REARRANGEMENT OF EXOGENOUS IMMUNOGLOBULIN V_H AND DJ_H GENE SEGMENTS AFTER RETROVIRAL TRANSDUCTION INTO IMMATURE LYMPHOID CELL LINES

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The variable regions of Ig and T cell receptor chains are encoded in the germ line by discrete elements that are brought together by site-specific recombination during lymphoid differentiation (1, 2). As a consequence of this process, a diverse set of variable regions can be specified by a relatively small number of gene segments. In the case of Ig H chains, for example, the variable regions are encoded by three germ line elements: V_H, D, and J_H. During B cell differentiation, individual segments from each group are joined to form a complete V_H-D-J_H variable region (3–9).

Unrearranged Ig and T cell receptor gene segments are accompanied by similar sequences that lie near the sites of recombination and are presumed to mediate rearrangement (3, 4, 10–15). These putative recombinational signals consist of three elements: a heptamer sequence, a spacer region, and a nonamer sequence. The heptamer and nonamer sequences are highly conserved, both among gene families and among vertebrate species (1, 2, 16, 17). The spacer regions are less well conserved with respect to sequence, but in length they fall into two classes of ~12 and 23 bp. Gene segments that are associated with 12-bp spacer regions normally recombine with segments that carry 23-bp spacer regions (3). The generality of these observations suggests that rearrangements of all Ig and T cell receptor gene segments are catalyzed by a common apparatus.

While the structural features of Ig gene segments have long been defined, the mechanism of rearrangement remains unknown. It has recently been demonstrated that exogenous Ig or T cell receptor gene segments can undergo rearrangement after they are stably introduced into lymphoid cells (18–22). In one experiment, a retroviral vector was used to deliver unrearranged kappa chain gene segments to a B cell precursor cell line; selection for integration was linked to the activation of a selectable marker, which in turn was dependent on rearrangement (18, 20). These results suggested a functional approach to the study of Ig gene rearrangement, by manipulation of model substrates in vitro and subsequent assay for their recombination in cultured cells. To facilitate this approach, we have devised an assay for the joining of exogenous Ig H chain gene segments in vivo. A retroviral vector is used to deliver the substrate to recipient

cells; selection for viral integration is independent of rearrangement. We demonstrate joining of the exogenous segments in cell lines that represent three distinct stages in B cell ontogeny. In addition, we directly observe that the joining of H chain gene segments occurs by means of a nonreciprocal recombinational mechanism. Our results suggest that Ig H and L chain gene segments are joined by a common mechanism that is more active in B cell precursors than in mature B cells. The assay described here, which permits selection for retroviral integration regardless of rearrangement, should be of particular use in defining the nucleotide sequence requirements for Ig gene assembly.

Materials and Methods

DNA Constructions and Probes. The retroviral vector pDOL⁻ (23) was a gift of Richard Mulligan, Whitehead Institute, Cambridge, MA. The vector (see Fig. 1 A) is based on sequences derived from the Moloney murine leukemia virus (Mo-MLV)[1], including: the 5' and 3' long terminal repeats (LTRs), which are required for initiation and polyadenylation of viral transcripts and for viral integration; the minus and plus strand primer-binding sites necessary for replication (24, 25); and sequences required for packaging of viral RNA (Ψ site) (26, 27). The 5' splice donor sequence has been mutationally inactivated to prevent the generation of subgenomic RNA. Located between the LTRs is a Bam HI–Hind III fragment containing the SV40 early promoter and origin of replication. Immediately adjoining this fragment is a 1,321-bp Hind III–Eco RI fragment containing the neomycin (*neo*) resistance gene of Tn5 (28, 29); in this vector the *neo* gene is under control of the SV40 early promoter. Also present is an Eco RI–Xho I fragment containing the pBR322 origin of replication.

The substrate for Ig H chain gene rearrangement, pLJHCR, was derived from pDOL⁻ by the insertion of a 2.0-kb cassette at the unique Bam HI site of the vector. This cassette is diagrammed in Fig. 1 B, and contains three elements. The 750-bp Bam HI–Eco RI fragment is the former Bgl II–Hind III fragment of p40E4-2-5.2 (30); it contains the recombined $D_{SP2.7}$ and J_H3 Ig gene segments of BALB/c mouse. The V_H-containing Eco RI–Bam HI fragment was derived from the 246-bp Eco RI–Pst I fragment of p40E4-2-26-8.0 (30); it contains the terminal 204 bp of the V_H81X coding sequence from BALB/c mouse (31) and 42 bp of 3' flanking DNA. Between these two segments of mouse DNA we inserted the xanthine–guanine phosphoribosyl transferase gene (*gpt*) of *Escherichia coli* (32–34), which was derived from the 1,057-bp Hind III–Bam HI fragment of pPT-1 (32) by the attachment of synthetic Eco RI linkers. In pLJHCR, the *gpt* gene lies in antisense orientation relative to the 5' LTR.

To prepare the *gpt* probe, pLJHCR was cut with a mixture of Eco RI and Kpn I, and the resulting 740-bp fragment was purified by electrophoresis through 1.0% agarose. To prepare the *neo* probe, pLJHCR was cut with Hind III and Eco RI, and the resulting 1,321-bp fragment was isolated by electrophoresis through 0.8% agarose. The locations of these probes with respect to the pLJHCR restriction map are indicated in Fig. 3.

All plasmids were propagated as monomers in the *E. coli* strain DH1 (35). Plasmid DNA was prepared by a detergent lysis procedure (36) and purified over two CsCl equilibrium gradients.

Cell Lines. The Ha-MSV–transformed cell line HAFTL-1 (37), the Ab-MuLV–transformed cell lines 40E4-2 (30) and PD31 (38), the B cell lymphomas M12.4.1 clone 4.5 (39) and A202J (40), and the B cell hybrid line SP2/0 (41) were cultured in RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-ME (RPMI-10). The cell lines Ψ-2 (26) (a gift of Richard Mann, Whitehead Institute, Cambridge, MA) and NIH-3T3 were

[1] *Abbreviations used in this paper:* LTR, long terminal repeats; Mo-MLV, Moloney murine leukemia virus.

propagated in DME supplemented with 10% FCS (DME-10). Lymphoid cells were cloned by limiting dilution at a concentration of 0.2 cell/microtiter well.

