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REVIEW

Alexander Spirin on Molecular Machines and Origin of Life

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Abstract—Once it was believed that ribosomal RNA encodes proteins, and GTP hydrolysis supplies the energy for protein synthesis. Everything has changed, when Alexander Spirin joined the science. It turned out that proteins are encoded by a completely different RNA, and GTP hydrolysis only accelerates the process already provided with energy. It was Spirin who first put forward the idea of a Brownian ratchet and explained how and why molecular machines could arise in the RNA world.

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INTRODUCTION

For all the apparent inconsistency of the topics indicated in the title, there is one principal character in the story – the ribosome, the main ribozyme of the Nature and the main passion of Alexander Sergeevich Spirin. In fact, he devoted to it his entire life in science, in order to find out how it is built, how it works and how it originated. One of his first papers [1] indicated that the ribosomal RNA, which makes up the bulk RNA of the cell, does not encode proteins, as was then thought, but plays a structural role. His last scientific publication, which came out 61 years later [2], is also devoted to the ribosome.

Studying the structure of ribosomes had helped A. S. Spirin to formulate the hypothesis that the ribosome is a machine that works due to a mutual mobility of its two subparticles. In order to figure out which "engine" sets the subparticles in motion, how it works, and what "fuels" it, he concerned himself with the general principles of functioning of the cellular mechanochemical systems. And when his long-cherished idea that it is the ribosomal RNA that catalyzes protein synthesis had been confirmed, he wanted to understand how such a machine could have arisen in the RNA world. This resulted in a holistic concept which I try to present here.

IN THE BEGINNING WAS RNA

Spirin started his scientific career under the guidance of Andrei Nikolaevich Belozersky by comparing nucleotide compositions of RNA and DNA from bacteria. This happened in 1954, a year after the publication of articles by J. Watson and F. Crick on the structure of DNA and its biological meaning [3, 4], which laid the foundation for molecular biology. As Alexander Sergeevich wrote [5], he was at that time a graduate student at the A. N. Bach Institute of Biochemistry of the USSR Academy of Sciences, but actually worked in the new building of the Biological (then – Biology and Soil) Faculty of the M. V. Lomonosov Moscow State University on the Lenin Hills, which was officially opened on September 1, 1954 [6].

Among his papers published at that time, one that appeared in 1957 in *Biochemistry (Moscow)* [1] stands out. In the review "Ab ovo usque ad mala" ("From the beginning to the end"), dedicated to the 50th anniversary of its publication [7], Alexander Sergeevich noted that this article gave rise to three new branches of molecular biology – **genosystematics**, **the study of mRNA** (informational, or messenger RNA), and, finally, **the study of noncoding RNA**. Nucleotide composition of the total RNA in organisms having highly different nucleotide composition of DNA was presented in this paper for the first time. Such attempts have been made earlier, but they were not successful due to the fact that the losses of RNA because of its degradation by ribonucleases during the isolation greatly distorted the results. Here, RNA was not isolated, but subjected to alkaline hydrolysis directly in the washed bacterial cells, which excluded errors caused by the RNA losses. A new method was also developed that allowed all four bases to be seen on one chromatogram, which improved the accuracy of determination of their relative content. It turned out that, although the ratio

Abbreviations: Aa, amino acid, aminoacyl.

(G + C)/(A + T) in the DNA of nineteen studied bacterial species differed more than 6-fold, the ratio (G + C)/(A + U) in their RNA differed slightly more than by 20%.

The significance of this result for molecular biology can be appreciated if considered from the point of view of the Crick's seminal paper "On Protein Synthesis" published in 1958 [8], where he for the first time presented the central dogma of molecular biology ("the transfer of information from nucleic acid to nucleic acid, or from nucleic acid to protein may be possible, but transfer from protein to protein, or from protein to nucleic acid is impossible") and the adapter hypothesis (an amino acid is carried to the template by an 'adaptor' molecule joined to that amino acid and containing 2-3 nucleotides complementary to the template). Along with these ingenious predictions, Crick put forward one more hypothesis – on the nature of "microsomal particles" (named "ribosomes" the same year [9]), consisting of RNA and protein. It was already known that these particles contain the bulk of cellular RNA and serve as sites for protein synthesis. According to Crick, the ribosomes resemble spherical RNA viruses; inside each there is a DNA-encoded RNA template that directs the synthesis of a specific protein, so there are as many different ribosomes in the cell as are different genes and different proteins. Accordingly, there must be a close correlation between the nucleotide compositions of cellular DNA and RNA. Therefore, the result published in *Biochemistry (Moscow)* [1] was totally unexpected: it refuted the postulate that ribosomal RNAs serve as templates for protein synthesis. Crick could not read the article published in Russian. However, he immediately responded to its main conclusions published next year in *Nature* [10], by characterizing the phase initiated by this publication as "confused" [11].

In fact, the articles in *Biochemistry (Moscow)* [1] and *Nature* [10] did clarify the situation. They indicated that RNAs that encode proteins and match the nucleotide composition of DNA do exist, but constitute just a small fraction of the total cellular RNA and are not contained in the isolated ribosomes; now they are known as mRNAs [12]. The bulk of cellular RNAs contained in the ribosomes is phylogenetically conserved and does not serve as a template for protein synthesis. Thus, this article, which formed the basis of the Spirin's PhD Thesis [13], became the first indication of the existence of both the coding mRNAs and the class of non-coding RNAs, which includes the ribosomal RNAs.

THE RIBOSOME STRUCTURE IS MAINTAINED BY RNA

Next A. S. Spirin began to study physical properties of high molecular weight RNAs. This work was carried out mainly in the Laboratory of Chemistry and Biochemistry of Nucleic Acids at the A. N. Bach Institute of Biochemistry, Spirin was appointed as the Head of this laboratory after he received the Doctor of Science degree in 1962 [14].

