

Ultrastructure of influenza virus ribonucleoprotein complexes during viral RNA synthesis

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2 **viral RNA synthesis**

3

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18

19 The single-stranded, negative-sense, viral genomic RNA (vRNA) of influenza A virus is
20 encapsidated by viral nucleoproteins and an RNA polymerase to form a ribonucleoprotein
21 complex (vRNP) with a helical, rod-shaped structure. The vRNP is responsible for
22 transcription and replication of the vRNA. However, the vRNP conformation during viral
23 RNA synthesis is not well understood. Here, using high-speed atomic force microscopy
24 and cryo-electron microscopy, we investigated the native structure of influenza A vRNPs
25 during RNA synthesis *in vitro*. Two distinct types of vRNPs were observed in association
26 with newly synthesized RNAs: an intact, helical rod-shaped vRNP connected with a
27 structured viral RNA product and a deformed vRNP associated with a looped, double-
28 stranded RNA, composed of a template vRNA and a nascent RNA. These results suggest
29 that while some vRNPs keep their helical structures during viral RNA synthesis, probably
30 for the repeated cycle of transcription and/or replication, others accidentally become
31 structurally deformed, which probably results in failure to commence or continue RNA
32 synthesis. Thus, our findings provide the ultrastructural basis of viral RNA synthesis and
33 advance our knowledge of the mechanism of viral transcription and replication.

34

35 Influenza A virus, a member of the *Orthomyxoviridae*, has eight, single-stranded,
36 negative-sense RNA segments (vRNAs) as its genome. Transcription and replication of
37 influenza A virus are carried out by ribonucleoprotein complexes called vRNPs in the
38 nucleus of infected cells. A vRNP consists of a vRNA, multiple copies of nucleoprotein
39 (NP), and a heterotrimeric, RNA-dependent RNA polymerase complex comprising PB2,
40 PB1, and PA subunits (1–4). Each vRNP adopts a helical, rod-shaped structure, in which
41 an NP-vRNA strand is folded back on itself and coiled, forming a double-stranded helix
42 with a loop structure at one end (5, 6). The heterotrimeric RNA polymerase is located
43 opposite to the loop end of the helical rod-shaped vRNP (7–9).

44 Although influenza virus vRNPs conduct both transcription and replication of the
45 vRNAs, mechanisms of the two processes are quite different. During transcription, the
46 PB2 subunit binds to the 5'-terminal methylated cap structure ($m^7GpppXm$) of host pre-
47 mRNAs (10, 11), and the PA subunit cleaves the pre-mRNA 10–13 nucleotides
48 downstream from the cap with its endonuclease activity (12–14). The resultant capped
49 RNA fragment is directed to the PB1 active site where it is used as a primer (15). After
50 elongation, a poly(A) tail is added to the 3' end of the transcript by stuttering of the
51 polymerase on the oligo-U stretch of the template vRNA (16–18). Hence, the 5'-capped
52 and 3'-polyadenylated viral mRNAs are synthesized in a primer-dependent manner. In

53 contrast, genome replication is thought to be primer-independent (19). Replication
54 involves generation of positive-sense complementary RNAs (cRNAs), which are
55 replication intermediates that act as templates for vRNA synthesis. Elongation of a
56 nascent cRNA by *cis*-acting RNA polymerase proceeds concomitantly with sequential
57 binding of free NPs, forming a rod-shaped, double-helical cRNP complex (20–22).
58 Afterward, *trans*-acting or *trans*-activating RNA polymerase generates vRNAs from
59 intermediate cRNAs (21, 23, 24).

60 Great progress has been made in delineating the molecular mechanisms by which
61 the RNA polymerase conducts transcription and replication based on its atomic structure
62 (15, 18, 24–30). However, it is the vRNP rather than the RNA polymerase that
63 accomplishes viral genome transcription and replication. Recently, Coloma *et al.*
64 investigated the structure of the vRNP during *in vitro* transcription using cryo-electron
65 microscopy (cryo-EM) and concluded that the vRNPs maintain their double helical
66 structures during transcription (31). However, because nascent RNAs were barely visible
67 together with potentially transcribing vRNPs, it remains unclear whether the vRNPs
68 observed were producing nascent RNA. Here, to further characterise transcription and
69 replication from an ultrastructural perspective, we analysed virion-derived vRNPs
70 producing nascent viral RNAs during *in vitro* RNA synthesis, using high-speed atomic

71 force microscopy (HS-AFM) and cryo-EM. The combination of these two techniques
72 enabled us to clearly visualize and characterise the native structures of vRNPs producing
73 nascent viral RNAs.

74

75 **Results**

76 **Virion-derived vRNPs produce both mRNA and cRNA *in vitro***

77 It has been reported that vRNPs isolated from influenza virions synthesize both mRNA
78 and cRNA *in vitro* by adding ApG or globin mRNA as a primer (32). To investigate the
79 structure of vRNP during transcription and replication, we purified vRNPs from influenza
80 A virions and performed *in vitro* RNA synthesis using primers. Autoradiography of the
81 RNA products after electrophoresis showed bands corresponding to the eight vRNAs of
82 influenza A virus in a 15-min incubation after the reactions (Fig. 1a, Supplementary Fig.
83 1a, b). Treatment of an influenza virus RNA polymerase inhibitor, 6-fluoro-3-hydroxy-2-
84 pyrazinecarboxamide-4-ribofuranosyl-5'-triphosphate (T-705RTP) (33), decreased the
85 band intensity in a dose-dependent manner (Fig. 1b), confirming that viral RNAs are
86 synthesized from virion-derived vRNPs.

87 Then, to determine whether vRNPs produce both cRNA and mRNA, we
88 performed strand-specific reverse transcription-quantitative real-time polymerase chain
89 reaction (RT-qPCR) for the NP and NA genes (34). Here, because 5' capped mRNA cannot
90 be generated using ApG as a primer, we defined positive-strand viral RNA containing
91 poly(A) tail at its 3' end as mRNA. Non-specific amplification was barely detectable
92 (Supplementary Fig. 2a, b). In the absence of the primers, cRNA and mRNA were hardly

93 detected, except for NP mRNA (Supplementary Fig. 2c, d). Addition of either ApG or
94 globin mRNA primer resulted in production of 1×10^5 – 5×10^6 copies μL^{-1} of cRNA and
95 mRNA of both NP and NA segments (Supplementary Fig. 2c, d). These results
96 demonstrated that virion-derived vRNPs produce both cRNA and mRNA in the presence
97 of primers. Since there was no significant difference in the level of viral RNA production
98 between ApG-primed and globin mRNA-primed samples, we used ApG as a primer in
99 subsequent ultrastructural analysis.

100

101 **During viral RNA synthesis, vRNPs show two distinctive structures**

102 Although vRNPs are helical rod-shaped structures in a static state, it is possible that they
103 may change their conformations during viral RNA synthesis. To investigate near-native
104 vRNP structures producing nascent viral RNA, we employed HS-AFM, which can
105 provide topographic images in solution by scanning sample surfaces with a probe tip,
106 without fixation or staining (35). After *in vitro* RNA synthesis, vRNPs were adsorbed
107 onto a mica substrate and were visualized in solution. Without the primer, after *in vitro*
108 RNA synthesis, vRNPs appeared as rod-shaped structures with helical grooves (Fig. 1c),
109 which is typical of vRNP structures visualized by negative-staining EM (5). Immediately
110 after adding ApG primer (0 min), vRNPs maintained helical rod-shaped structures (Fig.

111 [1d](#)). However, after a 15-min incubation with ApG primer, vRNPs showed distinctive
112 structures that were associated with potentially nascent viral RNA. On the basis of their
113 configurations, we classified vRNP-RNA complexes into two groups: helical rod-shaped
114 vRNPs associated with a structured RNA ([Fig. 1e, f](#), [Supplementary Fig. 3](#)), and deformed
115 vRNPs associated with a looped RNA ([Fig. 1g, h](#)). The configuration of vRNPs bound to
116 the structured RNA appeared similar to those of control vRNPs ([Fig. 1c](#)). Their diameters
117 were almost uniform and helical grooves could be observed along entire rod-shaped
118 vRNPs, suggesting no apparent conformational changes. Structured RNAs, which seem
119 to contain some secondary structures, were associated not only with the tip ([Fig. 1e](#),
120 [Supplementary Fig. 3a](#)), but also with the bodies of helical rod-shaped vRNPs ([Fig. 1f](#),
121 [Supplementary Fig. 3b](#)). By contrast, the configuration of vRNPs associated with looped
122 RNAs was substantially deformed, such that helical grooves of the vRNPs had
123 disappeared ([Fig. 1g, h](#)). vRNPs associated with looped RNA comprised 30.2% of all
124 vRNP-RNA complexes (N = 96).

125 To exclude the possibility that vRNPs became physically deformed after being
126 tapped with the AFM probe tip, we observed unstained frozen-hydrated samples of
127 vRNP-RNA complexes using cryo-EM. Both helical rod-shaped vRNPs associated with
128 structured RNA ([Fig. 2a](#)) and deformed vRNPs associated with looped RNA ([Fig. 2b](#))

129 appeared similar to those observed with HS-AFM, suggesting that deformation of vRNPs
130 occurs during RNA synthesis. To further investigate the configuration of vRNP-RNA
131 complexes in more detail, we performed low-dose cryo-electron tomography (cryo-ET)
132 to analyse their three-dimensional structure. A vRNP without *in vitro* RNA synthesis
133 showed a double helical structure with several grooves (Fig. 2c), consistent with vRNPs
134 reconstructed by single-particle cryo-EM (7, 8, 31). The tomographic reconstruction of a
135 deformed vRNP was able to resolve the continuous RNA loop associated with the
136 deformed vRNP (Fig. 2d), where both ends of the loop structure were located relatively
137 close to each other on the deformed vRNP, suggesting that the viral RNA polymerase
138 exists at the looped RNA-binding site, although its structure was not resolved.
139 Interestingly, we found that the vRNP partially maintained a double helical structure at
140 one end, and only the portion to which both ends of the looped RNA were bound was
141 deformed (Fig. 2d), suggesting that deformation of the helical rod-shaped vRNP is likely
142 participating in RNA synthesis. Unfortunately, the helical rod-shaped vRNP associated
143 with a structured RNA could not be technically reconstructed, because RNAs with
144 pleomorphic structure were not visible enough for cryo-ET due to low-contrast images of
145 cryo-EM, as shown previously (31).

