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Strategies for the Expression of Antibody Fragments in *Escherichia coli*

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This article summarizes the techniques for the expression of various antibody fragments in *Escherichia coli*. The properties of the various antigen binding fragments are discussed and several strategies for expression are compared. Emphasis is placed on the secretory approach, as it leads directly to functional fragments and thus forms the basis for all screening approaches, whether with cells or phages. Purification procedures of the fragments from *E. coli* are also discussed.

The genetic engineering of antibodies has received an important boost from the development of methods for the convenient production of antibody fragments in bacteria. In this article, some of the recent advances of this technology are summarized, and the rationale for the various parts of the methodology is discussed. This area of research is fairly new, with the inevitable consequence that the detailed methodology, including vectors, strains, growth, fermentation, and purification, is in a constant state of flux and improvement. Therefore, emphasis is placed on the background of and rationales for the procedures.

Why would one want to produce antibodies or antibody fragments in *Escherichia coli*? One reason is close at hand. Manipulations of *E. coli* are very simple, and the general procedures of working with these bacteria are established in the laboratories dealing with DNA manipulations. Growth of *E. coli* is inexpensive and can be scaled up with equipment much simpler than that necessary for the fermentation of higher cells. The transformation of DNA and the transfection with phage are extremely efficient. This has two important consequences. First, one may carry out genetic constructions directly in the expression vectors and second, one may create libraries with large diversity. Such a collection of plasmids or phage may be the product of random mutagenesis, a collection of synthetic fragments, or libraries from the repertoire of the immune response of an animal or a human being. There is currently no way to establish libraries with the same degree of diversity in higher cells.

The simplicity of genetic constructions is also very beneficial for the design and testing of hybrid molecules consisting of antibody fragments and other proteins, and it is very useful in the investigation of chimeric, humanized, and totally human antibodies. Furthermore, one may make use of the enormous knowledge that has accumulated about *E. coli* in terms of its metabolism, genetic manipulations, and infective phage.

CHOOSING THE APPLICATION

There are many uses for antibody fragments in *E. coli*. One should first distinguish those for which one specified fragment is desired. This might be the large-scale production of an antibody fragment for physical and biochemical studies or for medical use. The advantages of a production in *E. coli* are mostly simplicity, the rapid access to fragments and mutants, and the opportunity for biosynthetic labeling. Upon further development of this technology, the production costs should become significantly lower than those for all other production methods of antibodies. This might be of benefit in many diagnostic or therapeutic products based on antibodies. Another aspect worth mentioning is a safety aspect: a prokaryotic production process for antibodies, in which viral contaminations are not a problem, might facilitate the use of recombinant antibodies in affinity chromatography of pharmaceutical products, or even human use. The most obvious benefit of this new technology, however, is in providing a facile route to altering the fragments by any kind of protein engineering.

Although for the mere production of a single antibody fragment (or hybrid molecule) expression in the native state is not absolutely necessary (as one may develop a procedure for refolding the proteins from inclusion bodies), a secretion of native fragments is still extremely convenient. Such a technology has previously been developed (1–3) and is discussed in more detail below.

The second group of applications can be combined under the heading screening. Any kind of random mutagenesis, or any other library, will lead to a collection of dif-

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ferent antibody fragments. Usually one will be interested in testing this collection of molecules for function, be it for antigen binding, affinity, cross-reactivity, or even catalysis. In this case, it is *mandatory* to use a strategy leading to native fragments *in vivo* (1–3), as a very large number of different antibody fragments can clearly not all be refolded from inclusion bodies.

It is thus the desired application of the antibody that determines both the optimal fragment and the most suitable expression strategy. These points are discussed below.

CHOOSING THE FRAGMENT

The choice of the antibody fragment (Fig. 1A) depends on the desired application. To guide investigators in this decision, some of the known physical properties of the antibody fragments are described. It should be stressed that there are at least 10^{12} different antibodies, and obviously, they will differ in their detailed physical characteristics. Therefore, some range in properties must be expected.

The Whole Antibody

There are three features of the whole antibody that deserve mentioning in this context. First, it is bivalent. This means that a weak thermodynamic affinity can be compensated for by the entropic effect and may still lead to tight surface binding to appropriately spaced antigen. Notably, antibodies directed against protein-bound haptens and polymeric antigenic determinants show this effect. Second, the antibody is glycosylated. The nature and position of glycosylation depend on the class of the antibody and the species producing it. Most antibody species are glycosylated in C_H2, but others such as IgA and IgM can be glycosylated in C_H1 (i.e., in the Fab fragment) (4). There is no reported evidence that this glycosylation has any effect on antigen binding. The exception are rare cases, in which the hypervariable regions may fortuitously contain a glycosylation signal, and this may lead to an abolishment of antigen binding (5) or even an improvement in antigen binding (6). Glycosylation is, first of all, an intracellular sorting signal (often vital for the animal cell secreting it), but it does also play an important role in mediating the antibody effector functions (7–9). Glycosylation also influences the *in vivo* stability of the protein, but these effects are not yet predictable. Third, the Fc part of the antibody is itself responsible for many effector functions and the actions it elicits are an integral part of the animal immune response (10).

If whole antibodies are desired, *E. coli* may not be the preferred production host, as many of the advantages of having the complete molecule depend on glycosylation. For whole antibodies, efficient expression systems based on mammalian cells are available (11–13), and new strategies using other eukaryotic hosts are being developed (14–17). Furthermore, it has not yet been fully investigated whether the assembly of whole antibodies can be accomplished with satisfactory yields in *E. coli*. Protein engineering using *E. coli* as an expression host might instead be used to design alternative solutions for accomplishing the same objectives of biological effector functions and high avidity. For instance, immunotoxins or radiotoxins that accomplish the same goal of cell killing even more efficiently than natural antibodies might be designed. It should also be stressed that the design of the antibody combining site can be optimized using much smaller fragments in *E. coli*, and the final version can then still be transferred to a complete antibody molecule to be expressed in higher cells if this is desired. Therefore, *E. coli* and mammalian expression should complement each other.