Since the noncoding ribosomal RNAs turned out to be the major RNA species of the cell, they attracted the greatest Spirin's attention [5]. However, he began with a study of the genomic RNA of the tobacco mosaic virus (TMV) because it could easily be isolated in large amounts*; in addition, its integrity could unambiguously be proven by the infectivity test [15]. It was the studies of this RNA that resulted in the discovery of hidden internal breaks in the apparently intact high molecular weight RNA.

A mysterious property was known for TMV-RNA: unlike the RNA within the viral particle, the isolated RNA quickly lost its infectivity during storage even under aseptic conditions; at the same time, according to all applied physical tests (electron microscopy, spectral studies, sedimentation, viscometry) the stored RNA did not differ from the freshly isolated one [15]. However, it turned out that these RNAs began to behave differently when heated above 50°C: viscosity of the infectious RNA increased, with the transition being observed in a narrow temperature range; on the contrary, viscosity of the RNA that had lost its infectivity, not only did not increase, but even dropped during heating. Since the decreased viscosity indicated reduced size of macromolecules, it was concluded that the "continuous single-stranded structure of native RNA becomes discontinuous" during storage [16].

A similar story happened to the ribosomal RNAs. B. D. Hall and P. Doty reported that heating of 18S (600 kDa) and 28S (1300 kDa) RNAs isolated from the calf liver ribosomes resulted in a drop of their viscosity accompanied by dissociation into fragments with molecular weights of about 120 kDa. The authors concluded that 18S and 28S RNA are built from 5 and 10 RNA subunits, respectively, although they made a reservation that there remains a possibility that these subunits emerge as a result of "hidden scissions" of the RNAs by ribonuclease [17].

The fact that such possibility is indeed realized was shown in the Spirin's group the next year [18]. To prevent degradation by ribonucleases, RNA was extracted by phenol directly from the homogenized tissue, rather than from the ribosomes. Viscosity of the isolated high molecular weight RNAs, which were of the same size as the ribosomal RNAs studied by Hall and Doty, did not

* One liter of juice squeezed out of tomato tops infected with TMV, yielded up to 1 g of pure preparation of the virus [15]. When, in 1974, Tanya Vlasik, Kolya Rutkevich, and I joined the Spirin's laboratory at the Institute of Protein Research, we were shown a large flask with the ammonium sulfate-precipitated TMV, which has been stored in a cold room.

decrease on heating, but, like that of the intact TMV RNA, increased sharply. As in the case of TMV RNA, the drop in viscosity reported by Hall and Doty was only observed upon storage of ribosomal preparations that was accompanied by RNA degradation. Later, formation of the hidden RNA scissions not leading to a visible damage in the ribosomes or their subparticles, was directly demonstrated [19].

Thus, Spirin and his colleagues had demonstrated that ribosomal RNAs comprise covalently continuous polynucleotide chains. They had also shown that the isolated ribosomal RNAs acquire a compact configuration in solutions with high ionic strength [20], that the 3D structure of RNA is supported by hydrogen and electrostatic interactions [21-23], and that, by lowering ionic strength of the solution in the absence of Mg^{2+} ions, the ribosomal subparticles can reversibly unfold into ribonucleoprotein strands to which ribosomal proteins are bound [24]. In particular, it followed that, under physiological conditions, the ribosomal RNAs form rather rigid frameworks that support the structures of the ribosomal subparticles. This conclusion was of a key importance for understanding the principles of structural organization of the ribosomes and, apparently, had helped A. S. Spirin to formulate his model of the working ribosome. This had happened after he became the director of the Institute of Protein Research of the USSR Academy of Sciences, founded by him in June 1967.

A CYCLICALLY OPERATING BIOCHEMICAL MACHINE

This is how the ribosome was named in the famous model of locking and unlocking of the ribosomal subparticles published in 1968 [25]. By that time, the Crick's adapter hypothesis [8] was entirely confirmed: it was found that amino acids for protein synthesis enter the ribosome in the form of Aa-tRNAs synthesized by enzymes aminoacyl-tRNA synthetases from tRNA and amino acids activated by ATP; the Aa-tRNAs contain anticodons complementary to three-nucleotide codons (triplets) of the ribosome-bound mRNA and serve as substrates for the synthesis of a polypeptide chain in the form of peptidyl-tRNA.

It was also found that the ribosome consists of two loosely connected subunits, with mRNA and Aa-tRNA interacting with the smaller subunit (30S in bacteria, 40S in eukaryotes), whereas peptidyl-tRNA interacts with the larger subunit (50S in bacteria, 60S in eukaryotes) harboring the peptidyl transferase site that catalyzes synthesis of the peptide bonds. This synthesis occurs by attacking the peptidyl-tRNA (its ester bond between the carboxyl group of the last amino acid residue and the 3′-hydroxyl group of the 3′-terminal nucleotide of tRNA) by the amino group of the new Aa-tRNA. As a result, the polypeptide chain extended by one amino acid residue becomes bound to the new tRNA (and, hence, to the small subunit), whereas the tRNA that has been a part of peptidyl-tRNA, becomes deacylated. After the peptide bond synthesis, there occurs an act of translocation, which includes a shift of the elongated peptidyl-tRNA from the small to the large subunit (to which it has a greater affinity), accompanied by the movement of mRNA by one codon and dissociation of the deacylated tRNA from the large subunit into the solution. Now the Aa-tRNA-binding site of the small subunit with a new codon installed in it is ready to accept the next Aa-tRNA. Thus, the ribosome works cyclically: each cycle begins with the binding of Aa-tRNA, continues with the synthesis of the next peptide bond, and ends with the translocation (a detailed description of these events, taking into account new data, can be found in the Spirin's textbook [26] and in his latest papers on this issue [2, 5, 27]).

