Technique review

Ribosome display: Cell-free protein display technology

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Abstract

Ribosome display is a cell-free system for the *in vitro* selection of proteins and peptides from large libraries. It uses the principle of coupling individual nascent proteins (phenotypes) to their corresponding mRNA (genotypes), through the formation of stable protein–ribosome–mRNA (PRM) complexes. This permits the simultaneous isolation of a functional nascent protein, through affinity for a ligand, together with the encoding mRNA, which is then converted and amplified as DNA for further manipulation, including repeated cycles or protein expression. Ribosome display has a number of advantages over cell-based systems such as phage display; in particular, it can display very large libraries without the restriction of bacterial transformation. It is also suitable for generating toxic, proteolytically sensitive and unstable proteins, and allows the incorporation of modified amino acids at defined positions. In combination with polymerase chain reaction (PCR)-based methods, mutations can be introduced efficiently into the selected DNA pool in subsequent cycles, leading to continuous DNA diversification and protein selection (*in vitro* protein evolution). Both prokaryotic and eukaryotic ribosome display systems have been developed and each has its own distinctive features. In this paper, ribosome display systems and their application in selection and evolution of proteins are reviewed.

INTRODUCTION

Display systems are used for the selection of coding elements (DNA or RNA) from libraries in which the individual peptides or proteins as phenotypes are physically associated with their genetic material. They can also be used to alter the properties of the phenotype by evolution through cycles of mutation, selection and replication. The success of display selection relies on the ability to retrieve the genetic information along with the functional protein. Several methods have been devised and validated, both cell based, such as phage display[1] and cell surface display,[2,3] and cell free, such as ribosome display[4–7] and mRNA display.[8] Ribosome display is carried out fully *in vitro*, which overcomes some of the limitations of cell-based display systems. Through the formation of protein–ribosome–mRNA (PRM) complexes in cell-free systems such as *Escherichia coli* S30 or rabbit reticulocyte, individual nascent proteins are linked with their corresponding mRNA molecules, permitting selection of the genetic material through the functional properties of the protein, usually as a binding reaction. The mRNA can be amplified and recovered as DNA by reverse transcription polymerase chain reaction (RT-PCR) and further cycled, mutated or cloned. In this paper, ribosome display technology, including its applications in protein selection and evolution *in vitro*, will be reviewed.

RIBOSOME DISPLAY: PRINCIPLE AND ADVANTAGES

The key feature of ribosome display is the generation of stable PRM complexes via ribosome stalling such that the nascent protein and mRNA remain associated.[4–7]

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Two strategies which have been used are: (i) addition of antibiotics such as rifampicin and chloramphenicol (for prokaryotic ribosomes) or cycloheximide (for eukaryotic ribosomes) to halt translation at random,[4,9] or (ii) deletion of the stop codon, normally recognised by release factors which trigger detachment of the nascent polypeptide, to stall the ribosome at the 3′ end of the mRNA.[5,6]

Advantages of ribosome display

Ribosome display offers a number of advantages over cell-based methods such as phage or cell surface display. As has often been noted, the efficiency of transformation imposes a restriction on library diversity for cell-based systems, which does not apply in ribosome display. Thus, larger libraries can be screened in each cycle because PCR products are directly utilised as templates, avoiding the need for cloning. PCR libraries with huge potential diversity ($>10^{12}$ members) can be generated easily and the displayed library size is only dependent on the number of functional ribosomes in the reaction, which can be up to 10^{14}/ml.[6] The larger accessible library renders ribosome display a superior opportunity to select rare sequences and high-affinity combining sites. Moreover, using PCR, further diversity can be continuously introduced into the DNA pools after selection, providing an efficient route for protein evolution. In addition, cell-free systems can produce toxic and proteolytically sensitive or unstable proteins for which bacterial expression is often unsuccessful.[10] They also allow modified amino acids such as chemically labelled or unnatural amino acids to be incorporated into the protein at defined positions.[11] Eukaryotic cell-free systems are also capable of a variety of post-translational modifications,[10] expanding the possibility of displaying functionally relevant proteins.