Packaging of Recombinant Retrovirus. The plasmid pLJHCR was transfected into the Ψ-2 cell line by the calcium phosphate technique of Graham and van der Eb (42), as modified by Parker and Stark (43), except that salmon sperm DNA was omitted from the transfection cocktail. 2 d after transfection, the cells were trypsinized, suspended in fresh DME-10, and diluted 10-fold into DME-10 containing G418 at 1 mg/ ml (28). At 5, 8, and 11 d after transfection, cells were fed with fresh medium containing G418 at 1 mg/ml. Clones of G418-resistant (G418^r) cells were evident by 14 d after transfection; these were transferred to individual microtiter wells and propagated in DME-10 containing G418 at 1 mg/ml. To harvest recombinant LJHCR virus, stably transfected Ψ-2 cells were grown in a 78.5-cm^2 dish in the presence in G418 at 1 mg/ml until the cells had reached 75% confluence. The selective medium was then removed and 10 ml DME-10 was added. After incubation for 16 h, the culture supernatant was collected and filtered through nitrocellulose (0.45-μm pore size). The supernatant was stored in 1-ml aliquots at −70°C. Viral stocks were stable under these conditions for at least 6 mo.

Virus was quantitated by infection of NIH-3T3 cells followed by selection in G418, as described by Cepko et al. (44). Viral titers are expressed as the number of G418^r NIH-3T3 colonies obtained per ml Ψ-2 culture supernatant (G418^r cfu/ml).

Infection of Lymphoid Cell Lines. Cultures of lymphoid cells at 1–2 × 10^6 cells/ml were collected by centrifugation and suspended in RPMI-10 at a concentration of 10^7 cells/ml. To infect cells, 1 ml Ψ-2 culture supernatant and 0.1 ml (10^6) lymphoid cells were combined in a 20-cm^2 dish and incubated at 37°C in the presence of 8 μg/ml polybrene. After 2.5 h, 3 ml RPMI-10 was added and the culture was incubated for 48 h. Cells were then split 1:5 into selective medium containing G418 at 2 mg/ml. At 5, 8, and 11 d after infection, cells were transferred to fresh selective medium containing G418 at 2 mg/ml. Growth of G418^r cells was usually apparent by 14 d after infection.

Preparation and Analysis of Genomic DNA. Genomic DNA was prepared as described (45). DNA probes were labeled with ^{32}P by synthesis from random oligonucleotide primers as described by Feinberg and Vogelstein (46). Hybridization reactions were undertaken in 50% formamide, 0.6 M NaCl, 0.075 M sodium citrate, 0.065 M KH$_2$PO$_4$, 0.005 M Na$_2$-EDTA, 0.02% (each) BSA, Ficoll, and polyvinylpyrrolidone 360, 100 μg of denatured salmon sperm DNA per ml, and 10^6 cpm of heat-denatured probe per ml for 16–24 h at 42°C. Filters were washed in 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% SDS at 68°C for 45 min; ^{32}P was detected by autoradiography.

Isolation and Characterization of Genomic DNA Clones. Genomic DNA from the HAFTL-1/LJHCR-e-1 and PD31/LJHCR-m-10 cell lines was digested with Hind III and fractionated by electrophoresis through a preparative 0.8% agarose gel (Bull's Eye preparative gel unit, Hoefer, San Francisco). Selected fractions were pooled and DNA was ligated to λ2001 DNA (47), which had previously been digested with Hind III and Bam HI (Strategene, San Diego, CA). The products of ligation were encapsidated in vitro; the packaging extract (Gigapack, Stratagene, San Diego, CA) was used according to the supplier's instructions. The resultant bacteriophage were propagated in the *E. coli* strain LE392. Plaques were screened by the method of Benton and Davis (48). Bacteriophage were isolated by two additional rounds of plaque purification.

Results

Structure of the Retroviral Substrate for V$_H$-to-DJ$_H$ Joining. The recombination substrate used in these experiments, pLJHCR, was constructed in the retroviral vector pDOL$^−$ (see Materials and Methods and Fig. 1 A) (23). The vector contains the LTR sequences of the Mo-MLV, which are required for integration and transcription of the viral genome, and an element necessary for encapsidation of viral RNA (Ψ site) (26, 27). The vector also carries the *neo* gene of *E. coli* (28, 29) under control of the SV40 early promoter.
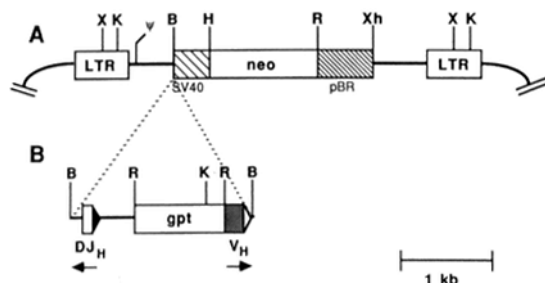
FIGURE 1. Structure of the model substrate for V_H-to-DJ_H recombination. The retroviral transcriptional unit of the vector pDOL⁻ is shown in A. The positions of the MoMLV LTRs and the *neo* gene are indicated by open boxes. Segments of DNA containing the SV40 early promoter and the pBR322 origin of replication are marked by hatched boxes. The approximate position of the site required for packaging of viral DNA is indicated (Ψ). Sequences surrounding the retroviral LTRs (~4,600 bp) are not included in the figure. The plasmid pLJHCR was derived from pDOL⁻ by the insertion of the 2.0-kb Bam HI cassette shown in B. The Ig DJ_H and V_H sequences are indicated by the small open box and the shaded box, respectively. The positions of the recombinational signal sequences of the Ig elements are marked by the filled and open triangles. The sense orientations of the DJ_H- and V_H-coding sequences are indicated by the arrows. The *gpt* gene is marked by the large open box. Restriction enzymes are abbreviated as follows: X, Xba I; K, Kpn I; B, Bam HI; H, Hind III; R, Eco RI; Xh, Xho I.

Two separate Ig sequences are present in pLJHCR; one contains the product of a D-to-J_H fusion and the other contains a portion of an unrearranged V_H gene, including 42 bp of its 3'-flanking DNA (Fig. 1B and Materials and Methods). These two segments have been shown to undergo V_H-to-DJ_H joining in cultured lymphoid cells (30). The Ig gene segments in pLJHCR lie in opposite transcriptional orientations; between them is located the *gpt* gene of *E. coli* (32), in antisense orientation with respect to the 5' LTR.

