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mRNA display: ligand discovery, interaction analysis and beyond

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***In vitro* peptide and protein selection using mRNA display enables the discovery and directed evolution of new molecules from combinatorial libraries. These selected molecules can serve as tools to control and understand biological processes, enhance our understanding of molecular interactions and potentially treat disease in therapeutic applications. In mRNA display, mRNA molecules are covalently attached to the peptide or protein they encode. These mRNA–protein fusions enable *in vitro* selection of peptide and protein libraries of $>10^{13}$ different sequences. mRNA display has been used to discover novel peptide and protein ligands for RNA, small molecules and proteins, as well as to define cellular interaction partners of proteins and drugs. In addition, several unique applications are possible with mRNA display, including self-assembling protein chips and library construction with unnatural amino acids and chemically modified peptides.**

Functional approaches, such as *in vitro* selection, currently provide the best means available for isolating peptides and proteins with desired chemical or biochemical properties. Over the past decade, display technologies have been essential tools in the discovery of peptide and protein ligands, and in delineating *in vivo* interaction partners. The phage and ribosome display systems [1,2] have been used principally for discovery, whereas the yeast two-hybrid method [3] has been used for *in vivo* interaction analysis.

Despite their power, technologies that require an *in vivo* step, such as phage display and the yeast two-hybrid system face certain limitations. In phage display, libraries must be transformed into bacteria, limiting the number of possible independent sequences to 10^9–10^{10}. The total number of sequences represented can be further decreased by other issues, including: degradation of unfolded molecules, poor expression in the bacterial host, failure in processing to the phage surface, failure to fold in the oxidizing periplasmic space of *Escherichia coli*, and toxicity of the gene product. Similarly, the two-hybrid system requires the interaction partners to be cloned into yeast, limiting the number of constructs examined to 10^6–10^7. In addition, in the two-hybrid approach, interactions must occur in the nucleus, limiting control over the binding stringency, appropriate binding partners and biochemical conditions.

Totally *in vitro* techniques, such as ribosome and mRNA display, overcome many limitations of phage display and the two-hybrid system. These approaches reduce biases due to expression and routinely generate libraries of $>10^{12}$ independent molecules because no transformation step is required. In addition, more control can be exercised over the binding conditions as well as the stringency of selection.

mRNA display

The 'mRNA display'* peptide- and protein-selection system provides an alternative method that can be applied to both ligand discovery and interaction analysis problems [4,5]. In this approach, encoded peptide and protein libraries are covalently fused to their own mRNA (Fig. 1). Fusion synthesis is possible because the message can act as both template and peptide acceptor if it contains a 3′-puromycin molecule. Puromycin serves as a chemically stable, small-molecule mimic of aminoacyl tRNA (Fig. 2). The selection cycle for a typical mRNA-display experiment is shown in Fig. 3.

Detailed descriptions of experiments and protocols have been published elsewhere [7–11]. Briefly, a synthetic oligonucleotide containing a 3′ puromycin is ligated to the 3′ end of an mRNA and the product is translated in rabbit reticulocyte lysate. The sequence present in the peptide is therefore encoded in the covalently attached mRNA, allowing the sequence information in the protein to be read and recovered after selection via reverse transcriptase (RT)–PCR. Thus, exceedingly small amounts of material can be amplified.

Since the original description of the mRNA-display system in 1997 [4,5], optimization has resulted in the ability to perform selection experiments on libraries that contain $>10^{13}$ molecules [7,8,12]. Routinely 10–40% of the input mRNA template can be converted to fusion product, resulting in $>5\times10^{13}$ mRNA–protein fusions per milliliter of translation reaction. Overall, the mRNA-display system allows libraries with sequence complexity ~10 000-fold that of phage display [1], 10^6-fold that of yeast display or yeast two- and three-hybrid systems [3,13–15], and $\sim10^9$-fold over colony screening approaches [16].

In the majority of mRNA-display experiments, polypeptides with relatively short chain lengths (10–110 amino

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* mRNA display has also been referred to as mRNA–protein fusions [4], *in vitro* virus and *in vitro* virus virion [5] and PROfusion™ technology [6].

