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In Vitro Selection Methods for Screening of Peptide and Protein Libraries

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1 Introduction

Protein ligand interactions form the basis of almost all cellular functions. The identification and improvement of the relevant ligand or receptor is therefore a

focus of much of current biochemical research and the prerequisite for most pharmaceutical applications. Despite great progress, computational methods have generally not produced the accuracy required for "designing" mutations which improve a function such as binding or stability. Over the last few years, however, enormous progress in molecular biology has made it possible to imitate nature's strategy to solve the problem, the strategy of evolution. Evolution is a continuous alternation between mutation and selection. In order to apply this strategy in the laboratory over many generations both components, mutation and selection, have to be easy to perform, robust to execute and powerful in order to succeed. In this

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chapter we will summarize the state of the art in carrying out both diversification and selection entirely in vitro, making use of cell-free translation.

All evolutionary methods must couple phenotype and genotype. Normally the carrier of the phenotype, proteins or peptides are undoubtedly the most versatile class of compounds and nature's choice in performing almost all tasks, except information storage, because of their modularity and chemical versatility. Almost all of the selection methods developed so far for peptides and proteins have either used living cells either directly, or indirectly by production of bacteriophages (PHIZICKY and FIELDS 1995). Two popular technologies illustrate the cell-based approach: the two-hybrid system (FIELDS and STERNGLANZ 1994) and phage display (RADER and BARBAS 1997).

These in vivo approaches are all limited by transformation efficiency (Dower and CWIRLA 1992). It is quite laborious to produce libraries of 10¹⁰ members (VAUGHAN et al. 1996), but enough of sequence space must be sampled to find reasonable "hits" or starting points for evolution, and such library sizes are required for undertaking difficult evolutionary tasks. Moreover, in an evolutionary approach, sequence diversification would normally take place in vitro, and thus ligation and transformation have to be repeated for every generation. Consequently, with a few exceptions (YANG et al. 1995; Low et al. 1996; CRAMERI et al. 1996; MOORE and ARNOLD 1996), real evolutionary approaches with several generations have rarely been reported using in vivo methods. In this chapter, we will summarize the state of the art for carrying out selection and diversification entirely in vitro, not using cells at any step and thereby circumventing the limitations of the in vivo methods summarized above. Libraries with more than 10¹² members can now rapidly be prepared and evolved with these methods.

2 History of In Vitro Based Selection Methods

Already in the 1970s a series of studies showed that specific mRNA could be enriched by immunoprecipitation of polysomes with antibodies directed against the protein product (SCHECHTER 1973; PAYVAR and SCHIMKE 1979; KRAUS and ROSENBERG 1982). Before the general advent of molecular cloning, this was an important means of enriching the mRNA for a particular protein. KAWASAKI (1991) suggested exploiting this observation as a method to enrich peptides and proteins from libraries, yet without publishing any experimental data. The idea was put into practice for the first time 3 years later by MATTHEAKIS et al. (1994), who reported an affinity selection of short peptides from a library by using polysomes from an *E. coli* system (1994), and later by GERSUK et al. (1997) by using a wheat germ system. Significant modifications and optimizations were necessary, however, to make this concept applicable to the selection of whole, folded proteins, such as single-chain Fv (scFv) fragments of antibodies (HANES and PLÜCKTHUN 1997). Subsequently, ribosome display also was used in a eukaryotic cell-free system (HE and TAUSSIG 1997). In a variation on this concept, it was reported that peptides could be attached to their encoding mRNAs after in vitro translation through a puromycin derivative synthetically coupled to mRNA (NEMOTO et al. 1997; ROB-ERTS and SZOSTAK 1997).

3 Two In Vitro Selection Schemes

The in vitro selection schemes can be divided into two groups: in the first, the polypeptide remains linked to the mRNA on the ribosome (Fig. 1). In this review, this method is referred to as "ribosome display", although other names such as polysome display (MATTHEAKIS et al. 1994), polysome selection (GERSUK et al. 1997) or ARM selection (HE and TAUSSIG 1997) have been used. In the second strategy, after in vitro translation of the mRNA, which has to be modified to carry a puromycin derivative at the end, a covalent RNA-peptide fusion is generated by the reaction of this puromycin derivative (Fig. 2). In this review, this technique is referred to as "RNA-peptide fusion" (ROBERTS and SZOSTAK 1997), although the name 'in vitro virus' has been also used (Nемото et al. 1997). For both strategies ribosomes and all other necessary components, especially initiation and elongation factors and a specially designed mRNA, are used for in vitro translation. In ribosome display, this is performed in such a way that neither the mRNA nor its encoded peptide leave the ribosome during the ligand binding reaction; similarly, in the RNA-peptide fusion, the mRNA and the encoded peptide have to stay on the ribosome long enough for the chemical reaction to occur.

