Association of Virus Specific Replicative Ribonucleic Acid with Nuclear Membrane in Chick Embryo Cells Infected with Japanese Encephalitis Virus

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SUMMARY

Using high resolution electron microscopic autoradiography and velocity sedimentation, RNA synthesis was examined in chick embryo cells infected with Japanese encephalitis virus (JEV). RNA was labelled with 3H-uridine for 5 min or 10 min at 15 h after infection in the presence of actinomycin D and d-glucosamine. Microautoradiography showed significant numbers of silver grains on the nuclear membranes of 5 min pulse-labelled thin cell sections.

The RNA species in membrane fractions obtained from the nucleus and cytoplasm of the infected cells were analysed by sucrose density gradient sedimentation. Radioactive 23S replicative form RNA and 8–12S RNA were obtained from the outer membrane fractions of the nuclear envelope. Labelled 42S virus RNA was obtained from the fractions containing large vesicle membranes and plasma membranes.

These results suggest that JEV-RNA synthesis is initiated in the perinuclear region in close association with the outer membranes of the nuclear envelope.

INTRODUCTION

It has been shown that virus RNA synthesis of toga-alphaviruses takes place on the cytoplasmic membranes of the host cells (Ben-Ishai, Goldblum & Becker, 1968, Sreevalsan, 1970). Friedman et al. (1972) and Grimley et al. (1972) reported that type I cytopathic vacuoles in the cytoplasmic fractions of cells infected with Semliki Forest virus served as important sites for virus RNA synthesis.

With toga-flaviviruses, Qureshi & Trent (1972) have shown that St. Louis encephalitis virus RNA replicative forms and RNA polymerase activity were found in cytoplasmic membranous structures. We reported previously that the synthesis of RNA in Japanese encephalitis virus (JEV), a toga-flavivirus, occurred in association with the nuclear region (Takeda, Yamada & Aoyama, 1965, Takeda et al. 1977). We reported that four species of virus-specific RNA were recognized: 42S RNase-sensitive RNA, 26S partially RNase-resistant RNA, 23S RNase-resistant RNA, and 8–12S RNase-sensitive RNA. The 23S RNA labelled in short pulse experiments and the 26S RNA labelled in pulse-chase experiments were found in a nuclear fraction separated with
mechanical agitation and treatment with NP-40 (Takeda et al. 1977). Zebovitz, Leong & Doughty (1974) reported that three JEV-RNAs, 45S, 27S and 20S, were associated with the nuclear envelope membranes that contained high virus RNA polymerase activity.

This communication is concerned with attempts to elucidate the involvement of nuclear structures in the replication of JEV-RNA.

METHODS

Virus. Japanese encephalitis virus (JEV) Nakayama strain was plaque-purified three times and grown in suckling mouse brain. Ten per cent suspensions of the brain were centrifuged at 8000 g for 30 min and the supernatant was used as seed virus. Infectivity of the supernatant was 1.2 × 10⁹ p.f.u./ml.

Cell culture. All experiments were performed with primary chick embryo fibroblasts (about 4 × 10⁶ cells/dish cultivated in 6 ml of Eagle's minimum essential medium containing 5% calf serum in Petri dishes (9 cm in diam.). Cells were infected at 28 to 30 h after seeding.

Virus assay. The infectivity of the JEV preparations was estimated by plaque assay on chick embryo (CE) cells according to the method described by Porterfield (1959), with slight modification.

Chemicals. Actinomycin D was purchased from Merck, Sharp and Dohme. D(+) glucosamine hydrochloride, uridine and thymidine were purchased from Sigma Chemical Company. Phenol and sodium dodecyl sulphate (SDS) were purchased from Nakarai Chemicals, Ltd. 5-³H-uridine (30 Ci/mmol, 1 mCi/ml) was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Procedures for labelling virus RNA. Monolayers of CE cells were infected with JEV at a m.o.i. of 10 p.f.u./cell. After adsorption for 90 min at 37 °C, 6 ml of warm medium was added and incubation was continued at 37 °C. Twelve h after infection, the medium was replaced with fresh medium containing 4 μg actinomycin D per ml, 5 mM-D-glucosamine and 2 × 10⁻⁵ M-thymidine. After an additional 3 h incubation, the medium was removed and replaced with the same medium containing 200 μCi/ml of ³H-uridine. The cells were further incubated at 37 °C for the times indicated in the text.