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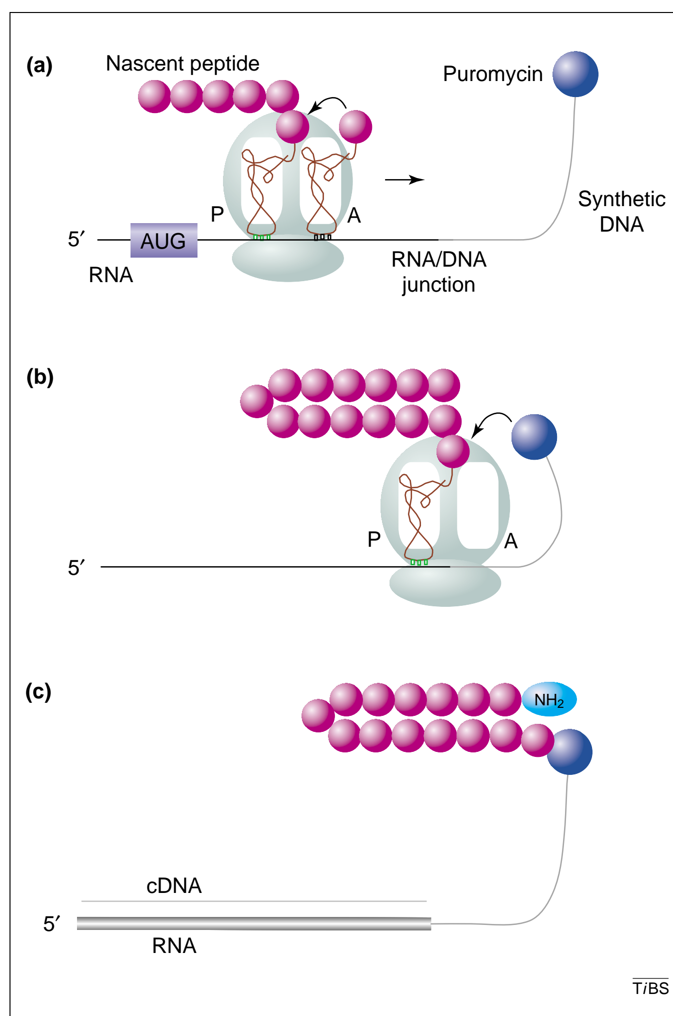

Fig. 1. Formation of an mRNA–protein fusion. (a) mRNA (black line) is ligated enzymatically [7] or photochemically [9] to a synthetic oligonucleotide (gray line) containing puromycin (blue) at its 3′ end. The ribosome (pale green) initiates synthesis of the template and reads in a 5′→3′ direction. tRNAs (brown) and amino acids (pink) are shown in the P- and A-sites of the ribosome. (b) Puromycin enters the ribosome, attaching the template to the C terminus of the nascent peptide. (c) Reverse transcription generates cDNA (gray line) that can be amplified by PCR.

acids) have been used. Larger proteins have also been studied (e.g. λ protein phosphatase, a 24 kDa enzyme [7]) but these typically form fusion products with somewhat reduced efficiency. Even for such proteins, libraries can still be readily constructed that are orders of magnitude larger than a typical phage display library. Another feature of mRNA-display constructs is that the mRNA appears to improve the solubility of the attached protein, enabling functional selection of sequences that can aggregate or are only partially soluble when expressed by themselves.

Ligand discovery with mRNA display

Selections to discover new peptides and proteins with desired features have now been completed. Sequences have been isolated that bind to RNA, small molecules and proteins; these results illustrate three important principles: (1) larger library size does, in fact, result in higher affinity molecules; (2) larger libraries result in a greater diversity of sequences with similar function; and (3) the

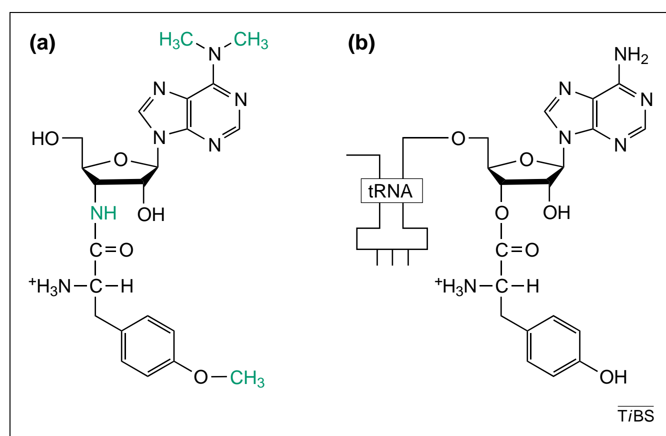

Fig. 2. Puromycin (a) is a small molecule analog of tyrosyl tRNA (b). Differences between the two molecules are highlighted in green text.

vast number of sequences recovered after selection can be analyzed using informational techniques, such as sequence co-variation analysis.