4 Ribosome Display

Ribosome display has been successfully performed using: (1) an *E. coli* S-30 system for display and selection of a peptides library (MATTHEAKIS et al. 1994) or of a library of folded proteins (HANES and PLÜCKTHUN 1997; HANES et al. 1998), (2) a wheat germ system for display and selection of a peptide library (GERSUK et al. 1997) and (3) a rabbit reticulocyte system for display and selection of folded proteins (HE and TAUSSIG 1997).

4.1 How Ribosome Display Works

The principle of ribosome display is shown in Fig. 1A. A DNA library, encoding a polypeptide in a special ribosome display cassette (discussed below), is either directly used for coupled in vitro transcription-translation, or first transcribed in vitro to mRNA, which is purified and used for the in vitro translation. This results in formation of ribosomal complexes (mRNA-ribosome-polypeptide), which are used for affinity selection. After removal of non-specifically bound complexes by intensive washing, RNA is isolated and used for reverse transcription and PCR. RNA can be isolated from bound ribosomal complexes either



directly, by removing Mg^{2+} with an excess of EDTA and thus causing dissociation of all bound complexes, or first by competitive elution of ribosomal complexes with free ligand followed by RNA isolation only from eluted complexes (Fig. 1B). On the one hand, the latter approach can be advantageous, because the RNA is isolated only from those ribosomal complexes which contain a functional ligand binding protein. But on the other hand, this approach might be difficult to apply for very tight binders.

4.2 Construction of a DNA Library

The ribosome display construct (Fig. 3) contains, at the DNA level, a T7 promoter for efficient transcription to mRNA. On the RNA level, the construct contains a prokaryotic ribosome binding site or a Kozak sequence, depending on the translation system used, followed by the protein coding sequence without a stop codon. In a prokarytic cell-free translation system the presence of a stop codon would result in the binding of the release factors (GRENTZMANN et al. 1995; TUITE and STANSFIELD 1994; TATE and BROWN 1992) and the ribosome recycling factor (JANOSI et al. 1994) to the mRNA-ribosome-protein complexes. This in turn would lead to the hydrolysis of peptidyl-tRNA between the 3'-ribose and the last amino acid of the polypeptide by the peptidyltransferase center of the ribosome (TATE and BROWN) 1992) (Fig. 4A). A similar mechanism is also operative in eukaryotic systems (FROLOVA et al. 1994; ZHOURAVLEVA et al. 1995). No equivalent to the prokaryotic ribosome recycling factor has been identified in eukaryotes so far. Obviously, no stop codon must be present in order to keep mRNA and the encoded protein in the ribosomal complexes. However, there is a backup-system in E. coli, involving the 10Sa-RNA (RAY and APIRION 1979), a stable bacterial RNA with tRNA-like structure (KOMINE et al. 1994). A polypeptide translated in vivo from mRNA without stop codon is modified by COOH-terminal addition of a peptide tag, encoded by the 10Sa-RNA (Tu et al. 1995) and subsequently released from the ribosome (Fig. 4B). The released protein tagged with this sequence is finally degraded by a tail-specific protease (KEILER et al. 1996).

In ribosome display constructs, the open reading frame coding for the protein comprises two portions: the NH₂-terminal part, which codes for the polypeptide to

Fig. 1. A Principle of ribosome display for screening protein libraries for ligand binding. A DNA library containing all important features necessary for ribosome display (for details see text) is first transcribed to mRNA and after its purification, mRNA is translated in vitro. Translation is stopped by cooling on ice, and the ribosome complexes are stabilized by increasing the magnesium concentration. Ribosomal complexes are affinity selected from the translation mixture by the native, newly synthesized protein binding to immobilized ligand. Nonspecific ribosome complexes are removed by intensive washing, and mRNA is isolated from the bound ribosome complexes, reverse transcribed to cDNA, and cDNA is then amplified by PCR. This DNA is then used for the next cycle of enrichment, and a portion can be analyzed by cloning and sequencing and/or by ELISA or RIA. **B** Two methods for mRNA isolation from bound ribosomal complexes. The bound ribosomal complexes can either be dissociated by an excess of EDTA and then RNA is isolated, or they can first be eluted specifically with free ligand followed by RNA isolation



