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Studies on the Infectivity of Foot-and-Mouth Disease Virus RNA using Microinjection

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SUMMARY

Foot-and-mouth disease virus (FMDV) RNA, isolated as virion RNA from purified virus particles or as total RNA from infected cells, has been microinjected into nuclei and cytoplasm of BHK cells. When injected directly into the nucleus FMDV RNA was not infectious, whereas cytoplasmic injection resulted in a high proportion of productive infections. Infectivity microinjection assays on dilution series of various FMDV RNAs showed that both single-stranded positive sense 35S RNA and double-stranded replicative form (Rf) RNA have an infectivity close to 1 p.f.u. per molecule, although only a minor fraction of BHK cells appeared able to support plaque formation following microinjection of Rf FMDV RNA. The infectivity of Rf FMDV RNA was not sensitive to inhibition by actinomycin D. The results are discussed in relation to the high virus particle to p.f.u. ratios observed for FMDV.

INTRODUCTION

Quantitative studies on the infectivity of picornaviruses have consistently shown that the majority of virus particles produced during an infection do not subsequently induce a new round of virus replication. Estimates of the number of virus particles required to form a p.f.u. vary considerably depending on the virus type and the particular experiment. For example, Bachrach et al. (1964) obtained estimates of between 83 and 2460 particles/p.f.u. for purified foot-and-mouth disease virus (FMDV). Furthermore, it is clear that many of the viral RNA molecules produced within an infected cell fail to be packaged into infectious virus particles. Baltimore (1969) showed that a poliovirus-infected cell can produce about 10^5 molecules of viral RNA, but that these yield only about 10^3 p.f.u. of virus.

There are several potential explanations for why a single virus particle might fail to initiate an infection in a cell and thus not be equivalent to a p.f.u. One commonly held explanation is based on the notion that a high proportion of RNA molecules carry lethal mutations resulting from error-prone RNA replication by RNA-dependent RNA polymerases (Holland et al., 1982; Domingo et al., 1985). Other factors which could contribute to the overall shortfall in infectivity might include inefficiency in the packaging of complete RNA genomes into mature virus particles or the defective entry of mature virus particles into the cell. Once inside the cell, events which lead to uncoating of the virus and release of its undamaged RNA into the cytoplasm could also contribute to the overall measured efficiency of the infectious process.

In principle it is possible to distinguish any effect due to defective RNA genomes, caused by accumulated mutations, from effects caused by failure or inefficiency in viral uptake and uncoating by studying the infectivity of purified viral RNA molecules introduced directly into the cytoplasm of cells. Isolated picornavirus RNA has been shown to be infectious following transfection, and up to 2 x 10^6 p.f.u./µg of genomic RNA can be obtained (see Koch & Koch, 1985). However, since this value represents a specific infectivity some 100-fold lower than that obtained with intact virus particles, i.e. about 10^8 RNA molecules are required for 1 p.f.u., it does not help to resolve the question of what is causing the relative inefficiency in the infectivity of virus particles.
We have approached this problem by directly microinjecting FMDV RNA into the cytoplasm of permissive BHK cells. These studies show that the single-stranded positive sense 35S FMDV RNA molecules have an infectivity close to 1 p.f.u. per molecule, and, consequently, that high virus particle number to p.f.u. ratios must reflect inefficiency in some component of the delivery of viral RNA to the cytoplasm rather than being due to a large proportion of defective viral genomes. We have also used the microinjection system to reanalyse the infectivity of the purified double-stranded replicative form (RF) of FMDV RNA.

METHODS

Cells. Primary bovine thyroid (BTY) cells were prepared as described by Snowdon (1966), approximately 1 week prior to use and passaged only once. BHK cells were used for all virus plaque assays and for microinjection experiments. Cells were grown in BHK-21 Glasgow-modified Eagle’s medium (Gibco), supplemented with 10% newborn calf serum and antibiotics, in an atmosphere of 5% CO₂ and 95% air. Actinomycin D (Sigma) was used at a concentration of 2 μg/ml where indicated. At this concentration, addition of actinomycin D immediately inhibited the incorporation by BHK-21 cells of [³H]uridine into TCA-insoluble material by a factor of 95% or greater.

