Diversity and Joining Segments of Mouse Immunoglobulin Heavy Chain Genes are Closely Linked and in the Same Orientation Implications for the Joining Mechanism

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Diversity and joining segments of mouse immunoglobulin heavy chain genes are closely linked and in the same orientation: Implications for the joining mechanism

(recombination/DNA tracking)

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ABSTRACT **We have mapped 12 diversity (*D*) gene segments of mouse Ig heavy chains between 80 and 1 kilobases 5′ to the four joining (J_H) gene segments by molecular analysis of DNA clones isolated from recombinant phage and cosmid libraries. All identified *D* and J_H segments are in the same 5′-3′ orientation. The significance of these findings with respect to the joining mechanism for Ig gene segments is discussed.**

The variable region of a heavy chain immunoglobulin is encoded by three DNA segments, V_H (variable), *D* (diversity), and J_H (joining) (1, 2). Somatic rearrangements of these DNA segments to form a joined *V–D–J* segment are required to generate a complete active heavy chain *V* gene (1–6).

The *D* segment encodes 1–13 amino acid residues composing the core of one of the three *V* subregions, referred to as complementarity-determining regions, that contain residues implicated for direct contact with antigens (3, 6). The *D* segments have been identified in mouse myelomas (4) and T-cell lines (5) in a form joined with J_H segments that are thought to be intermediates of *V–D–J* joining. These studies have led to the identification of a series of germ line *D* DNA segments (6).

There exist three known families of *D* segments in the mouse, members of a single family sharing extensive sequence homology (6). The first family, D_{SP2}, consists of nine members, each 12 base pairs (bp) long. The second family, D_{FL16}, consists of two members, one 23 bp long and the other 17 bp long. The third family, D_{Q52}, is a single-member family and is 10 bp long (4). The *D* segments belonging to the first two families form a cluster and are distributed in a 70-kilobase (kb) region: the two D_{FL16} and nine D_{SP2} segments occupy the 5′ and 3′ end regions of the cluster, respectively (6). While the D_{Q52} segment has been mapped precisely 696 bp upstream (5′ side) of J_{H1}, one of the four J_H segments, and has been shown to be in the same orientation as the J_H segments (4), the orientation of the D_{FL16}–D_{SP2} cluster relative to and its distance from the V_H or the D_{Q52}–J_H–C_H cluster have not yet been determined.

The purposes of the present study were to resolve these issues and at the same time try to identify any new *D* segments. We found that the 3′-most *D* segment of the D_{FL16}–D_{SP2} cluster is 19.5 kb 5′ to J_{H1}. There is no evidence for any additional *D* segments between the D_{FL16}–D_{SP2} cluster and D_{Q52}. In addition, all identified *D* segments were shown to be in the same orientation as the J_H segments.

MATERIALS AND METHODS

Bacteria, Phages, and Cosmids. Phage Charon 4A was obtained from F. Blattner of the University of Wisconsin (Madison) (7). *Escherichia coli* 803 (r_k^-, mk^-, $SuIII^+$) was from K. Murray (European Molecular Biology Organization Institute, Heidelberg, Federal Republic of Germany). Lysogens used for preparation of packaging mixtures, BHB 2688 [N205 *recA*$^-$(λimm_{434} cI_{ts} *b2 red3* Eam4 Sam7)/λ] and BHB 2690 [N205 *recA*$^-$(λimm_{434} cI_{ts} *b2 red3* Dam15 Sam7)/λ] were obtained from B. Hohn (Friedrich Miescher Institute) (8). Cosmid vector pJB8 was obtained from D. Ish-Horowicz and J. F. Burke (London) (9).

Preparation of Mouse Embryo Phage and Cosmid Libraries. The bacteriophage library used was prepared earlier (10). The library used was a partially *Eco*RI-digested mouse embryo DNA cloned into Charon 4A arms (11). The BALB/c mouse kidney DNA cosmid library was constructed according to the procedures described by F. G. Grosveld using pJB8 cosmid vector (12). A library of 2×10^5 transformants was constructed, and individual filters were replicated and stored at −70°C as described (13).

