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Camel heavy-chain antibodies: diverse germline V_HH and specific mechanisms enlarge the antigen-binding repertoire

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The antigen-binding site of the camel heavy-chain antibodies devoid of light chain consists of a single variable domain (V_HH) that obviously lacks the V_H–V_L combinatorial diversity. To evaluate the extent of the V_HH antigen-binding repertoire, a germline database was constructed from PCR-amplified V_HH/V_H segments of a single specimen of *Camelus dromedarius*. A total of 33 V_HH and 39 *VH* unique sequences were identified, encoded by 42 and 50 different genes, respectively. Sequence comparison indicates that the V_HHs evolved within the V_H subgroup III. Nevertheless, the V_HH germline segments are highly diverse, leading to a broad structural repertoire of the antigen-binding loops. Seven V_HH subfamilies were recognized, of which five were confirmed to be expressed *in vivo*. Comparison of germline and cDNA sequences demonstrates that the rearranged V_HHs are extensively diversified by somatic mutation processes, leading to an additional hypervariable region and a high incidence of nucleotide insertions or deletions. These diversification processes are driven by hypermutation and recombination hotspots embedded in the V_HH germline genes at the regions affecting the structure of the antigen-binding loops.

Keywords: antigen-binding repertoire/camel heavy-chain antibody/gene replacement/germline V_H/hypermutation hotspots

Introduction

The emergence of the *Camelidae* (camels and llamas) within the Artiodactyls is accompanied by a unique event in immunoglobulin (Ig) evolution, namely the appearance of additional classes of functional antibodies (Abs) composed solely of heavy chains (Hamers-Casterman *et al.*, 1993). These heavy-chain antibodies (HCAbs) lack the first domain of the constant region (C_H1), which is present in the genome but is spliced out during mRNA processing (Nguyen *et al.*, 1999; Woolven *et al.*, 1999). The antigen (Ag)-binding site of these HCAbs is composed of a single variable domain (referred to as V_HH). The V_HH structure resembles that of the heavy chain variable domain (V_H) of the conventional Abs. However, there are remarkable sequence differences at the second framework (FR2) and the third complementarity-determining region (CDR3) (Muyldermans *et al.*, 1994; Vu *et al.*, 1997). Most striking are the amino acid substitutions V37F (Val at position 37 in the V_H to Phe in the V_HH), or V37Y, G44E, L45R or L45C, and W47 most often to G [numbers refer to the amino acid positions numbered according to Kabat *et al.* (1991)]. In the conventional V_Hs, these FR2 amino acids interact with the variable domain of the light chain (V_L), and are conserved during evolution (Kabat *et al.*, 1991). The CDR3 of the V_HH is longer on average than that of a V_H domain (Vu *et al.*, 1997), and is often constrained by an interloop disulfide bond (Davies and Riechmann, 1996; Desmyter *et al.*, 1996).

A high titre and a complex repertoire of HCAbs can be obtained from immunized or infected dromedaries or llamas (Hamers-Casterman *et al.*, 1993; Ghahroudi *et al.*, 1997). Many HCAbs raised against enzymes are competitive inhibitors (Lauwereys *et al.*, 1998). This is surprising, since the active site of enzymes has a low antigenicity for conventional Abs (Novotny, 1991). Thus, the HCAbs recognize a broad range of epitopes, some of which differ from those for conventional Abs.

Previously, we identified germline V_H and V_HH segments indicating that the variable domain of the HCAbs is encoded by a distinct set of *V* genes (Nguyen *et al.*, 1998). In this study, we investigate the potential V_HH germline repertoire to gain insight into the ways by which the dromedary HCAbs acquire a complex repertoire of Ag-binding sites. In conventional Abs, the diversity of the Ag-binding site is generated at multiple levels. The V_H is generated by assembling variable (*V*), diversity (*D*) and joining (*J*) elements (Tonegawa, 1983), in which the *V*-gene segment encodes the CDR1 and CDR2; the CDR3 is generated by the *V–D–J* joining. In this joining process, great sequence variation is introduced by non-template addition of nucleotides at the V–D and D–J junctions (junctional diversity). Random association of a V_H and a V_L (combinatorial diversity) generates an immensely diverse Ag-binding repertoire. Additional diversification of the Ag-binding repertoire could be achieved by somatic hypermutation (Berek *et al.*, 1991) and gene conversion (Reynaud *et al.*, 1987; Becker and Knight, 1990). Thus, the primary Ag-binding repertoire of the HCAbs lacking the V_H–V_L combinatorial diversity relies on the innate number and sequence diversity of the V_HH germline segments and the junctional diversity.

The identification of the germline V_HH genes is not only of fundamental interest but also has a potential biotechnological benefit. At the moment, HCAbs with enzyme inhibiting activity can only be obtained after immunizing camels or llamas. Techniques have been developed to retrieve various binders from synthetic libraries of Ab fragments (Hoogenboom and Winter, 1992; Winter *et al.*, 1994). Single-domain Ab libraries have been constructed by adding a synthetic CDR3 region to the

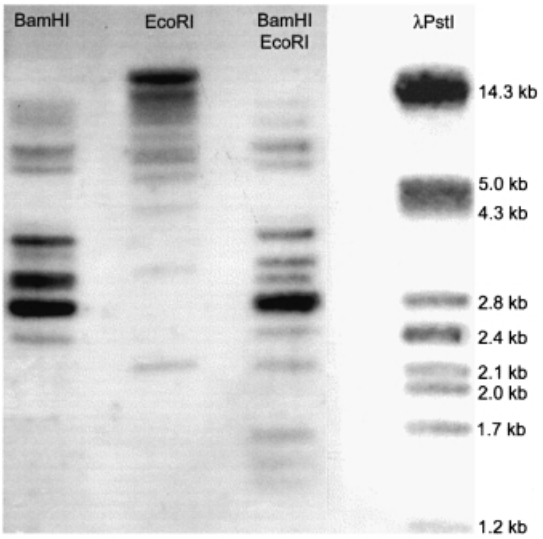


Fig. 1. Southern blot analysis of dromedary liver genomic DNA hybridized with a camel V_HH probe. Dromedary DNA was digested with *Bam*HI, *Eco*RI and *Bam*HI–*Eco*RI, as indicated on top of the lanes. Phage λ *PstI* restriction fragments are used as size marker.

known human V_H elements (Davies and Riechmann, 1995; Reiter *et al.*, 1999). It would be an asset if similar libraries of V_HHs were available to retrieve binders or inhibitors of interest. To be as successful as the dromedary in making good Ag binders we should start from a library that covers all potential V_HH segments. In addition, analysis of the amino acids that are mutated during the *in vivo* affinity maturation would provide a rational strategy for increasing the repertoire of the V_HH library or to improve the affinity of binders.

We cloned from a single dromedary the germline V_HH gene segments to analyse their complexity. The comparisons of the germline and cDNA V_H/V_HH sequences reveal the somatic diversification mechanisms used by the camelids to enlarge the primary Ag-binding repertoire of the HCAbs. The involvement of DNA signal sequences in these diversification processes is discussed.

