Chapter 1

Ribosome Display: A Perspective

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Abstract

Ribosome display is an in vitro evolution technology for proteins. It is based on in vitro translation, but prevents the newly synthesized protein and the mRNA encoding it from leaving the ribosome. It thereby couples phenotype and genotype. Since no cells need to be transformed, very large libraries can be used directly in selections, and the in vitro amplification provides a very convenient integration of random mutagenesis that can be incorporated into the procedure. This review highlights concepts, mechanisms, and different variations of ribosome display and compares it to related methods. Applications of ribosome display are summarized, e.g., the directed evolution of proteins for higher binding affinity, for higher stability or other improved biophysical parameters and enzymatic properties. Ribosome display has developed into a robust technology used in academia and industry alike, and it has made the cell-free Darwinian evolution of proteins over multiple generations a reality.

Key words: Directed evolution, Cell-free translation, Ribosome display, Protein engineering, Antibody engineering, DARPins, Designed ankyrin repeat proteins, Affinity maturation

1. Introduction: Ribosome Display in Context

All technologies of molecular evolution must couple phenotype and genotype. There are two fundamental possibilities for achieving this. The first one is compartmentalization. Nature's compartments are cells: they secure that the superior phenotype expressed by one cell's mutant genotype can be replicated, without the gene products from the wild type interfering. All selections based on microbial phenotypes use this principle. The second possibility is a direct physical coupling of genetic material to the protein product. Nature's example would be viruses: the virus coat and its receptor-binding properties are the phenotype whose genetic information is encoded on the viral genome, inside the virion.

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In order to use selection methods in biotechnology, i.e., for the enrichment of binders from libraries and their evolutionary improvement, one can exploit both compartmentalization and physical linkages. The most frequently used compartments are microbial cells, where intracellular interactions can be detected by genetic means. As exemplified by the yeast two hybrid system (1) or by the protein fragment complementation assay (2), only those cells grow in which a desired molecular interaction restores the critical factor, which has previously been split in two pieces. Alternatively, one can use enzymatic or optical means for detecting interactions (3–5). These examples are only meant to be illustrative – a large research field has developed around exploiting such phenomena.

Most popular, however, have been systems where the interaction itself occurs outside of the cell, even though the polypeptides are still being produced by cells. Thus, expression on the surface of bacteria (6) or yeast (7) has been used successfully, as has been the display of peptides (8–11) and polypeptides (12–14) on the surface of filamentous phages – probably still the most widely used display technology. In phage display, the bacterial cells are producing the phages, and thus the bacterial cells must first be transformed with the library, limiting the diversity. However, this will not be a comprehensive review on display technologies; such reviews can be found elsewhere (15–20).

In all these technologies that involve cells at any step, including phage display, the genetic information needs to be introduced into cells. This will usually limit the diversity present in the cells to below what has been present in the actual library of DNA molecules. The diversity reached in practice after transformation will depend on the host system (bacteria vs. yeast) and on the efforts made to transform the cells. This large-scale transformation step, while not difficult, can be quite laborious. It is this step that is avoided in technologies that are performed fully in vitro.

This chapter will summarize ribosome display, and mention other technologies that operate without any cells during the library selection. They are not restricted by the effort spent on the transformation step. This advantage is apparent in the primary library, which can be of bigger size, as all DNA (or mRNA) molecules present can in principle give rise to proteins that take part in the selection.

The most important advantage of ribosome display and other "full" in vitro technologies is, however, the easy combination with PCR-based randomization techniques, and thus the creation of a true Darwinian evolution process – in contradistinction to a mere selection from an existing "constant" library. In all technologies that require transformation of cells, after each randomization step the cells have to be transformed with the new library, and the workload is thus potentiated by the number of evolution steps.

In ribosome display, the workload to select binders in the absence or presence of randomization is almost identical. Thus, the more randomization steps are to be carried out in succession, the more attractive ribosome display becomes.

Of course, technologies have been developed to carry out randomization directly in cells as well (21–23). Nonetheless, in vitro methods give the user full control over where mutations should occur in the sequence (by using, e.g., a randomized cassette), which residue types are to be introduced (by using trinucleotide building blocks (24) or suitable mononucleotide mixtures), or how many random mutations should occur on average (25) – a level of control not yet within reach in cellular systems. Furthermore, the "shuffling" of the library (26, 27) can easily be introduced into the procedure if desired, to recombine mutants.

2. Development of Ribosome Display

Key to the development of ribosome display was the observation that rare mRNAs coding for a particular protein can be isolated from a pool of mRNAs by immunoprecipitation of stalled ribosomal complexes containing the nascent polypeptides (28, 29). Apparently, ribosomal stalling is frequent enough to be experimentally exploited.

To create RNA-based "aptamers", Tuerk and Gold (30) had developed a technology called SELEX (Systematic Evolution of Ligands by Exponential Enrichment), where multiple rounds of in vitro transcription of random nucleic acid pools, followed by affinity selection of the RNA aptamers and subsequent RT-PCR, lead to the selection of target-binding RNAs. In SELEX, genotype and phenotype are simultaneously represented by the same RNA molecule, since it exerts its function through its three-dimensional structure, which is in turn determined by its nucleotide sequence.

In their original publication about SELEX, Tuerk and Gold (30) already speculated that a similar approach might be adapted to protein selection, referring to the isolation of stalled translation complexes (28, 29).

The first experimental demonstration of the ribosome display technology was the selection of short peptides from a library using an *Escherichia coli* S30 in vitro translation system (31, 32). Kawasaki (33) had proposed a similar approach to enrich peptides from libraries in a patent application, however, without giving a detailed example, which was only published in 1997 (34).

Meanwhile, Hanes and Plückthun (35) had developed and first reported ribosome display for whole proteins, initially antibody scFv fragments, after significantly improving and modifying the system to increase its efficiency and to allow folding of scFv fragments during in vitro translation (36). The key observation with

these longer open reading frames (ORFs) was that mutations were found at great frequency (35) due to the large number of PCR cycles that every gene had undergone after several rounds. Because of the short encoded peptide sequences, Mattheakis et al. (31, 32) did not observe mutations. To play out its strengths, ribosome display needs to be carried out with whole proteins. The appearance of mutations, just by using a normal polymerase without proofreading activity, then made it clear that the unique advantage of ribosome display lies in its potential to do true *Darwinian evolution*, as opposed to mere *selection*, from a given library. This potential was demonstrated in the following year (37) by striving to select antibodies for improved affinity, and by adding additional diversity through random mutagenesis. The first selection from a non-immune synthetic antibody library followed soon thereafter (38).

