Assembly of Semliki Forest Virus Nucleocapsid: Detection of a Precursor in Infected Cells

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SUMMARY

The synthesis of Semliki Forest virus nucleocapsid in infected cells was studied by labelling the virus RNAs with $^3$H-uridine for different periods at various phases of infection. Short pulses (10 to 20 min) revealed the accumulation of 42S RNA in a ribonucleoprotein which sedimented at about 90S (90S RNP) and contained only small amounts of capsid protein. Only after longer pulses was the labelled 42S RNA found in the virus nucleocapsid, suggesting that the 90S RNP may be its precursor. The life time of the 90S RNP was long in the early phases of infection and short in the late phases, reflecting the increased rate of assembly of the nucleocapsid during infection. The 90S RNP was the only 42S RNA containing RNP found in cells infected with temperature sensitive mutants deficient in nucleocapsid formation or wild type infected cells treated with cycloheximide to inhibit nucleocapsid assembly.

INTRODUCTION

Semliki Forest virus (SFV) infected cells contain two main virus RNA species, the 26S and 42S RNAs (Friedman et al. 1966; Streevalsan & Lockart, 1966; Friedman & Berezesky, 1967; Sonnabend et al. 1967). The 26S RNA contains the sequences of the 3' end of the virus genome (Kennedy, 1976; Wengler & Wengler, 1976) and encodes the virus structural proteins; it is not assembled into virus particles (Simmons & Strauss, 1972a; Glanville et al. 1976; Strauss & Strauss, 1976; Ulmanen et al. 1976). 42S RNA has several functions: it is the genomic species and serves as a template in virus RNA replication and it is the messenger for the non-structural proteins which are synthesized early in infection (Simmons & Strauss 1972b; Martin & Burke, 1974; Sawicki & Gomatos, 1976; Glanville & Lachmi, 1977; Lachmi & Kääriäinen, 1977). In addition, the 42S RNA combines with the capsid protein to form the nucleocapsid of the virion (Kääriäinen et al. 1969; Acheson & Tamm, 1970; Laine et al. 1973).

We have shown previously that no soluble capsid protein can be detected in the infected cell and that after its synthesis SFV capsid protein associates with the large ribosomal subunit (Ulmanen et al. 1976). This association takes place already in the polysomes and the newly synthesized capsid protein is quite rapidly transferred into nucleocapsid (Söderlund & Ulmanen, 1977). However, a small proportion of the capsid protein was found to sediment, together with the 42S RNA, at about 90S. The 26S RNA sedimented at about 55S, apparently as a ribonucleoprotein (RNP), but capsid protein was not associated with this structure (Ulmanen et al. 1976).

In this report, the synthesis of the SFV nucleocapsid in infected cells is studied using
aH-uridine labelling. It is shown that the 90S RNP structure is a precursor of the intracellular nucleocapsid. When the synthesis of nucleocapsid was inhibited by cycloheximide or by using temperature sensitive mutants of SFV nearly all the 42S RNA was in the 90S RNP form.

METHODS

Viruses and cell cultures. The origin and cultivation of SFV, prototype strain, in HeLa and secondary specific pathogen free chick embryo fibroblasts (CEF II) has been described (Kääriäinen et al. 1969; Söderlund, 1973; Keränen & Kääriäinen, 1974), as has the propagation of temperature sensitive (ts) mutants in CEF II cells (Keränen & Kääriäinen, 1974). Cells were grown as monolayers in 5 cm plastic Petri dishes.

Isotope labelling. Confluent monolayers were infected at a multiplicity of infection of 50. After adsorption for 1 h at 37, or at 39 °C for mutant virus infection, the cells were washed and maintained in Eagle’s minimum essential medium (MEM) containing 0.2% bovine serum albumin and actinomycin D (1 μg/ml). To label the virus specific RNAs, 20 to 50 μCi of aH-uridine (28 Ci/mmol; the Radiochemical Centre, Amersham) per dish was added for the times indicated. Virus proteins were labelled with 35S-methionine (280 Ci/mmol; Radiochemical Centre, Amersham) as described earlier (Ulmanen et al. 1976).