RNA-binding peptides

RNA-binding proteins participate in the regulation of transcription, splicing and translation, and have been implicated in several diseases [17,18]. Selections for RNA-binding peptides also present a stringent functional test of mRNA display. Numerous mRNA-display selections have isolated >100 chemically distinct RNA-binding peptides [19,20] (T. Xia *et al.* unpublished observations). These selections demonstrate that even highly basic and unstructured molecules retain function and do not interact with the attached mRNA–cDNA hybrid. The majority of experiments have been conducted using the RNA-binding domain from phage λ N protein as a model system, owing to its small size (22 amino acids), high affinity (low nanomolar) and thorough characterization. Selections have resulted in the discovery of numerous peptides with nanomolar affinity for their cognate target [19]. The highest complexity RNA selection performed to date contained ten random residues (X_{10}, where X is any of the 20 amino acids) and $>9\times10^{12}$ different sequences in the initial library [19] (Fig. 4a). The selected peptides all bound the boxB RNA hairpin with high affinity ($K_d = 0.5$–5.0 nM) and most demonstrated equal or better specificity than the wild-type sequence. However, the selected peptides showed striking chemical diversity and bore little resemblance to the wild type. Only a single arginine residue at position 15 (glutamine in the wild type) showed any significant conservation. Despite the lack of homology, sequence co-variation analysis indicated that the molecules fold into helices, showing correlations between adjacent residues (i to i+1) and residues located one turn away (i to i+3 and i to i+4) [20].

ATP aptamers

Primordial proteins presumably evolved from random sequences and it is probable that one of these first proteins bound ATP. Keefe and Szostak [21] attempted to isolate a modern relative of these prebiotic proteins by selecting for ATP binding using a 109mer protein containing an 80-aa random region. Libraries containing such large numbers

