Polysomal RNA in Semliki Forest Virus Infected
Aedes albopictus Cells

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SUMMARY

Polysomes from Aedes albopictus cells were identified by their rapid labelling
with radioactive amino acids and their sensitivity to EDTA, RNase and puromycin. The major ribosome component in cytoplasmic extracts had a sedimentation coefficient of approx. 95S and may be a ribosome dimer.

In Semliki Forest virus infected Aedes albopictus cells, 42S and 26S RNA were
the major virus RNA species detected up to 10 h post infection. Virus RNA was
detected in association with pre-labelled ribosomes and banded at a buoyant
density of 1.55 g/ml. 42S, 38S, 33S and 26S virus RNA species were associated
with polysomes.

INTRODUCTION

Togaviruses replicate in the tissues of both vertebrate and invertebrate hosts and they
may grow to high titre in cultured invertebrate and vertebrate cells (Dalgarno & Davey,
1973; Davey, Dennett & Dalgarno, 1973). Infection of cultured vertebrate cells by toga-
viruses such as Sindbis or Semliki Forest virus (SFV), is typified by (1) the presence
of characteristic cytoplasmic vacuoles (Grimley, Berezesky & Friedman, 1968), (2) cytoplasmic
accumulation of virus nucleocapsids (Friedman & Berezesky, 1967; Dobos & Faulkner,
1969) and (3) eventual cell death. In contrast, in infected mosquito cells (1) cytoplasmic
vacuoles are not formed (Janzen, Rhodes & Doane, 1970; Whitfield, Murphy & Sudia, 1971;
Raghow et al. 1973), (2) nucleocapsids are formed early in infection but do not accumulate
(Raghow et al. 1973) and (3) the cell continues to divide and a persistent infection is estab-
lished (Stevens, 1970; Peleg, 1972).

There are two major and at least two minor single stranded virus RNA species detected
in mammalian cells infected with Sindbis or SFV (Levin & Friedman, 1971; Weiss &
4·0 × 10⁶ and 1·6 × 10⁶ respectively) are produced in large quantities throughout infection and
minor RNA species have designated sedimentation coefficients of 38S and 33S. Simmons &
Strauss (1974a) have shown that 33S RNA may be a conformational variant of 26S RNA.
Recent work has shown that 42S, 33S and 26S RNA species may be associated with poly-
somes in infected vertebrate cells (Kennedy, 1972; Moshowitz, 1973; Rosemond &
Sreevalsan, 1973; Soderlund, Glanville & Kääriäinen, 1974; Wengler & Wengler, 1974).

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Messenger function for 26S and 42S RNA species has been proved by *in vitro* translation (Clegg & Kennedy, 1974b; Simmons & Strauss, 1974b).

Studies on the replication of togaviruses have been carried out almost exclusively in vertebrate cells and little is known of the biochemical events occurring in infected insect cells. To understand the different manifestations of virus infection in mosquito and vertebrate cells, we have studied virus RNA and polysome-associated RNA in SFV-infected *Aedes albopictus* cells. The experiments of Stollar, Stevens & Shenk (1975) suggest that 33S may be the major single stranded RNA species produced in Sindbis-infected *A. albopictus* cells. We report that all the virus RNA species produced in virus-infected vertebrate cells are detected early after infection of *A. albopictus* cells with SFV. Polysome-associated RNA consists of 42S, 38S, 33S and 26S virus RNA species.

**METHODS**

**Materials.** Sodium dodecyl sulphate and puromycin were purchased from Sigma Chemical Company. Actinomycin D was obtained from Calbiochem Ltd. Triton X-100 was obtained from Laboratory Supply, Sydney. Heparin was purchased from Evans Medical, Boronia, Victoria, as a pyrogen-free solution ('Pularin'), containing 1000 international units of heparin/ml. [5,6-3H]-uridine (45 Ci/mmol), [2-14C]-uridine (514 mCi/mmol), [2-3H]-adenosine (20 Ci/mmol) and [14C]-protein hydrolysate (58 mCi/mAtom carbon) were obtained from the Radiochemical Centre, Amersham, England. [32P]-orthophosphate (48 Ci/mg phosphorus) was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney. 2,5-diphenylloxazole (PPO), 1,4-di-(2-(5-phenyloxazolyl)-benzene (POP-OP) and pure agarose were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England. Pancreatic ribonuclease (RNase) was obtained from Worthington Biochemical Corporation. Ultra-pure sucrose was obtained from Schwarz/Mann, Orangeburg, New York.

