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Immunoglobulin V_H gene sequence analysis of spontaneous murine immunoglobulin-secreting B-cell tumours with clinical features of human disease

D. ZHU,* C. VAN ARKEL,†‡ C. A. KING,* S. VAN MEIRVENNE,§ C. DE GREEF,§ K. THIELEMANS,§ J. RADL† & F. K. STEVENSON*

**Molecular Immunology Group, Tenovus Research Laboratory, Southampton University Hospitals, Southampton, UK, †Department of Immunological and Infectious Diseases, TNO-Prevention and Health, Leiden, the Netherlands, ‡Department of Immunology, Erasmus University, Rotterdam, the Netherlands, and §Laboratory of Physiology, Medical School, Vrije Universiteit Brussel, Laarbeeklaan, Brussels, Belgium*

SUMMARY

The 5T series of multiple myelomas (MM) and Waldenstrsöm's macroglobulinaemia-like lymphomas (WM), which developed spontaneously in ageing mice of the C57BL/KaLwRij strain, shows clinical and biological features that closely resemble their corresponding human diseases. In order to compare the patterns of somatic mutation in V_H genes of mouse tumours with those of human counterparts, we have determined and analysed sequences of immunoglobulin V_H genes in five cases of murine MM, two of WM and one of biclonal benign monoclonal gammopathy (BMG). Four of five MM and 2/2 WM cases used V_H genes of the large J558 family; one MM used a gene of the VGAM3.8 family, and both clones of the BMG used genes of the 36-60 family. N-region insertions were observed in all cases, but D-segment genes were only identified in 6/9 cases, which were all from the D-SP family and translated in reading frame 3. Compared with human MM, in which the V_H genes have been found to be consistently hypermutated (mean% $\pm$ SD = 8·8 $\pm$ 3·2), the degree of somatic mutation in the murine tumours was significantly lower (mean% $\pm$ SD = 2·9 $\pm$ 2·3). There was no significant evidence of clustering of replacement mutations in complementarity determining regions (CDR), a feature considered to be characteristic of antigen-selected sequences. However, one clone of the biclonal BMG case showed intraclonal variation, a feature described in some cases of human BMG. These results indicate that murine V_H genes in mature tumours differ from human counterparts in the level and distribution of somatic mutations, but support the concept that BMG may be distinct from MM.

INTRODUCTION

Monoclonal B-cell proliferative disorders are a common feature in the elderly, both in humans and in laboratory animals.[1] Benign and malignant forms are able to produce large quantities of monoclonal immunoglobulins, which are detectable in the serum. Multiple myeloma (MM) is the most common malignant plasma cell tumour, producing IgG, IgA, IgD or IgE. However, there is a benign counterpart of MM that also involves plasma cells, but with stable levels of the monoclonal immunoglobulin and no clinical characteristics of malignant disease. This benign form has been designated as BMG or monoclonal gammopathy of undetermined significance (MGUS), the latter term being used on the grounds that only time will show whether a monoclonal disorder is truly benign or will develop into malignant disease. In fact, the rate of conversion from MGUS to MM is approximately 15% within a median time of 9·6 years.[2] In humans, Waldenstrsöm's macroglobulinaemia (WM) is a relatively rare low-grade lymphoma involving lymphoplasmacytoid cells that secrete monoclonal IgM. The course of disease tends to be influenced by the molecular nature of the IgM, with hyperviscosity or cryoglobulinaemia as common features.

In human disease, immunoglobulin V gene sequences of various B-cell tumours have been investigated extensively. Recent data indicate that the malignant cell of MM is a B cell that has passed the germinal centre and undergone somatic hypermutation.[3–5] Both immunoglobulin V_H and V_L genes are extensively mutated and do not show intraclonal variation, indicating that the malignant plasma cells are no longer able to accumulate further mutations. Furthermore, there is clustering of replacement mutations in complementarity determining regions (CDR) in V_H genes in 25% of cases,[5,6] consistent with a role for antigen in selecting V_H sequences. Clustering

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Correspondence: Dr D. Zhu, Molecular Immunology Group, Tenovus Research Laboratory, Southampton University Hospitals, Tremona Road, Southampton SO16 6YD, UK.

has also been found in V_L in some cases.[6] The lack of intraclonal variation might be a distinctive feature of MM, since data from human MGUS studies demonstrated that a proportion of cases of MGUS showed intraclonal variation, indicating that the tumour cell may still be under the influence of a mutation mechanism.[7] There have been fewer studies of WM, but the limited sequence data on V_H and V_L genes show evidence for somatic mutation and intraclonal homogeneity.[8]

The 5T series of MM and WM, which developed spontaneously from ageing mice of the C57BL/KaLwRij strain, shows clinical and biological features that closely resemble those of the corresponding human diseases and could be used as experimental models.[9,10] Studies on the influences of T-cell function, antigenic stimulation and genetic background on monoclonal gammopathy (MG) development have been described previously.[11,12] In this paper, we report sequences of immunoglobulin V_H genes for five MM, two WM and one biclonal BMG. The sequences are analysed in comparison with human disease counterparts.

