Membrane Binding of Input Arbovirus Ribonucleic Acid: Effect of Interferon or Cycloheximide

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By 1 hr after infection, 36% of input Semliki Forest virus ribonucleic acid (RNA) which was cell associated was found in a membrane structure. This structure had many similarities to the membrane-associated replication complex (MRC) which had previously been identified in arbovirus infections. Interferon treatment did not affect the association of viral RNA with the MRC structure, but cycloheximide treatment inhibited it.

In a previously published report, the fate of input Semliki Forest virus (SFV) ribonucleic acid (RNA) was studied. Under normal conditions, a portion of the 42S virion RNA (7) was, by 1 hr after infection of chick cells, altered to 30 and 20S viral RNA forms. The latter was ribonuclease resistant. In cells treated with interferon before infection, or with cycloheximide or puromycin during infection, viral RNA probably was uncoated, as evidenced by the formation of 26S viral RNA, a form never found in the virion, but little or no 20S ribonuclease-resistant RNA was formed. It was concluded that all three treatments were similar in that they inhibited the transcription of input viral RNA, thereby preventing the formation of an RNA complementary to the input strand (6).

The results of a more detailed study are reported in this paper. The input SFV RNA rapidly became associated with a membranous cytoplasmic structure which had many of the properties of a replication complex, a structure which has been previously described in Sindbis virus and poliovirus infections (9, 15). The replication complex was membrane associated, as was that of Sindbis virus (15). A difference was noted, however, in the effects of treatment with cycloheximide during the course of infection or with interferon before infection. In cycloheximide-treated cells, entry of viral RNA into the membrane-associated replication complex (MRC) was decreased, whereas in interferon-treated cells no effect on this step was noted.

MATERIALS AND METHODS

Viruses. Pools of SFV and chick cell monolayer cultures were prepared by previously described

methods (8). An SFV pool was prepared in the presence of 10 μ Ci/ml each of tritiated adenosine (19 Ci/mmol) and tritiated uridine (20 Ci/mmol). The virus was partially purified by the method of Cheng (1) with the addition of a terminal banding in a 15 to 60% sucrose density gradient. Radioactivity was concentrated in two fractions at a density of 1.20 g/cm³. Infectivity [22×10^4 plaque-forming units (PFU)/ml] was highest in these fractions. The infectivity to radioactivity ratio was 2.4×10^5 PFU per count per min. All reported results were confirmed at least once with another pool of purified ³H-SFV and, in most cases, with a pool of the closely related Sindbis virus.

Infection procedure. Monolayers of chick cells containing about 2.5×10^7 cells were incubated at 37 C for 1 hr with 0.5 μ g of actinomycin D per ml and then for 1 hr at 4 C with 0.1 ml of ³H-SFV at a virus:cell multiplicity of 2:1. After this period, 5 ml of warm Eagle's medium with 10% fetal calf serum was added for 1 hr at 37 C.

Cell fractionation. The procedure employed for cell fractionation was based on previously described methods (15). After 1 hr at 37 C, the infected cells were washed five times with iced 0.85% NaCl and scraped into a small volume of the saline. After sedimentation at $800 \times g$ in the cold, the cells were resuspended in 1 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.2, and 0.001 M ethylenediaminetetraacetic acid (EDTA) and allowed to swell for 30 min before homogenization in a Dounce homogenizer. After homogenization, the cell extract was centrifuged at $800 \times g$ for 5 min, the fluid was removed, and the sediment was resuspended in 1 ml of Tris-EDTA and again sedimented. The supernatant fluids were combined and sedimented at $10,000 \times g$ for 15 min. The supernatant from this sedimentation was the postmitochondrial fraction. The pellet was gently resuspended in 1.0 ml of Tris-EDTA, and the differential sedimentation procedure was repeated. The postmitochondrial supernatants were combined, and the final pellet was resuspended in 0.5 ml of Tris-

EDTA. The suspensions were then layered over a discontinuous sucrose gradient in Tris-EDTA prepared as follows: 0.4 ml of 50% sucrose, 0.4 ml of 45% sucrose, 0.4 ml of 42.5% sucrose, 1.1 ml of 40% sucrose, 1.1 ml of 25% sucrose, and 1.1 ml of 15% sucrose. Sedimentation in the SW-50 head was for 30 min at $131,000 \times g$. The gradients were collected, and radioactivity was estimated by previously described methods (7).