Pulse-chase experiments. After 10 min pulse labelling with ³H-uridine, the cultures were incubated in medium containing 100 μg/ml non-labelled uridine for 20 min at 37 °C.

Isolation of nuclear membranes. Nuclear envelopes were isolated from the infected and labelled CE cells by use of discontinuous sucrose density gradients according to Kashnig & Kasper (1969), except that NaCl was used instead of KCl in the TKM buffer. In experiments for microautoradiography and analysis of RNA associated with membranes, the cells were suspended in 4 ml of TNM buffer (0.01 M-tris hydroxymethyl nitromethane, 0.1 M-NaCl, 0.005 M-MgCl₂, pH 7.5) containing 0.25 M-sucrose and allowed to swell for 15 min at 4 °C. The cells were disrupted with 20 strokes of a glass Dounce homogenizer using a tight-fitting pestle. The nuclei were washed with 0.25 M-sucrose-TNM buffer, and sedimented by centrifugation at 800 g for 10 min. The washed nuclei were disrupted by a sonic oscillator (Kubota Co. Model KMS250, Tokyo) at 10 Hz for 3 min. A smear sample was made for examination with a phase microscope to determine the degree of cell destruction.

An additional sonic treatment was performed if breakage of more than 70% of nuclei had not been achieved. Solid sodium citrate was added with stirring to make a final concentration of 10% (w/v). The lysate was then centrifuged at 39000 g for 45 min at 3 °C in a SW 50L (Spinco) rotor, and the supernatant was discarded. The pellet, including most of the membranous structures, was suspended by homogenization in 9 ml of sucrose-TNM...
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buffer–10% sodium citrate solution of density 1.22 g/ml and transferred to a 38 ml Spinco SW 27 rotor tube. The membrane suspension was overlaid serially with 9 ml each of sucrose–TNM buffer containing 10% citrate of densities 1.20, 1.18 and 1.16 g/ml. The discontinuous gradients were centrifuged at 100,000 g for 14 to 16 h at 3 °C. Nuclear membranes were recovered at interfaces of density 1.18 to 1.20 g/ml and 1.16 to 1.18 g/ml.

The membrane fractions from the gradient were dialysed against TNM buffer overnight. The membrane structures were sedimented by centrifugation at 100,000 g for 60 min.

Isolation of cytoplasmic membranes. The cytoplasmic membranes were obtained from the infected and labelled CE cells by the use of discontinuous sucrose density gradients according to the method of Caliguiri & Tamm (1969, 1970). The cytoplasmic fraction (5 ml) was mixed with an equal volume of 60% (w/w) sucrose–TNM buffer containing 20% citrate. The suspension was then placed on a discontinuous gradient prepared by layering sucrose-buffer (w/w) solution in the following order: 3 ml of 60%, 7 ml of 45% and 7 ml of 40%. Seven ml of 25% sucrose and 3 ml of TNM buffer were then overlaid on the extract. The gradient was centrifuged at 100,000 g for 14 to 16 h in a Spinco SW 27 rotor. After centrifugation, five visible bands of membrane fractions from the gradient were collected and prepared for further examination as described for the nuclear membranes.

RNA extraction and analysis. Membrane pellets were resuspended with a Dounce homogenizer in 2.5 ml of TNM buffer and 2.5 ml of 2% sodium dodecyl sulphate (SDS). The lysates were kept in a 37 °C water bath for about 10 min. Four ml of the lysate was layered on a 32 ml linear sucrose gradient from 6 or 15% to 30% over a 2 ml 40% sucrose cushion in a centrifugation tube of the Spinco SW 27 rotor. After centrifugation at 27000 rev/min (100,000 g) for 10 h or at 22,000 rev/min (65,000 g) for 16 h, the gradient was separated into 1 ml fractions which were monitored for u.v. absorbance. Bovine albumin (10 μg/fraction) was added to each fraction as a carrier. Trichloroacetic acid (TCA)-insoluble material was collected on a Whatman GF/C glass fibre filter. The filter was placed in a scintillation vial containing 5 ml of toluene base scintillation fluid, and counted with a Beckman liquid scintillation spectrometer.