Transfection of pLJHCR into Helper Cells and Production of Infectious Recombinant Virus. The virus encoded by pLJHCR is defective; its propagation requires that a number of viral functions be supplied in *trans*. The helper cell line Ψ-2 (26) expresses all Mo-MLV proteins necessary for viral packaging and replication. To propagate the recombination substrate as a retrovirus, pLJHCR was introduced into the Ψ-2 cell line by transfection in the presence of calcium phosphate (42, 43). Stable derivatives of Ψ-2 containing pLJHCR DNA were selected in medium containing G418 (28). The resulting G418^r cell clones were expanded to ~10^7 cells in the presence of G418; the cells were then grown for one doubling time in the absence of G418. The titer of virus in culture supernatants was measured by infection of NIH-3T3 cells and selection in G418 as described in Materials and Methods. Of eight independent G418^r Ψ-2 lines tested, three were found to be stable producers of recombinant retrovirus; viral titers in culture supernatants ranged from 1×10^3 to 9×10^3 G418^r cfu/ml. One such cell line, Ψ-2/LJHCR-1, was the source of the virus used in the experiments described here.

Transmission of the LJHCR Virus to Immature B Lymphoid Cells. To determine whether the LJHCR substrate could be transmitted by infection to immature lymphoid cells, we initially used two cell lines, HAFTL-1 and 40E4-2, representative of cells at early stages of B lymphoid differentiation. The HAFTL-1 cell line was established by transformation of mouse fetal liver with the Harvey murine sarcoma virus (Ha-MSV) (37, 49). This line expresses a number of surface markers characteristic of members of the B cell lineage (50); it undergoes continuing Ig D-to-J_H rearrangement during propagation in culture (37). The 40E4-2 cell line was established by transformation of mouse fetal liver with the
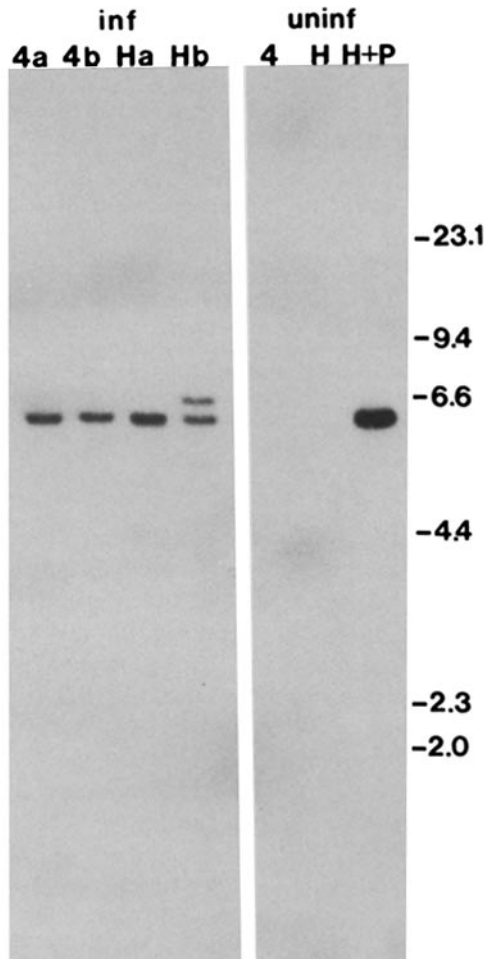
FIGURE 2. Detection of LJHCR proviral sequences in lymphoid cell populations. Genomic DNA (12 μg) from populations of G418^r cells or from uninfected cells was digested with Xba I and assayed for the presence of the LJHCR provirus by hybridization to the *neo* probe. Lanes *4a* and *4b*, DNA from 40E4-2/LJHCR, infections a and b; lanes *Ha* and *Hb*, DNA from HAFTL-1/LJHCR, infections a and b; lane *4*, DNA from 40E4-2; lane *H*, DNA from HAFTL-1; lane *H+P*, DNA from HAFTL-1 to which 23 pg of pLJHCR DNA was added before digestion. The positions of DNA standards (in kb) are indicated. •

Abelson murine leukemia virus (A-MuLV) (51). Like HAFTL-1, the 40E4-2 cell line expresses B lymphoid surface markers, but it possesses DJ$_H$ fusions on both chromosomes and undergoes V$_H$-to-DJ$_H$ joining in culture (30, 52).

These cell lines were infected with the LJHCR virus at a ratio of 1×10^2 (infections "a") and 1×10^3 (infections "b") G418^r cfu to 10^6 cells. After growth for 2 d in nonselective medium, cells were transferred to medium containing G418 at 2 mg/ml; G418^r derivatives of HAFTL-1 and 40E4-2 were evident at ~14 d. Samples of cells (~2×10^7) were taken from the G418^r populations for analysis of proviral DNA. Single cell clones were obtained from the same populations by limiting dilution.

To establish the presence of proviral DNA in G418^r derivatives of HAFTL-1 and 40E4-2, genomic DNA was digested with Xba I, fractionated by electrophoresis in agarose, transferred to nitrocellulose, and hybridized to a *neo*-specific probe (Fig. 2). The Xba I restriction enzyme was expected to cut LJHCR DNA once within each LTR to yield a single 6.0-kb fragment containing the entire internal proviral sequence (see Fig. 1). Each of the G418^r cell populations we
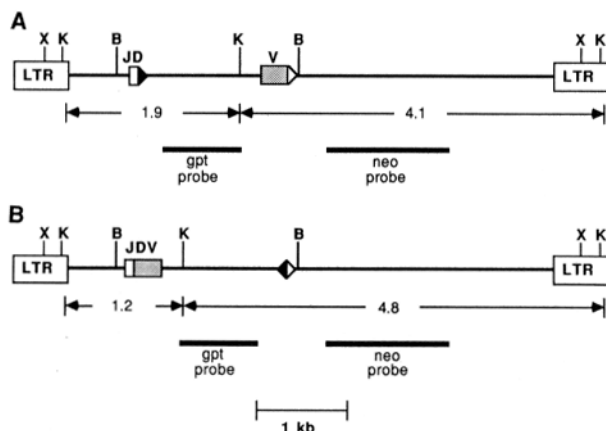
FIGURE 3. Assay for V_H-to-DJ_H joining within the LJHCR provirus. The V_H and DJ_H elements and the LTR sequences are indicated. The recombinational signal sequences of the DJ_H and V_H elements are marked by filled and open triangles, respectively. Regions corresponding to the *gpt*- and *neo*-specific probes are marked with heavy lines. The lengths (in kb) of Kpn I restriction fragments internal to unrearranged and rearranged proviruses are designated with arrows. (A) Unrearranged provirus. (B) Rearranged provirus. For description, see text.

