MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS

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72. Stocks, S.J., Brooks, D.E. Development of a general ligand for immunoaffinity partitioning in two phase aqueous polymer systems. Anal. Biochem. 173, 86–92 (1988).
73. Male, K.B., Nguyen, A.L., Luong, J.H.T. Isolation of urokinase by affinity ultrafiltration. Biotechnol. Bioeng. 35, 87–93 (1990).
74. Welling, G.W., Geurts, T., van Gorkum, J., Damhof, R.A., Drijfhout, J.W., Bloemhoff, W., Welling-Wester, S. Synthetic antibody fragment as ligand in immunoaffinity chromatography. J. Chromatogr. 512, 337–343 (1990).
75. Welling, G.W., van Gorkum, J., Damhof, R.A., Drijfhout, J.W., Bloemhoff, W., Welling-Wester, S. A ten residue fragment of an antibody (mini-antibody) directed against lysozyme as ligand in immunoaffinity chromatography. J. Chromatogr. 548, 235–242 (1991).

CHAPTER 3

GENETIC MANIPULATION AND EXPRESSION OF ANTIBODIES

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3.1. PROTEIN ENGINEERING

The techniques for genetic manipulation have expanded enormously over the past decade. As a result of the development of these new techniques it is now possible, for example, to change single amino acids of a protein selectively and to generate protein fragments with precision by inserting translational stop codons into the appropriate genes. The use of recombinant DNA methods frequently offers attractive alternatives to time-consuming and often inaccurate protein chemistry. A major step forward in technology has been made by the development of the now widely used polymerase chain reaction (PCR) [1,2]. For example, the use of this technique allows the isolation of a chosen gene from the genome or cDNA of a species in a matter of hours, and the only requirement for this to be feasible is that there be some preexisting knowledge of the nucleotide sequences that flank the gene to be isolated at either, or preferably both, of the 5′ and 3′ ends. Previously, the generation and screening of cDNA libraries for the isolation of a gene for which the sequence was known would have taken weeks or months.

Concomitantly with the development of techniques of genetic manipulation, the methods for expression of the proteins encoded by the engineered genes have been expanded and improved. During the early 1980s, mammalian cell expression systems based on lymphoid cells were developed for the efficient production of immunoglobulin molecules [3–5], and these systems have been widely used for the production of recombinant antibodies as both intact molecules and chimeras [6–13].

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It is now also possible to produce immunoglobulin fragments in both prokaryotic [14,15] and eukaryotic hosts in high yields. The expression hosts that can currently be used to produce antibodies or antibody fragments range from tobacco plants [16] to *Escherichia coli*, and include several different mammalian cell hosts (see section 3.6) and baculovirus-infected insect cells [17].

Thus, genes can be altered with precision and efficiency, and the encoded proteins can be expressed and purified for characterization. In the field of antibody engineering, immunoglobulins can now be tailor-made, and recently developed technology allows the isolation of antibody fragments that bind to almost any antigen. In addition, the affinity of a recombinant antibody for cognate antigen can be increased by using current techniques of molecular biology. The wide range of uses of antibodies in medicine and biology makes antibody engineering a particularly attractive prospect. In this review, recent developments in gene technology will be described. Subsequently, the application of these techniques to the engineering of immunoglobulins will be discussed (for reviews of these topics, see Morrison [18] and Winter and Milstein [19]).

3.1.1. The Polymerase Chain Reaction (PCR)

The PCR [1,2] is a method that is designed to selectively amplify discrete segments of DNA. The method usually consists of three steps and is shown schematically in Figure 3.1: (1) denaturation, (2) annealing of oligonucleotide primers which are designed to flank the DNA sequence to be amplified, and (3) extension of the primers by a processive polymerase (a processive polymerase synthesizes relatively long, continuous strands of single-stranded DNA, and its use is therefore important for the production of full-length products) in the presence of deoxynucleotide triphosphates (dNTPs). It is a widely used technique and in the past few years has been greatly simplified by the development of automated methods of temperature cycling, which are manifested in the large number of cycling blocks that are now commercially available. Temperature cycling is essential for the PCR, since the process comprises (1) a denaturation step at high temperature (usually about 95°C, to ensure that the template DNA, which is frequently double-stranded, is completely denatured into separate strands), followed by (2) annealing of the template DNA to the primer DNA at lower temperature (usually 30–60°C, depending on the length and nature of the oligonucleotide primers; generally the shorter the oligonucleotides, the lower the annealing temperature), and then (3) extension of the annealed products at the temperature for optimal activity of thermostable polymerases (usually about 72°C). Each cycling temperature is maintained for about 0.5 to 3 min. The use of a thermostable polymerase derived from the thermophilic bacterium *Thermophilis aquaticus*, and called Taq polymerase [2], has also greatly facilitated the PCR. Prior to this, the Klenow fragment of *E. coli* polymerase [1] was used, and the thermolability of Klenow polymerase meant that fresh enzyme had to be added at the end of each denaturation step. Furthermore, Klenow polymerase is optimally active at 37°C, and this low temperature (compare with 72°C for Taq polymerase) results, in some cases at least, in poor specificity of the primers

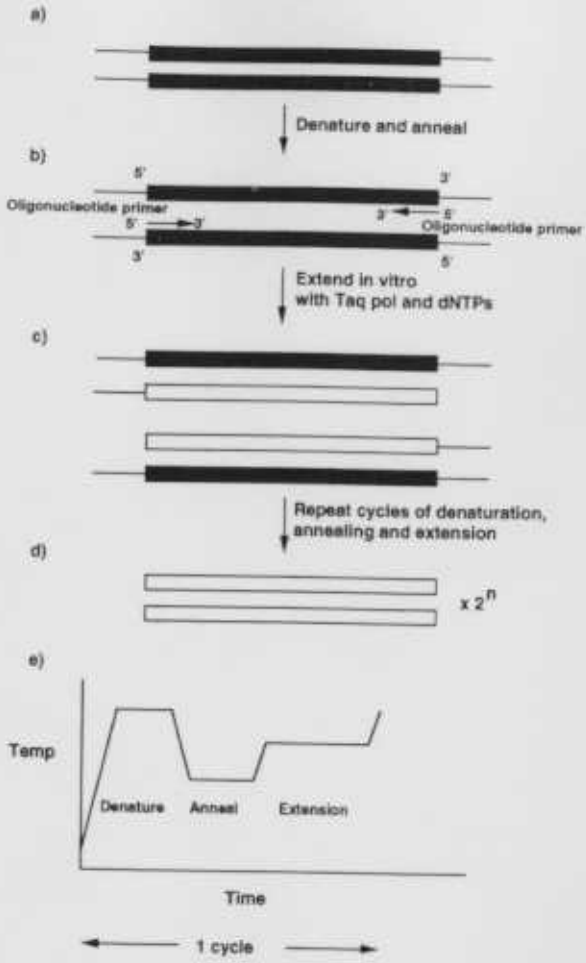

Fig. 3.1. The polymerase chain reaction (PCR) [1,2]. **a.** The region of DNA to be amplified is shown by filled-in boxes, and flanking sequences by single lines. **b, c.** The first round of PCR results in extension of the primers beyond the region to be amplified. **d.** Subsequent rounds result in extension within the region delimited by the primers. During each cycle the amount of DNA delimited by the primers is increased twofold, so that the total number of DNA molecules at the end of the cycling is $2^n \times$ starting number, where n = number of cycles. **e.** Schematic representation of the temperature changes that occur during a single PCR cycle. Denaturation is usually carried out at 94°C, annealing at 25–70°C (depending on the required specificity) and extension at 72°C (the optimal temperature for Taq polymerase activity).

due to annealing and extension at low temperatures. With the development of temperature cycling blocks and thermostable polymerases, the PCR can now be set up by mixing the components and then placing the reaction tube in a cycling block for a programmed period of time. This is therefore much less labor-intensive than the earlier method of carrying out the PCR, which involved transferring the reaction tube between water baths at different temperatures at 0.5- to 3-min intervals and adding fresh enzyme at each cycle; the total number of cycles (usually about 30) meant that this was tedious and also error-prone.

More recently, polymerases with improved properties (for example, with proofreading activity and with higher processivity) have been developed [20] and the use of these polymerases improves the fidelity of the PCR and therefore increases its utility further. The higher processivity allows longer stretches of DNA to be synthesized during the PCR. In addition to being used for the isolation of genes for which knowledge of the 5′ and 3′ sequences is already available, the PCR has the applications discussed below.

Tailoring Genes for Ligation Into Vectors With "Add-On" Oligonucleotides. Genes can be amplified with oligonucleotide primers that have 5′ sequences that are not complementary to the gene to be amplified, and contain sequences encoding restriction sites [21]. These oligonucleotides are often called "add-on" oligonucleotides because they add extra sequences to the ends of the target DNA during the PCR. The sites are usually chosen so that the recognition enzyme makes staggered cuts, usually with 5′ or 3′ overhangs of two to four nucleotides, rather than blunt ended, or "flush" cuts, which have no overhang. This facilitates the ligation (i.e., "sticking" the PCR product into a vector by using an enzyme called T4 DNA ligase to catalyze the reaction) of the genes into plasmid vectors following the PCR, since the PCR products can be cut with restriction enzymes to produce staggered ends that are complementary to the ends of a plasmid vector cut with the same restriction enzyme. The DNA therefore has cohesive ends and can be ligated into expression vectors that have been cut with the same restriction enzymes. This ligation event occurs with much higher efficiency than the ligation of the untreated PCR products into a vector that has been restricted with an enzyme that makes blunt-ended cuts. The relative inefficiency of the latter process is usually due, in part at least, to the fact that PCR products have ends that are not completely flush, that is, have a one- or two-base overhang. These ends can be converted into flush ends with no overhang by using T4 DNA polymerase to fill in, or S1 nuclease to remove, the single-stranded overhangs. It is generally more efficient, however, to incorporate restriction sites that can be cleaved to produce overhangs that are compatible with the vector cloning sites.

By the same approach, other useful sequences can be added to the PCR product by means of "add-on" oligonucleotides, which extend beyond the ends of the template DNA and encode additional sequences. With suitably designed oligonucleotides, therefore, DNA sequences other than those encoding restriction sites can be added at the 5′ or 3′ ends of the amplified DNA. For example, sequences encoding peptide tags that are in translational frame could be added to facilitate

detection or purification of the encoded protein, such as the c-myc peptide epitope [22]. Alternatively, promoter sequences for transcriptional initiation [23] or G + C clamps [24] can be inserted by judicious design of "add-on" oligonucleotides. G + C clamps are used in denaturing gel electrophoresis of lengths of DNA that differ by one or more bases; the addition of a GC-rich DNA segment (called a G + C clamp) results in enhancement of mobility differences.

Isolation of Genes for Which Sequences at Only the 3′ End Are Known. A number of PCR techniques now are available that allow the isolation of genes for which sequence information is available only for the 3′ end of the gene to be isolated [25,26]. Examples of such genes are the variable domain genes of immunoglobulins and T-cell receptors (TCRs). In both these cases, the sequences of the constant domains are well known for a number of species. For immunoglobulin genes it is possible to design degenerate and family-specific primers for their isolation [27–36], due to a reasonable amount of conservation of sequence in framework 1 and/or the secretion leader sequence (see Chapter 1). In contrast, TCR genes are considerably more variable in sequence at their 5′ ends [37], and although a number of reports of the use of family-specific primers have now been documented [38–42], the isolation of TCR variable domain genes can be carried out by inverse PCR [25,43] or anchor PCR [26,44].

Inverse PCR is a technique that has been developed to isolate genes for which sequence information is available only for either the 5′ or 3′ end of the coding region, or the sequences that flank the 5′ or 3′ sides of the coding region. Inverse PCR (Fig. 3.2) involves the use of three (or at least two) different primers, which in the case of TCR gene isolation anneal to the constant region of the gene to be isolated. cDNA synthesis is primed with oligonucleotide I, and second-strand cDNA synthesis is then carried out with RNase H and *E. coli* DNA polymerase I [25]. The resulting double-stranded cDNA is circularized and used as a template in the PCR with oligonucleotides II and III (Fig. 3.2). This will result in the isolation of a PCR product that contains the 5′ region of the variable domain gene, plus leader sequence and 5′ untranslated region. Oligonucleotides II and III can be designed to encode restriction sites within their sequence, to allow the restriction and ligation of the PCR products into appropriate vectors following amplification. The ligation of the PCR products into vectors facilitates DNA sequencing to determine the nucleotide sequence of the gene(s) that have been isolated. Alternatively, the PCR products can be sequenced by direct PCR sequencing [45,46], which avoids the need to ligate the amplified DNA into vectors prior to sequence analysis. To use direct PCR sequencing, however, the PCR products must be homogeneous so as to obtain unambiguous sequence information, and for this reason it cannot be used in situations where members of gene families are being isolated. An example of this is the isolation of repertoires of immunoglobulin or TCR variable domain genes, where the sequence differences from one variable domain gene to another mean that the genes have to be ligated into vectors and individual recombinant clones obtained prior to sequence analysis.

Anchor PCR is another technique that results in the isolation of genes for which

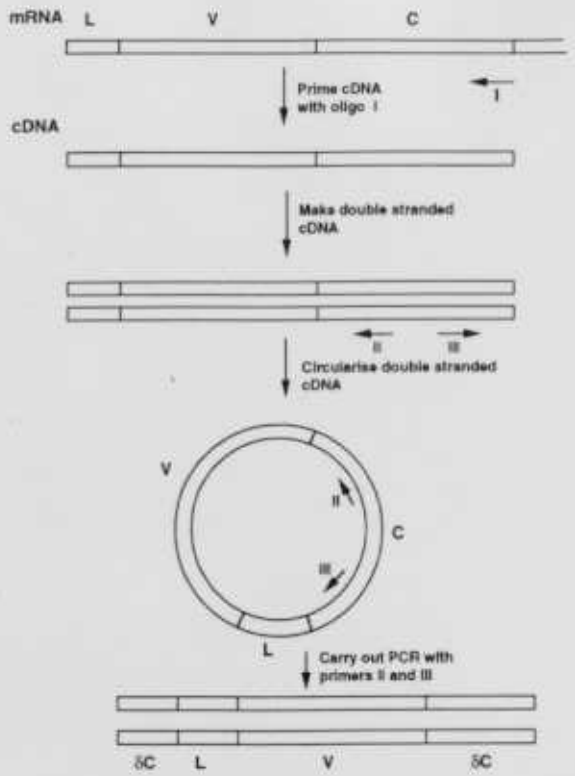

Fig. 3.2. Inverse PCR [25,43]. For the isolation of immunoglobulin or T-cell receptor genes, cDNA synthesis is usually primed with an oligonucleotide (I) that is specific for the constant (C) region. Double-stranded cDNA is synthesized in a reaction containing RNase H and *E. coli* DNA polymerase I and is then circularized with ligase. The resulting circular DNA is used as a template in the PCR with primers II and III to product the product shown. Note that restriction sites can be incorporated into the primers to facilitate cloning of the amplified genes. L, leader sequence gene; V, variable domain sequence gene; δC, part of constant region gene between priming site of oligonucleotide II and variable domain gene, and priming sites of oligonucleotides I and III.

sequence information is only available for either the 5′ or 3′ ends of the coding sequences or the sequences that flank the coding region. For anchor PCR (Fig. 3.3), cDNA synthesis is primed with a constant region primer (analogous to that used for inverse PCR). The single-stranded cDNA is subsequently tailed with deoxyguanosine triphosphate and terminal deoxynucleotidyl transferase (TdT). This tailing results in the addition of a run (of variable length) of G residues to the 3′ end of the first-strand cDNA. The tailed cDNA is then used as a template in the PCR with the cDNA synthesis primer and the anchor primer (see fig. 3.3). The anchor primer comprises a run of approximately 14 C's with an "add-on" sequence encoding a restriction site [26]. The cDNA synthesis primer can also have an internal restriction

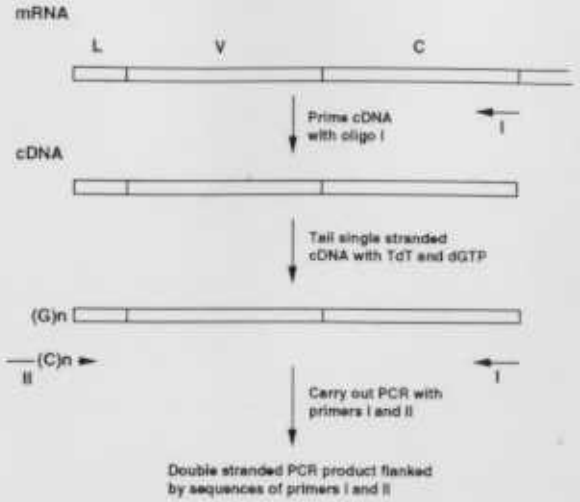

Fig. 3.3. Anchor PCR [26,44]. For the isolation of immunoglobulin or T-cell receptor genes, cDNA synthesis is primed with oligonucleotide I, and the single-stranded cDNA is tailed with TdT (terminal deoxynucleotidyl transferase) and dGTP. The resulting tailed cDNA is then used in the PCR with primers I and II, which usually have internal restriction sites to facilitate cloning of the PCR products. L, leader sequence gene; V, variable domain sequence gene; C, constant region gene.

site, so that the resulting PCR products can be restricted and efficiently ligated into vectors for sequencing.

Splicing by Overlap Extension. The PCR allows genes to be spliced together [47], using designed primers as shown in Figure 3.4. A situation in which it is desirable to splice genes together is in the generation of chimeric antibodies, in which one constant region gene is spliced to a particular variable domain gene. Splicing by overlap extension has the advantage that the splice site can be generated accurately at the nucleotide level, by design of suitable splicing oligonucleotides. The primers are designed to overlap the 3′ end of one gene and the 5′ end of the gene that is to be spliced downstream, as indicated in Figure 4. This approach was initially used to splice together genes encoding different domains of an HLA molecule [48]. More recently it has been extended to the generation of repertoires of randomly combined heavy and light chain immunoglobulin variable domain genes [34, 49] (Fig. 4). This is described in more detail in section 3.5, but it results in large numbers of different heavy chain variable domain genes being randomly combined with a similarly large number of light chain variable domain genes and is a critical step in the production of *in vitro* repertoires of genes encoding Fv fragments, from which fragments with the desired binding specificity can be selected.

