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Visualization of the reconstituted FRGY2–mRNA complexes by electron microscopy

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Abstract

Xenopus oocytes store large quantities of translationally dormant mRNA in the cytoplasm as storage messenger ribonucleoprotein particles (mRNPs). The Y-box proteins, mRNP3 and FRGY2/mRNP4, are major RNA binding components of maternal storage mRNPs in oocytes. In this study, we show that the FRGY2 proteins form complexes with mRNA, which leads to mRNA stabilization and translational repression. Visualization of the FRGY2–mRNA complexes by electron microscopy reveals that FRGY2 packages mRNA into a compact RNP. Our results are consistent with a model that the Y-box proteins function in packaging of mRNAs to store them stably for a long time in the oocyte cytoplasm.

Keywords: mRNP; Electron microscopy; Y-box protein; *Xenopus* oocyte

As the genome DNA is encompassed into a nucleosome structure, mRNA in eukaryotic cells is considered to be packaged into messenger ribonucleoprotein particles (mRNPs). The functions of the complex formation of genome DNA or mRNA with specific proteins include the compaction of long nucleic acids, protection from attack by nucleases, and most importantly, the impact on the regulation of gene expression.

Y-box proteins are known as major proteins that associate with mRNA in cytoplasmic mRNPs. Albeit they were originally identified as transcriptional factors that bind to the Y-box sequences in the promoter of the major histocompatibility complex class II genes, later studies revealed that they are implicated in various aspects of gene regulation [1–3]. Studies on cytoplasmic mRNPs in various cell types identified the Y-box proteins as core components of mRNPs. In *Xenopus* oocytes, the Y-box protein FRGY2/mRNP4 and its homologue mRNP3 are the major components of storage mRNPs and are involved in the translational repression of maternal mRNA [4–6]. FRGY2 represses translation through sequence-dependent and -independent interactions with mRNA [7,8]. The rabbit Y-box protein p50/YB1 is a core component of the globin mRNP and regulates its translation positively or negatively, depending on the molar ratio of p50 to mRNA [9]. Moreover, overexpression of the Y-box protein in cultured cells or in transgenic mice resulted in translational repression [10–12]. These results established the importance of the precise regulation of the amount or activity of the Y-box protein in the control of protein synthesis in a cell. Y-box proteins are thought to package mRNA into mRNPs, although the molecular nature of this packaging has not been clarified.

In this study, we prepared FRGY2–mRNA complexes using the purified components and found that the complex formation protects mRNA from the attacks by nucleases. Translation of mRNA was repressed, depending on the complex formation with FRGY2. The analysis of complexes by electron microscopy (EM) re-

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vealed the packaging of mRNA with proteins into compact structure as compared with naked mRNA. Our results of this study using this simple reconstitution system may provide initial clues to the organization and structure of mRNPs and will help us gain insight into how mRNPs are assembled and remodeled.

Materials and methods

Protein. The recombinant FRGY2 protein was expressed in *Escherichia coli* (*E. coli*) and purified as described previously [13].

Preparation of mRNA. To prepare capped *Xenopus* histone H1 mRNA, the pSPH1.11 plasmid was digested with *Eco*RI and the resulting DNA was used for in vitro transcription with SP6 RNA polymerase in the presence of the cap homologue and [α-^{32}P]UTP as described previously [14]. For *Xenopus* TFIIIA mRNA, an A_{30} tail was added to the TFIIIA full-length cDNA by PCR amplification with a primer set, GCACATGCATGCTGGCTGCGGGAAATGCTTTGC AAT and GCCGGTACCGTCGACTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTGCTGCACCTCACTTTTAATAGC. The PCR product was digested with *Sph*I and *Sal*I and then cloned into *Sph*I- and *Sal*I-digested pDTFL [14]. The resulting plasmid DNA, pDTFLpA, was linearized with *Sal*I and used for in vitro transcription with T7 RNA polymerase. The homogeneity of in vitro synthesized mRNA was confirmed by electrophoresis in an agarose gel containing formaldehyde. mRNA was denatured by incubation at 65 °C for 10 min and immediately chilled on ice just before use.

