Heavy Chain Variable (V\text{\text{H}}) Region Diversity Generated by V\text{\text{H}} Gene Replacement in the Progeny of a Single Precursor Cell Transformed 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.DJ) complex generated by V to V.DJ joining (V. 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. gene replacement in vitro generates immunoglobulin gene joints distinct from those generated by the usual V. to DJ. joining. Such joints keep the pentamer CAAGA at the 3' end of the donor VH segment and lack a recognizable D segment, as can be seen also in vivo. The results suggest that V. gene replacement participates in generating V. region diversity in vivo, as previously postulated. During the joining process, a unique V. 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.

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-
the three underlined nucleotides.

Figure 2. Sequence comparison between the nonfunctional VaDJH complex from /~m- 46-6 and 46-12 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.DJ. complex. The V. gene sequence in the newly formed V.DJ. complex is identical to that of V.L6 except for

\[ /~m + 46 - 12 \]

Figure 1. Ig gene rearrangement in 46-6 and 46-12. (A) Xbal-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 Jj probe. The Jj 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 Jj probe. (C) Xbal-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 Jj probe.

Results and Discussion

The analysis of IgH gene rearrangement by Southern blot analysis with the Jj probe showed that Jj 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 Xbal fragment disappeared and a newly rearranged 2.5-kb Xbal 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 Xbal fragment was a functionally rearranged

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Figure 2. Sequence comparison between the nonfunctional V_\alpha DJ_\alpha complex from µm- 46-6 and 46-12 (top) and the functional V_\alpha DJ_\alpha complex from µm+ 46-12 (bottom). The sequence of the functional V_\alpha DJ_\alpha 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.DJ_\alpha complex. The V. gene sequence in the newly formed V_\alpha DJ_\alpha complex is identical to that of V.L6 except for the three underlined nucleotides.
B and C). These results suggest that the same VH 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 μm in these clones was not the consequence of ordinary Vn to DJn rearrangement, but that it was possibly induced by Vn gene replacement, as already reported (3-5).

The nucleotide sequence analysis of genomic fragments containing the VnDJn complex in both 46-6 and 46-12 before and after maturation showed that the VnDJn complex in μm+ 46-6 and 46-12 was composed of Vn10, a Vn gene of the J558 Vn gene family (13), the Dsn1.1 segment, and the Jn1 segment (Fig. 2). However, this out-of-frame joining caused the VnDJn complex to be nonfunctional. In the newly formed VnDJn complex, the Vn10 gene of the nonfunctional VnDJn complex was completely replaced by a Vn gene identical to VnL6 (14), except for three nucleotides in μm+. 46-6 and 46-12. We called this Vn gene VnL6'. A nucleotide sequence comparison among the known IgH gene sequences by GenBank database showed that VnL6 and VnL6' belong to the VAGAM.3.8 family (15). The newly formed joint appears to be accompanied by a four-base deletion from the D segment and a single nucleotide insertion in 46-6 and 46-12. The results showed that μm expression in these clones was the consequence of Vn gene replacement, as reported previously (3-5). The DJn complex in the other allele of μm- 46-6 and 46-12 was composed of Dsp2 and Jn4, but the 5' heptamer sequence and a part of the 12-bp spacer sequence of the DJn joint were deleted (data not shown). Therefore, ordinary Vn to DJn recombination could not be generated in this allele.

The frequency of the use of VnL6' genes in the rearrangement as well as the junctional diversity generated in joints in the progeny cells were determined by sequencing six Vn-. VnDJn joints amplified by the PCR from each of 46-6, 46-11, 46-12, and 46-13, of which >95% of the cells expressed μm (Fig. 3). In the case of 46-12, functional VnDJn joints were isolated from three independent populations successively.

![Diagram](image-url)

**Figure 3.** Junctional diversity generated by Vn gene replacement. The nucleotide sequence of the nonfunctional VnDJn complex or germline VnL6' from position 419 is shown as a reference sequence (top). The asterisk denotes the nucleotide position of frame shift between the Vn and DJn sequences. The functional VnDJn complex, VnL6', DJn, and after maturation showed that the VnDJn complex in μm+ 46-6 and 46-12 was composed of Vn10, a Vn gene of the J558 Vn gene family (13), the Dsn1.1 segment, and the Jn1 segment (Fig. 2). However, this out-of-frame joining caused the VnDJn complex to be nonfunctional. In the newly formed VnDJn complex, the Vn10 gene of the nonfunctional VnDJn complex was completely replaced by a Vn gene identical to VnL6 (14), except for three nucleotides in μm+. 46-6 and 46-12. We called this Vn gene VnL6'. A nucleotide sequence comparison among the known IgH gene sequences by GenBank database showed that VnL6 and VnL6' belong to the VAGAM.3.8 family (15). The newly formed joint appears to be accompanied by a four-base deletion from the D segment and a single nucleotide insertion in 46-6 and 46-12. The results showed that μm expression in these clones was the consequence of Vn gene replacement, as reported previously (3-5). The DJn complex in the other allele of μm- 46-6 and 46-12 was composed of Dsp2 and Jn4, but the 5' heptamer sequence and a part of the 12-bp spacer sequence of the DJn joint were deleted (data not shown). Therefore, ordinary Vn to DJn recombination could not be generated in this allele.

