

Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation

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A mouse monoclonal antibody (mAb 425) with therapeutic potential was 'humanized' in two ways. Firstly the mouse variable regions from mAb 425 were spliced onto human constant regions to create a chimeric 425 antibody. Secondly, the mouse complementarity-determining regions (CDRs) from mAb 425 were grafted into human variable regions, which were then joined to human constant regions, to create a reshaped human 425 antibody. Using a molecular model of the mouse mAb 425 variable regions, framework residues (FRs) that might be critical for antigen-binding were identified. To test the importance of these residues, nine versions of the reshaped human 425 heavy chain variable (V_H) regions and two versions of the reshaped human 425 light chain variable (V_L) regions were designed and constructed. The recombinant DNAs coding for the chimeric and reshaped human light and heavy chains were co-expressed transiently in COS cells. In antigen-binding assays and competition-binding assays, the reshaped human antibodies were compared with mouse 425 antibody and to chimeric 425 antibody. The different versions of 425-reshaped human antibody showed a wide range of avidities for antigen, indicating that substitutions at certain positions in the human FRs significantly influenced binding to antigen. Why certain individual FR residues influence antigen-binding is discussed. One version of reshaped human 425 antibody bound to antigen with an avidity approaching that of the mouse 425 antibody.

Key words: antibody/anti-tumour/CDR-grafting/chimeric/molecular model-building

Introduction

The mouse monoclonal antibody 425 (mAb 425) was raised against the human A431 carcinoma cell line and found to bind to a polypeptide epitope on the external domain of the human epidermal growth factor receptor (EGFR). It was found to inhibit the binding of epidermal growth factor (EGF) at both low and high affinity EGFR sites (Murthy *et al.*, 1987). Enhanced expression of EGFR is found to occur on malignant tissue from a variety of sources, thus making mAb 425 a possible agent for the diagnosis and therapeutic treatment of human tumours. Indeed, mAb 425 was found to mediate tumour cytotoxicity *in vitro* and to suppress tumour cell growth of epidermoid and colorectal carcinoma-derived cell lines *in vitro* (Rodeck *et al.*, 1987). Radiolabelled mAb 425 has also been shown to bind to xenografts of human malignant gliomas in mice (Takahashi *et al.*, 1987).

Since mouse mAbs elicit an immune response in the human patient (Giorgi *et al.*, 1983; Jaffers *et al.*, 1986), two strategies for the humanization of mouse mAb have been developed. In the first, the mouse constant regions from both the light and heavy chain can be replaced with human constant regions. Such 'chimeric' mouse–human antibodies have been successfully constructed from several mouse mAbs directed against human tumour-associated antigens (Sun *et al.*, 1987; Whittle *et al.*, 1987; Liu *et al.*, 1987; Gillies and Wesolowski, 1990). This approach totally conserves the antigen-binding site of the mouse antibody, and hence the antigen affinity, while conferring the human isotype and effector functions. In the second approach, only the complementarity-determining regions (CDRs) from the mouse variable regions are grafted into human variable regions. It is reasoned that this technique will transfer the critical and major portion of the antigen-binding site to the human antibody (Jones *et al.*, 1986). CDR grafting has been carried out for several rodent monoclonals (Jones *et al.*, 1986; Richmann *et al.*, 1988; Verhoeyen *et al.*, 1988; Queen *et al.*, 1989; Co *et al.*, 1991; Gorman *et al.*, 1991; Maeda *et al.*, 1991; Tempest *et al.*, 1991). All retained their capacity to bind antigen, although the affinity was usually diminished. In most cases it was deemed necessary to alter certain amino acids in the human framework residues (FRs). It appears that at least a few changes in the human FRs are essential to ensure that the CDR-grafted or 'reshaped human' antibody has an antigen-binding site with the correct functional conformation. Both chimeric and CDR-grafted antibodies have proved superior to the mouse antibodies in the clinic (Hale *et al.*, 1988; LoBuglio *et al.*, 1989; Mathieson *et al.*, 1990).

