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Journal of Immunological Methods 201 (1997) 35–55

JOURNAL OF IMMUNOLOGICAL METHODS

Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system

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Received 5 July 1996; revised 23 September 1996; accepted 25 September 1996

Abstract

A prerequisite for the use of recombinant antibody technologies starting from hybridomas or immune repertoires is the reliable cloning of functional immunoglobulin genes. For this purpose, a standard phage display system was optimized for robustness, vector stability, tight control of scFv-ΔgeneIII expression, primer usage for PCR amplification of variable region genes, scFv assembly strategy and subsequent directional cloning using a single rare cutting restriction enzyme. This integrated cloning, screening and selection system allowed us to rapidly obtain antigen binding scFvs derived from spleen-cell repertoires of mice immunized with ampicillin as well as from all hybridoma cell lines tested to date. As representative examples, cloning of monoclonal antibodies against a his tag, leucine zippers, the tumor marker EGP-2 and the insecticide DDT is presented. Several hybridomas whose genes could not be cloned in previous experimental setups, but were successfully obtained with the present system, expressed high amounts of aberrant heavy and light chain mRNAs, which were amplified by PCR and greatly exceeded the amount of binding antibody sequences. These contaminating variable region genes were successfully eliminated by employing the optimized phage display system, thus avoiding time consuming sequencing of non-binding scFv genes. To maximize soluble expression of functional scFvs subsequent to cloning, a compatible vector series to simplify modification, detection, multimerization and rapid purification of recombinant antibody fragments was constructed.

Keywords: Phage display; Single-chain Fv; Monoclonal antibody; Antibody library

Abbreviations: BSA, bovine serum albumin; cam, chloramphenicol; CDR, complementarity determining region; cfu, colony forming units; DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane; EGP-2, epithelial glycoprotein-2; ELISA, enzyme linked immunosorbent assay; EMCS, *N*-(ε-maleimido-caproxyloxy)succinimide; FR, framework; gIIIp, wild-type geneIII protein of filamentous phage; IMAC, immobilized metal affinity chromatography; IPTG, isopropylthiogalactoside; LZ, leucine zipper; nt, nucleotide; OD, optical density; PBS, phosphate buffered saline; pelB, pectate lyase gene of *Erwinia carotovora*; PCR, polymerase chain reaction; scFv, single-chain Fv fragment; SD, Shine-Dalgarno sequence; SDS, sodium dodecylsulfate; SDT7g10, Shine-Dalgarno sequence of T7 phage gene10; SOE-PCR, splicing by overlap extension PCR; tet, tetracycline; V_H, heavy chain variable domain; V_L, light chain variable domain.

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PII S0022-1759(96)00208-6

1. Introduction

Molecular cloning and sequencing of antibody variable domains forms the basis of antibody modelling (Rees et al., 1994), antibody engineering (Plückthun, 1994; Nilsson, 1995) and experimental structure determination by NMR (Freund et al., 1994) or X-ray crystallography at high resolution (Ostermeier et al., 1995). Moreover, once the variable region genes have been cloned, the antibody domains can be further engineered in a multitude of ways to produce antibody variants with lower immunogenicity (Güssow and Seemann, 1991), higher affinity (Marks et al., 1992; Riechmann and Weill, 1993; Deng et al., 1994), altered antigenic specificity (Ohlin et al., 1996), or enhanced stability (Glockshuber et al., 1990; Reiter et al., 1994). Furthermore, genetic fusions of scFv fragments to effector proteins and toxins are powerful tools in the fields of medicine and diagnostics (Huston et al., 1993).

In all application areas, the demand for efficient generation of functional antibody fragments increases continuously. Although large prefabricated antibody libraries are gradually becoming a source of recombinant antibody fragments that cover a wide range of useful affinities (Vaughan et al., 1996), it may still be necessary to use the diversity of the immune system to create the most extensive panel of different antibodies against a given target possible. Furthermore, it is often of great interest and importance to clone V_H and V_L domains of the natural antibody response to a given antigen. In cases in which a large amount of experimental or clinical data is available on a given monoclonal antibody (mAb), it is frequently useful to base new constructs on this work and to determine its specific sequence and binding mode. Cloning and sequencing retains and immortalizes the unique and extensively characterized specificity of mAbs, which can be crucial for the rescue of unstable hybridoma cell lines.

One major problem in rapidly and simply obtaining sequence information about mAbs stems from the occurrence of aberrant mRNAs which are transcribed from rearranged, but non-functional, heavy and light chain genes in the hybridoma (Cabilly and Riggs, 1985; Strohal et al., 1987; Carroll et al., 1988; Kaluza et al., 1992; Kütemeier et al., 1992; Nicholls et al., 1993; Duan and Pomerantz, 1994; Yamanaka et al., 1995; Ostermeier and Michel, 1996). These non-productive chains are frequently preferentially amplified over the productive ones by sets of primers specific for the variable regions of antibody genes. The aberrant chains may greatly dilute the desired antibody sequences, which are the only ones binding the antigen in a pool of non-productive antibody-like sequences. Several attempts have been reported to overcome this problem, such as ribozyme cleavage of a known aberrant κ chain sequence (Duan and Pomerantz, 1994), treatment of aberrant mRNA/DNA hybrids with RNAseH (Ostermeier and Michel, 1996), or functional screening for full length scFv products in an in vitro transcription/translation system (Nicholls et al., 1993). Each of these methods is time consuming, depends on prior sequence information of the contaminating gene and fails to enrich binding molecules by selection procedures. Since antibody genes are usually amplified by PCR using degenerate sets of primers, mismatches and PCR errors will lead to point mutations or out-of-frame clones, which can also contribute to a background of non-functional scFv molecules. Therefore, it is absolutely vital, but often neglected (Miller et al., 1995; Kwak et al., 1996), that the binding specificity of the recombinant antibody sequence is demonstrated to be comparable with the binding characteristics of the parental monoclonal antibody, even when the deduced antibody sequence seems reasonable.

The inherent advantage of phage display is its direct link of DNA sequence to protein function (McCafferty et al., 1990; Winter et al., 1994). Thus, single clones can be rapidly screened for antigen binding and, even more importantly, selected from pools in the same experimental setup. This obviates the use of sequence specific methods to eliminate undesired sequences and leads to a more generally applicable procedure for hybridoma cloning.

However, phage display suffers from the fact that non-productive, aberrant chains are often very well expressed and non-toxic to the bacterial cell, whereas cells expressing functional scFv-geneIII fusions have a growth disadvantage and are selected against. The scFv-geneIII fusion protein can cause vector instability, creating deletions in the antibody fusion genes as occasionally observed (Courtney et al., 1995; Dziegiel et al., 1995; A. Krebber, unpublished obser-

vations; footnote 1). Thus, it is highly recommended to use a regulatable vector system allowing tight product suppression during all propagation steps as well as controlled expression of low amounts of scFv-geneIII fusion protein for phage display. Since a variety of serious technical problems concerning hybridoma cloning and enrichment of binding antibody fragments from phage display libraries have been reported [1], we have developed the reengineered phage display system described in this work. In order to provide a robust and straightforward methodology which ensures fast and reliable cloning, not only of hybridomas but also of larger antibody libraries, each step in the process was optimized. To illustrate the utility of our improved phage display system we report in detail several case studies of successfully cloned scFvs derived from monoclonal antibodies as well as enrichment of binding scFv sequences from cloned B cell repertoires.

2. Materials and methods

2.1. Isotyping

Isotypes of the mAbs were determined using the IsoStrip mouse monoclonal antibody isotyping kit (Boehringer Mannheim).

2.2. Preparation of mRNA

mRNA was extracted from $1–5 \times 10^6$ hybridoma or spleen cells using the QuickPrep mRNA purification kit from Pharmacia. In the case of hybridoma cell lines 13AD and 42PF total RNA was isolated essentially as described by Berger and Chirgwin (1989).

2.3. First strand cDNA synthesis

About 1 μg mRNA or 5 μg total RNA was reverse transcribed in a reaction volume of 33 μl using random hexamer primers according to the manufacturer's protocol (first strand cDNA synthesis kit (Pharmacia)).

2.4. PCR amplification of V_L and V_H

Various DNA polymerases (Taq (Perkin Elmer, Gibco), Pwo (Boehringer Mannheim), Pfu (Stratagene), Vent (New England Biolabs)) were successfully used for separate amplifications of V_L and V_H. For amplification of V_L from hybridomas either λ or κ primers were chosen according to the isotype. PCR reactions were performed in 50–100 μl volumes, containing 2–5 μl of cDNA reaction, 2 μM of LB and LF primer mixes (Table 1, Fig. 1B) for amplification of V_L or 2 μM of HB and HF primer mixes (Table 1, Fig. 1B) for amplification of V_H, 200 μM dNTPs, an optimized Mg^{2+} concentration (2–6 mM) and reaction buffer supplied by the manufacturers. After 3 min denaturation at 92°C, 2 U of DNA polymerase were added, followed by 7 cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C, 1 min at 72°C, and 23 cycles of 1 min at 92°C, 30 s at 63°C, 1 min at 72°C. One tenth of each PCR reaction was analyzed by agarose gel electrophoresis (Fig. 2).

2.5. Assembly PCR

The full length PCR products of V_L and V_H were purified by preparative agarose gel electrophoresis in combination with the QIAEX (Qiagen) or Jetsorb (Genomed) DNA extraction kit. Approximately 10 ng of each V_L and V_H DNA were combined by SOE-PCR (Fig. 1C; Ge et al., 1995). An initial denaturation step (3 min, 92°C) was followed by 2 cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C, 1 min at 72°C in the absence of primers. After adding the outer primers scback and scfor (Table 1, Fig. 1C; each 1 μM), 5 cycles of 1 min at 92°C, 30 s at 63°C, 50 s at 58°C, 1 min at 72°C, and 23 cycles of 1 min at 92°C, 30 s at 63°C, 1 min at 72°C were performed. One tenth of each PCR reaction was analyzed by agarose gel electrophoresis (Fig. 2).