Preparation and analysis of RNA from FMDV-infected cells. Cellular RNA was prepared from the cytoplasmic fraction of NP40-lysed cells as described by Xie et al. (1987). Samples of RNA were fractionated by the addition of an equal volume of 4 M-LiCl. After overnight incubation at 4 °C the precipitated LiCl-insoluble RNA was collected by centrifugation and the LiCl-soluble RNA was recovered by precipitation with ethanol. This procedure separated the double-stranded RNA and small soluble RNA (4S to 5S) from mRNA and large ribosomal RNA (rRNA). RNA samples were analysed by electrophoresis in 1% agarose gels in TBE buffer (100 mM-Tris, 100 mM-borate, 5 mM-EDTA).

To monitor the appearance of FMDV RNA during infection, RNA samples were run on denaturing RNA gels with formaldehyde (Maniatis et al., 1982), the RNA was transferred to nitrocellulose and probed with nick-translated FMDV-specific cDNA clones. The formation of viral RNA was quantified by densitometric scanning of the autoradiograph. The proportion of total cellular RNA corresponding to FMDV 35S RNA was estimated by densitometric scanning of photographic negatives of ethidium bromide-stained agarose gels. RNA transfections were performed as described by van der Werf et al. (1986).

Microinjection. The procedure for microinjection was similar to that described by Graessmann & Graessmann (1986).

Estimation of microinjected volumes. The delivered volumes were estimated by injecting a solution of [³⁵S]methionine (200 mCi/ml) directly into the cytoplasms of nuclei or nuclei of 50 BHK cells plated onto small pieces of glass coverslips. Coverglass chips carrying injected cells were then washed five times in ice-cold phosphate-buffered saline (PBS), air-dried and the retained [³⁵S]methionine was counted by liquid scintillation. In order to control for the possibility that some of the [³⁵S]methionine could have leached from the tip of the needle between injections and been taken up by cells via the medium, each piece of coverglass carrying injected cells was placed adjacent to a matched piece of coverglass carrying un.injected cells. Being in the immediate vicinity of the injected cells, any [³⁵S]methionine leached into the growth medium would also have been available to these uninjected cells, which therefore served as a control of any uninjected cell uptake of [³⁵S]methionine. These ‘control’ cells were processed and counted in the same way as the injected cells. Standard curves were determined by drying measured volumes of known concentrations of [³⁵S]methionine in PBS on glass coverslips which were then counted in the same way as the glass chips carrying cells.

RESULTS

FMDV RNA directly microinjected into the cytoplasm is infectious

The feasibility of the experimental approach was initially tested by microinjecting concentrated solutions of FMDV RNA from purified virus, or of total RNA from FMDV-infected cells, into the nuclei or cytoplasms of BHK cells, overlaying the monolayer with agar and staining the cell monolayer after 24 h. Fig. 1(a) shows clear plaques down two lines corresponding to the positions of cells which had been injected cytoplasmically with total RNA from cells infected with FMDV strain O₁ Kaufbeuren. Fig. 1(a) also shows that when saturating levels of RNA were used (see below) around 90% of injections led to the formation of a plaque. Injection into the cytoplasm, rather than the nucleus, has proved to be essential as no plaques were observed following injection of any FMDV RNA preparation (virion, total cellular, purified 35S or RF, see below) into the nuclei of several hundred BHK cells. For example, in Fig. 1(b) an example of O₁ Pacheco FMDV RNA injected cytoplasmically into one line of BHK cells
Infectivity of FMDV

Fig. 1. Efficiency of plaque formation following microinjection of FMDV RNA into the nucleus or cytoplasm of BHK cells. (a) Total RNA from cells infected with O1 Kaufbeuren FMDV was microinjected into the cytoplasms of two rows of 13 and 12 cells respectively. After overlaying with agar and incubation for 18 h at 37 °C the monolayer was fixed and stained in methylene blue to reveal the presence and location of plaques. The microinjected cells produced 13 and 10 plaques, respectively, giving an overall efficiency of 92%. (b) In one row, 10 BHK cells were microinjected with RNA from cells infected with O1 Pacheco FMDV directly into their nuclei (N) and in another row 10 cells were microinjected directly into their cytoplasms (C). Only those cells receiving cytoplasmic injection produced plaques.

