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Enzymatic RNA replication in self-reproducing vesicles: an approach to a minimal cell

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Summary: The replication of a RNA template catalyzed by Qß replicase was obtained in oleic acid/oleate vesicles simultaneously with the self-reproduction of the vesicles themselves. This was accomplished by entrapping the enzyme Qß replicase, the RNA template, and the ribonucleotides ATP, CTP, GTP, and UTP inside the vesicles. The water-insoluble oleic anhydride was then added externally. It binds to the vesicle bilayer where it is catalytically hydrolyzed yielding the carboxylate surfactant *in situ*, which then brings about growth and reproduction of the vesicles themselves. This experiment is presented as a first approach to a synthetic minimal cell, in which the reproduction of the membrane and the replication of the internalized RNA molecules proceed simultaneously.

Template-based, enzyme-free self-replication of short nucleotides (1,2), of nucleotide-analogs (3) or more recently of longer DNA sequences (4) has been developed under the underlying hypothesis that this is relevant for modeling the basic mechanisms of life. Eventually however in any project aimed at the synthetic rendering of the first forms of life, the compartmentation must be brought in, so as to mimick the cell as the fundamental expression of minimal life. It has been argued for some time that vesicles and liposomes are likely to be the first forms of shell in prebiotic cell structures (5,6) and more recently, the self-reproduction of micelles and vesicles has also been reported as a model for the shell reproduction of prebiotic cells (7,8). These considerations define what must be the next step in the design of a synthetic minimal life structure: a system in which the self-replication of DNA or RNA takes place within a double-layered spherical boundary - and in which also the shell is self-reproducing, so as to have a simultaneous "core-and-shell" replication. The description of such a system is

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the aim of this paper. In particular we will describe a system in which oleic acid/oleate vesicles host the enzyme Qß replicase, and which are able to self-reproduce while the enzyme replicates a RNA template.

Let us consider in turn the two main components of our system, namely the Qß replicase and the oleic acid/oleate vesicles.

Since the original experiments by Spiegelman *et al.* (9,10), Qß replicase has been widely used to investigate the process of replication and *in vitro* evolution of short ribonucleic acid chains (11,12). In this work we have used the so-called midi variant RNA (MDV-1 RNA) (13,14), a replicating RNA species originally isolated by Spiegelman and coworkers (9).

As far as the vesicles are concerned, it was shown by Hargraves and Deamer (15) and several other groups (16,17), that long-chain fatty acids are able to form vesicles in water depending on the pH. In particular, vesicles are formed when the pH is equal to the pK of the acid in the bilayer (so that an equimolar mixture of oleic acid/oleate results). The process of autopoietic self-reproduction (18) of caprylic acid/caprylate and oleic acid/oleate vesicles is discussed in detail elsewhere (19 - 21), but it is necessary to describe here the conditions of a typical experiment. Generally we start from a buffered water solution containing preformed vesicles, overlaid with a small amount of neat water insoluble oleic anhydride. Under those conditions, a significant increase of the hydrolysis rate of the anhydride can be observed with respect to a reference system which does not contain vesicles in the water phase (21). Since this increase of number resp. size of vesicles is due to an autocatalytic reaction taking place within the boundary of the parent structures, it meets the definition of autopoiesis (23 - 26).

MATERIALS AND METHODS

Materials: Sodium oleate was purchased from Fluka, oleic anhydride was from Sigma, ribonucleotides were from Pharmacia, [^{35}S]ATPαS (> 1000 Ci/mmol) was from Amersham, Qß replicase (27) and MDV-1 RNA (13) were purified as previously described.

Self-reproduction of oleic acid/oleate vesicles: A 20 mM oleic acid/oleate suspension (prepared by dispersing an oleate film in 200 mM BICINE buffer (pH 8.5) at room temperature) was overlaid with 0.015 Vol of neat oleic anhydride and allowed to react at 40°C. Aliquots were taken at given time intervals and diluted with 2.5 Vol 1 N HCl. The water-insoluble oleic acid was then extracted with 7.5 Vol isooctane and the concentration of oleic acid in the organic phase was analyzed by Fourier transform infrared (FTIR) spectroscopy on a Nicolet 5SXC FTIR spectrophotometer using a CaF_2 cell.

