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The leader sequence of tobacco mosaic virus RNA devoid of Watson–Crick secondary structure possesses a cooperatively melted, compact conformation

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Abstract

The 5′-untranslated region (5′-UTR) of RNA of tobacco mosaic virus (TMV), called omega sequence, is known as an mRNA leader promoting efficient initiation of translation. The central part of the sequence consists of many CAA repeats, which were reported to be mainly responsible for the enhancing activity of the omega leader. In this work we synthesized the polyribonucleotides containing either the natural omega sequence or the regular $(CAA)_n$ sequence, and studied them using UV spectrophotometry and analytical ultracentrifugation methods. It was demonstrated that the polyribonucleotides manifest significant hypochromicity, cooperative melting of their structures upon heating, high melting temperature, and the sedimentation coefficients typical of compactly folded RNAs of this size. Thus, the omega leader and its core $(CAA)_n$ repeat sequence devoid of secondary structure of the Watson–Crick type seem to be well structured elements of mRNA.

Keywords: Tobacco mosaic virus (TMV) RNA; 5′-Untranslated region; Omega sequence; Translational enhancers; Hypochromicity; Temperature melting; Sedimentation analysis

The rate of mRNA translation by ribosomes and the productivity of protein synthesis strongly depend on the rate of translation initiation. In eukaryotic protein-synthesizing systems 5′-untranslated regions (5′-UTRs) of mRNAs, called also leader sequences, can play a special role of translational enhancers due to their capability to attract (recruit) ribosomes with initiation factors and provide their correct setting at initiation site of mRNA. Among the enhancers are leader sequences of viral RNAs, including the RNAs of most plant viruses. The leader sequence of tobacco mosaic virus (TMV) RNA (the so-called ''omega sequence'') is one of the best studied [1–6]. The TMV RNA leader sequence is about 70 nucleotides long (varying from 60 to 73 in different strains) and contains from 10 to 13 CAA motifs, often in tandems, comprising about half of the leader sequence (see [1,6]). The central CAA-rich region was reported to represent the core regulatory element mainly responsible for the enhancing activity of the leader [6]. Moreover, it was demonstrated that the synthetic regular $(CAA)_n$ sequence also can be an efficient leader for initiation of translation [7]. As no stable secondary structure could be predicted for the omega leader, and especially for its functional CAA-rich core, it was believed that the leader is ''unstructured'' and that the lack of stable folds contributes to its enhancing activity in translation initiation [6]. At the same time it was mentioned that ''an absence of secondary structure alone does not explain translational enhancement'' [8].

Here, we synthesized the polyribonucleotide with omega sequence and studied its UV absorption and sedimentation properties. Unexpectedly, the polymer displayed a

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substantial hypochromicity, cooperative melting of its structure upon heating, high melting temperature, and the sedimentation coefficient typical of compactly folded RNA of this size. In other words, the omega sequence polyribonucleotide proved to be well structured. Taking into account the fact that the section with multiple CAA motifs and CAA tandems of the omega sequence was reported to be the most responsible for the enhancing activity, we also synthesized the regular $(CAA)_{19}$ polyribonucleotide, just flanked with short irregular sequences, and showed that this polymer manifests similar properties of cooperative melting and conformational stability, but being somewhat less compact.

Materials and methods

Materials. RNAase inhibitor RiboLock™, T7 RNA polymerase and NcoI restriction endonuclease were from Fermentas (Lithuania). Plasmids were amplified in *Escherichia coli* strain XL-1 (Promega, USA). Statistical (C,A) polyribonucleotide with C:A ratio of 1:2 was from Reanal (Hungary). $tRNA^{Phe}$ from brewers yeast was purchased from Sigma (USA). Exclusion chromatography was carried out on Superdex 200 10/300 HR column with ÄKTAbasic chromatography system (GE Healthcare, USA).