Complex formation and gel retardation assay. In standard reactions, 1 pmol of in vitro synthesized mRNA and various amounts of the FRGY2 protein were incubated at 30 °C for 20 min in 25 μl of buffer A (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, and 0.8 U/μl RNase inhibitor) [13]. Ten microliters of each reaction mixture was electrophoresed in a 1% agarose gel in 0.5× Tris–borate/EDTA buffer at room temperature. The gel was then dried and subjected to autoradiography.

In vitro translation. One picomole of mRNA was incubated with FRGY2 under the standard conditions (see above). An aliquot (12.5 μl) of this mixture was then incubated at 30 °C for 90 min in a 30 μl reaction mixture containing 15 μl rabbit reticulocyte lysate, 16.7 μM each of amino acids including 25 μCi [^{3}H]lysine, 5 μCi [^{3}H]arginine, and 20 U RNase inhibitor. The mixture was digested with RNase A and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) [8].

CsCl density gradient. One picomole of mRNA was incubated with 2.1 μg FRGY2 under standard conditions. The complexes were fixed by adding sodium phosphate buffer, pH 7.0, and formaldehyde at a final concentration of 0.1 M and 3.6%, respectively, and incubated on ice for 2 h [15]. Fixed mRNPs were then centrifuged through a 25–50% CsCl gradient containing 0.1 M sodium phosphate buffer, pH 7.0, and 3.6% formaldehyde for 20 h at 33,000 rpm in an SW55Ti rotar (Beckman) at 20 °C. The samples were collected from the top into 25 fractions of 200 μl. The refractive index and radioactivity of each fraction were determined. The protein/RNA ratio within the complexes was estimated from the buoyant density in CsCl solution as described by Preobrazhensky and Spirin [16].

Nuclease digestion. Ten femtomoles of mRNA was incubated with 1.4 μg FRGY2 at 30 °C for 20 min in 50 μl buffer A containing 30% glycerol. Ten microliters of the mixture was then incubated with micrococcal nuclease in the presence of 2.5 mM $CaCl_2$ at 30 °C for 5 min. The RNA was then purified and electrophoresed in a 6% polyacrylamide gel containing 8 M urea.

Electron microscopy. One picomole of mRNA was incubated with 2.1 μg FRGY2 at 30 °C for 20 min in 25 μl buffer B (10 mM triethanolamine, pH 7.9, 100 mM KCl, and 4 mM $MgCl_2$). To fix the complexes, 5 μl of 1% formaldehyde in buffer B was added and the mixture was incubated at 30 °C for 10 min, followed by the addition of 7.5 μl of 18.5% formaldehyde in buffer B and further incubation at 63 °C for 20 min. The sample was prepared on carbon-coated grids by a spreading method with benzalkonium chloride (BAC) and staining with uranyl acetate as described by Vollenweider et al. [17,18]. The grids were rotary shadowed at an angle of 3° with platinum and examined under a JEOL JEM-1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