examined (40E4-2/LJHCR infections a and b; HAFTL-1/LJHCR infections a and b) yielded a 6.0-kb *neo*-containing fragment (Fig. 2, lanes *4a, 4b, Ha,* and *Hb*) that comigrated with the fragment detected in a mixture of uninfected HAFTL-1 DNA and pLJHCR (Fig. 2, lane *H+P*). The fragment was not found in DNA from uninfected cells (Fig. 2, lanes *4* and *H*). In 3 of the 4 infected cell populations examined (40E4-2/LJHCR infections a and b; HAFTL-1/LJHCR infection a), this was the only *neo*-containing fragment detected (Fig. 2, lanes *4a, 4b,* and *Ha*). In one of the two infected HAFTL-1 pools (HAFTL-1/LJHCR infection b; Fig. 2, lane *Hb*) at least two *neo*-containing fragments were found that differed in length from the intact proviral fragment. The additional fragments may have arisen by recombination between internal proviral sequences and neighboring mouse DNA (S. V. Desiderio and K. Wolff, unpublished data). This observation prompted us to examine the stability of proviral sequences in other infected cell populations. In 14 clones derived from HAFTL-1/LJHCR infection a, and 23 clones derived from 40E4-2/LJHCR infection a, only the 6.0-kb *neo*-containing DNA fragment was seen (data not shown). Thus, the arrangement of the proviral DNA with respect to flanking sequences appeared to be stable in these cells.

Joining of Exogenous V_H and DJ_H Elements in the HAFTL-1 Cell Line. To detect specific recombination between the V_H and DJ_H sequences that had been introduced into the HAFTL-1 cell line, we used a direct physical assay, illustrated in Fig. 3. The LJHCR provirus contains three Kpn I restriction sites; one site lies within each LTR and a third site lies within the *gpt* gene, between the unrearranged V_H and DJ_H elements. Thus, digestion of unrearranged proviral DNA with Kpn I should create a 1.9-kb fragment that hybridizes to a *gpt*-specific probe and a 4.1-kb fragment that is detected by a *neo*-specific probe (Fig. 3A). Joining of the proviral V_H and DJ_H elements should result in an inversion of the intervening DNA. Digestion of proviral DNA with Kpn I after V_H-to-DJ_H rearrangement is therefore expected to yield two fragments; a 1.2-kb fragment and a 4.8-kb fragment that hybridizes to both the *gpt* probe and the *neo* probe (Fig. 3B).

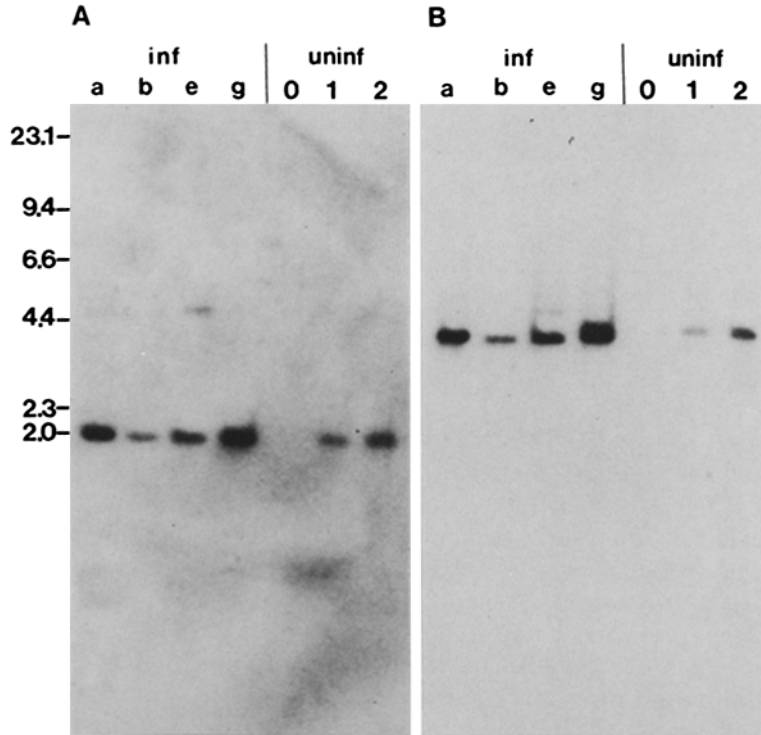Genomic DNA from clones of LJHCR-infected HAFTL-1 cells was cut with

FIGURE 4. Rearrangement of LJHCR proviral sequences in the HAFTL-1 cell line. Genomic DNA (12 μg) from clones of LJHCR-infected HAFTL-1 cells was digested with Kpn I and assayed for V_H-to-DJ_H joining by hybridization to DNA probes as outlined in Fig. 3. (A) Hybridization to the *gpt* probe. Lanes *a*, *b*, *e*, and *g*, DNA from HAFTL-1/LJHCR-a, HAFTL-1/LJHCR-b, HAFTL-1/LJHCR-e, and HAFTL-1/LJHCR-g, respectively. Lane *0*, DNA from HAFTL-1. Lanes *1* and *2*, DNA from HAFTL-1 to which 23 pg or 46 pg of pLJHCR, respectively, was added before digestion. (B) Hybridization to the *neo* probe. The filter used in A was washed in 0.5 M NaCl, 0.05 M NaOH, and 0.002 M Na_2EDTA for 30 min at 28°C to remove the *gpt* probe. The filter was neutralized and hybridized to the *neo* probe. Lanes are designated as in A. The positions of DNA standards (in kb) are indicated.

