J. Biochem. 2011;150(4):423–430 doi:10.1093/jb/mvr072

JB THE JOURNAL OF BIOCHEMISTRY

Cell-free RNA replication systems based on a human cell extracts-derived *in vitro* translation system with the encephalomyocarditisvirus RNA

Received April 7, 2011; accepted May 13, 2011; published online May 26, 2011

Tominari Kobayashi[1], Kodai Machida[1,2], Satoshi Mikami[3], Mamiko Masutani[1,3] and Hiroaki Imataka[1,2,3,*]

[1]Department of Materials Science and Chemistry; [2]Molecular Nanotechnology Research Center, Graduate School of Engineering, University of Hyogo, Himeji 671-2280; and [3]RIKEN Systems and Structural Biology Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

*Hiroaki Imataka, Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, Himeji 671-2280, Japan. Tel: +81-79-267-4021, Fax: +81-79-267-4885, email: imataka@eng.u-hyogo.ac.jp

To study the relationship between translation and replication of encephalomyocarditisvirus (EMCV) RNA, we established a cell-free RNA replication system by employing a human cell extracts-based *in vitro* translation system. In this system, a *cis*-EMCV RNA replicon encoding the *Renilla luciferase* (R-luc) or GFP and the viral regulatory proteins efficiently replicated with simultaneous translation of the encoded protein. To examine how translation of the replicon RNA, but not the translated products, affected replication, a *trans*-EMCV RNA replicon encoding R-luc and the RNA replication elements was next constructed. The *trans*-replicon RNA replicated only in the presence of the regulatory proteins pre-expressed in *trans*. Incubation with cycloheximide, puromycin or a dominant-negative eukaryotic translation initiation factor 4A following expression of the regulatory proteins almost completely inhibited not only translation of the *trans*-replicon RNA but also replication of the RNA, suggesting that EMCV RNA translation promotes replication of the RNA. In conclusion, the cell-free RNA replication systems should become useful tools for the study of the viral RNA replication.

Keywords: cell-free system/encephalomyocarditisvirus/RNA replicon/RNA virus/translation.

Abbreviations: DIG, digoxigenin; eIF, eukaryotic translation initiation factor; EMCV, encephalomyocarditisvirus; GFP, green fluorescent protein; IRES, internal ribosome entry site; ORF, open reading frame; PCBP, poly(rC)-binding protein; R-luc, Renilla luciferase; pfu, plaque-forming unit; TMEV, Theiler's murine encephalomyelitis virus; UTR, untranslated region.

The picornaviruses possess a positive-sense RNA genome of length 7.5–8.5 kb that is translated after entry into the cellular cytoplasm to produce the virally encoded polyprotein. The polyprotein is processed into structural (capsid) proteins and non-structural proteins by a series of proteolytic events. The non-structural proteins function in concert to replicate the genomic RNA, which is then packaged by the capsid proteins, consequently leading to assembly of infectious virions. *In vitro* synthesis of infectious poliovirus particles from their genomic RNA in HeLa cell extracts permitted direct experimental access to biochemical reactions ranging from viral translation to particle formation (*1*). Encephalomyocarditisvirus (EMCV) (*2*) and mengovirus (*3*) have also been synthesized successfully in Krebs-2 cell extracts, and these cell-free systems have facilitated study of the translational regulation of EMCV (*4*) and the mechanism of action of an anti-mengovirus drug (*3*).

Since the genome of the picornavirus serves as both the template for translation and replication of the RNA, the interplay between RNA translation and replication has been an issue of interest. Ribosomes that bind to the internal ribosome entry site (IRES) of the genomic RNA proceed to the 3′-end for translation, while the viral RNA polymerase complex synthesizes the negative RNA strand from the 3′-end of the genome. Both or either process would be impaired as a result of collision between the ribosome and the RNA polymerase, if both were on the same RNA at the same time. However, this conflict does not seem to exist *in vivo*, because protein synthesis and RNA synthesis of the picornavirus occur concomitantly in infected cells (*5*, *6*).

Biochemical analyses of translation and replication have been performed using the HeLa cell extracts programmed with the poliovirus genomic RNA. Synthesis of the negative-strand RNA increased upon incubation of pre-initiation RNA replication complexes with translation inhibitor puromycin (*7*, *8*). In contrast, treatment with other translation inhibitors such as cycloheximide and anisomycin decreased the synthesis of the negative-strand RNA; positive-strand RNA synthesis was not inhibited. As puromycin dissociates translating ribosomes from mRNA, while cycloheximide and anisomycin immobilize them on mRNA, the authors concluded that translating ribosomes inhibit the poliovirus negative-strand RNA synthesis (*8*). HeLa cell extracts and *Xenopus* oocytes were employed to formulate a proposed mechanism of the switch from translation to negative-strand synthesis: while the 5′-end of the poliovirus genomic RNA is bound by the cellular factor poly(rC)-binding

protein (PCBP), the viral translation is up-regulated, but when the viral protein 3CD accumulates and replaces PCBP bound to the 5′-end structure, 3CD represses translation and promotes negative-strand RNA synthesis (*9*).

The sub-genomic RNA replicon system has been widely used to analyse the mechanism of replication of RNA viruses (*10*, *11*). In this system, the replicon RNA is used for translation and RNA replication, but not for the assembly of virus particles, allowing researchers to isolate the former two events. However, the RNA replication systems depend on living cells, and hence it may be difficult to examine viral RNA translation and replication at the molecular level.

Here we report the development of a *cis*-cell-free RNA replicon that automatically replicates in HeLa cell extracts. We evolved this system into a *trans*-RNA replicon that replicates only in the presence of the regulatory proteins pre-expressed in *trans*. By combining this *trans*-RNA replication system with translation inhibitors, we explored the relationship between translation and replication of the EMCV RNA.

