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Antibody Framework Residues Affecting the Conformation of the Hypervariable Loops

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Rodent monoclonal antibodies have been "humanized" or "reshaped" for therapy by transplanting the antigen-binding loops from their variable domains onto the β-sheet framework regions of human antibodies. However, additional substitutions in the human framework regions are sometimes required for high affinity antigen binding. Here we describe antigen binding by a reshaped antibody derived from the mouse anti-lysozyme antibody D1.3, and several variants in which point mutations had been introduced into framework positions to improve its affinity. The affinities were determined from the relaxation kinetics of reactant mixtures using quenching of fluorescence that occurs upon formation of the antibody–antigen complex. The dissociation constant of lysozyme ranged from 3·7 nM (for D1.3) to 260 nM. Measurement of antibody–antigen association kinetics using stopped-flow showed that D1.3 and most of the reshaped antibodies had bimolecular rate constants of $1{\cdot}4 \times 10^6$ s^{-1} M^{-1}, indicating that differences in equilibrium constant were predominantly due to different rates of dissociation of lysozyme from immune complexes. Mutations in a triad of heavy chain residues, 27, 29 and 71, contributed 0·9 kcal/mol in antigen binding free energy, and a Phe to Tyr substitution of light chain residue 71 contributed an additional 0·8 kcal/mol. The combined effect of all these mutations brought the affinity of the reshaped antibody to within a factor of 4 of D1.3. All of these substitutions were in the β-sheet framework closely underlying the complementarity-determining regions, and do not participate in a direct interaction with antigen. The informed selection of residues in such positions may prove essential for the success of loop transplants in antibodies. Variation of these sites may also have a role in shaping the diversity of structures found in the primary repertoire, and in affinity maturation.

Keywords: humanized antibody; kinetics; site-directed mutagenesis; hypervariable loop; lysozyme

1. Introduction

The antibody is a Y-shaped molecule, in which the variable (V) domains forming the tips of the arms bind to antigen and those forming the stem (C-domains) are responsible for triggering effector functions that eliminate antigen. X-ray diffraction studies of crystalline antibody fragments reveal the V and C-domains as two layers of β-pleated sheet, with loops connecting the ends of the β-strands (Poljak *et al.*, 1973; Schiffer *et al.*, 1973; Segal *et al.*, 1974). In the heavy and light chain V-domains the loops at one end of the sheet are hypervariable in sequence (Wu & Kabat, 1970; Kabat & Wu, 1971), and form the antigen-binding site. The β-sheet provides a scaffold for mounting a diversity of loops, and indeed the antigen binding site can be transplanted from a rodent antibody to a human antibody, thereby "humanizing" the rodent anti-body, by transplanting these loops (Jones *et al.*, 1986; Verhoeyen *et al.*, 1988; Riechmann *et al.*, 1988). The clinical success of one such "reshaped" human anti-lymphocyte antibody, CAMPATH-1 (Riechmann *et al.*, 1988) in treating B-cell lymphoma (Hale *et al.*, 1988) and vasculitis (Mathieson *et al.*, 1990), has prompted the reshaping of antibodies directed against the interleukin-2 receptor (Queen *et al.*, 1989), CD4 (Gorman *et al.*, 1991) respiratory syncytial virus (Tempest *et al.*, 1991), herpes simplex virus (Co *et al.*, 1991), human immunodeficiency virus (Maeda *et al.*, 1991) and epidermal growth factor receptor (Kettleborough *et al.*, 1991).