146

147 **Both structured and looped RNAs associated with vRNPs are viral RNA products**

148 Next, to determine whether the observed structured and looped RNAs are products
149 synthesized by vRNPs, we used a nucleotide analogue, 5-bromo-UTP (Br-UTP), for *in*
150 *vitro* RNA synthesis. When Br-UTP was used for *in vitro* RNA synthesis instead of UTP,
151 both structured and looped RNAs associated with vRNPs were similarly observed (Fig.
152 3a, b, respectively). On incubation with the antibody, which reacts with Br-UTP present
153 only in a single-strand RNA, specific binding of the antibody to the structured RNAs was
154 observed (Fig. 3c, Supplementary Fig. 4), whereas the antibody did not react with
155 structured RNAs produced by *in vitro* RNA synthesis using UTP (Fig. 3e), indicating that
156 the structured RNAs are single-strand, nascent RNAs synthesized by the associated
157 vRNPs. By contrast, the antibody did not bind to looped RNAs produced during *in vitro*
158 RNA synthesis using Br-UTP (Fig. 3d) or UTP (Fig. 3f). Since the looped RNA has no
159 secondary structure, we presumed that it might be double-stranded RNA (dsRNA). To
160 test this hypothesis, RNase for digesting either single-stranded RNA (RNase A) or dsRNA
161 (RNase III) was added to the looped RNAs and examined *in situ* using HS-AFM. With
162 RNase A treatment, one end of the looped RNA was often detached from the vRNP and
163 became straight, but the looped RNA itself was not digested, suggesting that it is double-
164 stranded except at one end (Fig. 4a, Supplementary movie 1). In contrast, RNase III

165 digested looped RNAs (Fig. 4b, Supplementary movie 2). Upon binding, the RNase III
166 molecule immediately digested the RNA, which was further shortened by sequential
167 binding of more RNase III molecules, confirming that the looped RNA was dsRNA. This
168 finding was further verified using anti-dsRNA antibody. The antibodies efficiently
169 recognised the looped RNA associated with vRNPs (Fig. 4c, Supplementary movie 3),
170 while only a few antibody molecules were bound to parts of the structured RNA, probably
171 through stem-loop regions within the single-stranded RNA (Fig. 4d, Supplementary
172 movie 4). Then, do influenza viruses generate dsRNA during the transcription and
173 replication? To address this question, Vero cells were infected with PR8 virus and were
174 subjected to immunofluorescence assay (IFA) by using anti-dsRNA antibody. At 10 h post
175 infection, dsRNAs were detected in the nucleus of infected cells (Fig. 4e), but not in
176 mock-infected cells, suggesting that influenza viruses produce dsRNA and that the
177 deformed vRNP structures associating with the looped RNA may also be produced in the
178 infected cells.

179 Next, to determine whether double-stranded, looped RNA contains nascent RNA,
180 we used 5-ethynyl-UTP (EUTP) for *in vitro* RNA synthesis and evaluated its
181 incorporation into looped RNAs using Click chemistry. A Click reaction with biotin-azide
182 was followed by incubation with streptavidin, and specific binding of streptavidin to

183 looped RNAs was confirmed with HS-AFM (Fig. 3g). However, without streptavidin
184 (Supplementary Fig. 5a) or a Click reaction (Supplementary Fig. 5b), during *in vitro* RNA
185 synthesis using UTP as a substrate (Fig. 3h), binding of streptavidin to the looped RNAs
186 was not observed, suggesting that double-stranded, looped RNAs encompass nascent
187 viral RNAs produced by associated vRNPs. Taken together, these results indicate that
188 both structured and double-stranded looped RNAs associated with vRNPs are viral RNA
189 products formed during *in vitro* RNA synthesis.

190

191 **vRNA is partially dissociated from deformed vRNP associated with looped dsRNA**

192 Given that looped dsRNA contains a single-strand nascent viral RNA, it is likely that its
193 counterpart is the template vRNA dissociated from the deformed vRNP during RNA
194 synthesis. Hence, it is expected that deformed vRNPs have lower structural stability than
195 intact vRNPs due to loss of its vRNA as a structural component. To determine whether
196 deformed vRNPs lose the vRNA, at least in part, we examined the structural stability of
197 vRNPs by applying force with the cantilever tip during HS-AFM imaging (Fig. 5). Since
198 vRNAs within vRNPs are sensitive to RNase (36), vRNPs treated with a low
199 concentration of RNase A were prepared as control vRNPs that lacked intact residential
200 vRNA (Supplementary Fig. 6). Although vRNPs treated with RNase A maintained their

201 helical rod-shaped structures, they were easily broken with significantly less force (Fig.
202 5a, the bottommost panel) than those vRNPs untreated with RNase A (Fig. 5a, the
203 uppermost panel). vRNPs associated with structured viral RNA were physically stable,
204 much as intact vRNPs, whereas vRNPs associated with double-stranded, looped viral
205 RNA were broken with significantly less force, as is the case with RNase A-treated
206 vRNPs (Fig. 5a, b). Collectively, these results strongly suggest that at least some parts of
207 template vRNA are detached from NPs of vRNP during RNA synthesis, resulting in
208 formation of double-stranded, looped RNA with nascent viral RNA, and consequent
209 deformation of helical rod-shaped vRNPs.

210

211 **Discussion**

212 vRNP is responsible for transcription and replication of the influenza virus
213 genome. Thus far, details of its structure during viral RNA synthesis have been largely
214 unknown due to technical limitations. Here, we employed HS-AFM and cryo-EM to
215 visualise near-native vRNP structures during viral RNA synthesis. By virtue of combining
216 these techniques, we unambiguously demonstrated that two different types of vRNP-RNA
217 complexes are produced during RNA synthesis: helical rod-shaped vRNPs with structured
218 RNA and deformed non-helical vRNPs with looped dsRNA. Our results suggest that
219 some vRNPs probably maintain their helical structures for repetitive transcription and/or
220 replication; however, some vRNPs probably fail repetitive RNA synthesis due to
221 deformation of their helical structures.

222 vRNP synthesizing structured viral RNA was not greatly deformed, and
223 maintained its helical rod-shaped structure (Fig. 1e, f). The helical rod-shaped
224 configuration of vRNP associated with structured RNA was very similar to that of the
225 vRNPs in a static state. However, we presume that these two structures are essentially
226 different. In vRNP in a static state, the RNA polymerase complex exists at the tip of the
227 helical, rod-shaped vRNP, as reported (7–9). However, in vRNP synthesizing structured
228 RNA, localization of the RNA polymerase was likely not limited to the tip of the helical

229 rod-shaped vRNP, since the structured viral RNA was not only associated with the tip,
230 but also with the body of the rod-shaped vRNP (Fig. 1, Supplementary Fig. 3). In support
231 of this observation, Coloma *et al.* recently reported localization of RNA polymerase on
232 the body of the helical rod-shaped vRNP during transcription (31). Although the nascent
233 RNA structures in their cryo-EM images were not well resolved, vRNPs they observed
234 would correspond to vRNPs producing structured RNAs in our observations.

235 Although we could not determine whether structured RNA associated with the
236 helical vRNPs was mRNA or cRNA, the vRNPs with structured RNA are consistent with
237 the progressive helical track transcription model proposed by Coloma *et al* (31).
238 Therefore, we propose that helical vRNPs with structured RNA represent the correct
239 transcription mode, because maintenance of the helical, rod-shaped vRNP structure is
240 favourable to commence the next round of RNA synthesis (Fig. 6). Assuming that the
241 structured RNA is an mRNA, the 5'-terminus of vRNA is associated with RNA
242 polymerase throughout transcription (25, 37). If the 3' end of vRNA was released from
243 RNA polymerase in this process, the helical structure of the vRNP would be largely
244 loosened (Supplementary Fig. 7, pattern A). However, such a loosened vRNP structure
245 has never been observed, suggesting that the 3' end of the vRNA is not detached, which
246 is consistent with a recent finding that the 3' end of vRNA binds to the secondary binding

247 site of RNA polymerase after transcription (18). Therefore, we propose that in
248 transcription, vRNPs maintain the double-helical structure in which both 3' and 5' ends of
249 the vRNA are bound by the RNA polymerase throughout mRNA synthesis
250 (Supplementary Fig. 7, pattern B), which is consistent with a model that Coloma *et al.*
251 recently proposed (31).

252 In contrast, the structure of vRNP associated with the looped RNA was largely
253 deformed into non-helical structures, probably because the vRNA is at least partially
254 detached from the NPs of the vRNP (Fig. 1, 2). For the next round of transcription and/or
255 the next replication cycle, the looped dsRNA should be separated and the template vRNA
256 should rebind to the NPs of the vRNP, so that the deformed vRNP is refolded into its
257 native double-helical structure. Considering these complicated events, it is reasonable to
258 presume that deformed vRNPs associated with looped RNA represent a failure of RNA
259 synthesis (Fig. 6). If deformation of vRNPs sometimes occurs in virus-infected cells,
260 some vRNPs would fail to produce nascent RNAs and the encoded viral proteins, while
261 other vRNPs are successful in transcription and/or replication. Indeed, when influenza
262 viruses are infected at low multiplicity of infection, one or more virus proteins are not
263 expressed in some cells (38, 39). Deformation of vRNPs during transcription and/or
264 replication might be related to such observations.

265 Interestingly, looped RNAs associated with deformed vRNPs were dsRNAs,
266 which are probably composed of nascent RNA and template vRNA (Fig. 3–5). Although
267 it was previously thought that influenza viruses do not produce dsRNA in infected cells
268 (40, 41), dsRNAs were detected in the nuclei of virus-infected cells (Fig. 4e), suggesting
269 that in some cases dsRNAs can be generated during transcription and replication.
270 Although further analysis is needed, this finding suggests that looped dsRNAs observed
271 in this study may be produced in the nuclei of influenza virus-infected cells.

272 In conclusion, by combining HS-AFM and cryo-EM, we found two
273 morphologically different vRNPs during viral RNA synthesis. Our results suggest that
274 helical structures are prerequisite for successful repetitive transcription and replication,
275 while deformation of helical structures would represent abortive RNA synthesis. There
276 are still many questions to be resolved. Future investigations will attempt to identify the
277 determinants of looped RNA formation and of structured RNA synthesis, and to clarify
278 whether certain host factors prevent abortive dsRNA formation. Our present findings
279 provide novel insights into viral transcription and replication with strong evidence
280 regarding the composition and mechanism of production of viral RNAs.

281 **Methods**

282 **Purification of vRNP**

283 Influenza A virus, A/Puerto Rico/8/34 (H1N1) (PR8), was prepared as previously reported
284 (9). Purified PR8 virions ($\sim 5 \text{ mg mL}^{-1}$) were lysed in 50 mM Tris-HCl buffer (pH 8.0)
285 containing 100 mM KCl, 5 mM MgCl_2 , 1 mM dithiothreitol (DTT), 2% Triton X-100,
286 5% glycerol, 2% lysolecithin, and $1 \text{ U } \mu\text{L}^{-1}$ RNasin Plus RNase inhibitor (Promega) for
287 1 h at 30°C . The sample was ultracentrifuged through a 30–70% (w/v) glycerol gradient
288 in Tris-NaCl buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) at 45,000 rpm for 3 h at
289 4°C in a SW55Ti rotor (Beckman). Collected fractions were mixed with $2\times$ Tris-glycine
290 SDS sample buffer (Novex) and then subjected to SDS-PAGE, in which a 4–15% Mini
291 Protean TGX precast gel (Bio-rad) was used.

