Brief Definitive Report

Heavy Chain Variable (V_H) Region Diversity Generated by V_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_HDJ_H) complex generated by V_H to V_HDJ_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_HDJ_H rearrangements on both chromosomes (2). However, these cells can also perform a further V_H to V_HDJ_H joining using a 5′ V_H segment to replace the V_H sequence of the nonfunctional V_HDJ_H complex, leading to the generation of a functional V_HDJ_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 (μm^+) 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_HDJ_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-

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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_H fragments were isolated as V_HDJ_H joints from μm^- 46-6 and 46-12 by using λgt10. 2.5-kb XbaI fragments were isolated as V_H to nonfunctional V_HDJ_H joints from μm^+ 46-6 and 46-12 by using the λgt Wes. 4.6-kb XbaI fragments or 7.5-kb XbaI fragments were isolated as DJ_H joints from μm^- 46-6 and 46-12 by using the same phage. 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_H1 primer: 5′-AAA CGG TGA CCG TGG TCC-3′; synthetic J_H4 primer: 5′-AGA CGG TGA CTG AGG TT-3′; synthetic internal V_H10 primer: 5′-GAA GTT CCA GGG CAA GGC C-3′; or synthetic internal V_HL6' primer: 5′-CCT TTC CTG 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_H to nonfunctional V_HDJ_H junction were 5′-ACG GTT TGC CTT CTC TTT GG-3′ and 5′-TGG GGA GAT CTG AGA ATA TC-3′. 1 μg of genomic DNA was amplified as described elsewhere (12). After 30 amplification cycles, samples were subjected to electrophoresis on 2% agarose gel. Corresponding bands were purified from agarose gel and subcloned into Bluescript plasmids. The sequences of V_H joined to the nonfunctional V_HDJ_H complex were determined as described above.

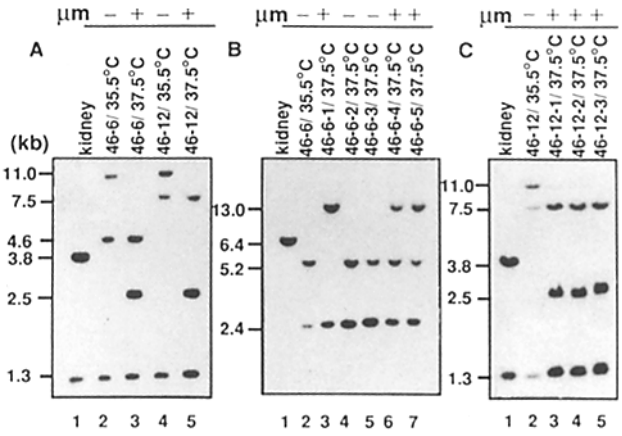

Figure 1. Ig gene rearrangement in 46-6 and 46-12. (*A*) XbaI-digested DNA samples (10 μg) from kidney, μm^- 46-6, μm^+ 46-6, μm^- 46-12, and μm^+ 46-12 were examined by Southern blot analysis with a J_H probe. The J_H probe was the 1.9-kb EcoRI/BamHI fragment of MEP203 (11). (*B*) EcoRI-digested DNA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

Results and Discussion

The analysis of IgH gene rearrangement by Southern blot analysis with the J_H probe showed that J_H 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_H 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

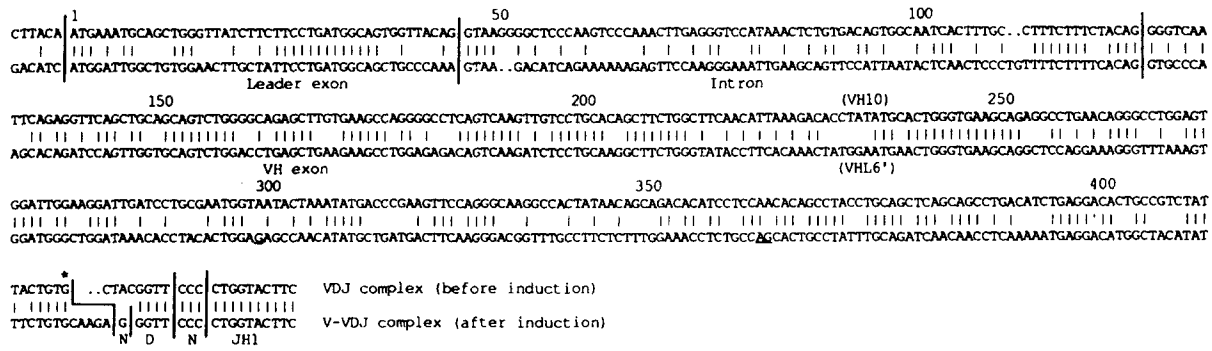

Figure 2. Sequence comparison between the nonfunctional V_HDJ_H complex from μm^- 46-6 and 46-12 (*top*) and the functional V_HDJ_H complex from μm^+ 46-12 (*bottom*). The sequence of the functional V_HDJ_H complex of 46-6 is identical to that of 46-12, except for an A nucleotide insertion in 46-6 at the boundary of V_HL6' 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_H and DJ_H regions of the V_HDJ_H complex. The V_H gene sequence in the newly formed V_HDJ_H complex is identical to that of V_HL6 except for the three underlined nucleotides.

