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Insight into the structural organization of the omega leader of TMV RNA: The role of various regions of the sequence in the formation of a compact structure of the omega RNA

Sultan C. Agalarov, Evgeny A. Sogorin, Nikolay E. Shirokikh, Alexander S. Spirin *

Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

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ABSTRACT

The 5′-untranslated sequence of tobacco mosaic virus RNA – the so called omega leader – is a well-known translational enhancer. The structure of the omega RNA has unusual features. Despite the absence of extensive secondary structure of the Watson–Crick type, the omega RNA possesses a stable compact conformation. The central part of the omega sequence contains many CAA repeats and is flanked by U-rich regions. In this work we synthesized the polyribonucleotides containing modified omega sequences, and studied them using analytical ultracentrifugation and thermal melting techniques. It was demonstrated that changes made in both the central and the 3′-proximal part of the sequence led to a strong destabilization of the omega RNA structure. We conclude that the regular $(CAA)_n$ core region and the 3′-proximal AU-rich region of the omega RNA interact with each other and contribute together to the formation of a stable tertiary structure.

1. Introduction

The 5′-untranslated sequence of tobacco mosaic virus (TMV) RNA – the so called omega leader – is known as a powerful translational enhancer [1]. In recombinant constructs the omega leader enhances translation of foreign RNAs both *in vivo* and *in vitro*; it can act as a translational enhancer in various cell types and different cell-free translational systems [2–7]. The TMV RNA leader sequence is about 70 nucleotides long and has an unusual primary structure [8]. The central part (about half of the sequence) contains only adenylic (A) and cytidylic (C) residues mainly grouped in CAA triplets. The CAA-containing part is flanked by U-rich regions, a shorter one at the 5′-end and a longer one at the 3′-end. There are no guanylic residues with the exception of the first residue at the 5′-end of TMV RNA. Based on the features of this nucleotide sequence no stable secondary structure with canonical Watson–Crick base pairing could be predicted for the omega leader [9,10]. On the other hand, it was shown by analytical centrifugation and thermal melting methods that the omega RNA possesses a stable compact structure [11]. Later chemical and enzymatic probing of the omega RNA structure indicated that the RNA has a secondary structure of a non-Watson–Crick type within its central part with CAA repeats and forms short regions of RNA helices of A form in its 3′-proximal part [12].

Here, we studied the contribution of particular RNA regions to the formation of a compact tertiary structure of the omega leader. For this purpose we synthesized omega sequences with altered either central, or 3′-proximal parts, and studied their sedimentation and thermal melting properties. Amazingly, the substitution of just three cytosines for three adenines within the region with CAA repeats led to essential decompactization and destabilization of the entire structure, thus supporting the model of a triple-helical arrangement of $(CAA)_n$ sequences proposed earlier [13]. On the other hand, when the AU-rich sequence in the 3′-proximal part of the omega RNA was replaced by GC containing sequence, the structure of the omega RNA also became less stable and less compact. We concluded that the regular $(CAA)_n$ region and the 3′-proximal AU-rich region of the omega RNA interact with each other and contribute together to the formation of a stable compact tertiary structure.

2. Materials and methods

2.1. Materials

RNAase inhibitor RiboLock™, T7 RNA polymerase and restriction endonucleases NcoI, HindIII were purchased from Fermentas (Lithuania). Plasmids were amplified in *Escherichia coli* strain XL-1 (Promega, USA). $tRNA^{fMet}$ from *E. coli* was from Sigma (USA).

2.2. Plasmids

To obtain RNA containing omega sequence the plasmid construct pTZ10Ω*luc* was used [7]. To obtain the polyribonucleotides

* Corresponding author. Fax: +7 495 514 0218.
E-mail addresses: spirin@vega.protres.ru, agafonov@vega.protres.ru (A.S. Spirin).