Materials and Methods

Plasmids and RNAs

To construct plasmids for the synthesis of EMCV *cis*-replicon RNA, pUC18 EMCV Rbz, which encodes full-length EMCV cDNA and a ribozyme sequence (*12*), was modified in the following ways. The leader and capsid-encoding region, L-1A-1B-1C-1D: 834–3535 nucleotide (nt), was replaced with the *Renilla luciferase* (R-luc) coding region (Promega) with the C-terminal myc sequence. The nucleotide number of the EMCV genome in this article follows GenBank M81861, but the poly (C) tract from 149 to 289 nt was shortened to nine cytidines (*13*). The non-structural protein-coding region (2A-2B-2C-3A-3B-3C-3D: 3536–7712 nt) plus the 3′-untranslated region (3′-UTR: 7713–7735 nt) was preceded by the EMCV IRES (EMCV IRES: 281–833 nt). The resulting plasmid, pUC18-T7-Rbz-5′ UTR-R-luc-EMCV IRES-2A-3D-A(40)-ter, encoded the T7 RNA polymerase promoter (T7), a ribozyme (Rbz), the EMCV 5′-UTR (1–833 nt), R-luc-myc, the EMCV IRES (281–833 nt), the 2A-2B-2C-3A-3B-3C-3D-3′-UTR (3536–7835 nt), a poly-A stretch (40 deoxyadenines) and the T7 RNA polymerase termination sequence (ter). A part of the 1B region (1250–1450 nt) was incorporated between R-luc and the EMCV IRES of this plasmid to construct the pUC18-*cis*-replicon. The R-luc-myc in the pUC18-*cis*-replicon was replaced with the myc-tagged green fluorescent protein (GFP) coding sequence to generate the pUC18-*cis*-replicon (myc-GFP).

To generate a plasmid for synthesis of the EMCV *trans*-replicon RNA, the EMCV IRES and a large part of the non-structural protein region (3536–7177 nt) were removed from the pUC18-c*is*-replicon to construct the pUC18-*trans*-replicon. To express non-structural proteins, the region encoding 2A-2B-2C-3A-3B-3C-3D (3536–7712 nt) was incorporated into pUC-T7-EMCV-MCS-ter (*14*), resulting in the construct pUC-T7-EMCV-2A-3D-ter. The mutation in the 3D RNA polymerase was generated by a 1-nt deletion at 7180 nt, causing a frame shift.

RNAs were synthesized with T7 RNA polymerase from a plasmid linearized with SalI or NotI, and purified using Chroma-spin30 (BD Bioscience). The genomic EMCV RNA was obtained as previously described (*12*).

Proteins

Recombinant eukaryotic translation initiation factor 4A (eIF4A) (wt) and a mutant eIF4A (DQAD) were obtained as previously described (*15*).

Cell culture and cell-free extracts

BHK-21 cells (RIKEN BRC) were cultured in an incubator (5% CO_2) at 37°C in Dulbecco's modified Eagle's medium (Nacalai) supplemented with 2.5% heat-inactivated fetal bovine serum (GIBCO), penicillin (1 U/ml) and streptomycin (0.1 mg/ml). HeLa S3 cells (RIKEN BRC) were cultured and cell extracts were prepared as previously described (*16*).

Virus infection

Confluent BHK-21 cells on 60-mm dishes were infected with EMCV at an MOI of 10. At every hour (1–8 h) post-infection, cells were scraped from a dish, and were suspended in 100 μl phosphate buffered saline. A cell extract was obtained by freezing and thawing an aliquot (10 μl) of the cell suspension for western blotting. RNA was extracted from the remaining 90 μl of the cell suspension for northern blotting.

Cell-free translation and replication of EMCV

EMCV genomic RNA (10 ng/μl) was incubated with the HeLa cell extract in the dialysis system as described (*12*) at 32°C for 2 to 10 h (final volume: 90 μl). The incubated mixture was analysed for protein synthesis by western blot and RNA replication by northern blot. To assay the titre of EMCV, an aliquot of the mixture was treated with RNase A (final concentration, 100 μg/ml) for 30 min at room temperature, and was serially diluted (10^{-2}–10^{-6}) with the culture medium. Confluent BHK-21 cells on a 60-mm dish were incubated with the diluted sample (1 ml) for 1 h. Following removal of the sample medium, an agar-containing medium [0.5 ml agar (2.5% in water, Difco) plus 2 ml medium] was overlaid on the cells. After the agar layer became solid (~30 min at room temperature), 2.5 ml of medium was overlaid and incubated for 30 h at 37°C. Plaques formed on each dish were counted, and plaque-forming units (pfu) per microlitre translation mixture were calculated.

Cell-free systems for *cis* and *trans* replicons

Cis-replicon RNA (1 ng/μl) was incubated with the HeLa cell extract in the dialysis system as described (*12*) at 32°C for 24 h (final volume: 90 μl). For the *trans*-replicon experiments, the HeLa cell extract was pre-incubated with the RNA (final concentration: 50 ng/μl) expressing 2A-2B-2C-3A-3B-3C-3D by the dialysis method at 32°C for 7 h. When required, the extract was further incubated with cycloheximide (final concentration: 100 ng/μl), puromycin (final concentration: 100 μM) or the eIF4A mutant (final concentration: 30 ng/μl) at room temperature for 10 min. The *trans*-replicon RNA (1 ng/μl) was then injected and incubated with the extract by the dialysis system at 32°C for 24 h. The R-luc activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Western blotting and antibodies

Western blotting was carried out using standard techniques. Anti-sera against EMCV 1B and 3D proteins were obtained by immunization of rabbits with the synthetic peptides DQNTEEMENLSDRVS-C and GALERLPDGPRIHVPRKT-C, respectively; one cysteine residue was added to the C-terminus of each peptide for conjugation to a carrier protein. Anti-S6 antibody (5G10) was purchased from Cell Signaling Technology. Anti-Calnexin (E-10) and anti-myc antibody (9E10) were purchased from Santa Cruz Biotechnology. Anti-eIF4G was previously described (*17*). Protein bands were visualized and quantified by LAS 4000 mini (Fuji).

