Heavy Chain Variable (V_{H}) Region Diversity Generated by V_{H} Gene Replacement in the Progeny of a Single Precursor Cell Transforme with a Temperature-sensitive Mutant of Abelson Murine Leukemia Virus

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Summary

Sequence analysis of a large number of DNA clones containing a functional heavy chain variable, diversity, and joining (V_{H}D_{J_{H}}) complex generated by V_{H} to V_{H}D_{J_{H}} joining (V_{H} gene replacement) in the progeny derived from a common precursor cell transformed with a temperature-sensitive (ts) Abelson murine leukemia virus (A-MuLV) indicates that endogenous V_{H} gene replacement in vitro generates immunoglobulin gene joints distinct from those generated by the usual V_{H} to DJ_{H} joining. Such joints keep the pentamer CAAGA at the 3′ end of the donor V_{H} segment and lack a recognizable D segment, as can be seen also in vivo. The results suggest that V_{H} gene replacement participates in generating V_{H} region diversity in vivo, as previously postulated. During the joining process, a unique V_{H} gene was selected in all progeny cells, together with a single A nucleotide dominantly added to the junctional boundaries. The basis of these regulatory processes is discussed.

The random joining of Ig heavy chain variable (V_{H}), diversity (D), and joining (J_{H}) segments, and the deletion/insertion of nucleotides at the boundaries of recombination sites, lead to the generation of V_{H} region diversity (1). The flexibility of the joining process, on the other hand, results in a considerable proportion of nonproductive rearrangements. Analysis of pre-B cell lines transformed with Abelson murine leukemia virus (A-MuLV) strongly indicates that the cells generated after V_{H} to DJ_{H} joining would be null cells with nonproductive V_{H}D_{J_{H}} rearrangements on both chromosomes (2). However, these cells can also perform a further V_{H} to V_{H}D_{J_{H}} joining using a 5′ V_{H} segment to replace the V_{H} sequence of the nonfunctional V_{H}D_{J_{H}} complex, leading to the generation of a functional V_{H}D_{J_{H}} complex. It has been suggested that this V_{H} gene replacement is mediated by a mechanism analogous to V_{H} to DJ_{H} recombination through the signal heptamer embedded in the 3′ end of the V_{H} coding region, which is identical to the signal sequence found at the 5′ end of D elements (3–5).

We have established immature B cell clones 46-6, 46-11, 46-12, and 46-13, generated from a common precursor cell transformed with a temperature-sensitive (ts) mutant of A-MuLV (6). All members of clones essentially became surface μ chain-positive (μ^{+}) pre-B cells as a result of V_{H} gene replacement when they were cultured at nonpermissive temperature. By using this system, we have recently observed that various intrachromosomal circular DNAs were generated in clone 46-6 cultured at high temperature (7). The structural analysis of the isolated circular DNA clones provided evidence that V_{H} gene replacement occurs by intramolecular DNA deletion, as seen in V-(D)-J joining (8, 9). In the present study, we analyzed the nucleotide sequences of genomic DNA clones of these progenies containing a functional V_{H}D_{J_{H}} complex generated by V_{H} gene replacement in order to determine the V_{H} region diversity generated by such a recombination process.

Materials and Methods

Cell Lines. A pre-B cell line, 46, was derived from a single colony of bone marrow cells on methylcellulose transformed with a ts mutant of A-MuLV at 35.5°C (10). The cell line was cloned by lim-
iting dilution (0.1 cell/well), and clones 46-6, 46-11, 46-12, and 46-13 were established. Since a single copy of proviral DNA was integrated at the same location of the chromosome in these clones as in the parental cell line, these clones were considered to be derived from a common precursor (our unpublished results). They were maintained at 35.5°C and cultured at 37.5°C when required. More than 95% of the cells usually became μm- within 3-6 wk after the temperature shift when these clones were cultured at 37.5°C.

**DNA Isolation and Southern Blot Analysis.** Cells were harvested, frozen on the day of surface staining, and stored at -70°C until use. High molecular weight DNAs were digested with XbaI, EcoRI, or PvuII, subjected to electrophoresis on 0.8% agarose gel, and blotted to a nitrocellulose membrane as described previously (11).

