Association of Foot-and-Mouth Disease Virus Replicase with RNA Template and Cytoplasmic Membranes

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The association of the replicase of foot-and-mouth disease virus (FMDV) with cellular membranes was suggested following attempts to produce a soluble enzyme by the use of detergents (Arlinghaus & Polatnick, 1967, 1969; Delagneau, 1971), as described for the purification of poliovirus replicase (Ehrenfeld, Maizel & Summers, 1970). Subsequently, the association with cellular membranes of the replicase and the single-stranded and replicative forms of RNA was reported for poliovirus (Caliguiri & Tamm, 1969, 1970a, b), for Semliki Forest virus (Friedman et al. 1972) and for tobacco mosaic virus (Ralph, Bullivant & Wojcik, 1971). In each case, the replicase-membrane complex contained endogenous RNA template (Arlinghaus & Polatnick, 1969; Caliguiri & Tamm, 1969; Ehrenfeld et al. 1970; Friedman et al. 1972). We show in this communication that FMDV replicase is associated with ribosome-containing membranes and requires glycerol to stabilize its activity during sedimentation in the presence of detergents. Furthermore, FMDV replicase remains intimately bound with its endogenous RNA template at high ionic strength (2.0 M-salt solution).

Foot-and-mouth disease virus (type SAT/1) was isolated following passage in BHK 21 cells (Barzilai, Lazarus & Goldblum, 1972). FMDV RNA was isolated according to Popescu, Lazarus & Goldblum (1971) and stored under alcohol at -20 °C (Lazarus & Itin, 1973). FMDV replicase was induced by infection of BHK cells at an input multiplicity of 100 p.f.u./cell, as previously described by Lazarus et al. (1972a). The cytoplasmic extract, containing FMDV replicase, was prepared by Dounce homogenization of the cells and clarification by low-speed sedimentation to discard nuclear residues (Lazarus et al. 1972b). The assay of replicase activity has been described (Lazarus et al. 1972a, b; Lazarus & Itin, 1973). In brief, the reaction mixture contained 10 mM-tris-HCl buffer solution, pH 8.1, with 0.08 mM each of ATP, GTP and CTP, 0.25 µmol [3H]-UTP (sp. act. 1.00 Ci/mmol; Radiochemical Centre, Amersham, England), 0.2 M-KCl, 20 mM-MgCl₂, 2 mM-2-mercaptoethanol, 2 mM-creatine phosphate and 5 µg phosphocreatine kinase. After 30 min at 38 °C, the reactions were terminated by adding sodium pyrophosphate (PPI) and TCA to 20 mM and 10 %, respectively. The precipitate was collected on Whatman GF/C glass fibre filters, washed with 5 % TCA-10 mM-PPI, dried with acetone and radioactivity determined by liquid scintillation. Discontinuous sucrose gradients were prepared according to Caliguiri & Tamm (1969).

A sample of the cytoplasmic extract from FMDV-infected BHK cells (in 10 mM-tris-HCl buffer solution, pH 7.8, 10 mM-KCl, 1 mM-MgCl₂, 1 mM-dithiothreitol, 0.1 mM-EDTA) was divided in two: one portion was the untreated control and 0.5 % DOC and 0.5 % NP-40 were added to the other. Sucrose was then added to 30 % (Caliguiri & Tamm, 1969) and the samples placed on the 40 % sucrose layer and overlaid with 20 % sucrose, both in the same buffer solution. After sedimentation (16 h at 40 000 rev/min at 1 °C in Spinco rotor SW 65Ti), the untreated FMDV-infected cytoplasmic extracts showed several light-scattering zones (Fig. 1) which were similar to those for non-infected control extracts (not shown). Replicase activity was located in a granular band in the 45 % sucrose layer just above the
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Fig. 1. Fractionation of cytoplasmic extracts from FMDV-infected cells by sedimentation in discontinuous sucrose gradients. Total recovery through the gradient was 101% for the untreated (○) and 9.9% for the DOC and NP-40 disrupted sample (●). Activity in the pellets was < 1% of the total.

bottom layer of 60% sucrose (Fig. 1). A band in this region has been identified as rough endoplasmic reticulum through the electron microscopic analyses of Caliguiri & Tamm (1969, 1970a, b); also the association of replicase with a ribosome-containing fraction may be anticipated from the elegant work on Qβ replicase by Kolakofsky & Weissmann (1971).

In contrast, the replicase from poliovirus was found in the 20% sucrose layer associated with smooth endoplasmic reticulum (Caliguiri & Tamm, 1969) while its single-stranded RNA and the double-stranded replicative forms were identified in the ribosome-containing bands (Caliguiri & Tamm, 1970a, b). The detergent-treated sample of FMDV replicase (Fig. 1) showed no activity on the discontinuous sucrose gradient. It thus appears that during sedimentation of the detergent-treated extract, replicase was either separated from the endogenous template and then not detected in the assay system, or inactivated by a combination of detergent and sedimentation through sucrose: it is known that replicase preparations retain their activity in DOC (Arlinghaus & Polatnick, 1967, 1969; Delagneau, 1971) as well as in NP-40 which prevents the formation of a Mg-DOC precipitate of low replicase activity. We failed to recover additional enzyme activity when the gradient was assayed with FMDV RNA, which was shown to stimulate a template-free FMDV replicase preparation (Delagneau, 1971).