Random and Site-Directed Mutagenesis of Genes. It is sometimes desirable to be able to change a particular amino acid in a protein by making a change at the corresponding codon or, alternatively, to insert mutations in a random way into a

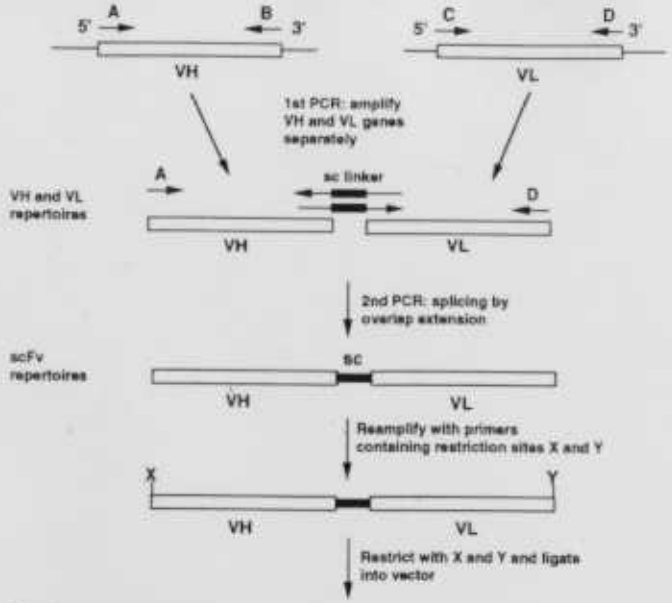

Fig. 3.4. Schematic representation of splicing by overlap extension (SOE) [47] for the generation of scFv genes from random combinations of repertoires of VH and VL domain genes [47,49]. The VH genes are isolated from antibody-producing cells by the PCR and primers A (5') and B (3'). The VL genes are isolated from antibody-producing cells in a separate PCR with primers C (5') and D (3'). The VH and VL gene repertoires are randomly combined by splicing together in a PCR with primers A and D, together with single chain (sc) linker primers. These sc linker primers encode a synthetic peptide linker [49,104] and also have the same or complementary regions as primers B (3' of VH genes) and C (5' of VL genes). Open boxes represent the VH and VL domain genes, and stippled boxes represent the sc linker sequence. Following the SOE reaction the assembled genes can be restricted with enzymes X and Y and ligated into an appropriately restricted vector.

gene. Site-directed mutagenesis, that is, alteration of a chosen amino acid, may be useful if this amino acid is believed to be critical for the function of a protein. It is clearly important that the function of the protein and the corresponding mutant can be assayed in a binding or enzymatic assay. Random mutagenesis is useful in cases where it is convenient to insert mutations at random sites within genes and then to screen the expressed proteins for altered properties; in the case of an antibody molecule, random mutagenesis could be used to generate mutants that have increased affinity for binding to antigen, for example. The PCR can be used to insert both random and specific mutations into genes. A requirement for this to be possible is that a convenient and preferably unique restriction site flank the region to be mutated; if no such site exists, it can generally be inserted by oligonucleotide-directed mutagenesis (Fig. 3.5) [50] without loss of translational sense. Oligonucleotide primers can then be designed to overlap the restriction site and to change one or more codons at the desired position in the gene. Mutations can be to a

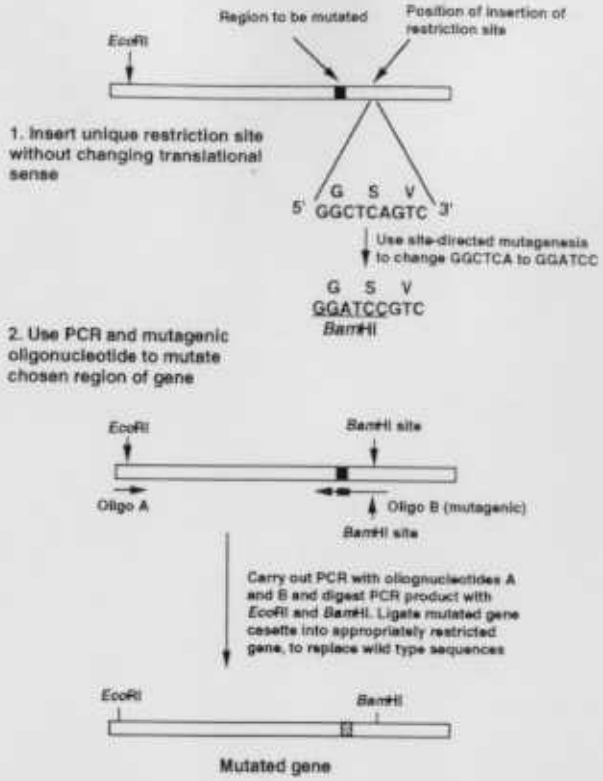

Fig. 3.5. Strategy for targeted mutagenesis of genes, using the PCR. First, a unique restriction site is inserted by oligonucleotide-directed mutagenesis in the vicinity of the region of the gene that is to be mutated. (This step is not necessary if such a site already exists.) The site is inserted without loss of translational sense (the corresponding amino acid sequences in one-letter code are shown above the nucleotide sequences). The wild-type gene containing the restriction site is then used as a template for the PCR with oligonucleotides A and B. Oligonucleotide A is complementary to the 5' end of the gene and contains an *Eco*RI restriction site (which is present in the wild-type gene). Oligonucleotide B contains a *Bam*HI restriction site and overlaps the region of the gene to be mutated. For this primer (shown by an arrow) the solid box represents regions that are mutagenic—that is, not complementary to the gene that is to be mutated, but encoding the desired mutations. The gene is represented by an open box, the region to be mutated by a filled-in box, and the mutated region in the context of the wild-type gene by a hatched box.

predetermined codon, or mixed oligonucleotides can be made that will randomize the sequence of the gene at the desired codon(s). One of the problems of this approach is that since some amino acids are encoded by as many as six different codons (e.g., serine), whereas others are encoded by only one (methionine), it is difficult to generate a random oligonucleotide that encodes the 20 different amino acids at the same frequency. Thus, the codons of the random primer will be biased

towards encoding amino acids that have the largest number of possible codons in the genetic code, for example, arginine and serine. Theoretically, it is possible to design the primers in such a way that there is an equal likelihood that each amino acid is encoded at each codon position. Whether it is necessary to design such oligonucleotides depends on the number or random mutants that can be screened, since the higher the number of mutants screened, the more likely it is that every possible amino acid at each mutated position will be inserted. A second problem is that if the random section of the primer is designed so that A, T, C, and G are incorporated in equimolar amounts, a significant number of the codons in the random primer will be stop codons. Thus, to minimize this, it is advisable to design the primers to insert only A and C at the third position of each codon in the sense strand, since two or three of the stop codons end in A (C is also not inserted at this position, to minimize the occurrence of biases towards particular amino acids in the random primer).

Error Prone PCR for Targeted Random Mutagenesis. Although for most purposes the PCR is carried out under conditions designed to minimize the error frequency, conditions have been developed to reduce the fidelity of Taq polymerase for the purpose of random mutagenesis [51]. These conditions involve the addition of low concentrations of manganese chloride and lowering the dATP concentration relative to the other three nucleotide triphosphates. Such conditions result in error frequencies of up to 2% at each base position [51,52]; that is, for a gene of length 400 bases, eight mutations on average can be inserted. The mutations are more frequently transversions/transitions than deletions/insertions [51], and this is of particular significance if the goal is to insert random point mutations in the region between the PCR primers. Deletions and insertions are undesirable in such random mutagenesis experiments, since they produce frameshifts that result in gross structural alterations of the encoded protein.

3.2. GENETICALLY ENGINEERED ANTIBODIES

3.2.1. Structure of the Immunoglobulin Molecule

The IgG molecule comprises two heavy and two light chains, which are linked to each other by an –S–S– bridge located at the C termini of the CH1 domain and the Cκ/Cλ domain [53] (Fig. 3.6; see also Chapter 1). The two heavy chains are also covalently linked to each other by one or more –S–S– bridges that are located in the hinge region. Crystallographic analyses of a number of antibodies [54–60] indicate that the immunoglobulin molecule is made up of strings of discrete domains, and each domain comprises two β sheets that pack against each other and are pinned together by an intramolecular –S–S– bridge. The individual β strands are connected to each other by relatively exposed loops. The peptide loops at the tips of the heavy and light chain variable domains (designated VH and VL domains respectively; shown schematically in Fig. 3.7) are hypervariable in sequence and are

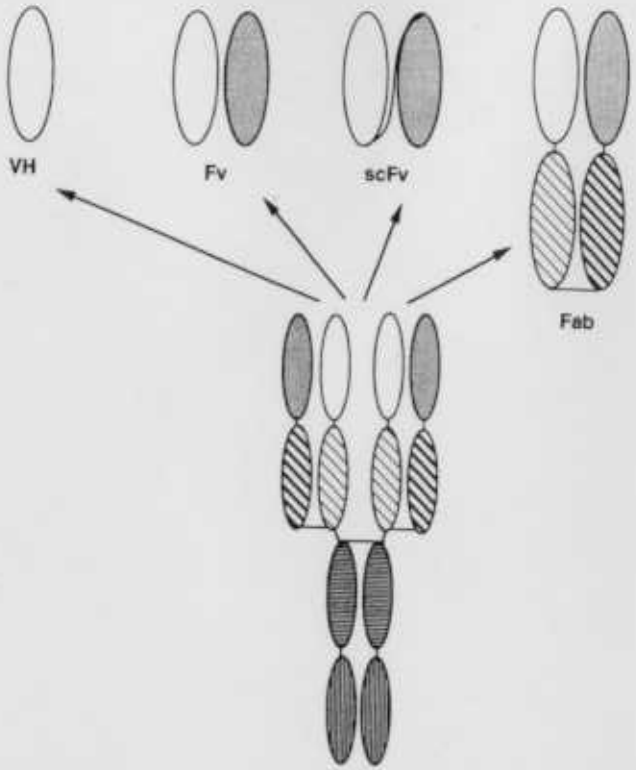

Fig. 3.6. Schematic representation of the immunoglobulin molecule. The immunoglobulin IgG molecule consists of strings of discrete domains, and comprises two heavy chains and two light chains [53]. The heavy and light chains are linked by an intermolecular disulfide bridge (indicated by a horizontal line between the hatched CH1 and CL domains). The heavy chains are also linked to each other by one or more intermolecular disulfide bridges. These –S–S– bridges are in the hinge region, which links the CH1 domain to the CH2 domain (indicated by horizontal hatching). The CH3 domain is indicated by vertical hatching. Immunoglobulin-derived fragments that can be expressed and secreted from recombinant *E. coli* cells are shown at the top of the figure. VH, heavy chain variable domain; Fv, VH and light chain variable (VL) domains; scFv, VH and VL domains linked by a synthetic peptide linker [104,105]; Fab, Fd (VH linked to CH1 domain) and paired light chain.

involved in contacting antigen during antibody–antigen interaction. In other words, it is residues within and flanking these loops that determine the specificity and affinity of a particular antibody for interaction with cognate antigen. Despite the variation in the sequences of the variable domains, and in particular their hypervariable loops, the overall main chain conformation is relatively well conserved [61,62]. This conservation is of relevance in CDR grafting, in which hypervariable loops are grafted at the genetic level from one antibody to another. The purpose of such CDR grafting experiments is to confer the binding specificity of an antibody of

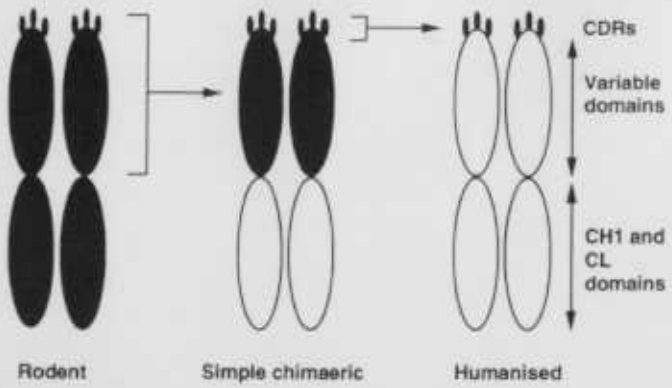

Fig. 3.7. Schematic representation of simple chimeric and CDR-grafted ("humanized") antibodies [9,11–13,70,71]. Rodent-derived regions are shown by filled ellipsoids, and human-derived regions by open ellipsoids. Only the Fab fragment of the immunoglobulin molecule is shown. For the simple chimeric molecule, the variable domains and CDR loops are derived from rodents, and the remainder of the antibody molecule is of human origin. In contrast, for humanized antibodies, only the CDR loops are derived from rodents.

rodent origin onto a human antibody framework, to produce "humanized" antibodies that are less immunogenic in human therapy. This is discussed in more detail in section 3.2.3. below.

The immunoglobulin can be fragmented into Fab and Fc portions, by virtue of the proteolytic sensitivity of the hinge region. For example, papain can be used to cleave the molecule into Fc and monovalent Fab fragments. It is more difficult, however, to produce Fv fragments (comprising VH and VL domains) by proteolysis, and although a method has recently been reported for this for a particular immunoglobulin molecule [63], it is generally more reliable and easier to generate these fragments by genetic engineering [15,64].

3.2.2. Gene Structure of Immunoglobulins

The domain structure of immunoglobulins is mirrored in the genetic organization, since each domain is encoded within a separate exon [65]. This allows domain-swapping experiments to be carried out with relative ease [6,9,11–13]. Domain-swapping experiments can be used to link antibody variable domains to constant regions of the isotype of choice, so that the effector functions of the antibody can be selected at will. In addition, it may be useful to link rodent variable domains to human constant regions to produce simple chimeric antibodies (discussed in section 3.2.3). For example, prior to the development of methods for the PCR and site-directed mutagenesis, restriction sites located within the introns were used to splice immunoglobulin genes together to produce simple chimeric antibodies. The relative advantage of the immunoglobulin gene organization, compared with many other genes that encode multidomain proteins, has now been reduced by the development

of methods that allow genes to be spliced together with precision and rapidity in the absence of conveniently located restriction sites.

3.2.3. Production of Chimeric Immunoglobulins and CDR Grafting

One of the problems associated with the use of rodent antibodies in human therapy is their immunogenicity, and until recently, it was difficult to produce human-derived monoclonal antibodies for both ethical and technical reasons [66–68]. The use of rodent antibodies in therapy results in an anti-immunoglobulin immune response (human anti-mouse antibody response, or HAMA response) [69,70]. This response is directed mainly towards the rodent constant regions and is highly undesirable, since it causes toxic reactions and rapid elimination/neutralization of the antibody. In the 1980s therefore, considerable effort was put into genetically manipulating rodent antibodies to make them less immunogenic for use in therapy [9,11–13,71,72]. The production of the genetically manipulated antibodies was made possible by the development of mammalian-based expression systems for efficient secretion of recombinant immunoglobulins [3–5]. The PCR-based approaches described above can all be applied to the manipulation of immunoglobulins. However, these techniques have been available only for the past few years. Prior to this, other methods of genetic manipulation were used to generate chimeric and humanized antibodies.

Simple Chimeric Immunoglobulins. In the early mid-1980s, the availability of the genes encoding antibody molecules allowed the formation of human–mouse chimeric molecules by the replacement of the rodent constant domains with human constant domains by relatively simple genetic manipulations. These spliced genes were then expressed in mammalian cells as secreted, chimeric proteins [9,11–13]. This approach links the specificity (determined by the variable domains) of the rodent antibody to human constant domains, and has been carried out for a variety of antibodies of potential therapeutic value. Such antibodies include those that recognize carcinoma antigen 17-1A [11] and L6.6 [9]. The binding specificity, as would be predicted from the discrete domain structure of the immunoglobulin molecule, is not affected by the chimerization process. Moreover, the replacement of the rodent constant domains with those of human origin allow the isotype and therefore the effector functions of the therapeutic antibody to be optimized [73] (see Chapter 1).

The use of human constant regions may not only generate less immunogenic antibodies for therapy, but the chimeras may have increased *in vivo* stability [74]. This is probably for two reasons: first, during prolonged therapy the HAMA response will increase the clearance rate of the therapeutic antibody. Second, the Fc region appears to be involved in determining the *in vivo* stability of the immunoglobulin molecule [75,76], and it is conceivable that human Fc regions confer higher stability in humans than murine Fc regions, owing to the presence of as yet unidentified species-specific residues in this region of the molecule that are involved in the control of immunoglobulin catabolism.

CDR-Grafted or Humanized Antibodies. For the chimeric antibodies described above, the rodent variable domains may also be seen as foreign, although they are less immunogenic than the rodent constant regions [74]. A refinement of the production of chimeric antibodies is the generation of humanized, or CDR-grafted, antibodies [71,72,77–83] (Fig. 3.7). The hypervariable, or CDR loops, of the immunoglobulin variable domains not only are the most variable regions of sequence from one antibody to another but, together with the flanking framework regions, they also determine the specificity and affinity of the antibody for binding to antigen [56,58–60].

To test the feasibility of transplanting the CDR loops, and concomitantly the binding specificity of a rodent antibody to a human framework, Jones and colleagues [71] initially "CDR-grafted" an antihapten antibody and demonstrated that the binding specificity could be transferred onto a human antibody with retention of affinity. This work was subsequently extended to the humanization of Campath-1 [72] and the antilysozyme D1.3 antibody [77]. Campath-1 has applications in therapy, and the humanized version has been used in the treatment of non-Hodgkin's lymphoma [84], systemic vasculitis [85], and rheumatoid arthritis [86]. More recently a number of other antibodies of possible therapeutic importance have been humanized—for example, anti-Tac [78], anti-HER2 [79], anti-respiratory syncytial virus [80], anti-herpes simplex [81], anti-HIV [82], and anti-EGF receptor [83] antibodies. All these humanized antibodies await the results of their use in controlled clinical trials to test their efficacy and immunogenicity.