RNA was extracted from the mitochondrial fraction by a sodium dodecyl sulfate (SDS)-phenol method previously described (7). It was sedimented in a 6 to 30% sucrose gradient for 1 hr at $300,000 \times g$ in an SW-65 rotor.

Interferon. Partially purified interferon was prepared and donated by Karl Fantes (3). The preparation contained 10,000 international units of chick interferon and 179 μg of protein per ml.

Incorporation of radioactivity. Incorporation of radioactivity from viral RNA into cell fractions was estimated by precipitation and washing with 2.5% perchloric acid. The washed precipitates were then solubilized with 0.33 N NaOH and counted. Protein content was estimated by the method of Lowry et al. (10).

Sarkosyl fractionation of the mitochondrial fraction. The method of Tremblay et al. (16) was employed to isolate a cell membrane-viral RNA complex as follows: 2.5 ml of 15% sucrose in 0.01 M Tris (pH 7.2), 0.1 M KCl, and 0.01 M $MgCl_2$ was layered over 2.5 ml of 40% sucrose in the same buffer, and 0.1 ml of the mitochondrial fraction was layered above the 15% sucrose. Sodium lauroyl sarcosinate was added to the mitochondrial fraction to a final concentration of 0.1%, and the upper layer was gently mixed. The tube was immediately centrifuged in an SW-50 rotor at $51,000 \times g$ for 30 min. The band of magnesium-Sarkosyl crystals which collected at the interface of the sucrose solutions was harvested by puncturing the side of the tube.

RESULTS

Membrane associated input viral RNA. To study the distribution of radioactivity after infection with 3H -SFV, chick cell monolayers were infected with a partially purified pool of 3H -SFV as described. After 1 hr at 37 C, the cells were washed and fractionated into nuclear and cytoplasmic extracts. Only 16% of the radioactivity applied was adsorbed to the cells, even at the low multiplicity of infection and small volume used (Table 1). Of the radioactivity associated with the cells, about 90% was present in the cytoplasmic fraction. Of this radioactivity, 36% was associated with the mitochondrial fraction and 64% with the postmitochondrial fraction.

When the mitochondrial and postmitochondrial fractions were analyzed in discontinuous 15 to 50% sucrose gradients (Fig. 1), most of the radioactivity present in the mitochondrial fraction was found to sediment at a density of 1.18 (41%

TABLE 1. *Distribution of radioactivity after infection of chick fibroblasts with tritiated Semliki Forest virus (3H -SFV)*

Determination	Acid-precipitable counts recovered
Total radioactivity added	38,300
Washed off cells	31,200
Adsorbed to cell	6,120
Nuclear fraction	680
Cytoplasmic fraction	5,300
Postmitochondrial fraction	3,300
Mitochondrial fraction	1,850

sucrose). Very little radioactivity was found at the top of the gradient (Fig. 1A). On the other hand, in the postmitochondrial fraction the reverse situation was seen. Most of the radioactivity was present at the top of the gradient, and a relatively minor fraction was present at its midpoint (Fig. 1B).

We were interested in the nature of the radioactivity in the mitochondrial fraction and sedimentation at a density of 1.18 since, later in infection, this region of the gradient in extracts of Sindbis virus-infected cells has been shown to contain an MRC with the replicative intermediate form of viral RNA and the viral RNA-dependent polymerase (15). The radioactivity of this fraction (Fig. 1A) was probably not due to unclipped virus or to nucleocapsids with their outer membrane coat removed. The peak of viral infectivity, although not sharp, did not coincide with that of the radioactivity (Fig. 1A). Also, although the uncoated viral RNA is completely ribonuclease resistant, that of the fraction under study was only partially resistant (Fig. 2). Moreover, when artificial mixtures were prepared from the cytoplasm of SFV-infected cells and 3H -SFV, 3H -SFV core or nucleocapsid, or the purified viral RNA forms (42S, 26S, and 20S RNA), only the 3H -SFV showed any tendency to adhere to the mitochondrial fraction (Fig. 3); however, that 3H -SFV which did adhere tended to band at a higher density than did the bulk of the radioactivity examined in Fig. 1A. The nucleocapsid and purified viral RNA forms remained with the postmitochondrial fraction.