Electron microscopy and microautoradiography. The pellets of labelled cells and fractionated membranes were fixed in 2% glutaraldehyde in 0.1% m-phosphate buffer at pH 7.3 for 1 to 2 h, and postfixed in 1% OsO4. The pellets were then dehydrated in ethanol and embedded in Epon 812. Sections of 50 nm in width were obtained with an LKB-ultratome, mounted on collodion-coated copper grids and stained with 6% uranyl acetate in ethanol. Melted (45 °C) Sakura NR-H2 emulsion was diluted 15 times with distilled water. A monolayer was applied to the specimens by the method of Mizuhira (1968). The preparations were stored in the cold room for 4 to 5 weeks, then developed according to the method of Mizuhira (1968), washed with distilled water, and fixed with Kodak D5 for 5 min. The specimens were stained with high pH lead solution. The autoradiograms were photographed at 75 kV with a Hitachi HU-IIB at magnifications 4000 and 5000.

RESULTS

Electron microscopic autoradiography with thin sectioned JEV-infected cells

CE cells infected with JEV at an m.o.i. of 10 p.f.u. were pulse labelled for 5 min, 10 min or 30 min with 3H-uridine at 15 h after infection in the presence of actinomycin D, thymidine and D-glucosamine. In some studies, 5 min-pulse labelled cells were chased with non-labelled uridine for 10 min. Autoradiography of ultrathin sections of the labelled cell specimens was performed. In the cells infected with JEV at an m.o.i. of 10 p.f.u./cell for
Table 1. *Distribution of grains on micro-autoradiography of JEV-infected cells*

<table>
<thead>
<tr>
<th>Group</th>
<th>Labelling time (min)</th>
<th>Nuclear membranes†</th>
<th>Nucleoli</th>
<th>Nucleoplasm</th>
<th>Matrix</th>
<th>RER†</th>
<th>Smooth vesicles</th>
<th>Mitochondria</th>
<th>Vacuoles</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected control</td>
<td>10</td>
<td>0.7±1.7</td>
<td>0.2</td>
<td>0.8</td>
<td>0.9</td>
<td>1.3±0.5</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.2±0.6</td>
<td>0.1</td>
<td>0.7</td>
<td>0.6</td>
<td>1.1±0.6</td>
<td>0.2</td>
<td>0.1</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Infected cells</td>
<td>5</td>
<td>4.5±0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.3</td>
<td>2.9±1.0</td>
<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>5+10 chased</td>
<td>2.6±0.6</td>
<td>0.4</td>
<td>1.1</td>
<td>1.3</td>
<td>5.3±1.5</td>
<td>0.4</td>
<td>0.5</td>
<td>0.1</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.9±1.4</td>
<td>0.9</td>
<td>1.4</td>
<td>0.7</td>
<td>6.0±2.6</td>
<td>1.0</td>
<td>0.9</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.4±1.8</td>
<td>0.2</td>
<td>1.6</td>
<td>0.5</td>
<td>5.0±1.8</td>
<td>0.8</td>
<td>0.2</td>
<td>1.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Mean grain number on organelles of 40 thin cross sections of different cells was counted. The cells containing Golgi apparatus and full sized nuclei were selected as an indication that the cells were sectioned through the centre or near to the centre of the nucleus.

† The standard error of the mean grain number is given.
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15 h and pulse labelled for 10 min with ³H-uridine, many silver grains were observed on the nuclei by light microscopy. In sections of 5 and 10 min pulse labelled cells, a significant number of silver grains were observed on nuclear membranes (Table 1 and Fig. 1a). There was also a significant difference in numbers of grains on rough surface endoplasmic reticulum (RER) of non infected cells and those of infected cells of 10 min pulse labelling. In the cases of chasing and 30 min-labelling, the numbers of grains on RER of infected cells were higher than those of non infected cells labelled for 30 min. After chasing with cold uridine, the grain number on RER was preferentially increased as shown in Table 1 and Fig. 2. In the specimens from 10 min and 30 min labelled cells, grains were distributed on nuclear membranes, RER, smooth surface vesicle membranes and the surface of cytoplasmic vacuoles.

