Effect of Virus Infection on the Stability and Synthesis of Actinomycin-Resistant Ribonucleic Acid in Baby-Hamster Kidney Cells

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1. The sucrose-gradient pattern of ^{32}P -labelled RNA synthesized in actinomycin-treated baby-hamster kidney cells infected with foot-and-mouth-disease virus depends greatly on the period of labelling. 2. Fractions are formed in infected cells that sediment at 12–20s and have the same base composition as similar fractions found in non-infected cells that have been treated with actinomycin. 3. In the presence of guanidine, which completely inhibits viral RNA synthesis, these fractions are labelled to a greater extent than in non-infected cells.

Brown & Cartwright (1964) reported that three peaks of viral specific RNA were formed in BHK cells* after infection with FMDV. Similar patterns have also been found in extracts from cells infected with encephalomyocarditis virus (Dalgarno & Martin, 1965), Western equine encephalomyelitis virus (Sreevalsan & Lockart, 1966), Semliki Forest virus (Sonnabend, Friedman, Dalgarno & Martin, 1964) and Maus-Elberfeld virus (Roberts, Newman & Rueckert, 1966), and it now seems to be recognized that, as well as the synthesis of progeny-RNA strands and the ribonuclease-resistant replicative form, there is a third fraction present in infected cells. The origin and function of this 'interjacent' fraction remains to be elucidated, but it has been suggested that it may represent a precursor or random-coil form of the progeny RNA (Martin, 1966; Kelly, Gould & Sinsheimer, 1965). A common feature of all these studies is that the infection and labelling is carried out in the presence of actinomycin. With a biological system of this nature a practical difficulty inevitably arises in designing experiments so as to include satisfactory controls. The possibility always exists that the process of inhibition of RNA synthesis by actinomycin in infected cells may be different from that in cells treated with actinomycin alone.

In our earlier work on FMDV, ^{32}P labelling of the virus-induced RNA was carried out during periods of 7–8 hr. in cells maintained on phosphate-deficient medium. Under these conditions ribosomal RNA synthesis in non-infected cells was found to be completely inhibited. In more recent studies of the synthesis of RNA during the lag phase before new virus is produced, we have used

much shorter periods of treatment with phosphate-deficient medium and actinomycin. Under these conditions it was frequently found that in non-infected cultures two fractions of RNA were labelled, which were coincident with the slower-sedimenting peaks present in RNA extracts from infected cells. These observations stimulated the investigation reported in the preceding paper (Martin & Brown, 1967).

The present paper examines the problem of whether all the RNA molecules formed after actinomycin-treated cells are infected with FMDV are specified by the virus. The use of guanidine, which inhibits some strains of FMDV (Pringle, 1964), has helped to elucidate some of these problems.

METHODS AND MATERIALS

Cell cultures. BHK cells were grown as monolayers as described in the preceding paper (Martin & Brown, 1967) and were used as 48 hr. cultures.

Virus production and assay. Virus (strain Tur. 43/62 type SAT₁ or strain 1 type 0 of FMDV) were grown in monolayers of BHK cells as described by Brown, Martin & Underwood (1966).

Extraction, sedimentation and analysis of RNA. These were performed as described in the preceding paper (Martin & Brown, 1967).

Materials. Guanidine was obtained as the hydrochloride from L. Light and Co. Ltd., Colnbrook, Bucks. In the experiments reported guanidine was used at 500 $\mu\text{g./ml.}$ The sources of [^{32}P]phosphate and actinomycin D were as described in the preceding paper (Martin & Brown, 1967).

RESULTS

Effect of viral infection on [^{32}P]phosphate incorporation into RNA of BHK cells grown in the presence of actinomycin. (a) At 7 hr. after infection.

*Abbreviations: BHK cells, baby-hamster kidney cells; FMDV, foot-and-mouth-disease virus.

Fig. 1. Sucrose-gradient centrifugation of RNA extracted from cells 7 hr. after infection with FMDV. The RNA was eluted from Sephadex G-200 columns and centrifuged in gradients (5–25% sucrose in 0.1M-acetate buffer, pH 5.0) for 14 hr. at 4° in a SW25 rotor of a Spinco model L ultracentrifuge at 20000 rev./min. (42000g): —, ^{32}P counts/min. after labelling for 7 hr. after infection with virus in the presence of actinomycin; ----, ^{32}P counts/min. after labelling non-infected cells for 7 hr. in the presence of actinomycin;, E_{260} .

