Semliki Forest Virus Replication Complex Capable of Synthesizing 42S and 26S Nascent RNA Chains

By PETER J. GOMATOS, 1 LEEVI KÄÄRÄINEN, 2 SIRKKKA KERÄNEN, 2 MARJUT RANKI 2 and DOROTHEA L. SAWICKI 1*

1Division of Animal Virology, The Sloan-Kettering Institute, New York, N.Y. 10021, U.S.A. and 2Department of Virology, University of Helsinki, 00290 Helsinki 29, Finland

(Accepted 31 January 1980)

SUMMARY

A complex synthesizing Semliki Forest virus (SFV)-specific RNAs was purified from infected HeLa cells. During purification, the RNA-synthesizing complex was monitored by the presence of RNA chains synthesized during a 1 min pulse in vivo and the ability to synthesize 42S and 26S RNAs in vitro. Finally, the protein composition of the replication complex was analysed. Thirty to 40% of the pulse-labelled RNAs and 10 to 25% of the polymerase activity present in the postnuclear supernatant were recovered in smooth membranes. At this stage of purification single stranded 42S and 26S RNA were synthesized and released from the replication complex in vitro. After treatment of the smooth membrane fraction with Triton X-100 the replication complex was solubilized. When analysed by sucrose gradient centrifugation, the solubilized replication complex distributed heterogeneously. It had reduced RNA polymerase activity, but was still able to synthesize both 42S and 26S nascent RNA chains which were not released from RNIs and RFs. The non-structural protein ns70 was the major virus-specified component associated with the replication complex.

INTRODUCTION

The 42S RNA genome of Semliki Forest virus (SFV) is infectious (Sonnabend et al. 1967) and thus does not require a virion-associated transcriptase to initiate infection. Addition of inhibitors of protein synthesis from the start of infection prevents RNA replication and virus formation (Wecker et al. 1962; Sreevalsan & Lockart, 1964; Ranki & Kääriäinen, 1970; Wengler & Wengler, 1976a), indicating a requirement for virus-induced protein synthesis to initiate the replication cycle. The parental 42S RNA is apparently translated into proteins which function as the virus RNA polymerase, synthesizing first 42S RNA negative strands (for review see Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1977; Kääriäinen & Söderlund, 1978), which in turn are used as templates for the synthesis of positive strands of 42S RNA and of 26S RNA (Simmons & Strauss, 1972). The latter RNA is a partial transcript of about one third of the 42S RNA negative strand from its 5' end (Kennedy, 1976; Wengler & Wengler, 1976b).

Four virus-specific non-structural proteins with mol. wt. of 70000 (ns70), 86000 (ns86), 72000 (ns72) and 60000 (ns60) have been demonstrated in SFV ts-1 mutant-infected cells.

* Present address: Department of Microbiology, Medical College of Ohio at Toledo, Toledo, Ohio 43699, U.S.A.
P. J. GOMATOS AND OTHERS

(Lachmi & Kääriäinen, 1976; Glanville et al. 1978). They are also found in wild type-infected cells, although in smaller amounts (Lachmi & Kääriäinen, 1977). It is probable that these proteins form the virus-induced components of the SFV RNA polymerase. Virus-specific RNA polymerase activity has been demonstrated in SFV-infected cells by several groups (Martin & Sonnabend, 1967; Martin, 1969; Sreevalsan & Yin, 1969; Friedman et al. 1972; Michel & Gomatos, 1973).

Clewley & Kennedy (1976) have purified a polymerase activity from SFV-infected BHK21 cells and identified two protein components with approximate mol. wt. of 90000 and 63000 by affinity chromatography on Sepharose-bound 42S RNA. The former is probably the same as ns86 and the latter equivalent to ns70 (Clegg et al. 1976; Lachmi & Kääriäinen, 1976).

Because isolation of a template-dependent polymerase from SFV-infected cells has proved difficult, we have approached the identification of the polymerase components by studying the complex formed between replicating RNA and polymerase. Recently we reported the isolation of a replication complex, the criterion of which was the labelling of nascent RNA chains in vitro by active polymerase (Ranki & Kääriäinen, 1979). In this paper we describe the isolation and partial purification of a replication complex which retains the capacity to synthesize RNA in vitro throughout the purification procedure. The final membrane-free and purified replication complex was composed of replicative intermediate RNA and of one major virus-specific non-structural protein.

