PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1165–1169 (1974)

Autoradiographic Localization of RNA Synthesis Directed by Arboviruses in the Cytoplasm of Infected BHK-21 Cells (37973)

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Electron microscopic studies of cells infected with arboviruses from groups A (alphavirus), B (flavivirus), or California (bunyavirus) have concluded that the replication of these agents appeared to be confined to the cytoplasm, without apparent involvement of the nucleus (1–3). RNA synthesis by the alphavirus Semliki Forest virus (SFV) has been reported to occur in the cytoplasm (4) while that of the flavivirus St. Louis encephalitis (SLE) virus has been reported to occur principally in the nucleus (5). The site of RNA synthesis by bunyaviruses has not been previously investigated, although acridine orange staining of cells infected with Guaroa virus (bunyavirus) indicated an increase in cytoplasmic RNA during infection (2). This communication presents evidence that RNA synthesis by all three arbovirus groups probably occurs in the cytoplasm, although different cytoplasmic regions appear to be involved for each virus group.

Materials and Methods. BHK-21 13S cells were obtained from Dr. K. Wiktor, Wistar Institute, Philadelphia, PA, and passaged and maintained as previously reported (6). Confluent monolayers grown on coverslips were infected with 10–100 PFU/cell of one of the following virus preparations: eastern equine encephalitis (EEE, Ets-4 mutant) (7); Japanese B encephalitis (JBE, OCT-541 attenuated strain) (8); Dengue type 2 (DEN-2, TH-36 isolate) (9); Powassan (POW, prototype) (10); and La Crosse (LAX, prototype) (11). EEE, JBE, and DEN-2 viruses were prepared in cell cultures as described in the above references; the POW and LAX viruses were used as low-passage suckling mouse brain materials (12). At 24 hr following infection with DEN-2, JBE, POW, or LAX, or 4 hr after EEE, cultures were treated with actinomycin D (2.0 μg/ml final concentration). Preliminary experiments had established that this dose irreversibly abolished 99.2% of host RNA synthesis after 1 hr of treatment. After 1 hr at 37°, duplicate coverslips were removed, washed in Hanks' balanced salt solution (HBSS), and exposed to ^{3}H-5,6-uridine (Schwarz Bioresearch) in HBSS supplemented with 0.4 mg/ml $NaHCO_3$ in Columbia staining dishes. Coverslips which were "chased" following labeling were washed with HBSS and transferred to medium containing 0.01 *M* uridine. All pulse and/or chase manipulations were performed at 37°. Coverslips were then thoroughly washed in ice-cold HBSS, fixed by 2 washes (2 min each) of ice-cold acid alcohol (1 part glacial acetic acid:4 parts ethanol), followed by 2 washes (20 min each) of ice-cold 5% (w/v) trichloracetic acid, and dehydrated in ice-cold 95% (v/v) ethanol. Coverslips were mounted on standard microscopic slides, dipped in NTB2 emulsion, and exposed in the dark for 30 days. The slides were developed in D-19 and then stained with haematoxylin and eosin. Grain counts were performed on the cytoplasm and nucleus of 50 cells per treatment group.

TABLE I. Average Grain Counts of Cytoplasm and Nucleus of Infected and Normal BHK-21 Cells Following Labeling with ^{3}H-Uridine (50 μCi/ml).

		Average grain counts per cell following			
Virus	Site[a]	5-min pulse	30-min pulse	5-min pulse 25-min chase	P^b
POW	C	29.6	44.9	15.3	<0.025
	N	7.6	9.8	2.6	
EEE	C	75.7	TNTC[c]	TNTC	<0.005
	N	8.3	15.2	4.1	
LAX	C	14.1	33.3	19.1	<0.005
	N	4.1	10.3	3.5	
DEN-2	C	15.1	33.2	12.0	<0.01
	N	3.7	12.2	3.3	
None	C	7.8	10.8	5.6	
	N	6.7	12.3	3.3	

[a] C – cytoplasm, N = nucleus.

