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Association of Viral Ribonucleic Acid with Cellular Membranes in Chick Embryo Cells Infected with Sindbis Virus

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Membranes from cells infected with Sindbis virus had associated with them viral ribonucleic acid (RNA) polymerase and about 60 to 70% of the viral RNA labeled when short pulses were used. This RNA contained most of the replicative intermediate and replicative form of viral RNA found in the infected cells. The use of "Mg^{2+} sarkosyl crystals" permitted the isolation of membrane-bound nucleic acids and allowed the demonstration that Sindbis virus RNA was synthesized on a membrane-viral RNA complex. Viral RNA from the infecting virions first became associated with the membranes during the latent period and, subsequently, slowly detached. The attachment of the viral RNA to the membranes did not require active viral RNA polymerase, since RNA from ts6, an RNA^- temperature-sensitive mutant of Sindbis virus, associated with cellular membranes at a nonpermissive temperature. However, the subsequent detachment of the RNA from the membranes was restricted in the absence of viral RNA synthesis. The results indicate that association of viral RNA with cellular membranes may represent an early step occurring during the replication of Sindbis virus RNA.

Several reports exist which implicate host-cell membranes as the sites of replication of many ribonucleic acid (RNA)-containing animal viruses. Poliovirus, Semliki Forest virus (SFV), and Sindbis virus are examples (2, 4, 5, 15). Analysis of the cytoplasmic extracts obtained from HeLa cells infected with poliovirus suggests that distinct membranous structures are involved with the transcription and translation of the viral RNA (2). Transcription of the poliovirus RNA has been shown to occur on a structure which sediments in sucrose density gradients with smooth cytoplasmic membranes (2). Translation, however, occurs elsewhere as the virus-specific polyribosomes are bound to membranous structures which sediment with the rough cellular membranes in a sucrose density gradient. Similarly, the viral RNA polymerase and nascent viral RNA have been recovered with membrane fractions of cells infected with Sindbis virus (8, 15). Electron microscopic and radioautographic observations of SFV-infected cells have revealed that the viral RNA is probably synthesized on the plasmalemma of the infected cells (8). However, despite the suggestion that cell membranes are somehow involved in the replication of these viruses, it is difficult to rule out the possibility that the observed association of virus-specific RNA or proteins with the membranes is due to some adventitious mechanisms. Rceently Tremblay et al. reported a novel method for isolating nucleic acids attached to a portion of the bacterial cell membrane (16). The method, termed the "M band" technique, consists of lysing spheroplasts of *Bacillus megaterium* with the detergent sodium lauryl sarcosinate. In the presence of magnesium, the detergent crystallized and the crystals banded by centrifugation through a sucrose gradient. The band of magnesium sarcosinate (M band) contained the cell membrane, deoxyribonucleic acid (DNA), and rapidly labeled RNA. The formation of the cell membrane-nucleic acid complex depended on the interaction of the cell membrane components with the crystals of detergent, since neither DNA or RNA interacted with the crystals directly, whereas partially purified cell membranes did (16). The above authors used the M band technique to demonstrate the attachment of the growing point of the bacterial DNA to cell membranes in *B. megaterium*. We have analyzed the cytoplasmic extracts obtained from chick embryo (CE) cells infected with Sindbis virus by the M band technique, and the results, to be presented, indicate that nascent viral RNA is asso-

ciated with the host membranes. Experiments will be reported which indicate that, besides the nascent viral RNA, the viral RNA polymerase is also associated with the host membranes. The association of the viral RNA with host membranes appears to be an essential step during the replication of the RNA.

MATERIALS AND METHODS

Cells and virus. The media used and the method of preparing CE cells have been described earlier (14). The HR and ts6 strains of Sindbis virus were kindly supplied by E. R. Pfefferkorn, Dartmouth Medical School, Hanover, N.H. Experiments in which cell cultures were incubated at 27 or 40 C were performed in incubators without a CO_2 atmosphere. The medium used to incubate the cell cultures in the absence of carbon dioxide was a modified Eagle's medium as formulated by Gardner (6). *N*-tris(hydroxymethyl) methyl glycine (Tricine) was used as the buffering agent instead of bicarbonate. The kinetics of replication and the final yields of infectious Sindbis virus in CE cells were similar regardless of the media used for incubation.