MATERIALS AND METHODS

Cells

Spontaneously developed mouse MM and WM lines have been maintained and propagated by intravenous transfer of bone marrow or spleen cells for several generations in syngeneic C57BL/KaLwRij recipients. Their cytogenetic abnormalities are relatively stable, as reported previously.[13] In this study, we used spleen cells of mice in terminal phase with high levels of serum monoclonal proteins, as detected by serum electrophoresis. The following established tumour lines were studied: 5T2, an IgG2aκ-secreting myeloma; 5T7, an IgG2bκ-secreting smouldering myeloma; 5T13 and 5T33, both IgG2bκ-secreting aggressive myelomas; 5T14, an IgG1κ-secreting moderately aggressive myeloma; and 5T10 and 5T16, both aggressive IgMκ-secreting WM.

The biclonal BMG arose in a long-term immunosuppressed old C57BL/KaLwRij mouse.[11] Both paraproteins, IgG2aκ and IgG2bκ, were detectable in the serum for more than 6 months without clinical signs of progression, and can therefore be considered as BMG rather than MGUS. Bone marrow cells were flushed out of the femura and tibiae of the mouse and subsequently used in this study.

cDNA synthesis and amplification

Total mRNA was extracted from the cells by RNAzol B (Cinna Biotex Labs Inc., Houston, TX). Single-stranded cDNA was synthesized using oligo (dT) primer in a reverse transcription system (RT) (Promega, Madison, WI). The immunoglobulin V_H genes were amplified by polymerase chain reaction (PCR) using Taq DNA polymerase and a 5′ universal FR1 V_H primer, or a mixture of 5′ primers specific for each of the V_H leader sequences in combination with a mix of J_H primers, or an appropriate 3′ constant region primer (Table 1). The conditions of the PCR amplification after initial denaturation of the cDNA at 94° for 5 min, comprised 30 cycles of 1 min at 94°, followed by 1 min at 60°, decreasing in the first five cycles with 1° each cycle to 55°, and a final 1 min at 72°. The PCR products were run in a 1·5% agarose gel, and the bands were cut out and purified using the Geneclean kit (Bio 101 Inc., Vista, CA). The purified products were ligated into pGEM-T vector (Promega). The ligation mixture was used to transform JM109 competent cells. Plasmid DNA was prepared from overnight cultures using QIAprep Spin Plasmid kit (QIAGEN GmbH, Hilden, Germany). At least six clones were sequenced for each case.

Table 1. Primers used in mouse V_H gene amplification

VH FR1 consensus	5′-AGG TSM ARC TGC AGS AGT CWG G-3′
L-J558	5′-ATG GRA TGG ASC TGG RTC TTT-3′
L-VGAM	5′-ATG GAA TGG CTG TGG AA-3′
L-IIc	5′-ATG AAA TKC AGC TGG RTY AT-3′
L-Va	5′-ATG ATR GTG YTR AKT CTT YTG-3′
MHALT1	5′-TGG RAT GSA GCT GKG TMA TSC TC-3′
MHALT2	5′-ATG RAC TTC GGG YTG AGC TKG G-3′
MHALT3	5′-ATG GCT GTC TTG GGG CTG CTC-3′
JH1	5′-TGA GGA GAC GGT GAC CGT GGT CCC-3′
JH2	5′-TGA GGA GAC TGT GAG AGT GGT GCC-3′
JH3	5′-TGC AGA GAC AGT GAC CAG AGT CCC-3′
JH4	5′-TGA GGA GAC GGT GAC TGA GGT TCC-3′
5T13 FR1	5′-GGC TGA GCT TGT GAA GCC TGG-3′
5T13 CDR1	5′-ACC AGC TAC TGG ATG CAC TGG-3′
VH heptamer	5′-ATG TGG TTR CAA CRC TGT GTC-3′
Cγ	5′-CAC AGG RRC CAG TGG ATA GAC-3′
Cμ	5′-GCT CTC GCA GGA GAC GAG GGG GA-3′

IUPAC-IUB codes for nucleotides are used.

Screening of 5T13 V_H-related germ line gene

C57BL genomic DNA was amplified by PCR using 5′ primers based on 5T13 V_H FR1 and CDR1 region sequences together with a 3′-primer based round the heptamer recombination sequence (Table 1). The PCR conditions were as above except that the final annealing temperature was 50°. The PCR products were purified, cloned and sequenced.

Sequencing and sequence assignments

The nucleotide sequences of amplified V_H genes were determined by the dideoxynucleotide chain termination reaction using the Sequenase® kit (United States Biochemicals Corp, Cleveland, OH) or the T7 Sequencing kit® (Pharmacia Biotech Inc., Uppsala, Sweden). Both T7 and SP6 promoter primers (Promega) were used for sequencing. Sequence alignment analysis was carried out by searching the Entrez databases of the National Center for Biotechnology Information (NCBI) using the BLAST program.[14]

RESULTS

Five MM, two WM and one BMG were studied. The disease phenotypes and V_H gene usage are summarized in Table 2. Comparison of the sequences with the NCBI's Entrez database was made, and the most homologous germ line genes were considered as the germ line donors. In some cases, sequences were aligned to rearranged genes for best match, since the closest germ line genes were far less homologous and probably not the actual germ line counterparts.