Kpn I, fractionated by electrophoresis, transferred to nitrocellulose, and hybridized to the *gpt* probe. Fig. 4 shows the results obtained with four of these clones, designated a, b, e, and g. In all four samples, a major 1.9-kb species was detected (Fig. 4A, lanes *a*, *b*, *e*, and *g*). In clone e (and faintly in clone a) a second, 4.8-kb species was found (Fig. 4A, lanes *a* and *e*). When the filter was stripped of the *gpt* probe and hybridized to the *neo* probe, a predominant species of 4.1 kb was found in all clones, as expected (Fig. 4B, lanes, *a*, *b*, *e*, and *g*). In addition, the minor 4.8-kb fragment that was detected with the *gpt* probe in clones a and e also hybridized to the *neo* probe (Fig. 4B, lanes *a* and *e*). These results are consistent with the occurrence of V_H-to-DJ_H joining within the LJHCR provirus. In all, 14 clones of LJHCR-infected HAFTL-1 cells were assayed; in two clones the rearranged provirus was detected. That these rearrangements represent joining of the V_H and DJ_H elements present within the LJHCR provirus was directly demonstrated by molecular cloning of the recombination products (see below).
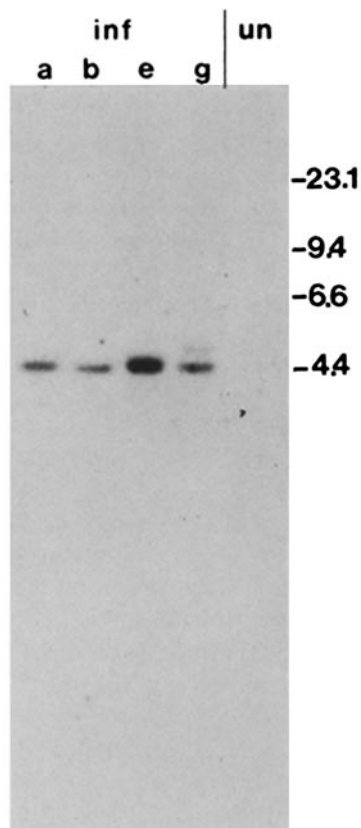
FIGURE 5. Analysis of viral integration sites in HAFTL-1/LJHCR cell clones. Genomic DNA (12 μg) from clones of LJHCR-infected HAFTL-1 cells was digested with Bam HI and viral integration sites were analyzed by hybridization to the *neo* probe, as described in the text. Lanes *a*, *b*, *e*, and *g*, DNA from HAFTL-1/LJHCR-a, HAFTL-1/LJHCR-b, HAFTL-1/LJHCR-e, and HAFTL-1/LJHCR-g, respectively. Lane *un*, DNA from uninfected HAFTL-1 cells. The positions of DNA standards (in kb) are indicated.

Integration of murine retroviruses occurs at a large number of sites in the host genome (53). Therefore, when two cells are infected independently they are likely to differ with respect to their proviral integration sites. Conversely, among the progeny of a single infected cell, the site of proviral integration will be the same. We used this fact to demonstrate that the rearrangement of exogenous Ig sequences occurred after their integration into the HAFTL-1 genome, rather than at an earlier stage in the experiment. Genomic DNA from clones a, b, e, and g was digested with Bam HI, fractionated by electrophoresis, transferred to nitrocellulose, and hybridized to the *neo* probe. The resulting *neo*-containing fragments are defined at one end by a Bam HI site within the LJHCR provirus and at the other end by a Bam HI site in the flanking mouse DNA. In the experiment shown in Fig. 5, all four clones share a common 4.4-kb *neo*-containing Bam HI fragment (sample g shows a minor band at 5 kb, and is probably not derived from a pure cell clone). Thus, at least clones a, b, and e are derived from the same infected cell. This fact, together with the presence of rearranged substrate in clones a and e, but not in b, indicates that rearrangement occurred after retroviral integration. In addition, homogeneous subclones of clone e containing rearranged or unrearranged provirus (see below) showed identical proviral integration patterns, further demonstrating that rearrangement occurred after retroviral integration (data not shown).

Assay for Rearrangement of the LJHCR Substrate in Other Cell Lines. Rearrangement of the LJHCR substrate was assayed in two cell lines that, together with HAFTL-1, represent intermediate states in B cell ontogeny (40E4-2 and PD31), in cell lines derived from mature B cells (M12.4.1 clone 4.5, A202J, and SP2/0), and in a fibroblastoid cell line (NIH-3T3). The 40E4-2 cell line, as described above, undergoes the second step in Ig H chain gene assembly: the joining of V_H segments to DJ_H elements (30, 52). The PD31 cell line represents a stage in B cell differentiation after the assembly of H chain genes, and undergoes continuing rearrangement of V_κ and J_κ segments in culture (38). The cell lines M12.4.1 clone 4.5 (39) and A202J (40) are derived from primary and secondary B cell lymphomas; they represent stages of differentiation after the assembly of Ig genes. The cell line SP2/0 (41) is derived from a hybrid between a mature B cell and a myeloma cell. Each of these lymphoid cell lines was infected with the LJHCR virus and $G418^r$ derivatives were obtained. Single cell clones were isolated from the $G418^r$ populations and assayed for rearrangement of the proviral substrate. Rearrangement was detected in 40E4-2 (4 positive clones out of 23 examined) and in PD31 (1 in 21 clones). We did not detect rearrangement of the substrate in the mature B cell lines M12.4.1 clone 4.5 (none in 32 clones) and A202J (none in 29 clones), or in the B cell hybrid line SP2/0 (none in 22 clones). We also failed to detect rearrangement of the provirus in the fibroblastoid cell line NIH-3T3 (none in 26 clones). Every cell clone that was positive for rearrangement contained a mixture of recombined and unrecombined substrate. Thus, the rearrangements we observed occurred during the expansion of the cell clones, and each positive cell clone represents at least one recombinational event.

Molecular Analysis of the Products of Recombination. To define precisely the products of recombination, we carried out molecular cloning of rearranged proviral DNA from LJHCR-infected HAFTL-1 and PD31 cells. For these experiments we used the HAFTL-1/LJHCR-e and PD31/LJHCR-m cell lines; these comprise a mixture of cells that contain either rearranged or unrearranged provirus (Fig. 6A, lane *e* and 6B, lane *m*). To obtain homogeneous populations of cells, subclones of HAFTL-1/LJHCR-e and PD31/LJHCR-m were isolated. Genomic DNA from these subclones was cut with Kpn I and assayed for recombination of proviral Ig segments. In some of these subclones we found only the 4.8-kb (rearranged) fragment (Fig. 6A, lane *e1* and 6B, lane *m10*), while in others only the 4.1-kb (unrearranged) fragment was detected (Fig. 6A, lane *e9* and 6 B, lane *m2*). Thus, from clones exhibiting a mixture of rearranged and unrearranged copies of the LJHCR provirus, we could obtain subclones in which all cells contained only the rearranged provirus.