METHODS

Viruses and cells. Wild type SFV, obtained from the second passage of SFV-infected mouse brain suspension in BHK cells was used in these experiments. The ts-1 mutant of SFV is that of Keränen & Kääriäinen (1974). The HeLa cells, from Dr H. Blough, Shy Eye Hospital, Philadelphia, were grown in 1-litre Corning tissue culture roller flasks in medium 199 supplemented with 10% calf serum.

Preparation and fractionation of infected cell extracts. Confluent monolayers were infected with 50 p.f.u. of SFV/cell in the presence of actinomycin D at 1 μg/ml. After adsorption for 1 h at 37 or 28 °C, the cells were washed and incubation was continued at 37 or 28 °C in the presence of Eagle’s MEM supplemented with 0.2% bovine plasma albumin and actinomycin D at 1 μg/ml. At the times indicated, the cells were collected and pelleted in cold phosphate-buffered saline (PBS), then swollen in hypotonic RS buffer (0.01 M-NaCl, 0.01 M-tris-hydrochloride, pH 8), disrupted by homogenization and the cytoplasmic extract obtained after pelleting the nuclei (Ranki & Kääriäinen, 1979).

To obtain the ‘mitochondrial pellet’ (P-15), the cytoplasmic extract was centrifuged at 15000 g for 20 min. The P-15 was resuspended in RS buffer, made 30% in sucrose and separated into various fractions by equilibrium centrifugation in discontinuous sucrose gradients as described by Caliguiri & Tamm (1970). Centrifugation was at 4 °C for 17 h at 24000 rev/min in an SW27 rotor. The material at each interface or collecting within a specific layer was diluted with RS buffer and obtained as a pellet by centrifugation in an SW50.1 rotor for 90 min at 45000 rev/min.

Radioactive labelling of virus components. To label nascent RNA chains only, 5,6-3H-uridine (26 Ci/mmol) at 100 μCi/ml in 5 ml was added at 1 min before harvest to one-fifth the number of roller flasks used. The cells at harvest were scraped on to frozen PBS for processing. The labelling of virus-specific proteins with 35S-methionine (800 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.), was carried out as described (Kääriäinen et al. 1978; Ranki & Kääriäinen, 1979).
**SFV replication complex**

*Assay conditions for SFV RNA polymerase.* In vitro RNA synthesis was carried out under conditions described previously except that polyvinylsulphate was omitted from the reaction mixture (Michel & Gomatos, 1973). Incubation was at 28 °C for 30 min. After synthesis, the mixture was made 0.02 m in EDTA, 1.95 % in sodium dodecyl sulphate and analysed in 15 to 30 % sucrose gradients in TNE buffer (0.01 m-tris hydrochloride, pH 7.4, 0.1 m-NaCl and 0.001 m-EDTA) after centrifugation in an SW27 rotor at 23000 rev/min for 16 to 17 h at 15 °C. The amount of radioactivity in acid-insoluble, *in vitro*-synthesized RNA was determined in a sample of each sucrose gradient fraction, and that in RNAs of known SFV RNA size was summed as a measure of synthesis *in vitro* of SFV-specific RNAs.

For analysis of replicative forms (RF), the material sedimenting from 20 to 30S was combined, extracted further with phenol-chloroform mixture, precipitated with ethanol and centrifuged in sucrose gradients after treatment for 15 min at 25 °C with RNase A at 0.02 μg/ml (Sawicki & Gomatos, 1976).

**Polyacrylamide gel electrophoresis in the presence of SDS.** Polyacrylamide slab gels with 10 % acrylamide in the main gel and 3 % in the spacer gel were made according to the method of Neville (1971), as described by Lachmi *et al.* (1975).

**Materials.** 5,6-3H-uridine (26 Ci/mmol) and 32P-GTP labelled in the α position were purchased from New England Nuclear Corp. (Boston, Mass.). 35S-methionine (800 Ci/mmol) was obtained from Amersham/Searle. All the other materials were obtained from previously described sources (Michel & Gomatos, 1973; Sawicki & Gomatos, 1976; Kääriäinen *et al.* 1978).