The methods used to infect the cells and to label the viral RNA were identical to those described earlier (14). Unless described otherwise, CE cultures were incubated with 3 ml of medium containing actinomycin D (5 μg/ml) for 2 hr. at 37 C. The cultures were incubated for 1 hr at 37 C with 0.5 ml of a suspension of the virus [10 plaque-forming units (PFU) per cell]. The cultures were washed three times with phosphate-buffered saline (PBS), followed by 5 ml of medium containing 2% dialyzed calf serum, and incubated at 37 C.

Chemicals and isotopes. Actinomycin D was a generous gift from E. Katz of this department. Acti-Dione (cycloheximide) was purchased from Sigma Chemical Co., St. Louis, Mo. Sarkosyl (NL-30 sodium lauryl sarcosinate) was a gift from Geigy Chemical Corp., Ardsley, N.Y. Isotopically labeled amino acids and uridine were purchased from Nuclear-Chicago Corp., Des Plaines, Ill. Tritium labeled guanosine triphosphate was purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y. Pools of Sindbis virus were prepared by methods similar to those described earlier (5). Virus labeled with ^{3}H-uridine was prepared by incubating infected cells in the presence of 25 μCi of ^{3}H-uridine (20 Ci/mmole) per culture. Pools of ts6 virus were prepared similarly by incubating infected cells at 27 C for 10 hr after the addition of virus to the cultures. The ts6 virus stocks contained less than 1% revertants at 40 C. Virus pools were purified by methods described earlier (5). Small samples of the partially purified virus preparations were frozen in portions at −40 C in the presence of 5% dimethyl sulfoxide. The infectivity to radioactivity ratio of the virus preparations ranged from 2×10^8 to 5×10^8 PFU per counts per min.

Preparation of cytoplasmic extracts. Cytoplasmic extracts were prepared by the method described previously (15). In some instances, the crude cytoplasmic extracts were fractionated as follows. Unbroken cells and nuclei were removed from the disrupted cell suspensions by centrifugation at 800 × *g* for 5 min at 4 C. The crude extracts obtained in the last step were separated into a mitochondrial and postmitochondrial fraction by centrifuging the extracts at 10,000 × *g* for 15 min. The supernatant fraction represented the postmitochondrial fraction. The pellet obtained was suspended in an appropriate volume of buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.2, with 10^{-4} M sodium ethylenediaminetetraacetate (EDTA)], and this represented the mitochondrial fraction. The mitochondrial fraction was further purified by one more cycle of centrifugation at 800 × *g* and 10,000 × *g*.

Isolation of membranes by the M band technique. Cytoplasmic extracts prepared by the methods described in the previous section were subjected to fractionation by the M band technique as described by Tremblay et al. (16). Sarkosyl in distilled water was layered on a gradient of 15 to 47% sucrose in 0.01 M Tris (*p*H 7.0), 0.1 M KCl, and 0.01 M magnesium chloride. Magnesium chloride was added to the cytoplasmic extract to a final concentration of 0.01 M. The cytoplasmic extract (1 ml) was layered immediately over the gradient and briefly mixed with the underlying layer of sarkosyl. The final concentration of sarkosyl in terms of the total layered volume of the cytoplasmic extract was 0.2%. The tubes were immediately centrifuged by using an SW27.2 Beckman rotor at 51,800 × *g* for 30 min. The magnesium sarcosinate crystals in the sucrose density gradient formed a sharp white band (M band) and possessed a buoyant density corresponding to that of about 30% sucrose solution. Fractions were collected from the gradient by the method described by Tremblay es al. (16).