Statistical analysis of the mean size and size distribution of vesicles after performing a self-reproduction experiment: A 20 mM oleic acid/oleate

suspension (prepared as described above) was forced through two polycarbonate filters with pores of 200 nm, overlaid with 0.015 Vol of neat oleic anhydride and allowed to react at room temperature for 8 h. The samples were frozen and freeze fracture electron microscopy was carried out (28). At the beginning (0 h) and after 8 h 25 electron micrographs (about 2000 vesicles at each time point) were analyzed.

Time-dependent MDV-1 RNA replication within oleic acid/oleate vesicles: A soldium oleate film was produced and dissolved at room temperature in 200 mM BICINE buffer (pH 8.5) containing 200 nM Qß replicase, 200 nM MDV-1 RNA, 500 µM CTP, GTP, and UTP each, 250 µM ATP, 25 µCi [^{35}S]ATPαS, 4 mM $MgCl_2$, and 0.25 mM DTT so that the final surfactant concentration was 50 mM. After having formed the lipid aggregates by shaking for exactly 30 sec, EDTA was added to a final concentration of 10 mM and the resulting suspension was forced through two polycarbonate filters with pore size of 200 nm using a Liposofast extruder (both from Avestin). Then the liposomes were separated from the external solutes by gel filtration column chromatography using a Bio-Gel A-15m column (29). The reaction mixture was overlaid with 0.015 Vol oleic anhydride and allowed to react at 37°C, then aliquots were taken at given time points (t = 0 was 20 min ater having prepared the liposomes) and the concentration of oleic acid/oleate was determined as described above. From other aliquots the MDV-1 RNA was isolated by phenol and chloroform extraction followed by ethanol precipitation, the RNA was analyzed by native PAGE (20% homogenous gel, PhastSystem from Pharmacia) and visualized on a PhosphorImager (Molecular Dynamics). Note that RNA replication by Qß replicase occurs at room temperature and therefore starts after having mixed all ingredients. Qß activity is totally inhibited by an excess of EDTA.

RESULTS AND DISCUSSION

Figure 1A shows the complete time course of a typical self-reproduction experiment of oleic acid/oleate vesicles under the conditions of this work. Note that the conversion of the oleic anhydride to oleate was completed after about 8 h (for more details see also 20,21). Figure 1B shows the corresponding statistical analysis of the size distribution based on 25 electron micrographs (with about 2000 vesicles) before and after the self-reproduction reaction (0 resp. 8 h). Note the significant increase of the smaller sized vesicles (of about 100 nm in diameter), which is attended by the presence of a smaller amount of large aggregates (from 200 to 750 nm in diameter). If we consider the overall increase of the vesicle number (of about 10 - 20%) with their size we obtain a good correspondence with the chemical yield increase of oleic acid/oleate concentration (of a factor of almost 4, see Figure 1A).

The RNA replication was carried out in oleic acid/oleate vesicles under conditions of vesicle self-reproduction, i.e. in the presence of overlaid water insoluble oleic anhydride. All reagents were entrapped inside the aggregates from the very start, so that the aqueous interior of the vesicles contained the enzyme Qß replicase, the MDV-1 RNA template, the substrates ATP, [^{35}S]ATP, CTP, GTP, and UTP, buffer and Mg^{2+}-ions. In particular,