However, reshaping requires that the rodent and human framework regions are structurally conserved, both in the orientation of the two β-sheets of each domain and in the packing of the heavy and light chain V-domains together; that the

hypervariable loops make the majority of contacts with antigen; and that the loops are supported in a similar way by the underlying β-sheet framework. Although these are likely to be true for some antibodies, the restitution of key contacts between loops and framework has proved necessary in others, and has been assisted by molecular modelling (Riechmann *et al.*, 1988; Tempest *et al.*, 1991) and systematic matching of rodent and human framework regions to minimize differences in primary sequences (Queen *et al.*, 1989; Gorman *et al.*, 1991; Maeda *et al.*, 1991). As a model we have reshaped a human antibody based on the hypervariable regions of mouse antibody D1.3 (Amit *et al.*, 1986; Bhat *et al.*, 1990) and the framework regions of human Vκ(I) family (Kabat *et al.*, 1987) and the myeloma protein NEW (Poljak *et al.*, 1973). The parent antibody structures have all been solved crystallographically. The Bence–Jones protein REI is in the Vκ(I) family (Epp *et al.*, 1974), and the D1.3 antibody was solved alone and in complex with the antigen lysozyme (Amit *et al.*, 1986; Bhat *et al.*, 1990). We achieved large enhancement of antigen affinity by substitution of several framework residues of a group that may exert a determining influence on the conformation of the CDRs†.

2. Antibody Design

The original distinction between hypervariable regions and framework residues (Wu & Kabat, 1970) had its basis in homologies between the primary sequences of immunoglobulins known at that time. However, as X-ray crystallographic structures became available, it became apparent that residues in the hypervariable regions formed the apical loops connecting the β-strands of the immunoglobulin fold, but could also extend part way along the β-strands themselves (Poljak *et al.*, 1973; Schiffer *et al.*, 1973; Segal *et al.*, 1974). Indeed structural analyses have invoked fewer, and in some cases different, residue positions as CDRs (for example, see Chothia & Lesk, 1987) In X-ray crystallographic structures of antigen–antibody complexes, all the CDRs do not necessarily make contact to antigen (Tulip *et al.*, 1989) and in particular the C-terminal portion of VH-CDR2 has never been shown to interact directly with antigen.

We based our designs on Kabat (Kabat *et al.*, 1987). The construction of the reshaped heavy chain has been described (Verhoeyen *et al.*, 1988). The framework amino acid sequences chosen for the light chain CDR acceptor were designed *de novo*. They are a consensus of human κ subgroup I sequences (Kabat *et al.*, 1987) and closely related (but not identical) to the sequence of the myeloma protein REI (Palm & Hilschmann, 1973). The framework sequences of the first reshaped antibody differ from D1.3 at 18 positions in the light chain and 30 in the heavy chain. The location of these residues is shown in the α-carbon traces of the variable domains of the D1.3 structure in Figure 1(a). The differences are dispersed over the entire variable region of the antibody: all eight framework segments have multiple replacements. The most numerous, half of the total, occur in framework 3 (between CDR2 and CDR3) of both the light and heavy chain. As expected, few differences are seen at the VH–Vκ framework interface, as this region is generally conserved. Two-thirds of the framework differences are located on the molecule's surface and are unlikely to affect antigen binding. There are also differences in buried residues, particularly at the interfaces with the hypervariable regions. We considered retaining these buried framework residues from mouse D1.3 in the reshaped antibody (Padlan, 1991), but even buried residues can form the critical element of a T-cell epitope if presented as a denatured peptide by a class II MHC molecule (Allen *et al.*, 1985). This could exacerbate any humoral response to the native antibody, for example an anti-idiotype reaction, and we therefore used the entire human framework regions, whether the residues were buried or not.

To probe the role of specific framework residues in supporting the CDR conformations, we introduced several additional framework substitutions into the reshaped antibody (VL: Phe71 to Tyr; VH Ser27 to Phe, Thr28 Phe29 Ser30 to Ser Leu Thr, and Lys71 to Val; see Fig. 1(b)).

Residue 71 of the light chain lies in a loop connecting β-strands and the tyrosine ring of D1.3 protrudes inward and is sandwiched between this loop and VL-CDR1 loop. The phenolic oxygen appears to be important: it hydrogen-bonds to the amide nitrogen and carbonyl of Gly68 and the backbone amide nitrogen of Asn31, thus forming a bridge between the two loops. The neighbouring residue Tyr32 is in intimate contact with lysozyme residue Gln121, previously identified as a central feature of the D1.3 recognition site (Amit *et al.*, 1986). Residue Phe71 was therefore changed to Tyr.

