Analysis of immunoglobulin heavy chain V-region genes belonging to the V_{NP} -gene family

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ABSTRACT

A method was devised to clone immunoglobulin V_H -region genes located on selected restriction fragments from genomic DNA directly into M13 vectors for subsequent nucleotide sequence analysis. Ten recombinant M13 clones representing four so far unknown V_H -region genes of the V_{NP} -gene family have been analysed. Sequence comparison shows that these genes are closely related to other V_H -genes of the V_{NP} gene family. One of the V_H -genes exhibits a so far unobserved unusual length of $100^{2/3}$ codons and appears to be functional. Analysis of the variation of the isolated V_H -genes suggests that framework and complementarity determining regions are exposed to separate types of selective pressures.

INTRODUCTION

Immunoglobulin V-region genes (V_H , V_K , V_L) are organized in multigene families whose diversity is essential for the efficient function of the immune system. It has been shown that the basic diversity of immunoglobulin V-regions is inherited in the germ-line and generated throughout evolution (1-3). This basic diversity is extended by somatic processes such as combinatorial V-(D)-J joining (4), flexibility in the joining site (4-8) and point mutations (1, 2, 5, 9-17). As recently shown somatic diversity also may be generated by recombination between related V-region genes (18, 19).

We here present the nucleotide sequences of four new germ-line V_H -region genes. A protocol was devised to clone V_H -region genes belonging to the V_{NP} -gene family (1, 20) directly from the genome of hybridoma B1-8.V1 (21) into M13 phage vectors for subsequent nucleotide sequence analysis. As many of these V_H -genes are located on PstI-BglII restriction fragments of ~ 2 kb length (22) we preferentially cloned such fragments. Ten recom-

binant M13 phages representing four closely related V_H-region genes were isolated and sequenced. As expected these genes belong to the V_H-gene family which operationally is defined as the V_{NP}-gene family by hybridisation to a V_{NP}-gene probe (1, 20). This gene family includes V_H-genes hybridising to others than V_{NP}-gene probes (23, 24). Comparison of the new V_H-gene sequences confirms the notion that complementarity determining regions are free to vary in evolution whereas strong selection appears to maintain the structure of the framework regions. One of the isolated V_H-region genes exhibits 100 2/3 instead of the common 98 codons. This finding suggests that a part of the 3rd complementary determining region (CDR3) of antibody heavy chain V-regions in some cases may be encoded by a V_H-segment.

MATERIALS AND METHODS

Vectors and bacterial hosts

CMK 603 (thr, leu, thi, supE, recBC, T₁^r, T₅^r, r^{-m+}, lac ZΔM15, lacY⁻ (F'⁺lacI^q lacZΔ M15 pro⁺)) was a gift of M. Koenen, Cologne. TB-1 (F'(traD36, proAB, lacI^q, lacZΔM15) (Δlac, pro), SupE, thi, recA, Sr1::Tn10^{TcR}) was given to us by A. Lamond, Cambridge, UK. M13mp701 is a gift of D Bentley, Oxford, UK.

Construction and screening of M13-libraries

High molecular weight DNA extracted from tissue-culture cells of the mouse hybridoma B1-8.V1 was digested to completion with the restriction endonucleases PstI and BglII. DNA fragments of an average size of 2 kb were enriched in a 10-40% sucrose gradient. The replicative form of M13mp701 was digested with PstI and BamHI and the 8 bp fragment originating from the polylinker segment of M13mp701 was removed from the vector DNA by sucrose gradient centrifugation. Transfection of CMK603 bacteria with B1-8.V1 derived PstI-BglII fragments ligated into M13mp701 and screening of the resulting phage libraries were performed as follows:

Protocol 1: 4.5 μg 2 kb PstI-BglII fragments were ligated to 5.4 μg vector DNA (molar ratio: 3/1). E.coli CMK603 hosts were rendered competent and transfected according to the protocol of Hanahan (25). A total of 50 ng DNA was added to 2.5x10⁸ competent cells in an Eppendorf tube. Transfected bacteria were

mixed with 0.2 ml of an exponentially growing CMK603 culture and plated with top-agarose on agar plates.