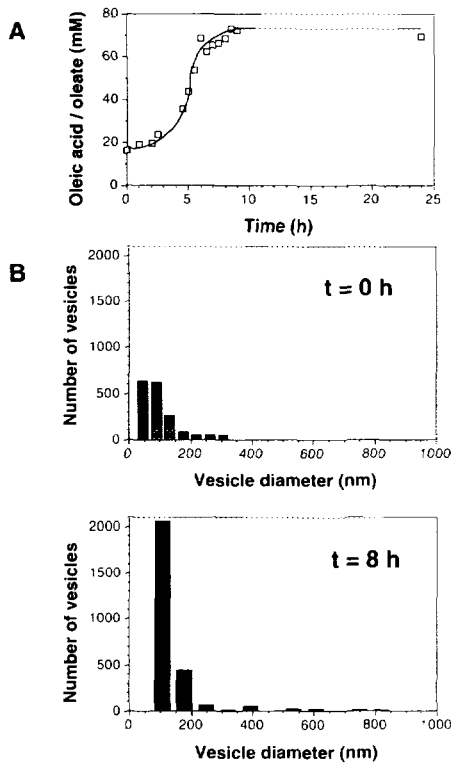

Figure 1. A: Self-reproduction of oleic acid/oleate vesicles. Time course of oleic anhydride hydrolysis measured by FTIR spectroscopy. B: Statistical analysis of the mean size and size distribution of vesicles after performing a self-reproduction experiment.

liposomes were prepared by first dispersing an oleate film in an aqueous buffered solution containing the whole reaction mixture. After shaking for 30 sec, EDTA was immediately added to the cloudy suspension of liposomes in order to inactivate the non-entrapped enzyme. This suspension was then extruded and liposomes were separated by gel filtration chromatography from non-entrapped material. After a given incubation time, the vesicles were destroyed by phenol and chloroform extraction and the newly synthesized, ^{35}S-labelled MDV-1 RNA was isolated and analyzed. The reaction has been followed up to six hours, and results are shown in Figure 2.

The fact that the so produced ^{35}S-labelled RNA was synthesized inside the vesicles and not outside has been established on the basis of the following observations: i) the presence of EDTA in the external bulk medium inhibits the enzyme totally ii) after incubation at 37°C for 2 h, the liposomal suspension was treated with pancreatic ribonuclease A, and nevertheless ^{35}S-labelled RNA could still be detected (data not shown) iii) the pooled chromatographed vesicle fractions showed a clear time dependence of RNA

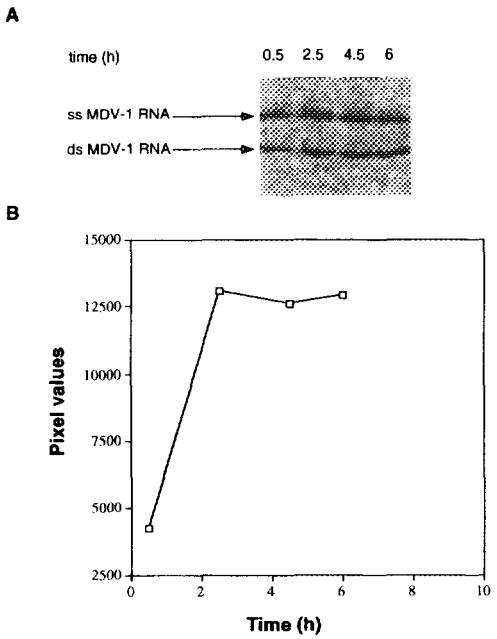

Figure 2. Time-dependent MDV-1 RNA replication within oleic acid/oleate vesicles (ss = single stranded, ds = double stranded). After indicated time intervals, aliquots were taken, analyzed by native PAGE and visualized (A) / quantified (B) using a PhosphorImager.

formation (Figure 2), i.e., the RNA synthesis keeps going on once the vesicles are separated from the original external medium.