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containing modified omega sequences the following pairs of commercially synthesized oligonucleotides (Syntol, Moscow, Russia) were used: 5′P_IAGCTTTATTTTTACAACAATTACCCACCACCACAAA CAACAAACAACATTACAATTACTATTTACAATTACAC and 5′P_ICATGGT GTAATTGTAAATAGTAATTGTAATGTTGTTTGTTGTTTGTGGTGGTGGG TAATTGTTGTAAAAATAA for 3A → 3C RNA (see Fig. 1 and the text below); 5′P_IAGCTTTATTTTTACAACAATTACCAACAACAACAAACAAC AAACAACGCCACCAGCACCAGCACCAGCACGAC and 5′P_ICATGGTCGT GCTGGTGCTGGTGCTGGTGGCGTTGTTTGTTGTTTGTTGTTGTTGGTA ATTGTTGTAAAAATAA for $(A,U)_n \rightarrow (G,C)_n$ RNA; 5′P_IAGCTTTATTTTT ACTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATTACAATTACTATTTA CAATTACAC and 5′P_ICATGGTGTAATTGTAAATAGTAATTGTAATGAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAGAAGTAAAAATAA for $(CAA)_n \rightarrow U_{3n}$ RNA.

The appropriate oligonucleotides were mixed in equimolar amounts, heated at 95° for 1 min, cooled down to their melting temperature and annealed for 3 min. The melting temperatures were calculated according to http://eu.idtdna.com/analyser/applications/oligoanalyzer/default.aspx The resulting double-stranded DNA fragments bearing at their opposite ends "hanging" sequences of recognition sites for the restriction endonucleases NcoI and HindIII were inserted into pTZ10Ω*luc* treated with the same restriction enzymes thus replacing the wild omega sequence. All of the plasmid constructs were verified by sequencing.

2.3. In vitro transcription

Transcription was performed as described in [14], with minor modifications. The incubation mixture contained 80 mM Tris-OAc pH 7.5, 10 mM KCl, 10 mM DTT, 2 mM spermidine, 0.01% Triton X-100, 4 mM ATP, GTP, UTP and CTP each, 22 mM $Mg(OAc)_2$, 1 U/μl of RNAase inhibitor, 12 U/μl of T7 RNA polymerase and 100 ng/μl of the plasmid hydrolyzed by NcoI. The reaction was performed at 37 °C for 2 h. The purification procedure of transcripts is described in detail elsewhere [12]. The purity of polyribonucleotides obtained was verified by electrophoresis in 6% polyacrylamide gel with 7% urea.

2.4. Analytical centrifugation

Analytical centrifugation was carried out in a rotor An-60Ti and Optima™ XL-1 Analytical Ultracentrifuge at 20 °C and 40,000 rpm. Sedimentation experiments were performed in 20 mM Tris–HCl buffer, pH 7.5, with 100 mM KCl and 0.5 mM EDTA. Sedimentation data were analyzed using Sedfit (V9.4c) software (http://analytical-ultracentrifugation.com/sedphat/download.htm).

2.5. UV spectrophotometry and temperature dependence of UV absorption

UV absorption measurements were made at 258 nm using a Hitachi model 200-20 spectrophotometer equipped with a heating block. Temperature control was carried out using a LKB 2219 Multi Temp II Thermostatic Circulator. Difference of temperatures of the circulating liquid and the measuring cell was carefully monitored by a thermocouple. The melting of polyribonucleotides was performed in Mg^{2+}-free 10 mM sodium cacodylate buffer, pH 7.5, with 100 mM NaCl and 0.5 mM EDTA. Prior to the experiments the buffer was carefully degassed. To prevent evaporation, silicone mineral oil was layered onto RNA samples and the optical cell was tightly closed with a lid. In all the experiments the thermal unfolding was shown to be reversible. The integrity of the samples was checked after melting experiments by polyacrylamide gel electrophoresis in the presence of urea.

3. Results and discussion

In order to test which elements of primary structure of the omega leader are responsible for the formation of a stable tertiary structure we constructed omega sequences modified either at its central CAA-rich part, or at the AU-rich 3′-proximal part. When creating these altered RNAs we took into account the already existing assumptions and experimental data on the possible structural organization of the omega RNA. We studied physical properties of these RNAs by velocity sedimentation and thermal melting techniques.