2.6. SfiI digest and cloning of scFv fragments into pAK100

The gel-purified scFv fragment and the phage display vector pAK100 were both digested with SfiI

[1] For typical examples, see the internet discussion forums, http://www.bio.net/hypermail/METHDS-REAGNTS, http://www.bio.net/hypermail/MOLECULAR-REPERTOIRES.

for 3 to 4 h at 50°C (Fig. 1D). After purification, the scFv fragment was ligated into the vector (molar ratio vector to insert 1.5 : 1) and transformed into *E. coli* XL1-Blue (Stratagene). For library construction, ligation mixtures precipitated with n-butanol (Thomas, 1994) were electroporated into XL1 Blue (Dower et al., 1988; yield approximately 5×10^7 clones per μg SfiI-cut insert DNA). After plating on NE medium (non expression medium: 2 × YT containing 1% glucose and 25 μg/ml chloramphenicol)

Table 1
Listing of the primers used for assembling mouse scFv fragments in the orientation VL-$(G_4S)_4$-VH, which are compatible with the pAK vector system presented in Fig. 1 and Fig. 4

Primer VL back:

	5' FLAG VL3'		
scback	ttactcgcggcccagccggccatggcggactacaaaG		
	5' FLAG VL 3'	d	µl Mix
LB1	gccatggcggactacaaaGAYATCCAGCTGACTCAGCC	2	1
LB2	gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC	4	2
LB3	gccatggcggactacaaaGAYATTGTGMTMACTCAGTC	8	5
LB4	gccatggcggactacaaaGAYATTGTGYTRACACAGTC	8	3.5
LB5	gccatggcggactacaaaGAYATTGTRATGACMCAGTC	8	4
LB6	gccatggcggactacaaaGAYATTMAGATRAMCCAGTC	16	7
LB7	gccatggcggactacaaaGAYATTCAGATGAYDCAGTC	12	6
LB8	gccatggcggactacaaaGAYATYCAGATGACACAGAC	4	1.5
LB9	gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC	4	2
LB10	gccatggcggactacaaaGAYATTGWGCTSACCCAATC	8	3.5
LB11	gccatggcggactacaaaGAYATTSTRATGACCCARTC	16	8
LB12	gccatggcggactacaaaGAYRTTKTGATGACCCARAC	16	8
LB13	gccatggcggactacaaaGAYATTGTGATGACBCAGKC	12	6
LB14	gccatggcggactacaaaGAYATTGTGATAACYCAGGA	4	2
LB15	gccatggcggactacaaaGAYATTGTGATGACCCAGWT	4	2
LB16	gccatggcggactacaaaGAYATTGTGATGACACAACC	2	1
LB17	gccatggcggactacaaaGAYATTTTGCTGACTCAGTC	2	1
LBλ	gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC	1	1

Primer VL for:

	5' (Gly4Ser)3-linker VL 3'		
LF1	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTGATTTCCAGCTTGG	1	1
LF2	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTTATTTCCAGCTTGG	1	1
LF4	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG	1	1
LF5	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG	1	1
LFλ	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG	1	0.25

Primer VH back:

	5' (Gly4Ser)2-linker BamHI VH 3'	d	µl Mix
HB1	**ggcggcggcggctcc**ggtggtggtggatccGAKGTRMAGCTTCAGGAGTC	8	4
HB2	**ggcggcggcggctcc**ggtggtggtggatccGAGGTBCAGCTBCAGCAGTC	9	4
HB3	**ggcggcggcggctcc**ggtggtggtggatccCAGGTGCAGCTGAAGSASTC	4	3
HB4	**ggcggcggcggctcc**ggtggtggtggatccGAGGTCCARCTGCAACARTC	4	4
HB5	**ggcggcggcggctcc**ggtggtggtggatccCAGGTYCAGCTBCAGCARTC	12	7
HB6	**ggcggcggcggctcc**ggtggtggtggatccCAGGTYCARCTGCAGCAGTC	4	2
HB7	**ggcggcggcggctcc**ggtggtggtggatccCAGGTCCACGTGAAGCAGTC	1	1
HB8	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGAASSTGGTGGAATC	4	2
HB9	**ggcggcggcggctcc**ggtggtggtggatccGAVGTGAWGYTGGTGGAGTC	12	5
HB10	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGCAGSKGGTGGAGTC	4	2
HB11	**ggcggcggcggctcc**ggtggtggtggatccGAKGTGCAMCTGGTGGAGTC	4	2
HB12	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGAAGCTGATGGARTC	2	2
HB13	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGCARCTTGTTGAGTC	2	1
HB14	**ggcggcggcggctcc**ggtggtggtggatccGARGTRAAGCTTCTCGAGTC	4	2
HB15	**ggcggcggcggctcc**ggtggtggtggatccGAAGTGAARSTTGAGGAGTC	4	2
HB16	**ggcggcggcggctcc**ggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG	8	5
HB17	**ggcggcggcggctcc**ggtggtggtggatccCAGGTCCAACTVCAGCARCC	6	3.5
HB18	**ggcggcggcggctcc**ggtggtggtggatccGATGTGAACTTGGAAGTGTC	1	0.7
HB19	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGAAGGTCATCGAGTC	1	0.7

Primer VH for:

	5'EcoRI 3'		
scfor	ggaattcggcccccgag		
	5'EcoRI VH 3'		
HF1	ggaattcggcccccgaggcCGAGGAAACGGTGACCGTGGT	1	1
HF2	ggaattcggcccccgaggcCGAGGAGACTGTGAGAGTGGT	1	1
HF3	ggaattcggcccccgaggcCGCAGAGACAGTGACCAGAGT	1	1
HF4	ggaattcggcccccgaggcCGAGGAGACGGTGACTGAGGT	1	1

in 530 cm^2 dishes (Nunc) and overnight incubation at RT, the colonies were scraped off the plates into 8 ml 2 × YT (Sambrook et al., 1989) and subsequently stored at −80°C after addition of 10% glycerol.

2.7. Rescue of scFv displaying phages

To rescue scFv displaying phages, 50 ml NE medium was inoculated with approximately 10^9 cells from the glycerol library stock. The culture was then shaken at 37°C. At $OD_{550} = 0.5$, 10^{11} cfu helper phage VCS (Stratagene) and 25 μl 1 M IPTG solution were added. After 15 min incubation at 37°C without agitation, the culture was diluted in 100 ml LE medium (low expression medium: 2 × YT containing 1% glucose, 25 μg/ml chloramphenicol and 0.5 mM IPTG). The culture was then shaken for 10 h at 26°C for phage production. 2 h after infection 30 μg/ml kanamycin was added. Phage particles were purified and concentrated 100-fold by two PEG/NaCl precipitations (Sambrook et al., 1989), resuspended in PBS and stored at 4°C. After overnight culture typically a phage titer in the range of 10^{11} cfu/ml was observed.

2.8. Selection of antigen binders by panning

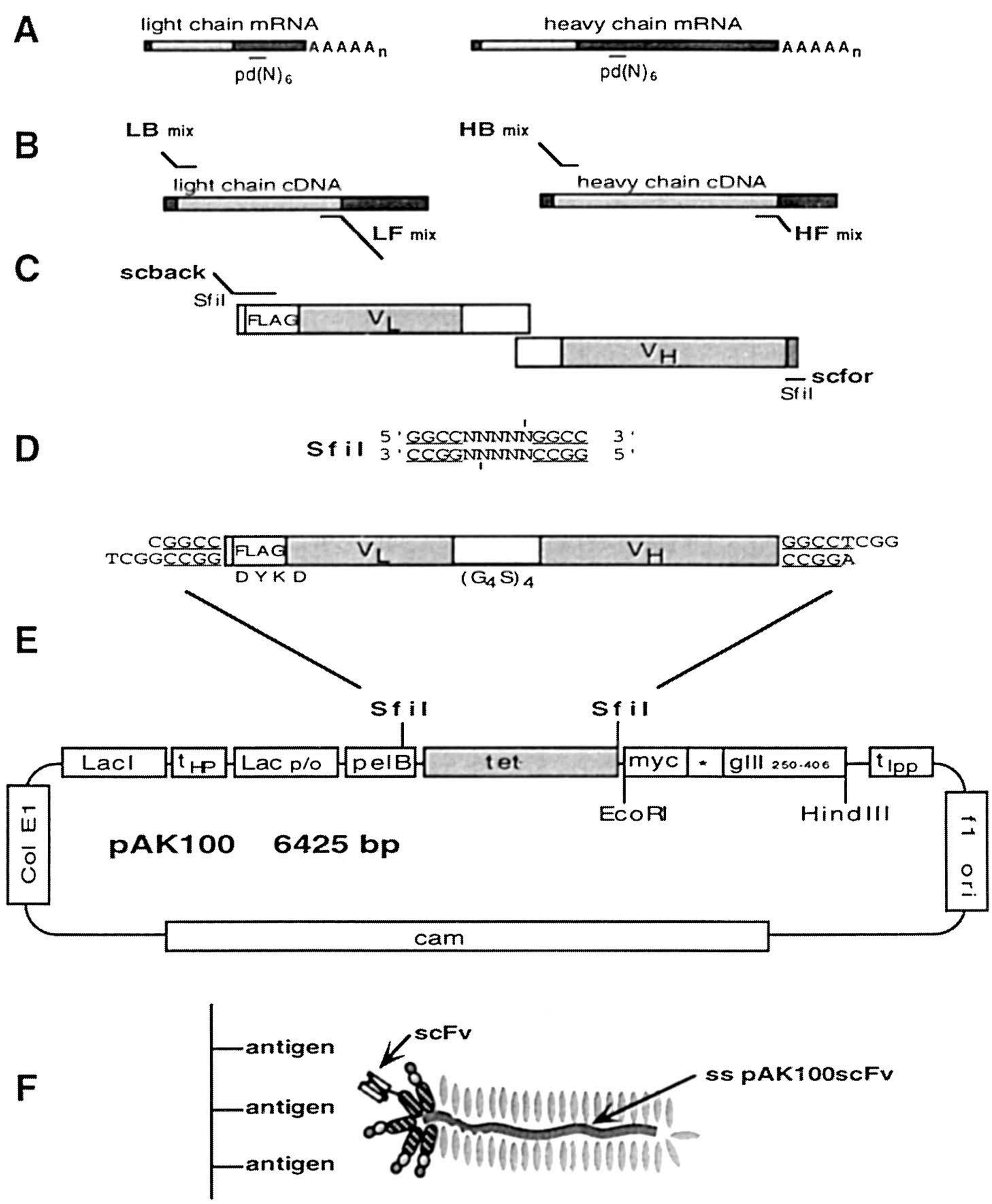
For selection, immunotubes (Nunc, Maxisorp) were coated overnight at 4°C with 4 ml of 10–100 μg/ml antigen solution (for the anti-ampicillin libraries: 100 μg/ml transferrin-EMCS-ampicillin in PBSU (1 : 1 mixture of PBS (20 mM NaP_i, 150 mM NaCl, pH 7.2) and urea-NaP_i buffer (8 mM urea, 50 mM NaP_i, pH 7.0)); for anti-leucine zipper mAb 13AD: 10 μg/ml biotin-LZ_{ss}/streptavidin complex (Leder et al., 1995) in 34.8 mM $NaHCO_3$, 15 mM Na_2CO_3, 0.02% NaN_3, pH 9.6; for the anti-EGP-2 mAb MOC31: 100 μg/ml EGP-2 in PBS). After blocking with 4% dried skimmed milk powder in PBS for 2 h at room temperature (RT), 10^{11} phagemid particles in 4 ml PBS containing 2% milk were added and incubated for 2 h with rocking at RT. Tubes were then washed 15 times with PBS/0.1% Tween and 15 times with PBS. Bound phages were eluted from the tube with soluble antigen (1 ml 10 mM ampicillin in PBS; anti-ampicillin libraries) or 800 μl 0.1 M glycine/HCl pH 2.2 for 10 min. The latter solution was neutralized with 48 μl 2 M Tris and the phages (typically 10^4 –10^6 cfu/ml) were used for reinfection (30 min at 37°C without agitation) of *E. coli* XL1-Blue cells in NE medium ($OD_{550} = 0.5$–0.8). In the case of elution with ampicillin, the solution was treated with 2 units of β-lactamase for 15 min before reinfection. This sublibrary was rescued as described above and subjected to further panning rounds or binding analysis by phage ELISA.