These results suggested to us that the bulk of the radioactivity sedimenting with the mitochondrial fraction was not due to unclipped virus. Extraction of radioactive RNA from this fraction tended to confirm this notion (Fig. 4). The extracted RNA was polydisperse with a peak at about 20S. It was partially resistant to ribonuclease treatment. Of the RNA forms of SFV, these properties are most similar to those of the par-

FIG. 1. Analysis in a discontinuous sucrose density gradient of cytoplasmic radioactivity after infection with ^3H -SFV. Gradient fractions from the (A) mitochondrial or (B) postmitochondrial fraction were analyzed for radioactivity and, in the case of the mitochondrial fraction, infectivity. The top of the gradient in this and subsequent figures is to the right.

tially double-stranded replicative intermediate (RI) form (5). No significant amount of 42S RNA, the RNA of the virion, was found. These results do not rule out the possibility that some 26S single-stranded viral RNA was present in the mitochondrial fraction since the method of analysis employed does not separate this form completely from the RI form (7). The bulk of the RNA, however, would appear to be in the RI form.

To study the kinetics of the entry of viral RNA into the MRC, RNA was extracted from the mitochondrial fraction of infected cells at earlier time periods. Cells were washed five times after virus adsorption at 4 C, and medium at 37 C was added. Mitochondrial fractions were prepared at 5 and 20 min after warming. RNA was then extracted from these fractions and analyzed on sucrose density gradients. The results (Fig. 5) showed that 5 min after warming, a peak appeared at 42S, corresponding to the RNA of the virion. Some slower sedimenting material was already present. At 20 min after warming, most of

FIG. 2. Ribonuclease resistance of radioactivity associated with the mitochondrial fraction after infection with ^3H -SFV. A mitochondrial fraction was prepared as in Fig. 1, and the rest was treated with ribonuclease (1 $\mu\text{g}/\text{ml}$, 20 min, 0 C) before sedimentation.

FIG. 3. Sedimentation in the mitochondrial fraction of radioactivity from artificial mixtures containing ^3H -SFV or ^3H -SFV cores or ^3H -SFV-RNA forms (42, 26, or 20S RNA). Preparations containing these were mixed with a cytoplasmic extract from SFV-infected chick cells after homogenization. Mitochondrial fractions were then prepared as in Fig. 1 and analyzed on 15 to 50% discontinuous sucrose density gradients. No radioactivity was found to be associated with the mitochondrial fractions from the mixtures of cytoplasm with ^3H -SFV cores (as shown) or ^3H -SFV-RNA forms (not shown).

the labeled RNA was of the slower sedimenting species, probably, as at 1 hr after warming (Fig. 4), in the RI form. This result suggested that some 42S RNA rapidly became membrane-associated and was then converted to a replicating RNA structure.

Finally, additional results indicated that the radioactive fraction found in Fig. 1 was not due to uncoated ^3H -SFV. In infection of chick cells with radioactive infectious RNA of the closely related Sindbis virus, the radioactivity of the

FIG. 4. Analysis of RNA extracted from the mitochondrial fraction of ^3H -SFV-infected cells. Cells were infected with ^3H -SFV, and after 1 hr mitochondrial fractions were prepared as described in the legend to Fig. 1. RNA was extracted in 0.1 M NaCl by an SDS-phenol method and analyzed ($300,000 \times$, 1 hr) on 6 to 30% sucrose density gradients. The designations 18S and 28S specify the peaks of added ribosomal RNA markers. One-half of the mitochondrial fraction was treated in 0.1 M NaCl with 1 μg of ribonuclease per ml for 20 min at 37 C before sedimentation.

FIG. 5. Kinetics of appearance of viral RNA forms in the mitochondrial fraction of ^3H -SFV-infected cells. Cells were infected as described in the legend to Fig. 4. After 5 or 20 min, mitochondrial extracts were prepared and RNA was extracted from these. Analysis by sucrose density gradients was also carried out as described in the legend to Fig. 4. Symbols: ●, RNA extracted after 5 min; ○, RNA extracted after 20 min.

mitochondrial fraction sedimented in the same manner as the result shown in Fig. 1A (T. Sreevalsan, *in preparation*). Also, analysis of the specific activity of the virus employed in the experiment shown in Fig. 1A (*see above*) demonstrated that the titer of infectious virus recovered could not

account for the level of radioactivity seen in the fractions containing the highest levels of tritium.

