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# Unusual Immunoglobulin Gene Rearrangement Leads to Replacement of Recombinational Signal Sequences

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**An unexpected immunoglobulin gene rearrangement, signal sequence replacement, was observed in which the recombinational signal sequences of a $V_H$ gene segment are fused intact to the 5′ end of a $DJ_H$ element. Nucleotides are not lost from the signal sequences, but they may be lost from the $DJ_H$ coding sequence. Signal sequence replacement may result from the alternative resolution of an intermediate in $V_H$-to-$DJ_H$ recombination. This type of rearrangement provides a means to alter the targeting of immunoglobulin gene segments and suggests a mechanism for the occurrence of $V_H$-$J_H$ junctions in vivo. Signal sequence replacement may represent an additional pathway for the generation of antibody diversity.**

THE VARIABLE REGIONS OF IMMUNOGLOBULIN (Ig) heavy chains are encoded in three separate DNA segments, $V_H$, D, and $J_H$, that are brought together by a series of site-specific recombinational events (*1*). In the unrearranged state these segments are associated with recombinational signal sequences, conserved heptamer and nonamer elements that are separated by a spacer region (*2, 3*). The spacer regions fall, on the basis of length, into two classes of 12 and 23 base pairs (bp); recombination normally occurs only between gene segments carrying spacers of different lengths (*2*). In the Ig heavy chain system, $V_H$ and $J_H$ segments are accompanied by 23-bp spacers, while D segments carry a 12-bp spacer at either end (*2, 3*). We now report a type of Ig gene rearrangement in which the recombinational signal sequences at the 5′ flank of a D gene segment are replaced by the recombinational signals of a $V_H$ segment. This reaction provides a means by which the targeting of Ig gene segments may be altered, and suggests an additional mechanism for generation of immunological diversity.

We previously assayed the joining of $V_H$ and $DJ_H$ segments that were stably integrated into the genomes of B-lymphoid cell lines by retroviral transduction (*4*). The assay relies on direct observation of the products of recombination; because the assay does not depend on biological selection for rearrangement, it places few constraints on the structures of the recombinants. We used a similar assay here. The substrate for recombination, pLJHCR-2 (Fig. 1A), was constructed in the retroviral vector pDOL$^-$ (*5*), and contains three murine Ig gene segments: $V_H$, $DJ_H$, and $J_H$ (Fig. 1A). Between the $V_H$ and $DJ_H$ segments lies the *gpt* gene of *Escherichia coli*. The Ig gene segments are arranged so that joining of $V_H$ to $DJ_H$ or to $J_H$ results in an inversion of the intervening DNA (Fig. 1B).

The pLJHCR-2 construct was packaged in the helper cell line ψ-2 (*6*) and the recombinant retrovirus was used to infect the B-progenitor cell line HAFTL-1 (*7, 8*), which undergoes continuing D-to-$J_H$ joining during propagation in culture (*8*). Derivatives of HAFTL-1 that contained integrated provirus were selected in G418.

Rearranged and unrearranged substrates can be distinguished by digestion with Kpn I and hybridization to probes specific for *gpt* or *neo* sequences. Digestion of unrearranged proviral DNA creates a 2.4-kb fragment that hybridizes to a *gpt*-specific probe, and a 4.0-kb fragment that hybridizes to a *neo*-specific probe (Fig. 1A). Proviral DNA that has undergone $V_H$-to-$DJ_H$ joining yields a 4.8-kb fragment that hybridizes to both probes (Fig. 1B). This assay was used to detect rearrangement of the integrated substrate in the HAFTL-1 cell line. Of 34 clones assayed, 21 yielded a 4.8-kb *neo*-containing fragment that also hybridized to the *gpt* probe, consistent with $V_H$-to-$DJ_H$ joining within the substrate [for example, clone a-15, Fig. 2, A and B, lanes d, and (*9*)]. This