Glycerol (25%) was incorporated into the sucrose and buffer solutions on the hypothesis
that a stabilizing cofactor was removed from the DOC and NP-40 treated preparation by sedimentation through sucrose. Cytoplasmic extracts in 25% glycerol containing 0.5% DOC and 0.5% NP-40 were then fractionated (Fig. 2a) on discontinuous gradients (Spinco rotor SW 25.1 at 24,000 rev/min for 22.5 h at 1°C) prepared in 1 mM-MgCl₂ or 20 mM-EDTA to further exclude Mg-DOC complexes. Replicase activity was stabilized by this treatment and appeared in light-scattering bands at the 30 to 40% and the 45 to 60% sucrose interphases (Fig. 2b) as well as in gelatinous pellets which contained 51% and 36% of the total replicase activity for the MgCl₂ and EDTA gradients, respectively. The total recoveries of activity were 98% from the EDTA gradient and 50% from the MgCl₂ gradient.

If replicase activity can be dissociated from its endogenous template at high ionic strength (Delagneau, 1971), then velocity sedimentation in a high salt-containing gradient should yield a template-dependent enzyme. Fractions from the EDTA discontinuous gradient were pooled from the peak region at the 30 to 50% interphase, pooled areas 1 and 2 (Fig. 2b) and concentrated by sedimentation in polyallomer tubes in Spinco rotor SW 50.1 at 45,000 rev/min for 10.5 h at 1°C, and redissolved in a buffer solution containing glycerol and both detergents. With other means of concentration, such as membrane filtration, pressure dialysis, or adsorption on DEAE- or CM-cellulose, or hydroxyapatite, or precipitation by protamine sulphate (Lazarus & Itin, 1973), we found only about 10% of the total and specific activity of the preparation. In contrast 75% of activity was recovered on sedi-

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Fig. 2. Fractionation by sedimentation in a discontinuous gradient of detergent-treated FMDV replicase preparation in the presence of glycerol. The FMDV replicase extract contained 25% glycerol in buffer solution with either 1 mM-MgCl₂ (○) or 20 mM-EDTA (●) and adjusted to 0.5% with DOC and 0.5% NP-40. All sucrose solutions contained 25% glycerol. †††, E₂₅₀. To counteract EDTA, 20 mM-MgCl₂ was added before assaying in addition to the Mg present in the reaction mixture.

(a) Light-scattering bands of the MgCl₂ and EDTA gradients. (b) Activities of fractions from above gradients. Areas denoted 1 and 2 were pooled and concentrated as indicated in the text.
Fig. 3. Sedimentation of FMDV replicase isolated from discontinuous gradients in glycerol gradients at high ionic strength. A parallel tube containing a cytoplasmic extract from HeLa cells was analysed for ribosome subunits (arrows denote positions). (a) Replicate activity in pooled area 1 (●) and pooled area 2 (○) samples from Fig. 2b. (b) On the parallel gradient another sample of FMDV replicase from pooled area 2 (Fig. 2b) was assayed without (●) or with added FMDV RNA (1 μg/assay) (○).

Our results conflict with those of Delagneau (1971) who obtained an RNA-dependent FMDV replicase preparation by treatment of cytoplasmic extracts at high ionic strength (0.5 M-ammonium sulphate). Differences may be due to the type of salt used rather than to ionic strength. However, our results and others (Arlinghaus & Polatnick, 1969; L. H. Lazarus & M. Popescu, unpublished observations) show that fractionation by ammonium sulphate failed to generate a template-dependent enzyme. Nor was template-dependent replicase found after application of a two-phase polymer system; a 90% loss in activity was observed (L. H. Lazarus, unpublished observations). Although 2.0 M-salt solutions readily solubilize DNA polymerase from endogenous template (Weissbach et al. 1971;
Lazarus & Kitron, 1973), the tenacity of the binding of replicase to RNA may reflect histone-like properties.

In conclusion, the following observations indicate that FMDV replicase is associated with cytoplasmic membranes containing ribosomes and remains bound to its RNA template: (1) replicase activity was found with a membrane fraction that banded at the position of rough endoplasmic reticulum in discontinuous sucrose gradients (Caliguiri & Tamm, 1969, 1970a, b); (2) detergent-treated cytoplasmic extracts were stabilized in the presence of glycerol and banded in the position of a mixed population of endoplasmic membranes (Caliguiri & Tamm, 1970a, b); (3) after sedimentation in glycerol gradients containing 2 o M-KCl, FMDV replicase and its endogenous RNA template remained tightly bound; and (4) the high sedimentation coefficients (92 to 126S) of the replicase complexes suggest association of the enzyme with RNA and membrane components (Arlinghaus & Polatnick, 1969; Caliguiri & Tamm, 1970b; Ehrenfeld et al. 1970).

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