Biochemical and Biophysical Research Communications **263,** 23–27 (1999)
Article ID bbrc.1999.1299, available online at http://www.idealibrary.com on IDEAL®

In Vitro Selective RNA Synthesis with L-A Virus Nanoparticles

Takashi Ebihara, Yasuko Yanagida, Eiry Kobatake, and Masuo Aizawa[1]
Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

Received July 28, 1999

New *in vitro* RNA synthesis has been performed with an L-A virus nanoparticles, in which the gene and polymerase are integrated. The specific recognition sequence (packaging site) of L-A virus was inserted within a gene of interest. Based on the intrinsic replication cycle, the exogenous RNA with the packaging site was encapsulated by an empty L-A virus nanoparticle. The packaging site worked as a recognition site even for exogenous RNAs. The recognized RNA was replicated to dsRNA, and was then transcribed by empty L-A virus nanoparticles. These results indicate that empty L-A virus nanoparticles recognize an exogenous RNA with the packaging site and synthesize RNA *in vitro*.

Recently, *in vitro* RNA synthesis by virus or phage enzymes have been demonstrated and utilized for various purposes, which includes RNA splicing, *in vitro* translation, and post-translational modifications of proteins (1–3). Especially, T7 RNA polymerase, SP6 polymerase and Qβ-replicase are effectively utilized for *in vitro* RNA synthesis. In these methods, however, the genes and polymerases are not so efficiently integrated in the reaction mixture, that the interaction of the gene and polymerase is a rate-determining step. To overcome the drawbacks, a new RNA synthesis system, in which gene and polymerase are encapsulated in a nanoparticle derived from a virus, has been proposed in this paper. A special attention has been paid to L-A virus, here.

L-A virus has been extensively studied in recent years by Wickner *et al.* (4). L-A is a double-stranded RNA (dsRNA) virus of *Saccharomyces cerevisiae* of which diameter is in the range of 40nm (5). The virus includes 4.6 kbp dsRNA as a genome and RNA polymerase inside of the particle. The genome encodes both the 80 kDa major coat protein (Gag) and a 180 kDa minor viral protein (Gag-Pol) (6). Gag-Pol has selective ssRNA-binding activity and plays a role in RNA synthesis, replication and transcription (7–11). L-A undergoes a replication by a conservative mechanism. The (+)-strands are made by transcription of dsRNA, while the (−)-strands are simply copied by themselves to form dsRNA. The (+)-single-stranded RNA(ssRNA) is recognized in a new coat to form new viral particles. Both (+) and (−)-strands are synthesized in a viral particle and newly synthesized (+)-strands are secreted out of particle.

Endogenous L-A virus RNAs, M1 and X (+) strands have been proved to be replicated by these empty L-A virus nanoparticles (12, 13). It was also reported that the 3′ terminal sequence and a nearby stem-loop structure are, along with a distant site, necessary for the *in vitro* replication reaction of X dsRNA (12, 14–16).

These facts encouraged us to construct an *in vitro* RNA synthesis system with L-A virus nanoparticles. It is our primary concern to design an *in vitro* RNA synthesis system with L-A virus nanoparticles, which replicates not only endogenous but exogenous RNA. As schematically illustrated in Fig. 1, both gene recognition and RNA polymerase domains are integrated in an L-A virus nanoparticles. A possible mechanism of the gene recognition should be the identification with the packaging site in the endogenous RNAs. To replicate any exogenous RNAs, the packaging site has been inserted to a gene of interest. The polymerase and gene are thus integrated in a nanoparticle for synthesizing RNA effectively. To demonstrate the *in vitro* RNA synthesis with empty L-A virus nanoparticles, luciferase RNA was selected as a reporter gene. The packaging site of L-A virus was inserted within the exogenous RNA encoding luciferase gene. The performance of the *in vitro* RNA synthesis with L-A virus nanoparticles is described in this paper.

MATERIALS AND METHODS

Materials. Yeast strain TF229 (*MAT***a** *his(3,4) leu2 ski2-2* L-A-HN) and plasmid pRE76-2-14 were kindly gifted by Prof. Reed B. Wickner. Restriction enzymes were purchased from TAKARA Shuzo

[1] To whom correspondence should be addressed. Fax: +81-45-924-5779. E-mail: maizawa@bio.titech.ac.jp.