In the above experiments, the bacterial cell-free translation extracts were home-made, as they needed to be free of reducing agents (because of the intramolecular disulfide bonds within the scFv domains), and they were required in large amounts. The feasibility of carrying out ribosome display in a eukaryotic cell-free translation extracts was subsequently demonstrated with an scFv-kappa fusion (39), and then by selecting an antibody in the same format from immunized transgenic mice (40).

3. The Ribosome Display Methodology

The principle of ribosome display is depicted in Fig. 1. A DNA cassette (typically a PCR fragment) is used that contains a promoter and an ORF, encoding a library of the protein of interest. It is transcribed in vitro, and the resulting mRNA does not contain a stop codon. Cell-free translation can run to the physical end of this mRNA, and complexes consisting of the protein of interest, the ribosome, and mRNA are formed. The ribosome itself serves as the connector. These ternary complexes are exposed to immobilized target molecules, the displayed proteins (library members) binding to the target are enriched on the target and others are washed out. From these bound complexes, the mRNAs are isolated, reverse transcribed and

Fig. 1. (continued) display construct is obtained by PCR amplification of both flanking regions and the library insert from the ligated vector. In vitro transcription of this PCR product yields mRNA that is used for in vitro translation. The ribosome stalls at the end of the mRNA and does not release the encoded and properly folded protein because of the absence of a stop codon. The ternary mRNA–ribosome–protein complexes are used for affinity selection on an immobilized target. The mRNA of bound complexes is recovered after washing from dissociated ribosomes, reverse transcribed and amplified by PCR. Thereby the selected pools of binders can be used directly for the next cycle of ribosome display or analysis of single clones after cloning into expression vectors, which are then used for *Escherichia coli* transformation and small-scale in vivo expression. Adapted from ref. 111; for the most current procedure and details see ref. 97.

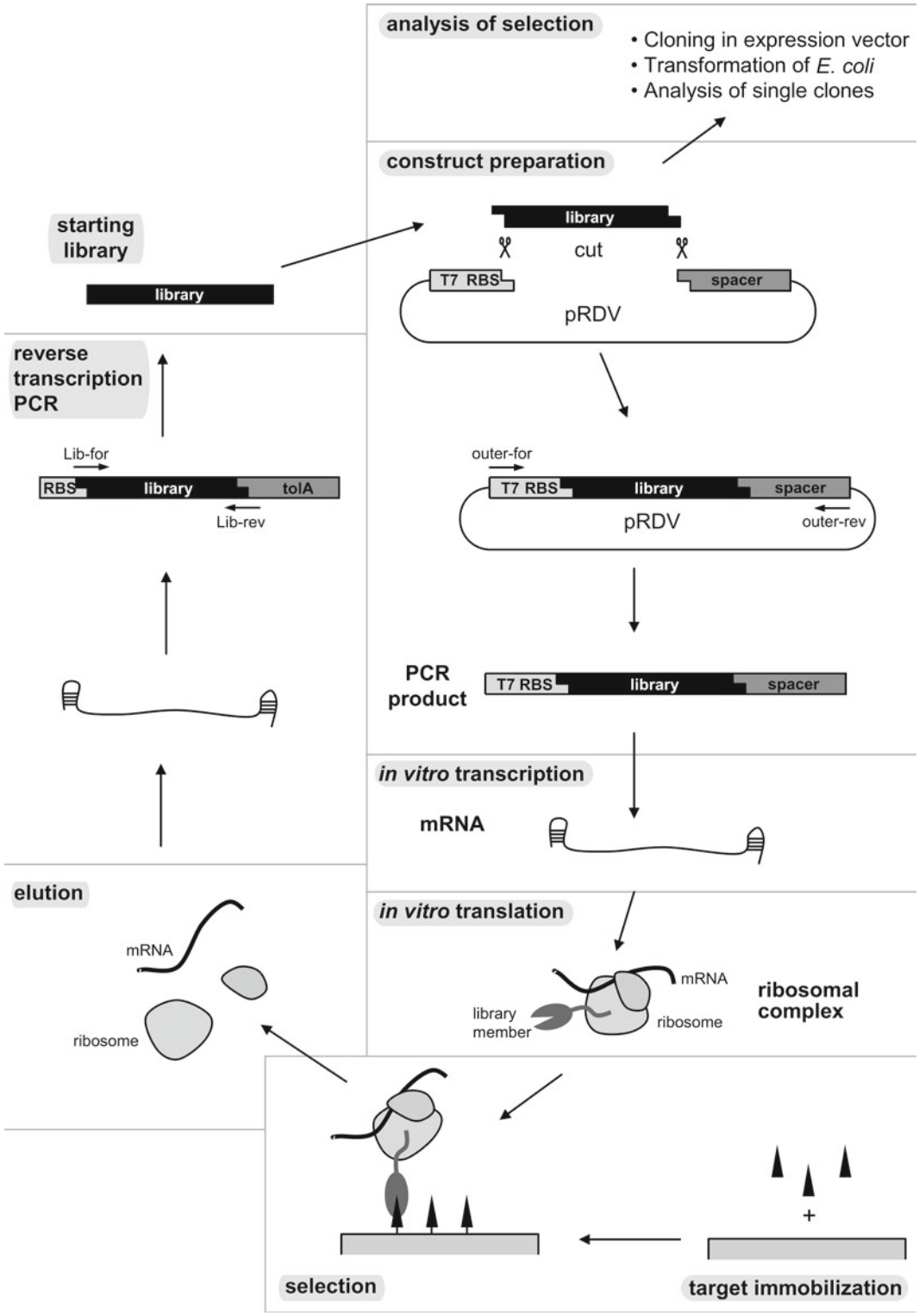

Fig. 1. Overview of the ribosome display selection cycle. A DNA library in the form of a PCR product, coding for binding proteins, is ligated into a ribosome display vector (pRDV), thereby genetically fusing it to a spacer sequence in-frame, and providing a strong promoter (T7) and translation initiation region (ribosome binding site, RBS) at the 5′ end. The final ribosome

PCR amplified to serve as the input of another round. After 3–5 rounds, the resulting DNA fragments are ligated into an expression vector and *E. coli* are transformed. The different proteins made by individual *E. coli* clones can then be further evaluated.

The logic of the individual steps will now be discussed. In vitro methods cannot rely on cells to link phenotype and genotype. Instead, a direct physical link between the genetic material and the protein product must be made. (An in vitro alternative is to create compartments in the form of a water-in-oil emulsion, which will be discussed below.) During protein biosynthesis, the encoding mRNA is read by the ribosome and parts of it are almost engulfed by the small subunit, which mediates codon/anti-codon contact to the tRNA. The protein emerges from the ribosomal tunnel within the large ribosomal subunit. During all these steps, the protein chain is covalently connected through an ester bond to the peptidyl-tRNA within the P-site, and thereby tightly maintained within the ribosome. Thus, during protein biosynthesis, neither protein nor mRNA can leave.

