Construction of nuclease resistant ribonucleotides by molecular evolution

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The molecular evolution by replicase (RNA-dependent RNA polymerase) was attempted for generating new nuclease-resistant RNAs, which realizes a cell free Darwinian molecular evolution. The Q β replicase replicates genome RNA of Q β virus, and additionally it can also synthesize other small RNAs using "minivariant" as a template. With the aid of Spiegelman's serial transfer method, which is a step by step molecular evolution system in a laboratory by RNA proliferation with frequent mutation using Q β replicase, high concentration ribonuclease T2-resistant fragments of RNA could be obtained.

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Recently, so called "Evolutionary Molecular Technology" proposed in 1984 by Eigen has been getting up, which is a novel paradigm including technologies of generating new functional biological macromolecules with the aid of evolving these molecules¹⁾.

As Eigen intimates, this new technology is mimicking molecular evolution in the laboratory, which furnish investigators with a powerful tool for a novel functionally biological macromolecules as drugs and so on in the near future. Of course there is much possibility that this concept will offer an attractive route to develop a novel molecules for drug delivery

Fig. 1 Darwinian evolution

systems. In biological macromolecules, nucleotides are much easily mutated than proteins. Recognizing the properties of DNA and RNA, there is much difference of their available structures. DNAs can usually uniform only a simple double helix structure. On the other hand, single-strand RNA is able to fold to construct a much variety of steric structure which leads the expression of molecular functions. Self-replicating RNAs replicated by Q β replicase which is a RNA-dependent RNA polymerase produced by bacteriophage Q β offer an adaptive *in vitro* system to investigate the molecular evolution^{2~4)}. This report deals with a construction of nuclease resistant ribonucleotides using molecular evolution with the aid of Q β replicase.

Evolutionary molecular technology

In various theories of molecular evolution, Darwinian evolution is frequently chosen for laboratory molecular evolution⁵⁾. The characteristics of Darwinian principle is a gradually rapid evolution. This theory fundamentally consists of refrained three ingredients which

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Fig. 2 Serial transfer method

A series of test chamber is filled with Q β replicase, RNA template, magnesium ion, ATP, GTP, UTP and CTP and RNase T2. Only first incubation chamber contains no RNase T2. A little bit of incubated fluid is transferred to next chamber which contains higher level of RNase and this cycle is repeated until 50 th generation of which chamber contains high level of RNase.

mean first selection of the fitness, secondly a multiplication of the selected and thirdly, a mutation for making variations. Last step of mutation succeeds the first step, that is, selection step of next cycle (Fig. 1). In these cycles genetic informations are evolved, and it leads a production of evolved biological macromolecules. Selection is a process distinguishing molecules which can clear natural or artificial barrier for survival. Multiplication is a step of generating progenies, strictly speaking, it is a process of making copies of inherited genes. Mutation leads the population to that of variations, which is indispensable for evolutionary development, because selection system can only function with variations.

The first successful trial of Darwinian evolution in a laboratory was done by Spiegelman et al⁶⁾. The virus Q β ⁷⁾ infecting *Escherichia coli* provides a experimental system in a laboratory for molecular self-replication. This virus possess a single-strand genome RNA about 4300 nucleotides. It's some region codes so called replicase which is a RNA polymerase dependent on RNA. A replicase has a function of making copies of virus genome RNA, and it is

essential for living of virus as well as its proliferation. Replicase makes much error than DNA polymerase, and this is very advantage for generation of mutants. Therefore, proliferation process of Q β RNA by Q β replicase possesses a included mechanism of mutant genesis inside^{8,9)}. According to the characteristics of selection, multiplication and mutation in Q β bacteriophage system, Spiegelman constructed a so called serial transfer experiment⁶⁾.

Fig. 2 demonstrates the principle of the serial transfer method. A typical experiment starts with a solution that includes magnesium ions, template Q β RNA, a purified Q β replicase and ATP, UTP, GTP and CTP. Spiegelman shortened time period which is available for Q β RNA proliferation. In this case shortened time for multiplication is a selection pressure in evolution cycle.

Various factors can be chosen as a selection pressure in the molecular evolution with the aid of serial transfer method. In this study, ribonuclease T2 (RNase T2) was chosen as a selection pressure in order to develop a nuclease resistant RNAs by molecular evolution using serial transfer method.

Fig. 3 Genesis of RNAs chains in second incubation chamber

A level of RNase T2 in second chamber fluid ;
(a) without RNase, (b) (c) tolerable level, (d) intolerable level.