[b] Probabilities, shown only if statistically significant ($P \leq 0.05$) from uninfected controls, were obtained by analysis of variance for 5-min, 30-min, and pulse-chase data of each infected group against that of uninfected controls (two-way classification with replication), treating cytoplasm and nucleus separately.

[c] Too numerous to count.

Results. Tables I and II depict the results of two experiments. All five arboviruses synthesized viral RNA in the cytoplasm of infected cells. Nuclear induction by these viruses was not evident, nor was there apparent transfer of labeled RNA from the cytoplasm into the nucleus of infected cells during the 25-min chase. Figure 1 shows that JBE virus produced most of its viral RNA in the perinuclear area, while EEE RNA synthesis was more diffuse and occurred closer to the cell periphery (Fig. 2). In some cases, grains present in EEE infected cells appeared to be arranged in circular or arc-shaped arrays, often associated with hypochromic areas (probably vacuoles). Uninfected controls displayed an even distribution of grains in the cytoplasm and nucleus (Fig. 3). Micrographs of POW- and DEN-2-infected cultures showed the same qualitative distribution of grains as JBE-infected cells, concentrated in the perinuclear area, but at a quantitatively diminished level. LAX-infected cells did not demonstrate a recognizable subcytoplasmic localization of RNA synthesis. It is also concluded that actinomycin D does not irreversibly abolish viral RNA synthesis at the dosage employed here.

Discussion. This work demonstrates di-

TABLE II. Average Grain Counts in the Cytoplasm and Nucleus of Infected and Normal BHK-21 Cells Following Labeling with ^{3}H-Uridine (100 μCi/ml).

		Average grain count per cell following		
Virus	Site	5-min pulse	10-min pulse	P
POW	C	60.4	56.9	<0.005
	N	18.9	19.2	
JBE	C	149.3	280.3	<0.005
	N	12.2	30.1	
EEE	C	101.8	TNTC	<0.005
	N	9.0	30.3	
None	C	26.5	38.9	
	N	13.7	25.3	