Replicas of the resulting M13-libraries on nitrocellulose filters were directly screened for phages carrying V_H -genes by in situ hybridization (26) to the nick-translated V-probe (27). Positively hybridizing plaques were replated and re-screened for purification and subsequent preparation of M13 single stranded DNA.

Protocol 2: 0.5 μ g PstI-BglIII fragments were ligated to 0.8 μ g PstI-BamHI cut M13mp701 (molar ratio: 3/1). The ligation mix was added to 10 ml of a suspension of competent CMK603 bacteria (10^9 /ml) and the mixture was distributed into the 96 wells of a chilled round-bottomed microtiter plate (NUNC, Roskilde, Denmark). After 15 min. incubation on ice the bottom of the plate was immersed for 100 seconds into a dry ice/isopropanol bath (-75°C). The cell suspensions then were allowed to thaw at room temperature for 5 min. Subsequently the cultures were heat pulsed by immersion into a 42°C water-bath for 2 min. Fresh medium then was added to each microtiter well and the plates were incubated at 37°C without agitation. After 1 hr bacteria were gently pelleted by centrifugation at 2000 rpm and culture supernatants were shaken off. The bacterial pellets then were re-suspended in 200 μ l per well of an exponentially growing E.coli TB-1 culture by gently vortexing the microtiter plate and M13 phage-growth was promoted by vigorously agitating the plate at 37°C for 8-12 hrs. After phage growth bacteria were pelleted and culture supernatants containing single stranded M13 phages were transferred to a fresh microtiter-plate by means of a multipipette. A replica of 96 phage cultures was spotted on a nitrocellulose filter utilizing the 96-teeth comb of the NUNC-TSP screening system (NUNC, Roskilde, Denmark). The filter was hybridized with the nick-translated V-probe following standard procedures (26). An aliquot of a positively hybridising phage culture containing 300 plaque forming units was plated on agar plates and phages were re-screened. A positively hybridizing plaque was amplified for preparation of single stranded phage DNA and subsequent nucleotide sequence

Figure 1:
Isolation scheme of recombinant M13 clones utilizing transformation in microtiter-wells.

analysis. Figure 1 gives a schematic picture of the cloning procedure described here.

Isolation of recombinant λ bacteriophages containing V_H -region genes

The λ charon 30 recombinant bacteriophage library containing EcoRI digested B1-8.V1 DNA has been described (19).

This library was screened with the nick-translated V-probe according to standard methods (26). DNA of positively hybridizing phages was isolated and restriction fragments containing V_H-region genes were subcloned into M13mp701 vectors as previously described (28).

Filter hybridisations

Filter hybridisations with nick-translated probes (27) were carried out as described (28).

DNA nucleotide sequence analysis

The dideoxy chain termination procedure (29, 30) was employed throughout all experiments.

DNA-probes

The V-probe used in this study is a 254 bp PstI-HinfI fragment isolated from plasmid pABu11 (1) which contains the cDNA encoding the heavy chain of anti-NP antibody B1-8.μ (31). The fragment was kindly provided by M. Siekevitz and F. Salblitzky, Cologne. Plasmid pABu11 is a gift of A.L.M. Bothwell, New Haven, CO.

Results

M13-libraries

A method was devised to clone genomic restriction fragments carrying antibody V_H-region genes directly into M13 vectors for rapid nucleotide sequence analysis. Antibody V_H-region genes belonging to the V_{NP}-gene family generally exhibit a PstI site at codon 4 (1, 20, 32) and are flanked by a BglII site 1.5-2.5 downstream of this PstI site (22). Cloning of V_{NP}-heavy chain genes on PstI-BglII fragments into PstI-BamHI digested M13mp701 vectors thus should allow us to directly sequence genes starting from codon 4 and extend sequence reading into the V-exon.

Transformation of competent bacterial hosts in order to generate a library of genomic B1-8.V1 derived restriction fragments in M13 phages initially was performed in Eppendorf tubes. We obtained 4.5×10^4 plaques/4.5 μg of enriched PstI-BglII fragments into M13mp701. The frequency of non-recombinant phages was 5%. Nine recombinant phages (MVAR1-4, 7-11) representing four different V_H-region genes were isolated from this library

utilizing plaque hybridisation to the V_{NP} -probe.