**RESULTS**

**Cellular distribution of labelled nascent SFV RNA**

In order to monitor the replication complex the nascent RNA chains were labelled with a short pulse of radioactive uridine before harvesting the cells. This revealed the site of the enzyme conveniently. When cells were pulse-labelled for 1 min with 3H-uridine before harvest, 80 to 90 % of the pulse-labelled RNA remained in the postnuclear supernatant. Fig. 1 shows that the pulse-labelled RNA was in replicative intermediates (RIs) which, after ribonuclease-treatment, had a double-stranded (ds) RNA core sedimenting at 22S (data not shown). No single-stranded (ss) 42S or 26S RNAs were labelled in 1 min.

From 60 to 85 % of the pulse-labelled RNA in the postnuclear supernatant was sedimented to the pellet (P-15) by centrifugation at 15000 g for 20 min. The ‘mitochondrial pellet’, P-15, was resuspended in RS buffer and fractionated by centrifugation on discontinuous sucrose gradients, following the procedure outlined by Caliguiri & Tamm (1970). The distribution of pulse-labelled SFV RNAs is shown in Fig. 2. About 40 % of the RNA label was associated with a fraction of smooth membranes at the 25/30 % interface (density of 1.16 g/ml). The remaining pulse-labelled RNA distributed with other membranous fractions of different density. The pulse-labelled RNAs in the fraction of smooth membranes at the 25/30 % interface sedimented in the same way as those in Fig. 1.

**Distribution of SFV RNA polymerase**

Essentially all the SFV polymerase activity present in the cytoplasmic extract was recovered in the P-15. No activity was detectable in the supernatant fraction. There was only a slight increase in specific activity in P-15 when compared to the cytoplasmic extract (Table 1). After further purification by equilibrium centrifugation the polymerase associated with smooth membranes had a twofold higher specific activity than the cytoplasmic extract. The distribution of the RNA polymerase activity in the discontinuous sucrose
Fig. 1. Sedimentation on sucrose gradients of pulse-labelled wild-type SFV RNAs. SFV-infected cells were labelled with $^{3}$H-uridine at 100 μCi/ml at 4.5 h p.i. for 1 min before harvesting. RNA from the cytoplasmic extract was analysed by centrifugation in a 15 to 30% sucrose gradient in the SW27 rotor at 23000 rev/min for 15 h at 15°C. Acid-insoluble radioactivity in 1 ml fractions was determined.

Fig. 2. Distribution of pulse-labelled SFV RNAs after equilibrium centrifugation in discontinuous sucrose gradients. SFV-infected cells were labelled as for Fig. 1. The ‘mitochondrial pellet’, P-15, was resuspended in RS buffer, adjusted to 30% with respect to sucrose and placed above sucrose layers of 65%, 45% and 40%. Above the 30% layer containing the sample were layers of 25% sucrose and RS buffer. All sucrose solutions were in RS buffer. Centrifugation was at 4°C for 17 h at 23000 rev/min in an SW27 rotor. The acid-insoluble radioactivity of 1 ml fractions was determined. The inset shows a polyacrylamide gel analysis of a similar smooth membrane fraction from infected cells pulsed with $^{35}$S-methionine 4 h p.i. The pulse was preceded by hypertonic treatment with 335 mM-NaCl and followed by 1.5 h chase (Ranki & Kääriäinen, 1979).

gradients followed closely that of the pulse-labelled RNAs shown in Fig. 2. The amount of polymerase activity found in the smooth membrane fraction varied in different experiments between 10 and 25% of that originally present in the cytoplasmic extract. Addition of the post-mitochondrial supernatant fraction to P-15 or to the smooth membranes did not increase the amount of SFV RNAs synthesized in vitro by either of them.

Most important for our analysis of the SFV RNA polymerase was the finding that the enzyme associated with smooth membranes yielded virus 42S and 26S ssRNAs containing label incorporated in vitro (Fig. 3). Thus, the components of the polymerase necessary for chain elongation and release of completed ssRNAs were still present in the replication complex at this purification step. The virus-specified proteins associated with the smooth
SFV replication complex

Table 1. Specific activity of SFV RNA polymerase at different stages of purification*

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>( ^{32}\text{P} ) incorporated/mg protein (ct/min ( \times 10^{-8} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnuclear supernatant (CE)</td>
<td>29</td>
</tr>
<tr>
<td>Mitochondrial pellet (P-15)</td>
<td>35</td>
</tr>
<tr>
<td>Smooth membrane pellet</td>
<td>73</td>
</tr>
</tbody>
</table>

* Fractions obtained as outlined in Methods were used for \textit{in vitro} synthesis of RNA for 30 min at 28 °C. \( \alpha^{32}\text{P}-\text{GTP} \) was the labelled precursor for \textit{in vitro} synthesis and was used at a specific activity of 29 900 ct/min/nmol. Protein in individual fractions was determined by the method of Lowry et al. (1951).