Plasmids. Plasmid pTZ10Ω*luc* was constructed by O.M. Alekhina (our laboratory) by cloning HindIII–PstI fragment of pΩ*luc* plasmid described in [9] between HindIII and PstI sites of pTZ19R vector (Fermentas). Plasmid p$(CAA)_{19}$-GUS containing β-galactosidase gene with $(CAA)_{19}$ leader sequence under T7 promotor was kindly provided by T.V. Pestova.

In vitro transcription. Transcription was performed as described [10], with minor modifications. The incubation mixture contained 80 mM Hepes–KOH pH 7.5, 10 mM DTT, 2 mM spermidine, 0.01% Triton X-100, 4 mM each of ATP, GTP, UTP, and CTP, 22 mM $MgCl_2$, 8 U/μl of T7 RNA polymerase, 0.2 U/μl of RNAase inhibitor and 200 ng/μl of the plasmid hydrolyzed with NcoI. The reaction was performed at 37 °C for 2 h. The two transcripts were to contain 67 nt omega sequence with flanking sequences GGGAAAGCUU and GUCGACCAUG at 5′- and 3′-ends, respectively, and regular $(CAA)_{19}$ sequence with flanking sequences GCAAGAA and CACCAUG at 5′- and 3′-ends, respectively. After transcription the reaction mixture was subjected to phenol/chloroform extraction, and polyribonucleotides were precipitated from aqueous phase with ethanol in the presence of 2 M NH_4OAc [11]. The transcripts were further purified by gel filtration chromatography with Superdex 200HR column in a buffer 10 mM Hepes–KOH, pH 7.5, 100 mM KCl, and 0.5 mM EDTA. The purity of polyribonucleotides obtained was verified by electrophoresis in 6% polyacrilamide gel with 7 M urea.

Statistical (C,A) polyribonucleotides. High-polymeric statistical (C,A) polyribonucleotide with C:A ratio of 1:2 (Boehringer–Mannheim) was used for preparation of samples with lengths of about 50 ± 10, 80 ± 10, 85 ± 10, and 110 ± 30 nucleotide residues. For this purpose a partial alkaline hydrolysis of the original high-polymer polyribonucleotide was carried out to obtain fractions of the shorter fragments. The hydrolysis was performed in 40 mM $NaHCO_3/Na_2CO_3$ buffer, pH 9.0 with 1 mM EDTA at 90 °C for 5 m [12]. The product was precipitated with 3 volumes of ethanol in presence 0.3 M NaOAc. The pellet was washed twice with 70% ethanol and dissolved in 7 M urea with xylene cyanol FF. Aliquots were loaded on 6% polyacrilamide gel with 7 M urea; 76 nt long $tRNA^{Phe}$ and the RNA with a length of 39 nucleotides were used as reference markers. After electrophoresis the gel was stained in 0.01% Toluidine Blue O solution. The gel segments corresponding to the polyribonucleotide fragments of proper lengths were excised, and the separated polyribonucleotides were eluted from the gel as described in [13].

UV spectrophotometry and temperature dependence of UV absorption. UV absorption measurements were made at 258 nm using Cary 100 spectrophotometer equipped with temperature controller (Varian Inc.). The melting of the polyribonucleotides under investigation was performed in Mg^{2+}-free 10 mM sodium cacodylate buffer, pH 7.5, with 100 mM NaCl, and 0.5 mM EDTA. The buffer was carefully degassed, and optical cells with tightly closing lids were used. In all the experiments the thermal unfolding was shown to be reversible. The integrity of the samples was checked after each melting experiment using polyacrylamide gel electrophoresis in the presence of urea.

Analytical centrifugation. Analytical centrifugation was carried out in the rotor An-60Ti and Optima™ XL-1 Analytical Ultracentrifuge (Beckman–Coulter) in 20 mM TrisOAc buffer, pH 7.5, with 100 mM KCl and 0.1 mM EDTA at 20 °C. Sedimentation data were analyzed using Sedfit (V9.4c) software (http://analyticalultracentrifugation.com/images/sedfit94.exe).