Results

Southern blot analysis of the genomic DNA

A rough estimate of the V_H/V_HH germline repertoire was first obtained by Southern blot analysis of dromedary liver DNA, probed by the PCR fragments from the upstream conserved octamer sequence to the FR3 of camel germline V_H or V_HH clones (Nguyen *et al.*, 1998). The hybridization patterns shown by these two different probes were identical. In single digestions with *Bam*HI or *Eco*RI, 11 bands of different intensity and ranging from 1.7 to >14 kb could be visualized (Figure 1). The double digest revealed at least 15 bands. Since we never observed an *Eco*RI or *Bam*HI restriction site between the octamer and FR3 sequences of V_H/V_HH (this work), we conclude that the dromedary V_H/V_HH germline gene segments are spread over a minimum of 15 different *Eco*RI–*Bam*HI size families.

Determination of dromedary germline V_H/V_HH sequences

A germline *V*-gene database was obtained by cloning and sequencing PCR fragments of V_H/V_HH elements obtained from liver DNA of a single dromedary. Out of 255 sequences we found 145 different sequences, which formed an initial germline V_H/V_HH database. Assuming a PCR error rate of 2×10^{-4} errors/base (Cha and Thilly, 1993), we estimated that up to four base errors might have accumulated within each 600 bp sequence. Hence, we only considered clones differing by at least 5 nucleotides (nt), which reduced the V_H/V_HH germline database from 145 to 94 sequences. The nucleotide sequences of these 94 clones have been submitted to the DDBJ/EMBL/GenBank databank (accession Nos AJ245107–AJ245200).

In an alternative approach, we screened the dromedary genomic library cloned in phage λ (Nguyen *et al.*, 1998). A total of 55 different phage clones were isolated and their V_H or V_HH regions were subcloned and sequenced. However, no new V_H/V_HH sequences were discovered, supporting that our database of 94 V_H/V_HH sequences obtained by PCR is representative to evaluate the potential V_HH repertoire.

Characterization of V_H/V_HH sequences and V_HH subfamilies

All 94 genomic V_H/V_HH sequences span from the conserved octamer to the Cys92 of FR3. They contain a TATA box 105 nt upstream of the ATG initiation codon. This ATG starts an open reading frame (ORF) of conserved sequences encoding a leader signal peptide, which is interrupted by an intron of 104 bp. The remaining sequence encodes the V_H or V_HH segment. Two sequences (cvhp51 and cvhp52) are interrupted by stop codons. These clones could be pseudo-genes and were excluded from further analyses. The remaining 92 clones probably encode functional genes as no aberrant amino acid occurs in the ORF at the critical positions for the Ig fold (Chothia *et al.*, 1988).

Identification of V_H/V_HH sequences. The nucleotide identity between any pair of the 92 V_H/V_HH encoding sequences is >80%. The amino acid sequences of FR1 and the beginning of FR3 are homologous to each other (Figure 2) and to the human or mouse V_H of family 3. Therefore, according to the family definitions (Brodeur and Riblet, 1984; Schroeder *et al.*, 1990), all these camel sequences could be assigned to the V_H family 3. Based on crucial amino acid differences in the FR2 region, we have previously distinguished the camel V_H3 family (used in the conventional Abs) and the V_H3H family (for the HCAbs) (Nguyen *et al.*, 1998). The current database can also be divided into these two families according to the presence of V37, G44, L45 and W47 (V_H3), and F/Y37, Q/E44 and R/C45 (V_H3H) (Figure 2). Out of 92 V_HHs, 50 sequences were clearly assigned to V_H3 (cvhp01–cvhp50) and 42 to the V_H3H family (cvhhp01–cvhhp42). This V_H/V_HH ratio (1.2/1) was also found among sequences obtained from the phage λ-isolated clones. We therefore conclude that a similar number of V_H3 and V_H3H genes reside in the dromedary genome.

Comparison of amino acid sequences revealed that a number of clones have an identical coding capacity (boxed in Figure 2) although their nucleotide sequence differed by >4 nt. As a result, the 50 V_H and 42 V_HH gene segments code respectively for 39 V_H and 33 V_HH unique amino acid sequences.

V_HH subfamilies. Multiple alignment of the V_HH amino acid sequences revealed the clustering of sequences with an additional cysteine at position 30, 32, 33 or 45, and with a CDR2 length of 16 or 17 amino acids (Figure 2). Based on these hallmarks, we further classified the 42 clones of the V_HH into seven subfamilies named 1a, 2a, 2b, 3b, 4b, 5a and 5b (Table I; Figure 2).

A neighbour-joining tree was constructed using the V_H/V_HH coding sequences to analyse the V_H and V_HH relationship. The dendrogram (Figure 3) shows clearly two clusters, one cluster contains only the V_H3 and the other the V_H3H genes, despite the fact that the pairwise sequence identity among V_Hs or among V_HHs may be lower than that of a V_H–V_HH pair. Moreover, the branches appear to correspond to the V_HH subfamilies defined above.

Determination of expressed V_HH gene segments

We assembled a V_HH cDNA database containing 103 sequences, 32 of which have a known Ag specificity. Thirty-one of the 103 cDNAs have a length deviating from any of the germline V_HH segments. While these clones could originate from a germline V_HH that has not yet been discovered, they could also originate from unequal DNA recombination or gene conversion (Reynaud *et al.*, 1987). The remaining 72 V_HH cDNA sequences (26 with known Ag specificity) were compared with all 42 germline V_HH sequences to reveal the closest sequence identity. The combination of the sequence identity score and the two V_HH subfamily hallmarks defined earlier leaves no doubt as to the identification of the germline subfamily. Among the 72 V_HH cDNA clones, there is no indication for the *in vivo* expression of members of subfamilies 1a and 5a (Table I). The result shows that at least five out of the seven V_HH subfamilies are used to generate productive V_HH domains found in HCAbs.

Identical D and J_H segments in V_H and V_HH cDNAs. A germline *D* mini-gene (similar to human *D4*) was identified with an RSS at both ends. We noted that some V_H and V_HH cDNAs employ this *D* element (Figure 4). In addition, our cDNA database also contains V_Hs and V_HHs that make use of the same J_H element. This would indicate that V_H and V_HH genes are within the same functional V region of the dromedary genome.

Structural repertoire of Ag-binding loops of camel germline V_H/V_HH

The structure of the Ag-binding loops of the camel germline V_Hs and V_HHs was predicted by algorithms of Chothia and Martin (http://www.biochem.ucl.ac.uk/~martin/abs/chothia.html) (Chothia *et al.*, 1989, 1992; Martin and Thornton, 1996), and by taking into account the crystallographic structures of dromedary and llama V_HHs (Desmyter *et al.*, 1996; Spinelli *et al.*, 1996; Decanniere *et al.*, 1999) (Table II; Figure 2).

The Ag-binding loops of the dromedary V_Hs are predicted to conform to the known canonical structures characterized by well-defined key amino acids. In contrast, the Ag-binding loops of the V_HHs are expected to deviate frequently from the known canonical structures, mainly due to the substitution of key amino acids. Notable substitutions are G26E and F29S in the H1 loop (the solvent-exposed part of the loop overlapping with the CDR1) and R71Q dictating the conformation of the H2 loop (the exposed part of the CDR2).

From crystallographic data on the V_HH structures, we infer that the combination Y27–Y29 (found in 22 germline V_HHs) can adopt either a type-1 or type-4 canonical structure, depending on the nature of the residue 31 (Decanniere *et al.*, 1999). Similarly, the six-residue H2 loops associated with R71 reveal either a H2 type-2 (Spinelli *et al.*, 1996) or a novel H2 structure (Decanniere *et al.*, 1999). These loops are therefore denoted as 'PS' in Table II for 'potentiality to switch'.

