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edited by

Carel J. van Oss

State University of New York at Buffalo
Buffalo, New York

Marc H. V. van Regenmortel

Institut de Biologie Moléculaire et Cellulaire du CNRS
Strasbourg, France

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9

Recombinant Antibodies

Andreas Plückthun *Universität Zürich, Zürich, Switzerland*

I. INTRODUCTION

The immune system is able to produce antibodies with essentially any specificity. What more then is there to do for the protein engineer? There are actually many answers to this question. The least imaginative one is that recombinant antibodies can be produced consistently, always with the same expression system and in unlimited quantities. More importantly, the researcher becomes independent of the animal. He or she can exchange constant domains to create any antibody subclass desired (independent of what the animal from which the antibody was derived chose to do). Alternatively, constant domains from humans may be used to minimize the human response against the foreign antibody protein in therapy. The most important development, however, is that completely new molecules can be made that do not occur in nature. This may entail the modification of the binding site itself; for example, to introduce residues that enhance binding to the antigen (or eliminate an undesired cross-reactivity). It may involve the conversion to human sequences of everything except the stretches of sequence contacting the antigen (again to minimize the human response, if the antibody is to be given in therapy). More ambitiously, engineering may be aimed at making more stable variable domains than nature does by altering the framework, and there is no reason to suppose that the natural sequence is of maximal stability. It may also be aimed at introducing a metal ion-binding site (e.g., to create a redox-sensitive biosensor). Finally, new and artificial domains may be added at the place the constant domains are in nature, to make homodimers, heterodimers, or higher oligomers; to fuse enzymes for easy detection in diagnostics; or to fuse small peptides for the same purpose.

To put some order into these many facets of antibody engineering, I will, in the first part, discuss the molecules to be made—the fragments and variants of natural antibodies—and the rationales for making them. In the next part, alterations of binding sites will be considered, followed by a discussion of how to make the recombinant antibodies in the laboratory. Finally, methods for circumventing classic methods of antibody production and selection, as well as general considerations for cloning, will be discussed.

II. FRAGMENTS OF THE ANTIBODY

A. Whole Antibodies

The antibody has an archetypical domain like structure (Fig. 1). The first question, therefore, is which of the domains are needed for which function, and what are the properties of molecules that are much smaller than whole antibodies, and may lack some of these domains, or parts thereof?

Antibodies of the IgG and IgM class contain in their Fc portion two important effector functions: antibody-dependent complement-mediated cytolysis (ADCMC) and antibody-dependent cellular cytolysis (ADCC). Both of these depend on binding of other proteins to the C_H2 domain (Shin et al., 1992), and in both functions, the binding is critically dependent on the exact conformation of the C_H2 domain. In the complement system, it is the C1q molecule that binds to the C_H2 domain surface. In IgG, the two C_H2 domains of the antibody do not have any protein–protein contacts (see Fig. 1b) (Deisenhofer, 1981; Sutton and Phillips, 1983; Harris et al., 1992) and the interdomain contacts are entirely mediated by sugars. If glycosylation is absent, the two domains are probably somewhat altered in conformation, or at least in relative distance, and C1q no longer binds (Leatherbarrow et al., 1985; Tao and Morrison, 1989; Gillies and Wesolowski, 1990). There is no evidence for direct binding to the sugars; the effects appear to be indirect (Lund et al., 1990). This is important information for the engineer, as it renders impossible the production of antibodies with intact effector functions in bacteria, and apparently also in yeast (Horwitz et al., 1988; Gillies and Wesolowski, 1990), since the absence of glycosylation or the yeast type of glycosylation does not maintain the required C_H2 conformation.

Similarly, the binding to cells triggering ADCC occurs through Fc receptors, which recognize the C_H2 domain, and this is again sensitive to glycosylation (Leatherbarrow et al., 1985; Tao and Morrison, 1989; Lund et al., 1990). It is now clear that the differences seen in cell binding by the Fc_γ-receptor for the closely related IgG subgroups is also dependent on the few residues that are different on the surface of this domain (Greenwood et al., 1993).

Subtypes of IgG and even allotypes have shown differences in ADCC or ADCMC (Brüggeman et al., 1987; Bindon et al., 1988; Gorman and Clark, 1990; Tao et al., 1991). If in a conventional production of monoclonal antibodies, a molecule that recognizes the antigen very well, but has the "wrong" IgG subclass, is obtained, it may then be easily converted to the desired subclass by genetic engineering. This is usually carried out simply by ligating the variable domains with the specificity of interest [cloned by the polymerase chain reaction (PCR), discussed later] into a vector containing the appropriate constant regions. By the same methodology, it is possible to produce recombinant IgA, IgD, and IgE with any specificity, but this has so far been done mostly for research purposes (Shin et al., 1992). Again, the same methodology is used to switch the constant domains from mouse to human, often referred to as *chimerization*. Vectors and methodologies used will be discussed later.

The rationale for chimerization (Morrison et al., 1984) has been to decrease the response of the human body to a foreign (murine) antibody as much as possible. Several therapeutically useful chimerized antibodies have been tested (Larrick and Fry, 1991). However, the variable domains are still totally murine in this case, and there may still be a human–antimouse–antibody (HAMA) response against framework regions of the variable domain. Unfortunately, it is not predictable whether a given variable domain would be immunogenic in humans. This is a complicated function of three-dimensional epitopes [generating both an antiframework and anticomplimentarity-determining region (CDR) response; see later], as well as linear T-cell epitopes, which are only beginning to be understood, but one is still far from being able to manipulate them experimentally. However, the reshaping or humanization of variable domains for converting as much as possible of the variable domains to human sequences is no longer a technical problem, and chimerization of antibodies will certainly be surpassed by this technology.

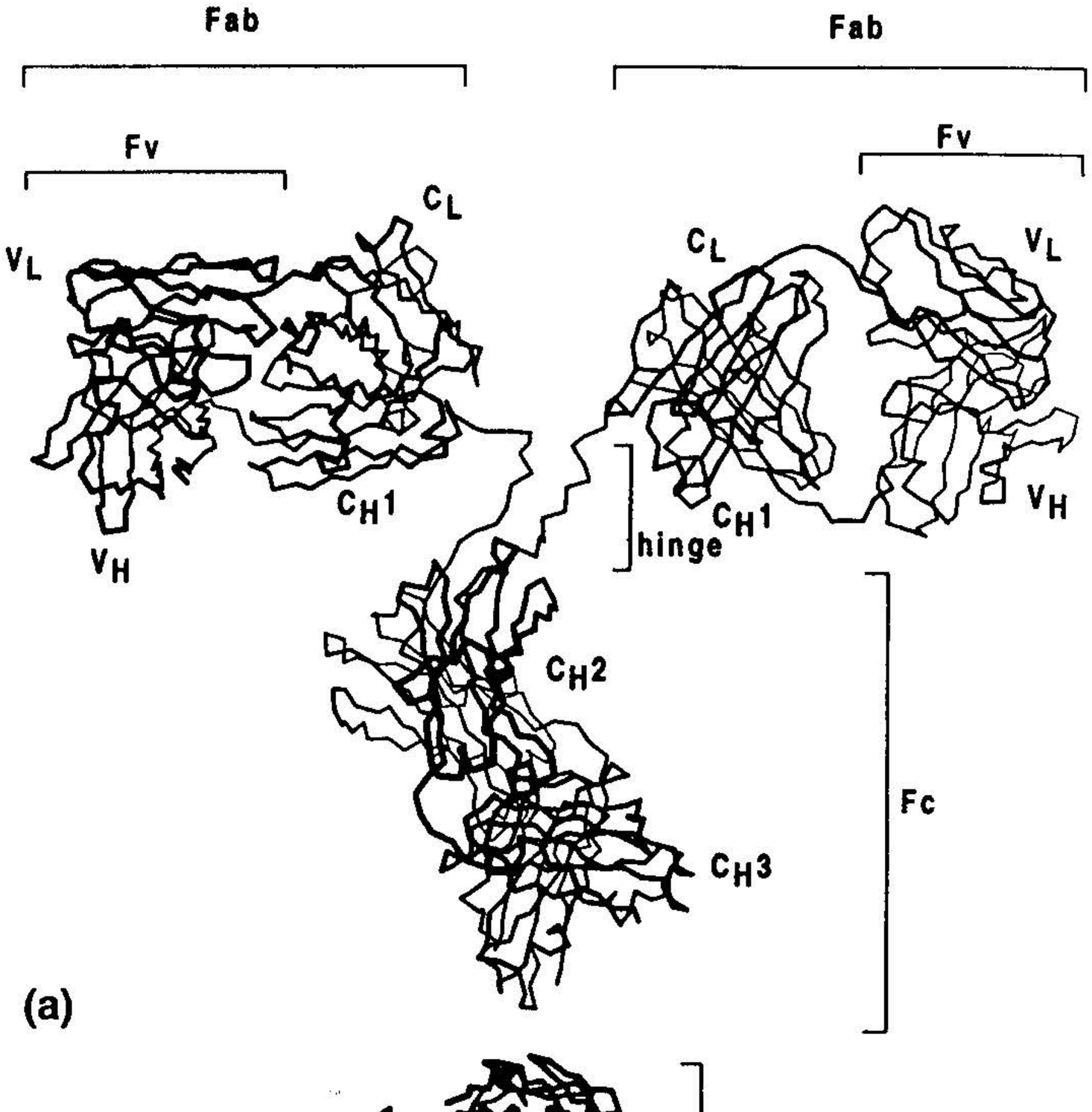


Figure 1 Structure of a whole antibody as determined from x-ray crystallography (Harris et al., 1992). This murine antibody is of the IgG2a class. The fragments and domains are labeled and the thickness of the lines is used as a depth cue. Note the lack of symmetry of the Fab fragments and the Fc part relative to each other, indicating flexibility in solution. The orientations of the Fab fragments indicate that some twisting motion around the hinge must be possible as well. (a) and (b) are two different orientations of the same molecule generated by turning around a vertical axis by about 90°. In (a) the overall dimensions are visible, whereas in (b) the lack of contacts between C_H2 is apparent. In (b) one Fab fragment points at the observer, the other into the distance.

The exclusive glycosylation of C_H2 is a hallmark of IgG.Other classes are glycosylated in other domains as well (summarized in Putnam et al., 1982); for example, IgM appears to be glycosylated in all constant domains, whereas some IgA subclasses are not glycosylated in C_H2, but are in C_H1 and C_H3. In addition to the respective biological functions, the stability and solubility of recombinant molecules which are not correctly glycosylated may be impaired. Therefore, if the relevant effector functions are not desired, instead of working with unglycosylated whole antibodies (e.g., through site-directed mutagenesis removing the glycosylation signal), it may be more efficient to use antibody fragments not containing these domains in the first place. There is no indication that glycosylation in the constant regions has any effect on antigen binding, and one would not be expected from the three-dimensional structure (see Fig. 1).

As always, there are exceptions. Very occasionally, the hypervariable regions may happen to contain a glycosylation signal (Asn—X—Ser or Asn—X—Thr) and this may abolish antigen binding (Margni and Binaghi, 1988) or even enhance it (Wallic et al., 1988). In general, however, we may summarize that glycosylation influences effector functions and stability, but not antigen binding.

Current applications of antibodies make use of these constant domains as a means of developing general reagents for *all* antibodies, no matter what the specificity or sequence of the variable domains may be. For instance, the constant domains have been used as a handle for purification, immobilization, or detection, using either anti-Fc antibodies or bacterial proteins, such as protein A from *Staphylococcus aureus* and protein G from streptococcal strains as a secondary reagent (Boyle and Reis, 1987). However, the same can be achieved with much smaller peptides (see later). Furthermore, the sugar residues have been used as a target for derivatization, often making use of aldehyde functions (generated by periodate oxidation of the sugar residues), and subsequent attack with amino groups or hydrazides present in the ligand to be coupled (reviewed in Blair and Ghose, 1983). Alternatively, the interdomain disulfide bonds from the hinge region have sometimes been mildly reduced (under conditions in which no reduction of the crucial intradomain disulfides occurs) and used as a derivatization handle (Blair and Ghose, 1983).

However, many other methods for linking other proteins (e.g., enzymes for detection) or small molecules to the antibody are now available (usually taking advantage of amino groups in the immunoglobulin). By using recombinant molecules, the linkage problem can be solved more elegantly. For instance, fusion proteins can be made at the genetic level (ensuring batch-to-batch consistency), and other molecules can be coupled by genetically engineered cysteines at the desired position, thereby eliminating the worry of cross-linking to residues in the binding site. Because the variable domains do not contain any free cysteines (again except in very rare occasions in the hypervariable loops), the derivatization at such a free cysteine would occur at a unique predetermined position.

A final consideration is the bivalency of the antibody. However, use the Fc part is not the only method by which this can be achieved, and there are now other, much smaller dimerization handles available, with which recombinant fragments of various kinds can be made to dimerize (discussed later).

We may conclude, therefore, that the presence of biological effector functions in the Fc portion (mediating ADCC and ADCMC) is the only reason for making whole antibodies, as all other functions can be obtained with smaller recombinant fragments, using a variety of different means.

B. Fab Fragments

Most antibody classes can be proteolytically cleaved in the hinge region to generate Fab fragments and the Fc portion. Depending on whether the cleavage occurs in front of or after the

interchain disulfides in the hinge region, the Fab fragment (usually obtained by papain digestion) or the $(Fab')_2$ fragment (by pepsin digestion) is obtained.

The Fab fragments (Fig. 2), can be made in recombinant form in a variety of host organisms. Particularly useful is their production in bacteria, and the technology will be described in a later section. It should be stressed again that the *inter*chain disulfide, with which C_H1 and C_L are linked in many subclasses, is not the cause of heavy–light pairing (Alexandru et al., 1980), and it can be taken out by mutagenesis, sometimes even slightly improving the homogeneity of the product, since this disulfide formation may not always be quantitative (Skerra and Plückthun, 1991).

What then is the function of C_H1 and C_L? It appears that their noncovalent association helps the stability of the heterodimer (Bigelow et al., 1974; Hochman et al., 1976; Klein et al., 1979; Horne et al., 1982), and nature thus has the possibility of allowing more variability in V_H and V_L, as these are not restrained by the demands on the V_H–V_L interaction energy.

Although C_H1 and C_L have also been used as handles for purification, detection and immobilization (usually with antibodies directed against these domains), much shorter tag sequences can now fulfill the same role in a much smaller molecule.

C. Fv Fragments and Covalent Derivatives

From the three-dimensional structure of antibodies (see Fig. 1), it is now clear that the Fv fragment (the heterodimer of V_H and V_L) contains the complete binding site (see also Chap. 1). Experimentally, this was first shown by proteolytic digestion of an antibody at about the same time as the first crystal structures became available (Inbar et al., 1972; Hochman et al., 1973; 1976; reviewed in Givol, 1991). Unfortunately, the proteolytic sites for cleaving off V_H and V_L are not particularly preferred except in special cases (Sharon and Givol, 1976; Takahashi et al., 1991). Therefore, the only practically useful access to Fv fragments is through gene technology (see Fig. 2).

This was facilitated by a functional *Escherichia coli* expression system (Skerra and

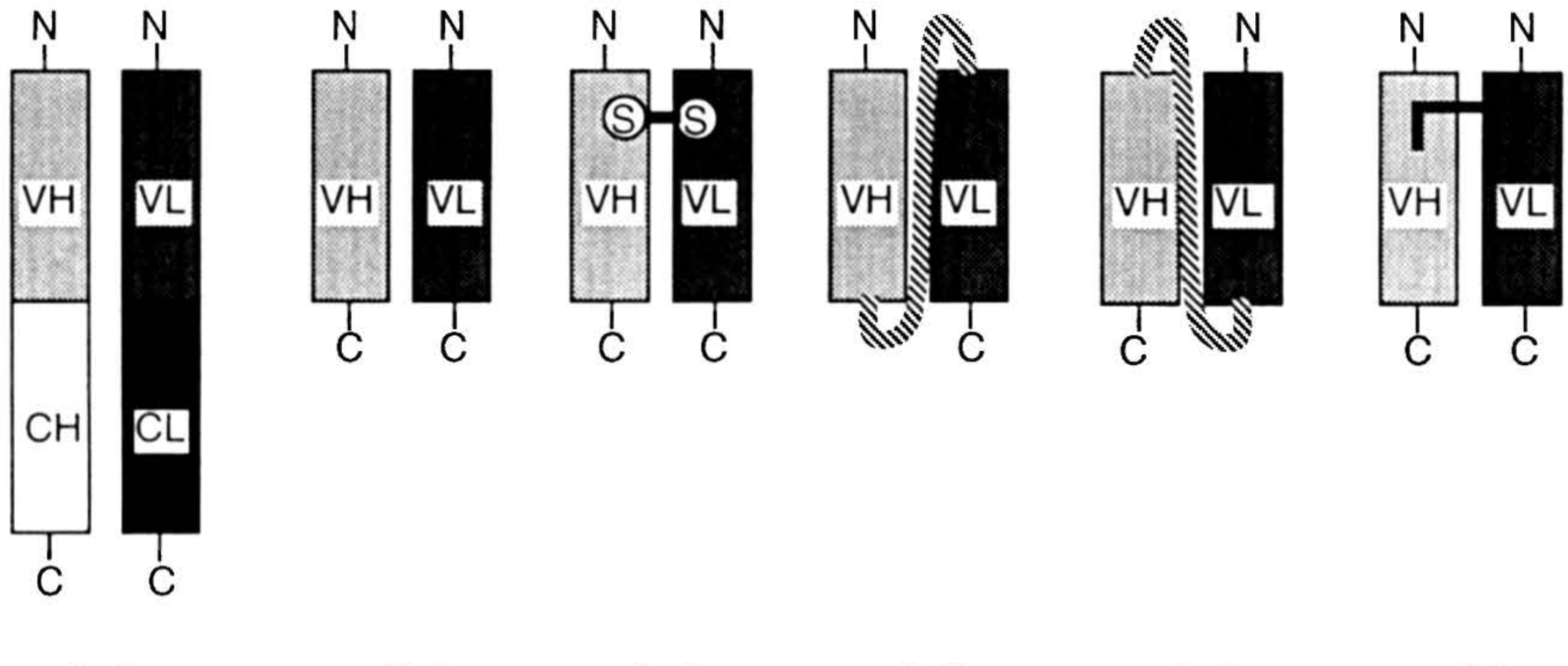


Figure 2 Monovalent fragments of the antibody shown schematically: (a) Fab fragment, (b) Fv fragment, (c) disulfide-linked Fv fragment containing an engineered intermolecular disulfide bond, (d) single-chain Fv fragment with the orientation V_H–linker–V_L, (e) single-chain Fv fragment with the orientation V_L–linker–V_H, (f) Fv fragment that has been stabilized by chemical cross-linking after purification. (c) to (f) indicate various possibilities of stabilizing the Fv fragment against dissociation (for details, see e.g., Glockshuber et al., 1990). Note that every antibody domain contains an internal disulfide bond (see also Fig. 3) which is crucial for stability, but is not shown here for simplicity. Depending on the antibody class, the two chains of the Fab fragment may also be (naturally) covalently linked, but this is of much lower importance for stability. All of these fragments have been functionally expressed in *Escherichia coli*. (From Plückthun, 1992.)

