

EVIDENCE FOR GENE CONVERSION AMONG IMMUNOGLOBULIN HEAVY CHAIN VARIABLE REGION GENES

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Immunoglobulin variable region diversity results from several mechanisms operating at different stages of B lymphocyte differentiation. Initially, a given V_H gene combines with one of 10–20 D genes and one of four J_H genes to form a complete heavy (H)¹ chain variable region gene (V_H-D-J_H) (1–4). Subsequent to this, a V_K gene rearranges with one of four functional J_K genes to form a complete light (L) chain variable region gene (V_K-J_K) (5–9). The assortment of each of these gene segments and the potentially random pairing of L and H chains account for considerable immunoglobulin variable region diversity. Further diversity arises as a result of variation in the joining sites of each of these gene segments, creating sequence differences within the third hypervariable region of both L and H chains (3, 4, 7–9, 10, 11). Finally, the process of somatic point mutation occurring in and around the rearranged V_L and V_H genes further expands the potential of the antibody repertoire (12–16).

V_H and V_K genes usually belong to groups of closely related genes whose members appear to be clustered along the chromosome (14, 17–22). Genes of a single group share extensive nucleic acid sequence homologies within their coding regions and may share homologies within their 5' and 3' noncoding regions as well. Thus, these closely related genes may potentially be involved in interactions, such as recombination or conversion, that may further contribute to immunoglobulin diversity (17). Such interactions have been observed in other mammalian multigene families (23, 24) and their occurrence has been postulated among immunoglobulin H chain constant region genes (25).

We have previously reported (26) the protein sequence of a phosphocholine (PC)-binding hybridoma antibody of CBA/J origin, HP101 · 6G6 (6G6), whose sequence appears to be derived by gene conversion involving three genes of the T15 V_H gene family (26). This gene family in BALB/c mice consists of four V_H genes, V1, V11, V13, and V3 (13). The V1 gene is used to encode the V_H regions of the majority of PC-binding antibodies. Hybridoma 6G6, however, is

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¹Abbreviations used in this paper: D region, diversity region; H chain, heavy chain; J region, joining region; L chain, light chain; NP, 3-nitro-4-hydroxyphenyl acetyl; PC, phosphocholine; V region, variable region.

an exception in that its V_H region is most closely homologous to the V11 translated sequence from which it differs at six positions. At three of these positions, the substituted amino acid is encoded at the same position by the V13 gene and a fourth substitution is coded at the same position by the V1 gene, suggesting that the 6G6 V_H sequence may have been generated by at least two gene conversion events involving the V11 gene interacting with the V13 and V1 genes. To more completely characterize the events involved in generating the 6G6 V_H sequence, we have determined the nucleic acid sequence of the rearranged gene. In addition, using coding and noncoding regions of the 6G6 clone as probes in a Southern blot analysis, we have established the presence in the CBA/J genome of members of the T15 V_H gene family believed to be involved in the generation of this V_H gene.

Materials and Methods

Genomic Blot Hybridizations. 6G6 tumor DNA and BALB/c and CBA/J liver DNA were isolated as described (27). 10 μ g of purified DNA was digested with the appropriate enzyme, electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) and hybridized with 32 P-labeled DNA probes at 65°C for ~18 h. After a final wash in 30 mM NaCl/3 mM sodium citrate/0.1% sodium dodecyl sulfate for 1.5 h at 65°C, the nitrocellulose was exposed to x ray film at -70°C.

Isolation of Recombinant Clones and Preparation of Plasmid Subclones. 400 μ g of 6G6 DNA was digested with 350 U of EcoRI (Bethesda Research Laboratories, Gaithersburg, MD) for 4 h. The DNA was then loaded into a trough on a 0.7% agarose gel and electrophoresed. Fractions of DNA were collected by washing the DNA from a second trough located 10 cm from the origin, using a peristaltic pump. Fractions containing the 2.4 kb rearranged J_H gene fragment were identified by genomic blot hybridization using a 32 P-labeled plasmid subclone of germline J_H DNA (pJ11) (28). DNA from the fraction containing the 2.4 kb rearrangement was cloned into the EcoRI site of λ gtw10. Recombinant phage were then screened with 32 P-labeled pJ11 and positive phage were plaque purified. DNA from one clone was purified and an 830 bp EcoRI-Hind III fragment containing the V_H -D- J_H genes was eluted from a 5% polyacrylamide gel. A restriction map was made by end labeling with α - 32 P-dATP and the Klenow fragment of Pol I (Boehringer Mannheim Biochemicals, Indianapolis, IN), digested with various restriction enzymes and electrophoresed on 5% polyacrylamide gels. Plasmid subclones were made of the EcoRI-Pst I fragment and an EcoRI-Sau 3A fragment by cloning into the appropriate restriction sites of pBR322. DNA was then isolated from recombinant plasmids and used as a probe in genomic blot hybridizations.