Thus, the dromedary V_H germline sequences have a structural repertoire of six different H1–H2 combinations (Table II), whereas the structural repertoire of the germline V_HHs is far more diverse, up to 10 different combinations of H1 and H2 loop conformations are observed. Moreover, it seems that both the H1 and H2 loop conformations of the V_HHs are apt to reshape due to minor modifications, thereby further enlarging the V_HH structural repertoire.

Variability of camel V_H/V_HH sequences

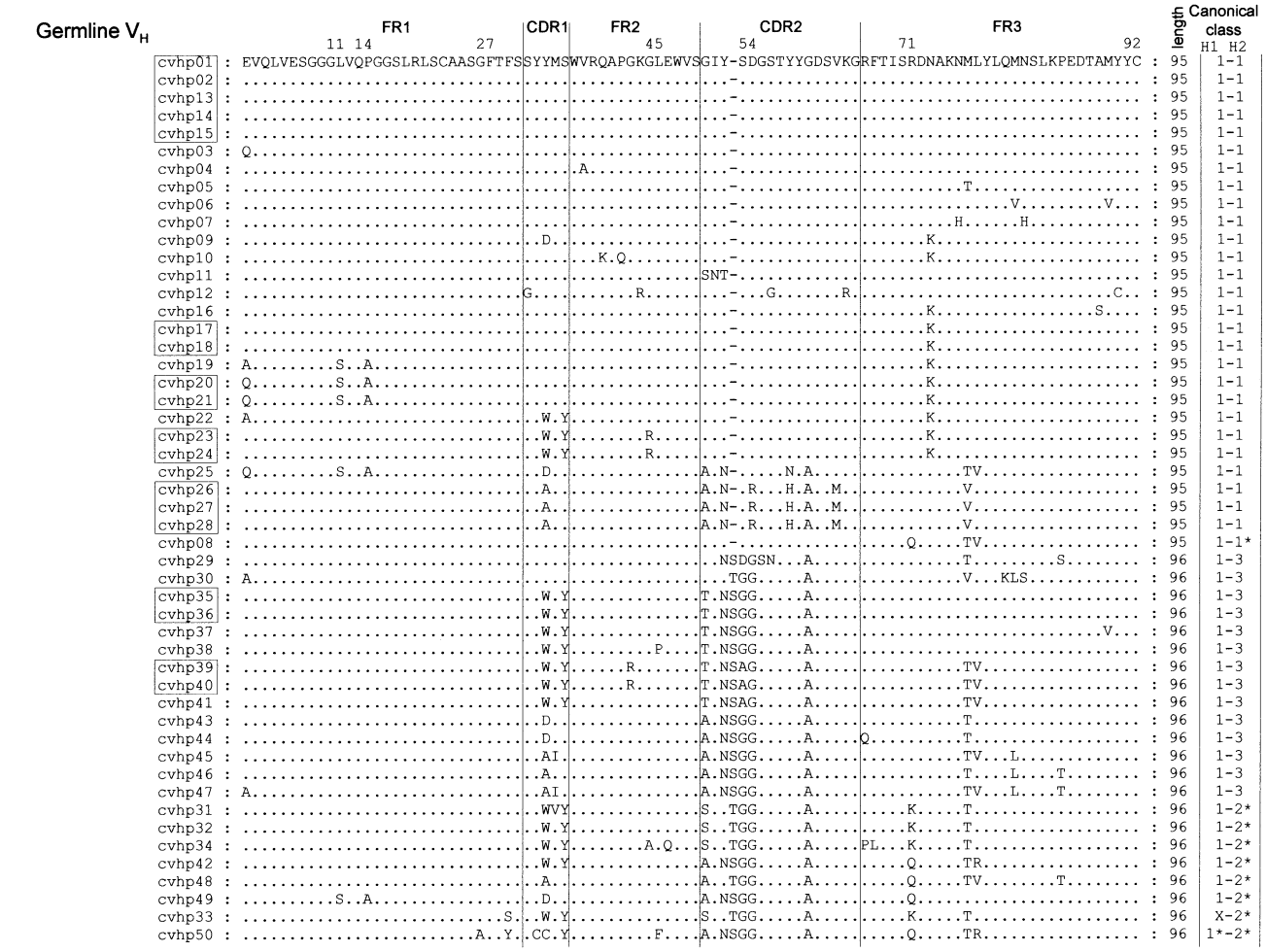
The variability plots (Kabat *et al.*, 1991) were constructed for the germline V_Hs and V_HHs (Figure 5, upper panels). Both histograms delineate the conventional CDR1 and CDR2 regions as the sites of greater variability.

Hypervariability in the H1 region of V_HHs cDNA. The variability of the V_H and V_HH cDNAs was plotted using sets of 50 V_H and 42 V_HH cDNAs, and compared with that of the germline sequences (Figure 5). The overall variability is much higher in cDNAs than in the germline, indicating that somatic mutation plays an important role in the generation of the camel V_H/V_HH repertoire. The most striking observation is the presence, exclusively in the V_HH cDNAs, of an additional hypervariable region (residues 27–30) located upstream of the conventional CDR1 region.

Hypermutational hotspots imprinted in the germline. Somatic diversification results from different mechanisms including gene conversion, and somatic hypermutation (Wagner and Neuberger, 1996; Neuberger *et al.*, 1998). The latter was proposed to be driven by hypermutational hotspots such as the AGY and TAY (Y = C or T) sequences (Yelamos *et al.*, 1995; Milstein *et al.*, 1998). The occurrence of these hotspots in the germline V_Hs and V_HHs was superimposed on the cDNA variability plots (Figure 6). Clearly, the occurrence of hotspots corresponds to the highest variability at the CDR1 and CDR2 of cDNA sequences. The hypervariable region found exclusively in the V_HH cDNA (residues 27–30) could result from the TAY hotspots that are present in the germline V_HHs, but absent in the germline V_Hs.

Putative recombination

As mentioned previously, the 31 V_HH cDNAs having lengths deviating from that of the germline could originate from unequal DNA recombination, gene conversion or gene replacement. Comparison of the nucleotide sequences of these off-size cDNA with germline V_HHs revealed four distinct regions in which nucleotides were inserted or deleted (Ins/Del). The four regions surround the residues 24 ± 1 (1 Del and 5 Ins), 30 ± 3 (9 Dels and 6 Ins), 54 ± 3 (2 Dels and 8 Ins) and 74 ± 1 (3 Dels). We noted


