Phage Display in Biotechnology and Drug Discovery

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A phage-derived synthetic antibody against human death receptor DR5. At the top, a phage-displayed antigen-binding fragment (Fab) was used as a framework to present synthetic CDR loops derived from a binary code that encodes only tyrosine and serine. At the center, synthetic Fab that recognizes human DR5 (red) with high affinity and specificity was selected and the X-ray crystal structure was determined (PDB ID code 1ZA3). At the bottom, the structure reveals that the third complementarity determining region (CDR) of the heavy chain plays a dominant role in antigen recognition. The CDR loop contains a biphasic helix with tyrosine and serine residues clustered on opposite faces, and the tyrosine face mediates contact with the antigen. The cover was designed by Frederic Fellouse and David Wood, and structures were rendered with PyMOL (DeLano Scientific, San Carlos, CA).

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Identification of Natural Protein–Protein Interactions with cDNA Libraries

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I. OVERVIEW

The genome projects provided us with a huge amount of information at the DNA level and lead to the identification of thousands of open reading frames. The demand for technologies allowing the functional analysis of gene products is, therefore, dramatically increased. Discovery and characterization of

interacting gene products, molecular recognition, and molecular modeling became central to life sciences. Surface display technology based on two pivotal concepts—physical linkage between genotype and phenotype and rescue of individual clones from large libraries by affinity selection—has the potential to substantially contribute to functional genomics. The expansion of surface display technology in biosciences is facilitated by the adaptability of the systems to high-throughput screening formats for automated library handling. While recombinant DNA techniques allow construction of highly complex molecular libraries, high-throughput screening allows rapid exploration of molecular diversity using combinatorial methods. These technologies are becoming increasingly important as molecular tools for the understanding of protein–protein interactions and for the generation of lead compounds, which, hopefully, will attract the business community to make investments in this novel segment of biotechnology.

II. INTRODUCTION

All surface display technologies exploit the concept of linking the phenotype as a gene product displayed on a surface to its genetic information integrated into the host genome (1). This concept is independent from the organism used and has been successfully applied to construct large molecular libraries in filamentous phage (2–4), phagemids (5–7), lytic bacteriophages (8,9), higher viruses (10,11) as well as prokaryotic (12–14), and eukaryotic (15,16) surface expression systems. When Smith (2) initially proposed the idea of phage display in 1985, he suggested that selection of genes from cDNA libraries could be one of the most significant applications of the technology. However, this potentially interesting area of research has lagged behind, despite the impressive progress of phage display technology achieved during the last years. Among the over 2000 papers describing the use of phage display available to date from the literature, only a few deal with selection of cDNAs.

One of the reasons thereof is a direct consequence of the capsid structure. Most phage or phagemid cloning vectors take advantage of the ability to assemble phage decorated with hybrid versions of the receptor protein pIII or the major coat protein pVIII (17,18). This strategy has proven to be useful for the N-terminal display of random peptide libraries (3,19–22), antibody fragments (23–26), and single proteins and protein domains (27–30) which are directly fused to the coat proteins (31) or to truncated forms thereof in phagemid vectors (32). These approaches have been very successful because they allow direct fusions of the gene products to be displayed to the N-terminus of the capsid proteins. However, the integrity of the C-terminus of pIII and pVIII is essential for efficient phage assembly and, therefore, the original vectors can only tolerate insertion of foreign DNA at the N-terminus (5,9,33). This represents the strongest limitation for the construction of cDNA display libraries. The cDNA inserts encoding the C-terminus of proteins as obtained after poly(A) -priming and reverse transcription (34) always contains translation stop codons, which prevent the synthesis of hybrid coat proteins (5,33,35). To overcome this limitation, several strategies, described in details below, have been devised. Fewer efforts have been invested in the use of the remaining three capsid proteins pVI (36–38), pVII, and pIX (39), but their adequacy as vectors to display cDNA libraries has not yet been tested extensively.

