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Evolution of catalytic function

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Abstract. An RNA-based evolution system was constructed in the laboratory and used to develop RNA enzymes with novel catalytic function. By controlling the nature of the catalytic task that the molecules must perform in order to survive, it is possible to direct the evolving population toward the expression of some desired catalytic behavior. More recently, this system has been coupled to an *in vitro* translation procedure, raising the possibility of evolving protein enzymes in the laboratory to produce novel proteins with desired catalytic properties. The aim of this line of research is to reduce darwinian evolution, the fundamental process of biology, to a laboratory procedure that can be made to operate in the service of organic synthesis.

INTRODUCTION

Organic chemists have enjoyed remarkable success in synthesizing a variety of natural products. Even more impressive is the success of nature in generating enzymes that produce the vast repertoire of natural products in the first place. Chemists are learning to employ enzymes to assist in organic syntheses and to modify enzymes to make them better suited for particular applications. Ultimately one would like to appropriate from nature not just the enzymes, but the process by which these enzymes are generated: the process of darwinian evolution based on natural selection.

Darwinian evolution involves the concerted operation of three processes, selection, amplification, and mutation, applied to a population of informational macromolecules. Selection is used to obtain the most advantageous individuals in a population, amplification provides copies of the selected individuals, and mutation introduces new variation among the copies. The power of laboratory evolution lies in the power of large numbers. It is not unusual to survey 10^{13} macromolecules at a time, and use the one-in-a-billion with the most desirable properties as "breeding stock" for the next generation. A generation can be carried out in one or two working days.

Thus far, laboratory evolution has been carried out successfully with RNA molecules. RNA lends itself most readily to this process because of its dual role as both a genetic molecule and a catalyst. A number of RNA enzymes (ribozymes) are known to exist in nature, and these serve as a starting point from which to begin an evolutionary search for novel catalysts. It has been possible, for example, to convert an RNA enzyme that cleaves single-stranded RNA to an RNA enzyme that cleaves single-stranded DNA (ref. 1). It has also been possible to evolve RNA metalloenzymes that have novel metal dependence (ref. 2).

It remains to be seen to what extent the range of RNA-based catalytic function can be expanded. If nature provides any indication, it is that the catalytic prowess of RNA is rather limited. After all, proteins carry out most of the catalytic functions in biological organisms. RNA has been shown to catalyze phosphoester transfer reactions (ref. 3,4), phosphoester hydrolysis (ref. 5-9), aminoacyl ester hydrolysis

(ref. 10), and peptide bond formation (ref. 11). Considering the functional groups that exist within RNA and the ability of RNA to adopt a well-defined tertiary structure, a number of other catalytic functions seem feasible. Nonetheless, proteins are more versatile catalysts, containing twenty dissimilar amino acid components rather than the four similar nucleotide components of RNA.

It is not yet possible to evolve protein enzymes in the laboratory. The major stumbling block is that proteins do not have genetic properties — they cannot be amplified and mutated directly. Instead, a gene encoding the protein must be amplified and mutated, and then expressed as the corresponding protein. If it were possible to perform "reverse translation", that is, revert selected protein molecules to their corresponding genes, then protein evolution would be straightforward. Nature solves the problem of protein evolution in a different way, by co-localizing genes and their corresponding proteins within a common cellular compartment. Some progress has been made toward the laboratory evolution of proteins by attaching short peptides and corresponding synthetic gene fragments to a common linker molecule, selecting on the basis of the peptide's function, and then amplifying the adjoined gene fragment (ref. 12). This technique, however, is limited to short peptides and relatively small populations of molecules.

Taking a different approach, we have learned to conduct both amplification of genetic molecules and translation of genetic molecules to their corresponding proteins under a common set of reaction conditions in the test tube. Methods must now be developed to co-localize a gene and its protein product so that the two can be selected together. This will require either post-translational attachment of the protein to its corresponding gene or physical isolation of gene / protein pairs.

EVOLUTION OF RNA ENZYMES

The first demonstration of darwinian evolution in the laboratory was carried out 25 years ago, using the RNA genome of Qß bacteriophage and purified Qß replicase protein (ref. 13). In the presence of the four nucleoside triphosphates, Qß replicase produces copies of Qß RNA in an autocatalytic fashion. Errors occur during replication, so that the copies contain roughly one or two mutations compared to their parents. The population of RNAs is under strong selection pressure to serve as an efficient substrate for the replicase. Those RNAs that are most efficient substrates will produce the most copies and therefore grow to dominate the population... that is, until a new mutant arises that is replicated even more efficiently and is able to take over the population. Over time, the evolving population improves its ability to be amplified by Qß replicase under the prevailing reaction conditions.