Germline V_H
FR1
CDR1
FR2
CDR2
FR3
length
Canonical class
H1 H2
cvhp01 : EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYYMSWVRQAPGKGLEWVSGIY-SDGSTYYGDSVKGRFTISRDNAKNMLYLQMNSLKPEDTAMYYC : 95 1-1
cvhp02 : ..... : 95 1-1
cvhp13 : ..... : 95 1-1
cvhp14 : ..... : 95 1-1
cvhp15 : ..... : 95 1-1
cvhp03 : Q..... : 95 1-1
cvhp04 : .....A..... : 95 1-1
cvhp05 : ..... : 95 1-1
cvhp06 : ..... : 95 1-1
cvhp07 : ..... : 95 1-1
cvhp09 : .....D..... : 95 1-1
cvhp10 : .....K.Q..... : 95 1-1
cvhp11 : ..... : 95 1-1
cvhp12 : .....G.....R.....-...G.....R..... : 95 1-1
cvhp16 : ..... : 95 1-1
cvhp17 : ..... : 95 1-1
cvhp18 : ..... : 95 1-1
cvhp19 : A.....S..A..... : 95 1-1
cvhp20 : Q.....S..A..... : 95 1-1
cvhp21 : Q.....S..A..... : 95 1-1
cvhp22 : A.....W.Y..... : 95 1-1
cvhp23 : .....W.Y.....R..... : 95 1-1
cvhp24 : .....W.Y.....R..... : 95 1-1
cvhp25 : Q.....S..A.....D.....A.N-.....N.A.....TV..... : 95 1-1
cvhp26 : .....A.....A.N-.R...H.A..M.....V..... : 95 1-1
cvhp27 : .....A.....A.N-.R...H.A..M.....V..... : 95 1-1
cvhp28 : .....A.....A.N-.R...H.A..M.....V..... : 95 1-1
cvhp08 : ..... : 95 1-1*
cvhp29 : .....NSDGSN...A.....T.....S..... : 96 1-3
cvhp30 : A.....TGG....A.....V...KLS..... : 96 1-3
cvhp35 : .....W.Y.....T.NSGG....A..... : 96 1-3
cvhp36 : .....W.Y.....T.NSGG....A..... : 96 1-3
cvhp37 : .....W.Y.....T.NSGG....A.....V... : 96 1-3
cvhp38 : .....W.Y.....P....T.NSGG....A..... : 96 1-3
cvhp39 : .....W.Y.....R.....T.NSAG....A.....TV..... : 96 1-3
cvhp40 : .....W.Y.....R.....T.NSAG....A.....TV..... : 96 1-3
cvhp41 : .....W.Y.....T.NSAG....A.....TV..... : 96 1-3
cvhp43 : .....D.....A.NSGG....A.....T..... : 96 1-3
cvhp44 : .....D.....A.NSGG....A.....Q.....T..... : 96 1-3
cvhp45 : .....AI.....A.NSGG....A.....TV...L..... : 96 1-3
cvhp46 : .....A.....A.NSGG....A.....T....L....T..... : 96 1-3
cvhp47 : A.....AI.....A.NSGG....A.....TV...L....T..... : 96 1-3
cvhp31 : .....WVY.....S..TGG....A.....K.....T..... : 96 1-2*
cvhp32 : .....W.Y.....S..TGG....A.....K.....T..... : 96 1-2*
cvhp34 : .....W.Y.....A.Q...S..TGG....A.....PL...K.....T..... : 96 1-2*
cvhp42 : .....W.Y.....A.NSGG....A.....Q.....TR..... : 96 1-2*
cvhp48 : .....A.....A..TGG....A.....Q.....TV.....T..... : 96 1-2*
cvhp49 : .....S..A.....D.....A.NSGG....A.....Q..... : 96 1-2*
cvhp33 : .....S...W.Y.....S..TGG....A.....K.....T..... : 96 X-2*
cvhp50 : .....A..Y..CC.Y.....F....A.NSGG....A.....Q.....TR..... : 96 1*-2*
Germline V_HH
FR1
CDR1
FR2
CDR2
FR3
length
Canonical class
H1 H2
Subfamily 1a
Cys⁻, 17CDR2
cvhhp42 : EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYDMSWVRQAPGREREGVAAINSGGGSTYYADSVKGRFTISQDNAKNTVYLQMNSLKPEDTAMYYC : 96 1-2*
cvhhp09 : Q.....S..A.....Y.Y..NC.G.F.....K.....YT.....R.....VL..K.S..... : 96 PS-PS
Subfamily 2a
Cys33, 17CDR2
cvhhp12 : D.....S..A.....EY.Y...C.G.F.....K.....H.....R.K...ML.....T..... : 96 PS-PS
cvhhp08 : Q.....S..A.....Y.Y..NC.G.F.....K.....YT..... : 96 PS-2*
cvhhp10 : .....D...H.....Y.Y...C.G.F.....K.....L..... : 96 PS-2*
Subfamily 5a
cvhhp11 : D.....S..A.....Y.Y...C.G.F.....K.....L..... : 96 PS-2*
Cys32, 17CDR2
cvhhp29 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.....R..... : 96 PS-2*
Subfamily 2b
cvhhp31 : Q.....S..A....K.....YI...CG.G.Y.....K...L.ST.....R.....L...L....T....R... : 96 1*-PS
Cys33, 16CDR2
cvhhp28 : Q.....S..A.....Y.Y..NC.G.F.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp06 : .....Y.YC.....Y.....K...F.S..D-SD...S..... : 95 PS-1*
Subfamily 3b
Cys30, 16CDR2
cvhhp07 : Q.....S..A.....Y.YC.....Y.....K...F.S..D-SD...S..... : 95 PS-1*
cvhhp16 : .....Y.Y..CS.G.Y.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp17 : .....Y.Y..CS.G.Y.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp18 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp19 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp20 : Q.....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp21 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD.....L..... : 95 PS-1*
cvhhp22 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD..... : 95 PS-1*
cvhhp26 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD.....ML.....S..... : 95 PS-1*
cvhhp27 : D.....S..A.....Y.Y..CS.G.YP....K...L.ST.I-SD.....R.....R..... : 95 PS-1*
cvhhp13 : Q.....S..A....K.....YI...CG.G.Y.....K...L.ST.S-SD...S.....L..... : 96 1*-1*
cvhhp14 : Q.....S..A....K.....YI...CG.G.Y.....K...L.ST.S-SD...S.....L..... : 96 1*-1*
cvhhp15 : Q.....S..A....K.....YI...CG.G.Y.....K...L.ST.S-SD...S.....L..... : 96 1*-1*
Subfamily 5b
Cys32, 16CDR2
cvhhp23 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD.....G.....R....S..... : 95 PS-1
cvhhp24 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD...H....M.....R.....VL..... : 95 PS-1
cvhhp25 : .....S..A.....Y.Y..CS.G.Y.....K...L.ST.I-SD.....R.K...ML..... : 95 PS-1
cvhhp32 : .....S..A.....Y.Y..CG.D.Y.....K...F.SS.S-TD.T.S.....K.K..D..... : 96 PS-1
cvhhp30 : D.....A.....Y..CC..Y.....K...L.SS.S-SD.....R.....K.....L..... : 96 1*-1
cvhhp34 : .....N.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.K..D..... : 96 1*-1
cvhhp33 : Q.K.E.....S..A.....T.P...SNRCG.D.Y.....K...F.SS.S-TD.T.S.....K.K..D..... : 96 X-1
cvhhp35 : Q.....S..A....K...T.P...SN.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.....L..... : 96 X-1
cvhhp36 : Q.....S..A.....T.P...SN.CG.D.Y..PA.KQ..W.SS.S-TD...S.....G....K.K..D..... : 96 X-1
cvhhp37 : .....S..A.....T.P...SN.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.K..D..... : 96 X-1
cvhhp38 : Q.....S..A.....T.P...SN.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.K..D..... : 96 X-1
cvhhp39 : Q.....S..A.....T.P...SN.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.K..D..... : 96 X-1
cvhhp40 : Q.....S..A.....T.P...SN.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.K..D..... : 96 X-1
cvhhp41 : Q.....S..A.....T.P...SN.CG.D.Y...A.KQ..W.SS.S-TD...S.....K.K..D..... : 96 X-1
Subfamily 4b
Cys45, 16CDR2
cvhhp01 : A.....S..A.....Y.....A.G.F.....K.C.L.ST.I-SD...N.....R..... : 95 1-1
cvhhp02 : A.....S..A.....Y.....A.G.F.....K.C.L.ST.I-SD...N.....R..... : 95 1-1
cvhhp03 : A.....S..A.....Y.....A.G.F.....K.C.L.ST.I-SD...N.....R..... : 95 1-1
cvhhp04 : A....D....S..A.....Y.....A.G.F.....K.C.L.ST.I-SD...N.....R..... : 95 1-1
cvhhp05 : .....Y.....A.G.F..P..K.C.L.ST.I-SD...N.....F.R..S..... : 95 1-1