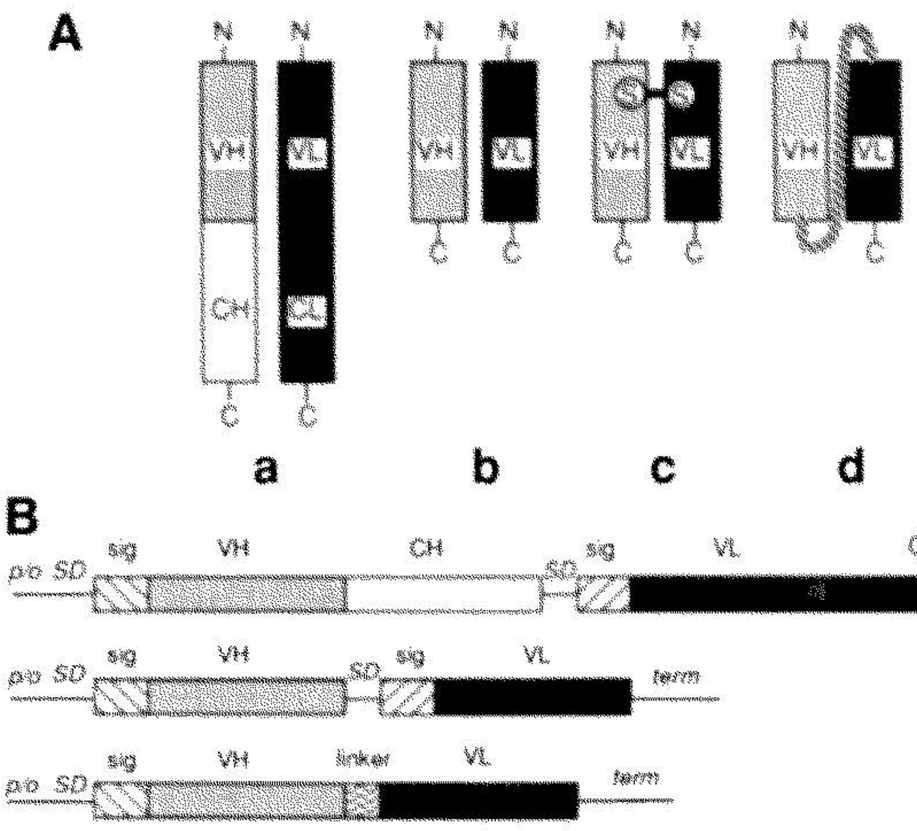

FIG. 1. (A) Schematic representation of antibody fragments that can be functionally expressed in *E. coli*. (a) The Fab fragment, (b) the Fv fragment, (c) an Fv fragment covalently crosslinked by the introduction of an artificial disulfide bond, and (d) the single-chain Fv fragment. (B) Arrangement of the operons for the co-secretion of native antibody fragments. p/o, a promoter/operator structure; SD, Shine–Dalgano sequences (ribosome binding sites); sig, signal sequences; term, a transcription terminator; and V_H, C_H, V_L, C_L, the antibody domains. (a) The expression unit for an Fab fragment (b) for an Fv fragment, and (c) for a secreted single-chain fragment.

The Fab Fragment

The Fab fragment of the antibody consists of the whole light chain and first two domains of the heavy chain, the so-called Fd fragment. The two constant domains C_H1 and C_L increase the association energy of the fragment compared to the Fv fragment. In many antibody classes, notably IgG, the light and the heavy chain are covalently linked, but covalent linking of the Fab fragment is not required for obtaining stable fragments. Mouse IgA, for instance, is not covalently linked, yet gives rise to stable Fab fragments in *E. coli* (18). This association of the two chains is an important factor for determining the stability of recombinant antibody fragments (18, 19). Additionally, the Fab fragment, by having constant domains, can be

detected using standard immunological reagents directed against the constant parts.

The Fab fragments of antibodies have been shown numerous times to have the same thermodynamic antigen binding affinities as the whole antibody (4). This has also been shown for Fab fragments produced in *E. coli* (2, 3, 18), even in a case where the C_H1 domain of the proteolytic material was glycosylated (18). Thus, this glycosylation in the constant domains clearly does not influence antigen binding.

An interesting and not fully understood property of Fab fragments is that they give lower yields than the Fv fragment of the same antibody under the same conditions in the *in vivo* assembly in *E. coli* (A. Skerra and A. Plückthun, unpublished). This fact offers room for improvements in yield, and its mechanistic basis is being investigated in the author's laboratory.

The Fv Fragment and Its Derivatives

From crystal structures of antibodies, it has been clear that the Fv fragment comprises the complete binding site of the antibody. Research on Fv fragments had been hampered by the fact that they are very difficult (and sometimes impossible) to prepare by proteolysis. Givol's group (20) first succeeded in preparing an Fv fragment by mild pepsin digestion, but the mouse antibody contained the unusual λ_2-chain. This procedure seems to be unsuccessful for other antibodies (21) and the use of a complicated multistep procedure must be attempted, or very rare mutant antibodies lacking the C_H1 domain (22) are required as the substrate. Clearly, the simplest way to obtain Fv fragments is by gene technology, and, while they can be prepared in myeloma cells (23), the simplest system to produce them is by using *E. coli* (1). Because the technology to produce Fv fragments of any antibody has become available only very recently, there is less knowledge about the range of physical properties and the stability of Fv fragments than of Fab fragments.

It has now become clear, due largely to the study of recombinant protein produced in *E. coli* (1, 18), that Fv fragments are indeed fully functional. Their only problem lies in the individuality of domain interactions, to which the hypervariable regions and the J segment contribute directly or indirectly. This results in different interaction energies between V_H and V_L for different antibodies. Some Fv fragments may therefore be fully stable under all conditions, whereas others dissociate at high dilution. For instance, in the case of the phosphorylcholine binding antibody McPC603, the dissociation constant of V_H and V_L is about 10^{-6} M (19), and the presence of the antigen improves this interaction energy due to its simultaneous binding to both chains.

To stabilize Fv fragments against dissociation, one may chemically crosslink them with glutaraldehyde (19). Alternatively, intermolecular disulfide bonds can be introduced by protein engineering (19) (Fig. 1A). To this goal, a strategy (24) based on finding two positions (one in each chain) in the three-dimensional structure, in which the backbone atoms of two residues can be superimposed on known disulfide bonds (19), was used. The positions for the new cysteines can be chosen such that the antigen binding site and proline residues (required for turns) are avoided. There are several such positions available (19). It remains to be investigated experimentally which of the suitable positions might be of most general use for variable domains, about which no structural information is available. Yet, in the investigated case of McPC603 (19), completely functional crosslinked fragments were obtained *in vivo*, with identical antigen binding properties and greatly increased stability.

Another solution to the dissociation problem is to link the two domains by a genetically encoded peptide linker, and create a so-called single-chain antibody (Figs. 1A and B). Both arrangements of the two domains in the gene have been constructed: In about half the reported cases, V_H was placed upstream of V_L (19, 25–28) and in the others, V_L was placed upstream of V_H (29–36). Generally, linkers that were found successful were around 14–16 amino acids in length. The required length depends of course on the choice of the endpoint of the upstream domain. The optimal linker length will also vary if the downstream domain is not started at the first amino acid of the mature antibody.

A number of different linker designs have been reported. The first variety, $(GGGGS)_3$, has been designed using the fact that glycine is able to accommodate a wider range of conformations than other amino acids and therefore should not be a folding nucleus or disturb the folding of the two domains. Serine makes the linker chain more soluble and prevents its internalization into the hydrophobic core during folding. Indeed, correct folding *in vitro* (25–29) and *in vivo* (19) has been obtained with the linker. In a modification of this design (30), some amino acids of the first β-strand of C_L were used as part of the linker, followed by zero to three repeats of (GGGGS). Too short a linker does of course not allow the folding to the native structure. In an alternative approach, a database search (31) has shown that the C-terminus of V_L can be connected to the N-terminus of V_H by a stretch of amino acids from carbonic anhydrase (29–33), which connects two parts of the structure in this enzyme with a distance similar to that required for the single-chain Fv fragment. New linkers (34–36) that consist of Ser, Gly, Lys, and Glu have now been reported, again aiming for solubility and flexibility.