292

293 ***In vitro* RNA synthesis using virion-derived RNPs**

294 Purified vRNP ($1\text{--}2 \text{ mg mL}^{-1}$ for cryo-EM and 0.01 mg mL^{-1} for the other experiments)
295 was incubated in 50 mM Tris-HCl buffer (pH 7.9) containing 5 mM MgCl_2 , 40 mM KCl,
296 1 mM DTT, $10 \text{ } \mu\text{g mL}^{-1}$ actinomycin D, 1 mM each of ATP, CTP, GTP, and UTP, 1 U
297 μL^{-1} RNasin Plus RNase inhibitor with a primer, 1 mM ApG (Iba). In some experiments,
298 $10 \text{ } \mu\text{g mL}^{-1}$ rabbit globin mRNA (Sigma) was used as a primer instead of ApG. The

299 reaction was performed at 30°C for 15 min (for ApG primer) or 30 min (for globin mRNA
300 primer) unless otherwise noted. For detection of newly synthesized RNA by radioisotope,
301 the same reaction mixture was used with the exception that 0.25 $\mu\text{Ci } \mu\text{L}^{-1}$ [α - ^{32}P] UTP
302 and 0.05 mM UTP were added. After *in vitro* RNA synthesis, RNA was purified with an
303 RNeasy Mini kit (Qiagen), mixed with equal volume of 2 \times RNA Loading Dye (New
304 England Biolabs), heated at 90°C for 2 min and immediately chilled on ice. The sample
305 was electrophoresed on 4% polyacrylamide gel containing 7 M urea in 0.5x TBE buffer
306 (Nacalai Tesque) at 120 V for 5 h. The gel was dried at 80°C for 2 h, exposed to an
307 imaging plate (BAS-MS 2025, Fujifilm) for 12–24 h and scanned with a Typhoon 3000
308 Phosphorimager (GE Healthcare). Labelling of nascent RNA with a nucleotide analogue
309 was also performed in the same reaction mixture using 1 mM 5-bromo-UTP (Br-UTP,
310 Sigma) or 1 mM 5-ethynyl-UTP (EUTP, Abcam) instead of UTP. Inhibition of RNA
311 synthesis was evaluated by adding 0.1–100 μM of 6-fluoro-3-hydroxy-2-
312 pyrazinecarboxamide-4-ribofuranosyl-5'-triphosphate (T-705RTP; kindly provided by
313 Furuta Y., Fujifilm Toyama Chemical Co., Ltd.) to the reaction mixture.

314

315 **Preparation of viral RNAs with T7 RNA polymerase**

316 RNA standards for NP and NA segments of PR8 virus were prepared as described

317 previously (34). Templates containing a T7 phage promoter sequence
318 (TAATACGACTCACTATAGGG) were amplified by PCR using sets of primers listed in
319 [Supplementary Table 1](#) and pPolII plasmid harbouring the sequence of each segment (42).
320 PCR products were purified with a Min Elute Gel Extraction kit (Qiagen) and were
321 transcribed *in vitro* with RiboMAX Large Scale RNA Production System-T7 (Promega)
322 according to the supplier's protocol. After RQ1 DNase I (Promega) treatment for 30 min
323 at 37°C, transcripts were purified using an RNeasy Mini kit. The concentration of purified
324 RNA was determined by spectrophotometry and the copy number was calculated from
325 the molecular weight of each RNA. 1×10^{10} copies of RNA were subjected to
326 electrophoresis (a 4% polyacrylamide gel containing 7 M urea) and visualised with silver
327 staining using a Silver Staining II kit (Wako). In addition, all 8 vRNA segments of
328 influenza A virus (A/WSN/33 strain) were similarly prepared and used as markers for
329 electrophoresis. Template DNAs were amplified with the primers listed in [Supplementary](#)
330 [Table 2](#) and transcribed by T7 RNA polymerase using $0.25 \mu\text{Ci } \mu\text{L}^{-1}$ [α - ^{32}P] UTP.
331 Transcribed RNAs were purified and mixed before electrophoresis.

332

333 **Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)**

334 RT-qPCR was performed according to the protocol of Kawakami *et al.* (34). RNA

335 standard or *in vitro* transcribed virus RNA was mixed with a quarter volume of 10 μM
336 tagged primer (for the sequence see [Supplementary Table 3](#)) and incubated at 65°C for 10
337 min. After immediately chilling on ice for 5 min, the mixture was pre-heated at 60°C for
338 5 min. Then 3 volumes of the reaction mixture (final concentration of 1 \times First Strand
339 buffer (Invitrogen), 5 mM DTT, 0.5 mM each dNTP mix, 10 U μL^{-1} Superscript III
340 reverse transcriptase (Invitrogen), 1 U μL^{-1} RNasin Plus RNase inhibitor: prepared with
341 saturated trehalose and preheated at 60°C for 5 min) were added to the RNA solution at
342 60°C, and the mixture was further incubated for 1 h at 60°C. The reaction was stopped
343 by heating the mixture at 85°C for 5 min, and the cDNA solution was stored on ice until
344 use. Fifty-fold diluted cDNA solution was mixed with forward and reverse qPCR primers
345 (each final 1 μM , for sequences see [Supplementary Table 3](#)), and an equal volume of
346 Thunderbird SYBR qPCR mix (Toyobo) was added. The qPCR reaction was performed
347 on Rotor-Gene Q (Qiagen) and the conditions were 95°C for 1 min, followed by 40 cycles
348 of 95°C for 15 sec and 60°C for 30 sec. For absolute quantitation, 10-fold serial dilutions
349 (1×10^9 – 1×10^4 copies μL^{-1}) of synthetic RNA standards prepared as described above
350 were used to generate a standard curve. The copy number was calculated from the
351 standard curve with a strong linear correlation ($R^2 > 0.99$) and amplification efficiency
352 between 95 and 105%.

353

354 **High-speed atomic force microscopy (HS-AFM)**

355 Samples for HS-AFM imaging were prepared in a microcentrifuge tube, dropped onto
356 freshly cleaved mica without surface modification, and incubated for the desired time
357 (generally 1–5 min) at room temperature. The mica surface was then washed sufficiently
358 with imaging buffer (50 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 40 mM KCl, 1 mM DTT).
359 The sample was observed in the imaging buffer at room temperature using an HS-AFM
360 System (Nano Explorer, RIBM). Images were taken at 2 images s⁻¹ using cantilevers with
361 a 0.1 N m⁻¹ spring constant and a resonance frequency in water of 0.6 MHz (BL-AC10DS,
362 Olympus). In order to acquire high-resolution images, the electron beam-deposited tips
363 were fabricated using phenol or ferrocene powder, as described in Uchihashi *et al* (43).
364 Incorporation of Br-UTP into nascent RNA was confirmed by incubating *in vitro*
365 transcripts with 0.1 mg mL⁻¹ monoclonal anti-5-bromodeoxyuridine antibody
366 (SAB4700630, Sigma) for 2 h at 4°C in a microcentrifuge tube and imaging the sample
367 with HS-AFM. Production of the dsRNA was examined by adding 1 µg mL⁻¹ J2 antibody
368 (10010200, Scicons) to the reaction mixture in a microcentrifuge tube, incubating for 2 h
369 at 4°C, and observing it by HS-AFM. *In situ* observation of RNA digestion by each RNase
370 was performed by adding 1/10 volume of the indicated concentration of RNase A

371 (Epicentre) or ShortCut RNase III (New England Biolabs) to the AFM liquid cell during
372 imaging. At least five independent experiments were performed for each RNase. All HS-
373 AFM images were viewed and analysed with Kodec 4.4.7.39 (44). A low-pass filter and
374 a flattening filter were applied to individual images to remove spike noise and to make
375 the xy-plane flat, respectively.

376

377 **Cryo-electron microscopy (Cryo-EM)**

378 One microliter of reaction solution of the vRNP was applied to a glow-discharged holey
379 carbon grid (Quantifoil R1.2/1.3, Cu 300 mesh) and blotted manually, followed by
380 application of 2 μ L of reaction solution, blotting and rapid freezing in liquid ethane on a
381 Vitrobot Mark IV (Thermo Fisher Scientific). Images were recorded close to focus with
382 a Volta phase plate on a Talos Arctica electron microscope equipped with a Falcon III
383 camera (Thermo Fisher Scientific) in integrating mode. The total dose during a single
384 exposure was ~ 40 electrons/ \AA^2 .

385 For cryo-electron tomography, 2 μ L of reaction solution of the vRNP mixed with colloidal
386 gold (1.9 nm or 5 nm diameter) were applied to glow-discharged holey carbon grids (C-
387 Flat CF-MH-2C, Protochips Inc.) and rapidly frozen in liquid ethane on a Vitrobot Mark
388 IV (Thermo Fisher Scientific). Images were recorded on a Titan Krios electron

389 microscope equipped with a Falcon II camera (Thermo Fisher Scientific). Tilt series were
390 acquired from -60° to 60° with 2° steps using the Legikon System (45). The total dose
391 during a single tilt series was $120 \text{ electrons}/\text{\AA}^2$. Tilt series data were processed in IMOD
392 (46) by using gold particles as fiducial markers for manual image registration after $2\times$
393 binning (final voxel size 4.4 \AA^3). A tomogram of the entire field of view was reconstructed
394 using SIRT (Simultaneous Iterative Reconstruction Technique). Volumes of interest were
395 extracted from the reconstructed 3D tomogram and visualised with IMOD and AMIRA
396 6.1 (Thermo Fisher Scientific). Consecutive Z-projections were generated using ImageJ
397 (47).

398

399 **Modification of RNA using Click chemistry**

400 A Click-iT RNA Imaging kit was purchased from Invitrogen. After *in vitro* RNA synthesis
401 with EUTP, the sample was deposited on mica and incubated for 3 min at room
402 temperature. The mica surface was washed with imaging buffer and the following
403 reactions were all performed on mica without drying the surface. Imaging buffer on the
404 mica surface was replaced with Click-iT reaction cocktail (Click-iT RNA reaction buffer
405 containing 4 mM CuSO_4 , 0.02 mg mL^{-1} biotin azide (Thermo Fisher Scientific), and $0.1\times$
406 Click-iT reaction buffer additive) and incubated for 30 min under light-shielding. The

407 mica surface was then washed with imaging buffer and the buffer was replaced with 0.1
408 mg mL⁻¹ of streptavidin solution (Jackson ImmunoResearch). After 15 min of incubation
409 at room temperature, the mica surface was washed again with imaging buffer for the HS-
410 AFM observation.

411

412 **Immunofluorescence assay (IFA)**

413 Vero cells (ATCC CCL-81) were grown in Eagle's minimum essential medium and
414 seeded on 35-mm glass bottom dish (Matsunami Glass) coated with rat collagen I
415 (Corning) one day before infection. Cells were infected with PR8 virus at MOI of 0.1 and
416 incubated for 10 h in minimum essential medium (Gibco) containing 0.3% BSA. Infected
417 cells were fixed in 4% paraformaldehyde (Nacalai Tesque) for 10 min and permeabilized
418 with 0.1% Triton X-100 in PBS for 10 min. Cells were then washed with PBS and blocked
419 with Blocking One (Nacalai Tesque) for 30 min. After blocking, cells were incubated with
420 anti-NP rabbit polyclonal (GTX125989, GeneTex) and anti-dsRNA mouse monoclonal
421 antibody J2 (10010200, Scicons) overnight at 4°C. Cells were washed with PBS and
422 incubated in Alexa Fluoro 488-conjugated anti-mouse antibody (A11001, Thermo Fisher
423 Scientific) and Hoechst 33342 (Thermo Fisher Scientific) for 1 h at 4°C. After incubation,
424 cells were washed with PBS and incubated in Alexa Fluoro 555-conjugated anti-rabbit

425 antibody (A21248, Thermo Fisher Scientific) for 1 h at room temperature. All antibodies
426 were diluted in PBS with 10% Blocking One. Section images were recorded and
427 deconvolved using DeltaVision Elite system (GE healthcare) with a 60× oil immersion
428 objective on an Olympus IX71 microscope.

