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Ribosome display: an in vitro method for selection and evolution of antibodies from libraries

Christiane Schaffitzel, Jozef Hanes, Lutz Jermutus, Andreas Plückthun *

Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, Zürich CH-8057, Switzerland

Abstract

Combinatorial approaches in biology require appropriate screening methods for very large libraries. The library size, however, is almost always limited by the initial transformation steps following its assembly and ligation, as other all screening methods use cells or phages and viruses derived from them. Ribosome display is the first method for screening and selection of functional proteins performed completely in vitro and thus circumventing many drawbacks of in vivo systems. We review here the principle and applications of ribosome display for generating high-affinity antibodies from complex libraries. In ribosome display, the physical link between genotype and phenotype is accomplished by a mRNA–ribosome–protein complex that is used for selection. As this complex is stable for several days under appropriate conditions, very stringent selections can be performed. Ribosome display allows protein evolution through a built-in diversification of the initial library during selection cycles. Thus, the initial library size no longer limits the sequence space sampled. By this method, scFv fragments of antibodies with affinities in the low picomolar range have been obtained. As all steps of ribosome display are carried out entirely in vitro, reaction conditions of individual steps can be tailored to the requirements of the protein species investigated and the objectives of the selection or evolution experiment.

Keywords: Ribosome display; scFv fragments of antibodies; In vitro selection; Directed evolution; Cell-free translation

1. Introduction

Köhler and Milstein established, more than 20 years ago, a powerful method to produce monoclonal antibodies from immunized mice by fusing murine B-cells to myeloma cells. The advent of this hybridoma technology (Köhler and Milstein, 1975) made possible the generation of a large number of antibodies that are currently in widespread use in biomedical research, diagnostics and more recently also in therapeutic applications (Holliger and Hoogenboom, 1998). Hybridoma technology, however, is limited by several shortcomings. For instance, the target of choice to be used as the antigen

Abbreviations: CDR, complementarity-determining region; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; RIA, radioimmunoassay; RT, reverse transcription; scFv, single-chain Fv antibody fragment; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELEX, systematic evolution of ligands by exponential enrichment; V, immunoglobulin variable region; V_H, immunoglobulin heavy-chain variable domain; V_L, immunoglobulin light-chain variable domain; VRC, vanadyl–ribonucleoside complexes

* Corresponding author. Tel.: +41-1-635-5570; fax: +41-1-635-5712; e-mail: plueckthun@biocfebs.unizh.ch

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needs to be immunogenic, but must not be lethal when injected into animals. Furthermore, the antibodies produced are, by necessity, animal antibodies, which may cause immunogenic reactions when administered to humans. A way out of this particular problem has been to resort to special mouse strains which are constructed to encode several human V regions (Lonberg et al., 1994; Jakobovits, 1995). Nevertheless, while the specificities and affinities of the antibodies can be checked and antibodies can be chosen accordingly, they cannot be improved in hybridoma technology. The intrinsic affinity of antibodies obtained in vivo usually does not exceed 10^{10} M^{-1} (Foote and Eisen, 1995), and it is usually at least one order of magnitude lower, as the generation of higher affinity molecules is not advantageous during the immune response; and thus, there is no selection pressure for further affinity maturation. Thus, in order to obtain antibodies with improved characteristics, it would be necessary to perform challenging cycles of structure-based engineering approaches. However, the predictive accuracy of even the most sophisticated approaches is currently insufficient for this task (Yelton et al., 1995; Dougan et al., 1998). Therefore, evolutionary methods are, at present, the only practical way to improve a given monoclonal antibody from a ''normal'' or a transgenic mouse. Here, we describe a methodology to do this.

The introduction of phage display technology (Smith, 1985; Scott and Smith, 1990) to the field of antibodies, in conjunction with methods of generating antibody libraries, allowed one to bypass some of these limitations (McCafferty et al., 1990; Winter et al., 1994). In particular, the need for animal immunization, as well as the lengthy process of repeated immunization, hybridoma generation and the screening for specific antibodies was replaced by library construction and affinity selection. In phage display, the phage particles, produced by individual bacteria, contain the DNA sequence coding for the protein that is displayed on the phage surface, thus providing a link between the genotype (DNA sequence) and the phenotype (protein), which is a prerequisite for any selection process. For instance, a single-chain Fv (scFv) fragment of an antibody consisting of the covalently linked variable domains of both heavy and light chains (Bird et al., 1988; Huston et al., 1988; Glockshuber et al., 1990) can be displayed on filamentous phage as a fusion to a minor coat protein from filamentous phage (McCafferty et al., 1990) and can be selected by binding to immobilized antigen. Alternatively, the display of Fab fragments of antibodies on the surface of filamentous phages can be used (Hoogenboom et al., 1991; Kang et al., 1991). In recent years, a wide array of antibodies has been selected by phage display, utilizing large, naive (i.e., not stemming from preimmunized organisms) recombinant libraries of human antibody genes (Winter et al., 1994; Vaughan et al., 1996; Aujame et al., 1997; Hoogenboom et al., 1998) or totally synthetic genes (Knappik et al., manuscript submitted), thereby providing a convenient direct access to human antibodies.

While phage display evidently represents a considerable progress over hybridoma technology, deficiencies still do exist. The necessary transformation step limits the library size to approximately 10^{10} (Dower and Cwirla, 1992), and even this size requires a substantial investment of labor. Also, selection in the context of the host environment cannot be avoided, possibly causing loss of potential candidates due to their growth disadvantage or even toxicity for *Escherichia coli*. Furthermore, difficulties in eluting phages carrying antibodies with very high (picomolar) affinity can be encountered (Schier and Marks, 1996). If a diversification step needs to be included in the selection strategy in order to evolve the antibody, either particular mutator strains can be used (Low et al., 1996), that can also create unwanted mutations in the utilized plasmid and in the host genome, or it is necessary to repeatedly switch between the selection procedure carried out in vivo (phage, bacteria) and the mutagenesis step for diversification (polymerase chain reaction [PCR] or recombination techniques) in vitro. This is a particularly laborious procedure, as after each diversification step, the newly created library has to be religated and retransformed. Consequently, evolution of antibodies over several cycles of diversification and selection has only rarely been reported (Yang et al., 1995; Schier and Marks, 1996; Moore et al., 1997), and it is not clear how this could ever become a routine procedure.