that most of these Ins/Del regions are within or at the border of peculiar DNA sequences in the germline V_HHs, suggesting an active involvement of these sequences in the Ins/Del events. For example, a palindromic sequence 'CAGTAGCTACTG' (corresponding to residues 30–33) borders the Del/Ins region 30 ± 3 (example in Figure 7A). Similarly, the Ins/Dels at region 54 ± 3 appear to coincide with the presence of another palindrome 'CTATTAATAG' (codons 51–52a) (Figure 7B). Furthermore, two different types of nucleotide insertions can be discerned. Among 17 insertion events, eight are clearly duplications of the bordering sequences, while nine are non-templated nucleotide insertions. An example of each case is shown in Figure 7B.

The high incidence of the heptamer-like sequence, a component of the Ig recombination signal (RSS) is another peculiar feature of the V_HH sequence (Nguyen *et al.*, 1998). The RSS occurrence is almost twice as high in the germline V_HHs (37/42) as it is in the V_Hs (21/50) (Figure 7C). The majority of these RSS in the V_HH are concentrated at the FR3 site corresponding to residues 76–78. Interestingly, this RSS location is downstream of a region with a modest increased variability (Figure 5), and it also abuts a deletion at position 74–75 found in three individual V_HH cDNA clones, indicating a possible causal relationship with the heptamer-like sequence in these deletion events (Figure 7D).

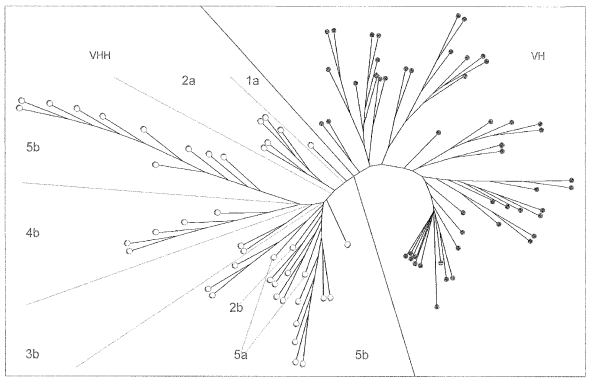


Fig. 3. Neighbour-joining phylogenetic tree of the dromedary germline V_H and V_HH segments. The tree was constructed by using clustalW, phylip packages with 1000 replicates for Bootstrap of nucleotide sequences encoding the V_H/V_HH portions. Filled and open circles denote a V_H and V_HH member, respectively. The two filled rectangles indicate pseudogenes. V_HH subfamilies are indicated.

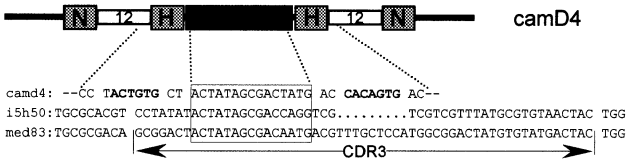


Fig. 4. The common usage of the *D* element in V_H and V_HH: a genomic *D* element (camD4) is flanked at both sides with the RSS containing a nonamer (N) separated from a heptamer (H) by a 12-nt spacer. The sequences derived from the germline camD4 (upper line), V_H-cDNA clone i5h50 (middle line) and V_HH-cDNA clone med83 (lower line) are boxed. The CDR3 region between the FR3 and FR4 is indicated.

Discussion

Dromedary V_H/V_HH gene segments

To evaluate the potential Ag-binding repertoire of the dromedary HCAbs, we established representative databases of the germline and cDNA V_H/V_HH sequences. The germline database contains 94 *V*-gene segments, of which the majority were found in two independent PCR experiments and confirmed by sequences derived from a genomic library. Statistical analysis taking into account the total number of sequenced clones and the number of doubles, triples, etc. also indicated that the dromedary may contain a total of 110 ± 20 V_H and V_HH genes. This estimation is compatible with the Southern blot analysis (Figure 1), in which the intensively labelled bands may correspond to V_H/V_HH genes residing in multiple fragments of similar size that overlap in our blot, and the longer fragments may contain more than one *V* element. The *V*-gene segments in our database have a high degree of sequence homology, several encoding an identical amino acid sequence. Some of these could be the result of a recent gene duplication, or alternatively, they might be allelic variants as the sequences were derived from a diploid genome. However, the allelic location of the genes has little relevance to our goal to evaluate the potential V_HH repertoire.

Clearly, the dromedary contains two distinct sets of *V* genes (~40 V_HHs and ~50 V_Hs), encoding the V domain of the conventional Abs and HCAbs, respectively. The V_H and V_HH genes appear to be within the same functional V region of the dromedary genome. The identification of an identical *D* mini-gene in a V_H cDNA and a V_HH cDNA (Figure 4) suggests the common use of the *D* segments for V_H and V_HH. Moreover, preliminary evidence shows that rearranged V_H and V_HH genes bear identical 3′ sequences downstream of the DJ_H segments (unpublished results). However, within this functional V region it is still unclear whether the germline V_Hs and V_HHs are interspersed or clustered.

Diverse germline V_HHs

All germline V_HHs reported here belong to a single family. However, the intrinsic structural repertoire of their Ag-binding loops is quite diverse. The prediction of the canonical structure shows that 10 combinations of the H1–H2 loops are possible in the V_HHs, which is higher than what is observed in the V_H of family 3, e.g. six in the dromedary, five in human and three in mouse (Tomlinson *et al.*, 1992; Almagro *et al.*, 1997). A total of 10 combinations of the H1–H2 loop structures were found in human and mouse germline V_Hs of all families (Almagro

Fig. 2. The deduced amino acid sequences of the dromedary germline V_H (upper) and V_HH segments (lower). Braces group the V_HH sequences in subfamilies as defined by their CDR2 length and the position of the additional Cys at indicated positions. Boxes indicate *V* genes that differ by at least 5 nt, but encode an identical amino acid sequence. The amino acid length is given in the column following the primary structure. The types of the predicted H1 and H2 canonical structures are in the last columns. Asterisks denote where the predicted loop may deviate from loop type defined by Chothia *et al.* (1992) due to novel residue at the key-site. X, unpredictable; PS, potentiality to switch (see text).

