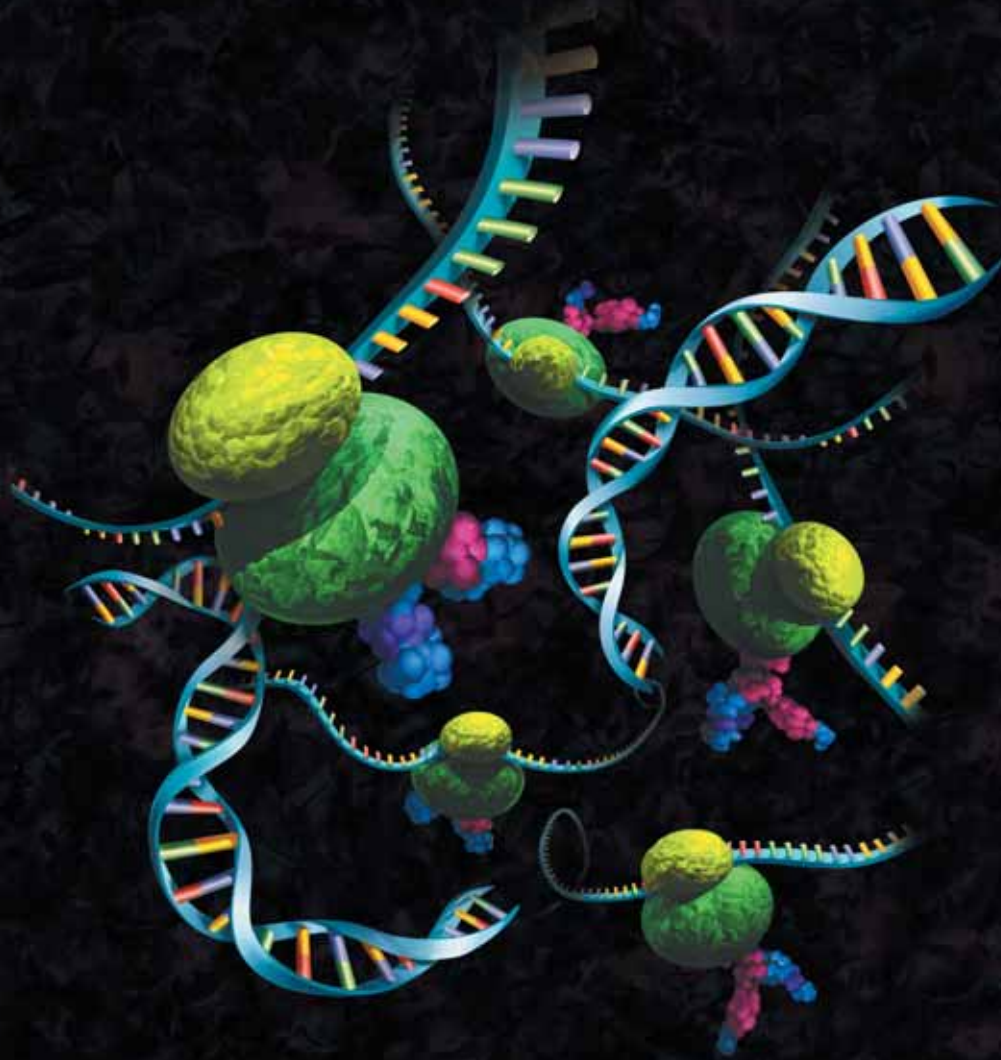


About the Image:

This illustration depicts antibody-ribosome-mRNA (ARM) complexes, which have been used as display selection particles in an in vitro eukaryotic method for selection of antibody-combining sites. An important feature of the ARM method is that it preserves the link between the peptide of interest and the gene that encodes it. The ARM strategy is based on two findings: i) rabbit reticulocyte lysates (cell-free systems used to generate proteins) produce functional single-chain antibodies; and ii) in a cell-free system nascent proteins and their mRNAs form stable ternary polypeptide-ribosome-mRNA complexes in the absence of a stop codon.



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Chapter Six: Ribosome Display

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Introduction

Combinatorial display technologies have proved to be valuable sources of diversity for identifying ligands to biological molecules (1). The technologies used are varied but are linked by a common approach of combining a very large amount of structural diversity with an effective selection to isolate those rare individuals in the diverse population that interact with the intended target. In vitro transcription/translation systems offer some unique advantages to this approach.