**Cloning and Characterization of Genomic DNA.** 5.2-kb EcoRI fragments containing J, fragments were isolated as V,D,J, joints from μm- 46-6 and 46-12 by using AatI0. 2.5-kb XbaI fragments were isolated as V, to nonfunctional V,D,J, joints from μm- 46-6 and 46-12 by using the Aat I Wes. 4.6-kb XbaI fragments or 7.5-kb XbaI fragments were isolated as DJ, joints from μm- 46-6 and 46-12 by using the same phase. Isolated clones were subcloned into Bluescript plasmids (Stratagene, San Diego, CA) and nucleotide sequences were determined by the dideoxy chain termination method by synthetic J,1 primer: 5'-AAA CGG TGA CCG TGG TCC-3'; synthetic J,4 primer: 5'-AGA CGG TGA CTG AGG TT-3'; synthetic internal V,L6 primer: 5'-GAA GTT CCA GGG CAA GTT TGC CTT CTC TTT G-3'; or synthetic internal V,L6' primer: 5'-CCT TTC GAG CCT GC-3'. 1 μg of genomic DNA was amplified as described elsewhere (12). After 30 amplification cycles, samples were subcloned into Bluescript plasmids (Stratagene, San Diego, CA) and nucleotide sequences were determined by the dideoxy chain termination method by synthetic J,1 primer: 5'-AAA CGG TGA CCG TGG TCC-3'; synthetic J,4 primer: 5'-AGA CGG TGA CTG AGG TT-3'; synthetic internal V,L6 primer: 5'-GAA GTT CCA GGG CAA GTT TGC CTT CTC TTT G-3'; or synthetic internal V,L6' primer: 5'-CCT TTC GAG CCT GC-3'. Homology search of nucleotide sequences was performed by GenBank (Rel. 68.0) and EMBL (Rel. 27.0) databases.

**Polymerase Chain Reaction.** High molecular weight DNAs from each μm- clone were subjected to PCR using Taq DNA polymerase (Cetus Corp., Norwalk, CT). The oligonucleotide primers flanking the V, to nonfunctional V,D,J, junction were 5'-AGG GTT CCA GGG CAA GTT TGC CTT CTC TTT G and 5'-TGG GGA GAT CTG AGG TT-3'; synthetic internal V,L6' primer: 5'-CCT TTC GAG CCT GC-3'. The analysis of IgH gene rearrangement by Southern blot analysis with the J, probe showed that J, loci of both alleles were already rearranged before maturation in 46-6, 46-12 (Fig. 1 A, lanes 2 and 4), and in the other clones (data not shown). When these clones fully changed their phenotype to μm+ cells, the formerly rearranged 10-kb XbaI fragment disappeared and a newly rearranged 2.5-kb XbaI fragment appeared as a discrete band (Fig. 1 A, lanes 3 and 5). This indicates that the μm+ cell population uses mostly a particular V, gene in the rearrangement. These changes coincided with the expression of μm, which indicated that the newly appearing 2.5-kb XbaI fragment was a functionally rearranged

![Figure 2](image)

**Figure 2.** Sequence comparison between the nonfunctional V,D,J, complex from μm- 46-6 and 46-12 (top) and the functional V,D,J, complex from μm+ 46-6-12 (bottom). The sequence of the functional V,D,J, complex of 46-6 is identical to that of 46-12, except for an A nucleotide insertion in 46-6 at the boundary of V,L6 and the D fragment instead of G nucleotide. The matched nucleotide and the gap are indicated by lines and dots, respectively. N and D denote the N sequence and D segment, respectively. The asterisked G nucleotide makes a frame shift in amino acid sequence between the V, and DJ, regions of the V,D,J, complex. The V, gene sequence in the newly formed V,D,J, complex is identical to that of V,L6 except for the three underlined nucleotides.

![Figure 1](image)

**Figure 1.** Ig gene rearrangement in 46-6 and 46-12. (A) XhaI-digested DNA samples (10 μg) from kidney, μm- 46-6, μm+ 46-6, μm- 46-12, and μm+ 46-12 were examined by Southern blot analysis with a J, probe. The J, probe was the 1.9-kb EcoRI/BamHI fragment of MEP203 (11). (B) EcoRI-digested DNA samples (10 μg) from kidney and 46-6 at five trials of independent temperature shift were examined by Southern blot analysis with the J, probe. (C) XbaI-digested DNA samples (10 μg) from 46-12 at three successive trials out of six independent temperature shifts were examined by Southern blot analysis with the J, probe.