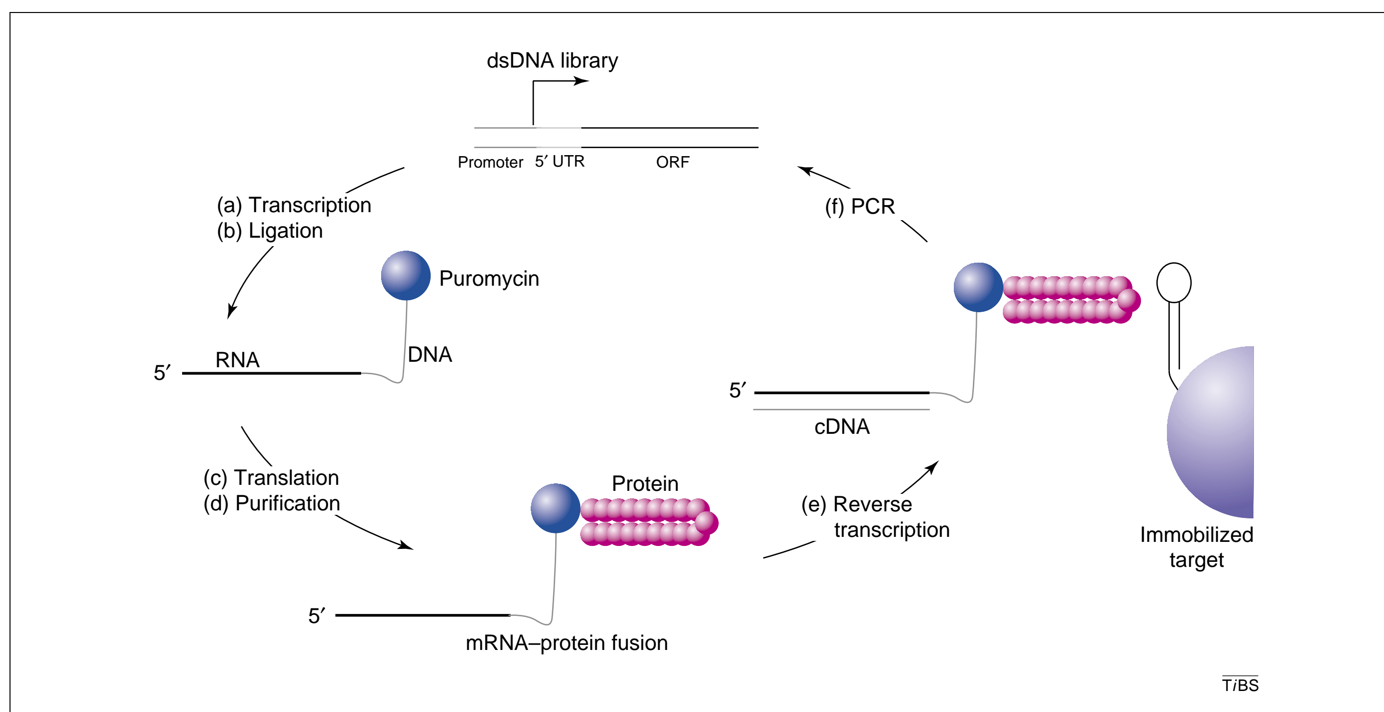

Fig. 3. A typical mRNA-display selection cycle. (a) A library of dsDNA sequences is transcribed to generate mRNA. (b) The mRNA is ligated to a puromycin oligonucleotide (blue) and used to program an *in vitro* translation reaction. (c) cDNA synthesis is performed and (d, e) the cDNA/mRNA–protein fusion is sieved using the target of interest. (f) PCR is used to regenerate the full-length DNA construct. For targets containing RNase or RNase-H activity, the cDNA can be crosslinked to the puromycin oligonucleotide to generate a cDNA–protein fusion [11].

of random positions present special problems because the probability of encountering a frameshift or stop codon can become substantial [7,22]. To solve these problems, the Szostak group utilized mRNA display to preselect library fragments for readability by selecting for the presence of N- and C-terminal epitope tags [12]. The readable fragments were then digested and assembled into full-length libraries that contained greatly improved open reading frames (ORFs).

After eight rounds of selection for ATP binding, four distinct protein-sequence families could be discerned [21]. Ten further rounds of selection, combined with mutagenesis, resulted in a clone (18-19) that bound ATP with high affinity ($K_d = 100$ nM) and could discriminate ATP from other nucleotide triphosphates with up to 2000-fold specificity. These protein aptamers contain a conserved Cys-Xaa-Xaa-Cys (CXXC) motif and function in a metal-dependent manner. The fact that the aptamer uses metals might indicate that chelation provides a simple way to create stable, functional protein structures, which is consistent with the large energy seen for protein–metal interactions [23]. One feature of these aptamers is that only a fraction of each clone appears folded and functional; the proteins themselves tend to aggregate when expressed as free proteins. Thus, selection of these proteins was most probably facilitated by the improved solubility imparted by the mRNA–cDNA tail, and argues that such sequences would not be found in a typical phage-display selection. The fact that the functional clones are not well behaved probably reflects the relative paucity of proteins that are both folded and functional in the vastness of sequence space. The structure of these totally novel polypeptide chains remains a tantalizing question that is yet to be solved.

Keefe and Szostak estimate that one in 10^{12} molecules in their initial library have the ability to bind ATP – approximately the same fraction seen for ATP-binding RNA aptamers [24]. This result is somewhat surprising given the greater chemical diversity of proteins (20 sidechains) relative to nucleic acids (four sidechains). Although functionally impoverished, nucleic acid aptamers might benefit from the ease of forming higher-order structures through simple base-pairing interactions, in contrast to proteins, which require a hydrophobic core for folding. It remains an open question whether catalytic proteins can also be found with similar frequencies to their nucleic acid counterparts.

Streptavidin aptamers

Szostak and coworkers also created long ORFs for a binary patterned library [12]. This library contained a random region of 87–88 amino acids with an initial complexity of $\sim 10^{13}$ sequences, and was assembled from two distinct 11-aa segments containing hydrophobic and polar amino acid patterning that results in either amphipathic α helices or β strands [25]. mRNA-display selections against streptavidin resulted in identification of several sequences that bind streptavidin with nanomolar affinity ($K_d \sim 5$ nM) [26] and bind 200–2200-fold better than the *Strep*-tag II peptide that was obtained previously by phage display [27,28].