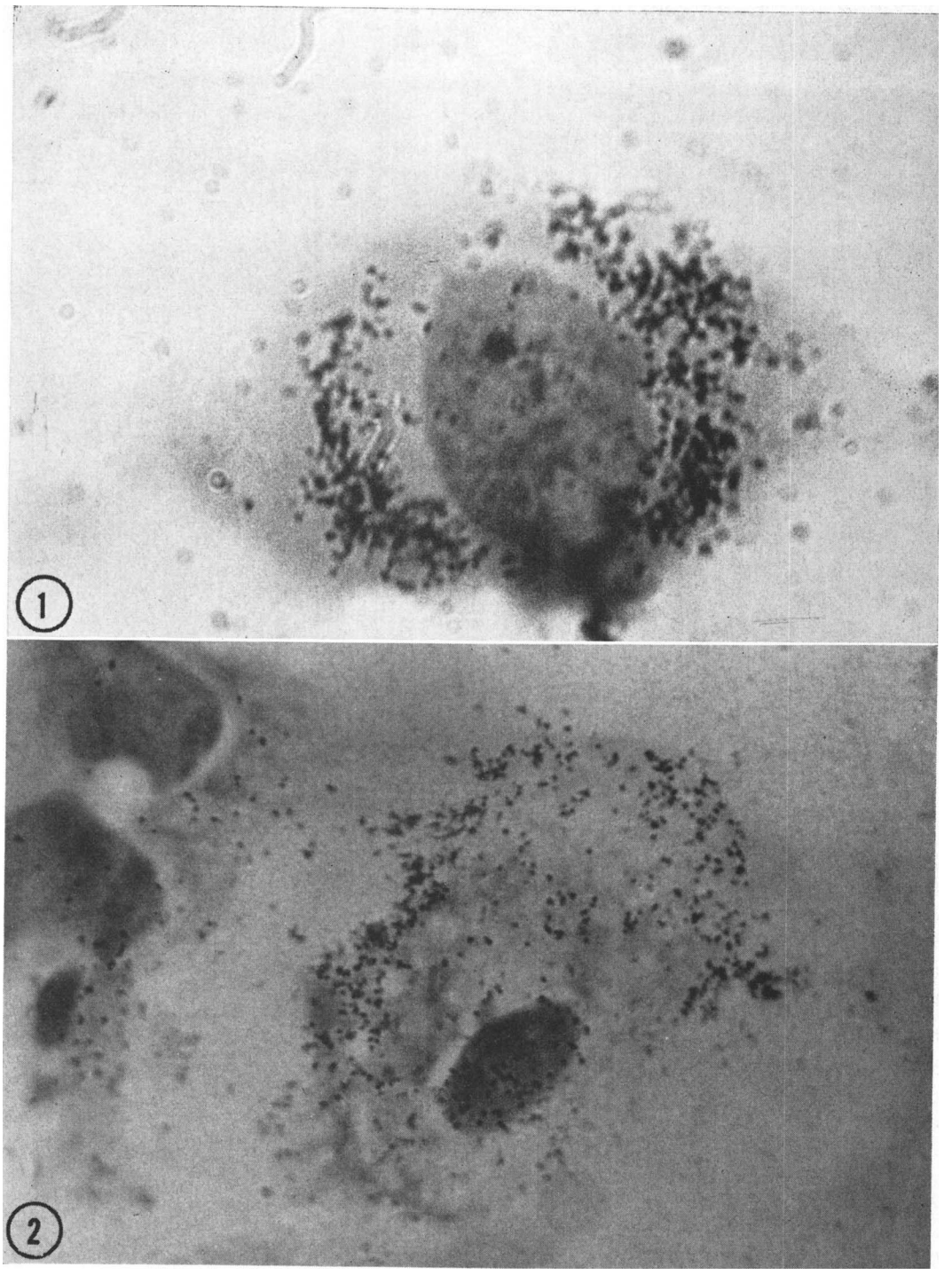

FIG. 1. Autoradiographic localization of RNA synthesis in BHK-21 cells infected with JBE. At 24 hr after infection, the cells were treated with Act-D (2.0 μg/ml) for 1 hr followed by a 5-min pulse of H^3-uridine (100 μCi/ml). Grains are localized in the perinuclear area of the cell. (×2400)

FIG. 2. Autoradiographic localization of RNA synthesis in BHK-21 cells infected with EEE. At 4 hr after infection, the cells were treated with Act-D (2.0 μg/ml) for 1 hr followed by a 5-min pulse of H^3-uridine (100 μCi/ml). Grains are localized in the cytoplasm in close association with vacuoles and the cell membrane. (×1400)

