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# Involvement of the Host Cell Nuclear Envelope Membranes in the Replication of Japanese Encephalitis Virus

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The distribution of viral ribonucleic acid (RNA) on various cell membrane fractions derived from a porcine kidney cell line infected with Japanese encephalitis virus was investigated. At 40 h postinfection, after virus growth had reached its peak, three viral RNAs, 45*S*, 27*S*, and 20*S*, were associated with the cytoplasmic membranes and intact nuclei. The amount of each RNA associated with the nucleus was two- to fivefold greater than that present with the cytoplasmic membranes. Treatment of washed infected nuclei with 1.0% Triton X-100, which removed the outer nuclear envelope membrane, also removed the viral RNA. When the nucleus was fractionated into nuclear envelope membranes and a large particle fraction which sedimented at 600 × *g*, nearly all of the viral RNA remained associated with the envelope membranes. The nuclear envelope membranes contained higher viral RNA polymerase activity than the cytoplasmic membranes derived from the same cells. These data suggest that major sites for Japanese encephalitis virus RNA synthesis may be localized on or in very close association with the nuclear envelope membranes.

Current concepts of viral replication visualize the cytoplasmic membranes of the host cell as the site of synthesis and assembly of various viral components (1, 2, 4-6, 8, 9, 17). In the case of the small ribonucleic acid RNA viruses, it appears that the cell nucleus plays little or no direct role in viral replication. However, for a wide variety of viral agents such as tobacco mosaic virus (11, 19), encephalomyocarditis virus (3, 7, 16), and influenza virus (13), the possibility that the nucleus has an important function in RNA replication has been suggested.

In a previous study (24), we described several virus-induced RNA species associated with Japanese encephalitis virus (JEV)-infected cytoplasmic membranes which were not found in uninfected cells. They were the 45*S* and 27*S* single-stranded forms and a 20*S* double-stranded RNA. In this paper, we present evidence which suggests that a major proportion of the viral RNA found in JEV-infected cells is synthesized either in close association with the host cell nucleus or on the nuclear envelope membranes.

## MATERIALS AND METHODS

**Cells.** A porcine kidney stable cell line designated as PS(Y-15) (12), cultivated as described previously (24), was used. Cell monolayers were prepared in 60-mm tissue culture plates.

**Infection and labeling of viral RNA.** A 10% mouse brain suspension (1.0 to 3.0 × $10^8$ plaque-forming units [PFU]/ml) of the Nakayama strain of JEV served as the virus seed. The relatively low virus titers of seed stocks did not permit the infection of cells with high multiplicities. However, in several preliminary experiments where cultures were infected with approximately 10 PFU per cell, the growth response was similar to that obtained with multiplicities of 1 to 5 used routinely in these studies. The virus was adsorbed for 1 h at 35 C, and the cultures were overlayed with 5 ml of Hanks lactalbumin hydrolysate medium (Grand Island Biological Co.) supplemented with 10% heat-inactivated calf serum. The cultures were incubated at 35 C in an incubator supplied with 5.0% carbon dioxide in air.

Replication of JEV compared with that of group A arboviruses is slow. Virus titers of JEV grown on PS(Y-15) cells begin to increase at 10 h and reach maximal titers ranging between $10^6$ to $10^7$ PFU/ml at 33 h. In most experiments described here, virus samples were taken during early (16 h) and late (40 h) growth stages. In several experiments where samples were taken at 33 h, sucrose density gradient sedimentation patterns of viral RNA were similar to those at 40 h. JEV replication is inhibited by actinomycin D (24). The drug, therefore, could not be added at the time of infection to restrict cellular RNA synthesis. To label viral RNA, 1 μg of actinomycin D per ml was added with 5 μCi of [5-$^3$H]uridine per ml at the appropriate time postinfection and was reincu-

bated at 35 C for the desired interval. Under these ditions, host cell RNA synthesis was sufficiently depressed to detect viral RNA production.

