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Abortive Infection of L Cells by Influenza Virus: Absence of Virion RNA Synthesis

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Influenza virus multiplies productively in chick cells and abortively in L cells. The infecting influenza virus RNA genomes are less stable in infected L cells than in infected chick cells. However, transcription of the virus genome in L cells, while reduced in rate, is not decreased in extent. There is no detectable synthesis of virion RNA in L cells, and this is the most likely cause of the abortive infection.

Certain mammalian cell lines (4, 7, 10, 15) are unable to support the growth of an infecting influenza virus, as judged by the lack of production of infectious progeny. The virus must enter these cells as the synthesis of virus-specified macromolecules has been demonstrated (7, 10, 15). Thus, the nonpermissive state cannot be attributed to the lack of appropriate cell surface receptor sites.

To elucidate the nature of the intracellular block in an abortive infection, I have chosen to study the infection of L cells with influenza virus. Evidence has been obtained (7) that some virus proteins are synthesized in L cells infected with influenza virus, but it has not been shown whether this synthesis includes all the proteins now known to occur in productively infected cells.

The fact that at least some virus proteins are made during abortive infection implies that at least some virus mRNA synthesis must also occur. It is important to ask whether all species of influenza virus RNA are found in abortively infected cells, particularly since control of transcription has recently been demonstrated in productively infected cells (1a). Ghandi et al. (7) have reported that RNA pulse-labeled in the presence of actinomycin D during abortive infection of L cells has a very similar electrophoretic profile to that synthesized during productive infection of chick cells. However, it is now well established (1, 13) that the synthesis of two types of virus-specific RNA occurs in cells productively infected with influenza virus. These are either of identical (virion RNA) or complementary (cRNA) nucleotide sequence to the virus genome. The bulk of the available evidence (5, 9, 12) suggests that cRNA acts as influenza virus mRNA in infected cells. Thus, it is clearly important to distinguish between these two RNA types in abortively infected cells.

In this report I describe experiments designed to answer two questions concerning influenza virus RNA synthesis in L cells. (i) Is transcription of the influenza genome into mRNA as complete as in productively infected chick cells? (ii) How does the synthesis of virion RNA compare with that found in productively infected chick cells?

MATERIALS AND METHODS

Viruses. Two influenza viruses, the Rostock strain of fowl plague virus [A/FPV/ROSTOCK/34 (Hav_1N_1)] and a recombinant virus between A/FPV/DUTCH 27 (Hav_1 Neq_1) and AP/BEL/42 (H_0N_1) (designated FPV/BEL; 14), were used. Stocks of both viruses were prepared by growth in embryonated eggs and purified as previously described (8).

Preparation of ^{32}P-labeled virus. ^{32}P-labeled FPV/BEL was prepared essentially according to the method devised by Stephenson and Dimmock (14). Twelve-day-old de-embryonated eggs were prepared as described by Bernkopf (3). Standard medium (6) was added, and each egg was inoculated with 0.1 ml of FPV/BEL virus containing approximately 10^4 PFU. The open end of the egg was sealed with aluminium foil and wax. Eggs were labeled from the start of incubation with 500 μCi of ^{32}P and incubated in a roller drum overnight at 37 C. Medium from the eggs was clarified by centrifugation at 1,500 $\times$ *g* for 10 min at 4 C, and then virus was precipitated by stirring at 4 C for 30 min in 60% saturated ammonium sulfate in phosphate-buffered Earle saline. After centrifugation at 18,000 $\times$ *g* for 30 min, the viral pellet was resuspended in 4 ml of phosphate-buffered saline–Ca/Mg (phosphate-buffered saline containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$). The suspension was loaded onto a 60-ml 15 to 45% linear sucrose gradient containing 0.1% bovine serum albumin and centrifuged at 60,000 $\times$ *g* for 1 h at 20 C. The gradient was fractionated into 2-ml aliquots and assayed for hemagglutination and total radioactivity. Where these

activities formed a coincident peak (normally half-way down the gradient), these fractions were pooled. The virus was precipitated with ammonium sulfate as before, resuspended in phosphate-buffered saline–Ca/Mg, and dialyzed overnight at 4 C against the same buffer solution.

Cells. Primary chicken embryo cells were prepared and cultured as described elsewhere (11).