RESULTS

Isolation of Cosmid Clones by Using a 5′ J_H Probe. Our general strategy is to isolate a series of overlapping genomic DNA clones from the cosmid and phage libraries and determine the distance between and the relative orientation of D_{FL16}–D_{SP2} cluster and the J_H cluster. *A priori*, the D_{FL16}–D_{SP2} cluster may be in either the 5′ or the 3′ side of the J_H cluster. Likewise, the *D* and J_H segments may be in either the same or opposite orientation. However, because the D_{Q52} segment was mapped 696 bp 5′ to the J_H cluster and is in the same orientation as the J_H segments (4), we thought that "chromosomal walking" toward the 5′ side from the J_H cluster and the 3′ side from the D_{FL16}–D_{SP2} cluster would be most promising.

We have previously isolated a genomic DNA clone, MEP200, from a phage library constructed by inserting BALB/c embryo DNA that had been partially digested with *Eco*RI. This DNA clone was shown to contain the J_H cluster-positive 6.4-kb *Eco*RI fragment, as well as *Eco*RI fragments of 1.5, 3.0, and 3.8 kb, all located 5′ to the 6.4-kb fragment (S. Tonegawa, unpublished data). We have now determined the order of the four *Eco*RI fragments (Fig. 1A). Screening of 200,000 cosmid clones with the 3.0-kb *Eco*RI fragment probe (Fig. 1A) yielded four clones. Each of the four clones contained the 6.4-kb J_H-positive, the 12-kb C_μ-positive, and the 10-kb C_δ-positive *Eco*RI fragments that had been identified previously (14, 15). In addition, each clone contained a few additional *Eco*RI fragments located 5′ to the 6.4-kb fragment, 3′ to the 10-kb fragment, or both. However, there was a general tendency that the cloned sequences

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Abbreviations: V, variable; D, diversity; J, joining; C, constant; kb, kilobase(s); bp, base pair(s); A-MuLV, Abelson murine leukemia virus.

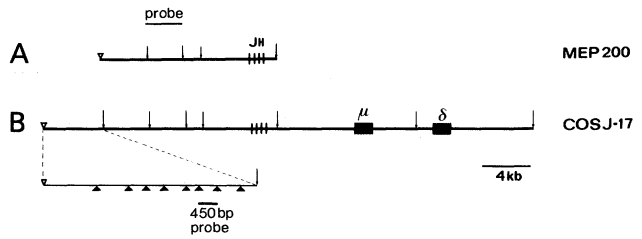

FIG. 1. (*A*) *Eco*RI map of phage clone MEP200; the 3-kb fragment shown was used as probe for cosmid screening. (*B*) *Eco*RI map of clone CosJ-17, which was positive to the 3-kb MEP200 5′ J_H probe shown in *A*. A 5′ CosJ-17 *Hin*fI 450-bp fragment was used as the probe for the second cycle of cosmid screening. Symbols: ↓, *Eco*RI; ▽, *Mbo* I; ▲, *Hin*fI.

extended more toward the 3′ side than toward the 5′ side relative to the position of the 3-kb *Eco*RI fragment used as the hybridization probe. The restriction enzyme map of clone CosJ-17, which extends most toward the 5′ side, is shown in Fig. 1*B*.

As the 3′ probe of the *D* cluster, we used a unique 1.1-kb *Bgl* II fragment isolated from the 3′ flanking region of the $D_{SP2.8}$ segment, the *D* segment that occupies the 3′-most position in the D_{FL16}–D_{SP2} cluster. However, we could not find any positive clones in the cosmid library.

Two Bands of 16- and 6.7-kb *Eco*RI Fragments Are Detected by a 5′-End Probe of Clone CosJ-17. The 5-kb *Mbo* I/*Eco*RI fragment located at the 5′ end of clone CosJ-17 contains repetitive sequences as judged by Southern gel blot analysis of embryo DNA (data not shown). Among the various shorter fragments generated by *Hin*fI digestion of the 5-kb *Eco*RI fragment, the 450-bp fragment shown in Fig. 1*B* was found to hybridize with *Eco*RI fragments of 16 and 6.7 kb present in an *Eco*RI digest of embryo DNA (Fig. 2*A*). We therefore concluded that one of the two *Eco*RI fragments overlaps the 5-kb *Mbo* I/*Eco*RI fragment from which the probe was isolated; the other *Eco*RI fragment must have been detected by cross-hybridization.