Results

Polyribonucleotide constructs

The full sequences of the polyribonucleotides synthesized for the purposes of their physical studies are presented in Fig. 1. The upper sequence is omega leader of TMV RNA, strain U1 [1], without cap structure and the first G; the sequence is flanked with decanucleotides GGGAAAGCUU and GUCGACCAUG at 5′-end and 3′-end, respectively, so that the omega sequence proper comprised about 80% of the full length of the polynucleotide. The lower sequence is the regular $(CAA)_{19}$ polymer flanked with heptanucleotides GCAAGAA and CACC AUG at 5′-end and 3′-end, respectively.

Hypochromism, melting temperature, and cooperative structural transition of the polyribonucleotides

Thermal unfolding of structured nucleic acids is accompanied by the so-called hyperchromic effect displayed as the rise of their ultraviolet (UV) absorption upon melting of their conformation [14,15]. As the decreased UV absorption of structured polynucleotides, or hypochromism, depends mainly on base stacking as a result of secondary and tertiary structure formation, the recording of the UV absorption in the course of heat denaturation of RNA sample provides information about the amount, stability and cooperativity of intramolecular interactions of a nucleic acid macromolecule.

GGGAAAGCUUUAUUUUUA$(CAA)_2$UUAC$(CAA)_4$A$(CAA)_2$ACAACAUUACAAUUACUAUUUACAAUUACAGUCGACCAUG

GCAAGAA$(CAA)_{19}$CACCAUG

Fig. 1. The sequences of polyribonucleotides comprising the natural 67 nt sequence of omega leader of RNA from tobacco mosaic virus (TMV), strain U1, flanked with 5′- and 3′-decanucleotide sequences (upper) and the synthetic regular $(CAA)_{19}$ sequence flanked with 5′- and 3′-heptanucleotide sequences (lower).

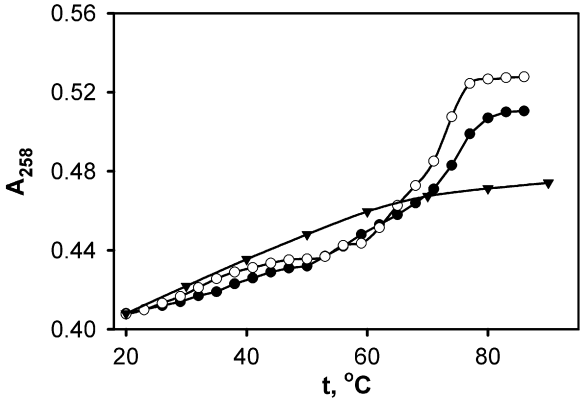

Fig. 2. Thermal melting profiles for the 87 nt polyribonucleotide comprising omega sequence (open circles), the 71 nt polyribonucleotide comprising regular $(CAA)_{19}$ sequence (filled circles), and statistical copolymer of the average length of about 80 nt (triangles) at ionic strength of about 0.1 in a Mg^{2+}-free buffer (10 mM sodium cacodylate, pH 7.5/100 mM NaCl/0.5 mM EDTA). UV absorption at 258 nm is plotted against temperature.