0006-291X/99 $30.00

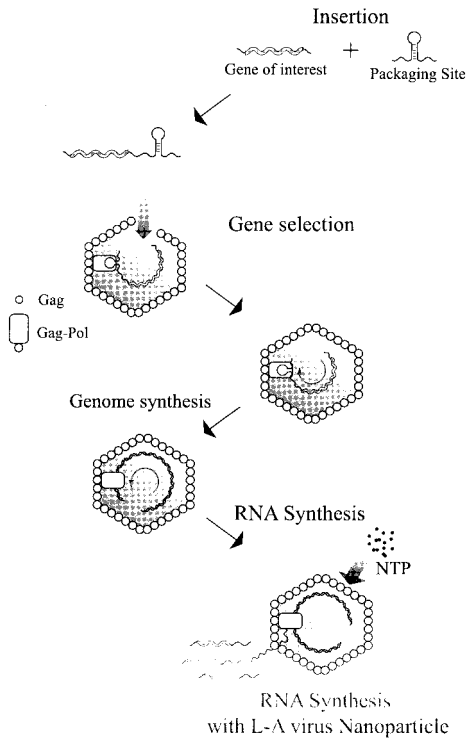

FIG. 1. The schematic illustration of *in vitro* RNA synthesis with an empty L-A nanoparticle.

(Shiga, Japan) for constructing plasmids. MEGAscript T7 was obtained from Ambion (Austin, Texas) for transcription of ssRNAs, and RNeasy was from QIAGEN (Hilden, Germany) to purify RNA products. ^{32}P-UTP was purchased from NEN Life Science Products, Inc. (Boston, MA).

Extraction of L-A virus and preparation of empty L-A virus nanoparticles. Yeast strain TF229 was used to purify mature L-A virus nanoparticles. Empty L-A virus nanoparticles were prepared according to the methods in the previous papers (7, 10, 14, 17) with a slight modification. TF229 cells were grown in YPAD medium (2% glucose, 2% peptone, 1% yeast extract, 0.04% adenine) for 3 days at 30°C and mature L-A virus nanoparticles were isolated from the stationary phase of strain TF229 cells. Cells were harvested, washed once with distilled water, suspended in a spheroplasting buffer (100 mM Tris-HCl, pH 7.6, 1 M sorbitol, 20 mM 2-mercaptoethanol, 1.5 mg/ml Zymolyase), and incubated at room temperature for 1 hr. All subsequent steps were carried out at 0–4°C.

The spheroplasts were collected, suspended in buffer A (50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 150 mM NaCl), and disrupted with a homogenizer. Cell debris was removed by low speed centrifugation (9600 × g, 30 min). Viral particles were collected by high speed centrifugation (100,000 × g, 40 min) and suspended in buffer A. The solution was cleared again by low speed centrifugation (9600 × g, 30 min). The density of the solution was adjusted to 1.35 g/ml by addition of CsCl, and viral particles made band by centrifugation at 130,000 × g for 20 hrs at 4°C.

Fractions containing the particles were dialyzed against buffer A containing 20% (v/v) glycerol for several hours and kept at −70°C. Purified L-A virus nanoparticles were dialyzed against 1000 vol of low ion strength buffer (2 mM Tris-HCl, pH 7.6, 1 mM Na EDTA, and 1 mM dithiothreitol) for several hours at 4°C.

The empty L-A virus nanoparticles were then separated from L-A dsRNA by density gradient centrifugation for 20 hrs at 130,000 × g at 4°C in CsCl at 1.31 g/ml. The empty L-A virus nanoparticle fraction was dialyzed against buffer A containing 20% (v/v) glycerol and kept at −70°C before use.