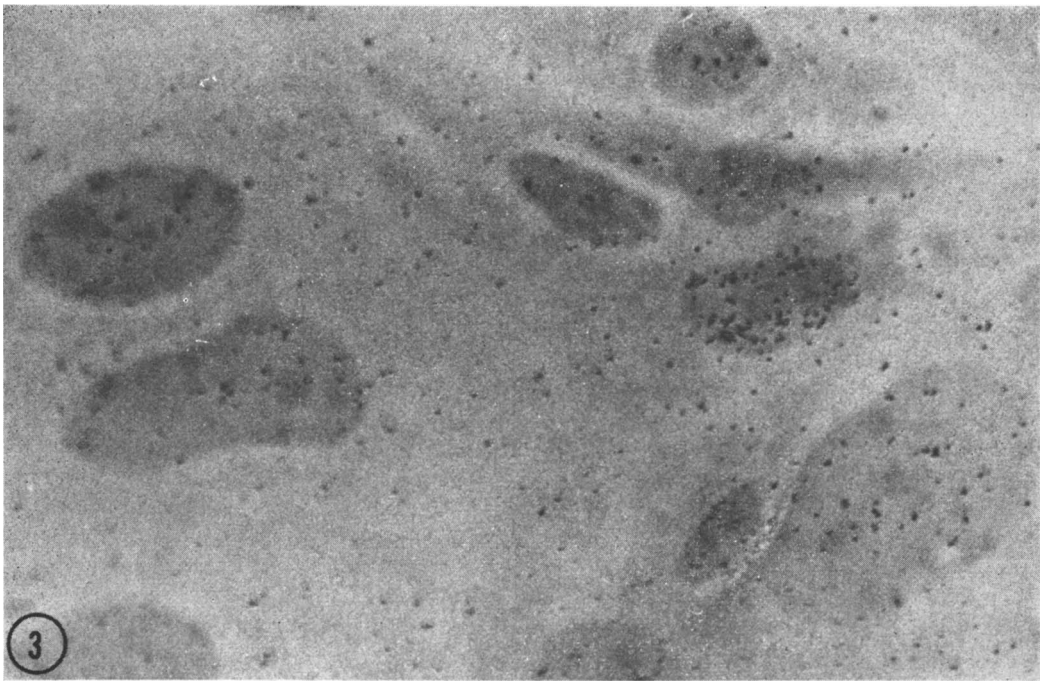

FIG. 3. Autoradiographic localization of RNA synthesis in uninfected BHK-21 cells. Cells were treated with Act-D (2.0 μg/ml) for 1 hr followed by a 5-min pulse of H^3-uridine (100 μCi/ml). Grains are approximately evenly distributed in cytoplasm and nucleus. (×1,200)

rectly that RNA synthesis directed by group B arboviruses occurs in the perinuclear area of the cytoplasm. This is in agreement with recent evidence based on JBE RNA polymerase distribution in subcellular fractions of infected porcine PS (Y-15) cells (E. Zebovitz, personal communication). Cardiff *et al.* (13) found the greatest DEN-2 polymerase activity present in a 10,000*g* membranous (mitochondrial) pellet. Interestingly, the earliest detectable dengue virus antigens appear in the perinuclear area also (14). In contrast, Trent and coworkers reported that the majority of SLE RNA visualized by radioautography was localized in the nucleus of PS (Y-15) cells following a pulse of radioactive uridine (5), although 1-hr pulse times were employed (D. Trent, personal communication).

Several explanations of these results are possible. The first is that the various group B agents may synthesize their RNAs in the nucleus and/or cytoplasm, depending on the virus and cell types. Differences in antigenic composition have already been reported between purified dengue virions grown in mammalian and arthropod cells (15). Alternatively, JBE and related agents may actually synthesize viral RNA in the cytoplasm, which is then transported into the nucleus for further processing. This might explain why Trent and coworkers found more grains in the nucleus than in the cytoplasm of SLE-infected PS (Y-15) cells following a 1-hr pulse (5). They also reported that the SLE RNA present in a nuclear fraction prepared from infected cells contained significantly more polyadenylic acid than that found in intact virions (5). However, we were unable to detect the transport of substantial amounts of DEN-2 or POW RNA from the cytoplasm into the nucleus during a 25-min chase or during a 30-min pulse.

The group A arbovirus employed here, EEE, synthesized its RNA in the cytoplasm. Much of this appeared to be associated with vacuoles probably similar to those reported for Semliki Forest virus-infected chick embryo fibroblasts (4). LAX, a California

group arbovirus, did not appear to follow either the alphavirus or flavivirus pattern of cytoplasmic grain distribution. Its cytoplasmic RNA synthesis was more or less evenly distributed throughout the cytoplasm. This is in agreement with the acridine orange staining pattern of Guaroa-infected cells (2). Whether this finding will extend to other members of the California virus group and the Bunyamwera supergroup bears further investigation.

Summary. RNA synthesis directed by five arboviruses representing three virus groups was shown by radioautography to occur in the cytoplasm of infected BHK-21 cells and was not detected in the nucleus. EEE, a group A arbovirus (alphavirus), synthesized viral RNA in the peripheral cytoplasm, often in association with vacuoles. JBE, DEN-2, and POW (group B arboviruses or flaviviruses) synthesized viral RNA in the perinuclear area of the cytoplasm. LAX, a California group arbovirus (bunyavirus), did not manifest any particular pattern of localization within the cytoplasm.

This work was supported in part by U. S. Public Health Service NIH Grant No. AI-02686. The technical assistance of Frank Brisset and Annie McCoy is gratefully acknowledged.

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Received Nov. 2, 1973. P.S.E.B.M., 1974, Vol. 145.