**Results and Discussion**

The analysis of IgH gene rearrangement by Southern blot analysis with the J, probe showed that J, loci of both alleles were already rearranged before maturation in 46-6, 46-12 (Fig. 1 A, lanes 2 and 4), and in the other clones (data not shown). When these clones fully changed their phenotype to μm+ cells, the formerly rearranged 10-kb XbaI fragment disappeared and a newly rearranged 2.5-kb XbaI fragment appeared as a discrete band (Fig. 1 A, lanes 3 and 5). This indicates that the μm+ cell population uses mostly a particular V, gene in the rearrangement. These changes coincided with the expression of μm, which indicated that the newly appearing 2.5-kb XbaI fragment was a functionally rearranged

![Figure 3](image)
V\(_{n}\)DJ\(_{h}\) complex. The newly rearranged fragments were identical in size in 46-6 and 46-12 (Fig. 1 A, lanes 3 and 5), and also in 46-11 and 46-13 (data not shown). Furthermore, every successful rearrangement in 46-6 and 46-12 at independent temperature shifts resulted in the generation of functional V\(_{n}\)DJ\(_{h}\) complexes carrying the same restriction sites (Fig. 1, B and C). These results suggest that the same V\(_{n}\) gene was selected in the rearrangements in these progenies. Since the D region gene segments were not involved in the rearrangement (data not shown), the results indicated that the expression of \(\mu m\) in these clones was not the consequence of ordinary V\(_{n}\) to DJ\(_{h}\) rearrangement, but that it was possibly induced by V\(_{n}\) gene replacement, as already reported (3-5).

The nucleotide sequence analysis of genomic fragments containing the V\(_{n}\)DJ\(_{h}\) complex in both 46-6 and 46-12 before and after maturation showed that the V\(_{n}\)DJ\(_{h}\) complex in \(\mu m^+\) 46-6 and 46-12 was composed of V\(_{n}\)10, a V\(_{n}\) gene of the J558 V\(_{n}\) gene family (13), the DJ\(_{h}1.5\) segment, and the J\(_{n}1\) segment (Fig. 2). However, this out-of-frame joining caused the V\(_{n}\)DJ\(_{h}\) complex to be nonfunctional. In the newly formed V\(_{n}\)DJ\(_{h}\) complex, the V\(_{n}\)10 gene of the nonfunctional V\(_{n}\)DJ\(_{h}\) complex was completely replaced by a V\(_{n}\) gene identical to V\(_{L}6\) (14), except for three nucleotides in \(\mu m^+\) 46-6 and 46-12. We called this V\(_{n}\) gene V\(_{n}\)L6'. A

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| Functional V.DJ complex | V.L6' DJH complex | V.DJ complex in both 46-6 and 46-12 before and after maturation |
|------------------------|------------------|---------------------------------------------------------------|
| VnL6' DJH complex      | V.DJ complex     | V.DJ complex generated by endogenous Vn gene replacement in vitro |
|                        |                  | from the present results (a) with those of Reth et al. (4) (b and c) and of Kleinfeld et al. (3) (d). Functional sequences of the VnDJh complex generated by Vn gene recombination substrate according to Covey et al. (5) are shown in e. The names of the Vn genes used in the replacement are listed and their 3' genomic sequences, including the internal heptamer (underlined), are shown. The 3' recombination heptamer sequence is double underlined. The joining sites in the donor V.DJ complex followed by nucleotide insertions. (B) Ig nucleotide sequences apparently generated by Vn gene replacement. VMu-1 and 264 belong to the VgAM3.8 family, and 3B9PC and 22.11 belong to the V186-2 family. |
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matured by temperature shifts (designated as 46-12-1, 46-12-2, and 46-12-3 in Fig. 3). A comparison between the nucleotide sequences of 36 independent joints from all four clones and those of the germline V~L6' (Dr. H. Sakano, personal communication) and the nonfunctional VnDJn complex showed that VnL6' appeared to be dominantly selected in the rearrangement. In addition, the Vn gene always joined with the target gene to keep the CAAGA sequence present at the border of the heptamer recombination signal. In 24 of the 36 joints, the junction appeared to be generated by a four-base deletion from the DHI61 segment of nonfunctional VnDJn joints and a one-base insertion of an A nucleotide at the junctional point, resulting in functional \( \mu \) chains carrying Arg at position 95. The joint carrying a C nucleotide instead of an A nucleotide was observed in seven cases, giving rise to a codon for Arg at position 95. In 4 of the 36 joints, VnL6' precisely joined downstream of the internal heptamer present in the target Vn10 without any base deletions and insertions. In the remaining case, the joint appeared to be generated by four nucleotide deletions from the D segment and a G nucleotide insertion at the junctional boundary. It has been revealed that some TCR and Ig coding joints contain recurrent mono- or dinucleotides (P nucleotides) that are preceded or followed by the neighboring V, D, or J segment with full coding capacity, and that P nucleotide and the immediately adjacent dinucleotide form a tetrancleotide palindrome (16). A predominant A mononucleotide in the junction could not be explained within the framework of the P nucleotide addition model.