Plasmid construction. The plasmid pBS5LAlucPS was constructed as a template for ssRNA synthesis as presented in Fig. 2. First, the region of T7promoter and multi cloning site was cut out from pBluescriptSKII$^-$ by digesting with BssHII, and the synthesized DNA fragment coding T7 promoter, 5′NCR (30 bp) of L-A virus and some restriction enzyme sites was ligated with the digested plasmid. Next, the packaging site from pRE76-2-14 (digested with DraI and SacI) was inserted into the plasmid. This plasmid was named pBS5LAPS, encoding the whole sequence of X in which some restriction enzyme sites were inserted to combine an exogenous gene. At last, the luciferase gene fragment from pT3T7luc (digesting with by PstI and KpnI) was inserted into pBS5LAPS. This plasmid was named pBS5LAlucPS.

Preparation of single-strand RNAs. The plasmid pBS5LAlucPS was digested at FspI site, and single-strand RNAs (ssRNA) were transcribed from the plasmid with T7 RNA polymerase. The transcription of ssRNAs was confirmed by 1.5% agarose electrophoresis. (+)LAPS ssRNA coding the packaging site was transcribed from pBS5LAPS, and (+)LAlucPS ssRNA coding luciferase and the packaging site at its 3′ end was transcribed from pBS5LAlucPS.

In addition, (−)LAPS ssRNA, (complement ssRNA of (+)LAPS ssRNA), and (+)LAluc ssRNA (without the packaging site) were prepared by transcription from pBS5LAlucPS digested with KpnI. ^{32}P-labeled RNAs were prepared by addition of ^{32}P-UTP to the transcription reaction according to the manual of transcription kit.

Recognition of specific RNA by an empty L-A virus nanoparticles. Empty L-A virus nanoparticles and ^{32}P-labeled RNA (50 ng) were incubated in 15 μl reaction mixture (50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 150 mM NaCl, 10 μg yeast t-RNA), at 30°C for 20 min. After incubation, the products were analyzed by electrophoresis to separate complex of ssRNA and empty L-A virus nanoparticle in a 1.5% agarose gel, and BAS2000 system (Fuji film) was used to acquire the image.

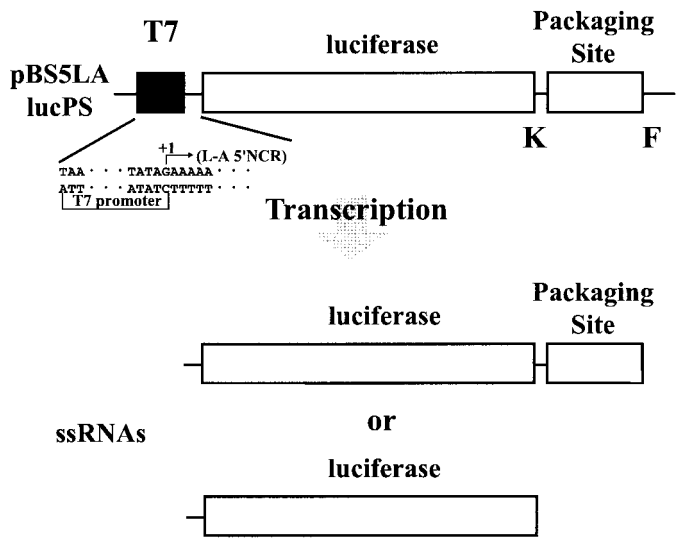

FIG. 2. Schematic representation of plasmid pBS5LAlucPS and transcribed ssRNA with/without the packaging site. pBS5LAlucPS encoded T7 RNA promoter and luciferase and packaging site from pRE76-2-14. K and F represent Kpn I and Fsp I sites, respectively. After digesting the plasmid with Kpn I or Fsp I, it was used as a template for transcription reaction by T7 RNA polymerase, and ssRNA was prepared.