RNA species associated with isolated nuclear membranes

The partially purified nuclear fractions obtained from the 10 min-labelled JEV infected cells were disrupted by sonication and subjected to isopycnic centrifugation in a discontinuous sucrose density gradient. Three distinct bands were usually formed after centrifugation. The upper band (N-1) present at the interface between a density of 1.16 and 1.18 g/ml contained high radioactivity (Fig. 3). The second band (N-2) was located at the interface between a density of 1.18 and 1.20 g/ml and the third band (N-3) was present at the interface between a density of 1.20 and 1.22 g/ml. The latter two bands contained very little radioactivity (Fig. 3). These three bands were dialysed against TNM buffer and sedimented at 100000 g for 60 min. A defined volume of the suspension of the pellet was used for analysis of RNA and the remainder was used for micro-autoradiography. As shown in Fig. 4, the radioactivity in the upper band (N-1) was predominantly associated with 23S and 8-12S species of RNA.

The results of electron microscopic observation revealed that the upper band (N-1) was mainly composed of the outer membranes of nuclear envelopes and some rough surface endoplasmic reticulum. The second band (N-2) contained the inner membranes of nuclear envelopes. The third band (N-3) contained ribosome particles, smooth surface membrane vesicles, electron dense granules and nucleoplasm.

RNA species associated with isolated cytoplasmic membranes

Five visible membrane bands were obtained from cytoplasmic fractions of the infected and pulse labelled cells by the method of Caliguiri & Tamm (1969, 1970). The upper two bands (C-1 and C-2), located between density 1.07 and 1.10 g/ml, mainly contained smooth membranes. The third band (C-3), located between density 1.14 and 1.15 g/ml, contained large vesicles and mixed rough surface and smooth surface membranes. The fourth band (C-4) located between density 1.17 and 1.18 g/ml, mainly contained rough surface endoplasmic reticulum. The lowest band (C-5) was enriched with plasma membranes.

In the cytoplasmic fraction prepared from 10-min pulse labelled cells, ³H-labelled 23S RNA was not present in any of these membrane fractions (Fig. 5). The 42S RNA present in the cytoplasmic fraction of 10 min pulse labelled cells was associated with bands C-3 and C-5 (Fig. 5b and d). The 8-12S RNA was partially associated with bands C-4 and C-5 (Fig. 5c and d). When the cells were pulse labelled and chased for 20 min, bands C-3 and C-4 contained 26S RNA as shown in Fig. 6.
DISCUSSION

In previous reports, we concluded that the nuclear or perinuclear region was a site of RNA synthesis of JEV from autoradiographic observations and the finding that nuclear extracts contained 23S replicative form RNA as well as 26S and 8–12S RNAs (Takeda et al. 1965, 1977). In this report, electron microscopic autoradiography of JEV-infected cells and biochemical analysis of nuclear membranes revealed that the outer membrane

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Fig. 1. Electron microscopic autoradiograph of thin-sectioned JEV-infected CE cells, labelled with \(^{3}\text{H}-\text{uridine}\) for 5 min at 15 h post infection in the presence of actinomycin D, thymidine and glucosamine. (a) In the thin cross section of 5-min pulse labelled cells, silver grains were observed on nuclear membrane (large arrows). (b) High magnification autoradiograph.

Fig. 2. Electron microscopic autoradiograph of thin sectioned JEV-infected CE cells, labelled with \(^{3}\text{H}-\text{uridine}\) for 30 min at 15 h post infection under the same condition as in Fig. 1. Silver grains were mainly observed on rough surface endoplasmic reticulum (small arrows).

