Infectivity of Mengovirus Replicative Form. Relationship to Cellular Transcription

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SUMMARY

Mengovirus-induced, double-stranded RNA (RF) is infective, but its infectivity, unlike that of mengovirus, is strictly dependent upon host cell macromolecular synthesis. The treatment of cells with actinomycin D, α-amanitin or cordycepin 1 h before infection with mengovirus RF results in a drastic reduction of virus yield, whereas the same treatment has no effect on mengovirus infectivity. The kinetics of sensitivity to inhibitors suggest that the cellular macromolecule necessary for RF to initiate its infective cycle is involved only during the very early steps of replication, and probably has a very rapid turn-over. The cellular uptake of the infecting molecule seems not to be altered by actinomycin treatment. Analysis of the intracellular distribution of [3H]- or [32P]-labelled mengovirus RF indicates that up to 40% of incoming molecules accumulate within the nuclear fraction (4 to 5% in nucleoli). Sedimentation velocity analyses of labelled RF recovered from each subcellular compartment show that the input molecule becomes heavier and polydisperse in gradients as the cycle of infection proceeds. A replication mechanism is proposed in which infective RF is transformed into replicative intermediate (RI), by a cellular RNA polymerase transcribing the first virus messenger RNAs with RF as abnormal template.

INTRODUCTION

Picornaviruses carry their genetic information in a molecule of single-stranded (ss), linear, non-segmented RNA. Virus RNA is a bifunctional molecule which can serve either as template RNA during the polymerization of a complementary strand, or as messenger RNA, when it binds cellular ribosomes and directs protein synthesis.

The replication of virus RNA (for a review see Baltimore, 1969) is a two-step transcriptional process, with the synthesis of a complementary (‘minus’) strand as the first step followed by the synthesis of new molecules having the original polarity, but using the ‘minus’ chain as template. Such a functional organization involves the appearance of three virus-induced RNA structures in infected cells: (1) the newly synthesized single-stranded RNA; (2) the replicative form (RF) (Montagnier & Sanders, 1963), a very stable, double-stranded molecule of two hydrogen-bonded complementary chains; (3) the replicative intermediate (Baltimore & Girard, 1966; Bishop & Koch, 1969), partially single- and partially double-stranded.
The replicative form of picornaviruses has been shown to be infective (Montagnier & Sanders, 1963; Bishop & Koch, 1967); this requires that, once in the permissive cell, RF expresses all its genetic content and originates new virus particles despite its well-defined secondary structure. This observation cannot be reconciled easily with present knowledge of the mechanisms involved in the replication of RNA viruses. On the one hand, RF should not be transcribed for there is no RNA-dependent RNA polymerase in the host cell. Such a polymerase appears in detectable amounts in picornavirus-infected cells at about 3 h after infection (Baltimore & Franklin, 1962, 1963; Baltimore et al. 1963; Baltimore, 1964). The polymerase is virus-coded and its ionic requirements for in vitro transcription are quite different from those for cellular RNA polymerases.

On the other hand, RF should not be translated for its secondary structure does not allow it to bind to ribosomes and direct protein synthesis. Furthermore, it has been demonstrated that RF blocks the in vitro protein synthesis of a 'cell-free' system (Ehrenfeld & Hunt, 1971; Hunt & Ehrenfeld, 1971; Robertson & Mathews, 1973) and actively promotes dissociation of formyl methionine-tRNA_{fmet}-30S ribosomal subunits (Darnbrough, Hunt & Jackson, 1972).

Once within the cell, the two strands of RF may separate and the 'plus' strands bind to ribosomes and start the normal infective cycle. But Koch, Quintrell & Bishop (1967) indicated that the events were less simple since actinomycin D reduced the number of infective centres developed by poliovirus RF, whereas the same treatment had no effect on the infectivity of virus ss RNA. These paradoxes prompted us to study the mechanisms responsible for the infectivity of RF and we now report results on the relationship between cellular transcription and the replication of RF.