Therefore, the association of viral RNA with a specific cell fraction (Fig. 1A) was probably not due to an artifactual interaction of viral RNA with cell membranes during the preparation of cytoplasmic extracts since mixing of viral RNA forms with such extracts failed to cause such a specific association. Additional evidence in favor of the idea that input viral RNA was present in an MRC was determined in experiments employing Sarkosyl magnesium precipitation of membrane fractions. It has been demonstrated that functional bacterial deoxyribonucleic acid (DNA) is associated with a membrane and the complex may be precipitated by magnesium with Sarkosyl (16). The precipitation is specific for membrane-bound functional DNA, as DNA or RNA forms artificially mixed with cell extracts are not precipitated. When the mitochondrial fraction of cells infected with ^3H -SFV was precipitated with Sarkosyl and magnesium and the precipitate banded at a 15 and 40% sucrose interface, most of the radioactivity recovered was found in the interface band (Table 2). The counts present in the postmitochondrial fraction or in artificial mixtures of viral RNA forms and cell sap did not band at the interface when subjected to the same treatment.

The results discussed so far suggested that, 1 hr after productive infection with ^3H -SFV, a small percentage of the input viral RNA was specifically associated with a membrane component of the mitochondrial fraction. By 1 hr after warming to 37 C, most of this RNA was probably in the RI form. This membrane component had, therefore, some of the characteristics of a viral MRC.

Effect of cycloheximide or interferon on association in input RNA with cell membrane. To study the effect of cycloheximide or interferon on input viral RNA, chick cells were treated with 1,000 units/ml of partially purified chick interferon before infection with ^3H -SFV, or, with 100 μg of cycloheximide per ml during infection. The dose of interferon employed inhibited virus growth by more than 200-fold; the dose of cycloheximide inhibited protein synthesis by 94%. The effect of these treatments on the distribution of cytoplasmic radioactivity was studied (Table 3). In the control cells, the distribution of cytoplasmic radioactivity between the mitochondrial and post-mitochondrial fractions was similar to that shown in Table 1. When the specific activity of the radioactivity in these fractions was studied, however, more than 2.5 times the activity of the postmitochondrial fraction was present in the mitochondrial fraction. In the cells treated with interferon, the distribution of radioactivity was approximately the same as in the control. In cells treated

TABLE 2. *Distribution of radioactivity after sedimentation of a sodium lauroyl sarcosinate-treated mitochondrial fraction*

Determination	Acid-precipitable counts recovered
Total radioactivity added.....	1,055
Top component.....	90
Interface precipitate.....	816

TABLE 3. *Distribution of cytoplasmic radioactivity after infection of chick fibroblasts with tritiated Semliki Forest virus*

Cytoplasmic fraction	Total radioactivity recovered (counts/min)	Specific activity (counts per min per μ g of protein)	Ratio of specific activities (mitochondrial fraction: postmitochondrial fraction)
Control			
Mitochondrial.....	8,339	11.9	2.6:1
Postmitochondrial..	17,596	4.65	
Interferon-treated			
Mitochondrial.....	8,425	14.0	2.4:1
Postmitochondrial..	13,443	5.85	
Cycloheximide-treated			
Mitochondrial.....	3,288	4.50	0.66:1
Postmitochondrial..	24,726	6.82	

with cycloheximide during infection, however, the bulk of the specific activity was in the postmitochondrial fraction.

These findings were confirmed in sucrose density sedimentation studies on the mitochondrial fraction (Fig. 6). Again, the control contained a peak of radioactivity sedimenting at a density of 1.18 (Fig. 6A). As expected, the interferon-treated cells contained a similar, but usually an even sharper, peak, as shown in Fig. 6B. On the other hand, in cells treated with cycloheximide during infection, the peak at this density was very much decreased (Fig. 6C). Therefore, input viral RNA appeared to enter a replication complex (RC)-like structure after interferon treatment, but this step was inhibited in the presence of cycloheximide.