Unlike conventional enzymes, the ribosome deals with large substrates (the masses of Aa-tRNA and peptidyl-tRNA are approximately two orders of magnitude greater than the masses of substrates of a standard biochemical reaction) and carries out a strictly ordered process: the ribosome reads the template from the 5′- to the 3′-end, and this determines the sequence of amino acids in the synthesized protein. The model of locking-unlocking of the ribosomal subparticles readily explains how these problems are solved. Locking of the subparticles brings the groups of Aa-tRNA and peptidyl-tRNA involved in the peptidyl transferase reaction in a close contact, while unlocking results in translocation of the template, which is dragged by the associated peptidyl-tRNA [25]. The main features of this model were later confirmed by the studies on crystal structure, cryoelectron microscopy, and dynamics of the ribosomes [2, 5, 27].

The same year a similar model was proposed by British scientist M. Bretcher [28], however, the Spirin's priority is obvious from comparison of the dates when the respective manuscripts were received in the editorial offices – January 29 [25] and April 26 [28]. During his speech at the conference dedicated to his 80th birthday, Alexander Sergeevich said that almost simultaneously with the Russian-language version of the paper submitted to *Doklady AN SSSR* (Reports of the USSR Academy of Sciences), he sent its English-language version to *Journal of Molecular Biology*, but that was rejected by the editors (many years later M. Bretcher confessed to him that he was a reviewer of the manuscript). Therefore, the English version was published in a little-known journal *Currents in Modern Biology* [29]. But just the next year A. S. Spirin successfully presented his model in English at the Symposium "The Mechanism of Protein Synthesis" in Cold Spring Harbor (USA) that gathered leading experts in the field [30].

THE RIBOSOME IS A MOLECULAR MACHINE, IT WORKS ONLY WITH THE HYDROLYSIS OF GTP, BUT IF THERE IS NO GTP, THEN WITHOUT

This is a paraphrased oriental parable that Alexander Sergeevich used as an epigraph for one of the chapters of his textbook on molecular biology [31]: "Hyena is a predatory animal, it hunts only on moon nights, but if there is no moon, then without." He also used to cite it at lectures on the ribosome energetics.

If the ribosome is a machine, what is then its engine and what is its fuel? When the model of locking-unlocking of the ribosomal subparticles was proposed, the answer to this question seemed obvious. It was known that protein synthesis on the ribosome requires GTP and protein elongation factors T (EF-Tu) and G (EF-G), which hydrolyze GTP and take part in the steps of Aa-tRNA binding and translocation, respectively. Thus, in addition to the ATP molecule used for the synthesis of Aa-tRNA, at least two GTP molecules are spent for the synthesis of each peptide bond. It was reasoned that, by analogy with the role of ATP hydrolysis in muscle contraction, this GTP hydrolysis could supply energy for the movements of mRNA and tRNAs on the ribosome [32]. Therefore, it was natural to assume that the elongation factors lock and unlock the ribosomal subparticles; they are the engines using GTP as a fuel [25, 29, 30].

However, shortly thereafter, S. Pestka showed that the synthesis of polyphenylalanine on a poly(U) template can occur in the absence of elongation factors and GTP [33, 34]. In Spirin's laboratory this result was confirmed; moreover, it was shown that the treatment of ribosomes with SH reagent *p*-chloromercuribenzoate, which completely inactivated elongation factors, did not suppress, and even stimulated the factor-free synthesis of polyphenylalanine [35, 36]. These observations dispelled any remaining suspicion that the observed synthesis was due to admixtures of the elongation factors and GTP. In subsequent works of the laboratory, it was shown that the mechanism of protein synthesis by ribosomes is exactly the same in the absence of elongation factors as in their presence; the only difference is that the synthesis in the presence of the elongation factors and GTP occurs at a higher rate and is more resistant to the action of inhibitors such as antibiotics (see reviews [37-39]).

Thus, by the mid-1970s, Spirin concluded that protein synthesis is provided with energy even without GTP – the energy of ATP used for the synthesis of Aa-tRNA covers all the needs – and that all the mechanical movements during the ribosome operation in the case of factor-free translation are due to the Brownian motion [37]. Why, then, are the elongation factors and GTP hydrolysis needed? Maybe, after all, they give the movement of the subparticles an additional impulse and thereby increase the power of the ribosome-machine?

OPERATION OF MOLECULAR MACHINES IS NOT ACCOMPANIED BY CHANGES IN THE PROTEIN FOLD

These questions attracted Spirin's interest to other mechanochemical systems, operation of which is accompanied by hydrolysis of a nucleoside triphosphate (NTP). At the Faculty of Biology of the Lomonosov Moscow State University, he initiated the studies on cell motility systems (carried out by V. I. Gelfand, V. A. Rosenblat, F. K. Gioeva, and N. A. Shanina) and of the Ca^{2+} pump (V. Melgunov). The breadth of his interests in this area can be judged by the topic of the term paper suggested to me by Spirin during the 1973-1974 academic year – "Molecular Mechanisms and Energetics of the Cell Mechanics" that included the ribosome, muscle contraction, contractile systems of viruses, bacteria and eukaryotic cells, as well as transmembrane transport.

In this regard, he was especially interested in the type of changes in protein conformation that might accompany operation of the molecular machines, since at that time it was believed that the energy of NTP hydrolysis could be stored in a strained protein conformation and later used for mechanical work. It was expected that this could be accompanied by "dramatic" changes in the protein secondary structure such as the α-helix → β-sheet transition observed in keratin [40, 41]. Accordingly, the goal of my diploma project [42] was the search for a difference in the conformations of the elongation factor EF-Tu before and after the GTP hydrolysis by comparing the structure of its complex with GTP (with or without Aa-tRNA) to that of its complex with GDP, and the goal of my Ph. D. Thesis [43] was comparison of the conformations of the alternative Na- and K-forms of Na,K-ATPase. However, no difference in the secondary structure of the proteins exceeding the experimental error (2-3%) was detected and it was concluded that even if the changes in the internal structure take place, they have extremely local character [44, 45].