Methods

Buffer solutions. These were: PBS (phosphate-buffered saline solution); tris buffer (10 mM-tris-HCl, pH 7.2; 50 mM-NaCl; 1 mM-EDTA); RSBK-Cα (10 mM-tris-HCl, pH 7.4; 10 mM-KCl; 1.5 mM-MgCl₂; 3.6 mM-CaCl₂); HSB (0.5 M-NaCl; 50 mM-MgCl₂; 10 mM-tris-HCl, pH 7.4); TSB (35 mM-tris-HCl, pH 7.0; 140 mM-NaCl).

Chemicals. Nonidet (NP-40, Shell) and Na deoxycholate (DOC) were purchased from Sigma and DEAE-dextran (DEAE-dx), mol. wt. 500000 from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation, purification and physico-chemical properties of mengovirus RF. The preparation and titration of the infectivity of RF on monolayers of mouse L cells have been described (Mechali et al. 1973).

Experiments with inhibitors of cell synthesis. Confluent monolayers of L cells in plastic dishes (60 mm diam.) were pre-treated with DEAE-dextran (200 μg/ml) with or without the blocking drug under study (37 °C, 1 h). Then cells were infected with 3 x 10⁸ p.f.u./dish of RF in 0.2 ml PBS-DEAE-dextran. Adsorption proceeded at 37 °C for 1 h. Inocula were removed, cultures carefully washed five times with 4 ml PBS and, after addition of 2 ml pre-warmed medium to each dish, incubation continued at 37 °C for a further 7 h to allow one replication cycle. After virus release by freezing and thawing the cultures three times, supernatant media were clarified by low speed sedimentation and the released virus titrated by plaque formation on L cells. Parallel experiments were performed with infection by 3 x 10⁸ p.f.u./dish of purified mengovirus.

Intracellular distribution of radioactively labelled RF. Ten L cell monolayer cultures in plastic dishes were pre-treated with medium PBS-DEAE-dextran, as above, infected with
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Table 1. Effect of actinomycin D on replication of mengovirus and mengovirus RF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus input</th>
<th>Virus yield (p.f.u., per input p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Mengovirus, 300 p.f.u./dish</td>
<td>3900</td>
</tr>
<tr>
<td>Actinomycin D, 5 μg/ml</td>
<td>Mengovirus, 300 p.f.u./dish</td>
<td>5700</td>
</tr>
<tr>
<td>None</td>
<td>Mengovirus RF, 300 p.f.u./dish</td>
<td>5070</td>
</tr>
<tr>
<td>Actinomycin D, 5 μg/ml</td>
<td>Mengovirus RF, 300 p.f.u./dish</td>
<td>200</td>
</tr>
</tbody>
</table>

Confluent monolayers of L cells treated or not with actinomycin D, were infected either with mengovirus or mengovirus RF. After adsorption for 1 h, inocula were removed and fresh media added. Incubation was continued at 37 °C for 7 h and virus production was determined by titration of the supernatant fluids.

[3]H]- or [32P]-RF in medium PBS-DEAE-dextran at a dilution of 3 × 10⁶ p.f.u. and 10,000 ct/min in 0·2 ml, and after 45 min at 37 °C, 2 ml pre-warmed Eagle’s minimum essential medium (MEM) were added to each dish. The end of the adsorption period was taken as zero time. Incubation at 37 °C was continued and at different times thereafter the distribution of radioactivity in the extra-cellular fluid and sub-cellular compartments was analysed for two monolayers.

Determinations of labelled RF remaining in the extra-cellular fluid. Nutrient medium was carefully withdrawn and a sample was counted with Bray’s solution in a Packard liquid scintillation spectrometer; monolayers were then washed four times with 3 ml ice-cold PBS and a sample of each wash was counted. Counts usually fell to the background level at the last wash.

