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Title: **Targeted *In vitro* Evolution of Protein Ligands**

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Targeted *In vitro* Evolution of Protein Ligands

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Abstract

The objective of this project was to create a diverse peptide library by exploiting the MS2 coat protein/RNA operator interaction. In order to accomplish this it was necessary to define the optimal scaffolding for the library in which DNA, coding for the stem loop operator, is fused to tandem coat protein monomers which, when translated, would form a covalent dimer subsequently binding the nascent RNA. Therefore, a randomized library region downstream of the coat protein monomers would also be translated and bound to the coding transcript allowing us to generate a widely diverse library as well as couple sequence to function. As proof of principle to define the scaffolding, we used the c-myc epitope to emulate our N-library region and performed binding and enrichment studies using an anti-myc immunoprecipitation followed by RT PCR. In order to simulate the library environment, which would contain upwards of 10^{11} different peptides, we used a competitor construct, which contained the covalent dimer/operator but lacked the myc epitope. To date, we have successfully competed our target transcript versus competitor at a molar ratio of $1:10^3$.

Background and Research Objectives

Several methodologies currently exist for the in vitro evolution of ligands. Among these are phage display, ribosome display[1] , RNA aptamer evolution, and puromycin linking[2]. All of these techniques offer the advantage of large diversity as well as coupling function to sequence. However, each has its own unique set of disadvantages. Among these are the toxicity of single chain ligands, lability of RNA aptamers, technically specialized requirements for puromycin linking, and the diversity limitations of the ribosome display system which is still under development. The advantages of the system we proposed include greater diversity, technically less demanding, quicker than aforementioned technologies, and end products in the form of small peptides which may or may not have inherent advantages over single chain or RNA ligands, depending on the target.

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Importance to LANL's Science and Technology Base and National R&D Needs

The evolution of small molecules to bind specified targets is important in several arenas. The ability to target a pathway molecule with an evolved ligand, which disrupts the pathway, gives rise to knockout mutations, an invaluable tool for bioresearch applications. An evolved ligand linked to a fluorescent reporter has inherent value as a molecular biosensor for pathogen detection or diagnostics, and, of course, the downstream therapeutic applications of these types of technologies are limited only by our ability to deliver and test them in an *in vivo* system.

Scientific Approach and Accomplishments

Our approach to defining the scaffolding necessary for a downstream library was to design and clone several DNA constructs within a specific framework to take advantage of the MS2 dimer/stem loop interaction during translation (Fig.1). Linear double stranded DNA was transcribed and translated *in vitro*. The translated proteins were then immunoprecipitated against a monoclonal anti-myc antibody immobilized on protein G agarose. The captured proteins were then denatured and the attached RNA was extracted via organic solvent and precipitated. The isolated RNA was reverse transcribed using a reverse primer to the myc epitope-coding region, the cDNA product was amplified by polymerase chain reaction (PCR), and the process repeated. In order to emulate the proposed library, each construct was competed with an identical construct lacking the myc epitope at various molar ratios of target construct : competitor. One of our initial concerns was the cooperativity of the MS2 monomers, as they have a propensity to dimerize spontaneously. Trans-dimerization would be extremely detrimental to our effort, as stem loop binding would be unconfined to nascent covalent dimers. We were fortunate enough to obtain a clone from Olke Uhlenbeck at the University of Colorado which coded for a mutated version of the MS2 coat monomer. This monomer (V75E;A81G) is shown to abolish all cooperativity while binding the stem loop operator with near wild type affinity[3]. Therefore, all constructs were cloned utilizing this mutated monomer.

Our initial success in proof of principle studies demonstrated that it was possible to isolate our target construct at 1:100 competition (Fig. 2) and that it is in fact possible to re-capture our target and enrich for it. We then experimented with varying sections of the scaffold and enlisted the aid of Paolo Catasti and Goutam Gupta from LANL's Theoretical Division (T-10).

It was determined through rational design that the linker region between the two monomers was integral to the proper dimerization and exposition of the stem loop binding site, and a linker was designed and cloned into the scaffold (Fig. 3). The improved linker allowed us to compete the construct at a molar ratio of $1:10^3$, while also demonstrating that the spacing and composition of the linker between the monomers was an important optimization parameter to be explored (Fig.4).

While our proof of principle studies showed promise, we have been unable to date enriched for our target product when competed beyond $1:10^3$. We expect that this may be due to several factors, not the least of which, may involve the kinetics of dimer/stem loop binding. It is possible that the off rate of the dimer -stem loop is such that recovery will not be possible at lower concentrations of target. While this remains to be tested, it illustrates the inherent disadvantages of the dimer-stem loop interaction being non-covalent. Further, more optimization is necessary via incorporating more elements of computer modeling and rational design in order to define the scaffolding of the library, which potentially would claim the highest diversity and make the system a viable alternative to current technologies.

References

[1] Hanes, J., Pluckthun, A., In vitro selection and evolution of functional proteins by using Ribosome Display. Proceedings of the National Academy of Sciences, 94, 4937-4942, 1997.

[2] Roberts, R., Szostak, J., RNA-peptide fusions for the in vitro selection of peptides and proteins., Proceedings of the National Academy of Sciences, 94, 12297-12302, 1997.

