Rhinovirus Multistranded RNA: Dependence of the Replicative Form on the Presence of Actinomycin D

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The multistranded and double-stranded RNAs synthesized in HeLa cells infected with rhinovirus in the presence and in the absence of actinomycin D have been characterized by polyacrylamide gel electrophoresis and hybridization studies. The replicative form is only found in infected cells treated with actinomycin D, whereas the replicative intermediate is found in both the presence and the absence of the drug. The significance of these results is discussed.

The genome of picornaviruses consists of one molecule of single-stranded RNA that is infectious under appropriate conditions (6). However, RNA complementary to the genome is not infectious (4, 5, 26). The genome of picornaviruses is present in polysomes of infected cells and has been shown to function as mRNA (16, 24, 29). It has been suggested that the input genome RNA is replicated in a two-step process, namely, the formation of a complementary template that is then preferentially copied to produce an excess of genomic RNA (SS RNA) (6).

Two replicative structures, the replicative intermediate (RI) and the replicative form (RF), have been obtained from human cells infected with rhinovirus in the presence of actinomycin D and characterized on polyacrylamide gels (17). The RI has been shown to be a multistranded structure, and the RF has been shown to be a double-stranded structure (17). In our study we investigated the effect of actinomycin D on rhinovirus multiplication. We found that although the virus multiplies with very similar kinetics in the presence or in the absence of actinomycin D, RF is only found in cells treated with actinomycin D. The inference that RF does not exist under natural conditions—i.e., in infection—is discussed.

MATERIALS AND METHODS

Propagation of cells and viruses. Monolayers of HeLa cells were grown in Eagle medium supplemented with 10% calf serum, 0.1% sodium bicarbonate, and antibiotics. The cells were infected with rhinovirus type 2 at 0.1 PFU/cell. Unlabeled infectious virus was prepared from these cells as previously described (17).

Preparation of radioactive rhinovirus-specified RNA. Monolayers of cells were infected with 10 PFU/cell of virus at 33 C. After 1 h the virus was removed and replaced with medium containing 2% calf serum and, when appropriate, 1 μg of actinomycin D per ml was added. After incubation for the required time, 200 μCi of [3H]uridine (specific activity, 24 Ci/mmole) (Radiochemical Centre, Amersham) in 2 ml of medium containing 2% calf serum was added. The RNA was extracted with phenol in the presence of sodium dodecyl sulfate (17) and then precipitated with 2 volumes of ethanol at -20 C for 16 h.

Chromatography of RNA on cellulose columns. Rhinovirus RNA species were fractionated by chromatography on Whatman CF11 cellulose, which separates single-stranded RNA from multistranded RNA (10). Columns (11 by 1.5 cm) were packed under gravity in TNE buffer solution (0.1 M NaCl-0.05 M Tris-0.001 M EDTA (sodium salt) adjusted to pH 7.0 at 20 C with HCl) containing 0.1% sodium dodecyl sulfate plus 35% (vol/vol) ethanol. The columns were loaded with RNA at concentrations not more than 0.5 mg/ml, and after elution at room temperature the flow rate was maintained at 1 ml/min. RNA species were eluted by stepwise washings of the columns with 0.1% sodium dodecyl sulfate in TNE buffer solution containing decreasing concentrations of ethanol (i.e., 35% ethanol, 15% ethanol, and 0% ethanol). Fractions (1 ml) were collected, and aliquots were assayed for radioactivity. The RNA species were concentrated by precipitation at -20 C with 100 μg of tRNA as carrier and 2 volumes of ethanol.