429

430 **Force measurement by HS-AFM**

431 To measure the force applied to the sample surface, two types of HS-AFM images were
432 taken simultaneously: topographic images and amplitude images. By measuring the
433 thermal noise and the InvOLS (inverse optical lever sensitivity) value [nm V^{-1}] of
434 deflection signal of a cantilever, we determined the spring constant (k_c) [pN nm^{-1}] and
435 quality factor (Q_c) of the cantilever as described previously (48). Determined k_c values
436 were in good agreement with nominal values reported by the manufacturer. During
437 observation, the vRNP was destroyed by gradually lowering the set point. Whether the
438 vRNP was destroyed was judged from the apparent change in the height of the vRNP.
439 After imaging, the amplitude value [V] at the frame in which vRNP was destroyed was
440 measured from the amplitude image, and the obtained value was converted into A_{sp} [nm]
441 using the InvOLS value described above. The free cantilever oscillation amplitude A_0 was
442 measured by releasing the cantilever from the sample surface. The average tip-sample

443 interaction force $\langle F_{ts} \rangle$ was calculated using the following equation, $\langle F_{ts} \rangle = k_c (A_0^2 -$
444 $A_{sp}^2)^{1/2} / Q_c$ (49). For preparation of the control vRNP, we treated 0.01 mg mL^{-1} of vRNP
445 with $0.05 \text{ } \mu\text{g mL}^{-1}$ of RNase A for 10 min at 37°C . Degradation of the vRNA was
446 confirmed by RT-PCR using primers for detecting the full-length NP segment.
447

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563

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565

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589

590 **Author contributions**

591 MN and TN designed the study. MN, YS, NK, SM, and YM performed the experiments.
592 MN, YS, NK, and TN analysed data. MN, YS, MW, and TN wrote the manuscript. All
593 authors reviewed and approved the manuscript.

594

595 **Competing interests**

596 The authors declare no competing interests.

597

598 **Additional information**

599 Supplementary information is available for this paper at <https://>

600 Correspondence and requests for materials should be addressed to T.N.

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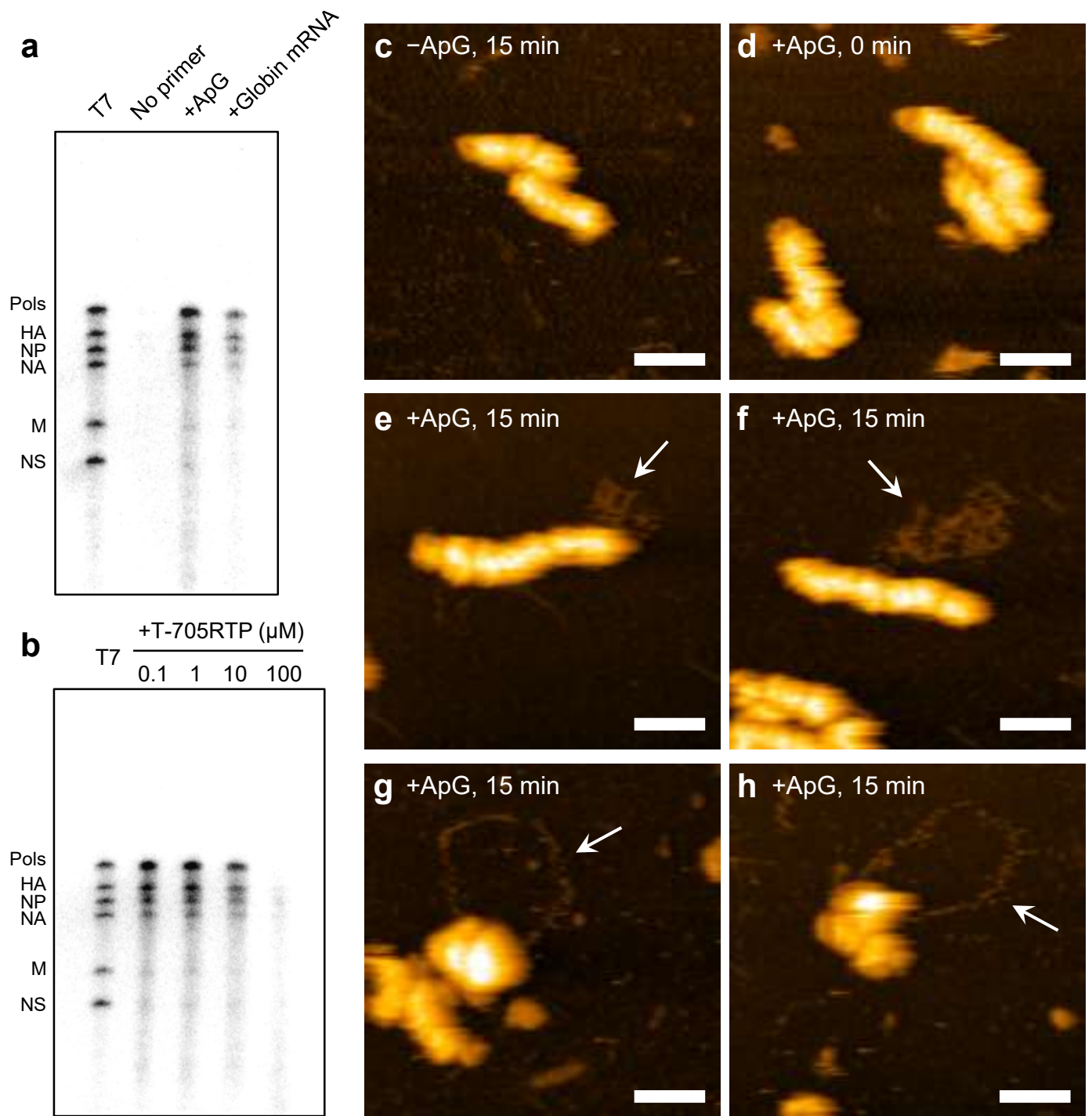


Fig. 1. HS-AFM observation of vRNPs during RNA synthesis.

a, Primer-dependent *in vitro* RNA synthesis using virion-derived vRNPs. RNA was synthesized *in vitro* using ApG or globin mRNA as a primer with 30 min incubation. As a negative control, the reaction mixture was used without primer. A mixture of eight influenza A virus vRNA segments (Pols indicates 3 polymerases, PB2, PB1, and PA) transcribed by T7 RNA polymerase was loaded in the leftmost lane (T7) for evaluation of sizes of the newly synthesized RNAs. **b**, Inhibition of *in vitro* RNA synthesis by T-705RTP. RNA was synthesized *in vitro* using ApG in the presence of the indicated concentration of T-705RTP. All purified RNA samples were analysed on a 4% polyacrylamide gel containing 7 M urea and detected by autoradiography. **c**, As a negative control for the HS-AFM observation, the reaction mixture omitting a primer was used. **d-h**, Virion-derived vRNPs were subjected to *in vitro* RNA synthesis using ApG as a primer. After incubation for 0 min (**d**) or 15 min (**c**, **e-h**), samples were observed with HS-AFM. Structured and looped RNAs associated with the helical (**e**, **f**) and deformed vRNPs (**g**, **h**), respectively, were observed as indicated by arrows at the different positions in the same samples. Scale bars on all images represent 50 nm.

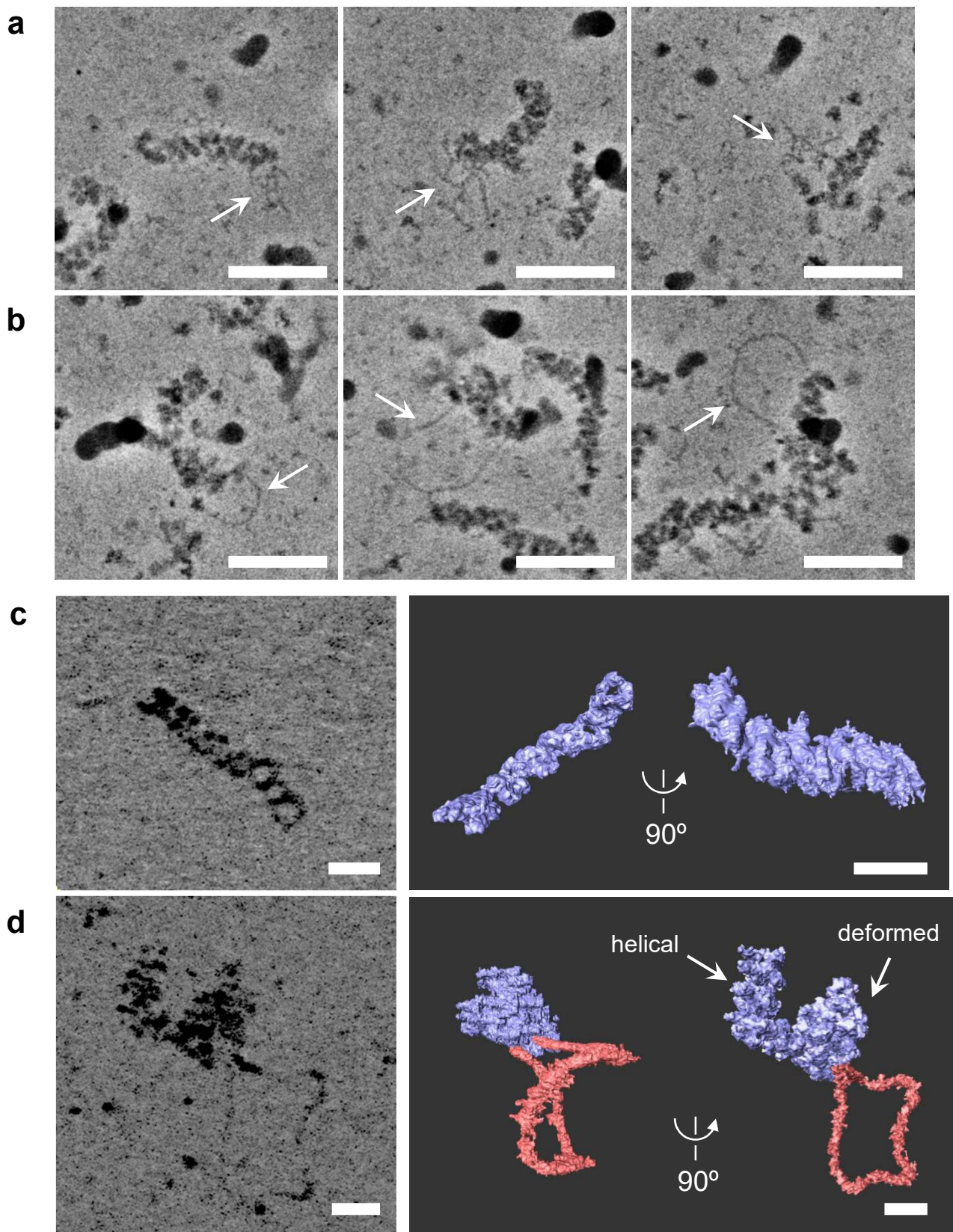


Fig. 2. Cryo-EM observation of vRNPs during RNA synthesis.