Cell fractionation and isolation of RNPs. Cells were harvested by washing the monolayers with ice-cold phosphate buffered saline containing cycloheximide (100 μg/ml) and the cells were scraped into about 0.7 ml of medium containing 0.15 M-NaCl, 0.01 M-tris, pH 7.4, 1.5 mM-MgCl2 (Iso B) and 0.65% Triton X-100 (Kumar & Lindberg, 1972). Cytoplasmic extracts were prepared as described earlier (Ulmanen et al. 1976). In some experiments the cells were lysed in low salt buffer containing 0.01 M-NaCl, 0.01 M-tris, pH 7.4 (tris-buffer) with Triton X-100. The cytoplasmic extracts were treated with EDTA (25 mM final concentration) when indicated and analysed on 15 to 30% (w/w) sucrose gradients (with a 0.5 ml cushion of 60% sucrose) made in either Iso-B buffer, tris-buffer or in a buffer containing 0.1 M-NaCl, 0.05 M-tris, pH 7.4, and 0.001 M-EDTA (TNE buffer). Gradients were collected using a peristaltic pump and cold acid-insoluble radioactivity was determined. The u.v.-absorbance (254 nm) was scanned with an automatic recorder (LDC LUV Monitor II) to determine the position of the ribosomal subunits, which were used as S-value markers. In some experiments 35S-methionine labelled SFV was added to the lysis buffer to follow the behaviour of virion-derived nucleocapsid undergoing similar treatment to the intracellular nucleocapsid.

Analysis of RNA and proteins was done using sucrose gradients and polyacrylamide-SDS gels as described earlier (Laemmli, 1970; Tuomi et al. 1975). The gels were impregnated with PPO (2,5-diphenyloxazole), dried and fluorographed. Protein concentrations were determined according to Lowry et al. (1951).

Determination of buoyant densities. Samples were fixed with glutaraldehyde (5%) and analysed on CsCl gradients according to Baltimore & Huang (1968).

RESULTS

Isolation of the intracellular nucleocapsid and 90S ribonucleoprotein

In order to establish a reliable method of isolating virus-specific RNPs from cells infected with SFV, the cells were lysed with Triton X-100 in isotonic and low salt buffers (0.15 M- and 0.01 M-NaCl, respectively). The polysomes were dissociated with EDTA and the RNPs, labelled with aH-uridine, were analysed by sucrose gradient centrifugation. Under isotonic
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Fig. 1. SFV-specific RNP's isolated from infected HeLa cells after treatment with EDTA. The cells were labelled with 3H-thymidine between 6 and 7 h p.i. Cytoplasmic extracts were prepared in three ways: (a) The cells were lyzed in isotonic buffer (SS-1B) containing Triton X-100 and the extract exposing to EDTA. (b) The cells were lyzed in hypotonic medium (SS-B) and the extract exposing to EDTA. (c) The cells were lyzed in isotonic buffer (SS-B) containing Triton X-100, the extract exposed to EDTA, and the extract exposing to EDTA. The cytoplasmic extract was labelled with 3H-thymidine and was added to each cytoplasmic extract corresponding to each cytoplasmic extract. RNP's were purified after the treatment. SFV isolated RNP's were centrifuged on 15 to 30% (w/w) sucrose gradients in buffers of medium strength and 0% EDTA. The RNA's were released with SDS from the pooled fractions indicated by bars A and B. They were analysed by centrifugation for 1.8 or 2.2 kV/cm for 1.8 or 2.2 kV/cm, and the absorbance was determined at 230 nm and 260 nm.
Conditions 42S RNA repeatedly sedimented as two peaks at 130S and 90S (Fig. 1a) as described earlier (Ulmanen et al. 1976). The 130S structure consists of intracellular nucleocapsids with a density of about 1.43 g/ml (Söderlund, 1973; Ulmanen et al. 1976), whereas the 90S material appears to be an RNP form of 42S RNA with a density ranging from about 1.45 to 1.58 g/ml and a peak at 1.48 g/ml in CsCl density gradients (not shown). If the EDTA treatment is performed in low salt medium, the virions, as well as the intracellular nucleocapsid, sediment at about 100S (Fig. 1b) as reported earlier (Söderlund & Kääriäinen, 1974; Söderlund et al. 1975). As Fig. 1(b) shows, the 100S structure sediments slightly faster than the 90S RNP. Lysing the cells in low salt buffer and restoring the ionic strength to isotonicity prior to the addition of EDTA yielded a nucleocapsid which again sedimented at 130S (Fig. 1c). These treatments did not affect the sedimentation behaviour of the 90S RNP. The 55S RNP, containing 26S RNA of SFV (Ulmanen et al. 1976) also remained unchanged in these treatments.
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An additional difference between the 130S nucleocapsid and the 90S RNP lay in their capsid protein content. As shown earlier, the capsid protein is the main virus specific protein co-sedimenting with the SFV RNP (Fig. 2 and Ulmanen et al., 1976). However, the amount of this protein in the intracellular nucleocapsids was much greater than in the 90S RNP as Fig. 6 illustrates. This difference was consistent in all experiments in which proteins were labelled with 35S-methionine pulses of 1 to 15 min duration followed by chases of 2 to 60 min (Söderlund & Ulmanen, 1977; and unpublished data). The experiments described above show that the 90S RNP structure is distinct from the 130S nucleocapsid and the 100S particle derived from nucleocapsid after exposure to EDTA at low ionic strength. The experiments described below were designed to shed light on the role of the 90S RNP in the infected cell.