Our laboratory developed recently a novel strategy for selection and evolution of antibodies entirely

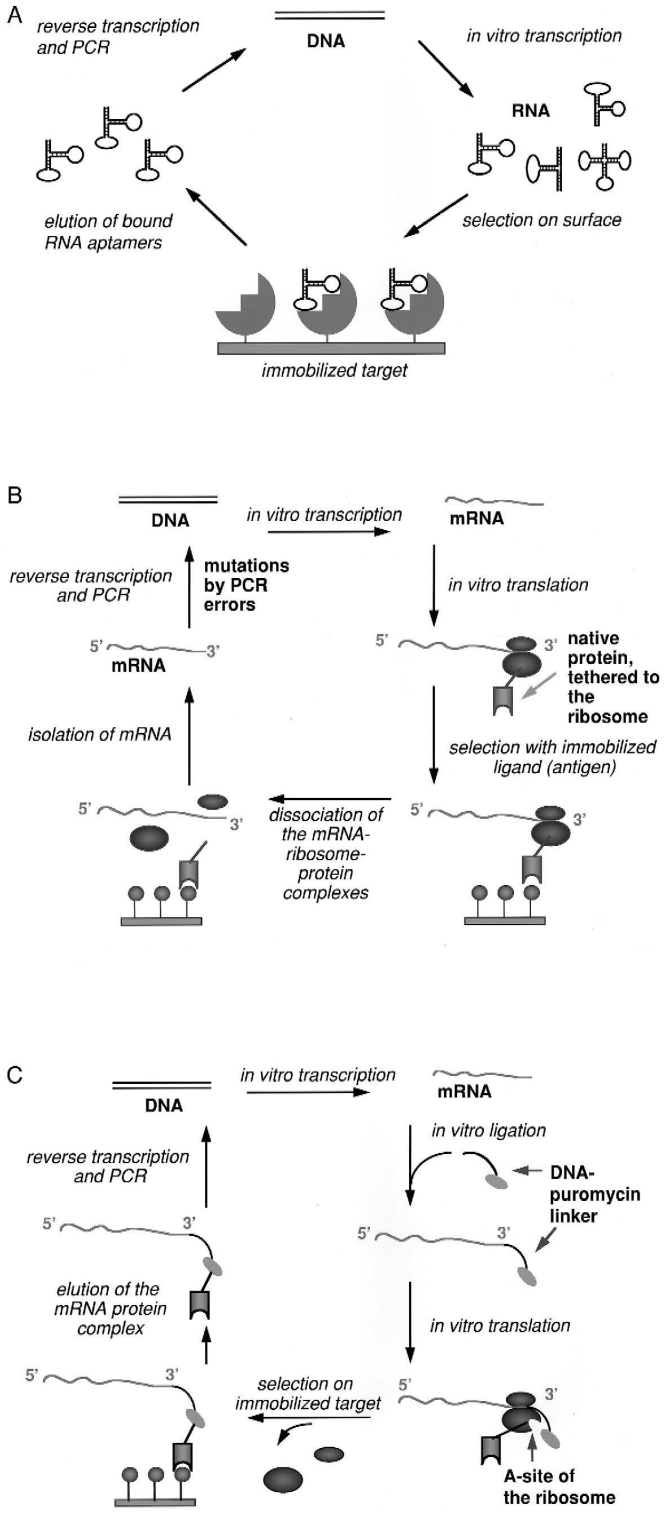
in vitro — ribosome display (Hanes and Plückthun, 1997). It is based on earlier studies with peptide display (Mattheakis et al., 1994), but required considerable improvements in the display and selection of folded proteins. In the improved ribosome display, many of the limitations of phage display are circumvented by utilizing a cell-free transcription, translation and selection system. Using this new technology, it has been possible to select and evolve high-affinity antibodies with dissociation constants as low as 10^{-11} M (Hanes et al., 1998) in a short time, starting from very large libraries which do not have to be transformed into cells. In the following sections, we describe the development and use of ribosome display, its present applications and future potential.

2. In vitro selection techniques

Tuerk and Gold (1990) introduced a technology, termed systematic evolution of ligands by exponential enrichment (SELEX), by which it is possible to exponentially enrich and to evolve ligands consisting of RNA in multiple rounds in vitro from a random oligonucleotide pool. Currently, this method is widely employed to screen for nucleic acid ligands (aptamers) binding to numerous targets, and also to isolate novel, nucleic-acid-based synthetic catalysts for a variety of potential applications in chemistry, diagnostics and, perhaps even therapy (Gold et al., 1995; Osborne et al., 1997). In SELEX, multiple rounds of in vitro transcription of random nucleic acid pools, affinity selection and reverse transcription (RT) PCR are performed, thus giving rise to exponential enrichment of high-affinity specimens. The specific binders can subsequently be cloned and sequenced, and then characterized. The principle underlying SELEX is schematically depicted in Fig. 1A. In SELEX, genotype and phenotype is represented by the same RNA molecule, which exerts its function through its three-dimensional structure, which in turn is determined by its nucleotide sequence.

Nevertheless, RNA molecules have some severe disadvantages as ligands, and they have been essentially replaced by peptides and proteins in evolution. For instance, RNA is extremely labile to ubiquitous RNases, and to actually use any aptamer in a real application requires first the stabilization of the RNA utilizing stable nucleotide analogues (Eaton et al., 1997; Ruckman et al., 1998). The initial (and remaining) appeal of SELEX was the very rapid generation of high-affinity binders from very large initial libraries. Since in ribosome display this problem has now been solved for peptides and proteins as well, the use of RNA aptamers appears less attractive in comparison. Already in their original publication (Tuerk and Gold, 1990), the authors speculated that their methodology could also be adapted for protein selection as well: Particular mRNAs had been isolated from a pool of variants by immunoprecipitation of the nascent polypeptides present in the mRNA–ribosome–polypeptide complexes (Korman et al., 1982; Kraus and Rosenberg, 1982). In fact, a patent application was filed soon afterwards (Kawasaki, 1991), proposing to utilize a similar approach to enrich peptides from libraries. Mattheakis et al. (1994) described the first experimental demonstration of this technology, termed polysome display by the authors. A synthetic DNA library encoding for peptides was used in an *E. coli* coupled transcription/translation system. After in vitro transcription/translation, the mRNA–ribosome–peptide complexes were isolated by centrifugation with a sucrose cushion. The ribosomal complexes were subsequently incubated together with the immobilized target for affinity selection. After several washing steps, the mRNA of the bound mRNA–ribosome–peptide complexes was eluted with EDTA, reverse-transcribed and PCR-amplified. The PCR products could be used for subsequent selection cycles or for analysis of the pool for specific binders. A peptide with nanomolar affinity to an immobilized monoclonal antibody was selected by this technology from a library of 10^{12} molecules; however, the true diversity of the library was not analyzed. Subsequently, binders for a particular prostate cancer marker were isolated using this technique (Gersuk et al., 1997).