The 6.7-kb Band Contains Previously Identified *D* Segments. We isolated four clones from the *Eco*RI partial phage library by using the 450-bp *Hin*fI/*Hin*fI probe. Three clones (R-3, R-5, and R-6) were identical and contained 6.7- and 5-kb *Eco*RI fragments. The fourth clone (R-2) carries *Eco*RI fragments of 5.4, 2.8, and 6.7 kb. When these clones were analyzed further, they were found to contain sequences that hybridize strongly with both SP2 and FL16 *D* probes; the 6.7- and 5-kb inserts of clones R-3, R-5, and R-6 and the 5.4- and 6.7-kb inserts of clone R-2 were positive. These clones were then compared with the *D* segment-positive phage clones previously isolated in our laboratory (6). Clones R-3, R-5, and R-6 were judged to be identical with the previously described clone RPI, and clone R-2, with clones R1-5, R1-6, and R1-9.

The above results suggest that the 6.7-kb band detected by the 450-bp *Hin*fI/*Hin*fI probe by Southern gel blot analysis (Fig. 2*A*) represents the *D*-containing 6.7-kb fragment previously described (6). Since the locations of the *Eco*RI sites in the region downstream (namely, the 3′ side) of the 6.7-kb *Eco*RI/*Eco*RI fragment (6) and the 5-kb *Mbo* I/*Eco*RI fragment (Fig. 1*B*) are clearly different, the 6.7-kb fragment must not overlap the 5-kb fragment and must have been detected in the Southern gel blot (Fig. 2*A*) by cross-hybridization. This in turn suggests that the 16-kb fragment detected in the same Southern gel blot (Fig. 2*A*) overlaps the 5-kb *Mbo* I/*Eco*RI fragment. Since the 3′-most *Eco*RI fragment of the D_{FL16}–D_{SP2} cluster is 16 kb, we suspected that we might have reached the *D* cluster.

The 5′ End Region of CosJ-17 Overlaps the 3′ End Region of the *D* Cluster. To test the above hypothesis, we carried out two types of experiment. The phage clone, B1-3, has previously been shown to contain a 16-kb *Eco*RI insert that contains the two SP2 type *D* segments that lie most 3′ in the D_{FL16}–D_{SP2} cluster (6). The partial restriction map of the 3′ end region of the 16-kb *Eco*RI fragment corresponds to that of the 5′ end region of CosJ-17 (Fig. 3*A*). To confirm that the two regions indeed overlap, the 2.5-kb *Bgl* II/*Eco*RI fragments (Fig. 3*A*) isolated from the B1-3 and CosJ-17 clones were digested with various restriction enzymes and the DNA fragments were fractionated side by side by acrylamide gel electrophoresis. The results (Fig. 2*B*) indicate that a total of 18 cleavage sites, for *Hin*fI, *Ava* II, and *Sau*3A, are distributed equally (within the limit of the resolution) in the two 2.5-kb fragments.

To further confirm the identity of the 3′ end region of the 16-kb *Eco*RI fragment and the 5′ end region of the CosJ-17 insert, we analyzed another phage clone, RIS73, in comparison