While the Qß system provides an elegant demonstration of darwinian evolution at the molecular level, it is highly constrained by the fact that the evolving RNAs must remain good substrates for the replicase enzyme. Qß replicase amplifies only those RNAs that closely resemble the Qß genome. This is sensible from the point of view of Qß bacteriophage because it would be wasteful for the polymerase to amplify unrelated RNAs that exist within the host cell. However, from the point of view of laboratory evolution, it is a severe restriction because most RNAs of biochemical interest, including all known ribozymes, cannot be amplified by Qß replicase.

An RNA amplification engine

Techniques are now available to amplify genetic molecules, regardless of their sequence. The best known of these techniques is the polymerase chain reaction (PCR), a reciprocal primer method that provides 10^6-fold amplification of a DNA sequence in a few hours (ref. 14,15). The PCR relies on a thermostable DNA-dependent DNA polymerase to copy both strands of a DNA duplex to produce additional DNA duplex molecules. A second gene amplification procedure is available to amplify RNA, again by a factor of 10^6 in 1-2 hours (ref. 16,17). This procedure makes use of two polymerases, reverse transcriptase

to copy the RNA to DNA, and T7 RNA polymerase to copy the DNA back to RNA. Unlike the PCR, which roughly doubles the number of gene copies with each reaction cycle, the RNA amplification procedure results in several hundred-fold amplification per cycle. Also unlike the PCR, the RNA amplification procedure operates at a constant temperature of 37 °C (ref. 18).

Both the PCR and the isothermal RNA amplification procedure can be used to amplify a population of RNA molecules. With the PCR, the RNA must first be reverse transcribed to DNA, then the DNA is amplified by the PCR, and the PCR products are transcribed back to RNA (ref. 19,20). With the isothermal RNA amplification procedure, the RNA is amplified directly (ref. 21). Both methods place no significant restrictions on the sequence of the RNA being amplified and are applicable to all known ribozymes. The two procedures can be made to operate in concert to achieve a combined amplification factor of greater than 10^9 (ref. 1).

There are a variety of laboratory techniques for introducing random point mutations into genetic molecules, including chemical mutagenesis (ref. 22-24), incorporation of mutagenic nucleotide analogues (ref. 25-27), incorporation of randomized synthetic oligonucleotides (ref. 28-31), and inaccurate copying by a polymerase (ref. 32-35). The last of these, inaccurate copying by a polymerase, has been applied to the PCR to produce mutations at a rate of 0.66% ± 0.13% (95% C.I.) per position (ref. 36). The resulting mutations are randomly distributed throughout the amplified sequence and show no strong preference with respect to the type of base substitution. Thus, selected RNA or DNA molecules can be amplified, subject to mutational error, to produce a descendant population of variant molecules.

Novel catalytic function

RNA molecules can be selected on the basis of their catalytic function (ref. 1, 37-40). The general requirement for selection is that catalytically active molecules become tagged in some way so that they can be distinguished from nonreactive molecules. If and only if an RNA molecule becomes tagged is it then amplified to produce "progeny" molecules.

One tagging procedure that has been applied to ribozymes that catalyze phosphoester transfer reactions is covalent attachment of the ribozyme to a target phosphate within the substrate. Group I ribozymes catalyze cleavage / ligation reactions involving nucleophilic attack by the 3′-hydroxyl of guanosine at a target phosphodiester bond within an RNA or DNA substrate (ref. 37,41). The products of this reaction are guanosine attached to the 3′ portion of the substrate and the released 5′ portion of the substrate (Fig. 1). If the guanosine nucleophile is placed at the 3′ end of the ribozyme, then the entire ribozyme becomes attached to the 3′ portion of the substrate via the guanylyl-(3′,5′)-phosphodiester linkage (ref. 42). Functional ribozymes that are tagged in this way can be selectively amplified by hybridizing a DNA primer across the ligation junction and using the primer to initiate reverse transcription and subsequent autocatalytic amplification.

The substrate specificity of a group I ribozyme is determined by complementary pairing between a template region within the ribozyme and the 5′ portion of the substrate (ref. 43,44). The target phosphodiester bond of the substrate lies immediately downstream from the region of complementary pairing. Group I ribozymes obtained from nature have the ability to bind and cleave a target RNA substrate with reasonable efficiency (K_m = 1 nM, k_{cat} = 0.1 min^{-1}, measured at 10 mM $MgCl_2$, pH 7.0, 50 °C (ref. 45)). Under the same reaction conditions, the ribozyme's ability to cleave a comparable DNA substrate is much lower (K_m = 30 μM, k_{cat} = 0.006 min^{-1} (ref. 46)). Under physiologic conditions (pH 7.5, 37 °C) DNA cleavage activity is almost undetectable (K_m = 6.6 μM, k_{cat} = 0.0002 min^{-1} (ref. 1)).

