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The unfolding of our understanding of RNA structure: a personal reflection ☆

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Abstract

In this article, I review how our research on RNA began, how it led us to demonstrate the single-stranded nature of RNA, and the ways in which it differs from double-stranded DNA. It was based on the development of a method for the isolation of undegraded rRNA and the observation that in rRNA preparations due to their viscosity behavior resemble a flexible, contractile coil. In support of this assumption, birefringence of flow measurements showed that rRNA solutions gave moderate positive values, which disappeared upon addition of salt. This is in contrast with DNA solutions where considerable negative birefringence persists even in the presence of salt. Further studies on RNA showed a close correlation of the ionic strength dependencies of optical rotation, optical density and hydrodynamic properties. These early results indicated that rRNA and tRNA possess a significant secondary structure. I then review the basis of the hairpin model for the secondary structure of RNA and finally, summarize current understanding of the tertiary structure of RNA.

Keywords: Ribosomal RNA; Transfer RNA; Secondary and tertiary structure

1. Introduction

It is a great pleasure to respond to the invitation of the Editor of this journal and contribute an article in honor of my dear friend Henryk Eisenberg.

I first met Heini at the Hebrew University in Jerusalem where we studied biochemistry and chemistry. I learned to know and appreciate him better during our war of independence (1947–1949). Heini was drafted into the Israeli army science unit (HEMED) of which I was one of its officers. When the war was over, we were ac-

☆ Dedicated to Heini Eisenberg.

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cepted as graduate students at the Weizmann Institute of Science in the little town of Rehovot 21 km south of Tel Aviv. In fact, Heini and Nutzi rented a room for a short period at my parent's home in Rehovot. Our friendship developed further upon my return from postdoctoral studies with Arthur Kornberg.

During 1955–1956, I was exploring the enzymatic synthesis of RNA with Arthur Kornberg in St. Louis, Missouri, USA. The enzyme that we partially purified from *E. coli* extracts, turned out to be polynucleotide phosphorylase (PNPase), which catalyses the condensation of nucleoside diphosphates to yield long oligoribonucleotides, with the release of phosphate groups. PNPase was the first enzyme to be discovered that catalyzes the synthesis of polyribonucleotides with a 3′,5′-phosphodiester bond. It was discovered by Marianne Grunberg-Manago and Severo Ochoa during a study of the mechanism of biological phosphorylation in *A. vinelandii* and our own studies in *E. coli* cell-free extracts (reviewed in [1]).

The ability of the enzyme to produce a variety of polynucleotides and triplet nucleotides turned out to be invaluable in determining the genetic code. The advances made in understanding the physicochemical properties of polynucleotides and their hybridisation reactions as well as the synthesis of polynucleotide inducers of interferon are additional examples of the role played by the enzyme. The reaction is reversible and, in the presence of phosphate ions, polynucleotides are phosphorolyzed by the enzyme and converted to nucleoside diphosphates. Subsequent research showed that degradation of RNA, rather than synthesis, was the cellular function of the enzyme. Renewed interest in PNPase arose recently with the finding that the enzyme is associated in a multiprotein complex that is involved in mRNA degradation.

2. The isolation of rRNA

In 1956 Alex Rich and Leon Heppel invited me to the NIH to introduce them to this enzyme. Leon was very generous in providing me with samples of his RNA collection, which I intended to use upon my return to the Weizmann Institute. I was soon faced with a problem: although the enzyme catalysed readily the phosphorolysis of synthetic oligoribonucleotides and TMV RNA, all the other RNA samples were not affected by the enzyme. It turned out that the RNA samples were degraded products that were resistant to phosphorolysis. My attempts to extract RNA from ribosomes yielded somewhat degraded preparations, while direct extraction of *E. coli* cells with a phenol–water mixture yielded RNA preparations that were heavily contaminated with polysaccharides. I therefore devised a method for isolating undegraded RNA from *E. coli* protoplasts [2]. This method yielded an excellent preparation, sedimentation in the ultracentrifuge revealing three boundaries with sedimentation constants of 4.1S, 16.5S and 23.7S. The two high-molecular-weight RNA components were separated from the low-molecular-weight fraction by ammonium sulfate precipitation and turned out later to be derived from ribosomes. As expected, the rRNA preparation was phosphorolyzed readily by PNPase.