Cells were harvested by scraping into the overlay medium with a rubber policeman and were washed twice by centrifugation with phosphate-buffered saline, pH 7.4 (PBS).

**Preparation of cytoplasmic membrane and nuclear fractions.** Procedures for the lysis of cells and isolation of cytoplasmic membrane fractions were described previously (24). The nuclear fraction remaining after the removal of the cytoplasmic membranes was subjected to a series of washes to remove cytoplasmic contamination (23). The nuclear fraction was suspended by means of a Dounce homogenizer in 10 volumes of 2.3 M sucrose containing $1.5 \times 10^{-3}$ M $CaCl_2$ and $10^{-2}$ M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5). The nuclei were sedimented by centrifugation at 35,000 $\times$ *g* for 30 min. The pellet was washed by resuspension in 3 to 4 volumes of 0.23 M sucrose containing $3.0 \times 10^{-3}$ M $CaCl_2$ and centrifuged at 600 $\times$ *g* for 5 min. Microscope examination of the nuclear pellet indicated that it consisted primarily of intact nuclei with a low level of particulate contamination. It was not possible to determine whether the contaminating material was cytoplasmic or due to the lysis of some nuclei.

Nuclear envelope membranes were obtained by disrupting the washed cell nuclei with a Dounce homogenizer after allowing them to swell for 30 min in 10 volumes of $2.0 \times 10^{-2}$ M phosphate buffer, pH 7.2 (23). The lysed nuclear suspension was centrifuged at 600 $\times$ *g* to remove intact nuclei and large-particle nuclear debris. The supernatant fluid containing the nuclear envelope was centrifuged at 47,000 $\times$ *g* for 20 min.

For the determination of the incorporation of [$^3$H]uridine by cell membranes, cytoplasmic and nuclear envelope membranes from uridine-labeled cells were suspended in 5 ml of reticulocyte standard buffer (0.01 M Tris-hydrochloride, 0.01 M KCl, $1.5 \times 10^{-3}$ M $MgCl_2$), and 1-ml samples were removed and precipitated with 10% trichloroacetic acid. Precipitates were collected on membrane filters, and radioactivity was measured.

**RNA extraction.** A cold phenol-sodium dodecyl sulfate procedure was used to extract RNA from the cytoplasmic and nuclear fractions as described previously (24).

**Sucrose density gradient analysis.** RNA samples in PBS were layered on 15 to 35% sucrose gradients and centrifuged for 18 h in an SW-25.1 rotor at 23,000 rpm in a model L Spinco centrifuge. Chick cell ribosomal RNA was added to provide 18*S* and 28*S* ribosomal RNA as sedimentation markers. Tubes were pierced from the bottom and 10-drop fractions were collected. Acid-insoluble radioactivity was measured by precipitating the fractions with 10% trichloroacetic acid. Precipitates were collected on 3.0-$\mu$m membrane filters which were dried and placed in 10 ml of a liquid scintillator solution (4.0 g of Omnifluor [New England Nuclear] in 1 liter of toluene) and counted with a Beckman LS-250 liquid scintillation spectrometer.

For the determination of ribonuclease resistance of viral RNA, the volumes of sucrose density gradient fractions were adjusted to 2 ml with PBS and divided into two 1-ml portions. One set was processed for acid-precipitable RNA without further treatment. Ribonuclease (1 $\mu$g) was added to each fraction of the second set, and the samples were incubated at 37 C for 30 min, treated with trichloroacetic acid, and processed as described above.