Table 2. Analysis of tumour-derived V_H gene sequences

Proliferative disorder	Isotype	V_H family	D_H	J_H	Most homologous V_H genes	% homology
MM5T2	IgG2aκ	VGAM3.8	D-SP2.5	J_H3	VGK3	97·5
MM 5T7	IgG2bκ	J558	–	J_H2	S1.2VH*	99·6
MM 5T13	IgG2bκ	J558	D-SP2.3	J_H3	VH124*	92·3
MM 5T14	IgG1κ	J558	–	J_H4	V102.1*	96·6
MM 5T33	IgG2bκ	J558	D-SP2.5	J_H2	17-1AVH†	99·3
WM 5T10	IgMκ	J558	D-SP2.2	J_H1	VH205.12*	95·9
WM 5T16	IgMκ	J558	D-SP2.2	J_H2	MUSIHCVRA†	98·5
BMG 41Ca	IgG2aκ	36-60	D-SP2.2	J_H2	6D6VH†	95·6
BMG 41Cb	IgG2bκ	36-60	–	J_H3	VHMRB9†	98·9

*The best matched germ line V_H genes in the Entrez database.
†The best matched rearranged V_H genes in the Entrez database.

MM V_H sequence analysis

V_H gene sequences. The V_H gene sequences derived from the five MM cases are shown in Fig. 1. 5T2 used a gene from the VGAM3.8 family, but the other four cases all used distinct V_H genes derived from the largest J558 family (for a review of mouse V_H gene family, see ref. 15). The best germ line match for 5T2 was VGK7.[16] There were six nucleotide substitutions, of which three were silent and three replacement. 5T7 was almost identical to the germ line gene S1.2 V_H,[17] with one silent substitution in FR2 and one replacement substitution in CDR2, changing Ser to Asn. 5T33 was highly homologous to an expressed V_H gene from a monoclonal antibody directed against carcinoma-associated antigen 17-1A.[18] One nucleotide difference was found in FR3, resulting in an amino acid change. Although 5T33 was aligned against an expressed gene, the fact that 5T33 V_H and 17-1A V_H shared very high homology strongly indicates that they were derived from a common germ line gene. 5T13 V_H and 5T14 V_H were less well matched to known germ line genes. The closest germ line match of 5T14 found in the Entrez database was the V102.1 gene.[19] 5T14 had nine nucleotide differences, introducing one silent and seven replacement mutations.

The known germ line gene in the database to which 5T13 V_H was most homologous was VH124.[20] Compared to VH124, 5T13 V_H had 14 nucleotide substitutions that resulted in 11 replacement amino acid changes. This low percentage homology between the 5T13-derived V_H gene and the closest germ line gene could be due either to somatic mutation of a germ line gene, or to the presence of novel germ line gene yet to be identified. Therefore, the corresponding germ line gene of 5T13 V_H was investigated further by screening PCR products amplified from genomic DNA isolated from a C57BL mouse using two 5T13-specific 5′-primers and the consensus heptamer 3′-primer. The first 5T13-specific primer is located in FR1, and the second in CDR1. Both primers had two mismatches with VH124 (Table 1 and Fig. 1). In total, 11 clones amplified with FR1 primer and eight clones with CDR1 primer were sequenced. Of the 11 FR1 clones, nine were identical to VH124; the others differed and had even lower homology to 5T13 V_H. Of the eight CDR1 clones, four were identical to VH124, one differed from VH124 by two nucleotides, and the remaining three clones differed and were less homologous to 5T13 V_H. VH124 was therefore considered to be the genuine donor of 5T13 V_H.

The distribution of the somatic mutations in MM-derived V_H genes is shown in Table 3. Analysis of the distribution of somatic mutations in each sequence was carried out by the method of Chang & Casali.[21] In this method, each V gene sequence is analysed codon by codon for significance of deviation from the germ line sequence. A modification of the binomial distribution model is then used to calculate whether the probability (P) of an excess (in CDR) or scarcity [in framework regions (FR)] of replacement mutations resulted by chance alone. 5T7 and 5T33 were not included, as the numbers of mutations were too small. In all three cases, the observed and expected numbers of mutations were not significantly ($P > 0.05$) different in either FR or CDR, indicating no evidence for antigen selection in the V_H sequences.