The two facts – the ability of the ribosome to operate without GTP hydrolysis and the lack of large conformational transitions in the NTPase proteins – let Spirin to question the validity of the popular ideas on the principles of operation of mechanochemical systems.

CAN A PROTEIN SERVE AS A MOTOR?

In the Fall of 1980, Alexander Sergeevich invited me to work together on this problem. The work went on for almost a year, we often met and had long conversations. Our conclusions and arguments we tested in lengthy and sometimes hot discussions with the experts in the field of protein and polymer physics Dmitry Sergeevich Chernavsky and Alexander Yulievich Grosberg. This resulted in the article "Bioenergetics and Protein

Synthesis" published in both English [38] and Russian [39] versions*.

The main idea of this article is that a protein molecule cannot serve as an energy transducer due to the nature of thermal (Brownian) motion; in particular, no energy can be stored in a "strained" protein conformation for a time period comparable with the duration of the working cycle of a molecular machine. Since we were not physicists, we were unable to convince D. S. Chernavsky using physics-based arguments. However, as biochemists, we put forward another argument – the principle of a common intermediate long known in biochemistry. It states that the only way to transfer energy from one biochemical reaction to another is to do that via a high-energy intermediate (pp. 403-404 in Ref. [46]).

The synthesis of Aa-tRNA, in which ATP is used to generate a high-energy ester bond between an amino acid and tRNA can be considered as an example. This synthesis occurs in two steps: Aa + AMP~PP → Aa~AMP + PPi; Aa~AMP + tRNA → Aa~tRNA + AMP, catalyzed by aminoacyl-tRNA synthetase. Here, the tilde (~) denotes high-energy bonds and ATP is shown as AMP~PP. In the first step, the amino acid is "activated", i.e., transformed into a high-energy derivative, aminoacyl adenylate (Aa~AMP), which reacts with tRNA in the second step. Thus, Aa~AMP is a high-energy intermediate for these two reactions. A similar approach (activation of compounds) is widely used in organic synthesis (e.g., in the synthesis of oligonucleotides), but no protein is involved in that case.

If a protein could serve as an energy transducer, then a direct condensation of amino acid and tRNA could occur by coupling it to the hydrolysis of ATP, without the intermediary formation of aminoacyl adenylate. Moreover, ATP hydrolysis could be the universal energy source for any endergonic biochemical reaction. However, this method of coupling the biochemical reactions has not been ever found in Nature. Therefore, we concluded that in the cyclically operating mechanochemical systems, the protein cannot transfer the energy of NTP hydrolysis to another process coupled in space and time. From this conclusion, in particular, it followed that elongation factors cannot serve as engines for the ribosome. What is, then, their function?

ENERGY-DEPENDENT CATALYSIS

Elongation factors increase the rate of protein synthesis by 1-2 orders of magnitude, i.e., they are catalysts. It is believed that an enzyme increases the rate of reaction by breaking it into a series of elementary steps, each of which has a lower energy barrier than the integral non-enzymatic reaction. This can be detected by a decrease in the value of the Arrhenius activation energy (E_a) when comparing the dependence of the rate of enzymatic and non-enzymatic reactions on temperature.

It turned out that if the temperature dependence of factor-dependent and factor-free translation is determined at an elevated concentration of Mg^{2+} ions, at which the rate-limiting step is translocation, then the values of E_a coincide [47]. This result confirmed the earlier conclusion that the elongation factors do not change the mechanism of protein synthesis on the ribosome, in particular, the mechanism of translocation, i.e., they do not break it down into several steps. It also indicated that the increase in the reaction rate is achieved through the decrease of the entropy of the system (which does not affect the temperature dependence, that is, the value of E_a); in other words, the elongation factors perform entropic catalysis [38, 39, 47].

According to A. S. Spirin, movements of the massive components of the translating ribosome (its subparticles, tRNAs, the template) occur at the expense of the energy of Brownian motion [37]. In the absence of elongation factors, they occur more or less randomly, so that productive movements (along the proper trajectories) are relatively rare. Binding of an elongation factor, in the right place and at the right time, greatly reduces the degrees of freedom: it fixes the translating ribosome in such a way that the movements occur along the selected trajectories. This increases the probability of the occurrence (and, hence, the rate and the resistance to inhibitors) of the corresponding steps.

However, would such fixation increase the rate of the entire process? The answer to this question was given by experiments with non-hydrolyzable GTP analogs [37, 48]. In the presence of GMP-P(CH_2)P or GMP-P(NH)P, the elongation factors catalyzed the codon-dependent binding of Aa-tRNA (EF-Tu) and translocation (EF-G) almost as efficiently as in the presence of GTP; however, the subsequent steps of the cycle (the peptidyl transferase reaction and the binding of a next Aa-tRNA, respectively) were blocked. To continue the cycle, the complex of an elongation factor with a non-hydrolyzable GTP analogue must have been washed away from the ribosome – by centrifugation through a sucrose cushion [49] or by passing the buffer through a poly(U)-cellulose column with the bound translating ribosomes [50]. Thus, the hydrolysis of GTP is required to detach an elongation factor from the ribosome; otherwise, the process of translation cannot go on.

It should be stressed that elongation factors set the trajectory of movement of the massive components of the translating ribosome, rather than the direction of this movement [38, 39]. After a factor loses its interaction

* This was our first joint publication, despite the fact that since 1973 A. S. Spirin was my scientific advisor, suggested scientific problems worth tackling for me, and discussed the results; earlier he refused to be my co-author saying that his contributions were not significant enough.

with the ribosome, everything might return to its original state if the next peptide bond would not be formed (and, hence, elongation of the polypeptide chain would not occur), or if the peptidyl transferase reaction would be reversed. Thus, it is not the elongation factors and GTP hydrolysis, but the growing peptidyl-tRNA that serves as a ratchet (see below) allowing the ribosome to utilize the Brownian motion for its operation. (An additional source of energy feeding the ratchet can be the process of folding the polypeptide chain into a protein globule, which occurs co-translationally as predicted by Spirin [51]).