Conditions for generating and screening of M13-libraries were improved by transforming bacterial hosts in microtiter wells and employing the NUNC-TSM screening system to identify phages carrying inserts which exhibit V_{NP} -related sequences. Transformation efficiency of CMK603 bacteria with supercoiled replicative forms of M13mp701 DNA in microtiter wells was 5×10^6 plaques/ μ g phage DNA. In a pilot experiment we determined the sensitivity with which a positive phage can be detected. 100 pg of recombinant M13 phages carrying a V_{NP} -gene were mixed with 1 μ g of M13mp701 DNA (molar ratio: 1:1.1 $\times 10^4$) and 20 ml of competent bacteria were transfected with this mixture in 2x96 microtiter wells (2 plates). An average of 30 000 plaques was counted per well when aliquots of the contents of 10 wells were plated directly after transformation but before phage amplification. 179 of 192 wells reacted positively in a spot-hybridisation test with a nick-translated V_{NP} -probe. According to Poisson-distribution one expects 10 of 192 wells to be negative (5%) and 30 wells (15%) to contain the progeny of 1 positive phage. This indicates that the screening procedure is sensitive enough to detect 1 positive phage among 30 000 unrelated phages. A library generated in a microtiter plate from 0.5 μ g PstI-BglII fragments ligated into M13mp701 contained one phage hybridizing to the V_{NP} -probe (MVARG2). The progeny of this phage was detected in a microtiter well among the progeny of 200 unrelated phages.

Some recombinant M13-clones showed the tendency to undergo internal rearrangements during phage propagation. MVAR4, 7, 8 twice deleted large parts of their inserts when the original phage stock was amplified for preparation of single stranded phage DNA. Employment of the recA host TB-1 for phage growth significantly improved the genetic stability of these phages.

Structures of V_{NP} -related genes derived from hybridoma B1-8.V1

The nucleotide sequence of the V_{NP} -related V_H -region genes cloned in ten recombinant M13 phages was determined according to the strategy depicted in Fig. 2.

Phages MVAR1 and MVAR9 carry identical inserts thus indicating that the same V_H -gene has been cloned into these phages. An-

Figure 2:

Restriction maps of V_{Np} -related V_H -region genes from the genome of B1-8.V1 cloned in M13 and Lambda-bacteriophages. B = BglII, E = EcoRI, P = PstI. protein coding regions are shown by raised boxes, L = leader region, V = V-region. Arrows indicate our sequencing strategy.

other V_H -region gene has been cloned 5 times. This gene is represented by phages MVARG2, MVAR3 4, 7 and 8. In clone MVAR2 the sequence of a V_H -region gene stops at the PstI site in codon 72. We think that a small PstI fragment carrying a segment of a V_H -gene coincidentally was cloned together with an unrelated PstI-BglII fragment. Phage clone MVAR10 carries a gene segment comprising codons 72-98 of a V_H -gene. The gene segments represented by phages MVAR2 and MVAR10, respectively, are part of the V_H -region gene cloned in λ -phage VAR108. The V_H -region gene cloned in MVAR11 also is represented by a λ -phage clone, namely VAR36 (see Fig 2). All nucleotide sequences of the V_H -region genes cloned in MVAR1, MVAR2/MVAR10/VAR108, MVARG2 and MVAR11 are summarized in Fig 3a.

The nucleotide sequences of the V_H -region genes isolated from the genome of B1-8.V1 extend through the V_H -gene segment into the 3' flanking region. The heptameric and nonameric recognition sites for V_H -D joining match the published consensus sequences (4-8). All V_H -genes cloned in VAR-clones appear to represent functional genes because no termination signal, deletion or insertion affects the reading frame and normal immunoglobulin domain folding appears not to be impaired. Hybridisation to the V_{Np} -probe and homology (82.8%-92.3%) to reference