**RNA polymerase activity.** The procedure described by Plagemann and Swim (18) was used to assay for RNA polymerase activity associated with cell membrane fractions. The reaction mixture consisted of Tris-hydrochloride buffer (pH 8.2), 50 $\mu$M; sodium phosphoenolpyruvate, 4 $\mu$M; magnesium acetate, 5 $\mu$M; adenosine, cytidine, and uridine triphosphates, 0.06 $\mu$M each; actinomycin D, 10 $\mu$g; pyruvate kinase, 10 $\mu$g; and [$^3$H]guanosine triphosphate, 1 $\mu$Ci. A 0.5-ml amount of cell membrane suspension prepared from normal or infected cells was added to the reaction mixture to a total volume of 1.0 ml. The reaction mixture was incubated for 30 min at 37 C. A 1-ml amount of 0.1 M sodium pyrophosphate containing 2 mg of bovine serum albumin and 2.5 ml of 1 M perchloric acid containing 25 mg of kieselguhr were added to stop the reaction. The resultant precipitate was washed twice by centrifugation with 5 ml of 0.5 M perchloric acid and once with 5 ml of 0.5 M trichloroacetic acid. The pellet was resuspended in 1.0 ml of 0.5 M trichloroacetic acid and heated in a 70-C water bath for 30 min. The precipitate was transferred to 20 ml of a dioxane-based scintillation fluid, and radioactivity was measured.

Protein content of the cell membranes was determined by the Lowry method (15), with bovine serum albumin fraction V as the protein standard.

## RESULTS

**Viral RNA species associated with the cytoplasmic membranes and nuclear fraction.** Figure 1 compares the sucrose density sedimentation patterns of RNA extracted from cytoplasmic membranes and washed intact nuclei at 16 and 40 h. Cells were incubated in the presence of [5-$^3$H]uridine and actinomycin D for 2 h before isolation of the cytoplasmic and nuclear fractions. Viral RNA synthetic activity was detected in JEV-infected cells at 16 h as well as at 40 h postinfection (Fig. 1). The distribution of viral RNA species between the cytoplasmic membranes and nuclear fraction appeared to be different at the 16-h stage of virus replication. The 27*S* and 20*S* viral RNA-forms were detected on the cytoplasmic membranes, but a 45*S* RNA peak was not seen. On the other hand, 45*S* RNA was present as a small but discrete peak associated with the nuclear fraction. At 40 h, both cellular fractions contained 45*S*, 27*S*, and 20*S* viral RNA species. The nuclear fraction, however, was found to contain two- to fivefold more viral RNA than

that associated with the cytoplasmic membranes. The 45*S* and 20*S* species were consistently predominant, but 27*S* RNA, shown as a shoulder in Fig. 1, was always a minor component.

The sedimentation pattern of RNA extracted from the nuclear fraction of uninfected cells is compared with that of infected cells in Fig. 2. At 16 and 40 h postinfection, a single prominent RNA with a sedimentation coefficient of approximately 4*S* was found in uninfected nuclear fractions, whereas the 45*S*, 27*S*, and 20*S* RNA forms were present only in RNA extracts of infected nuclei. In addition, the 20*S* RNA was resistant to ribonuclease and presumably represents the viral double-stranded RNAs (replicative form and replicative intermediate) (Fig. 2). These data strongly suggest that the 45*S*, 27*S*, and 20*S* RNAs found in infected cells were virus induced. Similar results were obtained for RNA associated with infected cytoplasmic membranes (24).

The 4*S* RNA was found associated with the host cell nucleus of both normal and virus-infected cells, but larger amounts were consistently formed in infected nuclei. This RNA

