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Members of novel V_H gene families are found in VDJ regions of polyclonally activated B-lymphocytes

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Four potentially productive and two non-productive VDJ gene segments were isolated from the DNA of mouse B-lymphocytes which had been polyclonally activated by bacterial lipopolysaccharide (LPS). Three VDJ regions exhibit V_H genes which stem from two novel V_H gene families. The complexity of these families is 5-9 genes. One of the non-productive VDJ regions exhibits a D segment which may have been generated by joining of two DSP2 segments. Both non-productive VDJ regions appear to contain rearranged pseudo V_H genes. Three potential somatic mutations distributed over two productive VDJ regions are observed.

Key words: V_H gene families/VDJ-rearrangement/somatic mutations

Introduction

Antibody diversity is partly based on the germ line-encoded repertoire of multiple variable, diversity and joining region gene segments (V_H, D and J_H in the heavy chain locus and V_L and J_L in the light chain locus). Joining of these segments generates combinatorial diversity. The resulting pool of V_HDJ_H and V_LJ_L gene segments is further diversified by somatic mutations (reviewed by Tonegawa, 1983).

To analyse rearrangement and somatic variation in VDJ regions from cells not selected by antigen we isolated rearranged V_H genes from splenic B-cells which had been activated polyclonally with lipopolysaccharide (LPS). This mitogen stimulates ~30% of the B-lymphocytes present in the spleen to differentiate into plasmablasts (Andersson *et al.*, 1977). Since we wanted to examine both active and silent Igh loci we established genomic libraries from DNA of LPS-blasts and sequenced six rearranged V_H genes.

Results

To isolate genomic fragments containing a (V)DJ-C_H gene, *BglII* restriction fragments of DNA from LPS-blasts were cloned into the lambda phage vector L47.1. The VDJ regions of recombinant phages positive for the universal J_H probe were subcloned into M13 phage vectors for nucleotide sequence analysis [MU designates phages from a library of $sIgM^+$ LPS-blasts; GAM3 designates phages from a library of $sIgG3^+$ LPS-blasts (Figure 1)]. Nucleotide sequences are shown in Figures 2, 3 and 5. None of the six sequences shows a DJ rearrangement indicating a silent allele of the Igh locus, but two VDJ segments are the result of an aberrant joining. The rearranged V_H genes come from four different V_H gene families; only two of these four families belong to the set of seven V_H gene groups described by Dildrop (1984) and Brodeur and Riblet (1984).

VMU-3.2 (Figure 2a) exhibits a V_H segment which belongs to the J558 family (group I). The V, D and J sequences of VMU-3.2 are in the correct translational reading-frame. D and J correspond to known germ line segments: DFL16.1 and J_H2 of the Igh^b locus (Kurosawa and Tonegawa, 1982; Krawinkel *et al.*, 1983); N sequences (Alt and Baltimore, 1982) are not present. The V_H sequence does not show stop codons or drastic deviations from the primary structure which is considered to be essential for normal heavy chain V domain folding and correct H-L chain interaction (Saul *et al.*,1977; Davies and Metzger, 1983). VMU-3.2 thus appears to represent an active gene. It is not possible to assign it to an already known germ line V_H gene from the J558 family. The nearest relative is gene V105 (Cohen *et al.*, 1982) which shows an overall homology of 88.5% to the coding region of VMU-3.2 (Figure 2a).

The 5'-flanking regions of both genes are very homologous (~85%) and show the $(TCA)_n$ repetitive sequence which is maintained among a subgroup of J558 family members and which is postulated to play a role in the interaction between those genes (Cohen *et al.*, 1982).

VMU-3.2 shows two single base exchanges upon comparison with a consensus sequence of 36 potentially functional germ line V_H genes from the J558 family (cons.mp Figure 2d). G at position 89 (codon 30) leads to a conservative threonine to serine exchange in CDR1. The same exchange is observed as a potential somatic mutation of germ line V_H gene IDCR.11 (Siekevitz *et al.*, 1983) in antibody 16.7 (Siegelman *et al.*, 1981). The second exchange at position 282 (C instead of T in the third base of codon 90) is silent, but affects a position which is highly conserved among germ line V_H genes of the J558 family: 48 out of 49 sequences have a T at this position. A C at position 282 occurs as a potential somatic exchange in the V_H region of antibody MPC11 (Givol *et al.*, 1981). Upon comparison with a consensus sequence of 36 potentially functional plus 13 pseudo germ line V_H genes (cons.all, Figure 2d) from V_H-family I VMU-3.2 still shows the exchanges described above, i.e., the latter do not occur in the known pseudo V_H genes. However, as long as the corresponding germ line gene to VMU-3.2 is not known the exchanges at positions 89 and 282 cannot be definitively attributed to somatic mutations.