Polyacrylamide gel electrophoresis of RNA. Polyacrylamide gels (2.2%) supported by 0.5% agarose were made by the procedure of Loening (18) with certain modifications (20). The gels were polymerized in 10-cm tubes with internal diameters of 0.7 cm. After a pre-electrophoresis of 30 min at 50 V, up to 60 μg of RNA was loaded on to each gel and subjected to electrophoresis for 3 to 5 h at 50 V. After electrophoresis the gels were extruded and analyzed on a Gilford gel scanner for material absorbing at 260 nm. The gels were frozen, sliced into 1-mm disks, and solubilized, and the radioactivity was measured as previously described (17).
Hybridization. The hybridization method used was essentially that of Avery (1). Radioactively labeled RNA preparations were dissolved in 0.02 × SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and denatured by heating at 98 C for 4 min and then rapidly brought to 6 × SSC and 65 C. When appropriate, excess unlabelled virus RNA was added at this stage. Samples were diluted after 4 h of incubation in water at 37 C to bring the salt concentration to 1 × SSC. Pancreatic and T. ribonucleases (Sigma Chemical Co. Ltd., London) were added to 50 µg/ml and 170 units/ml, respectively, and digestion was allowed to proceed for 20 min at 37 C. The reaction was stopped by the addition of 250 µg of yeast RNA and an equal volume of ice-cold 10% trichloroacetic acid. Other samples were precipitated without prior enzyme digestion to determine the total acid-precipitable radioactivity. Precipitates were collected on cellulose acetate filters (Millipore Ltd., London) and washed twice with cold 5% trichloroacetic acid and ether. The filters were dried under an infrared lamp and assayed for radioactivity.

Infectivity titrations. The infectivity of virus samples was titrated by plaque assay on monolayers of HeLa cells seeded into petri dishes (5 cm in diameter) (31). The dishes were inoculated with 0.2 ml of virus diluted in Eagle medium containing 4% calf serum, which was allowed to adsorb for 90 min at 33 C. The inoculum was removed, and the dishes were incubated with 5 ml of an overlay medium containing 0.5% (wt/vol) agarose (30) at 33 C for 4 days. The cells were fixed with formal saline, the agar was removed, and the cells were stained with 1% gentian violet in 20% ethanol.

Inhibition of protein synthesis in rabbit reticulocyte lysates by double-stranded RNA. Protein synthesis in rabbit reticulocyte lysates was measured according to established procedures (9). Double-stranded and multistranded RNAs from 8 × 106 cells were separated by cellulose CF11 chromatography. RNA from the peak fraction was serially diluted in 100 mM KCl, and 5-µl aliquots were added to 35 µl of reticulocyte lysate containing 25 µM hemin. Assays were preincubated at 30 C for 30 min before adding 10 µl of a solution containing 2.5 µCi of [3H]leucine (specific activity, 2.0 Ci mmol) (Radiochemical Centre, Amersham) and other components needed for protein synthesis (9). After a further 30 min of incubation, 10-µl samples were removed, hydrolyzed with alkali, precipitated with trichloroacetic acid, and assayed for radioactivity. Incubations in the absence of added RNA incorporated 39,000 counts/min for a 10-µl sample. The dilution of RNA acid inhibiting protein synthesis by 50% was calculated by interpolation on a standard curve. The kinetics of inhibition were characteristic of double-stranded RNA. Under the same conditions, a preparation of polynucleosinic-polynucleotidic acid had a 50% inhibitory concentration of 3 × 10−16 g/ml.

RESULTS

Effect of actinomycin D on rhinovirus multiplication. Certain strains of poliovirus have been shown to be sensitive to actinomycin D during their multiplication cycle in human cells (7, 13, 28). HeLa cells were infected with rhinovirus to see if actinomycin D affected the production of virions. Table 1 shows the effect of actinomycin D on the production of infectious virus in HeLa cells during the period of exponential increase (from 5 to 8 h postinfection). Actinomycin D (1 µg/ml) has only a slightly inhibitory effect during this time on the appearance of infectious virus. However, its rate of production or final yield after 12 h of infection was not affected.

Not only does actinomycin D have little effect on the production of infectious virus, but it also has a negligible effect on the total amount of viral RNA species synthesized during infection. However, the relative amounts of RI and RF produced under these conditions were difficult to calculate, as these species were obscured by cellular RNAs, presumably heterogeneous nuclear RNAs and rRNA precursors, when no actinomycin D was present during infection. To analyze RI and RF further, double-stranded and multistranded RNAs were separated from single-stranded RNAs by cellulose CF11 chromatography (17).