III. CLONING VECTORS

The basic idea of phage display technology consists in the synthesis of a recombinant protein as fusion with a phage coat protein, provided that the fusion does not interfere with phage infectivity or assembly. Historically, the first phage vectors allowing the fusion of polypeptides to pIII or pVIII contained the whole genome. According to the nomenclature proposed by Smith (40), these phage vectors can be described as type 3 and type 8 vectors (Fig. 1). The strongest limitation of these types of vectors is related to the short length of the inserts tolerated by the phage. The major coat protein pVIII

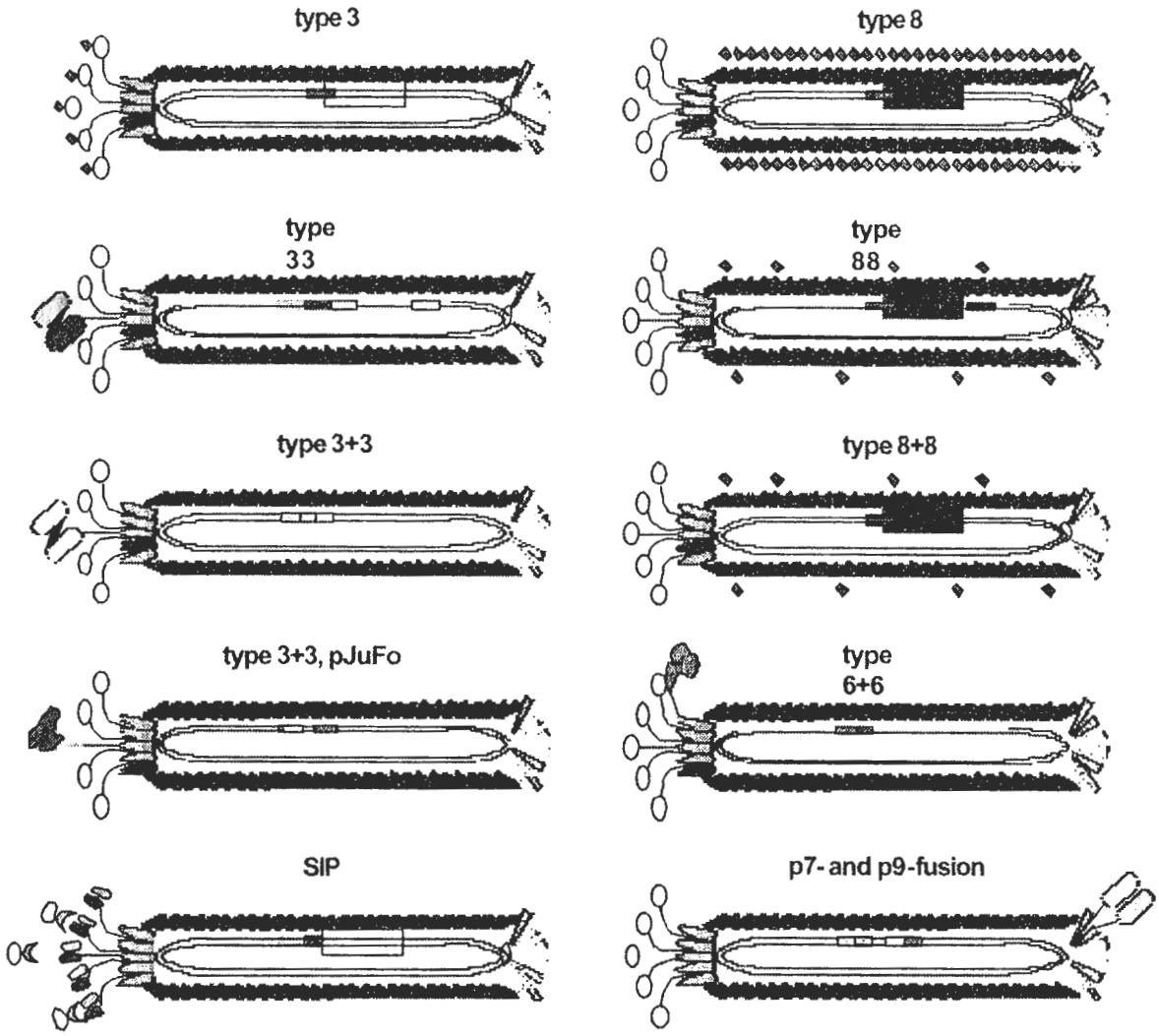

Figure 1 Different mono- and multivalent M13-based phage surface display systems. Vectors of the type 3, 8, 33 and 88 are modified wild type phage. All other systems are phagemid vectors and require co-infection with wild type phage for assembly of infective phagemid particles. See text for further explanations.