VGAM3-0 (Figure 2b) also contains a V_H segment belonging to the J558 family and is derived from the Igh^b locus as judged from the nucleotide sequence of the J_H 3'-flanking region. The recombinant phage GAM3-0 contains the C_{MU} gene (Figure 1). As it comes from a library of sIgG3-positive/sIgM-negative LPS-blasts (Radbruch and Sablitzky, 1983), it should be derived from the inactive chromosome. Indeed, sequence analysis of the VDJ segment VGAM3-0 shows an aberrant V_H to DJ rearrangement (Figure 2b): seven nucleotides that cannot be accounted for by known germ line sequences lead to a frameshift of the DJ sequence and introduce two in phase stop codons into the V_H to D transition region. Again, a V_H gene sufficiently similar to VGAM3-0 to be regarded as its germ line counterpart could not be found among the 49 published sequences. VGAM3-0 is

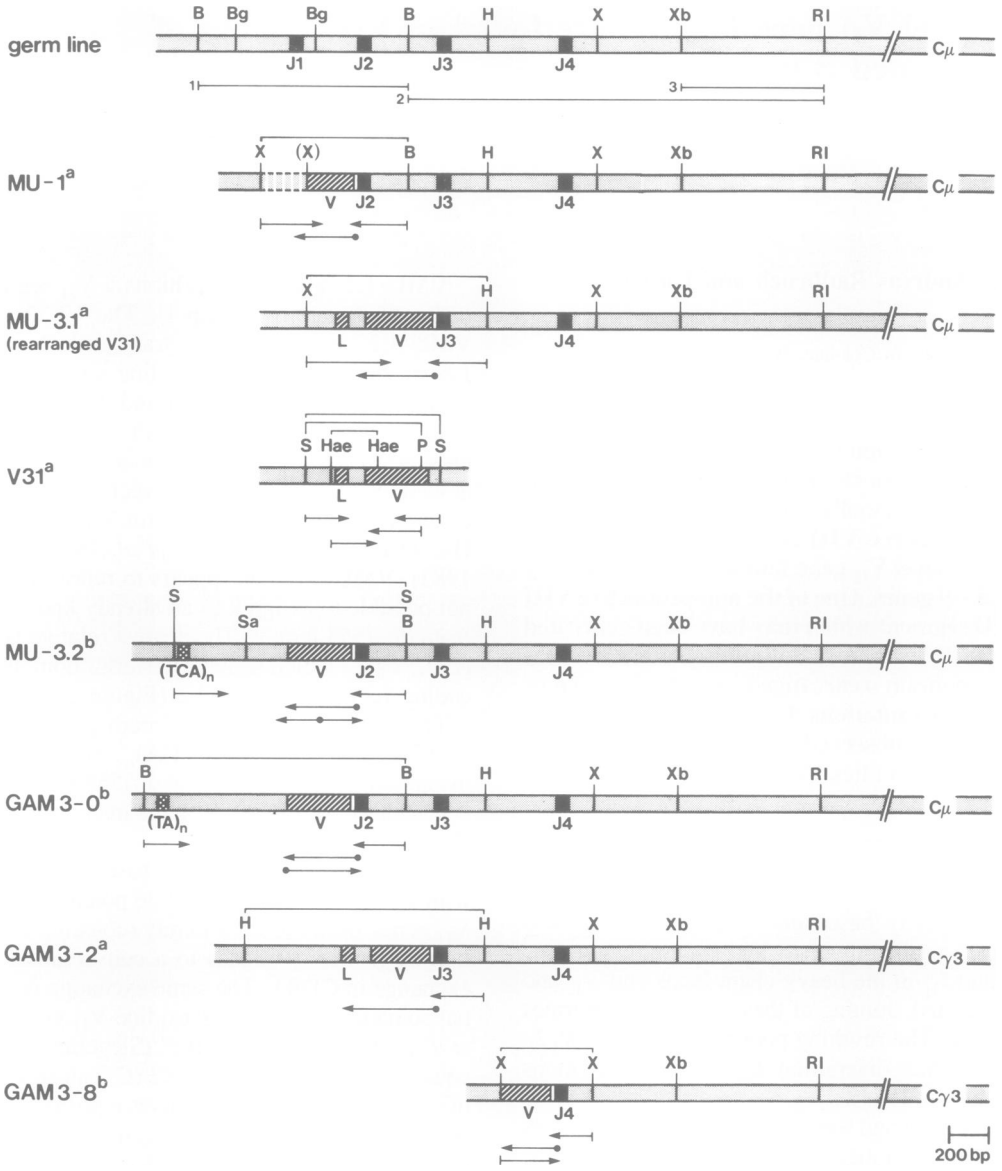

Fig. 1. Restriction map of the lambda phage clones analysed in this study. Fragments subjected to sequence analysis and orientations of sequencing are marked by arrows. J_H region probes no. 1, 2, 3 employed for detection of lambda and M13 phage clones are shown. Bg=*Bgl*II, B=*Bam*HI, RI=*Eco*RI, H=*Hind*III, Hae=*Hae*III, P=*Pst*I, S=*Sau*3A, Sa=*Sac*I, X=*Xho*II, Xb=*Xba*I.