In carrying out CDR grafting, it appears to be important for the retention of binding activity that the contacts of the CDRs with the donor framework residues be maintained in the acceptor framework. Thus, judicious choice of the human acceptor framework is essential if the binding affinity of the humanized antibody is to match that of the parent rodent antibody. The importance of maintaining the correct framework residues is exemplified in the work of Riechmann and colleagues [72], who showed that changing a single amino acid in the framework region of a humanized version of the Campath-1 changed the antibody from being almost inactive in binding antigen to having activity similar to that of the parent rodent Campath-1. Consistent with this, the importance of framework residues in orienting the CDR loops and/or contacting the antigen is demonstrated in the structures of antibody–antigen complexes which have now been solved by x-ray crystallography [56–60]. In addition, Tramontano and colleagues [87] have recently shown that framework residue 71 of the VH domain plays a major role in determining the conformation and orientation of the second CDR loop of this domain. The importance of this residue in maintaining the "correct" conformation of the CDRs in humanized antibodies, together with several other framework residues, has been analyzed extensively using the antilysozyme D1.3 system [88]. This study describes the effect of several framework changes on the binding affinity of the humanized antibody. In this respect it is interesting that in the antibody that was humanized by Riechmann and colleagues [72], the VH domain residue 71 is arginine in the parent antibody and valine in the human acceptor framework. Despite this, the humanized version maintains the binding affinity of the rat antibody; as suggested by Tramontano and colleagues [87], the smaller size of valine relative to arginine may allow the transplanted CDR2 loop to adopt the "correct" conformation in the recombinant antibody.

At the technical level, CDR grafting can now be carried out in several different ways: (1) Synthetic DNA that encodes the CDR loops can be grafted from the donor to the acceptor framework by using designed synthetic oligonucleotides and site-directed mutagenesis [50]. This involves the design of oligonucleotides that are complementary to the 5′ and 3′ framework regions that flank each CDR of the human acceptor antibody. The oligonucleotides have sequences that encode the rodent CDRs, and these sequences are flanked by the human framework sequences. (2) The PCR can be used, provided that restriction sites (that are preferably unique) flank the CDRs in the genes encoding the donor framework regions. Such sites can be inserted by site-directed mutagenesis (Fig. 3.5).

3.3. GENERATING MONOCLONAL ANTIBODIES *IN VITRO*

Recent developments in recombinant DNA technology, including the PCR, can be applied to the manipulation of antibody genes. The PCR can be used to isolate diverse repertoires of immunoglobulin variable domain genes from antibody-producing cells such as peripheral blood lymphocytes (PBLs) and splenocytes. This has led to the development of a new area of recombinant antibody work that involves the generation of vast numbers of different antibody variable domain genes in a relatively small number of PCRs. The genes can then be used as templates for the expression of recombinant antibody fragments in *E. coli*. The development of ways of efficiently expressing antibody fragments in this bacterial host allows the isolation of recombinant antibody fragments that have binding activities towards a chosen antigen. Thus, libraries of the variable domains can be expressed as either single VH domains (comprising a single heavy chain variable domain; Fig. 3.6), Fv fragments and Fab fragments using *E. coli* as a host, and the desired binding activity screened for [31,64]. More recently ways have been developed for the expression of recombinant antibodies on the surface of bacteriophage, which allows the selection of bacteriophage that bear immunoglobulin fragments that have antigen-binding activities using antigen coated surfaces [49,89,90] (Fig. 3.8). In this context, the term *screening* is used to describe the analysis of large numbers of bacterial colonies or plaques for the expression of the desired antibody fragments, using either ELISAs [64] or plaque screening [31]. Selection is used to describe the surface expression of antibodies on bacteriophage, and this has the advantage that the bacteriophage that bear fragments with the desired binding specificity can be isolated by selection ("panning") on antigen-coated surfaces (Fig. 3.8). The selected bacteriophage can then be used to reinfect *E. coli* to produce clones of cells that contain immunoglobulin genes with the desired antigen-binding activity (usually several rounds of selection are required, but this depends on the nature of the starting material; for example, if libraries of antibody genes generated from spleen cells and containing many different genes are expressed on the surface of bacteriophage, the

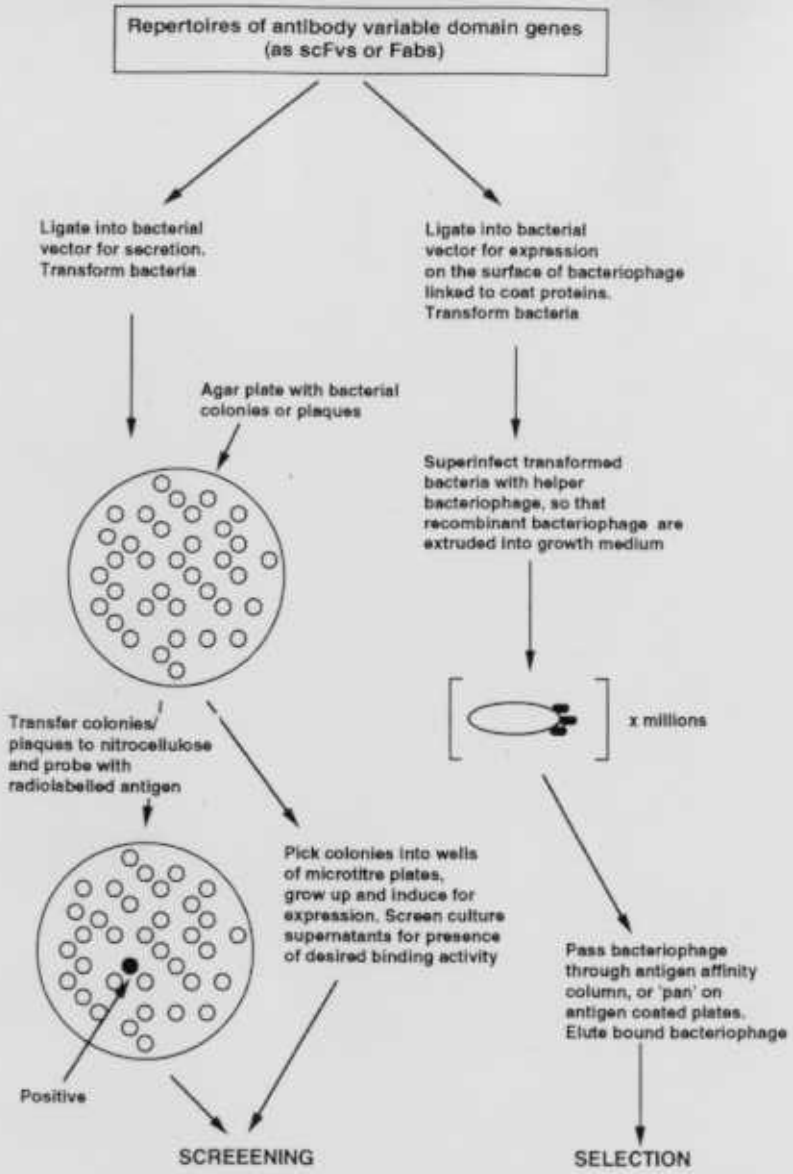

Fig. 3.8. Schematic representation of the screening and selection of immunoglobulin gene repertoires. The VH and VL gene repertoires are generated by the PCR and assembled as either scFv's or Fab's as described in the text. The genes can then be ligated into vectors for secretion or for surface expression on bacteriophage. The use of the former allows the antibody fragments that have the desired binding activity to be identified by screening (by either transferring the colonies to nitrocellulose and probing with radiolabeled antigen or by screening bacterial culture supernatants by ELISA). The use of bacteriophage surface expression allows the antibodies that have binding activity to be selected. The open ellipsoid represents a bacteriophage and the small filled ellipsoids represent antibody fragments expressed at the tip of the bacteriophage as fusion proteins with the gene III coat protein.

number of rounds of selection is usually three to four). Thus, selection clearly has advantages over screening, in that it is relatively easy to pan millions of bacteriophage on antigen-coated surfaces and, by reinfection of *E. coli* with the panned bacteriophage, to enrich for the desired clones. In contrast, screening of millions of clones is extremely tedious and would take weeks. It is now possible to isolate and express immunoglobulin gene repertoires in a matter of days, and this technology is an attractive alternative to the more time-consuming hybridoma technology [91]. This new approach and hybridoma technology each have their relative merits. Although the *in vitro* route is much more rapid, for the study of the development of an immune response *in vivo* the hybridoma route is still preferable, since the pairing between immunoglobulin heavy and light chains, as they exist within a cell *in vivo*, is maintained.

First, the developments that have led to this new technology will be described; they are (1) the use of the PCR to isolate repertoires of immunoglobulin genes, and (2) the generation of suitable prokaryotic expression systems for the production of the immunoglobulin fragments, and the screening or selection of clones that express fragments with the desired binding activity. Once fragments with the desired binding activity have been isolated, it is relatively straightforward to use recombinant DNA methods to link them to the constant regions of immunoglobulin molecules (for which the genes of many species and isotypes are now available) or to other proteins such as toxins (discussed in more detail in section 3.5). This combines the binding functions of the fragments with the effector functions of the desired properties. Second, the applications of these recombinant antibodies will be briefly discussed.

3.3.1. Use of the PCR

For the isolation of immunoglobulin variable domain genes (designated VH and VL for heavy and light chain variable respectively), several different types of PCR primers can be used [27–36]. As indicated in section 3.1.1, for the PCR it is necessary to have prior knowledge of the nucleotide sequences at the 5′ or 3′ ends of the genes (or flanking regions) that are to be isolated. The sequence knowledge does not have to be complete, as the PCR can be used with partially degenerate primers. The use of partially degenerate primers allows members of gene families to be isolated in a single PCR. These members may differ in one or more bases in the region to which the primer anneals. Clearly, the higher the primer degeneracy, however, the greater the degree of nonspecific binding and priming. Since immunoglobulin variable domain genes share considerable homology at their 5′ and 3′ ends, yet are not perfectly matched in sequence, they can be isolated en masse by the PCR and partially degenerate primers. This overcomes the problem of choosing a unique primer, and it is straightforward in programming oligonucleotide synthesizers to insert two or more bases at appropriate positions during syntheses.

There are now a number of different data bases that document the known sequences for the immunoglobulin genes of a variety of species. For example, the Kabat data base [37] contains sequence information for nine species including

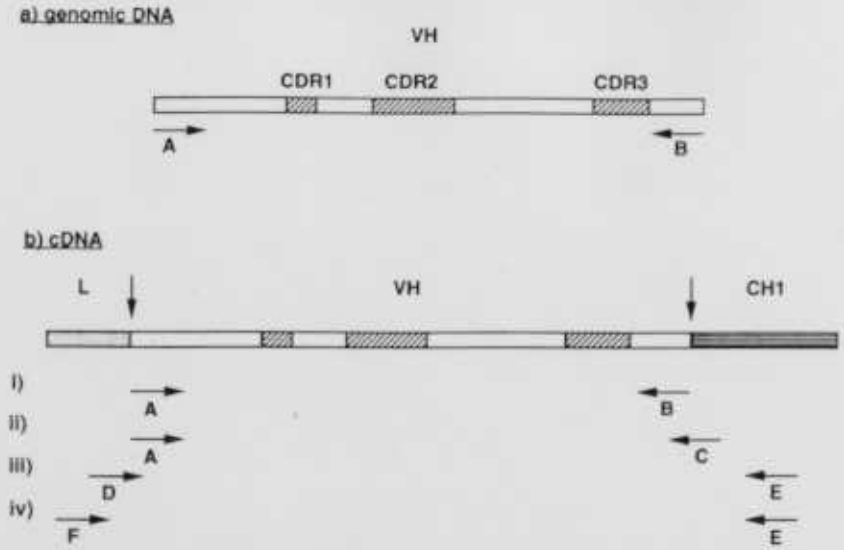

Fig. 3.9. Strategies for PCR amplification of immunoglobulin VH domain genes: **a.** From genomic DNA isolated from antibody-producing cells, primers A (5′) and B (3′) can be used to isolate the rearranged VH genes [27,34,64]. These primers anneal to the 5′ and 3′ ends of the coding sequences of the VH domain genes, and both productively and nonproductively rearranged genes (productively rearranged genes are those that give rise to complete open reading frames for translation) will be isolated. **b.** From cDNA isolated from antibody-producing cells, (i) primers A (5′) and B (3′) can be used, as in **a;** (ii) primers A (5′) and C (3′) can be used on cDNA only, since primer C overlaps both the J region and CH1 domain [33]; (iii) primers D (5′) and E (3′) can be used on cDNA only, since D overlaps the leader sequence and 5′ end of the VH domain genes, and E anneals to the CH1 domain [31,35,36]; and (iv) primers F (5′) and E (3′) can be used on cDNA only, since F anneals to the leader sequence (L) and E anneals to the CH1 domain [29,30,32]. All primers can be partially degenerate or family-specific. Hatched boxes represent CDR1, 2, and 3. The leader exon is represented by a stippled box, and the 5′ end of the CH1 domain by horizontal hatching. Vertical arrows indicate the location of intron–exon boundaries in the corresponding genomic DNA, which encodes rearranged immunoglobulin genes. Similar strategies can be used for the isolation of VL domain genes.

human, rat, mouse, and rabbit. This data base has been used to design oligonucleotide primers that anneal to the 5′ and 3′ ends of the variable domain genes of the mouse [27]. In addition, the primers were designed to have internal restriction sites, so that the resulting PCR products could be cloned directly into expression plasmids that have compatible restriction sites following amplification [27,64]. As an alternative approach, PCR primers have been designed for mouse and man that are specific for different VH and VL domain gene families at the 5′ ends and/or anneal to the constant domain genes [33] (Fig. 3.9). In addition, primers that are complementary to the leader sequences, or part of the leader sequence and the 5′ end of the VH domain gene, can be used [29–32,35,36].

Thus, there are a variety of primers that can be used in the PCR to isolate VH and VL domain genes as diverse repertoires from antibody-producing cells such as splenocytes or PBLs, or more simply, from homogenous populations of cells such as hybridomas. These genes can be cloned for expression as either Fv or Fab fragments, and the desired specificity isolated by screening or selection (described below).

3.3.2. Use of the PCR for the Isolation of the Immunoglobulin Genes

A variety of sources of immunoglobulin-producing cells can be used for the isolation of VH and VL domain genes. In the simplest case, isolation of the genes from the genomic DNA of preexisting hybridomas involves first washing the cells in phosphate-buffered saline [92]. Around 10^6 cells are the optimal number, but substantially smaller numbers can be used; in theory, one cell could be sufficient, but use of exceptionally small numbers of cells frequently results in both inefficient PCR and contamination problems. Following washing, the cells are resuspended in sterile water and boiled. Debris is pelleted by centrifugation, and aliquots of the supernatant containing the DNA are used in a standard PCR with the appropriate primers [92]. This method can clearly be used only with primers that anneal to positions within the VH and VL domain genes, that is, within one exon. In addition, the use of these primers on genomic DNA can result in the isolation of nonproductively rearranged genes [93]. In other words, during immunoglobulin gene rearrangement, due to the random nature of the process, some V, (D), and J elements are combined in a way that results in an interrupted (by a stop codon) reading frame. These genes will be present in the genomic DNA, but they are not efficiently transcribed into mRNA and would therefore be poorly represented in a cDNA library. Clearly the presence of a high proportion of nonproductively rearranged genes is a disadvantage for the generation of expression libraries of immunoglobulin genes derived from heterogenous populations of antibody-producing cells, and therefore for this purpose the use of cDNA may be preferable. As pointed out by Gherardi and Milstein [94], however, the use of cDNA (mRNA) biases the libraries toward isolation of the VH and VL genes from plasma cells at the expense of memory cells, and this may also be a disadvantage in some cases. The choice between using cDNA or genomic DNA depends on the goals of the individual experiment. For example, if the animal or human from which the genes are being isolated has been immunized (in the case of humans, by immunizations such as tetanus or HIV infection), it is preferable to use mRNA if the goal is to isolate antibodies that recognize the immunogen. This is because during active immune response, expanding B cells will be expressing high levels of antigen-specific mRNA.

To generate cDNA for use in the PCR, several approaches can be used. Lymphocytes are first isolated on Ficoll-Hypaque from either PBLs or splenocytes and then total RNA purified. The cDNA synthesis can be primed with either poly-dT, immunoglobulin constant region specific primers, or random hexanucleotide primers [32]. Random hexanucleotides are available commercially and are hexamers that anneal randomly throughout the genome. Priming with constant region specific

primers may be preferable in many cases, as it will result in the production of only immunoglobulin-derived cDNA for use in the PCR. If cDNA derived from all the different isotypes is desired, however, it may be preferable to prime cDNA with poly-dT or random hexanucleotides.

3.3.3. Expression of the Antibody Genes Using *E. coli* as a Host

In the early 1980s considerable effort was put into expressing immunoglobulin fragments in *E. coli*, and the protein was expressed as intracellular inclusion bodies [95–99]. Antibody fragments could be obtained from these inclusion bodies by solubilization and refolding, frequently with low yields of functional protein. Improved methods for refolding immunoglobulin fragments that are isolated from inclusion bodies are not available [100,101], but these methods are generally more tedious than the purification of fragments from either the culture supernatant or periplasmic space [14,15]. In addition, the secretion of immunoglobulin fragments into the periplasmic space, whence it leaks into the culture supernatant during prolonged induction periods, greatly facilitates the screening of clones for the production of binding activities [64].