DNA Sequencing. Restriction fragments of the 830 bp EcoRI-Hind III DNA fragment were labeled with γ - 32 P-dATP and polynucleotide kinase (Boehringer-Mannheim Biochemicals), digested with a second restriction enzyme, and sequenced by the method of Maxam and Gilbert (29). In addition, the EcoRI-Pst I fragment was subcloned into M13mp8 and sequenced by the dideoxy method (30).

Results

To characterize the events contributing to the generation of the 6G6 V_H region sequences, we have cloned and sequenced the rearranged V_H gene from this hybridoma. On the basis of Southern blot analysis of EcoRI-digested 6G6 DNA using a J_H plasmid clone (pJ11) as a probe, it was determined that this hybridoma contains a single rearranged J_H locus of ~2.4 kb in size (data not shown) that was then cloned into λ gtw10. 15 positive clones were identified and the nucleic acid sequence of an 830 bp fragment containing the rearranged V_H -D- J_H gene segment

from one was determined (Fig. 1). The coding region sequence determined for the V_H - D - J_H genes and its translated amino acid sequence is presented in Fig. 2 and compared with the germline nucleic acid sequence of the four genes of the BALB/c T15 V_H gene family (13), the germline D gene, D_{Q52} (31), and J_H3 (4). The nucleotide sequence agrees with the protein sequence previously determined (26) with a single exception. Residue 53 was originally identified as an asp. The nucleic acid sequence indicates an asn, making 6G6 identical at this position to all four genes of this V_H family. This sequence further indicates that the previously unidentified residue at position 71 is an arg that is also encoded by each member of the T15 V_H gene family. The coding region sequence of 6G6 is 97% homologous to V11, 95% homologous to V13, 94% homologous to V3, and 90% homologous to V1. Thus, as we previously concluded by amino acid sequence comparison (26), the 6G6 V_H sequence is most homologous to V11. There are a total of nine nucleic acid differences between 6G6 and V11, of which six result in a change in amino acid and three are silent. The substituted base at five of

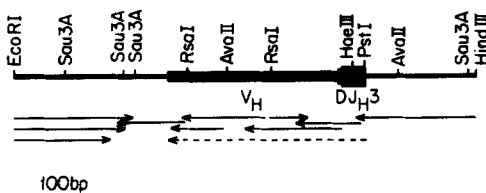


FIGURE 1. Restriction enzyme cleavage map of the 830 bp fragment containing the rearranged V_H , D , and J_H genes from hybridoma 6G6, illustrating the strategy used for sequencing. Solid arrows indicate sequencing by the method of Maxam and Gilbert (29) and the dashed arrow indicates dideoxy sequencing (30).

	1	10	20	30	40	50	60	70	80	90	100	110																
666	GAG	GTG	AAG	CTG	GTG	GAG	TCT	GGA	GGA	GGC	TTG	GTA	CAG	CCT	G	GGG	GGT	TCT	CTG	AGT	CTC	TCC	TGT	GCA	ACT	TCT	GGA	
V11
V13
V1
V3
666	TTC	ACC	TTC	ACT	GAT	TAC	TAC	ATG	AGC	TGG	GTC	CGC	CAG	CCT	CCA	GGA	AAG	GCA	CTT	GAG	TGG	TTG	GCT	TTC	ATT	ATT	AGA	
V11
V13
V1
V3
666	AAC	AAA	GCT	AAT	GGT	TAC	ACA	ACA	GAG	TAC	AGT	GCA	TCT	GTG	AAG	GGT	CGG	TTC	ACC	ATC	TCC	AGA	GAT	AAT	TCC	CAA		
V11	
V13	
V1	
V3	
666	AGC	ATC	CTC	TAT	LCT	CAA	ATG	AAT	ACC	CTG	AGA	GCT	GAG	GAC	AGT	GCC	ACT	TAT	TAC	TGT	GCA	AGA	GCT	
V11	
V13	
V1	
V3	
666	AAC	TGG	GAT	TTT	GCT	TAC	TGG	GGC	CAA	GGG	ACT	CTG	GTC	ACT	GTC	TCT	GCA	
DQ52	
JH3	