~83% similar to V186-2 (Bothwell *et al.*, 1981; Krawinkel *et al.*, in preparation) (Figure 2b). In the 5'-flanking region no significant homology could be found between these genes with the exception of a stretch of 44 alternating A/T bases. Such a structure may be involved in recombinations between members of a multigene family (Shen *et al.*, 1981). It may be a selective advantage for V_H genes to retain this structure in the germ line.

When compared with a consensus-sequence from 36 potentially functional germ line V_H genes (cons.mp) from family I, VGAM3-0 exhibits five base exchanges (Figure 2d). They are all located in the FR3 and without exception are replacement substitutions affecting highly conserved amino acid residues. Replacement exchanges occur at random in the FR regions of pseudo V_H genes, whereas functional genes show a bias in favour of silent exchanges in their framework regions, thus indicating a selective pressure towards maintenance of antibody structure (Givol *et al.*, 1981; Cohen and Givol, 1983; Loh *et al.*, 1983; Heinrich *et al.*, 1984; Blankenstein *et al.*, 1984). One therefore might argue that the V_H gene in VGAM3-0 represents a rearranged pseudo gene because of the drastic exchanges in the FR3. As also shown for VMU-3.2 no mutations are detected in the J_H coding sequence and ~200 bp of 3'-flanking region.

VGAM3-2 (Figure 2c) exhibits a V_H gene which belongs to family III and represents a VDJ region from the Igh^a locus. As judged from the sequence, VGAM3-2 encodes an intact antibody V_H region. DSP2.2 and J_H3 are involved in the VDJ rearrangement. The V_H gene is ~88% homologous to the BALB/c allele of V_H36-60 (Near *et al.*, 1984) which is the only germ line sequence known so far from group III. In contrast to V_H36-60, VGAM3-2 codes for an amino acid residue at codon 35a. This peculiarity is shared by the variable region of MOPC 315 (Padlan *et al.*, 1976) which from residue 1 to 47 is more similar to VGAM3-2 than to V_H36-60 (Figure 2c). From residues 59 to 94 VGAM3-2 and V_H36-60 match except for residues 78 and 79 where V_H36-60 shows two tyrosine codons instead of two phenylalanines in VGAM3-2 and MOPC 315. This segmental homology of VGAM3-2 to V_H36-60 and MOPC 315 possibly indicates that the germ line V_H gene of VGAM3-2 is the result