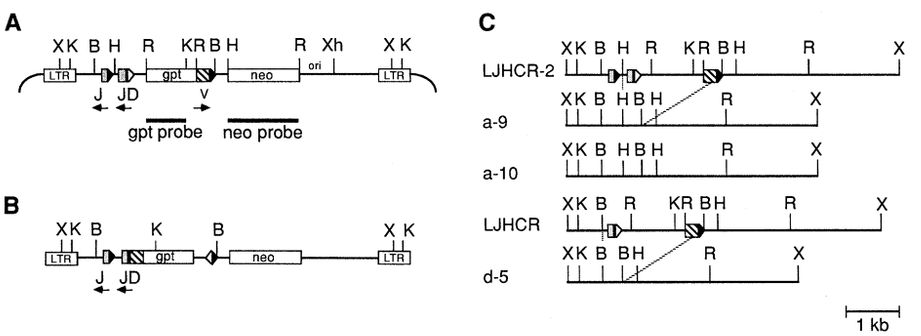


**Fig. 1.** (**A**) Structure of the pLJHCR-2 substrate for recombination. The retroviral portion of plasmid pLJHCR-2 is similar to pLJHCR (*4*), except for the insertion of a 407-bp Hind III–Bam HI fragment containing an unrearranged $J_H2$ gene segment. The Moloney murine leukemia virus long terminal repeats (LTRs), the *neo* gene, and the *gpt* gene are indicated by open boxes. The pBR322 origin of replication is indicated. The D sequence is indicated by a filled box; the $J_H$ sequence by a shaded box, and the $V_H$ sequence by a hatched box. Recombinational signal sequences carrying 23 and 12-bp spacers are indicated by filled and open triangles, respectively. The sense orientations of the Ig coding sequences are indicated by arrows. Sequences surrounding the retroviral LTR (about 7300 bp) are not included. Restriction enzymes are abbreviated as follows: X, Xba I; K, Kpn I; B, Bam HI; H, Hind III; R, Eco RI; and Xh, Xho I. (**B**) Structure of the LJHCR-2 substrate following $V_H$-to-$DJ_H$ joining. Sequence elements and restriction sites are indicated as in (A). Rearrangement of $V_H$ to $J_H$ would yield internal Kpn I fragments of about 1.2 and 5.2 kb. (**C**) Restriction maps of the recombined substrates from cell clones a-9, a-10, and d-5. Top line, the LJHCR-2 restriction map from the Xba I site in the 5′ LTR to the Xba I site in the 3′ LTR. Second and third lines, retroviral DNA from cell clones a-9 and a-10. Fourth line, the LJHCR restriction map from the 5′ Xba I site to the 3′ Xba I site (*4*). Fifth line, retroviral DNA recovered from cell clone d-5. Restriction sites, Ig gene segments, and recombinational signal sequences are indicated as in (A). Recovery of rearranged proviral sequences from clones of infected lymphoid cells was performed as described (*13*).

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interpretation was verified by molecular cloning and nucleotide sequence analysis of the recombinant from clone a-15 and of similar recombinants (*4, 9*). In contrast, two clones (a-9 and a-10) yielded a predominant *neo*-containing fragment of about 4.7 kb that did not hybridize to the *gpt* probe (Fig. 2, A and B, lanes a and b), indicating that rearrangement of the substrate in these cell clones resulted in deletion of the *gpt* marker.

To assess the integrity of the provirus in cell clones with evidence of rearrangement, genomic DNA was digested with Xba I and assayed for hybridization to a *neo*-specific probe (Fig. 2C). Most of the clones examined yielded a single 6.4-kb *neo*-containing fragment, as expected [Fig. 2C, lanes c and d, and (*9*)]. The clones a-9 and a-10, however, yielded a 4.7-kb *neo*-containing fragment (Fig. 2C, lanes a and b). While the 6.4-kb fragment also hybridized to a *gpt*-specific probe (Fig. 2D, lanes c and d), the 4.7-kb fragment present in clones a-9 and a-10 did not (Fig. 2D, lanes a and b). This result confirmed that the proviral DNA in these clones had suffered deletion of the *gpt* marker.