In contrast to the conventional Vn to DJn joining accompanying the various nucleotide deletions near the coding terminals (17), our results, as well as those of others (3, 4), show that in the endogenous replacement reaction the recombining donor Vn gene segment appears to be frequently cleaved precisely at the 3' end of the pentamer CAAGA present upstream of the internal heptamer (Fig. 4 A, a, b, and d). This feature was also observed in one of two cases in the recombination generated by the inversion Vn replacement substrate (5) (Fig. 4 A, a, c). This in Vn pentamer is highly conserved in most murine Vn gene segments. The recombinating segment of the nonfunctionally rearranged VnDJn complex appears to be cleaved precisely at the gene segment proximal to the border of the internal heptamer (7), although its 5' coding terminal is modified by exonuclease nibbling (Figs. 3 and 4). Therefore, Vn gene replacement sometimes generates Ig gene joints carrying a complete CAAGA pentamer and lacking a distinct D segment (Fig. 4 A, a and b).

Evaluation of published sequences of Ig gene joints generated in vivo (18) indicates that some joints are composed of Vn and Jn segments without a distinct D segment region, such as clones VMu-1, 264, 3B9PC, and 22.11 (15, 19-21) (Fig. 4 B). Interestingly, the Vn regions of VMu-1 and 264 are encoded by VGAM3.8-related genes highly homologous to VnL6', and their junctional segments appear to be derived from a part of DQ52 in VMu-1 and a part of DHI612 in 264 (15, 19, 22) (Fig. 4 B, a and b). The VnJn junctional segment in clone 3B9PC appears to be encoded by a part of DQ52 (20, 22) (Fig. 4 B, c), whereas 22.11 lacks a recognizable D segment (21) (Fig. 4 B, d). Although it cannot be excluded that the lack of a distinct D segment in these joints reflects exonuclease activity in the process of Vn,DJn joining, it could be argued that these Vn,DJn joints are generated by Vn to VnDJn joining, which keeps the pentamer CAAGA (or CAAGC) on the donor Vn segment of VMu-1, 264, 3B9PC, or 22.11, and which deletes most of the D segment on the target VnDJn complex.

We observed that in most progenies, Vn10 of the nonfunctional VnDJn complex in the VnJ558 family was replaced by the same Vn,L6' in the rearrangement. In concordance with this observation, the analysis of nucleotide sequences around the recombination sites in the clones of circular DNA generated in cell line 46-6 revealed that the internal heptamer of Vn10 joined most frequently to the signal heptamer of the germline Vn gene, whose 3' end of the sequence is identical to that of VnL6' (7). In a few exceptions, however, the internal heptamer of Vn10 joined to the signal heptamer of other germline Vn genes, which may belong to the VGAM3.8- or VnJ558-related family (7). This suggests that the VnL6' is selected as a donor gene in the rearrangement, although several Vn genes in the VGAM3.8- or VnJ558-related family remain on a nonfunctional VnDJn allele. We observed that VnL6' germline gene transcripts as well as those of VnB4, which belongs to the VnJ558 family (23), were synthesized in the cells before Vn gene replacement (data not shown). Therefore, the predominant use of the VnL6' gene in the rearrangement could not be attributable to chromatin activation limited to its locus.

It is generally thought that the use of a donor Vn gene in the replacement is limited by its physical linkage between donor and target genes, including the Eu region, which may contain a VnDJn recombination enhancing activity (24). Previous observations showed that Vn gene replacement occur within the Vn7183 or VnQ52 family, or between such families close to each other (6, 7) (see Fig. 4). Since, in preliminary experiments, mapping of Vn,L6' and the nonfunctional VnDJn complex by pulse field analysis suggests that VnL6' is probably mapped within 50 kbp upstream of Vn10 of the nonfunctional VnDJn complex (our unpublished observation), we believe that nonrandom selection of the VnL6' gene in the rearrangement is reflected mainly by its proximal location to the nonfunctional VnDJn complex. Further analysis of the physical linkage between donor and target genes should clarify this issue.