a)	V186.2	CAG GTC CAA CTG CAG CAG CCT GGG GCT GAG CTT	10
	MVAR1	--- --- --A --- --- T-- --- --G	
	MVAR2 *	--- --- --G --- --- T-- --A --- --G	
	MVARG2	--- --- --A --- --- C-- --- --G	
	MVAR11	--- --- --T-- --A --- --G-- --G	
	V186.2	GTG AAG CCT GGG GCT TCA GTG AAG CTG TCC TGC	20
	MVAR1	--- -G- --- --A --- --- --- --- ---	
	MVAR2	--A -G- --- A-- --- --- G-- --- ---	
	MVARG2	--- --- --- --- --- T-- --- ---	
	MVAR11	--- --A --C --- --A --- --- --- ---	
	V186.2	AAG GCT TCT GGC TAC ACC TTC ACC AGC TAC TGG	30 CDR 1
	MVAR1	--- --- --- --- --A --- --- --- ---	
	MVAR2	--- --- --- --A --- G-- --- --T --AT --- -T-	
	MVARG2	--- --- --- --- --A --- --- --A --- GAT	
	MVAR11	--- --- --- --- --- --- --T GAG --T ATT	
	V186.2	ATG CAC TGG GTG AAG CAG AGG CCT GGA CGA GGC	40
	MVAR1	--- --- --- --- --- --A --- --A ---	
	MVAR2	--A G-G --- --A --- --- --- --AG ---	
	MVARG2	--A A-- --- --- --- --- --AG --A	
	MVAR11	--A --- --- --A --- --- T-- --- --AG --T	
	V186.2	CTT GAG TGG ATT GGA AGG ATT GAT CCT AAT AGT	50
	MVAR1	--- --- --- --- --AT --- T-- GG- ---	
	MVAR2	--- --- --- --- GT- --- A-- --- GGA ---	
	MVARG2	--- --- --- --- T-- --- T-- --GA GA-	
	MVAR11	--- --- --- --- --G T-- T-- TCA --- GGA ---	
	V186.2	GGT GGT ACT AAG TAC AAT GAG AAG TTC AAG AGC	60 CDR 2
	MVAR1	--- A-- --- --C --- G-- --- --- ---	
	MVAR2	--- --- --- --C --- --- --- --- G--	
	MVARG2	--- A-- --- --- --- --- --- --- G--	
	MVAR11	--- A-- -TA --- --- --- --A --- --- GA-	
	V186.2	AAG GCC ACA CTG ACT GTA GAC AAA CCC TCC AGC	70
	MVAR1	--- -G- --- --- --- --C- T-- ---	
	MVAR2/10	--- --A --- --- --C- --- --- --A-	
	MVAR2	--- --- --- T-- --- --C- T-- ---	
	MVAR11	--- --- --- T-- --- --CG --- T-- ---	
	V186.2	ACA GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT	80
	MVAR1	--- --- --- --- --C --- --- ---	
	MVAR10	--- --- --- --- --- --- --- ---	
	MVARG2	--- --G --- --- G-- --- CA- ---	
	MVAR11	--- -T- --T --- G-- --T --T --A T-- ---	
	V186.2	GAG GAC TCT GCG GTC TAT TAT TGT GCA AGA	90 98
	MVAR1	--- --- --- --- --- --C --- A-- ---	
	MVAR10	--- --- --- --- --- -TC --- ---	
	MVARG2	--- --- --- --- --- -TC --- ---	
	MVAR11	--A --- --- --- --- -TC --- ---CACGAAGA	
	V186.2	CACAGTG TTGCAACCACATCCTGAGAGTGT CAGAAAACC	
	MVAR1	-----T-----T-----T-----	
	MVAR10	-----T-----T-----T-----	
	MVARG2	-----T-----T-----T-----	
	MVAR11	-----T-----A-C-----T-----C-----	
b)	VAR108	GGTAAGGGGCTTCCCATTTCCA.....A.ATCTGAAGATTATATAGGGC	
	V186.2	-----A--G-AG--GGCTTG--GG-----C-----	
	VAR108	CTGAGGTGACAATGGCAACCATTCGCCTTTCTCTCCACAGGT	
	V186.2	A-------A--T--C-T-----	
	VAR108	GGTAAGGGGCTTCCCATTTCCAATCTGAAGATTATATAGGGCCTGAGGT	
	V111	-----CA-----GG-----A	
	VAR108	GACAATGGCAACCATTCGCCTTTCTCTCCACAGGT	
	V111	-----C-----C-----C-----T-----	

gene V186.2 assign the V_H -genes presented here to the V_{NP} -gene family (1, 2). In addition, a proline codon (CCT) is present at amino acid position 7 in gene MVAR1, a feature confined to genes belonging to the V_{NP} -gene-family (1, 20) and not present in any other known V_H or V_L regions (23, 33).