Fig. 2. Principle of RNA-peptide-fusion strategy for screening peptide libraries. A DNA peptide library is first transcribed to mRNA, and after its purification mRNA is coupled to a DNA-puromycin derivative. The mRNA-DNA-puromycin derivative is purified and used for in vitro translation. The ribosome stalls at the first DNA residue and puromycin from the translated mRNA enters the ribosomal A-site, where it is covalently linked to the translated peptide with the help of the peptidyl-transferase center. Such an RNA-peptide-fusion no longer needs a ribosome. The desired RNA-peptides are affinity selected from the mixture by binding of the peptide to immobilized ligand. After intensive washing the bound RNA-peptides are isolated, reverse transcribed to cDNA, and cDNA is then amplified by PCR. This DNA is then used for the next cycle of enrichment, and a portion can be analyzed by cloning and sequencing and/

or by ELISA or RIA



Fig. 3. The scFv construct used for prokaryotic ribosome display. *T7* denotes the T7 promoter, *SD* the ribosome binding site, *spacer* the part of the protein construct connecting the folded scFv to the ribosome, *5*'sl and *3*'sl the stem loops on the 5'- and 3'-ends of the mRNA. The *arrow* indicates the transcriptional start

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Fig. 4A,B. Role of stop codon and 10Sa-RNA in *E. coli.* A Role of stop codon. A complex of two release factors (proteins), RF-1 and RF-3 or RF-2 and RF-3, binds in place of tRNA, when a stop codon is encountered. The release factor RF-1 recognizes the stop codons UAA or UAG while factor RF-2 recognizes the stop codons UAA or UGA. The binding of the release factor complex results in hydrolysis of peptidyl tRNA in the ribosome and the protein is released. **B** Role of 10Sa-RNA. Translation of mRNA without a stop codon results in the binding of 10Sa-RNA to the A-site of the ribosome. First alanine, which is the acyl group carried by this RNA, is added to the truncated protein. Then, this RNA is taken to be a messenger RNA, resulting in coupling of a peptide tag encoded by the 10Sa-RNA with the sequence indicated. Because this tag ends with a stop codon, the protein is released normally and then degraded by a protease specific for this COOH-terminal tag

be selected (the library), and the COOH-terminal part, which is constant and serves as a spacer. The spacer has several functions: (1) it tethers the synthesized protein to the ribosomes by maintaining the covalent bond to the tRNA which is bound at the P-site of the ribosome, (2) it keeps the synthesized polypeptide outside the ribosome and allows it to fold and to interact with ligands, despite the fact that the ribosome itself is thought to cover about 20–30 amino acid residues of the emerging polypeptide, and (3) it may slow down protein synthesis, since the spacer can contain rare codons, mRNA secondary structures or other stalling sequences (MATTHEAKIS et al. 1996). However, no beneficial effect of these translation retarding features for display efficiency has been experimentally demonstrated. A number of different spacers of various lengths have been used. For peptide libraries, spacers of 85 (MATTHEAKIS et al. 1994), 121 (MATTHEAKIS et al. 1996) and 72 amino acid residues (GERSUK et al. 1997) were reported. For protein display, spacers of 88–116 amino acid residues in length were used and found to increase the efficiency of *E. coli* ribosome display with the length of the spacer (HANES and PLÜCKTHUN 1997), and in a rabbit reticulocyte system a kappa domain of an antibody served as a spacer of 103 amino acids (HE and TAUSSIG 1997).

At the RNA level, additional important features which should be present in the ribosome display construct are 5'- and 3'-stem loops. They are known to stabilize mRNA against RNases and therefore increase the half life of mRNA in vivo as well as in vitro (BELASCO and BRAWERMAN 1993). The *E. coli* S-30 extract, which is used for in vitro translation during ribosome display, contains high RNase activity. The introduction of the 5'-stem-loop, derived from the T7 gene 10 upstream region directly at the beginning of the mRNA, and the introduction of the 3'-stem-loop, derived from the terminator of the *E. coli* lipoprotein, into the ribosome display construct were found to improve mRNA stability and therefore increased the efficiency of ribosome display approximately 15-fold (HANES and PLÜCKTHUN 1997). A similar improvement was observed when using the analogous 5'-stem-loop and the 3'-stem-loop derived from the early terminator of phage T3 (HANES and PLÜCKTHUN 1997).