Several experiments showed co-translational folding of proteins to occur in in vitro translation systems (Fedorov and Baldwin, 1995, 1997). Furthermore, the enzymatic activity of ribosome-bound nascent polypeptide chains (Kudlicki et al., 1995;


A
reverse transcription and PCR
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DNA
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mutations by PCR errors
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5'
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native protein, tethered to the ribosome
isolation of mRNA
selection with immobilized ligand (antigen)
dissociation of the mRNA-ribosome-protein complexes
C
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in vitro ligation
reverse transcription and PCR
DNA-puromycin linker
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in vitro translation
selection on immobilized target
A-site of the ribosome

Makeyev et al., 1996) could be detected, demonstrating that release from the ribosome is not a prerequisite for acquiring the native conformation of a nascent polypeptide chain, provided folding of the protein is not prevented by the ribosome tunnel. Thus, it appeared to be possible to obtain mRNA, ribosome and correctly folded functional protein in a linked assembly that could be used for screening and selection. The concept developed by Mattheakis et al. (1994) was used for the development of ribosome display of whole functional proteins in our laboratory (Hanes and Plückthun, 1997).

In ribosome display, a DNA library coding for particular proteins, for instance scFv fragments of antibodies, is transcribed in vitro. The mRNA is purified and used for in vitro translation. As the mRNA lacks a stop codon, the ribosome stalls at the end of the mRNA, giving rise to a ternary complex of mRNA, ribosome and functional protein. After in vitro translation, the ribosomal complexes, which are stabilized by high concentrations of magnesium ions and low temperature, are directly used for selection either on a ligand immobilized on a surface or in solution, with the bound ribosomal complexes subsequently being captured, e.g., with magnetic beads. The mRNA incorporated in the bound ribosomal complexes is eluted by addition of EDTA, purified, reverse-transcribed and amplified by PCR. During the PCR step, the T7 promoter and the Shine–Dalgarno sequence are reintroduced by appropriate primers. Therefore, the PCR product can be directly used for further selection cycles. Ribosome display is schematically depicted in Fig. 1B. The experimental procedures of this technique for the selection of scFv fragments of antibodies are commented on in detail below.

A 10^9-fold enrichment of scFv fragments of antibodies was achieved by five cycles of ribosome display, utilizing an *E. coli* S30 extract for in vitro translation. Subsequently, it was demonstrated that it is possible to select and evolve scFv fragments of antibodies from immune libraries by ribosome display even with K_d values in the low picomolar range (Hanes et al., 1998), and eukaryotic cell-free translation systems such as rabbit reticulocyte lysate can also be utilized (He and Taussig, 1997; Hanes et al., 1999). Finally, the selection of high-affinity antibodies from a library of synthetic antibody genes by using ribosome display has now also been demonstrated (Hanes, Schaffitzel, Jermutus and Plückthun, manuscript in preparation).

A somewhat different approach to couple phenotype and genotype was designed by Roberts and Szostak (1997) and independently by Nemoto et al. (1997), who linked a peptide covalently to its encoding mRNA. In this technology, called RNA–peptide fusion (Roberts and Szostak, 1997) or in vitro virus (Nemoto et al., 1997), mRNA is ligated at its 3′-terminus to a puromycin-tagged DNA linker in every cycle (Fig. 1C). The ribosome stalls upon reaching the RNA–DNA junction, the puromycin enters the ribosomal A-site, and the nascent peptide is thereby coupled to puromycin by peptidyl-transferase. The resulting covalently linked complex of polypeptide, puromycin and mRNA–DNA hybrid can be dissociated from the ribosome and used for selection experiments. This approach has so far only been demonstrated for peptides in a model enrichment. The covalent bond appeared attractive, when this method was first designed, as the link between peptide and mRNA seemed more stable than in ribosome display. However, we now know that the ternary ribosomal

Fig. 1. In vitro selection techniques. (A) SELEX. A DNA oligonucleotide pool is transcribed in vitro. The resulting RNA is directly used in affinity selection against an immobilized target. RNA molecules that bind (termed ‘‘aptamers’’) are subsequently eluted. By RT-PCR, an oligonucleotide pool enriched for binders can be regenerated and used for a new round of SELEX. (B) Ribosome display. A library of proteins, e.g., scFv fragments of antibodies, is transcribed and translated in vitro. The resulting mRNA lacks a stop codon, giving rise to linked mRNA–ribosome–protein complexes. These are used for selection on the immobilized target. The mRNA incorporated in bound complexes is eluted and purified. RT-PCR, which can introduce mutations, yields a DNA pool enriched for binders that can be used for the next iteration. (C) Protein–RNA fusion. Covalent RNA–protein complexes can be generated by ligation of a DNA–puromycin linker to the in vitro transcribed mRNA. The ribosome stalls at the RNA–DNA junction. Puromycin then binds to the ribosomal A site. The nascent polypeptide is thereby transferred to puromycin. The resulting covalently linked complex can be used for selection experiments.

complexes can be kept intact for at least 10 days under appropriate conditions, allowing very stringent off-rate selections.

3. Ribosome display of scFv fragments of antibodies

3.1. The ribosome display construct

The scFv construct used for ribosome display is depicted in Fig. 2. The construct does not contain a stop codon, and thus a quite stable mRNA–ribosome–polypeptide ternary complex can be formed during in vitro translation, provided the complexes are stabilized by high Mg^{2+} concentration and low temperature after translation. The 5′-untranslated region of the ribosome display construct contains the T7 promoter for efficient transcription by T7 RNA polymerase. The 5′-untranslated region of the RNA is derived from gene 10 of phage T7 and contains the Shine–Dalgarno sequence for efficient initiation of in vitro translation. This 5′-untranslated region is capable of forming a stable stem-loop structure on the mRNA level. The 5′-stem-loops were shown to protect mRNA against degradation by RNaseE (Bouvet and Belasco, 1992). A second stem-loop structure, present at the 3′-end of the mRNA, is of particular importance to protect mRNA from degradation by 3′-5′-exonucleases in the *E. coli* S30 extract. The sequence for the 3′-stem-loop was derived from the terminator of the *E. coli* lipoprotein or, alternatively, from the early terminator of T3 phage. The incorporation of both 5′- and 3′-terminal stem-loop structures in the construct led to an efficiency gain of ribosome display by a factor of 15 (Table 2; Hanes and Plückthun, 1997). A variety of other stem-loop sequences was also tested, but significant differences were not observed, leading to the conclusion that it is the existence rather than the sequence of these secondary structures that is decisive.