To determine the nature of the recombinational event that had occurred in a-9 and a-10, we cloned the rearranged proviral DNA from these cell lines. The restriction maps of the cloned proviral DNA revealed an internal deletion of about 1.7 kb that removed the *gpt* marker (Fig. 1C). A similar rearrangement was observed in a clone of the B-progenitor cell line 40E4-2 (*10*), into which the retroviral substrate LJHCR (*4*) had been introduced (clone d-5). This rearranged provirus was also recovered (Fig. 1C).

We determined the nucleotide sequences across the recombinant junctions recovered from clones a-9, a-10, and d-5, and found that in each instance the 3′ flanking sequences of the $V_H$ gene segment had been joined to the $DJ_H$ coding segment, replacing the original signal sequence at the 5′ flank of D (Fig. 3). In these cases, joining was precise with respect to the $V_H$ signal sequence. As a result, the heptamer-spacer-nonamer elements of the $V_H$ gene segment were fused intact to the $DJ_H$ coding sequence. In one recombinant, joining was imprecise with respect to the $DJ_H$ coding sequence, so that two nucleotides were lost at the junction (Fig. 3, a-9). We refer to the type of rearrangement seen in clones a-9, a-10, and d-5 as "signal sequence replacement," to distinguish it from $V_H$-D-$J_H$ joining. Similar rearrangements have been observed in experiments in which an extrachromosomal substrate was used for recombination (*11*).

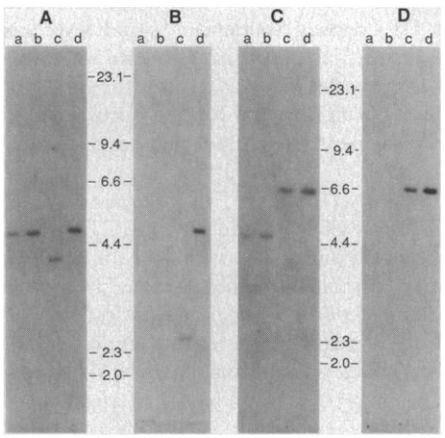


**Fig. 2.** Rearrangement of the LJHCR-2 provirus in the HAFTL-1 cell line. (Lanes a to d) DNA from clones a-9, a-10, a-13, and a-15. Clone a-13 contains predominantly unrearranged provirus, and serves as a control. (**A**) Hybridization of Kpn I–digested DNA to a *neo*-specific probe. Genomic DNA (15 μg) from clones of HAFTL-1 cells infected with LJHCR-2 was digested with Kpn I and hybridized to a *neo*-specific probe. (**B**) Hybridization of Kpn I–digested DNA to a *gpt*-specific probe. The filter used in (A) was washed in 0.5*M* NaCl, 0.05*M* NaOH, and 0.002*M* $Na_2EDTA$ for 1 hour at room temperature to remove the *neo* probe. The filter was neutralized and hybridized to the *gpt* probe. (**C**) Hybridization of Xba I–digested DNA to a *neo*-specific probe. Genomic DNA (15 μg) from clones of HAFTL-1 cells infected with LJHCR-2 was digested with Xba I, and proviral DNA was detected by hybridization to a *neo*-specific probe. (**D**) Hybridization of Xba I–digested DNA to a *gpt*-specific probe. The filter in (C) was washed as described in (B), neutralized, and hybridized to the *gpt* probe. The positions of DNA standards (in kilobases) are indicated. Culture of ψ-2 and lymphoid cell lines, packaging of recombinant retrovirus, and transmission of retrovirus to lymphoid cell lines were performed as described (*4*), except that selection for G418-resistant HAFTL-1 cells was performed at a G418 concentration of 1.5 mg/ml.