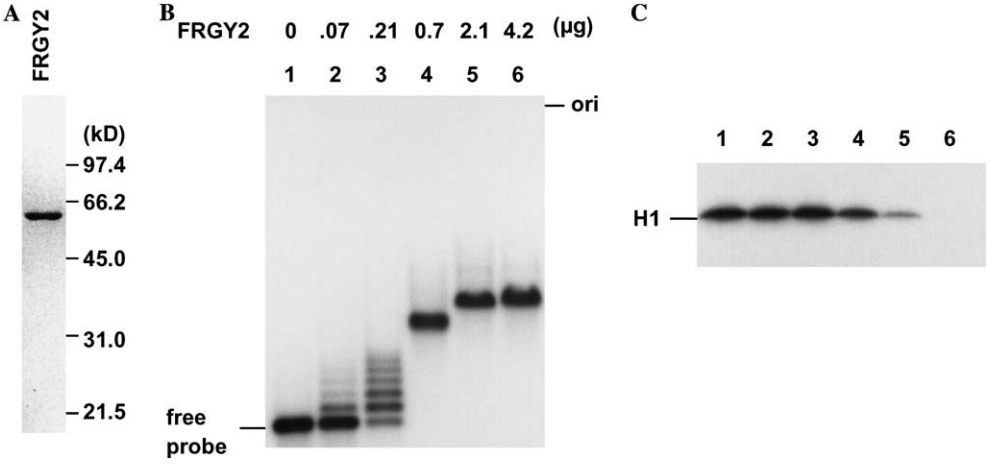

Fig. 1. Reconstitution of the FRGY2–mRNA complexes and translational repression. (A) Preparation of the recombinant FRGY2. The recombinant FRGY2 expressed in *E. coli* was purified and analyzed in SDS–PAGE. The gel was stained with Coomassie brilliant blue. (B) Gel retardation assay. One picomole of ^{32}P-labeled histone H1 mRNA and the indicated amounts of FRGY2 were incubated in a 25-μl reaction mixture. An aliquot (10 μl) was electrophoresed in an agarose gel. The gel was dried and subjected to autoradiography. Ori indicates the position of gel wells. (C) Translational repression by FRGY2. An aliquot (12.5 μl) of the reaction mixture prepared as in (B) was incubated with rabbit reticulocyte lysates as described in Materials and methods. The product was analyzed in SDS–PAGE and the gel was subjected to fluorography. The translation product was quantified by densitometry. Relative translational activities normalized by the value in lane 1, which was 100%, are 105% (lane 2), 104% (lane 3), 68% (lane 4), 8% (lane 5), and 0% (lane 6).

Results and discussion

To prepare FRGY2–mRNA complexes for EM analysis, we first performed the gel retardation assay (Fig. 1A). By titrating the purified recombinant FRGY2, a gradual mobility shift of ^{32}P-labeled mRNA was observed in an agarose gel (Fig. 1B). It should be noted that while the FRGY2–mRNA complexes were electrophoresed as a ladder of bands or a smear when the FRGY2 concentration was low, the complexes were detected as discrete bands in the case of using 0.7 μg of FRGY2 or higher, implying the formation of fairly uniform FRGY2–mRNA complexes and the cooperative RNA binding of FRGY2 proteins. Formation of the complexes in the presence of saturating amounts of FRGY2 well correlated with the translational repression of mRNA (Fig. 1C [8]).

Although we wished to increase the FRGY2 concentrations even after reaching the plateau, the available FRGY2 concentration was limited. We then instead used a lower concentration of mRNA in the gel retardation assay (Fig. 2A). Interestingly, the mobility of FRGY2–mRNA complexes at plateau levels was the same as that at the 200-fold decrease in RNA concen-