To explain the possible organization of the core of the omega RNA a model of folding of the regular $(CAA)_n$ sequence was proposed [13]. According to the model, a triple helix can be built by adjacent sections of the polyribonucleotide chain via formation of the nucleotide triads where A, A, and C are arranged in the same plane and joined together with pairs of hydrogen bonds of non-Watson–Crick type. The prediction was made that replacement of A by C in three consecutive CAA triplets should lead to an essential destabilization of the triple helix (A.V. Efimov, personal communication). As the CAA repeats may participate in the formation of a stable secondary structure of the omega RNA [12], we hypothesized that such a replacement should destabilize the entire tertiary structure of the omega leader. To test the prediction, we synthesized omega sequence where three cytosine residues were substituted for three adenine residues (Fig. 1). Fig. 2 shows a schematic representation of these substitution sites on the triple helix model [13]. The synthetic 3A → 3C omega RNA displayed a much lower degree of compactness as compared with the original omega RNA: the sedimentation coefficient of the 3A → 3C RNA was 3.4 S, whereas the sedimentation coefficient of the omega RNA was 4.2 S (Fig. 3). The strong effect, which resulted from only three nucleotide substitutions, implied a significant contribution of the $(CAA)_n$ sequence to the formation of compact tertiary structure of the omega RNA.

This implication was confirmed by experiments on thermal melting. Thermal unfolding of structural nucleic acids is accompanied by so-called hyperchromic effect displayed as the rise of their

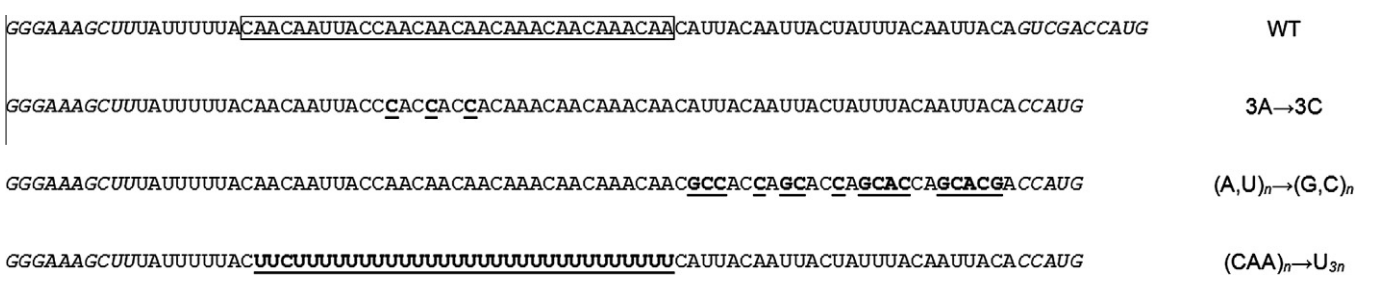

Fig. 1. The sequences of polyribonucleotides studied. WT, wild type omega sequence from TMV strain U1; the central (CAA)-containing part is boxed. 3A → 3C, the omega sequence, in which 3C are substituted for 3A. $(A,U)_n \rightarrow (G,C)_n$, the omega sequence, in which 3′ AU-rich region is replaced by GC sequence. $(CAA)_n \rightarrow U_{3n}$, the omega sequence in which CAA repeats of the central part are completely replaced by uridylic residues. All of the substitutions are shown in underlined bold. The flanked sequences generated from a vector are shown in italic.

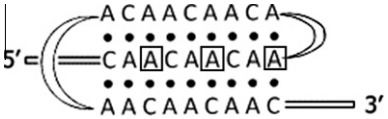

Fig. 2. Schematic representation of the $(CAA)_n$ polyribonucleotide of approximately the same length as central $(CAA)_n$ containing part of the omega RNA in terms of the triple helix model [13]. Three A, which were replaced by three C in this work, are shown in boxes.