Isolation of membranes by the "Tris" method. The method was similar to that described by Warren et al. (17). In brief, it consisted of the following steps. Approximately 2×10^8 to 3×10^8 CE cells were washed with 0.05 M Tris buffer (*p*H 7.4) and suspended in 10 ml of the same buffer. A 1.0-ml amount of 0.05 M $MgCl_2$ was added to the cell suspension. The cells were allowed to swell for 30 min at 3 C and then dimethyl sulfoxide (Matheson Chemicals) was added to obtain a final concentration of 2%. Cells were ruptured by using 18 strokes in a tight-fitting stainless-steel Dounce homogenizer. The homogenate was suspended in an equal volume of 20% sucrose in 0.005 M magnesium chloride. It was then layered onto a discontinuous gradient of sucrose solutions consisting of 15 ml of 50% sucrose and 30 ml of 30% sucrose in 0.005 M $MgCl_2$. The tubes were centrifuged at 1,600 × *g* for 15 min at 4 C. The Tris layer was removed and represented the postmembrane fraction. The membranes, contained in the 30% sucrose fraction, were recovered and centrifuged additionally at 5,860 × *g* for 20 min at 4 C. The pellet containing the membranes was resuspended in 2 ml of 30% sucrose solution in 0.005 M $MgCl_2$ and was placed on a discontinuous sucrose density gradient made up of 65, 55, 45, and 40% sucrose in 0.005 M $MgCl_2$. Centrifugation was in an SW27.2 Beckman rotor at 80,900 × *g* for 10 hr at 4 C. The membranes formed a band at

the top of the 45% sucrose. They were collected and recentrifuged on similar discontinuous sucrose density gradients.

Analysis of mitochondrial fraction on sucrose density gradients. The mitochondrial fraction was analyzed wherever required as follows. The suspension (0.5 ml) was layered over a discontinuous gradient in 0.01 M Tris, 10^{-4} M 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. Centrifugation was carried out in an SW50 Beckman rotor at 130,576 × *g* for 30 min at 4 C. The fractions were collected and assayed for radioactivity.

Isolation and analysis of RNA. Fractions to be analyzed for RNA were incubated for 10 min with 0.5% sodium dodecyl sulfate (SDS), and 10 μg of dextran sulfate per ml and then layered on 15 to 30% sucrose density gradients made in 0.01 M Tris (*p*H 7.4), 0.1 M NaCl, 10^{-3} M EDTA, and 0.5% SDS. The gradients were centrifuged at 80,900 × *g* for 14 hr at 23 C in an SW27.2 Beckman rotor. Fractions were collected and assayed for radioactivity.

Assay for radioactivity. Portions (100 μliters) of sample to be assayed were placed on Whatman no. 2 filter paper discs (4.25 cm) and the paper discs were dried. The discs were washed six times with 5% cold trichloroacetic acid, twice with cold 95% ethanol, and finally two times with ethyl ether. The ether was allowed to evaporate. The dried discs were placed in scintillation vials to which 10 ml of Liquifluor (New England Nuclear Corp.) was added and counted in a liquid scintillation counter.

Assay for viral RNA polymerase. The details of the assay system employed for detecting viral RNA polymerase have been described previously (15). The concentration of actinomycin D used was 2.0 μg per 0.5 ml of the assay mixture. The concentration of protein in samples used as the source of enzyme was assayed by the method of Lowry et al. (11).