V_HDJ_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_HDJ_H complexes carrying the same restriction sites (Fig. 1, *B* and *C*). These results suggest that the same V_H 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 V_H to DJ_H rearrangement, but that it was possibly induced by V_H gene replacement, as already reported (3–5).

The nucleotide sequence analysis of genomic fragments containing the V_HDJ_H complex in both 46-6 and 46-12 before and after maturation showed that the V_HDJ_H complex in μm^- 46-6 and 46-12 was composed of V_H10, a V_H gene of the J558 V_H gene family (13), the $D_{FI16.1}$ segment, and the J_H1 segment (Fig. 2). However, this out-of-frame joining caused the V_HDJ_H complex to be nonfunctional. In the newly formed V_HDJ_H complex, the V_H10 gene of the nonfunctional V_HDJ_H complex was completely replaced by a V_H gene identical to V_HL6 (14), except for three nucleotides in μm^+ 46-6 and 46-12. We called this V_H gene V_HL6'. A nucleotide sequence comparison among the known IgH gene sequences by GenBank database showed that V_HL6 and V_HL6' belong to the VGAM3.8 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 V_H gene replacement, as reported previously (3–5). The DJ_H complex in the other allele of μm^- 46-6 and 46-12 was composed of D_{SP2} and J_H4, but the 5′ heptamer sequence and a part of the 12-bp spacer sequence of the DJ_H joint were deleted (data not shown). Therefore, ordinary V_H to DJ_H recombination could not be generated in this allele.

The frequency of the use of V_HL6' genes in the rearrangement as well as the junctional diversity generated in joints in the progeny cells were determined by sequencing six V_H-V_HDJ_H 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 V_HDJ_H joints were isolated from three independent populations successively

				419	*				
former VDJ complex				TAC	TGT	G	CTA	CGG TCC CCC ...	
				419					
germline V_HL6'				TTC	TGT	GCA	AGA	CACAGTGTGAAAA....	
genomic clones									frequency
	46-6	TTC	TGT	GCA	AGA		AGG	TTC CCC	4/4
	46-12-1	---	---	---	---		G--	--- ---	3/4
		---	---	---	---		A--	--- ---	1/4
PCR clones									
	46-6	---	---	---	---		A--	--- ---	5/6
		---	---	---	---	CTA	C--	--- ---	1/6
	46-11	---	---	---	---		A--	--- ---	6/6
	46-12-1	---	---	---	---		A--	--- ---	5/6
		---	---	---	---		G--	--- ---	1/6
	46-12-2	---	---	---	---		A--	--- ---	5/6
		---	---	---	---		C--	--- ---	1/6
	46-12-3	---	---	---	---		C--	--- ---	5/6
		---	---	---	---		A--	--- ---	1/6
	46-13	---	---	---	---	CTA	C--	--- ---	3/6
		---	---	---	---		A--	--- ---	2/6
		---	---	---	---		C--	--- ---	1/6

Figure 3. Junctional diversity generated by V_H gene replacement. The nucleotide sequence of the nonfunctional V_HDJ_H complex or germline V_HL6' from position 419 is shown as a reference sequence (*top*). The asterisk denotes the nucleotide position of frame shift between the V_H and DJ_H sequence in the nonfunctional V_HDJ_H complex. Junctional sequences of four independent genomic clones from the genomic libraries of μm^+ 46-6 and 46-12 are shown at the top. Junctional sequences of the total 36 genomic clones from the PCR-amplified library of μm^+ 46-6, 46-12, 46-11, and 46-13 are given at the bottom. With μm^+ 46-12, junctional sequences were determined for three populations at independent temperature shifts. The nucleotide sequence from positions 419 to 439 of the V_HL6'-DJ_H junction are depicted. The V_H region sequence of all clones throughout 65 bp from the 3′ end was identical to that of V_HL6', except for one clone which carried a G nucleotide instead of an A nucleotide at the position 366 of germline V_HL6'. The underlined sequence represents the possible heptamer sequence. The double underlined represents the internal heptamer. Dashes denote the same nucleotides as indicated in the 46-6 genomic clone sequences. The frequency with which a particular sequence was found among DNA clones is shown in the last column.