UV absorption upon melting of their conformation, and the decreased UV absorption of structured polynucleotides at lower temperatures, or hypochromism, depends mainly on base stacking as a result of secondary and tertiary structure formation. The 3A → 3C RNA possessed less hypochromicity and manifested lower cooperativity in melting experiments as compared with the original omega RNA (Fig. 4). Thus, both sedimentation and thermal melting properties of the 3A → 3C RNA clearly demonstrated that the regular order of CAA nucleotides within the $(CAA)_n$ sequence is important for maintaining the compact and stable structure of the omega RNA. This fact is compatible with the triple helix model proposed for the conformation of the $(CAA)_n$ sequence in the central part of the omega RNA [13]. At the same time, the previously obtained data on sedimentation and thermal melting of the omega RNA and $(CAA)_{19}$ polymer revealed a greater compactness and stability of the omega RNA in comparison with the regular $(CAA)_{19}$ polyribonucleotide [11].

We assumed that the AU-rich sequence in the 3′-proximal part of the omega sequence may participate in compactization and stabilization of the whole structure of the omega RNA. The assumption was based on two experimental observations. First, 3′-proximal AU-rich sequences were found in many representatives of tobamoviruses. Second, nucleotide residues of the 3′-proximal AU-containing part of the omega RNA may form short regions of RNA helices of the A form [12]. In order to elucidate the possible contribution of the 3′-proximal AU-rich sequence of the omega RNA to stabilization of its structure, we synthesized a modified $(A,U)_n \rightarrow (G,C)_n$ RNA in which all of the uracils and some adenines of the 3′-proximal part were replaced by guanines and cytosines (Fig. 1). As with the 3A → 3C RNA, the $(A,U)_n \rightarrow (G,C)_n$ RNA was found to be essentially less compact than the omega RNA: its sedimentation coefficient was only 3.5 S (Fig. 3). In addition, the melting curve of the $(A,U)_n \rightarrow (G,C)_n$ RNA revealed the almost complete absence of cooperativity and significant reduction of hypochromicity (Fig. 4). It was quite a surprise that the replacement of adenylic and uridylic residues by guanylic and cytidylic ones in the omega sequence led to such changes: *ceteris paribus*, G and C would

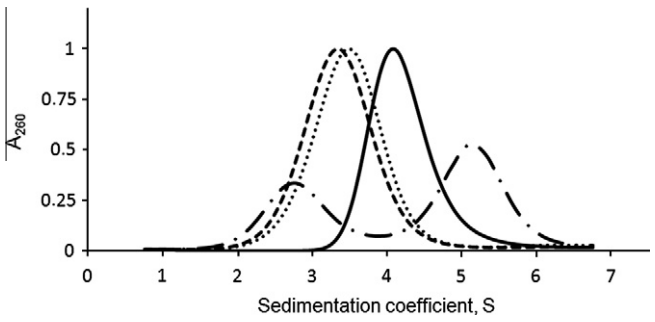

Fig. 3. Sedimentation profiles of RNA samples studied: wild type omega RNA, solid curve; 3A → 3C RNA, dashed curve; $(A,U)_n \rightarrow (G,C)_n$ RNA, dotted curve; $(CAA)_n \rightarrow U_{3n}$ RNA, dashed-dotted curve. Analytical centrifugation experiments were performed in 20 mM Tris–HCl buffer, pH 7.5 with 100 mM KCl and 0.5 mM EDTA. Distribution plots were calculated using Sedfit software.