Residues 27 to 30 of the heavy chain are part of a structural loop which includes VH-CDR1 (Kabat *et al.*, 1987). Indeed they are included within a structure-based hypervariable loop that comprises residues 26 to 32 (Chothia & Lesk, 1987) compared with VH-CDR1 that comprises 31 to 35 (Kabat *et al.*, 1987). These residues appear to make important interactions with CDR1 and CDR2. For example, in the D1.3 structure, Leu29 packs against Phe27 and Lys71 (see below), and Phe27 packs against residues 31 to 34. In turn Gly31 and Tyr32 of VH-CDR1 make contact with Lys16 of lysozyme. Residue Ser27 would be expected to create a cavity, and was therefore changed to Phe as in D1.3 and Riechman *et al.* (1988). Residues Thr28, Phe 29, Ser30 were changed *en bloc* to Ser, Leu, Thr.

Residue 71 of the heavy chain may fix the relative dispositions of CDR1 and CDR2, according to

† Abbreviations used: CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; K_{eq}, equilibrium dissociation constant; kb, 10^3 bases or base-pairs.

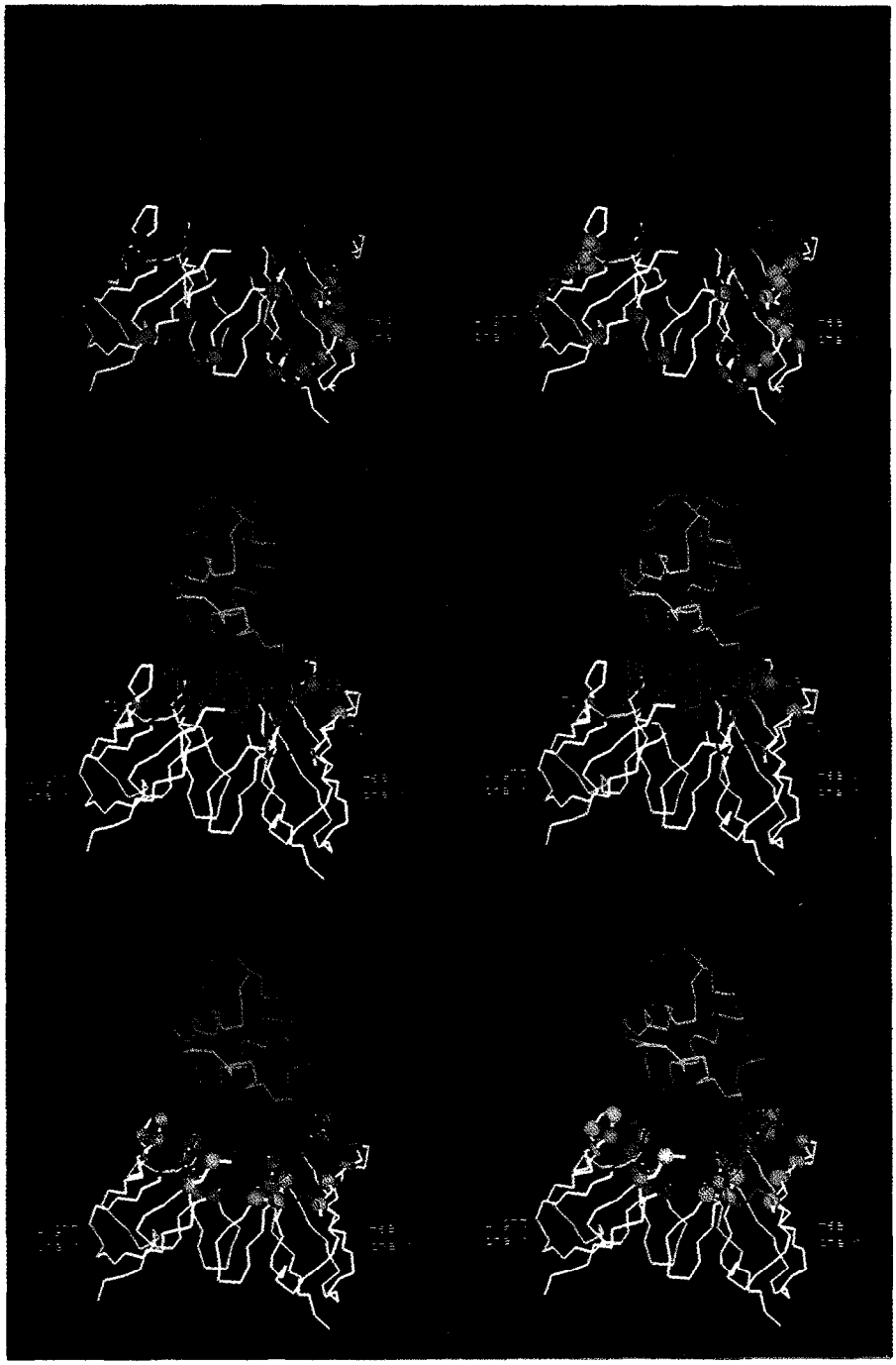