Translation normally ends at a stop codon. In *E. coli* ribosomes, the UAG and UAA stop codons are directly recognized by the Release Factor 1 (RF1), the UGA and UAA stop codons by RF2 (41). With the help of RF3, the ester bond between the synthesized protein and the last tRNA is positioned such that it is hydrolyzed, and the last amino acids of the finished protein, still within the exit tunnel, can slide out, leaving the empty tRNA behind. Now the ribosome recycling factor (RRF) and elongation factor G (EF-G) together help separate the large from the small subunit: they remove the tRNA, and after subunit dissociation, the mRNA can then leave as well (42). A similar mechanism also functions in eukaryotic ribosomes (43).

The absence of a stop codon in mRNA for ribosome display thus ensures that this "normal" course of events does not take place. The experimental conditions must furthermore minimize any early unwanted spontaneous hydrolysis of the ester bond between polypeptide and tRNA or any other spontaneous falling apart of the ternary complex. This is usually achieved by rather short translation times, subsequent cooling of the solution and addition of Mg^{2+}. These short translation times are also a compromise between efficient translation and degradation of the mRNA by nucleases present in the extract. It is believed that high Mg^{2+} "condenses" the ribosome by binding to the rRNA, making it difficult for the peptidyl-tRNA to dissociate or be hydrolyzed. While the details differ, the general strategy is the same for eukaryotic ribosomes (44).

The absence of a stop codon then causes "stalled" ribosomes. Once the ribosomes are at the end of the mRNA, they would have a peptidyl-tRNA in the P site and (presumably) an empty A site. As this situation can appear in the bacterial cell as well (if an mRNA molecule is missing its 3′ end containing the stop codon), bacteria

have devised a mechanism called *trans*-translation to rescue such stalled ribosomes, which would clog up protein biosynthesis (45, 46). The key molecule to act on the stalled ribosome is the transfer-messenger RNA (tmRNA, also called 10S RNA or SsrA), which has a tRNA-like domain charged with alanine and an mRNA-like domain. Peptide synthesis is resumed, with the additional help of the protein SmpB, by incorporating Ala, and thereby transferring the chain to the tmRNA. Up to now, the tmRNA molecule has acted like tRNA. Now its mRNA domain is being read, and the sequence ANDENYALAA* is appended to the protein, which ends with a stop codon (!), thus leading to regular termination and recycling of the ribosome. Even worse for ribosome display, this C-terminal sequence which has been added serves as a degradation tag in *E. coli*. Thus, the action of tmRNA would be detrimental for ribosome display. Starting from our very first experiments, an antisense oligonucleotide has always been added to titrate out tmRNA (35).

In order for the protein of interest to fold and be able to interact with a target, the whole protein of interest must be outside of the tunnel once the ribosomes have come to the physical end of the mRNA. Thus, to remain connected to the tRNA at the same time, the protein of interest must be fused to an unstructured region at the C-terminus, occupying the ribosomal tunnel. We have called this the "spacer" or "tether". This protein tail, which is the same in all library members, is thus fused in frame to the C-terminus of the randomized protein of interest in the ribosome display construct.

The features of the ribosome display construct are summarized in Fig. 2. On the DNA level, the construct requires a strong promoter for efficient in vitro transcription to mRNA. On the mRNA level, the construct contains, as a regulatory sequence for translation, either a prokaryotic ribosome-binding site (47, 48) if the *E. coli* system is used, or a Kozak consensus and enhancer sequence (49) if the eukaryotic ribosome display system is used. This sequence is followed by the ORF encoding the protein to be displayed, followed by a spacer sequence fused in frame to the protein of interest, as described above.

At both ends of the mRNA, the ribosome display construct should include stemloops. 5′- and 3′-stemloops are known to stabilize mRNA against RNases in vivo as well as in vitro. The presence of stemloops is important, especially in the *E. coli* ribosome display system because the extract used for in vitro translation contains high RNase activities. The efficiency of ribosome display was increased approximately 15-fold (35), when a 5′-stemloop derived from the T7 gene 10 upstream region and a 3′-stemloop derived from the terminator of the *E. coli lpp* lipoprotein were introduced into the ribosome display construct (35). A similar improvement in efficiency was observed when using the same 5′-stemloop and the

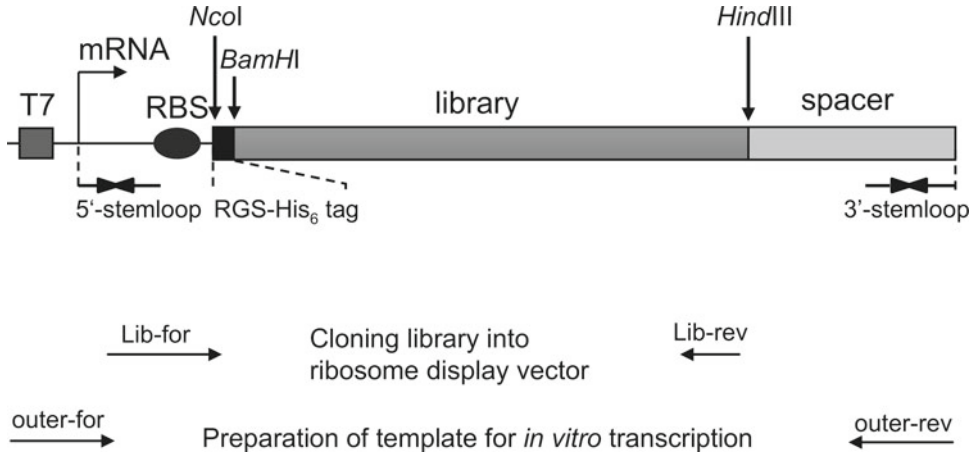

Fig. 2. The construct for ribosome display using *Escherichia coli* ribosomes. A T7 promoter and a ribosome-binding site (RBS) are necessary for in vitro transcription and translation. The coding sequence starts with Met-Arg-Gly-Ser-His_6 (the RGS-His_6 tag, or any other tag can be used), followed by the DNA library of the binding proteins and a spacer at the C terminus. The stop codon has been removed from the coding sequence. At the mRNA level, the construct is protected against RNases by 5′ and 3′ stem-loops. Fusion of the promoter and the spacer sequence can best be achieved by cloning into an appropriate vector providing these sequence elements and subsequent amplification by PCR. The oligonucleotides used for the cloning of the library into the ribosome display vector and for the generation of the template for in vitro transcription are indicated at the bottom schematically, the exact oligonucleotides can be found in the corresponding publications. Adapted from ref. 111; for the most current procedure and details see ref. 97.