2.9. Phage ELISA

Single colonies were grown separately in 2 ml NE medium at 37°C. After reaching $OD_{550} = 0.3$, 1 ml

Table 1 (continued). In this nomenclature, 'back' refers to 'toward the 3′ end of the antibody gene' and 'for' to 'toward the 5′ end of the antibody gene'. The sequences are given using the IUPAC nomenclature of mixed bases (shown in underlined capital letters, R = A or G; Y = C or T; M = A or C; K = G or T; S = C or G; W = A or T; H = A or C or T; B = C or G or T; V = A or C or G; D = A or G or T), with a column listing the d-fold degeneration encoded in each primer and the μl to be used to set up the PCR mix. The LB1-LB17 series encodes a stretch of 20 bases hybridizing to the mature mouse antibody κ sequences (in capital letters). Underlined is the preceding sequence which encodes the shortened FLAG sequence (Knappik and Plückthun, 1994). Since the FLAG tag uses the fixed N-terminal aspartate of the mature antibody (encoded by GAY), only three additional amino acids are necessary. The FLAG codons are then preceded by the codons specifying the end of the *pelB* signal sequence. The LBλ primer for mouse lambda chains is constructed in an analogous manner (the N-terminal glutamate of the mature mouse λ sequence is replaced by aspartate (encoded by GAT) to generate a FLAG tag). The VLfor primer sequences are complementary to the J elements of κ or λ chains (capital letters) and encode three repeats of the Gly_4Ser sequence, the terminal one (bold) of which has a different codon usage so that incorrect overlaps during the PCR assembly reaction are minimized. The VHback primers encode the other part of the linker as well as a BamHI recognition site (underlined), and the overlap with VLfor in the sequence shown in bold. The 20 bases given in capital letters hybridize with the mature mouse V_H sequences. The last 19 nt at the 3′ end of the VHfor primers hybridize with the J_H region. The first nt shown in capital letters will introduce a silent mutation at the end of V_H in order to code for the first nt of the second SfiI recognition site (underlined). The final assembly of the scFv gene by SOE-PCR is carried out with the scback and scfor primer set. The outer primer scback encodes the first SfiI site (underlined). All primers contain a phosphorothioate at the 3′ end, with the exception of scfor, to avoid potential interference with SfiI digestion.

NE medium complemented with 5×10^9 cfu VCS helper phage (Stratagene) and 1.5 mM IPTG was added. The cultures were allowed to produce scFv displaying phages during overnight incubation at 24°C. Phages from 1.6 ml culture supernatants were PEG precipitated and dissolved in 200 μl PBS. For anti-leucine zipper hybridomas 13AD and 42PF, scFv displaying phages were produced and concentrated 100-fold by PEG precipitation as described for the phage panning experiments. 10–100 μg/ml antigen (for the anti-his tag hybridoma 3D5: 100 μg/ml his-tagged citrate synthase in PBS; for the anti-leucine zipper hybridoma 42PF: 10 μg/ml BSA-LZ(7P14P) in 34.8 mM $NaHCO_3$, 15 mM Na_2CO_3, 0.02% NaN_3, pH 9.6; for the anti-DDT hybridoma 3D7: 70 μg/ml β-alaninamide-DDT conjugated to lysozyme; for the anti-ampicillin library and the hybridomas 13AD and MOC31: coating solutions as described for the panning procedure above) was coated onto NUNC plastic ELISA plates by overnight incubation at 4°C. 50 μl phage solution per well was mixed with 50 μl 4% dried skimmed milk powder in



PBS in the presence or absence of competing soluble antigen and incubated at RT for 10 min. After washing and blocking (blocking buffer: 4% dried skimmed milk powder in PBS) of the ELISA wells, the phage solution was added and incubated for 1 h at RT. After washing, 100 μl of 1/5000 diluted HRP/anti M13-conjugate (Pharmacia) in blocking buffer was added and incubated for 1 h at RT. For detection, 100 μl soluble BM blue POD-substrate (Boehringer Mannheim) was used.

2.10. Soluble expression of scFv fragments in pAK300 and pAK400

For soluble expression, 20 ml expression medium (2 × YT containing 25 μg/ml chloramphenicol) was inoculated with 200 μl of preculture (JM83 harboring the expression plasmid for the anti-ampicillin scFv antibody aL2, pAK300scFvaL2 or pAK400scFvaL2, respectively; grown overnight at RT in NE medium) and incubated in a shaking waterbath at 24°C. Expression was induced at OD_{600} = 0.5 with 1 mM IPTG and allowed to proceed for 4 h at 24°C. To monitor total scFv production, an aliquot of the culture was diluted in PBS to an OD_{600} = 1 and mixed with 5 × reducing SDS-PAGE sample buffer (Sambrook et al., 1989). The rest of the culture was adjusted to OD_{600} = 5 by centrifugation and resuspension in 2 ml PBS. The cells were disrupted three times by French press and separated into soluble and insoluble fractions by centrifugation (soluble fraction = supernatant; insoluble fraction = pellet vortexed in the same buffer volume). Aliquots of both fractions were mixed with 5 × reducing SDS-PAGE sample buffer. A 12.5 μl aliquot of each sample was used for 0.1% SDS-12% PAGE and subsequent Western blot detection.

2.11. Western blot

Gels were blotted onto PVDF membranes using standard protocols. The scFv fragments were detected using the anti-FLAG mAb M1 (Kodak), followed by an anti-mouse IgG peroxidase conjugate, essentially as described in Knappik and Plückthun (1994).

2.12. Sequencing

Nucleic acid sequences were determined either by manual Sanger dideoxy sequencing (USB Sequenase kit), or by cycle sequencing (Sequi Therm Long-Read Cycle Sequencing Kit-LC, Epicentre Technologies) with fluorescent primers using a DNA sequencer (LI-COR).

Fig. 1. Scheme of amplification and cloning procedure. *A*: cDNA synthesis. mRNA derived from spleen cells or hybridomas and random hexamer primer ($pd(N)_6$) or subclass specific primers (not shown) are used for cDNA synthesis. *B*: PCR amplification of V_L and V_H domains. The cDNA is used as the PCR template for the amplification of V_L and V_H domains by the primer mixes indicated (listed in Table 1). *C*: assembly SOE-PCR. V_L and V_H PCR products are first assembled into the scFv format (splicing by overlap extension) without primers and subsequently amplified by the outer primer pair scback and scfor. *D*: SfiI digestion of the amplified scFv fragment. The rare cutting enzyme SfiI is the only enzyme used for antibody cloning. *E*: ligation of SfiI digested pAK100 vector and insert. Note that directional cloning of the SfiI inserts is guaranteed because of the different SfiI sticky ends shown. In addition, self-ligation of insert or vector molecules is excluded by the asymmetry of the overhang. The phage display vector pAK100 contains a *tet* resistance cassette (*tetA* and *tetR*; 2101 bp) to facilitate monitoring of complete SfiI digestion by gel electrophoresis and by religating and subsequent plating on tet plates, the *lacI* repressor gene, a strong upstream terminator (t_{HP}), the *lac* promoter/operator and the *pelB* leader sequence, which has been modified to contain an SfiI site (for details see Fig. 3A). After ligation, the antibody fragment is fused in frame to geneIII250–406 (Fig. 3B). The in-frame fusion contains a myc-tag (Munro and Pelham, 1986) to act as a detection handle, in addition to the short 3-amino acid FLAG tag at the N-terminus (Knappik and Plückthun, 1994). The asterisk represents an amber codon. The geneIII portion starts at position 250 of the wt geneIII protein (gIIIp), thus avoiding extraordinarily long glycine linkers and, most importantly, any unpaired cysteine of gIIIp. The expression cassette is followed by a downstream terminator (t_{lpp}). The origins for phage replication and plasmid replication are as described in Ge et al. (1995). The chloramphenicol (cam) cassette is originally derived from pACYC184, but its expression strength has been modified by randomizing the promoter and selecting clones with optimal growth and selection properties (Krebber et al., 1995). *F*: detection and enrichment of binding scFv sequences by phage display. The scFv insert is displayed on the tip of filamentous phage whereas the genetic information encoding for the particular scFv fragment is packaged as single stranded DNA (ss pAK100scFv) in the phage interior. Panning of single-chain antibody displaying phages against the antigen allows the enrichment of functional antibody sequences.

3. Results

3.1. Design features of the improved phage display system

The reengineered phage display system and optimized methodology used in this work combines the following significantly improved features.

(i) In many cases, previously reported primer sets were too restricted to amplify either particular light or heavy chains (Table 2). Therefore, the set of mouse primers used in this study (Table 1) has been extended and optimized. It incorporates all mouse V_H, V_λ and V_κ sequences collected in the Kabat data base (Kabat et al., 1991) and combines extended primer sets described by Kettleborough et al. (1993); Ørum et al. (1993) and Zhou et al. (1994).

(ii) The V_L back primer set encodes a convenient, shortened version of the FLAG peptide, which introduces only three additional amino acids at the N-terminus of V_L. In this way, the scFv can be easily detected and purified by a commercially available mAb (Knappik and Plückthun, 1994; Ge et al., 1995; Kalinke et al., 1996).

(iii) To minimize PCR errors, polymerases with proof-reading capacity are used whenever possible (Marks et al., 1991; Yamanaka et al., 1995).

(iv) The scFv fragment is efficiently assembled by SOE-PCR (splicing by overlap extension; Horton et al., 1989) from two (Ge et al., 1995; Vaughan et al., 1996) rather than three pieces (Clackson et al., 1991; Ørum et al., 1993; recombinant phage antibody system (Pharmacia)).

(v) To avoid the occurrence of incorrect overlaps during assembly PCR, the four (Gly_4Ser) repeats in the single chain linker region are encoded by different codons (Table 1; Ge et al., 1995). In order to reduce the dimerization or aggregation tendency of scFv fragments (Desplancq et al., 1994; Huston et al., 1995), the linker between V_L and V_H is 20 amino acids in length rather than the frequently used 15 amino acids long variant.