We used a laboratory evolution procedure, maintaining a population of 10^{13} ribozyme variants over ten successive generations, to develop ribozymes with 100-fold improved activity compared to the wild type in cleaving a target DNA under physiologic conditions (K_m = 2.0 μM, k_{cat} = 0.006 min^{-1} (ref. 1)).

The procedure was initiated by randomly mutagenizing 140 nucleotide positions that encompass the catalytic center of the ribozyme at an error rate of 5% per position. Thus, the initial population of 10^{13} molecules included all possible 1-, 2-, 3-, and 4-error mutants, and a sampling of the higher-error mutants. The population was challenged to cleave a target DNA substrate, which was presented at 10 μM concentration for a period of 1 hour. Individuals that cleaved the DNA became tagged with the 3′ portion of the substrate and were selectively amplified, subject to mutational error, to produce progeny molecules. Over successive generations, DNA cleavage activity for the population as a whole improved progressively.

Unlike darwinian evolution in nature, which is an accomplished fact on the human time scale, darwinian evolution in the laboratory operates over a period of a few weeks. Individuals can be isolated from the population at any generation, past or present, and clonally expanded to provide material for genetic analysis and characterization of functional properties. Acquisition of RNA-catalyzed DNA cleavage activity, for example, is the result of specific mutations in a region of the ribozyme that binds and co-ordinates the guanosine nucleophile and in a region that allows ribozyme-bound DNA substrate to dock in close proximity to the nucleophile (ref. 1). In retrospect, these mutations appear sensible, but *a priori* they could not have been predicted. Such situations are ideal for the application of laboratory evolution technology, where the guiding principle is "blind wisdom" rather than "rational design".

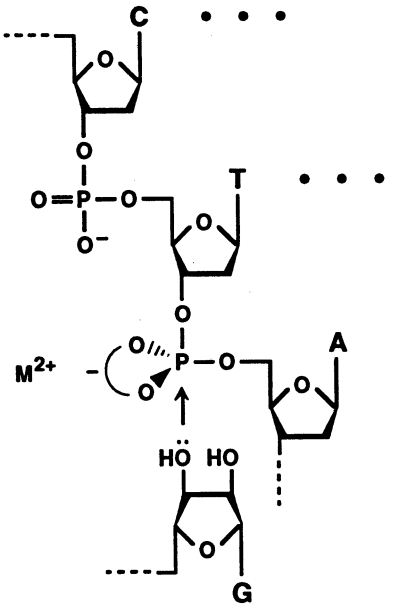

Fig. 1. Mechanism of RNA-catalyzed DNA cleavage, involving nucleophilic attack by the 3′-hydroxyl of ribozyme-bound guanosine at a target phosphodiester within the DNA substrate. The reaction proceeds via an $S_N2(P)$ mechanism involving a trigonal bipyramidal transition state. The negative charge on the phosphoryl oxygens is co-ordinated by a divalent metal cation. The portion of the substrate that precedes the cleavage site is bound to the ribozyme via base-pairing interactions (dotted lines).

The same selection strategy that led to the development of DNA-cleaving ribozymes was also used to change the metal dependence of a ribozyme (ref. 2). Group I ribozymes are catalytically active in the presence of Mg^{2+} of Mn^{2+}, but not Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Na^{+}, or K^{+} (ref. 47). However, Ca^{2+} and Sr^{2+} reduce the Mg^{2+} requirement of the ribozyme and promote folding of the ribozyme into its proper tertiary structure. Based on a Hill analysis, there appear to be four metal ion binding sites in the ribozyme, of which three are required for structural stabilization and one is required for catalysis (ref. 48).

A population of 10^{13} ribozyme variants was challenged to cleave a target RNA substrate in the presence of 10 mM $CaCl_2$ alone (pH 7.5, 37 °C). After eight generations, the evolved individuals acquired this activity, though still operating less efficiently in the presence of 10 mM $CaCl_2$ than the wild type does in the presence of 10 mM $MgCl_2$ (ref. 2). Ca^{2+}-dependent cleavage activity is attributable to a set of five specific mutations that accumulate within the catalytic center of the ribozyme. These mutations are different from those that result in RNA-catalyzed DNA cleavage activity. While it is likely that the Ca^{2+}-dependent ribozymes have learned to utilize Ca^{2+} rather than Mg^{2+} at the catalytic site, we cannot exclude the possibility that they have learned to do without a metal at the catalytic site, though still relying on Ca^{2+} for structural stabilization. It is also possible that the ribozymes have learned to scavenge minute amounts of Mg^{2+} and Mn^{2+} that exist as contaminants in the 10 mM $CaCl_2$ solution. This is unlikely because Mg and

Mn are present at concentrations of only 34 nM and 2 nM, respectively, as determined by inductively-coupled plasma emission spectrometry (ref. 2).