Double-stranded and multistranded RNAs synthesized in the presence and in the absence of actinomycin D. Figure 1 shows polyacrylamide gel profiles of [3H]uridine-labeled double-stranded and multistranded RNAs obtained from infected or uninfected HeLa cells incubated in the presence or absence of actinomycin D. The cells were labeled from 6 to 8 h during

<table>
<thead>
<tr>
<th>Table 1. Production of infectious rhinovirus in HeLa cells in the presence and absence of actinomycin D</th>
<th>Treatment</th>
<th>Incubation (hours postinfection)</th>
<th>PFU/ml (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No actinomycin D</td>
<td>5</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>340</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3,100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>5</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1,400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Monolayers were infected at a multiplicity of 10 PFU/cell for 1 h at 33C. The inoculum was removed by washing the cell sheet three times, and the incubation was continued in Eagle medium containing 2% calf serum and appropriate amounts of actinomycin D. At the times indicated cells were scraped into the medium and disrupted before the total infectivity was assayed.

* These results are averages of duplicate samples. A similar experiment produced similar results.
the exponential period of increase of infectious virus (17). All the manipulations were done at the same time under identical conditions, and each profile is derived from the same number of HeLa cells. RNA was extracted, and then double-stranded and multistranded RNAs were separated from single-stranded RNAs on cellulose CF11 columns by elution with different concentrations of ethanol (19). Single-stranded RNAs were eluted with 15% ethanol, and multistranded RNAs were eluted with 0% ethanol. The elution patterns of RNAs from cells treated with and without actinomycin D were the same. Treatment of the fractions of Fig. 1 with

![Polyacrylamide gel electrophoresis](image-url)

**Fig. 1.** Polyacrylamide gel electrophoresis of double-stranded and multistranded RNAs extracted from rhinovirus-infected and uninfected HeLa cells. These RNAs were separated from single-stranded RNAs on cellulose CF11 columns. (A) RNA from rhinovirus-infected cells labeled with [\(^3\)H]uridine from 6 to 8 h postinfection in the presence of actinomycin D; (B) RNA from rhinovirus-infected cells labeled with [\(^3\)H]uridine from 6 to 8 h postinfection in the absence of actinomycin D; (C) RNA from uninfected cells labeled with [\(^3\)H]uridine for 2 h in the presence of actinomycin D; (D) RNA from uninfected cells labeled with [\(^3\)H]uridine for 2 h in the absence of actinomycin D. The arrows are taken from the absorbance at 260 nm trace indicating the position of added "marker" HeLa DNA.
10 μg of DNase per ml for 1 h at room temperature did not alter the profiles on polyacrylamide gels.

Both RI and RF were found in infected cells treated with actinomycin D (Fig. 1A), but no RF was found in cells infected in the absence of actinomycin D, and the only multistranded species had a mobility similar to RI (Fig. 1B). We have called the latter species putative RI (pRI). It is interesting to note that this pRI appears to be larger in size than the RI obtained from infected cells treated with actinomycin D.

These profiles from infected cells were compared with parallel profiles of multistranded RNAs obtained from noninfected cells incubated with (Fig. 1C) or without (Fig. 1D) actinomycin D. Some multistranded RNAs were obtained under both conditions, and a greater amount was obtained from the culture that did not receive the drug. This RNA appeared to be very large, as it only just entered the gels, and it may be heterogeneous nuclear RNA, which is known to be partly double stranded (15, 27). In both cases the profiles of the peaks were different from those from infected cells, but the background radioactivity was identical in infected and noninfected cultures.

Similar results showing that RF is undetectable in the absence of actinomycin D (M. R. Macnaughton, unpublished data) have been obtained with rhinovirus-infected human embryo lung cells.

Both RI and RF species are infectious (6), and we have shown that RI + RF and pRI from infected HeLa cells are infectious to similar degrees when titrated by plaque assay on monolayers of HeLa cells. This result shows that pRI is at least partially virus specified. The following experiments were performed to show that RI and pRI are essentially the same species.

Treatment of pRI with pancreatic RNase (50 μg/ml) and T, RNase (170 units/ml), which digest single-stranded but not double-stranded RNAs (1), converted pRI into a double-stranded RNA species with a mobility similar to RF (Fig. 2). A similar result is obtained on treating RI with RNase (17). This result is consistent with the view that pRI is like RI, which comprises a template molecule of the same size as the genome and is partly hydrogen bonded to nascent molecules that have single-stranded "tails." These tails are removed by RNase treatment, leaving a double-stranded structure of the same size as RF consisting of one discrete and one segmented molecule (6).