RESULTS

Nascent viral RNA and the M band. Several cultures of CE cells were incubated with Eagle's medium containing actinomycin D and then infected with Sindbis virus. Cultures of uninfected cells which received medium containing actinomycin or no actinomycin were included as controls. The cultures were incubated for 10 min with 100 μCi of tritiated uridine per culture (specific activity, 20 Ci/mmole). Infected cultures received radioactive uridine at the 6th hr after the addition of virus. Cytoplasmic extracts were prepared from each set of cultures and analyzed by the M band technique. The results of one such experiment are shown in Table 1. It can be seen that about 10% of the total radioactivity incorporated into the uninfected cells was recovered from the band. There was no radioactivity associated with the M band in the extracts of uninfected cells receiving actinomycin D. However, most of the radioactivity in the infected cells was associated with the M band. This is seen from the results (Table 1) obtained when the mitochondrial and postmitochondrial fractions of the infected cells were analyzed. Most of the viral RNA which sedimented with the mitochondrial fraction was recovered from the M band, whereas only 7% of the viral RNA contained in the postmitochondrial fraction was present in the M band. The viral RNA found in the M band was not a result of nonspecific entrainment of the RNA and membranes to the crystals of magnesium sacosinate. This can be seen from the results (Table 1) on the analysis of the artifical mixtures prepared with the cytoplasm of Sindbis virus-infected cells and purified Sindbis RNA forms (40, 26, and 20*S* RNA), ^{3}H-uridine labeled or ^{14}C-amino acid-labeled virus. Only 0.2% of the added free viral RNA was entrained in the M band. Mature virus showed some tendency to adhere to the crystals of magnesium sarcosinate. This result agreed with that reported by Temblay et al. (16), who showed the absence of any significant entrainment of free nucleic acids or ribosomes in the M band. The above results suggested that the nascent RNA species from the infected cells may be attached to membranes since only membrane-bound structures attached to the M band (16). Prior incubation of cell extract with Triton X-100 or chloroform prevented the appearance of viral RNA in the M band (Table 2). Incubation with deoxyribonuclease under similar conditions resulted in little loss of radioactivity present in the M band. However, prior incubation of the cytoplasmic extract with ribonuclease resulted in the loss of about 70% of the radioactivity from the M band (Table 2). The simplest inference from the results presented in Table 2 was that viral RNA was found in the M band by virtue of its attachment with membranes. Previous reports have indicated that newly made RNA in Sindbis virus-infected cells consists of single stranded 40 and 26*S* and a large amount of ribonuclease-resistant RNA species (15). The ribonuclease-resistant RNA species were found to consist of the replicative intermediate and the replicative form of viral RNA species (Sreevalsan *unpublished data*). As seen from Fig. 1a, a large proportion of the pulse-labeled RNA isolated from the M band sedimented on a sucrose density gradient in the region of 28 to 18*S* and was resistant to ribonuclease. Of this RNA, about 5% sedimented in the region of the 40*S* and was sensitive to ribonuclease. In contrast, the majority of the viral RNA from the cytoplasmic extract which did not attach to the M band was ribonuclease-sensitive and sedimented on sucrose gradients at rates of 40 and 26*S* (Fig. 1b). The viral RNA attached to the M band was enriched with ribonuclease-resistant RNA, a result one might expect

TABLE 1. *Isolation of nascent viral RNA by M band*[a]

Sample	Total radioactivity in sample	Radioactivity in M band
	counts/min	*counts/min*
Uninfected cells, ^{3}H-uridine-labeled	6.4×10^3	6.2×10^2
Uninfected cells plus actinomycin D, ^{3}H-uridine-labeled	4×10^2	None
Infected cells plus actinomycin D, ^{3}H-uridine-labeled		
Mitochondrial fraction	3.3×10^3	2.8×10^3
Postmitochondrial fraction	1.8×10^3	1.2×10^2
Artificial mixture of infected cell extract plus		
^{3}H-uridine-labeled viral RNA	1.8×10^5	1.2×10^2
^{3}H-uridine-labeled virus	6×10^4	2.6×10^3
^{14}C-amino acid-labeled virus	3.8×10^4	2.7×10^3

[a] Cytoplasmic extracts were isolated from the uninfected and infected cells as described in Materials and Methods. The cytoplasmic fractions were fractionated by the M band technique. Artificial mixtures consisting of cytoplasmic extract from unlabeled infected cells and labeled mature virus or viral RNA were also analyzed by the M band technique.

TABLE 2. *Effect of some reagents on the composition of the M band*[a]

Reagent	Radioactivity recovered in M band (%)
Control	82
Triton X-100 (2.5 mg/ml)	0.01
Chloroform (1 volume)	None
Ribonuclease (10 mg/ml)	26
Deoxyribonuclease (10 μg/ml)	76

[a] CE cells were incubated with actinomycin D and then infected with Sindbis virus. At the end of 6 hr after virus addition, the cultures received ^{3}H-uridine (100 μCi/culture) for 10 min. Cytoplasmic extract was isolated from the infected cells. Portions (1 ml) of the cytoplasmic extract containing 4×10^4 counts/min were fractioned by the M band technique. Cytoplasmic extracts were incubated with the various reagents at 0 C before fractionation. Incubation with chloroform was performed by extracting the cytoplasmic extract three times with 1 volume of chloroform.