Northern blotting

A 10 μl aliquot of the incubated extract was diluted with 90 μl TE (40 mM Tris–HCl (pH 7.5), 0.2 mM EDTA) and treated with 0.25% sodium dodecyl sulphate at room temperature for 10 min. The samples were then mixed with phenol (100 μl saturated with citrate buffer pH 4.3) and chloroform (100 μl). Nucleic acids were precipitated with ethanol (250 μl) in the presence of 0.3 M sodium acetate (pH 5.2). The precipitate was dissolved in 10 μl TE, and used for northern blotting. Northern blotting was performed as described (*14*) using a digoxigenin (DIG)-UTP-labelled RNA anti-sense probe that was complementary to the R-luc or the EMCV 3C coding region. These probes were synthesized with T7 RNA polymerase from pcDNA3 anti-EMCV 3C or pUC-T7-anti-R-luc (*14*) in the presence of DIG RNA Labeling mix (Roche). RNA bands were

visualized and quantified by LAS 4000 mini (Fuji). The membrane was subsequently stained with toluidine blue (0.02%) to confirm that equal amounts of RNA were blotted in every lane on the membrane.

Results

Validation of the use of the cell-free system

We recently improved a cell-free system for EMCV synthesis by incorporating a dialysis technique (*12*). This system continuously supplies amino acids, nucleotides and energy-related molecules including ATP and creatine phosphate, and efficiently removes waste products through a dialysis membrane, thereby leading to a greater than eight-fold increased titre of synthesized EMCV compared with a conventional closed (batch) system (*12*). To verify that the EMCV synthesis *in vitro* reflects the *in vivo* situations, we examined chronological relationship between translation and replication of the EMCV RNA *in vivo* and *in vitro*.

We infected BHK-21 cells with EMCV, monitoring translation of viral proteins by western blot and RNA replication by northern blot. Viral proteins 1B (also in the form of 1AB) and 3D steadily accumulated from 5 to 8 h post-infection (Fig. 1A); 3CD was not detectable in this blot, possibly due to rapid processing of 3CD into 3C and 3D. Similarly, the EMCV RNA was progressively amplified from 4 to 8 h post-infection (Fig. 1B). Note that the timing of the appearance of the signal in these assay systems depended on the sensitivity of each probe. These results demonstrate that translation and replication of the EMCV RNA genome proceed simultaneously in the infected cells.

We next incubated the genomic EMCV RNA with the HeLa cell extracts in the dialysis system. Translation and replication of the EMCV RNA proceeded progressively and simultaneously (Fig. 1C and D), recapitulating the profile of translation and replication of the viral RNA in the infected cells. Infectious virus particles were generated after 4 h incubation, with the titre of the virus approaching 2×10^4 pfu/μl at 10 h (Fig. 1E) and $\sim 10^7$ pfu/μl translation mixture at 24 h of incubation (data not shown). These observations validate the use of the cell-free system to study the relationship between translation and replication of the viral RNA. There are a few differences in the profiles of the viral synthesis *in vivo* and *in vitro*. The level of the 1B protein relative to that of the 1A-1B protein in the cell-free system was much lower than that *in vivo* (Fig. 1A and C). Since the appearance of the 1B protein represents maturation of the picornavirus particles (*18*), the EMCV particles probably matures *in vitro* more slowly than *in vivo*. In the RNA analyses (Fig. 1B and D), there were multiple bands for the EMCV RNA with the RNA fraction from the cell-free system, whereas only the single band (the full-length RNA) was detected with the RNA fraction from the infected cells. We speculate that degradation of the EMCV RNA occurs in both systems, but the RNA *in vivo* degrades rapidly, leaving only the RNA in the viral particle intact. In contrast, the EMCV RNA *in vitro* may degrade more slowly than *in vivo*, resulting in the detection of the RNA fragments that are under degradation.

Cell-free *cis-RNA replicon*

Since the picornavirus genome RNA serves as the template for translation and transcription, and also is encapsulated to form progeny virions (*18*), any analysis should be complicated by the simultaneous occurrence of all three events. Thus, we next employed an RNA replication system in which the leader (L) and capsid-encoding region (1A-1B-1C-1D) were replaced with R-luc as a translation marker, thereby evading confounding effects of genome packaging on RNA translation/replication. Our initial construct contained (5′–3′) a ribozyme (Rbz: an RNA sequence to remove extra 5′ nucleotides derived from the template plasmid) (*12*), the EMCV 5′ UTR (1–834 nt), R-luc-myc, the EMCV IRES (241–834 nt), the functional protein-coding region (2A-2B-2C-3A-3B-3C-3D) and the 3′ UTR followed by a poly-A tail. This RNA construct was tested for the ability to replicate in the cell-free system, but it failed to do so (data not shown). We then inserted a portion of the 1B region between R-luc-myc and the EMCV IRES (Fig. 2A), because a *cis*-acting replication element (Cre), which is considered to be the scaffold for the replication machinery, resides in the 1B-capsid protein coding region of Theiler's murine encephalomyelitis virus (TMEV): TMEV belongs to the same genus (*Cardiovirus*) as EMCV (*19*). When this RNA (hereafter referred to as the *cis*-replicon RNA) was incubated with the HeLa cell extract in the dialysis system, the input RNA was largely degraded during the first 4 h of incubation, but the remaining RNA replicated between 12 and 24 h of incubation (Fig. 2B). Replication was due to the encoded 3D RNA polymerase, because a frame-shift mutation in the middle (at 7118 nt) of the 3D RNA polymerase abrogated replication (Fig. 2B and C). R-luc activity (Fig. 2D) along with the amount of the Renilla-myc protein (Fig. 2C) increased as the *cis*-replicon RNA replicated (Fig. 2B and C). Based on these results, we conclude that the *cis*-replicon RNA (Fig. 2A) acted as a bona fide cell-free replicon RNA, and that translation and replication of the replicon RNA occurred simultaneously in this system.

Compared to the virus synthesis system (Fig. 2B), the *cis*-replicon RNA replicated in a delayed manner (Fig. 2B compared to Fig. 1D), because the concentration of the input RNA was 1 ng/μl for the replication system compared to 10 ng/μl for the virus synthesis system; we wished to minimize the background level of the R-luc activity due to the input RNA, which resulted in a delayed production of the 3D protein (Fig. 2C compared to Fig. 1C). When we incubated increased concentrations of the *cis*-replicon RNA (10 and 50 ng/μl), the R-luc activity at 4 h was ~10 and 50 times that observed with 1 ng/μl, respectively. However, the R-luc activity reached similar levels at 24 h irrespective of the concentration of the input RNA (data not shown). Thus, the replication efficiency of the replicon RNA depends on the concentration of the input RNA.