Precursor role of the 90S RNP

To study the synthesis of the SFV nucleocapsid, infected HeLa and CEF II cells were grown in the presence of actinomycin D and 3H-uridine. Under these conditions only virus-specific RNAs were labelled. The cells were harvested and the RNP analysed at different phases of infection. This cumulative labelling reveals intracellular RNA-containing structures which have been synthesized up to the moment of harvesting of the cells. Incorporation of 3H-uridine was almost linear until 6 h post infection and the rate of RNA synthesis was quite similar in both cell types (Fig. 3, inset). The RNP were analysed at 3, 4 and 5 h post infection. At 3 h the bulk of labelled 42S RNA sedimented at 90S whereas the amount of nucleocapsid was small (Fig. 3a). As infection proceeded, relatively more radioactivity accumulated in the 130S peak (Fig. 3b and c). Apparently, in the early phases of infection the 42S RNA occurs mainly in the 90S RNP and later most of it is found in the intracellular nucleocapsid. Both cell types revealed this phenomenon.

Because these observations suggested that the 9cS RNP could be converted into nucleocapsids, infected cells were labelled with 3H-uridine at 5 h post infection (p.i.) for 10, 20 and 40 min. At this stage of infection the 42S RNA becomes rapidly incorporated into nucleocapsid as shown previously. During the shorter pulses (10 and 20 min), a large part of the newly synthesized RNA could be shown to sediment at 90S even at this phase of infection (Fig. 4a and b), although most of the 42S RNA made during the 40 min pulse was found in the 130S nucleocapsid (Fig. 4c). As the insets show, the short pulses mainly labelled full size virus RNAs. Quantification of the distribution of radioactivity in the 90S RNP and in nucleocapsid supports the view that 42S RNA, after its synthesis, is first found in the 90S RNP from which it is processed into nucleocapsids (Fig. 4c, inset).

The conversion of the 90S RNP into 130S nucleocapsids at different times after infection was studied by labelling infected cells with 3H-uridine for 20 min at 3, 5 and 7 h p.i. and analysing the amount of label sedimenting at 90S and 130S. Table 1 summarizes the results, showing that later in infection the nucleocapsid/90S RNP radioactivity ratio increased. These results can be explained by suggesting that the processing rate of 90S RNP to nucleocapsid increases during the late phase of infection.

To determine in which form newly synthesized 42S RNA occurs in infected cells when the synthesis of the nucleocapsid is blocked by cycloheximide, cells were labelled with 3H-uridine in the presence of cycloheximide (100 µg/ml) from 5 to 7 h p.i. This treatment does not prevent SFV RNA synthesis, but effectively inhibits the assembly of nucleocapsid (Friedman & Grimley, 1969; Söderlund, 1973). Analysis of RNP detected under these conditions showed that most of the labelled 42S RNA sedimented at 90S; no accumulation of radioactivity was seen at 130S (Fig. 5). When the infected cells were similarly labelled in
Fig. 3. Distribution of SFV RNPs isolated at different stages of infection. HeLa cells were labelled with \(^{3}H\)-uridine from 1 h p.i. and the RNPs isolated and analysed as for Fig. 1(a). (a) RNPs detected at 3 h, (b) at 4 h and (c) at 5 h p.i. The solid line in (c) shows the absorbance at 254 nm. Inset: incorporation of \(^{3}H\)-uridine into acid-insoluble form expressed as ct/min per \(\mu\)g protein in cytoplasmic extracts of HeLa and CEF II cells.
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Fig. 4. SFV-specific RNPs isolated from HeLa cells labeled with 3H-uridine for (a) 10 min, (b) 20 min, and (c) 40 min at 5 h p.i. The preparation and analysis of RNPs was as for Fig. 1(e). Inset in (a) and (b): the RNAs from the cytoplasmic extracts were analyzed as for the inset in Fig. 1(e). After treatment with SDS, inset in (c): percentage distribution of sedimenting radioactive at the position of the marker nucleocapsid (NC), from S-5-methylthioacetamide labeled SFV, see Fig. 1 and the 90 S RNPs.
Table 1. The distribution of sedimenting radioactivity (> 50S) in SFV infected HeLa cells after 20 min pulses with \(^3^H\)-uridine*

<table>
<thead>
<tr>
<th>Time of pulse (h) p.i.</th>
<th>NC†</th>
<th>% of radioactivity in</th>
<th>90S RNP</th>
<th>55S RNP</th>
<th>NC/90S ct/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>20</td>
<td>33</td>
<td>47</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>22</td>
<td>44</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>18</td>
<td>43</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

* The cells were harvested and RNPs analysed as described for Fig. 3.
† Nucleocapsid.