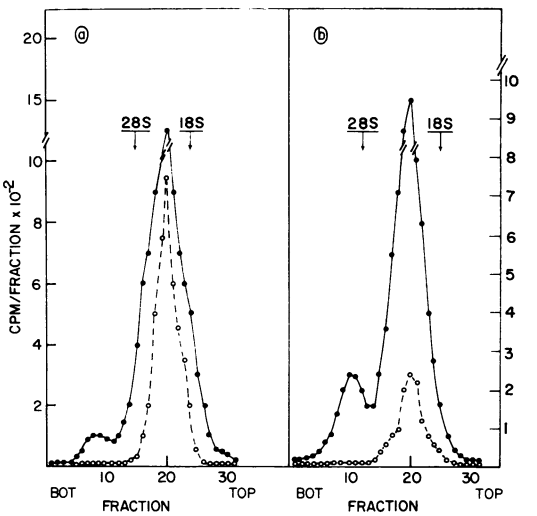

FIG. 1. *Infected cells were pulse-labeled with ^{3}H-uridine for 10 min. The total cytoplasmic extract was then fractionated by the M band technique. The fractions containing the M band and the fractions above the M band on sucrose density gradients were collected. Details on the extraction and analysis of the viral RNA are given in Materials and Methods. Alternate fractions were incubated with pancreatic ribonuclease (2 μg/ml, final concentration) for 10 min at 37 C, and trichloroacetic acid-insoluble radioactivity was determined. (a) RNA from the M band. (b) RNA from fractions above the M band. Symbols: ●, control; ○, incubated with ribonuclease. Arrows indicate the position where the 28 and 18S ribosomal RNA sediment on similar sucrose density gradients.*

if the membranes represented the site of viral RNA synthesis in the infected cells.

Viral RNA polymerase and cell membranes. If membranes were indeed the site of viral RNA synthesis, one would expect that viral RNA polymerase should also be found on them. We reported recently that the RNA polymerase in cells infected with Sindbis virus was found to be associated with the mitochondrial fraction (15). Preliminary experiments indicated that sarkosyl inhibited the activity of viral RNA polymerase. Therefore, we were unable to detect any viral RNA polymerase activity in the M band fractions. Other methods were investigated for isolating membranes from the infected cells. Warren et al. reported that the Tris method of isolating plasma membranes from cells was suitable for enzymatic studies (17). Therefore, we adopted the above method. Uninfected and infected cells were subjected to the

fractionation procedure of Warren et al. (17). The membranes obtained by the above method were found to sediment on sucrose density gradients as a single band and possessed a density of 1.20. The amount of pulse-labeled viral RNA as well as the viral RNA polymerase activity present on the isolated membranes is presented in Table 3. It can be seen from the results that about 70% of the pulse-labeled radioactivity present in the crude extract was recovered in the membrane fractions. About 50% of the radioactivity associated with the membrane was resistant to the action of ribonuclease. Most of the RNA polymerase activity found in the crude extract was also associated with the membrane fractions. Membranes isolated from uninfected cells under similar conditions did not contain any significant RNA polymerase activity or pulse-labeled RNA (Table 3). The increased specific activity of the viral RNA polymerase found in the membrane fraction over that found in the crude extract was due to the loss of contaminating proteins during the isolation procedures. About 3% of the total cellular protein was recovered in the membranes isolated under the above conditions.

The following conclusions can be drawn from the results described thus far. The site of viral RNA synthesis in the infected cells involves the host membranes. This conclusion is based on the fact that only membranous structures have been shown to attach to the M band (16). Most of the nascent viral RNA synthesized in the infected cells is, according to the above criterion, attached to the membranes. The membrane-viral RNA complex contains the viral RNA polymerase.