CDR3 and JH sequences. The CDR3 of 5T2 was composed of D-SP 2.5 D segment and a J_H3 gene. Both D and J segments were used in germ line configuration. N-region nucleotide insertions, either G or C, were observed at both V_H–D and D–J_H junctions. The CDR3 of 5T7 was short with six amino acids and the J_H2 gene was utilized. The nucleotides between V_H and J_H could be derived either entirely from N additions

Table 3. Distribution of mutations in tumour-derived V_H genes

		Observed			Expected	
Tumour	Region	R	S	R:S	R:S	P
5T2	FR	2	4	0·5	3·4	0·08
	CDR	1	0	–	3·6	0·39
5T13	FR	6	3	2	2·9	0·06
	CDR	4	3	1·3	5·4	0·21
5T14	FR	4	2	2	3·0	0·13
	CDR	3	1	3	4·8	0·18
5T10	FR	5	1	5	3·1	0·17
	CDR	3	2	1·5	4·9	0·22
5T16	FR	3	1	3	3·4	0·16
	CDR	0	0	–	3·8	0·43
41C-A	FR	5	2	2	2·9	0·10
	CDR	5	1	5	4·2	0·07
41C-B	FR	2	0	–	2·9	0·36
	CDR	2	0	–	4·7	0·15

The expected theoretical inherent R:S mutation ratio and the probability (P) that observed R mutations resulted from chance only were calculated according to Chang & Casali.

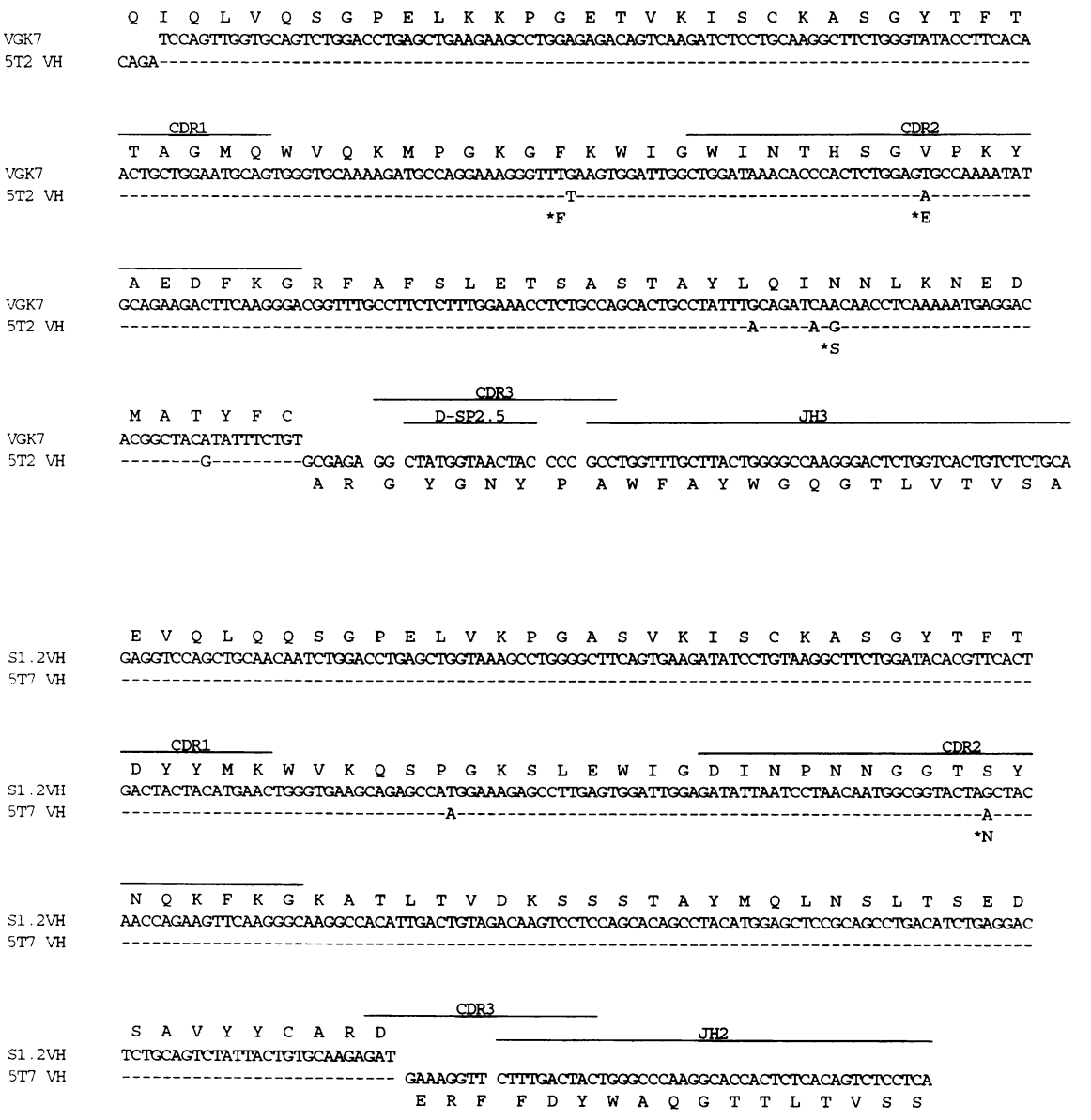

Figure 1. Nucleotide and deduced amino acid sequences of V_H genes derived from MM. Comparisons were made with the closest germ line or expressed V_H genes. Dashes represent identity with the representative germ line sequence. Replacement amino acids are starred.