BROWNIAN (THERMAL) RATCHET

The idea of a molecular machine driven by the Brownian (thermal) motion was published by Spirin back in 1978 [37]. In 1982, a detailed description of operation of such a machine was presented using the translating ribosome as an example, and it was concluded that all mechanochemical systems, including that of muscle contraction, should operate on a similar basis [38, 39]. Finally, it was shown that the direction of the process on the ribosome is determined by the energy of peptide bond synthesis; only the word "ratchet" was not said.

However, this idea was left almost unnoticed, although A. S. Spirin repeatedly returned to it in his subsequent publications [52-54]. Researchers dealing with mechanochemical systems continued to believe that operation of such systems is based on "power strokes" fueled by the energy of ATP hydrolysis [55, 56]. It was only by the mid-1990s that some scientists began to realize that the chemical energy cannot be converted into the mechanical one in this way: calculations showed that the total power of impacts on a molecular machine from all the sides as a result of the thermal (Brownian) motion of solvent molecules (the thermal noise) is nearly billion times greater than the power the machine may get from the ATP hydrolysis [57]. Hence, if the power strokes do occur at the molecular level, then only the Brownian motion can perform them.

As an alternative, the idea of a Brownian (thermal) ratchet was proposed – a functional analogue of the macroscopic ratchet (a gear with asymmetric teeth, equipped with a pawl that prevents reverse rotation), which converts the unordered thermal motion into the directed motion of a molecular machine. In order to prevent violation of the second law of thermodynamics, it was postulated that the Brownian ratchet is powered by the energy of a coupled chemical process [58-60]. In fact, this was a reiteration of the Spirin's idea, but only one paper on this issue [61] provided a reference to the article published in 1982 [38].

But what process does feed the Brownian ratchet with energy and, therefore, results in the useful work performed by the molecular machine? Nearly all the papers on muscle contraction claim that the energy source for this process is the hydrolysis of ATP, but no one provides any reference to works wherein that fact had been demonstrated. The literature search has led to the classic paper by V. A. Engelhardt and M. N. Lyubimova, which showed that myosin possesses the ATPase activity [62]. This result was then confirmed by A. Szent-Gyorgyi and I. Banga [63]*. However, the mere demonstration of the fact that ATP hydrolysis accompanies muscle contraction, does not prove that it is the energy source.

The analogy with muscle contraction allowed F. Lipmann to suggest that the GTP hydrolysis carried out by EF-G is the source of energy for the translocation of peptidyl-tRNA and mRNA on the ribosome [32, 64]. This statement seemed so natural and self-evident that no one would not ever doubt its validity, if it had not been discovered that the entire process of protein synthesis by the ribosome, including translocation, can occur in the absence of GTP hydrolysis. And, although no one has yet seen muscle contraction in the absence of ATP, the same analogy suggests that, perhaps, not the hydrolysis of ATP is the energy source for muscle contraction.

As discussed above, the elongation factors catalyze the process that is already provided with energy. This catalysis is energy-dependent as it consists of cyclic fixation-defixation of massive objects moving due to the Brownian motion. A similar cyclic fixation-defixation of massive objects (myosin and actin fibers) is observed during muscle contraction coupled with ATP hydrolysis [55, 56]. If biological processes follow the one logic (and, apparently, it was this consideration that Lipmann was guided by when drawing an analogy between the work of muscles and of the ribosome), then it is likely that in this case, too, the energy of NTP hydrolysis is spent not on the muscle contraction, but on its catalysis. A Brownian ratchet serving the muscle contraction could be powered, for example, by the energy of the cellular metabolism as a whole (including the ATP hydrolysis), which makes the contraction process energetically favorable [38, 39]**.

After the terms "thermal ratchet" and "Brownian ratchet" took the roots and became popular, A. S. Spirin published a series of papers in order to "translate" his

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* This is probably the shortest article in the history of science. It consists of only two phrases: "Our experiments corroborate the results of W. A. Engelhardt and M. N. Ljubimowa according to which Adenosine triphosphatase is bound to myosine. The enzyme is activated by Ca; Ca can be substituted by other bivalent metals."

** Those papers [38, 39] say: "A truly unique role of the coupled ATP hydrolysis in muscle contraction... consists, in our opinion, of a specific removal (or lowering) of potential barriers for the coupled process... this interpretation allows us to suggest the coupled ATP hydrolysis to be a regulator of muscle activity and muscle contraction velocity and, thus, of muscle power."

ideas on the mechanism and energetics of the ribosome into the new language, as well as to elaborate the model of locking-unlocking of ribosomal subparticles in view of the new data on the structure and function of the ribosome, while their essence remained unchanged [2, 5, 27, 65, 66].

THE RIBOSOME AND RNA POLYMERASE: TWO MACHINES IN A TEAM

In the issue of *Molecular Biology (Moscow)* dedicated to 80th anniversary of the birth of R. B. Khesin-Lurie, one of the pioneers in the studies of transcription, Spirin published a paper, where he showed that operation of the DNA-directed RNA polymerase can be entirely based on the Brownian ratchet model, without involving any power strokes.

According to Spirin, RNA polymerase is built and operates on the same principles as the ribosome: it contains several mobile structural blocks, whose mutual orientation changes during the elongation cycle due to the Brownian motion; this cycle includes translocation (a directed movement of the template and the growing RNA chain) and extension of a polymer with one unit (synthesis of the next internucleotide bond*) [67].

Unlike the ribosome, the operation of RNA polymerase is not associated with the hydrolysis of any NTP; therefore, here in principle no question can arise regarding its contribution to the energetics of the process, as well as what is the ratchet: it can only be the synthesized RNA chain growth of which determines direction of the entire process.