Plückthun, 1988), but Fv fragments can also be made in myeloma cells (Riechman et al., 1988b). For the Fv fragments reported, full functionality was observed. However, one complication was noted: The affinity of V_H and V_L for each other is lower than that of the two chains making up the Fab fragment (Glockshuber et al., 1990).

The affinity of V_H and V_L for each other depends on their primary sequence. This is immediately obvious from the three-dimensional structure (Fig. 3), since the parts of the domain, which are structurally most variable, CDR3 of the heavy and light chains are centrally located in the heterodimer and provide a number of binding interactions (Figs. 4 and 5). Consequently, some Fv fragments are "stable," whereas others are not. In binding analyses, this dissociation into V_H and V_L may lead to an *apparent* loss of binding, because the antigen (by binding to both V_H and V_L) stabilizes the heterodimer. In fluorescence titrations, curved Scatchard plots may result (Glockshuber et al., 1990).

Three strategies are available to combat this dissociation problem (Glockshuber et al., 1990). The first is chemical cross-linking. It does require, however, that the Fv fragment can be obtained in the first place, and it is somewhat dependent on the availability of suitably spaced lysine residues. As a result, the efficiency of this method may vary.

The most common solution is the use of the so-called single-chain Fv (scFv) fragment (see Fig. 2; Fig. 6) (Huston et al., 1988; Bird et al., 1988). In this case, V_H and V_L are not expressed as two separate proteins, but as a continuous protein, with a genetically encoded peptide linker. The COOH-terminus of V_H and the NH_2-terminus of V_L are typically about 35 Å apart, whereas the COOH-terminus of V_L and the NH_2-terminus of V_H are typically about 40–45 Å apart. Therefore, the two domains cannot be fused directly, but a peptide linker needs to be inserted, commensurate with the C—N distance in the native protein (see Fig. 6).

Numerous linkers have been tested and found to be functional (summarized in Huston, 1991). Apparently, few constraints exist except that the linker must have the right length and must not interfere with protein folding. It is useful, therefore, to avoid hydrophobic residues. The most popular linker has the sequence $(Gly_4Ser)_3$, and it has been used in both the orientations V_H–linker–V_L and V_L–linker–V_H,sometimes even for the same antibody (Anand et al., 1991; Knappik et al., 1993). Here, the orientation made no difference in stability (Knappik et al., 1993).

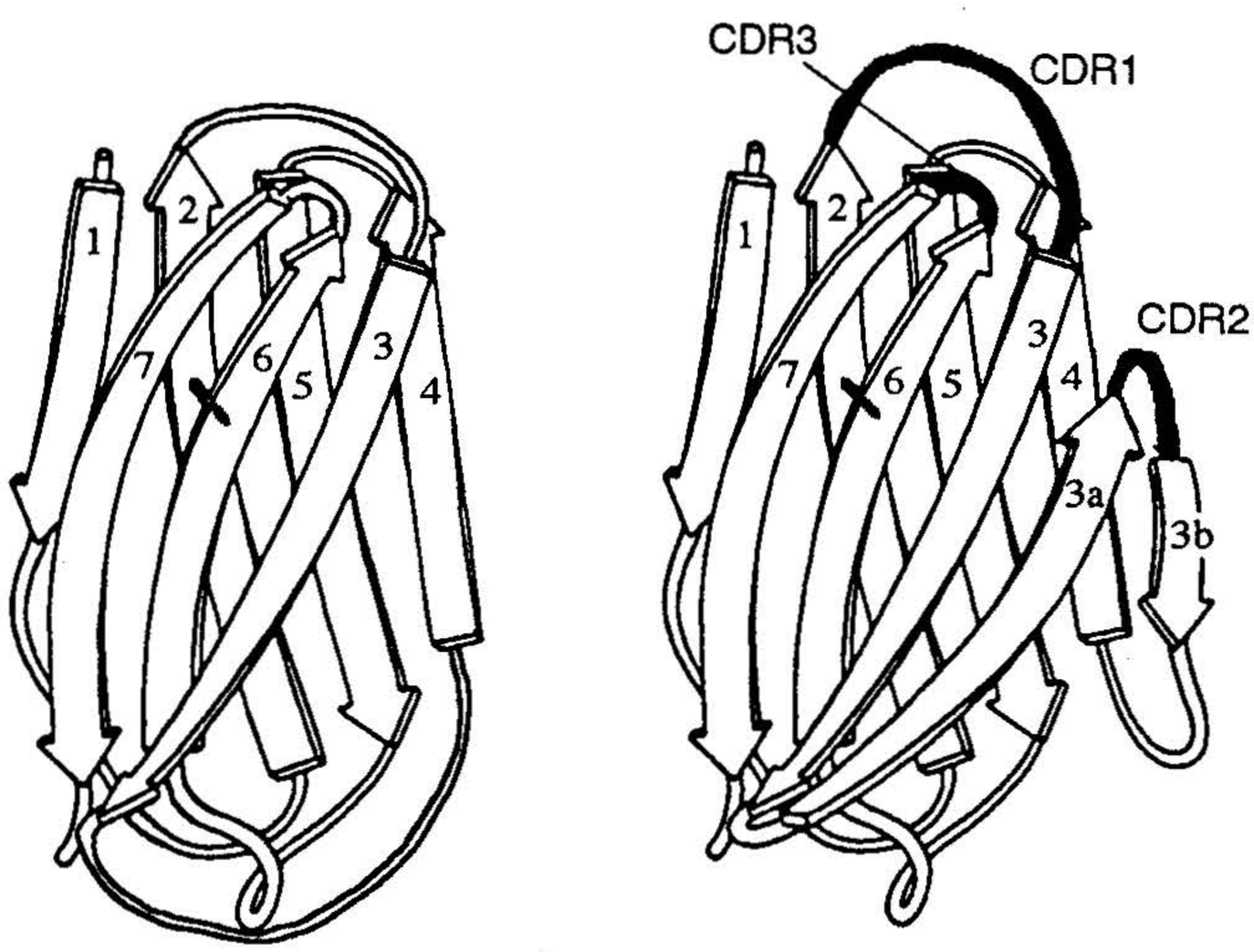


Figure 3 Topology of antibody constant and variable domains. The two additional strands in the variable domains can be clearly seen. The front face of the domain in this view is the dimerization interface. The central disulfide bond linking strand 2 to 6 is also indicated. (Adapted from Branden and Tooze, 1991.)

Recent nuclear magnetic resonance (NMR) results (Freund et al., 1993) now allow some conclusions to be drawn about the structure of the linker in the scFv fragment. By comparing the ^{1}H-NMR spectrum of an Fv fragment with the scFv fragment of the same antibody, it was found that there are essentially no differences in the structure, indicating that the linker does not interfere with the folded protein. Furthermore, the binding constants for several scFv fragments were very similar to the whole antibody (Huston et al., 1993). By labeling the scFv fragment with [^{15}N]serine and [^{15}N]glycine, it was possible to see the spectrum of the $(Gly_4Ser)_3$ linker directly, and deduce that it makes no permanent contacts with the rest of the protein, but is surrounded mainly by water and is much more flexible than the rest of the protein.

A picture thus emerges of scFv fragments consisting of two domains loosely held together. Indeed, the thermodynamic stability of the scFv fragment is rather low, and the peptide linker contributes little. Therefore, the weak V_H–V_L interaction energy of some antibodies is not automatically strengthened by using a scFv fragment. Instead, the dissociation is now converted to another reaction, an opening of a loosely linked fragment. This can lead to some problems of aggregation on concentration, and occasionally to some losses. However, many scFv fragments appear to be stable (Freund et al., 1993; Huston et al., 1993). The strategy to be discussed next however, disulfide linking of the variable domains, is more efficient at increasing thermodynamic stability (Glockshuber et al., 1990).

D. Disulfide-Linked Fv Fragments

The two variable domains in an Fv fragment can also be connected with a disulfide bond (see Fig. 2). In the initial demonstration of this effect (Glockshuber et al., 1990), the structures of the two domains were scanned using geometric search criteria (Pabo and Suchanek, 1986): Those positions were identified as potential candidates, for which a good overlap was possible between the main-chain atoms of the two amino acids to be tested and the main-chain atoms of any two cysteines forming a disulfide bond in the structural database. By using this approach, two positions were experimentally tested, which were found to be the most suitable ones. It was found that these S—S-linked Fv fragments could be made in *E. coli* and were significantly more stable toward irreversible denaturation at 37°C than the scFv fragment or the Fv fragment (Glockshuber et al., 1990). This directly shows that it is not sufficient to just covalently couple the two chains, but that the *method* of covalent linking is decisive. The "loose" coupling with the peptide linker in a scFv fragment is a less efficient guard against irreversible denaturation than the disulfide bonds, which may not allow reversible opening of the Fv fragment at the interface.

In the initial design, the positions found most suitable for disulfide bond formation linked CDR3 of the heavy chain to CDR2 of the light chain; however, because of the great structural variability of CDR3 of the heavy chain, these would not be generally suitable positions. In an effort to find a more general solution to this problem, the structures of ten Fv fragments were superimposed, and all positions at which a disulfide link would be possible were identified in as many fragments as possible. Indeed, two such positions were identified (Plückthun, 1993b). In both of them, framework region 4 of one chain is connected to framework region 2 of the other chain (Fig. 7) (for nomenclature, see Kabat et al., 1991). Because the structures of V_L and V_H are related to each other, and because the Fv fragment has something close to a pseudo–two-fold axis, these two positions are also structurally related. In the consensus numbering of Kabat et al. (1991), this would correspond to H-44 and L-100 for one S—S bond and H-103 to H-106 and L-43 for the other. Perfect matches could not be found with all Fv fragments because of some variation in the V_H–V_L association geometry, yet, a satisfactory fit was obtained for a large percentage of structures, and the variability may in itself hint at some flexibility in this area. Only future experience with a large number of Fv fragments may allow a judgment about the generality of this method.

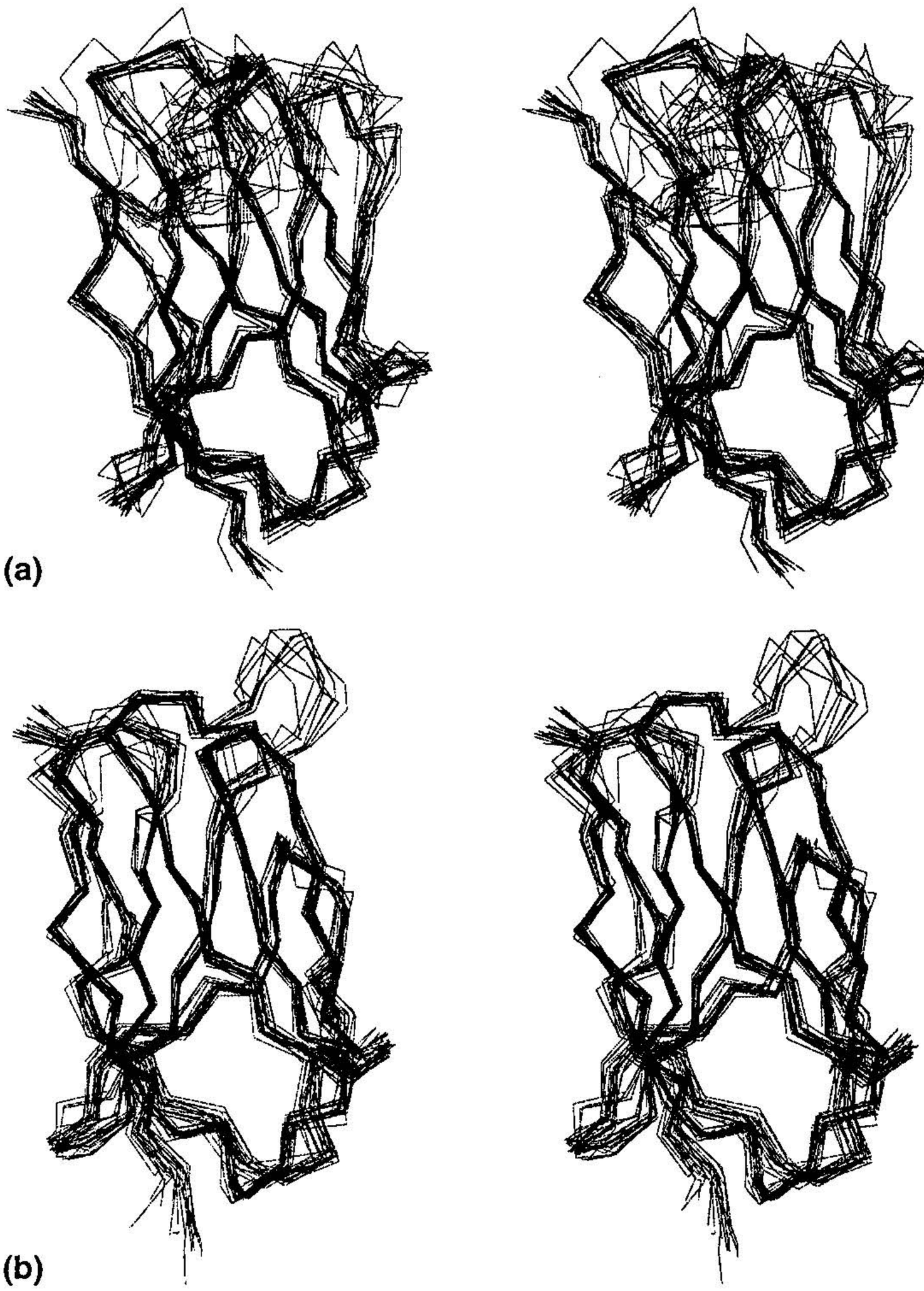
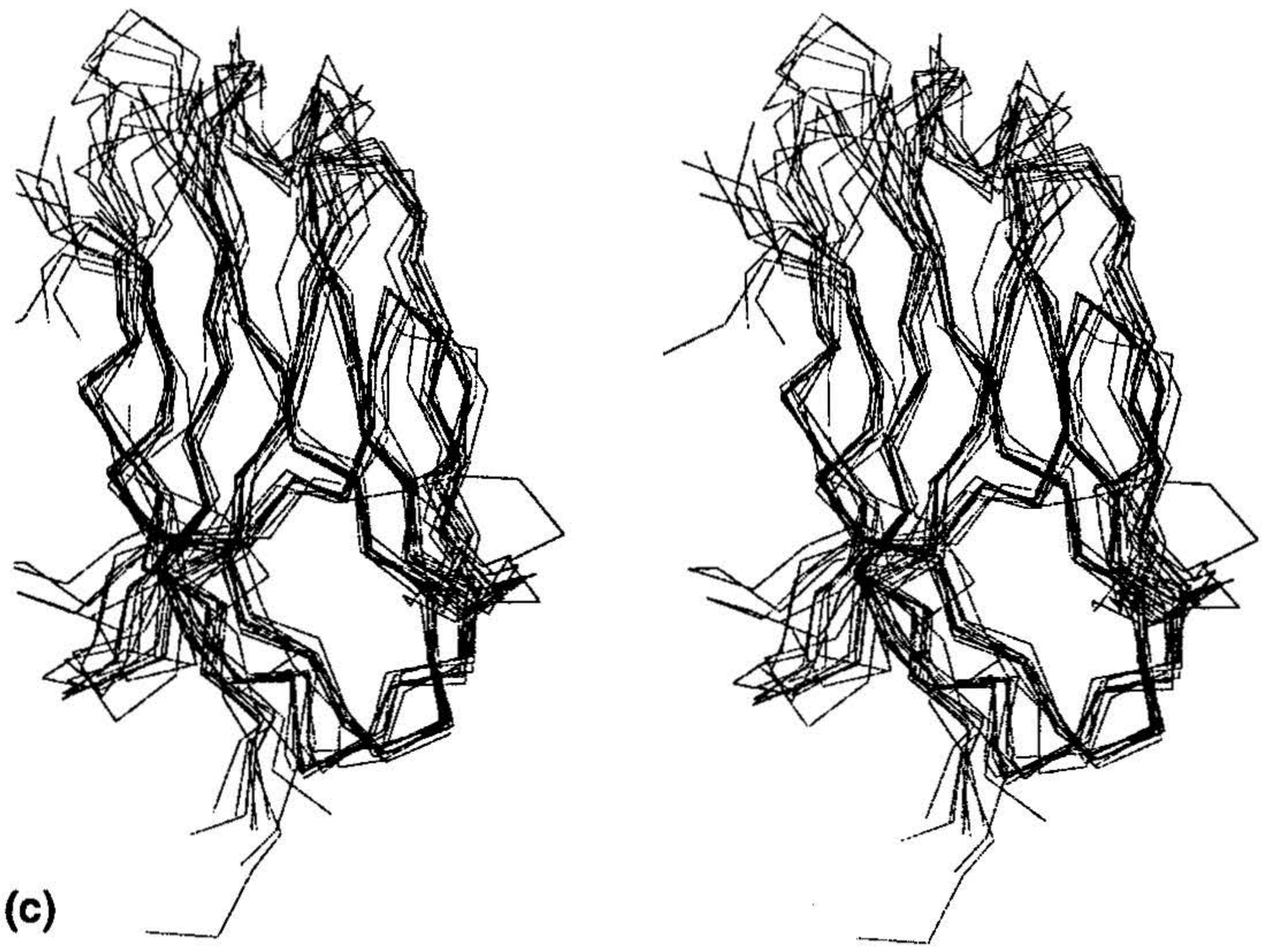


Figure 4 Stereodiagrams of the superposition of crystallized antibody domains. The orientation of the domains is approximately as in Fig. 3, and the view is again from the dimer interface. These diagrams should indicate the enormous structural variation in the hypervariable loops (CDRs) and the conservation of topology of the framework. This is the most important prerequisite for the humanization, as discussed in the text. However, the framework also shows variations leading to slightly different V_H–V_L orientations, which may profoundly affect the shape of the binding site. (a) V_H domains, (b) κV_L domains, (c) λV_L domains.