**Virus.** Semliki Forest virus was plaque purified on BHK cells and grown at an input multiplicity of 0.001 on BHK cells. Virus stock had a titre of 3×10⁹ p.f.u./ml and was stored at −20 °C.

**Cells and media.** Mosquito cells of Singh's *Aedes albopictus* cell line (Singh, 1971; obtained from the Arbovirus Research Unit, Yale University) were grown in monolayer cultures at 28 °C in the medium described by Mitsuhashi & Maramorosch (1964; M and M medium).

**Gel electrophoresis.** Agarose gels have been used to analyse high mol. wt. RNA (Daneholt et al. 1969). Agarose was dissolved in electrophoresis buffer (0.03 M-NaH₂PO₄·2H₂O, 0.036 M-tris-HCl, pH 7.8, 0.001 M-EDTA, 0.1 % SDS) by refluxing and 1.5 % w/v agarose gels were cast in 6×118 mm perspex tubes. Gels were pre-electrophoresed for at least 30 min at 5 mA/gel before the sample was applied in 50 μl of electrophoresis buffer diluted one in ten and containing 20 % sucrose. After electrophoresis for 4 or 4.5 h at 5 mA/gel at 20 °C the gels were immediately fixed in cold 5 % trichlороacetatic acid for at least 30 min and were sectioned unfrozen with a Mickle Gel slicer. 1 mm gel slices were dissolved in 0.2 ml dimethyl sulphoxide or hydrogen peroxide and counted in a toluene-Triton X-100 based scintillation fluid.

**Labelling of virus-specific RNA.** *Aedes albopictus* cells were seeded into 53 mm diam. plastic Petri dishes and grown in M and M medium containing either 0.03 M-NaH₂PO₄·2H₂O, 0.036 M-tris-HCl, pH 7.8, 0.001 M-EDTA, 0.1 % SDS) by refluxing and 1.5 % w/v agarose gels were cast in 6×118 mm perspex tubes. Gels were pre-electrophoresed for at least 30 min at 5 mA/gel before the sample was applied in 50 μl of electrophoresis buffer diluted one in ten and containing 20 % sucrose. After electrophoresis for 4 or 4.5 h at 5 mA/gel at 20 °C the gels were immediately fixed in cold 5 % trichlороacetatic acid for at least 30 min and were sectioned unfrozen with a Mickle Gel slicer. 1 mm gel slices were dissolved in 0.2 ml dimethyl sulphoxide or hydrogen peroxide and counted in a toluene-Triton X-100 based scintillation fluid.
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was added and incubation continued. Zero time for all experiments was taken as the time of virus inoculation. At times specified in each experiment, [3H]-uridine or [3H]-uridine and [3H]-adenosine were added at concentrations ranging from 10 to 50 μCi/ml. At the end of the labelling period, medium was removed and the monolayers were washed with ice cold PBS (0·13 m-NaCl, 0·007 m-Na2HPO4, o·0025 m-NaH2PO4, pH 7·3). The cells were scraped from the dishes and washed twice with PBS.

Extraction of total RNA. Washed Aedes albopictus cells were resuspended in 0·1 m-tris (pH 9·0) and the cells were lysed by addition of SDS to 1 %. One volume of water-saturated phenol was added and the mixture agitated in a 'Vortex' mixer at room temperature. The phases were separated by centrifuging and the phenol phase was re-extracted with 0·01 m-tris (pH 9·0). The aqueous phases were combined and extracted twice with phenol. 0·1 vol. of 3 m-sodium acetate (pH 6·0) was added to the aqueous phase and the RNA was precipitated overnight at −20 °C with 2·5 vol. of ethanol.

Isolation of RNA from components of cytoplasmic extracts. Selected fractions from sucrose gradients containing cytoplasmic components of infected cells were pooled and made 1 % in SDS by addition of 10 % SDS (w/v) in water. An equal volume of 0·1 m-tris (pH 9·0) was added and the RNA was phenol extracted and precipitated overnight with ethanol.