Fig. 2. Sucrose-gradient centrifugation of RNA extracted from cells 150 min. after infection with FMDV. The RNA was eluted from Sephadex G-200 columns and centrifuged in gradients as described in Fig. 1. —, ^{32}P counts/min. after labelling in the presence of actinomycin for 150 min. after infection; ----, ^{32}P counts/min. after labelling uninfected cells for 150 min. in the presence of actinomycin;, E_{260} .

BHK monolayers were infected with virus of strain 1 type 0 at a multiplicity of 100 plaque-forming units/cell, which, under the conditions used, caused destruction of the cell sheets after 7–8 hr. The cells were treated with actinomycin in phosphate-deficient medium containing [^{32}P]phosphate throughout the infection. Non-infected cultures were also treated with actinomycin and [^{32}P]phosphate under the same conditions. At 7 hr. after infection the tissue was extracted with phenol and the ethanol precipitate of the aqueous layer was filtered through Sephadex G-200 columns.

Sucrose-gradient analyses of the RNA from non-infected and infected cells are shown in Fig. 1. As described in the preceding paper (Martin & Brown, 1967) during prolonged treatment with nutritionally poor medium there is no significant incorporation of ^{32}P into the high-molecular-weight

RNA of control cells. The profile of ^{32}P radioactivity in the extracts from infected cells shows three peaks. The activity is predominantly at the 37s position, corresponding to viral RNA, but two smaller peaks are obvious between 20s and 12s values. FMDV of another immunological type (SAT₁) gave similar profiles. These results are consistent with previous reports by Brown & Cartwright (1964) and Brown & Martin (1965) that infection results in the formation of at least three RNA fractions separable by sucrose-gradient centrifugation. Ribonuclease treatment of this RNA before sedimentation degrades the 37s RNA completely, but a resistant fraction is present in the 16s position (Brown & Cartwright, 1964).

(b) At 150 min. after infection. When monolayers are infected with SAT₁ virus at a multiplicity of 100:1, maximum virus titre is achieved 3 hr. after infection. Cells were infected and subsequently treated with actinomycin and [^{32}P]-

Fig. 3. Sucrose-gradient centrifugation of RNA extracted from infected and non-infected cells after various labelling periods. The RNA was eluted from Sephadex G-200 columns and centrifuged in gradients as described in Fig. 1. —, ^{32}P counts/min. after labelling infected cells for 135 min.; ---, ^{32}P counts/min. after labelling infected cells for 105 min.; - · - ·, ^{32}P counts/min. after labelling non-infected cells for 105 min.; - · - · ·, ^{32}P counts/min. after labelling non-infected cells for 135 min.; · · · ·, E_{260} .

phosphate in phosphate-deficient medium for 150 min. from the time of infection. The cells were extracted with phenol and the RNA was purified by filtration through Sephadex G-200 and sucrose-gradient analysis.

Fig. 2 shows the profiles of ^{32}P radioactivity of RNA from infected and non-infected cultures. During this shorter period of exposure to nutritionally poor medium and actinomycin, as shown in the preceding paper (Martin & Brown, 1967), there is incorporation of ^{32}P into RNA that sediments in the 12–20s region of the gradient. Virus infection under these conditions produces, as well as the 37s RNA, more pronounced fractions in the 12–20s position. The total ^{32}P radioactivity in the gradients can be compared directly, as the radioactivities from different gradients have been correlated with the E_{260} values. From a number of experiments it appears to be significant that the relative amount of radioactivity in this region is greater in the infected cells than in RNA from control cultures.

Stabilization of actinomycin-resistant RNA by virus infection. The following experiment was

performed to show the effect of virus infection on the formation of actinomycin-resistant RNA at times before the appearance of new progeny viral RNA. Replicate monolayers of BHK cells in 4 oz. bottles were used in duplicate. Infected and non-infected cells were incubated with phosphate-deficient medium plus actinomycin and [^{32}P]-phosphate for 105 min. Cultures were then removed at 15 min. intervals and the RNA was extracted with phenol and purified by filtration through Sephadex G-200.