(c)

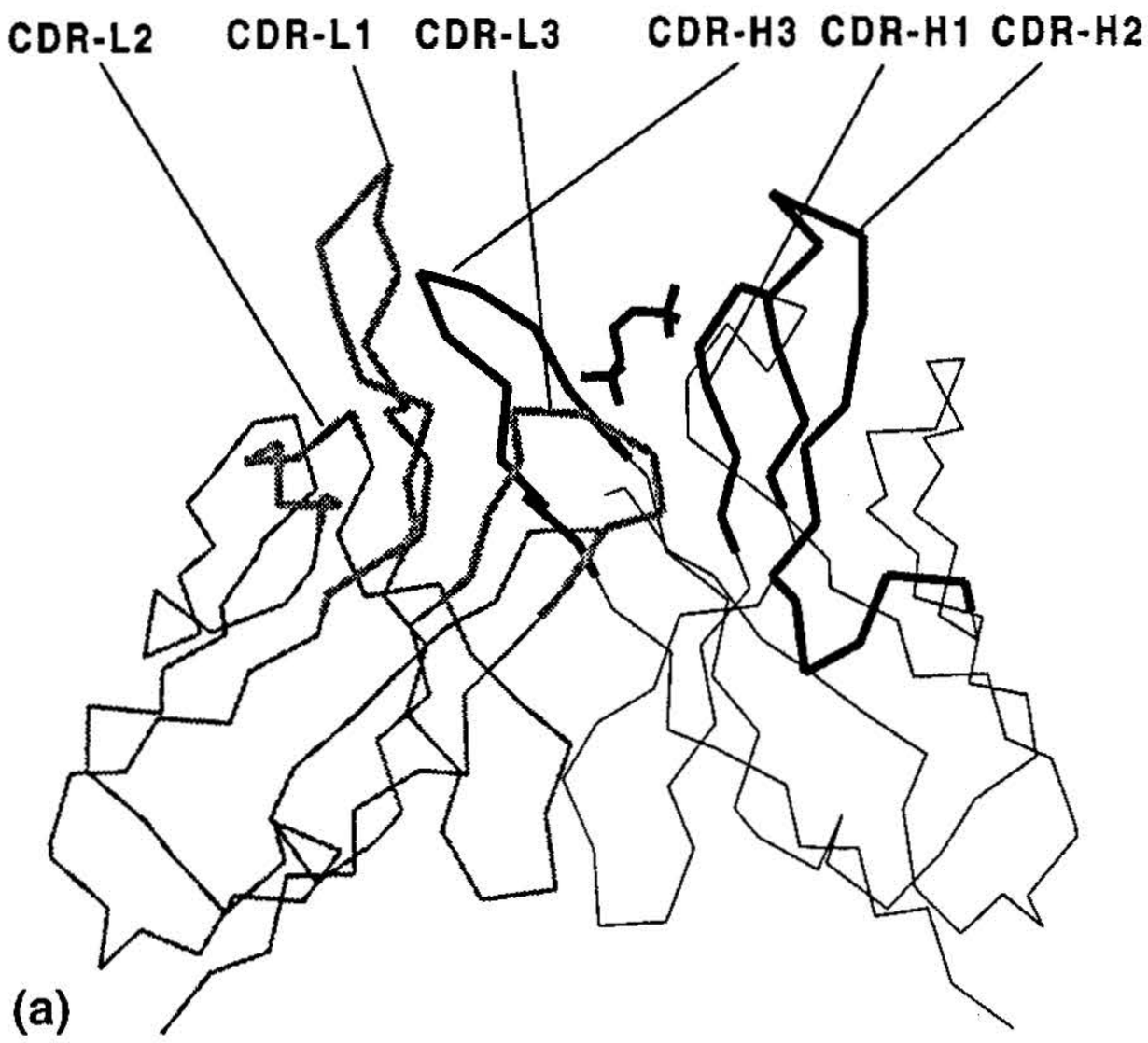
E. Why Would Anybody Want Small Antibody Fragments?

All of the attempts to solve the stability and association problems of Fv fragments are fueled by the desire to make very small antigen-binding molecules. There are many motivations for doing so. One is that it is generally more efficient to produce smaller molecules. The folding efficiency both in vitro and in vivo (that is, in bacteria; there seem to be no quantitative investigations about folding of antibody fragments in higher cells) seems to be often better for Fv-derived fragments than for Fab fragments or larger molecules. However, because of the extraordinary complexity of the underlying causes, there are certainly exceptions.

Another reason is that the constant regions may cause some of the nonspecific reactions of antibodies observed in applications such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA), and the best solution is the complete absence of this additional protein surface.

Yet another motivation is the desire to carry out structural studies with the antibody. Currently, complete NMR assignments with fragments larger than Fv are not possible. Similarly, Fv-sized fragments appear to give better-ordered crystals than Fab fragments (Bhat et al., 1990; Steipe et al., 1992), although it is dangerous to generalize from the few examples currently available.

The most important reason, however, may be the desire to use smaller molecules in antibody-based diagnostics and therapy. Usually, small fragments must be coupled to toxic agents, as they do not carry the effector functions residing in the Fc portion, and it is highly desirable that the damage caused by such toxic molecules be minimized when they are not bound to their target cell. Therefore, it may be advantageous if they clear rapidly through the kidney, and clearance has been shown to depend on the size of the protein (Colcher et al., 1990; Yokota et al., 1992). Additionally, solid tumors are often poorly vascularized, and the penetration of large molecules is very inefficient. Many researchers today hope that the use of antibodies in this type of therapy will be more successful if applied in the form of small Fv-sized fragments. Similarly, in diagnostic imaging, an antibody is coupled to a radioactive label, and the fast excretion of protein not bound to the target (causing the background signal) is advantageous (Huston et al., 1993).



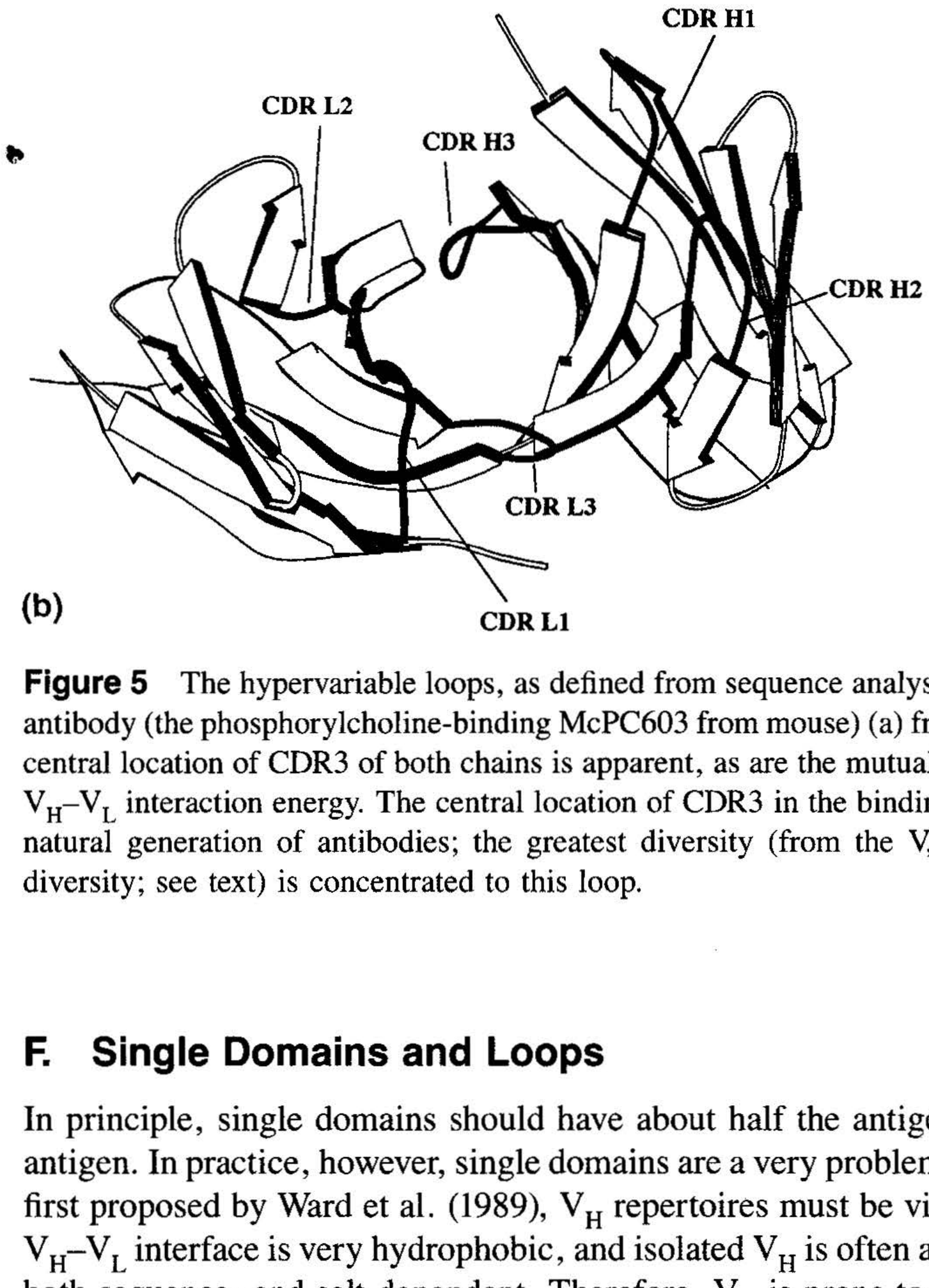


Figure 5 The hypervariable loops, as defined from sequence analysis (Kabat et al., 1991) shown for one antibody (the phosphorylcholine-binding McPC603 from mouse) (a) from the side and (b) from the top. The central location of CDR3 of both chains is apparent, as are the mutual contacts that may also influence the V_H–V_L interaction energy. The central location of CDR3 in the binding site is also why it is crucial in the natural generation of antibodies; the greatest diversity (from the V, D, J recombination, and N-region diversity; see text) is concentrated to this loop.

F. Single Domains and Loops

In principle, single domains should have about half the antigen-binding site and still bind the antigen. In practice, however, single domains are a very problematic substitute for antibodies. As first proposed by Ward et al. (1989), V_H repertoires must be viewed with some skepticism. The V_H–V_L interface is very hydrophobic, and isolated V_H is often at the limit of solubility, this being both sequence- and salt-dependent. Therefore, V_H is prone to nonspecific adsorption. This has been demonstrated experimentally: An Fv fragment used as an affinity ligand could separate two lysozyme variants, whereas the V_H domain from the same antibody could not (Berry and Davies,

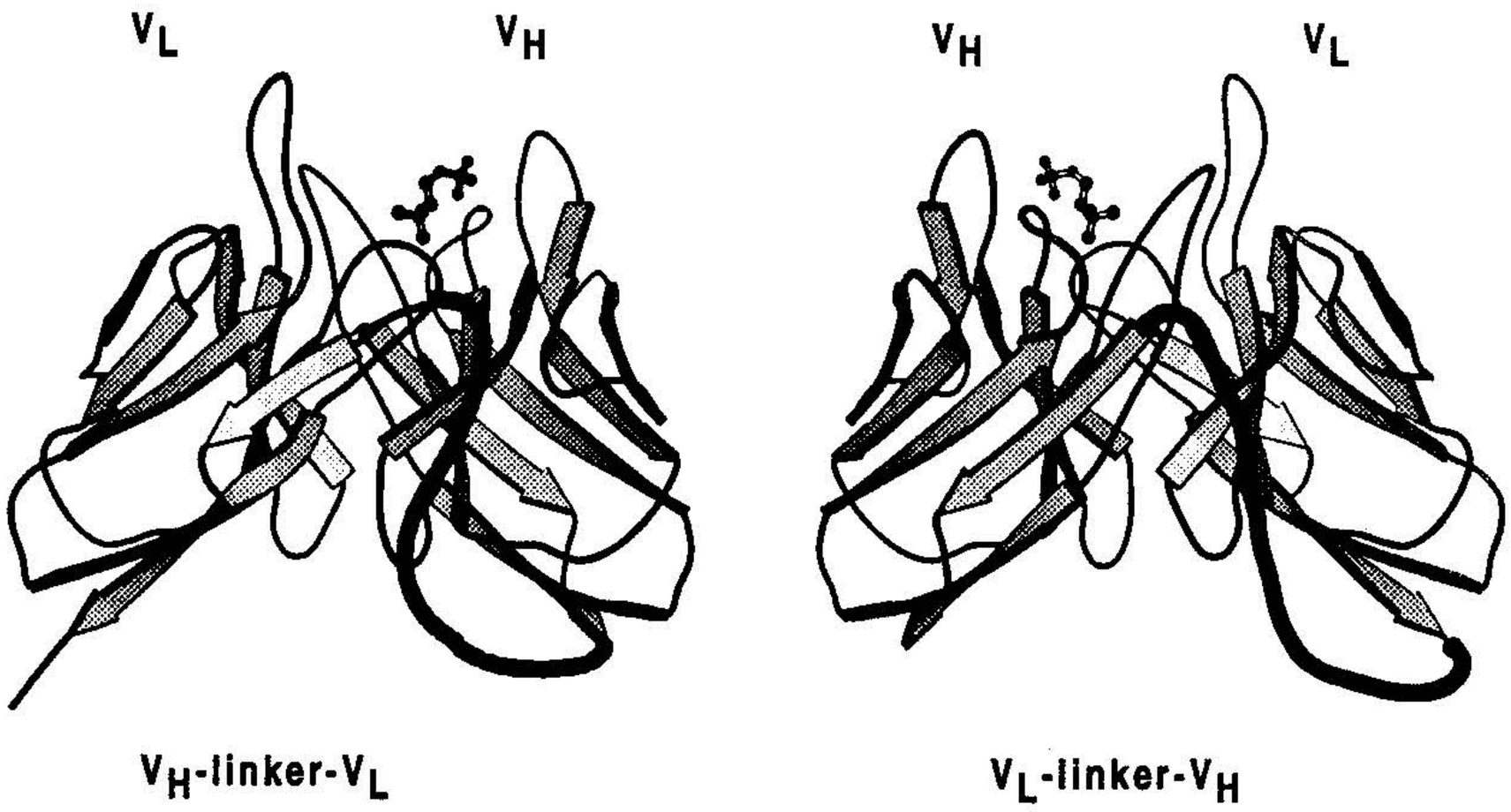


Figure 6 Schematic structure (monoview) of the two scFv fragments of the antibody McPC603. On the left, the scFv with the orientation V_H–linker–V_L is shown. On the right, the fragment is rotated 180° about the *y*-axis, and the other possibility of linking is shown (V_L–linker–V_H). The bold line indicates only the general topology of the linker. Recent NMR experiments (Freund et al., 1993) indicate that the linker is flexible and exposed mainly to water, and it may not have a preferred structure. The NMR experiments also suggest that the structure of the Fv portion is not influenced by the presence of the linker.

1992). Because of their poor solubility, V_H domains would also be difficult to produce, and a library may be very incomplete.

The V_L domains, on the other hand, may dimerize with themselves. Similar to the V_H–V_L association energy, the V_L–V_L association energy will also vary from antibody to antibody (Azuma et al., 1974; 1978; Maeda et al., 1976; Klein et al., 1979; Stevens et al., 1980). In the dimer state, the binding site would be obstructed, and V_L would not be generally useful for binding the antigen. Nevertheless, V_L domains have been useful models in structural studies (Steipe et al., 1992).

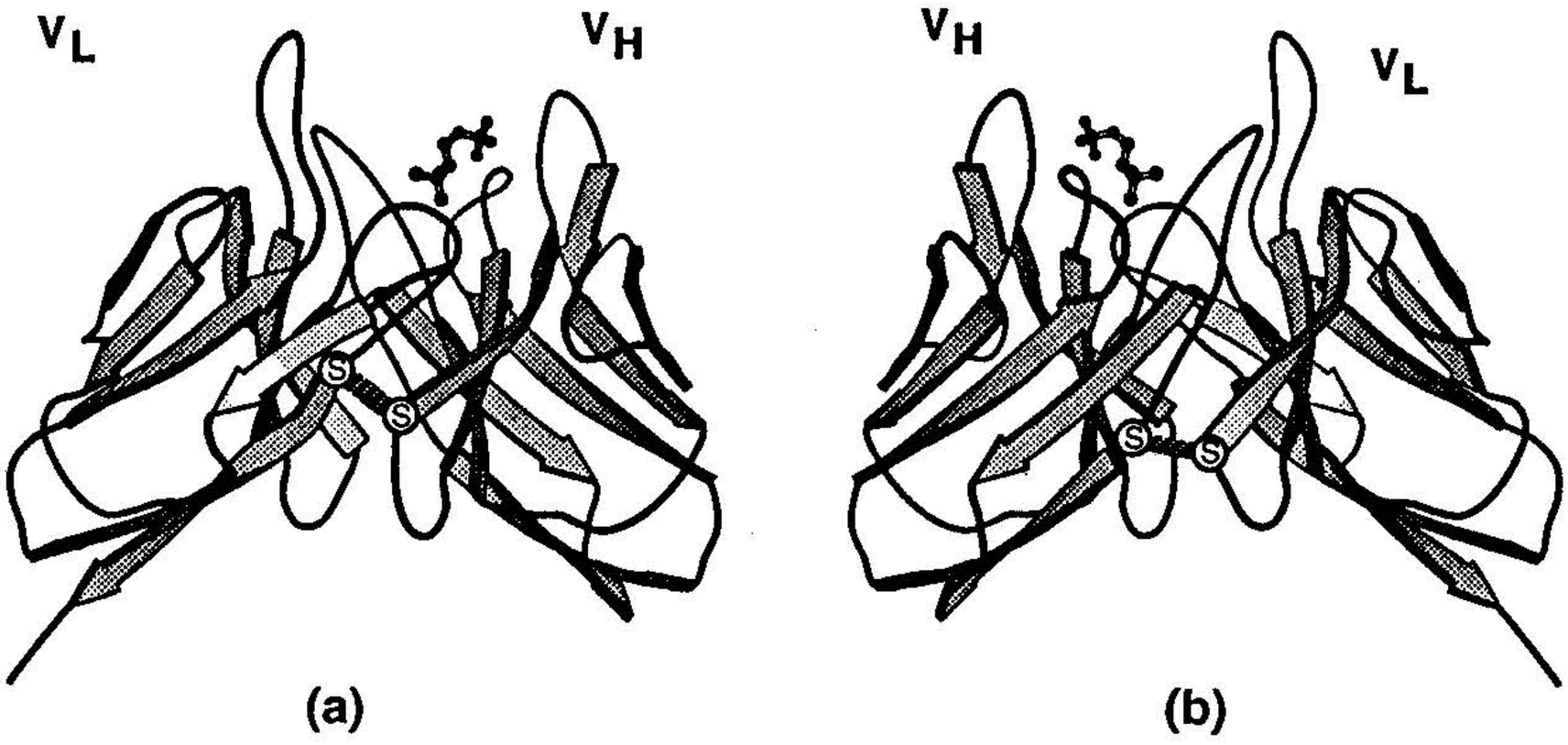


Figure 7 (a) Schematic structure (monoview) of the Fv fragment of the antibody McPC603, with the positions labeled that were found suitable for linking V_H and V_L in the framework in a variety of Fv fragments. (b) Same fragment as in (a) rotated 180° about the *y*-axis, indicating the other S—S bond. The similarity of the two possible positions, owing to the molecular pseudo–two-fold axis, is apparent. In each case, framework region 2 is connected to framework region 4 (for nomenclature, see Kabat et al., 1991).