3′-stemloop derived from the early terminator of phage T3 (35). The stemloop structures may protect the mRNA particularly from degradation by the exonucleases PNPase and RNaseII, which act from the 3′-end of the mRNA, and against RNaseE, which recognizes the 5′-end (50–52).

In the ribosome display cycle, the ribosomal complexes are then exposed to the target of interest. It is either free in solution (to be captured subsequently, e.g. by magnetic beads) or immobilized on plates. A very robust way for both strategies has been to biotinylate the target, as the interaction of biotin with streptavidin, neutravidin or avidin survives stringent washing steps and exposure to detergents. The many variations of this step will be discussed in subsequent chapters. Our laboratory has preferred enzymatic biotinylation at an engineered tail above all other methods (53).

Clearly, the details of how the library is exposed to the target will determine the selection outcome. The use of competitors (to avoid recognition of a similar target) and methods to select for high affinity (discussed below) as well as methods to select for properties other than high affinity (summarized below) are under constant development and refinement. Later chapters will discuss these aspects at length.

One of the great advantages of ribosome display is that the linkage between immobilized target and library member does not have to be broken. After selection on the target, only the mRNA is required from this point onwards. Therefore, it is sufficient to liberate

it by the dissociation of small and large ribosomal subunits by the addition of EDTA. This has the advantage that it is not more difficult to isolate complexes of very high affinity (which would be hard to dissociate) than those of lower affinity. Nonetheless, more specific elution procedures can be of interest for selecting binders to particular epitopes or with particular properties.

The mRNA needs to be reverse transcribed and the resulting DNA then amplified and brought back to the initial format containing a promoter for transcription of the next round. In the initial rounds, very few mRNA molecules will be obtained after selection. This step is perhaps one of the few technically demanding steps, as it requires attention to the fragility of RNA (in the presence of nucleases, which can also be introduced by careless laboratory handling). When designing a new ribosome display cassette (with different promoter, ribosome binding site, N-terminal tag on the protein, ORF, C-terminal tether and 5′ and 3′ stemloops), care must be taken in designing the primers needed for the PCR. Obviously, they must bind with very high specificity. These reverse transcription and PCR steps appear to be the most frequent focus of troubleshooting, when designing a new system from scratch. Even when taking a well-working system, and merely replacing the ORF, it must be considered that new hairpins might form unintentionally, e.g., engaging the start codon or the ribosome binding site, which would compromise translation efficiency. Fortunately, these issues are easily and rapidly evaluated.

4. Protein Folding in Ribosome Display

The most frequently used methods in ribosome display include the use of *E. coli* S-30 extracts for translation, which do contain ribosome-associated factors important for protein folding such as the trigger factor (54). In addition, molecular chaperones that are not associated to the ribosome are present in the *E. coli* extract, such as DnaJ/K/GrpE and GroEL/ES, as well as small heat shock proteins and others (55). Additional factors can be added, depending on the requirements of the proteins to be displayed (36). Antibody scFv fragments required the addition of eukaryotic protein disulfide isomerase (36).

Ribosome display has also been used as a tool to define a binding epitope, making use of the somewhat surprising finding that, while still on the ribosome, aggregation of a protein seems to be efficiently prevented. It was found that proteins, such as eukaryotic receptors that could not be expressed in functional form in *E. coli* nor efficiently refolded from inclusion bodies, nor expressed in functional form by in vitro translation, would fold while still attached to the *E. coli* ribosome (56). Perhaps the ribosome enhances solubility of the

ternary complex and sterically blocks aggregation. To define a binding epitope, a cell surface receptor was subjected to several rounds of random mutagenesis at high error rate, and this ribosome display library of receptor point mutants was selected on the target. The epitope could be recognized as an area devoid of surface mutations – evolutionary pressure in the experiment apparently maintained the residues in the epitope (57).

When using a eukaryotic translation system (39, 44, 58), the corresponding eukaryotic proteins would be expected to be present in the extract. On the other hand, if a system from pure components is used (59–62), it may be necessary to add these proteins relevant for folding, depending on the protein to be displayed (36).

5. Variations in the Ribosome Display System

5.1. Eukaryotic Cell Free Translation System

The ribosome display method can also be carried out with eukaryotic extracts, using a reticulocyte lysate (39, 58). Different methods of sequence recovery have been compared, and it was concluded that a similar procedure as used in the prokaryotic system also performs best in the eukaryotic system (44), even though in situ recovery can also be carried out (39, 58). The wheat germ in vitro translation system has also been used (34). While one might speculate that a eukaryotic translation system should perform better with eukaryotic proteins, there is actually no evidence for this (63). If particular factors are needed, such as, e.g., molecular chaperones and protein disulfide isomerase, they can (and have to) be added to either system (36).

5.2. PURE System

The use of a ribosome display system based on in vitro translation with purified components (PURE system) has also been described (59–62). In this system, no release factor is present. Matsuura et al. examined the efficiency of ribosome display in the absence and presence of a stop codon, as well as when using the *secM* stalling sequence (64) as an alternative means of trapping the ternary protein–ribosome–mRNA complex (60). Interestingly, the efficiency of display was almost identical in all cases. Another encouraging finding from the use of the PURE system is that the *intrinsic* stability of the ternary complexes is actually very high. Even after an incubation of the ternary complexes for 1 h at 50 °C, the display efficiency drops by less than a factor of 10. Presumably, both in the prokaryotic and eukaryotic extracts, RNAses set a practical limit on stability, rather than the intrinsic stability of the ternary complexes themselves.

The system with purified components may thus be of interest where the removal of a stop codon is inconvenient or high temperature is required in the selection procedure. It should be kept in mind, however, that the experiments were carried out with engineered

mRNA that had a C-terminal spacer, as in the standard system described above. In a natural non-engineered mRNA, one would expect that the last few amino acids of the protein of interest are still in the ribosomal tunnel, thereby hampering the folding of the protein. It will remain to be seen whether the PURE system is sufficiently cost-efficient to be used for standard selection and evolution experiments, in comparison to the use of translation extracts from *E. coli*.

5.3. Display of mRNA with Stop Codons

To address the display of natural mRNA (which of course all contain a stop codon), as an alternative to the use of the PURE system, engineered suppressor tRNAs have been used (65, 66). This could be another approach useful for future protein–protein interaction studies. Nonetheless, the problem remains that many (if not most) proteins will not fold, if part of their domain structure is still in the ribosomal tunnel. The critical question is therefore whether the translated spacer that results when suppressing the stop codons in natural mRNA will be long enough to allow folding of most proteins.