Isolation of polysomes from uninfected cells. Monolayer cultures of Aedes albopictus cells were grown in M and M medium and ribosomal RNA was labelled during a 24 h exposure to 2 μCi/ml [3H]-uridine. Radioactive medium was replaced with unlabelled M and M medium for 12 to 18 h and with Antheraea eucalypti medium containing one tenth of the normal concentration of amino acids for a further 2 h. Nascent proteins were labelled during a 2 min pulse with 10 to 20 μCi/ml [14C]-protein hydrolysate. At the end of the labelling period ice cold PBS was added and the cells scraped from the dishes and washed twice with PBS. All further operations were done at 4 °C. Cells were resuspended in polysome buffer (PB) (0·025 m-tris-HCl, 0·050 m-NaCl, 0·005 m-MgCl2, pH 7·2) containing 200 μg/ml heparin and 0·2 m-sucrose and lysed by addition of Triton X-100 to 0·5 %. The lysate was then made 0·5 % in sodium deoxycholate (DOC). Both Triton X-100 and DOC were added as 10 % (w/v) solutions in water. After removal of nuclei (at 1000 g for 3 min; heparin caused some nuclear lysis), the cytoplasmic extract was divided into three portions; one portion remained untreated, another was made 0·02 m in EDTA by addition of the required vol. of 0·1 m-EDTA (pH 7·0) in water and pancreatic RNase was added to the third portion to a final concentration of 1 μg/ml. The three cytoplasmic extracts were layered immediately over 16 ml pre-formed linear gradients of 0·5 to 1·5 m-sucrose in PB containing 150 μg/ml heparin and centrifuged at 22000 rev/min for 5 h at 4 °C in a SW27 rotor.

In those experiments in which puromycin was used to disrupt polysomes, the drug was added to cell monolayers in PBS at a concentration of 200 μg/ml at 28 °C immediately following a 2 min pulse with [14C]-amino acids. After 7 min at 28 °C, the cells were scraped from the dishes in ice cold PBS, washed twice in PBS and lysed in TNM (0·01 m-tris-HCl, 0·01 m-NaCl, 0·0015 m-MgCl2, pH 7·2) with Triton X-100 and DOC as described above. Puromycin-treated and control cytoplasmic extracts were analysed by centrifuging through 0·5 to 1·5 m-sucrose in TNM for 6 h in a SW27 rotor at 4 °C.

Gradient fractions were collected by puncturing the bottom of the tubes with a needle and withdrawing the contents directly into vials using an LKB peristaltic pump. Fractions were dissolved in Triton X-100 – toluene based scintillation fluid and counted in a scintillation counter.

Analysis of cytoplasmic extracts of infected cells. SFV-infected cells were labelled as described and were washed twice with ice cold PBS. In the experiments in which EDTA
was added to cytoplasmic extracts (Fig. 3 and 8), cells were resuspended in PB containing 200 µg/ml heparin. In other experiments, cells were resuspended in TMN. Cells were lysed by addition of Triton X-100 and DOC as described for uninfected cells. Cytoplasmic extracts were layered over pre-formed linear 16 ml gradients of 0.5 to 1.5 M sucrose in PB containing 150 µg/ml heparin or TMN and centrifuged at 26000 rev/min for 5 to 6 h in a SW27 rotor. Fractions of the gradient were collected and a 50 µl sample of each fraction was placed on a filter paper disc and precipitated with 10% trichloroacetic acid. At 10 min intervals the discs were washed twice with cold 10% tri-chloroacetic acid, twice with ethanol and once with ether. After drying, the discs were counted in a toluene-based scintillation fluid.