This paper describes the cloning, sequencing and humanization of mouse mAb 425. A chimeric antibody was constructed by fusing the mouse light chain variable (V_L) and heavy chain variable (V_H) domains of mAb 425 to human kappa and gamma-1 constant domains respectively. The human gamma-1 domains were selected because they have been demonstrated to be the most effective in complement and cell-mediated lysis (Brüggemann *et al.*, 1987; Reichmann *et al.*, 1988). CDR-grafted or reshaped human antibodies were constructed by transferring the mouse mAb 425 CDRs into human variable regions that show a high degree of homology to the mouse mAb 425 variable regions. It was found that changes at several positions in the human FRs were essential to ensure good antigen-binding. To help select potentially important FR residues, a molecular model of the mouse 425 V_L and V_H regions was constructed. The effect of specific changes in the FRs on antigen-binding was investigated and the influence of the FR residues on antigen-binding is discussed.

Materials and methods

Molecular cloning and sequencing

Total RNA was isolated from cell line W425-15 (Chirgwin *et al.*, 1979). Supernatant from the W425-15 cells used for total RNA isolation was assayed by ELISA to ensure that the cells were producing mAb 425 in high amounts. Poly(A)⁺ RNA was

Table I. Oligonucleotides used for cDNA cloning, construction of chimerics and mutagenesis

No.	Sequence	Description
1	5'-G T A G G A T C C T G G A T G G T G G G A A G A T G -3'	light chain primer for cDNA synthesis
2	5'-G T A G G A T C C A G T G G A T A G A C C G A T G -3'	heavy chain primer for cDNA synthesis
3	5'-C T C C A A G C T T G A C C T C A C C A T G G -3'	chimeric V _H front primer
4	5'-T T G G A T C C A C T C A C C T G A G G A G A C T G T G A -3'	chimeric V _H back primer
5	5'-A G A A A G C T T C C A C C A T G G A T T T T C A A G T G -3'	chimeric V _L front primer
6	5'-G T A G A T C T A C T C A C G T T T T A T T T C C A A C -3'	chimeric V _L back primer
7	5'-A G C G G T A C C G A C T A C A C C T T C A C C A T C -3'	primer to introduce F71Y into RV _L
8	5'-A T A C C T T C A C A T C C C A C T G -3'	primer to introduce S30T into RV _H
9	5'-C G A G T G G A T T G G C G A G T -3'	primer to introduce V48I into RV _H
10	5'-T T T A A G A G C A A G G C T A C C A T G A C C G T G G A C A C C T C T -3'	primer to introduce R66K, V67A, L71V into RV _H
11	5'-C A T G A C C G T G G A C A C C T C T -3'	primer to introduce L71V into RV _H

In primers 7–11, starred bases indicate base changes resulting in codon changes.

prepared from the total RNA (Aviv and Leder, 1972). Double-stranded cDNA was synthesized according to the methods of Gubler and Hoffman (1983), except that primers homologous to the 5' regions of the mouse kappa and gamma-2a constant regions were used to prime first-strand synthesis (Levy *et al.*, 1987). The design of the light chain primer (oligonucleotide 1; Table I) and heavy chain primer (oligonucleotide 2; Table I) was based on published data (Kaariten *et al.*, 1983; Kabat *et al.*, 1987; Levy *et al.*, 1987). Primers were synthesized on an Applied Biosystems 380B DNA Synthesizer and purified on urea-acrylamide gels. After second-strand synthesis, the blunt-ended cDNAs were cloned into *Sma*I-digested pUC18 and transformed into competent *Escherichia coli* cells, DH5- α . Clones were screened by hybridization using ³²P-labelled first-strand synthesis primers (Carter *et al.*, 1985). Sequencing of double-stranded plasmid DNA was carried out using Sequenase (United States Biochemical Corporation).