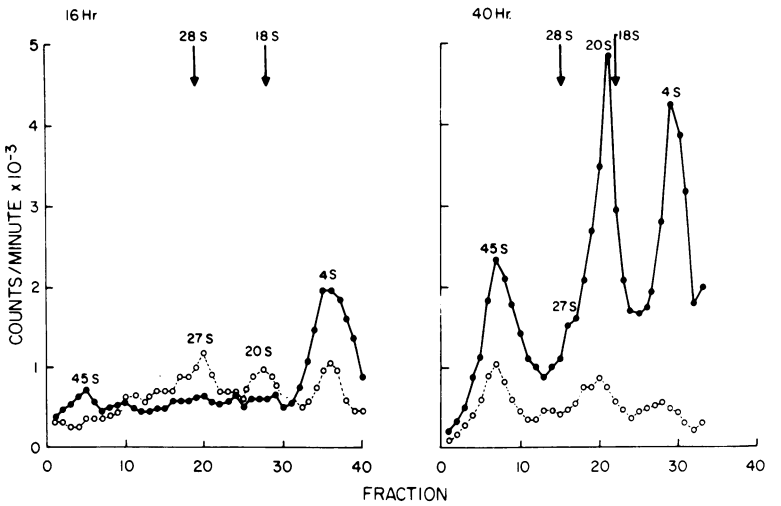


FIG. 1. *Sucrose density gradient sedimentation profiles of RNA extracted from cytoplasmic membranes and intact nuclei derived from PS(Y-15) cells infected with JEV for 16 and 40 h. Cytoplasmic membranes, O; nuclei, ●.*

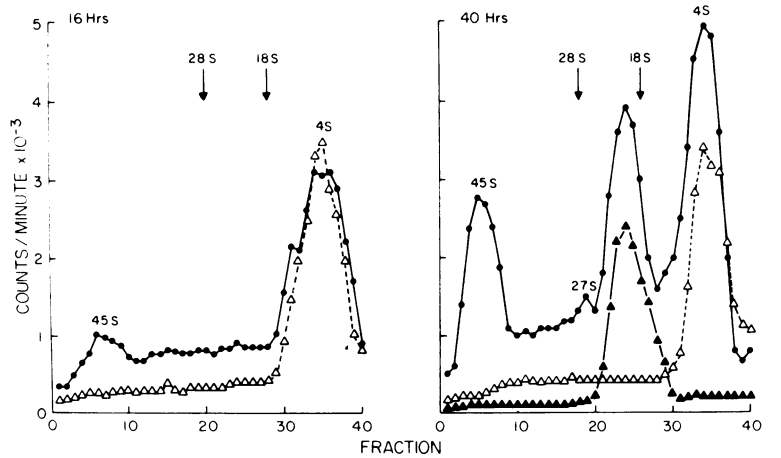


FIG. 2. *Sucrose density gradient sedimentation patterns of RNA extracted from nuclear fractions derived from uninfected and JEV-infected (PS-Y15) cells. Uninfected, Δ; infected, ●; infected and treated with 1 μg of ribonuclease for 30 min at 37 C, ▲.*

species seems to be a host cell product and its relationship, if any, to JEV RNA synthesis is not known.

**Viral RNA synthesis associated with the nuclear envelope.** The nuclear envelope is composed of two membrane layers. The outer membrane is directly connected to the endoplasmic reticulum and is usually regarded as a part of it. The inner membrane is a nuclear-derived structure (23).

When isolated nuclei are treated with nonionic detergents such as 1% Triton X-100, the outer membrane is removed, leaving the nuclei intact (22). By exploiting the property of nonionic detergents to selectively remove the outer nuclear membrane, we attempted to determine whether viral RNA found on the nucleus was associated with nuclear envelope membranes.

A suspension of washed nuclei obtained from 40-h infected cells was divided into two equal portions. One served as the untreated control and was processed for the extraction of RNA. The second sample of nuclei was washed once with 1.0% Triton X-100 before RNA extraction. Microscope examination of the detergent-washed nuclei indicated that they were still largely intact. The RNA sedimentation pattern of both samples is shown in Fig. 3. Treatment with 1.0% Triton X-100 removed virtually all of the viral RNA originally associated with the intact infected nucleus, indicating that the RNA may have been localized on the outer membranes.