[3] LeCuyer, K., Behlen, L., Uhlenbeck, O., Mutants of the Bacteriophage MS2 Coat Protein That Alter Its Cooperative Binding to RNA. Biochemistry, 34, 10600-10606, 1995.

Figure Captions

Fig. 1- Basic Scaffolding of MS2 constructs. Varied regions depicted by dashed lines.

Fig. 2- RT PCR after immunoprecipitation vs anti-myc antibody. A-uncompeted, B-1:1, C-1:10, D-1:100, E-1:10^8, F-Competitor only (negative control), G-0 template (negative control). Note lower band at ca.500bp was determined to be PCR artifact.

Fig. 3-Rational design linker connecting MS2 monomers to expose stem loop operator binding site. (Paolo Catasti, Goutam Gupta)

Fig. 4- Competition demonstrating product at 1:1000.

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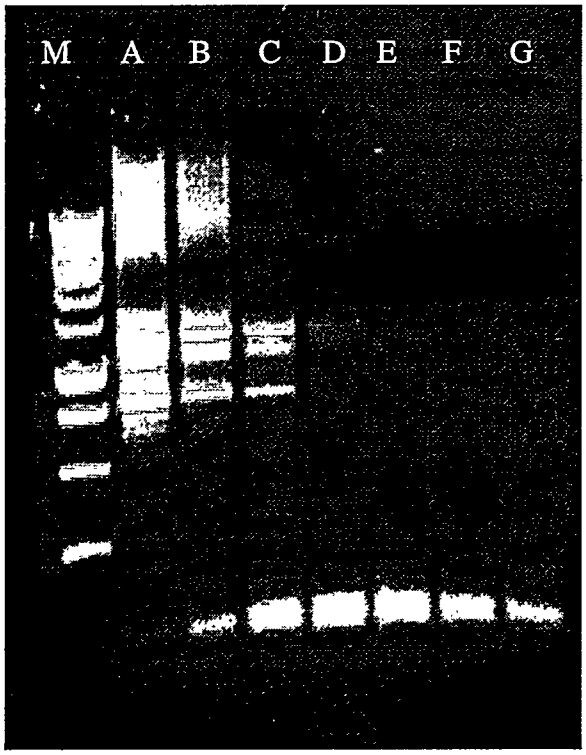

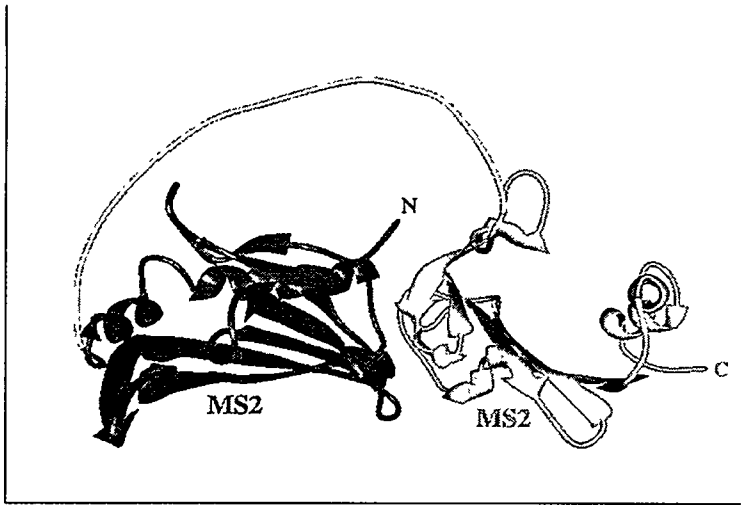

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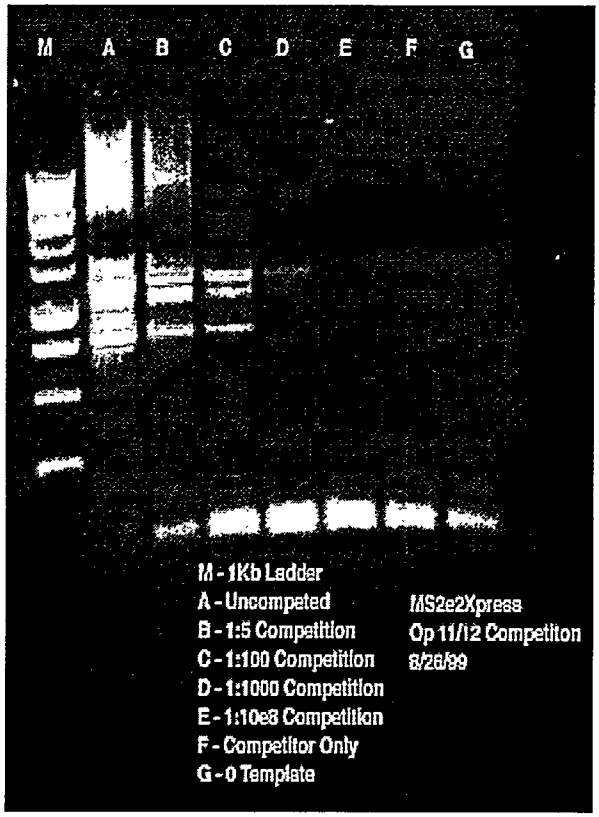

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