(vi) SfiI is the only enzyme used for directional cloning of scFv fragments into the optimized phage display vector pAK100 (Fig. 1E). The use of this enzyme has a number of distinct advantages: SfiI recognizes eight bases, interrupted by five non-recognized nucleotides (5′-GGCCNNNNNGGCC-3′). SfiI restriction sites are therefore very rare in antibody sequences, thus elimination of potentially interesting sequences by internal digestion is very unlikely. Two different sticky ends were designed to allow cloning of the scFv fragment in a directional manner. In contrast to the palindromic sticky ends, 3 bp overhangs derived from SfiI sites render impossible self-dimerization by either insert or vector. Finally, SfiI has the interesting property that it always cuts two sites at once (Wentzell et al., 1995), and therefore single-cut plasmids or inserts do not occur as intermediates. Digestion of vectors or inserts with single SfiI sites requires the binding of two different DNA molecules to the restriction enzyme and slows the turnover rate (Wentzell et al., 1995). While vectors with asymmetric SfiI sites have been described (Zelenetz, 1992; Barbas and Wagner, 1995; Yang et al., 1995), surprisingly this feature has not been used in antibody library cloning. Usually only one SfiI site, mostly in combination with NotI as a second site, is used (Hoogenboom et al., 1991; Ørum et al., 1993; Vaughan et al., 1996). Other systems employ a set of four enzymes to clone V_L and V_H independently of each other (Orlandi et al., 1989; Barbas et al., 1991; Johansen et al., 1995; Yamanaka et al., 1995). These systems thus run a significantly

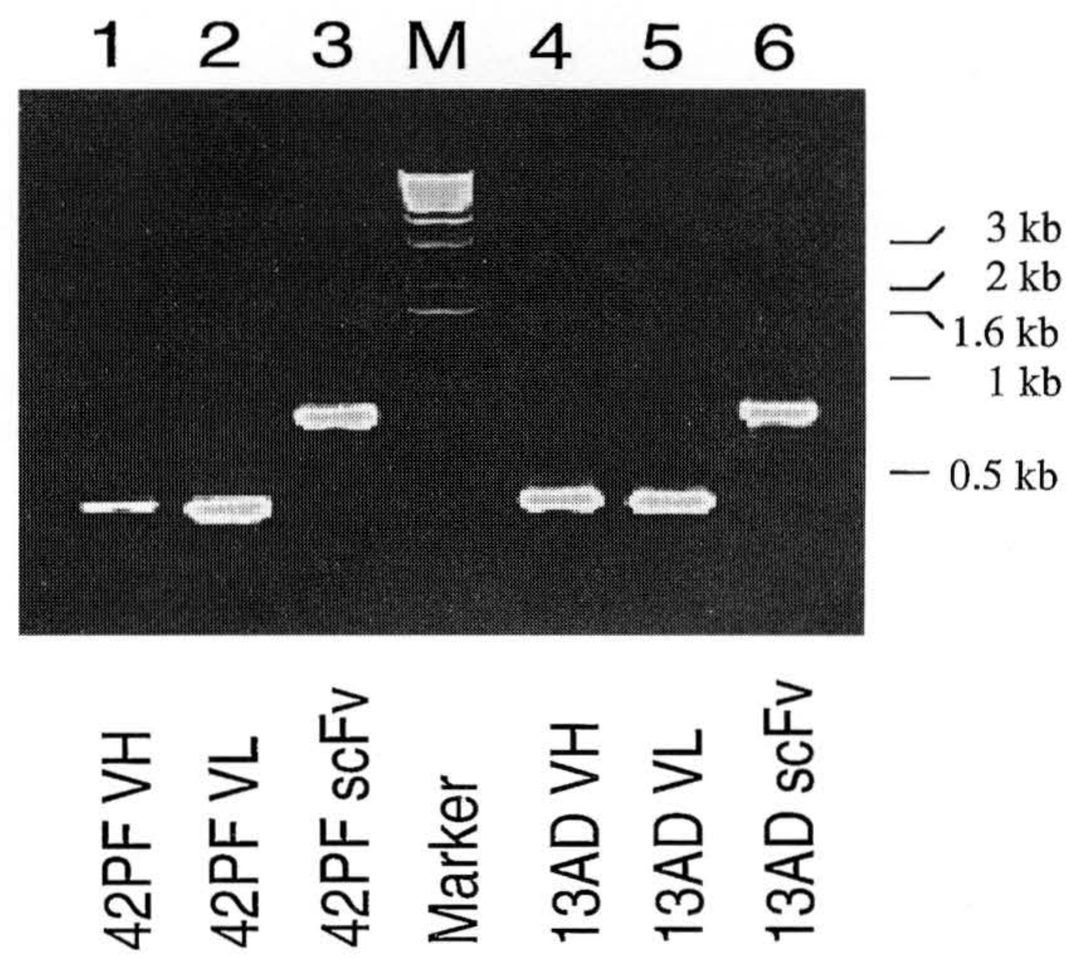


Fig. 2. DNA gel. PCR amplification of V_L and V_H from mouse hybridomas 13AD and 42PF using the primer sets listed in Table 1. Lane 1: V_H mAb 42PF; lane 2: V_L mAb 42PF; lane 3: assembled scFv 42PF; M: 1 kb marker (Gibco); lane 4: V_H mAb 13AD; lane 5: V_L mAb 13AD; lane 6: assembled scFv 13AD. A 1.5% agarose gel is shown.

higher risk of cutting in antibody genes and they also incorporate internal restriction sites in the variable region genes that create mismatches with the antibody template and bias amplification by poor primer hybridization. Furthermore, AvrII, SacI and SpeI sites, which are present in most sets of enzymes used to date, cut in the majority of mouse λ chains and are therefore not suitable for simultaneous cloning of λ and κ light chains.

(vii) A frequently observed phenomenon is the contamination of antibody libraries with uncut recipient vectors (Courtney et al., 1995; Johansen et al., 1995). Normally antibody-free vectors have a growth advantage over scFv-encoding ones and cause problems during the enrichment of antigen-binding antibody sequences by phage display. Therefore, the pAK100 vector (Fig. 1E), with a tetracycline resistance cassette (*tetA* and *tetR*; 2101 kb) inserted between the two different SfiI sites is used as the recipient. Digestion with SfiI yields a linearized vector which can be easily separated from the uncut one by gel electrophoresis. The loss of tet resistance can further ensure complete cutting of the recipient vector.

(viii) In order to avoid immunity to superinfection (Stengele et al., 1990), which is caused by expression of fusion proteins containing full length gIIIp, it is beneficial to use a truncated version of gIIIp. The truncated gIII 250–406 (Fig. 3B; Lowman et al., 1991), which is shorter than the more commonly used gIII 198–406 version, was chosen in order to eliminate a long glycine/serine rich linker stretch that favored instability. More importantly, the unpaired cysteine 201 at the end of the N-terminal domains, which reduces the folding yield of antibody-gIII fusions (data not shown) was also removed by this approach. Given that even background expression of truncated pIII fusions has been shown to be able to suppress superinfection to some extent (Ørum et al., 1993), it is furthermore an important

Table 2
Summary of cloned hybridomas

Hybridoma cell line	13AD	42PF	3D5	MOC31	3D7
Isotype	λ, IgG1	κ, IgG2b	κ, IgG2b	κ, IgG1	κ, IgG1
Tumor cell fusion partner	X63Ag8.653	X63Ag8.653	X63Ag8.653	X63Ag8.653	X63Ag8.653
Antigen	LZ	LZ(7P14P)	$(his)_5$ tag	EGP-2	DDT
Binders without panning	0/20	2/14	4/12	0/22	3/10
Binders after two panning rounds	5/6	nd	nd	8/10	nd
Identified aberrant or non-binding sequences	aVH13AD.1 aVH13AD.2	aVL42PF.λ	nd	nd	nd
PCR amplification by: Pharmacia Primer Mix					
V_L	No	No	nd	nd	No
V_H	Yes	Yes	nd	nd	Yes
Primers derived from Orlandi et al., 1989					
V_L	nd	nd	nd	No	nd
V_H	nd	nd	nd	aVHref	nd

Five hybridomas of three different isotypes have been cloned according to the scheme outlined in Fig. 1 and Fig. 9. For all hybridomas X63Ag8.653 (Kearney et al., 1979) was used as the tumor cell fusion partner. Hybridoma 13AD and 42PF produce antibodies directed against leucine zippers (Leder et al., 1995), 3D5 against C-terminal his tags (Lindner et al., 1997), MOC31 against the epithelial glycoprotein-2 (Souhami et al., 1988) and 3D7 against a derivative of DDT (Bürgisser et al., 1990). Functional binders (signal > 10 times background) are identified by phage ELISA as described in Section 2. The amino acid sequences of all identified aberrant chains are listed in Fig. 5. The sequences of aVH13AD.1 and aVHref are identical (except for amino acid 56; Fig. 5) to the aberrant V_H sequence published by Kütemeier et al. (1992). The same sequence was exclusively found (three independently sequenced clones) during V_H amplification of hybridoma MOC31 using primers derived from Orlandi et al. (1989), whereas V_L could not be amplified using this primer set. Amplification of V_L using the commercially available primer mix of Pharmacia failed in the case of hybridomas 42PF and 3D7, probably because appropriate sequences are absent from this mix, as well as for hybridoma 13AD, due to its λ isotype (nd = not determined; no = no PCR product detected; yes = PCR product detected).

A

pAK100scFv, pAK200scFv, pAK300scFv

```
                                          end lacI
...ATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGC
...M  Q  L  A  R  Q  V  S  R  L  E  S  G  Q  *

                        tHP terminator
GGTACCCGATAAAAGCGGCTTCCTGACAGGAGGCCGTTTTGTTTTGCAGC

                    CAP binding site
CCACCTCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGG

 -35                        -10          lac-operator
CTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGA
                                         |-> mRNA

                SD1          LacZ
TAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTTCTAGA
                             M  T  M  I  T  N  F  *

   SD2          pelB signal sequence
TAACGAGGGCAAATCATGAAATACCTATTGCCTACGGCAGCCGCTGGATT
                M  K  Y  L  L  P  T  A  A  A  G  L

                  SfiI            FLAG      VL
GTTATTACTCGCGGCCCAGCCGGCCATGGCGGACTACAAAGAY...
 L  L  L  A  A  Q  P  A  M  A  D  Y  K  D  ...
```

pAK400scFv

```
   SD2            pelB signal sequence
...GAAGGAGATATACATATGAAATACCTATTGCCTACGGCAGCC...
   T7g10          M  K  Y  L  L  P  T  A  A  ...
```

B

pAK100scFv

```
   VH     SfiI     EcoRI            myc tag
...CGGCCTCGGGGGCCGAATTCGAGCAGAAGCTGATCTCTGAGGAAGAC
...  A  S  G  A  E  F  E  Q  K  L  I  S  E  E  D

      geneIII 250-406
CTGTAGGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAG...
L  *  G  G  G  S  G  S  G  D  F  D  Y  E  K  ...
```

pAK200scFv

```
   VH     SfiI        geneIII 250-406
...CGGCCTCGGGGGCCGAGGGCGGCGGTTCTGGTTCCGGTGATTTT...
...  A  S  G  A  E  G  G  G  S  G  S  G  D  Y  ...
```

pAK300scFv, pAK400scFv

```
   VH     SfiI      his tag
...CGGCCTCGGGGGCCGATCACCATCATCACCATCATTAGT...
...  A  S  G  A  D  H  H  H  H  H  H  *
```

C

pAK500scFv

```
     VH     SfiI     EcoRI                dHLX
.....CGGCCTCGGGGGCCGAATTCCCCAAACCTAGCACCCCCCCTGGCA
.....  A  S  G  A  E  F  P  K  P  S  T  P  P  G  S

GCAGTGGTGAACTGGAAGAGCTGCTTAAGCATCTTAAAGAACTTCTGAAG
  S  G  E  L  E  E  L  L  K  H  L  K  E  L  L  K

GGCCCCCGCAAAGGCGAACTCGAGGAACTGCTGAAACATCTGAAGGAGCT
G  P  R  K  G  E  L  E  E  L  L  K  H  L  K  E  L

                                   his tag
GCTTAAAGGTGGGAGCGGAGGCGCGCCGCACCATCATCACCATTGACGTC
 L  K  G  G  S  G  G  A  P  H  H  H  H  H  *

 HindIII
TAAGCTT...
```

pAK600scFv

```
   VH     SfiI     EcoRI    alkaline phosphatase (AP)
...CGGCCTCGGGGGCCGAATTCCGGACACCAGAAATGCCTGTTCTG...
     A  S  G  A  E  F  R  T  P  E  M  P  V  L  ...
                       start AP
                                        HindIII
...CTCTTCTACACCATGAAAGCCGCTCTGGGGCTGAAATAAGCTT...
...L  F  Y  T  M  K  A  A  L  G  L  K  *
                                   end AP
```