To further establish the location of the viral RNA associated with the nucleus, washed intact nuclei obtained from 40-h infected cells were ruptured and the nuclear envelope membranes were isolated. The sedimentation patterns of RNA extracted from the nuclear membranes and from the heavy particles of the nucleus sedimented by low-speed centrifugation (600 × *g*) are shown in Fig. 4. The 45*S* and 20*S* viral RNA species were associated primarily with the nuclear envelope membranes. The large-particle nuclear fraction contained no demonstrable amounts of 45*S* RNA, very little, if any, 20*S* RNA, and almost all the 4*S* RNA.

The association of viral RNA with the nuclear envelope membranes suggests that the sites of viral RNA synthesis are localized on these membranes. It is conceivable, however, that viral RNA synthesis takes place primarily on the cytoplasmic membranes, but during the long periods of contact (2 h) with [$^3$H]uridine used in above-described experiments, viral RNA products were eventually transferred to the nuclear envelope membranes. To test this possibility, cells infected for 33 or 40 h were labeled with [$^3$H]uridine in the presence of actinomycin D for 5, 10, or 30 min. Cells were fractionated into cytoplasmic and nuclear envelope membranes, and the specific radioactivity of each fraction was determined. Variation in the amount of radioactivity incorporated by each membrane fraction was encountered among experiments (Table 1). The nuclear envelope membranes, however, incorporated 1.4 to 6.6 times as much as the cytoplasmic membranes derived from the same cells. Reducing the pulse interval did not change the distribution of the labeled uridine in favor of the cytoplasmic membranes.

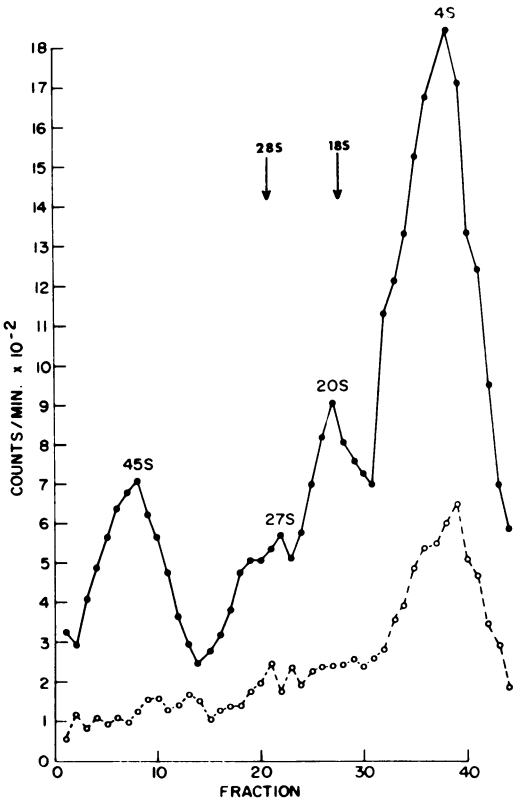


FIG. 3. *Effect of Triton X-100 upon RNA associated with intact nuclei derived from JEV-infected cells. Untreated nuclei, ●; Triton X-100-washed nuclei, ○.*

To determine whether the radioactivity measured in the short pulses (Table 1) indeed represented viral RNA, the following experiment was performed. RNA was extracted from cytoplasmic membranes and from washed intact nuclei labeled with [$^3$H]uridine for 10 min at 33 h postinfection and fractionated on sucrose density gradients (Fig. 5). The viral RNA