can only tolerate very small inserts of about six to eight amino acids between the N-terminal residues three and four (41,42). The pIII allows larger peptides and small proteins to be presented as fusion between the export leader sequence and domain D1 without dramatically affecting its function (19,43). Meanwhile, hybrid phage (type 33 and 88, Fig. 1) has been developed which contains both wild type and fusion coat proteins integrated into the genome (44). The possible drawback of these vectors consists in recombination events between the homologous wild type and fusion DNA regions, resulting in the loss of the information required for the production of fusion proteins (45).

By combination of the best features of phage and plasmids, new types of vectors termed phagemids were created (46,47). These vectors offer several advantages compared to filamentous phage, such as easy preparation of high yields of dsDNA for cloning and sequencing, simple maintenance as replicative plasmid form in bacteria, and adaptability to robot-assisted high-throughput screening technologies (48–50). Phagemids contain a bacterial and a phage origin of replication, the phage packaging signal, antibiotic resistance genes for selection of transformants, and the gene encoding a coat protein used to generate fusions to be displayed on phage surface. As a consequence thereof, they replicate in the host as plasmids and are able to be packaged in a phagemid particle, or recombinant phage, upon infection with a helper phage that provides the genes for the production of the structural, the packaging and assembly proteins needed for phage morphogenesis. Sophisticated helper phage carries mutations in the origin of replication or packaging sequences. Therefore, during replication, the phagemid genome is packaged more efficiently than the helper phage genome. The big advantage of phagemid over phage vectors consists in the possibility of displaying not only small, but also larger peptides (51), large molecules such as antibody fragments (52–54) and many other proteins including enzymes (55,56), enzyme inhibitors (57), and products of cDNA libraries (4,7,33,35,58–60). This becomes possible because the helper phage carries the full complement of capsid-encoding genes. As a competition during phagemid assembly, a mixture of wild type and fusion coat protein can be incorporated into the phage coat (vectors of type 3 + 3 and 8 + 8, Fig. 1). Moreover, the number of fusion protein copies incorporated into the recombinant phage particle (valency) can be influenced using inducible promoters inserted in front of the truncated coat protein gene on the phagemid genome (61).

More recently, other phage coat proteins have been exploited for the display of fusions including pVI (36) used to display cDNA products as C-terminal fusions (type 6 + 6, Fig. 1). The coat proteins pVII and pIX have been used as fusion partners for the display of heavy and light chain

antibody fragments (39); however, these approaches have so far been less commonly used. In addition to the mentioned "standard" phage display vectors, other pIII-based systems classified as "phage two-hybrid systems" have been reported. Formally these vectors have been termed SAP for selection and amplification of phage (62) and SIP for selectively infective phage (63,64). In these systems, the fusion proteins are expressed directly followed by the D2 and D3 domains of pIII rendering all phage infection defective. Infectivity, e.g., the ability of the phage to bind to the F-pilus and hence to infect *Escherichia coli* cells is restored by the selection target fused to the pIII domain(s) D1 or D1 and D2. The SIP technology may represent a powerful tool for rapid selection of protein–protein interactions (65) in spite of the few applications reported so far. Possible display strategies and vectors have been reviewed recently (66) (see also Chapter 2) and will not be discussed here in further detail.

IV. DISPLAY OF cDNA LIBRARIES ON PHAGE SURFACE

Highly diverse display libraries have been constructed by fusing either genomic (67,68) or cDNA fragments (5,7) (35,36,58,60) to gene *III* or gene *VIII* of filamentous phage. In both cases, the display can be a challenge as the presence of stop codons can hamper the generation of N-terminal fusions to the coat proteins, a direct consequence of the capsid structure (33,48,50). Since the integrity of the C-terminus of pIII and pVIII is considered essential for efficient phage assembly, insertions of foreign peptides can only be tolerated at the N-terminus. However, this problem has been alleviated using different strategies. Fusion of cDNAs to the C-terminus of the gene *VI* protein is compatible with phage propagation and packaging (36) as demonstrated in pilot experiments (37). The feasibility of this approach has been clearly demonstrated by the isolation of peroxisomal proteins from human cDNA libraries (38,69) and of a collagen-binding protein from a *Necator americanus* cDNA library (70). Moreover,