The protein coding sequence consists of a protein library and a spacer (tether), which is necessary for sufficient spatial separation of the nascent polypeptide chain from the ribosome and thus enables the polypeptide to fold correctly while being linked to the mRNA–ribosome complex. It was demonstrated by various studies that the ribosome covers between 20 and 30 C-terminal amino acids of a given polypeptide chain during protein synthesis (Malkin and Rich, 1967; Jackson and Hunter, 1970; Smith et al., 1978). Also, sufficient space and flexibility are provided by this tether for the protein to recognize and bind to the given target. Spacers derived from

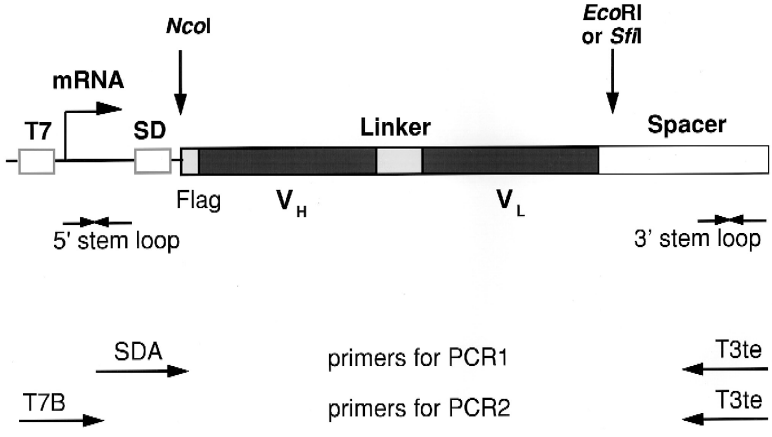

Fig. 2. The scFv construct used for ribosome display. The promoter (T7) is followed by a Shine–Dalgarno sequence (SD) and the scFv construct containing an N-terminal FLAG tag. Variable domains, V_H and V_L, are joined by a glycine/serine-rich linker. A spacer (tether) is cloned in frame behind the sequence of the scFv fragment of an antibody without a stop codon. Sequences encoding for RNA stem-loop structures are present at the 5′- and 3′-ends. Characteristic restriction sites are indicated. In ribosome display, after the RT step (primer T3te), two subsequent PCR steps are used to reintroduce the Shine–Dalgarno sequence (PCR1; primers SDA, T3te) and the T7 promoter (PCR2; primers T7B, T3te) to regenerate a complete scFv construct.

gene III of filamentous phage M13 encoding 88–116 amino acids were used for the display of scFv fragments of antibodies (Hanes and Plückthun, 1997), and it was shown that the efficiency of ribosome display increased with spacer length (Table 2). For ribosome display with oligopeptide libraries, a spacer length of 72 amino acids was used (Mattheakis et al., 1994). For ribosome display with a rabbit reticulocyte lysate (He and Taussig, 1997) and with an *E. coli* lysate (Hanes et al., 1999), the constant κ-domain of the light chain of an antibody (He and Taussig, 1997; Hanes et al., 1999) was also tested as a spacer, with similar results. In ribosome display utilizing *E. coli* cell extract for in vitro translation, spacers derived from the proline-rich sequence of TonB or the helical segment of TolA (Pellitier et al., unpublished results), two proteins spanning the periplasm of *E. coli*, were also used. However, the differences when using either one of these spacers, compared to the original gene III spacer (Hanes et al., 1999), will require a more detailed investigation.

Furthermore, rare codons can be incorporated in the spacer region (Mattheakis et al., 1996), with the intention of causing ribosome stalling and thus slowing down protein synthesis, perhaps additionally stabilizing the ribosomal complexes. However, a positive effect of these rare codons has not yet been experimentally demonstrated (Table 2).

Any library can be assembled into the described ribosome display format by ligation into the vector, pAK200 (Krebber et al., 1997), in frame with the gene III spacer sequence in vitro, without transforming *E. coli*. The ligation mixture is then used directly for PCR amplification. All necessary features, such as the Shine–Dalgarno sequence, the T7 promoter sequence and the stem-loops are introduced in two subsequent PCR reactions (for primers, see Table 1). As the PCR amplification terminates the gene before the stop codon of gene III protein, the construct has no stop codon.

3.2. In vitro transcription and translation

The initial DNA library is amplified by PCR and subsequently transcribed by T7 RNA polymerase and translated in vitro. In vitro transcription and translation can be carried out either in coupled or in separate reactions. Proteins containing disulfide bridges, such as immunoglobulins, in general, fold correctly only under oxidizing conditions, such that the crucial intradomain disulfide bridges can be properly formed (Goto and Hamaguchi, 1979; Glockshuber et al., 1992), and only a subset of antibodies can fold in the absence of disulfide bonds (Proba et al., 1998; Wörn and Plückthun, 1998a,b). On the other hand, T7 RNA polymerase requires β-mercaptoethanol to maintain its stability (Gurevich et al., 1991). Therefore, transcription and translation should be carried out separately for scFv fragments of antibodies (Hanes and Plückthun, 1997) and other proteins with disulfide bridges. However, a coupled rabbit reticulocyte system containing 2 mM DTT has been used for display of scFv fragments of antibodies (He and Taussig, 1997), but this approach would appear to be limited to the abovementioned subset of antibodies. If proteins are employed in the selection experiment that can fold correctly under reducing conditions, a coupled in vitro transcription/translation system may be preferred.

Table 1
Oligonucleotides used in ribosome display

Primer	Sequence	Remarks
T3te	5′-GGCCCACCCGTGAAGGTGAGCCTCAGTAGCGACAG-3′	introduces the 3′-stem-loop derived from the translated early terminator of phage T3; anneals to the gene III spacer
SDA	5′-AGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTT AAGAAGGAGATATATCCATGGACTACAAAGA-3′	introduces the ribosome binding site; used for the first PCR amplification step; underlined is the *Nco*I restriction site for cloning
T7B	5′-ATACGAAATTAATACGACTCACTATAGGGAGACCACAACGG-3′	introduces the T7 promoter and the 5′-stem-loop; used for the second PCR amplification step
Anti-*ssrA*	5′-TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCGACTA-3′	inhibits the 10*S*-RNA peptide tagging system

Conditions for efficient in vitro transcription have been described in detail (Gurevich et al., 1991). Approximately 0.1 mg mRNA is obtained after 2–3 h in a 200 μl transcription reaction, utilizing 1–2 μg DNA template. The transcribed mRNA can be purified by LiCl precipitation and a subsequent ethanol precipitation (Hanes et al., 1998) and then resuspended in DEPC-treated water. Alternatively, RNA purification kits can also be used.