Construction of chimeric genes

For each variable region, two polymerase chain reaction (PCR) primers (front and back) were synthesized (oligonucleotides 3–6; Table I). PCR reactions were set up using 1 ng of pUC18 plasmid DNA containing the cloned cDNA, front and back PCR primers at a final concentration of 1 μ M each, 1.25 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl and 0.01% gelatin (w/v). Amplitaq DNA polymerase (Perkin Elmer Cetus) was added at 2.5 U/assay. After an initial melt at 94°C for 1.5 min, 25 cycles of amplification were performed at 94°C for 1 min, 45°C for 1 min and 72°C for 3 min. A final extension step at 72°C was carried out for 10 min. PCR reactions were phenol/chloroform extracted twice and ethanol precipitated before digesting with *Hind*III and *Bam*HI. The PCR fragment coding for the V_L or V_H region was then cloned into an expression vector. This vector contains the human cytomegalovirus (HCMV) enhancer and promoter, the bacterial *neo* gene, and the SV40 origin of replication. A 2.0 kb *Bam*HI fragment of genomic DNA coding for the human gamma-1 constant region and containing the necessary signals for correct splicing and poly(A)⁺ sites (Takahashi *et al.*, 1982) was inserted in the correct orientation downstream of the V_H region fragment (see HCMV-CV_H425-gamma1 in Figure 1). This vector was later modified by removing the *Bam*HI site at the 3' end of the constant region

fragment, thus allowing variable regions to be directly inserted into the heavy chain expression vector as *Hind*III–*Bam*HI fragments (Maeda *et al.*, 1991). The fragment coding for the V_L region was inserted into a similar HCMV expression vector, in this case containing a *Bam*HI fragment of genomic DNA ~2.6 kb in size, coding for the human kappa constant region and containing a splice acceptor site and a poly(A)⁺ site (Rabbitts *et al.*, 1984) (see HCMV-CV_L425-kappa in Figure 1).

Molecular modelling of mAb 425 V_L and V_H

A molecular model of the variable regions of mouse mAb 425 was built on the solved structure of the highly homologous anti-lysozyme antibody, Hy-HEL5 (Sheriff *et al.*, 1987). The variable regions of mAb 425 and Hy-HEL5 have 90% homology.

The model was built on a Silicon Graphics Iris 4D workstation running under the UNIX operating system and using the molecular modelling package 'QUANTA' (Polygen Corp.). Identical residues in the FRs were retained; non-identical residues were substituted using the maximum overlap procedure (Snow and Amzel, 1986) incorporated into QUANTA's protein modelling facility. The main-chain conformation of the three N-terminal residues in the heavy chain were substituted from an homologous antibody structure (HyHEL-10; Padlan *et al.*, 1989) since their temperature factors were abnormally high (greater than the mean plus three standard deviations from the backbone temperature factors) and since they influence the packing of V_H CDR3 (H3) (Martin, 1990).

The CDR1 (L1) and CDR2 (L2) sequences of the V_L region and the CDR1 (H1) and CDR2 (H2) sequences of the V_H region from mAb 425 corresponded to canonical forms postulated by Chothia *et al.* (1989). L1 and L2 belong to canonical structural group 1 for both CDRs, and H1 and H2 belong to canonical structural groups 1 and 2 respectively. The main-chain torsion angles of these loops were kept as in HyHEL-5. The CDR3 (L3) sequence of the V_L region and the CDR3 (H3) sequence of the V_H region from mAb 425 did not correspond to canonical structures and, therefore, were modelled in a different way. The computer program of Martin *et al.* (1989) was used to extract loops from the Brookhaven Databank (Bernstein *et al.*, 1977). The loops were then sorted based on sequence similarity, energy and structure-determining residues (Sutcliffe, 1988). The top-ranked loops were inspected on the graphics and the best selected