Why then, unlike the RNA polymerase, the ribosome consumes two more GTP molecules in every elongation cycle? Probably because operation of the ribosome is associated with the movement of larger masses (in addition to its own mass, which is an order of magnitude greater, the ribosome has to move 2 to 3 tRNA molecules), as well as because it has to displace the template farther (three nucleotides instead of one in each cycle).

In the absence of GTP hydrolysis, the translation rate drops by 1-2 orders of magnitude [47], which is probably incompatible with the cell life. Furthermore, these processes are strictly coordinated in prokaryotic cells, where transcription and translation take place in the same compartment [68].

The mRNA begins to be translated while it is still synthesized, as soon as the initiation codon becomes available for landing of the ribosome. Duration of the elongation cycle of the ribosome, in which the mRNA is displaced by one triplet, is exactly 3 times greater than the time of the synthesis of one internucleotide bond by RNA polymerase; that is, the ribosome and RNA polymerase read their templates at the same rates. Moreover, reducing the translation rate (for example, by antibiotics or when the ribosome encounters rare codons) proportionally slows down the transcription. Usually, the translating ribosome stimulates transcription by suppressing the RNA polymerase "backtracking" (reversal of the translocation, during which the polymerase shifts back to the traversed portion of the DNA template, while the growing end of the mRNA annealed with the DNA template protrudes from the active center [68]). In other words, by pushing the RNA polymerase from behind, the translating ribosome helps the polymerase ratchet to avoid a slippage. Thus, the ribosome and RNA polymerase run in one team and together "drag a cart" of the gene expression by combining their energy resources.

In conclusion of the paper on the energetics of RNA polymerase, A. S. Spirin has written: "In further publications I will try to show that same principles can be applied to describe the movements of other molecular machines, such as ribosomes, kinesin, dynein, transmembrane ATP synthases, and bacterial flagella, without any "motors," "rotors", and "stators" [67]. Regretfully, he was not given enough time to do everything as planned.

IN THE BEGINNING WAS RNA – 2

If we would follow the chronology of Nature, then this episode – about the role of RNA in the origin of life – should have been the first in this series, since all the events developed here long before the emergence of cells, as well as of the ribosomes and other contemporary molecular machines. However, in the life of A. S. Spirin, it was a continuation of his passion for RNA that began almost half a century ago. What attention did he pay to this issue can be judged by the fact that he devoted to it almost half of his lecture on May 15, 2002, presented at the General Meeting of the Russian Academy of Sciences after he had received the Great M. V. Lomonosov Gold Medal, the major award of the Academy [69].

Apparently, his interest in the role of RNA in the origin of life Spirin inherited from his teacher, A. N. Belozersky, who said at the Moscow International Symposium "The Origin of Life on the Earth" in August 1957: "It seems that RNA, being associated with the most general processes of life, was formed at an earlier evolutionary stage, while the origin of DNA was associated with the development of more specialized and phylogenetically later features of organisms" [70]. According to Spirin [71], this was the first public presentation of the idea that the earliest form of life was based on RNA. Only a decade

* In fact, both additions of one unit to the polyribonucleotide (RNA polymerase) and to polypeptide (ribosome) use energy of the anhydride bond between the α- and β-phosphates of NTP: in the first case, during the polymer growth, and in the second – at the step of tRNA aminoacylation. In both cases, the equilibrium shifts toward the polymer synthesis due to hydrolysis of the liberated pyrophosphate (PPi).

later, Crick [72] and L. Orgel [73] published coordinated concepts on the role of RNA in the primordial life, and almost 30 years later, after the discovery of ribozymes [74, 75], when it became clear that RNA molecules can perform almost all vitally important functions (genetic, structural and catalytic) in the absence of DNA and proteins, W. Gilbert has coined the term "RNA world" [76]. Finally, in 2000, the peptidyl transferase site of the ribosome was shown to consists entirely of RNA and, hence, the ribosome must be a ribozyme [77]. This eliminated any last doubt that "omnipotent" RNAs [69, 78] originated prior to proteins and, apparently, fueled the Spirin's interest in the problem of the origin of life.

His first article on this issue Spirin published in the year of his 70th birthday [79]. Although by that time the idea of the RNA world had become commonly accepted, it was unclear how this world had come into being. Spontaneous synthesis of oligonucleotides from activated ribonucleotides has been demonstrated [80]. Several variants of the abiogenic origin of monomeric nucleotides have been proposed, including their synthesis at lightning discharges in a reducing (oxygen-free) atmosphere, arrival from the space along with meteorites, and synthesis in the deep-sea hydrothermal vents, also known as "black smokers" [81]. However, a large logical gap remained: how did the short random oligonucleotides evolve into RNA molecules with unique 3D structures, diverse catalytic functions, and ability to reproduce?

In search of an answer to this question, Spirin drew attention to the results obtained during the work on the project aimed at establishment of a cell-free RNA replication system, whose purpose was to develop a universal method for producing any desirable RNA in virtually unlimited amounts. To realize this project, in 1985 Spirin created a group at the Institute of Protein Research (later transformed into laboratory) of Viral RNA Biochemistry, and entrusted me to be its leader. That was completely unexpected for me who previously dealt with the problems of mechanochemical coupling and ion transport [43]. Thus, he introduced me to the RNA world, passing on the baton received from Belozersky.

This project was due to another Spirin's passion, the large-scale cell-free synthesis of proteins [82], which required large amounts of mRNAs. He hoped that those could be produced by Qβ replicase, the RNA-dependent RNA polymerase of bacteriophage Qβ, known for its unsurpassed ability to amplify RNA *in vitro*. Earlier, the attempts to use Qβ replicase for such a purpose were hampered by its extremely high selectivity (template specificity). But in 1985, it was believed [83] that this problem could be circumvented with the method invented at the Columbia University by the students of S. Spigelman. The idea was to embed a target RNA inside the molecule of an efficient natural Qβ replicase template [84]. Although certain success in this direction was achieved during the work on the project [85, 86], its main outcome appeared to be different.