For *E. coli* in vitro translation, the preparation of S30 extracts from *E. coli* MRE600 cells (Wade and Robinson, 1966) is carried out following a modified protocol, based on the procedure described by Pratt (1984). In particular, the reducing agents, DTT and β-mercaptoethanol, are omitted from all buffers for display of proteins containing disulfide bridges. The *E. coli* system used for ribosome display needs to be optimized according to Pratt (1984) with respect to the concentration of Mg^{2+} and K^{+} ions, the amount of S30 extract used and the translation time (Hanes et al., 1999). During in vitro translation, the protein synthesis follows a saturation curve reaching a plateau after approximately 30 min (Ryabova et al., 1997). At the same time, mRNA is continuously degraded. Thus, an optimal time exists, at which the concentration of intact mRNA–ribosome–protein complexes that can be used for selection is at a maximum. This optimal time for *E. coli* ribosome display in vitro translation is usually between 6 and 10 min after translation starts, as schematically depicted in Fig. 3 (Hanes and Plückthun, 1997). Although most proteins generally fold more efficiently at lower temperatures in vitro (Creighton, 1994), we found that, at least for scFv fragments of antibodies, more ribosomal complexes containing functional protein were obtained when the reaction was carried out at 37°C, which may be attributed to the chaperone activity in the *E. coli* extract. Endogenous transcription by *E. coli* RNA polymerase can be inhibited by addition of rifampicin (Mattheakis et al., 1996). It is noteworthy that supplementing protease inhibitors to the reaction did not have any effect, as judged by ^{35}S-methionine protein sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Hanes, Jermutus, Schaffitzel and Plückthun, unpublished results). Also, prolonged incubation of the scFv fragments of antibodies in S30 cell extracts after translation did not change the amount of synthesized protein significantly, when analyzed by SDS-PAGE (Hanes and Plückthun, 1997; Hanes et al., 1999).

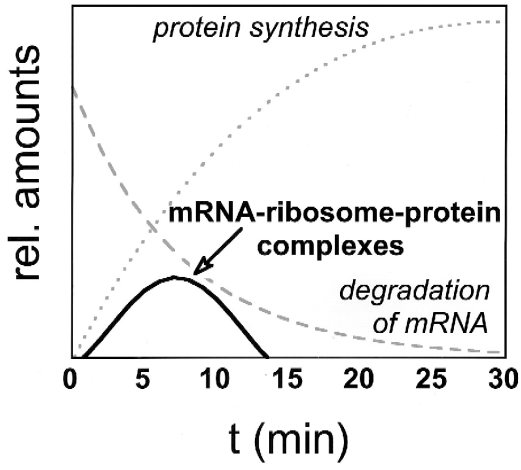

Fig. 3. Diagram showing the time course of protein synthesis and mRNA degradation during in vitro translation utilizing *E. coli* S30 cell extract and the resulting existence of a time optimum for intact mRNA–ribosome–protein complexes that can be used for selection. However, once the complexes bind to their immobilized target and are washed, the RNases are essentially removed and the ribosomal complexes are very stable.

3.3. Ribonucleases

In contrast to a coupled transcription/translation setup, where a constant level of mRNA is maintained through continuous transcription, particular attention needs to be paid towards RNases if the two steps have to take place separately. *E. coli* MRE600 is a strain that lacks RNaseI, one of the predominant ribonucleases that is involved in ribosomal RNA degradation (Meador et al., 1990). To date, five of the 20 *E. coli* ribonucleases have been shown to contribute to mRNA degradation (Hajnsdorf et al., 1996), many if not all of which are very likely to be present in the S30 cell extract. Thus, mRNA stability is regarded as the limiting factor for in vitro translation (Carrier and Keasling, 1997). mRNA half life can vary from as short as 30 s to up to 20 min in *E. coli*, depending on mRNA secondary structure and RNase activity (Ehretsmann et al., 1992). The RNase inhibitor, VRC (vanadyl–ribonucleoside complexes), a transition state analog, was shown to increase the

Table 2
Improvement of the efficiency of ribosome display

	Function	Effect in ribosome display[a]
Construct		
3′- and 5′-stem-loops	stabilization against RNases	15-fold improvement
Stalling sequences in the spacer (Mattheakis et al., 1996)	slows down the translation; mRNA is protected by the ribosomes	not determined
2′-Modified pyrimidines incorporated in the mRNA	mRNA stabilization	none
Phosphothioates	mRNA stabilization	none
Variation of the spacer length and sequence	tethers the polypeptide to the ribosome; allows folding to a functional protein	inceasing yield with increasing spacer length between 88 and 116 amino acids
Translation		
Anti-*ssrA* oligonucleotide	inhibition of the 10*S*-RNA peptide tagging system	fourfold improvement
Protease inhibitor cocktail without EDTA (Boehringer Mannheim)	protease inhibition	None
PDI	folding catalyst	threefold improvement
VRC	RNase inhibition	see text
RNasin (Mattheakis et al., 1996)	RNase inhibition	requires reducing conditions
Potassium glutamate (200 mM) instead of potassium acetate (100 mM)	required to maintain a steady state K^+ pool; glutamate is important in response to osmotic stress	several-fold improvement
Cycloheximide (Gersuk et al., 1997)	translation stop and stabilization of the mRNA–ribosome–peptide complexes in eukaryotic cell extracts	none in eukaryotic cell extracts (Jermutus, unpublished results)
Chloramphenicol (Mattheakis et al., 1996)	translation stop and stabilization of the mRNA–ribosome–peptide complexes	none
Purification of the mRNA–ribosome–peptide complexes by a sucrose cushion centrifugation (Mattheakis et al., 1996)	removing RNases and proteases	none

[a] Data from Hanes and Plückthun (1997), Hanes et al. (1998; 1999); unpublished results.

efficiency of *E. coli* ribosome display (Hanes and Plückthun, 1997), but also to inhibit the translation process (Table 2). More recently, it has been omitted from the protocol, as it was found that better results can be achieved if the reaction mixture is cooled immediately after stopping translation, and all subsequent steps are meticulously carried out on ice. Also, it has proved beneficial to have all substances, buffers and plastic precooled (Hanes et al., 1999). Furthermore, the stem-loop structures present at the 3′- and 5′-ends of the mRNA have improved the efficiency of ribosome display by a factor of 15 (Table 2). The 5′- and 3′-stem-loops may protect the RNA from degradation by exonucleases RNaseII and PNPase which act from the 3′-end of a message (Hajnsdorf et al., 1996) and against endonuclease RNaseE that recognizes the 5′-end (Bouvet and Belasco, 1992; McDowall et al., 1995). Fig. 4 schematically shows the generally assumed mode for mRNA degradation in *E. coli* (Carrier and Keasling, 1997).