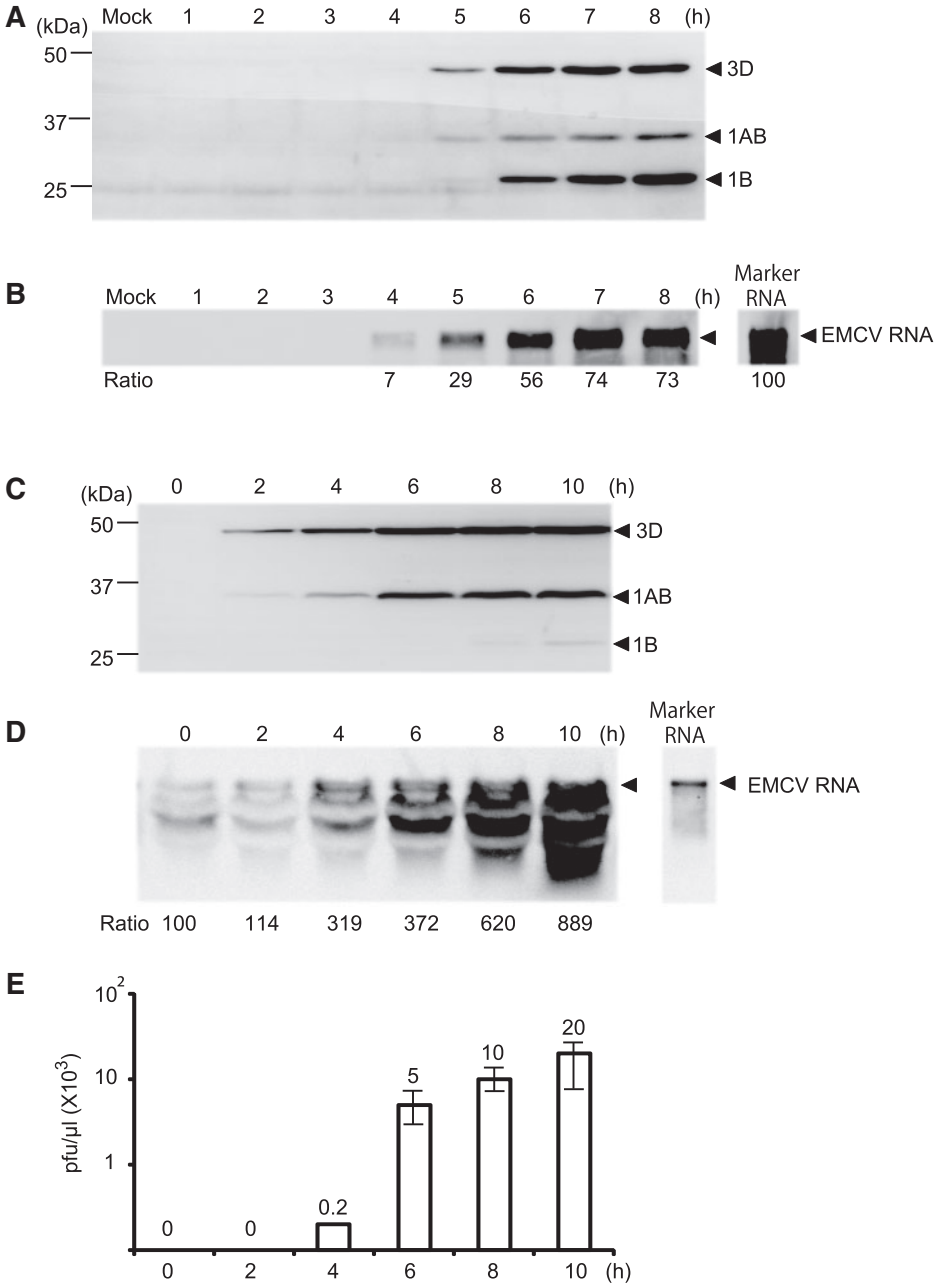

Fig. 1 Translation and replication of EMCV RNA *in vivo* and *in vitro*. (A) BHK-21 cells were infected with EMCV for 1–8 h, and cells were harvested at the indicated time. Mock: no infection. Extracts from the infected cells were resolved on 10% SDS–PAGE, and EMCV 1B, 1AB and 3D proteins were detected by western blot. (B) RNA extracted from the infected cells in (A) was northern blotted with the EMCV 3 C region (anti-sense) as probe. Bands were quantified, and values relative to that of the marker RNA (EMCV RNA, 10 ng) are reported below each lane. The arrowhead indicates EMCV RNA. (C) The EMCV genome RNA (10 ng/μl) was incubated with the HeLa cell extract by the dialysis method for 0–10 h. Western blot with antibodies against EMCV 3D and 1B proteins. (D) Northern blot was carried out with the RNA extracted from the incubated extracts in (C) using the EMCV 3C region (anti-sense) as probe. Bands were quantified, and values relative to that of the marker RNA (EMCV RNA, 10 ng) are reported below each lane. The arrowhead indicates EMCV RNA. (E) Aliquots of the incubated mixture were used to assay the titre of the synthesized EMCV. Each column and error bar indicate the mean and the standard error ($n = 3$), respectively.

To rule out the possibility that the replication of the replicon RNA was due to the synthesis of the reporter protein (R-luc-myc), we replaced the R-luc-myc with the myc-GFP in the replicon RNA, and the GFP replicon RNA was incubated with the HeLa cell extract. The GFP replicon RNA replicated along with the production of the myc-GFP protein (Fig. 2E). These results demonstrate that the cell-free *cis*-replicon replicates irrespective of the reporter protein, suggesting that this system may be used as an *in vitro* protein expression system starting with a limited amount of RNA.