or contributed partially by a D segment too short to be identified with certainty. The CDR3 sequence of 5T14 was also short, and again there was no clearly identifiable D gene. In this case, however, it was more likely that the V_H was rearranged directly to the J_H4 gene, and the six nucleotides between V_H and J_H were derived entirely from N-region insertions, since the nucleotides were exclusively C and G, which have been found to be predominant in N-regions.[22] The CDR3 of 5T13 was composed of the D-SP 2.3 D segment and J_H3 gene. There were N insertions at both V_H–D and D–J_H junctions. The CDR3 of 5T33 V_H was formed by the D-SP 2.5 D segment and J_H2 gene. N additions were only seen at the V_H–D junction. In all cases, there were no mutations in J_H segments, and N-region nucleotide insertions were predominantly G and C. In the three cases where the D segments were identifiable, reading frame 3 was used exclusively.

WM V_H sequence analysis

V_H gene sequences. Two WM cases, 5T10 and 5T16, were investigated. Again, genes from the J558 family were used in both cases. The V_H sequences aligned to the best matched sequences in the Entrez database are shown in Fig. 2. 5T10 was 96% homologous to the germ line gene VH205.12.[23] 5T10 had 11 nucleotide substitutions; two were silent mutations and nine replacement. 5T16 was closest to an expressed V_H sequence derived from a hybridoma producing anti-human procollagenase antibody.[24] There were four nucleotide differences, resulting in three amino acid changes.

The distribution of the somatic mutations in WM-derived V_H genes was also analysed according to Chang & Casali (Table 3).[21] Again, there was no significant evidence of antigen selection in either case (Table 3).