Characterization of pRI by hybridization. Multistranded and double-stranded RNAs prepared by CF11 cellulose chromatography from HeLa cells infected in the presence or absence of actinomycin D were used in these studies. We know by polyacrylamide gel electrophoresis that the preparations from actinomycin D-treated cultures contain only RI + RF and

![Fig. 2. Polyacrylamide gel electrophoresis of double-stranded and multistranded RNAs extracted from rhinovirus-infected HeLa cells without actinomycin D treatment. These RNAs were separated on cellulose CF11 columns from single-stranded RNAs. The RNAs were labeled with [3H]uridine from 6 to 8 h postinfection in the absence of actinomycin D. (A) Untreated RNA; (B) RNA incubated with 50 μg of pancreatic RNase per ml and 170 units of T, RNase per ml in 1 x SSC at 37 C for 20 min. The arrows are taken from the absorbance at 260 nm trace indicating the position of added marker HeLa DNA.](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
those from the untreated cultures contain only pRI. Table 2 shows the percentages of \(^3\)H-labeled RI + RF and pRI that self anneal or anneal with a large (50-fold) excess of unlabeled virion RNA. The data show that about 37% of RI + RF and about 21% of pRI hydridized to excess virion RNA and hence consist of viral complementary RNA. Thus, at least this proportion of the multistranded pRI consists of virus-specified RNA.

Assuming that the amount of labeled virion-type RNA present in the multistranded species exceeds the complementary RNA (see above), then, by definition, the amount of complementary RNA present is half the value that results from self annealing (RNase resistance counts per minute). One can thus derive the ratio of self-annealed RNA to the independent measurement of complementary RNA obtained by annealing with cold virion RNA. This value comes in all experiments close to the expected value of 2. This result shows that essentially all RI + RF and pRI are viral RNA species, since any contamination with cellular RNAs would have resulted in less annealing with unlabeled virion RNA and hence a ratio of self annealing to annealing with unlabeled virion RNA of less than 2.

The proportion of RNA complementary to virion RNA in RF is 50% (Macnaughton, unpublished data). Our preparation of multi- and double-stranded RNAs from actinomycin D-treated cells contain about equal proportions of RI and RF. By annealing the combined RI + RF with virion RNA, we find that the mixture contains 37% complementary RNA. Since this figure is less than 50%, we see that the majority of RNA in the RI is synthesized in the presence of actinomycin D of the same polarity as virion RNA. This result also argues that the proportion of viral complementary RNA in pure RI must be less than 37%. Hence our figure of 21% viral complementary RNA in pRI is consistent with it being a structure consisting largely of RNA of the same polarity as virion RNA. Our figure is close to the value of 19% complementary RNA reported for poliovirus RI (21).

**Inhibition of protein synthesis in reticulocyte lysates by double-stranded RNA.** We have estimated the amount of double strandedness in rhinovirus RI and RI + RF by measuring the inhibition of protein synthesis in reticulocyte lysates (9). Table 3 shows that there is considerably more inhibition of protein synthesis by RI compared to double-stranded RNA from uninfected HeLa cells, although this inhibition is less than for a mixture of RI + RF. Assuming that this inhibition is a measure of double strandedness (9, 14), then there is considerable double strandedness in RI.

**DISCUSSION**

During infection with picornaviruses, RI has been shown to be the intermediate precursor of progeny virus SS RNA. In vivo, it is the first RNA species to be labeled by brief exposure to radioactive precursors (3, 17), implying that it is the site of nascent RNA. Similarly, in vitro, using virus polymerase preparations, RI has been identified as the precursor of progeny SS RNA (12, 25). However, the role of RF in the synthesis of picornavirus RNA has never been readily understood, although it is now generally regarded as an end product of replication (2, 3, 12, 17), and evidence has accumulated showing that RF is a consequence of replication rather than an intermediate thereof (3, 22, 23). Nevertheless, RF has been shown to be infectious (6).