Table I. V_HH germline sub-families and their cDNA counterparts

Subfamily	Cys/CDR2[a]	Members	Total cDNA found	Binder found	Ags[b]
V_H3H–1a	Cys$^-$/17	1	0	0	
V_H3H–2a	Cys33/17	5	54	18	lysozyme, carbonic and anhydrase, cutinase, tetanus toxoid, RNase phenyl oxazolone
V_H3H–2b	Cys33/16	1	2	2	lysozyme, carbonic anhydrase
V_H3H–3b	Cys30/16	2	5	2	amylase
V_H3H–4b	Cys45/16	5	8	3	amylase, tetanus toxoid
V_H3H–5a	Cys32/17	2	0	0	
V_H3H–5b	Cys32/16	26	3	1	amylase
Total		42	72	26	

[a]The presence of an additional Cys at indicated position/the length of CDR2 (in amino acids).
[b]Reference from Desmyter *et al.* (1996), Ghahroudi *et al.* (1997), Lauwereys *et al.* (1998), Decanniere *et al.* (1999) and other unpublished Ag-binders provided by M.Lauwereys, K.Silence, T.Laeremans and M.T.M.C.Serrao.

Table II. Structural repertoire of the camel germline V_H and V_HH segments

V_H

H1			H2		V_HCSC members	
24	26	34	52	71		
A	GFTFSGY	M	Y-SDGG	R	1-1	27
A	GFTFSSY	M	Y-SDGS	Q	1-1*	1
A	GFTFSSY	@	NSDGSN	R	1-3	14
A	GFTFSSY	@	@TGGGS	K/Q	1-2*	6
A	GFTSSSY	M	YTGGGS	K	X-2*	1
A	AFTYSSC	M	NSGGGS	Q	1*-2*	1

V_HH

H1			H2		V_HCSC members	
24	26	34	52	71		
A	GYTFSSY	M	I-SDGS	R	1-1	5
A	GFT@@CS	M	S-TDGS	K	1*-1	2
A	GYIFSCS	M	S-SDGS	Q	1*-1*	3
A	GYTYSSC	M	@-@DG@	K	PS-1	4
A	GYTY@S@	M	D-SDGS	Q	PS-1*	12
A	GFTSN@C	M	S-TDG@	K	X-1	8
A	GFTFSSY	M	NSGGGS	Q	1-2*	1
A	GYIFSCS	M	NSGGGS	R	1*-PS	1
A	GYTYSS@	M	@@GGGS	Q	PS-2*	4
A	@YTYSS@	M	@@GGGS	R	PS-PS	2

V_HCSC or V_HHCSC: canonical structure class of V_H or V_HH, respectively. Asterisks denote that the predicted loop may deviate from the loop-type as defined by Chothia *et al.* (1992) due to a novel residue (underlined) at the key site. The @ denotes more than one residue occurring at that site. X, unpredictable; PS, potentiality to switch (see text).

et al., 1997). The higher complexity of the germline V_HHs within one family is due to the presence of novel residues at key sites for the loop conformation, the variable length of the CDR2 and the presence of an additional Cys located at position 30, 32, 33 or 45.

V_HH usage in the dromedary

A total of 42 V_HH gene segments were identified, which encode 33 unique sequences. In contrast to rabbits where one V_H germline is predominantly rearranged (Knight, 1992), members of at least five out of seven V_HH subfamilies are used to generate HCAbs in the dromedary (Table I). It also appears that the larger subfamilies were not necessarily the most frequently encountered in the cDNA clones. On the contrary, five members of subfamily 2a accounted for 75% of the inspected cDNAs, while 26 sequences of subfamily 5b matched only three cDNA clones (Table I). This preferential usage of particular V_HH segments could be due to their proximal location to the *D*-gene cluster, as observed in other mammals e.g. the human V_H3–23 (Stewart *et al.*, 1992) and mouse V_H10 (Schiff *et al.*, 1988).

We note that the Ag-binding loop structures of the V_HH subfamily 2a are predicted to switch readily. This subfamily appears to be the most prevalent to generate Ag binders, indicating that the paratope repertoire derived from these V_HH members could cope with various Ags. Therefore, the selection of these germline V_HHs as a scaffold to construct a synthetic single domain library would generate a sufficiently diverse repertoire to retrieve good Ag binders.

Somatic diversification of V_HHs

From a limited number of germline *V* genes, many species can generate a large Ag-binding repertoire through a somatic diversification process (Reynaud *et al.*, 1989; Knight, 1992; Sun *et al.*, 1994; Dufour *et al.*, 1996; Sinclair *et al.*, 1997). We note that the dromedary V_HH structural repertoire is readily diversified by the introduction of an additional disulfide bridge, the high incidence of nucleotide Ins/Dels, gene replacement and the extensive somatic hyperpoint mutations.

Extra disulfide bridge. The germline V_HH possesses a Cys in addition to the conserved Cys22 and Cys92 that form a disulfide bond in all Ig domains. This additional Cys is maintained in the vast majority of the V_HH cDNAs, which also acquired an additional Cys within the CDR3 (Muyldermans *et al.*, 1994; Vu *et al.*, 1997). The disulfide bonds between these additional cysteines cross-links the Ag-binding loops (Davies and Riechmann, 1996; Desmyter *et al.*, 1996). This has two consequences: it stabilizes the domain, and it induces constraints in the CDR1 or CDR3, which could lead to novel loop conformations and thereby increasing the paratope repertoire.

High incidence of insertions and deletions. The Ins/Dels of nucleotides found in 31 V_HH cDNAs cluster in four

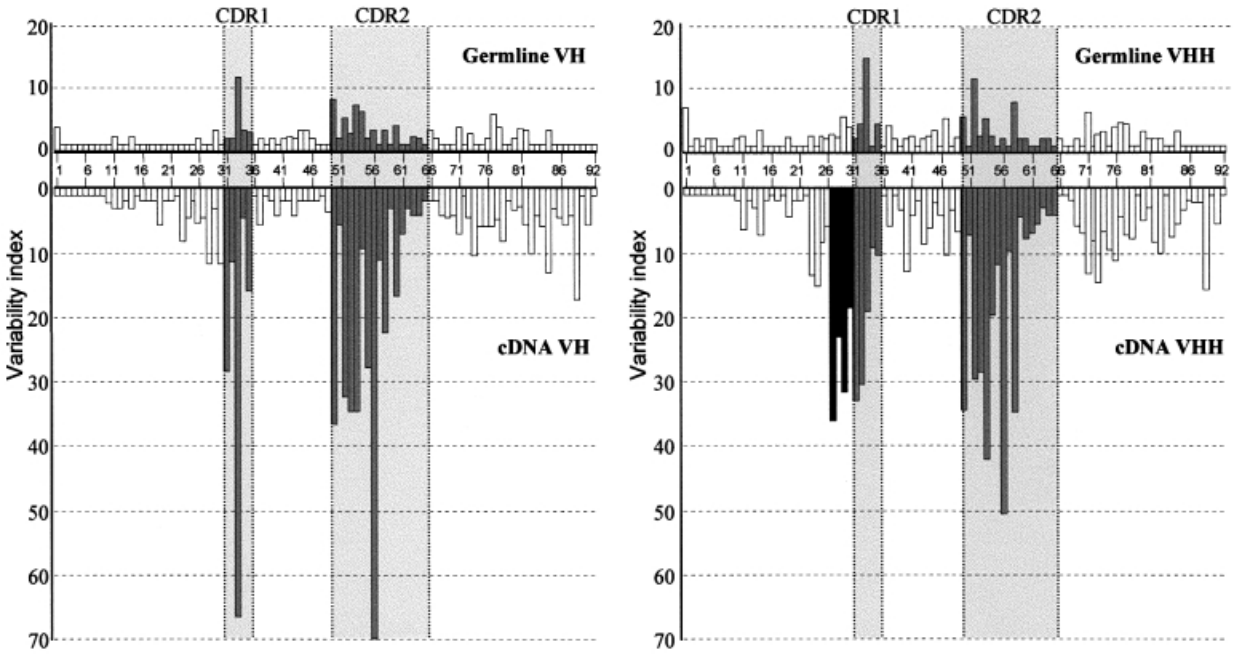


Fig. 5. Amino acid sequence variability plots for the V_H and V_HH germline (upper panels) and cDNA sequences (lower panels). The variability index (bars) was calculated from FR1 to FR3, as described in the results. The grey and open bars are for amino acid positions of the CDR and FR regions, respectively, and the black bars are for the extra hypervariable region in the V_HH. The CDR1 and CDR2, as defined by Kabat *et al.* (1991), are shaded and placed between dotted lines.