Fig. 3. Analysis of SFV RNAs synthesized \textit{in vitro}. SFV-infected cells were harvested at 4.5 h p.i. The smooth membranous fraction, isolated as described in Fig. 2, was used as a source of enzyme in the reaction mixture. Incubation was for 30 min at 28 °C and the \( ^{32}\text{P} \)-labelled GTP was used at a sp. act. of 23 000 ct/min/nmol. After synthesis, the SDS-EDTA mixture was added and the sample analysed by centrifugation on a 15 to 30% sucrose gradient at 15 °C for 17 h at 23 000 rev/min in an SW27 rotor. Acid-insoluble radioactivity from 1 ml fractions was determined.

membranes are shown in Fig. 2 (gel inset). The non-structural proteins ns70, ns72 and ns86 were all present in addition to the virus structural proteins.

\textit{Solubilized replication complex from smooth membranes}

The smooth membranes prepared from infected cells labelled with \( ^{3}\text{H}-\text{uridine for 1 min before harvest, or with } ^{35}\text{S}-\text{methionine, were solubilized with Triton X-100 and the replication complex was separated from the virus structural proteins by sucrose gradient fractionation (Fig. 4). Fig. 4(a) shows the distribution of } ^{35}\text{S}-\text{methionine labelled proteins and Fig. 4(b) the pulse-labelled RNA. The virus nucleocapsids and replication complex separate in the gradient and envelope proteins remain at the top as mixed micelles (Ranki & Kääriäinen, 1979).}

The sedimentation pattern of both proteins and the pulse-labelled RNA varied greatly. If analytical amounts (1 to 2 flasks of cells) were processed, RNA patterns as shown in Fig. 4(b) were regularly obtained. For preparative purposes, the processing took longer and less large-size material was obtained.
Fig. 4. Sedimentation analysis of solubilized smooth membrane-replication complex from SFV-infected HeLa cells. SFV-infected cells (incubated at 28 °C at which temperature the length of the growth cycle is twice as long as at 37 °C) were treated for 60 min with 0.5 M sucrose at 6 h p.i., followed by a pulse of 35S-methionine for 30 min (500 µCi/flask) and unlabelled methionine for 2.5 h further. At 10 h p.i., a 1 min pulse of 3H-uridine (1 mCi/flask) was given to one of the infected cultures before harvest. The smooth membranous fraction was prepared as for Fig. 2 and pelleted. The pellet was resuspended in TN buffer containing 1% Triton X-100 and then centrifuged through a 5 to 30% (w/w) sucrose gradient in TN buffer containing 0.1% Triton X-100 with a 1 ml cushion of 65% sucrose. Centrifugation was in an SW41 rotor at 39000 rev/min for 135 min at 4 °C. Purified Semliki Forest virus labelled with 35S-methionine was treated with Triton X-100 and analysed in a parallel gradient. The position of the nucleocapsid sedimenting at 140S is indicated. The bottom of the gradient is at the left. The protein composition of the fractions indicated by A to D was analysed by polyacrylamide gel electrophoresis (a, inset). The individual proteins were identified by comparing them with proteins from ts-1 mutant-infected cells electrophoresed in a parallel slot (Lachmi & Kääriäinen, 1976).

Polyacrylamide gel analysis of the different sucrose gradient fractions revealed that the major non-structural protein at the sucrose cushion and throughout the gradient, except the top, was ns70 (fractions A to C, Fig. 4a). The envelope proteins remained at the top of the gradient (fraction D) and capsid protein sedimented at 140S. In this gel ns70 and ns72 were not resolved but the identity of ns70 was confirmed by N-terminal labelling (Lachmi & Kääriäinen, 1976; data not shown). In some experiments trace amounts of ns72 and ns86 were found in association with the replication complex.