Fig. 5. The effect of cycloheximide on SFV specific RNPs. Infected HeLa cells were labelled with \(^3^H\)-uridine from 5 to 7 h p.i. in the presence (●—●) or in the absence (○——○) of cycloheximide (100 µg/ml). Isolation and analysis of RNPs was as for Fig. 1 (a).

the absence of cycloheximide most of the radioactive 42S RNA sedimented as nucleocapsids (Fig. 5, dotted line). This result supports the view that 42S RNA appears as a 90S RNP precursor before being converted into nucleocapsid.

If the polysomes were not dissociated before sucrose gradient analysis, most of the \(^3^H\) radioactivity sedimented faster than 100S (Fig. 6). The material with S-values larger than 130S consisted of polysomes and contained 26S and 42S RNAs together with nascent polypeptides (Table 2). The 130S peak contained mainly intracellular nucleocapsids but also small amounts of polysomes as evidenced by the presence of 26S RNA (Table 2) and
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Fig. 6. Distribution of radioactivity in cytoplasmic extracts from SFV infected HeLa cells with and without exposure to EDTA. Cells were labelled with ³H-uridine between 3.5 and 4.5 h p.i. and with ³⁵S-methionine for 15 min at 4.5 h p.i. Part of the cytoplasmic extract was treated with EDTA and centrifuged on 15 to 30% (w/w) sucrose gradients with a 60% cushion in TNE at 40,000 rev/min, for 2 h 20 min at 4°C in an SW 4I rotor. The other part of the cytoplasmic extract was analysed on a similar gradient made in Iso-B. ●—●, ³H-uridine and ○—○, ³⁵S-methionine in EDTA treated cytoplasmic extract which was not exposed to EDTA.

nascent chains in these fractions. The amount of 90S RNP was only about ¼ of that found in gradients loaded with EDTA-treated material suggesting that the 90S RNP was derived from large structures sedimenting with polysomes (Fig. 6 and Table 2). As reported previously, virtually all 26S RNA was associated with polysomes from which it was released by EDTA as 55S RNP (Wengler & Wengler, 1974; Ulmanen et al. 1976).

The 90S RNP accumulates in cells infected with ts-mutants defective in nucleocapsid synthesis

Two RNA positive ts mutants of SFV, ts 3 and ts-13, do not synthesize nucleocapsids when grown at the restrictive temperature (39 °C), although the virus RNAs are synthesized in the normal ratio (Keränen & Kääriäinen, 1974). The RNPs induced by these mutants at the restrictive temperature were analysed at 6 h p.i. (Fig. 7a and b). In both cases RNA label was found to be in two main peaks at about 90S and 55S, the former containing 42S RNA and the latter 26S RNA (Fig. 7, inset). This sedimentation pattern of RNPs was similar to that found in wild type infection early in the growth cycle and in cycloheximide treated cells.
Table 2. Distribution of $^3$H-radioactivity in the polysomes and RNPs analysed on the sucrose gradients presented in Fig. 6*

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Material</th>
<th>Ct/min</th>
<th>Ct/min in 42S RNA-containing structures</th>
<th>% in 42S RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysome gradient:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-4</td>
<td>Polysomes</td>
<td>13820</td>
<td>6357</td>
<td>46</td>
</tr>
<tr>
<td>5-9</td>
<td>Polysomes</td>
<td>8610</td>
<td>5682</td>
<td>66</td>
</tr>
<tr>
<td>10-16</td>
<td>Nucleocapsid</td>
<td>16520</td>
<td>14042</td>
<td>85</td>
</tr>
<tr>
<td>17-22</td>
<td>90–100S</td>
<td>5940</td>
<td>2791</td>
<td>47</td>
</tr>
<tr>
<td>25-32</td>
<td>50–60S</td>
<td>4210</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td>10-17</td>
<td>Nucleocapsid</td>
<td>13990</td>
<td>13990</td>
<td>100</td>
</tr>
<tr>
<td>18-23</td>
<td>90S RNP</td>
<td>10200</td>
<td>9180</td>
<td>90</td>
</tr>
<tr>
<td>25-32</td>
<td>55S RNP</td>
<td>16557</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The percentage amounts of 42S RNA were determined from analysis of the labelled RNA species on sucrose gradients.
† Not determined.