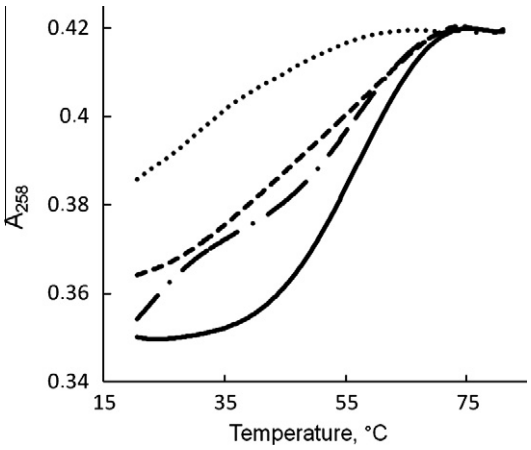

Fig. 4. Thermal melting profiles of RNA samples studied: wild type omega RNA, solid curve; 3A → 3C RNA, dashed curve; $(A,U)_n \rightarrow (G,C)_n$ RNA, dotted curve; $(CAA)_n \rightarrow U_{3n}$ RNA, dashed-dotted curve. The thermal melting experiments were performed in Mg^{2+}-free 10 mM sodium cacodylate buffer, pH 7.5, with 100 mM NaCl and 0.5 mM EDTA.

involve in more stable structure than A and U. In any case, our experiments indicated to the participation of the 3′-proximal AU-rich region of the omega RNA in the formation of its stable compact structure.

Finally, we decided to investigate physical properties of the polyribonucleotide, where CAA repeats of the central part of the omega RNA were completely replaced by uridylic residues (Fig. 1). In this case, we expected to get the most unfolded form of RNA. Fig. 3 demonstrates the sedimentation profile of the $(CAA)_n \rightarrow U_{3n}$ RNA. Unlike the other modified omega RNAs studied, the sedimentation profile of this RNA displayed two components with sedimentation coefficients of 2.9 S and 5.2 S (Fig. 3). We suggested that the slow peak corresponds to the monomeric form of the molecule, whereas the fast peak to its dimer. To test this suggestion we conducted electrophoretic separation under the same undenaturing buffer conditions, as used in sedimentation experiments. Under the undenaturing conditions the $(CAA)_n \rightarrow U_{3n}$ RNA also showed a division into two components (slow and fast migrated bands), while denaturing electrophoresis revealed a homogeneous band of an appropriate length of the monomer (data not shown). It should be also emphasized that both sedimentation and electrophoretic data clearly show that the $(CAA)_n \rightarrow U_{3n}$ RNA exists in solution mainly in the form of a dimer. At the same time, a low value of the sedimentation coefficient of the monomeric form (2.9 S) allowed us to conclude that the $(CAA)_n \rightarrow U_{3n}$ RNA is not compact and rather almost completely unfolded, as we expected. Unfortunately, the presence of mainly the dimeric form of this RNA in solution made the interpretation of the melting data to be extremely difficult. In any case, the melting curve of the $(CAA)_n \rightarrow U_{3n}$ RNA manifested a two-phase character: a low temperature phase and a relatively high temperature phase (Fig. 4). One can suppose that the first low temperature phase corresponds to the melting of the monomeric unfolded form, and the second corresponds to the melting of the dimer.

On the other hand, the possibility of the formation of dimers of the $(CAA)_n \rightarrow U_{3n}$ RNA may be considered as an evidence that the AU-rich 3′-proximal part interacts with the $(CAA)_n$ central part in the original omega RNA. We have reason to believe that the formation of dimers in the $(CAA)_n \rightarrow U_{3n}$ RNA occurs with the participation of the AU-rich 3′-proximal part. Indeed, it is quite difficult to assume that the dimerization involves only interactions between poly(U) sequences. The original omega RNA does not form dimers.

Hence, in the omega RNA, its AU-rich 3′-proximal part that involved in the formation of a stable structure seems to interact with the $(CAA)_n$ central region.

Summarizing the previous results [11–13] and the findings described here, we propose the following rough notion of the structural organization of the omega RNA. Apparently, the central $(CAA)_n$-containing part forms a secondary structure which is the basis (core) of the omega RNA. It is plausible to assume that this part forms the non-canonical triple helix proposed for the regular polyribonucleotide $(CAA)_n$ [13]. The regular central $(CAA)_n$ region and the 3′-proximal AU-rich region of the omega RNA interact with each other and contribute together to the formation of a stable compact tertiary structure, which is unique among the known structures of RNAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.102.

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