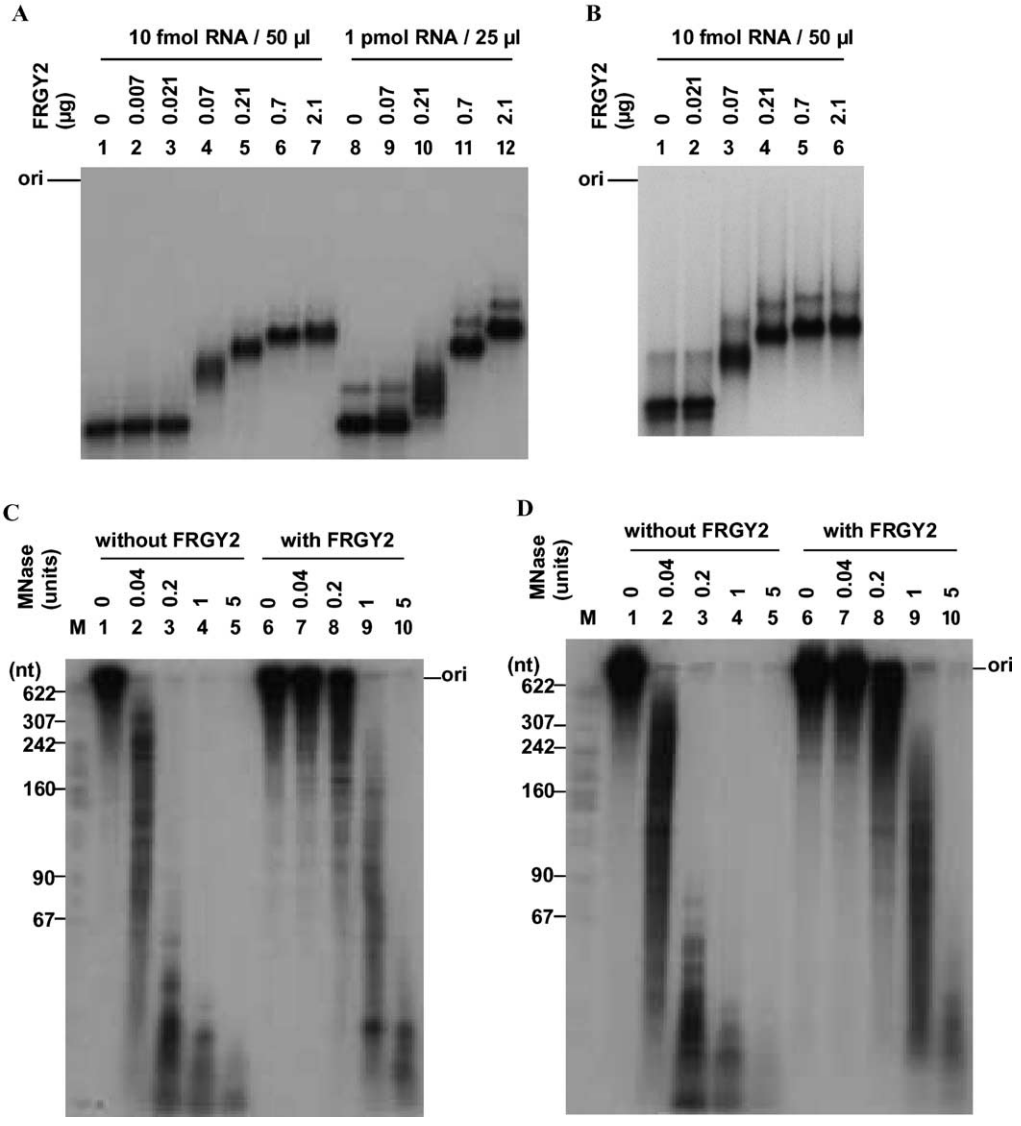

Fig. 2. Protection of mRNA from the nuclease attack by its complex formation with FRGY2. (A) Gel retardation assay with histone H1 mRNA. Ten femtomoles (lanes 1–7) or 1 pmol (lanes 8–12) of ^{32}P-labeled histone H1 mRNA was incubated with the indicated amounts of FRGY2 in a 50 μl (lanes 1–7) or 25 μl (lanes 8–12) reaction mixture. An aliquot (10 μl) was analyzed in an agarose gel. (B) Gel retardation assay with TFIIIA mRNA. Ten femtomoles of ^{32}P-labeled TFIIIA mRNA was incubated with FRGY2 in a 50-μl reaction mixture and an aliquot was analyzed in an agarose gel. (C,D) MNase digestion. Ten femtomoles of mRNA encoding histone H1 (C) or TFIIIA (D) was incubated with 1.4 μg of FRGY2 in a 50-μl reaction mixture. An aliquot (10 μl) was then digested with MNase. RNA was purified and analyzed in a polyacrylamide gel containing urea. Lane M is ^{32}P-labeled *Msp*I-digested pBR322. Ori indicates the position of gel wells.

tration in the binding reaction. At higher concentrations of FRGY2, aggregates of mRNA with proteins were not observed, which would otherwise be retained in gel wells. TFIIIA mRNA, the length of which is twice that of histone H1 mRNA, formed the complexes in the same FRGY2 dose-dependent manner as H1 mRNA (compare Fig. 2A lanes 1–7 with Fig. 2B).