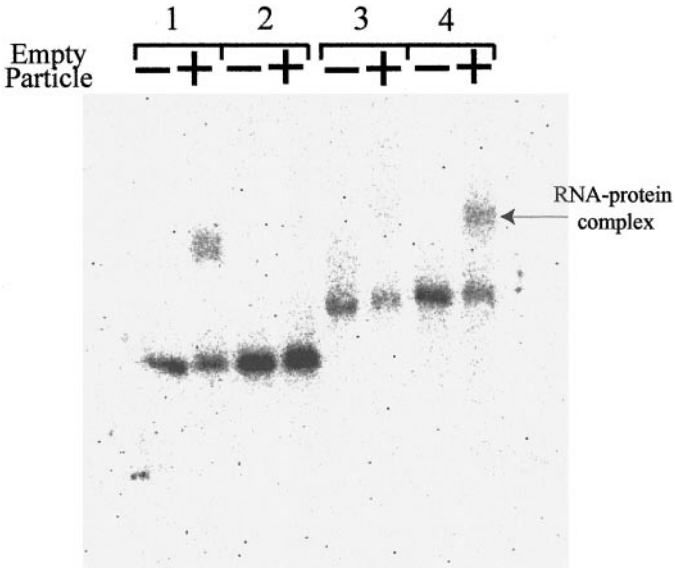

FIG. 3. Recognition of template ssRNA by an empty L-A nanoparticle. The ssRNA and empty L-A nanoparticles were mixed and incubated at 30°C for 20 min and electrophoresed in 1.5% agarose gel. (1) ssRNA encoding only packaging site. (2) ssRNA encoding complement sequence of packaging site. (3) ssRNA encoding luciferase sequence without packaging site. (4) ssRNA encoding luciferase sequence with packaging site. Each ssRNA was incubated with empty L-A nanoparticles (+), and without empty L-A nanoparticles (−) respectively, which was followed by application to gel electrophoresis.

Replication reaction. The RNA replication reaction was carried out according to the previous paper (13). The reaction mixture (25 μl) containing 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.1 mM Na EDTA, 20 mM NaCl, 5 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM each of ATP, CTP, and GTP, 0.2 μM [α-^{32}P]UTP, 10% PEG4000, added template ssRNA (1 μg), and empty L-A virus nanoparticles (10 μg of protein) was incubated for 90 min at 30°C. The RNA products were extracted and purified with RNeasy column, and separated in a 1.5% agarose gel, and detected as described above.

Denaturation of dsRNA. To separate double-strand RNA (dsRNA) from (+) ssRNA and (−) ssRNA, the RNA products were denatured for 1 min at 90°C in the presence of 30% DMSO, and 7 M urea. The sample was quickly chilled and applied to a 1.5% agarose gel.

Transcription reaction. The transcription reaction mixture was similar in component to that of the replication reaction except 20% (v/v) PEG4000. The RNA products were extracted and purified, and assayed in a 1.5% agarose gel.

Annealing test. ^{32}P-labeled samples were incubated for 5 min at 95°C in SSC buffer (150 mM NaCl, 15 mM Sodium Citrate, pH 7.0) containing 2% SDS and (+) or (−) non ^{32}P-labeled ssRNA (2 μg). After incubation, the sample was gradually cooled to room temperature.

RESULTS

Recognition of Specific RNA by Empty L-A Virus Nanoparticles

The ^{32}P-labeled RNA was mixed with the empty L-A virus nanoparticles and incubated at 30°C for 20 min. The products were analyzed by 1.5% agarose gel electrophoresis, which are shown in Fig. 3. RNA encoding only packaging site and empty L-A virus nanoparticles were mixed and incubated. Two bands are observed in the lane of ssRNA mixed with empty L-A virus nanoparticles (the (+) in lane 1, Fig. 3). The faster band must be ^{32}P-labeled RNA encoding only packaging site, since the same migration was observed in the lane, when only ^{32}P-labeled ssRNA was applied without empty L-A virus nanoparticles (the (−) in lane 1). There is another slower band in the lane with empty L-A virus nanoparticles. This slower band indicates that the ssRNA could make a complex with empty L-A virus nanoparticles. The ssRNA encoding complement sequence of the packaging site did not form a complex with empty L-A virus nanoparticles (the (+) in lane 2). Also there is a single band, when ssRNA encoding only luciferase is mixed and incubated with empty L-A virus nanoparticles (the (+) in lane 3). However, ssRNA encoding luciferase with the packaging site formed a complex with empty L-A virus nanoparticles, and slowly migrated band was observed (the (+) in lane 4). This result indicated that even an exogenous RNA encoding packaging site could be recognized by empty L-A virus nanoparticles.