Figure 1. FabD1.3 complex with lysozyme. The α-carbon trace of the FabD1.3 (Fischmann *et al.*, 1991) is marked in white (framework region) and red (CDRs), and the α-carbon trace of lysozyme in blue. Residues corresponding to (a) differences between framework residues of mouse (D1.3) and reshaped antibody, (b) point mutations introduced into the framework of the reshaped antibody and (c) the Vernier zone, are highlighted in green.

whether there is bulky side-chain (Lys or Arg), or a smaller side-chain (Val, Ala) present (Tramontano *et al.*, 1990). We therefore changed Val71 to Lys as in D1.3.

3. Methods

(a) *Construction of reshaped light chain variable gene and Phe71 → Tyr mutant*

A myeloma expression vector used to produce engineered immunoglobulin heavy chains (Neuberger, 1983; Neuberger *et al.*, 1985) was adapted for light chain expression. This entailed synthesis and cloning the reshaped light chain V-gene and its introduction into the vector M13-HuVNP (Jones *et al.*, 1986) to replace the heavy chain variable gene.

A set of oligonucleotides was designed to encode the reshaped light chain V-gene with codon usage of mouse immunoglobulin sequences. However, the sequence encoding residues beyond number 96 of the mature protein was taken directly from the human J1 segment (Hieter *et al.*, 1982) including 30 nucleotides 3′ to the