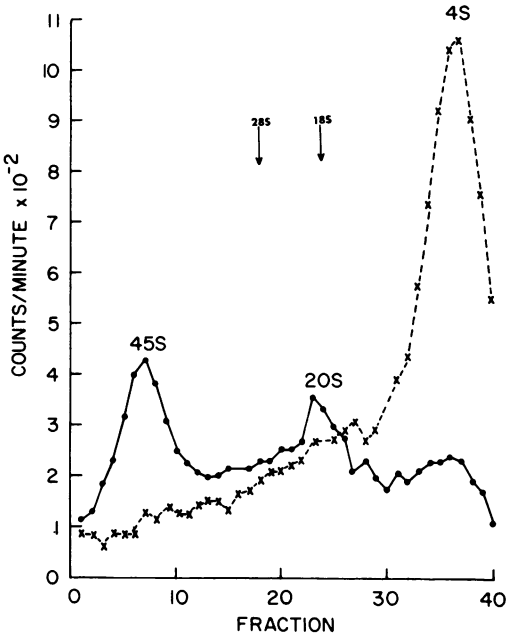


FIG. 4. *Distribution of viral RNA on the nuclear envelope membranes and large-particle nuclear debris. Nuclear envelope membranes, ●; large-particle nuclear fraction, ×.*

TABLE 1. *Incorporation of [5-³H]uridine into cytoplasmic and nuclear envelope membranes of JEV-infected cells*

| Period of pulse (min) | No. of expts | Sp act[a] | | Ratio[b] |
|---|---|---|---|---|
| | | Cytoplasmic membranes | Nuclear envelope membranes | |
| 5 | 3 | 1,164 | 3,793 | 3.2 |
| | | 160 | 914 | 5.7 |
| | | 12,140 | 38,000 | 3.2 |
| 10 | 5 | 1,520 | 6,331 | 4.2 |
| | | 1,930 | 12,850 | 6.6 |
| | | 29,300 | 48,000 | 1.6 |
| | | 3,049 | 4,248 | 1.4 |
| | | 14,485[c] | 20,558[c] | 1.4 |
| 30 | 1 | 4,500 | 13,550 | 3.0 |

[a] Based on three replicate samples and corrected by subtracting the specific activity of uninfected membranes. Expressed as counts per minute per milligram of protein.

[b] Ratio = (specific activity of nuclear envelope membranes)/(specific activity of cytoplasmic membranes).

[c] Samples were taken at 33 h postinfection.

species described earlier (Fig. 1) were present on both cell fractions, but the amount of each RNA associated with the nuclear fraction was three- to fourfold greater than that present on the cytoplasmic membranes. An RNA species with a sedimentation coefficient of 33*S*, not seen in previous experiments, was observed as a prominent peak on the nuclear fraction. It might be analogous to the RNA form described by Levin and Friedman (14) for group A arboviruses. Corresponding sucrose density gradient RNA profiles of uninfected PS(Y-15) cytoplasmic membranes and nuclei did not have the viral RNA peaks described above.

**Viral RNA polymerase activity associated with cell membranes.** The distribution of viral RNA polymerase on various cell fractions was examined in the experiments described below.

Cytoplasmic membranes and washed intact nuclei derived from normal and 40-h infected PS(Y-15) cells were tested for RNA polymerase activity. The resultant RNA products were extracted with phenol and analyzed on sucrose density gradients (Fig. 6). The major RNA species formed by the JEV-infected cytoplasmic membrane and nuclear fractions in vitro had a sedimentation coefficient of 20*S* and was resistant to ribonuclease. Corresponding fractions from uninfected cells did not synthesize this RNA species in vitro. Thus the 20*S* RNA appears to be a virus-induced double-stranded

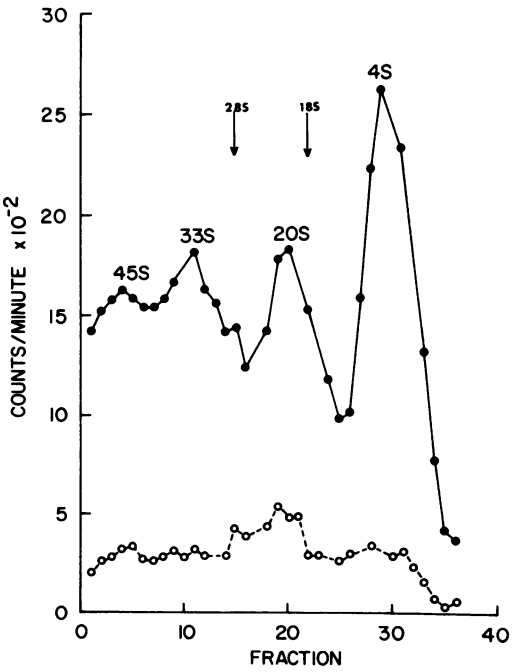


FIG. 5. *Viral RNAs associated with the cytoplasmic membranes and nuclear fraction after a 10-min pulse with [5-³H]uridine. Nuclear fraction, ●; cytoplasmic membranes, ○.*