Fuh and Sidhu (22) and Fuh et al. (71) have demonstrated that, in contrast to the common belief, polypeptides fused to the C-terminus of both the M13 pIII and pVIII coat proteins are functionally displayed on the phage surface. The C-terminal fusion approach, although not widely used until now, could be of considerable importance to phage display technology and would allow broad investigations of biological problems, which are not suited for N-terminal display. Main areas of interest in this field are the study of protein–protein interactions requiring free C-termini and functional screening of cDNA libraries.

More sophisticated approaches are based on the ability to separate the gene *III* product of filamentous phage into its functional domains: the N-terminal domains binding to the F pilus and mediating infection, and the C-terminal domain morphologically involved in capping the trailing end of the filament according to the vectorial polymerization model (72,73). Although cleavage of the gene *III* product into two separate functional entities is incompatible with phage propagation, infectivity can be restored by joining the segments through noncovalent protein–protein interactions (74,75). The so-called SIP technology (64,65) can be efficiently used to screen cDNA libraries for selection of proteins that interact with a target molecule as demonstrated in a few cases (76,77).

However, the most widely used systems for the construction and screening of cDNA libraries displayed on phage surface involve an indirect fusion strategy where cDNA inserts fused to the 3′ end of the Fos leucine zipper are coexpressed with a truncated form of the gene *III* product decorated with the Jun leucine zipper (5). The phagemid derived by modification of phagemid pComb 3 (78) and formally termed pJuFo (Fig. 2) has been widely used for the isolation of IgE-binding molecules from complex allergenic sources as reviewed elsewhere (7,48,79–81). Selective enrichment of IgE-binding molecules from cDNA libraries constructed using mRNA from *Aspergillus fumigatus* (82), *Malassezia furfur* (83), peanut (84,85), *Alternaria alternata* (86), *Cladosporium herbarum* (87), *Coprinus comatus* (88), storage mites (89), and wheat germ (90) yielded phage displaying hundreds of different

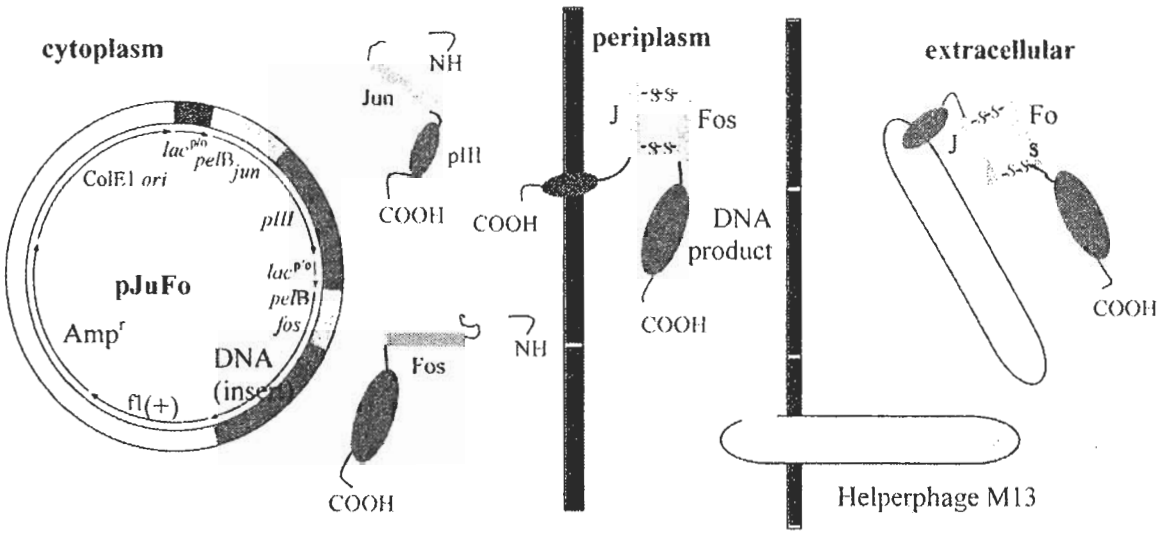

Figure 2 Genetic elements of the pJuFo phagemid and proposed pathway for the assembly of phage surface displayed cDNA libraries. (Modified from Ref. 5.)