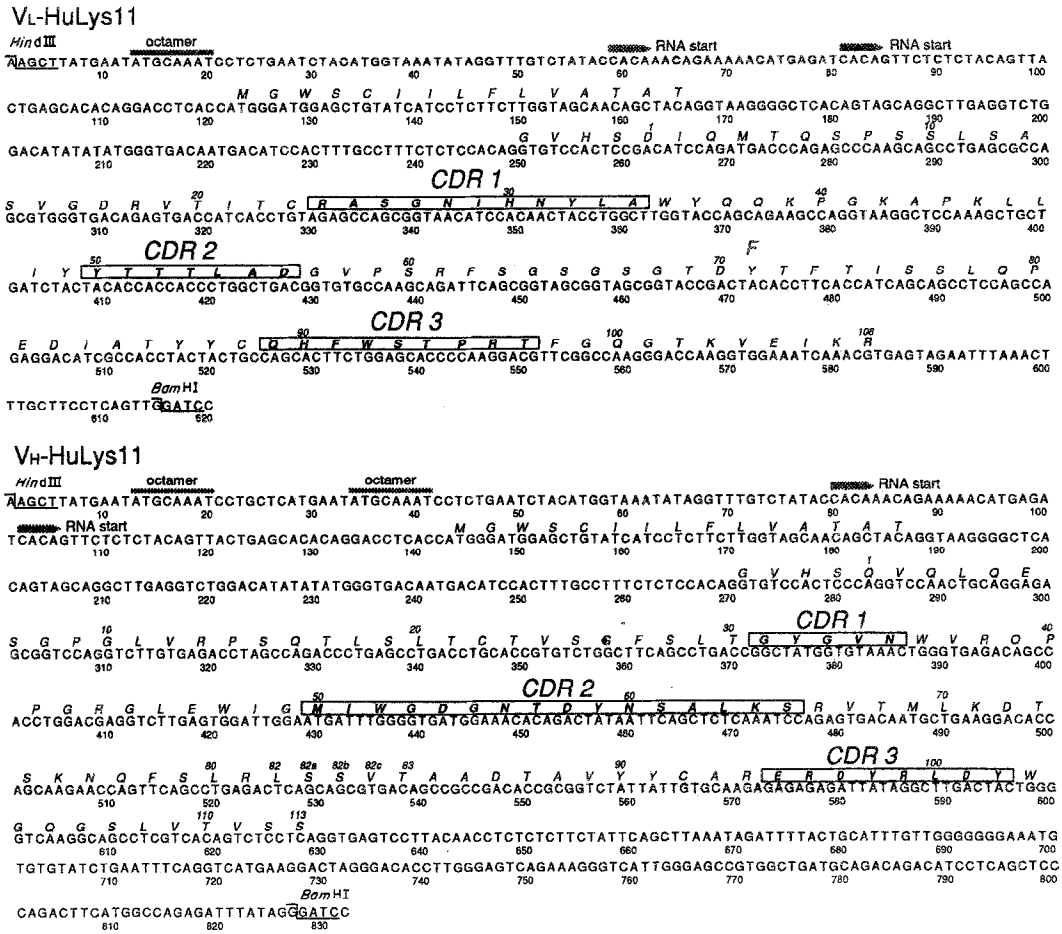

Figure 2. Nucleotide and encoded protein sequences of reshaped heavy and light chain variable domains. The DNA sequence encoding the highest-affinity reshaped antibody (HuLys11) is shown. The location of the mutations, transcriptional control motifs and protein translations appear above the corresponding nucleotide sequence, with nucleotide numbering underneath. Peptide numbering begins from the N-terminal residue of the mature protein. CDR sequences are boxed.

splice junction. Eighteen oligonucleotides were used to encode both strands of the 370 base-pair construct, and were assembled and cloned in three separate blocks, delimited by *Pst*I–*Kpn*I, *Kpn*I–*Kpn*I, and *Kpn*I–*Eco*RI sites. 50 pmol of each oligonucleotide was phosphorylated for 30 min at 37 °C in a 20 μl reaction mixture with 1 mM-ATP, 5 units polynucleotide kinase, 5 mM-dithiothreitol, 50 mM-Tris, 10 mM-$MgCl_2$ (pH 8). Portions (4 μl) of each phosphorylation mixture were annealed by heating together at 80 °C for 5 min, 67 °C for 30 min, and cooling gradually (30 min) to room temperature. 200 nl of annealing mix was ligated into 20 ng M13-mp19 vector (Yanisch-Perron *et al.*, 1985) in a volume of 10 μl (composition as above except for 120 units of T4 DNA ligase and a pH of 7·5). Recombinant clones were sequenced and the 3 blocks excised, gel-purified and assembled by ligation into M13-mp19 as a *Pst*I–*Eco*RI fragment.

The synthetic gene was introduced as a *Pst*I–*Bam*HI fragment into the vector M13-pHVNP and joined in-frame to the signal sequence with a mutagenic oligonucleotide (5′-TCA TCT GGA TGT CGG AGT GGA CAC CT-3′) (Zoller & Smith, 1982). Further derivatives were made: about 1·2 kb of sequence 5′ to the immunoglobulin octamer transcriptional control element (Falkner & Zachau, 1984) was deleted using the oligonucleotide 5′-TAG ATT CAG AGG ATT TGC ATA TTC ATA AGC TTG GGC TAA TCA T-3′, and a point mutation, Phe71 to Tyr, was introduced using the oligonucleotide 5′-GGT GAA GGT GTA GTC GGT ACC-3′. The annotated sequence of the mutated gene is given in Fig. 2.