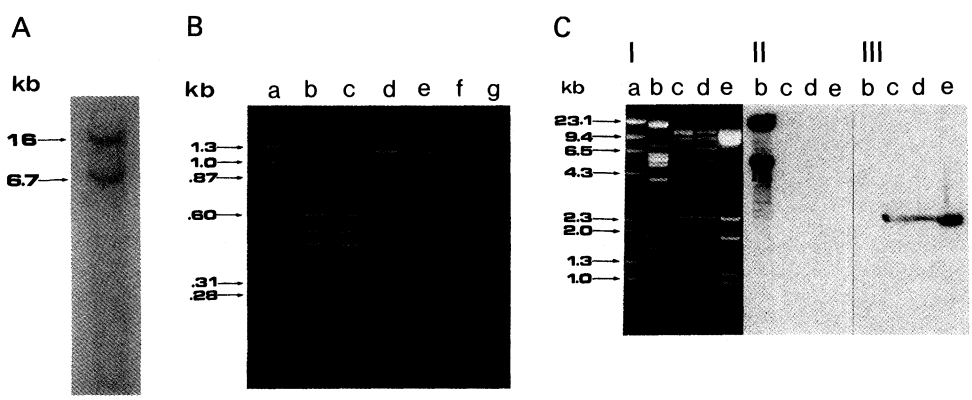

FIG. 2. (*A*) Southern blot hybridization of *Eco*RI-digested BALB/c mouse embryo DNA with the 5′ CosJ-17 *Hin*fI probe described in Fig. 1*B*. (*B*) Restriction enzyme pattern of a 2.5-kb *Bgl* II/*Eco*RI fragment isolated from both the B1-3 phage clone and clone CosJ-17 (see Fig. 3*A*); 0.2 μg of each DNA was restricted, electrophoresed on a 6% acrylamide gel, and stained with ethidium bromide (0.5 μg/ml). Lanes: a, *Hae* III-digested φX174 marker; b, *Hin*fI-digested B1-3 phage DNA; c, *Hin*fI-digested CosJ-17 fragment; d and e, *Ava* II-digested fragment from B1-3 and CosJ-17, respectively; f and g, *Sau*3A-digested fragment from B1-3 and CosJ-17. (*C*) Southern blot analysis of various cosmid clones. (*I*) Phage and cosmid DNAs were digested with *Bam*HI, electrophoresed on a 1% agarose gel, and stained with ethidium bromide (0.5 μg/ml). (*II*) DNA fragments were transferred to nitrocellulose membranes and hybridized to a mixture of nick-translated SP2 and DLF *D* probes. The hybridized membrane was washed twice under relaxed conditions with 0.75 M NaCl/0.075 M Na citrate/0.1% $NaDodSO_4$ at 42°C. (*III*) The same DNA fragments transferred to nitrocellulose membranes were hybridized to the Q52 *D* probe, and the membrane was washed under the same relaxed condition. Lanes: a, *Hin*dIII-digested λ DNA; b, B1-3; c, CosJ-11; d, CosJ-17; e, CosJ-20.

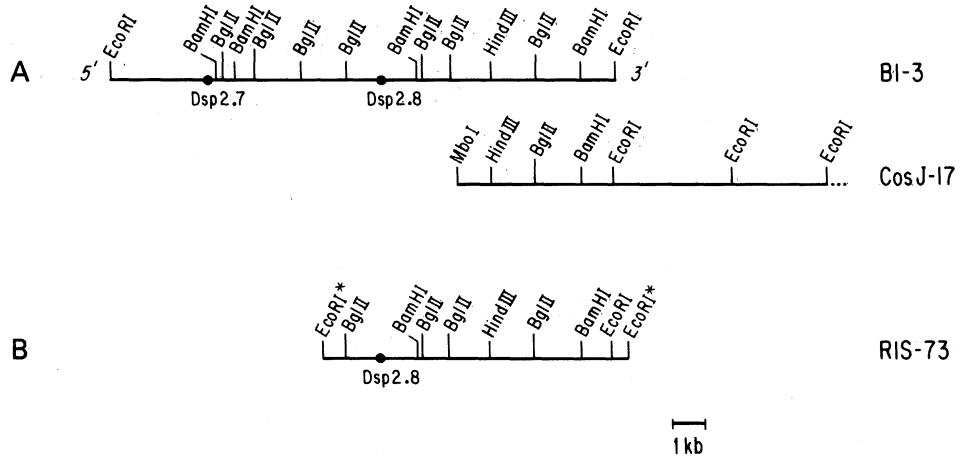

FIG. 3. (*A*) Map of the phage B1-3 16-kb clone and partial map of the CosJ-17 clone. The overlapping 5-kb region between clones B1-3 and CosJ-17 was mapped with *Bam*HI, *Bgl* II, and *Hin*dIII. ●, Positions of SP2-type *D* segments. (*B*) Map of phage clone RIS73.

with clone CosJ-17. This phage clone (mentioned in a previous report from this laboratory) was isolated from a partial *Eco*RI* library (6). Its insert was shown to contain the 3′ part of the 16-kb *Eco*RI fragment and an additional 500-bp *Eco*RI/*Eco*RI* fragment on its 3′ side. As shown in Fig. 3*B*, the partial restriction-site map of the 3′ region of clone RIS73 is indistinguishable from that of the 5′ region of clone CosJ-17. In addition, the 500-bp *Eco*RI/*Eco*RI* fragment of the former clone hybridizes with the 3.8-kb *Eco*RI fragment of the latter clone (data not shown).