Preparation of cytoplasmic extracts of chick embryo kidney cells. Chick embryo kidney cultures were prepared as described by Younghusband & Bellett (1971) and the cells were grown until confluent (3 days) in BHK medium containing 5 µCi/ml [32P]-orthophosphate. Cells were scraped from the plastic dishes in ice cold PBS, washed twice in PBS and resuspended in PB containing 0.2 M sucrose and 400 µg/ml heparin (used here as an RNase inhibitor; Palacios, Palmiter & Schimke, 1972). Triton X-100 and DOC were added to 1% and the cells were lysed by 10 to 20 strokes of a Dounce homogenizer. Nuclei and cellular debris were removed (at 3000 g for 10 min). Aedes albopictus cells were grown in M and M medium containing 5 µCi/ml [3H]-uridine for 24 h and a cytoplasmic extract was prepared in PB. The two cytoplasmic extracts were mixed and half of the mixture was made 0.025 M in EDTA by addition of 0.1 M EDTA (pH 7.0) in water. Both treated and untreated extracts were layered over 0.5 to 1.5 M sucrose in PB containing 150 µg/ml heparin and centrifuged for 6 h in a SW27 rotor at 4 °C.

Buoyant density analysis. The pH of 50% (w/w) glutaraldehyde was raised to pH 7.0 by addition of 5% (w/v) NaHCO3 and the RNP's in selected fractions of sucrose gradients containing cytoplasmic components of infected cells were fixed by addition of glutaraldehyde to 5%. Fractions were layered immediately in volumes up to 1 ml on to pre-formed 3.5 ml 23 to 55% CsCl gradients in PBS containing 0.8% BRIJ-58 (Baltimore & Huang, 1968) and centrifuged at 40000 rev/min in a SW50 rotor for 12 to 18 h at 4 °C. Fractions were collected from the bottom of the tube and 50 µl samples were assayed for acid precipitable radioactivity. A sample was removed from every fourth fraction and the refractive index was measured in a refractometer.

RESULTS

Isolation of polysomes from uninfected Aedes albopictus cells

Before analysing the polyosome-associated virus RNA species in SFV-infected cells, experiments were done to determine if undegraded polysomes could be isolated from uninfected A. albopictus cells. Ribosomal RNA was labelled during a 24 h exposure to [3H]-uridine. Radioactive medium was replaced with unlabelled medium for 12 h and with amino acid deficient medium for a further 2 h. Cells were then labelled with [14C]-protein hydrolysate for 2 min. A cytoplasmic extract was prepared as described in Methods and divided into three portions. One portion remained untreated, a second was made 0.02 M in EDTA and RNase was added to the third portion. Preliminary experiments indicated that A. albopictus ribosomal subunits released from polyosomes by EDTA, aggregated during zonal centrifugal analysis in sucrose gradients containing magnesium. This aggregation could be prevented by including EDTA or heparin in the gradient. Therefore, cytoplasmic extracts were analysed by zonal centrifugal analysis through sucrose gradients containing 150 µg/ml
Fig. 1. Sucrose density gradient analysis of *Aedes albopictus* polysomes: effect of EDTA and RNase. Ribosomal RNA and nascent protein in 1 to 2 × 10⁶ uninfected cells were labelled with [³H]-uridine and [¹⁴C]-amino acids respectively. A cytoplasmic extract was prepared, divided into three portions and analysed as described in Methods. The arrows indicate the position of the major ribosome component. (a) Remained untreated, (b) was made 0.02 M in EDTA and (c) was treated with 1 µg/ml RNase. , [³H]-uridine radioactivity; , [¹⁴C]-amino acid radioactivity.

heparin. As shown in Fig. 1, the large structures labelled during a 2 min pulse with radioactive amino acids, were sensitive to both EDTA and RNase. A proportion of the radioactive amino acid label remained associated with ribosomes after RNase treatment (Fig. 1 e). To prove that the large, rapidly-labelled ribosomal structures were polysomes, their sensitivity to puromycin was determined. Ribosomal RNA in *A. albopictus* cells was labelled with [³H]-uridine as described in Methods. Cells were pulsed for 2 min with [¹⁴C]-amino acids and immediately puromycin was added to half of the cells for 7 min. Comparison of the profiles in Fig. 2 indicated that puromycin, a drug known to specifically dissociate polysomes, caused a marked reduction in the proportion of rapidly sedimenting ribosomal RNA and protein. The lack of [¹⁴C]-amino acid label associated with the major ribosome peak in untreated cytoplasmic extracts, indicated that polysomes had not been degraded during extraction and analysis.