Fig. 3. *A*: upstream sequence of pAK100scFv, pAK200scFv, pAK300scFv and pAK400scFv. The region from the end of the *lacI* repressor gene to the beginning of the antibody V_L domain is shown. The *lacI* repressor gene, t_{HP} terminator sequence, CAP binding site, *lac* promotor/operator region (*lac p/o*) including the −35 and −10 sequence, Shine-Dalgarno (SD) sequence of *lacZ* (SD1), lacZ peptide, a second SD sequence (SD2), *pelB* signal sequence, N-terminal SfiI site, four amino acid FLAG tag and the start of the V_L domain (bold) are indicated above the sequence. For pAK400, the 15 bp upstream from the pelB start codon are replaced by a sequence including the SD sequence of the phage T7 gene10. *B*: downstream sequence of pAK100scFv, pAK200scFv, pAK300scFv and pAK400scFv. Relevant differences in the downstream sequences of pAK100, pAK200, pAK300 and pAK400 are shown. The last two bases of V_H (bold), SfiI and EcoRI restriction sites, myc or his tags and the start of geneIII(250–406) are indicated above the sequence. The stop codons are represented by asterisks. This corresponds to the region of the right-hand SfiI site in Fig. 4. *C*: sequences of EcoRI/HindIII fusion cassettes as used in pAK500 and pAK600. The dHLX dimerization motif was taken from Pack et al. (1993). The complete sequence of the mature *E. coli* alkaline phosphatase (AP) gene can be found in Shuttleworth et al. (1986). In order to provide a EcoRI/HindIII cloning cassette, the two internal EcoRI sites of the AP-gene have been removed by silent mutations (A. Knappik, unpublished data).

improvement to engineer the system such that complete product repression prior to helper phage infection can be ensured (see below).

(ix) A strong upstream t_{HP} terminator (Nohno et al., 1986) was incorporated between the *lacI* gene and the *lac* promoter region of pAK100 (Fig. 1E, Fig. 3A). This t_{HP} terminator sequence, in combina-

tion with glucose repression of the *lac* promoter (De Bellis and Schwartz, 1990), completely abolishes background expression before induction (for details see Krebber et al., 1996a). By these measures, selection against toxic scFv-gIII fusion proteins is avoided during propagation steps and plasmid maintenance is thus significantly improved.

(x) The *lac* repressor is encoded on the phagemid to ensure strain independent *lac* promoter repression.

(xi) Combining a synthetic SD sequence with a *pelB* signal sequence (Fig. 3A) leads to an only moderate level of translation allowing a low level of scFv-gIII expression upon induction by IPTG.

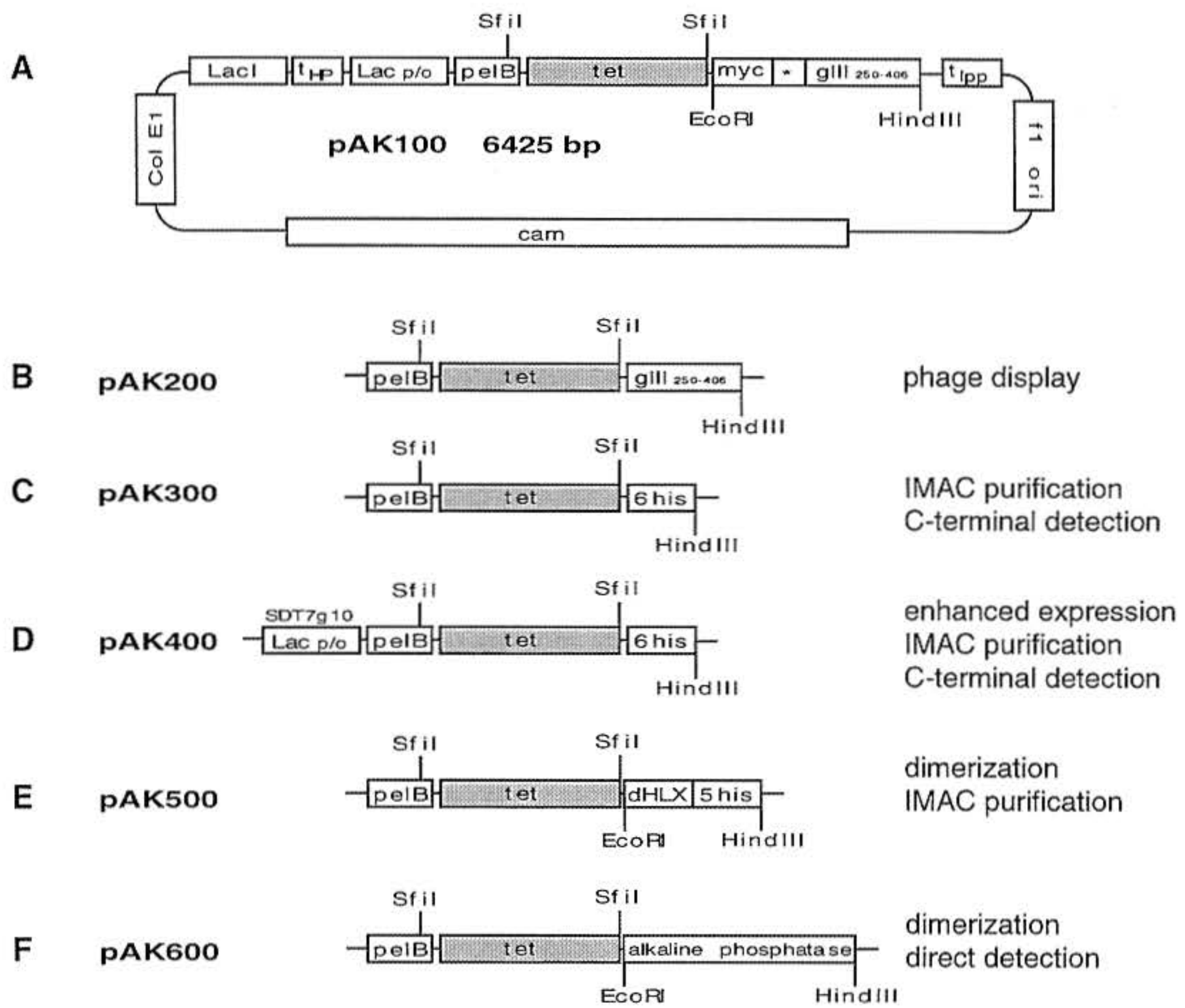
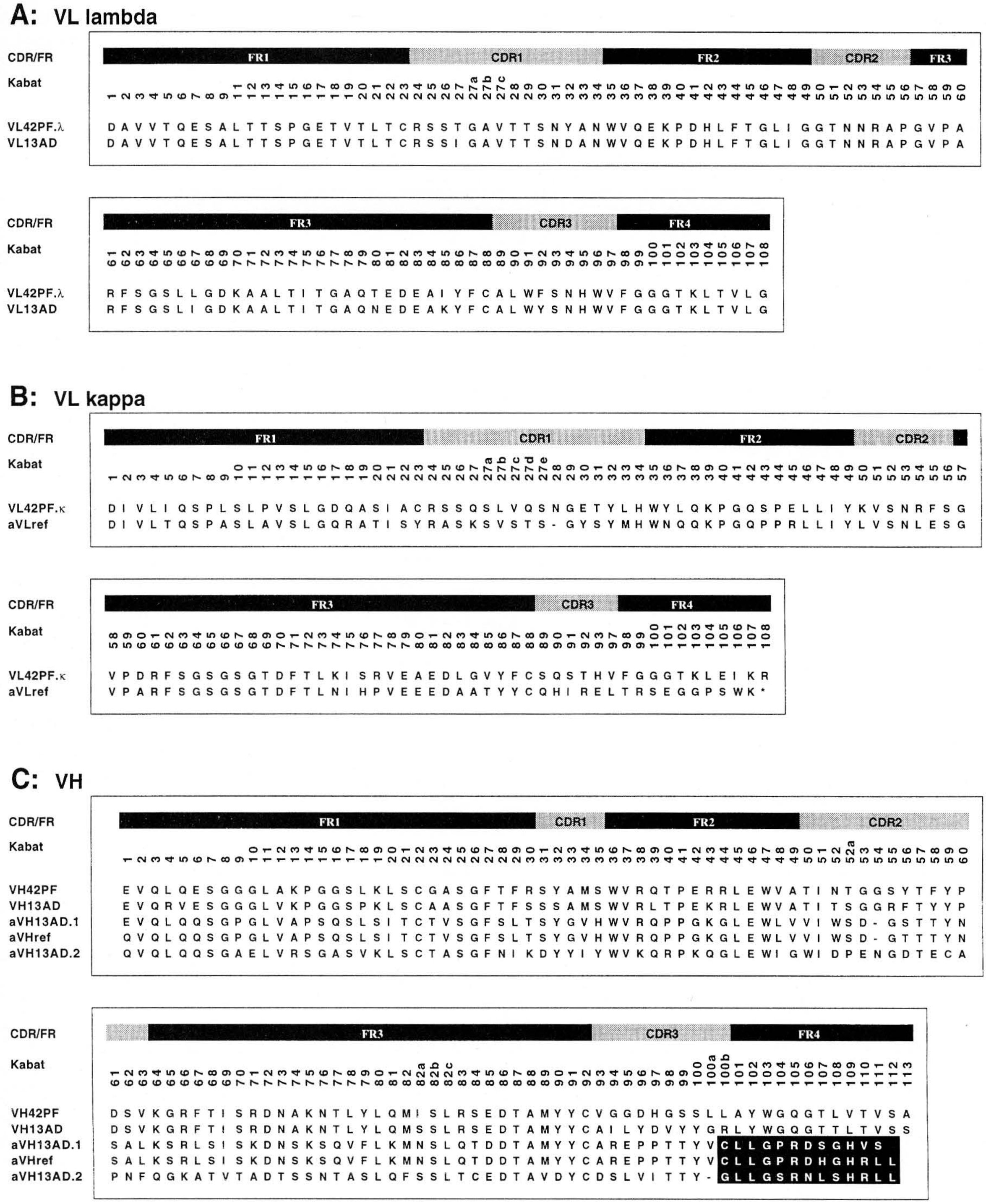


Fig. 4. pAK vector series. Phage display vector pAK100 (*A*) and related vectors (*B–F*) are useful to build modifications into antibody fragments cloned by the strategy outlined in Fig. 1 and Fig. 9. pAK200-pAK600 contain the same elements as described for pAK100 except for the modified cassettes shown. All vectors contain a *tet* resistance cassette (*tetA* and *tetR*; 2101 bp) to facilitate the monitoring of SfiI cutting. The in-frame fusion to geneIII250–406 using pAK100 (*A*) leads firstly into a myc-tag (Munro and Pelham, 1986) to be used as a detection handle, followed by an amber codon (asterisk). Depending on the strain used it is possible to switch between soluble expression of scFvs (in the case of non-suppressor strains such as JM83) and expression of scFv gene3 fusions (with suppressor strains such as XL1-Blue). The direct in-frame fusion to geneIII250–406 as in pAK200 (*B*) lacks the EcoRI site, myc-tag and amber codon. C-terminal his tag fusions can be used for purification by IMAC as well as for detection by an anti his tag antibody (Lindner et al., 1992, 1997; Kalinke et al., 1996) (*C*, *D*). Fusion partners, including helices for dimerization (Pack et al., 1993) (*E*) and alkaline phosphatase (AP) for direct detection of antigens by dimerized APscFv fusions (Lindner et al., 1997) (*F*), can be added to pAK100 or pAK400. The expression strength of either antibody or antibody fusions can be enhanced by replacing the original Shine Dalgarno (SD2) sequence by the stronger SDT7g10, as carried out in pAK400 (*D*).