A ribosome display library template can be conveniently prepared by ligation of a DNA library to the spacer and subsequent amplification of the ligation mixture in two PCRs with two pairs of oligonucleotides, which introduce all above-mentioned features important for ribosome display (e.g. HANES and PLÜCKTHUN 1997).

4.3 In Vitro Translation

The DNA library can either be directly converted to a ribosome-bound polypeptide library by coupled in vitro transcription-translation (MATTHEAKIS et al. 1994; HE and TAUSSIG 1997), or mRNA can be first prepared by in vitro transcription and subsequently used for in vitro translation (HANES and PLÜCKTHUN 1997; GERSUK et al. 1997). The coupled system is simpler than the uncoupled one, but it was observed that the efficiency of the coupled *E. coli* system is much lower than the uncoupled system (Hanes et al., unpublished experiments). Another disadvantage of the coupled system is an incompatibility of the redox requirements of transcription and translation when displaying proteins containing disulfide bridges. T7 RNA polymerase, which is necessary for transcription in this system, is usually stabilized by β -mercaptoethanol, which competes with disulfide bond formation. This problem may in principle be overcome by preparing T7 RNA polymerase without reducing agent, but the enzyme's activity must then be carefully monitored.

Translation is usually performed at 37°C when using the *E. coli* in vitro system (MATTHEAKIS et al. 1994; HANES and PLÜCKTHUN 1997). Despite the general tendency of proteins to fold with higher efficiency at lower temperature in vitro, the yield of functional molecules from in vitro translation was indeed found to be

higher at 37°C. This may be due to the action of chaperones in the extract, and it is a complicated function of the temperature-dependence of translation, folding, RNases and perhaps proteases. For in vitro translation in eukaryotic systems, lower temperatures are usually used, for example 30°C in the rabbit reticulocyte system (HE and TAUSSIG 1997) or even 27°C in the wheat germ system (GERSUK et al. 1997).

The time of translation is also an important variable and is more critical for uncoupled systems. At physiological temperatures, the absence of a stop codon is not sufficient to keep the mRNA and its encoded protein complexed to the ribosome forever. An in vitro translation of truncated lysozyme mRNA in a wheat germ system resulted only in free protein, and no protein present in the ribosomal fraction, after 80 min of translation (HAEUPTLE et al. 1986). The translated protein was only observed to be present in the ribosomal fraction after shortening of the translation time to 60 min, and its concentration increased when the translation was performed for only 30 min (HAEUPTLE et al. 1986). The translation necessary for ribosome display with an uncoupled wheat germ system was performed for 15 min at 27°C (GERSUK et al. 1997), and the optimal time for the uncoupled E. coli system is usually not longer than 10 min at 37°C (HANES and PLÜCKTHUN 1997). However, the complexes are very stable, as soon as they are cooled to 4°C (HANES et al., unpublished experiments). In a coupled transcription-translation system mRNA is continuously produced and therefore the reaction time can be extended to 30-60 min (MATTHEAKIS et al. 1996; HE and TAUSSIG 1997). Too long a translation, on the other hand, may lead to the depletion of some crucial component necessary for translation or transcription or the accumulation of low molecular weight compounds inhibiting translation (JERMUTUS et al. 1998), resulting in a subsequent decrease of the amount of ribosomal complexes. Several additional components can be used during in vitro translation which can improve the efficiency of ribosome display. RNasin, an inhibitor of RNases, was added during in vitro translation in the wheat germ system (GERSUK et al. 1997), but it was not reported whether it had any effect. RNasin was found to have no influence during in vitro translation in an E. coli system (HANES and PLÜCKTHUN 1997). However, vanadyl ribonucleoside complexes (BERGER and BIRKENMEIER 1979; PUSKAS et al. 1982), general RNase inhibitors which should act as transition state analogs, were found to increase the efficiency of E. coli ribosome display when used during in vitro translation (HANES and PLÜCKTHUN 1997). For displaying folded disulfide containing proteins such as scFv fragments of antibodies, eukaryotic protein disulfide isomerase (PDI), which catalyzes disulfide bond formation and rearrangement, was found to increase the efficiency of E. coli ribosome display three-fold when used during translation (HANES and PLÜCKTHUN 1997) (Table 1). The elimination of the 10Sa-RNA (the product of the ssrA gene) by an antisense oligonucleotide, which is responsible for tagging (see Sect. 4.2) and releasing truncated peptides from E. coli ribosomes (RAY and APIRION 1979; Ko-MINE et al. 1994; KEILER et al. 1996), yielded a four-fold improvement of ribosome display when using an antisense DNA oligonucleotide directed against this RNA (HANES and PLÜCKTHUN 1997) (Table 1).