3. The single-stranded nature of rRNA

Having at hand a high molecular preparation of RNA, it was only logical to approach my good friend Heini, and to suggest that we collaborate in an attempt to determine the physical properties of the high-molecular-weight rRNA preparation. Heini had already distinguished himself in the study of the solution properties of DNA, and he agreed immediately to my suggestion. It should be noted that at that time, the structure of RNA in solution, unlike that of DNA, was unknown, mainly because of the lack of good undegraded RNA preparations. We soon found that the viscosity of the rRNA solutions varied markedly with the concentration of added salt. On increasing the ionic strength of the rRNA solution the limiting viscosity decreased approximately 100-fold (Fig. 1). Similar results were obtained with rat liver RNA. Thus, rRNA behaves quite unlike DNA, where the limiting viscosity shows much smaller dependence upon ionic strength. We pro-

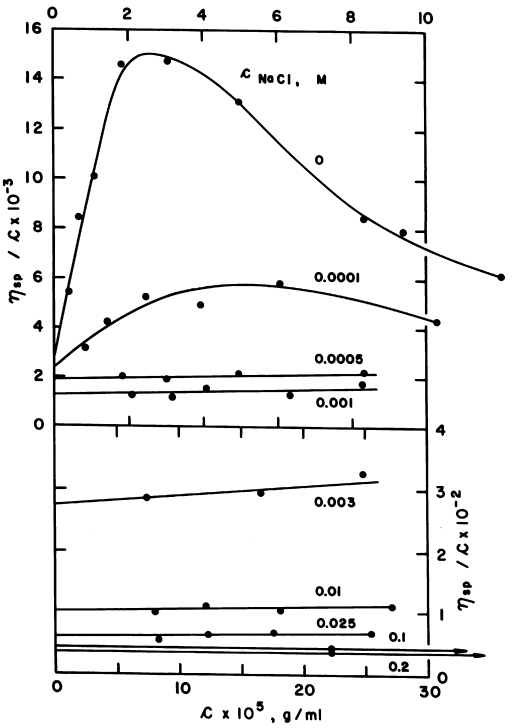

Fig. 1. Viscosity numbers of *E. coli* rRNA solutions at various NaCl concentrations [2].

posed therefore, that rRNA behaves as a flexible, contractile single-stranded coil [2]. In support of this assumption birefringence of flow measurements showed that rRNA in aqueous solution gave moderate positive values which disappeared upon addition of salt. This is in contrast with DNA solutions where considerable negative birefringence persists even in the presence of 2 M NaCl. The positive birefringence observed for rRNA could be compared with the measurements of Rosalind Franklin who found a positive contribution to birefringence of flow in TMV suspension, which disappeared upon removal of the RNA constituents from the virus particle. This gave a clear indication of the considerable difference between DNA and RNA structure [3].

In September 1958, I presented our results at the International Congress of Biochemistry in Vienna [4]. Our conclusion that rRNA is a contractile single-stranded coil was well received. After the lecture I met with Francis Crick, Paul Doty, Alex Rich and Vittorio Luzzati for lunch. Doty told me that they also had evidence that RNA is made of single stranded chains. He believed that rather than being a continuous chain, rRNA is an aggregate formed by a number of short fibers that are dissociated upon heating or urea treatment [5,6]. However, the correlation between the sedimentation velocity and viscosity data that we found for rRNA at different ionic strength concentrations did not support this idea. Crick made the interesting suggestion that the RNA fiber can, under certain circumstances, fold onto itself and give secondary structure characteristics which might explain the reversible curve obtained in our potentiometric titration measurements. By 1959 our work had been published in detail [2,7–9] as had that of Doty and his collaborators [10] (reviewed in [11]) and the single-stranded nature of RNA in solution was established.

4. Early studies on the secondary structure of RNA

In collaboration with Cox, the changes in configuration brought about by the addition of electrolyte were studied further. We noted that the abrupt fall in viscosity upon increasing the concentration of sodium chloride was greater than that found for simple polyelectrolytes [12]. Electron microscopical studies also supported the notion that rRNA is a molecule capable of a great degree of coiling [13]. The close correlation of the ionic strength dependencies of optical rotation, optical density and hydrodynamic properties, indicated that rRNA and tRNA possess a significant secondary structure (Fig. 2) [12,14–17]. Similar evidence that TMV RNA and rRNA are constrained by intramolecular bonds was obtained from studies of changes in hydrodynamic and optical properties with ionic strength and temperature [10,11,18–20].

