Intracellular Distribution of Virus-Specific RNA in Chick Embryo Cells Infected With Japanese Encephalitis Virus

(Accepted 3 September 1976)

SUMMARY

Japanese encephalitis virus (JEV) infected chick embryo (CE) cells were treated with 4 μg actinomycin D/ml and 5 mM-D-glucosamine at 2 or 3 h before harvesting. Production of JEV was not affected by the short-time treatment of these drugs. The radioactivity in virus-specific RNA in the glucosamine-treated cells was apparently higher than in non-treated cells. Nuclear and cytoplasmic extracts were prepared from the JEV-infected cells pulse-labelled with 3H-uridine at 15 h after infection. Analysis of virus RNA in nuclear extracts on sucrose density gradients showed that most of the radioactivity was in 23S RNA, 26S RNA and 8 to 12S RNA. The radioactivity of virus RNA in cytoplasmic extracts was found in 42S RNA and RNA fragments sedimenting at less than 8S.

We have previously shown, by autoradiographic techniques, that Japanese encephalitis virus (JEV) RNA synthesis was associated with the nucleus of PS(Y−15) cells (Takeda, Yamada & Aoyama, 1965). Further efforts to provide biochemical evidence to confirm this observation have been impeded by the low efficiency of 3H-uridine incorporation into virus RNA. This problem was overcome by treatment of the cells, before pulse-labelling with 3H-uridine, with glucosamine which reduced the intracellular UTP pools and greatly increased the amount of radioactive precursor incorporated into virus RNA (Scholtissek, Kaluza & Rott, 1972). It has been reported that glucosamine, if it is present throughout the replication cycle, inhibits the multiplication of Sindbis and Semliki Forest viruses (Kaluza, Scholtissek & Rott, 1972). JEV-infected CE cells were treated with 5 mM-glucosamine for 3 h from 12 to 15 h post-infection (p.i.). Virus yield at 15 h p.i. of the JEV-infected cells treated with glucosamine was almost the same as that of the non-treated cells (control titre: 4.2 × 10^7 p.f.u./0.4 ml; titre of the glucosamine-treated cells: 4.5 × 10^7 p.f.u./0.4 ml). It is suggested that the multiplication of JEV is not affected by the short-time treatment with glucosamine at later stages of replication.

Pulse-labelling experiments of 10^8 JEV-infected cells with 200 μCi 3H-uridine were performed with or without glucosamine. The labelled cells were extracted for RNA analysis with 1% sodium dodecyl sulphate (SDS: Sonnabend et al. 1964). The radioactivities in virus-specific RNA in the glucosamine-treated cells were apparently higher than those of non-treated cells (Fig. 1).

To suppress cellular RNA synthesis and detect virus-specific RNA, infected CE cells were treated with actinomycin D for 2 and 3 h before labelling. It has been reported that actinomycin has an inhibitory effect on the replication of JEV (Yamazaki, 1968; Zebovitz, Leong & Doughty, 1972; Kos, Osborne & Goldsby, 1975). In our experiment however, the short-time treatment with actinomycin after 12 to 15 h of virus replication did not cause reduction of JEV production. Perhaps the high m.o.i., the short time of treatment with actinomycin at 12 to 15 h after virus infection, or the origin of the drug which we used (Merck Sharp and Dohme) can account for the difference.
Fig. 1. Effect of d-glucosamine on incorporation of $^3$H-uridine into JEV-RNA. CE cells were infected with JEV at m.o.i. of 50 and were labelled with 200 µCi $^3$H-uridine/ml for 10 min or 20 min at 15 h p.i. in the presence and absence of 5 mM-d-glucosamine. Ten min-pulsed: △--△, without glucosamine; ▲--▲, with glucosamine; 20 min-pulsed: ○--○, without glucosamine; ●--●, with glucosamine.

The growth of JEV in CE cells infected at m.o.i. of 50 p.f.u. began to increase at 8 h and reached a plateau at 16 to 20 h p.i. (data not shown). RNA synthesis in CE cells infected with JEV was measured by 10 min pulse labelling with $^3$H-uridine. RNA synthesis was first detected at 3 h p.i. However, only RNase sensitive 8 to 12S RNA was detected at the first stage. RNA species of 23S, 26S which had been considered to be the main intermediate forms of JEV replication (Nishimura & Tsukeda, 1971; Zebovitz et al. 1972) could not be detected probably due to poor uptake of the radioactive precursor in our experiments. Sufficient amounts of 23S, 26S and virus 42S RNAs were observed at 15 h after infection.

As a consequence, analysis of RNA was carried out in the infected cells 15 h after infection, treatment with actinomycin and glucosamine as described above. Fig. 2(a) illustrates the results of sucrose density gradient analysis of RNA extracted from the infected whole cells after 10 min pulse labelling with $^3$H-uridine. Several RNA species are observed in the profile:
Fig. 2. (a) Sedimentation pattern of RNA species extracted from JEV infected CE cells. CE cells were infected with JEV at a m.o.i. of 10 and were labelled with 25 µCi ³H-uridine/ml from 13 to 15 h p.i. in the presence of actinomycin (4 µg/ml) and 5 mM-glucosamine. △···△, Uninfected; ●—●, infected; ▲—▲, infected and treated with 2 µg ribonuclease for 15 min at 37 °C. Spun at 50000 g for 15 h in the SW 27 rotor. (b) RNA sedimentation pattern of nuclear and cytoplasmic fractions from JEV infected cells. CE cells were infected with JEV at a m.o.i. of 50 and were labelled with 200 µCi ³H-uridine/ml for 10 min at 15 h p.i. in the presence of actinomycin and glucosamine. The labelled cells were disrupted by 5 strokes in a glass Dounce homogenizer after NP-40 treatment (0.05 %) at 0 °C for 15 min. After centrifuging at 800 g for 10 min, the supernatant was collected as cytoplasmic fraction. The nuclear pellet was washed with 1 M- and 0.25 M-sucrose-TNM buffer. ●—●, 10 min-labelled nuclear extract; ▲—▲, 10 min-labelled and chased with 2×10⁻² M-cold uridine for 10 min, nuclear extract; ○···○, 10 min-labelled cytoplasmic extract.
42S RNase-sensitive RNA, 26S partially RNase-resistant RNA, 23S RNase-resistant RNA and 8 to 12S RNase-sensitive RNA. The 42S peak corresponded to the single peak obtained from the RNA extracted from the purified labelled virus. RNA isolated from uninfected cells contained only RNA which sedimented in the 4 to 6S region.