(b) *Construction of mutants of reshaped heavy chain variable gene*

Mutants of the reshaped heavy chain variable gene (Verhoeyen *et al.*, 1988) were constructed by oligonucleotide-directed mutagenesis. As above, 1·2 kb of sequence 5′ to the octamer motif was deleted, but here it also resulted in spurious duplication of the motif. Residues 27 to 30, initially Ser-Thr-Phe-Ser, were changed to Phe-Ser-Leu-Thr with the mutagenic oligonucleotide 5′-TAC ACC ATA GCC GGT TAA GCT GAA GCC AGA CAC GGT-3′, Ser27 to Phe with the oligonucleotide 5′-GCT GAA GGT GAA GCC AGA CAC G-3′, and Val71 to Lys with the oligonucleotide 5′-TGC TGG TGT CCT TCA GCA TTG TC-3′. The annotated sequence of the mutated gene is shown in Fig. 2.

(c) *Eukaryotic expression vectors*

Fragments carrying heavy and light chain variable genes with signal sequences and promoters (as above)

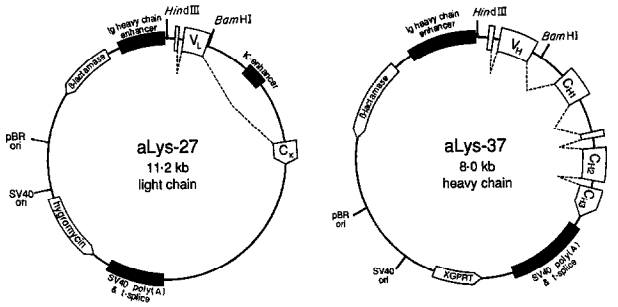

Figure 3. Vectors for expression of reshaped heavy (aLys37) and light (aLys27) chain variable regions as human γ1 and κ chains in myeloma cells. Immunoglobulin exons are indicated by wide boxes, the coding regions of β-lactamase, aminoglycoside phosphotransferase (hygromycin resistance) and bacterial xanthine-guanine phosphoribosyl transferase by narrow boxes, and splicing between immunoglobulin exons by broken lines.

were now introduced into expression vectors. A vector pSV-V_{NP}Hε (Neuberger *et al.*, 1985) based on the vector pSV2gpt (Mulligan & Berg, 1980), was further modified to facilitate rapid cloning of the variable genes and their co-expression as heavy and light chains in cultured myeloma cells. A human κ constant gene was built into a vector utilizing hygromycin resistance as a selectable marker, and a human heavy chain γ1 gene built into a vector utilizing mycophenolic acid resistance (Fig. 3).

The assembly of the vector for light chain expression involved a ligation of 3 fragments: a 2 kb *Hin*dIII–*Bam*HI fragment containing the synthetic variable domain described above; a *Hin*dIII–*Hin*dIII fragment containing the immunoglobulin heavy chain locus 5′ enhancer, β-lactamase gene, and SV40 origin of replication and promoters (M. S. Neuberger and L. Riechmann, unpublished results); a *Hin*dIII–*Bam*HI fragment containing the coding sequence of a hygromycin-specific aminoglycoside phosphotransferase, SV40 t-antigen splice site and poly(A) sequences, excised from the plasmid pSV2*hyg (A. Smith, D. Strehlow and A. Miyajima, unpublished results). Next, the vector was partially digested with *Hin*dIII, and the *Hin*dIII site at the end of the hygromycin fragment removed by fill-in extension with DNA polymerase I (Klenow fragment) and religation. The human Cκ constant region was then introduced as a *Bam*HI fragment at the unique *Bam*HI site of the vector. (A genomic fragment containing the human J and Cκ gene segments (Hieter *et al.*, 1982) was first cloned as a 10 kb *Bam*HI fragment in pUC7 (Vieira & Messing, 1982), the J-segments excised as a 5 kb *Hin*dIII fragment and the vector religated. The 5 kb *Bam*HI fragment containing the Cκ coding sequence and the κ enhancer, was used in the construction.) The *Bam*HI site at the 3′ end of the Cκ constant region, and a *Hin*dIII site internal to the κ fragment were then eliminated by fill-in and religation of partial digests.