From all these observations it was estimated that, in dilute salt solutions, RNA has a partial (40–60%) helical structure. Furthermore, the

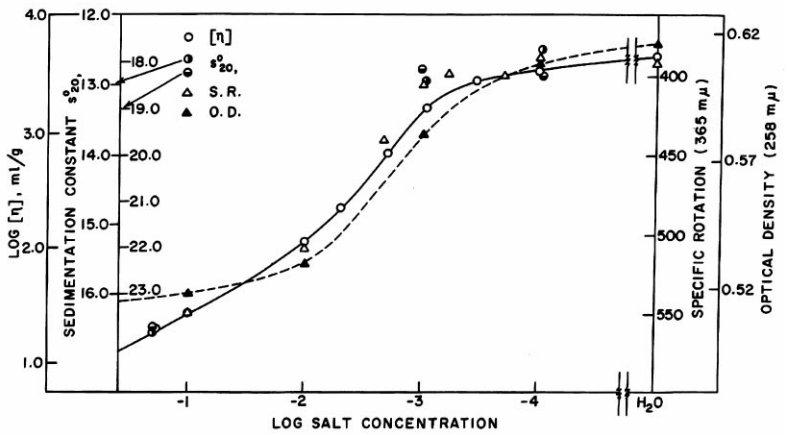

Fig. 2. The dependence of hydrodynamic and optical properties of *E. coli* rRNA on the concentration of added NaCl [15].

RNA molecule was considered as a composite of short DNA-like rigid helical regions connected by flexible single-stranded sections. To explain how half the bases in RNA could fit into helical regions, Jacques Fresco, Bruce Alberts and Paul Doty proposed that the double helical regions are not perfect helices but contain looped out residues. The predominant element of RNA secondary structure is the hairpin that is formed when a local region of polynucleotide chain folds back on itself to form a short, intramolecular base-paired helix, called the stem [21,22]. This model is still the basis of our understanding of the secondary structure of RNA. The functional importance of the secondary structure was indicated from phylogenetic comparison of rRNAs. These studies reveal that changes in primary sequence can occur as long as complementary changes preserve secondary structure. Comparative sequence analysis was also useful in identifying putative tertiary contacts in RNA chains. The number of unpaired loop residues enclosed by the stem can vary according to the type of RNA. Comparative sequence analysis revealed that the vast majority of all rRNA hairpins have four nucleotides in the loop. The tetraloops in rRNA are either composed of GNRA or UNCG (where N is any nucleotide and R is a purine) [23]. Tetraloops also occur in other types of RNA [24] such as mRNA of T4 [25], the catalytic RNA of bacterial ribonuclease P [26–28] and the self-splicing RNA [29–31]. The tetranucleotide loop sequences appear to stabilize the tertiary structure of an RNA molecule by making intramolecular contacts with a distal site in the molecule. Tetraloops also can serve as nucleation sites for intermolecular interactions with other nucleic acids and proteins [23,25].

We have extended our studies to the structure of rRNA and tRNA from various sources with similar results. Several years later, we demonstrated together with Inder Verma, Cyril Kay and Marvin Edelman the presence of rRNA in mitochondria from the fungus *Aspergillus nidulans* as well as the fungus *Trichoderma viride*. We also showed that the mitochondrial rRNA possesses a substantially ordered structure that differs from that of the homologous cytoplasmic rRNA species. Circular dichroism measurements (Fig. 3) suggested that both hydrogen bonding and base stacking play a role in stabilising mitochondrial rRNA and cytoplasmic rRNA structure, and indeed suggest that the two forces are not independent of one another [32,33].

The elucidation of the primary structure of $tRNA^{Ala}$ by Robert Holley et al. [34] and $tRNA^{Ser}$ by Hans Zachau et al. [35] provided the basis for the ‘cloverleaf’ model, and by 1969 it was clear that it could be fitted to several other tRNA sequences. In the late 1970s comparative sequence analysis of rRNA led to secondary-structure models for rRNA as well [36].

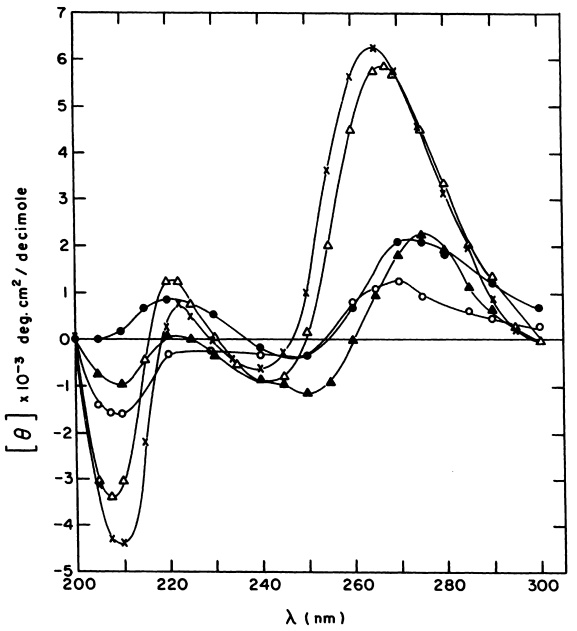

Fig. 3. Molar ellipticities per residue of mitochondrial rRNA of *Trichodema viride*. Experimental results in the presence of: 0.1 M phosphate, 5×10^{-3} M Mg^{2+} (×-×); 0.001 M phosphate (△-△); 80 vol.% ethylene glycol in 0.1 M phosphate buffer (●-●); 0.1 mM H_3PO_4, pH 3.3 (○-○) and RNA in 0.001 M phosphate, heated in the presence of 1% formaldehyde to 63°C for 10 min and then fast cooled to room temperature (▲-▲) [33].