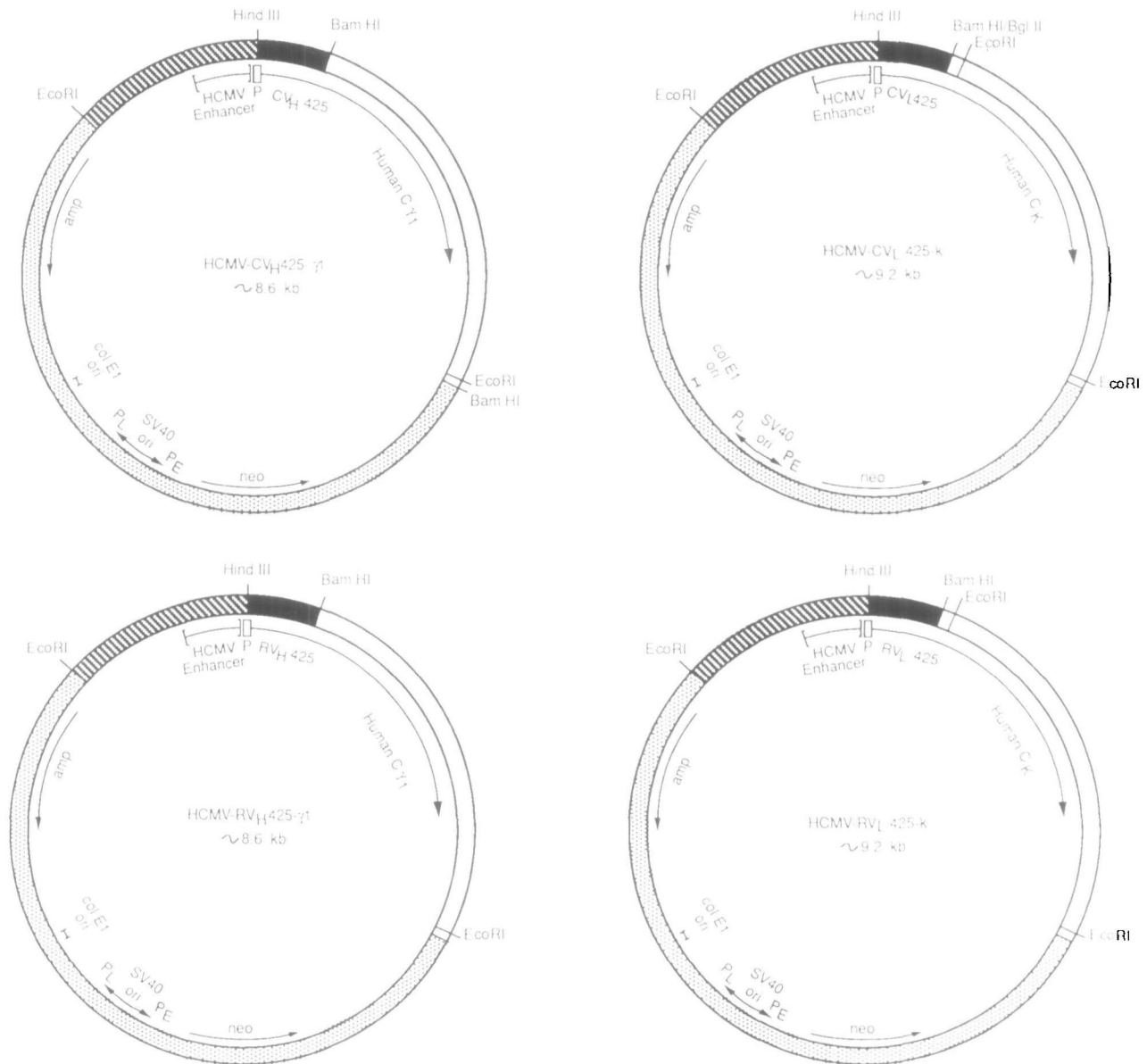


Fig. 1. Schematic representations of the vectors used for the expression of chimeric and reshaped human antibodies. Restriction sites used in the construction of the expression plasmids are marked. The variable region coding sequences are represented by the dark boxes, constant regions by the light boxes, the HCMV promoter and enhancer by the hatched boxes, and the nucleotide fragment from the plasmid pSVneo by the speckled boxes. The directions of transcription are represented by arrows.

by eye. H3 was modelled on bovine glutathione peroxidase (Epp *et al.*, 1983) in the region of residues 92–103. L3 was modelled on the murine IgA (J539) Fab fragment (Suh *et al.*, 1986) in the region of residues 88–96 of the light chain.