Determination of the intracellular distribution of labelled RF. The separation of cytoplasm, nuclei, nucleoplasm and nucleoli was as described by Penman (1969). Cells were scraped off with a small rubber policeman in 3 ml medium RSBK-Ca, allowed to swell for 5 min and disrupted by 10 strokes in a Dounce homogenizer with tight pestle. Nuclei were separated by low speed sedimentation (680 g, 2 min) and the molarity of cytoplasmic extract immediately corrected to 0·2 M-NaCl. Nuclei were washed once with medium RSBK-Ca, once with medium RSBK-Ca-NP-40(0·8%)-DOC(0·4%) and once with medium RSBK-Ca. A sample of each wash was counted in order to ascertain that nuclei were free of loosely bound material. No radioactivity was detected in the last wash. Nuclei were resuspended in 1 ml medium HSB with 50 μg RNAse-free DNAse. After incubation at 37 °C for 30 min the nucleoli were separated from nucleoplasm by sedimentation at 10,000 rev/min for 5 min. Nucleoli were resuspended in 0·5 ml medium TSB. All samples were stored at −80 °C until analysed.

RESULTS

Relationship to cellular transcription

The replication of picornaviruses is known to be independent of cellular transcription and the possibility of blocking cellular RNA synthesis with actinomycin D has been extensively exploited in biochemical studies of virus replication. However, nothing is known about the role of cell-directed synthesis in the infective cycle initiated by RF. To establish whether this cycle was also independent of cellular transcription, mouse L cells were treated with actinomycin D, infected either with mengovirus or with mengovirus RF (see Methods) and the amount of virus produced was determined after a single replication cycle of 8 to 9 h. As shown in Table 1, actinomycin D blocked the replication of mengovirus RF by more than 95% but, under identical conditions, failed to impair mengovirus replication.
Fig. 1. Effect of actinomycin D on the infective cycle initiated by mengovirus RF. L cell monolayers were infected with mengovirus RF as described in Methods but actinomycin D (5 μg/ml) was added at various times. After one replication cycle of 8 h the amount of virus produced was determined. Virus yields are expressed as percentages of those for untreated control monolayers.

Interaction between actinomycin D and double-stranded RNA

To rule out the possibility that this result might be due to a direct interaction between actinomycin D and double-stranded RNA, mengovirus RF was incubated in a final vol. of 50 μl at 30 °C for 25 min with or without an excess of actinomycin D (10:1, w/w). The unbound antibiotic (mol. wt. 1225) was then separated by gel chromatography in a small column of Sephadex G-50 (1 ml bed vol.) equilibrated with PBS: L cell monolayers were infected with the fractions of the void-volume eluate containing RF. Inocula were removed after adsorption and the amount of virus produced in a single infective cycle was determined. Incubation of RF with actinomycin D did not diminish its infectivity. Thus, the target of actinomycin D was the host cell and not RF.

Time-course of sensitivity to actinomycin D

To investigate the time-course of sensitivity to actinomycin D, L cell monolayers were blocked with actinomycin D at different times during the infective cycle initiated by mengovirus RF and the virus yield determined as already described. At 1 h after infection the infective cycle initiated by RF became completely resistant to actinomycin D (Fig. 1) and addition of the antibiotic 2 h after infection gave a slight increase in virus yield.

This indicated that treatment of cells with actinomycin D interfered at a very early stage of the replication cycle initiated by RF. It will be shown that penetration of RF is not involved.

Block of cellular transcription by cordycepin and α-amanitin

Actinomycin D blocks cellular transcription by intercalating into the dG-dC sequences of the DNA template (Haselkorn, 1964; Sobell et al. 1971), but the flow of genetic information in the cell can also be interrupted at other selected levels by appropriate inhibitors. For this purpose the two drugs cordycepin and α-amanitin were considered.

