ANTIBODY ENGINEERING

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CHAPTER 8

Expressing Antibodies in *Escherichia coli*

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There are many reasons why researchers may wish to work with recombinant antibodies: for answering basic questions of protein architecture, specific binding and protein folding, for applying the modified antibodies in biology and biotechnology (ranging from biosensors to affinity chromatography), or for use in medicine. At the beginning of each project there is the problem of producing the protein in sufficient quantities. This problem will still be encountered, even if the antibody is first selected from a phage library. This chapter will summarize methodology for cloning, expression, purification, and detection of recombinant antibodies, using *Escherichia coli*, the most familiar host in biochemical laboratories.

There are several attractive features about *E. coli*. Manipulations are quite simple and well established, growth is rapid, and new ideas about changes in the protein can therefore be tested in a very short time. Scale-up of fermentations is reasonably straightforward, and very large amounts of protein are thus accessible. The transformation efficiency of *E. coli* is probably unrivaled by any other microorganism; it is this last feature that

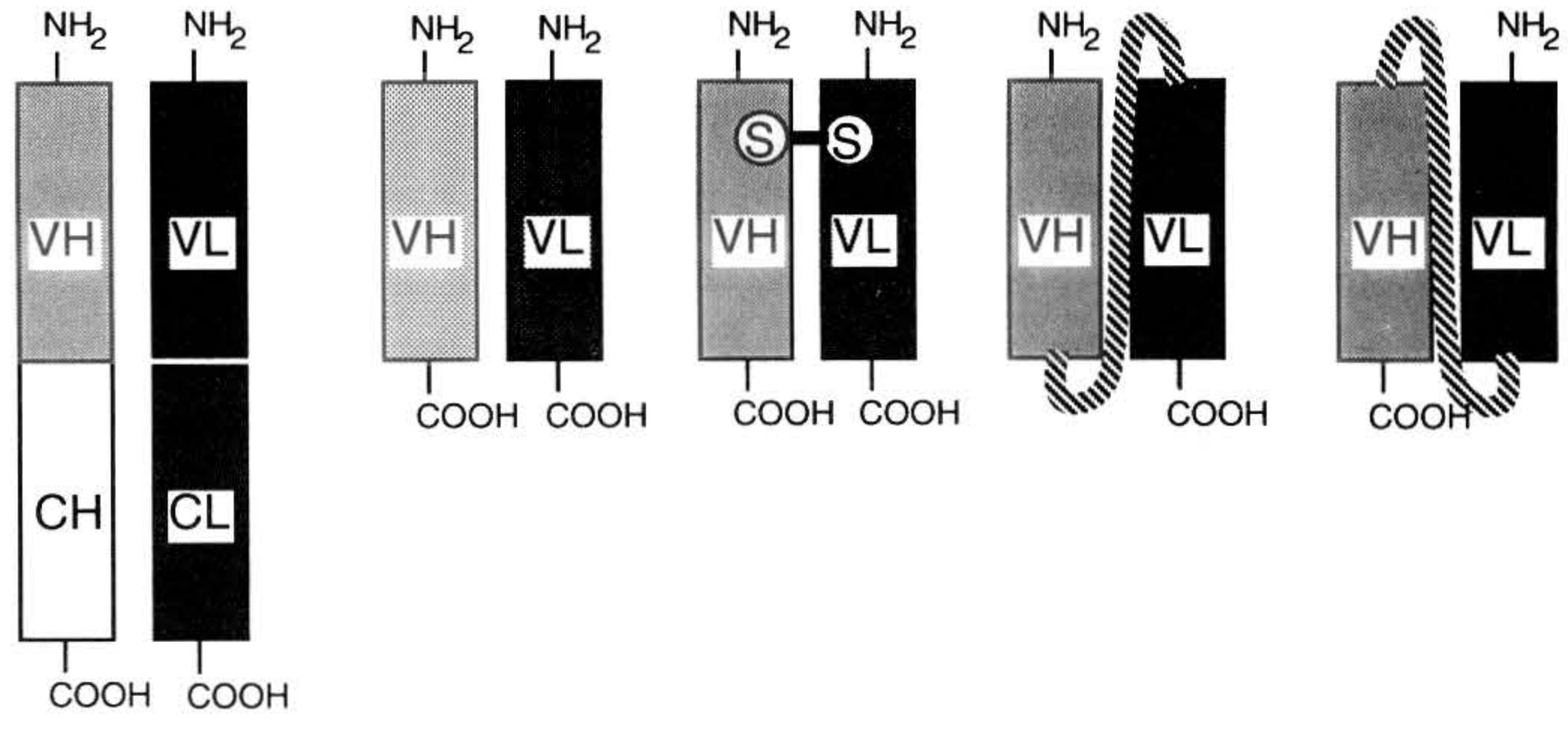
makes *E. coli* (together with its phages) particularly suitable for constructing and screening libraries.

Two decisions have to be made at the beginning of a recombinant antibody project. The first is which fragment of the antibody is desired and the second is the method of expression. Both points have been discussed in more detail elsewhere,[1–3] and therefore only a brief synopsis is given here.

CHOICE OF THE ANTIBODY FRAGMENT TO BE EXPRESSED

The antigen binding site is made up of the variable domains V_L and V_H. The smallest fragment containing the complete binding site is therefore the Fv fragment (Fig. 8–1).[4] Several investigations have shown that the Fv fragment has indeed the full intrinsic antigen binding affinity of one binding site of the whole antibody. The relative affinity of V_H and V_L for each other can be fairly low or sufficiently high to be stable, however, depending on the particular sequence of the antibody.[1,5] This may result in dissociation of the Fv fragment into its components in some antibodies, while others do not have this problem. On the other hand, there does not seem to be a severe kinetic problem of the two domains "finding" each other, as the heterodimers *do* form in *E. coli*, provided they are stable.[6]

The reversible dissociation of the Fv fragment can be counteracted in several ways. One may chemically cross-link the Fv fragment,[5] but this



Fab **Fv** **Fv (SS linked)** **scFv** **scFv**

Figure 8–1. Monovalent fragments of antibodies that can be functionally expressed in *E. coli*. The disulfide-linked Fv fragment is obtained by engineering.[5] Note that every antibody domain also contains an internal disulfide bond, which is usually 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.

requires obtaining the Fv fragment in the first place. A better approach is to engineer disulfide bonds into the fragment.[5] The latter strategy has a dramatic effect on increasing the stability against irreversible denaturation,[5] but more research is necessary to evaluate the generality of useful positions in all antibodies[7] and the stabilization effect caused by the various disulfide bonds.

The most popular method of covalently linked V_H and V_L is probably the single-chain Fv fragment (Fig. 8–1), in which the two domains are connected by a genetically encoded peptide linker.[5,8–10] A variety of linker peptides have been tested,[10] and the most frequently used one has the sequence $(Gly4Ser)_3$.[9] Recent NMR evidence[11] suggests that this linker is a flexible entity, which does not change the structure of the variable domains. It is compatible both with the orientation V_H-linker-V_L and V_L-linker-V_H, and both proteins show about the stability in reversible denaturation experiments.[12,13] The peptide linker does not stabilize the Fv fragment to the same extent as the disulfide bonds against irreversible denaturation,[5] and aggregation phenomena of scFv fragments have occasionally been noticed. Furthermore, some proteolysis within or at the edge of the linker is occasionally seen. Nevertheless, the scFv strategy secures the correct stoichiometry of the two domains, and only one expression module for the foreign gene is needed.

Closest to the naturally occurring antibodies is the Fab fragment. The constant domains make important contributions to the interactions between the light and the heavy chain and thus indirectly increase the stability against irreversible denaturation. On the other hand, several Fab fragments are more poorly expressed in functional form in *E. coli* than Fv-fragments and their derivatives[14] (Knappik and Plückthun, unpublished observations). It appears that a higher tendency to aggregate causes this problem.

Whole antibodies probably cannot be efficiently produced in functional form in *E. coli*, because they would lack the glycosylation in the Fc part, which seems to contribute to stability, and its absence would abolish all biological function.[15] In order to preserve the bivalency, which is a very effective means of increasing the functional affinity (avidity) to a surface or polymeric antigen,[16,17] another strategy can be used: the linking of scFv fragments by a small modular dimerization domain in the form of one or two amphipathic helices.[18,19] These "miniantibodies"[18,19] assemble in dimeric form in *E. coli*, and the binding performance of the best of them is indistinguishable from a whole antibody in avidity (see below).

EXPRESSION IN *E. COLI*: OVERVIEW

Two basic strategies can be employed to obtain antibody fragments from *E. coli*. The first is the functional expression of correctly folded fragments by

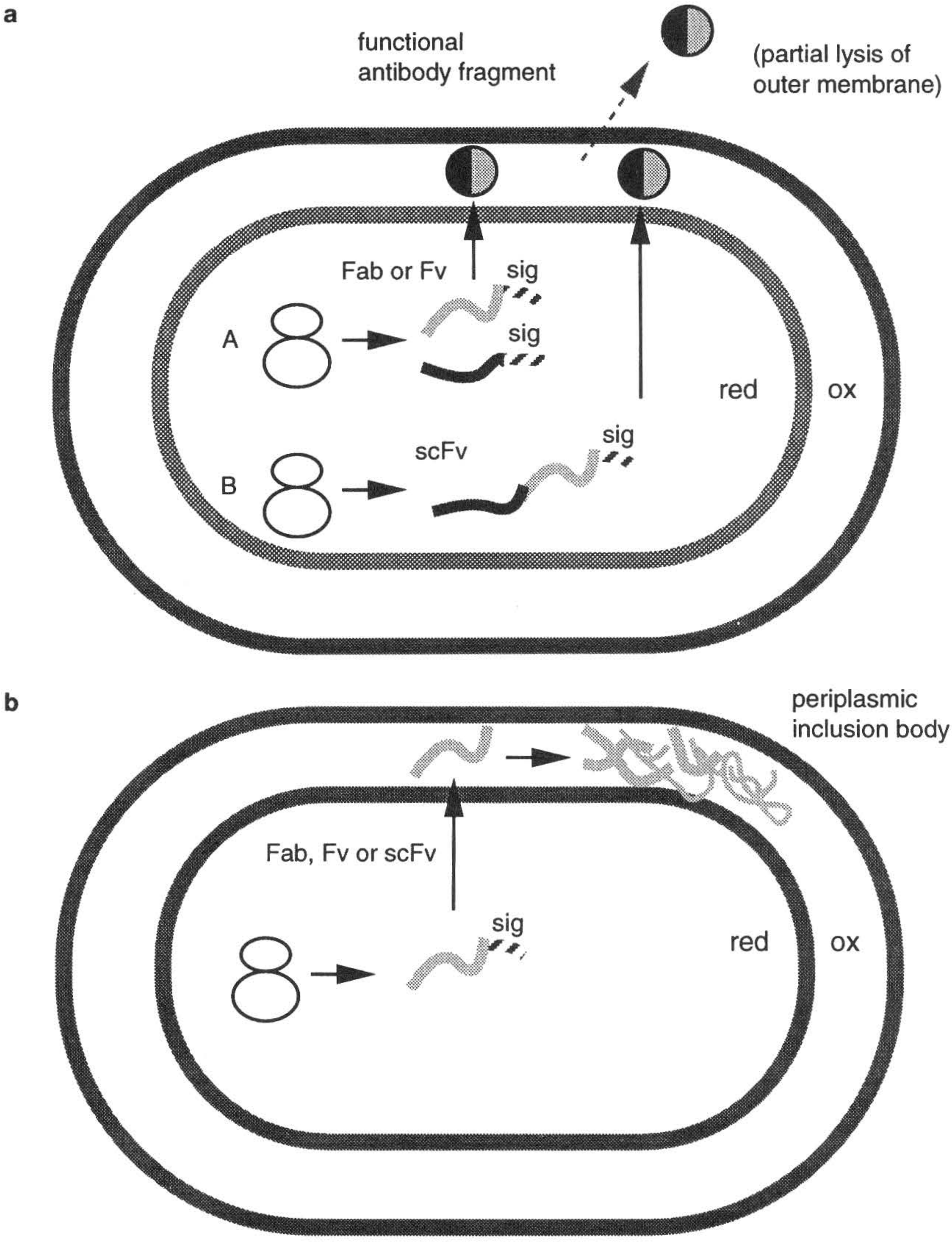


Figure 8–2. Schematic view of the different strategies found to be useful for antibody production in *E. coli*. (a) Functional expression: In this case, the two chains making up the antibody combining site are transported to the oxidizing milieu of the periplasm. In this compartment, there is a disulfide forming enzyme (DSbA) that facilitates the formation of the crucial intramolecular disulfide bonds. In (A), the pathway for two independent chains (as in Fv or Fab fragments is shown. Alternatively (B), both variable domains can be linked to form a continuous polypeptide chain (scFv fragment, see Fig. 8–1), which can also be secreted. (b) Periplasmic inclusion body formation. This is observed for many fragments

(continued)