Cell-free *trans*-RNA replicon

We wished to use the cell-free RNA replication system to explore how translation affected viral RNA replication. However, the replication of the *cis*-replicon RNA inarguably depends on its translation because it encodes its own functional proteins (2A–3D). Thus, we devised a cell-free *trans*-RNA replication system

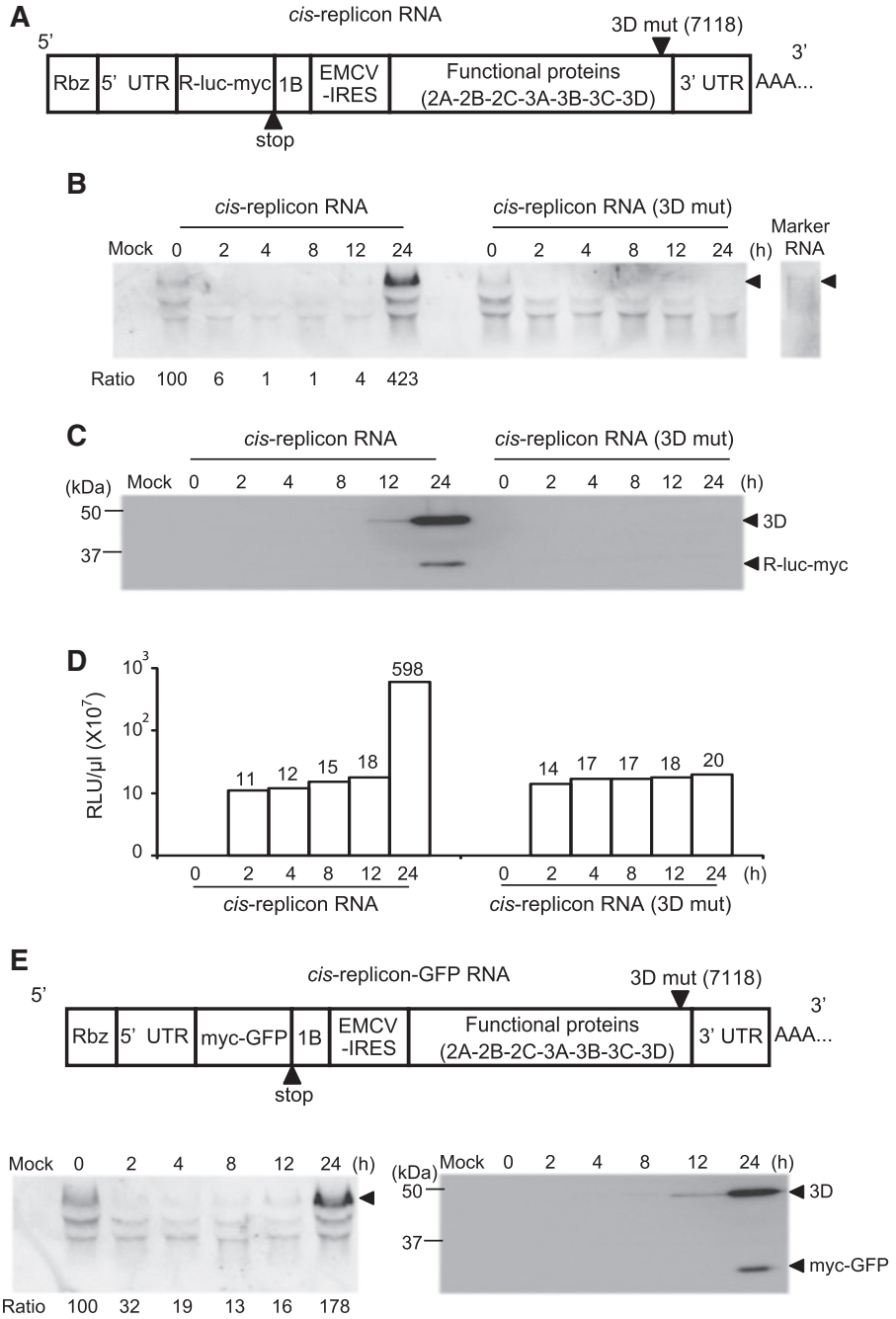

Fig. 2 A cell-free *cis*-RNA replication system. (A) Schematic of the *cis*-replicon RNA. Abbreviations are as defined in the main text. (B) RNA replication. The RNA depicted in (A) was incubated in the cell-free system for 0–24 h. Aliquots were removed from the incubation mixture at the indicated times for northern blots using the R-luc region (anti-sense) as probe. Bands were quantified, and values relative to that of the input RNA (Time 0) are reported below each lane. 3D mut: the RNA carrying the 3D mutation was employed. Mock: no RNA was incubated. Marker RNA: (*cis*-replicon RNA, 10 ng). The arrowhead indicates the *cis*-replicon RNA. (C) Western blot with antibodies against EMCV 3D and the myc-tag. (D) R-luc activity. Values are reported in *Renilla luciferase* units (RLU)/µl incubation mixture. (E) Schematic of the *cis*-replicon-GFP RNA (upper panel). The *cis*-replicon-GFP RNA was incubated in the cell-free system for 0–24 h. Northern blotting was carried out with the EMCV 3C region (anti-sense) as probe (lower left panel). Western blotting was performed with antibodies against EMCV 3D and the myc-tag (lower right panel).

(Fig. 3A), in which the region for the functional proteins (2A–3D) was removed from the *cis*-replicon RNA, and the proteins were supplied in *trans* by translation of another RNA (2A–3D RNA, Fig. 3A). Unlike a co-incubation system (*20*), the HeLa cell extract was pre-incubated with the 2A–3D RNA in the dialysis system for 7 h, and the *trans*-replicon RNA was then injected into the system (Time 0) with incubation continuing for 24 h. Samples obtained at 0, 4 and 24 h of incubation were analysed by northern blot (Fig. 3B) and the R-luc assay (Fig. 3C). The *trans*-replicon RNA was largely degraded during the first 4 h of incubation as observed with the *cis*-replicon (Fig. 2B and E), but the remaining RNA replicated up to the level of the input RNA over 20 h (Fig. 3B). R-luc activity increased between 4 and 24 h of incubation (Fig. 3C). Replication of the *trans*-replicon RNA depends on the pre-expressed proteins (2A–3D), because