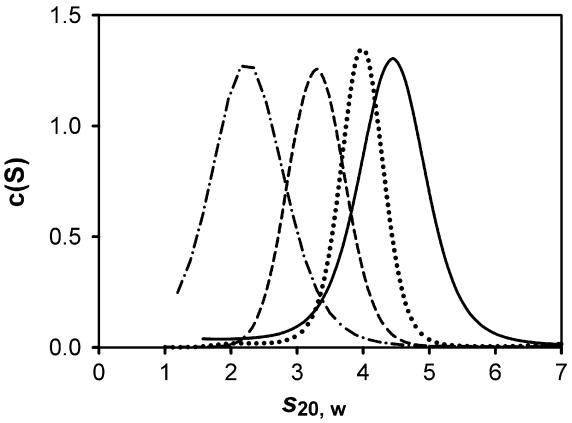

Fig. 3. Sedimentation profiles of the 87 nt polyribonucleotide comprising omega sequence (solid curve), the 71 nt polyribonucleotide comprising regular $(CAA)_{19}$ sequence (dashed curve) and 76 nt yeast $tRNA^{Phe}$ (dotted curve), as compared with that of statistical (C,A) polyribonucleotide of about 80 nt length (dashed-dotted curve) at ionic strength of about 0.1 in a Mg^{2+}-free buffer (20 mM Tris acetate, pH 7.5/100 mM KCl/0.1 mM EDTA) at 20 °C. Distributional plots were calculated from analytical centrifugation experiments using Sedfit software.

Fig. 2 demonstrates the experimental curves of the temperature dependence of UV absorption at 258 nm for three polyribonucleotides: (i) statistical (C,A) copolymer of average length of about 80 nt (fraction of 80 ± 10) with C:A ratio of 1:2, (ii) regular $(CAA)_{19}$ polymer with additional heptanucleotide sequences at its ends (the total length is 71 nt), and (iii) natural 67 nt omega sequence with additional decanucleotide sequences at its ends (the total length is 87 nt). It is seen that the statistical (C,A) polyribonucleotide possesses a relatively low hypochromism and indicates no cooperative melting. At the same time, both the regular $(CAA)_n$ polyribonucleotide and the polyribonucleotide with natural omega sequence display a substantial hypochromism and a cooperative transition in the high-temperature region, with melting temperature of about 70–75 °C (in Mg^{2+}-free buffers). The latter curves remarkably remind the temperature melting curves of tRNAs, very well structured polyribonucleotides, when recorded under similar ionic conditions [16]. It should be mentioned that all the polyribonucleotides studied here have a size (polynucleotide length) similar to that of tRNA molecules.

An attention should be paid to the fact that the melting curves of both the $(CAA)_{19}$ polyribonucleotide and the omega polyribonucleotide manifest a two-phase character: along with the high-temperature cooperative phase, a low-temperature non-cooperative phase is present (Fig. 2). Taking into account the homogeneity of the samples, as seen from the symmetrical sedimentation distribution (see below, Fig. 3), this fact may imply that a well-structured, stable core of the polyribonucleotides is supplemented by less structured, conformationally unstable sections of the molecules.

Folding compactness of the polyribonucleotides

It is well known that hydrodynamic properties, in particular sedimentation coefficients ($s_{20,w}$), reflect the mass and the shape of macromolecules, and in the case of the same chemical nature and similar molecular masses the value of $s_{20,w}$ indicates the level of compact folding of biological polymers [15].

In our experiments the sedimentation study of the 71 nt $(CAA)_{19}$ polyribonucleotide and the 87 nt omega polyribonucleotide was made in a comparison with the sedimentation behavior of a paradigmatic well-structured RNA of a similar molecular mass, namely 76 nt yeast $tRNA^{Phe}$ (Fig. 3). As seen from Fig. 3, all three samples displayed a homogeneous sedimentation distribution (symmetrical peaks) without the presence of denatured forms and other UV absorbing material. The $s_{20,w}$ values calculated from the sedimentation diagrams were as follows: 4.0S for $tRNA^{Phe}$, 3.3S for the $(CAA)_{19}$ polyribonucleotide, and 4.4S for the omega polyribonucleotide. At the same time, the $s_{20,w}$ value of the statistical (C,A) polyribonucleotide with the length of about 80 nt was 2.2S.