THE PUZZLE OF TEMPLATE-FREE RNA SYNTHESIS

Being an enzyme efficiently synthesizing RNA on an RNA template, Qβ replicase attracted attention of the researchers passionate about the origin of life. In 1975, M. Eigen's collaborators published a sensational paper reporting an incredibly high rate of RNA evolution in a test tube. It was declared that a large amount of a 200-nt long RNA accumulated within an hour in the reaction mixture containing only pure NTPs and pure Qβ replicase. The authors concluded that the RNA had been synthesized *de novo*: Qβ replicase started with a template-free synthesis of a variety of random polyribonucleotides, which then evolved within an hour in the same test tube into its efficient template [87].

This conclusion could make happy those preoccupied with the issue of the origin of life, since it had immediately eliminated the problem of the "geological paradox", which states that in the geological history of the Earth, no more than 100 million years could be allocated for the emergence, growth, and evolution of the RNA world [88]. This conclusion implied that once an RNA replicase happened to emerge in the primordial soup, the rest of the evolution would have occurred almost instantly. However, it reduced the chances of success in achieving the goal assigned to us by A. S. Spirin to nearly zero, since instead of the target RNAs, Qβ replicase would amplify those efficient templates that had originated *de novo*.

We observed RNA synthesis in the absence of added template in the very first experiment with Qβ replicase. However, sequencing of the major product RNA showed that it could not have been generated *de novo*: 80 nt of its total length of 120 nt perfectly matched a fragment of the Qβ phage coat protein gene, and another 33 nt – a fragment of the *Escherichia coli* aspartyl tRNA. It followed that this product was synthesized on a template formed as a result of recombination of the two RNAs [89].

The source of RNA templates in the reaction mixture had been identified by employing the method of molecular colonies invented by us. If the Qβ replicase reaction was carried out in an agarose gel rather than in a test tube (i.e., in liquid), then discrete colonies of RNA were formed, each of which contained 10^{10}-10^{11} copies of the parent molecule and could easily be detected by staining with ethidium bromide. The number of colonies increased if the gel was left open until the start of the reaction or if the experiment was carried out in a room where replicating RNAs were manipulated before. It followed that RNA templates entered the reaction mixture from air [90, 91].

The same method allowed us to investigate the process of RNA recombination. For this purpose, a mixture of fragments of a replicable RNA, none of which was capable of amplification by Qβ replicase, was added to the gel. Formation of the replicable molecules as a result of fragment recombination led to the appearance of RNA colonies [92]. We found that the replicable RNAs appeared when a mixture of the fragments was incubated without Qβ replicase or any other protein – the only requirement was the presence of Mg^{2+} ions – and that recombination occurred by a re-transesterification of internucleotide bonds [93, 94]. This demonstrated the possibility of spontaneous splicing of short RNAs into longer molecules.

The discovery of the ability of RNAs to spontaneously rearrange their primary structure, invention of the method allowing RNAs to be amplified in the form of molecular colonies, as well as the previously formulated principles of operation of molecular machines, formed a basis of the Spirin's concept of the origin and evolution of the RNA world.

ORIGIN AND EVOLUTION OF THE RNA WORLD

Nonenzymatic RNA synthesis. According to Spirin, the discovered spontaneous recombinations could be a solution to the problem of generation of sufficiently long RNAs. He suggested that oligoribonucleotides produced by abiogenic synthesis actively recombined by means of spontaneous nonenzymatic transesterification, leading to the formation of longer RNA chains and giving rise to their diversity. This could be the mechanism for generation, in a population of oligo- and polynucleotides, of catalytically active species (ribozymes) and other types of RNA with specialized functions. Moreover, nonenzymatic recombinations of oligonucleotides annealed side-by-side with a polynucleotide template, could result in stitching (splicing) of the oligonucleotides complementary to this template into a single strand. In this way, rather than by the catalysis of polymerization of mononucleotides, the primary copying (replication) of RNA could occur [79]. It can be added to the above that, unlike polymerization of monomeric nucleotides, spontaneous recombination of oligonucleotides does not require their preliminary activation [93].

And further: "The appearance of sufficiently long polyribonucleotides and generation of their variants due to spontaneous *cis*- and *trans*-rearrangements should have led to the spontaneous appearance of ribozymes, and a critical step would be the emergence, in the population, of a ribozyme capable of catalyzing the process of complementary RNA replication... As soon as such ribozymes had appeared – at least one molecule per the population of RNA molecules in a little pool – the RNA world acquired its entity as self-reproducing and developing matter on the ancient Earth" [71].

However, at this stage, there could be no biological evolution yet. Such ribozyme "should have been equally good at amplifying both the rare RNA molecules possessing some useful properties for the population... and the bulk of inactive ballast RNA molecules. The natural selection could only begin to operate if there was some form of compartmentalization, separation of individual ensembles of RNA, in which ribozymes and their products are held together. Only then the natural selection could distinguish those RNAs that give rise to better products, and the ensembles of those RNAs, whose functions better complement each other" [71].

Molecular colonies as a precellular form of compartmentalization. According to A. S. Spirin [95], the idea of "isolation from the environment" as a condition for the evolution of macromolecules was first formulated by A. I. Oparin [96]*. However, compartmentalization in the form of coacervate droplets proposed by Oparin could not be implemented in the RNA world due to the absence of polypeptides, polysaccharides or other polymers capable of coacervation [69]. Enveloping with a lipid membrane was also hardly probable, since there were neither lipids no transmembrane transport systems enabling exchange with the environment.