Table 1. Estimation of average volumes injected into a nucleus or cytoplasm*

<table>
<thead>
<tr>
<th>Sample</th>
<th>C.p.m.†</th>
<th>Average c.p.m./50 cells</th>
<th>C.p.m./injected cell</th>
<th>Vol. injected/cell (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nuclei</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>195</td>
<td>3.9</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>273</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>223</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 cytoplasms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>674</td>
<td>574</td>
<td>11.5</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>727</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>322</td>
<td></td>
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</table>

* Replicate 1 μl samples of serial dilutions of the injected solution of [35S]methionine, dried onto glass chips, showed that under these conditions of liquid scintillation counting the undiluted, injected solution contained 3 x 10⁸ c.p.m./μl. Therefore, 1 fl contained 0.3 c.p.m. [35S]methionine or 1 c.p.m. was contained in 3.3 fl.
† C.p.m. minus background radioactivity.

is shown, which resulted in the production of a line of clear plaques, compared with a neighbouring line of BHK cells which received an injection into the nucleus and which produced no visible plaques.

Most single molecules of FMDV RNA are infectious

In order to quantify the number of FMDV RNA molecules required to generate an infection after direct microinjection into a single cell it is necessary to know the concentration of full length FMDV RNA molecules, as well as the volume of the solution being injected. Estimates of these values were made as described in Methods.

Table 1 summarizes the volumes of cytoplasmic and nuclear injections, which averaged about 38 fl and 13 fl, respectively. These values are within the range of previous estimates of injected volumes (e.g. Graessmann & Graessmann, 1986). The proportion of total RNA from infected cells, which corresponded to FMDV 35S RNA, was estimated to be 5%.

Injection of a dilution series of FMDV RNA of known concentration allows the determination of the RNA concentration at which the efficiency of plaque formation begins to decrease. At this point a proportion of cells would not be receiving the minimum number of molecules in the 40 fl volume required to initiate plaque formation. Knowing the concentration of the RNA solution at which the infectivity can be diluted out allows the estimation of the average number of molecules being injected in a 40 fl volume. Thus a direct measure of
Fig. 2. Quantification of FMDV RNA infectivity by microinjection. Total cell RNAs from BTY cells infected with O\textsubscript{I} Pacheco ts13 (top row) or Ovi6 ts302 (bottom row) FMDV were prepared as described in Methods. In both cases the undiluted RNA was at a concentration of approximately 550 µg/ml. Dilutions, indicated at the top, of both RNAs were prepared and microinjected into the cytoplasms of 10 BHK cells in each of two rows. The monolayers were overlaid with medium containing 0.6% agarose, incubated overnight at 37 °C and then fixed and stained with methylene blue.
Infectivity of FMDV

Infectivity per RNA molecule can be established. Fig. 2 shows typical lines of plaques produced following cytoplasmic injection of dilution series of RNAs of two different temperature-sensitive mutants (O₁ Pacheco ts13 and Ovi6 ts302) of FMDV incubated at the permissive (37 °C) temperature. The measured concentration of total infected cell RNA was 550 μg/ml of which 5% was 35S RNA. Thus the estimated concentration of undiluted ts302 FMDV RNA was 27.5 μg/ml, so that at a dilution of 1:120, at which plaque formation began to drop, 40 fl would have contained approximately two molecules. Thus, each injected cell would have received, on average, two molecules. Assuming a skewed normal distribution in the frequencies of cells receiving different numbers of RNA molecules, at an average of two molecules per cell, 86% of cells would receive at least one molecule but only 50% would receive two or more molecules. The decline in frequency of plaque formation at an average of two molecules per cell therefore suggests that essentially each molecule of FMDV RNA must be capable of initiating an infection resulting in the formation of a plaque.

Low p.f.u. to particle ratios are not caused by defective RNA genomes

The analysis described above shows that at least half of the molecules of FMDV RNA can be considered as infectious, and thus defective RNA genomes must be a minor cause of low p.f.u. to particle ratios. We have extended this by monitoring the appearance of infectivity throughout the time course of an infection. Twenty-five cm² cultures of primary BTY cells were infected with O₁ Pacheco FMDV at high multiplicity and harvested at 30 min intervals up to 4 h post-infection, either to determine virus yield (as determined by plaque assay on BHK cells) or to extract total RNA. The results in Fig. 3 confirm those shown previously with poliovirus (Baltimore, 1969), namely that the appearance of 35S RNA slightly precedes the production of new virus infectivity. However, we have also microinjected a range of dilutions of each of the RNA samples prepared from cells at 30 min, 60 min, 120 min and 150 min post-infection. The number of infectious doses in each RNA sample was calculated from the mean of values obtained at limiting dilutions of RNA, and these are also shown in Fig. 3.