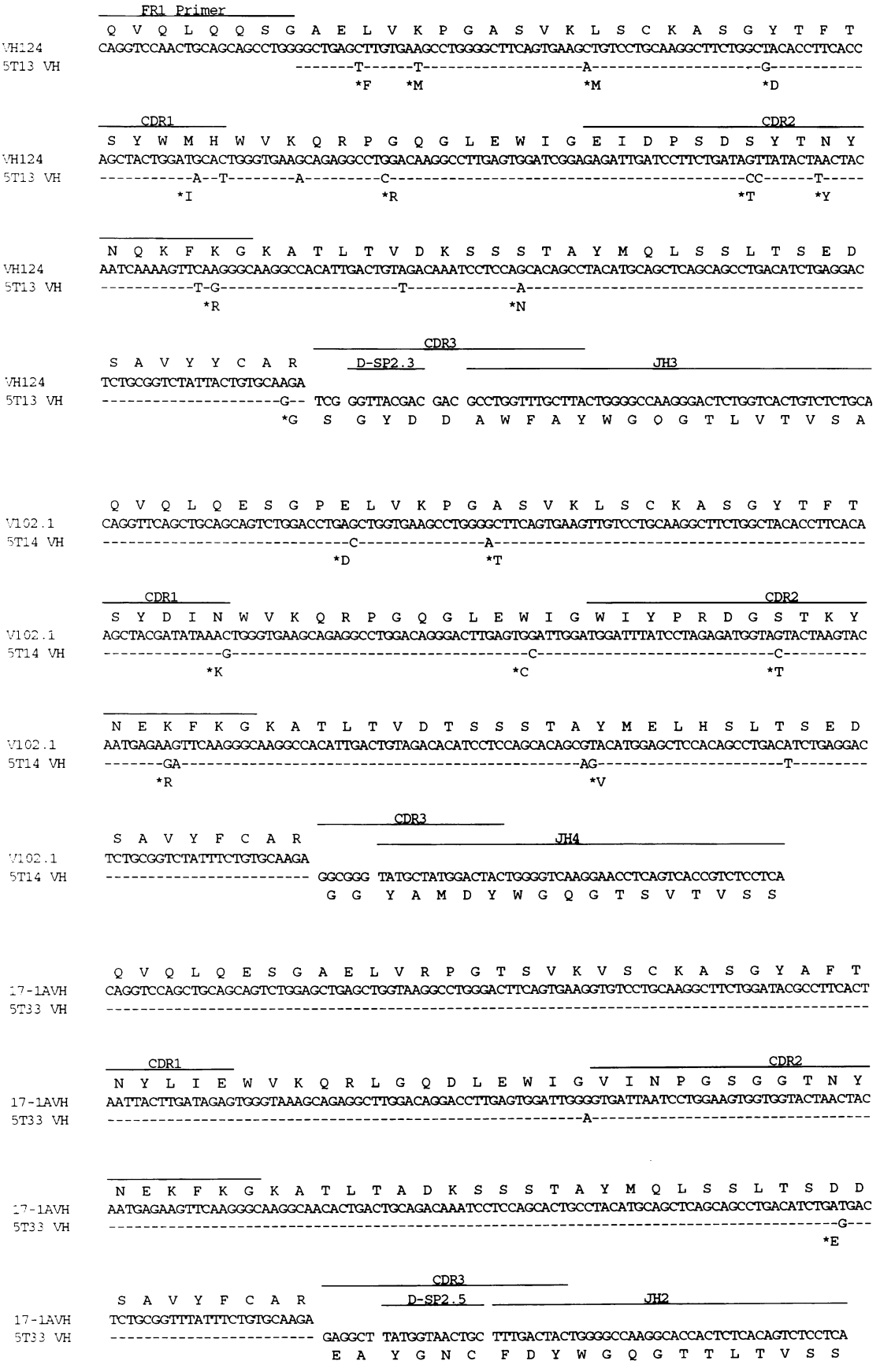

Figure 1. (Continued.)

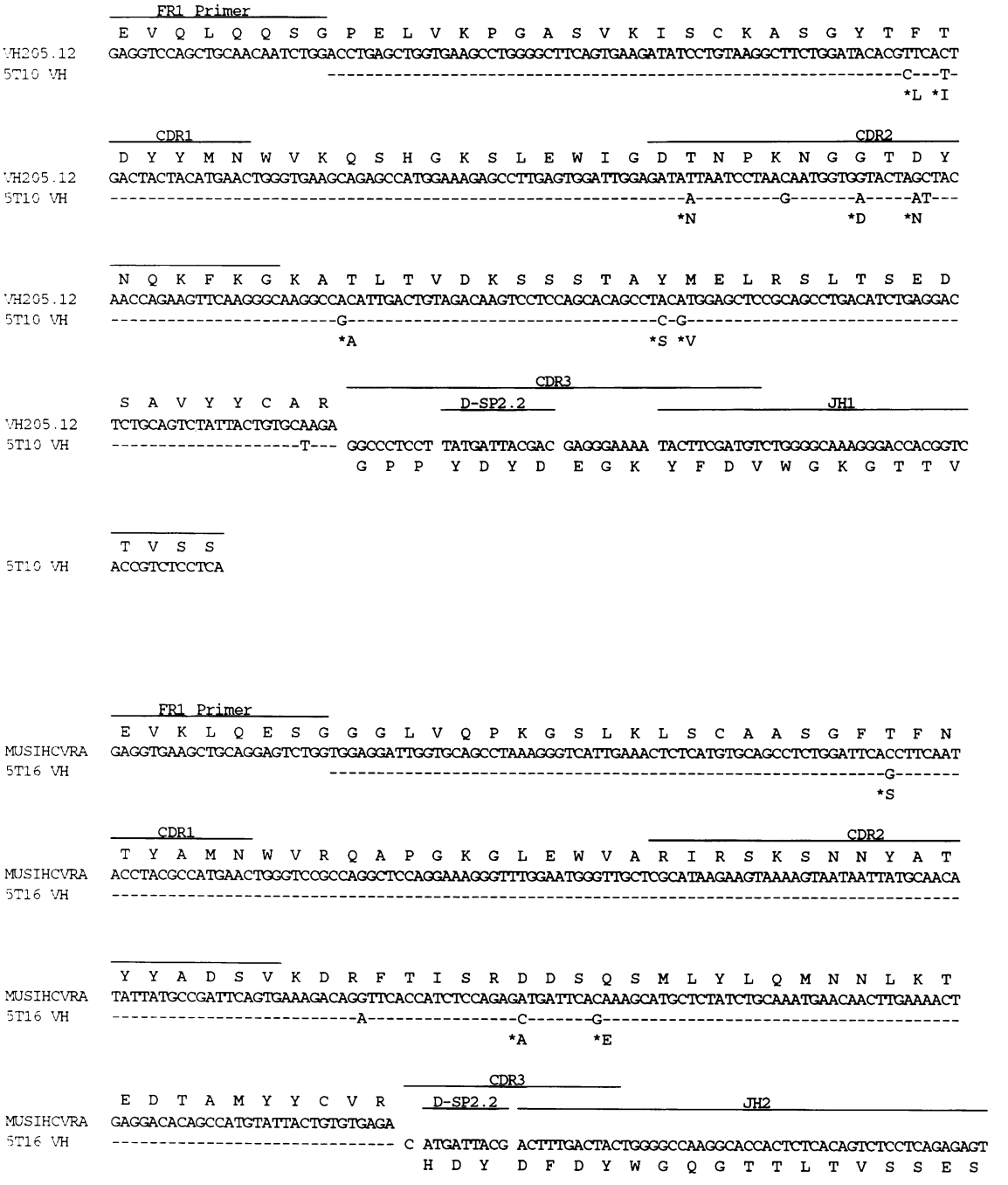

Figure 2. Nucleotide and deduced amino acid sequences of V_H genes derived from mouse WM-like lymphomas. Comparisons were made with the closest germ line or expressed V_H genes. Dashes represent identity with the representative germ line sequence. Replacement amino acids are starred.