The most commonly used technique of the display technologies is phage display (2–4). In phage display systems, diversity is created through recombinant DNA techniques that introduce a randomized peptide sequence as a gene fusion to a surface protein on the phage. Each phage is a separate biological unit that contains both the displayed peptide and the genetic information encoding the peptide. A physical separation of those phage interacting with the specific target is employed, typically by interaction on a microtiter plate, and those phage that have bound to the target are amplified by growth on the appropriate host strain. Following 2–3 rounds of this enrichment process, the isolated phage typically bind specifically to the target. The identity of the randomized portion in these clones is determined by conventional DNA sequencing of the obtained phage. Sequence analysis typically shows a number of related sequences, which can be aligned to determine a consensus sequence for the binding ligand. Recently in vitro methods have become more popular because of the potential advantages these approaches hold.

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In Vitro Display Libraries

In vitro display systems incorporate many of the same principles of the phage systems but have their own unique characteristics. One of the key parameters of an effective display system is having sufficient diversity in the total library of sequences. In biological systems this is typically limited by the efficiency of transformation of the initial library of recombinant phage into the host cell. This practical limitation forces the diversity of the library to be approximately 10^9 or less. The primary advantage of the in vitro systems utilizing transcription/translation is overcoming this limitation in library size by elimination of the transformation step. In the in vitro systems utilizing transcription/translation, DNA template encoding the randomized peptide is added directly to the reaction, and the diversity is simply a matter of translation efficiency and scale of reaction. Using in vitro systems, libraries of $>10^{12}$ sequences have been obtained. The increase in library size not only increases the probability of finding a high-affinity ligand within the population; it also increases the number of randomized amino acid positions that can be completely encompassed by the library. The randomized portion of the displayed peptide is typically encoded by

codons consisting of the sequence NNK where N represents any nucleotide and K represents G or T. This degenerate codon then encodes all of the 20 possible amino acids, as well as a single stop codon, through the use of only 32 possible nucleotide combinations rather than the 64 possible combinations obtained from a completely random NNN codon. By limiting the number of codons used, the diversity of amino acid sequences is increased within the library. With a phage library of 10^9 sequences, this diversity represents complete coverage of 6 amino acid positions (32^6). Using an in vitro library of 10^{12} sequences, this coverage increases to 8 amino acid positions (32^8).

Another key property of display libraries that is a challenge to any in vitro system is the physical linkage of the randomized peptide to the genetic information that encodes it. In biological systems this linkage is handled through the compartmentalization of the genetic material within the cell and viral particle. In an in vitro system where the various templates are translated in a single reaction another way must be found to link the peptide and the sequence encoding it. Several approaches to in vitro systems have been applied to achieve this linkage.

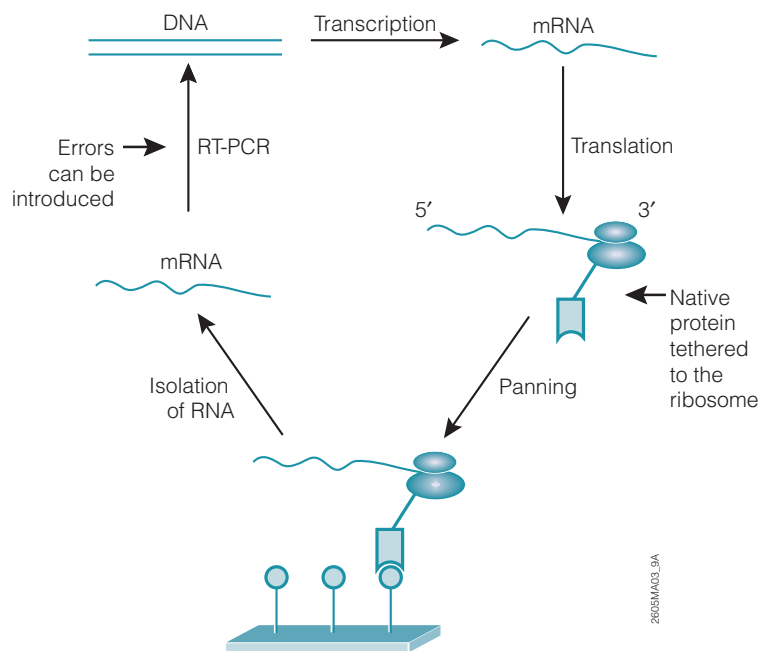


Figure 1. Schematic illustration of ribosome display (1). The ability to use proteins as the scaffolding structure for the presentation of the diversity in the libraries has a number of advantages. Scaffolding proteins can provide some rigidity to the displayed sequence. This rigidity helps to reduce the entropic penalty to binding and tends to give higher affinity ligands from library panning. Scaffolding proteins can remove the randomized region from the surface of the ribosome and provide better presentation of that sequence for affinity selection. Additional functionality can be provided by the scaffolding proteins and for creation of novel combinations of ligands and effector function. The diversity can also be applied to the activity of the scaffolding protein itself in the search for variants with novel properties.