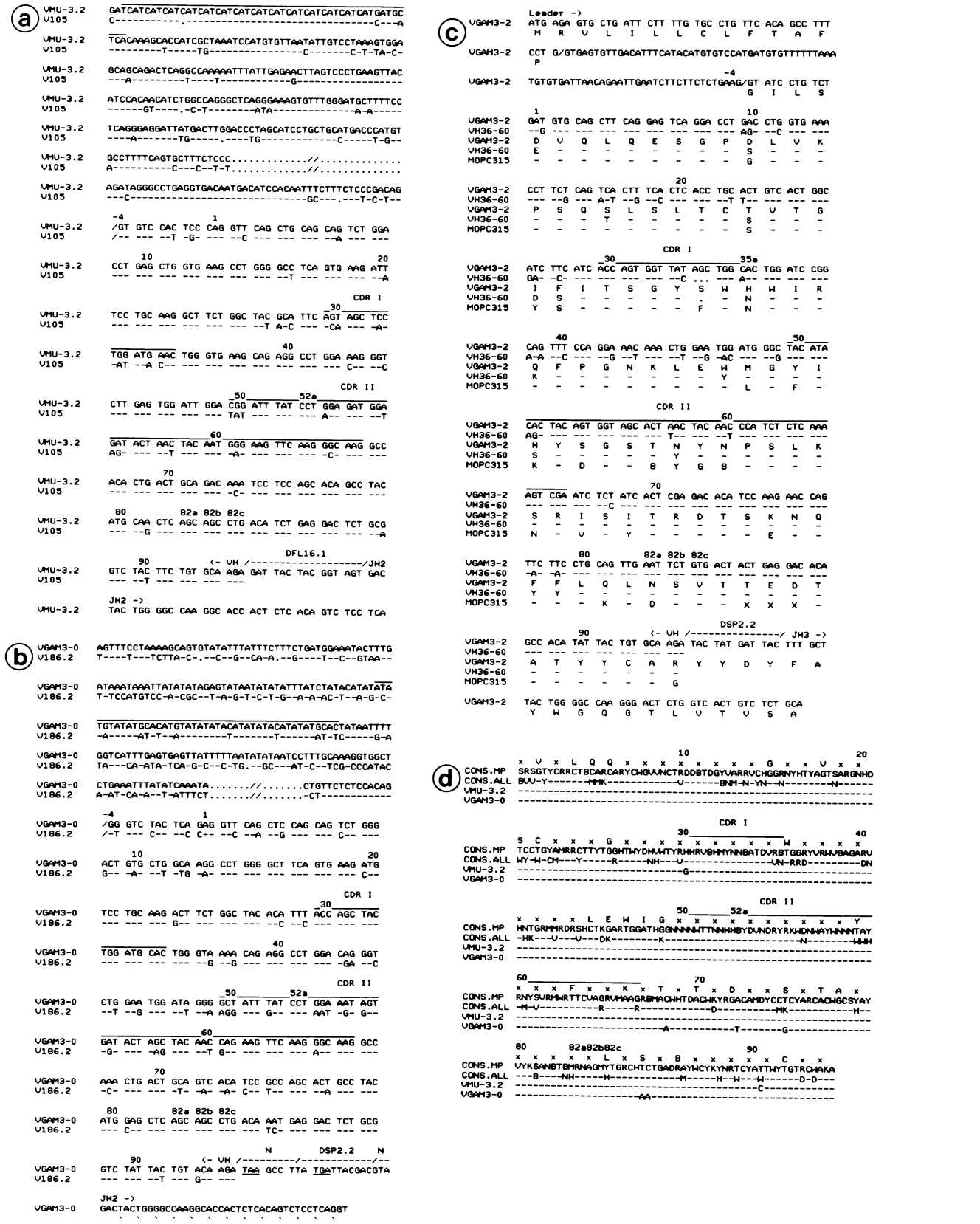

Fig. 2. Sequences of VDJ regions carrying V_H genes from V_H families I **(a,b)** and III **(c)**. Repetitive sequences in the 5′-flanking region are overlined. Protein coding sequences are written as triplets and numbered according to Kabat *et al.* (1976). **(a)** The sequence of VDJ region VMU-3.2 is compared with V_H gene V105 (Cohen *et al.*, 1982). **(b)** The sequence of VDJ region VGAM3-0 is compared with V_H gene V186.2 (Bothwell *et al.*, 1981). Stop codons in the N sequence and in the D region are underlined. The functional reading frame of J_H2 is marked by dashes. **(c)** Comparison of VDJ region VGAM3-2 to V_H genes V_H36-60 (Near *et al.*, 1984) and MOPC 315 (Padlan *et al.*, 1976) at the nucleotide level and/or at the level of amino acid sequence. The absence of codon 34 in V_H36-60 is marked by dots. **(d)** Comparison of VMU-3.2 and VGAM3-0 with consensus sequences of V_H genes from family I. Cons.all comprises 36 potentially functional and 13 pseudo V_H genes whereas cons.mp only comprises the 36 functional V_H genes. The consensus sequences are written in the IUB-IUPAC ambiguity code.