An *in vitro* RNA synthesis reaction was performed in the presence of ApG, and was observed with cryo-EM in vitreous ice. Structured RNAs (a, arrows) and looped RNAs (b, arrows) associated with vRNPs were observed. Scale bars represent 50 nm. (c, d) Cryo-ET analysis of vRNPs during RNA synthesis. c, Cryo-ET observations of vRNP without RNA synthesis. d, Cryo-ET observations of vRNP with RNA synthesis. Left panels: Consecutive Z-projections generated from tomograms; Thickness in Z is 44 nm (c) and 88 nm (d). Right panels: 3D reconstruction of vRNP segmented from the tomograms. The vRNP and RNA are coloured in blue and red, respectively. Scale bars on all images represent 20 nm.

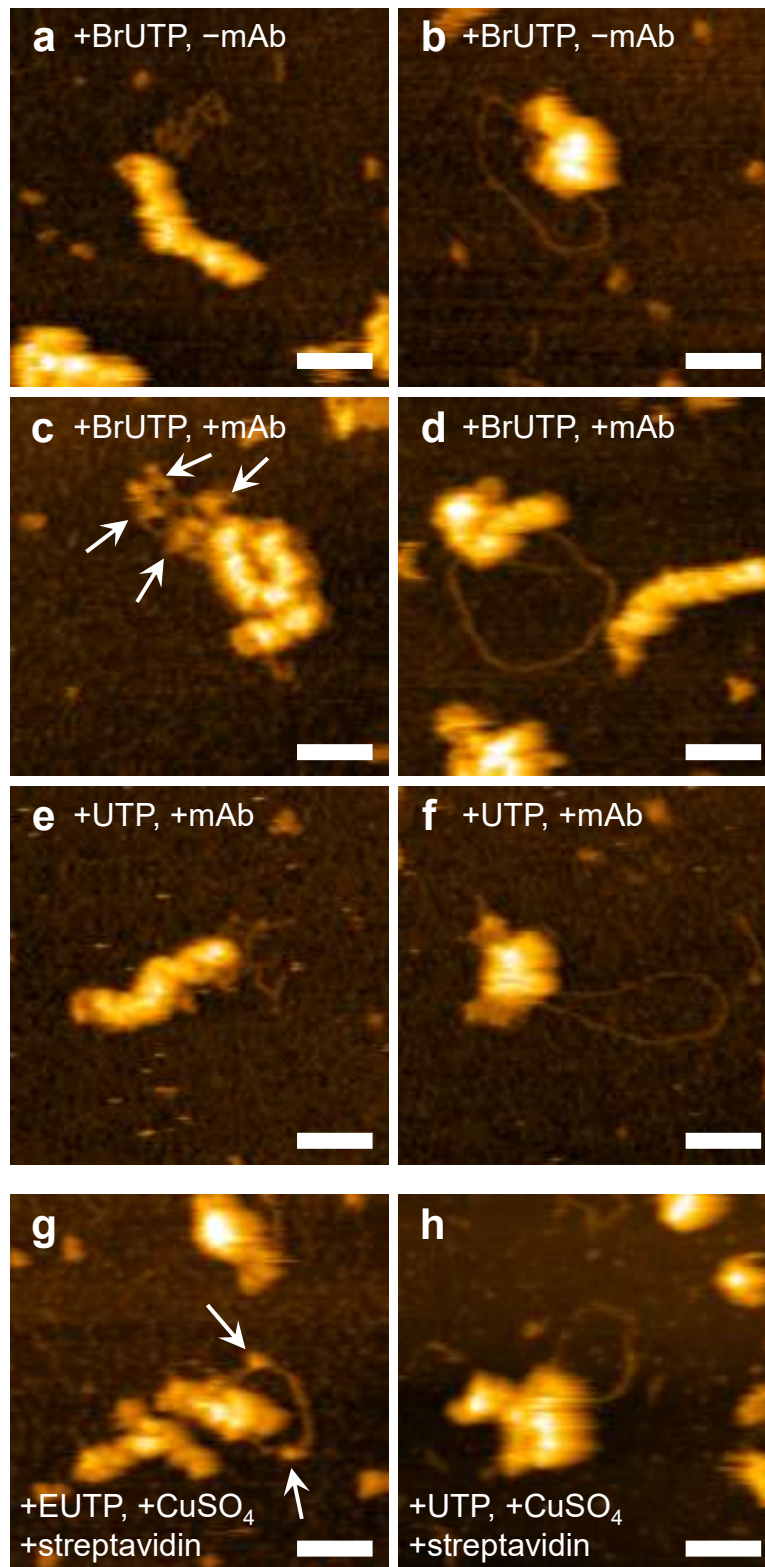


Fig. 3. Incorporation of nucleotide analogues into newly synthesized RNAs.

a-d, Br-UTP was used for *in vitro* RNA synthesis instead of UTP and HS-AFM images were taken without (**a**, **b**) or with (**c**, **d**) adding an antibody against Br-UTP. Binding of anti-Br-UTP antibodies was confirmed on structured RNAs (**c**, arrows) while no binding was observed on looped RNAs (**d**). Section analysis of the image (**c**) is shown in Fig. S4. **e**, **f**, vRNPs were *in vitro* transcribed using UTP and anti-Br-UTP antibody was added to the mixture. **g**, Confirmation of the incorporation of EUTP into looped RNA using Click chemistry. Streptavidin molecules binding to looped RNA are indicated by arrows. **h**, Negative control of the Click reaction. The sample was prepared using UTP instead of EUTP. Each of these results was reproduced at least 3 times. Scale bars, 50 nm.

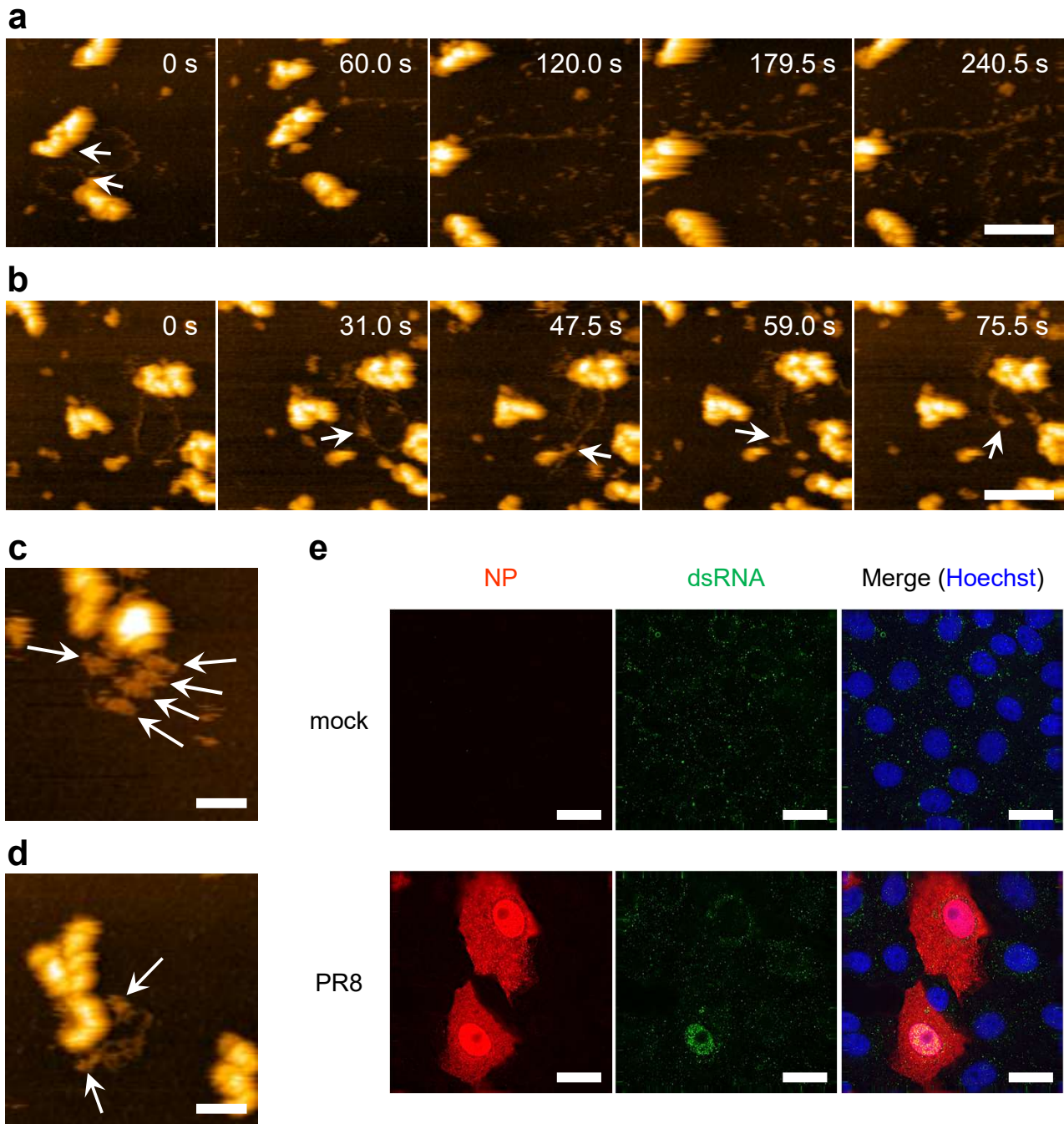


Fig. 4. Production of a double-stranded RNA by vRNP.

a, b, Digestion of looped RNAs with RNases. During HS-AFM observation of looped RNA associated with vRNP, RNase A (**a**) or RNase III (**b**) was added to the liquid chamber at a final concentration of $0.5 \mu\text{g mL}^{-1}$ or $0.02 \text{ U } \mu\text{L}^{-1}$, respectively. Five images were arbitrarily selected from each movie at the indicated times. One end of the looped RNA was detached from vRNP by adding RNase A at the position indicated by arrows (**a**). By contrast, RNase III digested looped RNA where the RNase bound (**b**, arrows). Scale bars represent 100 nm. **c, d,** Binding of anti-dsRNA antibodies to RNA associated with the vRNP. Antibodies bound to looped RNA (**c**) and to structured RNA (**d**) are indicated by arrows. Results were reproduced at least 5 times. Scale bars in **c, d** represent 50 nm. **e,** Detection of dsRNA in virus-infected cells by IFA. Vero cells were infected with influenza virus PR8 strain at MOI of 0.1. Infected cells were fixed at 10 h post-infection and double-stained with anti-NP and anti-dsRNA antibodies. Cell nuclei were stained with Hoechst. Scale bars, 20 μm .