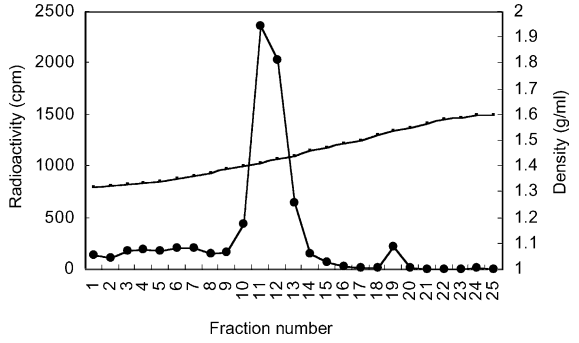

Fig. 3. Determination of the buoyant density of FRGY2–mRNA complexes in a CsCl gradient. The FRGY2–histone H1 mRNA complexes were fixed with formaldehyde and loaded on a 25–50% CsCl gradient. After ultracentrifugation, 25 fractions were collected from the top. The density (squares) was calculated from the refractive index and radioactivity (circles) of each fraction was determined. Note that naked mRNA, which was loaded on a separate CsCl gradient, was collected from the bottom of the tube after centrifugation (not shown).

Next, we examined whether the complex formation with FRGY2 protects mRNA from RNase digestion. Thus, naked mRNA and mRNA complexed with FRGY2 were digested with an increasing amount of micrococcal nuclease (MNase) and the resulting fragments were analyzed in a polyacrylamide gel (Figs. 2C and D). Titration of MNase indicates that the FRGY2–mRNA complex formation resulted in >5-fold decrease in the sensitivity to RNase digestion of mRNA (compare lanes 3 and 8 in Fig. 2C or D, for example). Our results support previous studies showing that the Y-box protein YB1 functions as an mRNA stabilizer in an AU-rich element-dependent or -independent manner [19,20]. We reasoned that digestion with MNase would reveal some structural units in the FRGY2–mRNA complexes, if any, since MNase preferentially recognizes the linker region of nucleosomes rather than the DNA region associated with histones; thus, digestion with MNase reveals nucleosomal structure. However, either with mRNA encoding histone H1 (Fig. 2C) or TFIIIA (Fig. 2D), we did not observe any repeating structures after MNase digestion. An RNA homopolymer may be used in order to test whether FRGY2 associates with RNA regularly as is the case with the poly(A) RNP containing the poly(A) binding protein, which also protects poly(A) from nuclease digestion [21].

To further characterize the FRGY2–mRNA complexes, we subjected them to ultracentrifugation in a