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mRNA structure	Additives ^a	Yield of mRNA after one round of affinity selection								
(5 and 3 -loops)		Percent of input mRNA	Number of molecules ^b	Relative amount						
	No	0.001	1.3×10^{8}	1						
+	No	0.015	2.0×10^{10}	15						
+	PDI	0.045	5.9×10^{10}	45						
+	Anti 10Sa-RNA ^c	0.060	7.9×10^{10}	60						
+	PDI, anti 10Sa-RNA	0.200	2.6×10^{11}	200						

Table 1. Summary of improvements for increasing the efficiency of ribosome display

PDI, protein disulfide isomerase.

^a 0.1% VRC during translation and 50 mM Mg²⁺ during affinity selection were used in all experiments. ^b Number of molecules isolated from 1 ml reaction.

^cAntisense oligonucleotide

4.4 Affinity Selection of Ribosomal Complexes

After in vitro translation, the reaction is stopped by rapid cooling on ice. In the eukaryotic system, cycloheximide can also be added (GERSUK et al. 1997), but the effect of this compound on the efficiency of the ribosome display has not been reported. The mixture is also diluted with a buffer containing magnesium acetate, which is present during the whole affinity selection. Concentrations of 5 mM magnesium acetate in a wheat germ system (GERSUK et al. 1997) or 10 mM magnesium acetate in an E. coli system (MATTHEAKIS et al. 1994) were used. Much higher concentrations (50 mM magnesium acetate) were found to be optimal and improved the efficiency of the E. coli ribosome display several-fold (HANES and PLÜCKTHUN 1997). A possible explanation for the need for high magnesium concentrations is that Mg²⁺ binds to the phosphates of the ribosomal RNA, the mRNA, and the peptidyl tRNA, thus stabilizing ribosome complexes. In the absence of magnesium, ribosome complexes may dissociate. No magnesium was used during the reported affinity selection in a rabbit reticulocyte system (HE and TAUSSIG 1997). It would be interesting to see if any improvement resulted from addition of Mg^{2+} to this system.

Chloramphenicol, an antibiotic which inhibits bacterial protein synthesis by binding to the 23S ribosomal RNA in the peptidyl transferase center, has been used throughout the entire affinity selection processes in an *E. coli* system (MATTHEAKIS et al. 1994). However, in a direct comparison, chloramphenicol was found to have no influence on the efficiency of *E. coli* ribosome display (HANES and PLÜCKTHUN 1997). While MATTHEAKIS et al. (1994) preparatively separated the ribosomal complexes by centrifugation through a sucrose cushion prior to affinity selection, all other reports used the translation mixture directly for panning (HANES and PLÜCKTHUN 1997; HE and TAUSSIG 1997; GERSUK et al. 1997). In a direct comparison, no improvement by the isolation of ribosomal complexes through a sucrose cushion was found (HANES and PLÜCKTHUN 1997). Affinity selection can be performed by using either ligands immobilized on a surface (such as panning tubes or microtiter wells) or biotinylated ligands bound to the ribosome-bound proteins which are subsequently captured by streptaridincoated magnetic beads. After extensive washing with a magnesium-containing buffer, mRNA can be isolated either from ribosome complexes dissociated with EDTA, or from complexes specifically eluted with an excess of a free ligand (Fig. 1B). The isolated mRNA is then used for RT-PCR, and the DNA thus obtained can be used for the next cycle of ribosome display. A portion of the DNA can be analyzed by cloning and sequencing and/or by ELISA or RIA after each round of ribosome display. When magnetic beads are used for selection, RT-PCR can also be directly performed with a portion of the beads (HE and TAUSSIG 1997).

The efficiency of ribosome display can also be improved by decreasing the nonspecific binding. Supplementing the diluted translation mixture before affinity selection with 2% sterilized milk and/or 0.2% heparin eliminates much of the nonspecific binding, perhaps by preventing binding of ribosome complexes to the

panning tube surface or to magnetic beads, and heparin probably acts also as an RNase inhibitor (HANES et al. 1998).