(xii) The insertion of an amber codon upstream of *ΔgeneIII* in pAK100, as described by Hoogenboom et al. (1991) and Lowman et al. (1991), allows switching between expression of membrane anchored scFv-geneIII250–406 fusion proteins and soluble scFvs simply by changing the expression host.



It must also be taken into account, however, that amber suppression is not complete and for this reason, an analogous phage display vector was constructed (pAK200; Fig. 4B), lacking the stop codon of pAK100. This leads to a higher proportion of displayed scFv-geneIII fusion protein, as monitored by ELISA and Western blot (data not shown). Direct competition of the same scFvs cloned into pAK100 or pAK200, however, reproducibly results in a complete enrichment of the pAK100 vector type after one round of phage panning (data not shown). This suggests that the lower level of fusion protein expression is a selective advantage.

(xiii) Chloramphenicol resistance (Kang et al., 1991) was used as the selective marker because it was found to allow more stringent selection than attainable with ampicillin, since the resistance protein does not leak into the medium. Furthermore, the use of chloramphenicol is advantageous over that of kanamycin or tetracycline because it does not reduce the phage titer as much (Johansen et al., 1995; Krebber et al., 1995; Krebber A., unpublished observations).

(xiv) The procedure can be used for library cloning, e.g. the repertoire from immunized mice, as well as for cloning of single sequences from hybridomas in a similar way. Furthermore, it is directly compatible with the recently introduced selectively infective phage system (SIP), which allows in vivo and in vitro selection of cognate protein/ligand interactions by strictly coupling the infectivity of filamentous phages to the binding event (Krebber et al., 1995; Krebber et al., 1996b).

(xv) Since the optimized phage display vector pAK100 is engineered to achieve low levels of expression, it is not a useful large scale production system for well folding and soluble antibody fragments. Thus, a compatible high-level expression plasmid has also been engineered. (Fig. 3A, Fig. 4D).

(xvi) A compatible vector series that facilitates various modifications of scFv fragments subsequent to cloning into pAK100 is also available (Fig. 3B, Fig. 3C, Fig. 4C–F; see also Ge et al., 1995).

3.2. Amplification of V region genes and assembly into the scFv format

The V_L back primer mix (LB1-17 and LBλ, representing a total of 131 variants) paired with five V_L forward primers (LF1, 2, 4, 5 and LFλ) and the V_H back mix (HB1-19, representing a total of 94 variants) paired with four V_H forward primers (HF1-4) have been used to amplify V_L and V_H domains from a variety of antibody cDNAs (Table 2).

Our improved primer set (Table 1) has been tested in different laboratories on cDNA derived from 12 hybridoma cell lines of different specificities and family sub-types to date. In all cases, the first PCR amplification yielded sufficient amounts of products for cloning, with a sharp band at the predicted size of 375–402 bp for V_L or 386–440 bp for V_H. Typical examples of V_H and V_L genes amplified from cDNA of two hybridomas, 42PF and 13AD, which secrete monoclonal antibodies directed against leucine zippers (Leder et al., 1995), are shown (Fig. 2). Using the same cDNA in combination with a commercially available primer mix (recombinant phage antibody system (Pharmacia)) or primers derived from Orlandi et al. (1989), amplification of V_L failed in several cases (Table 2), underlining the importance of an extended primer mix.

Fig. 5. Sequence alignment of functional and aberrant variable domains expressed by the hybridoma cell lines 13AD and 42PF. Residue numbers are according to Kabat et al. (1991). The 7 amino acids at each end are encoded by the PCR primer sequences. *A*: V_λ amino acid sequences. VL42PF.λ: non-binding V_λ found in clone 42PF; identical to germline $V_\lambda 1$ sequence (Weiss and Wu, 1987) except for F92Y. VL13AD (X99507): functional, antigen-binding V_λ sequence of hybridoma 13AD. *B*: V_κ amino acid sequences. VL42PF.κ (X99509): functional, antigen-binding sequence of clone 42PF. aVLref: aberrant V_κ transcript found in P3X63Ag8.653 (Carroll et al., 1988 (M35669); Duan and Pomerantz, 1994; Cabilly and Riggs, 1985; Strohal et al., 1987; Yamanaka et al., 1995). *C*: V_H amino acid sequences. VH42PF (X99508): functional V_H of hybridoma 42PF. VH13AD (X99506): functional V_H of hybridoma 13AD. aVH13AD.1: aberrant V_H.1 found in clone 13AD, showing a frameshift in CDR3. aVHref: non-functional V_H published by Yamanaka et al. (1995); this sequence is identical to the unpublished result of Mocikat (D50398). aVH13AD.2: aberrant V_H.2 found in 13AD. Sequence is different to aVH13AD.1, and also contains a frameshift in CDR3. EMBL accession numbers are given in brackets.

For further analysis the V_L and V_H PCR products of 42PF and 13AD have been cloned separately into the pCR-Script vector (Stratagene) and sequenced. For 42PF two plausible light chain sequences devoid of frameshifts, stop codons, deletions or atypical amino acids for murine V_L domains (VL42PF.κ, VL42PF.λ) were found, together with one heavy chain sequence (VH42PF) (Fig. 5). For hybridoma 13AD only 3 of 57 clones analyzed contained a bona fide functional heavy chain gene, denoted VH13AD, whereas two additional non-functional heavy chain sequences aVH13AD.1 (five clones) and aVH13AD.2 (49 clones) were found (Fig. 5). Both heavy chains are aberrantly rearranged at the DJ recombination site in CDR3 and contain several framework amino acids which deviate from the observed consensus of antibody sequences (Fig. 5). Sequencing of five V_L chains, amplified exclusively by the λ primer pair LBλ/LFλ, yielded a unique sequence denoted VL13AD (Fig. 5). Thus, both the 13AD and 42PF hybridoma produced more than one PCR-amplifiable heavy or light chain.

As outlined in Fig. 1, all amplified V_L and V_H domains have been linked by SOE-PCR, as shown for 13AD and 42PF (Fig. 2). These were subsequently digested by SfiI and ligated into the improved phage display vector pAK100.

3.3. Screening and enrichment of functional scFv sequences derived from hybridomas

After transformation of the ligation reaction into the recombination deficient *E. coli* strain XL1-Blue, 10–22 individual colonies were grown separately and infected by helper phage as described in Section 2. The recombinant scFvs, displayed on the surface of filamentous phage, were tested for antigen binding in a typical phage ELISA. In those cases where the parental hybridoma cell line did not produce large amounts of contaminating, non-functional light or heavy chain, about one third of the screened colonies contained the sequence information of the binding scFv fragments (examples are hybridoma 42PF amplified with a V_L κ mix, devoid of λ primers and hybridomas 3D5 and 3D7; Table 2). At the other extreme, an initial screening of phages derived from individual colonies of 13AD did not yield any functional binders. As previously demonstrated by the sequencing of individual V_H domains, functional sequences are greatly diluted by aberrant heavy chains in this hybridoma cell line. In order to identify and enrich functional binders, 10^5 *E. coli* colonies were pooled after transformation and subjected to two rounds of phage panning. After each round, six clones were tested for antigen binding in a phage ELISA. Two of six and five of six clones from the first and second panning rounds, respectively, were found to be positive for antigen binding. All positive clones had identical sequences (VL13AD paired with VH13AD), whereas all non-binding scFv sequences contained the aberrant heavy chains aVH13AD.1 or aVH13AD.2, occasionally in combination with point mutations in the light chain gene.

Clones which were found to be positive in phage

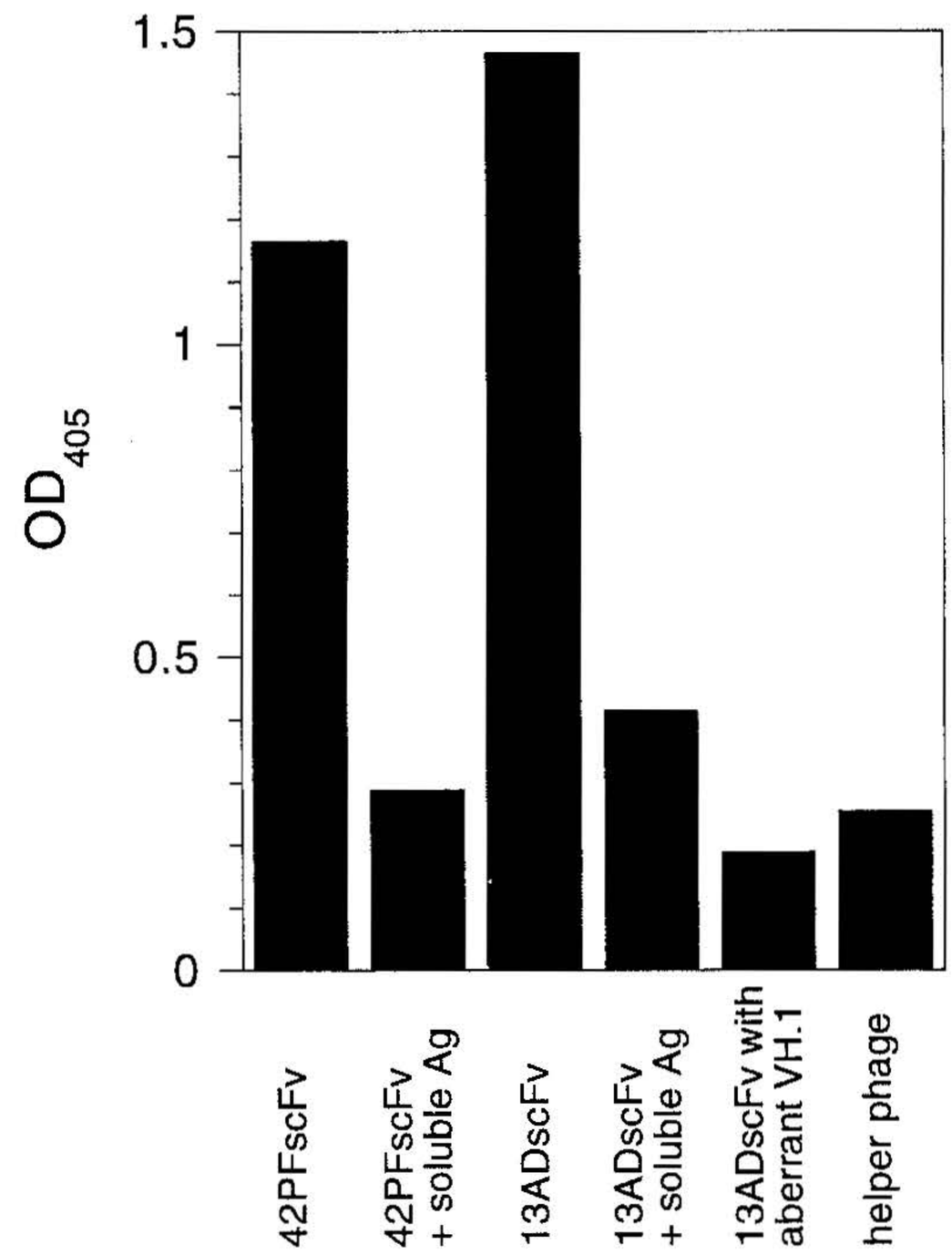


Fig. 6. Competition phage ELISA (13AD, 42PF). Competition phage ELISA with phages displaying functional and non-functional scFv fragments derived from hybridomas 13AD and 42PF. The ELISA was performed as described in Section 2. The non-binding 13ADscFv clone contains the aberrant aVH13AD.1 chain (Fig. 5C) and the functional VL13AD chain (Fig. 5A). For inhibition, phages were preincubated for 10 min with 10^{-4} M soluble peptide antigen before applying the mixture to the antigen-coated plate. As a negative control, an assay with VCS helper phage was performed.