In Vitro RNA Replication

Replication was carried out under the condition according to previous studies of R. B.Wickner *et al.* (13) with a slight modification. The LAlucPS ssRNA was used as a template for replication reaction. LAlucPS ssRNA encoded luciferase sequence and the packaging site. This ssRNA and empty L-A virus nanoparticles were mixed and incubated with some cationic ions and NTP as a substrate for replication reaction. The puri-

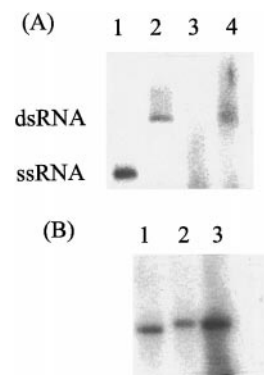

FIG. 4. Replication reaction of exogenous RNA with empty particles. The ssRNA encoding luciferase and packaging site were mixed and replicated with empty nanoparticle. The reaction mixture was assayed by 1.5% agarose gel electrophoresis. (A) Without denaturation before loading (lane 1, ^{32}P-labeled (+)LalucPS; lane 2, ^{32}P-labeled dsRNA of LalucPS; lane 3, replication reaction without template ssRNA (+)LalucPS; lane 4, replication reaction with template ssRNA (+)LAlucPS). (B) With denaturation before loading (lane 1, ^{32}P-labeled (+)LalucPS; lane 2, ^{32}P-labeled (−)LalucPS; lane 3, same sample of lane 4 in A with denaturation).

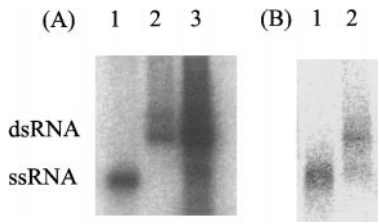

FIG. 5. The transcription reaction of exogenous RNA with empty particles. (A) The product of transcription reaction was applied on 1.5% agarose gel (lane 1, ^{32}P-labeled ssRNA (+)LalucPS; lane 2, ^{32}P-labeled dsRNA of LalucPS; lane 3, transcription reaction product). (B) Annealing test. The faster band of lane 3 in A was isolated and annealed with non-^{32}P-labeled (+)LAlucPS ssRNA (lane 1) or (−)LAlucPS ssRNA (lane 2).

fied RNA of the sample was analyzed in a 1.5% agarose gel. The results are shown in Fig. 4. First, electrophoresis without any treatment before loading is presented in Fig. 4-(A). The slower band is observed in the lane 4, compared with control ^{32}P-labeled LAlucPS ssRNA (lane 1). This band appears in the lane 3, where the product from the reaction without (+)LAlucPS ssRNA was applied. Therefore, this band was template dependent and RNA synthesis reaction was proceeded according to the exogenous RNA. This band was considered as dsRNA that was replicated by empty L-A virus nanoparticles. To confirm this, the same samples were applied in agarose gel with denaturation before loading (Fig. 4-(B)). In Fig. 4-(B), the template dependent band migrated up to the same level of ssRNA (lane 3). This suggests that the newly synthesized RNA is in double stranded structure and (−)LAlucPS ssRNA, because its position is comparable to ^{32}P-labeled (−)LAlucPS ssRNA (lane 2). These results indicate that an empty L-A virus nanoparticles is effective on replicating exogenous RNA to dsRNA.

In Vitro RNA Transcription

For transcription reaction, 20% PEG4000 was added to the replication reaction mixture and incubated at 30°C. After incubation, two bands appeared as shown in lane 3 of Fig. 5-(A). The slower band is ascribed to dsRNA that was produced by replication. And the faster band could be regarded as ssRNA that was transcribed by transcription reaction from its size. The RNA in the faster band was isolated and examined by annealing test. Annealing with (−)LAlucPS ssRNA resulted in shifting to the level of dsRNA (lane 2 in Fig. 5-(B)), while no difference was observed when the band was annealed with (+)LAlucPS ssRNA. This suggests that the faster band includes (+)LAlucPS sequence, and that empty L-A virus nanoparticles transcribe new RNA strands from the exogenous RNA with the packagin site.