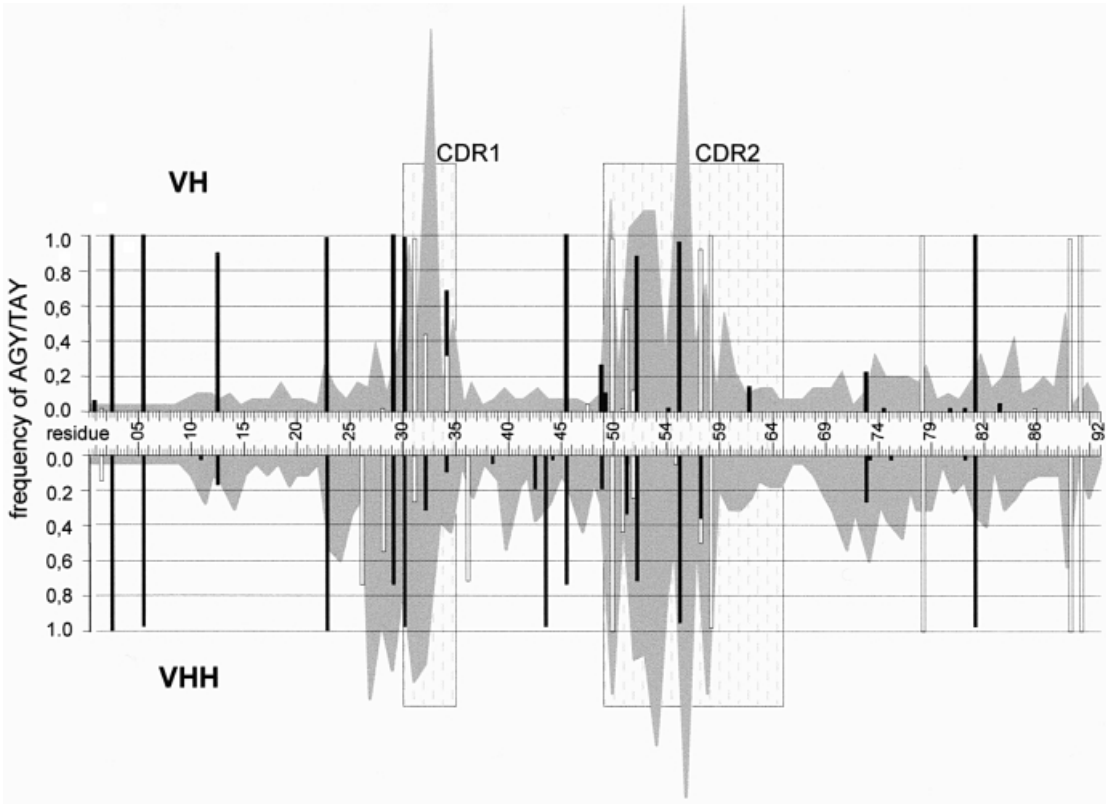


Fig. 6. Distribution and frequency of AGY (filled bars) and TAY (open bars) triplets in the germline V_H (upper) and V_HH (lower) segments. The occurrence of AGY and TAY triplets were scored in all reading frames. The numbers in between refer to the position of V_H codons (Kabat numbering). The shaded background originated from the cDNA variability plots shown in Figure 5. The dotted regions denote the CDR1 and CDR2, as defined by Kabat *et al.* (1991).

regions. The high incidence (30%) and the clustering feature of Ins/Dels suggest that they (at least in part) could be relics of a gene conversion-like event as described for chicken, rabbit and cattle (Reynaud *et al.*, 1987; Becker and Knight, 1990; Parng *et al.*, 1996).

The equal presence of two different types of nucleotide

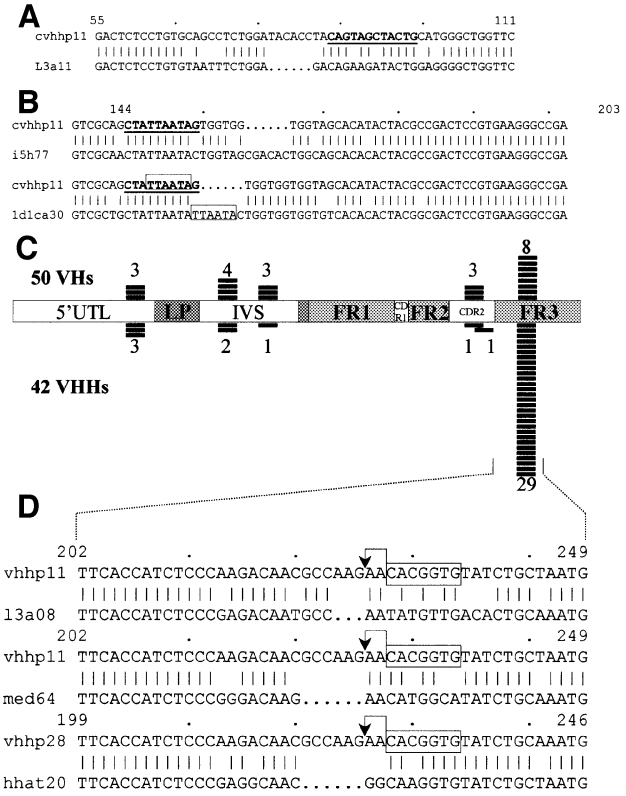


Fig. 7. (**A**) Nucleotide alignment of cDNA clone l3a11 (lower line) and its putative germline gene (cvhhp11, upper line), revealing a deletion of 6 nt (dotted). The palindromic sequence is in bold and underlined. Numbers indicate nucleotide positions of the germline V_HH element. (**B**) Alignment of the cDNA (lower lines) and the closest germline sequences (upper lines) indicates insertions, which are non-templated (pair 1) or are duplicated (boxed in pair 2). (**C**) Frequency and distribution of the RSS-like elements in the camel germline V_Hs and V_HHs. Numbers indicate the incidence of RSS found in 50 V_Hs (upper) and 42 V_HHs (lower). (**D**) Improper joining adjacent to the RSS signal resulting in clones with a deletion: the heptameric sequences are boxed and the expected cleavage site is indicated by an arrow. The three camel V_HH cDNA sequences (l3a08, med64 and hhat20) show a codon deletion when aligned with their putative corresponding genes (cvhhp28 and cvhhp11).

insertions indicates that there are at least two different modes of action leading to these sequence length variations. Half of the insertions are nucleotide duplications, indicating that these Ins/Dels could be by-products of somatic hypermutation (Wilson *et al.*, 1998). In the other half of the events, the inserted nucleotide sequences are different from their flanking sequences. Therefore, this later type could be referred to as non-templated nucleotide insertions as often found at the VD or DJ joints. Though the actual mechanism is still unknown, this observation opens the possibility that the nucleotide addition process could occur during the rejoining of double strand DNA breaks of the V_HHs.