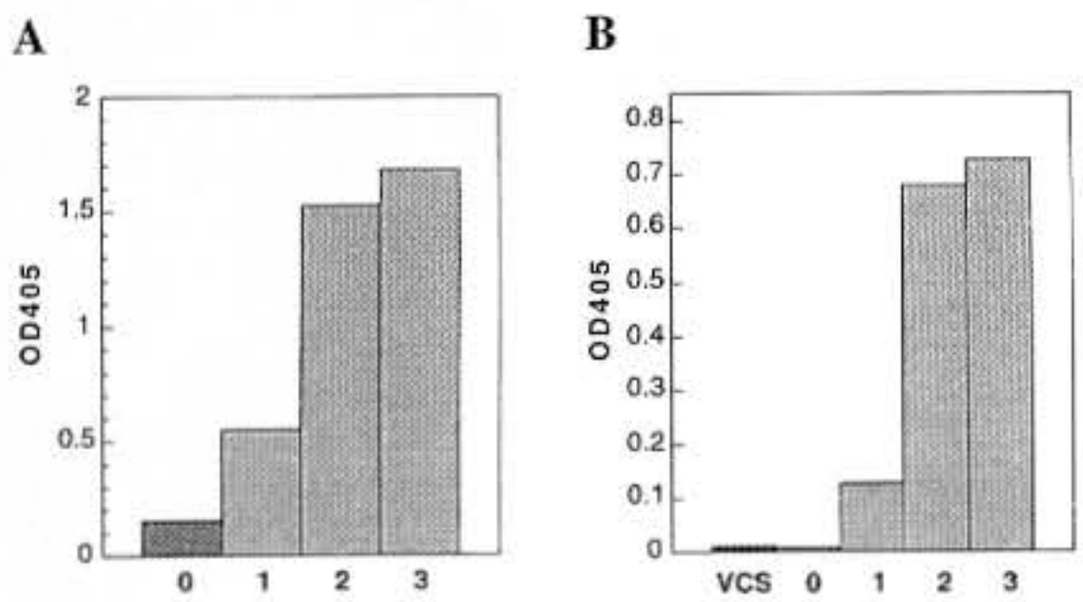


Fig. 7. Enrichment by panning. *A*: cloning of hybridoma MOC31. Enrichment of EGP-2 binding scFv by phage panning. *B*: repertoire cloning. Enrichment of ampicillin binding scFv displaying phages derived from anti-ampicillin library I. Phage pools ($5 \cdot 10^{10}$ cfu/well) prepared after 0, 1, 2 and 3 rounds of phage panning (column label), as well as VCS helper phage as a negative control, were tested for specific antigen binding in a phage ELISA as described in Section 2. Selective enrichment was also indicated by an increased number of phages eluted in subsequent panning rounds (data not shown).

ELISAs were further characterized by antigen inhibition studies to verify that the binding was antigen-specific (Fig. 6). In the case of hybridoma MOC31 (Souhami et al., 1988), again no binders were initially obtained, and two to three rounds of phage panning were required to enrich binding scFv fragments to a level that allowed identification of functional antibody sequences in individual clones (Fig. 7A; Table 2). This shows that the relevant sequences of numerous 'monoclonal' antibodies can be hidden in a pool of closely related antibody-like sequences and that, in the absence of panning, rigorous testing would be required in order to identify the correct sequence.

3.4. Cloning of the antibody response from immunized mice

The procedure described for hybridoma cloning was also applied to mRNA isolated from spleen cells of an immunized mouse. In addition, B-cells from the same mouse were fused to the tumor cell line X63Ag8.653 as in the case of monoclonal antibody production, but were kept as a pool for 10 days. This pool of hybridomas was subsequently used as a source of mRNA. The latter experiment was carried out because it might seem conceivable that B cells which have been stimulated by the antigen fuse preferentially (Köhler and Milstein, 1976). From fusion experiments, only a small number of a few thousand candidate clones is typically obtained, of which a high proportion usually codes for antigen binding antibody sequences. Since productive pairs of V_L and V_H domains are separated during the cloning process and are subsequently combined randomly to form scFvs, fairly large libraries are necessary to ensure that all original V_L and V_H pairings are represented (Gherardi and Milstein, 1992; Posner et al., 1994). A comparison of anti-ampicillin libraries derived from fused (library I) and unfused B-cells (library II) of the same immunized mouse should determine whether cell fusion prior to mRNA preparation is an advantageous enrichment step which enhances the probability of restoring functional V_L/V_H pairings in a small library. As outlined in Table 3, both libraries contained binding scFv fragments which could be enriched with a similar efficiency after two or three rounds of panning (Fig. 7B). Sequencing revealed that the same sequences were isolated simultaneously from both libraries (data not shown), indicating that B cell fusion to tumor

Table 3
Selection of ampicillin binding scFv fragments from B cell repertoires

	Library I	Library II
Source of RNA	Spleen cells fused to tumor cell line X63Ag8.653	B cells
Antigen	Ampicillin	Ampicillin
Library size	$4 \cdot 10^6$	$6 \cdot 10^6$
Clones containing SfiI insert	20/20	20/20
Clones expressing scFv	22/30	18/30
Binders before panning	0/12	0/12
Binders after two rounds	5/12	nd
Binders after three rounds	19/24	9/12

Spleen cells derived from the same BALB/c mouse immunized with ampicillin were taken for library construction before (library II) and after (library I) fusion to the tumor cell line X63Ag8.653. Both libraries were transformed into XL1-Blue cells by electroporation. The amount of SfiI insert-containing clones in the initial library was monitored at the DNA level by restriction analysis whereas the amount of full length scFv-expressing clones was analyzed by Western blot analysis, using the N-terminal FLAG detection system combined with C-terminal myc tag detection (data not shown). Binding scFv fragments were identified by phage ELISA. The enrichment process was followed by ELISA using phage pools as shown in Fig. 7B.

cells prior to mRNA preparation, at least in our experience, has no significant beneficial influence on library composition.

3.5. Soluble expression and modification of cloned scFv fragments

A scFv fragment obtained from the anti-ampicillin library I (scFvaL2) was sub-cloned into pAK300 and pAK400 (Fig. 4) for soluble expression in JM83. In comparison with the low expression medium (LE medium) used for phage display, changing to an expression medium devoid of glucose immediately increases the expression level of recombinant protein without any modifications to the vector system (De Bellis and Schwartz, 1990). Changing the translation initiation region present in pAK100 or pAK300 into a much stronger Shine-Dalgarno sequence (SDT7g10), such as that present in pAK400 (Fig. 3A, Plückthun et al., 1996), results in a further significant enhancement of protein expression. As shown in Fig. 8, the expression level strongly influences the ratio of soluble to insoluble scFv protein. While at the lower expression level of pAK300scFvaL2 100% of the scFv is soluble and

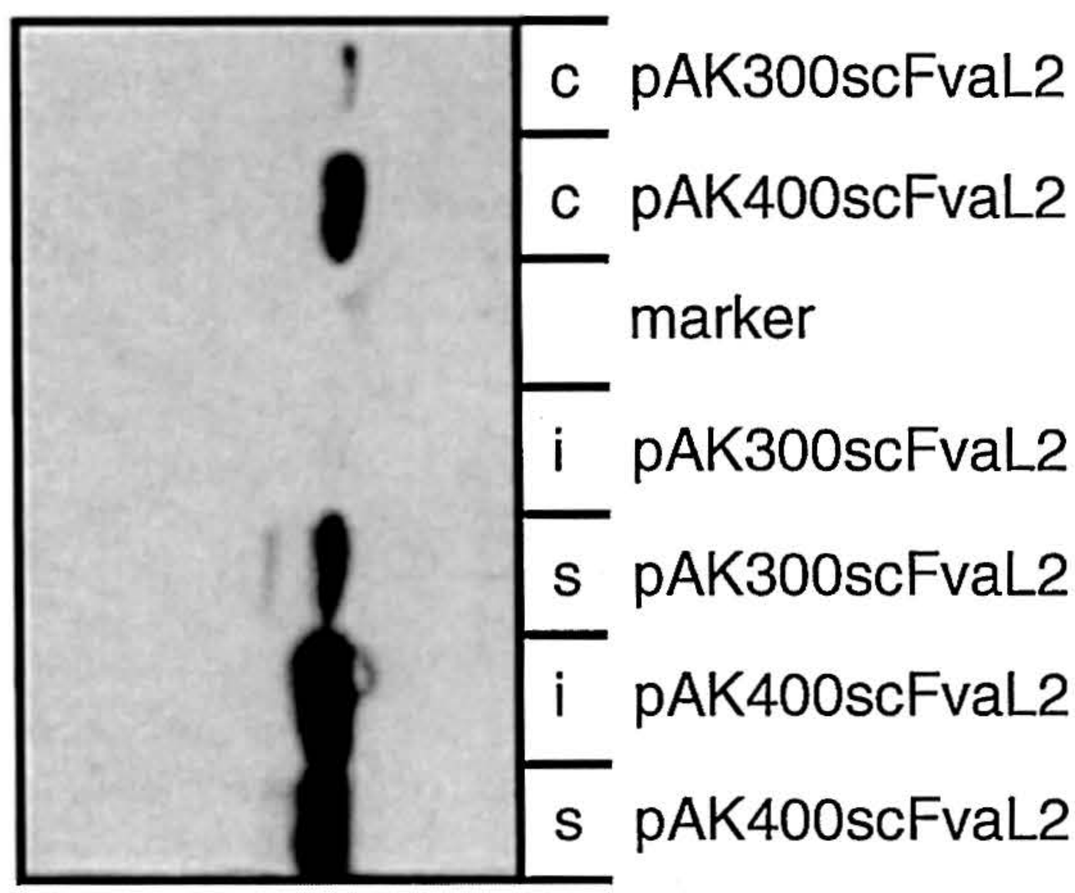


Fig. 8. Enhanced expression of aL2 in pAK400. The scFvaL2 was expressed in JM83 harboring pAK300scFvaL2 or pAK400scFvaL2 (Fig. 3 and Fig. 4). Expression levels were monitored by Western blot analysis as described in Section 2. c: whole culture (soluble fraction, insoluble fraction and culture supernatant), where the loaded sample corresponds to 1 ml of culture OD_{600} of 0.01; i: insoluble fraction; and s: soluble fraction, where the loaded sample corresponds to 1 ml culture at an OD_{600} of 0.05.