The model was subjected to steepest descents and conjugate gradients energy minimization using the CHARMM potential (Brooks *et al.*, 1983) as implemented in QUANTA in order to relieve unfavourable atomic contacts and to optimize van der Waals and electrostatic interactions.

Construction of humanized antibody genes

The construction of the first version of reshaped human 425 V_L region was carried out using a CDR-grafting approach similar to that described by Reichmann *et al.* (1988) and Verhoeyen *et al.* (1988). Single-stranded template DNA was prepared from a M13mp18 vector containing a HindIII–BamHI fragment coding for the human anti-lysozyme V_L region (Winter, 1988). Three oligonucleotides were designed which consisted of DNA

sequences coding for each of the mouse mAb 425 light chain CDRs flanked on each end by 12 bases of DNA complementary to the DNA sequences coding for the adjacent human FRs. Oligonucleotides were synthesized and purified as before. All three oligonucleotides were phosphorylated and used simultaneously in an oligonucleotide-directed *in vitro* mutagenesis system based on the methods of Eckstein and co-workers (Taylor *et al.*, 1985a,b; Nakamaye and Eckstein, 1986; Sayers *et al.*, 1988) (as supplied by Amersham plc). The manufacturer's instructions were followed throughout the exonuclease III digestion step. The reaction was then phenol/chloroform extracted, ethanol precipitated and resuspended in 100 µl of TE. A volume of 10 µl was used as template DNA in a 100 µl PCR-amplification reaction containing M13 universal primer and reverse sequencing primer to a final concentration of 0.2 µM each. Buffer and thermocycling conditions were as described previously with the exception of using a 55°C annealing temperature. The PCR reaction was phenol/chloroform extracted

twice and ethanol precipitated before digestion with *Hind*III and *Bam*HI and subcloning into pUC18. Putative positive clones were identified by hybridization to ³²P-labelled mutagenic primers (Carter *et al.*, 1985). Clones were confirmed as positive by sequencing. A V_L region containing all three mouse mAb 425 CDRs was cloned as a *Hind*III–*Bam*HI fragment into the light chain expression vector to create plasmid HCMV-RV_La425-kappa.

Version 'b' of reshaped human 425 V_L was constructed using the PCR mutagenesis method of Kammann *et al.* (1989), with minor modifications. The template DNA was the RV_La subcloned into pUC18. The first PCR reaction was set up in a total volume of 50 μl and contained 1 ng template, M13 reverse sequencing primer and primer 7 (Table I) at a final concentration of 1 μM. The reaction conditions were as described in Kammann *et al.* (1989). The PCR reaction was phenol/chloroform extracted and ethanol precipitated before isolating the PCR product from a TAE agarose gel. A tenth of the first PCR reaction was then used as one of the primers in the second PCR reaction. The second reaction was as the first, except the first reaction product and 20 pmol of M13 universal primer were used. Cycling was as described in Kammann *et al.* (1989). The *Hind*III–*Bam*HI fragment was cloned into pUC18 and sequenced. A correctly mutated DNA fragment was subcloned into the light chain expression vector to create plasmid HCMV-RV_Lb425-kappa.

The first version of the reshaped human 425 V_H region was chemically synthesized at British Bio-technology Ltd. A DNA sequence was designed coding for the required amino acid sequence and containing the necessary flanking DNA sequences (see Results). Codon usage was optimized for mammalian cells with useful restriction enzyme sites engineered into the DNA sequences coding for FRs. The 454 bp was synthesized and subcloned into pUC18 as an *Eco*RI–*Hind*III fragment. A *Hind*III–*Bam*HI fragment coding for the reshaped human 425 V_H region was then transferred into the heavy chain expression vector to create plasmid HCMV-RV_Ha425-gamma1.