3.4. Stabilizing mRNA by incorporating modified nucleotides

A further approach to stabilize mRNA involves incorporation of modified RNA nucleotides during in vitro transcription (Aurup et al., 1992). Besides using modified nucleotides, post-transcriptional modification by acetylation of the 2′-hydroxyl groups (Ovodov and Alakhov, 1990) present in mRNA can, in principle, have stabilizing effects against RNases. However, if not all nucleotides carry the substitution in their sugar moieties, but instead only one or two of the nucleotides have been replaced by modified ones, stabilization of 2′-modified mRNA is, by necessity, sequence-dependent. In this case, the template activity of modified mRNA is strongly dependent on which nucleotides were modified. Complete substitution of the nucleotides with nucleotide analogues can actually inhibit in vitro translation entirely, if either 2′-modified residues (Dunlap et al., 1971) or phosphothioates are used (Ueda et al., 1991). We observed diminished in vitro transcription and translation yields in our ribosome display experiments with 2′-amino- or 2′-fluoro-substituted pyrimidine nucleotides, while a significant stabilization of the modified mRNA against nucleolytic degradation was not observed (Table 2; Schaffitzel et al., unpublished results).

3.5. Protein expression and folding in ribosome display

Selection of mRNA–ribosome–protein complexes depends on correctly folded functional protein, in the present case scFv fragments of antibodies. It is well-established that antibody domains contain conserved disulfide bridges which are critical for their integrity (Goto and Hamaguchi, 1979; Glockshuber et al., 1992). In the endoplasmic reticulum of eukaryotes,

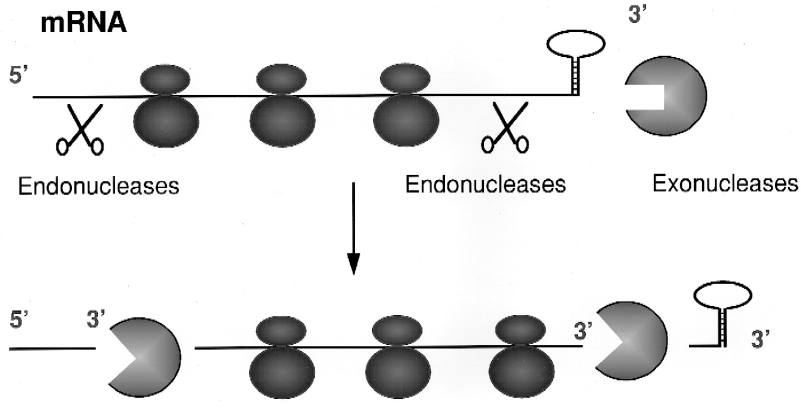

Fig. 4. Model for mRNA degradation in *E. coli.* Exonucleases (RNaseII, PNPase) are inhibited by 3′ terminal stem-loop structures on the mRNA. mRNA degradation is normally initiated by endonucleases (RNaseIII, RNaseE and others) that cut mRNA and can thereby remove structured regions on the mRNA such as stem-loops. Resulting mRNA fragments can then be degraded further by exonucleases.

disulfide bond formation and isomerization is catalyzed by the enzyme protein disulfide isomerase (PDI) (Gilbert, 1998) in the presence of glutathione (Hwang et al., 1992). In a systematic study of the effect of PDI and chaperones on in vitro translation (Ryabova et al., 1997), it was found that disulfide bond formation and rearrangement needs to take place cotranslationally. In fact, superior yields of functional antibody were obtained with eukaryotic PDI and a glutathione-redox shuffle. In the optimized batch system, about 8 μg of scFv antibody fragment was synthesized in 15 min/ml reaction volume, with at least 50% of the protein folded to functional scFv. Molecular chaperones present in the cell extract such as DnaK, DnaJ, GrpE, and GroEL/GroES, while increasing the solubility of scFv fragments of antibodies to nearly 100%, did not influence significantly the net amount of functional protein (Ryabova et al., 1997). We supplement our in vitro translation reaction with eukaryotic PDI and are able to improve ribosome display efficiency threefold (Table 2; Hanes and Plückthun, 1997).

3.6. 10S-RNA

In *E. coli*, peptides synthesized from mRNAs without stop codons are modified by the carboxy-terminal addition of an 11-amino-acid peptide tag (AANDENYALAA), which is encoded by the 10*S*-RNA, the product of the *ssrA* gene. The first alanine is not encoded by *ssrA*, but is transferred to the nascent polypeptide from the tRNA moiety of 10*S*-RNA, whose aminoacyl residue is alanine (Keiler et al., 1996). Addition of the peptide tag leads to degradation by specific carboxy-terminal proteases in the cytoplasm and periplasm. This system is thought to protect *E. coli* from conceivably harmful polypeptides derived from truncated mRNAs. The process of tagging an mRNA without a stop codon by 10*S*-RNA also occurs during in vitro translation (Himeno et al., 1997). For this reason, we decided to block the 10*S*-RNA system by means of an antisense oligonucleotide complementary to the tag encoding sequence of 10*S*-RNA (Table 1). Indeed, we observed a shortening of the longest translation product upon addition of this anti-*ssrA* oligonucleotide, consistent with the C-termini now being untagged, and a concomitant fourfold efficiency gain of ribosome display (Table 2; Hanes and Plückthun, 1997).

3.7. Affinity selection

Translation arrest in ribosome display occurs by fourfold dilution of the translation mixture in an ice-cold buffer containing 50 mM Mg^{2+}. This magnesium concentration is used also throughout the entire selection process to stabilize the mRNA–ribosome–protein complexes. Mattheakis et al. (1996) additionally supplemented the latter with chloramphenicol during the whole affinity selection. Chloramphenicol is thought to inhibit the elongation step of translation, and Mattheakis et al. also used it to stabilize the ribosomal complexes. However, we did not observe any beneficial effect of chloramphenicol addition (Table 2; Hanes and Plückthun, 1997).