RIBOSOME DISPLAY SYSTEMS

Prokaryotic ribosome (polysome) display

The first published description of ribosome display was for peptide selection using a coupled *E. coli* S30 system and termed 'polysome display'.[4] A synthetic DNA library encoding random peptide sequences was used to generate polysome complexes by adding chloramphenicol to stop translation. Specific complexes displaying interacting peptide epitopes were captured with immobilised antibody by panning on microtitre wells. The trapped polysomes were disrupted with EDTA to release the bound mRNA, which was then converted and amplified into cDNA by RT-PCR.[4] This procedure was subsequently modified to display folded single-chain antibody fragments.[5] Figure 1A outlines the display cycle. The modified prokaryotic method generated PRM complexes through deletion of the 3′ terminal stop codon from DNA. It also included a number of additional components, such as protein disulphide isomerase, vanadyl ribonucleoside complexes and anti-ssrA anti-sense oligouncleotide (to prevent the carboxyterminal addition of an 11 amino acid peptide encoded by the *ssrA* gene), in the translation mixture in order to promote folding of antibody fragments, stabilise mRNA and inhibit the action of ssrA RNA, respectively.[5] To avoid the disruptive effect of dithiothreitol (DTT) on the folding of antibody domains through reduction of disulphide bridges, transcription and translation were performed separately, and the mRNA was introduced into the uncoupled *E. coli* S30 translation system lacking DTT.[5]

Eukaryotic ribosome display

A eukaryotic ribosome display system — ARM (antibody–ribosome–mRNA) display — was developed for the selection of functional single-chain antibody fragments in a coupled rabbit reticulocyte lysate system (Figure 1B).[6,7] Deletion of the stop codon from the PCR fragment was again used to generate eukaryotic PRM complexes in a simple and rapid procedure. ARM complexes were generated by cell-free expression of PCR fragments followed by specific capture on