Although the library was patterned to form helices and sheets in reading frame one, all of the selected molecules were shifted into reading-frame three, effectively eliminating the patterning. The shifted frame seems to have

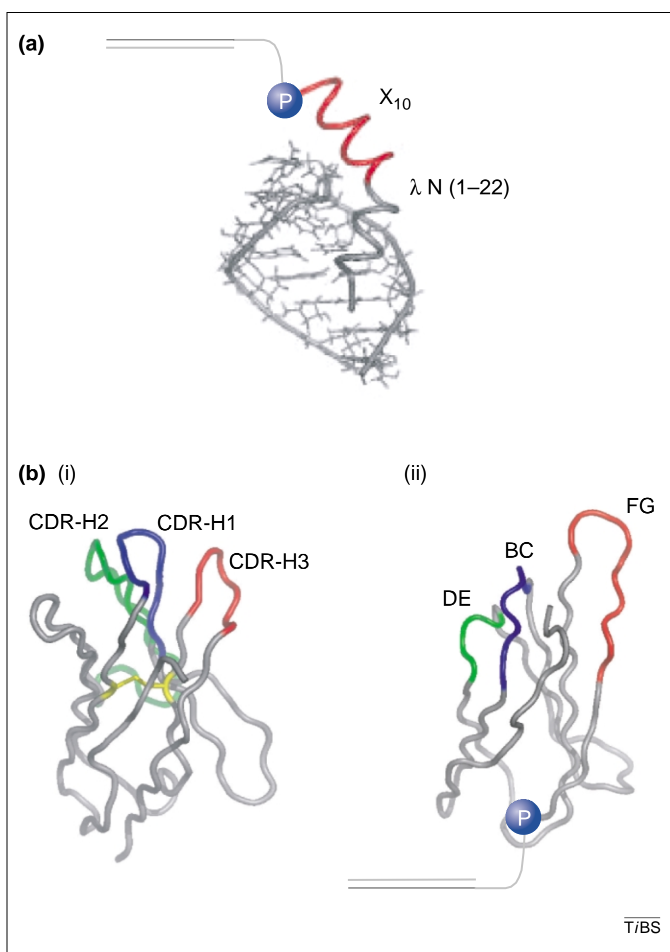

Fig. 4. Examples of mRNA-display libraries. (a) A library containing ten consecutive random residues (red) was constructed from the λ N RNA-binding protein and selected for binding to RNA hairpins [19]. (b) Structural comparison between the llama variable heavy chain domain V_{HH} (i) and the 10th fibronectin type III domain (^{10}Fn3) (ii). Three libraries derived from ^{10}Fn3 were constructed as antibody mimics of V_{HH} and selected for binding to TNF-α and leptin [32]. Residues of ^{10}Fn3 that were randomized are shown in color. Abbreviations: CDR, complementarity-determining region; P, puromycin.

been favored because of the presence of His-Pro-Gln (HPQ) tripeptide sequences. The HPQ peptide represents the minimal core of the *Strep*-tag II peptide and has been shown to bind streptavidin [27,28]. Frame one of the patterned library contained very few HPQ sequences (1/45 000 clones), owing to the library design, whereas in the third frame, HPQ peptides were present in 1/64 sequences.

The majority of the sequences in the library contained at least one HPQ motif and one similar tripeptide motif [e.g. HPQ, His-Pro-Ala (HPA) and Leu-Pro-Gln (LPQ)] and do not appear to contain any disulfide bonds. A 38-aa sequence, termed the 'SBP-tag' has been used for one-step affinity purification on streptavidin agarose and western blot detection using streptavidin–horseradish-peroxidase for visualization [29]. Despite frameshifting, the patterned library still contained ~10 000-fold greater sequence complexity than a standard phage-display selection, probably leading to the high affinity of the resulting aptamers. Finally, this experiment demonstrates the difficulty in designing random libraries with imposed structural features *a priori*.