Cordycepin (a 3' deoxy-substituted analogue of adenosine) arrests cell-directed synthesis by interfering with the synthesis of the poly A segment present in all mRNA of eukaryotes, with the only exception of histones mRNA (Adesnik et al. 1972). α-amanitin, an octapeptide
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Fig. 2. Effect of DEAE-dextran on α-amanitin-induced inhibition of RNA synthesis. L cell monolayers were pre-treated with (■—■) or without (□—□) DEAE-dextran (200 μg/ml). After 1 h at 37 °C the indicated amounts of α-amanitin were added and 1 h later 1 μCi [5-3H]-uridine (Radiochemical Centre, Amersham, sp. act. 27 Ci/mmol) was added to each culture (15 min 37 °C). The amount of TCA-precipitable radioactivity was then determined. In the absence of α-amanitin incorporation was 19043 ct/min (PBS) and 53586 ct/min (PBS-DEAE-dextran) and these values were taken as 100 % incorporation when computing the inhibitory effect of drug.

derived from the mushroom *Amanita phalloides*, specifically inhibits nucleoplasmic RNA polymerase II, which is the enzyme presumably responsible for synthesis of heterogeneous nuclear RNA. It has no effect on nucleolar RNA polymerase I (Chambon et al. 1972), or on bacterial RNA polymerase.

In early experiments we observed that the treatment of the cell cultures, even with high doses of α-amanitin (100 μg/ml), failed to inhibit RNA synthesis, as determined by [5'-3H]-uridine incorporation. This was in agreement with previous reports (Hastie, Armstrong & Mahy, 1972). Since α-amanitin at 0.01 μg/ml actually blocks RNA polymerase II *in vitro*, we interpreted this result as due to the slow penetration of the cell by α-amanitin. We tried to overcome this by treating cell cultures with a polycation, DEAE-dextran, known to facilitate the cellular uptake of other anionic molecules (Koch & Bishop, 1968; Dianzani et al. 1969; Pagano, 1970; Havliza & Koch, 1971). In the presence of DEAE-dextran, α-amanitin reduced RNA synthesis in the whole cell by more than 50 % (Fig. 2).

The effect of cordycepin and α-amanitin on the infectivity of mengovirus RF was studied as described in Methods. Neither drug had any effect on the infectivity of mengovirus, while under identical conditions the infectivity of mengovirus RF was inhibited by 95 % (cordycepin) and 58 % (α-amanitin; P ≤ 0.001).
Fig. 3. Distribution of radioactively labelled mengovirus RF. L cell monolayers were infected with [³H]- or [³²P]-labelled mengovirus RF and the distribution of radioactivity in the subcellular fractions at different times was determined as in Methods. (a) O—O, extra-cellular radioactivity; ■—■, cytoplasmic radioactivity; ▲—▲, radioactivity in the nuclear fraction. (b) Intranuclear distribution of [³H]-labelled mengovirus RF. ▲—▲, radioactivity in the nuclei at the end of DNAse-digestion; △—△, nucleoplasmic radioactivity; +—+, radioactivity in nucleoli (note different scale).

**Intracellular distribution of RF**

To investigate the fate of the incoming molecule of RF during a productive cycle, cells pre-treated with DEAE-dextran were infected with [³H]- or [³²P]-labelled RF and the intracellular distribution of RF was analysed. The penetration of mengovirus RF increased steadily (Fig. 3a) up to the 4th hour after infection. The further increase in the amount of radioactivity recovered in the extracellular fraction after 6 h probably reflected increased cellular fragility as the infective cycle proceeded. Alternatively this may be due to the release of reversibly bound RF.

Fig. 3(a) and (b) also shows that a considerable proportion of up to 48 % of total intracellular radioactivity accumulated within the nucleus. The bulk of nuclear radioactivity was found in the nucleoplasm; although the nucleoli acquired only about 10 % of nuclear radioactivity.