IgE-binding proteins. Interestingly, some of these structures represent phylogenetically conserved proteins and share a high degree of sequence identity to their human counterparts. Human proteins, including manganese-dependent superoxide dismutase (91), acidic P_2 ribosomal protein (92), and cyclopilin (93) could also be directly selected from a human lung cDNA library displayed on the pJuFo surface using sera of patients sensitized to *A. fumigatus* as ligand (94,95), thus demonstrating cross-reactivity to the environmental allergens (91,92).

Of course, filamentous phages and phagemids are not the vectors of choice for high-level expression of recombinant proteins. Therefore, all cDNAs isolated from phage surface display libraries need to be subcloned in high-level expression vectors and transformed to a suitable host if relevant amounts of protein are required, for example, for clinical studies (96). In general, inserts subcloned from selected phagemids into high-level expression vectors are well expressed because, for display on phage surface, the genetic information needs to be transcribed and translated by *E. coli*.

Filamentous phage display systems, like any other cloning system, are not universal as they are subjected to biological restrictions imposed by the host and by the codon usage of the cloned inserts. Possible serious biological

limitations derive from the characteristics of the phage life cycle. Filamentous phage particles are released from the host cell without breaking the integrity of the cell membrane. The proteins, which assemble to form the capsid in the periplasmic space, must therefore cross the lipid bilayer of the inner membrane. Therefore, any fusion peptide or protein with biochemical characteristics preventing transmembrane transport will not be integrated into the capsid. To be recognized by the ligand used for selection, displayed proteins need to adapt a conformation able to interact with the ligand. The chemical characteristics of the periplasmic environment, which affect the folding and stability of the recombinant proteins displayed, may influence the ability of hybrid coat proteins to interact with the ligand used for selection. Since cDNA libraries encode very diverse protein domains with different biochemical properties, it is probable that a subset of these proteins or protein fragments will not be displayed and thus not be present in the surface display library. In addition, cDNA libraries, like any other molecular library displayed on phage surface, suffer from host-specific biological limitations related to restriction in codon usage, refolding pathways, and potential toxicity of the expressed gene products for the heterologous host.

However, phage surface display of cDNAs allows for the survey of very large libraries using the discriminative power of affinity selection against homo- and heterogeneous ligands. Although the most successful applications of the pJuFo-based cloning technology are related to IgE-binding molecules using serum IgE from allergic patients as ligand, other successful applications have been reported. Examples are the mapping of protein–ligand interactions using whole genome phage display libraries (67), construction of vectors for stable immobilization of multimeric recombinant proteins (97), and detailed analysis of the C5a anaphylatoxin effector domain (98) followed by selection of a C5a receptor antagonist (99). Pereboev et al. (100) have used pJuFo to display adenovirus type 5 fiber knob as a tetrameric molecule able to bind to the coxsackievirus-Ad receptor, demonstrating the versatile applicability of the cloning system. More recently, pJuFo has been used