TNF-α aptamers using the ^{10}Fn3 domain

Monoclonal antibodies are useful both as a biochemical tool and as potential therapeutics. mRNA display has been used to isolate novel antibody mimetics based on a fibronectin domain. The tenth type III domain of human fibronectin (^{10}Fn3) displays an Arg-Gly-Asp (RGD) sequence involved in cell-surface recognition by integrins [30]. The ^{10}Fn3 domain has a β-sheet architecture similar to antibody V_H domains, with three structurally analogous loops (Fig. 4b). The antibody-like structure, exposure to the immune system, small size (94 aa), lack of disulfide bonds, high bacterial expression levels and high stability ($T_m = 90°C$) all make the ^{10}Fn3 domain an excellent potential scaffold. However, previous attempts to isolate ^{10}Fn3 derivatives using phage display resulted in molecules with only modest affinity ($IC_{50,\ ubiquitin} = 5\ \mu M$) and relatively non-specific binding [31].

Xu *et al.* constructed three libraries based on ^{10}Fn3, by randomizing either one loop (libraries 1 and 2) or all three loops simultaneously (library 3) [32]. An mRNA-display selection was then performed against tumor necrosis factor-α (TNF-α); after nine or ten rounds of selection, diverse high-affinity ($K_d = 1–24$ nM), and high-specificity ligands were isolated, primarily originating from library 3. Further selection for a total of 14 rounds resulted in clones with sub-nanomolar affinity ($K_d = 90–110$ pM). Returning to round eight, mutagenic PCR was added to the selection cycle, duplicating the affinity maturation process of antibodies. Further rounds of selection resulted in a clone with high affinity ($K_d = 20$ pM). Although less stable than the wild-type ^{10}Fn3, the best clone (12.21) was monomeric and showed good expression and protease resistance at 30°C. Immobilized versions of a round-nine clone (9.12) were used to capture TNF-α from a solution of 10% fetal-bovine serum, demonstrating the high specificity of these reagents, even when immobilized on a solid support.

Specificity and interaction analysis

Epitope recovery

In addition to enriching sequences containing a known epitope [1], mRNA display can also be used to determine which sequences are crucial for recognition. Baggio *et al.* used two random libraries to investigate the specificity of peptides binding the anti-*c-myc* antibody 9E10 [33] and bovine trypsin [34]. Selection against the 9E10 antibody with a 27-random-residue (x_{27}) library revealed a consensus sequence x[Q/E]xLISExx[L/M] (the *c-Myc* tag is EQKLISEEDLN), demonstrating that the Leu-Ile-Ser-Glu (LISE) sequence was the core element recognized by the antibody. In the same work, a six-random-residue library (positions 3–8) was created using the *Ecballium elaterium* trypsin-inhibitor two protein (EETI-II) as a scaffold [34]. EETI-II, a 28-residue protein with three disulfide bonds, is a member of the knottin family and inhibits bovine trypsin via interaction at positions 3–8 [35]. Selection against trypsin yielded a sequence consensus of Pro-Arg-Xaa-Leu-Xaa-Xaa (PRxLxx), with 20% of the selected clones matching the wild-type sequence of Pro-Arg-Ile-Met-Arg (PRILMR).

mRNA display has also been applied to define a

recognition epitope for the oncogenic v-abl tyrosine kinase, which is a target of great biological and therapeutic interest [36]. Initial experiments demonstrated that mRNA–peptide fusions containing a v-abl phosphorylation site (the tyrosine residue in EAIYAAPFAKKK) could be phosphorylated by the v-abl kinase and immunoprecipitated with α4G10, an anti-phosphotyrosine monoclonal antibody. Libraries of the form $GCGGx_5Yx_5GCG$ were subjected to phosphorylation with v-abl and precipitation with α4G10. The majority of clones contained an Ile/Leu/Val-Tyr-Xaa_{1-5}-Pro/Phe ([I/L/V]Yx_{1-5}[P/F]) consensus. Interestingly, despite the sequence variations, the kinase effectively phosphorylated all 12 of the consensus clones, indicating a broader specificity than previously thought.