Occasionally, single loops of the antibody may protrude into the antigen. When this occurs, weak binding of single domains is possible, and even the binding of isolated peptides is sometimes detectable (Williams et al., 1989; 1991; Saragovi et al., 1991). Generally, however, the affinities are in only the micromolar range, and this may be too low for many applications. Nevertheless, this is an area of active research. Neither proteins nor peptides are the ideal pharmaceutical compounds, but there is great interest in using them for finding general "lead substances" against a new target. Because selective antibodies against almost any target can be made, a *general* method from an antibody-binding site to a tight-binding small organic molecule would be a fundamental achievement.

G. Miniantibodies

As an example of new types of molecules accessible from genetic engineering, bivalent miniantibodies will be discussed. They combine the advantage of small size and the avidity gain from binding with two binding sites. Furthermore, they are readily available in functional form from bacteria by methods discussed in the following.

Why is bivalency advantageous and by how much? Any repetitive antigen structure (such as a cell or virus surface, an ELISA plate covered with antigen, or a polymeric antigen such as a polysaccharide or DNA) makes it possible that two binding sites of the same antibody bind simultaneously (Fig. 8). After the first binding site docks the antibody to the surface, the second one is now constrained to the neighborhood; its local concentration increases. Therefore, the effective affinity (also called avidity) of the bivalent molecule is the intrinsic affinity (i.e., monovalent binding) times a dimensionless number, which describes the docking of the second site. This second docking is more likely to occur if the intrinsic affinity is high, if there are many epitopes on the surface within reach, and if the linker arm connecting the two is short enough to constrain the second binding site close to the surface, but not so short that no second binding ever occurs. These relations have been quantitated (Crothers and Metzger, 1972; Karush, 1976; 1978), and this is depicted in Fig. 8. It is immediately obvious that the avidity gain by bivalency is dependent on many geometric factors and, consequently, that there will be enormous variability between different systems.

Nature has presumably optimized the distance between the two antigen-binding sites in the whole antibody (see Fig. 1) for the shortest distance likely to be useful for the antigens most frequently encountered. Single-chain Fv fragments (Bird et al., 1988; Huston et al., 1988; Glockshuber et al., 1990) have been connected by a long and flexible hinge region to a very small dimerization domain, such as one or two amphiphilic helices (Pack and Plückthun, 1992). Particularly useful are helix-turn-helix motifs, which form four-helix bundles (Fig. 9), as they lead to very stable fragments with avidity identical to whole antibodies, but only a third of their size (Pack et al., 1993). The molecules assemble by themselves in *E. coli* and can be produced in quantities of several hundred milligram per liter in this organism.

Similarly, bispecific antibody fragments can be constructed with similar techniques, and there is great interest in this application in the field of tumor immunology. For instance, more than one tumor marker may be combined to obtain greater tumor selectivity, or T cells may be recruited for killing (overview: Fanger and Guyre, 1991). Such dimerization devices can also be used to link other functionalities, such as toxins, marker enzymes, or enzymes that activate prodrugs.

Recombinant Fab fragments or Fv fragments can also be linked chemically (Carter et al., 1992; Cumber et al., 1992), most conveniently at cysteines introduced at the COOH-terminus. It appears, however, that disulfide formation does not occur with good yields in vivo, even for Fab′ fragments (to form $(Fab')_2$ fragments), neither in prokaryotes (Carter et al., 1992) nor in eukaryotes (Adair, 1992), as the disulfide formation across the hinge is not the *cause* of

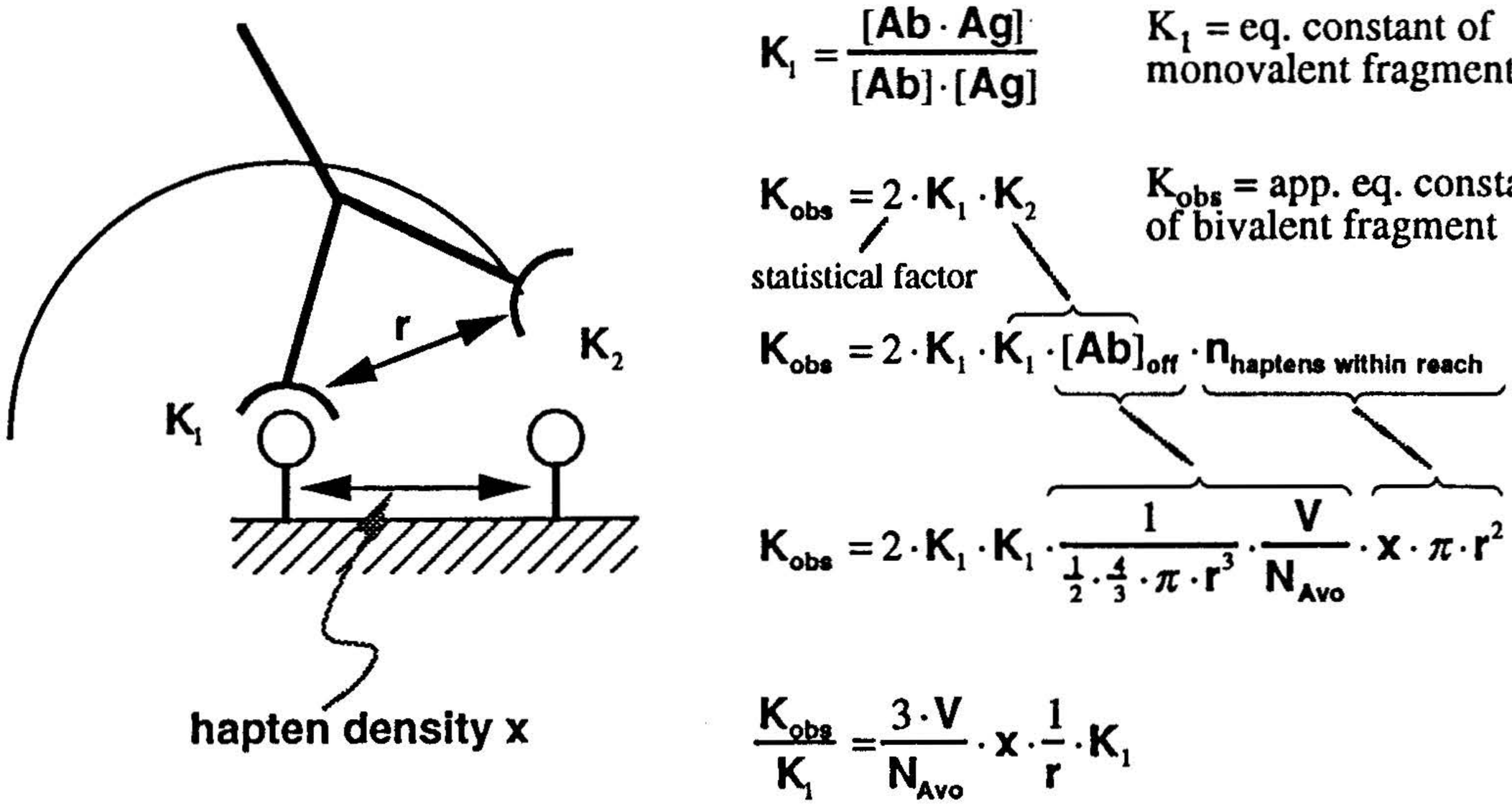


Figure 8 Enhancement of apparent equilibrium constant through bivalency effects, according to the treatment of Crothers and Metzger (1972). K_1 is the observed binding constant for a monovalent fragment; K_{obs} for a bivalent fragment. [Ab], [Ag] and [Ab·Ag]are the molar concentrations of antibody, antigen, and complex, respectively (as if the number of molecules in the reaction well were evenly distributed). K_2 is the binding constant of the second binding site, made unitless by the constant effective antibody concentration $[Ab]_{eff}$ that results from constraining it within the hemisphere of radius r. The hapten density x is a two-dimensional concentration (molecules per area). N_{Avo} is Avogadro's number, used to convert molecular into molar concentrations.

dimerization. Rather, in the whole antibody, the constant domains associate noncovalently, leaving time for covalent disulfide formation to occur in the hinge region.

H. Fusion Proteins

Fusion proteins, consisting of antibody fragments and other proteins, have been the focus of much interest in the field of recombinant antibodies. Fusion proteins are now being constructed in all areas where chemical immunoconjugates have been used. Both single-chain Fv fragments and Fab fragments have been used, and just as for the unfused antibody fragments, a variety of expression hosts have been investigated.

Of particular interest are fusions to enzymes (see, e.g., Neuberger et al., 1984; Wels et al., 1992b), since these enzymes are useful as detection reagents in ELISAs or similar applications, and also in prodrug activation therapy (Bosslet et al., 1992; Senter et al., 1993).

Additionally, toxins have been fused to antibody fragments in pursuit of the "magic bullet" that would kill only the target cell, but leave the rest of the body intact (see, e.g., Chaudhary et al., 1989; 1990; Batra et al., 1990; Wels et al., 1992a). Although this looks promising, many problems that limit both the specificity and the efficacy of the constructs described so far remain to be solved.

Protein domains have also been fused merely for simplifying purification and detection, but the same can be achieved with much smaller peptides, which do not have to be cleaved off afterwards (see, e.g., Ward et al., 1989; Lindner et al., 1992).

The list of fusion proteins is rapidly expanding, and it is not the function of this chapter to provide a comprehensive enumeration. There are many reasons why one may wish to fuse an

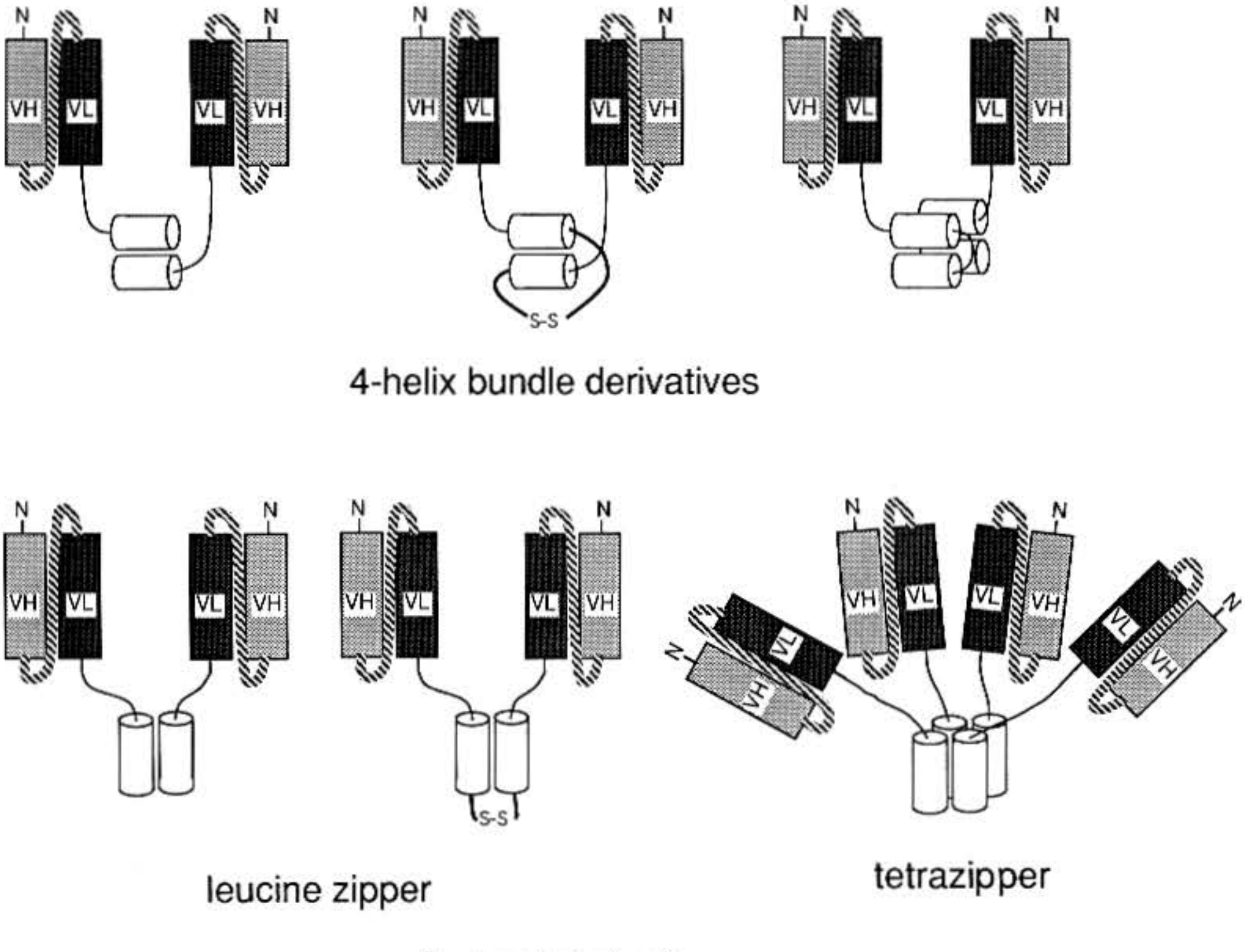


Figure 9 Bivalent fragments that have been shown to assemble in *E. coli*. In each example, a scFv fragment is connected to a hinge region, followed by an amphiphilic helix. In the top row, the helix comes from a four-helix bundle design by deGrado and co-workers. On the top left only one helix is fused, but the preponderant molecular species are dimers. In the middle, they are connected by a peptide, which ends in a cysteine. On the right, two helices are fused in tandem and a four-helix bundle is probably obtained, as very stable dimers are formed in vivo. In the bottom row, a parallel coiled-coil helix from a leucine zipper is used. This design is suitable for making heterodimers. On the right, the sequence of the zipper is changed for tetramerization by exchanging the amino acids at the interface (for details, see Pack and Plückthun, 1992; Pack et al., 1993; Pack et al., unpublished).

"effector molecule" to a targeting reagent in the form of an antibody, and this is probably one of the most immediately useful areas of recombinant antibody technology.

III. RESHAPING THE BINDING SITE: HUMANIZATION

An important application of reshaping the antibody variable domains is the humanization of the molecule (Fig. 10). The goal is to convert a given antibody (usually murine, when obtained by conventional monoclonal technology; see Chap. 4) to as fully human a form as possible. In this section, we will discuss only the design of the sequence. Many possibilities exist on how to actually assemble the gene in practice, involving either mutagenesis, total gene synthesis, PCR, or any combination thereof, and they will be only very briefly discussed in a later section.

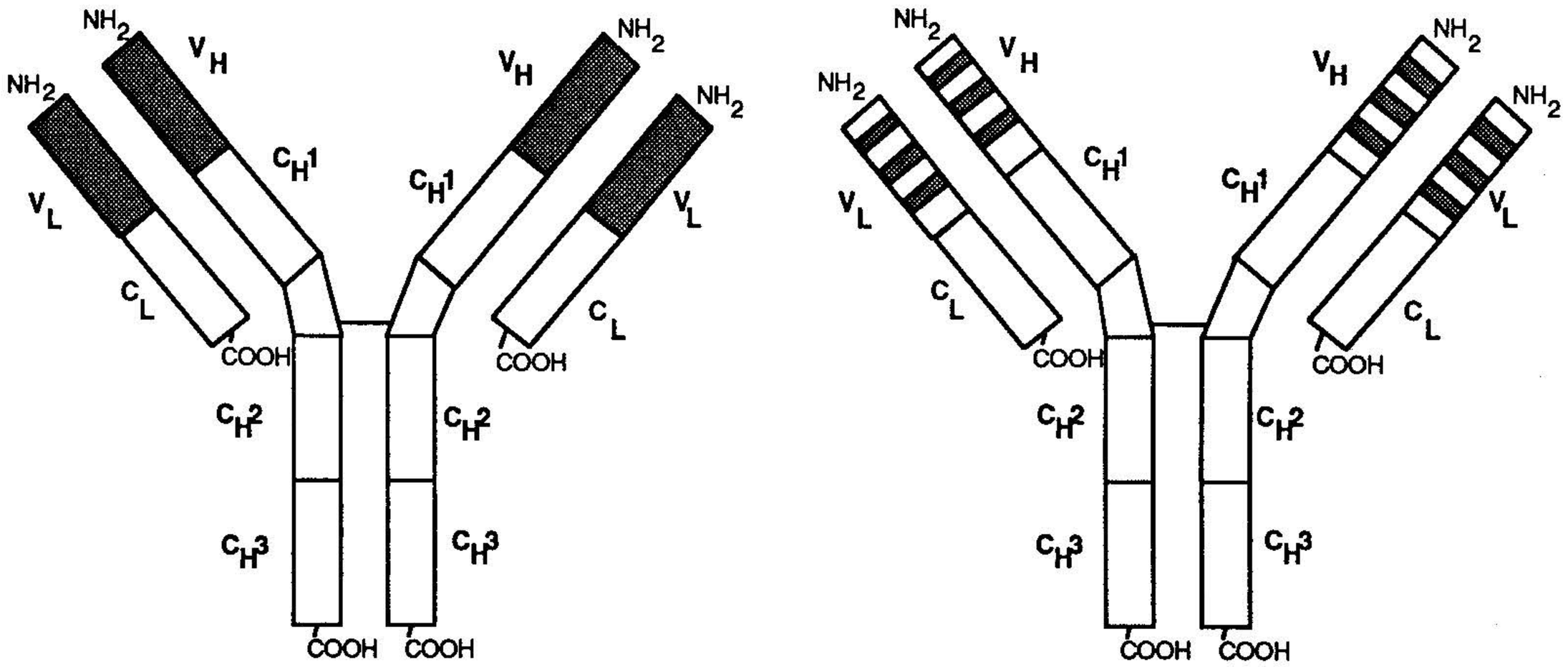


Figure 10 Chimerization and humanization. In chimerization (left), the whole variable domains would remain from the original (murine) antibody. In humanization (right), only the hypervariable loops would remain murine. In both diagrams, white are human sequences, gray are murine sequences.