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One approach is to mimic the in vivo cellular compartmentalization by creating reverse micelles, which serve as miniature reaction vessels for the in vitro reaction (5). Transcription/translation reactions are performed in emulsions with mineral oil. Individual DNA templates are contained within the aqueous micelles, and transcription and translation occurs and is retained within the aqueous environment of the micelle. Provided that these micelles can be effectively screened or selected, they provide a clonal segregation of the genetic material analogous to an individual phage or cell. The DNA template can be amplified by PCR^(f) and multiple rounds of screening or selection applied to isolate those individual clones with the desired properties.

A second approach to linking the genetic and structural information within display libraries is to use the genetic material as the structural diversity directly. The SELEX approach (6–10) creates diversity by creating an RNA library from a degenerate DNA template. The diversity of the secondary structure of the transcribed RNA, as well as the diversity of the primary sequence of that RNA, are used in the selection for sequences that bind to the intended target. Those sequences that bind are identified through reverse transcription and PCR amplification followed by DNA sequencing. The

primary advantages to this approach are the tremendous diversity that can be created, the fact that there is no need to link genetic and structural diversity and the speed at which the selection process can be performed. The primary limitation to this approach is the restriction to the four-base building blocks or a few base analogs that can be incorporated into the transcribed product.

Another approach that has been applied to in vitro systems is the use of polysomes for display (11–17; see Figure 1). Polysome display is one of a number of technologies in the area and offers some unique advantages. The method can be performed completely in vitro using a higher level of diversity than achieved through biological systems and can be used to express a variety of protein scaffolds. This method is a quick and flexible alternative to in vivo methods. Polysomes are the large macromolecular complexes of ribosomes, mRNA and translated protein that are formed during the translation process. Polysome display methods take advantage of the physical linkage of the genetic information encoded on the mRNA to the emerging translation product. The translation in the polysome complex is arrested either by addition of an antibiotic such as chloramphenicol, by secondary structure in the message, or by reduction of the reaction temperature. The

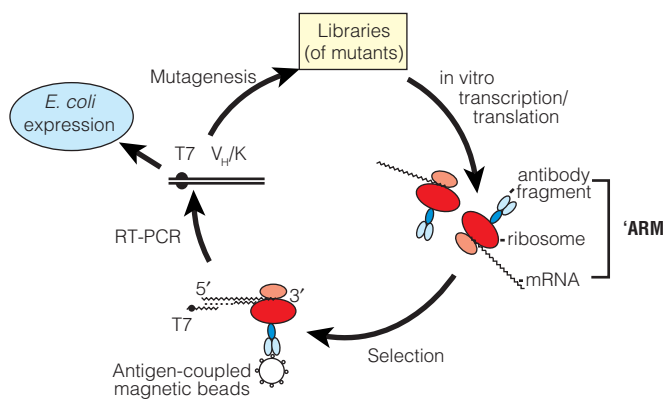


Figure 2. The ARM display cycle. The generation of an ARM library begins with mutagenesis of a V_H/K template, followed by antigen-selection of a specific binding ARM and then recovery of the genetic information by RT-PCR^(f).

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emerging translation product extends from the ribosome and is available for interaction with the target protein. After washing to remove unbound complexes, the complexes are eluted and the mRNA reverse transcribed and amplified for further rounds. In addition to the large diversity that can be screened by this method, it has the advantage that large proteins can be used as the scaffold for the diversity.

An example of using polysome display for isolating ligands is shown by the evolution and selection of antibody combining sites. The immune response of an animal to a presented antigen involves a process of selective amplification of those rare cells producing antibodies reactive to the antigen. This process can be copied via a polysome display approach (18,19,20; see Figure 2). A library of variants encoding mutations in the antigen combining sites of single-chain antibodies was expressed in vitro. Polysomes expressing those variants were incubated with magnetic beads coated

with the target antigen. Physical separation of the bound polysomes from unbound was performed and the process repeated for multiple rounds of enrichment. Using this method, both the specificity and the affinity of the antibody-antigen interaction can be modified. Enrichments of 10^4 – 10^5 per round of selection have been reported with the TNT[®] T7 Quick System^(a,b,c,d,e) (18).

Additional advantages of ribosome display approaches are that no cloning is required (PCR^(f) products are effective substitutes for T7- or T3- based TNT[®] Systems) and that flexibility is inherent with in vitro approaches. Unlike phage display, which is limited to an oxidizing periplasmic environment for protein folding, one can add components to in vitro systems to increase functionality, such as chaperonins, detergents or microsomal membranes. A recent review of in vitro protein selection approaches is presented by Roberts (21) and Hanes and Plückthun (22).

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