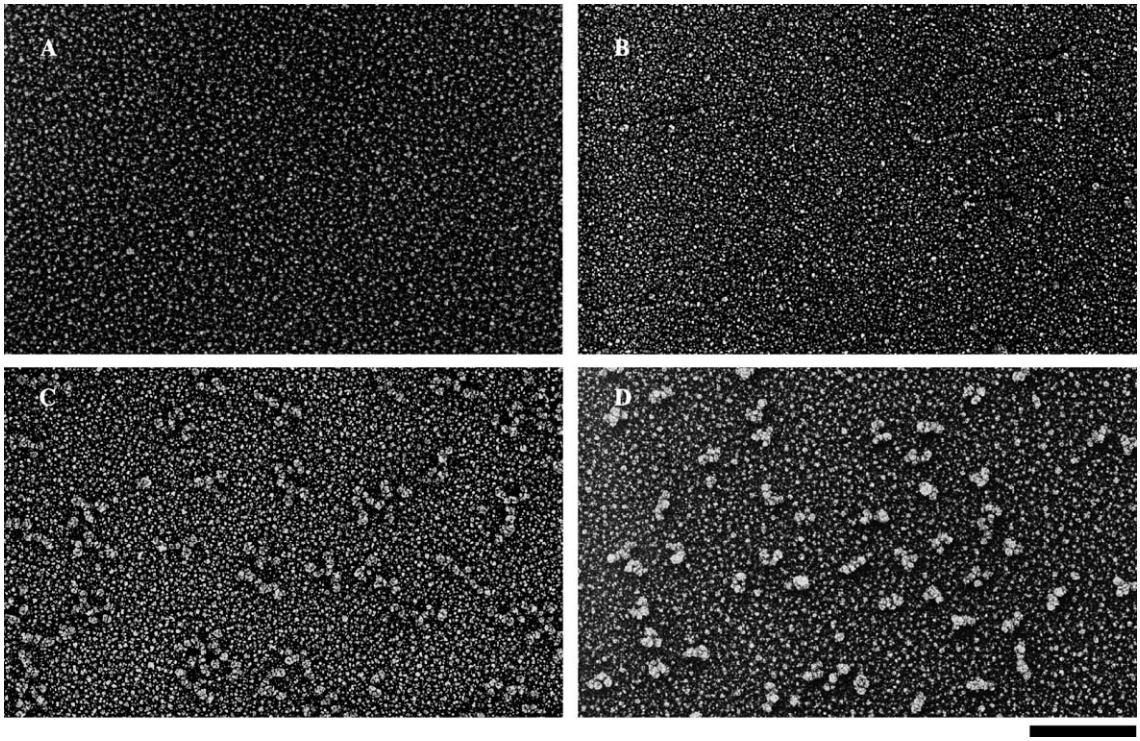

Fig. 4. Visualization of the FRGY2–mRNA complexes by electron microscopy. (A) A field of control BAC spreading without RNA and protein. (B) A field of naked TFIIIA mRNA. (C) A field of FRGY2–TFIIIA mRNA complexes. (D) A field of FRGY2–histone H1 mRNA complexes. The size bar indicates 200 nm.

CsCl gradient (Fig. 3). By determining the buoyant density of the complexes in CsCl solution, one can estimate the molar ratio of protein to RNA within ribonucleoprotein complexes [16]. The FRGY2–histone H1 mRNA complexes showed a rather sharp distribution with a peak at the density of 1.41 g/ml. This value suggests that these complexes contained 73% (w/w) protein. Since FRGY2 has a molecular mass of 35 kDa, every 35–40 nucleotides of RNA are bound by a single FRGY2 molecule in these complexes if one assumes that FRGY2 molecules are evenly distributed on mRNA. Maternal mRNPs purified from *Xenopus* oocytes have the buoyant density of 1.34–1.36 g/ml [15,22]. Reconstitution of mRNPs with oocyte poly(A)$^+$ RNA or in vitro synthesized cyclin mRNA and purified oocyte mRNP proteins results in the formation of particles with the density of 1.34–1.45 g/ml [15,23]. The reconstituted mRNPs with purified proteins generally have higher densities than the native mRNPs possibly because of the lack of other mRNP proteins ([23] and this study).

Based on the results showing that the FRGY2–mRNA complexes formed at the saturating amount of FRGY2 had a discrete mobility in gel retardation assay and showed a sharp distribution in a CsCl gradient, we concluded that under our conditions FRGY2 is capable of associating with RNA to form fairly uniform complexes. We prepared samples of naked mRNA and FRGY2–mRNA complexes by the BAC spreading method, followed by platinum rotary shadowing [17,18]. Representative fields of the FRGY2–mRNA complexes prepared with histone H1 mRNA (0.75 kb) and TFIIIA mRNA (1.5 kb) are shown in Fig. 4. Under our conditions, naked RNA in an extended structure was visualized as a very thin filament, with almost the same width as those of platinum particles (compare Figs. 4A and B). The FRGY2–mRNA complex appeared to be a consecutive series of several particles and the size of the complexes depended on the length of mRNA used (Figs. 4C and D).