No Additional *D* Segments Can Be Identified in the 20-kb Region Between $D_{SP2.8}$ and D_{Q52}. Our results have shown that the 3′-most *D* segment of the D_{FL16}–D_{SP2} cluster, $D_{SP2.8}$, and the D_{Q52} segment are about 20 kb apart. To examine the possibility that this region of DNA might contain additional *D* segments related to the known *D* segments, we analyzed clone CosJ-17 and other cosmid clones by Southern gel blotting. The hybridization probes used for this experiment were a mixture of three genomic DNA fragments, each of which contains a representative *D* segment and its 5′ and 3′ flanking sequences of one of the three *D* segment families (4–6). We have previously shown that all 12 known *D* segments can be detected by the mixed probes by Southern gel blot analysis of total cellular DNA. As shown in Fig. 2*C*, neither the 5-kb *Mbo* I/*Eco*RI fragment residing between $D_{SP2.8}$ and D_{Q52} nor any of the three *Eco*RI/*Eco*RI fragments (3.8, 3, and 1.5 kb) showed any indication of sequence homology to the probes, even under relatively relaxed hybridization conditions (Fig. 2*C*) and even after a prolonged exposure.

DISCUSSION

Linkage of *D*, J_H, and C_H Segments. On the basis of the work reported here, we now know the relative locations of the *D* segment cluster and the J_H segment cluster. Thus, the 11 germ line *D* segments previously described have been mapped between 80 to 19.5 kb 5′ to the J_{H1} segment, the 5′-most J_H segment of the four known J_H segments. In addition, as previously shown, the 12th known germ line *D* segment (D_{Q52}) lies about 700 bp 5′ to the J_{H1} segment. Since the positions of the J_H segments relative to the C_H segments coding for the *C* regions of eight different heavy chains (i.e., μ, δ, γ_3, etc.) is known (14, 15), the present study allows a complete mapping of 12 *D* segments, 4 J_H segments, and 8 heavy chain-coding DNA sequences that span as a whole about 280 kb (Fig. 4). The precise location of germ line V_H segments relative to any of these gene segments is currently unknown.

There Probably Exist Only About a Dozen *D* Segments. Our extensive search by hybridization of the cloned genomic DNA fragments with *D* sequence probes did not reveal any new *D* segment that might be located between the D_{FL16}–D_{SP2} cluster and the J_H cluster. An analogous search in the upstream region of the D_{FL16}–D_{SP2} cluster has not yet been carried out and there may exist additional *D* segments in this region. Nevertheless, the above negative finding supports the hypothesis that there

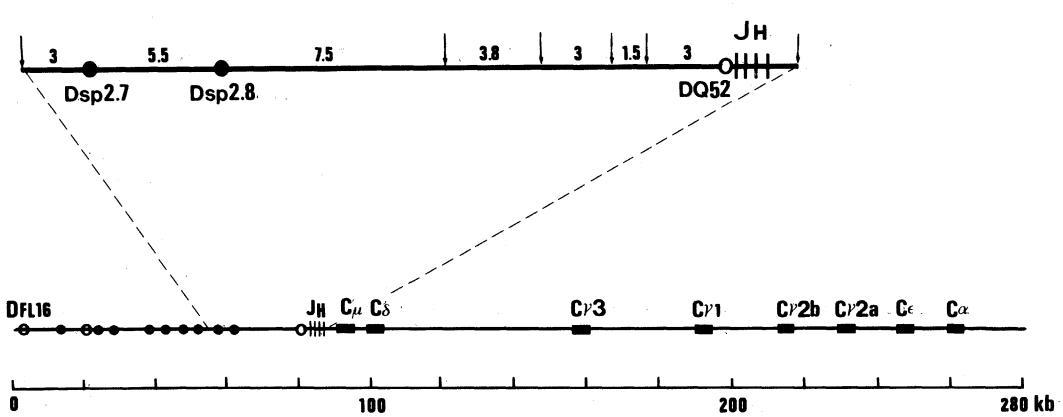

FIG. 4. Complete linkage map of the D–J_H–C_H cluster, occupying a 260-kb region. (Insert) Detailed *Eco*RI linkage map of the 3′-most SP2-type *D* segment with D_{Q52} and J_H (2, 6, 14, 15).

may not be very many more *D* segments than the known 12, as suggested previously (6).