6. Applications of Ribosome Display to Complex Libraries

6.1. scFv Fragments

Antibody scFv fragments were the first complex library with which ribosome display was tested for selection and affinity maturation (37), initially from a library of immunized mice, later from a synthetic library (38). At that time, the recreated synthetic repertoire of the antibodies was the only general binding protein scaffold available with great diversity.

The folding of antibody fragments in an in vitro translation system must be commensurate with their oxidative folding (many if not most antibody domains need the intradomain V_H and V_L disulfide bond to fold properly), and thus this reaction must be catalyzed (36). In addition, the β-sandwich architecture of antibodies can lead to aggregation, and this may be part of the reason, why more rounds of enrichment appear to be necessary than for some other scaffolds that fold extremely well in an in vitro translation system.

There are more publications on using phage display than ribosome display in the selection from naive antibody libraries, but there simply may be no necessity to break with tradition. Filamentous phage display (12) works very well with secreted one-chain disulfide-containing proteins such as scFv (67), and there is always the option of combining the two methods, as opposed to directly combining selection and affinity maturation in one procedure, as in the ribosome display selection from naive or synthetic libraries (68).

The analysis of ribosome display selection from the fully synthetic antibody library HuCAL leads to the conclusion that the selection is not exhaustive, and the outcome is governed by the occurrence

	Vκ1	Vκ2	Vκ3	Vκ4	Vλ1	Vλ2	Vλ3
VH1A	C(9)					C(2)	
VH1B							
VH2							
VH3		A(9) A(1) B(2)		A(1)	A(1) A(1)	A(1) A(1)	
VH4							
VH5							
VH6					B(6)		B(1)

Fig. 3. Framework usage of the insulin-binding HuCAL scFv fragments in three different ribosome display experiments. The *vertical* and *horizontal axes* denote the HuCAL heavy-chain and light-chain variable domains. ScFvs isolated in experiments (**A**), (**B**), or (**C**) are denoted accordingly. *Numbers in parentheses* represent the number of closely related scFvs with the same CDRs, but different point mutations. It is apparent that in different experiments random mutations lead to the proliferation of particular sequence families, but that this phenomenon occurred in different families in the three experiments. Adapted from ref. 38.

of random mutations; in selections which were repeated against the same target, different frameworks were dominant in the different selections. This suggests that an early beneficial mutation may have given rise to a lot of (further mutated) progeny of a particular clone, while in the next selection experiment on the same target, another framework combination may have acquired such a beneficial mutation (Fig. 3).

6.2. Designed Ankyrin Repeat Proteins

Combinatorial libraries of a new class of small proteins, termed "Designed Ankyrin Repeat Proteins" (DARPins) (69, 70) were developed that can act as an alternative to antibodies, as they are particularly robust to engineering. They are based on a very different structure and are built from consecutive 33-amino acid repeats, each forming a β-turn followed by two antiparallel α-helices. In each repeat, seven residues were randomized, and these internal repeats are flanked

by constant capping repeats, to give one contiguous polypeptide chain with a randomized concave, groove-like binding surface, which is randomized in the library. The proteins contain no cysteine, can be expressed in soluble form in the cytoplasm of *E. coli* at very high levels, and are very stable and resistant to aggregation (refs. 71, 72 and references therein).

It may be these favorable biophysical properties, combined with the fact that high affinity binders are obtained at high frequency, that cause the direct selection of binders from the diverse library to work very well with DARPins. Thus, binders against many targets, including difficult ones such as, e.g., detergent-solubilized GPCRs (73) or conformers of DNA (O. Scholz, unpublished), have been selected directly by ribosome display (e.g., see refs. 70, 74–80).

6.3. Other Scaffolds

Binders based on the camelid VHH domains with micromolar affinity have been isolated by ribosome display from a naive library (81), and with nanomolar affinity from an immunized llama (82).

7. Combining Ribosome Display with Other Selection Technologies

Ribosome display has been combined with other selection technologies. It has been used as the affinity maturation step of a phage display library (e.g., see ref. 68), and thus used as the second stage in binder selection.

However, one can also use ribosome display as the first step and follow it up by another technology to simplify the evaluation of individual clones. At the end of the ribosome display procedure, the final selected pool is usually cloned in *E. coli*, and crude extracts of individual *E. coli* expression cultures are then analyzed by ELISA. Instead of going through enough rounds such that most of these clones will be positive, an earlier round can be cloned, and an in vivo selection can be applied to this selected pool. For this purpose, the pools of ribosome display were cloned after the first, second, and third round in a protein fragment complementation assay (PCA) (2), a split enzyme selection system using DHFR. This technology has a low discrimination power for affinity, but essentially serves as a convenient qualitative screen of binding. It can be seen that even after one round of ribosome display, binders can be obtained, albeit with micromolar affinity. These correspond to a random sampling of the library which has been enriched, perhaps 10^3- to 10^4-fold, and high affinity binders are too rare to be expected to be found in this small sampling. However, already after the second round of ribosome display, binders with nanomolar affinity are found (77). This combination of ribosome display with PCA might become of interest in high-throughput applications of ribosome display.

8. Comparison of Ribosome Display with Related In Vitro Methods

8.1. mRNA Display

The first steps of mRNA display are identical to ribosome display. A DNA library, encoding promoter, ribosome-binding site, and the randomized open reading frame of interest without a stop codon is transcribed to yield a library of mRNA molecules. This library is then ligated to a C-terminal linker consisting of DNA which contains at its end a puromycin molecule. In vitro translation is carried out as in ribosome display. The ribosomes are thought to stall at the 3′ end of the mRNA where it is linked to DNA. The attached puromycin molecule then enters the P-site and takes on the role of a tRNA, and the growing peptide chain is