Cellular interaction partners

mRNA-display libraries that are constructed from cDNA offer the potential for isolating biologically relevant interaction partners. Hammond and co-workers used a random priming approach to create mRNA-display libraries from several different human tissues [37]. This approach yields libraries of various lengths and in three reading frames, and it also enables the experimenter to construct libraries with tissue-specific primer tags. After selection, PCR using these primers can be used to deconvolute the library and obtain binders from specific tissues. Sieving cellular libraries against the anti-apoptotic protein Bcl-X_L resulted in isolation of >20 different proteins, including the known interaction partners Bim, Bax and BCL2L12. The diversity in the cellular mRNA-display libraries suggests that hundreds to millions of fragments of various lengths will be present from each gene. In that vein, the Bcl-X_L selection demonstrates that alignment of multiple positive clones is equivalent to typical deletion analysis, providing a clear indication of the sequence boundaries necessary for recognition.

Cellular libraries can also be used to characterize and discover cellular proteins or receptors that interact with a drug of interest. McPherson *et al.* used the immunosuppressive drug FK506 as a target for cellular libraries [38]. This work resulted in isolation of the known target FKBP (FK506-binding protein) and defined a region within FKBP that was necessary and sufficient for its interaction with FK506 [38].

Unique applications of mRNA display

Self-assembling protein microarrays

The mRNA–protein fusions used in mRNA display can also be used for high-throughput screening applications. Protein chips offer the promise of quick analysis of the expressed-protein content in a sample and the ability to perform *in vitro* interaction analysis. Weng *et al.* demonstrated that a standard DNA chip could be converted into a protein chip by hybridization of mRNA–protein fusions (Fig. 5) [3]. mRNA–protein fusions encoding the MYC, FLAG or HA11 epitopes were synthesized and incubated with a DNA chip. The chip was imprinted with DNA that was complementary to a unique (MYC, FLAG or HA11) or common (5′ or 3′) nucleic acid portion of the fusions. Hybridization of the fusions to cDNA directs the self-assembly of the protein chip. The experiments demonstrate that, at least for antibody–epitope interactions, these protein arrays preserve the functionality of the displayed proteins, present them in a uniform orientation and have sub-attomole detection limits.

Non-natural libraries

Phage display and the yeast two-hybrid system contain an obligate *in vivo* step and, thus, are generally limited to display only the 20 natural amino acids. Recently, Li *et al.* demonstrated that the suppressor tRNA strategy for incorporating unnatural amino acids [39,40] can be used to create mRNA-display libraries bearing an unnatural residue [41] (Fig. 6). The combination of these two powerful technologies increases the chemical