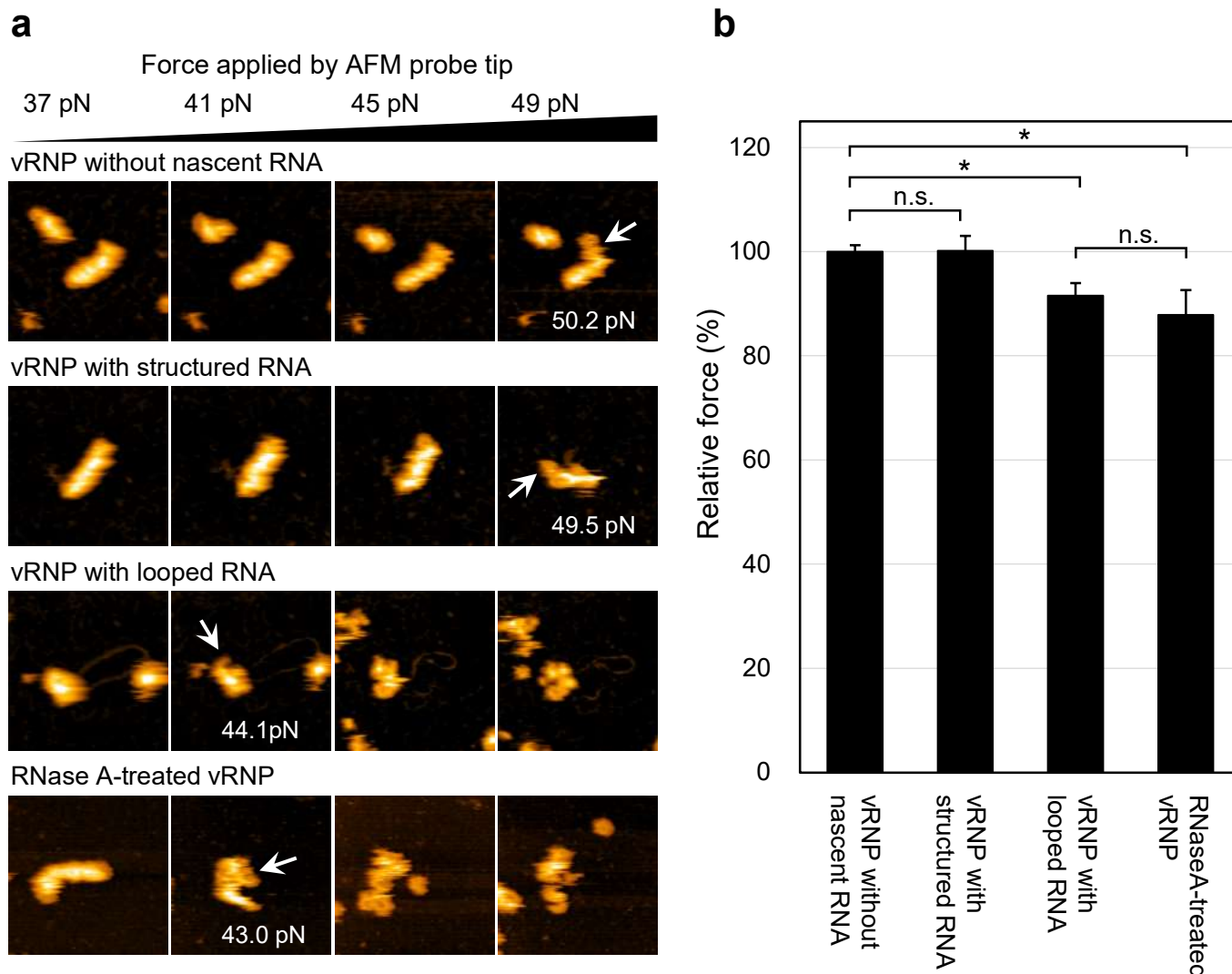


Fig. 5. Deformation of vRNP by releasing the residential vRNA.

a, Deformation of vRNPs with the AFM probe tip. vRNP without a nascent RNA, with a structured RNA or with a looped RNA was deformed by applying force with the cantilever tip during the HS-AFM observation. As a control for the vRNP lacking its intact vRNA, the vRNP pre-treated with RNase A was also deformed. When the vRNP was confirmed as deformed (arrows), the force was measured as described in the Methods. Image sets are representative of 5 vRNPs of each sample and average forces required for deforming vRNPs are calculated. **b**, Structural stability of vRNP during RNA synthesis. The force required for deforming the vRNP without nascent RNA was set as 100% and the relative force of each sample is shown. Significance was determined using the Tukey-Kramer multiple comparison test (*, $P < 0.05$) in R software. Error bars represent the standard deviation of five independent measurements (n.s. = “not significant”).

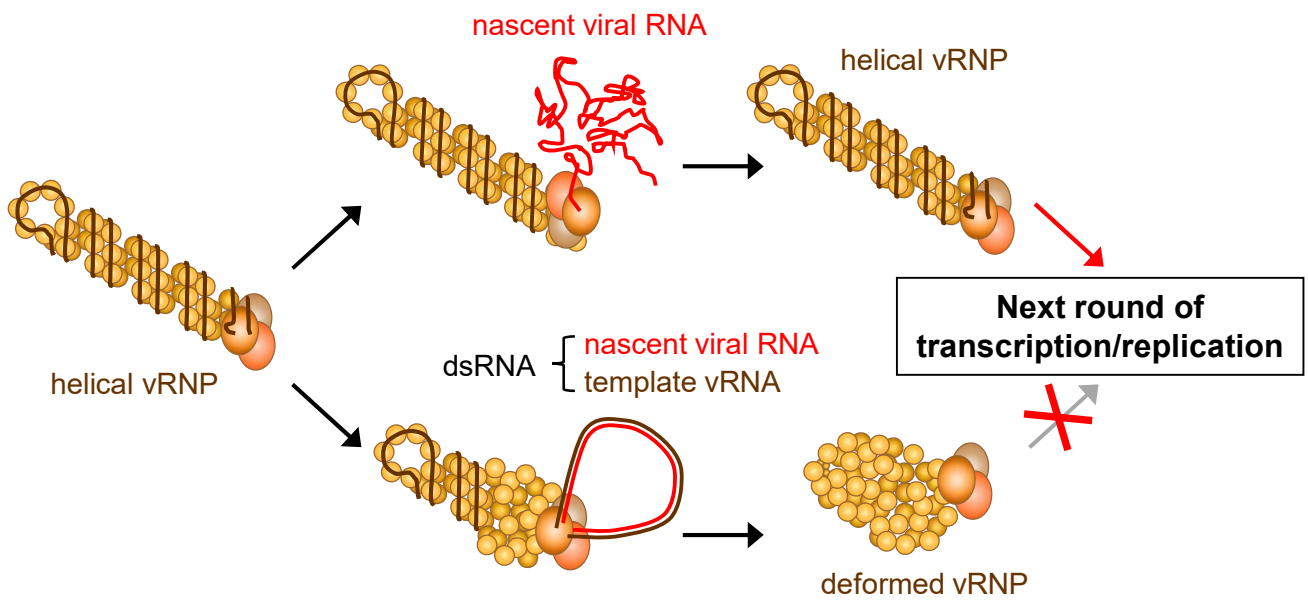


Fig. 6. Model for synthesis of nascent viral RNAs by influenza vRNPs.

When structured viral RNA is synthesized, the vRNP keeps its helical rod-shaped structure and the vRNP is used in next round of transcription and/or replication (upper). In contrast, when looped dsRNA is produced, the vRNP disrupts its helical rod-shaped structure because it loses the residential vRNA. As a result, such deformed vRNP cannot proceed to the next round of transcription and/or replication cycle (lower).

Figures

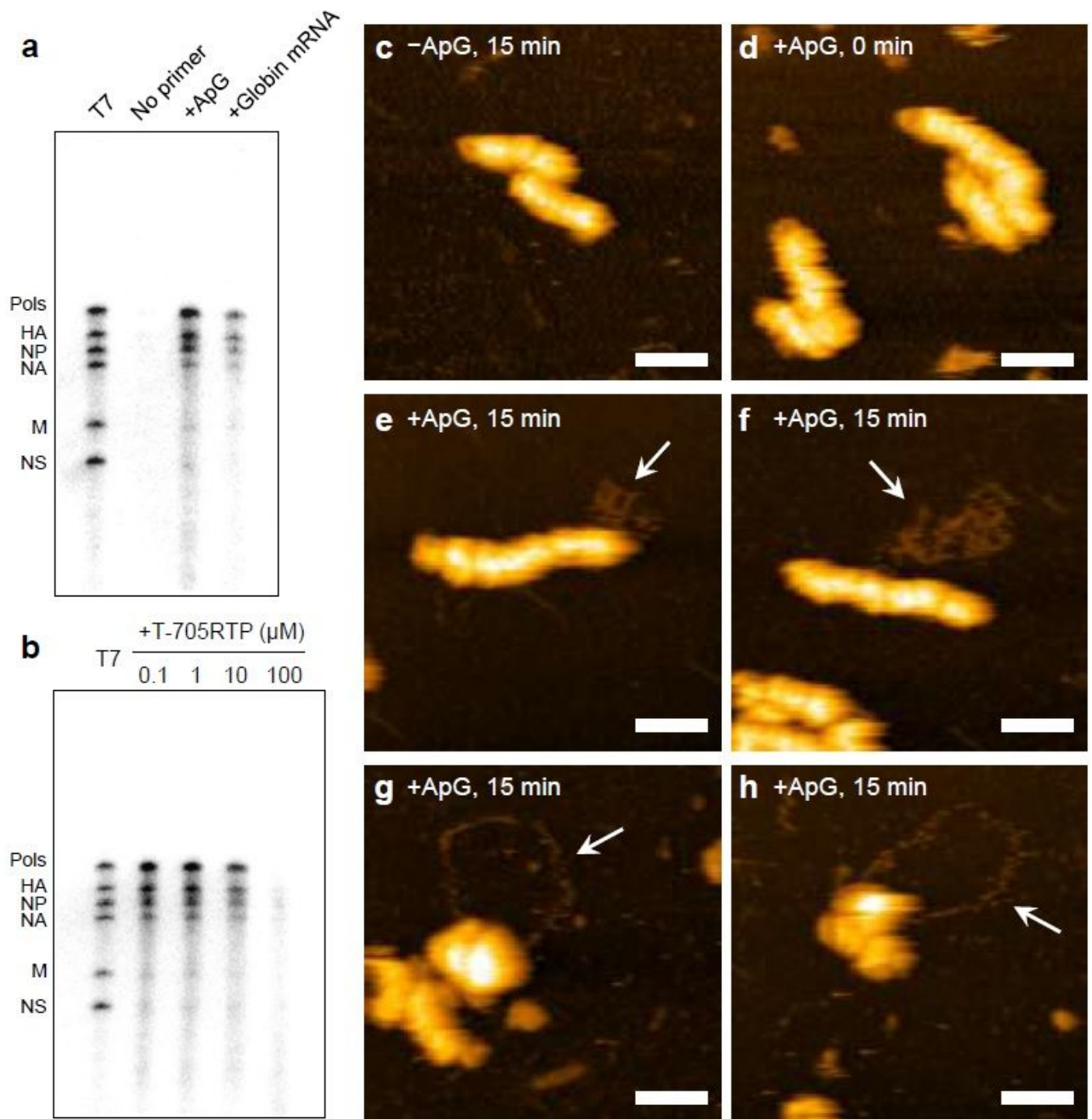


Figure 1

HS-AFM observation of vRNPs during RNA synthesis. a, Primer-dependent in vitro RNA synthesis using virion-derived vRNPs. RNA was synthesized in vitro using ApG or globin mRNA as a primer with 30 min incubation. As a negative control, the reaction mixture was used without primer. A mixture of eight