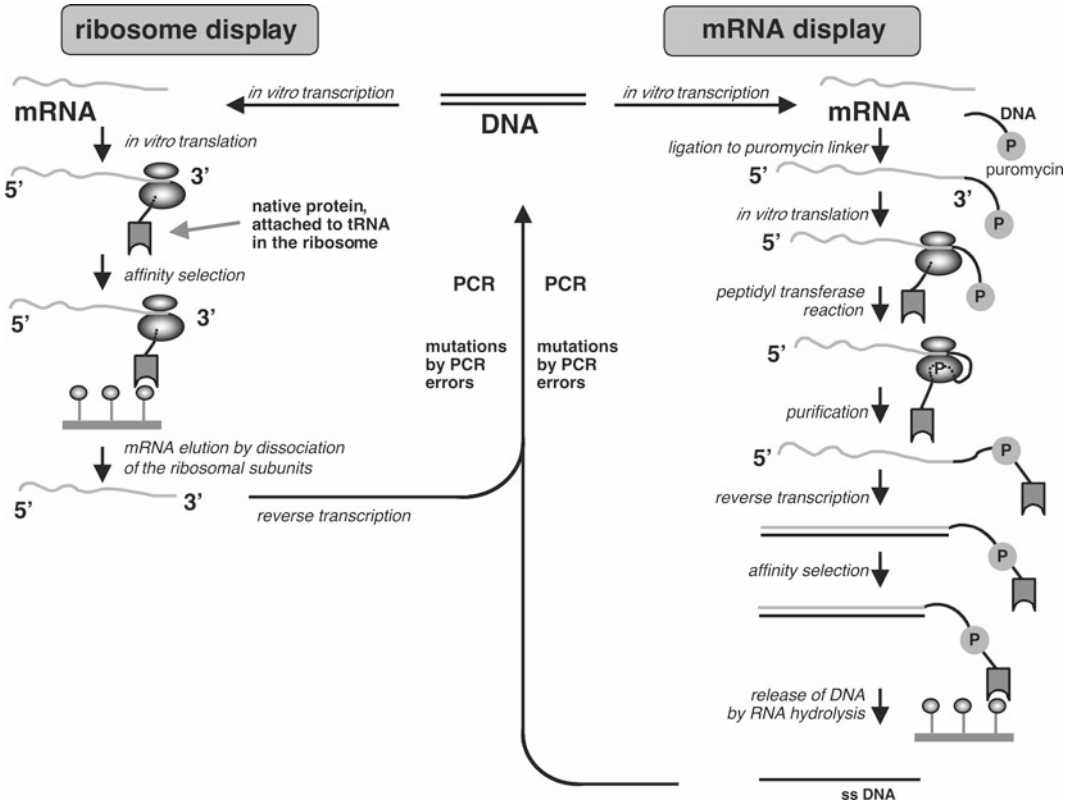

Fig. 4. Comparison of ribosome display (*left*) and mRNA display (*right*). For both, the DNA encoding the library is first transcribed in vitro. In ribosome display, the resulting mRNA lacks a stop codon, giving rise upon translation to linked mRNA–ribosome–protein complexes, which can be directly used for selection against an immobilized target. The resulting mRNA is obtained upon dissociating the ribosomal subunits, reverse transcribed and amplified for the next round. In mRNA display (*right*), the mRNA is first ligated to a DNA linker connected to puromycin. The mRNA is translated in vitro, and the ribosome stalls at the RNA–DNA junction. Puromycin then binds to the ribosomal A-site, and attacks the peptidyl-tRNA at the P-site. The nascent polypeptide is thereby transferred to puromycin, as if it were an aminoacyl-tRNA. The resulting covalently linked mRNA–protein complex has the puromycin-linker-mRNA on one side of the tunnel, the protein on the other side of the tunnel. The mechanism, by which this complex purified from the ribosome is not entirely clear (cf. Fig. 5). It is then reverse transcribed and used for selection experiments. The DNA strand is recovered from target-bound complexes by hydrolyzing the complementary mRNA at high pH, then it is amplified by PCR. Adapted from ref. 112.

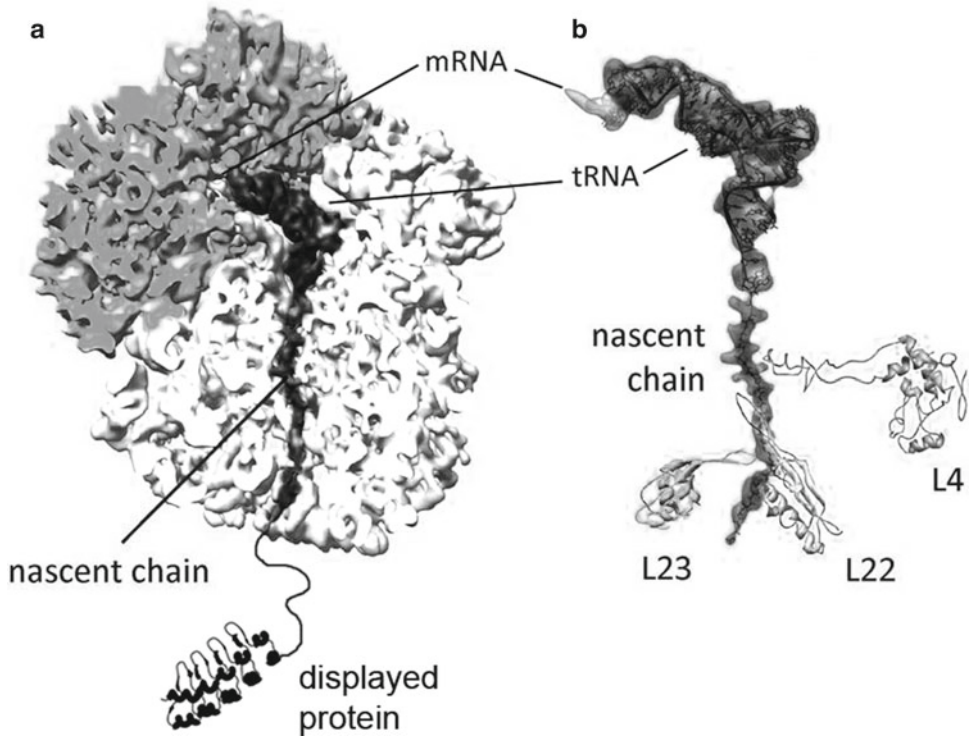

Fig. 5. Schematic illustration of the topology relevant for ribosome display. This figure is adapted from Seidelt et al. (113). (**a**) CryoEM reconstruction of the *Escherichia coli* ribosome 70S complex with the TnaC stalling sequence is shown. For illustration, the folded domain of a DARPin has been added at the N-terminus. The unstructured connector outside the ribosome and the part that is within the tunnel (*nascent chain*) would correspond to the "spacer" or "tether" region in Figs. 1 and 2. After selection, the addition of EDTA disassembles the two subunits, and only the mRNA must be recovered. Note that the mRNA is not shown here – it contacts the tRNA where indicated and is otherwise partially within the small subunit in this representation and thus not highlighted. (**b**) tRNA, attached nascent chain and the contact point of the mRNA are shown. The ribosome has been left out, except for three proteins from the large subunit, *L4*, *L22,* and *L23* which contact the nascent protein. In ribosome display, the mRNA can be recovered and purified after disassembling the ribosome into small and large subunit. There is no need to elute or recover the protein–tRNA complex. By contrast, in mRNA display, a puromycin molecule, covalently attached to the nascent protein would take the place of the tRNA in this picture. This puromycin is also covalently connected to a DNA spacer, which itself is covalently connected to the mRNA. It is apparent that this will create two large structural units on either side of the ribosomal tunnel: on one side the puromycin, attached to DNA and RNA, on the other side the folded protein, which has emerged from the ribosomal tunnel. Thus, in order to remove the large ribosomal subunit before panning, the ribosome has to be unfolded (potentially unfolding the protein of interest as well), or the protein of interest has to be unfolded (in order to thread backwards through the tunnel), or the whole linker-mRNA has to thread forwards through the tunnel. Alternatively, the large subunit might stay just in place during the selection, such that mRNA display works actually like ribosome display.

transferred to puromycin, and thereby covalently connected, via the linker, to the mRNA (Fig. 4).