CDR3 and JH sequences. 5T10 had a long CDR3 sequence that was composed of the D-SP2.2 D segment and J_H1 gene. The stretch of nucleotides between V and D and D and J could not be assigned to any D segments, and were presumably derived from N additions. By contrast, the CDR3 sequence of 5T16 was very short and formed by D-SP2.2 and J_H 2.

BMG V_H sequence analysis

The BMG had two paraprotein bands, and V_H gene analysis also showed that there were two distinct groups of sequences, both derived from the small 36-60 family (Fig. 3). In the first group, 41Ca, a number of sequences were closely similar to each other, with an identical V–D–J junction sequence, indicating that they originated from the same clone. It is unlikely that the nucleotide heterogeneity was introduced by Taq DNA polymerase, since it was not seen in the other cases, and the Taq DNA polymerase error rate in our system is much lower (<1/5000 bases). All intraclonal nucleotide variations led to a change in amino acid sequence. The best matched gene found in the database for this group of sequences was a gene used

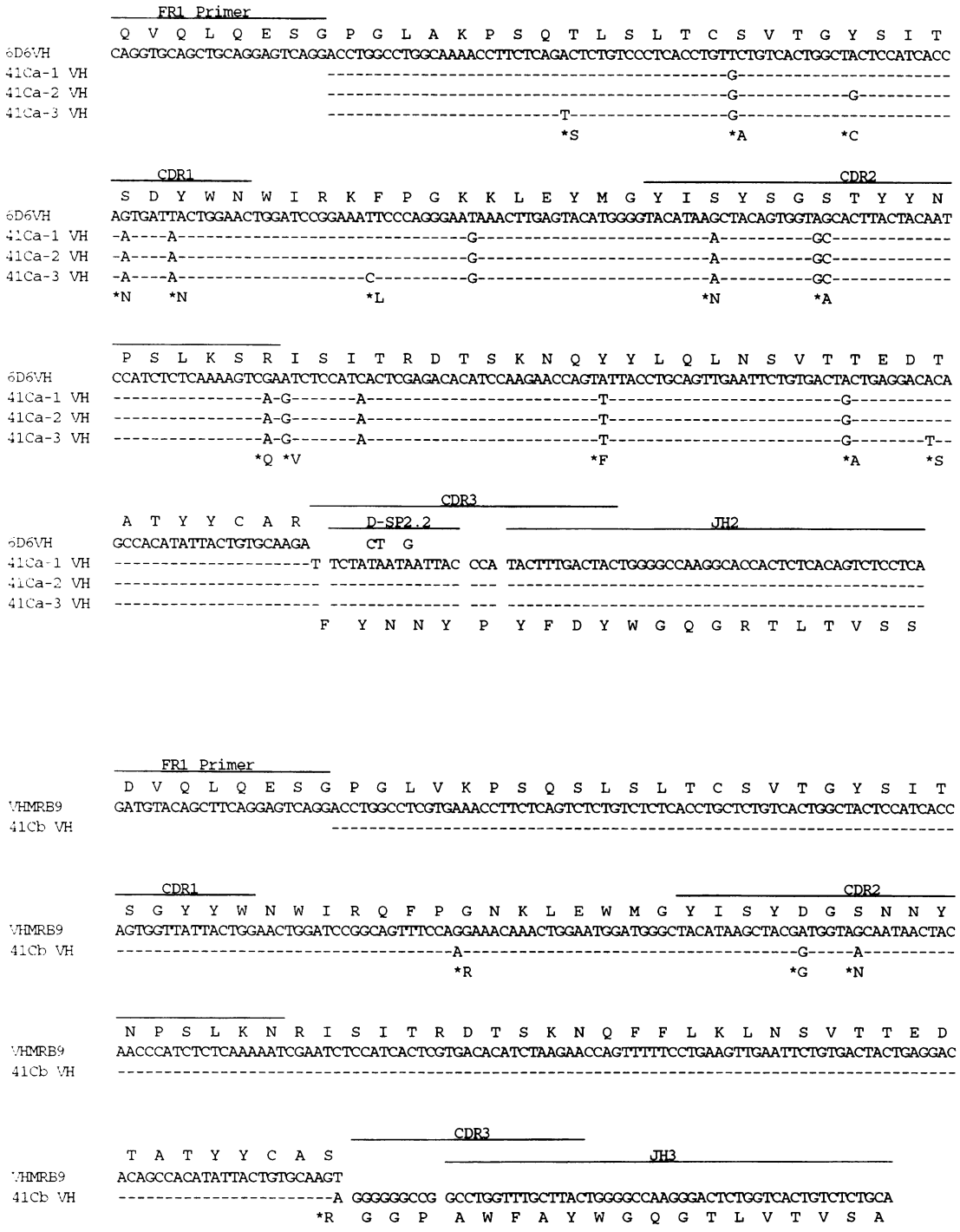

Figure 3. Nucleotide and deduced amino acid sequences of V_H genes derived from mouse BMG. Comparisons were made with the closest germ line or expressed V_H genes. Dashes represent identity with the representative germ line sequence. Replacement amino acids are starred.