*Sedimentation coefficient of Aedes albopictus* ribosomes

Ribosomal RNA in primary chick embryo kidney and *A. albopictus* cells was labelled with [³²P]-orthophosphate and [³H]-uridine respectively and cytoplasmic extracts were prepared. The extracts were pooled and half of the mixture was treated with EDTA. Treated and untreated extracts were analysed by zonal sedimentation through sucrose gradients containing heparin. The data in Fig. 3(a) show that the major ribosome component in extracts of *A. albopictus* cells had a sedimentation coefficient of approx. 95S (the S value of chick ribosomes was taken as 74S). The ribosome peak retained its sedimentation coefficient of 95S in the absence of heparin (Fig. 6). The ribosomal subunits from chick and mosquito cells sedimented at approx. the same rates (Fig. 3b).
Fig. 2. Sucrose density gradient analysis of *Aedes albopictus* polysomes: effect of puromycin. Ribosomal RNA and nascent protein in $6 \times 10^7$ uninfected cells were labelled with $[^3H]$-uridine and $[^{14}C]$-amino acids respectively. Puromycin was added to half of the cells and cytoplasmic extracts were prepared in TNM and analysed as described in Methods. (a) Without puromycin; (b) with puromycin. •, $[^3H]$-uridine radioactivity; ▲, $[^{14}C]$-amino acid radioactivity.

Fig. 3. Comparison of the sedimentation coefficients of ribosomes and ribosomal subunits from *Aedes albopictus* and chick embryo kidney cells. Cytoplasmic extracts were prepared from $2 \times 10^7$ $[^{32}P]$-orthophosphate labelled chick embryo kidney cells and $5 \times 10^7$ $[^3H]$-uridine labelled *A. albopictus* cells and analysed as described in Methods. (a) Without EDTA; (b) with EDTA; •, $[^3H]$-uridine radioactivity; ▲, $[^{32}P]$-orthophosphate radioactivity.
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**Fig. 4.** Growth curve of SFV in *Aedes albopictus* cells infected at a multiplicity of infection of 20, kindly supplied by Dr Mary Davey. Virus was assayed on Vero cells: ▼—▼, extracellular virus; •—•, cell-associated virus.

**Species of virus RNA in infected cells**

Figure 4 shows the kinetics of growth of SFV in *Aedes albopictus* cells. The length of the latent period was approx. 4 h.

Ribosomal RNA in *Aedes albopictus* cells was labelled with $^{32}$P-orthophosphate prior to infection with SFV. Actinomycin D was added at the time of infection and virus RNA was labelled with $^3$H-uridine from 4·5 to 5·5 and from 4·5 to 10 h post infection. Uninfected cells were labelled with $^3$H-uridine for 5·5 h starting 4·5 h after addition of actinomycin D. RNA was extracted and analysed by agarose gel electrophoresis as shown in Fig. 5. The slowly migrating $^{32}$P-labelled component from both infected and uninfected cells is sensitive to DNase and must therefore be cell DNA (Davey, 1973). Only one $^{32}$P-ribosomal RNA peak was detected. It is known that 26S ribosomal RNA from cultured *Aedes aegypti* and *A. albopictus* cells splits on heating at 60 °C into two pieces similar in mol. wt. to 18S ribosomal RNA (Stollar *et al.* 1971; Dalgarho, Hosking & Shen, 1972). It is not clear why ribosomal RNA is broken down when RNA is extracted directly from cells under the conditions of phenol extraction used in these experiments. The RNA synthesized in uninfected cells in the presence of actinomycin D is heterogeneous and has an electrophoretic mobility similar to that of 18S ribosomal RNA (Fig. 5c). The $^3$H-uridine profiles in Fig. 5(a, b) show that double stranded RNA and single stranded 42S and 26S RNA forms were the dominant virus species labelled during 1 and 5·5 h periods from 4·5 h post infection. From the rates of migration compared with BHK ribosomal RNA, the mol. wt. of the two
Fig. 5. Species of virus RNA in *Aedes albopictus* cells infected with SFV. Ribosomal RNA in $2 \times 10^8$ cells was labelled with $^{32}$P-orthophosphate. Cells were infected with SFV and virus RNA was labelled with 20 $\mu$Ci/ml $[^3H]$-uridine in the presence of 4 $\mu$g/ml actinomycin D. Infected cells were labelled (a) 4·5 to 5·5 h and (b) 4·5 to 10·0 h post infection. An uninfected control (c) was labelled for 5·5 h starting 4·5 h after addition of actinomycin D. RNA was extracted and analysed by agarose gel electrophoresis. • •, $[^3H]$-uridine radioactivity; \(\forall\) \(\forall\), $[^32P]$-orthophosphate radioactivity.