FIGURE 2. Comparison of the nucleic acid sequence of the V_H - D - J_H gene segment of 6G6 with the germline nucleic acid sequence of the four members of the T15 V_H gene family, D_{Q52} and J_H3 . Included also is the deduced amino acid sequence of the 6G6 V region. Numbering of codons is according to Kabat et al. (37).

these positions in 6G6, three coding (codons 24, 49, 50) and two silent (codons 26, 42), is also present at these same positions in the V13 gene. However, the substituted bases at codons 42 and 49, in addition to being present in V13, are also present at these same positions in V1 and V3, and the change at codon 50 is also present in V3. In addition, the silent and coding changes at codons 82a and 82b, respectively, are both present at these same positions only in the V1 gene. The remaining two differences at codons 19 and 95 are not found in any other gene of this V_H family and are therefore unique to the 6G6 V_H gene. The D and J region nucleic acid sequence of 6G6 is identical to the BALB/c D_{Q52} and J_{H3} germline genes with the exception of a single base change at the D_H - J_H junction.

The comparisons described above involve a rearranged V_H gene of CBA/J origin and four germline genes of BALB/c origin. To establish that the CBA/J genome contains a family of V_H genes homologous to the genes of the BALB/c T15 V_H family, we have performed a Southern blot analysis of CBA/J DNA using as probes plasmid subclones of either 6G6 coding region DNA (p6G6) or 5' noncoding DNA (p5'-6G6) (Fig. 3). Fig. 3 compares BALB/c and CBA/J liver DNA after either EcoRI or BamHI digestion followed by hybridization with these probes. Four major bands can be seen with BALB/c DNA after hybridization with p6G6 (Fig. 3, lanes 1 and 2) that correspond to the T15 V_H family

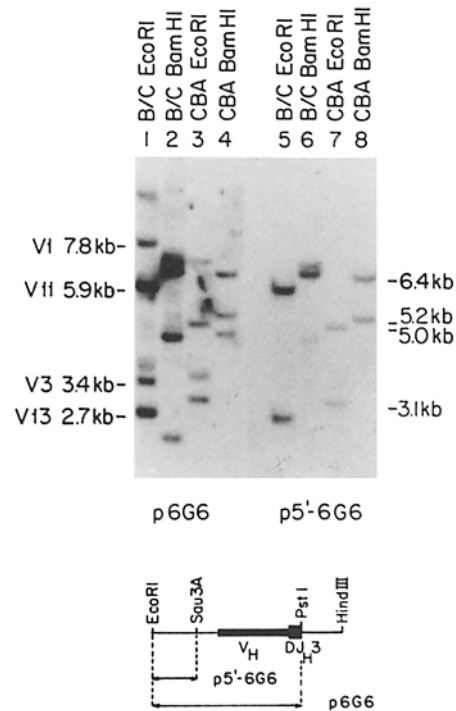


FIGURE 3. Southern blot hybridization of the p6G6 (lanes 1-4) and p5'-6G6 (lanes 5-8) probes to BALB/c and CBA/J DNA after either EcoRI or BamHI digestion. Assignment of the V1, V11, V3, and V13 genes to separate fragments of EcoRI-digested BALB/c DNA (lane 1) is according to Crews et al. (13).

previously described by Crews et al. (13) using an S107 cDNA probe (S107 is a PC-binding myeloma protein whose H chain is encoded by the V1 gene). Four bands can also be seen with CBA/J DNA after EcoRI digestion and hybridization with p6G6 (Fig. 3, lane 3), although after Bam HI digestion there appear to be only three (Fig. 3, lane 4). The reason for the discrepancy in the number of bands after EcoRI and Bam HI digestion is not clear. These data do suggest, however, that the CBA/J genome contains V_H genes homologous in sequence to the genes of the BALB/c T15 V_H family and indicate that this family in mice of both strains is the same size. The 5'-6G6 probe, when used to hybridize to either EcoRI- or Bam HI-digested BALB/c DNA (Fig. 3, lanes 5 and 6), identifies two fragments. The 2.7 kb EcoRI (Fig. 3, lane 5) and 6.4 kb Bam HI fragments (Fig. 3, lane 6) have been previously identified by Crews et al. (13) to correspond to the V13 and V11 gene segments, respectively. Thus, the p5'-6G6 probe only recognizes a sequence associated with the V11 and V13 genes. This probe also hybridizes to two CBA/J DNA fragments (Fig. 3, lanes 7 and 8), suggesting that the CBA/J genome contains only two genes homologous to the BALB/c V11 and V13 genes.