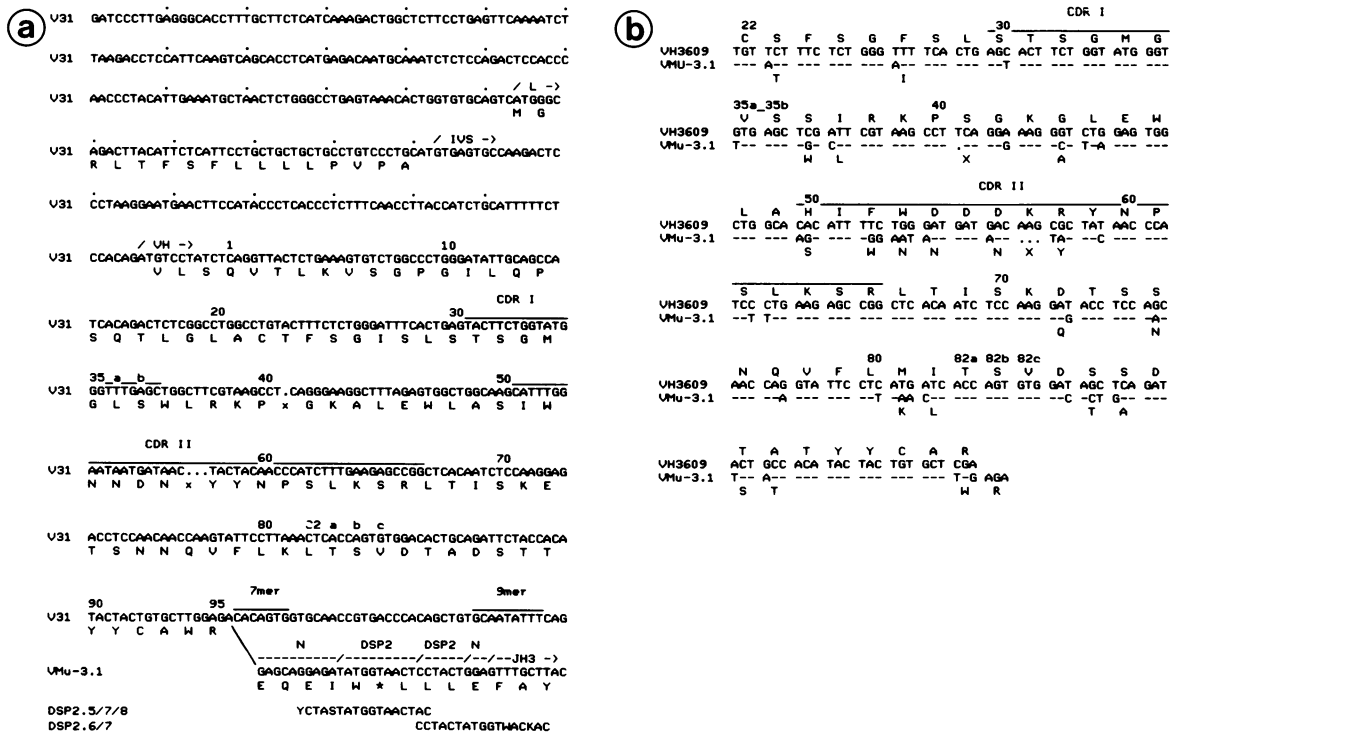

Fig. 3. **(a)** Sequence of V_H gene V31 which belongs to V_H family VIII and is rearranged in VDJ region VMU-3.1. The reading frame of V31 is deduced from conserved regions of other V_H genes (Dildrop, 1984) and from the reading frame of V_H gene V3609 (Brodeur *et al.*, 1984, see **b**). The heptameric and nonameric recognition sequences for VD joining are overlined. The D portion of VMU-3.1 possibly comprises two DSP2 segments whose corresponding germ line sequences (Kurosawa and Tonegawa, 1982) are shown. Potential N sequences are separated from the V, D and J region by dashes. The stop codon is marked by an asterisk. Nucleotide deletions are presented as dots. **(b)** Comparison of VMU-3.1 (V31) with V_H gene V3609 which serves as a reference sequence for V_H family VIII (Brodeur *et al.*, 1984).

of an ancient recombination between V_H36-60 and the germ line gene of MOPC 315.

Lambda clone MU-3.1 represents another example of an inactive VDJ segment (Igh^a). The D to J_H3 rearrangement is aberrant because of the non-productive reading frame of the J_H3 of exon. Ten nucleotides of N sequence (Alt and Baltimore, 1982) are inserted between the V and D segment. The D portion of VMU-3.1 may represent a rearrangement involving two D segments, namely DSP2.5/7/8 and DSP2.6/7. The latter is separated by another three nucleotides of N sequence from J_H3 (see Figure 3a).

The rearrangd V_H gene of MU-3.1 exhibits several unusual features: (i) the leader sequence encodes 16 instead of 15 amino acids; (ii) the CDR1 is two amino acids longer than the ones encountered normally (positions 35a and b); (iii) whilst the 3′ ends of most V_H genes encode the amino acids C-A-R, VMU-3.1 shows the 'insertion' of a tryptophan residue into this sequence (C-A-W-R); (iv) a deletion of one nucleotide in FR2 (codon 41) leads to a frameshift in the V_H sequence.

VMU-3.1 shows <70% homology to any of the seven known V_H gene families. Dildrop (1984) and Brodeur and Riblet (1984) report that members of a V_H gene family share sequence homology of at least 80% whereas unrelated V_H genes, i.e., genes from different families, are <70% homologous. Only recently Brodeur *et al.* (1984) presented the partial mRNA sequence of the NZB plasmacytoma PC3609 which shared not more than 60% homology to any given V_H sequence. The authors proposed that V_H3609 represents the eighth V_H family. The comparison of VMU-3.1 and V_H3609 (Figure 3b) reveals that both sequences are 80.3% homologous and thus can be considered to be members of the same V_H gene family. Since the CDR2 of V_H3609 contains 17 residues in contrast to the 16 amino acids encoded by the CDR2 of VMU-3.1 a deletion of one triplet in VMU-3.1 has to be assumed.

To estimate the size of the new V_H gene family we probed genomic Southern blots of *Eco*RI-digested liver DNA with the radioactively labelled M13 subclone of MU-3.1 (Figure 4). In both BALB/c and C57BL/6 DNA 7−9 bands are detected. The pattern of restriction fragments differs between the haplotypes. If one assumes that every *Eco*RI band corresponds to one V_H gene, the complexity of the V_H3609 family would fall in the range of 10. We also isolated the germ line gene corresponding to the V_H portion of VMU-3.1; *Bam*HI fragments from BALB/c liver DNA were cloned into L47.1 phages. Screening of this library was performed under high stringency conditions and one clone showed the restriction fragments predicted by the sequence of VMU-3.1 (data not shown). The V_H gene of this clone (designated V31) was sequenced. As there is no difference between the sequences of V31 and VMU-3.1 we conclude that VMU-3.1 contains a rearranged pseudo gene which did not undergo somatic mutations after joining to a DJ segment. The peculiar amino acid sequence C-A-W-R mentioned above is obviously the true 3′ end of V31.