The diluted translation mixture can be used for selection experiments directly without further purification of the ribosomal complexes by a sucrose cushion centrifugation as described by Mattheakis et al. (1994). The ribosomal complexes are stable for at least 10 days at 4°C; however, their amount gradually decreases over this time (Jermutus et al., unpublished results). Selection is carried out in the presence of sterilized skim milk (1%–2%) and heparin (0.2%), which successfully prevent non-specific binding of ribosomal complexes to surfaces and therefore decrease the background signal. Heparin additionally inhibits nucleases.

Selections can be performed both with ligands immobilized on a surface or in solution, if a tagged ligand is used, which can then be captured, e.g., with magnetic beads. Immobilization of protein antigens on plastic surfaces, however, may lead to partial unfolding of the protein due to hydrophobic interactions with the plastic. In extreme cases, this can lead to the selection of antibodies against protein epitopes that are not accessible in the native form (Schwab and Bosshard, 1992). This can be circumvented by selecting in solution. Here, a tag needs to be fused to the antigen that allows subsequent capture of the mRNA–ribosome–scFv complexes bound to the antigen. We used biotin-labeled antigen for selection in solution which allows capture of the antigen-bound ribosomal complexes on streptavidin-coated mag-

netic beads. It has been demonstrated previously with phage display that optimal selection of higher-affinity scFv fragments of antibodies can be achieved when selection is performed in solution against biotinylated antigen (Schier et al., 1996a,b). If biotin is used as a tag, the milk added to the selection reaction needs to be depleted of biotin beforehand. We typically alternate between streptavidin-coated magnetic particles and avidin–agarose, to avoid selecting streptavidin or avidin binders.

The high stability of the ribosomal complexes at 4°C allows for intensive washing of the bound ribosomal complexes with ice-cold Mg^{2+}-containing buffer. The desired mRNA species are then eluted with EDTA-containing buffer. EDTA dissociates the ribosomal complexes by chelating Mg^{2+}. Alternatively, the bound ribosomal complexes can also be eluted by adding soluble, untagged antigen, which offers the advantage that non-specifically bound ribosomal complexes do not co-elute. However, high-affinity binders may be eluted less efficiently than lower affinity ones. Cleavable linkers between antigen and biotin can also be used to elute ligand-bound mRNA–ribosome–scFv complexes. Nevertheless, the direct extraction of mRNA, which avoids all of these steps, is one of the advantages over phage display in isolating very high-affinity binders.

The eluted mRNA is then purified with commercial mRNA purification kits, including DNaseI treatment to remove any DNA template bound non-specifically, which may still be present after in vitro transcription, translation, selection and elution. The purified mRNA is utilized for RT-PCR and can also be used for Northern analysis to quantify the efficiency of the system. For RT-PCR, a primer is used that anneals to the 3′-terminus of the mRNA (Table 1). The Shine–Dalgarno and T7 promoter sequences at the 5′-end are reintroduced by subsequent PCR steps with appropriate primers. Thus, only intact mRNA is reverse-transcribed and PCR-amplified. The PCR-amplified DNA can now be directly transcribed in vitro. The mRNA can either be used for the next selection cycle, or be translated in vitro in the presence of radioactive amino acids and tested by radioimmunoassay (RIA) for specific binders present in the pool. To obtain individual sequences, the DNA pool is ligated in a plasmid, transformed into *E. coli* and the plasmids of single clones are isolated. The individual clones can be analyzed by further RIA experiments and sequencing of single clones. Antigen-specific scFv fragments are generally enriched by a factor of 100–1000 per cycle of ribosome display (Hanes and Plückthun, 1997; Hanes et al., 1999). After five rounds, enrichments of up to 10^9 have been achieved (Hanes and Plückthun, 1997).

If large amounts of specific scFv fragments are required for further experiments, overexpression in the *E. coli* periplasm (Skerra and Plückthun, 1988; Plückthun et al., 1996) or in inclusion bodies (Huston et al., 1995) can be performed. Good yields of functional scFv fragments, selected in vitro by ribosome display, were observed on expression in *E. coli* (Hanes et al., 1998), indicative of a selection for proteins that are expressed at a reasonable level during ribosome display using the S30 cell extracts. This also indicates that the fundamental rules for efficient protein folding must be the same in the cell and in vitro.

4. In vitro evolution by ribosome display

If screening and selection of an unaltered library is required, proofreading polymerases can be used for the PCR amplification step in ribosome display. On the other hand, if a non-proofreading polymerase like *Taq* DNA polymerase is used, which introduces on average one mutation per 20,000 nucleotides (Cline et al., 1996), each selection round leads to a diversification of the DNA sequence pool. Thus, through combined mutation and selection, a process resembling affinity maturation of scFv fragments of antibodies can occur. This was demonstrated when selecting scFv fragments of antibodies against a mutant of the yeast transcription factor GCN4 from an immune mouse library. A monomeric scFv with a k_d of 4×10^{-11}M was selected that apparently was not present in the initial immune library and had undergone a 65-fold affinity maturation in the process of ribosome display (Hanes et al., 1998). This suggests the enormous potential of ribosome display not only as a screening technique but also as an efficient method for real protein evolution.

Diversification during PCR can be further enhanced by mutagenesis methods such as oligonucleotide-directed mutagenesis (Hermes et al., 1990), er-

ror prone PCR in the presence of non-physiological metal ions such as Mn^{2+} (Lin-Goerke et al., 1997) or dNTP analogues (Zaccolo et al., 1996). Other PCR methods that can be used for diversification involve homologous recombination in vitro, such as DNA shuffling (Stemmer, 1994) and StEP (staggered extension process; Zhao et al., 1998). As both mutagenesis and selection take place entirely in vitro, comparatively minor efforts are required to carry out many evolution cycles. After a first selection of the initial library, only the scFv encoding region is amplified and diversified in vitro. This second-generation library is inserted into the ribosome display format by assembly PCR and subsequently transcribed into mRNA. The evolution of the sequence pool can be monitored by RIA. Since the ternary ribosomal complexes are stable for several days and selection pressure can be applied both during and after translation, numerous applications for directed protein evolution are conceivable (Jermutus et al., manuscript in preparation).