COUPLED RNA AMPLIFICATION AND TRANSLATION

Cell-free translation of synthetic mRNAs is relatively straightforward. The eukaryotic translation machinery can be obtained as a wheat germ extract or rabbit reticulocyte lysate (ref. 49,50), both of which are available from commercial sources. A coupled transcription-translation system prepared from *E. coli* is also available (ref. 51). We tested each of these systems in an attempt to find reaction conditions that enable *in vitro* translation and isothermal RNA amplification to proceed simultaneously. This would allow autocatalytic amplification of a messenger RNA (mRNA) that in turn is translated to protein (Fig. 2). If the system is designed such that the functional protein product is necessary for RNA amplification, then the protein is placed under selection pressure and becomes the target of laboratory evolution.

The problem of coupling isothermal RNA amplification and *in vitro* translation turned out to be more difficult than we had anticipated. The RNA amplification system contains 16 components (input RNA, two synthetic DNA primers, four deoxyribonucleoside triphosphates, four ribonucleoside triphosphates, reverse transcriptase, T7 RNA polymerase, $MgCl_2$, dithiothreitol, and Tris buffer) and operates within a narrow range of temperature, pH, Mg^{2+} concentration, and overall ionic strength (ref. 21). Likewise, the *in vitro* translation systems contain many components (input RNA, intact ribosomes, tRNAs, amino acids, aminoacyl synthetases, initiation factors, elongation factors, $Mg(Ac)_2$, KAc, creatine phosphate, creatine phosphokinase, hemin, dithiothreitol, and Tris buffer) and operate within a narrow range of reaction conditions (ref. 52). For the wheat germ extract and *E. coli* extract translation systems, we were unable to find reaction conditions that are compatible with isothermal RNA amplification. However, working with a commercial preparation of rabbit reticulocyte lysate (TNT™, Promega), we are able to operate isothermal RNA amplification and *in vitro* translation simultaneously.

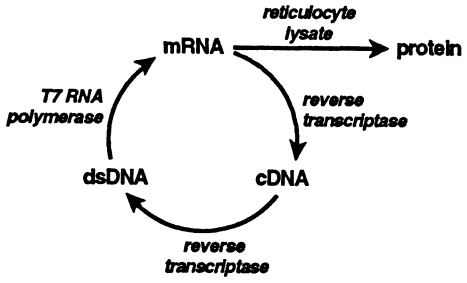

Fig. 2. Coupled isothermal RNA amplification and *in vitro* translation. The mRNA is amplified autocatalytically by the combination of reverse transcriptase and T7 RNA polymerase and is simultaneously translated to protein. See text for reaction conditions.

The reaction conditions for coupled RNA amplification and translation are as follows: 0.01 - 1 pmol input mRNA, 1 μM (each) oligodeoxynucleotide primers, 0.2 mM (each) dNTPs, 2.4 mM (each) NTPs, 4 $U/\mu l$ Moloney murine leukemia virus (MoMLV) reverse transcriptase, 5 $U/\mu l$ T7 RNA polymerase, 1 mM (each) amino acids, 7.5 - 10.0 mM $MgCl_2$, 3 mM KAc, 0.2 mM spermidine, 5 mM dithiothreitol, 40 mM Tris•HCl (pH 7.5), and 50% (v:v) Promega TNT™ rabbit reticulocyte lysate, incubated at 37 °C for 2 hours. Under these conditions, isothermal RNA amplification proceeds nearly as well as in the absence of amino acids and reticulocyte lysate, as judged by the incorporation of [α-^{32}P] GTP into newly-synthesized RNA (Fig. 3). The yield of full-length RNA is lower at 7.5 mM than at 10.0 mM $MgCl_2$, and drops precipitously below 7.5 mM $MgCl_2$. *In vitro* translation of RNA, in this case the genomic RNA of brome mosaic virus, proceeds under the same set of reaction conditions, as judged by the incorporation of [^{35}S] methionine into newly-synthesized protein (Fig. 4). The yield of full-length protein is somewhat lower under these conditions than at 0.5 mM $MgCl_2$ and in the absence of the components required for RNA amplification.