to select autoantigens from human cDNA libraries derived from patients suffering from vitiligo (101) and prostate cancer (102). In the first study, purified IgG from serum of vitiligo patients was used to screen a melanocyte cDNA-phage surface display library resulting in the discovery of the melanin-concentrating hormone receptor 1 (MCHR1) as a novel autoantigen related to this autoimmune disorder. Immunoreactivity against the receptor was demonstrated in sera of vitiligo patients using radiobinding assays. Among sera from healthy controls and from patients with other autoimmune diseases, no immunoreactivity to MCHR1 was found, indicating a high disease specificity of autoantibodies raised against the receptor. In the second study, a cDNA library constructed from mRNA isolated from a lymph node metastasis of a patient suffering from hormone refractory prostate cancer (HRPC) was screened with purified autologous and heterologous IgG of patients suffering from prostate cancer. Sequencing of single clones after four rounds of biopanning yielded different cDNAs depending on the amount of IgG used for screening, some of them corresponding to already known cancer-associated antigens. These results, together with the isolation of colorectal-tumor-associated antigens from a primary colorectal tumor cDNA library displayed on the surface as fusion to the gene *VI* protein (37), demonstrate the applicability of phage surface display for identification of cDNA expression products in such diseases as cancer and autoimmune disorders. The pJuFo vector was also used to clone proteins directly interacting with the cytoplasmic tail of the murine IgE-antigen receptor from a murine B cell cDNA library displayed on phage surface (103). In contrast to the previous examples, which used heterogeneous ligands, a homogeneous synthetic 28 amino acid long peptide derived from the cytoplasmic tail of IgE was used as selection bait. Among the inserts from 30 randomly chosen clones sequenced after five rounds of biopanning, two carried cDNA fragments coding for the hematopoietic protein kinase 1 (HPK1). The BIACORE measurements showed that HPK1 interacts in vitro with the cytoplasmic tail of IgE as expected. The binding of HPK1 to the cytoplasmic domain of IgE indicates the

existence of an isotype-specific signal transduction and may represent a missing link to upstream regulatory elements of HPK1 activation.

These examples clearly demonstrate that phage-displayed cDNA expression cloning can be a powerful tool for the isolation of unknown genes. The great advantage of cDNA surface display compared to conventional lambda phage-based methods is that in many cases the functional activity of a protein structure can be used to select interaction partners together with the genetic information required for their production. Thus, sequencing of the DNA of the integrated section of the phage genome can readily elucidate the amino acid sequence of a displayed gene product.

V. PROBLEMS ASSOCIATED WITH THE DISPLAY OF cDNA LIBRARIES ON PHAGE SURFACE

A growing number of observations, published or not, indicate that filamentous phage display technology is subjected to several limitations, some of these already discussed. Obviously, the quality of any cDNA library depends directly on the quality of the cDNA ligated into the vector, which, in turn, is determined by the quality of the mRNA. Although the methods for the isolation of mRNA available are quite reliable, oligo(dT)-priming might generate a high frequency of truncated cDNAs through internal poly(A)-priming during reverse transcription (104). Therefore, for genome wide gene identification, reverse transcription should be done using anchored oligo(dT) primers, which diminish the generation of truncated cDNAs caused by internal poly(A)-priming. During the construction of a cDNA-phage surface display library, every step should be optimized to create the highest frequency of potentially expressible full-length cDNA inserts. Transformation with empty phagemids or phagemids containing short inserts that have a growth advantage over large insert-containing phagemids may result in these undesirable clones becoming over-represented during library amplification. Avoiding overgrowth by defective clones is especially

important for large and highly heterogeneous cDNA expression libraries.

Translational problems related to the codon usage might be alleviated by the use of hosts harboring genes encoding limiting tRNA species like argU, ileY, and leuW to attain reasonable expression levels of proteins affected by rare codon usage (105). Like other prokaryotic-based expression systems, phage display may not be suitable for selection of proteins that require post-translational modifications (e.g. glycosylation, phosporylation, etc.) or heterodimeric assembly for functional activity. Moreover, due to the absence of mammalian chaperonins, conformationally dependent structures may not be efficiently expressed or refolded in these bacterial expression systems and therefore not recognized by the ligand used for selection.

However, a successful screening does not only depend on biological factors, but also on the biopanning strategy used (1,18). Selective enrichment of clones of interest becomes necessary since phage surface display libraries contain large numbers of cognate and uncognate phage. In a standard amplified library with a diversity of 10^8, each single clone is present in several thousand copies among a population of 10^{12}–10^{13} phage molecules. Therefore, the use of efficient selection and screening procedures is one of the key elements which determine the success of the combinatorial approach as discussed in details elsewhere (106). Phage display techniques require immobilization of the target protein to a solid support during biopanning. The immobilization process must maintain the target in a native or native-like conformation for phage selection (107). The commonly used method of protein immobilization through direct adsorption to plastic surfaces denatures many proteins making them unsuitable targets for phage selection. Indirect immobilization of biotinylated ligands on streptavidin coated surfaces or chemical cross-linking to bifunctional resins (108) has been reported to be more successful for the generation of native-like ligand surfaces and should be considered whenever possible.