Fig. 4 represents the theoretical dependences of sedimentation on logarithm of molecular masses for compact particles (the upper solid line, $s \propto M^{2/3}$) and random coils (the lower dashed line, $s \propto M^{0.5}$ (see Ref. [15], p. 1027). The experimental values for the regular $(CAA)_{19}$ polyribonucleotide, omega sequence and tRNA, as well as for 5S RNA [17], together with those of statistical (C,A) copolymers with average lengths of 50, 80, 85, and 110 nucleotides are also plotted in Fig. 4. It is seen that the sedimentation coefficients of statistical (C,A) polyribonucleotides correspond rather to random coil molecules, just demonstrating a slightly higher compactness, as compared with ideal random coils, probably due to some inter-purine stacking interactions quite possible in such a A-rich polymer. On the contrary, all the other polyribonucleotides studied, including omega and $(CAA)_{19}$, manifest their closeness to the compact particles line. It is remarkable that the compactness of the omega

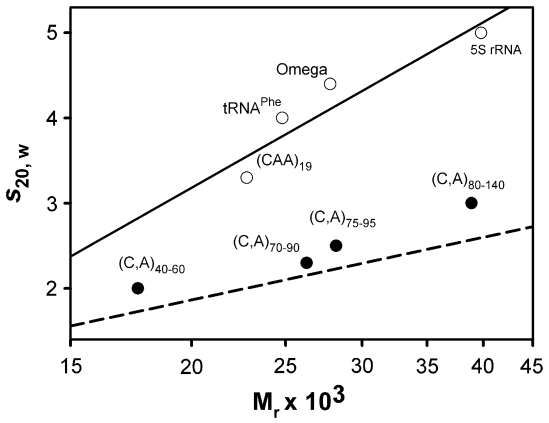

Fig. 4. Relationship between molecular masses and sedimentation coefficients. Theoretical line for random coil molecules is dashed; line for compact near-spherical particles is solid. $(CAA)_{19}$, omega, $tRNA^{Phe}$ and 5S rRNA polyribonucleotides are symbolized by empty circles, statistical copolymer fractions—by filled circles.

polyribonucleotide proved to be not less than that of tRNA. The regular $(CAA)_{19}$ polymer is found less compact than omega and tRNA, but still sufficiently more compact than the statistical (C,A) copolymer.

Discussion

It is quite obvious that both sequences presented in Fig. 1 are unable to form a secondary structure through complementary base interactions of the Watson–Crick type. Correspondingly, the methods and programs used for prediction of folding patterns of the polyribonucleotides [18–20] show very low or zero capacity for folding in these polymers. At the same time, our experiments demonstrate that the polyribonucleotides are well-folded and possess an essential conformational hypochromism (at room temperature and ionic strength of about 0.1, even in a Mg^{2+}-free buffer). Temperature melting experiments reveal a significant stability, at least for some core structure, which cooperatively melts in the region of 70–75 °C. Moreover the dependence of sedimentation coefficients on the molecular masses of omega and $(CAA)_{19}$ polyribonucleotides are typical of compact molecules.

Our attempts to find extended CAA-rich tracks and their conformations among known secondary and tertiary structures of RNAs were unsuccessful. The only observations was that CAA motif looks "anti-helical", as it is rarely found inside double-helical regions of known RNA structures, but it is often present in end loops of helical hairpins (see, e.g., helices H9, H15, H21, H27, H31, H42 of 16S ribosomal RNA of *E. coli* [21,22]) and, less frequently, in interhelical strands. Thus, by now the secondary and/or tertiary structures of the $(CAA)_n$ polyribonucleotide and the CAA-rich omega leader seem to be a puzzle that requires special attention and further experimental and theoretical studies.

In any case, the generally accepted notion that leaders of mRNA must be "unstructured" in order to efficiently initiate translation [23–25] and that omega leader of TMV RNA represents such a case [4,26] should be reconsidered. It is a unique secondary/tertiary structure of the omega leader that may provide specific binding of initiation factors and other proteins which can be relevant to high rate of translation initiation. In this connection it seems worthy to mention the protein called 102-kD or p102 that was reported to be a specific omega-binding protein directly interacting with the poly (CAA) region and actively contributing to the enhancing function of the TMV RNA leader [25,26].

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