In the Ig joints, a single nucleotide was added to the boundary of Vn to VnDJn joining, but this did not resemble random N segments (25). The dominant type of joints that carry an A nucleotide insertion at the joining boundary were observed in various combinations with other minor types in each progeny matured from independent pre-B cell clones or in the same clone at independent inductions of maturation. This implies that heterogeneous junctions may have been initially generated during maturation, whereas the junctional restriction was later selected under unknown pressure. The observation that Vn to VnDJn joining produced specific Arg...
codons, AGG and CGG, at high frequency raises the possibility that the junctional restriction could be selected at the amino acid level, as has been proposed in another system (26). Alternatively, these features of Vn gene replacement may reflect some unique mechanism operating in the joining, or simply low activity of terminal deoxynucleotidyl transferase and exonuclease at the stage of cells where Vn gene replacement would take place.

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References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature (Lond.) 302:575.
2. Reth, M.G., S. Jackson, and F.W. Alt. 1986. VDJH1 joining and DJ replacement during pre-B differentiation: non-random usage of gene segments. EMBO (Eur. Mol. Biol. Organ.) J. 5:2131.
3. Kleinfield, R., R.R. Hardy, D. Tarlinton, J. Dangl, L.A. Herzenberg, and M. Weigert. 1986. Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly 1+ B-cell lymphoma. Nature (Lond.) 322:840.
4. Covey, L.R., P. Ferier, and F.W. Alt. 1990. V4 to VDJH rearrangement is mediated by the internal Vn heptamer. Int. Immunol. 2:579.
5. Takemori, T., I. Miyazoe, T. Shirasawa, M. Taniguchi, and T. Graf. 1987. A temperature-sensitive variant of Abelson murine leukemia virus confers inducibility of IgM expression to transformed lymphoid cells. EMBO (Eur. Mol. Biol. Organ.) J. 6:951.
6. Usuda, S., T. Takemori, M. Matsuoka, T. Shirasawa, K. Yoshida, A. Mori, K. Ishizaka, and H. Sakano. 1992. Immunoglobulin V gene replacement is caused by the intramolecular DNA deletion mechanism. EMBO (Eur. Mol. Biol. Organ.) J. 11:611.
7. Okazaki, K., D.D. Davis, and H. Sakano. 1987. T cell receptor beta gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. Cell. 49:477.
8. Fujimoto, S., and H. Yamagishi. 1987. Isolation of an excision product of T-cell receptor alpha-chain gene rearrangements. Nature (Lond.) 327:242.
9. Tanimoto, T., J. Mizuguchi, I. Miyazoe, M. Nakanishi, K. Shigemoto, H. Kimoto, T. Shirasawa, N. Maruyama, and M. Taniguchi. 1990. Two types of mu complex are expressed during differentiation from pre-B to B cells. EMBO (Eur. Mol. Biol. Organ.) J. 9:2493.
body V regions as determinants of clonal persistence and malignant growth in the B cell compartment. *EMBO (Eur. Mol. Biol. Organ.)* J. 7:3693.

21. Maizels, N., and A. Bothwell. 1985. The T-Cell-independent immune response to the hapten NP uses a large repertoire of heavy chain genes. *Cell.* 43:715.

22. Kurosawa, Y., and S. Tonegawa. 1982. Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. *J. Exp. Med.* 155:201.

23. Yancopoulos, G.D., and F.W. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged V\(h\) gene segments. *Cell.* 40:271.

24. Ferrier, P., B. Krippl, T.K. Blackwell, A.J.W. Furley, H. Suh, A. Winoto, W. Cook, L. Hood, F. Costantini, and F.W. Alt. 1990. Separate elements control DJ and VDJ rearrangement in a transgenic recombination substrate. *EMBO (Eur. Mol. Biol. Organ.)* J. 9:117.

25. Desiderio, S.V., G.D. Yancopoulos, M. Paskind, E. Thomas, M.A. Boss, N. Landau, F.W. Alt, and D. Baltimore. 1984. Preferential utilization of the most JH-proximal V\(h\) gene segments in pre-B-cell lines. *Nature (Lond.)* 311:752.

26. Sanz, I., and J.D. Capra. 1987. V\(k\) and J\(k\) gene segments of A/J Ars-A antibodies: Somatic recombination generates the essential arginine at the junction of the variable and joining regions. *Proc. Natl. Acad. Sci. USA.* 84:1085.