Construction of a reshaped anti-lysozyme antibody with human γ2 constant region has been described (Verhoeyen *et al.*, 1988); our interest shifted to the γ1 isotype, as it is more potent in activation of the complement cascade and of antibody-dependent cell-mediated cytotoxicity (Brüggeman *et al.*, 1987; Riechmann *et al.*, 1988). A clone of the human γ1 constant region exons (Takahashi *et al.*, 1982), was provided as a 2 kb *Bgl*II fragment in the M13 phage tg131 (Kieny *et al.*, 1983) by M. Brüggemann. After *Hin*dIII digestion, fill-in and religation to destroy the internal *Hin*dIII site, the *Bgl*II fragment was introduced into the *Bam*HI-digested pSVgpt-HuV_HLYS-HulgG2 vector (Verhoeyen *et al.*, 1988) to replace the γ2 constant region exons. Although the ligation destroys the *Bam*HI sites of the vector backbone, an internal *Bam*HI site (present in the polylinker of the M13-tg 131 vector) remains at the V-proximal end of the insert.

(d) *Construction of transfectoma lines*

Light and heavy chain constructs were introduced into myeloma cells by electroporation (Potter *et al.*, 1984), and stably transformed cells selected on the basis of drug resistance and cloned. Generally we co-transformed cells with both light and heavy chain constructs simultaneously, but we also tried transforming first with light chain, cloning the intermediate light chain producing transfectoma, then transforming with the heavy chain construct. The sequential method gave higher transfection frequencies, and was a convenient strategy in creating a family of antibodies with identical light chains, but similar yields of antibody were purified from clones obtained either through sequential or cotransformation. The level of antibody production by individual clones of cells varied widely: the better clones were identified by ELISA and picked for large scale growth.

Plasmids were purified from 1 l of bacterial culture by alkaline detergent lysis and CsCl/ethidium bromide equilibrium density gradient centrifugation (Ish-Horowicz & Burke, 1981). Heavy chain constructs were linearized with *Pvu*I, a step which increased transfection frequency by more than 10-fold. Light chain constructs do not have a convenient restriction site for linearization, so the circular form was used. The cell line NS0 (Galfré & Milstein, 1981), a myeloma which produces neither endogenous immunoglobulin chain due to abolition of heavy chain transcription and a defect in the κ transcript (Carroll *et al.*, 1988) was grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 110 mg sodium pyruvate/l, 100 mg streptomycin/l, and 100,000 units penicillin/l. Cells were harvested at a density of 10^6 cells/ml, and held on ice. 10^7 cells were suspended in 0·1 ml PBS or medium, placed in a sterile plastic 0·4 cm × 1 cm cuvette and mixed with 10 μg of each DNA construct. After several minutes, three 2 kV pulses from an Apelex (Bagneux, France) cell porator were applied, 1 s apart, then the cells were returned to ice. The electroporation mixture was washed into a flask with 25 ml medium, and grown overnight. An equal volume of selective medium was added, and the suspended cells distributed over a 24-well plate. For selection of hygromycin resistance alone, cells were exposed to a drug concentration of 0·4 g/l. For co-selection of hygromycin and mycophenolic acid resistance, culture medium contained 0·2 g hygromycin/l, 0·8 mg mycophenolic acid/l, and 0·25 g xanthine/l. As stock solutions of xanthine were made in 0·1 M-NaOH, an equivalent of HCl was also added to preserve the pH of the growth medium.

An antigen-based ELISA procedure was devised to facilitate screening of transformants. Microtitre plates (Dynatech) were coated overnight with 0·3 g lysozyme/l in 50 mM-NaHCO3 (pH 9·6). The plates were washed with PBS, then blocked with 1% (w/v) bovine serum albumin in PBS for 5 min. 0·1 ml portions of culture supernatant were allowed to react for 2 h, and after washing, adsorbed antibody was quantified with peroxidase-conjugated rabbit anti-human IgG (Dakopatts, Denmark) diluted to

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