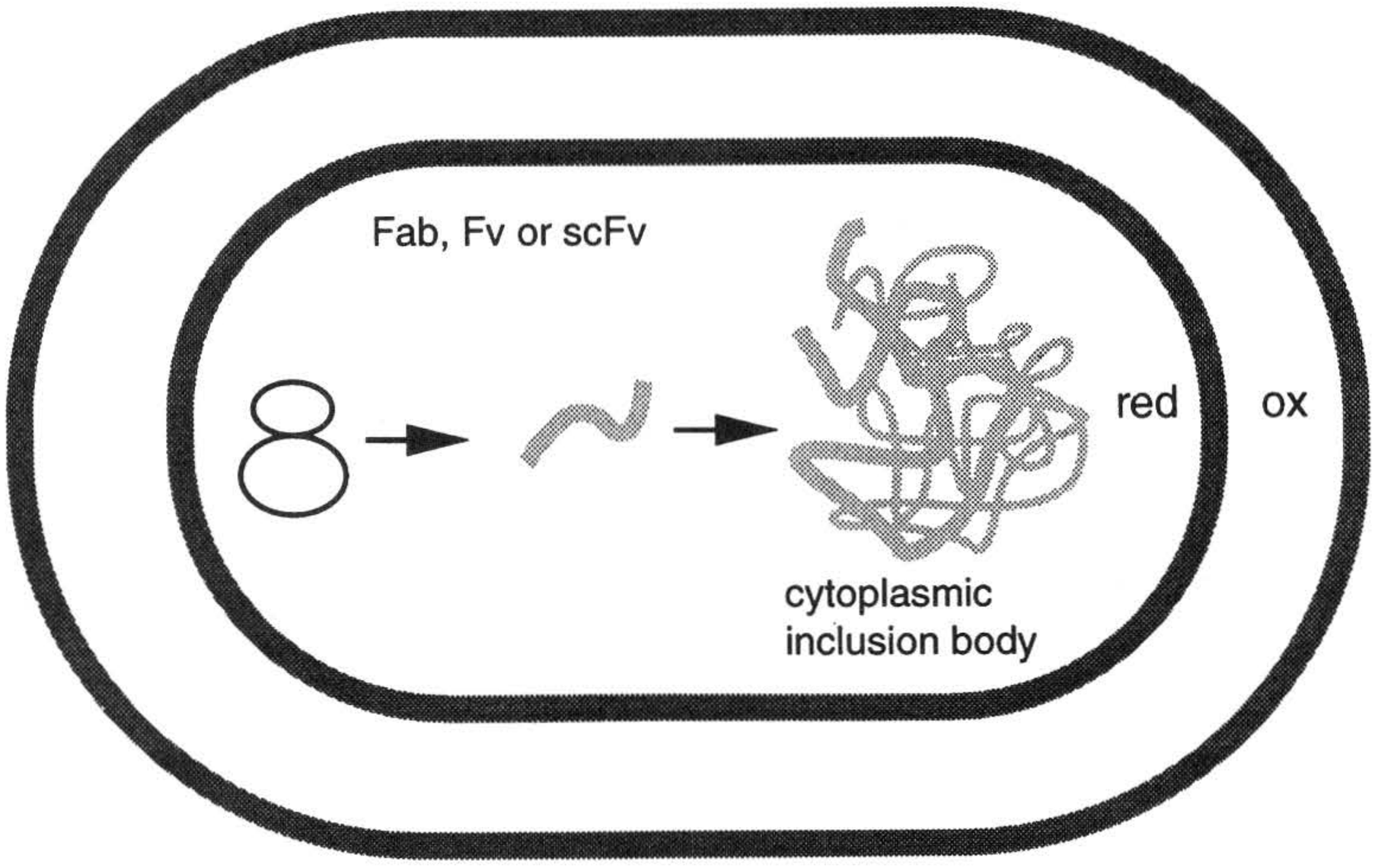


Figure 8–2. *(cont.)* as a side reaction to correct folding, and its extent depends on the sequence and the growth conditions. It can be also exploited preparatively. The protein is apparently transported, processed, and then precipitates. The protein must be refolded *in vitro* as in (c). (c) Cytoplasmic inclusion body formation. In this case, the protein is expressed without a signal sequence under a strong promoter and translation signal. Inclusion body formation appears to be more successful at temperatures of 37°C and higher. The protein must be refolded *in vitro*.

secretion into the periplasmic space,[6,20] the second the *in vitro* refolding of protein obtained from inclusion bodies, either from the cytoplasm or the periplasm (Fig. 8–2). Before going into details about both approaches, some general remarks about the various strategies are necessary.

The intramolecular disulfide bond of antibody domains appears to be crucial for stability.[21] Most antibody domains do not have a sufficient free energy of folding to tolerate removal of this stabilizing element. Consequently, in order to form the intradomain disulfide bonds correctly, which is the prerequisite to obtain folded antibodies from *E. coli*, a cosecretion of both chains (in the case of Fv or Fab fragments) must be achieved.[6,20] In the case of the single-chain Fv fragment, secretion also leads to the correct folding and assembly of both chains.[5] Nevertheless, there is not a quantitative yield of folded material, but a competition between folding and aggregation occurs, whose main determinant is the primary sequence (Knappik and Plückthun, unpublished).

To achieve secretion of antibody fragments, both chains must be equipped with signal sequences. A variety of bacterial signal sequences were shown to lead to transport and correct cleavage.[1–3] Notably, the leakage of the antibody protein to the medium appears to be linked to the sequence of the

mature antibody tested and also depends on the growth physiology (see below).

In order to cosecrete both chains to the bacterial periplasm, they are advantageously arranged in an artificial operon.[6,20] Thereby, they can be coordinately regulated. This is crucial for maintaining stability of the host-vector system, since the secretion of many (if not most) antibody fragments presents a stress to the cell. For phage display vectors, expression systems comprising two plasmids[22] or two promoters on one plasmid[23] have also been used, but the rigorous plasmid maintenance and the minimization of promoter leakiness is probably more easily controlled in a two-cistron system with only one promoter.

As will be discussed below in more detail, the folding of periplasmic protein does not proceed quantitatively for most antibody fragments.[12,14] The yield of this process depends on external factors such as temperature and growth physiology, but also on the primary sequence and the type of fragment used (Fv, scFv, Fab). It is also possible to isolate the periplasmically precipitated protein and use it as the starting material for refolding experiments.[24–26]

Finally, one may not even attempt to secrete the antibody fragment, but directly aim for cytoplasmic inclusion bodies. They can be refolded *in vitro*, but experiments with a number of fragments have shown that the refolding procedure has to be adapted to the particular fragment under study.[10] Therefore, folding optimization work is almost certainly necessary. Additionally, one must separate correctly folded from soluble, but incorrectly folded, molecules for any critical application.

Most important for deciding which strategy to use is the desired application of the antibodies. If a series of mutants is to be analyzed or a number of binding constants to be established, secretion is almost certainly the faster method. Using properly designed vectors and high-cell-density fermentation, very high-volume yields can now be obtained from secretion,[19,27] and no refolding is necessary. However, if one particular fragment is needed repeatedly in very large quantities, and the work of optimizing the refolding conditions can be justified, the expression from cytoplasmic or periplasmic inclusion bodies may be considered as an alternative. An example is the production of isotope-labeled protein required for NMR investigations: The high cost of the isotopic label may justify the optimization of the refolding protocol.[11]

To evaluate different "expression systems," two points must be kept in mind. First, the only relevant quantity is the amount of *correctly folded*, *purified* protein obtainable at the end. Second, because of the individuality of the different antibody sequences and their dramatic influence on the yield of *in vivo* and *in vitro* folding, a comparison of two expression systems with two *different* antibody fragments may be close to meaningless. Obviously,

the cell density at which a volume yield is reported must be considered as well.

In this chapter, we will go through all steps from cloning an antibody to the actual expression, purification, and detection of the fragment, using the secretion strategy. We will also describe the refolding of an scFv fragment from cytoplasmic inclusion bodies in order to obtain isotopically enriched protein for NMR purposes.

VECTORS FOR ANTIBODY SECRETION

General Considerations

The vectors described here have been in constant "evolution," and there may never be a single solution for every possible expression experiment. Therefore, the latest generation (pIG) vectors (Fig. 8–3) have a modular architecture allowing convenient changes of most elements.

a.

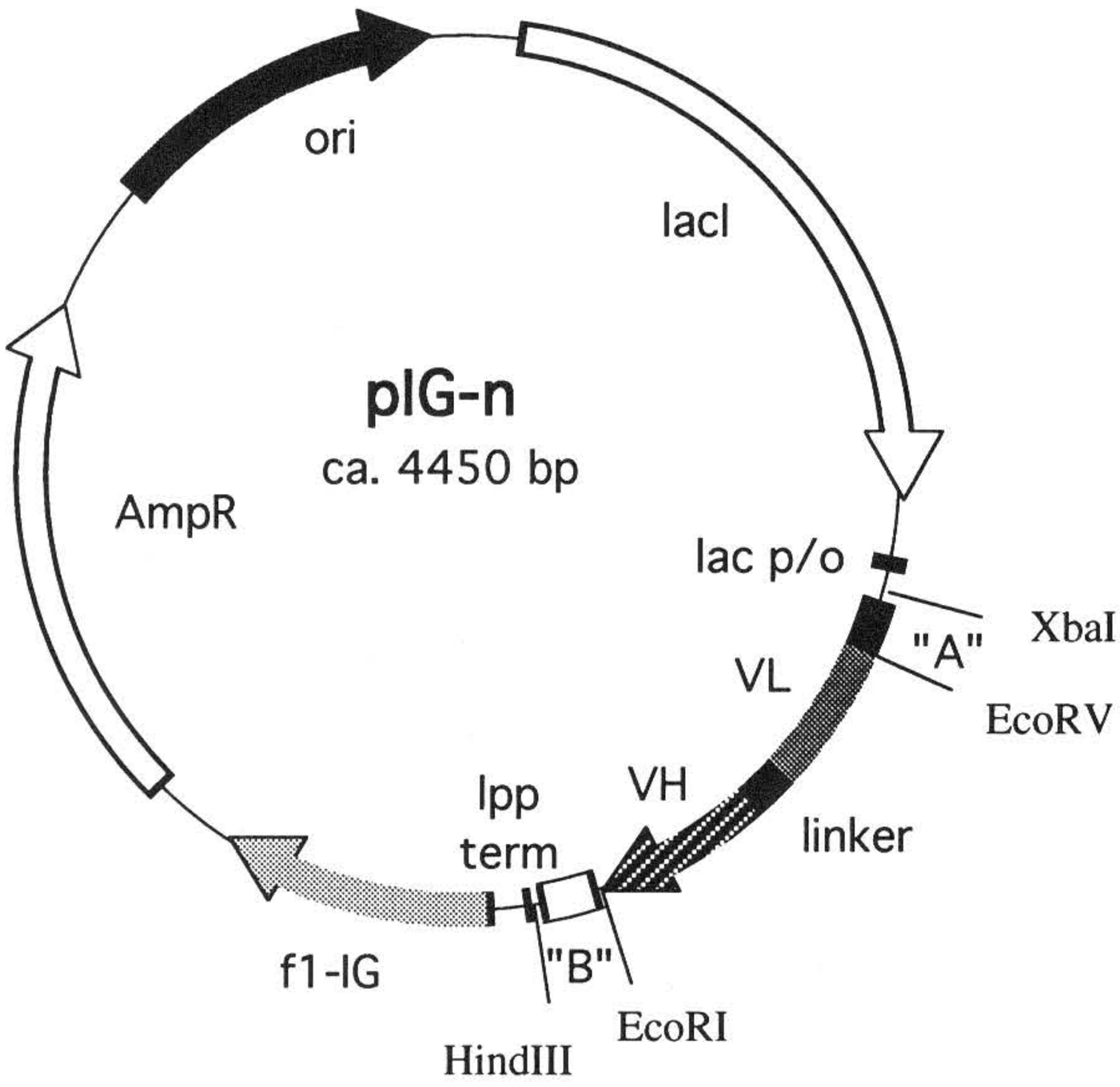


Figure 8–3. (a) Schematic drawing of the pIG vector series. *lac p/o* denotes the *lac* promoter operator, *lacI* the *lac* repressor gene, *lpp term* the terminator of the *E. coli* lipoprotein, *f1-IG* the intergenic region (origin of replication) of the f1 phage, Amp^R the ampicillin resistance and *ori* the origin of replication of the plasmid. For details, see the text and Skerra et al.[34] The "A" and "B" cassettes are explained below. *(continued)*

b **"A" cassette** **"B" cassette**

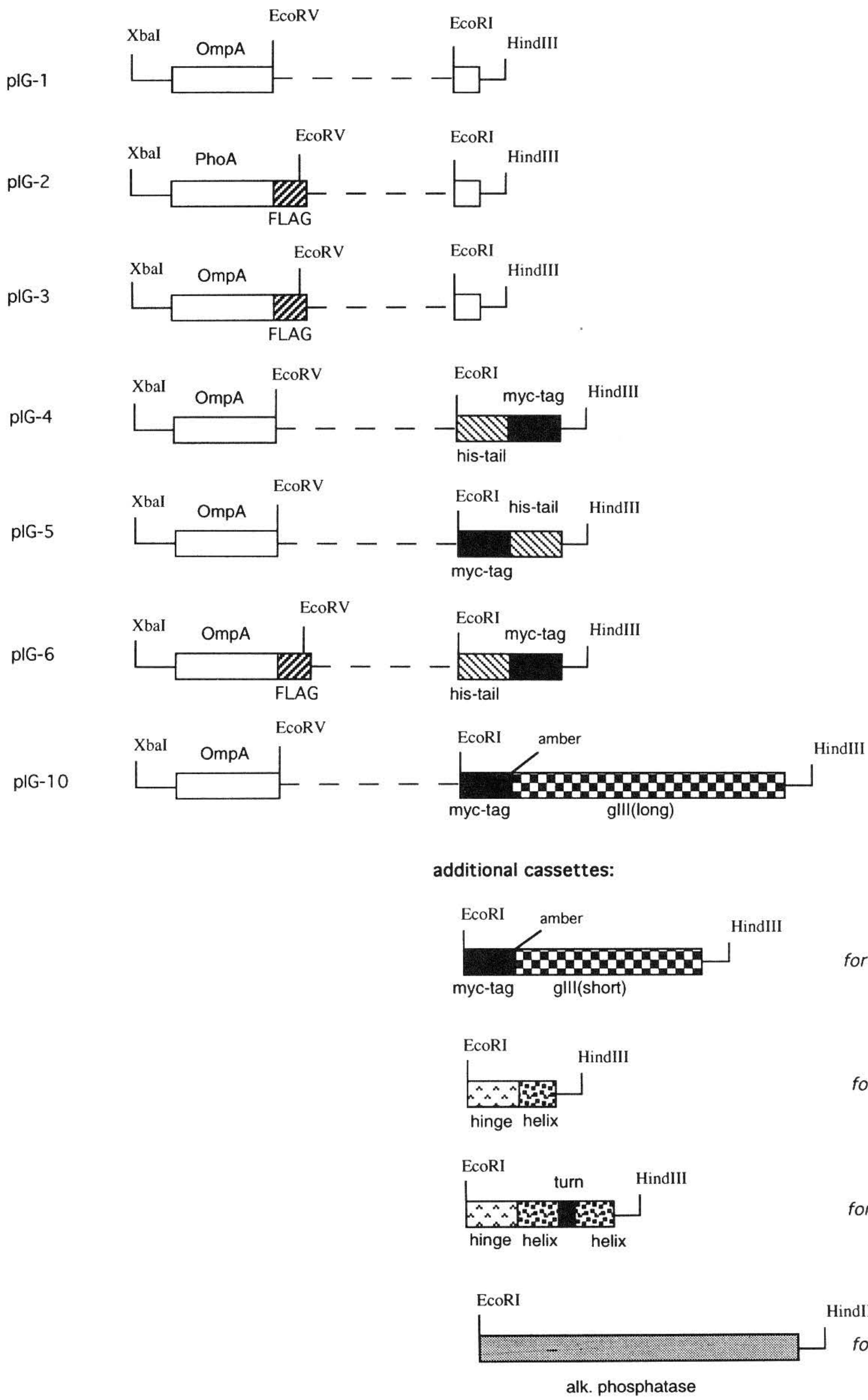


Figure 8–3. (*cont.)* (b) Schematic drawing of the N- and C-terminal cloning cassettes ("A" and "B" cassettes) of the pIG vectors, as well as additional cassettes useful in antibody work. The various fragments are not drawn to scale.