Unidirectional Joining of *D* Segments. As previously pointed out, germ line Ig gene segments carry, in the vicinity of the joining end(s), conserved heptameric and nonameric sequences separated by a 12- or 23-bp spacer of unconserved sequence (1, 2, 16). In addition, a legitimate joining occurs only between two segments, one characterized by a short spacer and the other by a long spacer (1, 2). Since these rules are so universally followed that these sequences are thought to constitute a "signal" to guide enzyme catalyzing the recombination (the putative "recombinase") to the joining ends for the recombinational reaction. Since the signal sequence present in one end of a *D* segment is a nearly perfect complement of that present in the other end (both are of a short spacer type) (4–6), a question arises as to whether a *D* segment might be used bidirectionally in the formation of complete heavy chain genes. Indeed, this may be advantageous for the organism because it will increase the diversity of finished genes. However, comparison of the nucleotide sequences of known germ line *D* segments and the somatically rearranged *D* segments of expressed heavy chain genes indicate that *D* segments are expressed only in one orientation (4–6). Furthermore, this unidirectional expression of *D* segments correlates equally with unidirectional joining of germ line *D* segments in *D*–J_H segment joining: In eight out of eight cases studied, germ line *D* segments have been joined with one of the four J_H segments only in one of the two orientations, which is the same orientation as in expressed genes (6). Since these joined *D*–J_H segments are unexpressed, an explanation based on selection applied at the level of protein is not sufficient. Clearly a proper explanation for the unidirectional use of *D* segments must be sought in the joining mechanism. One conclusion that can be drawn from the present study may be important in considering this mechanism—namely, that the orientations of *D* segments relative to J_H segments are identical without an exception (at least to date) in the germ line configuration and in the somatically rearranged configuration. In light of this finding, we suggest (see below) a joining mechanism that can explain the unidirectional joining of *D* segments with J_H segments.

Associative Tracking Model. As suggested previously, we assume that the recombinase is composed of two structurally related DNA binding subunits or domains, one having the capacity to bind the signal sequence with a 23-bp spacer and the other, with a 12-bp spacer (1, 2). As postulated for other specific DNA-binding proteins including the "resolvase" encoded by a transposon Tn5 (17, 18), we suggest that the recombinase binds nonspecifically on any DNA sequence (Fig. 5, step 1) and then travels along the DNA by a one-dimensional walk until one of the two subunits or domains finds a properly spaced signal sequence where it binds tightly (Fig. 5, step 2). We further suggest that, while the recombinase is bound tightly and specifically to this signal sequence via one subunit or domain, its second subunit or domain continues DNA tracking (Fig. 5, step 3) until it finds a signal sequence with a spacer of the proper length (Fig. 5, step 4) (a short one if the first signal sequence was the long spacer type and vice versa). In this second phase, tracking only those signal sequences that lie in the orientation opposite to the first, one can bind tightly with the second subunit or domain because the asymmetry of the combining site does not permit its tight binding with the signal sequence of the same polarity. One addendum to the above scheme is that, when the recombinase subunit finds a proper signal sequence, it does not necessarily bind tightly: the subunit may skip that signal and continue tracking by weak binding. In this way, *D* segments distal to J_H segments can participate in *D*–J_H segment joining and *vice versa*.

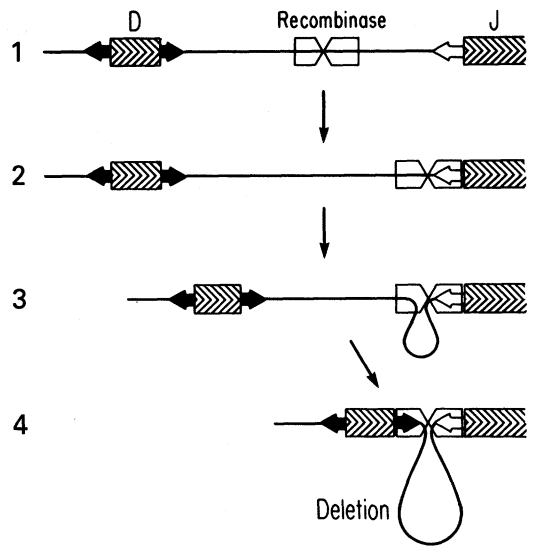

FIG. 5. Associative tracking model. *D* and J_H segments are in the same orientation. ◊, Signal sequence with a 23-bp spacer; ◆, signal sequence with a 12-bp spacer.