**Characterization of radioactive material found in nuclei**

This was assumed to be the incoming RF, and not a product of re-utilization of the label in the synthesis of DNA or RNA, because it resisted digestion by DNAse (100 %) or RNAse.
Table 2. Penetration of mengovirus \[^{3}H\]-labelled RF into actinomycin D-treated L cells

<table>
<thead>
<tr>
<th>Time (hours since adsorption)</th>
<th>Intracellular [^{3}H]-labelled RF (ct/min)</th>
<th>Control</th>
<th>Actinomycin D treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8310</td>
<td>6648, 80%</td>
<td></td>
</tr>
<tr>
<td>1(\frac{1}{2})</td>
<td>6893</td>
<td>7513, 109%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5998</td>
<td>7437, 124%</td>
<td></td>
</tr>
<tr>
<td>4(\frac{1}{2})</td>
<td>4595</td>
<td>6203, 135%</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4674</td>
<td>6590, 141%</td>
<td></td>
</tr>
</tbody>
</table>

Confluent monolayers of L cells pre-treated or not with actinomycin D (5 \(\mu g/ml\)) were infected with \[^{3}H\]-labelled RF and the amount of intracellular radioactivity was determined as in Methods.

Fig. 4. Cytoplasmic extracts and nucleoplasm of \[^{3}H\]-labelled RF infected L cells were extracted with phenol and sedimented through a sucrose density gradient of 5 to 20% in tris buffer solution at 36000 rev/min for 45 min at 5 \(^\circ\)C. Fractions of 0.3 ml were collected and precipitated with ice-cold TCA (5%) after addition of 100 \(\mu g\) yeast RNA. Precipitates were collected into glass-fibre discs (Whatman GF/C), dried and counted in a Packard liquid scintillation spectrometer (counting time: 20 min; accuracy level 0.7%). Arrows indicate the position of 18 S and 28 S ribosomal RNA added as marker. Sedimentation is from left to right.

Penetration of RF into actinomycin D-treated L cells

We further examined the effect of actinomycin D on the cellular uptake of mengovirus RF. RF penetrated actinomycin D-blocked L cells (Table 2) as effectively as untreated control cells.

Modifications in the structure of incoming RF

We looked for modifications of the structure of the incoming molecule of RF during the replication cycle. Cells were infected by \[^{3}H\]-labelled RF and the sub-cellular fractions separated as already described. Cytoplasm and nucleoplasm from \[^{3}H\]-labelled RF-infected cells were extracted with phenol twice at room temperature (Koch & Bishop, 1968) in the (97 to 100%) but was completely hydrolysed by alkaline treatment (95 to 98%). Sedimentation analyses confirmed this (see below).
presence of 1% SDS, and the [3H]-labelled RF recovered by this procedure was analysed by velocity sedimentation through a density sucrose gradient of 5 to 20% in tris-HCl buffer. All procedures were at 0 to 4°C unless otherwise stated.

The sedimentation profile of RF in the cytoplasm was modified as soon as 45 min after infection (Fig. 4). A shoulder appeared on the heavier side of the peak and, as the infective cycle proceeded, the [3H]-labelled RF behaved as if part of some heavier, poly-disperse structure. Radioactivity was found in a wide band (9 to 40 S at 3 h after infection) with a main peak at 18 to 20 S. These changes were not due to partial denaturation of the parental molecule of RF. In parallel tests partially and fully heat-denatured mengovirus RF conserved the original sedimentation pattern, with a single peak at 18 to 20 S.

Alterations in the nucleoplasm were less marked and similar modifications were observed only towards the end of replication.

DISCUSSION

The results presented in this paper demonstrate that, unlike that of mengovirus, the infectivity of its replicative form (RF) depends on the integrity of cell-directed RNA synthesis.

The mechanism by which cellular transcription is blocked may not be relevant: treatment of cells with actinomycin D, which acts on template DNA, with α-amanitin, which blocks RNA polymerase II, or with cordycepin, which affects post-transcriptional addition of the poly A segment to mRNA, led in each case to a similar result in which the infectivity of mengovirus was unaffected whereas the replication of its RF was depressed drastically.