DISCUSSION

Genomic M13 libraries

We devised a rapid procedure to clone eucaryotic genes directly into M13 bacteriophages for subsequent nucleotide sequence analysis. The method was applied to immunoglobulin V_H -region genes flanked by a PstI site at the 5' end and a BglII site at the 3' end. Ten recombinant M13-phage clones containing V_H -region genes were identified by hybridisation to the V_{NP} -probe. Nucleotide sequences were obtained starting at the PstI site at codon 4 which is strongly conserved in V_H -genes belonging to the V_{NP} -family (1, 20).

The cloning procedure described here originally was devised to analyse a particular V_H -gene from the genome of hybridoma B1-8.V1 (21) namely gene V102.1 (1). This gene very likely was involved in a recombination with V_H -gene V186.2 (1) thus generating the recombinant VDJ-segment (18, 19) which encodes the heavy chain V-region of hybridoma B1-8.V1. In spite of exhaustive screening we failed to obtain gene V102.1 from the M13-phage library generated from PstI-BglII digested genomic DNA of hybridoma B1-8.V1. We finally isolated the gene from a λ -phage library representing the complete B1-8.V1 genome. M13 subclones containing the 1.6 Kb PstI-BglII fragment which carries gene V102.1 with high frequency gave rise to deletions during phage

Figure 3:

a) Comparison of nucleotide sequences of VAR clones with V186.2, which represents the reference gene of the V_{NP} gene family (1). Protein coding regions are numbered according to amino acid positions. Sequences involved in V_H -D joining are underlined.

b) Comparison of nucleotide sequences of the intron between leader region and V_H -region: VAR108 is compared to V186.2 (1) and $V_{MPC11-111}$ (32). Gaps (.....) are introduced to maximize sequence fit. Signals for RNA-splicing are overlined.

propagation (unpublished data). The 3' flanking region of gene V102.1 appears to exhibit sequences which interfere with the replication of M13. This may explain why gene V102.1 could not be isolated from a M13 library of B1-8.V1 derived genomic PstI-BglIII fragments.

Although we restricted our analysis to V_H -region genes located on size selected fragments flanked by particular restriction enzyme cleavage sites the method of isolating a desired gene from genomic M13 phage libraries can be applied universally and thus should be considered when cloning strategies are devised.

V_{NP} -related V_H -region genes derived from B1-8.V1

Four so far unknown, V_{NP} -related V_H -region genes represented by phages MVAR1/9, MVAR3/4/7/8/G2, MVAR2/10/ λ VAR108 and MVAR11/ λ VAR36 were isolated from the genome of hybridoma B1-8.V1 (21). This hybridoma originates from a fusion between a BALB/c derived X63.Ag8 cell and a C57BL/6 lymphocyte (31). It is therefore difficult to assign the B1-8.V1 derived V_H -genes to a particular haplotype of the Igh-locus as there are no characteristic sequence differences between the V_H -region genes belonging to either the V_{NP}^b or the V_{NP}^a gene family (20). Members of both families are present in the genome of B1-8.V1 albeit most of the V_{NP}^b -genes appear to have been deleted when V186.2 rearranged to the D_{FL}16 segment, as indicated by Southern-hybridization experiments (F. Sablitzky, T. B., unpublished data). The V_H -gene represented by MVAR2 may be assigned to the Igh^b equivalent of the B1-8.V1 genome as it is identical to germ-line V_H -gene V104.1 which previously has been isolated from the C57BL/6 genome (A.L.M. Bothwell, pers. communication).

MVAR11 appears to be the Igh^a-allele of the germ-line V_H -gene that is expressed in hybridomas A25.9.7 and A31.90 which secrete C57BL/6 derived anti-idiotope antibodies (F. Sablitzky, K. Rajewsky, to be published). It should be noted that MVAR11 exhibits 100 2/3 instead of 98 codons. The codons following the AGA at position 98 never have been observed so far in any other V_H -gene (23, 33). It cannot be resolved here whether the third base of codon 101 (GAC) in the V_H -region of A25.9.7 stems from the V_H -D joinig recognition sequence of MVAR11. The uncommon

length of MVAR11 effects the interpretation of the N-sequences (34) which often are observed between codon 98 of the V_H -segment and the beginning of the D-segment in V_H -D- J_H -regions. N-sequences have been attributed to the action of terminal transferases in the somatic process of V_H -D-joining (34). In some V_H -D- J_H -regions, however, N-sequences or at least part thereof may be germ-line encoded as additional codons of the V_H -segment instead of being inserted somatically.