If *D*–J_H segment joining occurs according to the above scheme, only one of the two signal sequences flanking a *D* segment—namely, that lying proximal to J_H segments—can be used for *D*–J_H joining and hence the unidirectional joining of *D* segments is achieved. A natural consequence of the scheme is looping-out and deletion of the DNA lying between the two signal sequences (inclusive) participating in the recombination. This prediction seems to be generally met by experimental findings; the sequence 5′ to the joined J_H segment is almost always deleted from Abelson murine leukemia virus (A-MuLV) transformants, B-cell lymphomas, and myelomas that have undergone J_H rearrangement on both chromosomes (ref. 19; unpublished data). In addition, it has been shown that only those *D* segments that are mapped 5′ to the *D* segment participating in *D*–J_H segment joining are retained in almost all myelomas and A-MuLV transformants (unpublished data). These results indicate that *D*–J_H joining almost always occurs by looping-out deletion mechanism.

Dissociative Tracking Model. In contrast to *D*–J_H segment joining, often V_κ–J_κ segment joining does not occur by the simple looping-out deletion mechanism. Many myelomas and A-MuLV transformants contain an apparently reciprocal recombination product—namely, the J_κ signal sequence joined back to back with the V_κ signal sequence (20–24). Both the ratio of the reciprocal products to *C* region rearrangements and the isolation of lines that bear reciprocal fragments apparently unrelated to their accompanying assembled κ genes initially led to the presentation of sister chromatid exchange model (22, 23). However, recent findings of a truly reciprocal product in a myeloma (25) and of the existence of secondary rearrangements in an A-MuLV transformant (24) suggest that V_κ–J_κ joining may occur by a combination of inversion and deletion. Whichever of the two mechanisms turns out to be correct, the DNA-tracking mechanism as outlined above cannot easily explain the persistence of the reciprocal product.

Therefore, we suggest that the recombinase exists in cells in a dissociated form as well as in the associated form and that each of the two subunits independently (as well as in the associated form as discussed above) has the capacity to bind DNA weakly, to track on it, and to bind tightly and specifically with its respective signal sequence. The pair of signal sequences tightly

bound to the subunits are brought together for the joining reaction by virtue of diffusion and affinity of the two subunits. Unlike the associated subunits, the dissociated subunits are free from each other in choosing the orientation of the signal sequence with which they bind. Likewise, the dissociated subunits can bind signal sequences in separate DNA molecules under proper conditions. This "dissociative tracking" mechanism, in contrast to the "associative tracking" mechanism outlined above, can result in inversion or sister chromatid exchange in addition to deletion.

Distance Between Joining Segments May Affect the Joining Mechanism. Why then does D–J_H segment joining occur by "associative tracking" and V_κ–J_κ segment joining by "dissociative tracking"? The key to this question may lie in the difference in the distance between the joining gene segments in the germ line genome. As shown in this study, all identifiable D segments lie between 80 to 1 kb from J_H segments. The distance between the J_κ segments and the most proximal V_κ segment is unknown. However, the number of V_κ segments per haploid genome has been estimated to be 90–300 (26) and, in several cases studied, the distance between two adjacent V_κ segments is about 10 kb (unpublished data). These values suggest that the most distal V_κ segments are at least 900–3,000 kb from J_κ segments, values at least one order of magnitude higher than that for the most distal (identifiable) D segments. If the distance between the joining segments is below a certain limit (as in D–J_H segment joining) we assume that associative tracking is intrinsically more efficient than dissociative tracking. However, if this distance exceeds a certain value (as in V_κ–J_κ segment joining), the efficiency of associative tracking decreases rapidly. This is because, during the second-phase tracking, the loosely bound subunit will fall off the DNA at a predetermined frequency. Thus, dissociative tracking becomes the major mechanism. The above consideration suggests that D–J_H segment joining could occasionally occur by dissociative tracking. The rare inverted joining of D and J_H segments discovered in one A-MuLV transformant may be such an example (27).

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