Recombination-based methods have the advantage that deleterious or non-essential mutations can be suppressed by recombination with the original sequence, while beneficial mutations persist and accumulate (Moore et al., 1997). These methods can further be combined with random mutagenesis. A further approach to create mutant scFv repertoires is to replace the V_H or V_L genes of a set of antibodies with a V gene repertoire (chain shuffling); here, a single fixed cross-over site is utilized (Schier et al., 1996a) in contrast to DNA shuffling. These methods are fully compatible with ribosome display and the procedures can easily be incorporated into the protocol.

5. Discussion and outlook

A cognate antibody is generally enriched 100-fold to 1000-fold during one cycle of ribosome display (Hanes and Plückthun, 1997; Hanes et al., 1999). The mRNA yield after in vitro translation, affinity selection and elution amounts to a total of 0.2%; i.e., for every 10 μg mRNA (about 1.5×10^{13} molecules) initially employed in a 100 μl in vitro translation reaction, 20 ng of mRNA (about 3×10^{10} molecules) encoding a specific antibody is recovered from ribosomal complexes. Therefore, in a 1-ml in vitro translation reaction, a library of 3×10^{11} independent members is screened. Note, however, that the actual sequence space explored is much larger, because for each selected variant, many thousand single, double and triple mutants are explored, due to the error rate of the polymerase employed. For phage display to sample the same sequence space, the single-pot library would have to encode all these variants at once and then be of a size of 10^{16}, which is not feasible.

At mRNA-to-ribosome ratios close to those present in our ribosome display experiments (approximately 1:1), predominantly monoribosomal complexes are obtained (Hanes, unpublished results), as evidenced by sucrose gradient centrifugation. For this reason, we prefer the term ''ribosome display'' over ''polysome display''. A significant part of the mRNA in the ribosomal complexes can be degraded by RNases. Also, some RNA molecules can be part of complexes with misfolded and thus non-functional polypeptides. For these reasons, in vitro translation constitutes a critical step in ribosome display. An unresolved issue is the presence of rather significant amounts of translation products shorter than expected (Hanes and Plückthun, 1997), which are not a consequence of proteolytic degradation but possibly of ribosome stalling prior to the end of the mRNA. After sucrose gradient centrifugation, the same pattern of radioactive-labeled shorter peptides was found to be associated with ribosomes (Hanes, unpublished results). Still, some premature dissociation of either the synthesized polypeptide chain from the ribosomal complex or of the ribosome from the mRNA during in vitro translation cannot be excluded. In previous studies, it was found that up to two thirds of nascent polypeptide chains may dissociate during translation (Fedorov and Baldwin, 1998). Dissociated polypeptide chains could act as competitors to the mRNA–ribosome–antibody complexes used for selection, if low amounts of antigens are used. Therefore, off-rate selections may be more advantageous than equilibrium competition for small amounts of antigen.

Recently, it was reported that condensation of *E. coli* cell extracts combined with dialysis during translation (continuous-flow system) can increase protein yields dramatically (Kigawa et al., 1999). Yields of up to 6 mg/ml protein were reported by

using this system. As was detailed above, significant improvements of ribosome display efficiency can be achieved by utilizing more functional ribosomes and by variation and amelioration of in vitro translation conditions. Furthermore, by substituting potassium acetate by potassium glutamate during in vitro translation, we were able to considerably improve mRNA yields during ribosome display (Table 2; Hanes et al., 1999). Thus, it may well be that there are further useful additives missing in the cell extract. By the same token, other components may be present that are inhibitory for the in vitro translation reaction.

By the absence of transformation steps in ribosome display, very large libraries ($\geq 10^{11}$) have become amenable for in vitro selection. Furthermore, ribosome display is not only a powerful screening method, but simultaneously allows for sequence evolution by mutagenesis during the iterative selection process. The combination of large library sizes and mutagenesis provides a possibility to cover a very large sequence space during evolution. Thus, synthetic combinatorial antibody libraries, which encode the entire structural human repertoire (Knappik et al., manuscript submitted), can now be efficiently screened by ribosome display, and a variety of high-affinity binders, which even have not been part of the initial library, have been selected for (Hanes, Schaffitzel, Jermutus and Plückthun, manuscript in preparation).

Ribosome display can be utilized to obtain ultra-high affinity (picomolar and better) antibodies since it is the mRNA–ribosome–antibody complex that is dissociated during the elution step rather than the antibody–antigen complex, as for instance in phage display. Therefore, an elution is not absolutely necessary in ribosome display as the antibody can be ''left behind'' — only the mRNA is needed. By adjusting the selection conditions, it is possible to generate not only high-affinity antibodies but also to improve specificity for the recognized epitope, the stability of the antibody and other molecular properties. The selection conditions can be further varied by adding competitor antigens and increasing selection time (off-rate selection) during the experiment (Jermutus et al., manuscript in preparation).

In vitro translation conditions can also be varied in ribosome display. For example, the redox state of the system can be chosen by altered DTT. Chaperones and enzymes that support folding such as PPIase (protein-prolyl *cis–trans* isomerase) or PDI (protein disulfide isomerase) can be added, and reaction conditions similar to those present in the periplasm or, alternatively, the cytoplasm of *E. coli* can be created according to the needs of the selected protein species. If disulfide-free functional antibodies (Marasco, 1995; Proba et al., 1998; Cattaneo and Biocca, 1999) for use as ''intrabodies'' need to be generated, appropriate mutagenesis steps can be combined with ribosome display selection in an environment similar to the *E. coli* cytoplasm. These intrabodies than can be expressed intracellularly to bind to their target and exert a variety of functions.

For selection experiments with certain proteins, requiring for instance the presence of eukaryotic chaperones, it may prove to be of value to test eukaryotic cell extracts such as rabbit reticulocyte lysate or wheat germ extract for in vitro translation. We and others were able to demonstrate that for ribosome display, both eukaryotic and prokaryotic extracts can be used, each with its own merits (He and Taussig, 1997; Hanes et al., 1999). So far, for the antibody fragments tested, the *E. coli* extract proved to result in better enrichments (Hanes et al., 1999).

6. Conclusion

Ribosome display is a powerful method for screening very large antibody libraries. Each step of ribosome display is carried out in vitro, thus circumventing limitations associated with in vivo systems. Libraries can be further diversified during PCR steps in ribosome display using low-fidelity polymerases. Thus, high-affinity antibodies initially not present in libraries can be generated and selected against a large array of antigens. Diversification can additionally be increased with mutagenesis and in vitro recombination techniques that are well compatible with ribosome display, avoiding cumbersome alternations between in vitro and in vivo steps. We anticipate that ribosome display will be of particular importance in the future for directed evolution of proteins through many generations, yielding versatile molecules for a large variety of applications.

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