The mRNA display procedure leaves us with an interesting topological conundrum. On one side of the ribosomal tunnel is the folded protein, on the other side is puromycin, connected via a linker with the mRNA (Fig. 5). The cartoons of the mRNA display procedure (14) implicitly suggest that the ribosome is removed. There appear some potentially denaturing steps in the procedure after translation (14), even though it is not clear whether this would be sufficient to unfold the large subunit, and especially what

effect these conditions would have on the covalently bound protein of interest.

There are thus four possibilities: First, the large subunit of ribosome unfolds, thereby opening the exit tunnel, such that the protein can slide out sideways. It is unclear to what degree the protein of interest would unfold as well. Second, the DNA linker and the whole mRNA thread through the protein tunnel. Third, the protein of interest unfolds before the large subunit and threads backwards through the protein tunnel. Fourth, the large subunit of the ribosome is actually not completely removed, and is still present during panning, similar as in ribosome display. This question is not only of academic interest, as it may have some effect on the protein to be displayed.

A number of protein scaffolds have been used with mRNA display, e.g. some stabilized by metal ions (83–85), which would become unfolded by adding EDTA. However, selections have also been carried out using the fibronectin scaffold (86–88) which probably folds and unfolds reversibly, or scFv fragments (89), as well as diverse other proteins (90) where it is not clear whether selection did require folded domains. The problem of topology in mRNA display has apparently not been solved.

8.2. Water-in-Oil Emulsions

The packaging of the translation extract into small droplets in the form of a water-in-oil emulsion combines the in vitro approach with the compartment concept of coupling genotype and phenotype (91–93). If, on average, each droplet contains only one mRNA molecule, the protein content of each droplet is monoclonal. The most persuasive application of this technology is for enzymatic reactions (94, 95). The basic challenge for evolving enzyme turnover is that the phenotype, namely the production rate of the enzymatic reaction product, cannot be easily linked to the enzyme molecule itself in a mixture of mutants in solution, at least not for reactions with multiple turnovers with high rates. The cellular confinement solves this problem. Nonetheless, the full potential of this approach can be reached only if the reaction can be followed in the compartments directly, requiring optical detection and sorting.

On the other hand, the selection for a binding event in a compartmentalized system is somewhat less compelling. Emulsions have to be broken and selections can be carried out in the bulk phase as in ribosome display and mRNA display. The generic detection of binding events *within* a droplet remains a challenge, and it is less clear how to achieve this in a semiquantitative way for binding *strength*, i.e., affinity.

However, an interesting application of the emulsion technology for the selection of binding proteins was described by Sumida et al. (96), in which they used the emulsion format to express both chains of a Fab fragment from a bicistronic operon within the same droplet. By fusing the heavy chain to streptavidin, and attaching biotin to

the DNA fragment via a photocleavable linker, both chains of the Fab fragment and the corresponding DNA stay together after breaking the emulsion. After panning, the DNA has to be recovered by photocleavage and can be amplified. It will be interesting to see how well this system will perform with complex libraries.

9. Comments on Library Size

The absolute functional library size in ribosome display is given by the number of different ternary complexes that are formed from mRNA and ribosomes and give rise to a nascent protein that can fold. This requires that the protein of interest is translated at least to the point that the relevant domain is outside the ribosomal tunnel.

From the amount of PCR fragment that is used as the input for transcription, we can calculate that under standard conditions (97) about $2–3 \times 10^{12}$ molecules input DNA are used. If the library template DNA to make this PCR is of good quality and highly diverse we can assume that these DNA molecules are all different. Also, further mutations will have been introduced while carrying out this very PCR.

The transcription of this linear PCR fragment will usually create multiple copies or mRNA per DNA molecule, and only an aliquot of the resulting mRNA is used for translation. In a standard ribosome display reaction about $1–3 \times 10^{13}$ mRNA molecules are used. It is entirely possible that additional errors are introduced by the RNA polymerase, such that they will contain a greater diversity than the input DNA.

The other critical variable is the number of functional ribosomes. The number of assembled ribosomes in an *E. coli* cell depends on its growth rate and is between 18,000 and 72,000 (98). From the amount of S30 extract used and the number of assembled ribosomes per cell, there should be about $1–4 \times 10^{14}$ assembled ribosomes in the standard ribosome display reaction. Thus, even if not all ribosomes are functional, there should be a sufficient excess to translate most mRNA molecules. Also, while the number of ribosomes per mRNA will follow a binomial distribution, there should be a significant proportion of monosomes (one ribosome per mRNA).

Using multi-ml quantities of S30 extract and more mRNA, almost arbitrarily large numbers for the library size can be stated. For example, in several review articles the library size of ribosome display and mRNA display have been "compared", where the latter has usually referred to an experiment where extreme amounts of S30 extract have been used. This simple, direct relationship between the amount of extract used and the functional diversity of the library has not been recognized by all authors.

More importantly, it remains to be seen whether the best use of the rather valuable S30 extract (and the even more valuable eukaryotic extract, or the truly precious purified components) is to use it all at once in the first round, as opposed to using it in smaller aliquots over a multi-step selection with built-in affinity maturation.

Another important aspect is the functional fraction of the library, and whether the non-functional part is merely inactive, or instead "sticky" and thus becoming enriched during selections. This is a quantity that can almost not be objectively determined. This issue also sets a limit to the number of random mutations that is practically useful before the population becomes extinct.

10. Selections for Higher Affinity

The most attractive exploitation of the built-in possibility of generating mutations is to improve affinity. Affinity is usually quantified by the equilibrium dissociation constant K_D, which is the ratio of the dissociation rate constant k_d (loosely referred to as off-rate) over the association rate constant k_a (loosely referred to as on-rate). The association rate constant for protein–protein complexes falls in a remarkably small window, typically between 1×10^5 and 1×10^6 M^{-1} s^{-1}, as summarized from various experimental studies by Northrup et al. (99) and further computationally analyzed by these authors. The net association rate is often visualized as the collision rate times the fraction of "successful" collisions, in other words, where the two proteins have productive orientations. This means that affinity is largely determined by off-rate, and that measures to improve affinity should normally attempt to decrease the off-rate.