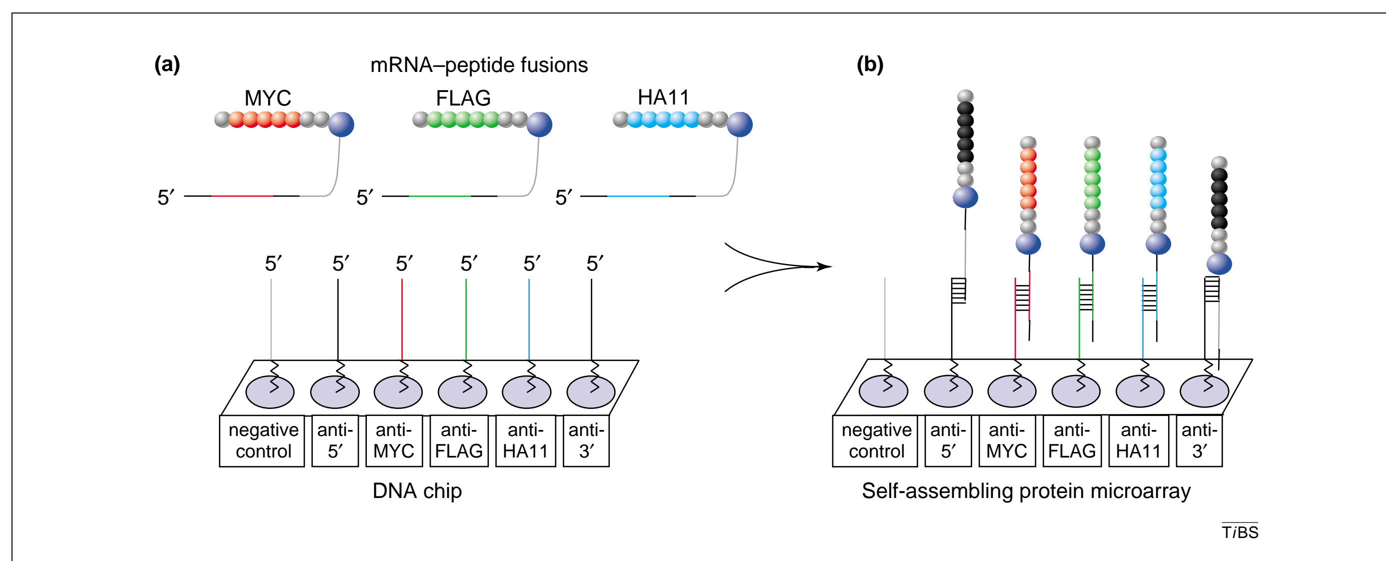

Fig. 5. A self-assembling protein chip. (a) A mixture of mRNA–protein fusions containing the MYC (red), FLAG (green) or HA11 (light blue) (black) epitope was incubated with a standard DNA chip; black circles indicate amino acids that can be MYC, FLAG or HA11. The nucleic acid component directs the fusions to regions on the chip containing cDNA. (b) A DNA complementary to the 5′ or 3′ sequence hybridizes to all three fusions, whereas the anti-MYC DNA will isolate only fusions containing the MYC epitope. This results in peptide microarrays that can be spatially addressed and recognized by monoclonal antibodies [32].

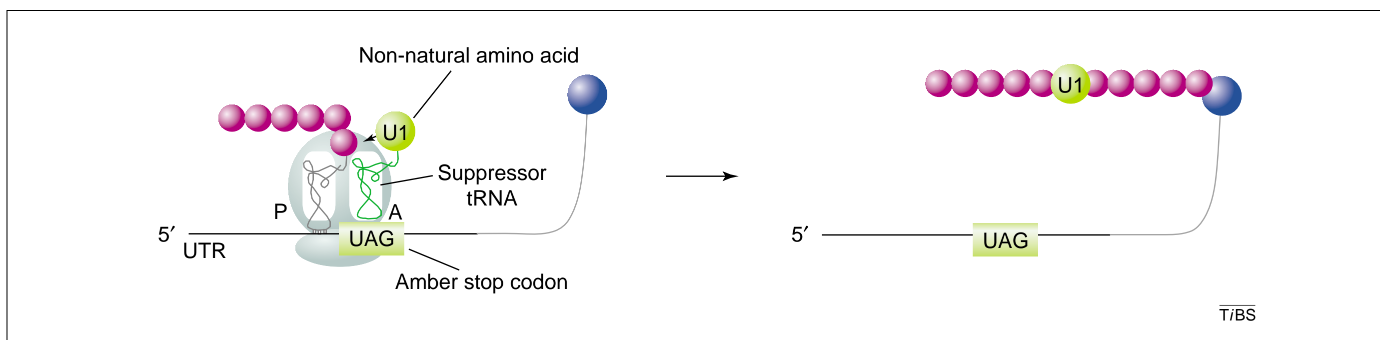

Fig. 6. Inserting non-natural residues into mRNA-display libraries. *In vitro* nonsense suppression by a chemically amino-acylated suppressor tRNA was used to insert biocytin into mRNA-display libraries and select for the presence of the non-natural residue [41].

diversity that can be displayed and should facilitate discovery of ligands with improved affinity, specificity, stability or reactivity. Indeed, recent work indicates that chemically derivatized libraries bearing a pendant penicillin sidechain can be used to discover novel potential antibiotics (S. Li and R. W. Roberts, unpublished observation).

Concluding remarks

Techniques for performing mRNA display are now well established and enable facile synthesis and selection of mRNA–protein fusion libraries [7,8,12]. Completed selections demonstrate that mRNA display is a powerful tool for both ligand discovery and interaction analysis. Notable features of the resulting ligands are high affinity (nanomolar to picomolar) and striking sequence diversity [19,26,32]. The *in vitro* nature of the system provides a unique opportunity for *in vitro* affinity maturation and evolution [21,32], inclusion of non-natural residues [41], chemical derivatization of libraries and *in vitro* recombination experiments [42]. Future applications point toward the isolation of new catalysts and the creation of libraries that are composed entirely of unnatural sidechains or non-peptide backbones.

Acknowledgements

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Characterization and role of protozoan parasite proteasomes
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