Before going to the sequence design, it should again be emphasized that the humanization of an antigen-binding domain is independent of the choice of the fragment size of the antibody. One may thus humanize whole antibodies, scFv fragments, Fab fragments, or any fusion protein. Furthermore, it may be advantageous to test new variants (using, for example, an Fab fragment) with a convenient expression system such as *E. coli* (Plückthun, 1991), even when the final version is a whole antibody to be made in eukaryotic cells.

Humanization has also been called "CDR grafting," "reshaping," "hyperchimerization," and "veneering," (see, e. g., Jones et al., 1986; Verhoeyen et al., 1988; Riechman et al., 1988a; Queen et al., 1989; Kurrle et al., 1990; Tempest et al., 1991; Law et al., 1991; Shearman et al., 1991; Co et al., 1991; 1992). All of these are slightly different approaches aimed at introducing as much human sequence as possible without compromising the binding efficiency. A recent compilation of such experiments is given in Adair (1992).

All of this is possible, for crystallography has shown that the framework of the antibody is much better structurally conserved than the CDRs (see Fig. 4). Importantly, this is also true across species boundaries. Therefore, the initial attempt was to simply graft the CDR sequences from the murine to a human framework (Jones et al., 1986). However, the definition of "CDR" and "framework" is somewhat arbitrary and is based on the statistics of sequence variations (Kabat et al., 1991). There are frequently residues outside the CDR that need to be transferred as well because they may (1) have direct interactions with the antigen, or (2) may themselves have interactions with the CDRs, thereby influencing their conformation. Hence, it may not be surprising that the simple grafting of the CDRs will not normally lead to the same binding affinity as for the murine antibody, unless further residues are changed to the murine version as well. These residues will generally be (1) adjacent to the CDRs in the linear sequence; or (2) adjacent to any residues of the CDR in space (Padlan, 1991; Foote and Winter, 1992). From a comparison of loop conformations in crystal structures, a few framework residues have also been identified that may determine loop conformations (Chothia and Lesk, 1987; Chothia et al., 1989).

While paying attention to these general considerations, a sequence of the humanized antibody has to be designed. In general, a three-dimensional model should be built, which has become easier, not so much because of progress in theoretical understanding, but because the number of crystal structures is continuously growing, and a similar loop can now more often be found.

Three basic strategies for designing the humanized sequence can be delineated, all of which have led to success, but which are not mutually exclusive and can certainly be combined.

The first possibility is to use as the basis of the human framework one of the human antibodies for which the x-ray structure has been solved, usually REI and NEWM. By using extensive sequence tabulations (Kabat et al., 1991), positions in the sequence are then identified that constitute likely somatic mutations in the murine and the human sequence of each chain. The aim is to keep the murine somatic mutations (under the assumption of positive selection for tight binding) and to reject the human ones (to convert them to the most prevalent and perhaps least immunogenic version) in the final sequence to be synthesized. In the final model, it must then be decided, somewhat arbitrarily, which of the human framework residues should be additionally changed back to the mouse version based on a hypothesis of where the antigen binds. In practice, usually more than one mutant version is synthesized, and it is convenient to use *E. coli* expression for this purpose, as mutants can be built and checked rapidly (Plückthun, 1991; Carter et al., 1992).

The second strategy is very similar, except that one searches for *any* human sequence on the basis of it being most similar to the murine one, regardless of whether its structure is solved. This then allows evaluation of whether some of the murine residues or even sequence stretches also occur in humans. The hope is that these then may not be immunogenic and need not be changed. Nevertheless, a model should be built and similar (arbitrary) decisions have to be made and, again, several variants are usually constructed.

A third strategy would be to exchange only those surface-exposed residues not presumed to be involved in binding (Padlan, 1991). This is the most conservative change and the one most likely to keep all molecular packing and all antigen interactions intact, while disguising the murine antibody under a "human coat." However, this approach has been criticized as not alleviating T-cell immunogenicity, as the peptides generated and presented from such an antibody would be rather different from those of humans.

Numerous antibodies have been humanized using all of these methods (Adair, 1992). As far as affinity is concerned, it was usually possible to achieve the same binding constant as in the mouse antibody and, in a few examples, it was even possible to increase the affinity of the murine antibody (see, e.g., Co et al., 1992).

Another equally important question is whether the effort is actually worth it; that is, whether the humanized antibodies are nonimmunogenic. There may not be a general answer to this question. At the time of writing, no in vivo comparison between the human response to chimerized and humanized versions of the same antibody was available, but dramatic differences between the immunogenicity of several chimerized antibodies are apparent (Adair, 1992). In one case, it appears that the antigen-binding region is itself immunogenic (Meredith et al., 1992), which it would be in a humanized antibody as well. Nevertheless, since humanization is no technical problem today, it may still be the best method of decreasing the likelihood of an adverse response.

IV. PRODUCING RECOMBINANT ANTIBODIES

A. Expression in Mammalian Cells

The functional expression of whole antibodies is still best carried out in mammalian cells. Several general reviews are available in which the peculiarities of high-level protein expression in mammalian cells are discussed, with emphasis on promoter selection, transcriptional and translational signals, enhancers, gene amplification, and suitable host cells (Kaufmann, 1987; Bendig, 1988; Kane, 1991). For antibodies, in particular, a number of recent reviews on the

technology (Morrison and Oi, 1989; Shin and Morrison, 1989; Wright and Shin, 1991; Bebbington, 1991) are available.

In the past, many considerations in expression strategies and vector design were governed by concerns about ligating different constructs efficiently and precisely into the fairly large eukaryotic expression vectors. In the age of the polymerase chain reaction (PCR), however, convenient restriction sites are rarely a concern, since any fragment can be amplified with suitable primers, encoding the restriction sites of choice in the overhanging parts of the primers. Also, the enormous increase in sequence information (Kabat et al., 1991) now allows the use of consensus primers in the variable domains (discussed later), permitting the convenient shuffling of domains between antibodies.

The domainlike structure of the antibody (see Fig. 1) is reflected at the gene level (see also Chap. 1), with all domains being on a separate exon (Fig. 11). Most workers have preferred the use of genomic constructs for mammalian expression, with a variety of promoters. However, the use of cDNA-based expression plasmids has also been reported (see, e.g., Weidle et al., 1987; Liu et al., 1987a,b; Cattaneo and Neuberger, 1987). The intron requirement appears to depend on the promoter used (summarized in Wright and Shin, 1991), but high-level production has now been achieved with genomic and cDNA constructs.

In mammalian cells, we must distinguish between transient expression and stable expression. Transient expression is the closest mammalian cells come to the simplicity of bacterial systems. Usually, COS cells are used, which are derived from the green monkey kidney cell line CV-1, transformed with an SV40 virus defective in its origin of replication. These cells can replicate SV40-based plasmids to very high copy numbers, until the cell dies. This method is still useful for preparing very small amounts of protein rapidly, to test the correctness of the vector construction, before investing in the work required for stable cell expression (Whittle et al., 1987; Daugherty et al., 1991; Kettleborough et al., 1991; Maeda et al., 1991).

Alternatively, a CHO-K1 cell line, which is stably transfected with the adenovirus E1A *trans*activator has been described. In these cells, genes under the control of the human cytomegalovirus major immediate early promoter and enhancer (hCMV-MIE) can be transiently expressed (Cockett et al., 1991).

For stable mammalian expression, both lymphoid (Wright and Shin, 1991) and nonlymphoid cells (Bebbington, 1991) have been used successfully. In this case, the vector DNA inserts into the genome, and the insertion locus is a decisive factor determining the expression yield. The production of antibody from hybridoma (i.e., the traditional monoclonal antibody production) is highly variable, and the use of optimized recombinant protein expression may increase the yields and lead to more consistent results.

Two nonproducing lines are commonly used as myeloma hosts for recombinant antibodies: p3X63Ag8.653 (or P3 for short) and SP/0. The choice of cell line is often governed by transfection frequencies. In lymphoid cells, viral promoters are usually combined with an IgH enhancer. The most widely used vectors are derived from pSV2 (Mulligan and Berg, 1981), and they use the SV40 origin of replication as well as the early promoter, driving a selectable marker gene, usually the *neo* gene (conferring resistance to neomycin or G418) or the *gpt* gene (encoding xanthine–guanine phosphoribosyltransferase, allowing the cell to survive on xanthine, when purine biosynthesis is blocked by mycophenolic acid). To encode both chains of the antibody, either different vectors (see, e.g., Shin and Morrison, 1989), or one vector with two transcription units, can be used (see, e.g., Page and Sydenham, 1991; Bebbington et al., 1992). Polycistronic messages do not seem to be efficient in eukaryotes (Hasemann and Capra, 1990).

As nonmyeloma hosts, Chinese hamster ovary (CHO) cells have been most promising (Bebbington, 1991). Particularly attractive is the possibility of gene amplification using the dihydrofolate reductase (DHFR) inhibitor methotrexate and the *dhfr* gene encoded in the vector, in

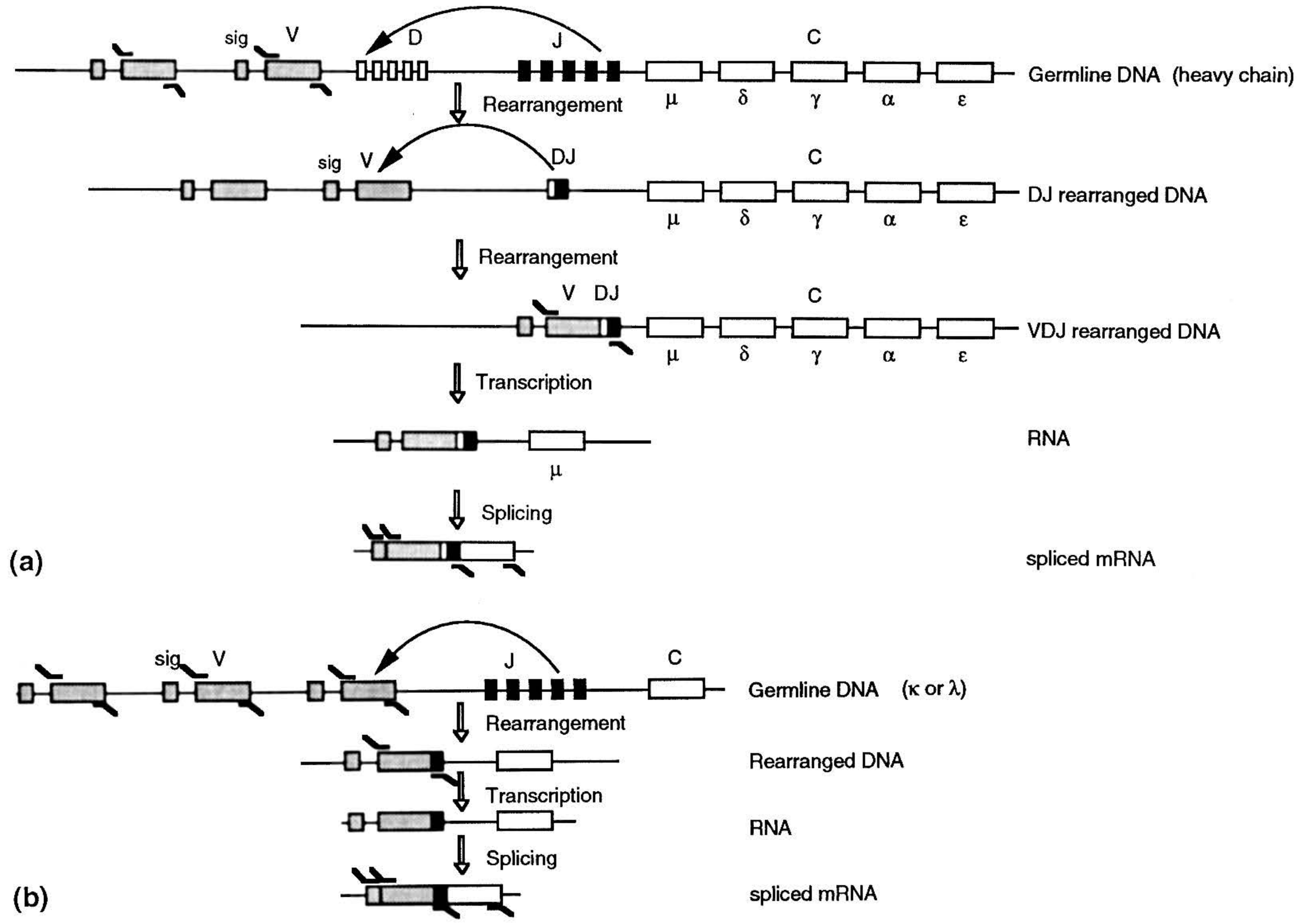


Figure 11 Assembly of antibodies from their genes in the immune response, and suitable location of PCR primers for amplifying various fragments. (a) The situation in the mouse heavy chain locus is shown schematically. In reality, there are four Cγ-genes (γ1, γ2a, γ2b, γ3). A further rearrangement ("class switching") can couple the VDJ-gene to C-genes other than Cμ, giving rise to the other antibody classes, such as IgG (not shown). For amplifying genomic V genes, the primers must be located in the V gene, as there is an intron between each signal sequence and each V gene. Since the V gene does not encode a full protein domain, the missing parts (D and J) must be encoded on the PCR primer, which must then be a random mixture itself. If rearranged DNA is to be amplified, the downstream primer should be located in the J segment. If mRNA is to be amplified (e.g., in the cloning of a defined monoclonal antibody) the upstream primer may be located either in the beginning of the mature V gene or in the signal sequence. The downstream primer may also be located in the constant domain (e.g., to amplify particular antibody classes). (b) The situation in the mouse light chain Iκ-locus is shown analogously.

combination with DHFR-deficient CHO cells. More recently, glutamine synthetase has been used for amplification (Bebbington et al., 1992), since it is an essential enzyme in the absence of added glutamine. Analogous to the methotrexate poisoning of DHFR, glutamine synthase can be poisoned with methionine sulfoximine, leading to further gene amplification. The glutamine synthase amplification has been used in both myeloma and nonmyeloma cells.

Although the preponderant use of mammalian expression is the production of whole antibodies, small fragments can also be made. Indeed, Fab′ fragments (King et al., 1992a) and Fv fragments (Riechmann et al., 1988b; Owens et al., 1991; King et al., 1992b) have been expressed in eukaryotes, the latter, however, in only very modest yields. Antibody fragment production in eukaryotes must compete with the much simpler and more efficient prokaryotic systems (see later section).

B. Other Eukaryotes

Plant cells have been used for antibody expression (Hiatt et al., 1989; 1992). Although there may be interesting options for manipulating plant metabolism by the binding of cellular components or, eventually, even the catalysis of metabolic reactions, plants are an unlikely competitor as production hosts for protein-based therapeutics. There is no specific advantage apparent that would justify the difficult workup and the incorrect glycosylation.

Similar arguments might also be made against insect cells as hosts, infected with baculovirus-based vectors (Hasemann and Capra, 1990; zu Putlitz et al., 1990; Reis et al., 1992). Antibodies for nontherapeutic use will probably be made largely in bacteria or other easily fermentable microbial hosts. Yeast has also been investigated as an antibody host. However, the yields in *Saccharomyces cerevisiae* have so far been rather disappointing (Horwitz et al., 1988; Better and Horwitz, 1989), and the different glycosylation again makes it undesirable for whole-antibody production. More recently, a single-chain Fv fragment was expressed in *Schizosaccharomyces pombe* (Davis et al., 1991) and an Fab fragment in the filamentous fungus *Trichoderma reesei* (Nyyssönen et al., 1993). Undoubtedly, there will be more research on using other fungi as production hosts, since the fermentation of these microorganisms is well established.

C. Expression in *Escherichia coli*

For many researchers, *E. coli* suffers from the reputation of being able to produce heterologous proteins in only insoluble form. However, it has been possible to express completely functional antibody fragments by secretion (Better et al., 1988; Skerra and Plückthun, 1988) (Fig. 12). This way, no in vitro refolding is necessary, and antibody fragments with binding activity can be isolated directly. Production of inclusion bodies, with subsequent refolding, is also possible, and the pros and cons of both approaches will be discussed.