Clearly, there are many ways to link the two domains. It remains to be investigated which linkers other than $(GGGGS)_3$ (19) are compatible with *in vivo* transport and folding, and to what degree they are resistant to proteases in *E. coli*. In the course of these investigations, the determination of crystal structures of various single-chain Fv proteins will be an important guide for further improvements. The linker may also be designed to contain additional features useful for the detection of the construct

(i.e., containing the epitope for an antibody made against the linker peptide) or features useful for its purification. For instance, a stretch of histidine residues can be incorporated into the linker residues for immobilized metal affinity chromatography (I. and A. Plückthun, unpublished).

The stability toward irreversible denaturation seems to be correlated directly with the association of the two chains (19). Thus, disulfide-bonded Fv fragments are vastly more stable than unlinked Fv fragments, possibly because of forcing a very tight interaction upon the fragment. The single-chain variant tested (containing the linker $(GGGGS)_3$) and the chemically crosslinked Fv species are significantly more stable toward irreversible denaturation, albeit not as dramatically as the disulfide-linked one (19).

In conclusion, the smaller Fv fragment will emphasize the individuality of each particular antibody in terms of stability and dissociation energy. Yet, the prospects in tumor biology and medicine with such small fragments (34) possibly showing improved tumor penetration, faster loss of unbound molecules from the body, and less immunogenicity make it worthwhile to consider the production of the Fv fragment of the antibody under study. Three strategies for stabilization are now available (19), and for labile Fv fragments, the covalent linking of the domains should be undertaken. With single-chain antibodies, the possibility that the connecting linker may disturb the binding of some antigens cannot be excluded. However, the reported binding constants (see above) for single-chain antibodies were all within one order of magnitude of the parent antibody, provided that the linker was chosen appropriately, and almost identical in some cases (19).

Isolated Domains

Even more than with the Fv fragments, the physical properties of individual domains will vary from antibody to antibody. The domain interface makes especially the V_H domain "sticky" (37). This makes V_H domains difficult to work with, as some precipitate easily at room temperature (Glockshuber and Plückthun, unpublished). The solubility problem may be solved by protein engineering, but the main problem will remain, namely, that the single domain contains only half the antigen binding site and therefore a lower affinity (or no affinity at all in many antibodies) and, more importantly, a low *selectivity*.

The V_L domain may dimerize with itself. Dissociation constants are usually between 10^{-3} and 10^{-6} M. V_L is unlikely to bind to the original antigen significantly as a dimer for steric reasons alone. Again, protein engineering may be used to alter dimer dissociation energies, but it is unlikely that *any* single domain will be able to compete with antibodies in selectivity. Single antibody domains are therefore no antibody substitute.

CHOOSING THE EXPRESSION STRATEGY

After choosing the fragment to be expressed, the experimenter is confronted with the selection of the strategy. There are currently four available strategies for obtaining antibody fragments from *E. coli:*

(i) The direct expression in the cytoplasm.
(ii) The expression of cleavable cytoplasmic fusion proteins.
(iii) The expression of secreted fusion proteins.
(iv) The secretion of both chains in the same cell, (the only strategy leading to *functional* antibody fragments).

Each strategy can in principle be used with any of the fragments discussed above. Any procedure aimed at screening, whether in cell supernatants, on colonies, or in phage plaques, however, must use the secretion strategy (1–3) because functional folded antibody fragments are crucial. Even the expression on the surface of a filamentous phage makes use of the same *E. coli* secretion apparatus (38) in order to assemble the antibody domains in an oxidizing milieu. Thus, the experiment envisaged also determines the strategy. If merely large-scale production is desired, all four strategies are in principle available.

(i) Cytoplasmic Expression

The direct expression of antibody fragments in the cytoplasm (reviewed in (39)) will normally lead to inclusion bodies. While this may facilitate a partial purification using differential centrifugation steps (for detailed protocols see, e.g., (40–42)) it does leave the investigator to work out a refolding protocol. A study of the parameters for a particular Fab fragment has appeared (42), but no detailed optimization of the procedures for other types of fragments has been reported. The published data often do not contain detailed protocols, and sometimes give no indication of the folding yield at all. It is possible that each fragment has a different folding optimum of the parameters to be considered in the folding reaction. First, the protein concentration must be considered, as too high a concentration will lead to aggregates, whereas too low a concentration will prevent the two domains from finding each other (in addition to being cumbersome). Too low a concentration should not be a problem in covalently linked fragments, however. Also, the redox buffer, the pH, the temperature, and the salt concentration must be optimized. Yet, since correctly folded material can often be selectively purified by antigen affinity chromatography, this strategy can be still used successfully and has been used in most reported work on single-chain antibodies (25–36). It is more laborious than the secretion strategy discussed below and requires careful quantitation of the success of refolding.

(ii) Cytoplasmic Fusion Proteins

In the expression of single domains, fusion proteins can be constructed with highly expressed cytoplasmic

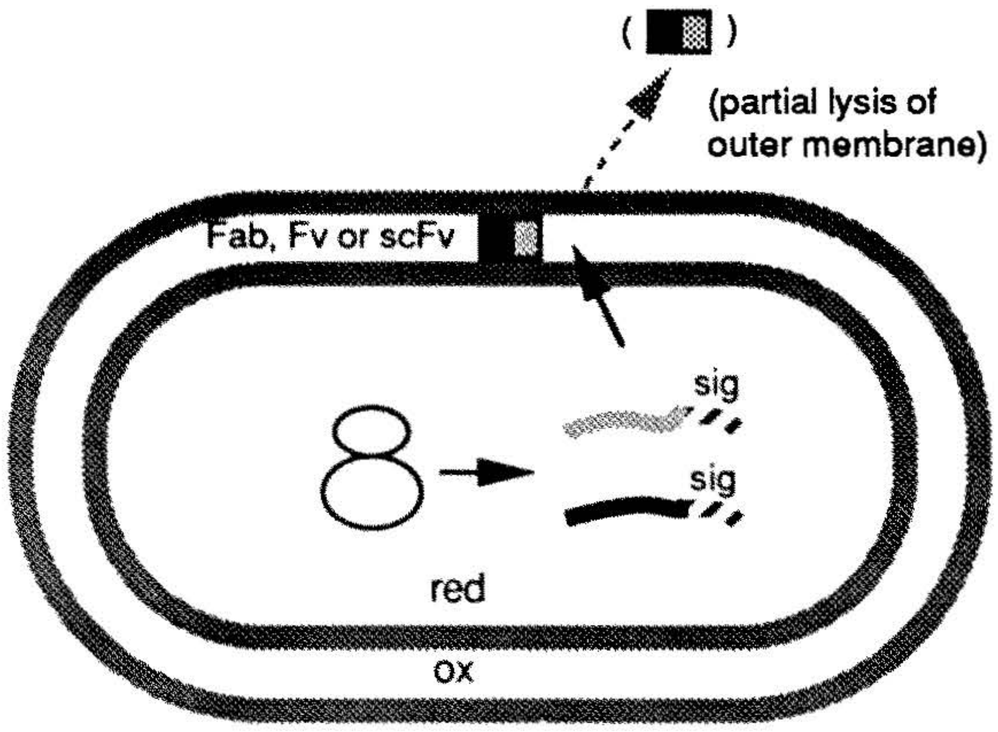

FIG. 2. Schematic representation of the secretory process. The antibody protein is allowed to reach the oxidizing periplasmic compartment, where disulfide bonds can form and each domain may act as the folding template for the other. During this process, the outer membrane starts to become leaky at higher temperature (37°C), but less so at room temperature.

proteins, helping the domain to precipitate and form inclusion bodies (43, 44). In addition to the refolding steps detailed above, the cleavage procedure must be optimized. For this purpose, the blood clotting protease factor Xa has been used (43, 44). Detailed protocols for the use of factor Xa have been reported (45). Depending on the proteolytic sensitivity and efficiency of translational initiation of a particular domain, this strategy can prove advantageous, even though it contains additional steps.