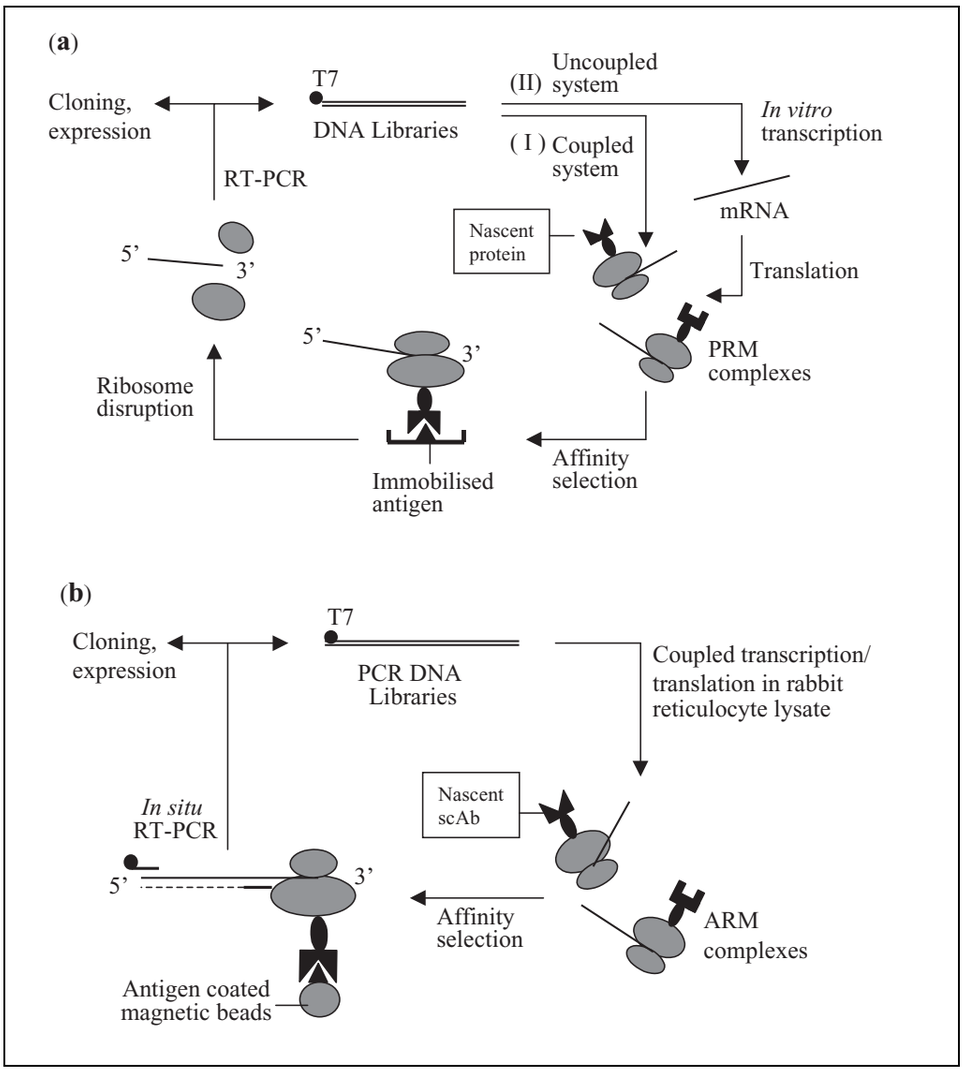

Figure 1: Prokaryotic and eukaryotic ribosome display cycles. (A) *E. coli* ribosome display: (I) using coupled *E. coli* S30 extract and (II) using uncoupled *E. coli* S30 extract. (B) ARM ribosome display for single-chain antibodies. T7: T7 promoter; PRM: protein–ribosome–mRNA; RT-PCR: coupled reverse transcription polymerase chain reaction; scAb: single chain antibody fragment; ARM: antibody–ribosome–mRNA

antigen-coated magnetic beads. DNA was recovered from the trapped mRNA by amplification with a novel *in situ* RT-PCR procedure without dissociation of the ribosome complex (Figure 1B). Starting from an anti-progesterone hybridoma cell line (DB3), ARM complexes of a three-domain antibody fragment (V_H/K) were generated which retained the detailed specificity of the DB3 monoclonal antibody, implying correct folding of the nascent protein.[6] Specific progesterone-binding complexes could be enriched from a mutant library through antigen selection by a factor of about 10^4-fold in a single display cycle.[6] A modification of ARM display has been described in which oxidised/reduced glutathione and Qβ RNA-dependent RNA polymerase are included in the translation mixture in order to display folded proteins and to introduce mutations into mRNA for *in vitro* protein evolution.[12] A eukaryotic display system has also been used to select an enzyme, sialyltransferase II, from a cDNA library in a single well of a microtitre plate.[13]

KEY ISSUES

DNA construct design

In general, ribosome display constructs should contain a promoter (T7, SP6 or T3) and a translation initiation signal such as a Shine–Dalgarno (prokaryotic) or

Spacer required for protein display

Kozak (eukaryotic) sequence.[14] A consensus sequence for protein initiation in both *E. coli* and eukaryotic systems has also been described.[15] To enable the complete nascent protein to be displayed and fold into its active conformation, a spacer domain of at least 23–30 amino acids' length is required at the C terminus, to allow the protein to exit completely from the ribosome 'tunnel'.[16] The spacer also provides a known sequence for the design of primers for RT-PCR recovery. A number of different spacers have been successfully used, including the constant region of immunoglobulin kappa chain (Cκ),[6,7] gene III of filamentous phage M13[5] and the C_H3 domain of human IgM.[12] Spacer length has been shown to affect display efficiency: a spacer of 116 amino acids was more efficient in displaying proteins than its shorter partners.[14] To remove the stop codon from DNA, a 3′ primer lacking the stop codon is used during PCR construction. Constructs designed for prokaryotic *E. coli* display should incorporate sequences containing stem–loop structures at the 5′ and 3′ ends of the DNA to stabilise mRNA against degradation by RNase activities in *E. coli* cell-free systems.[5]

Cell-free systems for protein expression

The choice of a cell-free system is generally based on the origin of the proteins to be displayed and the particular application. Rabbit reticulocyte lysate, wheatgerm and *E. coli* S30 extracts have been successfully used for ribosome display of peptides, antibodies and other proteins (sialyltransferase II, bovine heart fatty acid-binding protein) in either coupled or uncoupled systems.[4–7,9,12,13,17]

Coupled and uncoupled systems

Coupled cell-free systems use DNA as the template, whereas uncoupled systems require mRNA obtained from native sources or *in vitro* transcription.[18] In general, coupled systems are simpler and more efficient; they also avoid problems of mRNA degradation and mRNA secondary structure.[19] A higher expression yield and more stable mRNA have been observed in a coupled *E. coli* S30 system compared with its uncoupled equivalent.[19] Parallel expression of five different coding sequences of bacterial and eukaryotic origin in either *E. coli* S30 extract, wheatgerm extract or rabbit reticulocyte lysate systems revealed that, while the two eukaryotic systems generated predominantly full-length products for all five sequences tested,[20] *E. coli* S30 produced incomplete nascent polypeptides bound to pausing ribosomes[20] and formed polysomes. The presence of polysomes raises the possibility of multivalent attachment of nascent proteins or peptides and selection of lower affinity binding sites.