Eight other versions of the reshaped human 425 V_H regions were constructed by a variety of methods. The *Hind*III–*Bam*HI fragment coding for the version 'a' of the reshaped human 425 V_H region was transferred to M13mp18 and single-stranded DNA prepared. Using oligonucleotides 8–10 (Table I), PCR-adapted M13 mutagenesis, as described above, was used to generate DNA coding for reshaped human 425 V_H regions versions 'd', 'e', 'f' and 'g' in pUC18. These versions were subcloned into the heavy chain expression vector as *Hind*III–*Bam*HI fragments to create plasmids HCMV-RV_Hd425-gamma1, HCMV-RV_He425-gamma1, HCMV-RV_Hf425-gamma1 and HCMV-RV_Hg425-gamma1.

Reshaped human 425 V_H regions versions 'b' and 'c' were generated using the PCR-mutagenesis method of Kammann *et al.* (1989) as described above. The template DNA was reshaped human 425 V_H region version 'a' subcloned into pUC18, and the mutagenic primer used in the first PCR reaction was either primer 10 or 11 (Table I). After mutagenesis and sequencing, sequences bearing the desired changes were subcloned into the heavy-chain expression plasmid to create plasmids HCMV-RV_Hb425-gamma1 and HCMV-RV_Hc425-gamma1.

Reshaped human 425 V_H regions versions 'h' and 'i' were constructed from the pUC-based clones of existing versions. A 0.2 kb *Hind*III–*Xho*I fragment from version 'e' was ligated to a 2.8 kb *Xho*I–*Hind*III fragment from either version 'b' or 'c' producing the new versions 'h' and 'i' respectively. The *Hind*III–*Bam*HI fragments coding for these versions were

subcloned into the heavy chain expression vector to produce the plasmids HCMV-RV_Hh425-gamma1 and HCMV-RV_Hi425-gamma1.

Transfection of DNA into COS cells

COS cells were electroporated with 10 μg each of the expression vectors bearing the genes coding for the heavy and light chains. Briefly, 10 μg of each plasmid was added to a 0.8 ml aliquot of a 1 × 10⁷ cells/ml suspension of COS cells in PBS. A Bio-Rad Gene Pulser was used to deliver a pulse of 1900 V, at a capacitance of 25 μF. The cells were left to recover at room temperature for 10 min before plating into 8 ml DMEM containing 10% fetal calf serum. After 72 h incubation, the medium was collected, centrifuged to remove cellular debris and analysed by ELISA.

Quantification of IgG production and detection of antigen binding

Human IgG present in COS cell supernatants was detected by ELISA. In the ELISA assay for human IgG, 96 well plates were coated with goat anti-human IgG (whole molecule) (Sigma) and human IgG in the samples that bound to the plates was detected using alkaline phosphatase-conjugated goat anti-human IgG (gamma-chain specific) (Sigma). Purified human IgG (Sigma) was used as a standard. Binding to the antigen recognized by mAb 425 was determined in a second ELISA. Plates were coated with an EGFR protein preparation and antibodies binding to EGFR were detected using either an anti-human IgG (gamma-chain specific) peroxidase conjugate (for chimeric and reshaped human antibodies) or an anti-mouse IgG (whole molecule) peroxidase conjugate (for the mouse mAb 425 antibody) (both conjugates supplied by Sigma). Purified mouse mAb 425 was used as a standard.

Competition binding assay

Mouse mAb 425 was biotinylated using a kit supplied by Amersham plc. ELISA plates were coated with an optimal dilution of the EGFR protein. Dilutions of the COS cell supernatants, in a volume of 50 μl, were mixed with 50 μl of the biotinylated mouse mAb 425 (estimated by ELISA to be 1.75 μg/ml each). Each COS cell supernatant was tested in duplicate. Plates were incubated at room temperature overnight. Bound biotinylated mouse mAb 425 was detected by the addition of streptavidin horseradish peroxidase complex (Amersham plc). A control with no competitor present allowed a value of percentage of inhibition or blocking to be calculated for each COS cell supernatant as follows: 100 – [(OD₄₅₀ of sample/OD₄₅₀ of control) × 100].