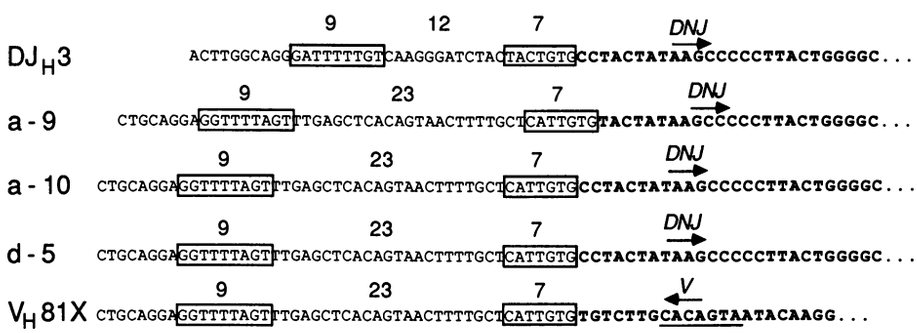


**Fig. 3.** Signal sequence replacement in the LJHCR-2 substrate. Top line and bottom line, partial sequences of the unrearranged $DJ_H$ ($D_{SP2.7}$-$J_H3$) (*10*) and $V_H$ ($V_H81X$) (*12*) elements. Middle lines, nucleotide sequences at the recombinant junctions recovered from the LJHCR-2–infected HAFTL-1 clones a-9 and a-10, and from the LJHCR-infected 40E4-2 clone d-5. Sequences from the $V_H$ and $DJ_H$ coding regions are boldface, and the sense orientations of the coding sequences are indicated by arrows. The conserved heptamer and nonamer signal sequences are boxed. The lengths of the spacer regions (23 or 12 bp) are indicated. The heptamer-like sequence present within the $V_H$ coding region is underlined. Nucleotide sequences were determined by the dideoxynucleotide chain termination method (*14*), by the use of $^{35}$S-dATP and Sequenase (United States Biochemicals).

Signal sequence replacement is apparently performed by the same apparatus that normally carries out the joining of Ig gene segments because the same specific sites are involved, namely the Ig coding segments and their accompanying recombinational signals. The joints described above are precise with respect to the heptamer sequences; joining of heptamer signals without loss of nucleotides accompanies Ig and T cell receptor gene assembly (*4, 15–17*). At one of the joints, nucleotides from the $DJ_H$ coding sequence are lost, as has been observed at the junctions between Ig gene segments (*1*). Thus these new rearrangements may result from the alternative resolution of an intermediate in $V_H$-to-$DJ_H$ joining (Fig. 4A). The initial pairing and cleavage of Ig gene segments may yield an intermediate in which four DNA ends (two coding ends and two flanking ends) are held in proximity. Commonly, this intermediate would ultimately be resolved when the two coding sequences and the two flanking sequences are joined (Fig. 4A, a). Less frequently, the coding sequences of one segment may become joined to the flanking sequences of the other, resulting in signal sequence replacement (Fig. 4A, b).

We have considered the possibility that the rearrangements described above may have been mediated by a heptamer-like element that is present within the $V_H$ coding segment, near its 3′ end (Fig. 3, line 5). In two instances, such elements were shown to mediate recombination between $V_H$ gene segments and a preexisting $V_HDJ_H$ unit (*18, 19*). This mechanism is not likely here, because in all three cases the $DJ_H$ coding sequence has been joined to the 5′ terminal nucleotide of the heptamer element of the $V_H$ recombinational signal sequence. If recombination had been mediated by the internal heptamer-like element of $V_H$, the $DJ_H$

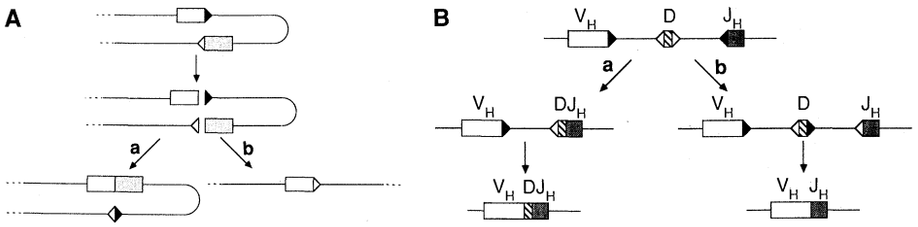


**Fig. 4.** (**A**) model for signal sequence replacement by alternative resolution of an intermediate in Ig gene rearrangement. Open and shaded boxes represent different Ig coding segments; filled and open triangles represent recombinational signal sequences bearing 23- and 12-bp spacer regions. For explanation, see text. Loss of nucleotides from the coding sequences and addition of novel nucleotides at the junctions are not included in the model, but these processes are presumed to occur after cleavage of DNA and before joining. (**B**) Altered targeting of Ig heavy chain gene segments as a consequence of signal sequence replacement. Boxes representing $V_H$, D, and $J_H$ segments are indicated. Filled triangles represent recombinational signals carrying 23-bp spacers; open triangles, recombinational signals with 12-bp spacers.

coding sequences would likely have been joined to sites within the $V_H$ coding sequence, rather than to a specific site at its 3′ flank.