(iii) Secreted Fusion Proteins

The third strategy is to export a hybrid protein. In this case, the antibody domain or fragment is fused to the C-terminus of a normally transported protein. The *Staphylococcus aureus* Protein A has been used for this purpose (26). It has been shown that a single-chain Fv fragment can retain its function and that cleavage is not always required, although for functional and medical studies, this should be undertaken and has been investigated (I. and A. Plückthun, unpublished).

Fusions can also be made to the C-terminus of the antibody domain. In the case of secreted antibody fusion proteins, this transport, however, would then be dependent on the secretion of the antibody domains, and is therefore discussed in the next section. The use of the C-terminal secretion signal from hemolysin has also been reported (46), but the method has not yet reached the maturity required for large-scale protein production.

(iv) Secretion of Functional Antibody Fragments

The fourth strategy is to secrete the antibody domains (1–3) or a single-chain fragment (19). This is the only strategy that results in functional antibody fragments *in vivo* and therefore is the basis of all procedures relying on screening binding activity directly with bacteria or phage. The strategy was first developed for Fv fragments (1) and Fab fragments (2, 3) and has been extended to single-chain fragments (19). Why must an antibody chain be secreted to be functional? The main reason seems to be the disulfide bond that provides stability to the anti-

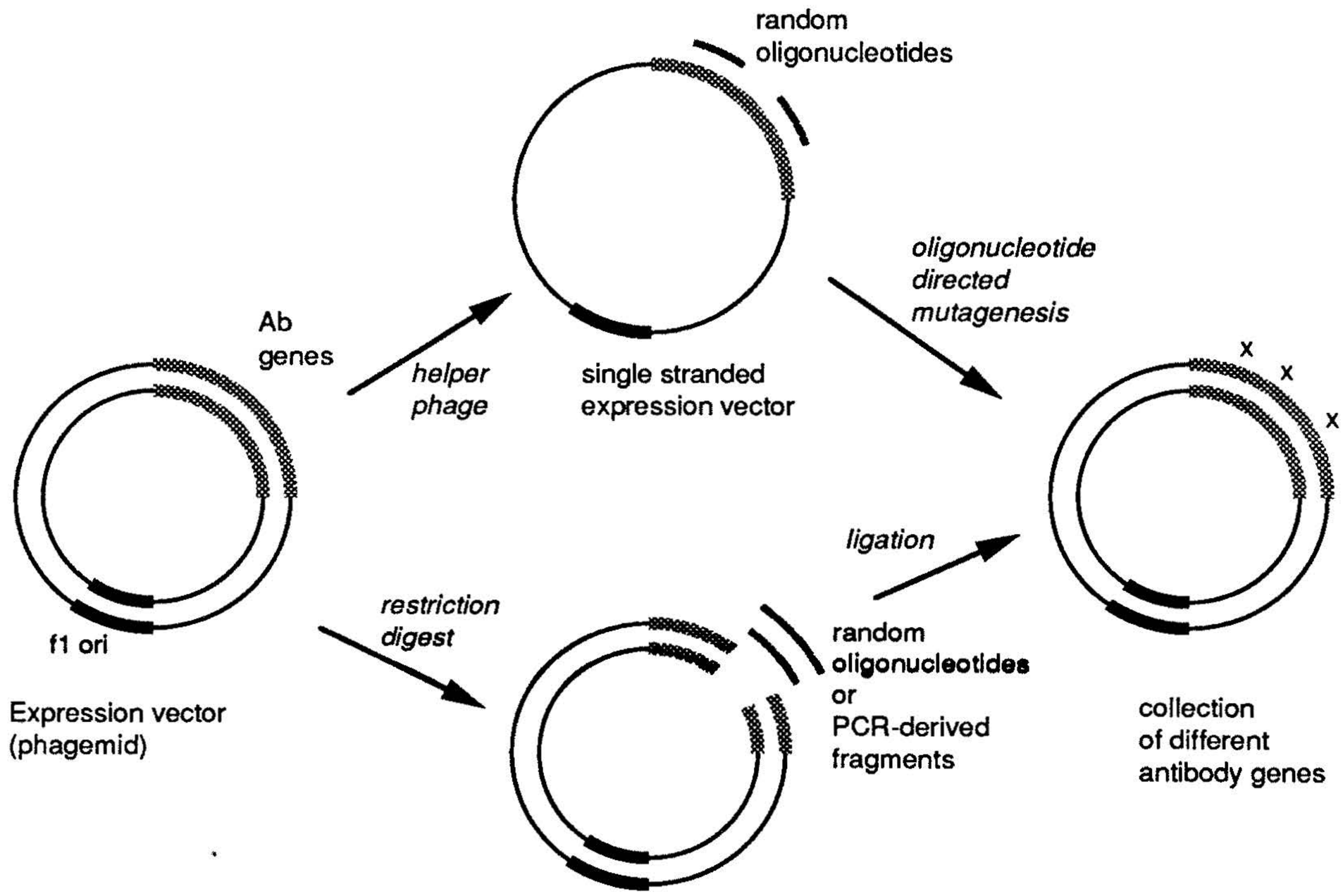

FIG. 3. Strategies for random mutagenesis made possible by using expression phagemids.

body domain. Without the disulfide bond in the variable domains, no functional molecules of any antibody fragment tested can be made (44). Therefore, the folding must take place in an oxidizing environment (Fig. 2). Also, the presence of both chains during folding may allow each chain to act as the folding template for the other. It should be noted that functional fragments can be obtained whether the two domains are linked or not, and therefore secreted heterodimers can clearly be produced in *E. coli.* Even when a functional fragment is to be expressed on the surface of a phage (38), the secretion strategy must be used. The biochemistry of filamentous phage coat assembly makes this type of phage particularly suitable for surface expression of antibody domains, since the coat proteins of filamentous phages use the same transport apparatus as secreted *E. coli* proteins, and therefore fusion proteins are straightforward constructions.

The vectors developed in the author's laboratory have been described in detail elsewhere (1, 3, 47). Briefly, an artificial operon was designed (Fig. 1B), where both chains are under the control of one promoter, and each chain (either V_H and V_L for the Fv fragment or V_HC_H and V_LC_L for the Fab fragment) is preceded by Shine–Dalgano sequences. The secretion vector for the single-chain Fv fragment (19, 47) is completely analogous, but the intergenic region is replaced by the linker region. Desirable features of such vectors include an inducible promoter with a resident repressor gene on the plasmid, as the bacteria become sensitive to lysis when secreting antibody (see below). Also, further vector constructions and all mutagenesis strategies are greatly simplified by the presence of a filamentous phage origin on the plasmid (47) (Fig. 3).