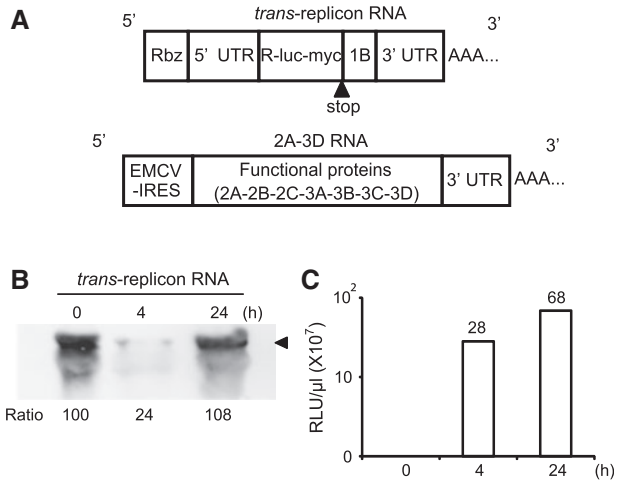

Fig. 3 A *trans*-RNA replication system. (A) Schematics of the *trans*-replicon RNA and the RNA that expresses the polyprotein 2A–3D (2A–3D RNA). Abbreviations are as defined in the main text. (B) RNA replication. The HeLa cell extract was pre-incubated with the 2A–3D RNA for 7 h, and then the *trans*-replicon RNA was added to the system at Time 0. Incubation was continued for 24 h. At the indicated time of incubation, an aliquot was removed for northern blots with the R-luc region (anti-sense) as probe. Bands were quantified, and values relative to that of the input RNA (Time 0) are reported below each lane. The arrowhead indicates the *trans*-replicon RNA. (C) Translation. R-luc activity was measured in each sample. Values are reported in *Renilla luciferase* units (RLUs)/μl incubation mixture.

neither incubation of *trans*-replicon RNA without prior expression of the functional proteins nor the pre-incubation with 2A–3D RNA that possessed the 3D mutation allowed replication of the *trans*-replicon RNA (data not shown).

Use of translation inhibitors

Having established the *trans*-RNA replication system (Fig. 3), we examined how translation of the replicon RNA affected replication. The HeLa cell extract was pre-incubated with the 2A–3D RNA in the dialysis system for 7 h, and the extract was further incubated with cycloheximide or puromycin for 10 min. The *trans*-replicon RNA was then injected into the dialysis system (Time 0), and incubation was continued for 24 h. Samples obtained at 0, 4 and 24 h of incubation were analysed by the R-luc assay (Fig. 4A) and by northern blot (Fig. 4B). Both compounds completely inhibited translation of the *trans*-replicon RNA (Fig. 4A), and simultaneously prevented its replication (Fig. 4B). Cycloheximide inhibits translation elongation by binding to the E-site of the ribosome (*21*), thereby immobilizing translating ribosomes on the mRNA, while puromycin inhibits translation elongation by occupying the ribosomal A-site in place of an aminoacyl–tRNA, leading to dissociation of the translating ribosome (*22*, *23*). Despite the different mode of inhibition, both compounds abrogated RNA replication. To further confirm that the effect of the translation inhibitors on RNA replication was not due to properties other than translation inhibition, we used an eIF4A mutant as an additional translation inhibitor. eIF4A is an RNA helicase that is essential for translation initiation. eIF4A forms a complex (eIF4F) with eIF4G and eIF4E; dominant negative eIF4A mutants such as eIF4A (DQAD) inhibit translation initiation by forming non-functional eIF4F complexes (*15*, *24*). When eIF4A (DQAD) (*24*) was added after expression of the viral functional proteins (2A–3D), translation of the *trans*-replicon RNA was suppressed being consistent with the observation that translation initiation from the EMCV IRES depends on eIF4A (*25*), and its replication was abrogated simultaneously (Fig. 4C and D). Incubation with either translation inhibitor did not affect the concentration of 3D RNA polymerase in the system (Fig.4E; the 2A–3D RNA was pre-incubated for 7 h, and therefore this RNA was almost degraded at Time 0, producing no more 3D protein). Since treatment with these three different translation inhibitors brought about essentially the same results, we suggest that translation of the replicon RNA promotes its replication.

Discussion

Here we have reported the development of *cis* and *trans* cell-free EMCV RNA replication systems using the HeLa cell-based *in vitro* protein synthesis system that includes dialysis. In this HeLa cell-based cell-free system, translation and replication of the EMCV RNA mirror the processes in infected cells (Fig. 1), validating the use of the cell-free system to explore the mechanism of EMCV RNA replication. The *cis*-replicon RNA containing the open reading frame (ORF) for R-luc or GFP in place of the capsid proteins, the region encoding functional proteins (2A–3D) and the RNA elements necessary for replication was translated and replicated efficiently in the cell-free system (Fig. 2). Since the use of a cell-free replication system does not require the labour and time required to establish cellular RNA replication systems, the cell-free replication system may replace the cellular systems as a tool for studies of viral replication and for screening anti-virus compounds, especially for chemicals that cannot pass the plasma membrane. Also, this system can be used as an *in vitro* protein-expression system. Noteworthy is that only a small amount of mRNA is needed at the start of incubation in this system because the input RNA automatically replicates.

Replication of the *cis*-replicon RNA obviously depends on its own translation, as its translation provides the virally encoded functional proteins necessary for replication. Since we wished to examine how translation, not the translated products, affected replication of the EMCV RNA, we generated a cell-free *trans*-replicon RNA that contained the R-luc ORF and the RNA elements necessary for replication (Fig. 3). This RNA replicates only when the functional proteins are supplied in *trans*. We chose to express these proteins prior to incubation of the *trans*-replicon RNA in the cell-free system, such that subsequently added translation inhibitors did not affect the expression levels of the functional proteins. We used three different translation inhibitors: cycloheximide, puromycin and an eIF4A mutant. The former two chemicals inhibit translation elongation, while the third molecule (a recombinant protein) inhibits translation initiation. All