(continued)

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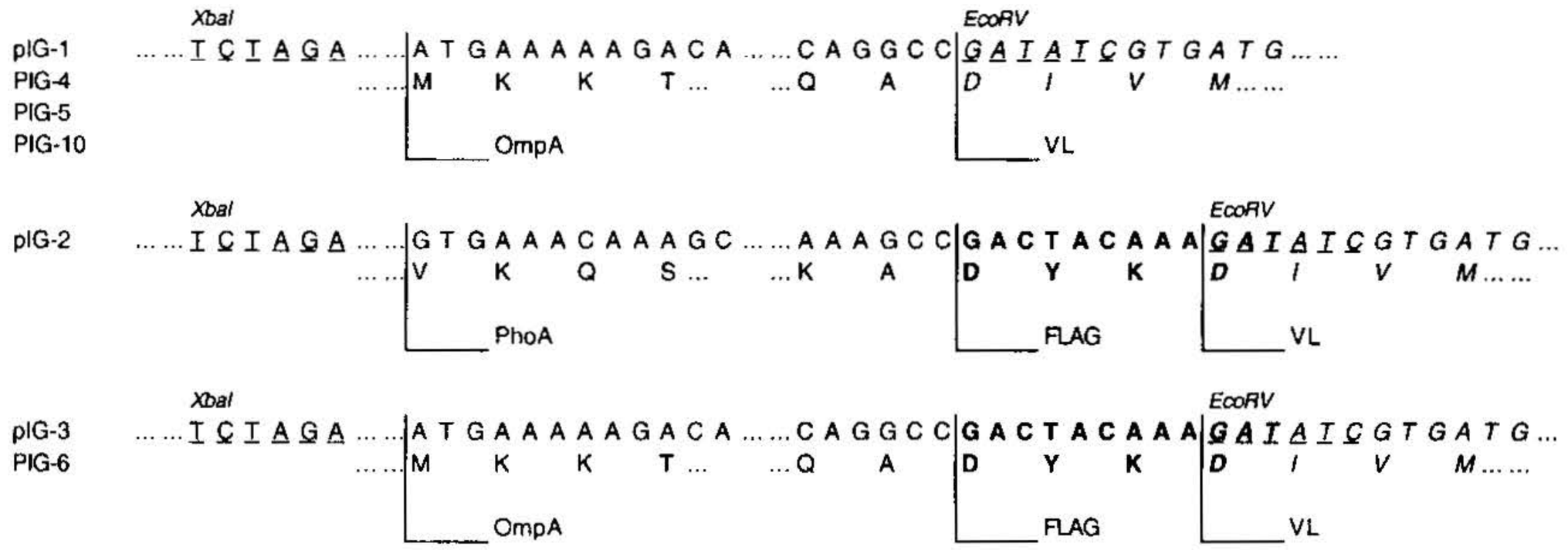
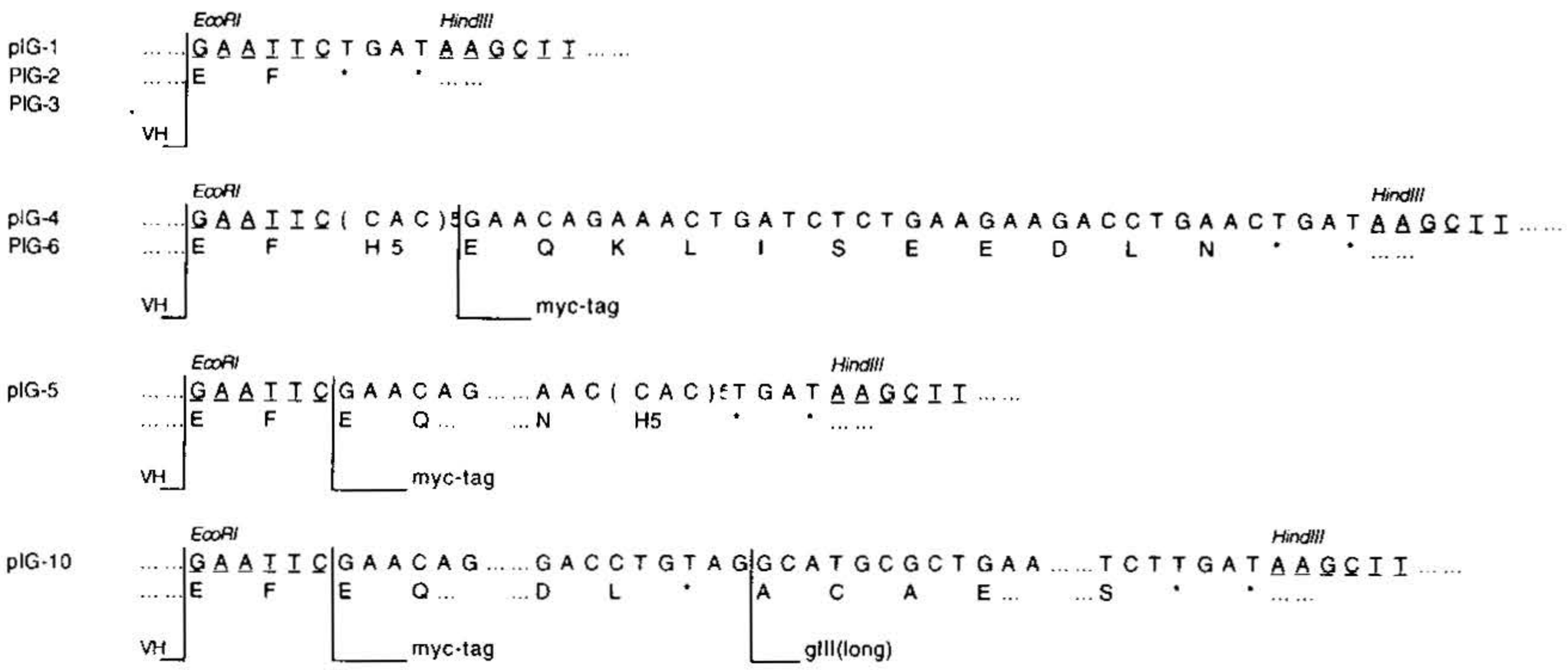
"A" Cassette



"B" Cassette



Further cassettes

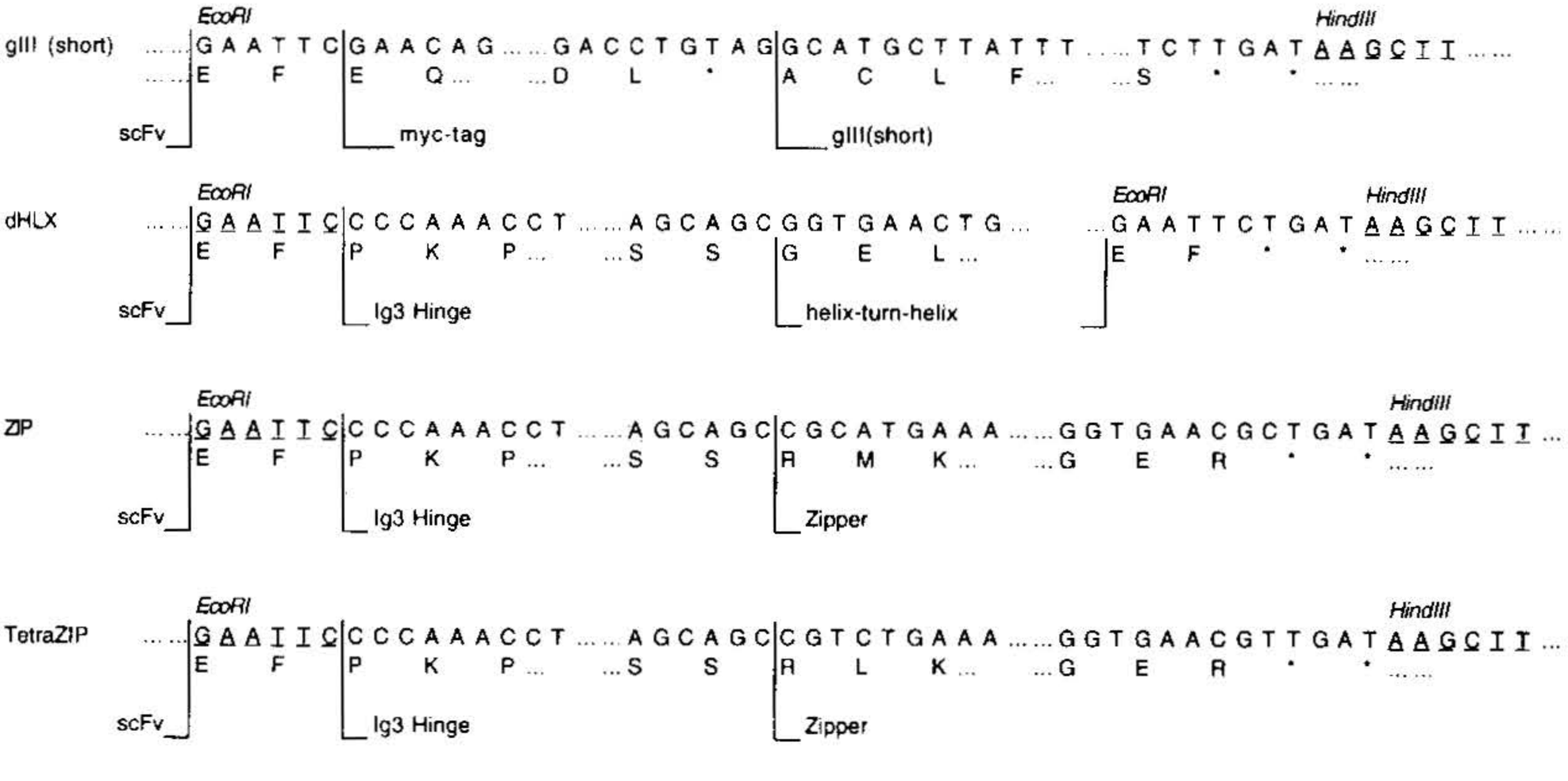


Figure 8–3. *(cont.)* (c) Detailed drawing of the "A" and "B" cassettes as shown in (b). Only the N- and C-terminal sequence of OmpA, PhoA (pIG-1 to -3) and gene III (gIII) (pIG-10) are shown. The C-terminal Asn (N) residue in the myc-tag is

(continued)

We will first summarize some general features of the vectors. The origin of replication is that of the pUC family of vectors, giving rise to a high copy number,[28] which is, however, much lower at room temperature.[29] The vectors contain the origin of the f1 phage, which allows packaging of the plasmid for site-directed mutagenesis[30] and for use in phage-display of the antibody fragments.[31] Three different antibiotic resistances have been tested. Ampicillin resistance is not advantageous in extended fermentations[19] or at high growth temperatures,[32] since the leakage of periplasmic proteins through the outer membrane of the bacterial cells, resulting from antibody expression, leads to very rapid hydrolysis of the antibiotic by leaked-out β-lactamase. In the shorter shake-flask experiments, and especially at low growth temperature, this resistance can be used, however. The activity of β-lactamase has been very helpful to diagnose the plasmid content and the integrity of the outer membrane of the bacterial cells. Tetracyclin resistance has been used in large-scale experiments.[27] Kanamycin has also been very useful in fermentation experiments,[19] but in selection and cloning experiments, a background problem may appear if streptomycin-resistant cells are used, since they can mutate to become kanamycin resistant.[33]

The promoter favored in this laboratory has been the *lac* promoter,[6,34] since it can be induced independent of any external variables. Particularly, this allows induction experiments to be carried out at any temperature and growth phase or in any strain. Strain independence is secured by the plasmid-encoded *lac* repressor. The *phoA* promoter has also been used in antibody fermentation,[27] but it requires defined media, calculated for phosphate to be depleted at a desired point.

In the dicistronic constructs, we usually prefer two *different* signal sequences.[6,34] While this is not absolutely necessary, it guards against homologous recombination, which has been observed under certain conditions (G. Wall, personal communication), since the expression of antibodies by secretion does constitute a stress for *E. coli.*

The pIG series of vectors (Fig. 8–3) contain an antibody expression cassette flanked by one set of unique restriction sites external to the coding sequence (*Xba*I and *Hind*III) and a different unique set at the edge of the coding sequence, but within it (*Eco*RV and *Eco*RI). This permits convenient shuffling of elements, fused at the N-terminus to the antibody coding

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Figure 8–3 *(contd.)* replaced by the amber codon (TAG) in pIG-10, such that the expression of the complete fusion protein is only possible in an amber suppressor strain, and the scFv form is obtained in nonsuppressor strain. The underlined bases are the restriction sites of the restriction enzymes indicated above. The bases and amino residues *in italics* are of the scFv. An asterisk (*) denotes a stop codon. The "FLAG" sequence is written in **bold**. Further "B" cloning cassettes are available for mini-antibodies (ZIP, dHLX and tetraZIP), phage-display (gIII-(short)), and alkaline phosphatase fusion (AP) and are explained in the text.