We have operated under conditions of excess of Qß replicase/RNA template over the number of vesicles (the calculated ratio at the beginning is about 3:1) so that, at least in principle, the replication of RNA can proceed for a few generations of vesicles (a larger excess of enzyme/template is technically not possible at this stage). The increase of the surfactant concentration following the hydrolysis of oleic anhydride was determined by Fourier transform infrared (=FTIR) spectroscopy, simultaneously with the detection of the ^{35}S-labelled RNA as described above. In these experiments the oleic acid/oleate concentration increases from the initial 19 mM to a final value of 74 mM (Figure 1A), while MDV-1 RNA is synthesized simultaneously inside the vesicles (as shown in Figure 2).

These processes can be pictorially illustrated as shown in Figure 3. This drawing should not be taken too literally, as the nature of the intermediates and the values of the equation coefficients have not yet been elucidated. In addition, the system, as it is shown here, presents some limitations which must be overcome in the next phase of the work: first of all, we are dealing

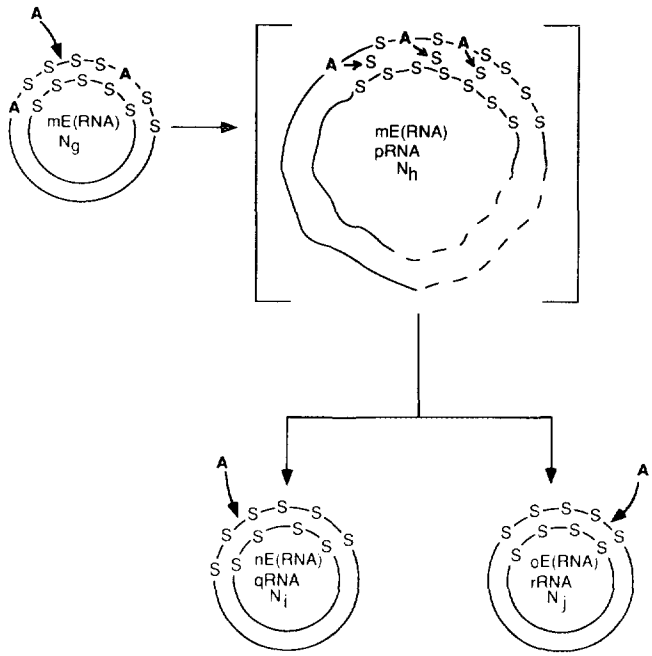

Figure 3. Schematic illustration showing the simultaneous reproduction of vesicles and enzymatic replication of nucleic acids. S stands for the surfactant, A for the oleic anhydride, E(RNA) for the Qß-RNA complex, and N for the ribonucleotides.

with an enzymatic RNA replication rather than with a self-replication. Furthermore, the two processes of "core-and-shell" replication proceed at this stage independently from one another (replication of the RNA does neither enhance "cellular division" nor does the RNA contain any information regarding the shell; all information it has is used for directing its own replication).

On the other hand, some positive points can be extracted from the work as it stands now. We could show that it is possible to realize under normal laboratory conditions a compartimentalized RNA replication system which is able to self-reproduce simultaneously. Since the reproduction of the boundary is due to a reaction which takes place within the boundary and is catalytically induced by the boundary itself, the process can be defined as autopoietic. Notice also that the boundary acts as a chemically reactive species capable of binding and catalysis. This may suggest that also in the evolution of the first minimal life forms the boundaries may have performed much more than a simple protective shell function. More in general, by combining the RNA-replication with the principles of autopoiesis, we have operated a bridge between the two more accepted views on the theory of minimal life, the one

based on the "RNA-world" (30 - 32) and the other based on the cellular autopoietic view. This has interesting implications for the work currently going on in which the self-replication of RNA is seen as the basic event leading to mutation and evolution. It will then be possible to carry out these experiments under the constraints of a growing bilayered compartment, which will add a new dimension to the simulation of laboratory evolution.

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18. Following a recent proposal (22), we will use the term **self-replication** for linear information sequences which replicate according to a template-based process. The term **self-reproduction** is a more general one, valid for all other structures (e.g. micelles and vesicles). The term **autopoietic self-reproduction** refers to a process taking place from within the spherical boundary of the structure itself.
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