Fig. 3 shows the sucrose profiles of RNA extracted from infected and non-infected cells after 105 min. and 135 min. labelling periods. In non-infected cells the amount of ^{32}P incorporated decreases with the time of exposure to phosphate-deficient medium. In contrast, however, there is an increase in the amount of these fractions present when cells have been infected with virus. These observations could be explained in two ways. First, the increase in radioactivity in the RNA from infected cells could suggest that synthesis of virus-induced RNA was taking place. On the other hand, it is possible that the degradation process that seems to be occurring in control cultures is inhibited after infection.

Effect of guanidine on the replication of SAT₁ virus. Guanidine is a potent inhibitor of certain strains of picornaviruses. Pringle (1964) and Brown *et al.* (1966) have shown that it can be used to inhibit the replication of the SAT₁ strain of FMDV. In the present experiments guanidine was used at a concentration of 500 $\mu\text{g./ml.}$ and the yield of virus was inhibited by 99.8%, since the number of plaque-forming units/ml. was decreased from about 1×10^8 when guanidine was absent to 2.5×10^5 or less when guanidine was present.

Effect of guanidine on the synthesis of actinomycin-resistant RNA. Monolayers of BHK cells in 4 oz. bottles were treated with actinomycin in phosphate-deficient medium with or without guanidine. [^{32}P]-Phosphate was added to each bottle 15 min. after addition of the phosphate-deficient medium, and incubation at 37° continued for 3 hr. The cells were extracted with phenol and the RNA was purified by filtration through Sephadex G-200.

The sucrose-gradient-sedimentation profiles (Fig. 4) show that RNA from guanidine-plus-actinomycin-treated cells is very similar to that formed in cells treated with actinomycin alone.

Effect of guanidine on the synthesis of viral RNA. Monolayers of BHK cells in 4 oz. bottles were infected with virus in the presence or absence of guanidine. All cultures were treated with actinomycin. Labelling with ^{32}P was continued for 150 min. in phosphate-deficient medium supplemented with actinomycin with or without guanidine. The cells were extracted with phenol and

Fig. 4. Sucrose-gradient centrifugation of RNA extracted from non-infected cells treated with guanidine. The RNA was eluted from Sephadex G-200 columns and centrifuged in gradients as described in Fig. 1. —, ^{32}P counts/min. after labelling for 3 hr. in the presence of actinomycin; ----, ^{32}P counts/min. after labelling for 3 hr. in the presence of actinomycin plus guanidine ($500\text{ }\mu\text{g./ml.}$);, E_{260} .

Fig. 5. Sucrose-gradient centrifugation of RNA extracted from infected and non-infected cells that had been treated with guanidine. The RNA was eluted from Sephadex G-200 columns and centrifuged in sucrose gradients as described in Fig. 1. —, ^{32}P counts/min. after labelling for 150 min. after infection with FMDV in the absence of guanidine; ----, ^{32}P counts/min. after labelling for 150 min. after infection in the presence of guanidine; ^{32}P counts/min. after labelling non-infected cells for 150 min. in the absence of guanidine. Actinomycin was present in all cultures., E_{260} .

the RNA was purified by filtration through Sephadex G-200.

Fig. 5 shows that the presence of guanidine inhibits the formation of viral progeny RNA (37s), but similar radioactivities were recorded in the 20–12s region of the gradients, which were significantly greater than the radioactivity of the RNA from non-infected cells. The results demonstrate that guanidine, when present throughout the period of infection, completely suppressed the formation of virus RNA. The proportion of the ^{32}P radioactivity in the 20–12s fractions increased compared with the non-infected cells.

This increase in the 20–12s fraction after infection indicates that the actinomycin-resistant RNA is not degraded as it is in non-infected cells, even though new viral RNA is not formed. It does not, however, eliminate the possibility that some viral induced RNA is formed in the presence of guanidine and is present in this fraction.

Effect of ribonuclease on viral and actinomycin-resistant RNA. The profiles obtained when RNA preparations described in the previous section were treated with $0.01\text{ }\mu\text{g.}$ of ribonuclease/ml. before sedimentation are shown in Fig. 6. RNA from both the control and guanidine-plus-virus-treated cells is completely degraded to low-molecular-weight fractions. In the virus RNA there is a ribonuclease-resistant fraction that sediments at approx. 16s.

The ability of guanidine to suppress the synthesis of viral specific ribonuclease-resistant structures indicates that the increase in ^{32}P incorporation into RNA sedimenting in the 20–12s region of gradients is most probably due to the stabilization of the actinomycin-resistant RNA that is formed in non-infected cells.