Results

Cloning and sequencing of variable region genes of mAb 425

From the cDNA synthesis and cloning using the kappa chain primer, 314 colonies were picked for screening. From the cDNA synthesis and cloning using the gamma-2a primer, 252 colonies were picked for screening. After screening by hybridization using the two respective cloning primers, 21 light chain colonies and 12 heavy chain colonies gave strong signals. Plasmid DNA was isolated from these colonies and analysed by restriction enzyme digests to determine the size of the cDNA inserts. Clones that appeared to have inserts 400–500 bp or 500–600 bp for the V_L and V_H respectively were selected as candidates for sequencing. Three V_L clones and three V_H clones were sequenced on both strands using M13 universal and reverse sequencing primers. Of the three possible V_L clones sequenced, one coded for a complete variable region and the others appeared

to code for unrelated peptides. Two of the V_H clones coded for identical V_H regions, while the other appeared to code for the V_H region with the intron between the leader sequence and FR1 still present. Apart from the intron, the third V_H clone contained coding sequence identical to that of the first two clones. To verify the sequence of the V_L region, three more cDNA clones containing inserts of the appropriate size were sequenced. Two of these gave sequences in agreement with the first V_L clone. The third was an unrelated DNA sequence. In the clones sequenced, not all of the original primer sequence was present. The extent of the deletions varied from clone to clone. These deletions, which probably occurred during cDNA synthesis and cloning, may have decreased the efficiency of the colony screening. Indeed, other groups have reported the need to use different oligonucleotides for the priming and screening (Hoogenboom *et al.*, 1990).

The V_L and V_H genes for mAb 425 are shown in Figure 2. For patent reasons, the DNA sequences coding for CDRs are represented by Xs. The amino acid sequence of the 425 V_L and V_H regions were compared with other mouse variable regions in the Kabat data base (Kabat *et al.*, 1987). The V_L region could be placed into the mouse kappa chain variable region subgroup IV or VI. Within the FRs, the 425 V_L region has an 86% identity to the consensus sequence for mouse kappa subgroup IV and an 89% identity to subgroup VI. The 425 V_L region

appears to use the JK4 segment. The 425 V_H region has a 98% identity to the FRs of the consensus sequence for mouse heavy chain subgroup II(B).

Construction and expression of chimeric 425 antibody

To construct the chimeric genes, front and back PCR primers were synthesized for each variable region. The front primer was designed to incorporate a *HindIII* site for cloning into the expression vector and a Kozak sequence to optimize translation (Kozak, 1987). The back primer was designed to incorporate a *BamHI* site (V_H) or a *BglII* site (V_L) for cloning and the necessary splice donor site. The PCR reaction was carried out as described in Materials and methods.

The PCR-modified V_L region DNA was cloned into the *HindIII*–*BamHI* sites of the HCMV light chain expression vector as a *HindIII*–*BglII* fragment. The entire PCR-modified V_L fragment was sequenced to ensure that no errors had been incorporated during the PCR step. The PCR-modified V_H DNA was cloned to the HCMV heavy chain expression vector as a *HindIII*–*BamHI* fragment and also sequenced to confirm the absence of PCR errors.

The expression vectors containing the chimeric 425 V_L and V_H regions were co-transfected into COS cells. After 72 h of transient expression, the COS cell culture medium was assayed by ELISA for the production of human IgG and for the ability