One point of interest was the nature of the viral RNA present in the RC-like structure in interferon-treated cells. As shown in Fig. 4, under ordinary conditions the predominant RNA species present in the mitochondrial fraction of control cells after 1 hr was the RI form. It has also been shown that, in cells treated with high concentrations of interferon, little or no ribonuclease-resistant RNA was formed from input

FIG. 6. *Effect of interferon or cycloheximide treatment on association of input ^3H -SFV with mitochondrial fractions. (A) Untreated cells; (B) cells treated with 1,000 units of interferon per ml for 14 hr before infection; (C) cells treated with 100 μ g of cycloheximide per ml during infection. The cells were infected with ^3H -SFV and, after 1 hr, mitochondrial fractions were prepared and analyzed on discontinuous 15 to 50% sucrose density gradients as described in the legend to Fig. 1.*

SFV RNA (6). The presence of viral RNA in the MRC structure of interferon-treated cells seemed paradoxical. RNA was, therefore, extracted from the mitochondrial fraction of interferon-treated cells. The results (Fig. 7) show that the major peak of RNA present was in the 42S form, in marked contrast to the predominant 20S peak in the control cells (Fig. 4). In addition, as expected, little or no ribonuclease-resistant RNA could be demonstrated in the mitochondrial fraction of the interferon-treated cells (Fig. 7). In this respect, the interferon-treated cells after 1 hr resembled the control cells very early after warming to 37°C (Fig. 5).

DISCUSSION

The results indicated that in untreated, infected cells a small fraction of the input ^3H -SFV RNA enters an MRC structure. Most, if not all, of this

FIG. 7. Analysis of RNA extracted from the mitochondrial fraction of ^3H -SFV-infected cells which had been treated with interferon. Cells were infected with ^3H -SFV after 14 hr of treatment with 1,000 units of interferon per ml. Mitochondrial fractions were prepared, and RNA was extracted from them and analyzed as in Fig. 4.

RNA is in the RI form (5), suggesting that the structure is indeed associated with virus RNA synthesis. Later in infection, functional viral RNA polymerase is present in a very similar structure (15).

Results from cycloheximide-treated cells suggest that entry of input RNA into this structure requires protein synthesis, since this viral function is inhibited. On the other hand, no such inhibition was seen in interferon-treated cells, but the viral RNA present in the MRC structure remained for the most part in the 42S form present in the virion.

The results with interferon indicate that, whatever the site of its action is, this must lie beyond the step in which the input RNA is integrated into the MRC structure. Interferon treatment has no effect on viral uncoating (2), except in the case of vaccinia virus in which this is partially dependent on viral protein synthesis (11). Since treatment with 1,000 units of interferon per ml appears to block translation of input RNA in the SFV-infected cell (4), the integration step probably does not require the viral RNA polymerase. This conclusion was strengthened by findings with a temperature-sensitive mutant of the closely related Sindbis virus, TS-6. The temperature-sensitive step of TS-6 is related to viral RNA polymerase production, but TS-6 RNA has been found to enter the RC-like structure at permissive (27°C) and nonpermissive (39°C) temperatures. At both temperatures, however, cycloheximide treatment blocks this step (T. Sreevalsan, *in preparation*).

In all cases studied, therefore, the action of cycloheximide was to inhibit viral RNA entry in the MRC structure; the nature of the protein required for this function is of some interest. The current concept of interferon action is that translation of viral, but not cellular, messenger RNA is blocked in interferon-treated cells (14). The present results, taken together, suggest that it may be the synthesis of a cell protein which is required for the integration of input viral RNA into the RC-like structure. If the latter hypothesis is correct, some of the properties of this cell protein can be deduced. The protein would not be one specifically induced by virus infection, since the virus replicates quite well in actinomycin D-treated cells. Also the protein must have a fairly rapid turnover rate, a conclusion suggested by the result obtained with cycloheximide. The existence of a host protein controlling virus replication in C-M cells persistently infected with mumps virus was recently reported. This protein also seems to have the properties noted above (13).

An alternative explanation for our observations would be that the integration step requires viral protein synthesis and that cycloheximide is a more efficient inhibitor of virus protein synthesis than is interferon. In the latter case, residual virus protein synthesis in interferon-treated cells would be responsible for the integration step. Interferon has been shown to inhibit some steps in the synthesis of viral RNA more effectively than others (12).

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