To demonstrate directly V_H-to-DJ_H recombination within the LJHCR substrate, we carried out molecular cloning of rearranged proviral sequences from HAFTL-1/LJHCR-e-1 and PD31/LJHCR-m-10. The restriction maps of the fragments recovered from these cell lines were identical to the restriction map expected after joining of the Ig gene segments in LJHCR (Fig. 7, *a–c*). In each example, V_H-to-DJ_H joining gave rise to two products: a coding joint and a noncoding joint. We determined the nucleotide sequences of these regions. From this analysis it was clear that the LJHCR rearrangements observed in the HAFTL-1/LJHCR-
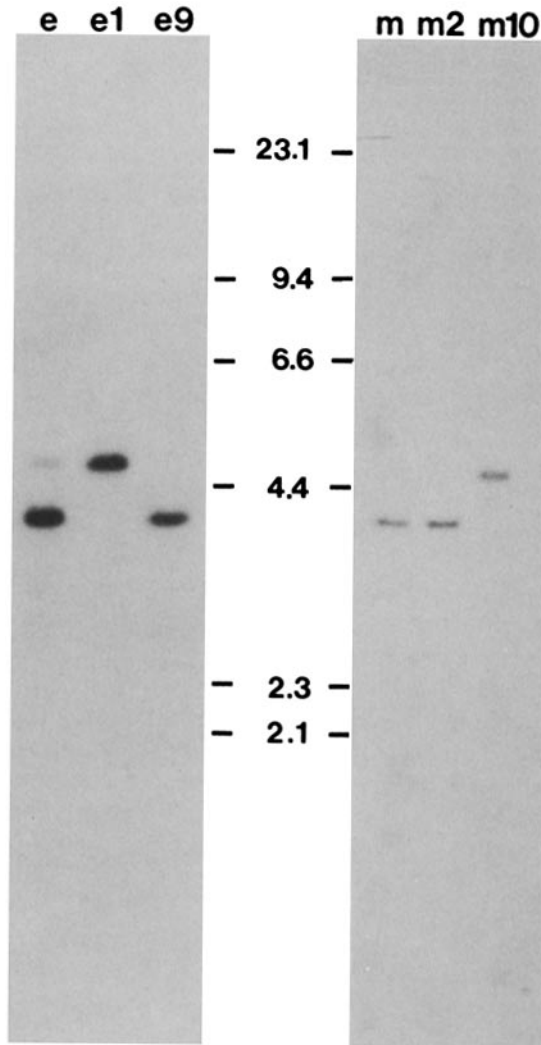
e   e1   e9   m m2 m10

— 23.1 —

— 9.4 —

— 6.6 —

— 4.4 —

— 2.3 —

— 2.1 —

FIGURE 6. Assay for rearranged and unrearranged LJHCR provirus in secondary clones of LJHCR-infected lymphoid cells. Genomic DNA (12 µg) was digested with Kpn I and assayed for V_H-to-DJ_H joining by hybridization to the *neo* probe. (A) Analysis of subclones of HAFTL-1/LJHCR-e. Lane *e*, DNA from HAFTL-1/LJHCR-e; lanes *e1* and *e9*, DNA from HAFTL-1/LJHCR-e-1 and HAFTL-1/LJHCR-e-9, respectively. (B) Analysis of subclones of PD31/LJHCR-m. Lane *m*, DNA from PD31/LJHCR-m; lanes *m2* and *m10*, DNA from PD31/LJHCR-m-2 and PD31/LJHCR-m-10, respectively. The positions of DNA standards (in kb) are indicated.