MVAR2 and MVAR10 as represented by VAR108 (see Fig. 2) appear to belong to the V_{NP}^a -gene family because the intron between leader region and V_H -region of VAR108 is very homologous (89.5%) to the corresponding region of the Igh^a -derived gene V_{MPC11} -111 and its related genes (32) (Fig. 3b). No gaps need to be introduced into the intron sequence to obtain optimal sequence fit. Sequence homology is far less pronounced (72.0%) when the intron of VAR108 is compared to the intron of V186.2 which represents the reference gene of the V_{NP}^b -gene family (1). MVAR1 cannot be assigned either to the Igh^b or the Igh^a haplotype.

The MVAR and VAR-genes very likely do not encode NP-binding V_H -regions because amino acid residues His-35, Arg-50 and Asp-52 which are predicted to be involved in NP-binding (35) are not expressed in the CDR-regions.

A comparison of sites at which the MVAR and VAR genes differ from each other shows a striking localization of the differences in the CDRs (Table 1) as had been seen previously by comparison of other genes from the V_{NP} - or its equivalent, the V_{MPC11} -gene family (1, 3). CDRs are more than twice as different from one another as are the FRs. By separating the changes into ones that affect the encoded amino acid (R, replacement differences) and ones that do not (S, synonymous differences) (36) a variation of R/S values is evident in spite of statistical fluctuations: FRs show a relative deficit of replacement differences (R/S: 1-2) whereas CDRs show an excess (R/S: 3.5-7). A random difference between two related sequences would produce an R/S of about 3 (Table 1). Similar differences of R/S values have been reported by others (3, 20, 37). This suggests that the different parts of the V_H -region have been

Table 1:
Variation of framework-regions (FRs) and complementarity determining regions (CDRs) of MVAR genes.

Regions compared	Total exchanges %		R-exchanges %		S-exchanges %		R/S	Genes compared
FRs	22/216	10.2	13/216	6.0	7/216	3.2	1.86	MVAR1 vs MVAR2/10
CDRs	15/69	21.7	12/69	17.4	3/69	4.4	4	
FRs	21/216	9.7	13/216	6.0	8/216	3.7	1.63	MVAR1 vs MVAR2
CDRs	16/69	23.2	14/69	20.3	2/69	2.9	7	
FRs	32/216	14.8	17/216	7.9	15/216	6.9	1.13	MVAR1 vs MVAR11
CDRs	23/69	33.3	18/69	26.1	6/69	7.3	3.6	
FRs	18/216	8.3	12/216	5.6	6/216	2.8	2	MVAR2/10 vs MVAR2
CDRs	16/69	23.2	14/69	20.3	2/69	2.9	7	
FRs	24/216	11.1	12/216	5.6	12/216	5.6	1	MVAR2/10 vs MVAR11
CDRs	19/69	27.5	16/69	23.2	3/69	4.4	5.33	
FRs	23/216	10.7	12/216	5.6	11/216	5.1	1.09	MVAR2 vs MVAR11
CDRs	18/69	26.1	14/69	20.3	4/69	5.8	3.5	

(*) Codons 1-3 are excluded. The frequency of replacement exchanges (R) and synonymous exchanges (S) is compared.

under different selective pressure in evolution. The relative deficit of R differences in FRs suggests selection for maintenance of V_H -region structure. A similar constraint on replacement variations indicating selection for maintenance of structure and function has been observed when coding regions of rat preproinsulin genes or rabbit and mouse β -globin genes were compared (36). Most interestingly, comparison of V_H -genes to a non-functional V_H -gene which has accumulated 14 crippling mutations yielded R/S values in FR regions close to the random value 3 thus indicating that R/S values <2 indeed reflect selective pressure (38). It seems to be a rather unique phenomenon that CDRs are free to vary as no selection against R differences can be observed. Unbiased diversification of the antigen-binding site (determined by the CDRs) appears to be highly favourable when the function of the immune system is taken into account.

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