Recently, Friedman and Sreevalsan reported that interferon did not inhibit the association of input SFV to host cellular membranes (5). However, it was found that in the presence of cycloheximide, an inhibitor of protein synthesis, there was significantly no association between the input viral RNA and the membranes (5). It was possible that the synthesis of viral RNA polymerase was necessary for the attachment of parental RNA with the membranes, and cycloheximide prevented the above step as a result of its inhibitory effect on the synthesis of the enzyme. Therefore, it was interesting to find out whether the viral RNA from ts6, an RNA$^-$ mutant of Sindbis virus, can attach to cell membranes at 40 C in the presence of cycloheximide. The mutant ts6 has been found to synthesize active viral RNA polymerase and RNA at 27 C but not at 40 C (1). Therefore, the fate of the input viral RNA from ts6 at 27 and 40 C was investigated (Fig. 2). It can be seen from the results that the input RNA was associated with the membranes regardless of the temperature of incubation. However, in the presence of cycloheximide, very little of the input radioactivity was associated with membranes at both temperatures. Most of the radioactivity from the cultures receiving cycloheximide was recovered from the postmitochondrial fraction of the infected cells. These results suggest that the association of the viral RNA with membranes requires a step involving protein synthesis at 27 or 40 C. The results also suggest that the protein(s) required for the association of the viral RNA with cellular membranes does not consist of the active form of viral RNA polymerase. The results presented in Table 4 show the fate of the radioactivity associated with the cellular membranes when cultures infected with ^{3}H-uridine-labeled ts6 were incubated at 27 and 40 C. It can be seen that there is a gradual loss of label from the membranes as incubation time increased at 27 C. However, there

TABLE 3. *Viral RNA and RNA polymerase activity associated with plasma membranes from uninfected and infected cells*

Sample	Pulse-labeled RNA[a]		Viral RNA polymerase activity (^{3}H- GTP/mg of protein)[b]	
	Uninfected cells	Infected cells	Uninfected cells	Infected cells
Crude extract	1×10^3	4.4×10^4	9×10^2	2.0×10^4
Membrane fraction	2×10^2	3.1×10^4	1.3×10^2	2.0×10^5
Postmembrane fraction	7.4×10^2	1.0×10^4	1.1×10^2	2.2×10^2

[a] Cultures were incubated with actinomycin D and were infected with the virus or left uninfected. Tritiated uridine was added to the cultures (100 μCi/culture) at the 6th hr after the addition of virus to cultures and incorporation was allowed for 10 min. Cytoplasmic extracts were prepared and membrane fractions were isolated by the Tris method (17). Trichloroacetic acid-insoluble radioactivity was determined for various fractions.

[b] Cultures were incubated with actinomycin D and were infected or left uninfected. Six hours after the addition of virus to the cultures to be infected, the cells from both infected and uninfected cultures were harvested and the membrane fractions were isolated by the Tris method (17). The viral RNA polymerase activity shown represents counts per minute of ^{3}H-guanosine triphosphate (GTP) incorporated per milligram of protein for 30 min.

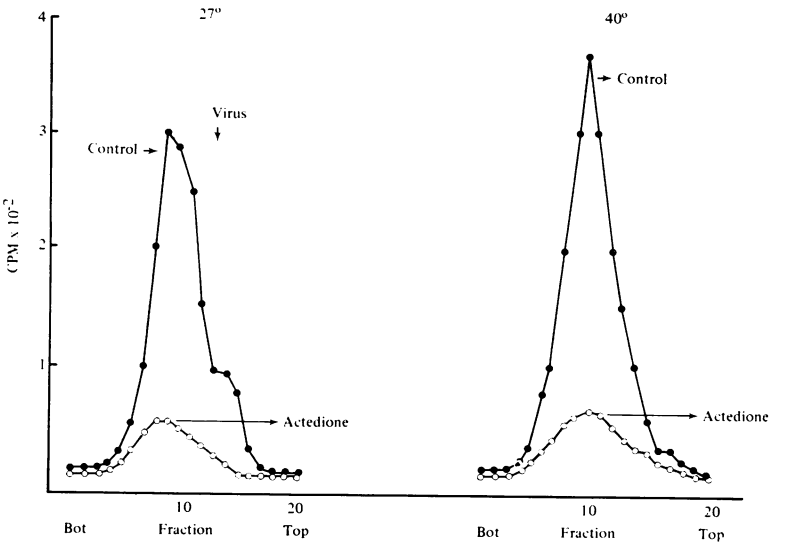

FIG. 2. *CE cell cultures were incubated with actinomycin D for 1 hr at 37 C. Then some cultures received medium containing 100 μg of Acti-Dione (cycloheximide) per ml. After an additional incubation period of 30 min, all cultures were infected with ^{3}H-ts6 virus at 27 or 40 C in the presence or absence of 100 μg of cycloheximide per ml. The virus to cell multiplicity was about 20:1. At the end of 1 hr, the cultures were washed and received medium or medium containing cycloheximide. Cultures were incubated for 60 min at 27 or 40 C. Cytoplasmic extracts were prepared and fractionated to obtain the mitochondrial fraction. The mitochondrial fractions were analyzed individually on discontinuous sucrose density gradients. Fractions were collected and analyzed for acid-insoluble radioactivity. The position at which mature ts6 virus sediments on a similar sucrose density gradient is shown by an arrow.*