Discussion

Based on amino acid sequence analysis of a PC-binding hybridoma, 6G6, we have previously suggested the occurrence of gene interaction (conversion) among immunoglobulin V_H regions. To more precisely assess this possibility, we have cloned and sequenced the 6G6 V_H gene and analyzed this structure in terms of genetic events that may have contributed to its origin. The nucleotide sequence confirms, with one exception, the amino acid sequence previously determined and verifies the conclusion that the coding region is most homologous to the BALB/c V11 gene, a member of the PC- V_H family not commonly used in the production of PC-binding antibodies. The PC family in BALB/c mice consists of four genes, one of which (V1) encodes H chains for most anti-PC antibodies. We have also determined 275 bases 5' of the 6G6 coding region (data not shown) and compared it to the 5' sequences of the V11, V13, V3, and V1 genes (Gerald Siu, Stephen Crews, and Leroy Hood, personal communication). The 6G6 and V11 5' sequences differ by only 3 base changes compared to 21 base changes between 6G6 and V13, and even more extensive differences between the 6G6, V3, and V1 5' sequences, further suggesting that the 6G6 V_H region was originally derived from the V11 homologue in CBA/J mice. The 6G6 coding region sequence additionally identifies nine nucleotide differences, relative to V11, seven of which are surprisingly found in other members of the T15 V_H family.

There are several possible explanations for the nucleotide substitutions of the 6G6 sequence. The first is point mutation occurring either somatically or in the germline. However, we had previously argued against this explanation since the probability of accumulating four parallel mutations in 6G6 that are shared by other members of the T15 V_H family was considered unlikely. The observation of three additional shared silent base pair changes, relative to V11, argues further against point mutation since the occurrence of seven parallel mutations is even less likely. A second explanation for the 6G6 sequence is that it was

derived by a series of crossover events between V11, V13, and V1. However, this mechanism would require at least six crossovers within the length of one V_H gene and therefore also appears to be unlikely.

A third possibility is gene conversion. Gene conversion is a process whereby two closely related genes interact such that a portion of the sequence of one gene is converted to the sequence of the other, possibly by mismatch pairing followed by excision and repair (32). This process has been hypothesized (23) to have occurred in several mammalian multigene families including the murine heavy chain constant region genes (25) and the murine class I histocompatibility genes (24). The nucleic acid sequence of the 6G6 V_H gene differs from that of V11 by nine substitutions, seven of which are found encoded in other members of this family. Since the data presented indicate that 6G6 is encoded by the V11 homologue in CBA, gene conversion becomes an attractive mechanism to explain these seven substitutions. Five of these substitutions (three coding and two silent) can be generated by conversion between V11 and V13 (Fig. 2). Although the changes at codons 42, 49, and 50 could also be derived by conversion of the V11 gene by the V1 or V3 genes, this would require two or more separate conversion events to account for these five substitutions, since the substitutions at 24 and 26 can only be derived by events involving V13. The changes in codons 82a (silent) and 82b (coding) in 6G6, relative to V11, could be generated by a second conversion involving only V11 and V1. The fact that three silent substitutions are included among the seven which are shared further argues that this sequence can not result merely from some selection process, since there should be no such pressure at silent positions.