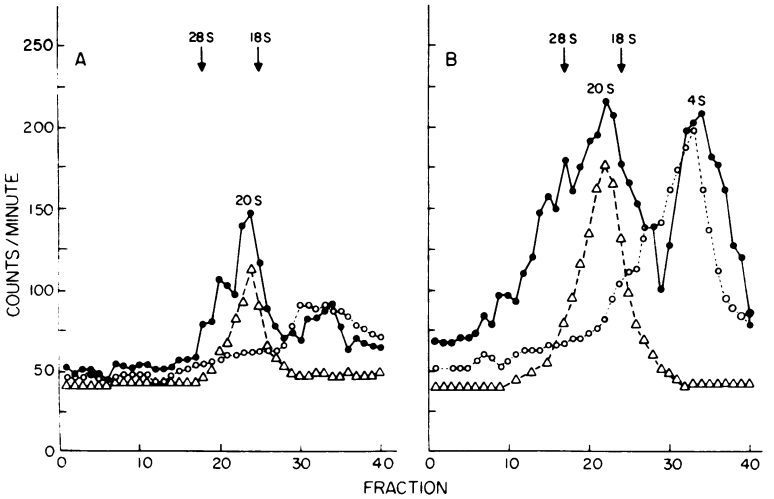


FIG. 6. *Sucrose density gradients of RNA synthesized by RNA polymerase associated with cytoplasmic membranes and with washed intact nuclei derived from normal and JEV-infected PS(Y-15) cells. A, Cytoplasmic membranes; B, nuclear fraction. Infected, ●; uninfected, ○; infected and treated with 1 μg of ribonuclease for 30 min at 37 C, △.*

RNA. Other viral RNA forms seemed to be formed by viral RNA polymerase in vitro but were not clearly resolved on sucrose gradients (Fig. 6).

Large amounts of 4S RNA were also formed in vitro by nuclei derived from normal and virus-infected cells. These results support our earlier observation (Fig. 1) that the 4S RNA is probably not virus induced.

Our studies have suggested that viral RNA synthesis can take place on cytoplasmic and nuclear envelope membranes, but the latter appeared to play a more significant role. If the major site of viral RNA synthesis occurs at or in close association with the nuclear envelope membranes, it would be expected that these membranes would have a higher level of RNA polymerase activity than the cytoplasmic membranes. This was tested in the next experiment.

Cytoplasmic and nuclear envelope membranes derived from 40-h infected cells were tested for RNA polymerase activity. Since the yield of nuclear envelope membranes was generally much smaller than that of the cytoplasmic membranes, the values were corrected on the basis of protein content (counts per minute per milligram of protein). To correct for the contribution of residual actinomycin D-resistant cellular RNA polymerase activity, the corresponding activity of uninfected cell membranes was subtracted (Table 2).

The nuclear envelope membranes exhibited 1.7- and 4.3-fold greater RNA polymerase activity than did cytoplasmic membranes from the same cells. This observation appeared to indicate that the membranes associated with the infected cell nucleus had a higher specific activity of viral RNA polymerase than the cytoplasmic membrane.