The choice of the signal sequence deserves some comment. A. Skerra and A. Plückthun (unpublished) have shown that a variety of *E. coli* signal sequences will function. Other workers (2, 37) have used still different signal sequences with success, notably the one from the *pelB* gene of *Erwinia carotovora.* Independent of the particular signal sequence chosen, the outer membrane becomes leaky as a consequence of the induction of protein production, especially at high temperatures (2) (Fig. 2). Therefore, it would be incorrect to speak of a "secretion to the medium," as there is no selectivity about the secretion. Rather, the periplasmic content seems to be partially emptied out. After prolonged times at higher temperature, the whole cell may start to lyse. These facts give

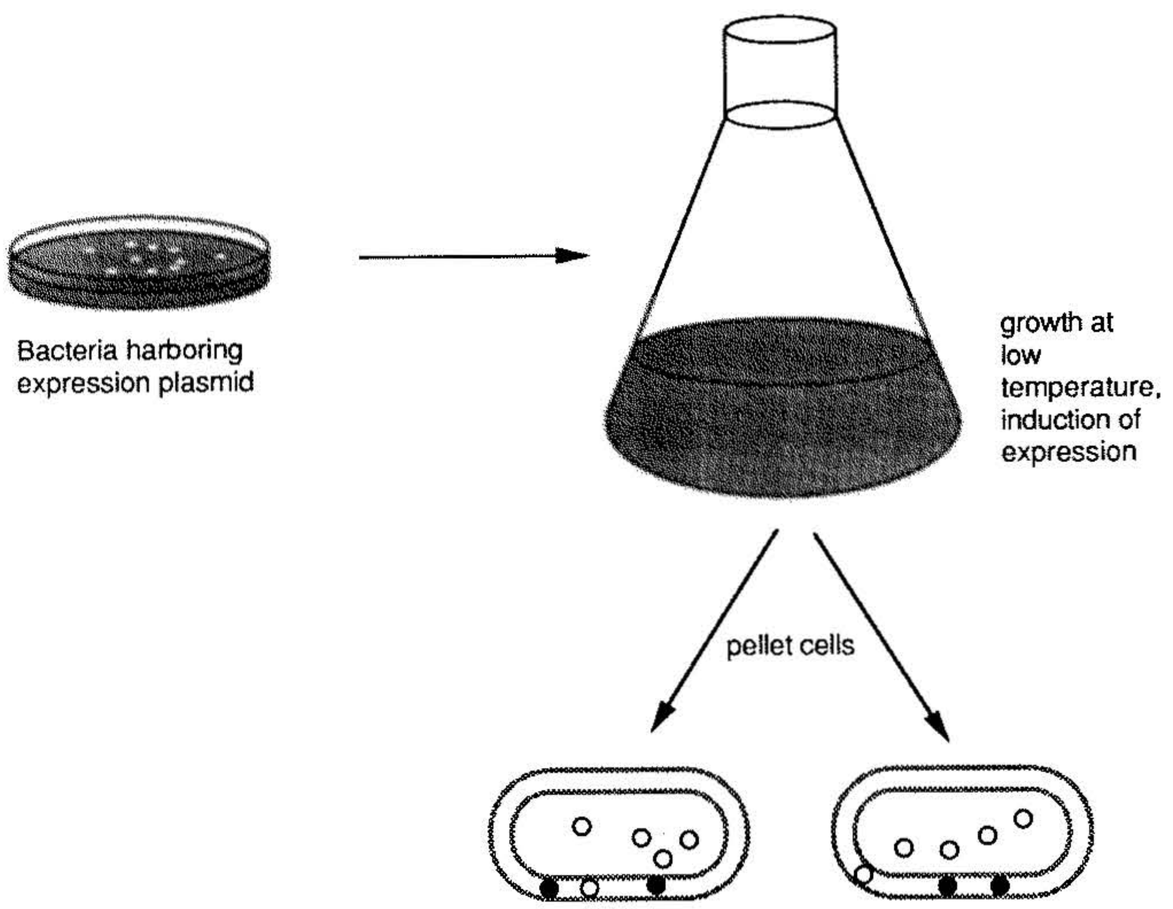

FIG. 4. Purification scheme for antibody fragments secreted in *E. coli.* Two alternative strategies are shown: lysis of whole cells by French press or preparation of the cytoplasmic fraction.