TABLE 4. *Fate of ^{3}H ts6 at 27 and 40 C*[a]

Time of incubation at 27 or 40 C	Radioactivity (counts/min) associated with membranes at	
	27 C	40 C
min		
60	1,340	1,835
90	725	1,721
120	325	1,635
150	210	1,625

[a] Cultures were infected with ^{3}H-uridine-labeled ts6 under conditions similar to those described in Fig. 2. Cultures were harvested at various times after incubation at the respective temperatures; the cytoplasmic extracts were prepared and then analyzed individually on sucrose density gradients by methods identical to those shown in Fig. 2. Values of the radioactivity associated with membranes at different intervals were obtained by adding up the radioactivity contained in fractions 6 to 12 of sucrose density gradients similar to those described in Fig. 2.

was no appreciable loss of label at 40 C under identical conditions. The radioactivity found in the membranes from cells infected and incubated at 40 C did not represent uneclipsed virus since little radioactivity was present in cultures incubated with cycloheximide at 40 C (Fig. 2). The synthesis of viral RNA in ts6-infected cells can occur only at 27 C and not at 40 C (1). Therefore, the above results may suggest that the input RNA detaches from the membranes during the synthesis of new viral RNA.

DISCUSSION

In recent years, the cellular membranes of bacteria and mammalian cells have come under closer scrutiny and a surprising number of metabolic functions have been found to be associated with the membranes. Investigations on the replication of both DNA- and RNA-containing phages and animal viruses have been carried out, and in a number of cases host membrane interactions have been implied (3, 10, 18; C. F. Earhart and M. Schaechter, Bacteriol. Proc., p. 162, 1969). The data presented here provide evidence for the involvement of host membranes with the replication of Sindbis viral RNA. One of the criteria employed here for surmising a membrane-viral RNA complex is the observation that newly synthesized Sindbis viral RNA is associated with the M band. Convincing evidence has been presented indicating that only the membrane constituents of bacterial cells attached to the M band (16). It has been shown that the nucleic acids (both DNA and

RNA) appearing in the M band were not a consequence of nonspecific entrainment of nucleic acids by membranes (16). Our results also support the above findings since the M band contained little or no RNA when an artificial mixture of free viral RNA and cytoplasmic extract was fractionated. Also, the data indicate that most of the nascent viral RNA found in the M band contained the ribonuclease-resistant form of viral RNA. At present, it is thought that the ribonuclease-resistant RNA is an immediate precursor of single-stranded viral RNA. Thus, it is logical to suggest that membranes may be the sites of viral RNA replication in the infected cells. Also, the experiments indicate that the viral RNA polymerase is associated with the host membranes. Thus, the membranous structure has many similarities to the replicative complex which has been identified in infections with many RNA-containing viruses (7, 12, 13).