In principle, signal sequence replacement can alter the targeting of Ig gene segments. The assembly of heavy chain genes, for example, normally involves the joining of a D segment to a $J_H$ segment and subsequent joining of a $V_H$ segment to the $DJ_H$ unit (*2, 3, 20*) (Fig. 4B, a). If the signal sequences of a $J_H$ segment were replaced by the signal sequences of a D segment, this would permit the direct joining of a $V_H$ segment to $J_H$ (Fig. 4B, b).

The existence of heavy chain genes that lack D segments would be consistent with the occurrence of signal sequence replacement in vivo. The nucleotide sequence of one functional μ gene, 22.11, contains a precise fusion of $V_H$ and $J_H$ coding segments (*21*). The Ig heavy chain genes that lack D sequences are not necessarily the result of direct $V_H$-to-$J_H$ joining, in that $V_H$-to-$DJ_H$ joining can be so imprecise as to completely remove the D sequence (*10*). Nevertheless, because of the precision of the $V_H$-$J_H$ junction, it seems unlikely that the 22.11 μ gene was formed by imprecise $V_H$-to-$DJ_H$ joining. Our observations suggest an alternative possibility—that the gene was assembled by direct $V_H$-to-$J_H$ joining, via signal sequence replacement.

*Note added in proof*: Analysis of a fourth signal sequence replacement product revealed precise joining with respect to the $V_H$ heptamer sequence and loss of one nucleotide from the $DJ_H$ coding sequence, in agreement with the results presented above.


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# Structural Rearrangement of the Retinoblastoma Gene in Human Breast Carcinoma


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**Structural changes of the human retinoblastoma gene have been demonstrated previously in retinoblastoma and some clinically related tumors including osteosarcoma. Structural aberrations of the retinoblastoma locus (RB1) were observed in 25% of breast tumor cell lines studied and 7% of the primary tumors. These changes include homozygous internal deletions and total deletion of RB1; a duplication of an exon was observed in one of the cell lines. In all cases, structural changes either resulted in the absence or truncation of the RB1 transcript. No obvious defect in RB1 was detected by DNA blot analysis in primary tumors or cell lines from Wilms' tumor, cervical carcinoma, or hepatoma. These results further support the concept that the human RB1 gene has pleiotropic effects on specific types of cancer.**


TUMOR DEVELOPMENT IN CERTAIN types of cancer is thought to be the result of the unmasking of one or more recessive genes by two mutations. The mutations can result in a reduction to homozygosity of the altered allele, as has been demonstrated in a number of human cancers (*1–11*). Retinoblastoma is the best studied example in which the mutation of a recessive gene is suspected to play a role in malignant transformation. Complementary DNA (cDNA) clones of the putative RB1 gene have recently been isolated (*13–15*). Structural aberrations at RB1, including examples of internal deletions of both alleles of the RB1 gene (*15*), were demonstrated in many primary retinoblastoma tumors and cell lines. The RB1 transcript is either absent or truncated in most of the tumor samples. The complete inactivation of both alleles of the RB1 gene in retinoblastoma appears to be required for tumorigenesis.

Clinically, surviving patients with the familial form of the disease are at increasing risk for other specific types of primary malignancies such as fibrosarcoma, melanoma, and osteosarcoma (*16*), which suggests that a single pleiotropic recessive locus is the link between retinoblastoma and osteosarcoma. While breast tumors have seldom been associated with retinoblastoma, mothers of children with osteosarcoma are at higher risk for breast tumors (*17*). Studies of restriction


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