Although being a pseudo gene that exhibits two deletions and one insertion, V31 does not show gross deviations from its NZB relative or from the coding and 5′-flanking sequences of other V_H genes. This suggests that V31 is a 'young' pseudo gene having evolved only recently from a functional gene. V31 is rearrangd in VMU-3.1 and hence the recognition signals which are necessary for the V to DJ joining process must be functional. The sequence of V31 (Figure 3a) shows the highly conserved CACAGTG heptamer and − following the 23-bp spacer − a

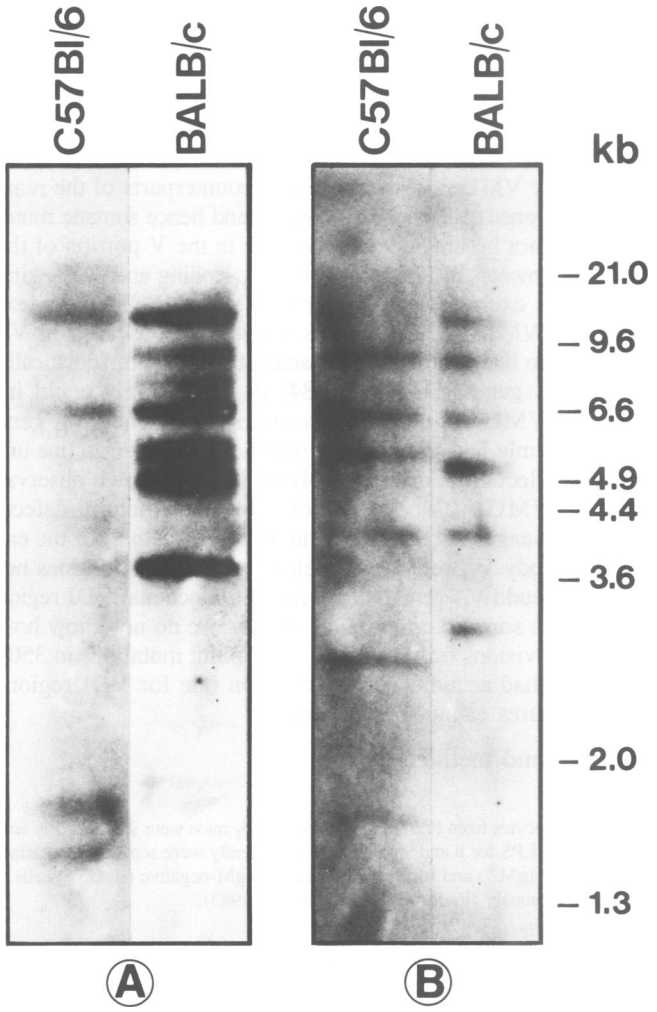

Fig. 4. Southern hybridization analysis of *Eco*RI-digested liver DNA from BALB/c and C57BL/6 mice. **(A)** VMU-3.1 and **(B)** VGAM3-8 are used as probes representing V_H families VIII and IX, respectively. The J_H locus is detected by these probes as a 6.6-kb band.

GCAATATTT nonamer. The latter differs considerably (in five out of nine positions) from the ACAAAAACC consensus nonamer (reviewed by Tonegawa, 1983). This means that the nonameric signal sequence can diverge to a large extent without impairing the ability of the V_H gene to fuse to a D segment.

The V_H gene sequences of lambda clones MU-1 and GAM3-8 were found to be identical except for three base substitutions in the CDR2. Again, the sequences could not be assigned to any of the seven known V_H gene families. As concluded from the flanking sequences of the corresponding J_H segments, VMU-1 comes from a BALB/c and VGAM3-8 from a C57BL/6 chromosome. We could not determine the 5′ end of VMU-1/VGAM3-8 because 55 nucleotides are cut from the V_H exon by the *Bgl*II site which was used as a cloning site (Figure 5).

Two VDJ regions derived from the H chain mRNAs of hybridomas which were generated by fusing myeloma cells to LPS-activated splenic B-lymphocytes are strongly homologous to the ones exhibited by VGAM3-8/VMU-1 and thus also represent V_H genes from family IX (R.Dildrop, unpublished). The three replacement exchanges between VMU-1 and VGAM3-8 very likely represent allotypic differences, because the mRNA sequences which stem from LPS-blasts of the b-haplotype are identical to the VGAM3-8 sequence at the positions where the latter