The data presented here do not permit any conclusions on the nature or the type of host membranes involved. However, evidence has been presented here which suggests that the membrane-viral RNA complex does play an essential role in the replication of the viral RNA. This conclusion is a consequence of the experiments reported here on the behavior of the input RNA ts6 in the infected cells at 27 and 40 C. The input RNA attaches to the membranes at 27 C, the permissive state, and thereafter it progressively detaches. The detachment of the input RNA is restricted at 40 C, the nonpermissive state where no new viral RNA synthesis occurs. Results similar to those reported here have been obtained in the replication of T4 viral DNA in infected bacteria (C. F. Earhart and M. Schaechter, Bacteriol. Proc., p. 162, 1969). Our results also indicate that the attachment of the input viral RNA to the host membranes does not necessarily depend on the presence of an active viral RNA polymerase in the infected cells. This is seen from the data showing the association of the input ts6 RNA with membranes under conditions in which both active and inactive viral RNA polymerases are induced in the cells. It is not known whether there exists any specific site(s) on the cellular membranes for the attachment of the viral RNA. However, the experiments with cycloheximide indicate that the association of the viral RNA with the membranes requires a step involving the synthesis of proteins. The nature of this step is of some interest. This step may be under the control of the cellular or viral genome. In this context, it is significant to note that interferon has no effect on the association of the viral RNA with membranes (5). Based on the current concepts on the mechanism of action of interferon, it was concluded that the above step may be controlled by the cellular genome and not by the viral genome (5). This conclusion is supported by our results on the behavior of the input RNA from ts6 at 27 and 40 C. The nature of this hypothetical cellular protein needed for integration of the viral RNA with membranes has been discussed already (5). However, direct evidence on the origin and nature of the above protein is still lacking.

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LITERATURE CITED

1. Burge, B. W., and E. R. Pefferkorn. 1966. Isolation and characterization of conditional lethal mutants of Sindbis virus. Virology 30:204–213.
2. Caliguiri, L. A., and I. Tamm. 1969. Membranous structures associated with translation and transcription of polio virus RNA. Science 166:885–886.
3. Frankel, F. R. C., C. Majumdar, S. Weintraub, and D. M. Frankel. 1968. DNA polymerase and the cell membranes after T4 infection. Cold Spring Harbor Symp. Quant. Biol. 33:495–501.
4. Friedman, R. M. 1968. Replicative intermediate of an arbovirus. J. Virol. 2:547–552.
5. Friedman, R. M., and T. Sreevalsan. 1970. Membrane binding of input arbovirus ribonucleic acid: effect of interferon or cycloheximide. J. Virol. 6:169–175.
6. Gardner, R. S. 1969. The use of Tricine buffer in animal tissue cultures. J. Cell Biol. 42:320–321.
7. Girard, M., D. Baltimore, and J. E. Darnell. 1967. The poliovirus replication complex: site for synthesis of poliovirus RNA. J. Mol. Biol. 24:59–74.
8. Grimley, P. M., I. K. Berezesky, and R. M. Friedman. 1968. Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. J. Virol. 2:1326–1338.
9. Hallick, L. R., R. Boyce, and H. Echols. 1969. Membrane association of bacteriophage lambda DNA: possible direct role of regulator gene. Nature (London) 223:1239–1242.
10. Haywood, A. M., J. H. Cramer, and N. L. Shoemaker. 1969. Host virus interaction in ribonucleic acid bacteriophage-infected *Escherichia coli*. I. Location of "late" MS2-specific ribonucleic acid synthesis. J. Virol. 4:364–371.
11. Lowry, O. H., N. J. Rosenbrough, A. F. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
12. Penman, S., H. Becker, and J. E. Darnell. 1964. A cytoplasmic structure involved in the synthesis and assembly of polio virus components. J. Mol. Biol. 8:541–555.
13. Polatinck, J., and R. B. Arlinghaus. 1967. Foot and mouth disease virus induced RNA polymerase in baby hamster kidney cells. Virology 31:601–608.
14. Sreevalsan, T., and P. T. Allen. 1968. Replication of Western equine encephalomyelitis virus. II. Cytoplasmic structure involved in the synthesis and development of the virus. J. Virol. 2:1038–1046.
15. Sreevalsan, T., and F. H. Yin. 1969. Sindbis virus-induced viral ribonucleic acid polymerase. J. Virol. 3:599–604.
16. Tremblay, G. Y., M. J. Daniels, and M. Schaechter. 1969. Isolation of a cell membrane-DNA nascent RNA complex from bacteria. J. Mol. Biol. 40:65–76.
17. Warren, L., M. C. Glick, and M. K. Wass. 1966. Membranes of animal cells. I. Methods of isolation of the surface membrane. J. Cell. Physiol. 68:269–288.
18. Yarus, M. J., and R. L. Sinsheimer. 1967. The process of infection with bacteriophage ϕX174. XIII. Evidence for an essential bacterial "site." J. Virol. 1:135–144.