The appearance of infectious RNA paralleled that of 35S RNA, but the estimated maximum number of infectious doses of RNA (5 × 10¹¹ infectious doses/25 cm² flask) exceeded the virus yield (8.2 × 10⁸ p.f.u./25 cm² flask) by a factor of approximately 600. Furthermore, we calculated that 5 × 10¹¹ infectious doses corresponded to 7.2 × 10¹¹ FMDV 35S RNA molecules in this preparation. This confirmed our earlier conclusions that a single infectious dose of FMDV RNA corresponds to a single FMDV RNA molecule and that, to a first approximation, each FMDV RNA molecule is capable of initiating an infection.

In an analogous way the specific infectivity of purified virion RNA can be compared with the specific infectivity of the virus particles from which the virion RNA was isolated. We purified two batches of O₁ Pacheco FMDV and isolated the virion RNA. The infectivity of these RNAs was measured using the microinjection assay. Parallel aliquots were used to measure the number and infectivity of intact virus particles. The results in Table 2 show that, while the specific infectivity of virion RNA is not as high as that of FMDV 35S RNA in preparations of cellular RNA, in both cases the infectivity of virion RNA determined by microinjection was approximately 20-fold higher than the infectivity of virus particles measured by plaque assay. It is likely that the lower infectivity of virion RNA results from RNA degradation occurring during the purification procedure.

The replicative form of FMDV is less infectious than 35S RNA but infectivity is dependent on a host cell component

In all of the above experiments involving infectivity of FMDV RNA in preparations of total cellular RNA we considered the FMDV 35S RNA component to be the only infectious agent. However, the double-stranded Rf of picornavirus RNA has been shown to be infectious by transfection, by e.g. Montagnier & Sanders (1963), and could therefore be contributing to the infectivity of microinjected total infected cell RNA. Any contribution would have to be small since the Rf component does not usually exceed 50% of FMDV RNA in an infected cell, and,
Fig. 3. Time course of infection by FMDV on BTY cells. Twenty-five cm$^2$ cultures of BTY cells were infected with O1 Pacheco FMDV at zero time and incubated at 37 °C. At 0.5 h intervals one culture was frozen and stored for subsequent assay of virus production by plaque assay on BHK cells (○). At each sampling interval a second culture was used to extract total cell RNA. The appearance of FMDV RNA (Q) was assessed by densitometric scanning of an autoradiograph following hybridization with $^{32}$P-labelled type O FMDV cDNA to a Northern blot of the RNA samples (see Methods). The number of infectious doses in each RNA preparation (□) was estimated by microinjection of dilution series of each RNA sample into the cytoplasms of BHK cells.

Table 2. Specific infectivity of virion RNA

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>No. of particles*</th>
<th>No. of p.f.u. †</th>
<th>No. of infectious doses‡</th>
<th>Particles/p.f.u infectious dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$1.2 \times 10^{12}$</td>
<td>$5.3 \times 10^9$</td>
<td>$8.5 \times 10^{10}$</td>
<td>226</td>
</tr>
<tr>
<td>B</td>
<td>$1.5 \times 10^{12}$</td>
<td>$7.2 \times 10^9$</td>
<td>$2 \times 10^{11}$</td>
<td>208</td>
</tr>
</tbody>
</table>

* Particle number was estimated from $A_{260}$ of the virus suspension assuming 1.0 unit contains $9.6 \times 10^{12}$ virus particles (Rueckert, 1976). $A_{260}$ measurements on extracted RNA showed essentially 100% recovery of virion RNA.
† Determined by plaque assay on BHK cells.
‡ Determined by microinjection of serial dilutions of extracted RNA.

being double-stranded, an equal weight of Rf would contain only half the number of positive sense RNA molecules which alone can participate in the initial translation event.

Fig. 4(a) shows that, as the infection proceeded, the ratio of Rf to 35S FMDV RNA increased to approximately 1 after 4 h as judged by ethidium bromide staining (Hewlett et al. (1977) showed that poliovirus single-stranded RNA and double-stranded RNA gave similar yields of
Fig. 4. Agarose gel analysis of RNA from FMDV-infected BTY cells. (a) RNA samples extracted from O1 Pacheco-infected BTY cells, at 0.5 h (lane 2), 1 h (lane 3), 1.5 h (lane 4), 2 h (lane 5), 2.5 h (lane 6), 3 h (lane 7), 3.5 h (lane 8) and 4 h (lane 9) post-infection, together with RNA from uninfected cells (lane 1), were analysed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Purified FMDV RNA was run in lane 10 as a marker. These RNA samples were also used to determine the percentages of total infected cell RNA that was 35S FMDV RNA. (b) Total cytoplasmic RNA prepared from O1 Pacheco-infected BTY cells at 3 h post-infection was fractionated with LiCl as described in Methods. Total cytoplasmic RNA (lanes 1 and 4), RNA soluble in 2 M-LiCl (lanes 2 and 5) and RNA precipitated by 2 M-LiCl (lanes 3 and 6) were analysed by electrophoresis in the presence of 0.5 µg/ml ethidium bromide directly (lanes 1 to 3) or following treatment with 20 µg/ml RNase A in 3 x SSC at 37 °C for 30 min.