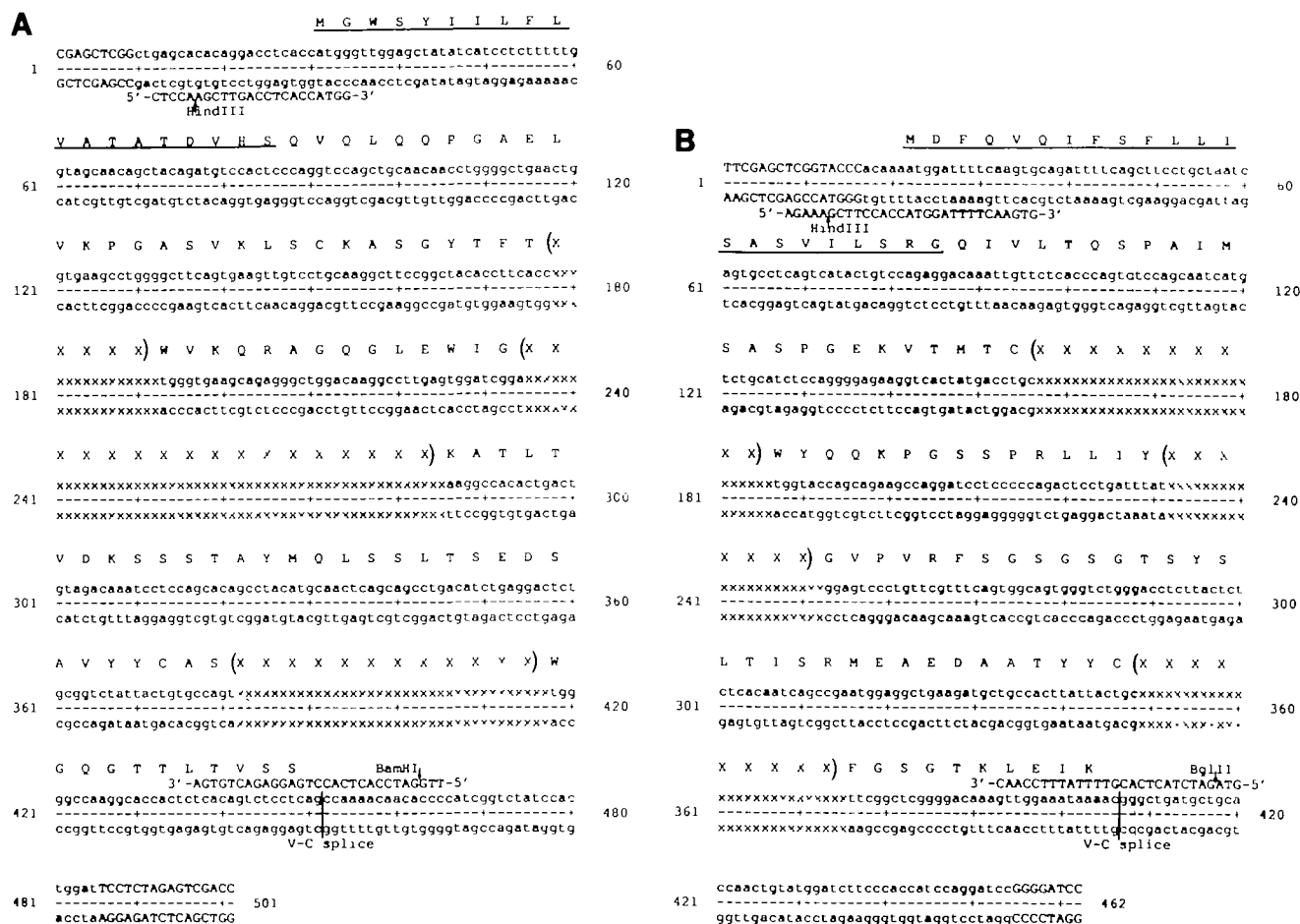


Fig. 2. The nucleotide and amino acid sequences of the V_H 425 (panel A) and V_L 425 (panel B) cDNA as cloned into pUC18. Nucleotides from the pUC18 vector are in upper case lettering. The amino acids contributing to the leader are underlined and CDRs are indicated by brackets; information concerning the CDRs has been removed for patent reasons. The splice sites between the variable regions and constant regions are also shown. The front and back PCR-primers and their annealing sites, used in the construction of the genes coding for the chimeric antibodies, are shown.

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