In spite of these limitations, phage display technology has significant advantages over other screening methods.

Compared to conventional bacterial or lambda phage-based expression systems, which are submitted to the same biological limitations, phage display technology enables rapid and selective enrichment of desired clones in small volumes using minimal amounts of ligand molecules which can be, indeed, a limiting factor for selection.

VI. ADAPTABILITY OF PHAGE DISPLAY TO HIGH-THROUGHPUT SCREENING TECHNOLOGY

The identification of the proteins produced in a given biological system started years ago with the discovery and improvement of recombinant DNA technologies that allowed controlled expression of genes in many different hosts (109). However, cDNA-cloning technology including DNA sequencing can not be directly used to study protein–protein interactions; the challenge of functional genomics aimed to turn sequence information into function. Estimates of the total number of proteins resulting from transcription of the approximately 35,000 human genes vary from 300,000 to millions (110), thus allowing for a much greater number of potential protein–protein interactions. Although many phenotypes can already be pinpointed to their genetic origin through the sequence information of genome projects, many others remain unknown. Unfortunately, sequence information in itself is neither sufficient to provide significant knowledge of the underlying mechanisms of life, nor of the biology of organisms. It rather provides a sound basis and framework for further investigations.

The rapid identification of complex networks of interacting molecules in cells and tissues requires technologies that provide logistic and/or physical links between proteins and the genes that encode them. The complex nature of molecular interactions and the large numbers of diverse molecules involved in biological processes require high-throughput technology, allowing a sufficient degree of parallelization (50). New technologies for large scale analysis of genes and proteins have been devised such as differential display, RNA/DNA microarrays, and mass spectrometry (111) which,

however, suffer from the lack of a physical link between sequence information and function. Phage display provides a physical link between genotype and phenotype (2,5,6) and allows the handling of large libraries based on the power of affinity selection (78). This physical link can easily be combined with a logistic protein–DNA link provided by robot technology, enabling high-throughput picking and high-density arraying of single clones (50). These high-density arrays have the advantage that each clone has a unique position defined by the coordinates on the microtiter plate and allow an unequivocal identification of each clone in later stages.

It has been shown that a human fetal brain cDNA expression library can be screened in parallel for either DNA hybridization, protein expression, and for antibody screening in a high-density array format on filter membranes (112,113). This robot-based high-throughput screening technology has been successfully applied to phagemid libraries expressing complex allergen repertoires preselected with serum IgE of allergic individuals (48,79,81,95). The potential of the combination of cDNA-phage surface display with selection for specific interaction by functional screening and robotic technology is illustrated by the isolation of more sequences potentially encoding IgE-binding proteins than postulated from Western blot analysis using extracts derived from raw material of complex allergenic sources (114). Moreover, robot-based high-throughput screening technology has been applied to recombinant antibody arrays displayed on phage surface to detect antibody–antigen interactions (49). Therefore, high-throughput screening technology applied to complex surface displayed cDNA libraries will play an important role in the postgenomic era by identifying potential ligands against large numbers of diverse molecules expressed by cell cultures, tissues, or organisms.

VII. CONCLUSIONS

The major challenge in the postgenomic era is to turn sequence information into function. The key molecular players

in cells and tissues, which are instrumental for the functioning of an organism, are the proteins. Built up from 20 different amino acids encoded by the DNA of a limited number of genes, proteins are produced through complex translational and post-translational pathways generating a great deal of functional structures. This diversity enables complex networks of molecular interactions governing the functioning of an organism. The characterization of large numbers of genes, their expression patterns, and protein interactions demands the use of high-throughput technologies able to link information, deposited in the genome, to function exerted by the proteins themselves.

Phage surface display of cDNAs as a biological approach linking genotype and phenotype, although subjected to intrinsic biological limitations, can be used for the efficient identification of gene products based on protein–protein interactions. Thus, the technology has the potential for substantially contributing to rapid developments in functional genomics. The basic knowledge accumulated from successful and unsuccessful applications of cDNA-phage surface display will improve our understanding of the biological limitations of the systems currently used, and thus will help further improve the technology.

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