Experimental

(1) Materials

Nucleoside triphosphates were purchased from Boehringer Mannheim, acrylamide and bisacrylamide were from Pharmacia. Q β replicase was isolated from Q β bacteriophage-infected *Escherichia coli* Q13 by the procedure of Eoyang and August¹⁰. RNase T2 was purchased from U. S. Biochemical Corp. Silver Stain Plus was purchased from Bio-Rad.

(2) Synthesis of Minivaliant Template and Separation

RNA (MDV-1 RNA) was synthesized according to the procedure by August et al.¹¹; in 1 ml reaction in which the final level of components were 12 mM MgCl₂, 84 mM Tris HCl (pH 7.4), 400 μ M ribonucleoside triphosphates, 50~500 pg/ml MDV-1 RNA and 40~160 μ g/ml Q β replicase. Reactions were incubated at 37°C. Synthesized MDV-1 RNA was isolated according to the method by Kramer et al.¹².

(3) Synthesis of evolved fragments of ribonucleotides

With the aid of Q β replicase system, evolution of RNAs was performed as followings¹³; a 200 μ l reaction solution including 10 ng MDV-1(+) RNA template, 15 μ g Q β replicase, 400 μ M (each) ATP, UTP, GTP and CTP, 84 mM Tris HCl and 12 mM MgCl₂ was incubated at

37°C for 20 min. In first incubation, no RNase T2 was added, however, second tube contained 1 pg/ml RNase T2, increasing its level up to 5 μ g/ml in last incubation.

The reaction was terminated by the addition of 500 μ l of a solution containing 100 mM EDTA, 400 mM NaCl and 1 mg/ml SDS. These synthesized RNA fragments were fractionated on 10 % polyacrylamide slab gels containing 7 M urea. The RNA was suspended with xylene cyanol and bromphenol blue and was layered over the gel. Electrophoresis was carried out at 250 V and separated bands were located by silver stain method¹⁴.

(4) Kinetics of proliferation of RNA fragments

The RNA fragment of each solution which was periodically sampled was isolated and analyzed by electrophoresis under denaturing conditions as above described. Strength of stained area on the gel was scanned and relative concentration was calibrated by standard oligoribonucleotides.

Results and Discussion

Fig. 3 shows the typical proliferation pattern of RNA fragments in second incubation chamber which was synthesized by Q β replicase with or without RNase T2. When there is no RNase in the incubation tube, synthesis of RNA started immediately from the template MDV-1(+) RNA as well as in first incubation chamber which contains no RNase as demonstrated in Fig. 3a. Although, an adequate low concentration of RNase T2 delayed the appearance of RNA fragments, their decreased proliferation rate can be seen (Fig. 3b • c). A too much level of RNase which was intolerable for RNAs in a chamber easily destroyed all the RNA including template, and no multiplication was not observed as shown in Fig. 3d. Selecting an appropriate higher level of RNase in next chamber which increases slowly step by step and repeat-

Fig. 4 Proliferation rate of RNase-Sensitive and -Resistant RNAs

ing this procedure enable a genesis of resistant RNAs variants against high level of RNase. At 50th generation of incubation, RNA fragments which could not be digested by 5 $\mu\text{g}/\text{ml}$ RNase T2 were obtained.

A comparative proliferation kinetics of the 50th variants replicated with or without RNase T2 were indicated in Fig.4. It will be noted that the resistant variants indicate the stable proliferation in incubation medium containing high level (5 $\mu\text{g}/\text{ml}$) of RNase T2, nevertheless lower slope of linear portion of its synthesis and much lag time until its first appearance.

Although, variant of 50th generation incubated without RNase throughout all the cycles showed a much faster proliferation speed, it was entirely digested by such a high concentration RNase T2. Therefore, it is considered that these former mutants at 50th generation accepted the lower amplification rate in compensation for getting a nuclease resistant ability. RNase T2 selectively cleaves the phosphodiester bond at 3' site of adenylic acid residue of RNAs¹⁵. Although there is no data on sequence analysis of obtained RNase T2 resistant RNAs in this study, it should be that these resistant variants possess very less cleavage sites. And when there are a few cleavage sites

in the resistant variants, there is a possibility that these sites should be hidden inside by their configuration. In this case, protection way from the nuclease digestion by configuration change of macromolecules might also be evolved by the serial transfer method.

The observations in this study elucidate that serial transfer method is a useful way for evolving RNAs to provide a certain new function to these macromolecules and this technology might be the platform for studying evolutionary dynamics of biological macromolecules such as nucleotides.

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