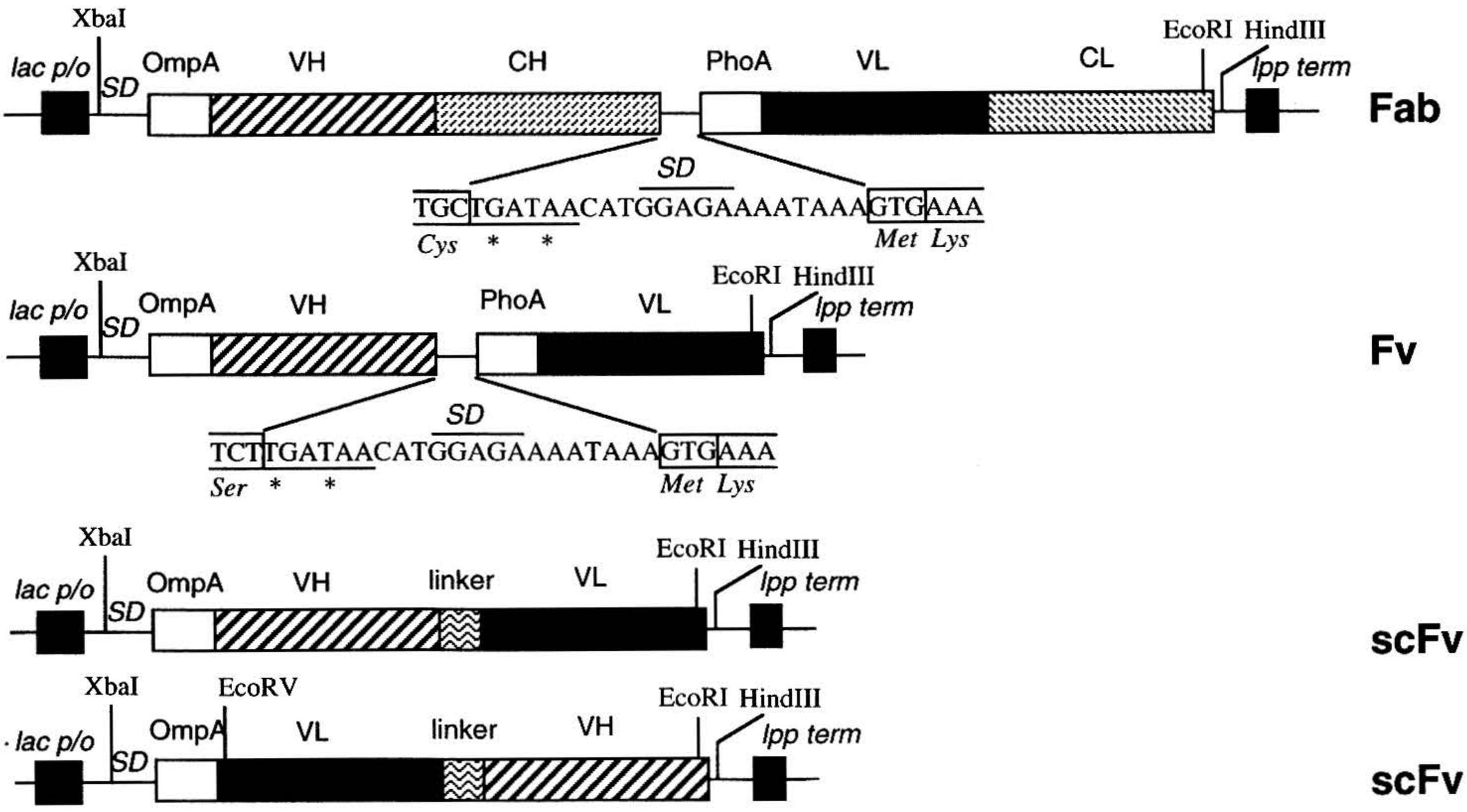


Figure 8–4. Schematic arrangement of the genes for functional expression of other antibody fragments in these vectors. The independent chains of the Fab fragment or the Fv fragment are expressed in a dicistronic operon, with the intergenic region shown. The two different orientations of the scFv fragment are also shown, both of which have been shown to function. *p/o* denotes a promoter/operator structure, *SD* the Shine-Dalgarno sequence, and *lpp term* the lpp transcription terminator. For details, see text and Skerra et al.[34].

sequence (the "A" cassette containing different signal sequences and N-terminal "tag" sequences), or C-terminal to the antibody sequence (the "B"-cassette containing C-terminal "tag" sequences, fusion domains for enzymatic activity, e.g., alkaline phosphatase or protein III for phage display, or an oligo-his tail for purification). Furthermore, the "B" cassette may contain the dimerization elements for making bivalent or bispecific "miniantibodies."[18,19] Having the option of using either a C-terminal and/or an N-terminal "tag" sequence for detection increases the flexibility for constructing fusion proteins of any kind and comparing different fragments with the same tag. The internal restriction sites (*Eco*RV and *Eco*RI) also permit the convenient ligation of PCR products. Further internal restriction sites (see below) permit domain exchange in the expression vectors of the pHJ series.

The vectors are equally suited to accommodate Fab, Fv, or single-chain Fv fragments (Fig. 8–4). The unlinked fragments (Fab and Fv) are arranged as a dicistronic operon, controlled under one promoter. The intergenic region is shown, and the upstream region has been described.[34] In Fig. 8–5, an example of the pHJ series is shown. Here the goal was to make domain exchange very convenient, e.g., to exchange variable and constant domains and interconvert Fab, Fv and scFv fragments. Since scFv fragments are

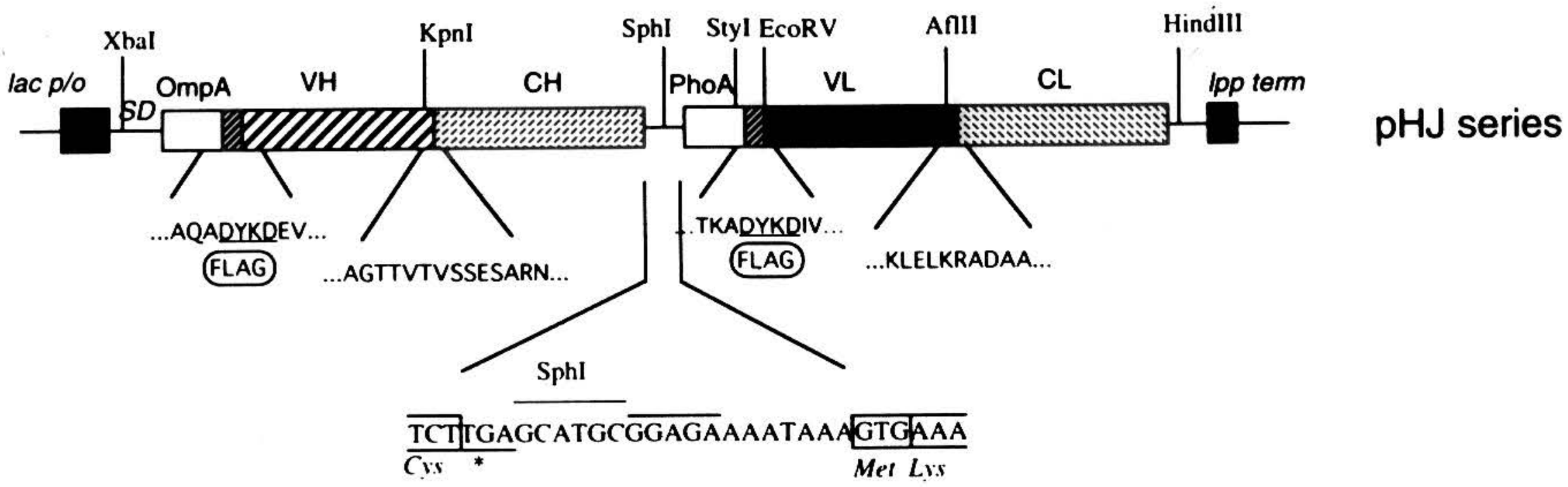


Figure 8–5. Schematic drawing of the pHJ vector series, which was created to facilitate domain switches as well as versatile detection of all chains. They are compatible with the pIG vectors. Both chains carry FLAG epitopes (see text) for easy detection. As an example, the Fab fragment is shown; Fv and scFv vectors carry analogous restriction sites to convert between these antibody fragments conveniently.

currently used in most laboratories, we will take those as an example and describe their cloning, expression, purification, and detection.

pIG Vectors

The pIG vector series is derived from pASK-lisc,[34] where the unique *Eco*RV site in the *lacI* gene in the natural gene was removed by site-directed mutagenesis. As the first vector in the series (Fig. 8–3), we have introduced a single *Eco*RV site behind the OmpA signal sequence, and there is a unique *Eco*RI site downstream from the *Eco*RV site. The genes for scFv fragments (in V_L-linker-V_H arrangement) can be easily inserted between these sites.

For detection of the scFv fragments, it is preferable to clone the fragments into the vectors containing a detection "tag": pIG-2 (with the PhoA signal sequence) and pIG-3 (with the OmpA signal sequence) contain a short version of the FLAG epitope[35–37] sequence (Asp-Tyr-Lys-Asp) (see below) and can thus be used for this purpose. Since the majority of mouse V_κ starts with the sequence Asp-Ile or Glu-Ile, we could conveniently use the last Asp residue in the shortened FLAG sequence as the first amino acid of V_L (hence the V_L-linker-V_H arrangement) and *Eco*RV (GAT ATC, encoding Asp-Ile) was used as the N-terminal cloning site for the scFvs. Therefore, the scFv constructs from pIG-2 and pIG-3 need only an additional three amino acids at the N-terminus for sensitive immunological detection (see below).

For combining detection and purification on large scales, the vectors pIG-4 or PIG-5 are recommended. Instead of the N-terminal FLAG, they contain a C-terminal his_5-tail/myc-tag or myc-tag/his_5 tail. pIG-6 contains both FLAG tag and his_5-tail/myc-tag. The scFv fragments in these constructs containing the oligo-histidine stretch can be purified in one step by immobilized metal affinity chromatography (IMAC) (see below). By using

the latter construct, we have routinely purified scFv fragments of several antibodies and their mutants. The use of the myc-tag for immunological detection has been reported,[38–39] and the use of the FLAG tag in very sensitive Western blots is described below.

Another vector in this series is pIG-10, which has been used for phage-display of the ScFv fragments.[31] Production of functional scFv after phage selection is also possible by transforming a non-amber-suppressor strain of *E. coli*. The scFv fragments produced contain a C-terminal myc-tag for detection.

The advantages of choosing *Eco*RV and *Eco*RI for scFv cloning are their robustness as restriction enzymes and the relatively low occurrence of those sites in mouse and also in human variable domains (Table 8–1). By using *Eco*RI as the C-terminal cloning site, we can also insert any C-terminal tail for modification of the scFvs (Fig. 8–3).

Obtaining the Genes of the Antibody

At the beginning of any antibody project, there are several typical starting scenarios.

1. A hybridoma line exists as the source of the antibody genes, but they have not yet been cloned. In this case, it is necessary to first isolate mRNA and then obtain cDNA using primers specific for the constant regions or located at the C-terminus of the variable domains. A PCR can be carried out using either set of primers in conjunction with primers specific for the 5′-end of the variable domain. For the cDNA synthesis, the poly-d$(T)_n$ primer used in most kits can be used, when a PCR with specific primers follows.
2. The antibody has already been cloned, either in a nonexpression vector or a eukaryotic vector or as a fragment other than the one desired. In this case, the sequence would already be known and precise custom primers should be used. In most instances, PCR is the fastest way to reclone a gene if restriction sites are not compatible. It is strongly recommended that any PCR is followed by DNA sequencing, and that a polymerase with low error rate is used.
3. The sequence of the antibody is known, but the gene is not easily available. In this case, a total synthesis of the genes is most practical.[41] If the genes of a related antibody are available, the new gene can be made by site-directed mutagenesis,[42] making use of the f1 origin in the plasmids.
4. Only the antigen is available. While we will not describe the construction of libraries, as this is done elsewhere in this volume, such construction is possible with display vectors such as pIG-10. The

Table 8–1. Frequency of Restriction Enzyme Recognition Sites in Variable Domains of Human and Mouse Antibodies

	V_H (%)		V_L (%)	
Enzyme	*Human*	*Mouse*	*Human*	*Mouse*
Aat II	8.9	0.9	0	0.3
Afl II	0.4	2.4	0	0.4
Apa I	11.7	0.6	12.2	0
Apa LI	1.2	2.5	1.0	1.4
Asc I	0	0	0	0
Ase I	2.0	20.8	1.0	2.9
Bam HI	16.1	8.3	11.2	9.9
Bcl I	0.8	0.4	6.1	1.8
Bgl I	0.4	0.3	3.1	0.3
Bsp HI	2.0	0.6	0	0.9
Bst EII	47.6	8.5	18.4	23.1
Bst XI	5.6	2.5	1.0	1.5
Bsu 36I	1.6	3.2	16.3	6.4
Cla I	0	1.4	0	0.1
Drd I	5.6	25.7	2.0	2.0
Eag I	36.7	0.1	0	0
Eco RI	0	6.1	9.2	0
Eco RV	6.9	8.1	1.0	6.9
Hind III	0.8	4.8	1.0	3.7
Hpa I	0	0.2	0	0.1
Kpn I	0	1.9	64.3	56.4
Mlu I	0.8	0	0	0
Nar I	0	3.2	3.1	0.1
Nco I	2.4	6.2	0	5.6
Nde I	4.4	2.0	0	0.9
Nhe I	0.4	0.1	1.0	0
Not I	0	0	0	0
Nru I	0.4	0	0	0
Pac I	0	0	0	0
Pfl MI	20.2	13.4	6.1	19.5
Pme I	0	0.1	0	0.4
Pst I	32.3	42.6	74.5	53.4
Pvu II	79.8	19.0	7.1	1.9

(*continued*)

Table 8–1. (*Cont.*)

	V_H (%)		V_L (%)	
Enzyme	*Human*	*Mouse*	*Human*	*Mouse*
Sac I	10.5	5.3	1.0	4.7
Sac II	11.7	0.2	0	0
Sal I	1.6	0.2	0	0.1
Sfi I	0	0	0	0
Sph I	2.4	0.3	5.1	0.4
Sma I	8.9	0.6	7.1	1.0
Spe I	1.6	0.9	0	6.2
Sty I	19.8	30.7	73.5	6.1
Tth111 I	14.5	5.0	77.6	30.4
Xba I	0	1.2	0	3.8
Xho I	1.2	7.4	0	0.5

Note: 1271 V_H, 785 V_L of mouse, 164 V_H and 98 V_L of human immunoglobulin sequences in the computer readable Kabat data base were scanned with the restriction sites listed using a word processor. An additional 84 human germline V_H sequences (Marks et al., 1993) were scanned with the same set of restriction sites using the UWGCG MAPSORT program. The frequencies given in the table indicate the percentage of sequences having *at least* one occurrence (but possibly more) of the respective restriction site.

expression and purification of a defined fragment is then carried out as described here.