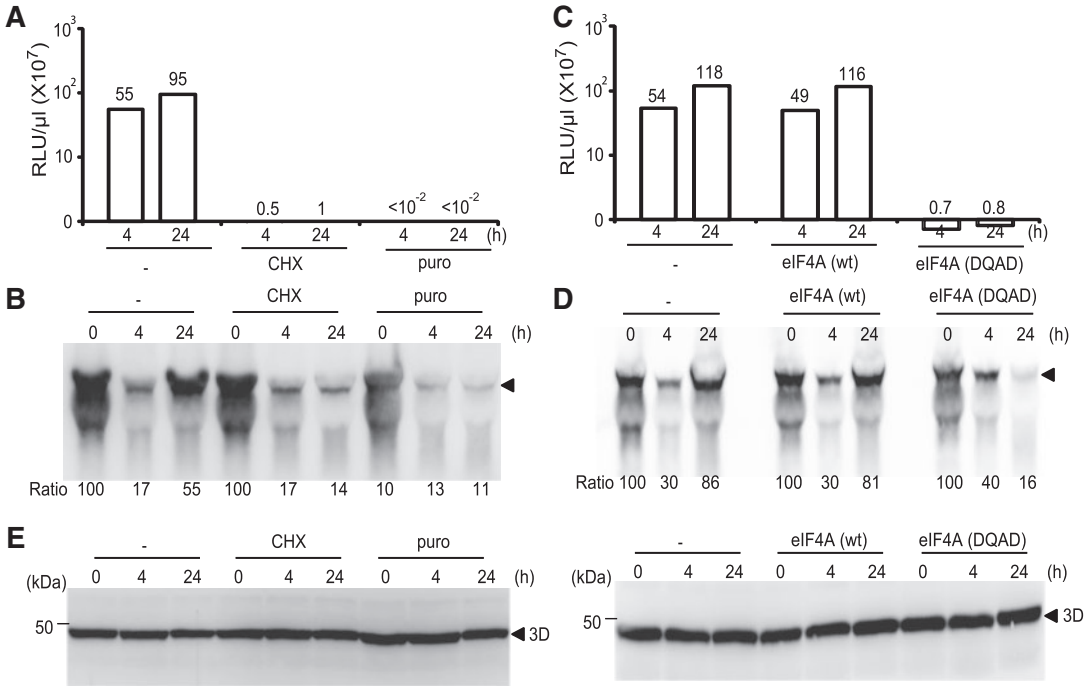

Fig. 4 Inhibition of translation of the *trans*-replicon RNA abrogates its replication. The HeLa cell extract was pre-incubated with the 2A–3D RNA for 7 h. Following treatment with cycloheximide (CHX) or puromycin (puro) for 10 min, the *trans*-replicon RNA was incubated for 4 or 24 h. The incubated mixtures were analysed by the R-luc assay (A) and northern blot using the R-luc region (anti-sense) as probe (B). Bands were quantified, and values relative to that of the input RNA (Time 0) are reported below each lane. The arrowhead indicates the *trans*-replicon RNA. (C) and (D). Experiments were performed as in (A) and (B) except that wild-type eIF4A (wt) or eIF4A mutant (DQAD) was employed instead of cycloheximide and puromycin. (C) R-luc assay. (D) Northern blot. The arrowhead indicates the *trans*-replicon RNA. (E) Western blot for 3D RNA polymerase.

suppressed replication of the *trans*-replicon RNA as well as translation of the RNA in the cell-free system (Fig. 4). We therefore infer that *cis*-translation of the replicon RNA enhances replication of the RNA. Coupling between genome translation and replication of poliovirus was previously proposed from *in vivo* experiments (*26*).

How does translation promote replication? The picornavirus RNA replicates in association with the intracellular membranes (*27*, *28*), and it has been suggested that translation and transcription of poliovirus RNA occur in distinct membranous structures (*29*). We examined whether translation affected association of the *trans*-replicon RNA with membranous structures by sucrose floatation gradient analysis, and found that treatment with any of three translation inhibitors (cycloheximide, puromycin or an eIF4A mutant) reduced the amount of the *trans*-replicon RNA in the membranous fraction (T. Kobayashi *et al*., unpublished data), suggesting that translation of the replicon RNA assists association of the RNA with the membranous structures, thereby promoting RNA replication. Thus, it is likely that the replicon RNA is firstly translated so as to be associated with the membrane for efficient replication.

Cycloheximide and puromycin inhibit translation elongation by different mechanisms. Cycloheximide seems to bind to the E-site of the ribosome, immobilizing the ribosome on the mRNA (*21*), while puromycin mimics the aminoacyl–tRNA in the A-site, resulting in dissociation of the ribosome from the mRNA (*22*, *23*). Our results showed that both compounds inhibited replication of the *trans*-replicon RNA as well as translation, suggesting that translation and replication do not occur at the same time on the same replicon RNA: if the same replicon RNA was translated and replicated at the same time, puromycin would have enhanced replication due to dissociation of the ribosome.

Contradictory results have been reported regarding activities of cycloheximide and puromycin in cell-free systems. For example, puromycin stimulated negative-strand RNA synthesis and overall RNA synthesis of poliovirus in isolated preinitiation replication complexes, while cycloheximide inhibited them, consistent with the idea that the same RNA is used for translation and transcription *(8)*. However, in a separate study, cycloheximide stimulated RNA synthesis of a poliovirus RNA replicon in a HeLa cell extract supplemented with purified poliovirus 3D polymerase (*9*). Our protocol differs from previous work in two respects. First, we used northern blots to monitor RNA replication and show bona fide changes in the RNA amount, while other studies measured incorporation of a radiolabelled nucleotide; this latter technique does not necessarily reflect the absolute amount of the RNA. Secondly, we employed dialysis to maintain conditions similar to those found *in vivo*. Our cell-free system allowed translation and replication of the EMCV RNA as in infected cells (Fig. 1), and we thus believe that our system adequately reflects replication of EMCV in cells. We failed to detect and quantify the negative-strand RNA synthesized from the replicon RNAs in the cell-free system by using

RT–PCR or northern blotting, as it was difficult to accurately distinguish a very small amount of the negative-strand RNA from an ample amount of the positive-strand RNA.

In conclusion, the cell-free RNA replication systems should become useful tools for the study of the viral RNA replication and also for the expression of recombinant proteins *in vitro*.