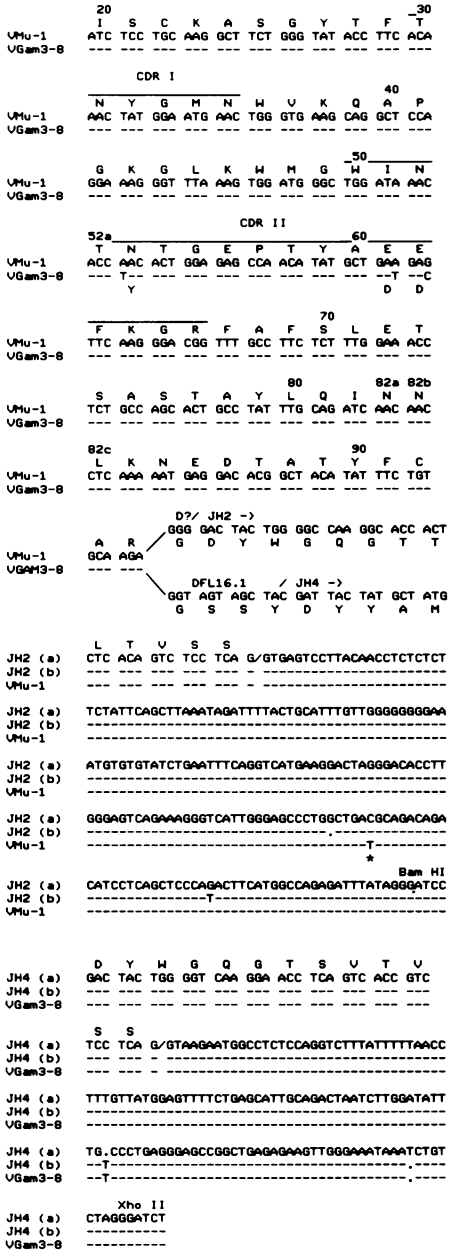

Fig. 5. Comparison of VDJ regions VMU-1 and VGAM3-8 which are reference genes for V_H family IX. D and J regions are separated by dashes. The 3′-flanking regions of VMU-1 and VGAM3-8 are compared with the corresponding regions from a- and b-haplotype J_H loci. The somatic mutation in VMU-1 is marked by an asterisk.

differs from VMU-1 (Figure 5).

As shown by Southern blotting experiments the complexity of V_H gene family IX is rather low: in both BALB/c and C57BL/6 liver DNA five bands could be detected with the radiolabelled VGAM3-8 probe upon digestion with *Eco*RI (Figure 4) and seven bands hybridized in *Hind*III-digested DNA (not shown).

It is unexpected that members of such a small V_H gene family are independently isolated twice from different populations of LPS-blasts. The conclusion that VMU-1-/VGAM3-8-like genes are expressed predominantly in an LPS-blast population, however, does not hold because only a few per cent of LPS-blast-derived hybridomas express genes of the new family (R.Dildrop *et al.*, 1985).

VMU-1 shows an example for a somatic mutation. The C to

T exchange in the 3'-non-coding region of J_H2 cannot be accounted for by the germ line sequences of either the a- or the b-haplotype (Gough and Bernard, 1981; Krawinkel *et al.*, 1983) of the J_H locus (Figure 5).

Discussion

In this study we investigated the structure of six rearranged V_H genes from active and silent heavy chain loci of polyclonally stimulated B-lymphocytes. Four sequences (VMU-1, VMU-3.2, VGAM3-2 and VGAM3-8) show an open reading frame with V, D and J segments in correct phase whilst two sequences (VMU-3.1 and VGAM3-0) represent aberrantly rearranged VDJ genes from the inactive chromosome.

Finding only VDJ and no DJ rearrangements among six J_H probe-positive phage clones is unexpected in the light of the result that a splenic B-cell population selected for expression of immunoglobulin contains 60% B-cells carrying a productive VDJ region on the active allele and a DJ region on the inactive allele of the Igh locus. Assuming that the remaining fraction carries one productively and one non-productively rearranged VDJ-region per genome the overall ratio of DJ- to VDJ-regions is 1:2.3 (Alt *et al.*, 1984). We are not aware of any selection against phages carrying DJ regions in our cloning procedures. Cloning *Bgl*II restriction fragments rather should have provided a bias in favour of such clones since most D segments have a *Bgl*II site in their 5'-flanking region. In accordance with low DJ to VDJ ratios found in 15 AMULV-transformed B-cell lines and 32 myelomas, 1:7.5 and 1:5, respectively (Alt *et al.*, 1984), our data suggest that cessation of V to DJ rearrangement once a productive VDJ region has been formed is not an absolute rule. Secondary rearrangements upon stimulation with LPS should be considered.

A D to D fusion seems to be expressed in VMU-3.1 which exhibits in a tandem array the DSP2.5/7/8 segment and six nucleotides which match the 5' end of the DSP2.6/7 segment (Figure 3a). The chance to generate at random six nucleotides as an N sequence which happens to match a hexamer in any of the known D segments (Kurosawa and Tonegawa, 1982) is not high, namely $60/4^6 = 1/68$.

One thus has to consider the possibility that the D portion of VMU-3.1 either was generated by recombination of DSP2 elements in the germ line or by somatic D to D rearrangement. The latter possibility is difficult to reconcile with the canonical rule of VDJ-joining (see Siu *et al.*, 1984a, for discussion): V gene assembly always joins a one helical turn recognition element (12 bp between heptamer and nonamer) to a two helical turns recognition element (23 bp between heptamer and nonamer) in the three immunoglobulin families and in the T-cell receptor beta chain gene family (Siu *et al.*, 1984b). However, all known DSP2 segments are flanked by one-turn recognition elements (Kurosawa and Tonegawa, 1982) and this should render D to D joining impossible.