The observation that mengovirus normally replicates under conditions in which its RF is blocked, indicates that such an effect is not due to a mere cytotoxic action of the drugs, but that mengovirus and mengovirus RF initiate their replication cycle in different ways.

As far as the mechanism of infectivity of RF is concerned, the distinct effects of inhibitors have three main implications: (1) the possibility is excluded that some undetectable contaminant virus single-stranded RNA may account for the infectivity of RF; (2) they rule out the hypothesis that RF strands may separate spontaneously to allow the ‘plus’ chain to bind ribosomes and, (3) they strongly suggest that some cellular macromolecule is involved in the replication cycle initiated by RF.

The kinetics of sensitivity to actinomycin D suggest that such a macromolecule should be necessary only during the very early steps of RF replication. Similar results have been reported for the replication of influenza virus (Mahy, Hastie & Armstrong, 1972): the time-course of the sensitivity of influenza virus to α-amanitin resembles that shown in Fig. 1, especially if the different length of the replication cycles of mango and influenza viruses are considered.

The inability of cells to support RF replication shortly after treatment with actinomycin D indicates that such a macromolecule has a very rapid turn-over. The role of an unknown cellular factor is speculative: a first possibility is its participation in the direct conversion of RF into mRNA involving the active separation of both strands.

A second possibility, which we consider the more likely, is that such a factor is necessary for a cellular RNA polymerase to start transcription with RF as template. Cytoplasmic factors that specifically stimulates RNA polymerase II activity have been described (Seifart, Benecke & Juhasz, 1972; Sudgen & Keller, 1973; Natori et al. 1973) and it has been shown that they enhance in vitro RNA polymerase activity with double-stranded, but not single-stranded, DNA as template. These or similar factors may allow a cellular RNA polymerase to transcribe RF.
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The fate of the incoming molecule of RF was studied in terms of its intracellular distribution and its modifications in each sub-cellular compartment.

Replication of picornaviruses is known to occur in the cytoplasm though a considerable amount of RF accumulates in the nuclear fraction: between 3 and 4 h after infection 40% is found in the nucleoplasm and 4 to 5% in nucleoli. The observed late reduction of nuclear RF may be a result of increased nuclear fragility (Ascione, Arlinghaus & Vande Woude, 1972). The role of such a nuclear fraction of RF is not clear at present and requires further investigation.

Actinomycin D neither interacts directly with RF, nor interferes with its penetration in the host cell.

As expected for a virus that replicates in the cytoplasm, the more striking changes in the structure of incoming RF occur in this sub-cellular fraction. RF seems not to be modified within the nuclei: it conserves its double-stranded structure throughout the cycle and remains 96 to 98% RNAse-resistant (data not shown), and sediments as a major peak at 18 S. By the end of the cycle, however, its sedimentation profile is similar to that of RF recovered from 'early' cytoplasmic fractions. On the other hand, the analysis on sucrose gradients of the [3H]-labelled RF re-extracted from the cytoplasm of [3H]-labelled RF-infected cells shows that from the beginning of the replication cycle, the incoming molecule modifies its sedimentation behaviour: RF becomes heavier and a shoulder appears in the leading edge. At 3 h after infection [3H]-labelled RF sediments as a wide band (9 to 40 S) and 3 h later is polydisperse. These findings are consistent with a replication model in which the parental molecule of RF is being transformed into replicative intermediate, and we postulate that within 1 h after infection the first virus messengers are synthesized by a cellular RNA polymerase, the RF (d-s RNA) serving as template for this abnormal transcription. This assumption is further supported by the finding that a cellular, α-amanitin-sensitive RNA polymerase is able to bind to mengovirus RF in vitro and that the double-helical structure of the RNA template is essential for binding to occur (Pérez-Bercoff et al. 1974). Since the complex 'cellular polymerase-RF' may be non-functional, experiments are in progress to test whether or not it is able to synthesize in vitro new chains of RNA using requires further RF as template.

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