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**Figure 4.** (A) Comparison of the junctional sequences of the functional VnDJn 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 Kleinfield et al. (3) (d). Junctional sequences of the VnDJn complex generated by Vn gene recombination substrate according to Covey et al. (5) are shown in c. 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' recombinational heptamer sequence is double underlined. The joining sites in the donor Vn gene and target VnDJn complex are indicated by arrows. The single and double arrows represent the joining sites in the target VnDJn complex followed by nucleotide insertions. (B) Ig nucleotide sequences apparently generated by Vn gene replacement. VMU-1 and 264 belong to the VGA3.8 family, and 3B9PC and 22.11 belong to the V186-2 family.
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_{μ}L_{6}' (Dr. H. Sakano, personal communication) and the nonfunctional V_{μ}D_{J_{μ}} complex showed that V_{μ}L_{6}' appeared to be dominantly selected in the rearrangement. In addition, the V_{μ} 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 D_{PHS6} segment of nonfunctional V_{μ}D_{J_{μ}} joints and a one-base insertion of an A nucleotide at the junctional point, resulting in functional μ 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, V_{μ}L_{6}' precisely joined downstream of the internal heptamer present in the target V_{μ}10 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 the P nucleotide and the immediately adjacent dinucleotide form a tetranucleotide 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 V_{μ} to DJ_{μ} 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 V_{μ} 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 V_{μ} replacement substrate (5) (Fig. 4 A, c). This intra-V_{μ} pentamer is highly conserved in most murine V_{μ} gene segments. The recombining segment of the nonfunctionally rearranged V_{μ}D_{J_{μ}} 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 exonucleotic nibbling (Figs. 3 and 4). Therefore, V_{μ} 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 V_{μ} and J_{μ} 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 V_{μ} regions of VMu-1 and 264 are encoded by VGAM3.8-related genes highly homologous to V_{μ}L_{6}', and their junctional segments appear to be derived from a part of D_{Q_{2}} in VMu-1 and a part of D_{PHS6.2} in 264 (15, 19, 22) (Fig. 4 B, a and b). The V_{μ}J_{μ} junctional segment in clone 3B9PC appears to be encoded by a part of D_{Q_{2}} (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 V_{μ}D_{J_{μ}} joining, it could be argued that these V_{μ}D_{J_{μ}} joints are generated by V_{μ} to V_{μ}D_{J_{μ}} joining, which keeps the pentamer CAAGA (or CAAGC) on the donor V_{μ} segment of VMu-1, 264, 3B9PC, or 22.11, and which deletes most of the D segment on the target V_{μ}D_{J_{μ}} complex.

We observed that in most progenies, V_{μ}10 of the nonfunctional V_{μ}D_{J_{μ}} complex in the V_{μ}J558 family was replaced by the same V_{μ}L_{6}' 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 V_{μ}10 joined most frequently to the signal heptamer of the germline V_{μ} gene, whose 3' end of the sequence is identical to that of V_{μ}L_{6}' (7). In a few exceptions, however, the internal heptamer of V_{μ}10 joined to the signal heptamer of other germline V_{μ} genes, which may belong to the VGAM3.8- or V_{μ}J558-related family (7). This suggests that V_{μ}L_{6}' is selected as a donor gene in the rearrangement, although several V_{μ} genes in the VGAM3.8- or V_{μ}J558-related family remain on a nonfunctional V_{μ}D_{J_{μ}} allele. We observed that V_{μ}L_{6}' germline gene transcripts as well as those of V_{μ}B4, which belongs to the V_{μ}J558 family (23), were synthesized in the cells before V_{μ} gene replacement (data not shown). Therefore, the predominant use of the V_{μ}L_{6}' 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 V_{μ} gene in the replacement is limited by its physical linkage between donor and target genes, including the Eu region, which may contain a V_{μ}D_{J_{μ}} recombination enhancing activity (24). Previous observations showed that V_{μ} gene replacement occur within the V_{μ}7183 or V_{μ}Q52 family, or between such families close to each other (6, 7) (see Fig. 4). Since, in preliminary experiments, mapping of V_{μ}L_{6}' and the nonfunctional V_{μ}D_{J_{μ}} complex by pulse field analysis suggests that V_{μ}L_{6}' is probably mapped within 50 kbp upstream of V_{μ}10 of the nonfunctional V_{μ}D_{J_{μ}} complex (our unpublished observation), we believe that nonrandom selection of the V_{μ}L_{6}' gene in the rearrangement is reflected mainly by its proximal location to the nonfunctional V_{μ}D_{J_{μ}} 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 V_{μ} to V_{μ}D_{J_{μ}} 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 maturational restriction was later selected under unknown pressure. The observation that V_{μ} to V_{μ}D_{J_{μ}} 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 V<sub>n</sub> 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 V<sub>n</sub> gene replacement would take place.

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