The hypothesis of gene conversion to derive the 6G6 V_H sequence from the V11, V13, and V1 genes would require that the V11, V13, and V1 homologues are present in the CBA/J genome. To test this we have used 6G6 coding region (p6G6) and 5' noncoding region (p5'-6G6) plasmid subclones as probes against CBA/J DNA to establish the presence of the members of the T15 V_H gene family in the CBA/J genome. The p6G6 probe hybridizes to fragments containing the four major genes of the T15 V_H family in BALB/c mice (Fig. 3, lanes 1 and 2) (13). It also hybridizes to four fragments of CBA/J DNA, suggesting that CBA/J mice possess a family of closely related V_H genes homologous to the members of the BALB/c T15 V_H family. The p5'-6G6 subclone, when tested with BALB/c DNA, only hybridized to fragments containing the V11 and V13 genes (Fig. 3, lanes 5 and 6). This probe similarly hybridized to two fragments of CBA/J DNA (Fig. 3, lanes 7 and 8), suggesting that CBA/J mice possess V_H genes homologous to the BALB/c V11 and V13 genes. These data, along with our previous amino acid sequence analysis of CBA/J hybridomas that establish the presence of an allelic form of the V1 gene in CBA/J mice which differs by only four amino acid substitutions (33), indicate that the V11, V13, and V1 genes postulated to be involved in the generation of the 6G6 V_H sequence by gene conversion are present in the CBA/J germline.

The Southern blot analysis also indicates that the size of the T15 V_H family is equivalent in mice of each strain, and therefore it does not seem likely that there has been a gene duplication in CBA/J mice to create an additional form of V11 which encodes the 6G6 V_H region. However, the V11 homologue in CBA/J mice

might exactly encode the 6G6 V_H region, indicating that the gene conversion to create this sequence has occurred among germline genes after the separation of the BALB/c and CBA/J haplotypes. Alternatively, the V11 homologue in CBA/J may be more homologous to the BALB/c V11 gene than to the 6G6 V_H gene, indicating that the gene conversion has occurred somatically. In either case, it is strongly suggested from these data that a "conversion" relative to BALB/c germline sequences has occurred to yield the 6G6 V_H . The conversion events proposed have occurred over relatively short segments of the V_H gene and in this way are similar to the conversion proposed among the class I histocompatibility genes (24). Bentley and Rabitts (34) have postulated the occurrence of conversion among germline V_k genes that also involves a relatively short segment of the V_k gene. In contrast, a rearranged anti-3-nitro-4-hydroxyphenyl acetyl (NP)^b V_H gene has been described by Dildrop et al. (35) and Krawinkel et al. (36) that appears to have undergone either conversion or a double crossover with a second closely related V_H gene involving a considerably larger segment of DNA. In this example, the recombinational event may have involved over half of the V_H gene and as such may be more consistent with a double crossover. However, regardless of the mechanism of recombination the anti-NP^b V_H data does indicate that recombinational events can occur somatically and involve rearranged V genes.

The consequences of gene conversion may be of importance to both the individual and the species. If gene conversion occurs in the germline, it will not only create new V gene sequences that will then be retained in the germline but it may serve a more important function by correcting certain sequences that have been altered by point mutation occurring during evolution. These sequences would presumably be of biological importance and a mechanism to preserve them may be of survival value. Thus, gene conversion among germline genes may be an important means in establishing and maintaining the germline V gene repertoire. Gene conversion that occurs somatically involving a rearranged V gene will create new sequences not encoded in the germline and may make a contribution to the generation of somatic diversity.

Summary

We have previously reported (26) that the V_H region amino acid sequence of a phosphocholine (PC)-binding hybridoma antibody of CBA/J origin, HP101 · 6G6 (6G6), differs extensively from the V_H regions of other PC-binding antibodies. The sequence of 6G6 V_H appears to be derived from a gene homologous to the BALB/c V11 gene, a member of the PC V_H (T15 V_H) gene family not normally used to encode PC-binding antibodies. The 6G6 V_H sequence differs from the translated sequence of V11 by six amino acids, four of which occur at the same position in other members of this gene family. This coincidence led to the proposal that the 6G6 V_H gene was derived by gene conversion involving three genes of the PC V_H gene family. We report here the nucleic acid sequence of the rearranged V_H gene of hybridoma 6G6. This sequence supports our previous suggestion of gene conversion by confirming those differences, relative to the BALB/c V11 gene sequence, that are encoded by other members of this gene family, and extends this correlation to include three silent base pair substitutions as well. In addition, 5' noncoding region sequence and Southern blot analysis

using probes derived from the coding and 5' noncoding regions confirm that the 6G6 V_H gene is likely to be derived from the V11 homologue in CBA/J mice, and suggest that all three genes believed to be involved in the generation of the 6G6 V_H gene are present in the CBA/J genome, a prerequisite for their involvement in gene conversion.

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