Conditions for isolation of nuclei from chick embryo cells with non-ionic detergent (NP-40) were determined by studying the effect of detergent both on the retention of labelled DNA in undamaged nuclei and on the content of remaining intact cells. Twenty-seven % and less than 10 % of treated cells remained intact after treatment with 0.025 % and 0.05 % of NP-40 respectively. Ninety-four % of labelled DNA was retained in the nuclear fraction when cells were treated with NP-40 at a concentration of 0.05 %.

Therefore 0.05 % was chosen as an optimum concentration of NP-40 to isolate nuclei. The cell suspension in TNM buffer (0.01 M-tris, 0.1 M-NaCl, 0.005 M-MgCl₂, pH 7.5) containing 0.05 % NP-40 was placed in an ice-water bath for 15 min with occasional shaking on a vortex mixer outside the bath. The suspension was disrupted with 5 strokes in a glass Dounce homogenizer using a tight-fitting pestle, and centrifuged at 800 g for 10 min. The supernatant fluid was collected as the cytoplasmic fraction. The nuclear pellet was washed twice with 1 M- and 0.25 M-sucrose-TNM buffer, and collected by centrifuging at 800 g for 10 min at 3 °C. Light microscopic observation revealed that about 95 % of the cells in the culture were ruptured. The electron micrograph of the nuclei revealed that nuclear components were retained in the isolated nuclei. The nuclei were enclosed by outer and inner membrane with some rough surface endoplasmic reticulum and cytoplasmic particles.

Enzymic analysis was performed according to the method of Mackler & Green (1956) on the cytoplasmic fractions and the nuclear fractions separated under the conditions with or without NP-40 treatment. NADH-cytochrome c reductase, known to be one of the integral components of the endoplasmic reticulum, was used as a marker enzyme when nuclear fractions were tested for cytoplasmic contamination. The enzymic activity was followed spectrophotometrically by measuring the increase in extinction at 550 nm. NADH-cytochrome c reductase was found in the nuclear fractions without NP-40 treatment at 19 % of total activity obtained for the whole cells. As NADH-cytochrome c reductase activity was inhibited when NP-40 concentration increased above 0.025 %; we could not test the concentration of NP-40 which was used for extraction of nuclear fractions. Since the total enzymic activity of the nuclear fractions from CE cells treated with NP-40 at the concentration 0.01 % was 14.5 % of the activity of whole cells, it was suggested that the nuclear fractions used for RNA analysis contained 15 % or less cytoplasmic material.

JEV-infected cells were labelled with 200 μCi ³H-uridine for 10 min at 15 h after infection in the presence of actinomycin and glucosamine. The nuclear as well as cytoplasmic fractions were subjected to RNA analysis. Nuclear extracts on sucrose density gradients showed that most of the radioactivity was in 23S RNA, 26S RNA and 8 to 12S RNA. The amount of radioactivity at each peak was 26, 14 and 17 % of the total count respectively (Fig. 2b). When a shorter labelling time of 5 min was adopted, 21 % and 29 % of the total counts were in 23S RNA and 8 to 12 S RNA (data not shown). When the cells were chased for 10 min with cold uridine after the 10 min labelling, most of the radioactivity was found in 26S RNA. The radioactivity was not found in nuclear extracts when the chasing period was prolonged to 20 min or longer. On the other hand, the radioactivity of cytoplasmic extracts was found predominantly in 42S RNA and in smaller sized RNA fragments of less than 8S (Fig. 2b).

The precise sequence of synthesis of these RNA species is not yet clear. The presence of 8 to 12S RNA has also been reported by Zebovitz (Zebovitz et al. 1972).

We have not yet determined whether 26S RNA is a replicative intermediate or acts as
a messenger RNA as reported in group A togavirus (Simmons & Strauss, 1972, 1974). Similar RNAs of sedimentation coefficients 20S, 26S and 43S have been reported in St Louis encephalitis virus-infected cells (Trent et al. 1969) though the precise sequence of synthesis of these RNA species was not clarified.

The results of our experiments suggest that JEV-RNA synthesis is associated with the portion of the cell which is isolated as the nuclear fraction, confirming the previous autoradiographic findings (Takeda et al. 1965). Since the nuclear fraction was contaminated with a small amount of cytoplasmic component, as shown above, further analysis is required to determine whether JEV-RNA synthesis occurs inside the nucleus or in the perinuclear regions. Our further analysis of the nuclear fraction suggested that the outer membranes of the nuclear envelopes of the infected cells were mainly involved in JEV-RNA synthesis (unpublished data).

We wish to express our appreciation to Dr D. W. Trent for his criticisms and comments in the preparation of this manuscript and to Mr Masanori Unoki for his excellent technical assistance.

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REFERENCES


(Received 8 June 1976)