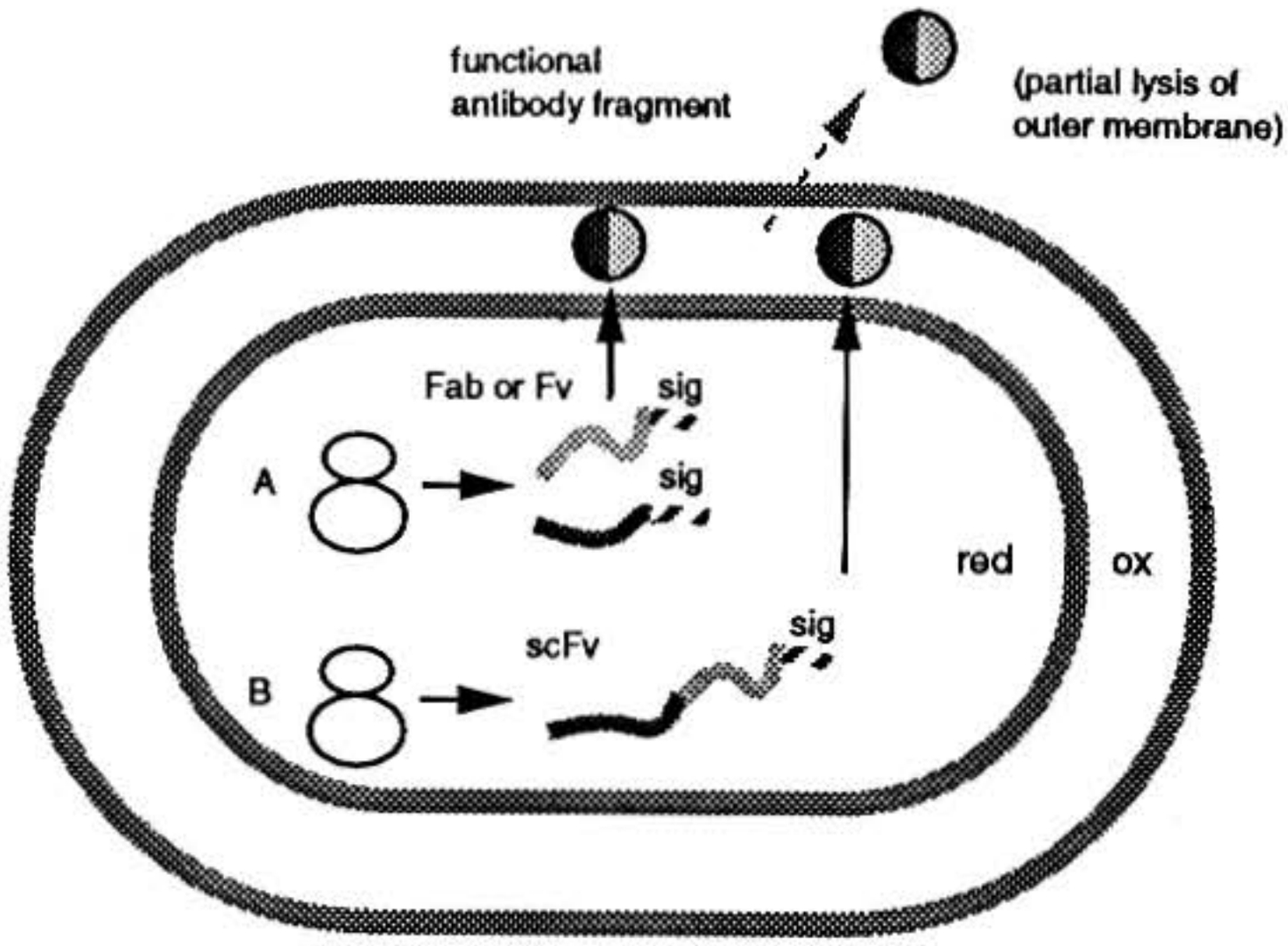
The main virtue of *E. coli* as the expression host lies in its extreme simplicity of handling in the laboratory, its fast growth (leading to a fast realization of a new idea for producing a particular mutation), but most importantly, its spectacular transformation efficiency. It is this property that is vital for making libraries.

The variable domains crucially depend on the conserved intradomain disulfide bonds for stability (Glockshuber et al., 1992). Therefore, any functional expression must be able to generate them in vivo. In the B cell or plasma cell, this occurs by transport to the endoplasmic reticulum (ER) (Wall and Kuehl, 1983), where the eukaryotic disulfide isomerase is involved in the formation and perhaps reequilibration of the disulfides. In *E. coli*, disulfide formation occurs in the periplasm, where the *E. coli* disulfide isomerase DsbA (Bardwell et al., 1991) has a similar role. In fact recombinant antibodies have been found to make use of the *E. coli* enzyme (Knappik et al., 1993).

Transport to the bacterial periplasm is the functional analogue of transport to the ER. In the periplasm, cleavage of the signal sequence, folding of the domains, formation of the disulfide bonds, and domain association occur. This strategy has been successful for Fv fragments, scFv fragments, Fab fragments, and many derivatives thereof, such as bivalent miniantibodies (detailed in Plückthun, 1992).

For this assembly to be successful, equimolar amounts of both the heavy and light chains have to be produced. It is not possible, in general, to express them separately and simply mix them, because the separate heavy chains (V_H or V_H–C_H) are frequently insoluble. Instead, the heavy chain must be coexpressed with the light chain in the same cell. For the single-chain Fv fragment, this is ensured by the protein linker. For the unlinked fragments, three different possibilities exist: (1) two plasmids, (2) two promoters on one plasmid, and (3) a dicistronic gene.

(a)


functional
antibody fragment
(partial lysis of
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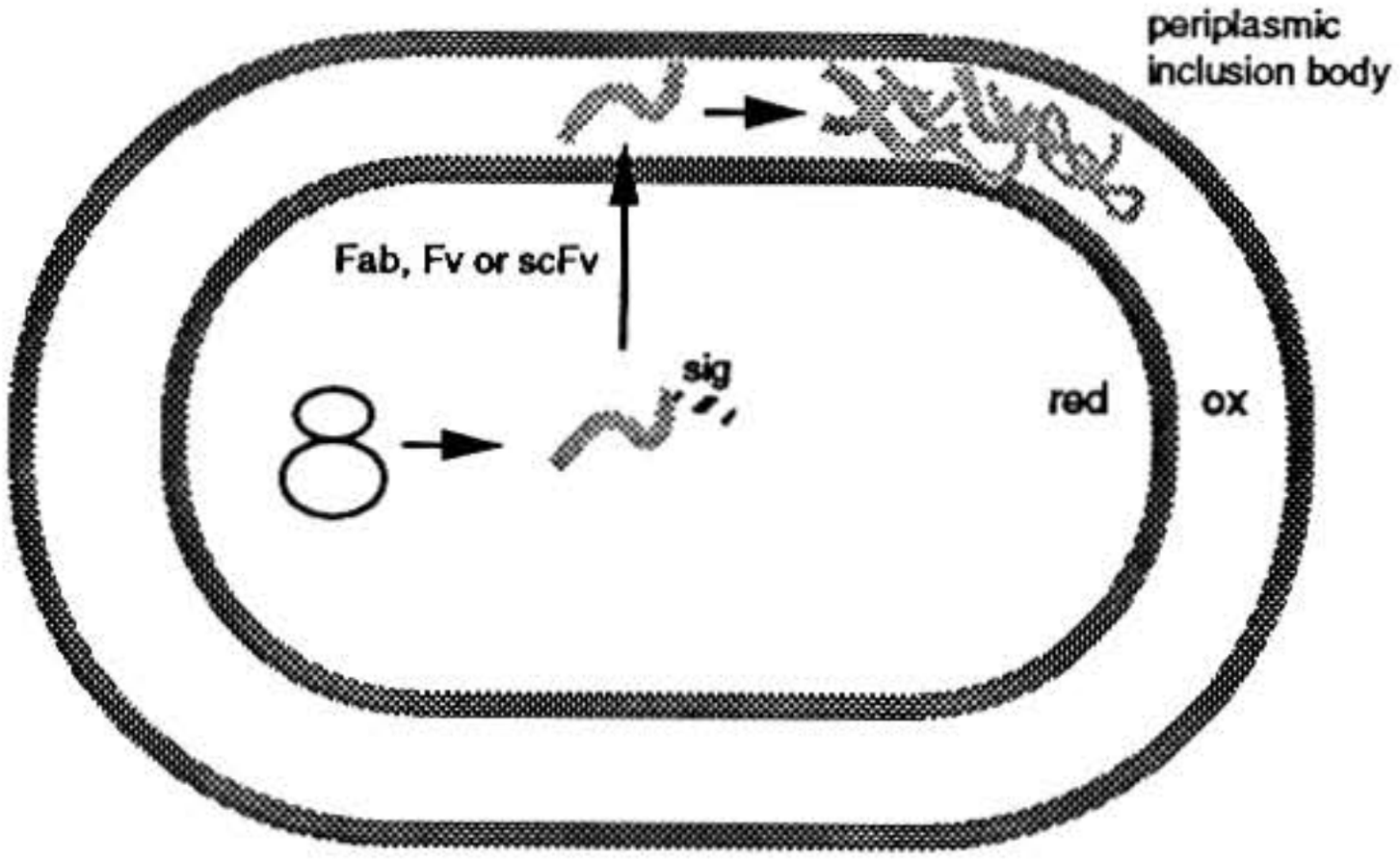
(b)


periplasmic
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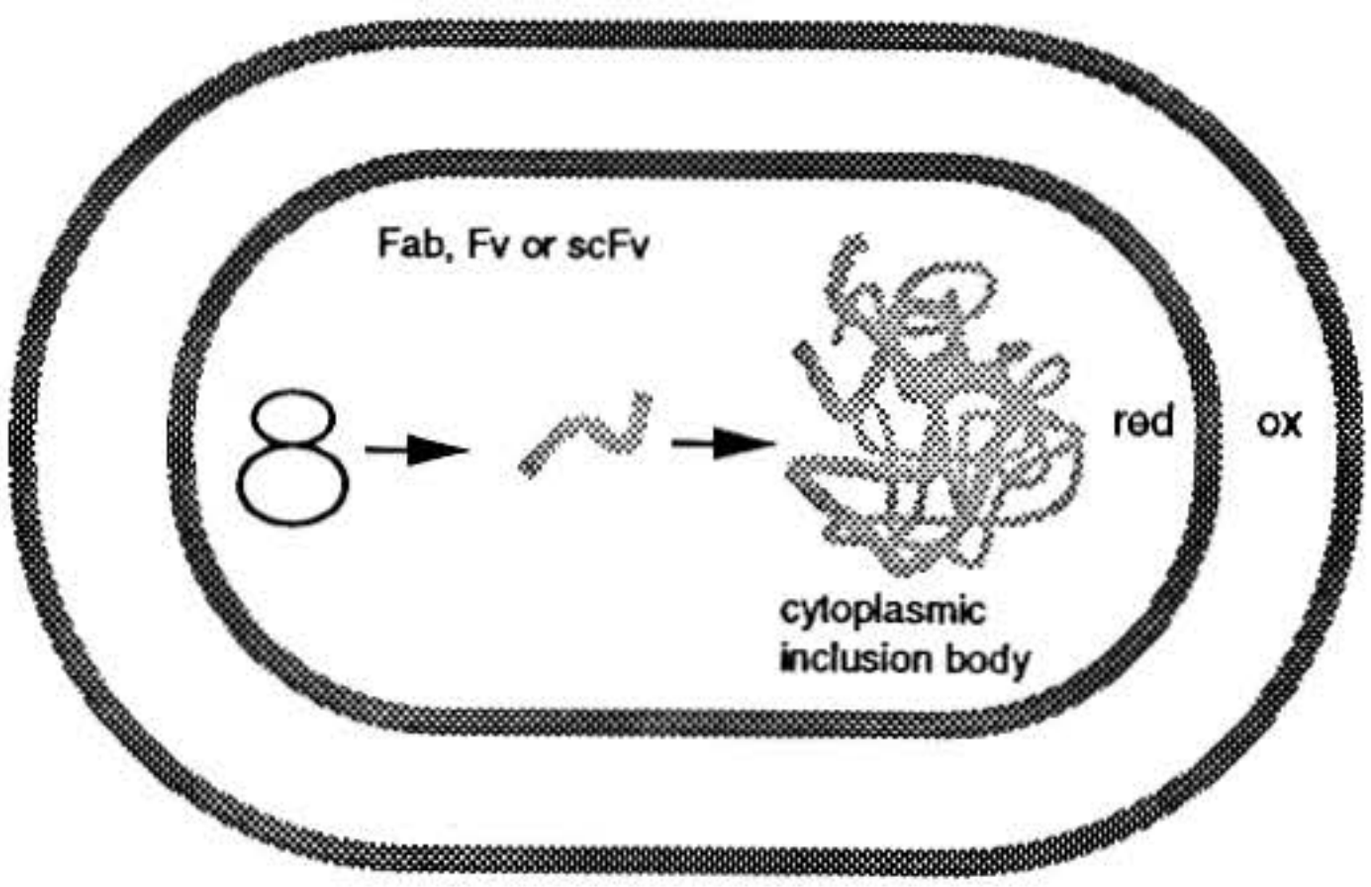
(c)


Fab, Fv or scFv
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inclusion body

To understand the virtue of the third approach (Better et al., 1988; Skerra and Plückthun, 1988) it is important to realize that the secretion of antibodies is a stress for *E. coli* (the exact molecular causes of which remain unknown) and that, for the production of antibodies, the host–vector system needs to be stable. Therefore, it is useful to have a tightly controlled expression system for both chains to reduce the stress during the growth phase, and this is most easily achieved with the dicistronic approach. With this strategy, hundreds of milligrams of functional antibody fragment per liter of *E. coli* are now achievable in fermentation (Carter et al., 1992; Better et al., 1993; Pack et al., 1993). In the construction of libraries (see Sec. V), the other two systems have been used as well (Barbas et al., 1991; Collet et al., 1992). In eukaryotes, in contrast, dicistronic expression is very inefficient (Hasemann and Capra, 1990).

Technical details about this process have been described elsewhere (Plückthun, 1993a). Vectors developed and found useful in our laboratory are shown in Fig. 13. A few general remarks, however, might illustrate the current state of knowledge. It appears that the periplasmic folding process frequently limits the yield, giving rise, in addition to the folded protein, to some precipitated protein in the periplasm. This ratio depends on the type of fragment and its primary sequence (Skerra and Plückthun, 1991; Carter et al., 1992; Knappik et al., 1993).

The amount of folded protein can often be increased simply by lowering the growth temperature (Glockshuber et al., 1990). Employment of stronger promoters is not automatically useful, as the "folding bottleneck" cannot be overcome by producing more protein, and a substantial leak rate will lead to plasmid loss and, in extreme cases, to plasmid rearrangements (Wülfing and Plückthun, 1993). An interesting, but not molecularly understood, observation has been that on induction of antibody expression, the outer membrane of *E. coli* becomes leaky, and the periplasmic proteins leak into the medium (Plückthun and Skerra, 1989). This phenomenon is dependent on the physiological state of the bacteria (Pack et al., 1993), but independent of the choice of signal sequence.

The precipitated protein in the periplasm can also be used as the starting material for in vitro refolding (Colcher et al., 1990) (see Fig. 12). Alternatively, one may eliminate the signal sequence and directly produce inclusion bodies in the cytoplasm (Buchner and Rudolph, 1991). In both