Single stranded RNA species have been estimated to be approx. $4\cdot0 \times 10^6$ and $1\cdot6 \times 10^6$ (B. T. Eaton & R. L. Regnery unpublished data). Small amounts of 33S and 38S RNA were also detected in infected *A. albopictus* cells (Fig. 2a and Fig. 11).

**Nucleocapsids in infected cells**

In cytoplasmic extracts of togavirus-infected vertebrate cells a major virus-specific ribonucleoprotein (RNP) is the nucleocapsid which sediments at 140S and contains 42S RNA...
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Fig. 6. Sucrose density gradient analysis of cytoplasmic extracts from SFV-infected Aedes albopictus cells. Ribosomal RNA in 9 x 10^7 cells was labelled with [14C]-uridine. Cells were infected with SFV and labelled with 50 μCi/ml [3H]-uridine in the presence of 4 μg/ml actinomycin D from 4 to 8 h post infection. A cytoplasmic extract was prepared in TNM and analysed as described in Methods.

- - - - - [3H]-uridine radioactivity; V — — — V, [14C]-uridine radioactivity.

(Dobos & Faulkner, 1969). Experiments were done to determine if nucleocapsids could be detected in SFV-infected Aedes albopictus cells at 8 h post infection. Virus-specific RNA in infected cells was labelled with [3H]-uridine from 4 to 8 h post infection. The major virus-specific RNP in cytoplasmic extracts sedimented at 140S (Fig. 6) and contained single stranded 42S RNA (data not shown). The buoyant density of the 140S RNP (fractions marked ‘b’ in Fig. 6) was 1.46 g/ml (Fig. 7 b). This value was in close agreement with that of 1.47 g/ml for the buoyant density of nucleocapsids isolated from SFV-infected chick embryo cells (Acheson & Tamm, 1970).

In Sindbis virus-infected vertebrate cells it has been shown that some of the proteins labelled during a 90 s pulse with [14C]-amino acids become associated with virus nucleocapsid and sediment at 140S after treatment of cytoplasmic extracts with EDTA or after incubation of infected cells with puromycin (Moshowitz, 1973). The data in Fig. 8 (a) show that all of the rapidly sedimenting radioactivity incorporated in uninfected cells during a 4 min [14C]-amino acid pulse was associated with EDTA-sensitive structures. When cytoplasmic extracts of infected cells were treated with EDTA, some radioactivity sedimented in the same position as nucleocapsids (Fig. 8 b). A radioactivity profile identical to that in Fig. 8 (b) was obtained with cytoplasmic extracts of infected cells which had been exposed to puromycin for 7 min immediately after labelling with [14C]-amino acids. These experiments confirmed that a proportion of the polysomes synthesized nucleocapsid protein.

Virus polysomes from infected cells

The next experiment was done to determine if virus RNA could be detected in association with polysomes from infected cells. The gradient in Fig. 6 contained virus components labelled with [3H]-uridine from 4 to 8 h post infection. Analysis of the buoyant density of RNPs sedimenting at approx. 250S (fractions marked ‘a’ in Fig. 6) showed that virus RNA...
Fig. 7. Buoyant density analysis of virus RNP's in cytoplasmic extracts of infected cells. Fractions in the gradient in Fig. 6 were combined according to the horizontal lines marked 'a', 'b', and 'c' and were fixed with glutaraldehyde. The buoyant density profiles obtained with fractions 'a', 'b' and 'c' are shown in (a), (b) and (c) respectively. •, [3H]-uridine-labelled virus RNA; •, [14C]-uridine labelled ribosomal RNA; •, buoyant density.