According to Spirin, under these conditions compartmentalization would most naturally had been achieved by formation of molecular colonies as a result of RNA amplification on the surface of a wet clay exposed upon drying out of a puddle inhabited by RNA molecules and containing activated nucleotides or their precursors (a Darwin's pond). This way of "temporary" compartmentalization would provide conditions, on the one hand, for natural selection, and, on the other hand, for evolution of the entire population of the puddle by systematic enrichment with successful RNA variants by analogy with the SELEX technology (Systematic Evolution of Ligands by EXponential enrichment [97]).

The following scenario can be envisioned [71, 95, 98]. If, upon drying a puddle, molecules of a replicating ribozyme and several other RNA species capable of binding the necessary substances and catalyzing the necessary reactions would occur together at some location, then a mixed RNA colony would grow. The colonies comprising more active and better complement-

* In particular, Oparin wrote: "The moment when the gel was precipitated or the first coagulum formed, marked an extremely important stage in the process of the spontaneous generation of life. At this moment... the transformation of organic compounds into an organic body took place... at the same time the body became an individual... and set itself apart from the environment surrounding it." And then: "Only the most complicated and efficient could grow and develop, all the rest either ceased to develop or perished."

ing each other variants of RNA molecules, would outgrow other colonies. During the next watering of the puddle resulting in a partial dissolution of the colonies, the progeny of the most densely populated colonies would begin to dominate the population. At the same time, the watering would allow the colonies to "interbreed". Along with intra- and intermolecular recombinations of RNA molecules and replication errors, this would lead upon the next drying of the puddle, to the appearance of RNA colonies with new features. In addition to amplification of RNA molecules, the consecutive drying/watering cycles would lead to a better adaptation of the colonies to the environment, that would imply the launch of the natural selection. RNA colonies, as linked ensembles within pieces of clay, could spread over long distances by water flows (in the form of clay suspensions) or by the wind (in the form of dry particles).

Such RNA colonies meet all the requirements of a "universal precursor" of living beings on the Earth, as defined by K. Woese [99]: high level of mutations due to imperfect mechanisms of replication of the genetic material, free exchange of the genetic material between progenotes (cell precursors), and communal way of life of these predecessors, when any products and innovations of one become the property of all [69, 71, 95, 98].

The need for molecular machines. According to Spirin, evolution of the RNA world inevitably lead to the emergence of molecular machines. The need for such machines was critical during the replication of RNA whose product was a duplex – a double helix formed by complementary RNA strands. On the one hand, RNA in this form is much more resistant to hydrolysis than in the single-stranded state, and this ensures its better preservation. On the other hand, duplex cannot serve as a template: for replication, it must be unwound into the composite complementary strands [100].

Like PCR (polymerase chain reaction), RNA replication could occur due to cyclic temperature changes: the duplex would melt upon temperature increase, while the strands would be copied upon temperature decrease. Probably, the required extremes of the day and night temperatures could had been attained at that time at some locations on the Earth, but replication would occur at a very low rate: just one cycle a day. This may have been the case at the dawn of the RNA world, but replication had to be faster to ensure further development.

A duplex melts (gets unwound) at higher temperature because the integral power of impacts of the solvent molecules on it (i.e., of the Brownian motion) becomes greater. However, increase in the integral power of impacts on a target can be achieved not only by increasing temperature, but also by increasing the target size. Apparently, this is the principle of operation of molecular machines: they "catch" the Brownian motion like a sail catches the wind and share it with a bound object; in this case, with the section of the duplex that is to be melted.

Probably, as the replicating ribozyme became increasingly larger and the specificity of its interactions with RNA became higher, it acquired the ability of using the Brownian motion to locally unwind the duplex at a progressively lower temperature, until it finally began to replicate RNA under isothermal conditions. In other words, the replicating ribozyme evolved into a molecular machine capable of moving along the duplex and synthesizing the RNA complementary to the template strand of the duplex due to the energy of Brownian motion. As in the case of contemporary RNA polymerase, the synthesized RNA served as the ratchet determining direction ("rectification") of the process.

Another possibility, considered by Spirin, involved cooperation of the replicating ribozyme (elongase) with the ribozyme-helicase capable of locally unwinding a duplex when bound to it in a complex with a cleavable ligand of the NTP type, and leaving the unwound region after cleavage of the ligand, clearing the way for the following elongase [100].

CLOSING THE RING

Further development of the RNA world would had led to the emergence of yet another molecular machine comprised of proto-ribosomal RNA possessing the peptidyl transferase activity and one more proto-ribosomal RNA capable of interacting with both the first proto-ribosomal RNA and a proto-mRNA, as well as with a set of proto-tRNAs specifically binding amino acids or short peptides. Such a machine would be able to translate the nucleotide sequence of proto-mRNAs into the amino acid sequence of polypeptide chains, which ensured the transition of life onto a higher level [79, 98].

Wherever the described events would take place – on the planet Earth, on other space objects, or in the nuclei of comets – the following conditions for the emergence of the communal RNA world must have been met: (i) presence of liquid water and RNA-adsorbing surfaces; (ii) existence of cycles of drying and wetting (or flooding), heating and cooling, freezing and thawing; (iii) protection against cosmic radiation [88, 94].

In conclusion of his Lomonosov lecture Spirin said: "What happened to the RNA world after the collapse of the commune? Although the commune collapsed, the world of RNA was preserved in every cell of every living organism. The basis of contemporary life is the inherited biosynthesis of proteins, which determines all the characteristics of living organisms nowadays. The central component of this process of protein biosynthesis is a set of RNA molecules of various types interacting with each other: first of all, the ribosomal RNA, which makes up the apparatus of protein synthesis; tRNA, which delivers activated amino acids to the ribosome, in order to build polypeptide chains of proteins; and mRNA, which carries the program

for protein synthesis in its nucleotide sequence... One may say that the totality of RNA molecules – the RNA world – still constitutes the core of life" [69].

Obviously, the potential of Spirin's ideas is far from being exhausted and, certainly, they will continue to play an important role in the development of molecular biology and related fields of science.

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