5. The tertiary structure of RNA

It was obvious at that time, that in addition to the secondary structure of RNA, tertiary interactions must also take place. Early understanding of the tertiary RNA folding was based on the X-ray analysis of tRNA [37,38]. These studies showed that many interactions which maintain the tRNA tertiary structure are of a novel type, such as base triples and interactions between bases and the phosphodiester backbone and may also occur in rRNA.

A major development in our understanding of RNA structure is its ability to form pseudoknots. RNA obtained from various plant viruses such as TYMV, TMV and BMV are terminated with a tRNA-like structure which is recognized by tRNA-specific enzymes such as aminoacyl-tRNA synthetases [39]. The tRNA-like structures appear to be involved in TYMV RNA replication [40] and deletion of 5–10 nucleoside residues from the 3′-terminus of TMV RNA results in loss of infectivity as well as its aminoacylation capacity [41]. Sequence analysis of the 3′-terminal region of the viral RNAs, showed that the cloverleaf structure as found in tRNA appeared to be absent. However, three-dimensional folding of the 3′-domain based on pseudoknotting, imposes tRNA-like mimicry that shows a great resemblance with the L-shape configuration of tRNA and thus could explain the amino acid acceptance of these plant RNA viruses. The pseudoknot conformation originates from a hairpin structure, in which a single-stranded region folds back to pair with residues in the hairpin-loop region [42]. Pseudoknots have since been found in many classes of RNA. Sequence comparisons, chemical modifications, enzymatic digestions, site-directed mutagenesis and other techniques have established the importance of pseudoknots in ribosome functioning, in catalytic and self-splicing RNA, in the *trans*-translation process of tmRNA and as motifs for recognition of proteins [43,44]. Many viruses regulate translation of polycistronic mRNA using a −1 ribosomal frameshift induced by a pseudoknot [45–48]. It is postulated that the process of frameshifting requires a slippery sequence, where the ribosome changes reading frame and a complex mRNA structure located immediately downstream, in many cases a pseudoknot. It is thought that the folded pseudoknot structure stimulates the event at least partially by causing a translational pause [49,50]. The crystal structure of the beet western yellow virus pseudoknot was recently elucidated. It reveals that the pseudoknot is stabilized by more tertiary hydrogen bond interactions than secondary interactions in Watson–Crick base pairs [48] and mutational analyses show that triplex interactions are required for frameshifting function [51].

Many approaches have been employed to elucidate the tertiary structure of rRNA. It appears that at least 60% of the 16S RNA is present in double-stranded helices that contain bulges and non-canonical base pairs [52,53]. Thus, a model for the three-dimensional folding of *E. coli* 16S rRNA has been proposed that incorporates re-

sults obtained from intra-RNA cross-linking, RNA–protein cross-linking, foot printing, cryo-electron microscopy, immuno-electron microscopy, neutron scattering, modeling and energy calculations [54,55]. Moreover, the overall tertiary organization obtained is consistent with recent crystallographic data obtained from the *Thermus thermophilus* small ribosomal subunit [56].

The discovery of RNA enzymes in several systems has intensified interest in rRNA function. Early ideas on ribosome function held that the catalytic activity resides in the protein components and the rRNA was viewed as an inert scaffold on which proteins are organized [57]. The demonstration of the ability of RNA to catalyze enzymatic reactions [58,59] has drawn attention to the functional importance of rRNA. The observation that a large portion of 16S rRNA is found unexposed at the interface between the 30S and 50S ribosomes would be consistent with an active role for the RNA in the functional process of protein synthesis [60–62]. Indeed, the 23S rRNA appears to participate in vitro in peptide bond formation in the apparent absence of most of the ribosomal protein components [63]. Moreover, RNA molecules have been shown to perform many functions that were commonly attributed to proteins. For example, an RNA transcript specifically in steroid target tissue, functions as a component of a large multiprotein complex to selectively enhance transcriptional activation by steroid receptors [64].

Heini and I started as colleagues, neighbors and friends and now many years later we are neighbors in the same apartment house and even closer friends.

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