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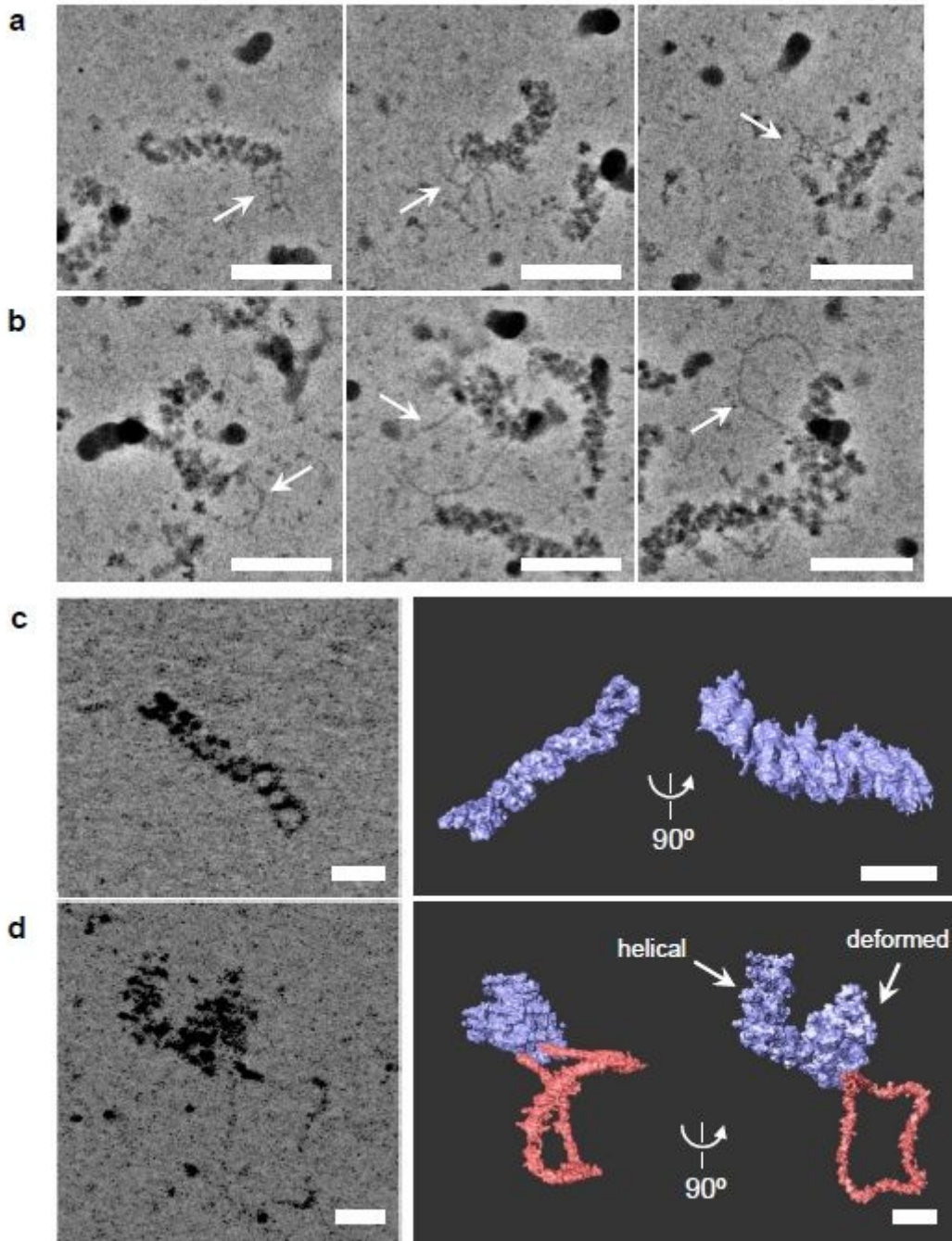


Figure 2

Cryo-EM observation of vRNPs during RNA synthesis. An in vitro RNA synthesis reaction was performed in the presence of ApG, and was observed with cryo-EM. Scale bars represent 50 nm. (c, d) Cryo-ET analysis of vRNPs during RNA synthesis. c, Cryo-ET observations of vRNP without RNA synthesis. d, Cryo-ET observations of vRNP with RNA synthesis. Left panels: Consecutive Z-projections generated from tomograms; Thickness in Z is 44 nm (c) and 88 nm (d). Right panels: 3D reconstruction of vRNP segmented from the tomograms. The vRNP and RNA are coloured in blue and red, respectively. Scale bars on all images represent 20 nm.

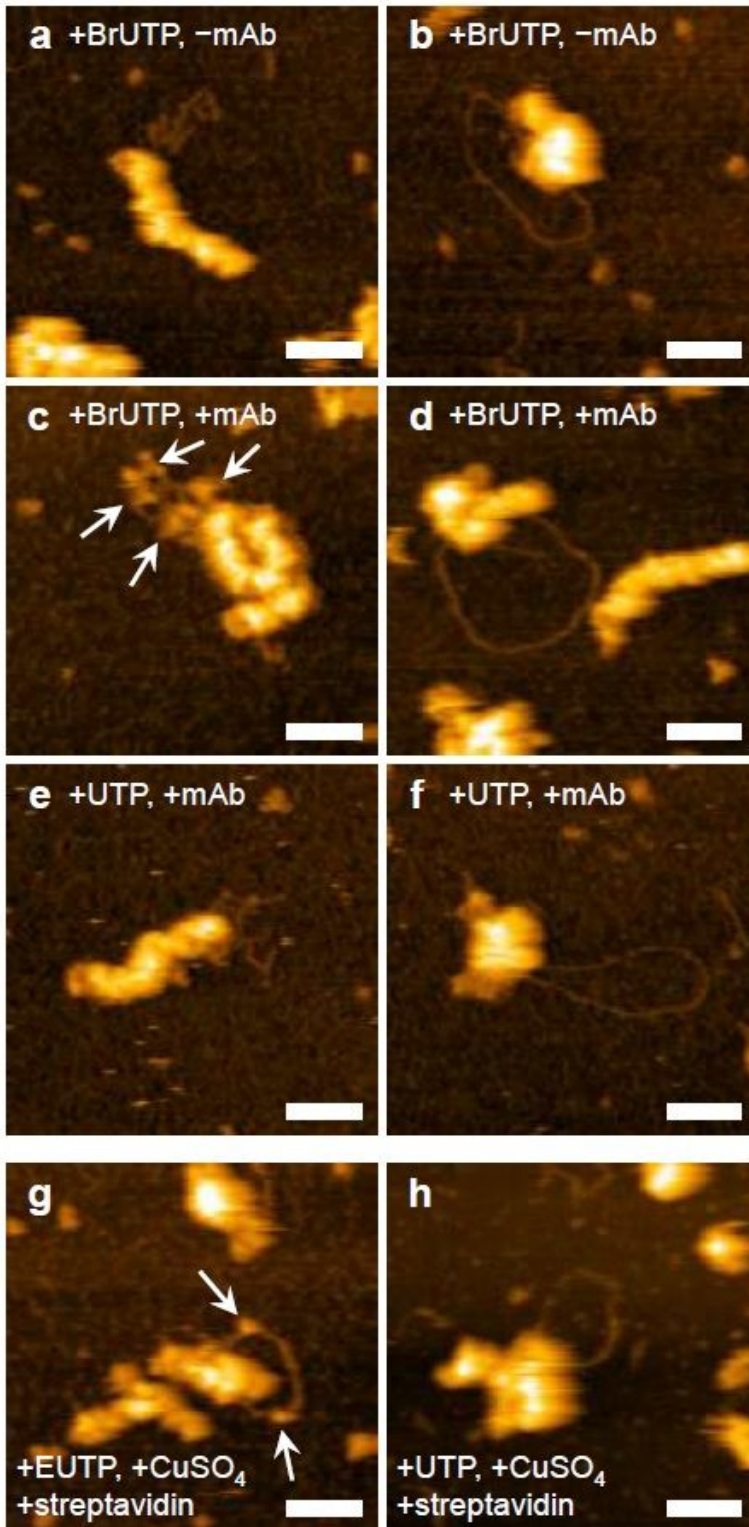


Figure 3

Incorporation of nucleotide analogues into newly synthesized RNAs. a-d, Br-UTP was used for in vitro RNA synthesis instead of UTP and HS-AFM images were taken without (a, b) or with (c, d) adding an antibody against Br-UTP. Binding of anti-Br-UTP antibodies was confirmed on structured RNAs (c, arrows) while no binding was observed on looped RNAs (d). Section analysis of the image (c) is shown in Fig. S4. e, f, vRNPs were in vitro transcribed using UTP and anti-Br-UTP antibody was added to the mixture. g, Confirmation of the incorporation of EUTP into looped RNA using Click chemistry. Streptavidin molecules binding to looped RNA are indicated by arrows. h, Negative control of the Click reaction. The sample was prepared using UTP instead of EUTP. Each of these results was reproduced at least 3 times. Scale bars, 50 nm.