DISCUSSION

An effective integration of molecules leads to effective expression of function, even for RNA synthesis system. From this aspect, an *in vitro* RNA synthesis system has been constructed with empty L-A virus nanoparticles as schematically illustrated in Fig. 1. The insertion of the packaging site was confirmed to be effective on selective recognition of exogenous gene of interest by empty L-A virus nanoparticles. A template ssRNA was designed to encode the packaging site and exogenous gene information of interest (luciferase). The experimental results are completely supported by the findings reported elsewhere (12, 13). In the empty L-A virus nanoparticles, RNA synthesis was catalyzed at the RNA polymerase site with reflecting the template ssRNA. The product was identified to dsRNA, which was replicated from the exogenous RNA. It is pointed that excess addition of host factors should not be required for replication and transcription reactions, though it was reported that native M1 RNA was replicated in the presence of host factors (13). Our results indicate that empty L-A virus nanoparticles prepared in this paper include host factors that are required for replication. Figure 5 shows that empty L-A virus nanoparticles transcribed exogenous RNA encoding luciferase and packaging site. This reaction needs 20% PEG4000. PEG4000 enhanced the RNA synthesis reaction by empty L-A virus nanoparticles, which was previously reported (13).

These results show that L-A virus can be a useful material to construct new RNA synthesis system. Empty L-A virus nanoparticles can recognize RNA encoding interest sequence with packaging site, and replicate and transcribe new RNA strand. These facts prompt us to construct general RNA selection system and RNA synthesis system based on L-A virus.

Here, we showed only the main point of this system, that is, the empty L-A virus nanoparticles can work as an RNA synthesis machine for exogenous RNA. Now we are studying details of this system, for example, assemble process, particle form RNA secretion, and also applying this system with cell-free protein synthesis system.

ACKNOWLEDGMENT

We thank Dr. R. B.Wickner, National Institute of Health, Bethesda, for his kind providing us with L-A virus and its cDNAs.

REFERENCES

1. Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) *Nucleic Acids Res.* **15,** 8783–8798.
2. Gurevich, V. V., Pokrovskaya, I. D., Obukhova, T. A., and Zozulya, S. A. (1991) *Anal. Biochem.* **195,** 207–213.
3. Wu, Y., Zhang, D. Y., and Kramer, F. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11769–11773.

4. Wickner, R. B. (1992) *Annu. Rev. Microbiol.* **46,** 347–375.
5. Fujimura, T., Ribas, J. C., Makhov, A. M., and Wickner, R. B. (1992) *Nature* **359,** 746–749.
6. Icho, T., and Wickner, R. B. (1989) *J. Biol. Chem.* **264,** 6716–6723.
7. Fujimura, T., Esteban, R., and Wickner, R. B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4433–4437.
8. Fujimura, T., and Wickner, R. B. (1987) *Mol. Cell. Biol.* **7,** 420–426.
9. Fujimura, T., and Wickner, R. B. (1988) *Cell.* **55,** 663–671.
10. Fujimura, T., and Wickner, R. B. (1988) *J. Biol. Chem.* **263,** 454–460.
11. Fujimura, T., Esteban, R., Esteban, L. M., and Wickner, R. B. (1990) *Cell.* **62,** 819–828.
12. Esteban, R., and Wickner, R. B. (1988) *J. Virol.* **62,** 1278–1285.
13. Fujimura, T., and Wickner, R. B. (1989) *J. Biol. Chem.* **264,** 10872–10877.
14. Esteban, R., Fujimura, T., and Wickner, R. B. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4411–4415.
15. Esteban, R., Fujimura, T., and Wickner, R. B. (1989) *EMBO J.* **8,** 947–954.
16. Fujimura, T., and Wickner, R. B. (1992) *J. Biol. Chem.* **267,** 2708–2713.
17. Esteban, R., and Wickner, R. B. (1986) *Mol. Cell. Biol.* **6,** 1552–1561.