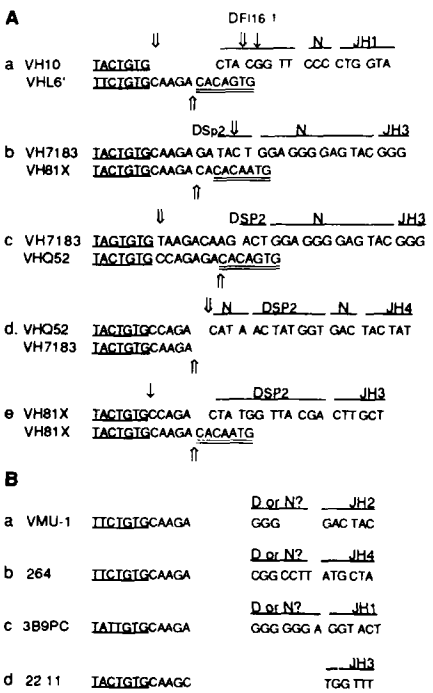

Figure 4. (*A*) Comparison of the junctional sequences of the functional V_HDJ_H complex generated by endogenous V_H gene replacement in vitro from the present results (*a*) with those of Reth et al. (4) (*b* and *c*) and of Kleinfield et al. (3) (*d*). Junctional sequences of the V_HDJ_H complex generated by V_H gene recombination substrate according to Covey et al. (5) are shown in *e*. The names of the V_H 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 V_H gene and target V_HDJ_H complex are indicated by arrows. The single and double arrows represent the joining sites in the target V_HDJ_H complex followed by nucleotide insertions. (*B*) Ig nucleotide sequences apparently generated by V_H 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_HL6' (Dr. H. Sakano, personal communication) and the nonfunctional V_HDJ_H complex showed that V_HL6' appeared to be dominantly selected in the rearrangement. In addition, the V_H 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 $D_{FI16.1}$ segment of nonfunctional V_HDJ_H 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_HL6' precisely joined downstream of the internal heptamer present in the target V_H10 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_H to DJ_H 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_H 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_H replacement substrate (5) (Fig. 4 *A*, *e*). This intra-V_H pentamer is highly conserved in most murine V_H gene segments. The recombining segment of the nonfunctionally rearranged V_HDJ_H 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_H 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_H and J_H 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_H regions of VMu-1 and 264 are encoded by VGAM3.8-related genes highly homologous to V_HL6', and their junctional segments appear to be derived from a part of D_{Q52} in VMu-1 and a part of $D_{FI16.2}$ in 264 (15, 19, 22) (Fig. 4 *B*, *a* and *b*). The V_H-J_H junctional segment in clone 3B9PC appears to be encoded by a part of D_{Q52} (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_H-DJ_H joining, it could be argued that these V_HDJ_H joints are generated by V_H to V_HDJ_H joining, which keeps the pentamer CAAGA (or CAAGC) on the donor V_H segment of VMu-1, 264, 3B9PC, or 22.11, and which deletes most of the D segment on the target V_HDJ_H complex.

We observed that in most progenies, V_H10 of the nonfunctional V_HDJ_H complex in the V_HJ558 family was replaced by the same V_HL6' 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_H10 joined most frequently to the signal heptamer of the germline V_H gene, whose 3′ end of the sequence is identical to that of V_HL6' (7). In a few exceptions, however, the internal heptamer of V_H10 joined to the signal heptamer of other germline V_H genes, which may belong to the VGAM3.8- or V_HJ558-related family (7). This suggests that the V_HL6' is selected as a donor gene in the rearrangement, although several V_H genes in the VGAM3.8- or V_HJ558-related family remain on a nonfunctional V_HDJ_H allele. We observed that V_HL6' germline gene transcripts as well as those of V_HB4, which belongs to the V_HJ558 family (23), were synthesized in the cells before V_H gene replacement (data not shown). Therefore, the predominant use of the V_HL6' 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_H gene in the replacement is limited by its physical linkage between donor and target genes, including the Eμ region, which may contain a V_HDJ_H recombination enhancing activity (24). Previous observations showed that V_H gene replacement occur within the V_H7183 or V_HQ52 family, or between such families close to each other (6, 7) (see Fig. 4). Since, in preliminary experiments, mapping of V_HL6' and the nonfunctional V_HDJ_H complex by pulse field analysis suggests that V_HL6' is probably mapped within 50 kbp upstream of V_H10 of the nonfunctional V_HDJ_H complex (our unpublished observation), we believe that nonrandom selection of the V_HL6' gene in the rearrangement is reflected mainly by its proximal location to the nonfunctional V_HDJ_H 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_H to V_HDJ_H 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 V_H to V_HDJ_H 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_H 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_H gene replacement would take place.

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