Protein folding on ribosomes

For the selection of functional PRM complexes, the ribosome-bound protein must fold into an active conformation. There is evidence that nascent proteins, either *in vivo* or in a cell-free system, fold co-translationally on ribosomes.[21] For some nascent proteins, molecular chaperones appear to be involved in the folding process.[22] Ribosomes themselves or ribosomal RNA from either prokaryotic or eukaryotic sources also contribute to the folding process.[23] The recently published crystal structure of the large ribosomal subunit has provided the basis for understanding co-translational folding of a nascent protein during synthesis.[24] In addition, active ribosome-bound enzymes have been generated in cell-free systems after an extension of 23 or more amino acids at the C terminus,[25] demonstrating that correct folding of nascent proteins occurs without requiring release from the ribosome. The successful selection of antigen-specific antibody fragments from ribosome display libraries also confirms that folding takes place while bound to the ribosome.[5,6,26–29]

As in most cell-free systems, the coupled rabbit reticulocyte lysate system contains 2 mM DTT. Different antibodies may have various folding stabilities that could be affected by DTT concentration

in cell-free systems, the main concern being possible reduction of intra-chain disulphide bridges.[14] While one antibody fragment showed no binding activity after production in an uncoupled *E. coli* S30 system containing 1 mM DTT,[30] a number of other soluble, functional antibody fragments, including engineered mutants, were generated in either the S30 system or rabbit reticulocyte lysate containing 2 mM DTT.[31–33] Similarly, in ribosome display, whereas the prokaryotic system yielded fewer functional antibody–ribosome complexes in the presence of DTT,[34] rabbit reticulocyte lysate containing 2 mM DTT led to the selection of functional antibody fragments and a single-domain scaffold, which also contains intra-chain disulphide bridges.[6,11,26,35] Moreover, similar conditions have been used for the production of a wide range of proteins up to 400 kilodaltons, including oligomers and cysteine-containing proteins.[10] Rabbit reticulocyte lysate containing 2–5 mM DTT has also been used to refold denatured proteins[36] and to express active proteins that failed to be produced by *in vivo* expression systems.[37]

Effect of DTT on protein folding *in vitro*

Affinity selection of ribosome complexes

PRM complexes have been selected using ligands immobilised either on magnetic beads or microtitre wells[5,6] or by soluble biotinylated proteins followed by capture on streptavidin-coated surfaces.[27] It is essential to keep the PRM complexes intact during selection. This is achieved by maintaining the complexes at 4°C with elevated magnesium concentrations, eg 5 mM for eukaryotic ribosome complexes[26,28] and 50 mM for *E. coli* complexes.[5] Under such conditions, complexes can be stable for at least two weeks.[34] To obtain effective selection, background binding needs to be eliminated, and various blocking reagents have been applied to reduce non-specific sticking.[29,35]

***In situ* RT-PCR for DNA recovery**

A defined selection condition or strategy often results in the production of proteins with desired properties.[38] In general, *in vitro* selection strategies such as 'off-rate selection' and 'stability selection', which have been used in phage display, can also be applied in ribosome display.[38]

Recovery of genetic information after selection

Retrieving genetic information after selection is a critical step for efficient display. A sensitive procedure is required to allow the recovery of rare species from a large library. A novel *in situ* RT-PCR recovery procedure has been developed for the eukaryotic ARM display system and has been shown to be efficient for DNA recovery (Figure 1B).[6,7] In this method, a primer hybridising slightly upstream of the 3′ end covered by the ribosome is used for RT-PCR, which is carried out on the intact ligand-bound complexes.[6,7] Not only does this simplify the rescue process, but it avoids losses incurred in disrupting PRMs to release mRNA. It also makes it highly likely that only monosome complexes are recovered, since it is difficult to see how a polymerase would be able to copy a complete mRNA carrying intervening ribosomes. With *in situ* RT-PCR, it may be possible to analyse ribosome-bound mRNA quantitatively by the Taqman method[39] and to automate the display process for proteomics applications (see below).