Secretion systems for the production of antibody Fab and Fv fragments were first reported in 1988 [14,15] and involved the linkage of the antibody genes in translational frame to prokaryotic leader sequences such as OmpA, phoA, and pelB (the former two leaders are derived from *E. coli* genes, and the latter leader from the pectate lyase gene of *Erwinia carotovora*). These leaders direct the expressed protein into the periplasmic space, where the leader sequences are cleaved from the recombinant protein by signal peptidase. By means of such systems, yields of 2–5 mg/L and 5–10 mg/L of culture for Fab and Fv fragments, respectively, can be obtained [14,15,64]. Plasmids for the expression and secretion of antibody fragments are shown in Figure 3.10. Although Fv fragments may have advantages due to their relatively small size (approximately one sixth of the size of a complete immunoglobulin molecule), the stability of the association of the VH and VL domain appears to vary considerably from one Fv to another. In contrast, for Fab fragments the association of the CH1 and CL (Cκ or Cλ) domains appears to be stable, which in turn acts to stabilize the VH–VL domain interaction. The variable stability of the VH–VL interaction in Fv fragments is presumably due to the differences in the sequences of the CDR3s from one antibody to another, as these loops form the core of the antibody–antigen combining site and bridge the interface of the VH/VL domain interaction [102]. The sequences of these loops may be directly involved in the interaction of the two domains or, alternatively, may affect the conformation of the flanking framework residues, which in turn affects the stability of the interaction of the VH and VL domains. In a high proportion of cases the genes encoding the antibody fragments will be used to rebuild complete antibody molecules, in which the VH–VL domain interaction is stable. Thus, the instability is usually only a problem during screening or selection for the Fv fragment of the desired specificity. In some instances, however, it may be that smaller fragments such as Fv's are attractive for use. For example, it is conceivable that the rapid

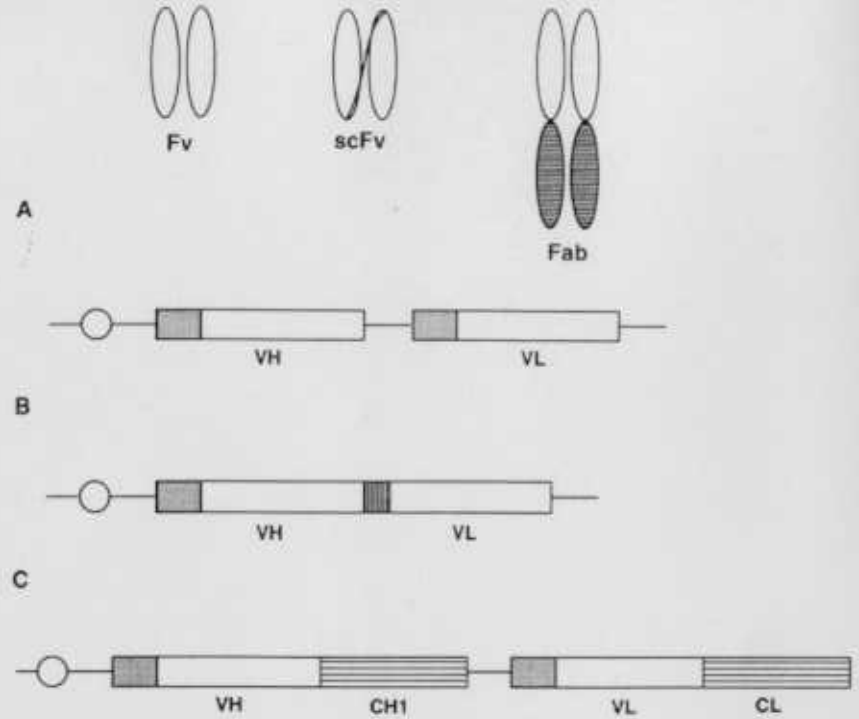

Fig. 3.10. Plasmic vectors for the secretion of immunoglobulin fragments [14,64]. **A.** Fv fragments. **B.** scFv fragments. **C.** Fab fragments. The restriction sites in these plasmids can be modified to accommodate genes tailored with different restriction sites. These sites are incorporated during the PCR [27,31,64]. The pelB leader sequences [14] are represented by stippled boxes, VH and VL genes by open boxes, the single chain linker sequence [104] by vertical hatching, and the antibody CH1 and CL domains by horizontal hatching. Open circles represent the *lacz* promoter.

clearance of the Fv fragment may make it a useful reagent for binding to and rapidly clearing toxic substances from the body—for example, in the case of drug overdoses. There are several possible ways of stabilizing the VH–VL domain interaction, as follows.

1. An intramolecular –S–S– bridge can be inserted between the two domains by genetic engineering [103]. Thus, light chain residue 55 and heavy chain residue 108, or light chain residue 56 and heavy chain residue 106, can be changed to cysteine residues that are close together in the three-dimensional structure of the associated VH–VL domains. This has been carried out for an antiphosphorylcholine Fv and shown to result in the expression of functionally active Fv fragments that are stably associated [103]. Due to the conservation of the structure of the immunoglobulin fold, this approach is probably general for a wide range of Fv fragments; it is difficult, however, to see how such an –S–S– bridge can be incorporated into repertoires of antibody genes during the generation of expression libraries.

2. Glutaraldehyde treatment has been used to chemically cross-link the VH and VL domains of an antiphosphorylcholine Fv fragment, to improve the stability without loss of functional activity [103].

3. An approach that can readily be incorporated into synthetic libraries of antibody genes, unlike (2) and (2) above, is to express the VH and VL domains as single chain Fv fragments (scFv's) (Fig. 3.6). In scFv's, the VH domain is linked by a peptide linker to the VL domain [104,105]. The use of a number of different peptide linkers has now been reported, and the scFv's can either be expressed as intracellular inclusion bodies or be secreted into the periplasmic space [101,104–110]. The secretion yields are usually lower than those of inclusion bodies, but the ease of purification when secretion systems are used may offset the advantage of high yields. For most purposes, the secretion yields are sufficient for further analyses of binding specificity and affinity of the scFv fragments prior to their being used to rebuild complete antibodies (section 3.5). The presence of the linker peptide may result in a lowering of affinity of the antibody fragment for interaction with antigen, possibly due to steric interference of the linker with the binding site and/or distortion of the CDR loops by the linker peptide. The isolation of the scFv's from inclusion bodies by denaturation followed by renaturation may also result in a decrease in affinity due to incomplete refolding. The potential disadvantage of a reduction in affinity is usually outweighed by the advantage of producing the Fv as a stably associated VH–VL heterodimer, and if binding activities are being selected from recombinant libraries, this becomes irrelevant, since it is possible to select the highest-affinity scFv from a background of lower-affinity fragments (section 3.5 below).

For optimization of the expression yield and affinity, testing of a variety of peptide linkers and/or switching the order of the VH and VL domains in the expression plasmid is advisable. For an anticarbohydrate Fv fragment, much higher levels of secretion were obtained when the VL domain was located 5' to the VH domain gene [110], indicating that the order of VH and VL domain genes with respect to each other may have a drastic effect on the expression levels.

In summary, for the production and isolation of Fv's with the desired binding characteristics from libraries of antibody genes, the insertion of a single chain linker peptide is useful for the screening/selection steps as this stabilizes the VH–VL domain interaction. If the ultimate aim is to rebuild a complete immunoglobulin with the desired effector functions (section 3.5), it is easy to remove the synthetic linker by using designed synthetic primers and the PCR.

3.3.4. Small Units of Antigen Binding

Single VH Domains ("dAbs"). The observation that the VH domain of the antilysozyme D1.3 antibody has high affinity for lysozyme binding in the absence of the paired VκD1.3 domain prompted the generation of diverse repertoires of immunoglobulin VH domain genes by using the PCR [64]. These genes were isolated from the splenocytes of mice that had been immunized with hen egg lysozyme or keyhold limpet hemocyanin (KLH). The VH domain genes were cloned into expression plasmids containing the pelB leader for secretion, and the supernatants of the clones screened for the presence of VH domains with antigen-binding activities. The diversity of the repertoires were checked by nucleotide sequencing; and of more than 50 VH gene sequences, all were unique, and members of each of the murine DH (diversity segment) and JH (joining segment) families were represented [64,93]. The recombinant *E. coli* clones were grown up and induced for expression, and the culture supernatant was screened with ELISA for the presence of binding activities towards lysozyme and KLH. VH domains with antigen-binding activities against these two antigens were isolated, and two of the antilysozyme VH domains characterized further in terms of affinity, nucleotide sequence, and specificity. These VH domains had affinities of the order of 10^{-8} M for binding lysozyme in solution, and also did not show binding affinities for a variety of antigens other than hen egg lysozyme. This affinity is reasonable, and is similar to that of a significant proportion of complete immunoglobulin molecules. More recently, VH domains that have specificity for mucin and for influenza neuraminidase have also been isolated (D. Allen and P. Hudson, personal communication).

The expression levels of VH domains as secreted proteins from recombinant *E. coli* cells are significantly lower than those of Fv and Fab fragments, and this is probably a reflection of their rather hydrophobic and insoluble character [64]. The exposure of the conserved hydrophobic residues of VH domains that are buried in an Fv or Fab fragment by association with the paired VL domain presumably contributes to the rather insoluble characteristics of these single domains. It may also be that the VH domains, in the absence of associated VL domains, are rather unstable and denature easily. Thus, these rather unattractive features may offset the advantages of the small size of VH domains. It may be possible, however, to use protein engineering to improve the properties of these domains. Such engineering would involve the insertion of residues to stabilize the immunoglobulin fold and residues of more hydrophilic character at the exposed positions. An alternative approach may be to design a stable framework structure onto which one or more CDR loops, which are known to play an important role in binding to cognate antigen, could be mounted in a suitable conformation for binding. This approach requires a significant amount of protein modeling and design, and the technology for the generation of such "de novo" antibodies may be available in the near future.

Minimal Recognition Units (MRUs). CDR-derived peptides have recently been isolated from antibodies and shown to retain binding affinity for cognate antigen [111,112]. These peptides were designed on the basis of modeling and/or sequence analysis and have potential as therapeutic reagents as blocking peptides. This approach will probably work only for antibodies for which one of the six CDRs makes the major contribution to the binding of the antigen to the antibody, and for this reason it may not be general. It does, however, offer an new immunotherapeutic approach in the cases in which individual CDRs have significant binding affinities.

3.3.5. Combinatorial Libraries of Immunoglobulin Genes

One of the potential problems that may occur during the isolation of antibody VH and VL domain genes from antibody-producing cells is that the matching that exists between a VH and a VL domain within a particular B cell is lost once the cells have been lysed (as a mixed population) and their nucleic acids extracted. To reconstitute an Fv or Fab fragment with binding activity, the genes need to be recombined in such a way that functional binding units can be produced. This may not necessitate the matching of VH and VL domains as they existed *in vivo*, as VH domains appear to be able to combine with a number of different VL domains and still retain antigen-binding activity [113]. There is therefore a need to be able to recombine the libraries (or repertoires) of VH and VL domain genes in as random a way as is possible. Recombinant DNA technology now allows the *in vitro* generation of randomly combined repertoires of VH and VL domain genes. The VH and VL domain genes can be combined either by ligation at a unique restriction site [31], or by splicing by overlap extension (SOE) (Fig. 3.4) [49,114]. The expression of randomly combined VH and VL domain genes as Fab fragments using the lambda Zap expression plasmid was reported by Huse and colleagues [31]. In this study, Fab fragments with reactivities towards the immunogen p-nitrophenyl phosphonamidate (NPN; a transition state analogue for carboxamide hydrolysis) were isolated. More recently, the approach has been used to isolate Fabs from both murine and human-derived repertoires [31,35,36,116]. In all cases, the genes were isolated from "immunized repertoires"; that is, even for the human-derived fragments the human donor had recently been boosted with tetanus toxoid, the antigen used in these studies for subsequent selection for binding fragments. For the isolation of Fv or Fab fragments from unimmunized or naive repertoires, it is generally predicted that extremely large numbers of recombinant clones will have to be screened. This is due to the fact that exposure of the immune system to antigen results in the clonal expansion and affinity maturation of the antigen-specific B-cell clones with extremely high efficiency [116,117]. Thus, for the isolation of very-low-frequency clones from naive libraries, the use of selection systems is desirable to avoid tedious screening.

A major step in the development of selection systems was reported by McCafferty and colleagues in 1990 [89] (Fig. 3.8). This system involves the expression of antibody scFv fragments on the surface of the filamentous bacteriophage fd. Genes encoding the D1.3 scFv were inserted in translational frame into the gene III coat protein of the bacteriophage. The recombinant bacteriophage were shown to bind specifically to lysozyme-coated surfaces, and this opened the door for the surface expression of libraries of immunoglobulin variable domain genes, followed by selection of phage bearing antigen-binding scFv's.

The phage expression systems have now been extended to the expression of libraries of scFv's derived from immunized mice, and scFv's that recognize the immunogen pHOx (2-phenyloxazol-5-one) have been isolated by selection by binding to antigen-coated surfaces (panning) and characterized [49]. Interestingly, from

a library size of 10^6 clones derived from a naive mouse, no pHOx-specific clones could be identified, indicating that for the isolation of antigen-specific clones from naive libraries the numbers of clones generated should be extremely large. In this respect, Marks and colleagues [34] have recently extended the use of the phage expression system to the isolation of anti-turkey-egg lysozyme and anti-pHOx clones from naive human-derived libraries. The library sizes in this study were 2.9×10^7 to 1×10^8, and four rounds of panning (i.e., bacteriophage isolated from the first round of panning were used to reinfect *E. coli*, the resulting clones grown up, and the extruded bacteriophage panned again on antigen-coated surfaces) were required to isolate antigen-specific, and presumably low-frequency, clones.

Phage expression systems similar to those used for the display of scFv fragments have been reported for the surface expression of Fab fragments using the gene III and gene VIII coat protein genes of filamentous bacteriophage such as fd or M13 [90,118,119]. Using these phage display systems, Fab's with binding specificities toward tetanus toxoid (human-derived) [119], HIV (human-derived) [120], hepatitis antigens (human-derived [121], and progesterone (murine) [52] have been isolated. The gene VIII coat protein is expressed in up to 24 molecules per phage particle, in contrast to the gene III coat protein that can be expressed in up to 4 copies per particle. Thus, for the isolation of low-affinity, high-avidity clones it may be preferable to use the gene VIII system, in which Fabs are expressed in multivalent form on the surface of the bacteriophage. In contrast, for the selection of higher-affinity, low-avidity clones, surface expression using antibody–gene III fusions is suitable.

The bacteriophage display systems greatly facilitate the isolation of low-frequency, low-affinity clones from immunoglobulin fragment expression libraries. Prior to the development of these selection systems, it was impractical to screen the extremely large numbers of clones necessary to isolate the desired specificity from naive repertoires. The use of the phage display systems for the isolation of human-derived antibodies is particularly attractive, since to date the production of human monoclonal antibodies by hybridoma technology has proved difficult due to both technical and ethical barriers [66–68].

For the purification and further characterization of the antibody fragments, following identification of phage that bear scFv's or Fab's with the desired binding activity, it is facile to express the fragments as secreted proteins in one of the two following ways.

1. The pHEN1 vector [122] has a suppressible amber codon that is located between the 3′ end of the antibody gene and the 5′ end of the gene III coat protein gene. Thus, by switching between a suppressor and a nonsuppressor strain of *E. coli*, the fragment can be surface-expressed on bacteriophage or secreted as soluble fragments into the culture supernatant.

2. The Fab fragment-gene III/VIII genes in the pCOMB vectors can be modified by restriction and religation, to remove the gene III/VIII gene, causing the Fab fragment to be secreted as a soluble protein, rather than attached to the surface of bacteriophage particles in linkage to coat protein genes [119].

3.3.6. Improvement of the Affinities of the Fragments Isolated From Naive Repertoires

The binding affinity of the antibody fragments derived from naive libraries will generally be lower than those derived from immunized repertoires, due to the lack of affinity maturation [116,117] for naive repertoires. There are several ways in which the affinities could be improved. The genes encoding the low-affinity fragments could be used as templates for rounds of *in vitro* mutagenesis followed by surface expression and selection [52] (Figs. 3.8 and 3.11), in an attempt to mimic somatic mutation *in vivo*. Alternatively, severe combined immunodeficient (SCID) mice that have been populated with human PBLs [123] or transgenic mice that have the human immunoglobulin loci [124] can be used. These mice can be immunized and the antibody-producing cells used to generate VH and VL gene libraries for surface expression and selection. In addition, *in vitro* immunization of human PBLs

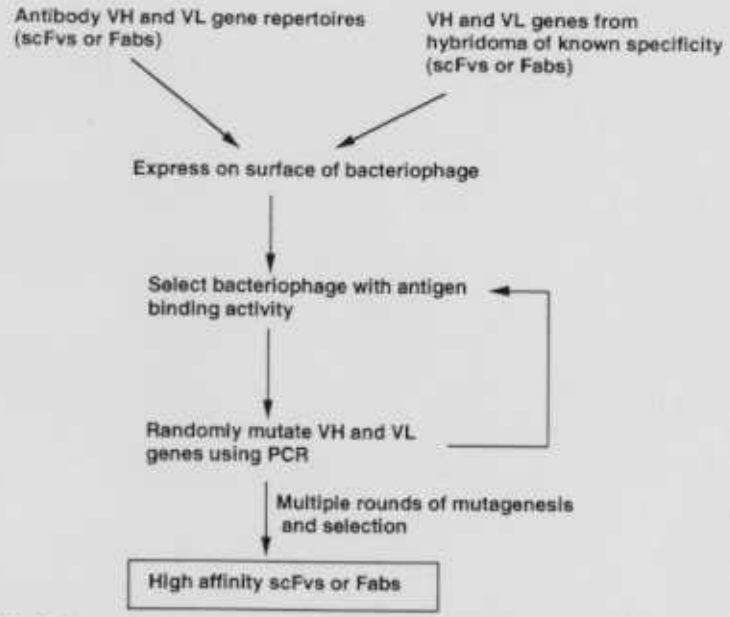

Fig. 3.11. Scheme for the improvement of antigen-binding activities of immunoglobulin scFvs or Fabs expressed on the surface of bacteriophage. The scFv or Fab can be derived from either VH and VL gene libraries or from a hybridoma of defined specificity. For the former, antibodies that have the desired binding activity have to be first selected from the libraries. For the latter, selection of binding fragments from a background of nonbinders is unnecessary due to the clonal nature of hybridomas. The VH and VL genes can then be randomly mutated by a number of different methods (see text) and the antibody variants that have improved affinity can be selected from a background of lower-affinity variants, by bacteriophage surface expression. After each round of mutagenesis and selection, the fragments can be characterized as secreted scFvs or Fabs (see text) and further rounds of mutagenesis/selection can be carried out until the desired affinity is reached.