PCR Assembly

N and C-terminal Primers

We are describing the use of a degenerate N-terminal primer (SC-1) to amplify several murine monoclonal antibodies for expression in the pIG vectors as an example. This type of degeneracy can certainly be extended, and the expanded primer set can be used for direct scFv cloning or repertoire cloning. There are several tabulations of useful PCR primers,[43–50] but consultation of on-line databases is recommended to take advantage of the many new sequences that are continuously being reported.

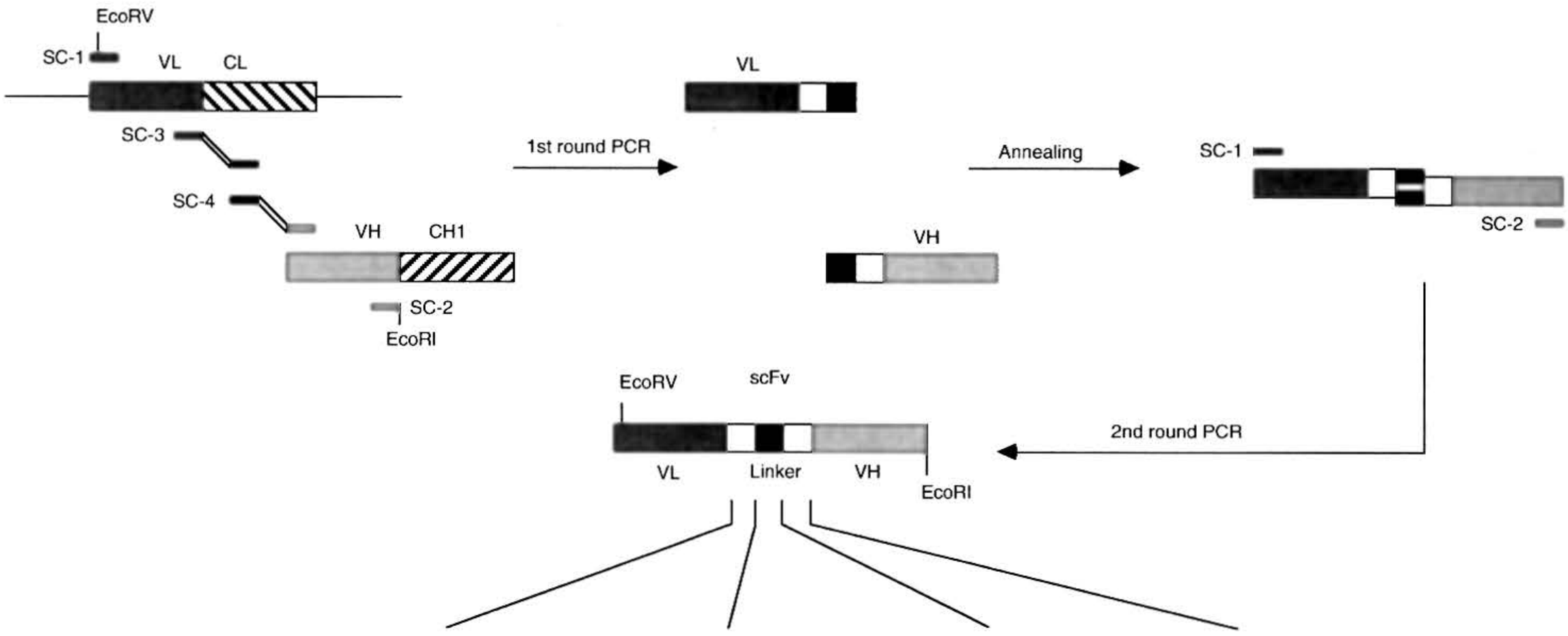


Figure 8–6. Schematic representation of the assembly reaction of the scFv. The bases that are involved in the final assembly of the individual V_L and V_H fragments to yield the scFv (annealing region), as well as the amino acids they code for, are shown in *italics*.

N- and C-Terminal Primers

SC-1: 5′- GCC *GAT ATC* **GT(GT) ATG AC(CA) CAG TCT CCA GCA** -3′
SC-2: 5′- ATG *GAA TTC* **TGA GGA GAC TGT**-3′

The restriction sites (*Eco*RV for SC-1 and *Eco*RI for SC-2) are shown in *italics*. The sequences for scFv are in **bold**. Note that due to the *Eco*RV cloning site, the first two amino acids of the scFv are always Asp-Ile.

Linker Primers

We have used the most common linker sequence $(Gly4Ser)_3$.[9] Since this is a repeated sequence, the primers have to be chosen such that incorrect annealing (leading to, say $(Gly4Ser)_2$) is avoided. We chose to use different codons at the annealing site to fine-tune the annealing temperature of the PCR (Fig. 8–6). Because this annealing is independent of the internal V_H or V_L sequences, the pairing of V_H and V_L is completely random, and therefore this strategy is also suitable for repertoire cloning.

Sequence of the Linker Primers

SC-3: 5′- *GGA GCC GCC GCC GCC* **AGA ACC ACC ACC ACC AGC** CCG TTT TAT TTC-3′
SC-4: 5′- *GGC GGC GGC GGC TCC* **GGT GGT GGT GGT TCT** GAA GTT AAA CTG GT-3′

The sequences shown in italics are involved in the anneling of the amplified V_H and V_L fragments (T_m = 58°C). The linker sequences are shown in **bold**.

Cloning and Assembly Procedure

In the following, the assembly of an scFv fragment is given as an example. The assembly of other fragments, using other vectors described in the text, is carried out analogously. The methods generally follow ref. 52.

Protocol 1: PCT Cloning of an scFv Fragment

(A) PCR amplifications of individual domains

1. Mix the following in order:

   5 µl 10X PCR buffer (0.2 M Tris-HCl, pH 8.8, 0.1 M KCl, 0.1 M $pNH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100)
   0.5 µl 100X BSA (10 mg/ml)
   1 µl of a solution 10 mM in each dNTP
   0.2 µg of the DNA encoding the antibody fragment
   1 µl of primer (50 pmol/µl stock solution) SC-1 and SC-3 for V_L and SC-2, SC-4 for V_H respectively
   1 µl of vent polymerase (2 u/µl) (New England Biolabs)
   H_2O to give final volume of 50 µl

2. Overlay the mixture with approximately 50 µl of mineral oil (Sigma, heavy white oil).
3. 30 cycles of PCR: 92°C, 1 min.; 60°C, 1 min.; 72°C, 1 min. We use a Perkin Elmer DNA Thermal Cycler TC1.

   As a negative control, the DNA template solution or the PCR primers are omitted. The 0.2 µg of the DNA listed above is given for using a cloned gene as the template. When starting from mRNA, we use the RNA extraction kit (No. 27-9270-01), the mRNA purification kit (No. 17-9258-02) and the first strand cDNA synthesis kit (No. 27-9261-01) from Pharmacia.

(B) PCR assembly of the V_H and V_L genes to an scFV fragment

1. Mix the following in order:

   5 µl 10X PCR buffer
   0.5 µl 100X BSA (10 mg/ml)
   1 µl of 10 mM dNTP
   1 to 1.5 µl of V_H and V_L mixture (in 1:1 proportion, as estimated from the agarose gel of reaction mixture from step 3 above)
   1 µl of vent polymerase
   H_2O to give final volume of 50 µl

2. Overlay the mixture with approximately 50 µl of mineral oil.
3. 2 cycles of OCR: 92°C, 1 min.; 60°C, 1 min.; 72°C, 1 min.
4. Add 1 ml of primer SC-1 and SC-2 under the mineral oil, respectively.
5. Continue PCR for 30 cycles under the same conditions.

As a negative control, the V_H and V_L mixture or the PCR primers are omitted. Although it is possible to assemble the scFv fragment using the crude PCR mixture of V_H and V_L as the template, it usually yields better results in our hands when the V_H and V_L PCR-bands are first purified (Fig. 8–6).

Protocol 2: Cloning of scFv into the pIG Vectors

(A) Purification of PCR product

1. The PCR mixture containing the assembled scFv gene is purified on a 1% LMP agarose gel using TAE as the running buffer.
2. The band with the correct molecular weight as detected under long-wavelength UV light is excised (see Fig. 8–6) and mixed with TEN buffer (0.1 M Tris-acetate, pH 7.5, 0.5 M NaCl, 5 mM EDTA).
3. The suspension is heated to 65°C until the agarose is completely molten and dissolved in the buffer (approximately 5 min.).
4. The solution is extracted 2X with Roti-Phenol (Roth), 1X with phenol-chloroform-isoamylalcohol (25:24:1) and 2X with chloroform-isoamyl-alcohol (24:1). Each organic layer can be back-extracted with a small volume of TE buffer.
5. To the final aqueous layer, 5 M NaCl is added to give a final concentration of 0.3 M. Two volumes of absolute ethanol are added and the suspension is left at −20°C for over 2 hr (it is preferable to keep it overnight at this temperature).
6. The suspension is centrifuged (14,500 × *g*, 4°C, 45 min), washed with 70% ethanol (kept at −20°C) and dried (in a speed-vac).
7. The pellet is dissolved in 20 µl of TE.

(B) Restriction digest of the PCR product and the cloning vector

1. Mix the following:

   5 µl of buffer "H" (Boehringer-Mannheim)
   approximately 0.2 µg of the vector or 0.5 mg of the PCR product
   2 µl of *Eco*RV (20 u/µl)
   2 µl of *Eco*RI (20 u/µl)
   H_2O to give a final volume of 50 µl

2. The mixtures are incubated at 37°C overnight.
3. The fragments of the desired size are purified via LMP agarose gel and extracted with phenol-chloroform (see steps 4 to 7 above).

(C) Ligation and transformation of competent *E. coli* cells

1. Mix the following on ice:

   2 µl of 10X ligase buffer (containing 10 mM ATP)
   50 fmol of the *Eco*RV–*Eco*RI fragment of the vector
   150 fmol of the *Eco*RV–*Eco*RI digested PCR fragment
   1 µl of concentrated T4 DNA ligase (2,000 u/µl, New England Biolabs units)
   H_2O to give a final volume of 20 µl

   As a control, the PCR fragment is omitted.
2. The mixtures are incubated at 16°C overnight.
3. 5 µl of each mixture is used to transform 200 µl of thawed (or freshly prepared) competent JM83 cells from a frozen stock. The JM83 cells are made competent by the "Simple Efficient Method" (SEM).[40]
4. Ten percent of the transformed cells (approximately 100 µl) is spread onto an LB agar plate (containing streptomycin and ampicillin), and the rest is centrifuged and most of the supernatant is removed. The cell pellet is resuspended in the remaining medium and spread on another LB plate, containing the antibiotics.
5. The agar cultures are incubated at 37°C for approximately 12 hr.

(D) Screening the correct clones

1. Ten to 20 colonies are picked (depending on the number of colonies on the control plate) and are used to inoculate 2 ml of LB media (containing the antibiotics).
2. The cultures are shaken at 37°C for 6 to 12 hrs.
3. The plasmids are isolated following the mini-boiling method.[53] Alternatively, commercial DNA preparation kits (e.g., Quiagen from Diagen GmbH) can be used.
4. The screening of the correct clones is carried out by *Eco*RV, *Hind*III double digest (see Fig. 8–3).

Expression by Secretion: Procedure

As an example, the expression and purification of an scFv fragment is described. Here, immobilized metal affinity chromatography (IMAC) is described as a general purification method, but antigen-affinity chromatography is another powerful purification method.

Protocol 3: Expression and Purification of Recombinant scFv Fragments

1. An overnight culture of *E. coli* JM83 (50 ml) containing the plasmid encoding the scFv fragment is used to inoculate 2 l of LB medium

(25 μg/ml streptomycin and 100 μg/ml ampicillin). The culture is shaken at room temperature (RT) until $OD_{550} = 0.6$ is reached (2 to 3 hr.).

2. 2 ml of 1 M IPTG solution (1 mM final concentration) is added and the shaking is continued for another 4 hrs.
3. The culture is harvested by centrifugation (Sorval GS-3, 5,000 rpm, 4°C, 30 min.) and the supernatant is thoroughly removed.
4. The cell pellets are suspended in 15 ml of TBS buffer (0.1 M Tris-HCl, pH 8.0, 1 M NaCl), and more TBS is added to reach a final volume of 20 ml.
5. 0.5 M EDTA (pH 8.0, 40 μl) is added (final concentration of 1 mM), and the suspension is stirred vigorously on ice for at least half an hour.[67]
6. The suspension is centrifuged (Sorval SS-34, 15,000 rpm, 4°C, 30 min) and the supernatant is carefully collected.
7. The supernatant is loaded onto a Diagen Ni-NTA agarose column (3 ml) preequilibrated with TBS buffer at 4°C. The supernatant is allowed to pass through the column with a flow rate of approximately 0.5 ml/min.
8. After the supernatant has completely entered into the gel bed, the column is washed with TBS buffer until the baseline is reached (about 20 column volumes) or overnight.
9. A washing solution containing 8 mM imidazole in TBS buffer (60 ml) is applied, followed by an 8 to 130 mM imidazole gradient (120 ml), and 5 ml fractions are collected.
10. Each fraction or every other fraction is analyzed by 12.5% silver-stained SDS-PAGE.
11. The fractions containing the pure scFv fragments are collected and concentrated by ammonium sulfate precipitation. The precipitate is collected (centrifuged at 15,000 × *g*, SS-34, 4°C, 30 min).
12. The precipitated scFv fragment is dissolved in 1 ml of TBS buffer and dialyzed against 2 × 2 l of TBS buffer. The protein solution is stored at 4°C (0.03% sodium azide is added to prevent bacterial growth) or at −20°C.