e-1 and PD31/LJHCR-m-10 cell lines were the result of V_H-to-DJ_H joining (Fig. 8). At the V_H–DJ_H junction recovered from HAFTL-1/LJHCR-e-1, the coding sequences of the V_H region and part of the DJ_H region are identical to the sequences that had been introduced into the cell line (Fig. 8 A, *a*). As is typical of V_H–DJ_H joints (9), some nucleotides were lost from the DJ_H coding sequence. The V_H–DJ_H junction obtained from PD31/LJHCR-m-10 was similar (Fig. 8 B, *a*); nucleotides were lost from both the V_H and DJ_H coding sequences. The sequences at the noncoding junctions from HAFTL-1/LJHCR-e-1 and PD31/LJHCR-m-10 revealed that the flanking sequences, in contrast to the coding sequences, had been precisely fused (Fig. 8 A, *b*, and B, *b*). No loss or addition of nucleotides was observed in either case.
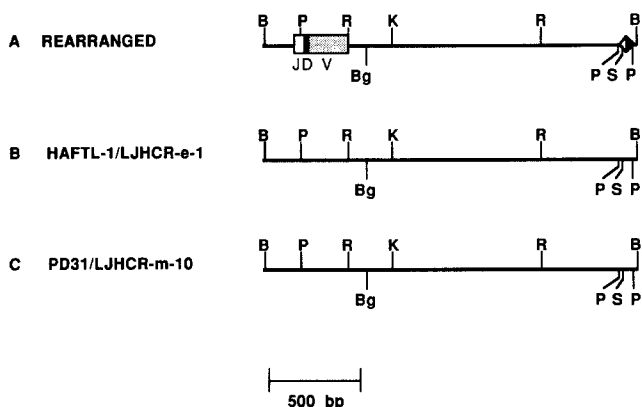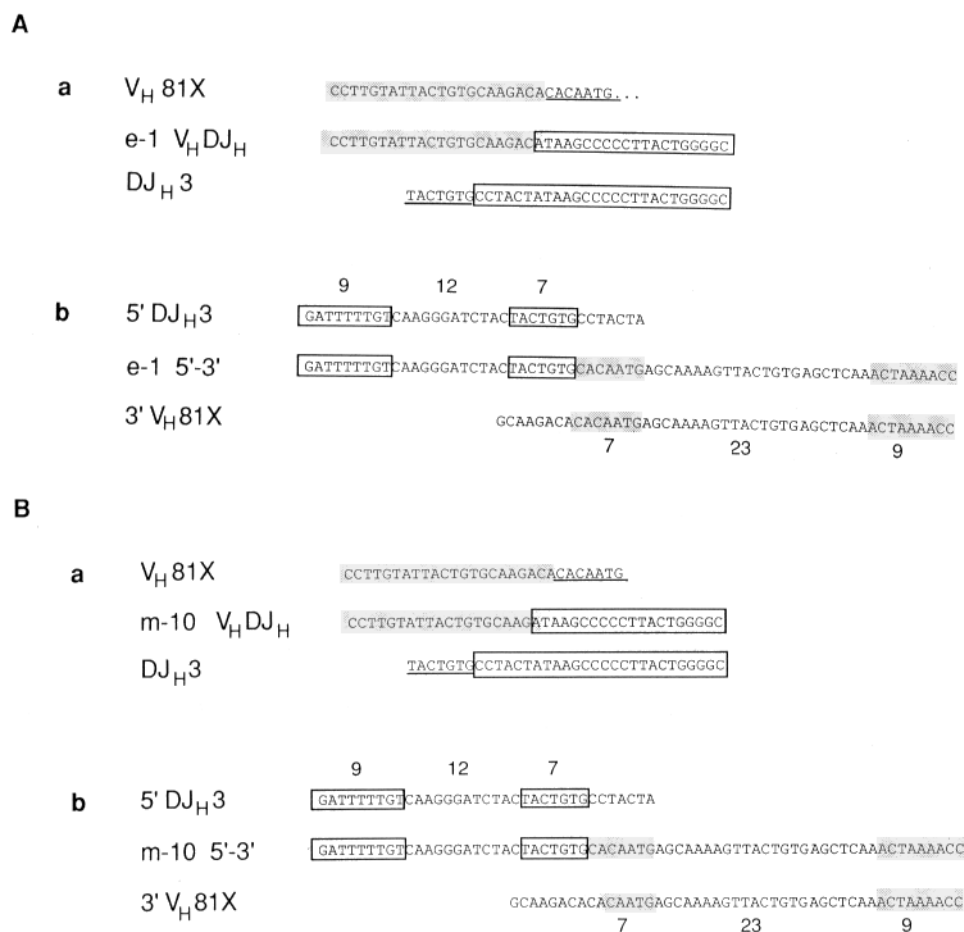
FIGURE 7. Joining of exogenous V_H and DJ_H elements in the HAFTL-1 and PD31 cell lines. Size-selected genomic DNA libraries for the HAFTL-1/LJHCR-e-1 and PD31/LJHCR-m-10 cell lines were constructed in λ2001 and screened for hybridization to the *gpt* probe. After initial propagation in λ2001, the 2.0-kb Bam HI fragments containing rearranged Ig sequences were subcloned into the Bam HI site of pUC13. (*A*) Predicted map of the LJHCR Bam HI cassette after V_H-to-DJ_H joining, shown for comparison. The positions of the recombined V_H, D, and J_H segments are shown as shaded, filled, and open boxes, respectively. The joined recombinational signal sequences of the V_H and DJ_H elements are marked by open and filled triangles, respectively. (*B*) Map of the 2.0-kb *gpt*-containing Bam HI fragment from HAFTL-1/LJHCR-e-1. (*C*) Map of the 2.0-kb *gpt*-containing Bam HI fragment from PD31/LJHCR-m-10. Restriction enzymes are abbreviated as follows: *B*, Bam HI; *P*, Pst I; *R*, Eco RI; *Bg*, Bgl II; *K*, Kpn I; *S*, Sac I.

Discussion

We have used a recombinant retroviral substrate to examine the rearrangement of exogenous Ig V_H and DJ_H elements in a series of cell lines. The retrovirus carries a neomycin resistance marker whose expression is independent of the arrangement of the resident Ig gene segments. The ability to select for retroviral integration independently of rearrangement made it possible to quantitate the virus present in culture supernatants of the transfected helper cell line. More importantly, this feature permitted us to introduce the substrate into a variety of cell lines regardless of their ability to support rearrangement. Joining of the exogenous gene segments was detected by a stringent hybridization assay that allowed us to simultaneously observe the rearranged and unrearranged substrate present in a cell population.

The assembly of Ig genes occurs by means of an ordered program of rearrangement (38, 52, 54, 55). The earliest step is the joining of a D segment to a J_H segment at the H chain locus; this is followed by the appendage of a V_H segment to the DJ_H element, to produce an intact μ transcriptional unit. The assembly of L chain genes from separate V and J segments begins only after productive H chain gene assembly has occurred. Thus, at each of these stages, rearrangement appears to be restricted to particular gene segments. The retroviral substrate for V_H-to-DJ_H joining was introduced into three cell lines that exhibit continuing rearrangement of endogenous gene segments: HAFTL-1, 40E4-2, and PD31. In each of these cell lines, rearrangement of endogenous genes is representative of a specific stage in Ig gene assembly. All of these cell lines supported rearrangement of the exogenous substrate, regardless of the nature of their endogenous rearrangements. Our results suggest that specific V_H and DJ_H sequences, removed from their normal environments and placed within the context of a Mo-MLV transcriptional unit, are able to escape the regulation imposed on the rearrangement of intact gene segments in situ. This escape may

A

a $V_H 81X$ CCTTGTATTACTGTGCAAGACACACAATG...

   e-1 $V_H DJ_H$ CCTTGTATTACTGTGCAAGAC ATAAGCCCCCTTACTGGGGC

   $DJ_H 3$ TACTGTG CCTACTATAAGCCCCCTTACTGGGGC

                          9   12   7

b $5' DJ_H 3$ GATTTTTGT CAAGGGATCTAC TACTGTG CCTACTA

   e-1 5'-3'   GATTTTTGT CAAGGGATCTAC TACTGTG CACAATGAGCAAAAGTTACTGTGAGCTCAAACTAAAACC

   $3' V_H 81X$ GCAAGACACACAATGAGCAAAAGTTACTGTGAGCTCAAACTAAAACC
                                                              7   23   9

B

a $V_H 81X$ CCTTGTATTACTGTGCAAGACACACAATG

   m-10 $V_H DJ_H$ CCTTGTATTACTGTGCAAG ATAAGCCCCCTTACTGGGGC

   $DJ_H 3$ TACTGTG CCTACTATAAGCCCCCTTACTGGGGC

                          9   12   7

b $5' DJ_H 3$ GATTTTTGT CAAGGGATCTAC TACTGTG CCTACTA

   m-10 5'-3'   GATTTTTGT CAAGGGATCTAC TACTGTG CACAATGAGCAAAAGTTACTGTGAGCTCAAACTAAAACC

   $3' V_H 81X$ GCAAGACACAATGAGCAAAAGTTACTGTGAGCTCAAACTAAAACC
                                                              7   23   9