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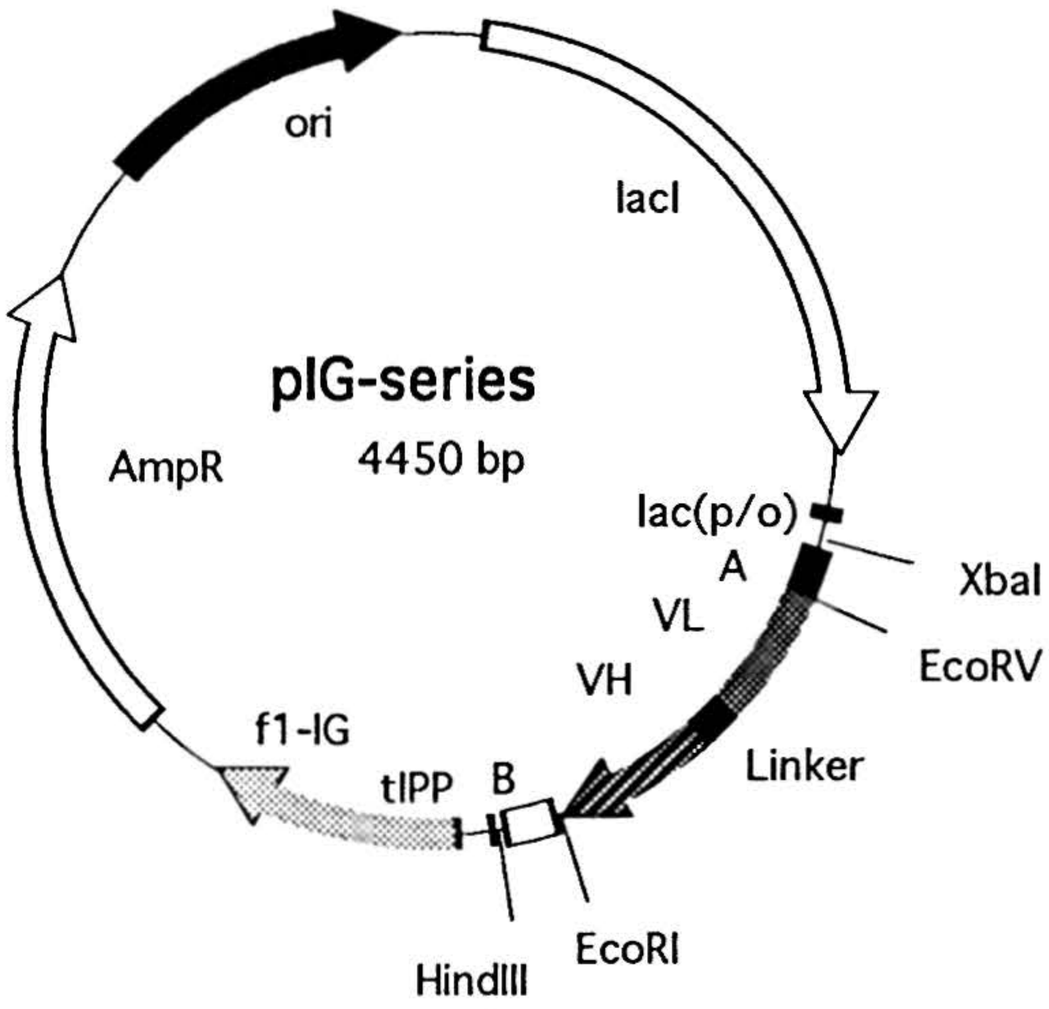
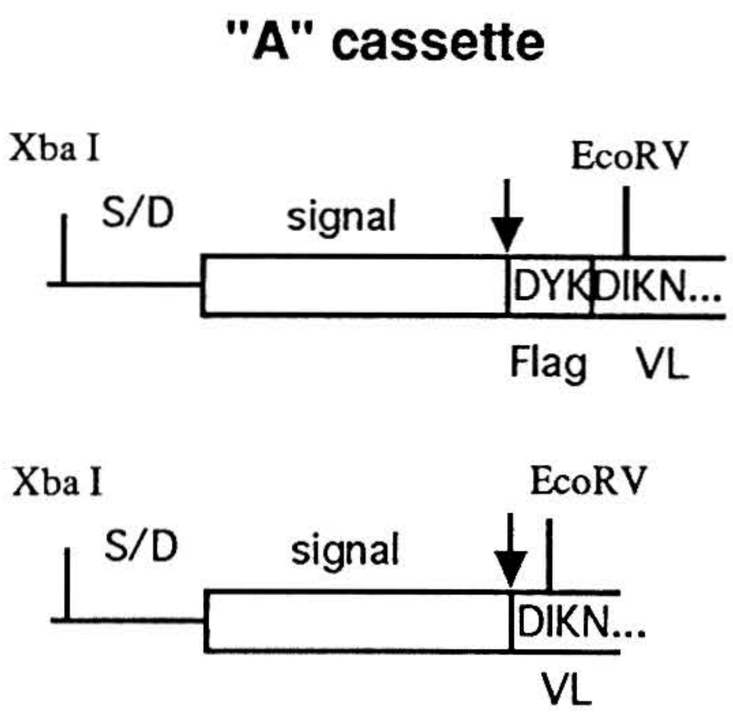
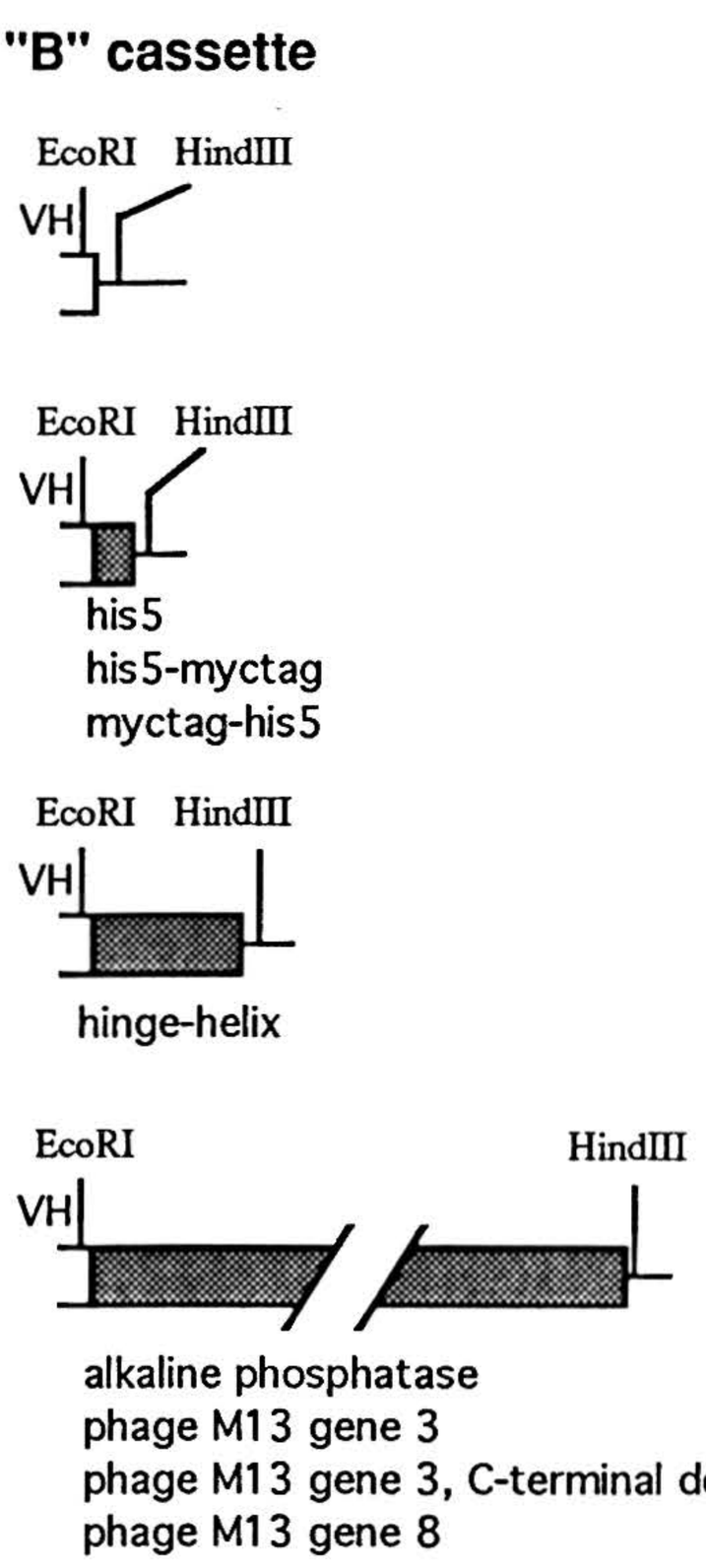
Figure 12 Schematic view of the different expression strategies found to be useful for antibody production in *E. coli*. (a) Functional expression: here, the two chains making up the antibody-combining site must be transported to the oxidizing milieu of the periplasm. In this compartment, there is a disulfide forming enzyme (DsbA) that allows the formation of the crucial intramolecular disulfide bonds, which each domain has and requires for stability. In (A), the pathway for two independent chains (as in a Fv or Fab fragment) is shown. Both are made as precursors containing a signal sequence and are then transported to the periplasm, where the signal sequence is cleaved off. There, folding, disulfide formation and assembly takes place. Alternatively (B), both variable domains can be linked to form a continuous polypeptide chain (scFv fragment) which can then also be secreted.

For reasons not understood in mechanistic detail, secretion of these proteins presents a stress to *E. coli* and results in lysis of the outer membrane after a time. The phenomenon is observed more readily in shake flasks than in controlled fermentation conditions, during which it can be minimized. To prevent leakage and periplasmic inclusion body formation, it is crucial to grow cells at low temperatures (e.g., 25°C). (b) Periplasmic inclusion body formation. This is observed for many fragments, but has been exploited preparatively mostly for scFv fragments. This phenomenon is temperature-dependent and is most easily induced at temperature of 37°C or higher. The protein is apparently transported, processed, and then precipitates. The protein must be refolded in vitro as in (c). (c) Cytoplasmic inclusion body formation. Here, the protein is expressed without a signal sequence under as strong a promoter and translation signal as possible. Inclusion body formation appears to be more successful at temperatures of 37°C and higher. The protein must be refolded in vitro.



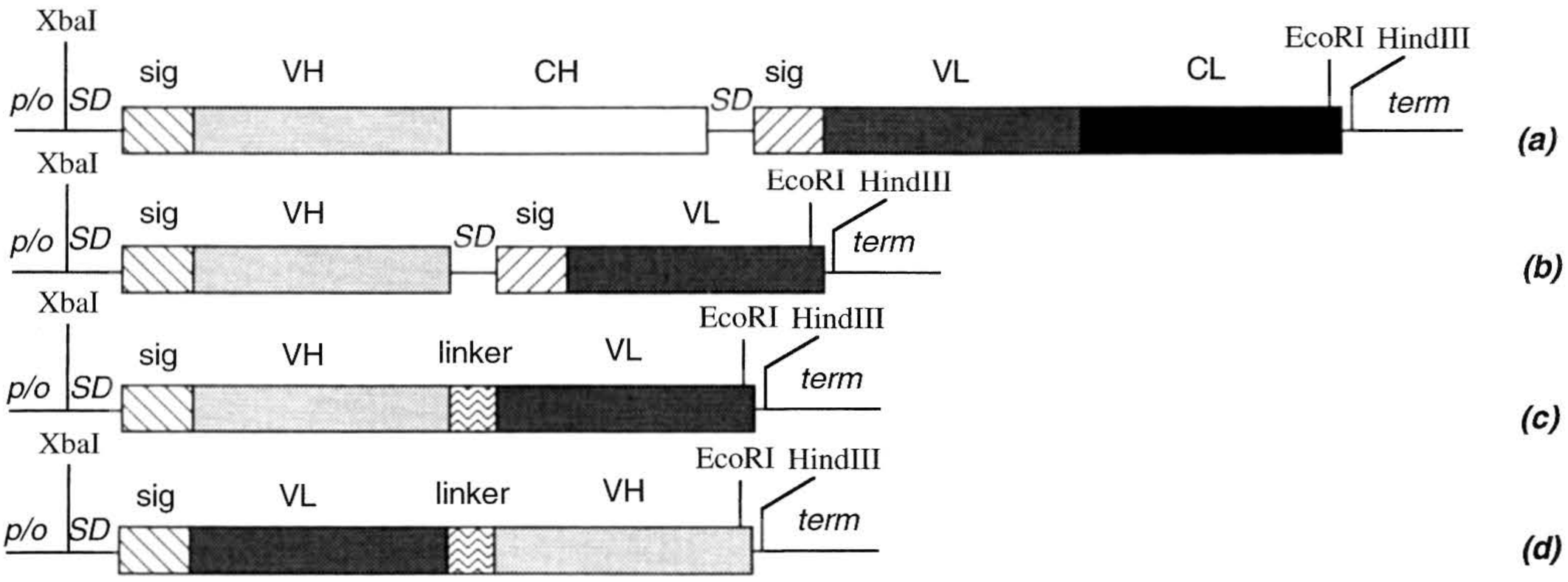




(a)

Figure 13 (a) Secretion vectors for antibody expression in *E. coli*. As an example, a new series of vectors suitable for expressing scFv, Fv, and Fab fragments is shown (L. Ge, A. Knappik, and A. Plückthun, unpublished), which incorporate restriction sites within the antibody gene for convenient PCR cloning, as well as cassettes for detection, purification, and phage display.

The design is very modular, allowing easy exchange of antibiotic resistance, the fragment of the antibody and the cassettes "A" and "B." The A cassette contains a bacterial signal sequence, directly fused to the mature antibody part or preceded by only three additional "Flag" amino acids, which can be detected with extremely high specificity and sensitivity using a Ca^{2+}-dependent antibody (A. Knappik, L. Ge, and A. Plückthun, unpublished), using a much shorter epitope than originally presumed to be necessary (Prickett



(b)

et al., 1989). The B cassette may be used to introduce a COOH-terminal purification or detection tag, to fuse a hinge and helix for dimerization, to fuse a phage gene for surface display or an enzyme for easy detection of the antibody. PCR of antibody genes can be carried out with primers carrying extensions using *Eco*RI and *Eco*RV sites, which are both rare in antibody sequences. (b) The schematic arrangement of genes for functional expression is shown. Since, in this case, both chains of the antibody must be secreted to the same periplasmic space to assemble, they must be produced either as two different secreted protein chains in the same cell (*a*, *b*), or they must be linked by a peptide linker (*c*, *d*). It is advantageous to express the independent chains of the Fab fragment (*a*) or the Fv fragment (*b*) in a dicistronic operon (*a*, *b*), as discussed in the text. Two different orientation of the scFv fragment are shown that have both been shown to function (*c*, *d*). *p/o* denotes a promoter–operator structure, *SD* a Shine–Dalgarno sequence, and *term* a transcription terminator.

methods, in vitro refolding is necessary, and the problem lies in the individuality of the optimal refolding protocol for every individual antibody (Huston et al., 1991). However, if massive amounts of one particular antibody are needed (e.g., for clinical studies) refolding may be a viable alternative.

V. OBTAINING DIVERSITY: ANTIBODY LIBRARIES

Other than making defined mutations, recombinant technology coupled with bacterial expression lends itself to the construction of antibody libraries. A particularly useful method is to randomly combine collections of heavy and light chains, but many other variations are possible and will be discussed. But how does one test the functionality of such a large collection of molecules?

The first approach was to use λ-phages (Huse et al., 1989; Caton and Koprowski, 1990; Persson et al., 1991; Duchosal et al., 1992). The virtue of this phage is its spectacular infectivity of *E. coli*, thus generating progeny of a large proportion of DNA molecules assembled in vitro. The phage itself is not taking part in the production of antibodies; therefore, it would be confusing to speak of a "λ-expression system," as is occasionally done. The phage only delivers the DNA, in the form of an expression plasmid (as described in the previous section) integrated into its genome (Huse et al., 1989), and the antibody is then made by the bacterial cells while they are lysing. Screening is carried out by filter overlays with radioactive antigen and, after a candidate clone has been found, the expression plasmid must be excised from the phage, and bacterial production is carried out as described earlier.

A different strategy is the use of filamentous phage (Fig. 14). Here, it is possible to express the antibody fragment on the surface of the phage and to trap phages with the "right" binding properties to a solid support carrying the antigen and then reinfect them. This is possible because

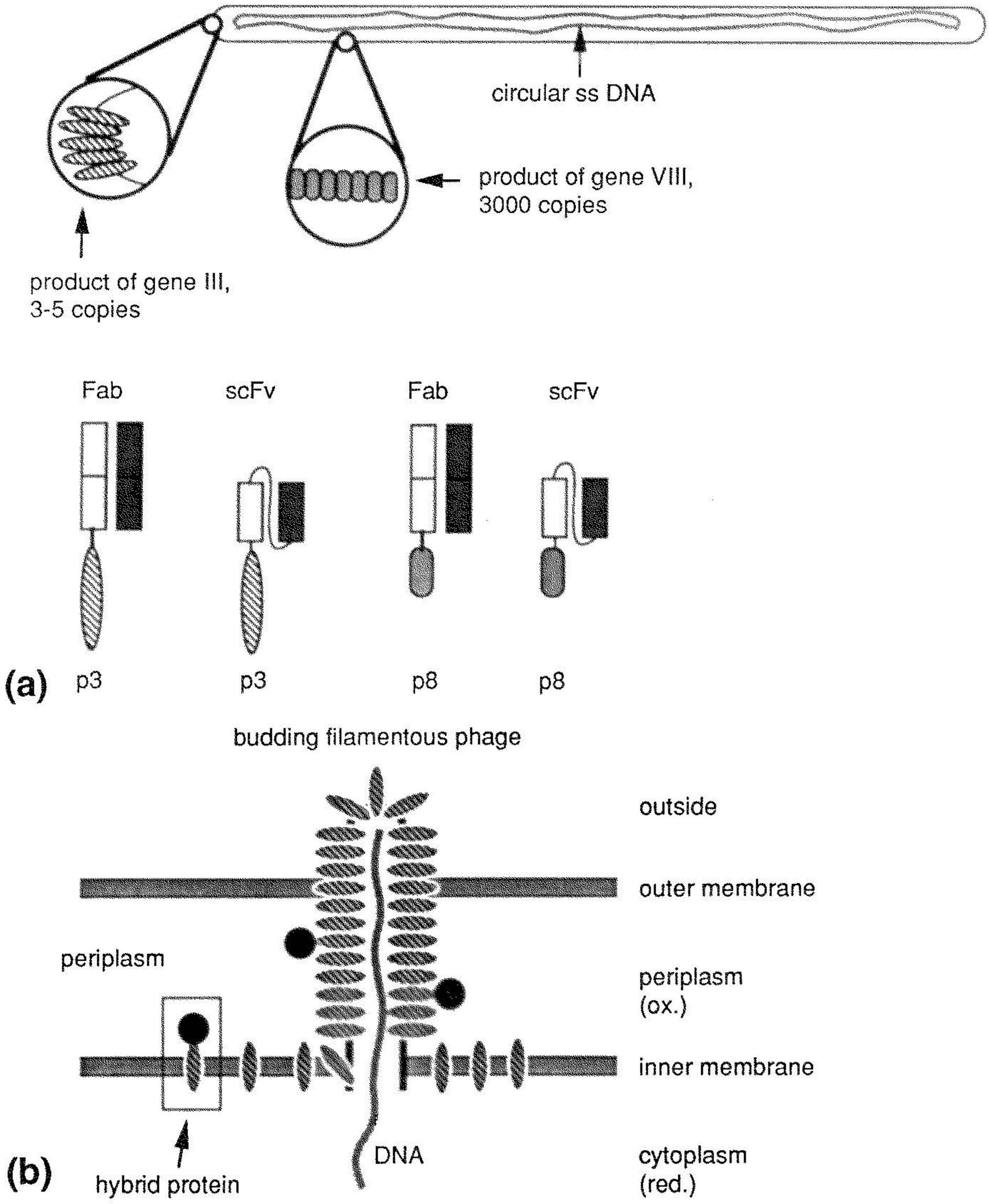
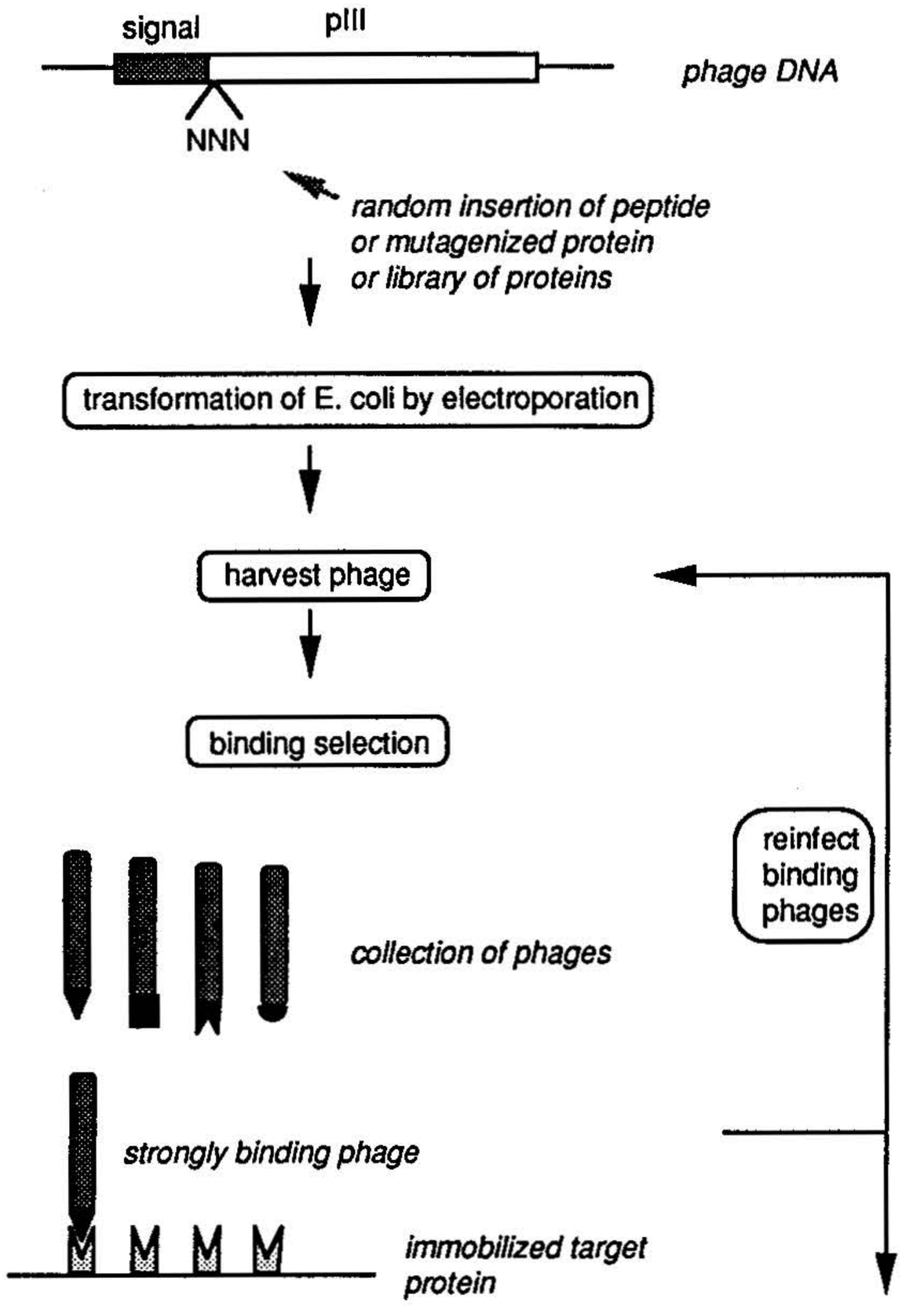


Figure 14 (a) Schematic drawing of a filamentous phage, indicating the product of gene *III* at the tip, and the major coat protein, product of gene *VIII*. At the bottom, fusion proteins of one chain of an Fab fragment or single-chain Fv fragment are schematically depicted. (b) Schematic drawing of the incorporation of a hybrid protein into the phage coat. The strategy makes use of the correct folding of the antibody domains in the periplasm. The hybrid protein folds in the periplasm, having a COOH-terminal extension in the form of a phage coat protein, while attached to the inner membrane. The w.t. coat protein and the hybrid coat protein then attach themselves to the DNA of the budding filamentous phage. (c) Screening strategy exemplified for gene *III* fusions. The "random insertion" is a collection of peptides or antibodies, from which variants that bind to the immobilized target structure are selected.