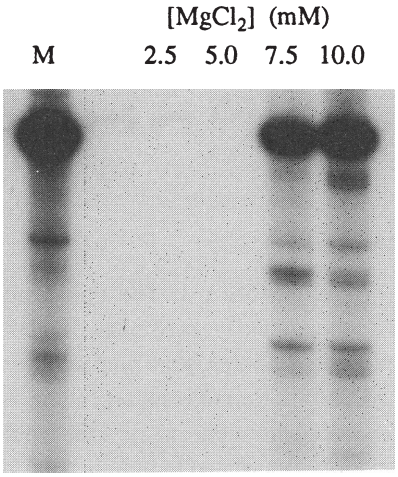

Fig. 3. Isothermal amplification of a 393-nucleotide RNA in the presence of varying concentrations of $MgCl_2$, under reaction conditions as described in the text. Marker lane (M) shows products obtained at 10 mM $MgCl_2$ in the absence of amino acids and reticulocyte lysate. Reaction products were separated in a 5% polyacrylamide / 8 M urea gel, an autoradiogram of which is shown.

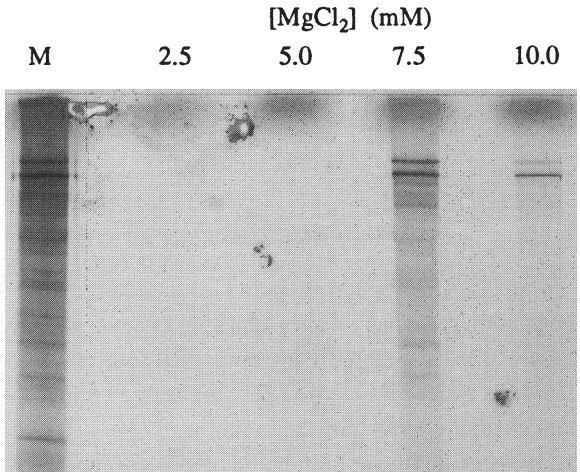

Fig. 4. *In vitro* translation of brome mosaic virus genomic RNA in the presence of varying concentrations of $MgCl_2$, under reaction conditions as described in the text. Marker lane (M) shows products obtained at 0.5 mM $MgCl_2$ in the absence of oligodeoxynucleotide primers, dNTPs, NTPs, MoMLV reverse transcriptase, and T7 RNA polymerase. Reaction products were separated in a SDS / 10% polyacrylamide stacking gel, an autoradiogram of which is shown.

It is somewhat surprising that translation does not occur below 7.5 mM $MgCl_2$ in the coupled reaction system, even though *in vitro* translation alone operates efficiently at 0.5 mM $MgCl_2$. This apparent discrepancy may be due to complexation of Mg^{2+} with deoxyribo- and ribonucleoside triphosphates that are present in the coupled reaction system. Such complexation could substantially reduce the available amount of Mg^{2+}. Because of the highly multivariate character of the coupled reaction system, it is possible that there is some entirely different set of reaction conditions that we have not tested that allows simultaneous RNA amplification and translation, perhaps over a broader range of $MgCl_2$ concentration. We are content, at present, to operate under the conditions described above.

As one begins to think about the laboratory evolution of protein enzymes, several targets immediately come to mind. The most obvious would be to employ a mRNA that encodes MoMLV reverse transcriptase, so that the product of translation is a protein that is required for RNA amplification. Reverse transcriptase is a special case because the completed protein has preferential access to its corresponding mRNA, the two being held together within the same translation complex. The enzyme has the opportunity to convert its own mRNA to complementary DNA, thereby promoting autocatalytic amplification of its own genetic information. There would be strong selection pressure favoring the development of a variant form of reverse transcriptase that best recognizes and amplifies its own mRNA.

Perhaps more interesting would be to employ a dicistronic mRNA that encodes both MoMLV reverse transcriptase and T7 RNA polymerase. In this case the RNA would contain all of the genetic information necessary for its own replication — in a sense, the system would operate as an artificial retrovirus. But unless there is some way to ensure co-localization of T7 RNA polymerase and its corresponding mRNA, it is difficult to see why the polymerase should operate exclusively to assist in the amplification of its own genetic information. So too, any protein enzyme that has a "biosynthetic" function, for example an NTP synthetase, would operate for the benefit of all individuals in the population unless there is some way to achieve microcompartmentalization. The problem of co-localization of gene and gene

product, which was solved in nature over 3.5 billion years ago (ref. 53), remains to be solved for evolution in the laboratory.

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