5 Applications of Ribosome Display

5.1 Display of Peptides on Ribosomes

An *E. coli* ribosome display system was used for displaying peptides from a decamer random library. This library was selected for binding to the monoclonal antibody D32.39, which has been raised to bind dynorphin B, a 13-residue opioid peptide, with 0.29 nM affinity (MATTHEAKIS et al. 1994). A library of 10^{12} DNA molecules was used for ribosome display using coupled in vitro transcription-translation. After five rounds of ribosome display, several different peptides ranging from 7.2 to 140 nM affinity to the antibody were found. However, a peptide similar to dynorphin B, or any peptides possessing a similar affinity, were not obtained.

A wheat germ ribosome display system was used for displaying a 20-mer random library, which was selected for binding to a prostate-specific antigen (PSA) (GERSUK et al. 1997). After four rounds of selection, several peptides showing higher affinity to PSA than to bovine serum albumin or gelatin were isolated, but no quantitative data were reported.

5.2 Display of Folded Proteins on Ribosomes

Two scFv fragments of an antibody were used as a model system: scFv-hag, derived from the antibody 17/9 (specific for hemagglutinin peptide) (SCHULZE-GAHMEN et al. 1993), and scFv-AL2 (specific for ampicillin) (KREBBER et al. 1996). mRNAs of these two scFvs were mixed in a ratio of 1:10⁸ (scFv-hag:scFv-amp) and applied for

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affinity selection by using ribosome display on a hag-surface (HANES and PLÜCKTHUN 1997). After five rounds of selection, a pool of enriched sequences was cloned and single clones were analyzed. Of 20 scFvs, 18 were scFv-hag and two were scFv-amp, demonstrating that the 10⁹-fold enrichment was successful. The average enrichment per cycle of ribosome display was thus about 100-fold, and this number is now known to depend both on the antibody, antigen and type of surface (Hanes et al., unpublished experiments). All 18 scFvs with the anti-hag sequence were analyzed by RIA, and it was shown that all but one of them bind hag peptide and can be inhibited by it. Sequence analysis showed that all of them had mutated during the five cycles of ribosome display and possessed between zero and four amino acid substitutions with respect to the original scFv-hag. All changes were independent of each other. It was also shown that from a binary mixture of scFvhag and scFv-amp mRNAs, mixed in a ratio of 1:1, either scFv could be enriched, depending on which antigen was used for affinity selection (HANES and PLÜCKTHUN 1997).

E. coli ribosome display was applied to affinity selection of scFv antibody fragments from a diverse library generated from mice immunized with a variant peptide of the transcription factor GCN4 dimerization domain (HANES et al. 1998). The E. coli ribosome display system using uncoupled in vitro translation and all the improvements reported by HANES and PLÜCKTHUN (1997) were used. After three rounds of ribosome display, an enriched pool of scFv genes were cloned and single clones were analyzed by RIA. Twenty-six different scFvs binding to a GCN4variant peptide were isolated. Several different scFvs were selected, but the largest group of 22 scFvs was closely related to each other and differed in zero to five amino acid residues with respect to their consensus sequence, the likely common progenitor. The other four scFvs were different from each other and also from the group of closely related 22 scFvs, and showed lower affinity to the GCN4-variant peptide than the 22 related scFvs, based on RIA analysis. The best scFv was found among the related ones and had a dissociation constant of $(4 \pm 1) \times 10^{-11}$ M, measured in solution. The scFv identical to the consensus sequence, a likely common progenitor of the 22 related scFvs, was identified and had a dissociation constant of only $(2.6 \pm 0.1) \times 10^{-9}$ M. Detailed analysis showed that for the 65-fold higher affinity of the best scFv to the antigen only one amino acid change in

the "progenitor" scFv was responsible. It was also shown that this high-affinity scFv was selected from mutations occurring in vitro during ribosome display rounds and that it was not present in the original library. Thus, this selected scFv had evolved throughout the rounds of ribosome display (HANES and PLÜCKTHUN 1997). The in vitro selected scFvs could be functionally expressed in the E. coli periplasm with good yields, or prepared by in vitro refolding in equally good yields. Rabbit reticulocyte ribosome display was also used to select a scFv derivative of an antibody, of the type V_H-linker-V_L-C_L, binding to progesterone. A minilibrary was prepared by mixing the DNA coding for this construct derived from the progesterone specific antibody DB3^R (carrying the mutation Trp100Arg in V_H which does not influence antigen binding), and a DNA of several mutant scFvs in position H35 which do not bind this antigen (HE and TAUSSIG 1997). The enrich-