The *E. coli* strain JM83 (*ara*, Δ[*lac*, *pro*], *str*A, *thi*-1 [Φ80d*lac*ZΔM15])[51] has been found useful for expression experiments under shake flask conditions, since it shows comparatively little leakage of periplasmic proteins or lysis before and after induction. Many other strains tested do not perform as well, while a few others do. The genetic basis for this relative robustness is unclear; we found no obvious correlation to the genetic markers indicated. In the controlled culture conditions of a high-cell-density fermentation, leakage and lysis are suppressed,[19] perhaps because growth is artificially

slowed by the feeding regime, giving the cells the time for more extensive cell wall synthesis. The considerations for choosing a strain in fermentation are therefore different ones: It should be prototrophic (to avoid the feeding of special nutrients) and have no defects preventing the growth to high cell densities.[19] Some workers find the introduction of a phage resistance useful.

Optimizing the Yield in Secretion

The major goal in expression is to optimize the yield. For this purpose, it is necessary to define the bottleneck in the overall process. There are several areas where a problem could conceivably lie: (1) plasmid copy number, (2) amount and stability of mRNA, (3) translation, (4) transport, (5) folding and assembly, and (6) protein degradation. We will now discuss the relevance of each of these points to antibody secretion.

1. Plasmid copy number: Most modern vectors, especially those based on modified ColE1 origins as in the pUC series,[28] have high copy numbers. However, this copy number is a function of temperature.[29] At the lower temperature recommended for secretion, this copy number is about equal to pBR322. One problem not completely understood is the spontaneous plasmid loss from *E. coli*, on which secreted proteins impose a stress. It appears that this is not due to the decrease in the steady state copy number,[53] but rather to a total loss of plasmid in a subpopulation of cells.[19] Stable antibiotics are helpful to combat this phenomenon before induction, and constitutive plasmid-encoded functions (e.g., the antibiotic resistance protein) can be used to measure this quantitatively. However, since growth is slowed or even stops after induction, plasmid loss during the expression phase cannot be prevented with antibiotics.[19]

2. Amount of mRNA: The amount of mRNA is mostly determined by initiation frequency and degradation rate. The initiation rate (i.e., the promoter strength) is crucial, but easily remedied by the choice of any suitable "strong" promoter, as this is now well understood.[54] There is no indication that promoter strength is dependent on the gene itself. In principle, any strong promoter should be suitable, but other criteria such as complete repressibility narrow down the choices.

The mode of induction is also crucial, but for protein folding reasons. Antibody folding proceeds better at low temperature, and thus a heat-inducible promoter is less suitable for functional expression. Apparently, even if the temperature is lowered to 25°C after the heat pulse, some nucleation of protein precipitation can occur, decreasing the amount of soluble protein in some systems (Wülfing and Plückthun, unpublished observations). The degradation of mRNA in *E. coli* is only beginning to be understood.[55]

Therefore, even if an antibody gene were chemically synthesized completely, as has been done,[56] it is not clear what features should be avoided or accumulated within the coding region to increase the half-life of the mRNA. The sequence outside the coding region is also important, but there is probably still no better strategy than to use 5′ regions (i.e., the promoter and Shine-Dalgarno sequence) and 3′ regions (i.e., the transcription terminator) of well-expressed genes to guard against exonucleolytic attack.

3. Translation: Translation efficiency is mostly determined by translation initiation.[57] Generally, elongation plays less of a role, as the amounts achievable with secretory systems will reach other limits much sooner than that of the translation elongation.

Despite intensive research, translation initiation is still not rationally understood, although several trends have become apparent. Since translation initiation efficiency is a complex function of mRNA secondary structure, Shine-Delgarno sequence, its distance from the AUG start codon, but also the primary sequence of the 5′-untranslated region itself and even that of the beginning of the translated region,[57] cassettes of other well-expressed genes are normally used, and the whole sequence from the promoter to the start codon is left unchanged. In the case of a transported gene, the signal sequence may also come from the same gene as the Shine-Dalgarno sequence. To increase translation efficiency further, a two-cistron strategy has been used in our laboratory, in which a strongly expressed gene (e.g., β-galactosidase) is present only as a very short peptide, immediately followed by a stop codon and another Shine-Dalgarno sequence preceding the signal sequence of the protein of interest.[34,58] The intention is to gather ribosomes via the first and second ribosome binding site, each one optimized in its natural context. However, other efficient upstream regions are now available, which may be even more potent than the two-cistron approach. In using the extremely potent upstream region of gene 10 of phage T7 with a secreted protein, occasional problems of plasmid rearrangements have been noticed (Freund and Plückthun, unpublished).

4. Transport: The expression limit is probably not restricted by translocation through the membrane. When plasmids containing the w.t. *lac* promoter and the *lacUV5* promoter are compared, the amount of soluble protein remains remarkably constant. Yet, the amount of processed but insoluble protein dramatically increases (Knappik and Plückthun, unpublished experiments).[14] In one example,[21] it has been directly demonstrated that this insoluble protein can be degraded by externally added protease after spheroplasting the cells. Therefore, this insoluble and processed protein must have been transported and then precipitated. Only much smaller amounts of precursor can be seen in these experiments, which are soluble and cannot

be degraded by externally added protease, and thus must be cytoplasmic. These observations suggest that it is periplasmic folding, not transport, that is the bottleneck of antibody expression.

5. *Folding and assembly:* Taking all data together, it appears that periplasmic protein folding most frequently limits the yield of functional expression of antibody fragments. The following observations support this notion: (1) in the same vector, antibodies with different primary sequences differ dramatically in the distribution between periplasmic soluble and periplasmic precipitated protein (Knappik and Plückthun, unpublished experiments), (2) the same is true for different fragments of the same antibody,[12,14] (3) variations in promoter strength usually increase the amount of periplasmic precipitated proteins, but not of folded protein.[12 14]

Empirically, the simplest measure to improve the folding yield is to lower the growth temperature of the cells to about room temperature. Probably the distribution of folding intermediates between pathways of folding and aggregation is more favorable at lower temperature. Depending on the primary sequence of the antibody, these effects may be more or less pronounced.

The most effective way to improve folding may be to change the primary sequence of the antibody. Analyzing the sequences of well-expressed and poorly expressed antibodies as a basis, it was indeed found to be possible (1) to improve the ratio of folded to unfolded protein and (2) to diminish the lysis of the cells, usually caused by the onset of antibody expression, by simple point mutations of the antibody (Knappik and Plückthun, in preparation). However, the mechanism of how these mutations exert their effects is not yet known.

Two slow steps of antibody folding, which is taking place in the periplasm of *E. coli*, could conceivably be rate limiting: disulfide formation and proline *cis-trans* isomerization. Both steps occur during the folding process of antibody fragments and, for both, periplasmic proteins exist in *E. coli* that are known to catalyze these processes: proline *cis-trans* isomerase (rotamase) and disulfide isomerase (DsbA).[59,60] Nevertheless, the overexpression of neither was found to have a dramatic effect, nor was there any synergistic effect noted when both were coordinately overexpressed.[12] It is possible that the aggregation tendency of the folding intermediates just cannot be overcome by catalyzing these steps. This does not exclude that some antibody fragments that suffer less from aggregation of early folding intermediates (because of their primary sequence) are further helped along the folding pathway by these putative folding catalysts. Interestingly, antibody fragments *do* make use of the *E. coli* disulfide formation machinery, as no functional fragments can be obtained in the absence of the DsbA gene.[21] However, the availability of large amounts of DsbA does not help further.

6. Degradation: No comprehensive study on the effect of *E. coli* proteases on series of antibody fragments has yet been reported. It is likely that many recombinant fragments are being somewhat degraded by proteases, but to various extents, and depending on the sequence. Occasionally, there are degradation products seen on SDS-PAGE; however, small peptide fragments may be removed much faster than they are made, and they probably do not accumulate.[61] Protease-deficient strains have been tested in our lab, yet without dramatic effects on the yields of folded antibody fragments (Schroeckh and Plückthun, unpublished experiments). On the other hand, even bivalent miniantibodies with very extended hinge regions between the antibody domain and the dimerization domain can be prepared from *E. coli* with only small amounts of degradation in the hinge region.[18,19]

DETECTION: USING THE FLAG "TAG" FOR IMMUNODETECTION OF ANTIBODY FRAGMENTS

In many experiments involving the expression of antibody fragments in *E. coli* it is desirable to detect and quantify the products using standard immunological methods. Therefore, a system that allows highly sensitive and specific detection regardless of the particular antibody fragment expressed would be necessary. The costly and time-consuming production of antisera against particular fragments can thus be avoided.

The FLAG epitope was originally described as consisting of a highly charged and therefore soluble eight-amino-acid peptide (DYKDDDDK), which is recognized by a commercially available monoclonal antibody in a calcium-dependent manner.[35,36] The fusion of the peptide sequence to any antibody fragment allows the rapid and sensitive detection of the expressed protein by immuno-blotting or ELISA, and even one-step purifications using an anti-FLAG affinity column.[35–37] There is no observable cross-reactivity with *E. coli* proteins present in the crude extract.

The C-terminal four amino acids of the FLAG sequence (DDDK) were originally designed as an enterokinase cleavage site to allow the specific removal of the tag after purification. We found that these four amino acids are not necessary for the sensitive and specific recognition of the anti-FLAG antibody, and they can be omitted for all applications where there is no need for the removal of the FLAG peptide,[37] leading to the four-amino-acid tag DYKD. We then investigated the influence of the amino acid at the fifth position, which is an aspartic acid in the original FLAG epitope used as the immunogen,[35,36] and we found that the sensitivity of recognition is increased about ten-fold if this position contains a glutamic acid instead of an aspartic acid. A change of the positions six to eight gave only minor differences in sensitivity of detection.[37] Therefore, we suggest the five-amino-acid tag

DYKDE as the optimal sequence. So far, this is the shortest high-affinity tag sequence for which an antibody is commercially available. However, even when the fifth amino acid is Ile, as results from fusing Asp-Tyr-Lys to the N-terminus of the V_L domain in the pIG vectors (Fig. 8–3), very good sensitivity is obtained. This design creates an appendage of only three additional amino acids.[37]

The FLAG sequence can be fused either to the N- or C-terminus of a given antibody fragment. The fusion to the N-terminus has several advantages:

1. It was found by competition ELISAs that the anti FLAG antibody binds three to four orders of magnitude better under conditions where the α-amino group of the first amino acid is freely accessible.[35,36] The expression of antibody fragments in native form by transporting them to the periplasmic space of *E. coli*[6,20] generates such a free N-terminus, since the signal sequence is cleaved off after transport. We found that the FLAG sequence fused between the signal sequence and the mature part of the antibody fragment maintains correct processing of the signal peptide after transport, leading to the desired free N-terminal FLAG peptide.[37]
2. The FLAG sequence at the N-terminus is stable and is not removed by *E. coli* proteases, which was confirmed by N-terminal sequencing of purified antibody fragments produced in *E. coli*. Furthermore, at least the short FLAG sequence does not interfere with the binding of the antibody, regardless of which particular fragment is used (Fab, Fv, or scFv fragment) and regardless of which chain carries the FLAG (light chain or heavy chain or both).[37]
3. After cloning and successful expression of an antibody fragment in *E. coli*, there is often the need of constructing several variants of this antibody, for example, connecting the two chains to an scFv, constructing a Fab fragment from an Fv fragment by insertion of the constant domains or vice versa, or fusing the antibody gene to other genes (protein III of phage M13, alkaline phosphatase, toxins) or peptides (*his-tag* for purification or helix peptides for dimerization). All of these constructions involve one or both C-termini of the antibody chains. A FLAG peptide at the N-terminus does not interfere with additional cloning steps at the C-terminus and facilitates direct comparisons of these constructs.

The only disadvantage of the N-terminal location of the epitope appears to be that in functional ELISAs (where the plate is coated with antigen, antibody fragment is bound and detected with an anti-*tag* antibody), antigen binding may make the N-terminal epitope inaccessible to the anti-FLAG antibody. We prefer C-terminal *myc-tag* sequences[38,39] for this purpose (Fig. 8–3).

Protocol 4: Western Blot Using the FLAG Peptide as Detection Tag

This procedure is designed to detect as few as 1 ng of an antibody fragment containing the FLAG sequence with the sequence DYKDE at its N terminus. Therefore, 10 µl of an *E. coli* culture with an $OD_{550} = 1$ (about 10^6 cells) expressing the fragment can be loaded directly on the gel, or an equal amount can be used after harvest and fractionation of the *E. coli* culture. Generally, the amount of antibody fragment loaded on the gel should be in the range of 1 to 100 ng, if this protocol is used.

1. Perform the SDS gel electophoresis and blot the gel using standard protocols. Both nitrocellulose or PVDF membranes can be used.
2. All following steps are carried out at room temperature. Block the membrane by soaking it for 30 min in 50 ml blocking solution (1% PVP-40 in TBST) with shaking.
3. Wash the membrane 3 times with 50 ml TBST.
4. Dilute 0.75 µg per lane of the anti-FLAG antibody M1 in blocking solution (15 µg antibody in 50 ml blocking solution for a gel with 20 slots). Soak the membrane for 60 min in this antibody solution. The solution can be stored at 4°C (add 0.05% thimerosal to prevent bacterial growth) and used several times.
5. Wash the membrane 3 times with 50 ml TBST.
6. Perform a second blocking step by soaking the membrane for 30 min in 50 ml 1.5% gelatine in TBST.
7. Wash the membrane 3 times with 50 ml TBST.
8. As the second antibody, use an Fc-specific anti-mouse antiserum conjugated to horseradish peroxidase (POD). Dilute the antiserum in 50 ml blocking solution. To avoid a high background, a dilution up to 1:25,000 is recommended. Soak the memrane for 45 min in this solution.
9. Wash the membrane 3 times with 50 ml TBST, then 3 times with 50 ml TBS. These final washing steps also reduce the background and can be extended up to several hours without losing the intensity of the signal.
10. Detect the POD-conjugate by using enhanced chemoluminescence with luminol as substrate and an X-ray film as detection medium. Follow the protocol given by the supplier. Start with an exposure time of one minute and then reduce or increase the time accordingly.