In the prokaryotic display systems, mRNA is released by the disruption of ribosome complexes with EDTA.[4,5] A side-by-side comparison of *E. coli* and rabbit reticulocyte lysate systems has shown that the disruption procedure generates poor recovery and enrichment from rabbit ribosome complexes.[28] Similarly, a comparison of *in situ* RT-PCR and the disruption procedures also revealed that the disruption procedure was five-fold less efficient in recovering DNA from rabbit ribosome complexes (authors'unpublished data). This may indicate that disruption is inefficient for eukaryotic complexes. It remains to be determined if *in situ*

RT-PCR recovery can be applied to *E. coli* complexes. Within the last two years, a method has been described to disrupt eukaryotic ribosome complexes by heating.[13]

APPLICATIONS

Selection of peptides from designed libraries

Ribosome display was used to select high affinity, specific ligand-binding peptides from combinatorial libraries. Peptides binding to a monoclonal antibody, D32.39, with affinities ranging from 7.2 to 140 nanomoles (nM) were selected from a random decapeptide library of 10^{12} members using a coupled *E. coli* S30 system.[4] Similarly, peptides binding to prostate-specific antigen with affinities of 0.8–20 nM were also obtained from a random 20-mer peptide library (10^{12} members) using an uncoupled wheatgerm system.[9] It was suggested that selection of the high-affinity peptides was attributable to the use of a very large ribosome display library.[4,9]

Selection of single-chain antibody fragments

Ribosome display has been utilised to select single-chain antibody fragments against a variety of targets. Using an *E. coli* display system, picomolar affinity antibody fragments against peptides and proteins were selected from either an immunised mouse library[29] or the human synthetic antibody library, HuCAL.[40] Antibody fragments, which recognise specific different DNA structures/conformations formed by identical sequences, were also obtained.[41] Eukaryotic ARM display was able to select fully human anti-progesterone antibody fragments from an immunised transgenic mouse library.[26]

Selection of other proteins

Both the prokaryotic and eukaryotic ribosome display systems have been used for the selection of non-antibody proteins. With the eukaryotic rabbit reticulocyte lysate system, variants of the single-domain CTLA-4 scaffold specifically binding to lysozyme were selected in a single cycle.[12] This system has also been used to display a cDNA library, from which sialyltransferase II was specifically isolated by its substrate in a single cycle.[13] Using the *E. coli* system, a His-tagged bovine heart fatty acid-binding protein was enriched with anti-His antibody by a factor of 10^8-fold over nine cycles from a library containing a large excess of an equivalent non-His–tagged protein.[17] The method has also been used for the selection of enzymatic activity.[38]

In vitro antibody evolution

Ribosome display is a powerful means for bringing about protein evolution *in vitro*, since mutation can be continuously introduced into DNA pools in subsequent cycles. Successive DNA diversification, followed by ribosome display/selection under defined selection conditions, has produced antibody mutants with improved affinity and stability.[27,29,40] By using a low fidelity DNA polymerase for DNA amplification, ribosome display has selected antibody mutants with up to a 40-fold improvement in affinity over the original antibody fragment.[40] This shows that protein evolution occurs during ribosome display cycles. Various PCR strategies, such as error-prone PCR,[29,40] DNA shuffling[27] or a combination of site-directed mutagenesis, error-prone PCR and H-CDR3 shuffling (authors' unpublished data), have been used to increase mutation rate, leading to the selection of evolved antibody fragments.[27,29,40] Mutation can also be accumulated in mRNA during display cycles by the inclusion of Qβ RNA-dependent RNA polymerase in the translation mixture.[12]

Use of a defined selection condition has also resulted in the isolation of proteins with improved properties. An 'off-rate' selection strategy, in which selection was carried out over a period of ten days, increased the affinity of an anti-fluorescein antibody fragment a further

30-fold when compared with its original high affinity of 1.1 nM.[27] Inclusion of DTT in the translation mixture during selection cycles led to the isolation of evolved antibody fragments that were stable in the absence of intra-domain disulphide bonds and which can be functionally expressed in the reducing environment of *E. coli* cytoplasm.[27]

Proteomics applications

Since ribosome display avoids the problems of cytotoxicity, soluble protein expression and secretion bias in cell-based systems, it could be an ideal means by which to display functional (single chain) proteins for applications such as target discovery and functional identification. With the completion of genome sequences, it is possible to design general cDNA libraries for ribosome display. In combination with high throughput protein arrays,[42,43] the screening power of ribosome display may be further increased, permitting library-versus-library screening and genome-wide analysis of protein–protein interactions. As the ARM display procedure is contained within a single tube,[6,7] it may be developed into an automated process for proteomics applications where high throughput manipulation is necessary. A reconstituted cell-free system has been described[44] which could be useful for further optimisation and improvement of ribosome display technology.

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