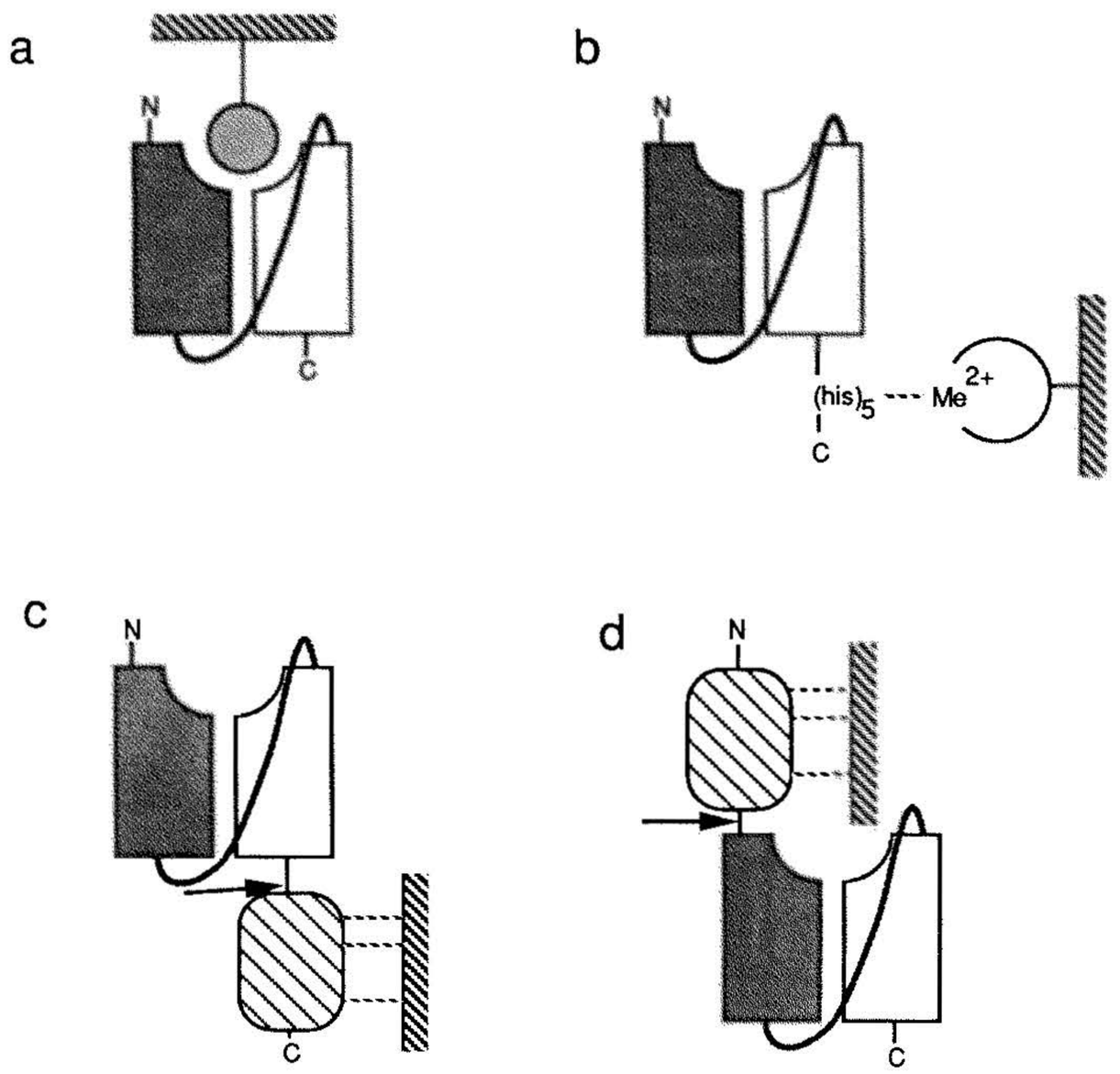

FIG. 5. Purification strategies especially suitable for recombinant antibody fragments (in addition to conventional purification techniques). (a) Antigen affinity purification; (b) Immobilized metal affinity chromatography, in fragments containing a $(his)_5$-tail; (c) hybrid protein with affinity-selectable domain or peptide at the C-terminus; (d) hybrid protein, with affinity selectable domain or peptide at the N-terminus. The arrows denote cleavage at engineered sites for proteases or chemical cleavage.

the investigator the choice of either letting the cells lyse and purifying the protein from the medium or suppressing the lysis phenomenon and purifying the protein from the cell.

In the author's laboratory, there is a strong preference for working with the smaller volume of centrifuged cells rather than the supernatant medium, especially on larger scales. The medium from lysed cells also contains membrane fragments, which sometimes may be difficult to remove (46). The protein can be prepared either from the cells by French press lysis or by a periplasmic fractionation (Fig. 4), and detailed protocols for both have been given elsewhere (1, 2, 47). One of the essential variables controlling lysis of the growing cells and the efficiency of folding is the temperature (19). It is generally found advantageous to grow the cells at room temperature.

CHOOSING THE PURIFICATION STRATEGY

If the periplasmic secretion system is used, an enrichment of the antibody protein can be achieved by a cell fractionation, i.e., the selective lysis of the outer membrane (47). The periplasm of *E. coli* contains a much smaller number of proteases and also only a small fraction of the total cell protein. For this reason alone, purifications can be simplified. The antibody protein in the secretion system can also be purified from a cell homogenate (1, 3). In this case, the whole cell is passed through a French press (Fig. 4).

If the protein is produced in the native state, the antibody fragments can be directly purified by antigen affinity chromatography (Fig. 5) (1, 3, 19). This technique is essentially the same as that used in the purification of whole antibodies from cell culture or serum. In the case of anti-hapten antibodies, the fragments can be eluted with soluble hapten, whereas for protein antigens other techniques are required. Detailed protocols for antigen affinity chromatography are available elsewhere (48).

A new strategy of completely general use is the design of an affinity handle. In antibody fragments, the C-terminus is the location of choice (Fig. 5). Single-chain fragments can be purified to homogeneity in a single step from *E. coli* using immobilized metal affinity chromatography (IMAC), and only a minimal structural perturbation of three additional residues on the C-terminus is necessary (Fig. 6) (47). The affinity tail found most advantageous contained five consecutive histidine residues, did not disturb the binding of the antibody fragment to the antigen, and did not interfere with transport to the periplasm. In contrast, clusters of arginine residues, which might be useful in ion-exchange chromatography, were not compatible with secretion (47). The exact design of the affinity tail and the detailed protocols for the chromatography of the native fragment have been given elsewhere (47).

FACTORS INFLUENCING YIELD

The quest for increasing the yield must begin with an analysis of the problem. From experience with different antibody fragments and related molecules it has become apparent that along the expression pathway the bottlenecks for different fragments are at different steps. All factors influencing gene expression in *E. coli* of course also apply to antibodies. Protein-independent factors in-

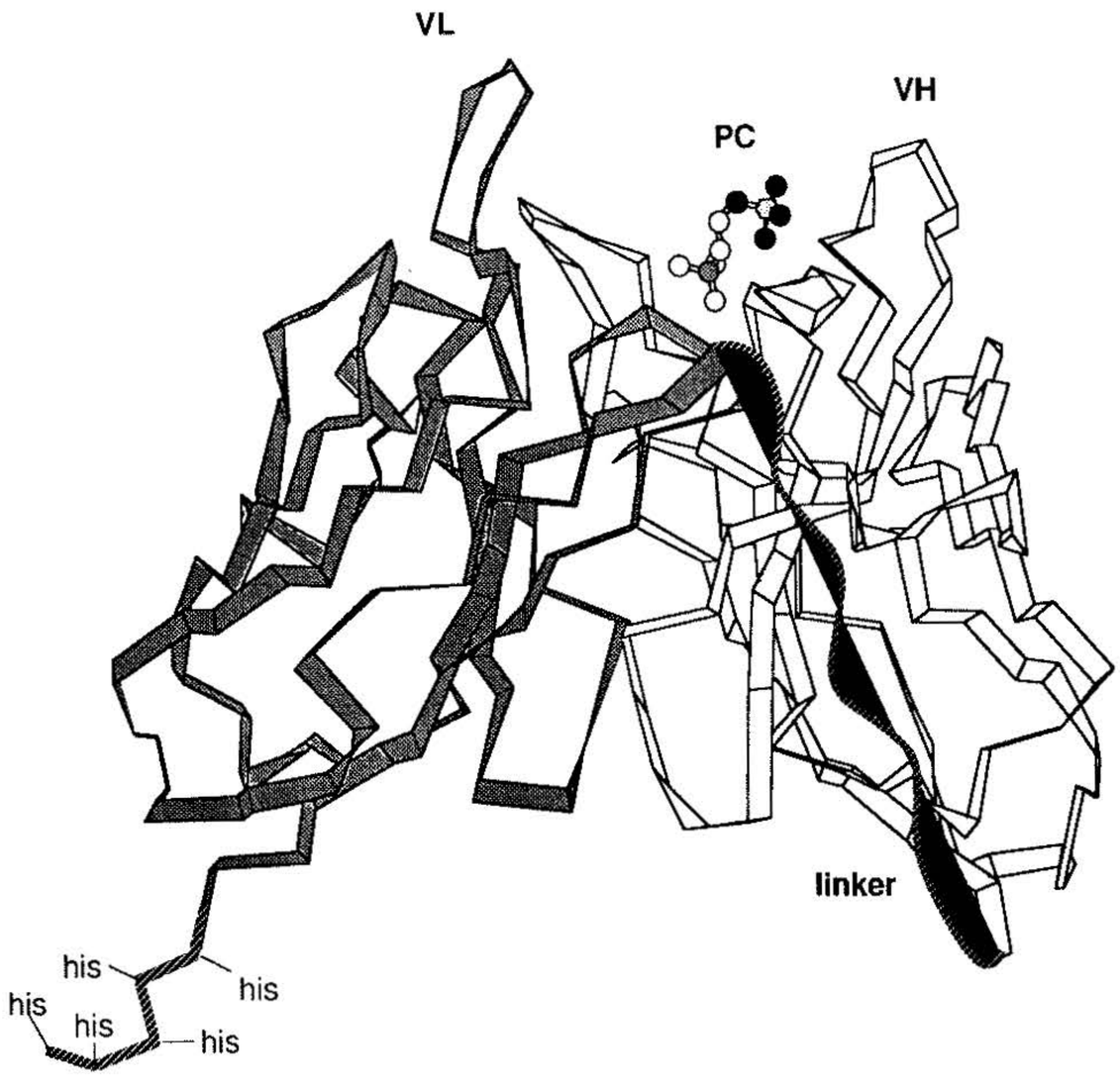

FIG. 6. Structural view of the single-chain Fv fragment containing a $(his)_5$ tail.