TABLE 2. *Distribution of viral RNA polymerase activity on cytoplasmic and nuclear envelope membranes*

| Expt | Sp act[a] | | Ratio[b] |
|---|---|---|---|
| | Cytoplasmic membranes | Nuclear envelope membranes | |
| 1 | 3,314 | 14,358 | 4.3 |
| 2 | 483 | 823 | 1.7 |

[a] Specific activity was corrected for RNA polymerase activity present in corresponding membranes of uninfected cells. Expressed as in Table 1.

[b] Expressed as in Table 1.

## DISCUSSION

Evidence of nuclear involvement in JEV RNA replication was obtained by Takeda et al. (20), who demonstrated viral RNA association with the nucleus by radioautographic techniques and by Takehara (21), who reported that 15 to 30% of the total RNA polymerase found in infected Vero cells resided in the nuclear fraction.

A special role of the nucleus in the synthesis of viral RNA by group B arboviruses was indicated by Brawner et al. (Abstr. Annu. Meet. Amer. Soc. Microbiology, 1973, p. 202). They found that viral RNA was localized on the

nucleus, and they detected in detergent-washed nuclei a 45*S* viral RNA enriched in polyadenylate [poly(A)]. They suggested that a nuclear function may be involved in viral RNA processing and in the addition of poly(A) to 45*S* RNA.

Our initial experiment (Fig. 1) indicated that cytoplasmic membranes and nuclear fractions were involved in the synthesis of viral RNA but that a major proportion of the virus-induced RNA was associated with the nuclear fraction. Subsequent experiments (Tables 1 and 2) suggested that this association reflected the site of viral-induced RNA synthesis. The ratio of RNA associated with the nuclear fraction, or nuclear membrane, to that recovered in the cytoplasmic membrane fraction did not materially change when the pulse of exposure to labeled uridine was changed from 2 h to 5 or 10 min; in addition, viral RNA polymerase activity was higher in the nuclear envelope fraction than in the cytoplasmic membranes.

The possibility that the radioactivity and RNA polymerase activity associated with infected cell nuclei and nuclear envelope membranes was due to contamination with cytoplasmic constituents was considered. The procedure for the isolation of cell nuclei probably does not totally exclude cytoplasmic elements, but light and electron microscopic examination of the nuclear preparations revealed the presence of limited cytoplasmic material. To account for the level of RNA synthesis shown in Fig. 1, 5, and 6, it is necessary to assume that our nuclear preparations were grossly contaminated with cytoplasmic constituents or that these constituents had a remarkably high specific activity.

The nature of the 4*S* RNA found in large amounts associated with JEV-infected nuclei (Fig. 1-6) is not known. Control uninfected nuclear fractions contained this RNA species, but infection substantially increased its level. In a previous study (24), we found that actinomycin D inhibited ribosomal RNA synthesis of uninfected PS(Y-15) cells but increased the 4*S* RNA content. Actinomycin D is known to cause a conversion of nuclear RNA to acid-soluble products (10). One possible explanation of the large amount of 4*S* RNA is that it represents cellular and, perhaps, degraded viral RNA resulting from the action of actinomycin D.

The results shown in Fig. 3 and 4 provide indirect evidence that the outer layer of the nuclear envelope may be the site of virus-induced RNA synthesis. The nuclear envelope membrane contained nearly all the recovered radioactivity associated with the viral RNA species, whereas the large nuclear fractions contained almost exclusively 4*S* RNA; however, when the outer nuclear envelope was removed with detergent, the viral RNA was also removed.

The relationship between RNA synthesis associated with the cytoplasmic membrane to that found on the nucleus is not known. Early (16 h) in the virus infection, we detected the 45*S* viral RNA on the nucleus, but not on the cytoplasmic membranes (Fig. 1). On the other hand, the 27*S* and 20*S* RNAs were associated largely with the cytoplasmic membranes at this early stage of virus replication. This observation suggests that the sequence of RNA synthesis and the amount of various viral RNAs made may vary with the type of cell membrane involved. The relationship between the membranes of the nucleus and cytoplasm in JEV replication deserves further investigation.

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