Our results show that in rhinovirus-infected cells not treated with actinomycin D, the only

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**Table 2. Annealing of \(^3\)H-labeled double-stranded and multistranded RNAs from rhinovirus-infected HeLa cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total counts/ min in viral RNA (a)</th>
<th>Annealing with excess unlabeled virion RNA: RNase resistance (counts/min) (c)</th>
<th>RNA complementary to virion RNA (%) (c/a)</th>
<th>Self-anealing RNase resistance (counts/min) (b)</th>
<th>Self-anealing RNase resistance (%) (b)</th>
<th>Ratio (b/c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation with actinomycin D (RI) + RF</td>
<td>14,621</td>
<td>5,760</td>
<td>39.4</td>
<td>10,311</td>
<td>70.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Incubation without actinomycin D (pRI)</td>
<td>34,079</td>
<td>11,849</td>
<td>34.8</td>
<td>22,579</td>
<td>66.3</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>28,089</td>
<td>10,725</td>
<td>38.2</td>
<td>18,960</td>
<td>67.5</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>17,414</td>
<td>4,009</td>
<td>23.0</td>
<td>7,791</td>
<td>44.7</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>10,772</td>
<td>1,991</td>
<td>18.5</td>
<td>3,943</td>
<td>36.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>52,386</td>
<td>11,762</td>
<td>20.5</td>
<td>20,861</td>
<td>39.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*The RI + RF and pRI were obtained from infected cells labeled 6 to 8 h postinfection with \(^3\)H\)uridine and separated from single-stranded RNAs by chromatography on CF11 cellulose.*
**TABLE 3. Inhibition of protein synthesis in reticulocyte lysates by double-stranded and multistranded RNAs from rhinovirus-infected and noninfected HeLa cells**

<table>
<thead>
<tr>
<th>RNA species*</th>
<th>Total counts/min in undiluted RNA</th>
<th>Reciprocal of RNA dilution producing 50% inhibition of protein synthesis</th>
<th>Uncorrected</th>
<th>Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI + RF from infected cells treated with actinomycin D (1 μg/ml)</td>
<td>1,126</td>
<td>720</td>
<td>1,280</td>
<td></td>
</tr>
<tr>
<td>RI from infected cells not treated with actinomycin D</td>
<td>3,916</td>
<td>820</td>
<td>420</td>
<td></td>
</tr>
<tr>
<td>Double-stranded RNA from uninfected cells treated with actinomycin D (1 μg/ml)</td>
<td>168</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Double-stranded RNA from uninfected cells not treated with actinomycin D</td>
<td>8,414</td>
<td>280</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

* RNA in each case was extracted from 8 × 10⁶ cells.

**RHINOVIRUS MULTISTRANDED RNA**

RI consists of genomic strands, and thus the greater part of this RI consists of positive RI. At present, we are trying to identify negative RI, which we would expect to be found during earlier stages of rhinovirus replication.

On infecting HeLa cells with poliovirus type 1 (strain LSc 2ab) in the presence and absence of actinomycin D (1 μg/ml), we have observed that poliovirus RF exists in the infected cells whether or not the cells had been treated with actinomycin D. Thus, in this case RF is not an artifactual structure caused by actinomycin D.

However, the effect of actinomycin D on poliovirus multiplication is known to vary with the strain of poliovirus used (7, 13, 28). Thus, although we are suggesting that during natural infections with rhinovirus type 2 no detectable RF is produced, this hypothesis appears not to hold for all picornaviruses.

Our results may have implications for certain aspects concerning the multiplication of other viruses, since double-stranded RNA molecules are suggested to have specific effects, such as the induction of interferon (32), cytopathic changes (11, 30), and inhibition of protein synthesis (8, 9, 14). We have shown by inhibition of protein synthesis in reticulocyte lysates with double-stranded RNA that there is considerable double strandedness in RI, and this may be enough to induce the effects currently ascribed to RF.

However, before considering the implications of these results further, we need to know how general are these phenomena and in particular to what extent the RFs of other picornaviruses depend on actinomycin D for their existence.

**ACKNOWLEDGMENTS**

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