fluorescence after staining with ethidium bromide. This is somewhat higher than observed with poliovirus, where up to 20% of poliovirus RNA is Rf (Baltimore, 1969), but it is similar to previous observations with FMDV (Arlinghaus et al., 1966). Fractionation on the basis of LiCl solubility yielded a LiCl-soluble Rf RNA component which, as judged by ethidium bromide staining, was free of 35S and cellular RNA except 4S to 5S RNA, and a LiCl-insoluble fraction which contained the 35S FMDV RNA component together with cellular rRNA and mRNA (Fig. 4b). Treatment of total RNA and these LiCl fractions with ribonuclease A (Fig. 4b) confirmed the reported RNase resistance of the double-stranded Rf RNA in both the total infected cell RNA preparation and the LiCl-soluble fraction, and the RNase sensitivity of the 35S RNA component. Infectivity studies confirmed the relative resistance of Rf to RNase inactivation (data not shown).

Microinjection of a dilution series of the LiCl-soluble and -insoluble fractions showed two features (see Fig. 5). Firstly, whereas microinjection of 35S RNA gave a high proportion (> 50%) of plaque formation at concentrations of RNA that deliver at least one FMDV RNA molecule per cell, microinjection of Rf produced, on average, only 7% plaque formation. This was the case even at high concentrations of Rf RNA at which greater than 100 molecules were delivered per injection. The simplest interpretation of this concentration-independent low percentage is that only a minor fraction of the BHK cells can support an infection by Rf.
Fig. 5. Infectivity of Rf and 35S FMDV RNAs. BHK cells were injected as described for Fig. 2 with serial dilutions of purified LiCl-insoluble Rf FMDV RNA (O) or LiCl-soluble 35S FMDV RNA (●) and the percentage of injections resulting in plaques was measured. With 35S RNA preparations the percentage plaque formation was sufficiently high that often individual plaques merged to produce a continuous line, making precise quantification difficult. In these cases percentages have been scored as >50%.

Fig. 6. Effect of actinomycin D on the infectivity of Rf (O) and 35S (●) FMDV RNAs. Actinomycin D was added to the medium to a final concentration of 2 μg/ml at zero time. Rf and 35S FMDV RNA preparations were microinjected into the cytoplasms of BHK cells, as described for Fig. 2, at various times after addition of actinomycin D and the efficiency of plaque formation was scored after incubation for 20 h at 37 °C.

Table 3. Virus yields from transfection of Rf and 35S RNAs into actinomycin D-treated cells

<table>
<thead>
<tr>
<th>RNA preparation</th>
<th>Untreated BHK cells</th>
<th>Actinomycin D-treated BHK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total infected cell</td>
<td>7 × 10⁵</td>
<td>4-6 × 10⁵</td>
</tr>
<tr>
<td>LiCl pellet (35S RNA)</td>
<td>7-8 × 10⁵</td>
<td>6-0 × 10⁵</td>
</tr>
<tr>
<td>LiCl-soluble (Rf)</td>
<td>4-4 × 10⁵</td>
<td>3-6 × 10⁵</td>
</tr>
</tbody>
</table>

* Cells were transfected with RNA preparations containing between 50 and 75 ng of FMDV RNA using DEAE-dextran (van der Werf et al., 1986). Cells were incubated in the absence or presence of actinomycin D (2 μg/ml) and harvested after 7 h. The virus yields were determined by plaque assays on BHK cells. The yields are expressed as p.f.u. per μg of FMDV RNA used in the transfection.

The second conclusion to be drawn from this experiment was that on a per molecule basis, Rf was as infectious as 35S RNA. Our data showed that, to a first approximation, single molecules of both 35S RNA and Rf could cause the formation of a plaque. This appears to be contrary to the results of Bishop and Koch (1967), which were based on transfection experiments involving poliovirus RNAs. We believe that, as suggested by these workers, the discrepancy lies in the differential resistance to degradation of Rf and 35S RNAs during transfection, whereas both RNAs would be protected from any degradative processes by direct microinjection into the cytoplasm of the cell (see Discussion).