Fig. 6. Sucrose-gradient centrifugation of RNA shown in Fig. 5 after treatment with $0.01 \mu\text{g.}$ of ribonuclease/ml. The gradient conditions were described in Fig. 1 and centrifugation was carried out at 25000 rev./min. (65000g) for 14 hr. —, ^{32}P counts/min. after treatment of virus-induced RNA with ribonuclease; ----, ^{32}P counts/min. after treatment of RNA formed in the presence of virus plus guanidine with ribonuclease; -.-.-, ^{32}P counts/min. after treatment of RNA formed in the presence of actinomycin;, E_{260} after treatment with ribonuclease.

Characteristics of RNA present in cell debris after release of virus 12 hr. after infection. Monolayers were infected with a low multiplicity (0.1 plaque-forming unit/cell) of virus and incubated for 12 hr. in the presence of actinomycin, phosphate-deficient medium and [^{32}P]phosphate. By this time the cell sheet had been completely disrupted and was easily shaken into the medium. This is the procedure we usually adopt in preparing virus harvests for the preparation of radioactively labelled virus. The suspension was centrifuged at 1000 rev./min. in an MSE bench centrifuge for 5 min. to deposit the cell debris, and the supernatant was removed. The supernatant contains over 99% of the virus. The cell debris, which is generally discarded at this stage, was washed once with phosphate-buffered saline and then extracted with phenol as already described for the treatment of whole cells. The aqueous layer was mixed with 2 vol. of ethanol and the resulting precipitate dissolved in 0.1 M-acetate buffer, pH 5.0, and filtered through a column of Sephadex G-25. The first radioactive peak emerging

Fig. 7. Sucrose-gradient centrifugation of RNA extracted from the cell debris remaining 12 hr. after infection with FMDV. The RNA was eluted from Sephadex G-25 columns. Sucrose-gradient conditions were described in Fig. 1. (a) —, ^{32}P counts/min. present in RNA extracts of cell debris after destruction of monolayers by virus infection. The gradients were centrifuged at 20000 rev./min. (42000g). (b) ^{32}P radioactivity associated with the high-molecular-weight RNA shown in (a) (fractions 10–18) was precipitated with 2 vol. of ethanol along with BHK-cell ribosomal RNA, and recentrifuged at 24000 rev./min. (62000g). —, ^{32}P counts/min. (c) Individual fractions (6, 10 and 14) from the gradient shown in (b) were precipitated with 2 vol. of ethanol and BHK-cell RNA and centrifuged again at 25000 rev./min. (65000g). ----, Fraction 6; —, fraction 10; -.-.-, fraction 14 (^{32}P counts/min.). In (a), (b) and (c), represents E_{260} .

from the Sephadex was analysed by sucrose-gradient centrifugation. Fig. 7(a) shows the profiles of ^{32}P radioactivity in the RNA extracted from the cell debris remaining 12 hr. after infection. The activity is predominately associated with two peaks in the 20s and 16s positions, relative to ribosomal RNA. To obtain a better separation of these fractions another gradient was centrifuged at higher speeds (24000 rev./min.; 62000g) and

Table 1. *Base composition of RNA*

The RNA was prepared and fractionated as described in the text, and analyses were carried out in duplicate as described by Martin & Brown (1967).

Treatment of cells and source of 12-20s RNA	Base composition (% of ^{32}P radioactivity)			
	Adenosine	Uridine	Guanosine	Cytidine
Cells plus actinomycin	26.1	22.5	31.0	20.3
Cells + actinomycin and guanidine	26.4	23.2	30.6	19.8
Cells infected for 14 hr. with FMDV	26.8	21.7	29.7	21.6
Cells infected for 3 hr. in the presence of guanidine	25.6	23.7	31.5	19.0
Cell debris after 12 hr. infection	26.8	20.4	29.9	22.8
37s Viral RNA	26.8	21.8	23.6	28.8

the profiles shown in Fig. 7(b) confirm that at least two distinct fractions are present. Further, recycling of the individual fractions showed that the fractions retained their initial positions with regard to ribosomal RNA (Fig. 7c).