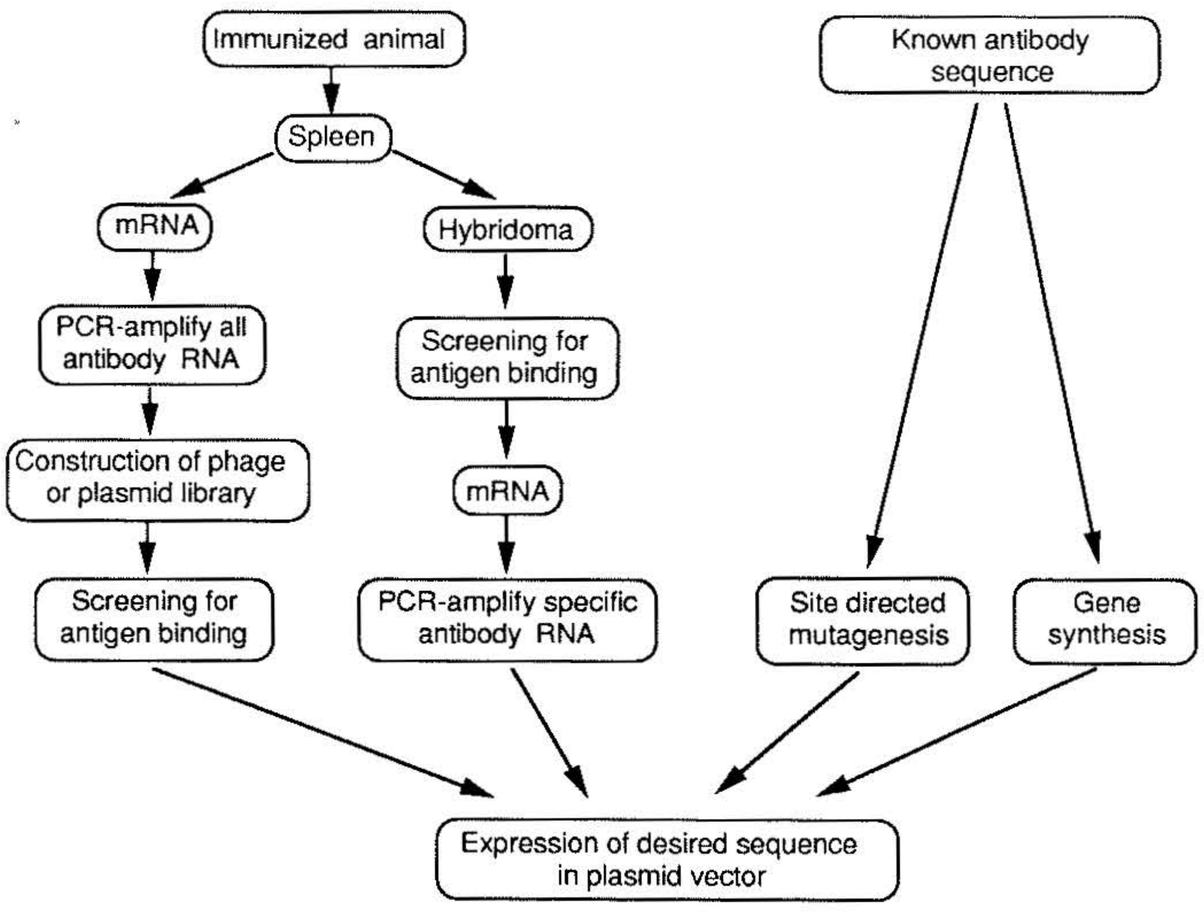

FIG. 7. Alternative pathways for obtaining the DNA for the antibody expression.

clude the gene dosage (i.e., the plasmid copy number) and the mRNA production rate (i.e., the promoter strength). These are reasonably well understood (49, 50, and references therein) (at least compared to the other factors) and should normally not provide any problem. The steady-state level of mRNA, however, is also dependent on its degradation rate, and the signals determining this are only beginning to be unraveled (51). More importantly, translational efficiency in turn also determines the mRNA stability by protecting the mRNA with ribosomes. Translational initiation is usually considered to be the major problem (52), although the pragmatic use of the 5′-untranslated and translated region of a well-translated protein like OmpA (in the form of its signal sequence) alleviates some of the lack of theoretical understanding. The translational elongation is unlikely to be limiting at the levels at which antibodies can be expressed in the correct folding state (52).

The real challenge in the manipulation of the system lies in the complicated protein folding fate that a transported protein suffers. First, it is prevented from folding before being transported through the membrane. At this point, it will interact with cytoplasmic molecular chaperones (53), and be sensitive to proteases. Then it must be transported, the signal sequence must be cleaved correctly, and folding and assembly must occur on the periplasmic side of the membrane. Clearly, the importance of various kinds of molecular chaperones on the periplasmic side of the membrane will vary from fragment to fragment, and also their sensitivity to cytoplasmic, periplasmic, and membrane bound proteases. These factors are only poorly understood and are now being investigated in the author's laboratory to provide rational approaches to the convenient production of various antibody fragments and related molecules.

The best available tactic is therefore to work on increasing the cell density. Procedures reporting yields of functional recombinant proteins of "a gram per liter" are usually high-cell-density fermentations, where not so much the yield of protein *per* cell but the yield *of* cells is very high. Densities corresponding to OD_{550} of 400 can be reached today (54). In evaluating expression yields, it is very important to be aware of these facts, and not to directly compare experiments done at 500-fold different cell densities to judge expression strains, vectors, or procedures.

While much research remains to be done in the further investigation of protein folding *in vivo* and the physiology of the producing cell, even now the production of antibody fragments of *E. coli* can be used advantageously. Engineering studies that made use of *E. coli* expression (reviewed in (55)) illustrate this point. Furthermore, the expression technology was the basis for work with libraries and screening technology (Fig. 7 and discussed elsewhere in this issue). The comparative simplicity of the methodology will undoubtedly act synergistically in the further rapid development of the field of antibody engineering.