To demonstrate polymerase activity, material from fractions sedimenting between 20 and 70S was pooled and used for in vitro RNA synthesis. As a result of the in vitro synthesis 85 pmol of 32P-GTP were incorporated into SFV-specific RNA. Sucrose gradient analysis revealed that only replicative forms (RF) and replicative intermediates (RI) were labelled. We were able to determine whether both 42S and 26S RNA nascent chains were synthesized by analysing the RFs created by mild ribonuclease treatment of the RIs. RFI (22S) is the double-stranded form of 42S RNA and RFIII (15S) represents the double-stranded form of 26S RNA (Simmons & Strauss, 1972; Sawicki & Gomatos, 1976). Fig. 5 shows that...
**SFV replication complex**

Fig. 5. The RFs obtained from SFV RNAs synthesized *in vitro* by the solubilized replication complex. Solubilized replication complex derived from the smooth membranes of infected cells was prepared as described for Fig. 4. The material containing the pulse-labelled RNAs corresponding to fractions 20 to 30 was pooled and used as a source of enzyme for incubation in the assay mixture at 28 °C for 30 min. α-32P-GTP was used at a sp. act. of 35,000 ct/min/nmol. After synthesis the RNA was isolated as described in Methods. The RNAs were subsequently treated with pancreatic RNase at a concentration of 0.02 μg/ml for 15 min at 25 °C. The resulting RFs were analysed by centrifugation in a 15 to 30 % sucrose gradient (SW41 rotor at 30,000 rev/min for 16 h). The radioactivity in 0.7 ml fractions was determined. 3H-labelled reovirus RNAs were used as markers for 15', 35' and 10'5S. ● — ●, 32P; ▲ — ▲, 3H.

RFI and RFIII were labelled *in vitro* indicating synthesis of 42S and 26S RNA chains. Thus the solubilized replication complex differed from that in the smooth membranes since it had lost the capacity to release ssRNA.

**DISCUSSION**

The non-structural proteins specified by alphaviruses are probably responsible for the synthesis of virus RNAs in the infected cell based on both genetic and biochemical evidence (Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1977; Kääriäinen & Söderlund, 1978). Large precursors of non-structural proteins accumulate in cells infected with RNA-negative mutants when cultures are shifted to the restrictive temperature (Kääriäinen et al. 1978; Keränen & Kääriäinen, 1979). In our attempts to identify virus-specific components of the SFV RNA replication complex, we used two criteria: (i) presence of nascent positive strands labelled *in vivo* immediately before harvest of the cells; and (ii) presence of RNA polymerase activity *in vitro*.

We purified our SFV replication complex largely as Clewley & Kennedy (1976) had reported, but used a different buffer for disruption of cells and for assay of enzyme activity. During *in vitro* incubation our smooth membrane fraction yielded nanogram quantities of SFV-specific RNAs. It can thus be regarded as a very active enzyme preparation capable of elongating and releasing ssRNAs. We did not investigate whether it initiated synthesis of new RNA chains *in vitro*. The replication complex of Clewley & Kennedy (1976) lost the capacity to release ssRNAs after the first purification step.

After solubilization, we obtained replication complexes which sedimented heterogeneously in a sucrose gradient, but they were free from structural proteins. These complexes
had reduced polymerase activity but analysis of the in vitro product for labelled replicative forms confirmed that both 42S and 26S nascent RNA chains had been synthesized. The solubilized replication complex was thus able to polymerize the RNAs, but not release them from the complex. The major non-structural protein associated with the replication complex was ns7o.

In our recent paper we reported the isolation of an RNA replication complex from P-15 which was allowed to synthesize RNA in vitro and the complex was recognized by its labelled nascent RNA chains. The virus non-structural protein ns7o, in addition to ns72 and ns86 as minor components, were associated with the complex (Ranki & Kääriäinen, 1979). The results of both of these approaches are in conformity and show that ns7o is the major virus-specified protein present in the replication complexes synthesizing both 42S and 26S RNA. Because polymerase activity of such complexes has been shown here, it is suggested that ns7o is the polymerase component responsible for RNA chain elongation. Ns86 and ns72, present in the smooth membrane fraction, were apparently lost during the solubilization and fractionation of the membrane-bound replication complex. These proteins may play a vital role, for example, in chain initiation, termination and possibly in the regulation of 26S RNA synthesis.

We thank Ingrid Kuechenthal for excellent technical assistance. This work was supported by grants from NIAID, no. AI-15528; from NCI, Core Grant CA-08478; from the Sigrid Jusélius Foundation; and from the Finnish Academy.

REFERENCES


*(Received 9 November 1979)*