FIGURE 8. Nucleotide sequences at the recombinant junctions recovered from the HAFTL-1/LJHCR-e-1 and PD31/LJHCR-m-10 cell lines. (A) Products of V_H-to-DJ_H joining in the HAFTL-1/LJHCR-e-1 cell line. (a) *Top line and bottom line*, partial sequences of the unrearranged $V_H 81X$ (31) and $D_{SP2.7}$-$J_H 3$ (30) elements; *middle line*, nucleotide sequence at the coding joint in HAFTL-1/LJHCR-e-1. Sequences derived from the V_H-coding region are shaded; sequences from the $DJ_H 3$ coding region are boxed. (There is an ambiguity of one nucleotide in the assignment of the crossover point, because the *A* residue at the junction could have arisen from either coding region.) Conserved heptamer sequences are underlined. (b) *Top line and bottom line*, partial sequences of the unrearranged $D_{SP2.7}$-$J_H 3$ 5' flank and the $V_H 81X$ 3' flank; *middle line*, sequence at the noncoding joint in HAFTL-1/LJHCR-e-1. Conserved heptamer and nonamer sequences derived from the DJ_H^3 flank are boxed; those from the V_H flank are shaded. The lengths of spacer regions are marked in bp. (B) Products of V_H-to-DJ_H joining in the PD31/LJHCR-m-10 cell line. (a) Top line and bottom line are as described in *A*; *middle line*, sequence at the coding joint in PD31/LJHCR-m-10. (b) *Top line and bottom line*, as described in *A*; *middle line*, sequence at the noncoding joint in PD31/LJHCR-m-10. Sequences are marked as in *A*. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00642.

be directly related to the unusual transcriptional setting of the exogenous segments (56), or may result from the removal of *cis*-acting elements required for developmental ordering of rearrangement. We have so far observed no rearrangement of the LJHCR substrate in three cell lines representative of

mature B cells, or in the fibroblastoid cell line NIH-3T3. The present data support the idea that Ig H and L chain gene segments are joined by a common apparatus that is more active in B cell precursors than in mature B cells.

The V_H81X gene segment is one of the most J_H-proximal V_H segments in the BALB/c mouse germline (31). Joining of the V_H81X segment to a DJ_H unit is accompanied by deletion of the intervening DNA (31). Thus, in the unrearranged state, the V_H81X gene segment appears to lie in the same transcriptional orientation as the J_H locus. In the LJHCR substrate, the V_H81X and DJ_H3 sequences are inverted with respect to their orientation in the germline; nonetheless, they are able to undergo joining when introduced into appropriate cells. This result agrees with the observation that exogenous V_κ and J_κ gene segments are joined when they are introduced in opposing orientation into a lymphoid precursor cell line (18, 20). Examples of inversional recombination in vivo at the κ locus (57) and the T cell receptor β locus (58) have recently been described. Taken together, the evidence suggests that the joining reaction can occur independently of the relative orientation of the participating gene segments. The available data do not, however, rule out the possibility that the frequency of rearrangement may be orientation dependent.

In experiments that used a model substrate for recombination, the joining of V_κ and J_κ segments was shown to be asymmetric; the coding joints are generally variable, while the reciprocal joints are invariant (20). We were able to examine the coding joints and the reciprocal joints produced by rearrangement of the H chain segments present in the LJHCR substrate. We observed that these rearrangements are similar to V_κ-to-J_κ rearrangements in that nucleotides may be lost at the coding joints, but not at the reciprocal joints. Thus, rearrangement of the V_H and DJ_H elements in the LJHCR substrate is asymmetric. Our data are consistent with models in which reciprocal joints are formed by site-specific cleavage at the borders between the coding sequences and the conserved heptamer sequences, followed by direct joining of the heptamers (20).

Junctions between V_κ- and J_κ-flanking sequences have been isolated at random from a variety of mouse and human lymphoid cell lines (57, 59–62). In general, these resemble the reciprocal joints formed during recombination of exogenous substrates, in that the heptamer sequences are precisely fused. In one case, however, the heptamer sequences are separated by several nucleotides (62). Another exception to the precise joining of heptamer sequences was found in a product of rearrangement at the T cell receptor β locus (58). It is difficult to tell whether these exceptional sequences are products of the normal mechanism of rearrangement, however, because the recombination events that gave rise to these structures were not directly observed and can only be inferred.

The coupling of site-directed mutagenesis with a functional assay for recombination is likely to be a powerful approach to the problem of Ig gene rearrangement. The LJHCR substrate is suited for such an approach, in that mutations that affect rearrangement should not interfere with selection for stable integration of the provirus. This feature has recently enabled us to introduce a series of mutant substrates into lymphoid cells (Morzycka-Wroblewska, E., and S. V. Desiderio, unpublished results). Thus, it seems likely that the assay described

here will be of use in defining the nucleotide sequences that direct rearrangement of Ig gene segments.

Summary

A model substrate for the joining of Ig V_H and DJ_H elements has been constructed in a retroviral vector carrying a selectable marker whose expression is independent of the arrangement of the resident Ig gene segments. The substrate was introduced into lymphoid and nonlymphoid cells, and site-specific recombination between the V_H and DJ_H elements was monitored by a direct hybridization assay. Joining of the exogenous gene segments was observed in cell lines representative of three distinct stages in early B cell differentiation. Rearrangement was not observed in three cell lines derived from mature B cells, or in a fibroblastoid cell line. The V_H and DJ_H elements were initially arranged so that the V_H–DJ_H junction and the recombined flanking sequences could be recovered after rearrangement. By molecular cloning and nucleotide sequence determination, V_H–DJ_H junctions formed upon rearrangement of the substrate were found to resemble closely similar junctions in functional H chain genes. The joining of V_H and DJ_H elements was observed to be asymmetric; loss of nucleotides occurred at the coding joints, but not at the junctions between flanking sequences. Our results suggest that Ig H and L chain gene segments are joined by a common mechanism that is more active in B cell precursors than in mature B cells. These observations provide further evidence that the rearrangement of Ig gene segments occurs by a nonreciprocal recombinational mechanism. The model substrate described here is likely to be of use in defining the nucleotide sequences that mediate rearrangement and in examining the developmental specificity of this process.

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