Fig. 8. The effect of EDTA on polysomes in cytoplasmic extracts from uninfected and SFV-infected cells. 1.0 x 10⁶ uninfected and 1.0 x 10⁶ SFV-infected Aedes albopictus cells were incubated in M and M medium containing 4 μg/ml actinomycin D for 7 h and amino acid deficient medium containing actinomycin D for a further 2 h. Infected cells were labelled with 10 μCi/ml [3H]-uridine from 5 to 9 h post infection. Monolayers were exposed to 20 μCi/ml [14C]-amino acids for 4 min and cytoplasmic extracts were prepared in PB containing 200 μg/ml heparin. Half of each extract was made 0.02 M in EDTA and the extracts were analysed through sucrose gradients in PB containing 150 μg/ml heparin. The arrow indicates the position of [3H]-uridine-labelled nucleocapsids. (a) Uninfected; (b) infected cells. •, untreated; •, EDTA-treated.
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Fig. 9. Sucrose density gradient analysis of cytoplasmic extracts from SFV-infected cells labelled 2 to 3.75 h post infection. Ribosomal RNA in 2 x 10^6 cells was labelled with [32P]-orthophosphate. Cells were infected with SFV and labelled with 50 μCi/ml each of [3H]-uridine and [3H]-adenosine in the presence of 4 μg/ml actinomycin D from 2.0 to 3.75 h post infection. A cytoplasmic extract was prepared in TNM and analysed as described in Methods. ○—○, [3H]-uridine and [3H]-adenosine radioactivity; □—□, [32P]-orthophosphate radioactivity.

was present in two components (Fig. 7a). Nucleocapsids were the major RNP with a buoyant density of 1.46 g/ml and the remainder of the virus RNA banded with ribosomal RNA at a buoyant density characteristic of polysomes, 1.55 g/ml.

In an attempt to label virus-specific polysome-associated RNA in the absence of nucleocapsid synthesis, actinomycin D-treated infected cells were labelled with [3H]-uridine and [3H]-adenosine from 2 to 3.75 h post infection. Cytoplasmic extracts were prepared and analysed by zonal centrifuging through sucrose gradients (Fig. 9). Fractions marked 'a', 'b' and 'c', sedimenting at approx. 210S, 135S and 90S respectively, were fixed with glutaraldehyde and the buoyant density of constituent RNPs was determined. The data in Fig. 10(b) show that only a small proportion of the material sedimenting at 140S had a buoyant density of 1.47 g/ml. Therefore few nucleocapsids were labelled during this time interval. The major virus RNP sedimenting at 210S had a buoyant density of 1.54 g/ml (Fig. 10a). No RNP's of buoyant density 1.46 g/ml were detected in fractions sedimenting faster than 210S. EDTA is known to disrupt SFV-specific polysomes from infected mammalian cells and bring about a reduction in the buoyant density of polysome-associated virus RNA (Soderlund et al. 1973). EDTA treatment of polysomes in fractions sedimenting faster than 200S (Fig. 10) caused a reduction in buoyant density of virus RNA to 1.42 g/ml (data not shown).

Polysome-associated virus RNA

To determine which species of virus RNA were associated with polysomes, SFV-infected Aedes albopictus cells were labelled with [3H]-uridine and [3H]-adenosine from 2 to 3.75 h post infection. A cytoplasmic extract was prepared and the RNA which was isolated from
Fig. 10. Buoyant density analysis of virus RNP's in cytoplasmic extracts from infected cells. Fractions in the gradient in Fig. 9 were combined according to the horizontal lines marked 'a', 'b' and 'c' and fixed with glutaraldehyde. The buoyant density profiles obtained with fractions 'a', 'b' and 'c' are shown in (a), (b) and (c) respectively: •, [\(^{3}H\)]-uridine and [\(^{3}H\)]-adenosine radioactivity; ○, [\(^{32}P\)]-orthophosphate radioactivity; ■, buoyant density.

Fig. 11. Species of RNA in polysomes sedimenting faster than 200S in cytoplasmic extracts of SFV infected Aedes albopictus cells. Fractions 1 to 13 from a gradient identical but different to that in Fig. 9 were pooled and the extracted and analysed by gel electrophoresis. •, [\(^{3}H\)]-uridine and [\(^{3}H\)]-adenosine radioactivity; ○, [\(^{32}P\)]-orthophosphate.
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polysomes sedimenting faster than 200S was analysed by agarose gel electrophoresis. The data in Fig. 11 show that in addition to the large and small ribosomal RNA species, 42S, 38S, 33S and 26S species of virus RNA were also associated with polysomes.