[123,125] can be used to increase the proportion of antigen-specific B cells that are used to repopulate the SCID mice. These approaches are described in more detail below.

Error-Prone PCR. A possible route for the production of high-affinity antibodies from naive repertoires has recently been reported by Gram and colleagues [52]. This involved the isolation of antiprogesterone (IgM) antibodies in the form of Fab fragments from the bone marrow cells of unimmunized mice using the high-avidity pCOMB8 (gene VIII coat protein) vector. The genes encoding several antiprogesterone specificities were then expressed as scFv's using the low-avidity pCOMB3 (gene III coat protein), and the scFv genes were used as templates for error-prone PCR [51,52]. By this approach, it was demonstrated that the affinity could be improved 13- to 30-fold after one round of mutagenesis and selection. Further rounds of mutagenesis and selection could presumably be used to increase the affinity further. What the limits to improvement of affinity by this approach might be is not clear at present.

Immunization of Transgenic Mice and Severe Combined Immunodeficient (SCID) Mice

Transgenic Mice. Transgenic mice have recently been described that have a human immunoglobulin heavy chain minilocus (an incomplete human heavy chain locus in germline configuration [124]). These genes can be rearranged in functionally active form, presumably due to the conservation of the recombinase activity between mouse and man. Immunoglobulin molecules that make up human heavy chains and murine light chains can be isolated from the serum of the mice. This is a step towards creating the entire human immunoglobulin repertoire in mice; it would provide an invaluable tool for generation of "pure" human antibodies. Moreover, the transgenic mice could be immunized with antigens of choice, allowing affinity maturation of the immune response to occur *in vivo*, rather than using the *in vitro* approaches described above. The main advantage of using the *in vivo* route rather than *in vitro* mutagenesis is that it is clearly less labor-intensive. There may, however, be disadvantages, insofar as the extent of somatic mutation *in vivo* depends on the immunogenicity of the antigen. In contrast, *in vitro* mutagenesis is not limited by the immunogenicity of the antigen for which high-affinity antibodies are being generated.

SCID mice. It has recently been demonstrated that it is possible to repopulate SCID mice with human PBLS [123,126,127], and to isolate human-derived antibodies from these mice by the *in vitro* approaches described above. In this approach, human PBLs were immunized *in vitro* prior to being injected into mice, and then the mice were immunized with antigen *in vivo* after transfer of the PBLs [123]. Fabs with specificities for hepatitis B core antigen and tetanus toxoid were isolated and characterized further. This route promises to have potential in the isolation of antibodies from PBLs derived, for example, from HIV-seropositive

donors and from autoimmune patients, and the resulting antibodies have obvious applications in therapy and diagnosis.

Semisynthetic Immunoglobulins. For the structures of antibody–antigen complexes solved to date, the CDR3 region of the immunoglobulin VH domain usually plays a significant, if not major, role in the interaction [56,59,60,128]. Thus hypermutation of this region of an immunoglobulin may not only result in clones that have higher affinity for antigen binding, but may also result in the generation of novel specificities. In this respect, hypermutation of the CDR3 of the antilysozyme D1.3 VH domain, using the PCR and a degenerate oligonucleotide (with equimolar mixes of A, T, C, and G inserted at each position corresponding to CDR3 residues during synthesis), resulted in the generation of higher-affinity variants [129]. These were identified by screening. It is now much more convenient to combine random PCR-directed mutagenesis with the phage display systems, and to use this combination of PCR-based technology with selection to identify phage that bear novel binding specificities.

Recently, the PCR, in combination with a highly degenerate oligonucleotide primer ("randomized" in the region corresponding to CDR3 and complementary at the 5′ and 3′ ends to the flanking framework regions; Fig. 3.12), has been used to generate a library of human-derived antibodies that differ only in their VH CDR3 region [130]. The maximum number of different CDR3 sequences in this library was estimated to be 10^{20}. For this study, the gene encoding an anti-tetanus-toxoid Fab was used as the template, and the mutated genes were cloned for phage display using pCOMB3. The resulting phage were panned on fluorescein-coated plates, and phage bearing antifluorescein specificities were selected and isolated under a variety of elution conditions. Thus, this approach can be used to generate new specificities from an antibody fragment that originally had a specificity towards tetanus toxoid.

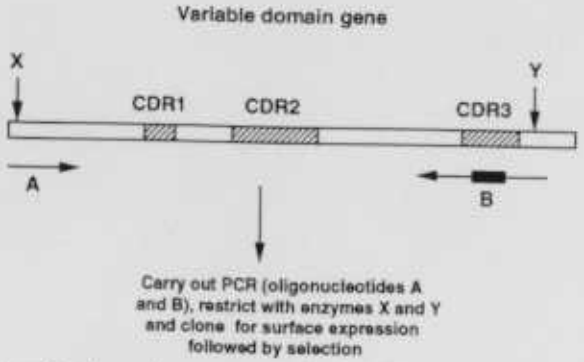

Fig. 3.12. Strategy for the random mutagenesis of a VH domain gene. The PCR is carried out with two primers: A anneals to the 5′ end of the VH domain gene and has an internal restriction site X. B is complementary to the framework regions that flank CDR3 and has a region of random nucleotides (shown schematically by the filled box) that overlaps, but is not complementary, to CDR3. B also has an internal restriction site Y.

To date this approach has been used to generate antihapten antibodies, and haptens are known to interact with substantially smaller numbers of CDR residues than protein antigens. Because of the crystallographic structures of antibody–antigen complexes [56,58–60], protein antigens usually contact residues that are located in all six CDRs, and the surface area of the interaction is extensive. It is probable that to generate fragments that have specificities for protein antigens (as opposed to haptens) rather more extensive mutagenesis of the CDR loops will have to be carried out—that is, up to all six CDRs may have to be hypermutated. This is technically possible using the PCR, for example, but the numbers of mutants that will have to be generated is enormous; thus, if this is to be feasible, the bacteriophage surface expression system needs to be able to select higher-affinity variants out of a large background of lower-affinity variants, and as yet the limits of this system in this respect have not been rigorously tested.

3.3.7. Immunogenicity of Antibodies Generated *In Vitro*

One of the goals of engineering antibodies for use in therapy is to reduce their immunogenicity. Initially, simple chimeric antibodies were made [9,11–13], followed by CDR-grafted or humanized immunoglobulins, in which only the CDR loops are of rodent origin [71,72,77–83]. Using current developments in technology, it is possible to isolate antibody fragments of human origin, and these have considerable potential as therapeutic reagents. However, hypermutation of human antibody variable domains with the aim of improving the binding affinity may generate sequence motifs that are immunogenic *in vivo*. The likelihood that this will occur probably depends on the extent of hypermutation and on the regions of the variable domains that are hypermutated. *A priori* it would be predicted that mutations in the CDR loops (particularly the highly diverse CDR3) are less likely to be immunogenic than mutations in the more conserved framework regions. The latter mutations are less likely to be selected, since mutations in these regions have (1) a lower probability of improving the binding affinity and (2) a higher probability than mutations in the CDRs of disrupting the immunoglobulin fold structure. In addition, the production of antibodies with completely novel CDR loops may generate fragments that have autoreactivities *in vivo*, and are representative of specificities that are normally "forbidden." Whether these concerns are valid awaits the testing of these new recombinant antibodies *in vivo*.

3.4. FRAGMENTS FOR THERAPY AND DIAGNOSIS

The rapid *in vivo* clearance rates [131–135] of immunoglobulin Fv and Fab fragments makes them attractive reagents for use in clinical situations in which short half-lives are either advantageous or required. A prerequisite for an Fv or Fab to be useful *in vivo* is for it to be retained in the location where it is needed, and this will clearly depend on factors such as the affinity of the fragment for antigen, and the

tissue in which the target antigen is located. The fragments, like the complete immunoglobulin molecule, have biphasic clearance curves. For an Fv fragment the half-life for the α phase is of the order of 0.15 h and that for the β phase is 1.4 h; and after 4 h, less than 1% of the injected dose is present in the serum [135]. In contrast, the complete IgG molecule has a serum half-life that is of the order of 6–9 days [136,137]. The reasons for the rapid clearance of the fragments are that (1) they do not contain the Fc portion of the molecule, and a considerable amount of experimental data indicate that this is important in stabilizing the immunoglobulin molecule *in vivo* [75,76]; and (2) they are small relative to a complete immunoglobulin. The role of size in determining *in vivo* clearance rates is indicated by the finding that an $(Fab)_2$ fragment is cleared twice as slowly as an Fab fragment [131]. The *in vivo* half-life of the $(Fab)_2$ fragment, however, is still substantially less than that of the complete immunoglobulin molecule and even that of the smaller Fc fragment derived by proteolysis [138], indicating that sequences in the Fc region are involved in stabilizing immunoglobulins *in vivo*. Fab fragments may be preferable to Fv fragments for *in vivo* use, as Fv fragments have a tendency to dissociate (see section 3.3 above). For example, dissociation of the D1.3 Fv fragment was observed in an *in vivo* clearance study carried out in rats [135]. Using a combination of genetic manipulation and protein chemistry [135], a bivalent Fv fragment has been constructed that has improved stability *in vivo*. In addition, the bivalent nature of this protein may improve the avidity of the antibody–antigen interaction. An alternative way to prevent dissociation of the VH and VL domains of Fv fragments for *in vivo* use might be to covalently link the domains using recently described peptide linkers to generate scFv's (see section 3.3). However, these linkers may be immunogenic, which is clearly a disadvantage for use in therapy in which repeated doses are necessary.

Rapid clearance of immunoglobulin fragments is particularly attractive if they are to be used as reagents for the imaging of tumors [131,132]. Clearly for an Fab to be a useful imaging reagent, it needs to localize efficiently to the tumor site. The rapid clearance reduces the immunogenicity, which is of significance if the imaging is to be followed by therapy using the same antibody as a complete immunoglobulin molecule. A further advantage of the use of Fab or Fv fragments in imaging is that they appear to give high tumor : normal tissue localization ratios [131,132], and they may be more penetrative into tumor masses [139]. The relatively high tumor : normal tissue localization is presumably due to the absence of Fc receptor-mediated binding [140,141] and rapid clearance. Interestingly, a recent study [132] has indicated that scFv fragments may be preferable to the use of Fab or $(Fab)_2$ fragments for imaging, since the smaller fragments do not accumulate in the kidneys.

Fragments of immunoglobulins may also have uses in situations in which binding followed by rapid clearance is desirable, and such a situation occurs in the treatment of drug overdoses. The fragments would be predicted to bind to the drug and clear it rapidly from the circulation. For such an antibody fragment, it would be attractive if it were also to block the binding of the drug to its receptor, and the new *in vitro* approaches to generate antibody fragments of defined specificities may facilitate the development of such reagents.

3.5. REBUILDING THE FRAGMENTS AND EXPRESSION AS COMPLETE IMMUNOGLOBULIN MOLECULES OR AS ANTIBODY-CHIMERAS

Clearly antibody Fv and Fab fragments have no effector functions, and therefore for effective treatment of tumors and infections it is necessary to link the fragments to moieties such as the immunoglobulin Fc fragment or bacterial/plant toxins. In addition, the fragments may be linked to radioisotopes, but as recombinant DNA techniques are not used to generate radiolabeled antibody (fragments), this will not be discussed further. The Fc portion of the antibody is known to be involved in the effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement fixation [140–142]. The Fv and Fab fragments that are generated by the methods described above can be used as building blocks for complete antibody molecules. Fabs may, however, have uses in the treatment of infections in situations in which neutralization of the infectious agent is all that is required to block progression to disease. To use Fv or Fab fragments as building blocks, the genes that encode high-affinity Fv/Fab fragments of the desired specificity can be linked to the genes encoding the desired isotype. The choice of isotype depends on the effector functions that are required. For example, using a set of matched chimeric antibodies that differ only in their constant regions, it has been shown that the human IgG1 isotype is the most effective in complement-mediated lysis and ADCC [73,143,144], and this may therefore be the one of choice for human therapy. In this respect, the humanized version of the antibody Campath-1 was linked to the human IgG1 isotype prior to effective use in the therapy of non-Hodgkin's lymphoma [72,84]. The complete antibodies can be expressed in a variety of mammalian expression hosts, and these are described in more detail in section 3.6 below.

The antibody fragments can also be linked to toxins such as *Pseudomonas* exotoxin [145,146], ricin A chain [147], or phospholipase C from *Clostridium perfringens* [148] to generate immunotoxins (ITs). For example, in the case of *Pseudomonas* exotoxin, a truncated form that lacks the cell-binding domain and therefore has no toxicity if used unlinked to an antibody has been linked to a scFv fragment. This scFv recognizes a carbohydrate antigen expressed on the surface of many human carcinomas [107]. The exotoxin also has a lysine–aspartic acid–glutamic acid–leucine (KDEL) tetrapeptide at the carboxy terminus, and this peptide has been demonstrated to improve the efficacy of the IT, presumably due to an effect on the intracellular routing of the toxin [149]. This Fv–toxin chimera can be expressed in high yields in recombinant *E. coli* cells, and it has recently been demonstrated that it is effective in the treatment of tumors in immunodeficient mice [107]. A potential caveat about the use of ITs in prolonged therapy is that problems may be associated with the immunogenicity of the toxin moiety. It may be possible to overcome this by using different toxin moieties in cycles, and this depends on the availability of toxins with the desired therapeutic efficacies. Clearly, if the IT has immunosuppressive activity, the immunogenicity is not of as much concern as for ITs without this activity.

Generally the toxins that are attached to antibodies or antibody fragments have extremely high potencies, and for this reason it is particularly important to ensure that the antibody fragment to which they are attached is specific, to avoid the potentially disastrous consequences of nonspecific cytotoxicity. For example, neurological toxicity occurred when a therapeutic IT was constructed using a monoclonal antibody that unexpectedly cross-reacted with a component of the central nervous system [150]. This problem could be circumvented by the generation of antibodies of high affinity and specificity to epitopes that are not shared by other tissues in the body. This is frequently difficult to do, however, since in practice it is obviously very difficult to screen all the tissues of a human or animal for cross-reactivities.

A further route for the attachment of effector functions to an antibody fragment is an indirect one. Shalaby and colleagues [79] recently reported the construction of a bispecific Fab, for which one arm is specific for the HER2 protooncogene and the other arm is specific for the CD3 complex expressed on T cells. The bispecific antibody has the capacity both to bind to tumor cells and to capture T cells in the vicinity of the tumor cells. This bispecific reagent was produced by expressing the two Fabs individually in *E. coli* cells, followed by purification and chemical linkage [79]. This approach may be generally applicable for the production of bispecific antibodies, and is particularly attractive since it is much more rapid than the more conventional route of fusion and screening of hybridomas [151]. In addition, it allows an Fab of a particular specificity to be coupled to a range of Fabs of different specificities to generate a panel of bispecifics with relative ease and rapidity. Thus, mixing and matching experiments can be carried out with relative ease using purified Fabs of different specificities.

3.6. EXPRESSION OF ANTIBODY GENES IN MAMMALIAN CELLS

Rebuilt antibody genes, in which the V-regions are linked to a C-region with the desired effector function, cannot be efficiently expressed in *E. coli*. Several attempts have been made to produce intact immunoglobulins in bacteria (reviewed in Better and Horwitz [152], and in all cases inefficient reassembly of the heavy and light chains *in vitro* was required to produce active antibody, since the immunoglobulins were not correctly assembled or secreted from the cell. Recombinant antibodies have, on the other hand, been successfully produced from the cells of diverse eukaryotes, including yeast [153], insects [17], and plants [16,154], generally by secretion into the medium. However, mammalian cell expression currently provides the most efficient route for production of intact antibodies. Fragments such as Fabs, and perhaps some toxin conjugates, can be secreted from either *E. coli* or mammalian cells, and in some cases the productivities of these fragments of intermediate size may be sufficiently similar from the two host cell types that the choice of expression system is not entirely straightforward. In such cases, other factors such as the end use of the antibody and the downstream processing required may have to be taken into consideration. In this section, the technology for expression in mammalian cells is first described and then a comparison of expression systems is made.

Cloned DNA can be introduced into mammalian cells by a number of transfection techniques and can enter the nucleus, where it may persist for a period of a few days in a high proportion of the cell population and result in transient expression of genes present on the vector. Alternatively, low-frequency integration events can be selected, by using an appropriate marker gene, in which the introduced DNA becomes inserted into apparently random sites in the host cell genome. In this case, the DNA replicates along with the rest of the genome and a permanently transfected cell line is established.

3.6.1. Transient Expression

The ability to produce small amounts of antibody (up to about 1 mg) within a few days from transient expression experiments is very valuable in the analysis of multiple genetically engineered forms of an antibody. Recombinant antibodies have been shown to assemble efficiently in a number of nonlymphoid as well as lymphoid types of cells and this has provided the possibility of exploiting a number of transient expression systems for Ig production (reviewed in Bebbington [155]). COS cells (monkey kidney cells transformed with SV40 T-antigen) support the replication of virtually any bacterial plasmid containing a 340-bp sequence of SV40 including the origin of replication (provided that particular sequences from pBR322 that inhibit replication are removed). A strong promoter such as the MIE promoter from cytomegalovirus (CMV) is used to direct transcription of a heavy chain gene and a light chain gene on separate vectors, which are cotransfected into the same COS cell population. This system can be used to produce recombinant antibodies secreted into the culture medium at levels of about 1 μg/ml within 2–3 days from transfection from cells grown to confluence (i.e., about (2–5) × 10^6 cells/ml) [156].