REFERENCES

1. Skerra, A., and Plückthun, A. (1988) *Science* **240,** 1038–1041.
2. Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) *Science* **240,** 1041–1043.
3. Plückthun, A., and Skerra, A. (1989) *in* Methods in Enzymology (Langone, J., Ed.), Vol. 178, pp. 497–515, Academic Press, San Diego.
4. Nisonoff, A., Hopper, J. E., and Spring, S. B. (1975) The Antibody Molecule, Academic Press, New York.
5. Margni, R. A., and Binaghi, R. A. (1988) *Annu. Rev. Immunol.* **6,** 535–554.
6. Wallick, S. C., Kabat, E. A., and Morrison, S. L. (1988) *J. Exp. Med.* **168,** 1099–1109.
7. Nose, M., and Wigzell, H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6632–6636.
8. Leatherbarrow, R. J., Rademacher, T. W., Dwek, R. A., Woof, J. M., Clark, A., Burton, D. R., Richardson, N., and Feinstein, A. (1985) *Mol. Immunol.* **22,** 407–415.
9. Walker, M. R., Lund, J., Thompson, K. M., and Jefferis, R. (1989) *Biochem. J.* **259,** 347–353.
10. Morgan, E. L., and Weigle, W. O. (1987) *Adv. Immunol.* **40,** 61–134.
11. Morrison, S. L., and Oi, V. T. (1989) *Adv. Immunol.* **44,** 65–92.
12. Shin, S. U., and Morrison, S. L. (1989) *in* Methods in Enzymology (Langone, J., Ed.), Vol. 178, pp. 459–476, Academic Press, San Diego.
13. Page, M. J., and Sydenham, M. A. (1991) *Biotechnology* **9,** 64–68.
14. Horwitz, A. H., Chang, C. P., Better, M., Hellstrom, K. E., and Robinson, R. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8678–8682.
15. Hiatt, A., Cafferkey, R., and Bowdish, K. (1989) *Nature* **342,** 76–78.
16. Hasemann, C. A., and Capra, J. D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3942–3946.
17. Putlitz, J., Kubasek, W. L., Duchêne, M., Marget, M., von Specht, B. U., and Domdey, H. (1990) *Biotechnology* **8,** 651–654.
18. Skerra, A., Glockshuber, R., and Plückthun, A. (1990) *FEBS Lett.* **271,** 203–206.
19. Glockshuber, R., Malia, M., Pfitzinger, I., and Plückthun, A. (1990) *Biochemistry* **29,** 1362–1367.
20. Inbar, D., Hochman, J., and Givol, D. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2659–2662.
21. Sharon, J., and Givol, D. (1976) *Biochemistry* **15,** 1591–1594.
22. Takahashi, H., Igarashi, T., Shimada, I., and Arata, Y. (1991) *Biochemistry* **30,** 2840–2847.
23. Riechmann, L., Foote, J., and Winter, G. (1988) *J. Mol. Biol.* **203,** 825–828.
24. Pabo, C. O., and Suchanek, E. G. (1986) *Biochemistry* **25,** 5987–5991.
25. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R., and Oppermann, H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5879–5883.
26. Tai, M.-S., Mudgett-Hunter, M., Levinson, D., Wu, G.-M., Haber, E., Oppermann, H., and Huston, J. S. (1990) *Biochemistry* **29,** 8024–8030.
27. Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. (1989) *Nature* **339,** 394–397.
28. Kreitman, R. J., Chaudhary, V. K., Waldmann, T., Willingham, M. C., FitzGerald, D. J., and Pastan, I. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8291–8295.
29. Batra, J. K., FitzGerald, D., Gately, M., Chaudhary, V. K., and Pastan, I. (1990) *J. Biol. Chem.* **265,** 15198–15202.

30. Condra, J. H., Sardana, V. V., Tomassini, J. E., Schlabach, A. J., Davies, M.-E., Lineberger, D. W., Graham, D. J., Gotlib, L., and Colonno, R. J. (1990) *J. Biol. Chem.* **265,** 2292–2295.
31. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S.-M., Lee, T., Pope, S. H., Riordan, G. S., and Whitlow, M. (1988) *Science* **242,** 423–426.
32. Batra, J. K., Chaudhary, V. K., FitzGerald, D., and Pastan, I. (1990) *Biochem. Biophys. Res. Commun.* **171,** 1–6.
33. Chaudhary, V. K., Batra, J. K., Gallo, M. G., Willingham, M. C., FitzGerald, D. J., and Pastan, I. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1066–1070.
34. Colcher, D., Bird, R., Roselli, M., Hardman, K. D., Johnson, S., Pope, S., Dodd, S. W., Pantoliano, S. W., Milenic, D. E., and Schlom, J. (1990) *J. Natl. Cancer Inst.* **82,** 1191–1197.
35. Bedzyk, W. D., Weidner, K. M., Denzin, L. K., Johnson, L. S., Hardman, K. D., Pantoliano, M. W., Asel, E. D., and Voss, E. W., Jr. (1990) *J. Biol. Chem.* **265,** 18615–18620.
36. Iverson, B. L., Iverson, S. A., Roberts, V. A., Getzoff, E. D., Tainer, J. A., Benkovic, S. J., and Lerner, R. A. (1990) *Science* **249,** 659–662.
37. Ward, E. S., Güssow, D., Griffiths, A. D., Jones, P. T., and Winter, G. (1989) *Nature* **341,** 544–546.
38. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) *Nature* **348,** 552–554.
39. Plückthun, A. (1991) *Biotechnology* **9,** 545–551.
40. Rudolph, R. (1990) *in* Modern Methods in Protein and Nucleic Acid Analysis (Tschesche, H., Ed.), pp. 149–171, de Gruyter, Berlin/New York.
41. Laminet, A., and Plückthun, A. (1989) *EMBO J.* **8,** 1469–1477.
42. Buchner, J., and Rudolph, R. (1991) *Biotechnology* **9,** 157–162.
43. Baldwin, E., and Schultz, P. G. (1989) *Science* **245,** 1104–1107.
44. Glockshuber, R., Schmidt, and Plückthun, A. Submitted.
45. Nagai, K., and Thogersen, H. C. (1987) *in* Methods in Enzymology (Berger, S. L., and Kimmel, A. R., Eds.), Vol. 152, pp. 461–481, Academic Press, San Diego.
46. Holland, I. B., Kenny, B., Steipe, B., and Plückthun, A. (1990) *in* Methods in Enzymology (Deutscher, M. P., Ed.), Vol. 182, pp. 132–143, Academic Press, San Diego.
47. Skerra, A., Pfitzinger, I., and Plückthun, A. (1990) *Biotechnology* **9,** 273–278.
48. Dean, P. D. G., Johnson, W. S., and Middle, F. A. (eds.) (1985) Affinity Chromatography, a Practical Approach, IRL Press, Eynsham, UK.
49. Knaus, R., and Bujard, H. (1990) *in* Nucleic Acids and Molecular Biology (Eckstein, F., and Lilley, D. M. J., Eds.), Vol. 4, pp. 110–122, Springer Verlag, Berlin.
50. Balbás, P., Soberón, X., Merino, E., Zurita, M., Lomeli, H., Valle, F., Flores, N., and Bolivar, F. (1986) *Gene* **50,** 3–40.
51. Belasco, J. G., and Higgins, C. F. (1988) *Gene* **72,** 15–23.
52. McCarthy, J. E. G., and Gualerzi, C. (1990) *Trends Genet.* **6,** 78–85.
53. Fischer, G., and Schmid, F. X. (1990) *Biochemistry* **29,** 2205–2212.
54. Riesenberg, D. (1991) *Current Opinions Biotechnol.* **2,** in press.
55. Plückthun, A. (1991) *Current Opinions Biotechnol.* **2,** 238–246.