(c)

of the life cycle of the filamentous phage (reviewed in Russel, 1991). Its single-stranded DNA is covered in the cytoplasm with the protein pV (the product of gene *V*), and on extrusion through the inner membrane, covers itself with the coat proteins. The major coat protein pVIII (present in about 2700 copies) and the minor coat protein pIII (present in about two to five copies) are both made from precursor proteins and go through an intermediate state in the inner membrane. The NH_2-terminus is exposed to the periplasm, and later to the outside of the phage; therefore, anything attached to the NH_2-terminus will fold in the periplasm. This is why functional antibody fragments can be expressed on the surface of phage.

This technology was first invented by Smith for "displaying" peptides (Smith, 1985; Parmley and Smith, 1988) on phage and later applied to peptide libraries (Scott and Smith, 1990; Cwirla et al., 1990; Devlin et al., 1990). Several different whole proteins have subsequently been displayed on the phage surface, among them antibody fragments (Bass et al., 1990; McCafferty et al., 1990; 1991; Roberts et al., 1991). Both single-chain Fv (McCafferty et al., 1990; Clackson et al., 1991; Marks et al., 1991a) as well as Fab fragments (Hoogenboom et al. 1991; Barbas et al., 1991; Bruton et al., 1991; Zebedee et al., 1992) fused to gene *III* have been used. Additionally, fusions to gene *VIII* have been constructed (Chang et al., 1991; Kang et al., 1991). The single-chain Fv fragment is usually genetically inserted between the signal sequence of pIII and the mature portion of the protein (see Fig. 14). For an Fab fragment, only one chain is inserted, the

other is periplasmically expressed, and will then assemble in the periplasm to the pIII fusion protein, which is still attached to the inner membrane.

The product of gene *III* binds to the F-pili of *E. coli* and, therefore, is responsible for infectivity. In the "display phages," this infectivity must be maintained, or otherwise the enrichment of antigen-binding phages with cycles of "panning" and infection is impossible. However, if at least one or two NH_2-terminal domains of pIII are accessible, the phage appears to remain infective.

The panning of antigen-binding phages can be carried out on columns carrying immobilized antigens (Clackson et al., 1991); with ELISA plates or tubes to which the antigen is adsorbed (Marks et al., 1991a); or with magnetic beads carrying the soluble antigen (Hawkins et al., 1992). The phage is then eluted and reinfected, and correct binders are enriched after each cycle. This technology is thus able to "fish out" antigen-binding phages from a library of diverse molecules. In the following, we will discuss different possibilities of generating this diversity.

In nature, the variability of antibodies is achieved by three factors: (1) recombination of a

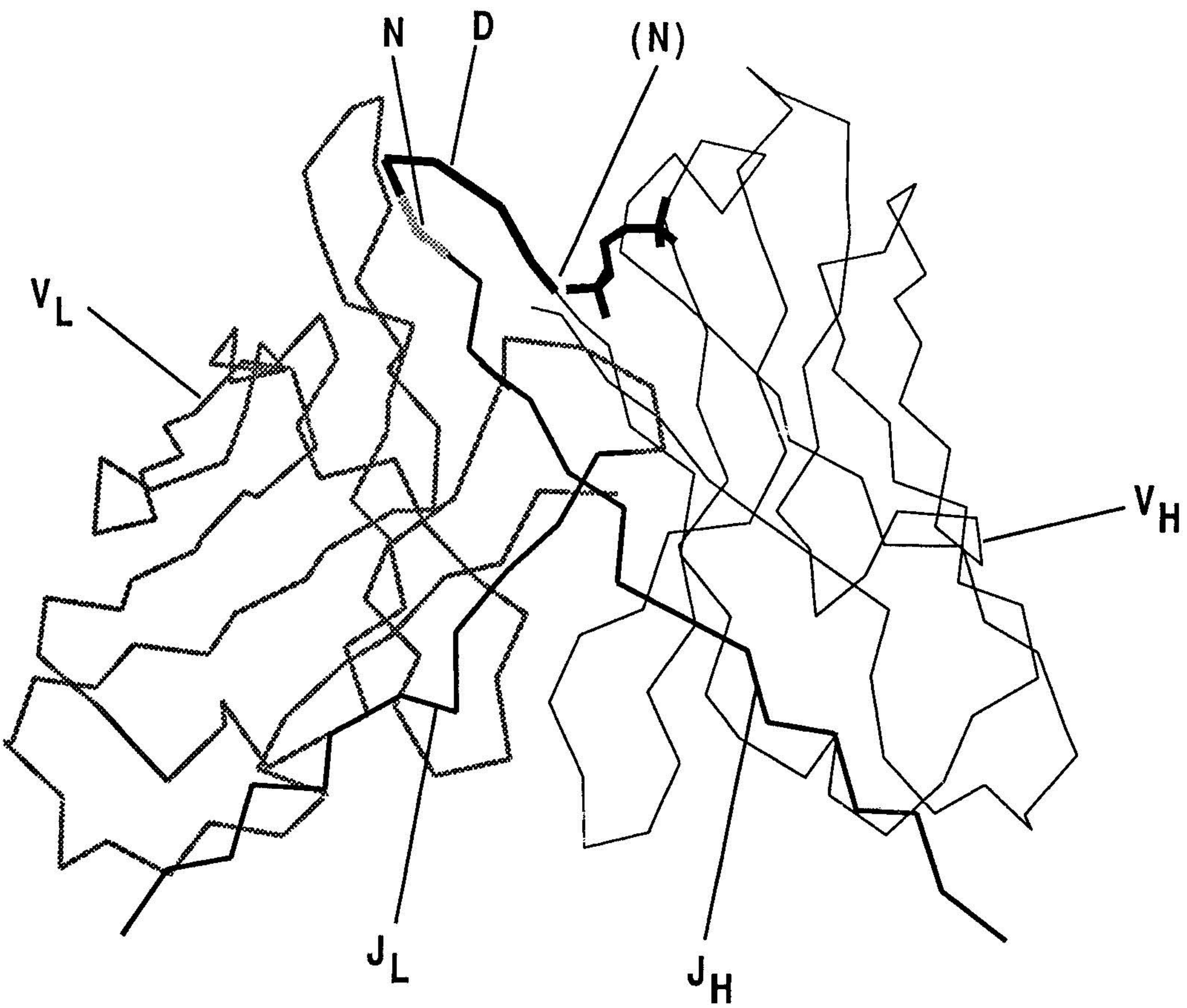


Figure 15 Location of the genetic elements making up the antibody variable domain using the phosphorylcholine antibody of McPC603 as an example. V denotes the V (variable) gene, D the D (diversity) element, J the J (joining) segment, and N the N-region (noncoded) diversity. The parenthesis denotes that there is no N-region diversity in this particular antibody at this point.

limited number of gene segments (see Fig. 11) (V_H, D, J_H as well as V_κ, C_κ, or V_λ or C_λ), giving from the *sum* of components the *product* of combinations by DNA rearrangement; (2) an "imprecise" joining of V_H, D, and J_H, giving rise to the so-called N-region diversity (see also Chap. 1); and (3) the "somatic" mutation of candidate clones, improving promising antibodies by random point-mutation. Localization of the gene segments in the final structure is shown in Fig. 15.

The first option is thus to amplify with suitable PCR primers (discussed later) the antibody mRNA, transcribed from the rearranged DNA that is obtained after the immunization of an animal (Clackson et al., 1991) (see Fig. 11). This is, in a sense, equivalent to making monoclonal antibodies by the conventional way (Fig. 16) (see Chap. 4). In the latter, B cells that have been stimulated by the antigen seem to fuse preferentially, and the selection is done by the animal, leaving a fairly small number of candidate clones, a high proportion of which are usually functional. In the phage library, many mRNA molecules will be amplified and many nonfunctional combinations will be obtained, among which the functional ones must be found. It now appears that both technologies generate *different* subsets (which may or may not overlap) of the total set of potentially functional H–L combinations, because neither method is currently exhaustive.

The use of this approach without prior immunization has usually been unsuccessful for finding high-affinity antibodies, since the frequency of potentially useful H–L pairings among mRNA molecules is too small. However, low-affinity antibodies have been found when the reper-

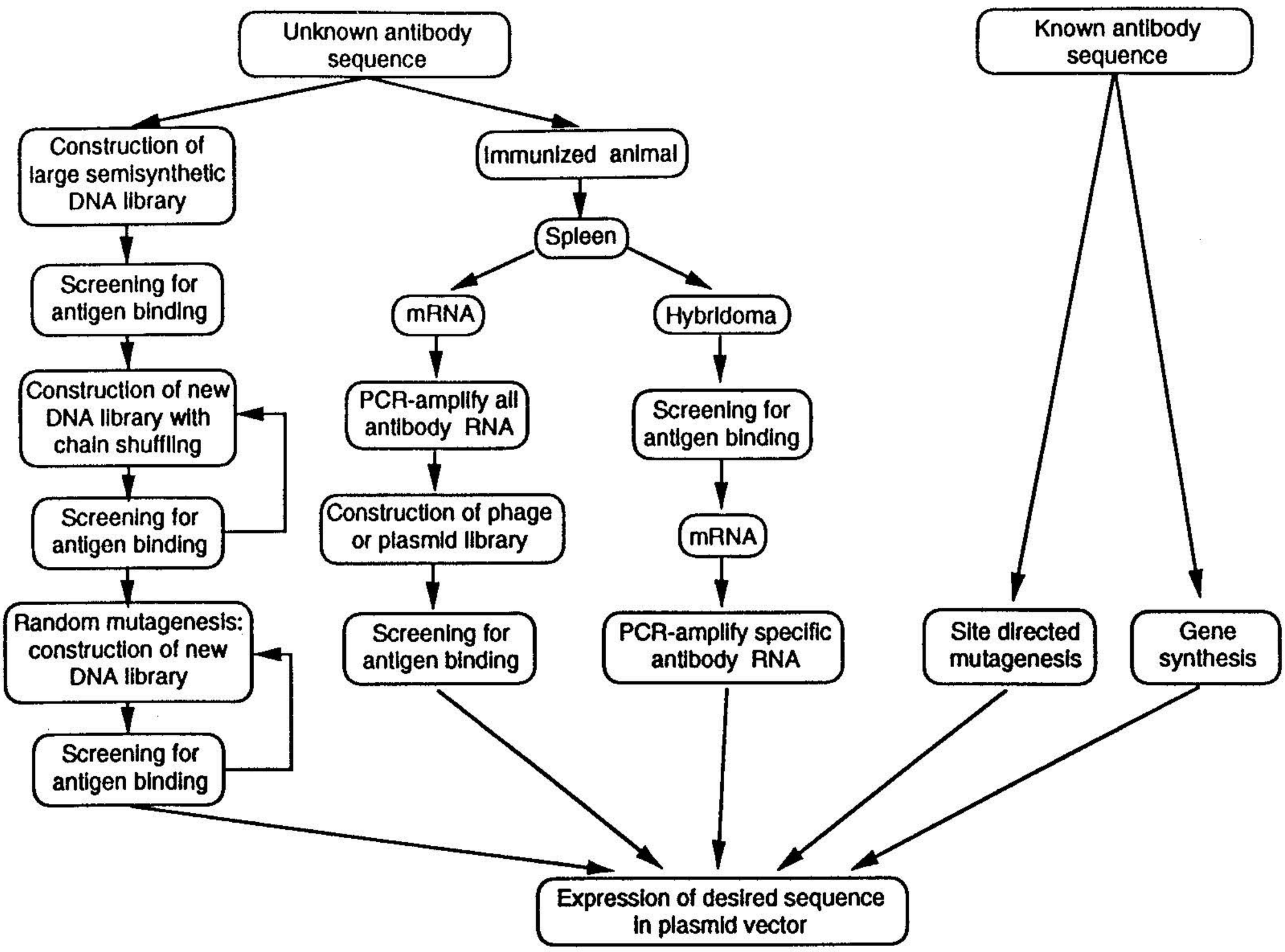


Figure 16 Strategies for obtaining a cloned antibody.

toire was large enough (Marks et al., 1991a). Immunization is generally not possible for ethical reasons in humans except in special cases (Zebedee et al., 1992). However, human beings suffering from a human immunodeficiency virus (HIV) infection have produced antibodies against a variety of infectious agents (presumably from opportunistic infections), and mRNA for the corresponding human antibodies could be isolated from mRNA of bone marrow (Burton et al., 1991).

Currently, several laboratories are attempting to use genomic variability of the V segments directly, by using PCR primers encoding the variability that would normally be introduced by fusing D, J_H, or J_L (see, e.g., Gram et al., 1992; Hoogenboom and Winter, 1992). Again, low-affinity antibodies have been obtained. However, extremely large libraries may already contain antibodies of useful affinities. To improve affinities, the chains of a collection of useful antibodies may be shuffled (Marks et al., 1992). More promising may be the development of evolutionary strategies mimicking the somatic point mutations.

In summary, high-affinity antibodies from synthetic libraries (i.e., without using animals or humans at some stage) is the declared goal of this research. Library techniques, together with rational engineering, are a focus of very active research, and it seems likely that only a combination of all these approaches will be able to create antibodies of the same quality as natural ones in the test tube. When combined with progress in engineering more stable frameworks adapted to a particular need, it may be possible to surpass nature, and avoid the use of animals altogether.

VI. OBTAINING THE DNA: ANTIBODY CLONING

All of the foregoing depend on obtaining the genes of the desired antibody (or collection of antibodies) (see Fig. 16). During the last decade, a large number of antibodies have been cloned by classic techniques (i.e., making DNA libraries and screening them with suitable DNA hybridization probes). From this work, enough sequence information could be assembled (Kabat et al., 1991) to design primers for PCR. Today, PCR is almost exclusively used in antibody cloning because of its simplicity.

If mRNA is used as a template, and the variable region is to be amplified, two possibilities exist both for the 5′ and the 3′ ends (see Fig. 11). The primer at the 5′ end may be located either in the signal sequence, or at the beginning of the mature gene. Both primer families have their advantages and disadvantages. If the "true" NH_2-terminus of the antibody is considered crucial, the signal sequence primers have to be used. In bacterial expression (including phage display), however, additional cloning steps would be necessary to introduce the bacterial signal sequence. At the 3′ end, primers within the constant domain can be used (for making Fab fragments). Alternatively, primers within the J segment can be used. If rearranged DNA (as opposed to mRNA) is the template, only the "inner" primers can be used, since the V domain is separated by introns from the signal sequence and the constant domain. Finally, if genomic V genes are to be used, the 3′ primer would itself have to encode D and J (see Fig. 11). Tabulations of primers for mice and humans are given elsewhere (Orlandi et al., 1989; Sastry et al., 1989; Larrick et al., 1989; Jones and Bendig, 1991; Persson, et al., 1991; Marks et al., 1991b; Campbell et al., 1992; Kettleborough et al., 1993), but researchers may wish to make use of the on-line databases to be most up to date about new consensus sequences, since more and more sequences are continuously being reported.

Many workers will, however, already have an antibody expression clone and wish to alter it (see Fig. 16). A typical example would be humanization. This can be achieved with site-directed mutagenesis, and all six CDRs can be simultaneously exchanged. Alternatively, very efficient ways to synthesize genes are now available (Prodromou and Pearl, 1992) and may actually be the fastest way to create a new gene for expression.

VII. CONCLUSIONS

Genetic engineering has been vital for establishing structure–function relationships in antibodies. Today, this knowledge is already being applied in the construction of "unnatural" antibodies for laboratory or medical use: humanized antibodies, small fragments, bivalent and bispecific constructs. New access routes are being developed that may in the future obviate the need of immunizing animals. Intensive research is directed at finding rational and evolutionary methods for increasing affinities beyond what nature can achieve. New production methods based on bacteria and other microorganisms are being developed, which may make antibodies into relatively cheap bulk reagents for biotechnology. Still at the beginning, research is now being carried out to stabilize antibodies for new and demanding technical applications. All of this would be impossible without the distinct interdisciplinary character of research in this field, combining the state of the art in molecular biology, structure prediction and experimental determination, protein chemistry, and immunology. Because of this enormous progress, it is to be feared that this chapter may be outdated far too soon.

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