The CMV–MIE promoter can also be used effectively for transient expression in an adenovirus-transformed human embryo cell line, 293. One of the early proteins of adenovirus (an E1A protein) enhances transcription from the CMV–MIE promoter when coexpressed in the same cell, and yields of 7–15 μg/ml of recombinant antibody have been obtained a week after transfection by this system [157].

3.6.2. Establishing Permanent Transfected Cell Lines

To select for retention of the vector within the host genome, a number of marker genes exist. The most widely used are two bacterial genes provided with mammalian transcription signals: neo, conferring resistance to an antibiotic, G418 [158]; and gpt, conferring resistance to mycophenolic acid, which is an inhibitor of IMP dehydrogenase, an enzyme involved in nucleoside biosynthesis [159]. Vectors containing such selectable marker genes can be introduced into mammalian cells by various techniques. The method that is most appropriate depends largely on the cell type but electrophoretic methods (in which a millisecond pulse of high-voltage electricity is applied across the suspension of cells and DNA) tend to be highly efficient for many types of cells and are the preferred choice for myeloma cells [160]. However, fragments of cloned genomic DNA containing complete Ig genes

that are introduced in this way into the genome of a myeloma cell are inefficiently expressed relative to the same genes in their natural chromosomal locations in a hybridoma cell. Thus a 9.5-Kb genomic fragment containing a mouse γ2b gene has been expressed at only up to 20% of the level in the parental hybridoma [161], and genomic light chain genes tend to be even more poorly expressed [162,163]. Expression levels are also highly variable among different transfectant lines. Such low and variable expression levels are generally ascribed to two factors: the likelihood that not all the DNA sequences necessary for complete expression are isolated when the genes are cloned, and the profound influence on gene expression of the chromosomal structure surrounding the integration site [164].

Use of Immunoglobulin Promoters and Enhancers. In the early 1980s, several transcription control elements were characterized in immunoglobulin genes. Promoter regions were identified and tissue-specific downstream enhancers were isolated from the heavy chain J–C intron [161,165] and the κ-gene intron [166] (see Chapter 1). This enabled versatile expression vectors to be constructed with immunoglobulin promoters and enhancers to direct transcription of cDNA or genomic clones encoding antibodies or their fragments in myeloma-type cells. Chimeric antibody expression vectors assembled from murine genomic V-region clones, fused upstream of human constant region sequences, make use of the natural promoter and enhancer present in the V-region DNA (Fig. 3.13A). The use of a genomic V-region fragment also serves to provide an intron, which is an absolute requirement for the formation of stable mRNA from a transcript initiated at an immunoglobulin promoter [167]. Typically the genes for the heavy and light chains have been placed on separate vectors and cotransfected into a myeloma cell line [3–13,168,169], most commonly the SP2/0 hybridoma cell line. Expression levels from such cotransfected vectors can be up to 35 μg/ml of antibody accumulated in the medium once the cells have overgrown and died [168], although much lower figures are more common. Specific production rates of 1–7 μg/10^6 cells per day have been obtained [168] or, in one case, up to 10 μg/10^6 cells per day [169]. This is considerably less than the levels at which hybridomas typically express antibody from genes in their natural chromosomal loci, as hybridomas have production rates between 10 and 100 μg/10^6 cells per day and accumulate between 50 and 500 μg/ml, depending on the culture medium used and hence the cell biomass attainable [170] (and see Chapter 5).

A better approach may be to use sequential transfection, in which a light-chain-producing cell line is established first and the heavy chain vector is subsequently introduced into this cell line. This approach has yielded 32 μg/ml at small scale and up to 100 μg/ml in serum-free fermentation [171]. Although it is not possible to compare expression levels for different antibodies and different fermentation conditions, yields resulting from retransfection can be expected to be higher due to the importance of balancing the levels of synthesis of the two chains. Many heavy chains are not secreted in the absence of light chain but are retained in the endoplasmic reticulum (ER) by association with the ER protein grp78 [172]. Accumulation of certain heavy chains in the ER can be toxic to the cell [173], so synthesis of

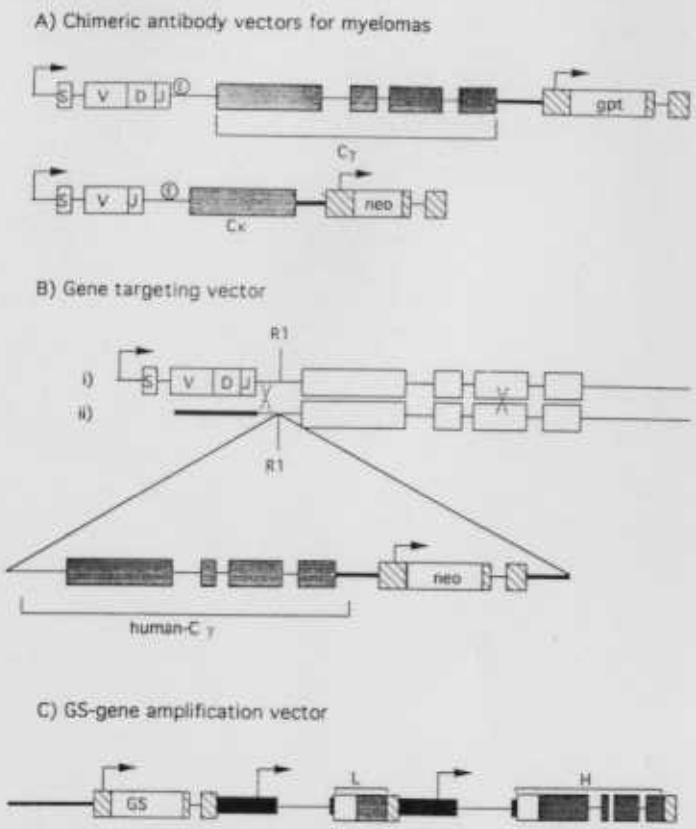

Fig. 3.13. Mammalian expression vectors for recombinant antibody genes. Vectors are shown as linear molecules, as they appear in the host genome. Promoters are marked with arrows. Immunoglobulin enhancers are marked E. R1 represents a convenient restriction enzyme site. Thin lines denote introns and plasmid backbones. Hatched segments are from SV40. Speckled segments are human immunoglobulin DNA regions. Black segments are from the CMV-MIE upstream region. L and H represent light and heavy chain coding sequences. In **B** the chromosomal allele is marked (i) and the vector DNA (ii). Representative recombination sites are marked with crosses.

excess heavy chain may limit expression. In a cotransfection experiment, the probability that both vectors will integrate such that synthesis of the two chains is balanced will inevitably be small, whereas by selecting light-chain-producing lines first, it is relatively straightforward to screen heavy chain transfectants for high levels of intact antibody. Nevertheless, any such vectors are still limited by the inefficient transcription of the transfected genes. There are, however, several newly identified elements from regions distant from the genes in the heavy chain [174], κ [175], and λ [176] loci, which show enhancer activity. These have yet to be evaluated fully for the expression of recombinant antibodies in cultured cells, and it therefore remains to be seen whether it will be possible to assemble all the control

elements necessary for full expression of immunoglobulin genes on a practically useful vector.

Targeting Genes to Transcriptionally Active Sites in the Genome. An attractive alternative to the use of expression vectors containing immunoglobulin transcription control elements might be to target the required coding regions to an active, rearranged immunoglobulin locus so that the control elements in their natural chromosomal locations are exploited to achieve full expression. If there is an extended region of homology between sequences on a vector and a region of the host cell genome, homologous recombination can occur between them to introduce the DNA at a precisely defined site in a chromosome. An example of a targeting vector is shown in Figure 3.13B. Although random integration by nonhomologous recombination still predominates, a number of reports of detection of the rare gene-targeting events exist, leading to high-level expression of heavy and light chain genes [177–179]. In principle, separate targeting events could be carried out to introduce light and heavy chain genes into their respective loci, but such experiments have not been reported, perhaps because the procedures are so lengthy and laborious. Nor has the technology been extended to introduce DNA sequences other than constant regions into immunoglobulin loci.

Vector Amplification. A more versatile approach for the efficient expression of immunoglobulin-type molecules has been to use selection for gene amplification to obtain increased copy numbers of vectors randomly integrated into the genome. Gene amplification is a common mutation in mammalian tissue culture cells in which a large region of a chromosome comes to be represented in multiple tandem repeats as a result of a poorly understood sequence of events (perhaps associated with "illegitimate" recombination between regions of two sister chromatids that are not precisely homologous [180]). Amplification events can be readily detected in regions of the genome containing a gene that encodes an essential enzyme whose activity can be inhibited by a tight-binding selective inhibitor. Mutants arising in a cell population with increased resistance to the inhibitor commonly result from overproduction of the enzyme and this in turn most commonly results from amplification of the corresponding structural gene. Thus a gene coding for such an enzyme can act as a selectable marker for amplification events. An example of a suitable enzyme is dihydrofolate reductase (DHFR), a key enzyme in nucleoside biosynthesis, which can be inhibited by methotrexate (MTX). DHFR has been widely exploited as an amplifiable marker in a DHFR-deficient mutant chinese hamster ovary (CHO) cell line (reviewed in Kaufman [181]). Transfectants are selected in nucleoside-free medium and amplification of the region of the chromosome containing the vector can be selected with MTX. The procedure has also been adapted for use in other cell types, such as myelomas, that have endogenous DHFR activity, either by using a mutant DHFR marker gene encoding an enzyme partially resistant to MTX or by using a DHFR gene expressed from a strong promoter [181]. In either case, transfectants are selected on the basis that the vector confers resistance to elevated levels of MTX and amplification is selected by using progressively higher levels of MTX.

A MTX-resistant DHFR gene has been used as the selectable marker to produce a chimeric antibody in SP2/0 cells by vector amplification [182]. In this case, genes for the heavy and light chains expressed from the promoters and enhancers present in the murine V-region genomic DNA were inserted into separate vectors, each containing a DHFR gene. After cotransfection of both plasmids and multiple rounds of selection in gradually increasing concentrations of MTX, cell lines were obtained that secreted up to 35 $\mu g/10^6$ cells per day of antibody. This is similar to the productivity of typical hybridomas, but the selection procedures were comparatively lengthy (taking up to 6 months) and the levels of MTX required are exceptionally high. No data on stability are given but many amplification events are known to be unstable if MTX is removed. If high levels of MTX are indeed required continuously to maintain productivity, the high cost of MTX may cause problems in deriving an economically feasible process for producing antibody on a large scale. In other cases, MTX selection has been reported to lead to increased productivity of recombinant antibody without gene amplification [183,184]. The mechanism is unclear but will presumably also require the continued presence of the drug.

An alternative amplifiable marker that is particularly attractive for use in myeloma cells is glutamine synthetase (GS) [155,185]. GS provides the only pathway for synthesis of glutamine in mammalian cells, so for cells in glutamine-free culture media it is an essential enzyme. The levels of GS vary between different cell types and are particularly low in myelomas. Consequently, such cells are unable to grow without glutamine in the culture medium, and a GS gene can act as a selectable marker in these cells by conferring glutamine-independence [185]. Vector amplification can subsequently be selected using a the specific GS inhibitor, methionine sulfoximine (MSX). Using a GS vector as shown in Figure 3.13C, in with the immunoglobulin genes are under the control of CMV-MIE promoters, a chimeric B72.3 antibody has been produced in the NSO myeloma cell line at secretion rates of 10–15 $\mu g/10^6$ cells per day in exponentially growing cultures and up to 35 $\mu g/10^6$ cells per day towards the end of a batch fermentation [185]. By using an enriched serum-free medium to grow the cells in a fed-batch air lift fermenter (see Chapter 5), it is possible to obtain accumulated yields of 560 mg/L of chimeric antibody from this system. This is higher than typically found for murine hybridomas grown in similar fermentation systems, suggesting that it will be readily feasible to obtain an economically viable process from such cell lines. Only a single round of selection for amplification was required to obtain these high-producing lines, in contrast to the multiple rounds of selection usually required with DHFR selection. The productivity of the highest-producing cell line was also maintained for at least 30 cell generations in the absence of MSX, which may be a significant advantage in the development of a manufacturing process. More recently, cell lines have been obtained with this system, which can accumulate up to 1 g/L antibody in enriched serum-free medium after a single round of selection for GS gene amplification (H. Finney, unpublished) or 500 mg/L without selection for gene amplification (C.R.B., unpublished).

Recombinant antibodies have also been produced from CHO cells. There is extensive experience in the use of this cell type for the production of other recombinant proteins for therapeutic use. Efficient expression systems based mainly on

DHFR gene amplification are well established in these cells and have the attraction that, at least in some cases, gene amplification is stable for extended periods in the absence of continued MTX selection. Like myeloma cells, CHO cells can be grown on a large scale in suspension culture using serum-free media. Immunoglobulin transcription control elements do not function efficiently in CHO cells but there are a number of strong promoters available. For instance, a humanized antibody has been produced from CHO cells by placing the heavy and light chain genes under the control of separate β-actin promoters [186]. In this case, the DHFR gene and both immunoglobulin genes were all on the same plasmid. Although MTX selection did lead to increased productivity, the levels of antibody achieved after a single round of amplification were low (4 μg/ml). Better results were obtained in this case by cotransfection of a plasmid containing just the β-actin/heavy chain transcription unit with a separate plasmid containing the DHFR gene and the β-actin/light chain gene. Selection for amplification of the light chain plasmid led to cell lines secreting up to 100 $\mu g/10^6$ cells per day and accumulating 200 μg/ml of antibody. It is not reported whether the heavy chain gene was coamplified.

GS gene amplification has also been used to express immunoglobulin genes efficiently in CHO cells, by using the CMV-MIE promoter to express the immunoglobulin genes [155]. The vector used is essentially similar to that shown in Figure 3.13C, but a GS minigene (containing part cDNA and part genomic sequences) expressed from an SV40 late promoter is used in CHO cells, since this seems to amplify more readily. Cell lines secreting up to 15 $\mu g/10^6$ cells per day and accumulating 200 μg/ml of a chimeric B72.3 antibody have been obtained (see Table 3.1).

An alternative approach is to use two amplifiable markers, one to select for efficient expression of the light chain gene and another for the heavy chain gene. An IgM antibody has been made (although without the J chain) by selecting for amplification of a light chain vector containing a DHFR gene and amplification of the heavy chain with an adenosine deaminase-selectable marker in a separate transfection [192]. A cell line making the complete antibody was then formed by fusion of

TABLE 3.1. Expression of Recombinant B72.3 Antibodies

Molecule	Promoter	Host	Selection	Yield (mg/L)	Reference
IgG1	Ig	SP2/0	gpt + neo	60	[184]
	Ig	SP2/0	gpt + neo	20	[187]
	Ig	SP2/0	DHFR[1]	150	[184]
IgG4	CMV-MIE	CHO	gpt + neo	100	[188, 189]
	CMV-MIE	CHO	GS[1]	200	[155]
	CMV-MIE	NSO	GS[1]	560	[185]
Fab	CMV-MIE	CHO	gpt + neo	120	[190]
Fv	CMV-MIE	CHO	gpt	2	[191]

[1]Selection for gene amplification.

the cell lines expressing heavy and light chains. The fusion product secreted approximately 30 $\mu g/10^6$ cells per day of assembled antibody. In a variation of this procedure, Fouser et al. [193] used sequential amplification of these two marker genes in the same cell line, thus avoiding the necessity of carrying out cell fusion. In a further modification, they also included untranslated regions of the κ gene (from the J/C intron and the 3′-untranslated region of the mRNA), which enhanced expression even in the CHO cells. By this means, cell lines were generated after amplification of each selectable marker that secreted 80–110 μg antibody per 10^6 cells per day. Any approach that uses two sequential amplification regimes is clearly rather time-consuming, and the stability of these cell lines in the absence of continued selection is not reported. However, it is clear from these very high expression levels that CHO cells can secrete antibody at least as efficiently as myeloma-type cells. Figures of 100–110 $\mu g/10^6$ cells per day [186,193] are in fact higher than have been reported for either natural hybridomas or transfected myelomas. Whether these high production rates measured at laboratory scale will allow CHO cell expression to compete with the yields obtained from transfected myelomas at manufacturing scale remains to be seen.

Other Factors Influencing Expression in Mammalian Cells. Mammalian expression systems continue to be developed. One factor that influences expression levels but has frequently been overlooked is the possibility of interference between closely adjacent genes (reviewed in Bebbington [155]). Although genes in the expression vectors are equipped with polyadenylation signals, they do not generally act as transcription terminators, so transcription from one promoter may extend beyond the end of the desired gene. Interference between neighboring promoters may therefore contribute to imbalanced synthesis of the two chains. If, however, all genes are present on a single plasmid, they may be arranged so as to minimize this effect.

Although much of the emphasis has been placed on vectors that are directed to optimizing transcription, the effect on protein synthesis of postranscriptional processes should also be considered. Indeed gene amplification can be used to increase vector copy numbers to such an extent that transcription rates are no longer limiting. The stability of the immunoglobulin mRNA will of course affect its steady-state concentration in the cytoplasm and hence the rate of protein synthesis. RNA stability can be influenced by sequences in the 3′-untranslated region, often in a cell-type-specific manner [194], as well as by the presence of introns. The influence of particular introns and untranslated RNA sequences on antibody expression has been suggested as a contributing factor in the high yields of recombinant antibody obtained from vectors containing immunoglobulin κ genomic sequences in CHO cells [193]. There is also an absolute requirement for introns in immunoglobulin mRNA if the Ig H promoter is used but not if the CMV-MIE promoter directs transcription in myeloma cells [168]. In high-yielding cell lines it is possible that the secretory pathway may ultimately limit the rate of antibody production from the cell. At present, however, little is known about precisely which parts of the secretion apparatus might limit immunoglobulin secretion, apart from the involvement of the ER

protein grp78 described above in section 3.6.2.1. Lastly it should be noted that fermentation conditions can have a profound effect on the amount of product obtained and there is often considerable scope for increasing the concentration of cells in the fermentation system and hence the overall yield (see Chapter 5).