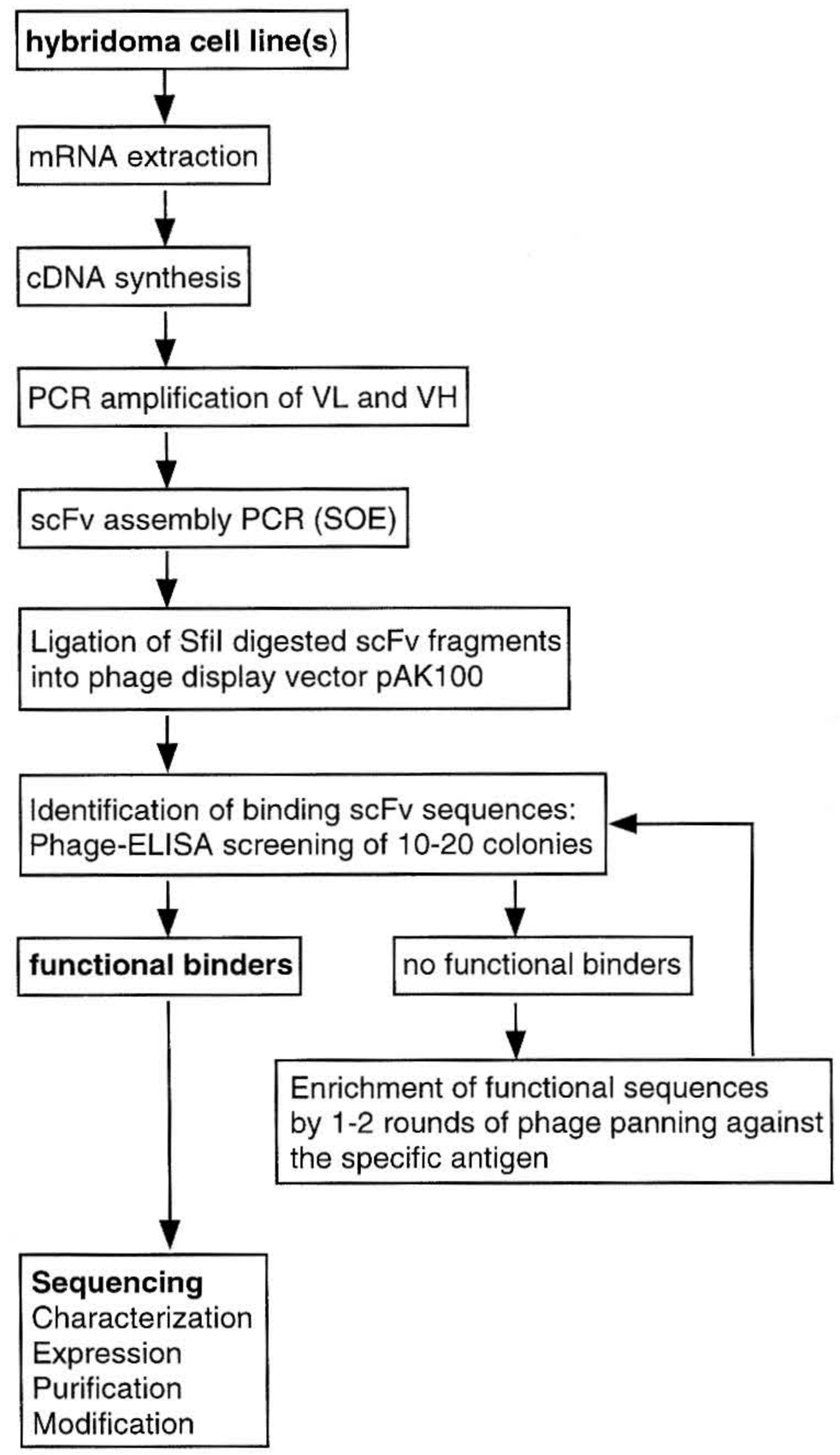


Fig. 9. Outline: Generation of scFv antibodies from hybridomas. A flow diagram summarizing the most important steps is shown.

functional, the enhanced expression in pAK400scFvaL2 causes production of more soluble but also large amounts of insoluble material (Fig. 8).

The scFvaL2 has comparatively favorable folding properties (A. Krebber, unpublished). For scFv fragments, which are already mainly found in the insoluble fraction after expression in pAK100 or pAK300, sub-cloning into pAK400 does not improve the yield of functional antibody fragment (data not shown). Instead, cell lysis problems, caused by such poorly folding proteins, are enhanced in the stronger expression vector since higher expression levels normally lead to a higher proportion of non-functional aggregates (Le Calvez et al., 1995), which are likely to impair growth of the expression host. Therefore, it has proved to be advantageous to adapt the expression level to the particular scFv sequence which has

to be expressed by the choice of vector and induction conditions.

Moreover, SfiI cassettes of scFv fragments can be fused directly in frame with oligo-histidine tags for purification by IMAC (Lindner et al., 1992), with dimerization or tetramerization modules to obtain dimeric or multimeric scFv antibodies (Pack et al., 1993, 1995) and with enzymes such as alkaline phosphatase to produce dimeric scFv molecules which can be detected directly by virtue of their enzymatic activity (Lindner et al., 1997; Fig. 3B and C and Fig. 4). If IMAC does not directly yield pure scFv antibody, an immunoaffinity column using an anti-FLAG mAb can also be employed (Kalinke et al., 1996). Combination of the N-terminal FLAG tag and the C-terminal myc or his tags allows monitoring of full length scFv product formation, since antibodies against all three tags are available (Munro and Pelham, 1986; Knappik and Plückthun, 1994; Lindner et al., 1997). Therefore, Western blot detection of N- and C-terminal degradation and of proteolysis of particular scFv sequences becomes easily possible. The whole spectrum of compatible modification cassettes (see also Ge et al., 1995; Plückthun et al., 1996) combined with the pAK vector series creates a highly versatile system, allowing easy characterization and further genetic engineering of scFv fragments initially obtained.

4. Discussion

The improved phage display system based on the pAK vector series (Fig. 4), an extended primer mix (Table 1) and a very straightforward cloning procedure (Fig. 1) proved to be robust and reliable both in a library setting and for hybridoma cloning. Following the scheme outlined in Fig. 9 all hybridomas tested to date could be cloned, characterized for functional antigen binding and sequenced with a reasonable effort, in as few as 10 days (hybridoma 3D5).

The optimized phage display system was suitable for eliminating high amounts of non-functional chains that are transcribed from various aberrant mRNAs in some hybridoma cell lines. In contrast to other methods (Nicholls et al., 1993; Duan and Pomerantz, 1994; Ostermeier and Michel, 1996), only functional and binding antibody genes will be sequenced. After RNAseH treatment of aberrant RNA/DNA hybrids (Ostermeier and Michel, 1996), eight out of 12 sequenced clones were still derived from aberrant pseudogenes, whereas without mRNA treatment all nine clones tested carried pseudogenes. Duan and Pomerantz (1994) used ribozyme treatment to improve the ratio of aberrant to functional sequences from three positive clones in 150 to 12–34 in 150. Both methods depend on the availability of sequence information of the aberrant chain prior to cloning, whereas phage display simply enriches binding sequences over any kind of contaminating chain. This has been found to be particularly important, as both of the hybridoma cell lines we have characterized in detail (13AD and 42PF) produced aberrant mRNAs which seem to be specific for the individual hybridomas and could not be found in the published literature or any database (Fig. 5). The origin of some of the aberrant mRNAs could not be traced to the myeloma cell lines originally utilized for cell fusion. Hybridoma 13AD, for example, contained three different heavy chain mRNAs, of which only one is known to be derived from the tumor cell line X63Ag8.653. It seems plausible that many additional non-binding chains originate from aberrant rearrangements of the second allele of the B-cell involved in the generation of the particular hybridoma. Termination of rearrangement in the immunoglobulin loci takes place only after synthesis of a functional membrane-bound immunoglobulin. Thus, a given B-cell may produce aberrant mRNAs that contain stop codons or frameshifts in addition to the functional mRNA that is translated into the mature immunoglobulin chain. Furthermore, some hybridomas may be the result of the fusion of more than one B-cell with the myeloma cell. This gives rise to the possibility that more than one typical heavy and light chain gene is expressed, as observed for hybridoma 42PF. Such typical in-frame but non-binding sequences cannot be distinguished from the binding chain by sequencing. This underlines the importance of a functional test involving antigen binding in order to avoid the risk of isolating an incorrect sequence.

When this is taken in combination with possible PCR errors or mutations introduced by the primer mix, it can be envisaged how cloning of a hybridoma

can very easily generate a diverse collection of different scFv fragments. Thus, functional screening is always superior to sequence analysis of individual clones.

If more than one monoclonal antibody against the same antigen is available, pooled hybridoma cell lines can be used as a source for mRNA extraction, as carried out in the case of the anti-ampicillin library I. This allows for fast and inexpensive simultaneous cloning of several different antibodies in one experimental setup. Alternatively, a small fraction of B-cells prepared for traditional monoclonal antibody generation can be set aside for phage library construction. It has been reported, however, that parallel screening of hybridomas and phage libraries, as described by Gherardi and Milstein (1992); Kettleborough et al. (1994) and Ames et al. (1995), has led to the discovery of different antibody sequences from the two sources. This may simply reflect the fact that neither hybridoma generation nor phage libraries provides an exhaustive sampling of the immune response. The findings may suggest in addition that recombinant expression of antibody fragments combined with phage display selects against certain antibody sequences (see also Riechmann and Weill, 1993; Posner et al., 1994; Jackson et al., 1995). The absence of certain sequences in phage libraries might be due to insufficient library size, PCR amplification of only a subset of binding variable genes or selection against phagemids expressing less stable or less well folding antibody fragments, particularly if they are incorporated into phage particles less frequently or impair growth of *E. coli* (Knappik and Plückthun, 1995; Krebber et al., 1996a). We believe, however, that our optimized cloning procedure and the tightly regulatable phage display vector will contribute to overcoming some of these biases and will therefore facilitate the construction of more diverse antibody libraries. Given the stress that antibodies fused with gIIIp impose on the cell, a high expression level is of no importance and actually serves as a burden for phage display. In contrast, the absence of background expression before induction is of utmost importance if loss of clones from the library or gene deletions are not to quickly accumulate. Thus, the phage display vector pAK100 was optimized by lowering the expression level for phage display and, more importantly, by eliminating background expression before helper phage infection (Krebber et al., 1996a). In contrast to many previous vector constructs, therefore, even in a library setting, no deletions, empty vectors, recombination events or other symptoms of instability have been detected to date. Moreover we claim that it is advisable to sub-clone selected scFvs into related, but more powerful, expression vectors subsequent to cloning, since unbiased library screening by phage display and maximized functional production of a particular antibody fragment are likely to require different expression optima.

Acknowledgements

We wish to thank Friederike Ackermann, André Gerber, Barbara Klinger and Dr. Stefan Frey for helpful contributions and expert technical assistance and Dr. Gerard Wall for critically reading the manuscript.

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