Of course, there are exceptions. A protein pair can be properly oriented upon approach by electrostatic forces, leading to a higher fraction of successful collisions (100), and this can also be engineered (101). However, this electrostatic steering will greatly diminish in importance in physiological buffers with high ionic strength and may thus not be so useful for practical applications. A second class of exceptions will be those interactions which are characterized by an unusually slow observed association rate, much slower than 10^5 M^{-1} s^{-1}. This can be due to two things: either one of the partners is not in a productive conformation, and only a small fraction of molecules are able to interact (conformational selection) (102, 103), or a slow conformational change must occur in one of the partners before or upon binding (induced fit) (104). In summary, if the on-rate of the protein to be improved is not *unusually* slow (say, only 10^3–10^4 M^{-1} s^{-1}), and if high affinity should be achieved also under physiological conditions with considerable ionic strength, then an improvement of off-rate is the most likely route to success.

Nonetheless, we still have at two strategic options. In the first, in round to round, less target is immobilized. The underlying assumption is that the binding molecules to be selected, of different affinities, will equilibrate and the ones of low affinity will be displaced by the ones of high affinity that will eventually occupy all the sites. This approach becomes difficult once the affinities are already quite high, say with K_D in the low or subnanomolar range. Equilibration then becomes slow (see below) and once the amount of immobilized species becomes too low, background binding to the blocked surface or other present molecules such as streptavidin becomes a significant problem. The second, more attractive approach is thus to select for the off-rate directly (105–107).

The typical set-up is to expose the library of binders (the ribosomal complexes in ribosome display, the phages in phage display) to biotinylated target in solution. After some time, an excess of non-biotinylated target is added, with the assumption that a fast dissociating binder (one with fast off-rate) will expose an unoccupied binding site and immediately rebind to soluble competitor target, present in excess. The binders with slow off-rate, on the other hand, remain on the biotinylated target and can thus be isolated by adding capture beads carrying, e.g., streptavidin.

Initially, one might think that for selecting an off-rate as slow as possible one should compete and thus wait as long as possible before collecting the binders with the beads. However, after experimentally finding that this does not lead to the desired outcome, we have recently computationally analyzed this process and found that the optimal selection works quite different (108). Here only a very qualitative summary is given; the interested reader is directed to the original publication.

Let us assume that the initial biotinylated target is sufficient to capture all binders and that the capture beads are sufficient to capture all biotinylated target. If we incubate this library with non-biotinylated target in excess for a very long time, *all* the binders will equilibrate between both forms of target. After sufficiently long time, the distribution of binders on the biotinylated target and on the non-biotinylated target becomes identical – no affinity enrichment at all is achieved!

The enrichment of the binders with the slowest off-rate is thus a transient phenomenon (108). At intermediate times, the immobilized target will indeed carry a population that is enriched for the binders with slow off rate. This enrichment will be highest at the time that is the reciprocal of the best off-rates. When in doubt, it is better to err on the side of shorter times.

The selection pressure is dominated by the ratio of non-biotinylated to biotinylated target, which should be as high as possible. There is usually a practical upper limit, given by the availability of the target. It is not useful to decrease the amount of immobilized target because of the danger of selecting background binders as explained above.

Given a limited amount of target, the practitioner has the choice between few rounds of very stringent selections (using the target in large excess) or many rounds with less stringent selections (using the valuable target over more rounds). The less selective strategy will keep a higher diversity and thus potentially binders with a greater range of biological properties and effects. Yet, when the target is severely limited, one selective round with high target excess is probably the best strategy.

After any highly selective step, the number of binders becomes, by necessity, very small. Thus, background binding by non-functional clones can become significant. In order to rectify this problem, a non-selective round directly following the stringent one has been found to be highly useful (108) (and references therein). In this case, all remaining binders are "collected" and amplified, which thereafter greatly outnumber the non-functional molecules.

11. Selections for Properties Other than Affinity

11.1. Selectivity

Besides high affinity, selective discrimination of a particular target, and non-recognition of a similar molecule, is often desired. This can involve recognition of a particular mutant, a posttranslational modification or a conformation. Like in other display technologies such as phage display, this can best be achieved by immobilizing the desired target, and adding the non-desired target as a competitor, such that all members of the library which recognize both, and thus do not discriminate, will be washed out by binding to the non-immobilized competitor.

11.2. Catalysis

The use of display technologies such as phage display (reviewed in ref. 109) and ribosome display (reviewed in ref. 110) for selecting enzymatic turnover has been attempted. While a number of approaches have been found for carrying out selections to identify active catalysts from among many inactive molecules, it is less apparent how to select for the quantitative improvement of enzymatic turnover with display technologies that ultimately select only for a binding event. Ribosome display might thus play a role in the initial selection of very large libraries to identify active molecules. At the present time, it appears that the use of emulsion techniques might be better suited to select for improvements of in vitro turnover rates (94, 95). Ultimately, a direct sorting of the aqueous compartments as a quantitative measure of turnover will be needed.

11.3. Stability

It is a widespread assumption that ribosome display is unsuitable for evolution of protein stability, and thus its inherent advantages

of large library size and facile interfacing with random mutagenesis cannot be exploited for this problem. Fortunately, this is not true. The recent discovery that the intrinsic stability of the ribosomal complexes, e.g., as found in the PURE system (60–62), is rather high is further encouraging for such experiments. Even with the standard *E. coli* system, such experiments have been successfully carried out: Using an antibody scFv fragment of medium stability as a model system, its stability was improved by a succession of random mutagenesis and selection for specific binding in the presence of a suitable buffer favoring unfolding (106). The antibody derives a significant part of its stability from its intradomain disulfide bonds. By increasing the level of reducing agents from round to round, scFv fragments were selected which could fold in the complete absence of disulfides. More importantly, when the disulfides were allowed to form again, the free energy of folding gained by the selected mutations was almost additive. It is likely that similar scenarios can be designed for the selection of high stability variants of other proteins. The main prerequisite will be to select for binding that is strictly coupled to correct folding, and does not allow partially folded "sticky" molecules to become enriched.

12. Conclusions and Future Prospects

Ribosome display has proven to be a robust procedure, used now in academic and industrial laboratories, which comes rather close to experimental protein evolution in the test tube. Un doubtedly, the procedure will be further improved and applied to many new targets and selection goals. Progress in automation, selection on complex targets such as whole cells, as well as applications of deep sequencing are the obvious developments that can be expected to contribute to the further development of this powerful in vitro evolution. Undoubtedly, this evolution technology will itself evolve.

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