DISCUSSION

Polysomes have been isolated from Aedes albopictus cells and identified by their rapid labelling with [14C]-amino acids (Fig. 2), their buoyant density of 1.55 g/ml (Fig. 7) and sensitivity to puromycin (Fig. 2), EDTA and RNase (Fig. 1). Ribosomal subunits released from polysomes and ribosomes by EDTA were found to aggregate in concentrations of magnesium as low as 0.0015 M (B. T. Eaton & R. L. Regnery, unpublished data). This aggregation could be prevented by including the polyanion heparin in sucrose gradients. Heparin has been used as an RNase inhibitor in the preparation of undegraded polysomes from chick oviduct cells (Palacios et al. 1972) and chick embryo kidney cells. It causes no detectable disruption of A. albopictus polysomes (Fig. 1, 3 and 8).

The tendency of Aedes albopictus ribosomes to aggregate in the buffers used in these experiments is also shown by the finding that the major ribosome component in cytoplasmic extracts sediments at approx. 95S, ahead of a chick embryo kidney 74S ribosome marker (Fig. 3). The following results suggest that this component may be a ribosome dimer: (1) a sedimentation coefficient of 95S is consistent with this suggestion, (2) the buoyant density of the 95S component is 1.55 g/ml (Fig. 7c), (3) the mol. wt. of the large and small ribosomal RNA species from A. aegypti cells are 1.5 x 10^6 and 0.7 x 10^6 respectively (Dalgarno et al. 1972). Radioactivity in the large and small ribosomal RNA species extracted from the 95S region of the gradients containing cytoplasmic extracts is in approx. a 2:1 ratio respectively (data not shown). This indicates that the two subunits may be present in the complex in equal proportions.

All the virus RNA species produced in togavirus-infected vertebrate cells are also detected early in SFV-infection of Aedes albopictus cells (Fig. 5 and 11). The experiments of Stollar et al. (1971) indicated that 33S RNA was the major virus RNA species detected in Sindbis-infected A. albopictus cells. One reason for the discrepancy between this result and those reported here may lie in the fact that Stollar et al. labelled infected cells from 24 h post infection. We are presently investigating the species of RNA produced at different times post infection.

Virus nucleocapsids, sedimenting at 140S and with buoyant density 1.46 g/ml (Fig. 10b), were detected in cytoplasmic extracts of infected cells as early as 3-75 h post infection (Fig. 9). Protein is very rapidly incorporated into virus nucleocapsids in infected mammalian cells (Moshowitz, 1973) and this is also true early in the infection of Aedes albopictus cells (Fig. 8). Neither of these two findings regarding nucleocapsid synthesis in SFV-infected A. albopictus cells explain the failure of nucleocapsids to accumulate in togavirus-infected mosquito cells (Raghow et al. 1973).

Virus RNA was detected in association with ribosomes in polysomes of buoyant density 1.55 g/ml (Fig. 7 and 10). EDTA treatment of polysomes released virus RNA as a RNP with buoyant density 1.42 g/ml (unpublished data). Virus RNP sedimenting at approx. 95S and with a buoyant density of 1.42 g/ml was detected in cytoplasmic extracts of infected cells which had not been treated with EDTA (Fig. 7c).

Polysomes were the only labelled component sedimenting faster than 200S when infected cells were labelled from 2-0 to 3-75 h post infection (Fig. 10). 42S, 38S, 33S and 26S single stranded virus RNA species were isolated from polysomes sedimenting faster than 200S.
All of these RNA species in infected vertebrate cells have been shown to contain poly(A) (Clegg & Kennedy, 1974a), a property consistent with their identification as messenger RNA. The nature of the material sedimenting at 100S in cytoplasmic extracts of infected cells labelled from 2.0 to 3.75 h post infection is not known (Fig. 9). A buoyant density of 1.51 g/ml (Fig. 10c) suggests that it may be virus messenger RNP associated with one or two ribosomes.

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REFERENCES


Togavirus mRNA’s in A. albopictus cells


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