The TBST listed in step 2 consists of 50 mM Tris Cl, pH 7.4; 15 mM NaCl; 1 mM $CaCl_2$; and 0.05% Tween-20. TBS is TBST without Tween. PVP-40 is polyvinylpyrrolidone, MW 40,000 Da.

The POD in step 8 together with luminol as substrate gives a high sensitivity for the detection. If different detection systems are used, the

amount of antibody fragment loaded on the gel should be increased. The optimal dilution depends on the antiserum used and should be checked by performing serial dilutions from 1:2,500 to 1:25,000.

PURIFICATION OF NATIVE ANTIBODY FRAGMENTS

General Considerations

Purification of whole antibodies has usually relied on classical chromatograph, antigen-affinity chromatography, or affinity chromatography using bacterial immunoglobulin-binding proteins such as proteins A, B, G, or L.[62–65] However, the usefulness of this strategy for Fv or scFv fragments is fairly limited, as these bacterial proteins bind mostly to the constant domains, and only few subgroups of V domains are recognized.[64,66]

However, using affinity tails, any fragment can now be purified by rather convenient and reproducible procedures, and—in contrast to immunoaffinity purifications—this technology can be carried out on a very large scale. The most convenient strategy is probably the use of a stretch of histidines at the C-terminus[34,37] (Fig. 8–3). This has been successfully applied to scFv fragments (Protocol 3) either of the form V_H-linker-V_L-his_5 or V_L-linker-V_H-his_5, to V_L domains (V_L-his_5), and also a Fab fragments, where the his_5 tail was fused only on C_H1. Since the theavy chain of most Fab fragments is practically insoluble, if not paired with a light chain, this amounts to a purification of assembled Fab fragments (Knappik, Bauer, and Plückthun, unpublished), even when the two chains are not covalently linked. In the mouse kappa V_L domain, the last two amino acids Arg-Ala (number 108 and 109 according to Kabat) are replaced by histidines in our standard constructs, and only three additional His residues had to be added to the end. X-ray crystallography showed that this had no influence at all on the structure of the V_L domain.[67] In the case of V_H, the histidines were added behind residue 113 (Kabat numbering), separated by Glu-Phe for cloning purposes (Fig. 8–3).

If the yield of a particular fragment is very poor or contains some unusually sensitive sites, it is possible that small amounts of a putative copurified protease become noticeable. Having a better ratio of antibody fragment to protease contaminant, this degradation is insignificant. Nevertheless, this copurification can occur both on Ni^{2+}-NTA and Zn^{2+}-IDA, irrespective of whether the procedure is carried out under nondenaturing or denaturing conditions.[67] This suggests that this protease can itself be easily refolded. Its identity is not yet clear, and it cannot be inhibited by the protease inhibitors EDTA or PMSF. Yet, for most fragments studied, this has not been a problem.

Factors Influencing IMAC Purifications

Influence of the Histidine Tail Sequence

We recommend the use of his_5 and his_6 tails. Other tails for IMAC have been investigated with antibody domains,[34] but the retardation was found to be worse than with his_5. A dynamic equilibrium involving all juxtaposed imidazoles of the tail may contribute to the binding and favor oligo-his sequences as the best ligand (Hochuli, personal communication). Longer oligo-histidine tails may bind better, but appear to lead to lower yields of purified protein, perhaps because of proteolytic degradation. We cannot rigorously exclude problems with the transport through the membrane using very long histidine tails, but no toxic effects nor significant precursor bands were observed with a his_9 tail in the case of the scFv fragment.

Influence of the Chromatography Conditions

A number of chromatography conditions were previously tested using V_L domains as a model.[67] It was found that the buffer composition itself is of secondary importance and can thus be adapted to other requirements of the procedure. We prefer elution with an imidazole gradient at constant pH. The choices of the ligand and the metal, however, are very important, as they are interrelated. Iminodiacetic acid (IDA) is used most advantageously with Zn^{2+}, as Ni^{2+} leads to a long tailing of host proteins and thus exceedingly long washes. Nitrilotriacetic acid (NTA), on the other hand, must be used with Ni^{2+}, since the recombinant protein elutes too early with Zn^{2+}. If the expression of a particular fragment is very poor (e.g., because of severe folding problems), it is possible that homogeneity cannot be achieved in a single step. In such cases, a fractionated ammonium sulfate precipitation or an additional ion exchange chromatography step have been found useful.

SPECIAL ANTIBODY FRAGMENTS: MINIANTIBODIES

Bivalency is a very efficient means of inreasing the functional affinity of an antibody to a surface or to a polymeric antigen. Its physical basis[16,17] is that, once the first binding site is bound to the surface, the local concentration of the second is dramatically increased. This can be quantified, based on simplifying assumptions such as neglecting the energy to "bend" either antigen or antibody, inhomogeneous surface distributions, or already occupied sites. Importantly, this gain from a second binding site is inversely

proportional to the distance of the binding sites, provided this distance is long enough to ever reach a second antigen. Furthermore, it is proportional to the surface concentration of epitopes. Nature has presumably optimized the geometry of antibodies for the antigens typically encountered. Yet, no successful attempts to make functional bivalent whole antibodies in *E. coli* have been reported so far. Part of the problem is that, at least in IgG, and C_H2 domains contact each other only via the oligosaccharide residues, which are of course absent in *E. coli.*

Functional analogs of bivalent whole antibodies have been designed that assemble in *E. coli.*[18,19] They have been termed "miniantibodies" because of their small size. They are based on scFv fragments, linked via a hinge region to a small dimerization domain, which consists of amphipathic helices (Fig. 8–7). The hinge region has been taken from the long upper hinge of mouse IgG3, giving the miniantibody enough flexibility for the dimerized binding sites to adapt to the same surface.

Two different principles of dimerization domains have been tested. One is based on parallel coiled-coil helices, as they occur in eukaryotic transcription factors (leucine zippers) (Fig. 8–7(a)). However, this was not found to be the optimal design. These constructs have lower functional affinities, and there have been problems with nonspecific binding and, consequently, scattering of binding data.

The best construct found so far is based on anti-parallel amphipathic helices, arranged as a helix-turn-helix module (Fig. 8–7(b), dHLX), presumably forming a four-helix bundle.[19] A larger percentage of the hydrophobic surface of each helix is shielded in the 4-helix bundle than in the coiled-coil helices. This construct (the sequence is given in Fig. 8–7(c)) was found to give rise to an identical functional affinity (avidity) as a whole antibody and to be very stable. It could be produced in high-cell density fermentation at about 200 mg/l,[19] in a model construct with the phosphorylcholine binding scFv fragment of the antibody MCPC603.

Most experiments have been carried out with single-chain Fv fragments of the orientation V_H-linker-V_L, and in this context, there was only negligible proteolysis in the hinge. These miniantibodies are most efficiently constructed from synthetic DNA encoding the dimerization domain introduced between the *Eco*RI and *Hin*dIII site of the expression vectors ("B" cassette, Fig. 8–3).

The expression procedures of the miniantibodies follow the expression of all other fragments (Protocol 3). The assembly of the molecules occurs spontaneously in the bacterial periplasm, and functional dimers can be directly isolated. However, purification has to rely on antigen-affinity chromatography or N-terminal tags, because additional C-terminal tags behind the dimerization domains were found to lead to proteolytic instability.

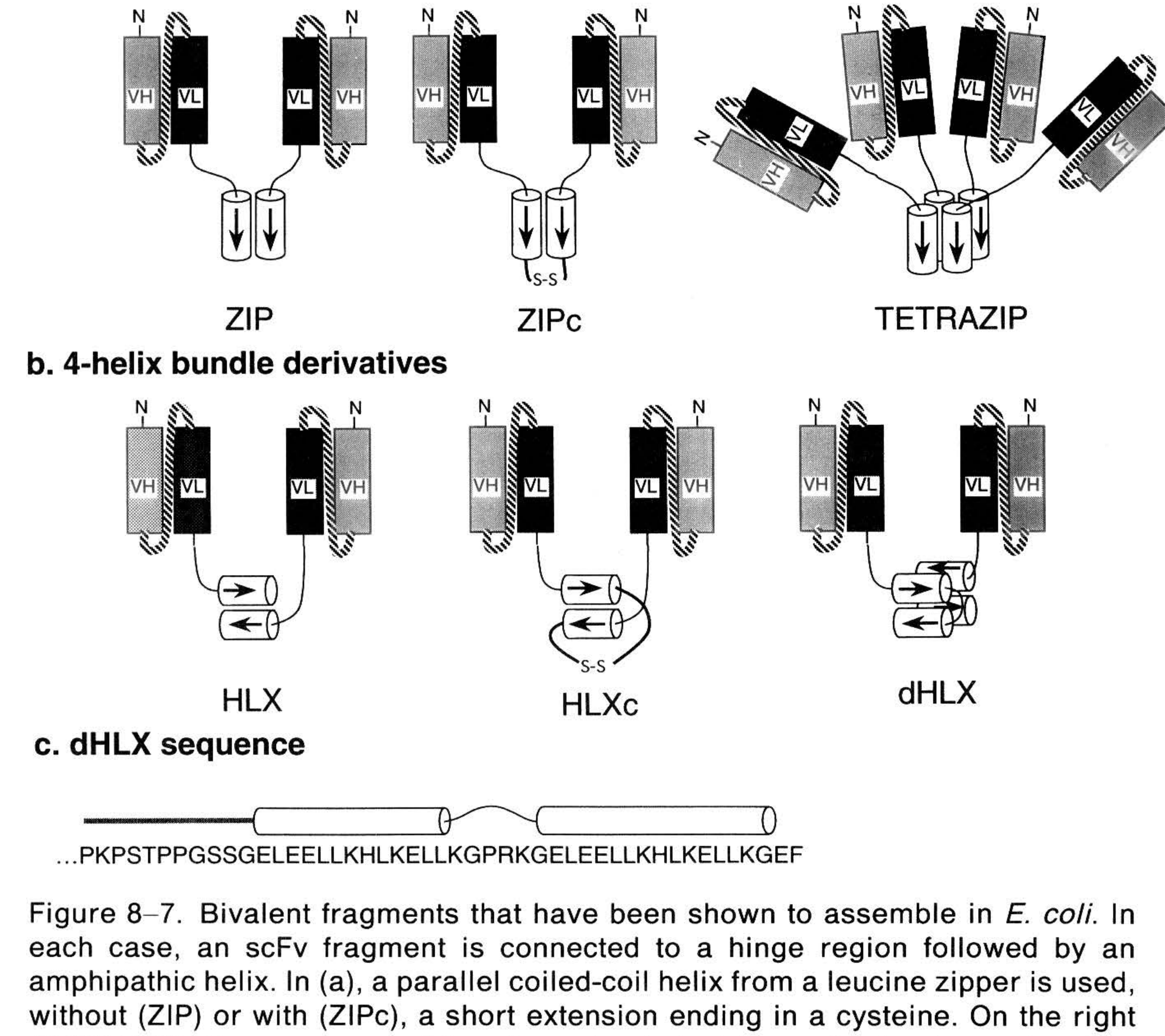


Figure 8–7. Bivalent fragments that have been shown to assemble in *E. coli*. In each case, an scFv fragment is connected to a hinge region followed by an amphipathic helix. In (a), a parallel coiled-coil helix from a leucine zipper is used, without (ZIP) or with (ZIPc), a short extension ending in a cysteine. On the right (TETRAZIP), the sequence of the zipper leads to tetramerization by exchanging the amino acids at the interface (Pack and Plückthun, in preparation). In (b) the helix comes from a 4-helix bundle design by deGrado and co-workers. On the top left (HLX) only one helix is fused, but the predominant molecular species are dimers. In the middle (HLXc), they are connected by a peptide, which ends in a cysteine. On the right, two helices are fused in tandem and a 4-helix bundle is probably obtained (dHLX), as very stable dimers are formed *in vivo*. For details, see Pack and Plückthun,[18] Pack et al.,[19] and Pack and Plückthun, submitted. (c) Amino acid sequence of the hinge and the helix-turn-helix module used for dimerization in the dHLX construct. The helical regions are indicated diagrammatically.

EXPRESSION OF ANTIBODY FRAGMENTS AS INCLUSION BODIES

General Considerations

The production of antibody proteins as cytoplasmic inclusion bodies in *E. coli* is also possible as an alternative. All types of antibody fragments (Fab,

Fv, scFv, and even the chains for the whole antibody) have been produced this way,[8–11,68–70] and a variety of strains, plasmids, and promoters have been used. The use of the T7 system[71] as a particularly strong but regulatable system was found useful by several investigators.[10,11]

Usually, the inclusion body approach is carried out using genes not encoding signal sequences. Therefore, the antibody fragments stay in the cytoplasm and largely precipitate. Since precipitation is desired, it is useful to do exactly what needs to be avoided when secreting the antibody, namely to grow the cells at higher temperature, e.g., 37°C.