in an anti-MPO antibody (Gilbert *et al.*, unpublished data, accession Z37144). 41Ca clones were different from this gene by between 12 and 15 nucleotides, which were located in FR as well as in CDR. The D segment used matched equally well to D-SP2.2 and D-SP2.9, both with one silent and two replacement nucleotide changes. The D segment was rearranged to a J_H2 gene in germ line configuration. The second clone, 41Cb, was most homologous to the gene VHMRB9, which was used in an anti-histone antibody.[25] There were three nucleotide differences, one in FR2 and two in CDR2, all resulting in amino acid changes. No intraclonal variation was observed. The CDR3 was short, with no identifiable D segment. The J_H3 germ line gene was used. The nucleotides between V and J were exclusively G and C, probably derived entirely from N additions.

In both cases, there was no evidence of antigen selection,

since no significant difference was found in the observed and theoretically expected numbers of mutations in either FR or CDR.

DISCUSSION

We have determined and analysed immunoglobulin V_H gene sequences derived from five cases of MM, two of WM and one of biclonal BMG. In contrast to corresponding human diseases, in which V_H genes have consistently been found to be extensively mutated with a mean% $\pm$ SD of $8{\cdot}8 \pm 3{\cdot}2$,[5] the rate of somatic mutation in mouse tumours was significantly lower (two-sample t-test, $P < 0{\cdot}01$), with a mean% $\pm$ SD of $2{\cdot}9 \pm 2{\cdot}3$. In the five mouse MM, 5T2, 5T7 and 5T33 had only a limited number of mutations. In contrast, 5T13 and 5T14 were considerably different from the closest germ line genes, with 11 and seven amino acid replacement mutations, respectively. Although the mouse immunoglobulin V_H repertoire has not been fully sequenced and seems to be more complicated, with estimates varying between 100 and 1000 members,[15] we could not find a better-matched germ line donor for 5T13 by sequencing PCR products amplified with 5T13-specific primers. We believe therefore that the low homology between 5T13 V_H and VH124 is due to somatic hypermutation.

Recent studies indicate that in humans the neoplastic cell of MM is a B cell that has passed the germinal centre and undergone somatic hypermutation.[3–5] Both immunoglobulin V_H and V_L genes are extensively mutated and in a proportion of cases ($\sim$25%) there is evidence for antigen selection, as significant clustering of replacement mutations has been observed in CDR regions in either V_H or V_L.[6,7] In the mouse, however, the origin of MM cells is less clear, since the mutation rate of V_H genes varies from case to case. 5T13 and 5T14, which have numerous nucleotide substitutions, may originate from B cells that have been exposed to the hypermutation mechanism in the germinal centre, possibly memory cells. However, there is no clear evidence for antigen selection, as the mutations were not clustered in the CDR regions. In contrast, 5T2, 5T7 and 5T33 have only a small number of mutations. It is possible therefore that they originated from naive cells that had not undergone somatic mutation process. On the other hand, it is generally believed that the rate of somatic mutation of mouse V genes is lower, especially in old animals.[26] It cannot be ruled out that the precursor cells of 5T7 and 5T33 were at the same differentiation stage as 5T13 and 5T14, but failed to accumulate further mutations. It is interesting to note that in mouse B-cell lymphomas, BCL1,[27] 38C13 and A31,[28] the V_H genes have just one (BCL1) and no (38C13 and A31) somatic mutation (our unpublished data).

Compared to human MM, the CDR3 regions of mouse MM are less complex. Unlike human MM, no D–D fusion and D segments in reverse orientation were observed and the D segments identified were all in germ line configuration. There was evidence of N-region additions in all the murine tumours investigated; however, it is difficult to compare the degree of N additions to that of the human tumours because of the complexity of the D segment usage of the latter. Interestingly, in two murine MM cases the CDR3 regions seemed to be contributed mostly by N insertions with no apparent D segments involved. However, the possibility that very short or mutated D segments were used could not be ruled out.

Similar variations in the somatic mutation rate of V_H genes were also observed in the two cases of WM and the biclonal BMG. The two BMG clones both used V_H genes from the much smaller 36-60 family. The clone 41Ca was more mutated and intraclonal nucleotide variations were observed, suggesting that the tumour cells may still be undergoing somatic mutation. In this aspect, mouse BMG is very similar to the human counterpart, as intraclonal variation has been observed in a number of human cases.[7] The degree of somatic mutation in the clone 41Cb was much lower, with only three nucleotide substitutions. Similar low frequency of somatic mutation in one case of BMG has been observed previously.[29]

The 5T series of MM and WM-like lymphomas is, in many aspects, closely similar to corresponding human diseases. However, the rate and pattern of somatic mutation of V_H genes in some cases are quite different. Obviously, the findings have implications in using the 5T series of mouse tumours as experimental models, especially in anti-idiotypic therapy studies, where it might be anticipated that the number of idiotypic determinants arising from somatic mutations and genetic complexity in CDR3 will be more limited than in the human disease counterparts.

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