Analysis of individual complexes at higher magnification revealed that they consisted of particles of 10–20 nm in diameter (Fig. 5). The length of the complexes had a relatively wide range of distribution (90–150 nm), implying that the proteins bind to different positions of RNA resulting in the distinct folding of RNA, although the number of protein molecules that bind to a single RNA molecule is rather constant between the complexes (see Fig. 3). The average length of the complexes was about 125 nm. Given that the length of 1.5 kb TFIIIA mRNA is approximately 500 nm (1500 nt $\times$ 3.5 Å/nt = approximately 5000 Å), its complex formation with FRGY2 likely results in three or fourfold compaction of the length when compared to the naked RNA. Portions of naked RNA were not clearly observed in most of the complexes, except in a small number of examples shown at the bottom of Fig. 5, suggesting the packaging of mRNA with FRGY2. *E. coli* CspD, which is highly homologous to the cold shock domain of the eukaryotic Y-box proteins, binds to single-stranded DNA in patches, forming unlinked dot-like structures [24]. The carboxyl-terminal tail domain, which has been shown to mediate the multimerization of FRGY2 and is absent in

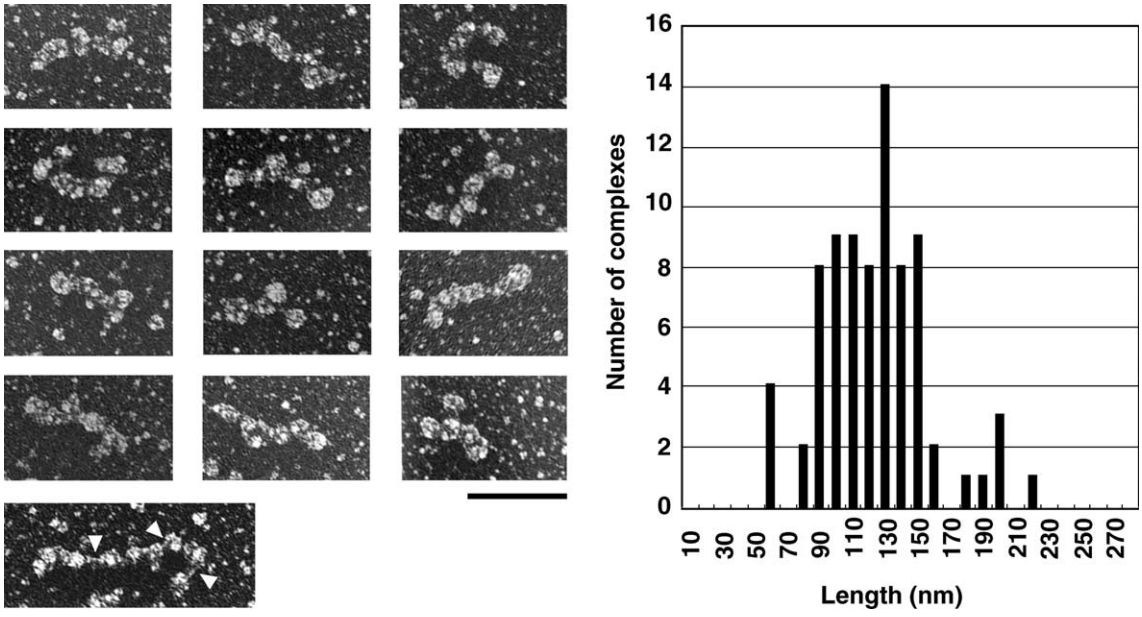

Fig. 5. EM of individual FRGY2–mRNA complexes. Representative micrographs of the FRGY2–TFIIIA mRNA complex are shown. The size bar indicates 100 nm. The length of each complex was measured and summarized in a histogram. A micrograph at the bottom shows an example of the complexes with the naked RNA portion, which were not included in the histogram. Portions of naked RNA are indicated by arrowheads.

CspD, might be required for the packaging of RNA [3,25]. In fact, the tail domain is essential for the incorporation of FRGY2 into mRNPs in the oocytes [8].

The reconstituted FRGY2–mRNA complexes prepared in this study appear to satisfy the three possible functions of storage mRNPs in oocytes: packaging of mRNA into a compact structure, protection of mRNA from the attack by nucleases, and repression of translation. Formation of mRNPs with Y-box proteins may be therefore a prerequisite to storage of maternal mRNA in a translationally inert state in the oocyte cytoplasm for a long period of time. Purification of cytoplasmic mRNPs, and their visualization and characterization are the next steps to gaining more insights into the structure of mRNPs.

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