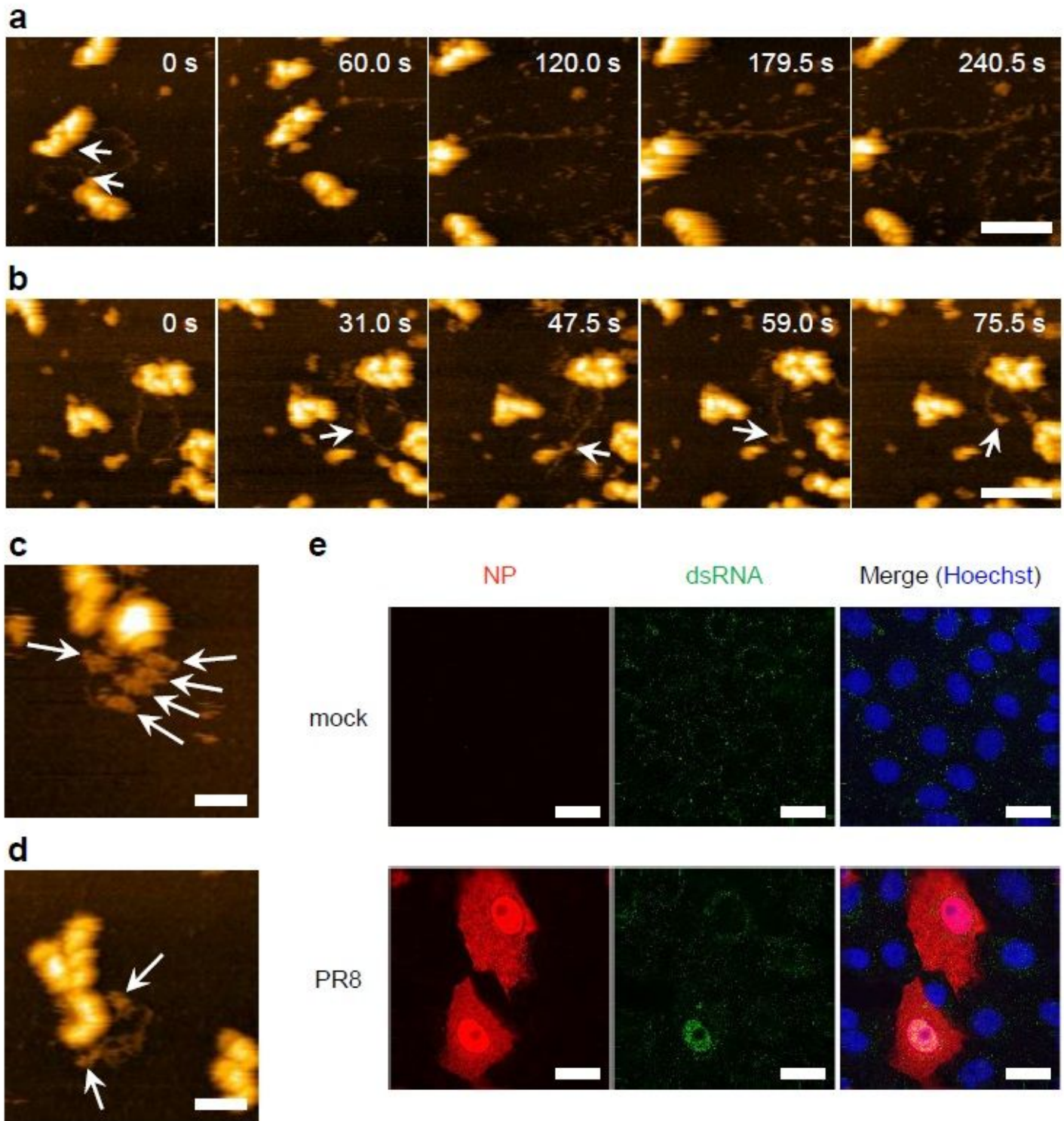


Figure 4

Production of a double-stranded RNA by vRNP. a, b, Digestion of looped RNAs with RNases. During HS-AFM observation of looped RNA associated with vRNP, RNase A (a) or RNase III (b) was added to the liquid chamber at a final concentration of $0.5 \mu\text{g mL}^{-1}$ or $0.02 \text{ U } \mu\text{L}^{-1}$, respectively. Five images were arbitrarily selected from each movie at the indicated times. One end of the looped RNA was detached from vRNP by adding RNase A at the position indicated by arrows (a). By contrast, RNase III digested

looped RNA where the RNase bound (b, arrows). Scale bars represent 100 nm. c, d, Binding of anti-dsRNA antibodies to RNA associated with the vRNP. Antibodies bound to looped RNA (c) and to structured RNA (d) are indicated by arrows. Results were reproduced at least 5 times. Scale bars in c, d represent 50 nm. e, Detection of dsRNA in virus-infected cells by IFA. Vero cells were infected with influenza virus PR8 strain at MOI of 0.1. Infected cells were fixed at 10 h post-infection and double-stained with anti-NP and anti-dsRNA antibodies. Cell nuclei were stained with Hoechst. Scale bars, 20 μ m.

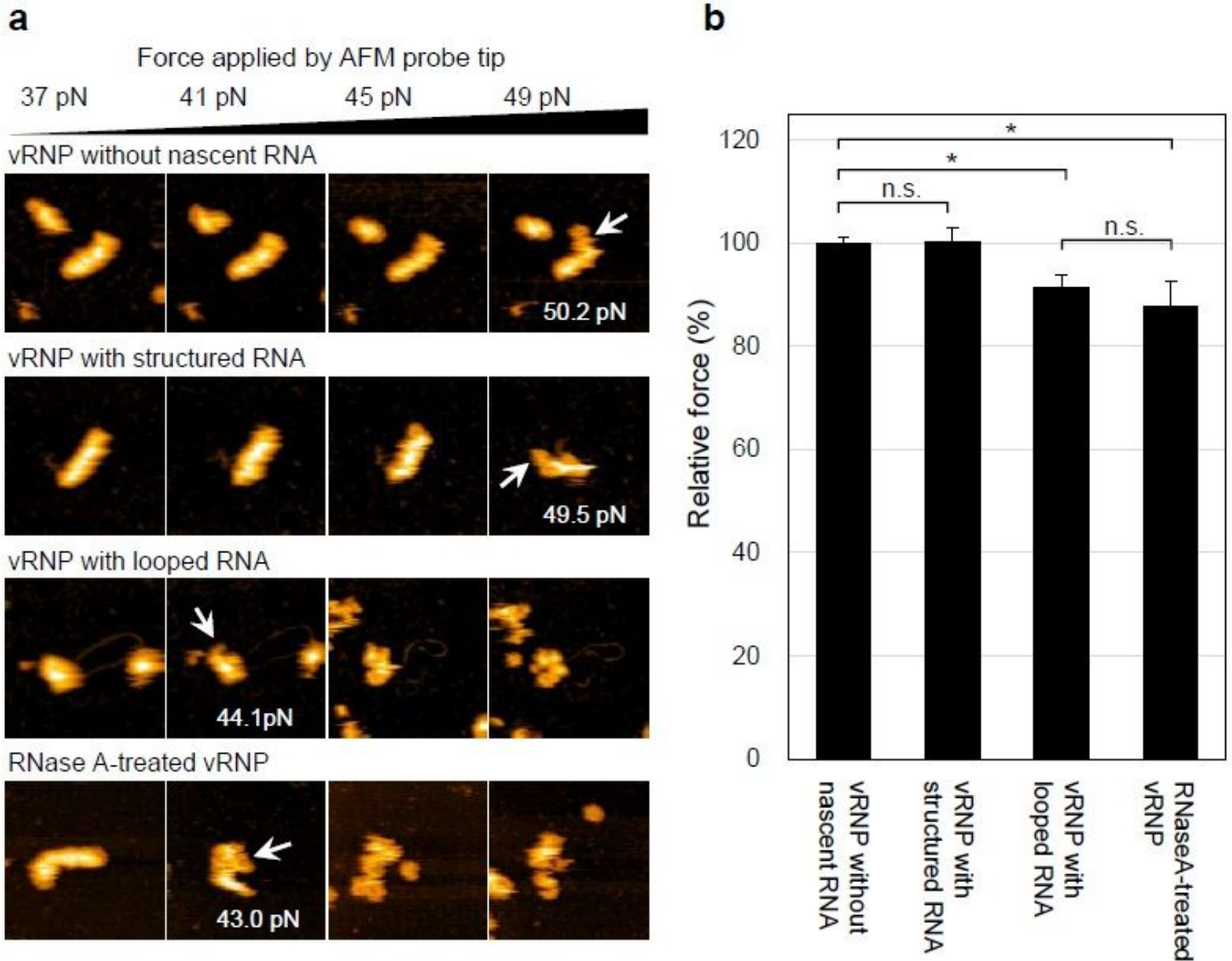


Figure 5

Deformation of vRNP by releasing the residential vRNA. a, Deformation of vRNPs with the AFM probe tip. vRNP without a nascent RNA, with a structured RNA or with a looped RNA was deformed by applying force with the cantilever tip during the HS-AFM observation. As a control for the vRNP lacking its intact vRNA, the vRNP pre-treated with RNase A was also deformed. When the vRNP was confirmed as deformed (arrows), the force was measured as described in the Methods. Image sets are representative of 5 vRNPs of each sample and average forces required for deforming vRNPs are calculated. b, Structural stability of vRNP during RNA synthesis. The force required for deforming the vRNP without nascent RNA

was set as 100% and the relative force of each sample is shown. Significance was determined using the Tukey-Kramer multiple comparison test (*, $P < 0.05$) in R software. Error bars represent the standard deviation of five independent measurements (n.s. = “not significant”).

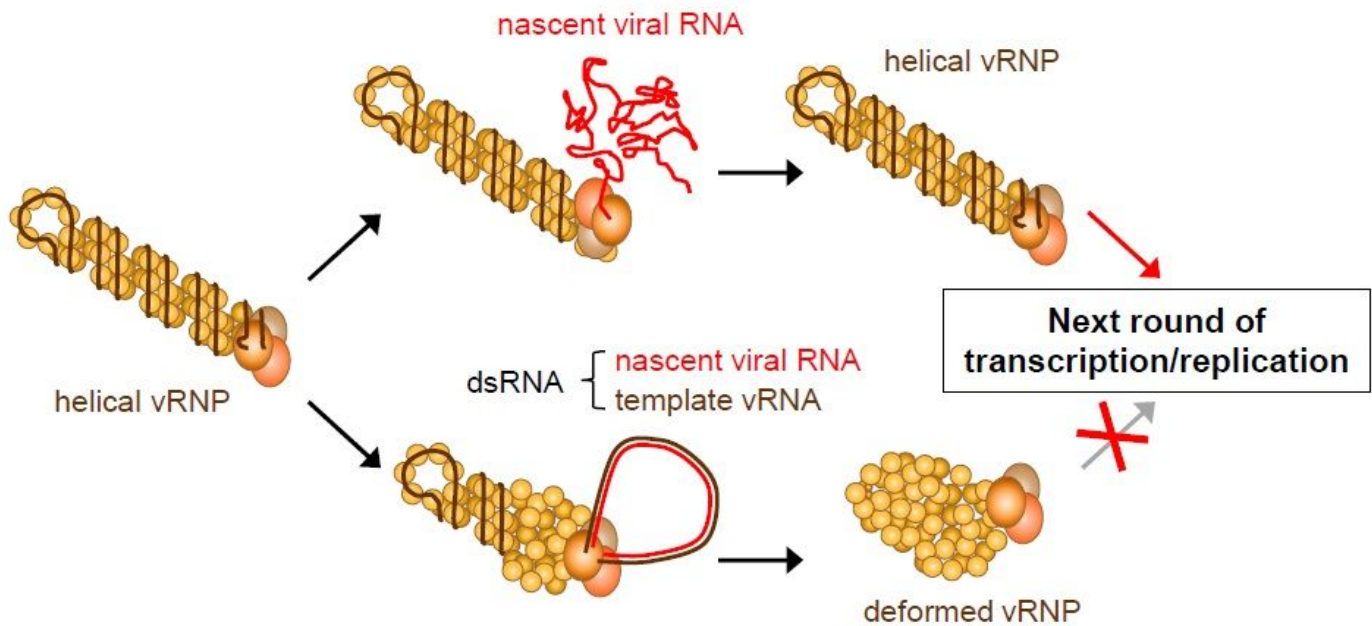


Figure 6

Model for synthesis of nascent viral RNAs by influenza vRNPs. When structured viral RNA is synthesized, the vRNP keeps its helical rod-shaped structure and the vRNP is used in next round of transcription and/or replication (upper). In contrast, when looped dsRNA is produced, the vRNP disrupts its helical rod-shaped structure because it loses the residential vRNA. As a result, such deformed vRNP cannot proceed to the next round of transcription and/or replication cycle (lower).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymovie1.mp4](#)
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- [Supplementarymovie3.mp4](#)
- [Supplementarymovie4.mp4](#)
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