Funding

Grant-in-Aid for Scientific Research on Innovative Areas (20200056) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.I.); Grant-in-Aid for Scientific Research (C) (22560774) from the Ministry of Education, Culture, Sports, Science and Technology (to H.I.).

Conflict of interest

None declared.

References

1. Molla, A., Paul, A.V., and Wimmer, E. (1991) Cell-free, de novo synthesis of poliovirus. *Science* **254**, 1647–1651
2. Svitkin, Y.V. and Sonenberg, N. (2003) Cell-free synthesis of encephalomyocarditis virus. *J. Virol.* **77**, 6551–6555
3. Fata-Hartley, C.L. and Palmenberg, A.C. (2005) Dipyridamole reversibly inhibits mengovirus RNA replication. *J. Virol.* **79**, 11062–11070
4. Svitkin, Y.V., Herdy, B., Costa-Mattioli, M., Gingras, A.C., Raught, B., and Sonenberg, N. (2005) Eukaryotic translation initiation factor 4E availability controls the switch between cap-dependent and internal ribosomal entry site-mediated translation. *Mol. Cell. Biol.* **25**, 10556–10565
5. Belsham, G.J. and Normann, P. (2008) Dynamics of picornavirus RNA replication within infected cells. *J. Gen. Virol.* **89**, 485–493
6. Egger, D. and Bienz, K. (2005) Intracellular location and translocation of silent and active poliovirus replication complexes. *J. Gen. Virol.* **86**, 707–718
7. Barton, D.J., Black, E.P., and Flanegan, J.B. (1995) Complete replication of poliovirus in vitro: preinitiation RNA replication complexes require soluble cellular factors for the synthesis of VPg-linked RNA. *J. Virol.* **69**, 5516–5527
8. Barton, D.J., Morasco, B.J., and Flanegan, J.B. (1999) Translating ribosomes inhibit poliovirus negative-strand RNA synthesis. *J. Virol.* **73**, 10104–10112
9. Gamarnik, A.V. and Andin, R. (1998) Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes Dev.* **12**, 2293–2304
10. Kaplan, G. and Racaniello, V.R. (1988) Construction and characterization of poliovirus subgenomic replicons. *J. Virol.* **62**, 1687–1696
11. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113
12. Kobayashi, T., Mikami, S., Yokoyama, S., and Imataka, H. (2007) An improved cell-free system for picornavirus synthesis. *J. Virol. Methods* **142**, 182–188
13. Hahn, H. and Palmenberg, A.C. (1996) Mutational analysis of the encephalomyocarditis virus primary cleavage. *J. Virol.* **70**, 6870–6875
14. Mikami, S., Kobayashi, T., Masutani, M., Yokoyama, S., and Imataka, H. (2008) A human cell-derived in vitro coupled transcription/translation system optimized for production of recombinant proteins. *Protein Expr. Purif.* **62**, 190–198
15. Fukao, A., Sasano, Y., Imataka, H., Inoue, K., Sakamoto, H., Sonenberg, N., Thoma, C., and Fujiwara, T. (2009) The ELAV protein HuD stimulates cap-dependent translation in a Poly(A)- and eIF4A-dependent manner. *Mol. Cell.* **36**, 1007–1017
16. Mikami, S., Masutani, M., Sonenberg, N., Yokoyama, S., and Imataka, H. (2006b) An efficient mammalian cell-free translation system supplemented with translation factors. *Protein Expr. Purif.* **46**, 348–357
17. Imataka, H., Gradi, A., and Sonenberg, N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.* **17**, 7480–7489
18. Arnold, E., Luo, M., Vriend, G., Rossmann, M.G., Palmenberg, A.C., Parks, G.D., Nicklin, M.J., and Wimmer, E. (1987) Implications of the picornavirus capsid structure for polyprotein processing. *Proc. Natl Acad. Sci. USA* **84**, 21–25
19. Lobert, P.E., Escriou, N., Ruelle, J., and Michiels, T. (1999) A coding RNA sequence acts as a replication signal in cardioviruses. *Proc. Natl Acad. Sci USA* **96**, 11560–5
20. Barton, D.J., O'Donnell, B.J., and Flanegan, J.B. (2001) 5' cloverleaf in poliovirus RNA is a cis-acting replication element required for negative-strand synthesis. *EMBO J.* **20**, 1439–1448
21. Schneider-Poetsch, T., Ju, J., Eyler, D.E., Dang, Y., Bhat, S., Merrick, W.C., Green, R., Shen, B., and Liu, J.O. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat. Chem. Biol.* **6**, 209–217
22. Blobel, G. and Sabatini, D. (1971) Dissociation of mammalian polyribosomes into subunits by puromycin. *Proc. Natl Acad. Sci. USA* **68**, 390–394
23. Starck, S.R. and Roberts, R.W. (2002) Puromycin oligonucleotides reveal steric restrictions for ribosome entry and multiple modes of translation inhibition. *RNA* **8**, 890–903
24. Pause, A., Methot, N., Svitkin, Y., Merrick, W.C., and Sonenberg, N. (1994) Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. *EMBO J.* **13**, 1205–1215
25. Pestova, T.V., Shatsky, I.N., and Hellen, C.U. (1996) Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol. Cell. Biol.* **16**, 6870–6878
26. Novak, J.E. and Kirkegaard, K. (1994) Coupling between genome translation and replication in an RNA virus. *Genes Dev.* **8**, 1726–1737
27. Hsu, N.Y., Ilnytska, O., Belov, G., Santiana, M., Chen, Y.H., Takvorian, P.M., Pau, C., van der Schaar, H., Kaushik-Basu, N., Balla, T., Cameron, C.E., Ehrenfeld, E., van Kuppeveld, F.J., and Altan-Bonnet, N. (2010) Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell* **141**, 799–811
28. Netherton, C., Moffat, K., Brooks, E., and Wileman, T. (2007) A guide to viral inclusions, membrane rearrangements, factories, and viroplasm produced during virus replication. *Adv. Virus Res.* **70**, 101–182
29. Caliguiri, L.A. and Tamm, I. (1969) Membranous structures associated with translation and transcription of poliovirus RNA. *Science* **166**, 885–886