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ment was analyzed by DNA sequencing of the pool. No antigen binding data (RIA or ELISA) of the translated protein have been reported to date. DB3^R DNA diluted 10^2 - to 10^6 -fold with mutant DB3^{H35} DNA was applied for ribosome display using progesterone-BSA, covalently immobilized on magnetic beads. After one round of ribosome display DB3^R was reported to be the dominant species, recovered from 10^2 - to 10^4 -fold dilution, and comprising about 50% when enriched from a 10^5 -fold diluted mixture. The reported nominal enrichment for one cycle of ribosome display was therefore about 10^4 - 10^5 , even though the translation mixture contained 2 mM DTT, and the ribosomes were not stabilized by Mg²⁺. Yet, in a direct comparison of the eukaryotic and the *E. coli* ribosome display system with the same scFv fragment, lower enrichments were found for the eukaryotic system (Hanes et al., submitted).

6 RNA-Peptide Fusion

The selection principle of the RNA-peptide fusion approach is directly related to the ribosome display technology, but uses a puromycin-tagged mRNA and several additional steps to achieve covalent coupling of mRNA to its encoding polypeptide (Fig. 2). The method has so far been applied using the rabbit reticulocyte in vitro translation system (ROBERTS and SZOSTAK 1997; NEMOTO et al. 1997). The RNApeptide fusion construct consists of the mRNA coding for the peptide sequence, fused either to a DNA spacer of the sequence dA₂₇dCdC coupled to puromycin (ROBERTS and Szostak 1997) or to a DNA-RNA hybrid spacer of 125 deoxynucletides and four ribonucleotides coupled to puromycin (NEMOTO et al. 1997). The principle of this system is similar to ribosome display: an mRNA–DNA–puromycin hybrid is used for in vitro translation in a rabbit reticulocyte system. When the ribosome reaches the DNA portion of the template, translation stalls. At this point the ribosome complex can either dissociate, or puromycin, which is part of the template, can enter the ribosome and attach itself to the synthesized peptide. In this way the genotype, mRNA, can be directly attached to the phenotype, its encoded peptide. For testing the system, the template encoding a myc-epitope was used. It was shown that the RNA-myc-peptide fusion can be isolated from an in vitro translation mixture by immunoprecipitation with a monoclonal antibody recognizing a myc-epitope (ROBERTS and SZOSTAK 1997). The myc-peptide-mRNA fusion was also enriched by immunoprecipitation from a mixture prepared by translation of myc-encoding template, diluted with template encoding a random peptide pool. The reported enrichment factor was 20- to 40-fold. Nonspecific RNA-peptide fusions (peptides coupled not to their encoding RNA) were not observed, and thus the enrichment is probably limited by nonspecific binding of peptides and unprotected RNA or DNA to the target. About 1% of RNA was converted to the RNApeptide fusion (ROBERTS and SZOSTAK 1997).

7 Conclusions and Perspectives

Two closely related in vitro selection methods for screening of peptide and protein libraries, ribosome display and RNA-peptide fusion, have been reported so far. Both are based on a cell-free translation system, and all steps are performed in vitro without using cells in any step. The phenotype, the synthesized protein or peptide, is attached to its genotype, encoded by mRNA, either by complexing it with the ribosome in the ribosome display system or, in the RNA-peptide fusion method, by subsequent covalent attachment of the synthesized peptide to its puromycin-derivatized mRNA. The ribosome display system was shown be effective in the selection of peptides and also of functional, folded proteins from complex libraries, while selection experiments with the RNA-peptide fusion system have so far been reported for peptides.

The main advantage of in vitro selection methods is, as already mentioned, that no cloning is necessary and therefore very large libraries can be used for screening and selection. The ribosome display system can be performed very rapidly; one cycle of selection can be achieved within one day, which compares well with in vivo selection methods, when a library has to be prepared. In the RNA-peptide fusion approach the mRNA-puromycin derivative must be synthesized after each cycle, and therefore this method is somewhat slower than ribosome display. Another advantage of in vitro selection methods is the automatic introduction of mutations during the procedure, when non-proofreading polymerases are used, and thus proteins can also affinity-mature during selection. It appears that in vitro selection methods have a great utility in compound identification and optimization, and they can thus help to answer fundamental questions of protein structure and evolution.

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