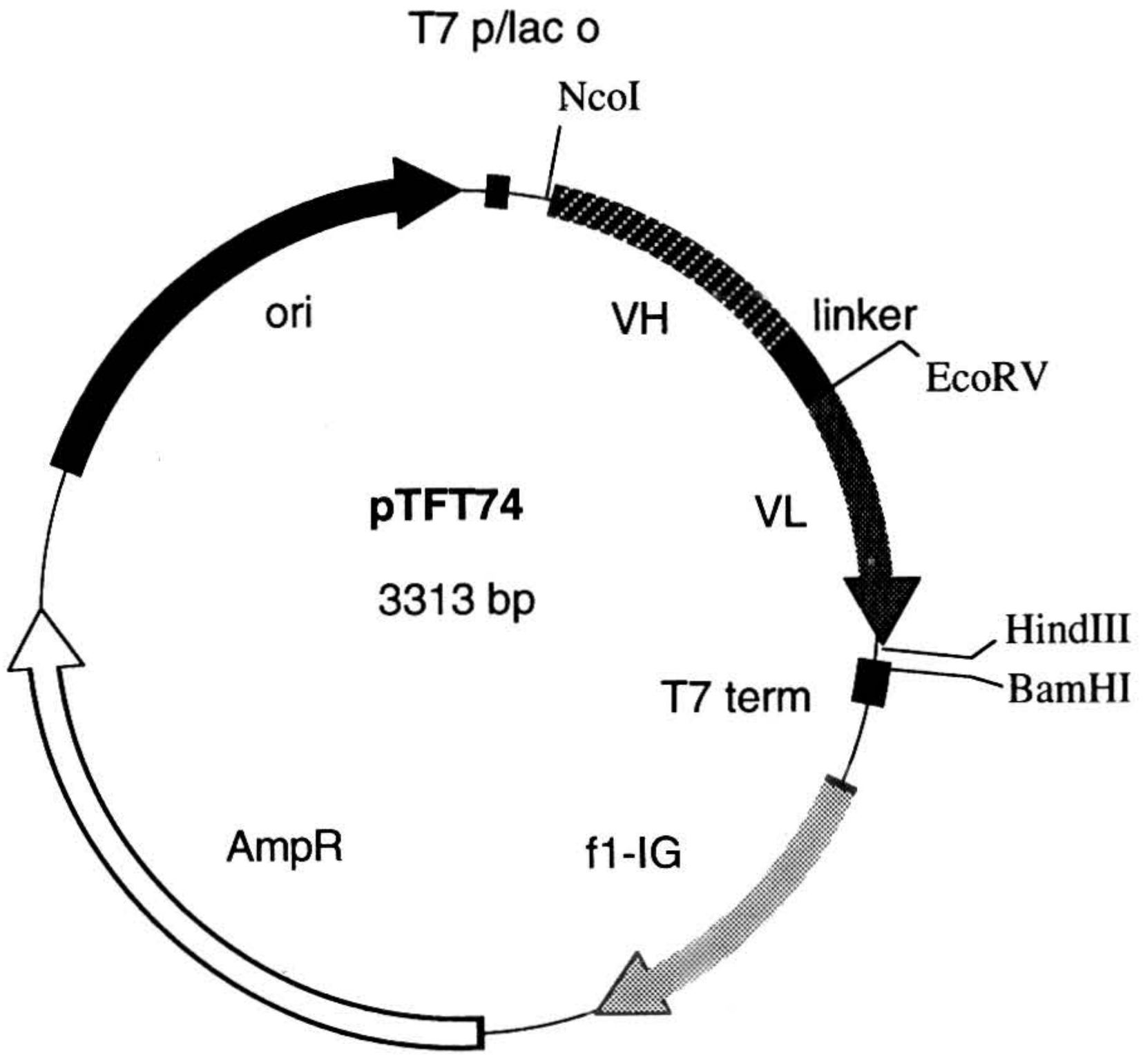
Alternatively, one may also isolate that portion of the secreted protein which precipitates after transport to the periplasm. This has been described for scFv fragments[24–26] and Fab fragments.[72] At higher temperatures (37°C), the protein can still be transported, but the folding in the periplasm is often severely impaired, although apparently not for all antibody sequences.[27] In the oxidizing milieu of the periplasm, some of the precipitated protein already has disulfide linkages, but it is not known what percentage of molecules have them, and how many of them are correct.

A vector for cytoplasmic inclusion body formation is shown in Fig. 8–8. Cloning of the antibody fragments (without signal sequences) can be carried out analogously to Protocols 1 and 2. For expression experiments, the plasmid must be transformed into *E. coli* BL21 (DE3),[71] which carries the T7-RNA polymerase in the chromosome under the control of the lacUV5 promoter.

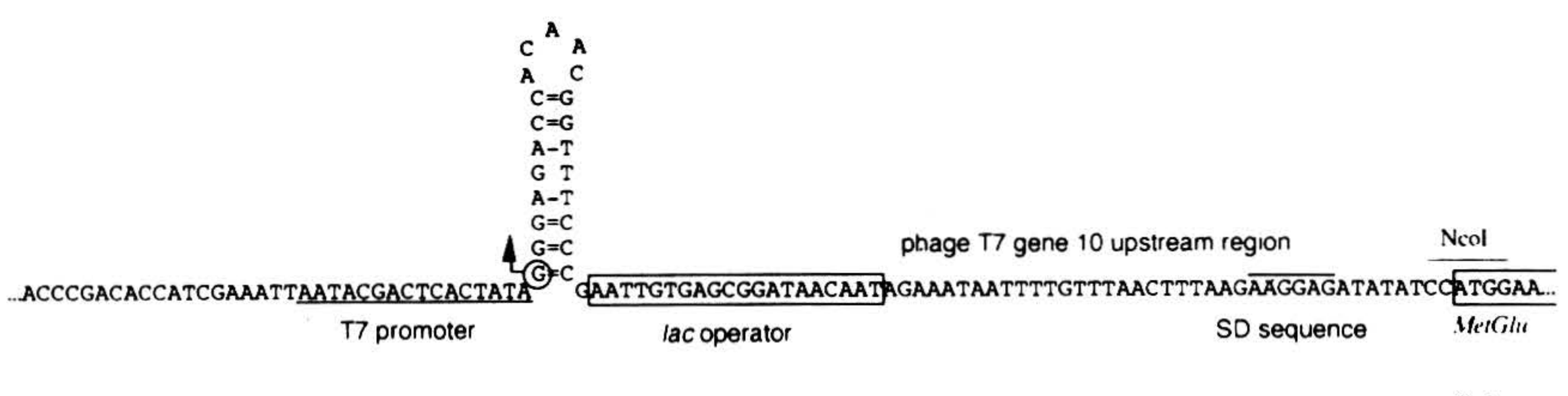
Protocol 5: Refolding of scFv Fragments from Inclusion Bodies

1. Grow BL21 (DE3) cells containing the plasmid of interest at 37°C. In isotope labeling studies, the limiting component would lead to early starvation, and growth is carried out only to an OD_{550} of 0.5. In other experiments without limiting isotopic nutrient, much higher ODs can be used. Induce for about 4 h. This recipe is given for 2 liters of cells.
2. Centrifuge the cells at 3,000 × g for 10 min, and resuspend them in 100 ml of 10 mM TrisHCl, 2 mM $MgCl_2$, pH 8.0.
3. Lyse the cells by two-fold passage through a French Pressure Cell at 1.1 kbar.
4. Treat the lysate with DNAse (approximately 5,000 u) and RNAse (approximately 1,000 u) for 30 min at 37°C.
5. Add 0.5 M EDTA, pH 8.0 (20 ml) and Triton X-100 (1 ml) and store the solution in an ice-bath for aboout 30 min.
6. Centrifuge the sample at 16,000 × g for 10 min and wash the pellet with 0.5 M GdmCl, 0.1 M TrisHCl, 20 mM EDTA, pH 6.8 once and then two more times with 0.1 M TrisHCl, 5 mM EDTA, pH 6.8.
7. Solubilize the pellet in 50 ml of 5.5 M GdmCl, 0.2 M Tris, 2 mM EDTA, 0.14 M DTE, pH 9.5 and stir under argon at room temperature for 2 h.

a.



b.



c.

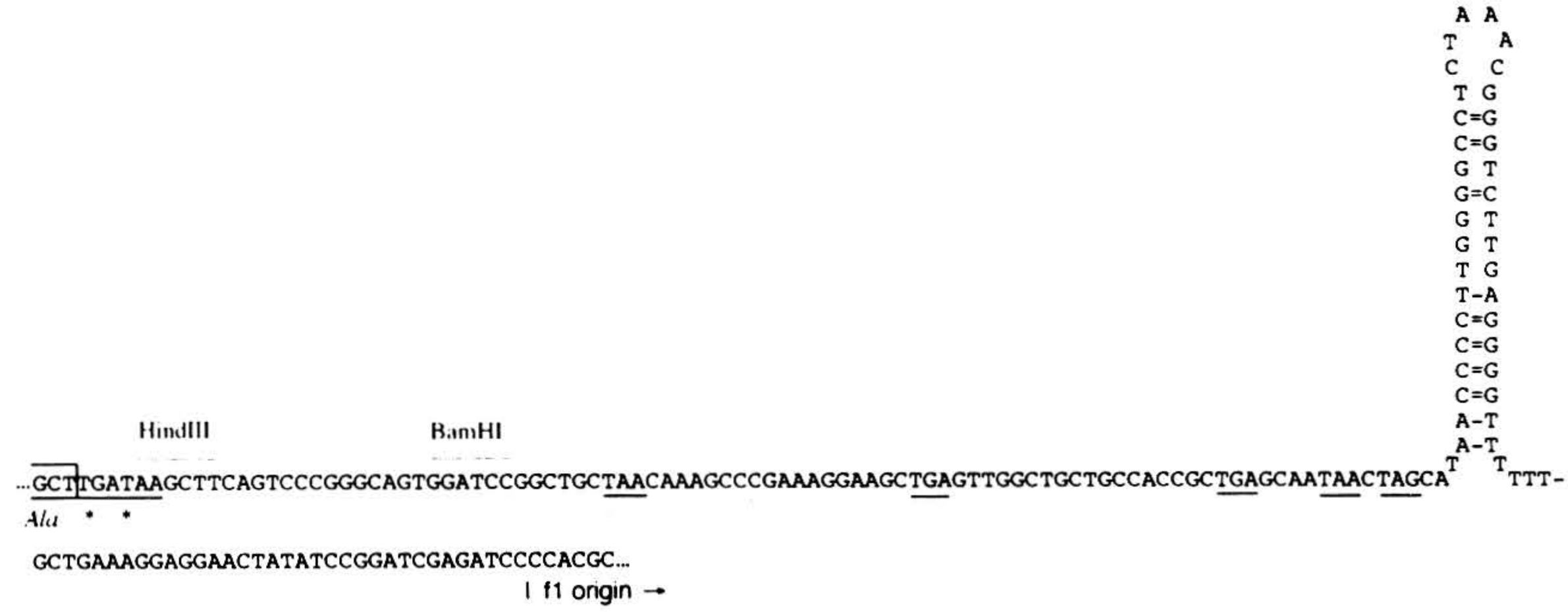


Figure 8–8. Vector for inclusion body formation. (a) Schematic drawing of the pTFT74 vector. *T7p/laco* denotes the *T7* promoter with the *lac* operator, *T7 term* the phage T7 terminator of gene 10, *f1-IG* the intergenic region (origin of replication) of the f1 phage, Amp^R the ampicillin resistance and *ori* the origin of replication of the plasmid. The vector does not contain a *lacI* gene, and no other *E. coli* terminator is present beside the T7 terminator behind the antibody gene. (b) Details of the promoter/operator region. The promoter, operator, and Shine-Dalgarno sequence of phage T7 gene 10 are indicated, the transcriptional start is circled, and a putative hairpin structure is shown.[71] (c) Details of the terminator region. The end of the antibody gene is shown, stop codons are underlined, and restriction sites are overlined. A putative hairpin structure is shown.[71].

8. Centrifuge at 30,000 × g for 30 min to remove insoluble material, and dialyze the supernatant three times against 1 l of the same buffer not containing DTE.
9. Renaturation of the protein now takes place after diluting the solubilisate into 5 l of renaturation buffer (0.8 M arginine, 0.2 M Tris, 2 mM EDTA, 0.2 mM reduced glutathione, 1 mM oxidized glutathione, 0.2 mM benzamidine hydrochloride, 0.2 mM ε-caproic acid, pH 9.5). We have also included hapten at 100-fold the K_D value in renaturing hapten-binding antibodies. **It is crucial to let this reaction continue for more than 50 h at 10°C, as it is very slow.** It is useful to add the denatured protein in several small aliquots to the refolding buffer. At higher temperatures, more precipitation occurs.
10. Concentrate the protein with an Amicon RA2000 cell to a final volume of 300 ml.
11. Dialyze the solution against buffer for affinity chromatography (e.g., 0.2 M sodium borate, 0.4 M NaCl).
12. Purify functional scFv by antigen affinity chromatography, if possible.

Factors Influencing *in vitro* Refolding

The refolding of antibodies is not principally different from that of other disulfide containing proteins.[73–75] The disulfide formation must be kinetically catalyzed and thermodynamically allowed, and at the same time, aggregate formation must be suppressed. The ratio of reduced to oxidized glutathione has to be optimized for the particular antibody to be refolded, covering the range of 1:5 to 5:1 molar ratios. Because of the importance of S–S formation, it is useful to carry out refolding at high pH, in order to speed up the disulfide exchange reactions, since the reactive species is the thiolate anion.

The aggregation of folding intermediates is a severe problem and an important side reaction lowering the yield *in vitro* and *in vivo*. Thus, rather low protein concentrations have to be used. It is useful to add the unfolded protein to the refolding mix in aliquots, since the folded protein has a much higher solubility. Additionally, additives such as arginine are useful, as they appear to increase the solubility of intermediates.[73–75] Too low a final protein concentration, on the other hand, may lead to gigantic volumes, and prevents chain association when refolding heterodimeric Fab fragments.

CONCLUSIONS

The expression of various antibody fragments in *E. coli* has been a useful technology in the study and continued engineering of antibodies. It greatly

lowers the activation barrier to work with recombinant and engineered antibodies and should therefore be autocatalytic in the further development of the field. Many of the initial difficulties of scale-up, sufficient yields, and simple work-up procedures are being solved now and should add to the advantages of the technology. While there are still many fundamental questions of protein folding, structure, and stability unsolved, which have a direct bearing on the expression technology itself, there is justified hope that the rational understanding will catch up with the technological advances, and they will cross-fertilize.

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