Under any hypothesis, it is important to note that the Ins/Dels are not randomly distributed, but often occur near or within the paratope. Therefore, they substantially reshape the V_HH loop structure. This is true for both the conventional V_H and the V_HH. However, in dromedary the incidence of off-size clones is much higher in the V_HH cDNA than in the conventional V_H cDNA (30 versus 1.5%), indicating that the V_HH is more prone to these changes. Moreover, whereas in human most Ins/Dels were found in non-functional Ig genes (Klein *et al.*, 1998), in the dromedary, six out of 31 off-size cDNAs have a known Ag specificity.

Gene replacement. While the exact Ins/Del mechanism involving palindromes remains unknown, the high incidence of the embedded RSS at V_HH-FR3 abutting the Del/Ins region 74 ± 1 [Figure 7D and Ins at position 75A of two llama V_HHs (Vu *et al.*, 1997)] supports the gene replacement mechanism (Reth *et al.*, 1986) involving recombination-activating gene (RAG) proteins as observed in human and mouse (Kleinfield *et al.*, 1986; Komori *et al.*, 1993). The involvement of a RAG-like activity to initiate the gene replacement predicts a DNA cleavage two bases upstream of the heptamer sequence (Ramsden *et al.*, 1996). This is exactly the position where the deletions are observed in these individual clones (Figure 7D). It is possible that improper joining (Melek *et al.*, 1998) causes these Ins/Dels, and proper joining retains the sequence length but induces nucleotide replacements (Weinstein *et al.*, 1994). The latter possibly leads to the increased variability observed in this region of the V_HH (Figure 5). The region around position 75 lies in a solvent exposed loop immediately adjacent to the H2 loop in the folded Ig domain. In monomeric T-cell receptors, the corresponding region is also hypervariable and reported to interact with ligand (Howell *et al.*, 1991). By analogy, we suppose that this V_HH region can be involved in the Ag–Ab interaction by either directly contacting the Ag or inducing structural changes in the Ag-binding site. Taken together, it is plausible that gene replacement at this region, in which a new V_HH rearranges to the pre-existing V_HH-D-J_H, results in a novel Ag-binding site composed of the incoming V_HH and the existing CDR3.

Extended hypervariable CDR1 region. The patterns of the cDNA variability plots (Figure 5) are basically consistent with the classification of FR- and CDR-regions as defined by Kabat *et al.* (1991). However, the extra hypervariable region (residues 27–30) present exclusively in the V_HH is most remarkable. Crystallographic studies of V_HH–antigen complexes demonstrated that amino acids located in this area interact with Ag (Desmyter *et al.*, 1996; Decanniere *et al.*, 1999). Obviously, the V_HH uses this region together with a long CDR3 to increase the surface area interacting with Ags. Surprisingly, to attain new amino acids at these positions the germline V_HHs accumulated two new hypermutation hotspots (Figure 6) by two single point mutations. The non-hotspot triplet TTY in the V_H is substituted by TAY in the V_HH, a well-known hotspot for hypermutation (Milstein *et al.*, 1998). These mutations in the germline lead to the amino acid substitutions F27Y and F29Y, two key-elements for the H1 loop conformation (Chothia *et al.*, 1992). Further mutations of Y27 or Y29 in the V_HHs has two pronounced effects. Some mutations will lead to complete new loop conformations and thereby will expand the structural repertoire of the V_HHs. Other mutations might lead to amino acid substitutions provoking subtle surface modifications that might improve the V_HH–antigen fit. For these reasons, we propose to vary these amino acids in libraries of synthetic single domain V_HHs or human V_Hs (Davies and Riechmann, 1996; Reiter *et al.*, 1999) to increase the potential repertoire and to search for more potent Ag binders.

In conclusion, the data presented here demonstrate that the Ag-binding repertoire of HCAb, derived from ~40 germline V_HHs, is largely diversified by specific mechanisms. These include the introduction of a variety of interloop disulfide bridges, the increased surface area of hypervariable regions and a high rate of paratope reshaping. The use of these specific mechanisms also results in novel paratopes different from those of the conventional Abs. This could explain the large proportion of HCAbs acting as competitive enzyme inhibitors. It is clear that particular DNA signals with a high incidence in the V_HHs trigger these somatic diversification processes. It is possible that the absence of the light chain provides the freedom to make these changes possible and to allow the V_HH to explore new structures.

Materials and methods

Southern blots

High MW genomic DNA was prepared from liver tissue of a single camel (*Camelus dromedarius*) from Morocco. Dromedary genomic DNA (10 µg) was digested with *Bam*HI, *Eco*RI or *Bam*HI–*Eco*RI and electrophoresed in a 0.8% agarose gel. The DNA was transferred to Hybond-N (Amersham) membranes. Two homologous V_H probes of 0.6 kb corresponding to the dromedary germlines V_H and V_HH (Nguyen *et al.*, 1998) were obtained by PCR and labelled with ^{32}P (Radprime, Gibco-BRL). After hybridization, the membranes were washed at 65°C for 15 min/wash (twice with 2× SSC, 0.1% SDS, once with 2× SSC, 1% SDS, and finally with 0.1× SSC), and autoradiographed.

Genomic DNA amplification, cloning and DNA sequencing

The primers used to amplify V_H/V_HH genes were derived from the V_H and V_HH genomic sequences (Nguyen *et al.*, 1998). One specific primer of 26 nt corresponds to the upstream conserved Ig octamer sequence (V_HOCTB: 5′-TCTATATATCTAGATGACATGCAAAT-3′). The other primer anneals at the FR3 sequence and starts from the Cys92 codon (V_HFR3F: 5′-ACAGTAATACATGGCCGTGTCCTC-3′). PCRs (50 µl) were performed on 0.5 µg liver genomic DNA with 2.5 U of *Taq* DNA polymerase (ROCHE) (30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C with a final incubation of 10 min at 72°C). PCR products were gel-purified, ligated into TA-PCRII cloning vector (Invitrogen), and subsequently transformed into *DH5*α cells. Randomly chosen clones with inserts of the expected size were sequenced from both ends. Two sets of PCR and cloning were carried out independently to exclude possible PCR errors.

Screening of a genomic library

A camel liver genomic library (Nguyen *et al.*, 1998) was screened by plaque hybridization with the same probes used in Southern blot experiment. The V_H/V_HH element from the putative positive phage clone was amplified using the V_HFR3F primer and a primer that corresponds to the leader-peptide sequence: V_HLB 5′-GGCTGAGCTCGGTGGT-CCTGGCT-3′. The phage-derived PCR fragments were cloned, and two clones were sequenced for each experiment.

Camel V databases

Germline V_H DNA sequences were aligned and grouped according to sequence identity. DNA sequences (600 bp) differing by no more than four bases were considered identical as these differences might be due to PCR or sequencing errors. The camel germline V_H/V_HH database contains the remaining unique sequences. The camel cDNA data were collected from the V_H and V_HH cDNA sequences available within our group.

Sequence analysis

All DNA and protein sequence analyses were performed with version 10 of the University of Wisconsin Genetics Computer Group package (Devereux *et al.*, 1984).

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