination. This recombination results in the deletion or inversion of a prokaryotic transcription terminator. In the absence of recombination, the terminator interferes with the expression, in *Escherichia coli*, of a downstream chloramphenicol acetyltransferase gene. Upon recovery from the 1-8 cells (rapid alkaline/SDS lysis method) and transformation into *E. coli*, recombinant plasmids will confer resistance to both ampicillin and chloramphenicol while unrecombined substrates will confer only ampicillin resistance. The ratio of ampicillin–chloramphenicol-resistant (AC) colonies to ampicillin-resistant (A) colonies reflects the fraction of substrate that underwent recombination while resident in the eukaryotic cells. Typically, the number of AC and A colonies from a single bacterial transformation was 50–200 AC and 5×10^3 to 20×10^3 A

colonies. All lymphoid transfections were performed in triplicate, and the AC/A ratio was consistent for each substrate. A small number (1–10) of AC colonies were picked from each of several separate bacterial transformations taken from each lymphoid transfection. These recombinant substrates were subjected to restriction analysis to determine which pair of recombination signals was used in the recombination reaction. A *Hind*III–*Eco*RI double digest (or a *Pvu*II single digest, which yields a similar restriction pattern) was used for recombinants from $p3 \times 12$, $p3 \times 23$, and the *DJ* substrates. These restriction digests were examined by using 3.5% and 5% polyacrylamide gels. Inversional recombinants from the *DJ* substrates were confirmed by the presence of the signal joint by use of a *Sall*–*Bam*HI double digest and electrophoresis on 12% polyacrylamide gels. This series of digests allow sufficient resolution (± 5 bp) to rule out non-*V(D)J*-mediated deletions. Representative samples from each of the various types of recombinants were sequenced to confirm the restriction analysis. AC colonies from unrecombined substrates were undetectable (<1 AC colony for every 2×10^6 unrecombined substrates transformed directly into *E. coli* without passage through 1–8 cells). When picking colonies, the plates were sectorized and all colonies in a sector were picked regardless of colony size. The eukaryotic transfection method and plasmid harvest protocol have been described previously (Hesse et al. 1987). In this study we used electrotransformation (Bio-Rad Gene Pulser) and electrocompetent *E. coli* DH10B (Hsieh et al. 1991) instead of heat shock transformation as in previous studies (Hesse et al. 1987).

V(D)J recombination products were examined for their replication rate relative to substrate and to the other reaction products by cotransfecting the plasmids at 1 : 1 ratios and determining their relative ratio at the time of harvest 48 hr after transfection. There were no differences found between the plasmids. Therefore, reaction outcomes are not altered by propagation in the eukaryotic cells.

Hybrid joint formation (Lewis et al. 1988) occurred at a frequency approximately 14-fold below the frequency of deletions plus inversions. We have not included these in the tabulations of deletions and inversions. Double events (inversion followed by deletion) occurred at a frequency below 1% of the sum of single-event deletions and inversions. We also have excluded these from the tabulations of single-event deletions or inversions. These exclusions do not affect our analysis of single-event deletion versus inversion.

Normalization of growth differential in transformed *E. coli*

Depending on which pair of recombination signals is used on our substrates, the recombinant products have different distances between the promoter and the chloramphenicol acetyltransferase gene. When transformed into *E. coli*, we observe that this variation in distance gives rise to measurable and reproducible differences in the plating efficiency of the bacterial transformants. When comparing deletional and inversional recombinants, deletional recombinants had higher plating efficiencies than inversions. Plating efficiency was measured by the following method. Bacteria transformed with a recombinant plasmid were plated in equal numbers onto AC and A plates. Plating efficiency was calculated by dividing the number of colonies on AC plates by the number on A plates. All data in this study have been normalized to 100% plating efficiency. Plating efficiency data from 17 different deletions from 6 *DJ* model substrates were pooled, and the average was used as the standard for normalizing deletions. Using the same method, data averaged from 15 inversions were used as the standard for normalizing inversions. We established a standard curve for plating efficiency as

a function of incubation time (either 16 or 24 hr). At 16 hr incubation time the plating efficiency is $44\% \pm 5.5\%$ (s.d.) and $26\% \pm 3.0\%$ (s.d.) for deletions and inversions, respectively. At 24 hr, the plating efficiency is $68\% \pm 6.8\%$ (s.d.) and $51\% \pm 4.7\%$ (s.d.) for deletions and inversions, respectively. The plating efficiencies for the three products of $p3 \times 12$ were so similar (56–66%) that we did not adjust the raw data (Fig. 2). This was also the case for $p3 \times 23$ (73–85%) (Fig. 1).

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The basis for the mechanistic bias for deletional over inversional V(D)J recombination.

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