3.6.3. Choosing an Expression System

Comparisons between different reports of antibody expression in mammalian cells must be treated with caution because of the effects of random integration on expression levels ("position effects"), variability in secretion of different antibodies, and the effects of culture conditions on antibody yield. Nevertheless, from the results reported for expression of various genetically engineered antibodies containing V-region sequences of the murine B72.3 antibody (Table 3.1), it is clear that gene amplification systems currently provide the most efficient expression of whole antibodies in both CHO and myeloma cells. On a per cell basis it is possible to produce antibodies equally efficiently from CHO or myeloma hosts. Indeed the highest quoted production rates are from CHO cells. However, the process advantages associated with myeloma cells (higher biomass attainable in currently available media) mean that these have so far produced the highest reported yields in batch fermentations. It may also be worth noting when designing an expression system that vectors using CMV promoters and containing a selectable marker gene expressed from a SV40 early promoter can generally also be used in COS cells or 293 cells for transient expression. This facilitates the analysis of novel engineered molecules before the establishment of transfected cell lines is undertaken.

Because of the higher yields, mammalian-cell expression systems are generally preferable for producing whole antibodies. For the production of fragments or other engineered molecules, it is often less clear which system will provide the highest yields. Fv fragments have been produced with CMV-MIE promoters and gpt selection in NS0 myeloma cells at yields of 8 mg/L [195], but it is likely that *E. coli* expression systems will be more suitable for such molecules, since yields of up to 500 mg/L Fv have been obtained from bacterial expression [196]. For Fab fragments, it is not clear whether bacterial or mammalian expression systems will prove more productive: mammalian expression systems can produce at least 100 mg/L of Fab but the highest yields so far reported are from *E. coli* expression [196,197]. However, it should be borne in mind that comparisons between different antibodies and fragments are not always valid. Secretory pathways in mammalian cells, for instance, may recognize certain recombinant proteins as foreign and fail to secrete them efficiently. Even single amino acid changes can profoundly affect the secretion of immunoglobulin chains [198], so for any chosen molecule it may make sense to try more than one expression system.

It may also be appropriate to consider a number of other factors in addition to productivity if, for instance, the antibody is intended for therapeutic use in humans. Here we consider briefly two significant issues that are discussed further in Chapters 5 and 6: the effect of host cell type and culture conditions on glycosylation, and the implications of host cell type on the downstream processing of the product.

Recombinant antibodies differ from their natural counterparts with respect to the detailed oligosaccharides attached because of host cell differences in glycosylation patterns and possibly the effects of different culture conditions. For instance, mouse cells possess a glycosyl transferase, not present in human or CHO cells, that adds a terminal α (1,3)-gal residue [199,200]. The effects of such differences on the behavior of antibodies *in vivo* have so far not been characterized. Experience with other recombinant proteins suggests that carbohydrate microheterogeneity in general may not significantly affect the properties of the molecules [199]. Furthermore, for most antibodies, the carbohydrate is buried between the two heavy chains and so may not be accessible, for instance, to immune complex formation. Nevertheless, it remains to be seen whether antibodies produced in different host cell types differ in their immunogenicity, rates of clearance from the circulation, or effector functions.

Purification procedures are likely to differ significantly for molecules produced from different host cell types and this may be a contributory factor in the choice of expression system. For instance, antibodies derived from *E. coli* require downstream processing steps to eliminate bacterial toxins such as lipopolysaccharide. Proteins produced intracellularly in bacteria may also be subject to proteolytic degradation. On the other hand, an important issue in the purification of antibodies from mammalian cells is the ensuring of removal of infectious agents. Both CHO and murine myeloma cell lines contain endogenous retroviruses [201,202,203], which can produce viral particles shed into the culture medium. Although there is no evidence that such retroviruses could be harmful to humans, downstream processing steps to remove these and other adventitious agents will inevitably be required.

Although for many purposes, none of the expression systems described would appear to be excluded, the choice of production process will inevitably take into account the costs associated with the entire expression system, including the requirements for downstream processing.

3.7. SUMMARY

It is now possible to use genetic manipulation to generate antibodies with improved properties. For example, rodent antibodies can be humanized to produce antibodies that promise to be less immunogenic than their rodent counterparts in human therapy. Recently developed technology involving the use of *E. coli* as an expression host provides almost unlimited potential for the isolation and generation of antibody fragments of the desired specificity and affinity. Such recombinant antibody fragments can be produced efficiently from *E. coli* or can be linked at the genetic level to an isotype that carries the effector function of choice or to a toxin molecule. The rebuilt antibody genes can then be introduced into mammalian cells. Advances in expression systems for mammalian cells, particularly using vector amplification in rodent myeloma or CHO cells, means that it is now feasible to produce many engineered antibodies for human therapeutic use economically from mammalian cell fermentations.

The "designer" antibodies that result from the application of these recombinant techniques could provide invaluable reagents for therapy and diagnosis. The next few years promise to be an exciting era for the field of antibody engineering.

3.8. REFERENCES

1. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N. Science 230, 1350 (1985).
2. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A. Science 239, 487 (1988).
3. Neuberger, M.S. EMBO J. 2, 1373 (1983).
4. Oi, V.T., Morrison, S.L., Herzenberg, L.A., Berg, P. Proc. Natl. Acad. Sci. USA 80, 825 (1983).
5. Ochi, A., Hawley, R.G., Hawley, T., Shulman, M.J., Traunecker, A., Köhler, G., Hozumi, N. Proc. Natl. Acad. Sci. USA 80, 6351 (1983).
6. Neuberger, M.S., Williams, G.T., Fox, R.O. Nature 312, 604 (1984).
7. Schnee, J.M., Runge, M.S., Matsueda, G.R., Hudson, N.W., Seidman, J.G., Haber, E., Quertemous, T. Proc. Natl. Acad. Sci. USA 84, 6904 (1987).
8. Sharon, J., Gefter, M.L., Manser, T., Morrison, S.L., Oi, V.T., Ptashne, M. Nature 309, 364 (1984).
9. Liu, A.Y., Robinson, R.R., Hellström, K.E., Murray, E.D., Jr., Chang, C.P., Hellström, I. Proc. Natl. Acad. Sci. USA 84, 3439 (1987).
10. Morrison, S.L. Science 229, 1202 (1985).
11. Sun, L.K., Curtis, P., Rakowicz-Szulczynska, E., Ghrayeb, J., Chang, N., Morrison, S.L., Koprowski, H. Proc. Natl. Acad. Sci. USA 84, 214 (1987).
12. Boulianne, G.L., Hozumi, N., Shulman, M.J. Nature 312, 643 (1984).
13. Morrison, S.L., Johnson, M.J., Herzenberg, L.A., Oi, V.T. Proc. Natl. Acad. Sci. USA 81, 6851, (1984).
14. Better, M., Chang, C.P., Robinson, R.R., Horwitz, A.H. Science 240, 1041 (1988).
15. Skerra, A. Plückthun, A. Science 240, 1038 (1988).
16. Hiatt, A., Cafferkey, R., Bowdish, K. Nature 342, 76 (1989).
17. Hasemann, C.A., Capra, J.D. Proc. Natl. Acad. Sci. USA 87, 3942 (1990).
18. Morrison, S.L., Oi, V.T. Adv. Immunol. 44, 65 (1989).
19. Winter, G., Milstein, C. Nature 349, 293 (1991).
20. Mattila, P., Korpela, J., Tenkùanen, T., Pitkänen, K. Nucleic Acids Res. 19, 4967 (1991).
21. Higuchi, R. In Erlich (ed.): "PCR Technology: Principles and Applications for DNA Amplification (p. 61). New York, London, Tokyo, Melbourne, Hong Kong: Stockton Press, (1989).
22. Evan, G., Lewis, G.K., Ramsay, G., Bishop, J.M. Mol. Cell. Biol. 5, 3610 (1985).
23. Stoflet, E.S., Koeberl, D.D., Sarkas, G., Sommer, S.S. Science 239, 491 (1988).
24. Scheffield, V.C., Cox, D.R., Lerman, L.S., Myers, R.M. Proc. Natl. Acad. Sci. USA 86, 232 (1989).

25. Ochman, H., Gerber, A.S., Hartl, D.L. Genetics 120, 621 (1988).
26. Loh, H.Y., Elliott, J.F., Cwirla, S., Lanier, L.L., Davis, M.M. Science 242, 217 (1989).
27. Orlandi, R., Güssow, D.H., Jones, P.T., Winter, G. Proc. Natl. Acad. Sci. USA 86, 3833 (1989).
28. Chiang, Y.L., Sheng-Dong, R., Brow, M.A., Larrick, J.W. BioTechniques 7, 360 (1989).
29. Larrick, J.W., Danielsson, L., Brenner, C.A., Wallace, E.F., Abrahamson, M., Fry, K.E., Borrebaeck, A.K. Biotechnology 7, 934 (1989).
30. Larrick, J.W., Danielsson, L., Brenner, C.A., Abrahamson, M., Fry, K.E., Borrebaeck, C.A.K. Biochem. Biophys. Res. Commun. 160, 1250 (1989).
31. Huse, W.D., Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton, D.R., Benkovic, S.J., Lerner, R.A. Science 246, 1275 (1989).
32. Sanz, I., Kelly, P., Williams, C., School, S., Tucker, P., Capra, J.D. EMBO J. 8, 3741 (1989).
33. Marks, J.D., Tristem, M., Karpas, A., Winter, G. Eur. J. Immunol. 21, 985 (1991).
34. Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G. J. Mol. Biol. 222, 581 (1991).
35. Mullinax, R.L., Gross, E.A., Amberg, J.R., Hay, B.N., Hogrefe, H.H., Kubitz, M.M., Greener, A., Alting-Mees, M., Ardourel, D., Short, J.M., Sorge, J.A., Shopes, B. Proc. Natl. Acad. Sci. USA 87, 8095 (1990).
36. Persson, M.A.A., Caothien, R.H., Burton, D.R. Proc. Natl. Acad. Sci. USA 88, 2432 (1991).
37. Kabat, E.A., Wu, T.T., Perry, H.M., Gottesmann, K.S., Foeller, C. Washington, DC: U.S. Department of Health and Human Services, U.S. Government Printing Office, 1991.
38. Choi, Y., Kotzin, B., Herron, L., Callahan, J., Marrack, P., Kappler, J. Proc. Natl. Acad. Sci. USA 86, 8942 (1989).
39. Oksenberg, J.R., Stuart, S., Begovich, A.B., Bell, R.B., Erlich, H.A., Steinman, L., Bernard, C.C.A. Nature 345, 344 (1990).
40. Bragado, R., Lauzurica, P., López, D., López de Castro, J.A. J. Exp. Med. 171, 1189 (1990).
41. Wucherpfennig, K.W., Ota, K., Endo, N., Seidman, J.G., Rosenzweig, A., Weiner, H.L., Hafler, D.A. Science 248, 1016 (1990).
42. Panzara, M.A., Gussoni, E., Steinman, L., Oksenberg, J.R. BioTechniques 12, 728 (1992).
43. Uematsu, Y., Wege, H., Straus, A., Ott, M., Bannwarth, W., Lanchbury, J., Panayi, G., Steinmetz, M. Proc. Natl. Acad. Sci. USA 88, 8534 (1991).
44. Ohara, O., Dorit, R.L., Gilbert, W. Proc. Natl. Acad. Sci. USA 86, 5673 (1989).
45. Innis, M.A., Myambo, K.B., Gelfand, D.H., Brow, M.A.D. Proc. Natl. Acad. Sci. USA 85, 9436 (1988).
46. Lee, J.-S. DNA Cell Biol. 10, 67 (1991).
47. Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R. Gene 77, 61 (1989).
48. Horton, R.M., Cai, Z., Ho, S.N., Pease, L.R. BioTechniques 8, 528 (1990).
49. Clackson, T.C., Hoogenboom, H., Griffiths, A.D., Winter, G.P. Nature (London) 352, 624 (1991).

50. Carter, P., Bedouelle, H., Winter, G. Nucleic Acids Res. 13, 4431 (1985).
51. Leung, D.W., Chen, E., Goeddel, D.V. J. Methods Cell. Mol. Biol. 1, 11 (1989).
52. Gram, H., Marconi, L.-A., Barbas, C.F. III, Collet, T.A., Lerner, R.A., Kang, A.S. Proc. Natl. Acad. Sci. USA 89, 3576 (1992).
53. Porter, R.R. Science 180, 713 (1973).
54. Saul, F.A., Amzel, L.M., Poljak, R.J. J. Biol. Chem. 253, 585 (1978).
55. Marquart, M., Deisenhofer, J., Huber, R. J. Mol. Biol. 141, 369 (1980).
56. Amit, A.G., Mariuzza, R.A., Phillips, S.E.V., Poljak, R.J. Science 233, 747 (1986).
57. Satow, Y., Cohen, G.H., Padlan, E.A., Davies, D.R. J. Mol. Biol. 190, 593 (1986).
58. Colman, P.M., Laver, W.G., Varghese, J.N., Baker, A.T., Tullock, P.A., Air, G.M., Webster, R.G. Nature 326, 358 (1987).
59. Sheriff, S., Silverton, E.W., Padlan, E.A., Cohen, G.H., Smith-Gill, S.J., Finzel, B.C., Davies, D.R., Proc. Natl. Acad. Sci. USA 84, 8075 (1987).
60. Padlan, E.A., Silverton, E.W., Sheriff, S., Cohen, G.H., Smith-Gill, S.J., Davies, D.R. Proc. Natl. Acad. Sci. USA 86, 5938 (1989).
61. Chothia, C., Lesk, A.M. J. Mol. Biol. 196, 901 (1987).
62. Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith-Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R., Colman, P.M., Spinelli, S., Alzari, P.M., Poljak, R.J. Nature 342, 877 (1989).
63. Inbar, D., Hochman, J., Givol, D. Proc. Natl. Acad. Sci. USA 2659 (1972).
64. Ward, E.S., Güssow, D.H., Griffiths, A.D., Jones, P.T., Winter, G. Nature 341, 544 (1989).
65. Blackwell, T.K., Alt, F.W. In Hames, Glover (eds.): Molecular Immunology (p. 1), Oxford, Washington, DC: IRL Press, 1988.
66. Carson, D.A., Freimark, B.D. Adv. Immunol. 38, 275 (1986).
67. Borrebaeck, C.A.K. Immunol. Today 9, 355 (1988).
68. Thompson, K.M. Immunol. Today 6, 113 (1988).
69. Meeker, T., Lowder, J., Maloney, D., Miller, R., Thielemans, K., Wainke, R., Levy, R. Blood 65, 1349 (1985).
70. Khazaeli, M., Saleh, M., Wheeler, R., Huster, W., Holden, H., Carrano, R., LoBuglio, A. J. Natl. Cancer Inst. 80, 937 (1988).
71. Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., Winter, G. Nature 321, 522 (1986).
72. Riechmann, L., Clark, M., Waldmann, H., Winter, G. Nature (London) 332, 323 (1988).
73. Brüggeman, M., Williams, G.T., Bindon, C.I., Clark, M.R., Walker, M.R., Jefferis, R., Waldmann, H., Neuberger, M.A. J. Exp. Med. 166, 1351 (1987).
74. LoBuglio, A.F., Wheeler, R.H., Trang, J., Haynes, A., Rogers, K., Harvey, E.B., Sun, L., Ghrayeb, J., Khazaeli, M.B. Proc. Natl. Acad. Sci. USA 86, 4220 (1989).
75. Mueller, B.M., Reisfeld, R.A., Gillies, S.D. Proc. Natl. Acad. Sci. USA 87, 5702 (1990).
76. Ellerson, J.R., Yasmeen, D., Painter, R.H., Dorrington, K.J. J. Immunol. 116, 510 (1976).
77. Verhoeyen, M., Milstein, C., Winter, G. Science 239, 1534 (1988).