Another feature which reportedly distinguishes picornavirus Rf and 35S RNAs is the sensitivity of Rf infectivity to inhibition by actinomycin D (Koch et al., 1967) and other inhibitors of RNA transcription and processing (Perez-Bercoff et al., 1974). We have investigated the effect of actinomycin D on the infectivity of 35S and Rf RNAs of FMDV and found both forms insensitive to the action of the inhibitor. Fig. 6 shows that pretreatment of the cells for up to 2-5 h with 2 μg/ml actinomycin D had no detectable effect on the percentage of cells that could support virus replication following injection with either 35S or Rf RNA. Furthermore, the yield of FMDV 7 h after transfection of BHK cells with either 35S or Rf RNA was not significantly different in the presence or absence of actinomycin D (see Table 3).
should be noted that the yield of virus from the transfection of Rf RNA was similar to that obtained with 35S RNA. Cells exposed to actinomycin D were clearly inhibited by the drug since the density of cells in treated monolayers was significantly lower than in untreated monolayers. We conclude that the ability of FMDV Rf RNA to mediate an infection in BHK cells is not inhibited by actinomycin D.

**DISCUSSION**

We have shown that a single molecule of FMDV 35S RNA is able, with a high probability, to initiate a cycle of virus replication. This demonstrates that the initial translation and subsequent replication of this RNA within the cell is a very efficient process. Furthermore, this observation demonstrates that a large proportion of the viral RNA molecules within an infected cell is infectious. Hence the relatively high particle to p.f.u. ratios observed with picornaviruses must be due to inefficiencies in the delivery of the intact RNA genome to the site of translation and replication within the cytoplasm of the cell. Postulated mutations resulting from errors in RNA replication by the RNA-dependent RNA polymerase must be few, or, at least, do not appear grossly to affect the viability of the total RNA population (Holland *et al.*, 1982; Domingo *et al.*, 1985).

Our studies of the infectivity of FMDV Rf using the microinjection system reveal novel features in the biology of these molecules. Molecules of Rf RNA are as infectious as 35S RNA molecules, but only in those cells capable of expressing the infectivity of Rf RNA. This is a minor fraction of the cell population, as over 90% of cells do not yield virus when injected with Rf. At this stage we do not know the basis for this cell selectivity. It is unlikely to be related to the intracellular concentration of a short-lived host cell factor, as has been proposed for other picornaviruses (e.g. Perez-Bercoff *et al.*, 1974; Detjen *et al.*, 1978), since actinomycin D pretreatment of the cells for up to 2.5 h did not affect the ability of cells to produce virus from Rf RNA.

The transfection experiments described show that FMDV Rf RNA generates amounts of virus similar to those induced by FMDV 35S RNA. This contrasts with results from other workers using other picornaviruses (e.g. Bishop & Koch, 1967) which produce higher yields of virus following transfection with Rf RNA. We suggest that three different and opposing effects are operating in these transfection experiments. First, the well documented resistance of Rf RNA to degradation by RNase will tend to enhance preferentially the apparent infectivity of Rf RNA relative to 35S RNA. Second, variations in the transfection technique may favour entry of Rf RNA over the 35S RNA form (Kuhn & Wimmer, 1987). Third, the small and perhaps variable proportion of cells permissive for Rf RNA infectivity will depress the apparent relative infectivity of the Rf and tend to counterbalance the nuclease effect noted above. Thus the measured infectivity of Rf RNA relative to 35S RNA is the net result of several interacting effects and will inevitably vary according to the virus type, experimental conditions and the host cell used.

We have been unable to demonstrate any relative sensitivity of FMDV Rf RNA infectivity to inhibition by actinomycin D, either in the transfection assay or by microinjection. This is at variance with previous reports on the infectivity of Rf RNA of poliovirus (Koch *et al.*, 1967) and mengovirus (Perez-Bercoff *et al.*, 1974). At this stage it is not possible to say whether this represents a distinctive property of FMDV, either in general or of the O₁ Pacheco strain in particular, or the cells used.

In summary, our results show that the microinjection system is a useful tool in the study of the interactions between a viral RNA molecule and its host cell and in the analysis of interactions between different RNA molecules in the same host cell, for example in RNA recombination.

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