Fig. 7. SFV specific RNPs in CEF II cells infected with ts-mutants (a) ts-3 and (b) ts-13 of SFV and grown at 39 °C. The infected cells were labelled with $^3$H-uridine from 3 to 5 h p.i. and the RNPs isolated and analysed as for Fig. 1 (a). The bars (A to D) refer to the pools from which RNAs were analysed in sucrose gradients as described in Fig. 1 but centrifugation was for 12 h at 24,000 rev/min in an SW 27 rotor (insets). ———•, Ts-mutants; ○——○, RNPs from control cells infected with wild type virus.
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DISCUSSION

The assembly of SFV nucleocapsid in infected cells was studied mainly by labelling the virus RNAs with $^3$H-uridine. The cellular membranes were solubilized with Triton X-100 and the polysomes were dissociated with EDTA prior to sucrose gradient analysis. When the disruption of cells and subsequent EDTA treatment was carried out at low ionic strength the sedimentation value of the nucleocapsid was reduced from 130S to 100S as has been described previously (Söderlund & Kääriäinen, 1974). Under isotonic conditions this change was not observed. Thus the 42S containing 90S RNA described here is probably not derived from the nucleocapsid during the isolation procedure. The small amount of capsid protein in the 90S RNP compared to the nucleocapsid also supports this view because, as shown by Söderlund et al. (1975), the 100S structure has the same RNA-protein ratio as has intact nucleocapsids. Furthermore, the 100S particle sediments again at 130S after addition of Mg$^{2+}$ (unpublished data). Because $^3$H-uridine cannot be chased in a proper way, pulses of various lengths were used to study the fate of the 90S RNP.

More labelled 42S RNA is found in the 90S RNP after short pulses than after longer pulses, when most of the label is found in the nucleocapsid. This suggests that the 90S RNP is a precursor of the intracellular nucleocapsid. The results obtained with the temperature sensitive mutants ts-3 and ts-13 support this notion. For these mutants the assembly of the nucleocapsid is inhibited at restrictive temperature, apparently due to a mutation in the capsid protein (Keränen & Kääriäinen, 1974; Lachmi et al. 1975), leading to the accumulation of the 90S RNP. In ts-3 mutant infected cells only small amounts of capsid protein are formed since the cleavage of the structural polyprotein is impaired at 39 °C. The reason for the inability of ts-13 to form nucleocapsid is so far unknown.

Conversion of the 90S RNP into the nucleocapsid must include addition of capsid protein to the RNP since the 90S structure contains only small amounts of it. Early in infection the amount of available capsid protein is small and so the transfer process might be slow, whereas later it would take place rapidly. The labelling kinetics of 90S and 130S structures during the 20 min pulses given at different times of infection support this prediction as do the results obtained from cumulative labelling with $^3$H-uridine; early in infection the amount of the 90S RNP was dominant, whereas later the 42S RNA was rapidly encapsidated.

The pathway of the capsid protein to the 90S RNP is still largely unknown. Our previous studies have shown that the nascent capsid protein attached to the 60S ribosomal subunit, from which it is rapidly transferred to the intracellular nucleocapsids (Söderlund & Ulmanen, 1977). Binding of capsid protein to the 90S RNP must be rapid, since we have been unable to detect structures with capsid protein content, intermediate between the 90S RNP and nucleocapsid. It may be that encapsidation of the 90S RNP takes place by binding of numerous capsid proteins in a short time. Most of the 90S RNP is associated with large structures sedimenting with polysomes unless EDTA is used. It is possible that the transfer of capsid protein takes place in these structures. Whether the 90S RNP is actually used as a messenger at the time of transfer remains open. It could equally well be associated with the polysomes via the capsid protein. In any case the fact that cycloheximide inhibits the assembly of nucleocapsid shows that protein synthesis is firmly coupled to this process.

Many details of the assembly of the Semliki Forest virus nucleocapsid remain to be solved. Current knowledge already shows that the assembly process differs from other RNA-viruses which have been studied in more detail, e.g. picornaviruses (Baltimore, 1969; Fernandez-Tomas & Baltimore, 1973), small bacteriophages (Hung, 1976) and TMV (Butler, 1971; Richards & Williams, 1976).
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