78. Queen, C., Schneider, W.P., Selick, H.E., Payne, P.W., Landolfi, N.F., Duncan, J.F., Avdalovic, N.M., Levitt, M., Junghans, R.P., Waldmann, T.A. Proc. Natl. Acad. Sci. USA 86, 10029 (1989).
79. Shalaby, M.R., Shepard, H.M., Presta, L., Rodrigues, M.S., Beverley, P.C.L., Feldmann, M., Carter, P. J. Exp. Med. 175, 217 (1992).
80. Tempest, P.R., Bremner, P., Lambert, M., Taylor, G., Furze, J.M., Carr, F.J., Harris, W.J. BioTechnology 9, 266 (1991).
81. Co, M.S., Deschamps, M., Whitley, R.J., Queen, C. Proc. Natl. Acad. Sci. USA 88, 2869 (1991).
82. Maeda, H., Matsushita, S., Eda, Y., Kimachi, K., Tokiyoshi, S., Bendig, M.M. Hum. Antibodies Hybridomas 2, 124 (1991).
83. Kettleborough, C.A., Saldanha, J., Heath, V.J., Morrison, C.J., Bendig, M.M. Protein Eng. 4, 773 (1991).
84. Hale, G., Dyer, M.J., Clark, M.R., Phillips, J.M., Riechmann, L., Waldmann, H. Lancet 2, 1394 (1988).
85. Mathieson, P.W., Cobbold, S.P., Hale, G., Clark, M.R., Oliveira, D.B.G., Lockwood, C.M., Waldmann, H. N. Engl. J. Med. 323, 250 (1990).
86. Isaacs, J.D., Watts, R.A., Hazleman, B.L., Hale, G., Keogan, M.T., Cobbold, S.P., Waldmann, H. Lancet 340, 748 (1992).
87. Tramontano, A., Chothia, C., Lesk, A.M. J. Mol. Biol. 215, 175 (1990).
88. Foote, J., Winter, G. J. Mol. Biol. 224, 487 (1992).
89. McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J. Nature (London) 348, 552 (1990).
90. Kang, A.S., Barbas, C.F., Janda, K.D., Benkovic, S.J., Lerner, R.A. Proc. Natl. Acad. Sci. USA 88, 4363 (1991).
91. Kohler, G., Milstein, C. Nature 256, 52 (1984).
92. Jackson, R.H., McCafferty, J., Johnson, K.S., Pope, A.R., Roberts, A.J., Chiswell, D.J., Clackson, T.P., Griffiths, A.D., Hoogenboom, H.R., Winter, G. In Rees, Sternberg, Wetzel (eds.): Protein Engineering (p. 277). Oxford, Washington, DC: IRL Press, 1992.
93. Güssow, D., Ward, E.S., Griffiths, A.D., Jones, P.T., Winter, G. Cold Spring Harbor Laboratory Quant. Biol. 54, 265 (1989).
94. Gherardi, E., Milstein, C. Nature 357, 201 (1992).
95. Cabilly, S., Riggs, A.D., Pande, H., Shively, J.E., Holmes, W.E., Rey, M., Perry, L.J., Wetzel, R., Heyneker, H.L. Proc. Natl. Acad. Sci. USA 81, 3273 (1984).
96. Boss, M.A., Kenten, J.H., Wood, C.R., Emtage, J.S. Nucleic Acids Res. 12, 3791 (1984).
97. Kurokawa, T., Seno, M., Sasada, R., Ono, Y., Onda, H., Igarashi, K., Kikuchi, M., Sugino, Y., Honjo, T. Nucleic Acids Res. 11, 3077 (1983).
98. Ishizaka, T., Helm, B., Hakimi, J., Niebyl, J., Ishizaka, K., Gould, H. Proc. Natl. Acad. Sci. USA 83, 8323 (1986).
99. Liu, F., Albrandt, K.A., Bry, C.G., Ishizaka, T. Proc. Natl. Acad. Sci. USA 81, 5369 (1984).
100. Buchner, J., Rudolph, R. BioTechnology 9, 157 (1991).

101. Whitlow, M., Filpula, D. Methods: A Companion to Methods in Enzymology (Vol. 2, 97), 1991.
102. Chothia, C. Novotny, J., Bruccoleri, R., Karplus, M. J. Mol. Biol. 186, 651 (1985).
103. Glockshuber, R., Malia, M., Pfitzinger, I., Plückthun, A. Biochemistry 29, 1362 (1990).
104. Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R., Oppermann, H. Proc. Natl. Acad. Sci. USA 85, 5879 (1988).
105. Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufmann, B.M., Lee, S.L., Pope, S.H., Riordan, G.S., Whitlow, M. Science 242, 423 (1988).
106. McCartney, J.E., Lederman, L., Drier, E.A., Cabral-Denison, N.A., Wu, G.-M., Batorsky, R.S., Huston, J.S., Opperman, H. J. Protein Chem. 10, 669 (1991).
107. Brinkmann, U., Pai, L.H., FitzGerald, D.J., Willingham, M., Pastan, I. Proc. Natl. Acad. Sci. USA 88, 8616 (1991).
108. Gibbs, R.A., Posner, B.A., Filpula, D.R., Dodd, S.W., Finkelman, M.A.J., Lee, T.K., Wroble, M., Whitlow, M., Benkovic, S.J. Proc. Natl. Acad. Sci. USA 88, 4001 (1991).
109. Laroche, Y., Demaeyer, M., Stassen, J.-M., Gansemans, Y., Demarsin, E., Matthyssens, G., Collen, D., Holvoet, P. J. Biol. Chem. 266, 16343 (1991).
110. Anand, N.N., Mandal, S., MacKenzie, C.R., Sadowska, J. Sigurskjold, B., Young, N.M., Bundle, D.R., Narang, S.A. J. Biol. Chem. 266, 21874 (1991).
111. Williams, W.V., Moss, D.A., Kieber-Emmons, T., Cohen, J.A., Myers, J.N., Weiner, D.B., Greene, M.I. Proc. Natl. Acad. Sci. USA 86, 5537 (1989).
112. Taub, R., Gould, R.J., Garsky, V.M., Ciccarone, T.M., Hoxie, J., Friedman, P.A., Shattil, S.J. J. Biol. Chem. 264, 259 (1989).
113. Kang, A.S., Jones, T.M., Burton, D.R. Proc. Natl. Acad. Sci. USA 88, 11120 (1991).
114. Mullinax, R.L., Gross, E.A., Hay, B.N., Amberg, J.R., Kubitz, M.M., Sorge, J.A. BioTechniques 12, 864 (1992).
115. Caton, A.J., Koprowski, H. Proc. Natl. Acad. Sci. USA 87, 6450 (1990).
116. Berek, C., Milstein, C. Immunol. Rev. 105, 5 (1988).
117. French, D.L., Laskov, R., Scharff, M.D. Science 244, 1152 (1989).
118. Chang, C.N., Landolfi, N.F., Queen, C. J. Immunol. 147, 3610 (1991).
119. Barbas, C.F. III, Kang, A.S., Lerner, R.A., Benkovic, S.J. Proc. Natl. Acad. Sci. USA 88, 7978 (1991).
120. Burton, D.R., Barbas, C.F. III, Persson, M.A.A., Koenig, S., Chanock, R.M., Lerner, R.A. Proc. Natl. Acad. Sci. USA 88, 10134 (1991).
121. Zebedee, S.L., Barbas, C.F. III, Hom, Y.-L., Caothien, R.H., Graff, R., DeGraw, J., Pyati, J., LaPolla, R., Burton, D.R., Lerner, R.A., Thornton, G.B. Proc. Natl. Acad. Sci. USA 89, 3175 (1992).
122. Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P., Winter, G. Nucleic Acids Res. 19, 4133 (1991).
123. Duchosal, M.A., Eming, S.A., Fischer, P., Leturcq, D., Barbas, C.F. III, McConahey, P.J., Caothien, R.H., Thornton, G.B., Dixon, F.J., Burton D.R. Nature 355, 258 (1992).

124. Brüggemann, M., Caskey, H.M., Teale, C., Waldmann, H., Williams, G.T., Surani, M.A., Neuberger, M.S. Proc. Natl. Acad. Sci. USA 86, 6709 (1989).
125. Borrebaeck, C.A.K., Danielsson, L. Möller, S.A. Proc. Natl. Acad. Sci. USA 85, 3995 (1988).
126. McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Lieberman, M., Weissman, I.L. Science 241, 1632 (1988).
127. Carlsson, R., Martensson, C., Kalliomaki, S., Ohlin, M., Borrebaeck, C.A.K. J. Immunol. 148, 1065 (1992).
128. Stanfield, R.L., Fieser, T.M., Lerner, R.A., Wilson, I.A. Science 248, 712 (1990).
129. Ward, E.S., Güssow, D.H., Griffiths, A., Jones, P.T., Winter, G.P. In Melchers, et al. (eds.): Progress in Immunology (p. 1144). Berlin: Springer-Verlag, 1989.
130. Barbas, C.F. III, Bain, J.D., Hoekstra, D.M., Lerner, R.A. Proc. Natl. Acad. Sci. USA 89, 4457 (1992).
131. Covell, D.G., Barbet, J., Holton, O.D., Black, C.D.V., Parker, R.J., Weinstein, J.N. Cancer Res. 46, 3969 (1986).
132. Colcher, D., Bird, R., Roselli, M., Hardman, K.D., Johnson, S., Pope, S., Dodd, S.W., Pantoliano, M.W., Milenic, D.E., Schlom, J. J. Natl. Cancer Inst. 82, 1191 (1990).
133. Sealy, D., Nedelman, M., Tai, M.-S., Huston, J.S., Berger, H., Lister-James, J., Dean, R.T. J. Nuclear Med. 31, 776 (1990).
134. King, D., Mountain, A., Harvey, A., Weir, N., Owens, R., Proudfoot, K., Phipps, A., Adair, J., Lisle, H., Bergin, S., Lawson, A., Rhind, S., Pedley, B., Boden, J., Begent, R., Yarranton, G. Antibody Immunoconj. Radiopharm. 4, 210 (1991).
135. Cumber, A.J., Ward, E.S., Winter, G., Parnell, G.D., Wawrzynczak, E.W. J. Immunol. 149, 120 (1992).
136. Pollock, R.R., French, D.L., Metlay, J.P., Birshtein, B.K., and Scharff, M.D. Eur. J. Immunol. 20, 2021, (1990).
137. Tao, M-H., and Morrison, S.L. J. Immunol. 143, 2595 (1989).
138. Spiegelberg, H.L., Fishkin, B.G. Clin. Exp. Immunol. 10, 599 (1972).
139. Sutherland, R., Buchegger, F., Schreyer, M., Vacca, A., Mach, J. Cancer Res. 47, 1627 (1987).
140. Burton, D.R. In Calabi, Neuberger (eds.): Molecular Genetics of Immunoglobulins. Amsterdam: Elsevier, 1987.
141. Duncan, A.R., Woof, J.M., Partridge, L.J., Burton, D.R., Winter, G. Nature (London) 332, 563 (1988).
142. Duncan, A.R., Winter, G. Nature (London) 332, 738 (1988).
143. Steplewski, Z., Sun, L.K., Shearman, C.W., Ghrayeb, J., Daddona, P., Koprowski, H. Proc. Natl. Acad. Sci. USA 85, 4852 (1988).
144. Lucisano. V.Y.M., Lachmann, P.J. Clin. Exp. Immunol. 84, 1 (1991).
145. Chaudhary, V.K., Queen, C., Junghans, R.P., Waldmann, T.A., FitzGerald, D.J., Pastan, I. Nature 339, 394 (1989).
146. Chaudhary, V.K., Batra, J.K., Gallo. M.G., Willingham, M.C., FitzGerald, D.J., Pastan, I. Proc. Natl. Acad. Sci. USA 87, 1066 (1990).
147. O'Hare, M., Brown, A.N., Hussain, K., Gebhardt, A., Watson, G., Roberts, L.M., Vitetta, E.S., Thorpe, P.E., Lord, J.M., FEBS Lett. 273, 200 (1990).

148. Chovnick, A., Schneider, W.P., Tso, J.Y., Queen, C., Chang, C.N. Cancer Res. 51, 465 (1991).
149. Seetharam, S., Chaudhary, V.K., FitzGerald, D., Pastan, I. J. Biol. Chem. 266, 17376 (1991).
150. Gould, B.J., Borowitz, M.J., Groves, E.S., Carter, P.W., Anthony, D., Weiner, L.M., Frankel, A.E. J. Natl. Cancer Inst. 81, 775 (1989).
151. Milstein, C., Cuello, A.C. Nature 305, 537 (1983).
152. Better, M., Horwitz, A.H. Methods Enzymol. 178, 476 (1989).
153. Horwitz, A.H., Chang, P., Better, M., Hellstrom, K.E., Robinson, R.R. Proc. Natl. Acad. Sci. 85, 8678 (1988).
154. Benvenuto, E., Ordas, R.J., Tavazza, R., Ancora, G., Biocca, S., Cattaneo, A., Galeffi, P. Plant Mol. Biol. 17, 865 (1991).
155. Bebbington, C.R. Methods 2, 136 (1991).
156. Whittle, N., Adair, J., Lloyd, C., Jenkins, L., Devine, J., Schlom, J., Raubitschek, A., Colcher, D., Bodmer, M. Protein Eng. 1, 499 (1987).
157. Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B.B., Henner, D., Wong, W.C.T., Rowland, A.M., Koch, C., Carver, M., Shepard, H.M. Proc. Natl. Acad. Sci. 89, 4285 (1992).
158. Southern, P., Berg, P. J. Mol. Appl. Genet. 1, 327 (1982).
159. Mulligan, R.C., Berg. P. Science 209, 1422 (1980).
160. Toneguzzo, F., Keating, A., Glynn, S., McDonald, K. Nucleic Acids Res. 16, 5515 (1988).
161. Gillies, S.D., Morrison, S.L., Oi, V.T. Cell 33, 717 (1983).
162. Rice, D., Baltimore, D. Proc. Natl. Acad. Sci. USA 79, 7862 (1983).
163. Potter, H., Weir, L., Leder, P. Proc. Natl. Acad. Sci. USA 81, 7161 (1984).
164. Plon, S.E., Groudine, M. Curr. Biol. 1, 13 (1991).
165. Banerji, J., Olson, L., Schaffner, W. Cell 33, 729 (1983).
166. Picard, D., Schaffner, W. Nature 307, 80 (1984).
167. Neuberger, M.S., Williams, G.T. Nucl. Acids Res. 16, 6713 (1988).
168. Shearman, C.W., Kanzy, E.J., Lawrie, D.K., Li, Y., Thammana, P., Moore, G.P., Kurrle, R.J. J. Immunol. 146, 928 (1991).
169. Weissenhorn, W., Weiss, E., Schwirzke, M., Kahnze, B., Weidle, U.H. Gene 106, 273 (1991).
170. Caulcott, C.A., Boraston, R., Hill, C., Thompson, P.W., Birch, J.R. In Collins (ed.): Complementary Immunoassays. New York: John Wiley.
171. Beidler, C.B., Ludwig, J.R., Cardenas, J., Phelps, J., Papworth, C.G., Melcher, E., Sierzega, M., Myers, L.J., Unger, B.W., Fisher, M., David, G.S., Johnson, M.J. J. Immunol. 141, 4053 (1988).
172. Hendershot, L.M., Ting, J., Lee, A.S. Mol. Cell. Biol. 8, 4250 (1988).
173. Kohler, G. Proc. Natl. Acad. Sci. USA 77, 2197 (1980).
174. Petterson, S., Cook, G.P., Bruggemann, M., Williams, G.T., Neuberger, M.S. Nature 344, 165 (1990).
175. Meyer, K.B., Neuberger, M.S. EMBO J. 8, 1959 (1989).
176. Eccles, S., Sarver, N., Vidal, M., Cox, A., Grosveld, F. New Biologist 2, 801 (1990).

177. Fell, H.P., Yarnold, S., Hellstrom, I., Hellstrom, K.E., Folger, K.R., Proc. Natl. Acad. Sci. USA 86, 8507 (1989).
178. Wood, C.R., Morris, G.E., Alderman, E.M., Fouser, L., Kaufman, R.J. Proc. Natl. Acad. Sci. USA 88, (1991).
179. Baker, M.D., Schulmann, M.J. Mol. Cell. Biol. 8, 4041 (1988).
180. Smith, K.A., Gorman, P.A., Stark, M.B., Groves, R.P., Stark, G.R. Cell 63, 1219 (1990).
181. Kaufman, R.J. Methods Enzymol. 185, 537 (1990).
182. Dorai, H., Moore, G.P. J. Immunol. 139, 4232 (1987).
183. Gillies, S.D., Dorai, H., Wesolowski, J., Majeau, G., Young, D., Boyd, T., Gardner, J., James K. Bio/Technol. 7, 799 (1989).
184. Gillies, S.D., Lo, K-M., Wesolowski, J. J. Immunol. Methods, 125, 191 (1989).
185. Bebbington, C.R., Renner, G., Thomson, S., King, D., Abrams, D., Yarranton, G.T. Bio/Technology 10, 169 (1992).
186. Page, M.J., Sydenham, M.A. Bio/Technology 9, 64 (1991).
187. Hutzell, K., Kashmiri, S., Colcher, D. Cancer Res. 51, 181 (1991).
188. Colcher, D., Milenic, D., Roselli, M. Cancer Res. 49, 1738 (1989).
189. King, D.J., Adair, J.R., Angal, S., Low, D.C., Proudfoot, K.A., Lloyd, C., Bodmer, M.W., Yarranton, G.T. Biochem. J. 281, 317 (1992).
190. King, D.J., Mountain, A., Adair, J.R., Owens, R.J., Harvey, A., Weir, N., Proudfoot, K.A., Phipps, A., Lawson, A., Rhind, S.K., Pedley, B., Boden, J., Boden, R., Begent, R.H.J., Yarranton, G.T. Antibody Immunoconj. Radiopharm. 5, 159 (1992).
191. Owens, R.J., King, D.J., Howat, D., Mountain, A., Harvey, A., Lawson, A., Rhind, S., Pedley, B., Bode, J., Begent, R., Yarranton, G.T. Antibody Immunoconj. Radiopharm. 4, 459 (1991).
192. Wood, C.R., Dorner, A.J., Morris, G.E., Alderman, E.M., Wilson, D., O'Hara, R.M., Kaufman, R.J. J. Immunol. 145, 3011 (1990).
193. Fouser, L.A., Swanberg, S.L., Lin, B-Y., Benedict, M., Kelleher, K., Cumming, D., Riedel, G.E. Bio/Technology 10, 1121 (1992).
194. Cox, A., Emtage, J.S. Nucleic Acids Res. 17, 10439.
195. Riechmann, L., Foote, J., Winter, G. J. Mol. Biol. 203, 825 (1988).
196. Adair, J.R. Immunol. Rev. 130, 5 (1992).
197. Carter, P., Kelley, R.F., Rodrigues, M.L., Snedcor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourell, J.H., Shepard, H.M., Henner, D. Bio/Technology 10, 163 (1992).
198. Nakaki, T., Deans, R.J., Lee, A.S. Mol. Cell. Biol. 9, 2233 (1989).
199. Liu, D.T-Y. Trends Biotechnol. 10, 114 (1992).
200. Bebbington, C.R. In Zola, H. (ed.): Monoclonal Antibodies: The Next Generation (p. 163). Bios. Scientific, 1994.
201. Manley, K.F., Givens, J.F., Taber, R.L., Ziegel, R.F. J. Gen. Virol. 39, 505 (1978).
202. Stoye, J.P., Coffin, J.M. J. Virol. 61. 2659 (1987).
203. Risser, R., Horowitz, J.M., McCubrey, J. Annu. Rev. Genet. 17, 85 (1983).