We find three VDJ regions that do not use V_H genes belonging to the seven 'classical' V_H gene families (Dildrop, 1984; Brodeur and Riblet, 1984). One thus has to assume that the known myelomas and hybridomas are not representative as far as their V_H gene usage is concerned. Even more than nine V_H gene families may exist. The fact that a member of a new V_H gene group is found twice (VMU-1/VGAM3-8) may well be attributed to selection by the molecular cloning strategy. We used DNA completely digested with *Bgl*II and VMU-1/VGAM3-8 carry a *Bgl*II site at codon 19. In contrast, finding two V_H genes from the V_H family I is in accordance with the estimated complexity (60 genes, Brodeur and Riblet, 1984) of this family. One would have expected that at least one of the two group I genes – VMU-3.2 or VGAM3-0 – matches one of the 49 known germ line sequences. Since this is not the case one wonders whether a gene family comprises more members than estimated from the bands in a Southern blot.

Except for VMU-3.1, the germ line counterparts of the rearranged V_H genes could not be identified and hence somatic mutations could not be unequivocally shown in the V portion of the six VDJ segments. In ~1.5 kb of total J_H-coding and 3'-flanking regions one exchange was observed (VMU-1). The two exchanges in VMU-3.2 at positions which are conserved in V_H family I germ line genes are a characteristic feature of somatically mutated V_H genes (Dildrop, 1984) or alternatively could indicate that VMU-3.2 represents a rearranged pseudo V_H gene which randomly had accumulated mutations in the germ line unbiased by selection (Cohen and Givol, 1983, and own observations). As VMU-3.2 does not show obvious structural defects and the exchanges at codons 30 and 90 are not lethal for the encoded antibody V_H region, we believe that VMU-3.2 does not contain a pseudo V_H gene but represents a functional VDJ region carrying two somatic point mutations. As we do not know how many cell divisions occurred until three point mutations in 3500 nucleotides had accumulated, a mutation rate for VDJ regions in LPS-cultures cannot be calculated.

Materials and methods

Cells

Splenic lymphocytes from (C57BL/6 × BALB/c)F_1 mice were stimulated *in vitro* with 40 μg/ml LPS for 8 and 9 days and subsequently were separated in surface IgM-positive ($sIgM^+$) and surface IgG3-positive/IgM-negative ($sIgG3^+$) cells as described previously (Radbruch and Sablitzky, 1983).

λ Phage libraries

L47.1 bacteriophage libraries were constructed from *Bgl*II-digested DNAs of either $sIgM^+$ or $sIgG3^+$ LPS-blasts harvested on day 8 after stimulation. Recombinant phages carrying VDJ segments were isolated with the ^{32}P-labelled 600-bp *Xba*I-*Eco*RI fragment 3' to J_H4 or the 2100-bp *Bam*HI-*Eco*RI fragment comprising J_H3 and J_H4 (Figure 1). Both fragments were purified from pBR328 clones and nick-translated. Positively hybridizing phages were propagated on NM539 bacteria (Frischauf *et al.*, 1983). Phage V31 was isolated from an L47.1 bacteriophage library of *Bam*HI-digested BALB/c liver DNA using the M13 clone VMU-3.1 as a probe. M13 clones were ^{32}P-labelled by primer extension (Messing and Vieira, 1982). Isolated phage clones were mapped using single and double restriction endonuclease digestions.

Southern hybridization of genomic DNAs

Southern hybridization utilizing ^{32}P-labelled M13 clones of VDJ regions was performed following standard procedures (Maniatis *et al.*, 1982) in 1 × NET (150 mM NaCl, 30 mM Tris, pH 8.0, 1 mM EDTA), 1 × Denhard solutions, 0.2% SDS, 200 μg/ml denatured herring sperm DNA, 10% dextran sulphate at 65°C. Washes were performed with 1 × NET and 0.1 × NET at 65°C.

DNA sequencing

Restriction fragments containing VDJ segments were cloned into M13 vectors and their sequence was determined on both strands utilizing the chain termination procedure (Sanger *et al.*, 1980). Subclones of VDJ regions in M13 vectors were identified utilizing the nick-translated J_H region fragments shown in Figure 1. Sequence strategies are also depicted in Figure 1. As primers for dideoxynucleotide-interrupted chain extension we used either commercially available sequence primers or a J_H2-4 region primer, a V_H region primer which hybridizes to most of the V_H genes of group I (Dildrop, 1984) at codons 45–49 (Sablitzky and Rajewsky, 1984), a V_H primer hybridizing to the opposite strand at the same positions and a primer homologous to the splice acceptor site at the 5' end of V_H exons (5'-TTCTCTCCACAGG-3'). Oligonucleotides were synthesized by K.Otto and kindly provided by B.Müller-Hill.

Computing

Nucleotide sequence data were processed utilizing the programs of the University of Wisconsin Genetics Computer Group on a VAX11/750 computer. Consensus sequences were plotted for 49 germ line V_H genes belonging to V_H family 1 (Dildrop, 1984) which is identical to the J558 family (Brodeur *et al.*, 1984). The letter code for nucleotide ambiguity is taken from the IUB compendium

'Biochemical Nomenclature and Related Documents', 1978, The Biochemical Society, London WC1R 5DP. The certainty level of the consensus at each position is 99%, i.e., unique exchanges are taken into account.

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