Base composition of RNA. RNA from ^{32}P -labelled cells has been analysed for the distribution of radioactivity in the four nucleotides adenosine, cytidine, guanosine and uridine. Table 1 compares the base ratios of the various fractions studied in the present paper. As reported in the preceding paper (Martin & Brown, 1967) the RNA formed in the presence of actinomycin is characterized by a high guanosine content with low amounts of cytidine and uridine. Four further points may be made about the results in Table 1. (1) The presence of guanidine does not alter the composition. (2) The RNA formed in infected cells at short periods after infection, before the appearance of 37s viral RNA, has similar base ratios to the RNA in the non-infected cells. (3) The RNA extracted from cell debris remaining after prolonged exposure of tissue to virus also has ratios similar to those found in non-infected cells. The presence in cells 12 hr. after infection of RNA that has a base composition identical with that of the labile RNA formed in control cells supports the view that some cellular RNA may be stabilized by the infective process. (4) Finally, the composition of RNA formed in the presence of virus plus guanidine is identical with that in the control cells. This would seem to eliminate the possibility that the increase in radioactivity of this RNA is due entirely to virus-specific synthesis.

DISCUSSION

Brown & Cartwright (1964) and Brown & Martin (1965) reported that three peaks of virus-specific RNA were found in BHK cells after infection with FMDV. These earlier experiments were carried out under conditions with long periods of labelling during which no incorporation of ^{32}P occurred in

uninfected actinomycin-treated BHK cells. Thus the earlier conclusions that multiple species of RNA were formed after infection seemed at that time justified. The evidence presented in the present paper, however, makes it imperative to reassess the significance of the various RNA fractions formed in infected cells that have been treated with actinomycin.

Inhibition of viral replication by guanidine succeeds in inhibiting only two detectable RNA fractions, mainly the 37s viral progeny RNA and the 16s ribonuclease-resistant RNA, which corresponds to a duplex form of the viral RNA (Brown & Martin, 1965). The remaining RNA fractions, which are synthesized in the presence of guanidine plus virus, are identical in sedimentation behaviour and base composition with the actinomycin-resistant RNA found in non-infected cells, and it would therefore seem unlikely that they are directly coded for by the ingoing viral RNA strand. As shown in the preceding paper (Martin & Brown, 1967), the actinomycin-resistant RNA is degraded on prolonged exposure to nutritionally poor medium. The present results suggest that infection by virus, either in the presence or in the absence of guanidine, causes a stabilization or inhibition of this degradation process.

It has previously been suggested that actinomycin-resistant RNA synthesis may represent metabolically active messenger RNA synthesis (Roberts & Newman, 1966). Although the present work does not define precisely the function or origin of this RNA in BHK cells one may note the effect that virus infection can have on it. If it does in fact represent messenger RNA, the present experimental system would point to a useful approach to the investigation of the effect of viral infection on cellular messenger RNA.

Picornaviruses cause rapid inhibition of protein synthesis in infected cells and this process requires the action of the ingoing viral RNA strand. The rate at which host-cell protein synthesis is inhibited by viral infection is much more rapid than the

inhibition of cellular protein synthesis caused by actinomycin treatment (Franklin & Baltimore, 1962; Penman, Scherrer, Becker & Darnell, 1963), suggesting that the inhibition of cellular protein synthesis by the virus is not only due to suppression of messenger RNA synthesis. Host-cell protein synthesis is also rapidly inhibited by FMDV or poliovirus even when viral RNA replication is prevented by guanidine (Brown *et al.* 1966; Holland, 1964; Bablanian, Eggers & Tamn, 1965; Penman & Summers, 1965), and Willems & Penman (1966) have shown that it is possible that virus inhibits host-cell protein synthesis by inactivating the cellular messenger RNA, so that it is incapable of associating with new ribosomes.

In this context it may be found that the stabilization of the actinomycin-resistant RNA by virus infection may be linked with the rapid inhibition of host-cell protein synthesis. On the basis of the model proposed by Willems & Penman (1966) these results may represent the stabilization of messenger RNA on single ribosomes with a concomitant loss of protein-synthesizing activity. This is in contrast with the observation in the preceding paper (Martin & Brown, 1967) that degradation of the RNA occurred after inhibition of protein synthesis by cycloheximide.

Finally, the present results point to the difficulty of deciding, when actinomycin is used, whether all the RNA components formed after infection are in fact virus-specific. Our present studies with FMDV infection of BHK cells shed doubt on our previous

claims that at least three virus-specific peaks are produced. Whether or not this situation exists in other virus-cell systems remains to be ascertained.

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