Fig. 3. Sedimentation pattern of the radioactive components of a disrupted nuclear extract. The membrane fractions of nuclear envelope were obtained from the 10-min labelled JEV-infected cells by the method of Kashnig & Kasper (1969). The upper band of three district bands contained high radioactivity. The solid line shows radioactivity and the broken line shows u.v. absorbance at \(E_{280}\) nm.
fraction of the nuclear envelope contained pulse labelled 23S and 8–12S species of RNAs which were not found in the fractions of the inner membranes of nucleoli and other nuclear components (data not shown). As we could not obtain purified outer membranes of the nuclear envelope, the fraction of the outer membranes is probably contaminated by perinuclear endoplasmic reticulum. The outer membrane fraction, however, contained 23S RNA alone and not 26S RNA. From this finding, it is most reasonable to assume that the replicative 23S RNA is synthesized on the outer membranes of the nuclear envelope. In the prolonged labelling or labelling and chasing experiments, the labelled 23S RNA was not detected in the outer membrane fraction of the nuclear envelope, though silver grains were observed on nuclear membranes in microautoradiograms (Table 1). Radioactive 26S RNA was detected in the fraction of rough surface endoplasmic reticulum and in the fraction of smooth and rough surface membranes containing large vesicles.

Zebovitz et al. (1974) reported that three JEV-RNAs, 45S, 27S and 20S, were associated with the nuclear envelope membrane. There is a difference between our results and theirs in the location of the 45S (42S) RNA. We have not obtained an appropriate explanation for this difference which may be due to differences in the fractionation technique.

Zebovitz et al. also reported that 4S RNA was found associated with the host cell nucleus of both normal and virus-infected cells, thus regarding it as a host cell product. We found 8–12S RNA located in cytoplasmic membrane bands C-4 and C-5 and also in
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Fig. 5. RNA species associated with cytoplasmic membranes. The membrane fractions were obtained by the method of Caliguiri & Tamm (1969, 1970) from the cytoplasmic extract of JEV-infected cells labelled with 3H-uridine for 10 min. Five visible turbid bands were usually formed. The membrane fractions were dialysed against TNM buffer to remove sucrose. The lysates of the membrane pellets were analysed by centrifugation at 65000 g for 16 h on 15 to 30% (w/v) sucrose gradients containing 1% SDS. (a) Bands C-1 and 2; (b) band C-3; (c) band C-4; (d) band C-5.

nuclear membrane band N-1. The 8–12S RNA species are considered virus specific because larger amounts are formed in infected cells in the presence of actinomycin D.

Although the role of double-stranded replicative form RNA in virus replication is still vague, some investigators reported that the double-stranded replicative form plays an intermediate role in toga-alphavirus RNA synthesis (Michel & Gomatos, 1973; Martin & Burke, 1974). Since the radioactivity of 23S RNA could be chased to 26S RNA, 23S RNA appeared to be a precursor of 26S RNA in JEV-RNA synthesis. If this is the case, it must be explained why 26S RNA is found mainly in the cytoplasmic fractions. Since 26S RNA is distributed in the fractions of rough surface endoplasmic reticulum and mixed membranes of rough and smooth surface, it seems to be likely that 26S RNA is a messenger RNA (mRNA) associated with polyribosomes. Simmons & Strauss (1972, 1974) have already pointed out that the virus mRNA is 26S RNA in the cells infected with toga-alphavirus. However, Naeve and Trent (personal communication) have suggested that 42S (45S) RNA alone has a messenger role in toga-flavivirus replication. Therefore, further investigation is required to clarify what sort of role 26S RNA plays in JEV replication.

It is likely, at any rate, that JEV-RNA synthesis begins in the structure closely associated with the outer membranes of nuclear envelope and spreads outwards through the perinuclear region when the virus RNA synthesis is at maximum rate. Kos, Osborne & Goldsby (1975) and D. W. Trent (personal communication) have also suggested that some early essential step in the replication of toga-flavivirus may occur in the nucleus.
Fig. 6. RNA species associated with cytoplasmic membranes of pulse labelled and chased JEV-infected cells. The cytoplasmic membrane bands (C-3, C-4) were obtained from JEV-infected cells labelled with 3H-uridine for 10 min and chased with cold uridine for 20 min. The lysates of the membrane pellets were subjected to isopycnic centrifugation in the discontinuous sucrose gradient system described in Methods. •...•, Band C-3 (density 1.14 to 1.15); ⨃-○, band C-4 (density 1.17 to 1.18).

REFERENCES


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