L929 cells were obtained from I.C.I. Ltd. (Alderley Edge). Growth of L cells was carried out in bicarbonate-buffered Eagle medium (Flow Laboratories) containing 7% fetal calf serum (Gibco-Biocult).

Growth of virus in cells. Chick cells or L cells (both 2×10^7 cells/14-cm petri dish) were infected with 1 ml of purified ^{32}P-labeled FPV/BEL containing approximately 5×10^2 hemagglutination units, 10^7 PFU, and 10^5 counts/min of ^{32}P. The virus was allowed to absorb for 45 min at room temperature. Unabsorbed virus was removed, and cells were washed with, and subsequently incubated in, maintenance medium at 37 C. Chick cell maintenance medium consisted of 2% calf serum in medium 199 buffered with bicarbonate. Infected L cells were maintained in the medium described above. At the end of the required growth period, cells were scraped off the petri dishes and washed twice with phosphate-buffered saline.

In other experiments L cells in 14-cm petri dishes (2×10^7 cells/dish) were infected with fowl plague virus at a multiplicity of 20 PFU/cell. Absorption and growth in maintenance medium at 37 C were as described above. At various times postinfection, this medium was discarded and replaced by 10 ml of bicarbonate-buffered Earle medium containing 5% dialyzed calf serum. One hour later [^{3}H]uridine (200 μCi/plate) was added, and cells were harvested after a further hour.

Preparation of RNA. RNA was purified from virus and infected cells as described elsewhere (1).

RNA-RNA hybridization. All RNA samples to be hybridized were dissolved in 0.04× SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and processed as described previously (1), except that T_1 RNase was used at a concentration of 850 U/ml.

RESULTS

Stability of infecting viral genomes. ^{32}P-labeled influenza virus, prepared and purified as described in Materials and Methods, was used to infect chick or L cells at a multiplicity of infection of 0.5 PFU/cell. At various times postinfection cells were harvested and RNA was prepared. Table 1 shows the trichloroacetic acid-insoluble radioactivity extracted in two such experiments. In chick cells there is a constant amount of trichloroacetic acid-insoluble radioactivity. In L cells, however, the radioactivity becomes increasingly soluble.

Decrease in trichloroacetic acid solubility to 20 to 30% of the starting value was seen by 6 to 8 h postinfection in all L-cell experiments. In some cases, however, the decrease did not commence until 3 to 4 h postinfection.

TABLE 1. *Stability of infecting ^{32}P-labeled influenza virus RNA*

Hours post-infection	Trichloroacetic acid-insoluble radioactivity (counts/min)	
	Chick cells	L cells
1	4,164	1,465
2	4,228	1,114
3	4,060	873
4	3,838	713
5	3,928	431
6	4,034	ND[a]

[a] ND, Not determined.

Transcription of infecting virus genomes. The extent of transcription of influenza virus RNA in chick or L cells was measured by the technique first described by Bean and Simpson (2).

This involves infecting cells with virus containing radioactively labeled RNA and subsequently extracting total intracellular RNA. As more of the genome is transcribed into cRNA, more of the input radioactivity can be made RNase resistant by annealing the RNA extracts. Thus, if the entire genome is transcribed, it is theoretically possible to convert 100% of the input counts into an RNase-resistant form.

Purified RNA preparations extracted from chick cells at various times after infection with ^{32}P-labeled virus were annealed as described above. The RNase resistance of the ^{32}P radioactivity after annealing is shown in Fig. 1 as a function of time postinfection.

A maximum of about 70% of the input genome can be made RNase resistant by annealing the sample prepared at 2 h postinfection. Subsequently, the amount of annealing obtained decreased with time.

An identical experiment was performed on L cells infected with ^{32}P-labeled virus, and the results are shown in Fig. 2. RNase resistance rose more slowly than in chick cells and reached a maximum at 4 h postinfection. The time of maximum annealing varied between experiments, but the RNase resistance always rose more slowly and reached a maximum later than in chick cells. The level of maximum annealing attained with L cells was very similar to that obtained with chick cells.

Virus RNA synthesis in infected cells. If radioactive uridine is added to cells infected with virus, both cellular and viral RNA will be labeled. Labeled RNA which is specified by the virus can be identified by annealing procedures. Thus, labeled virus cRNA can be detected by its

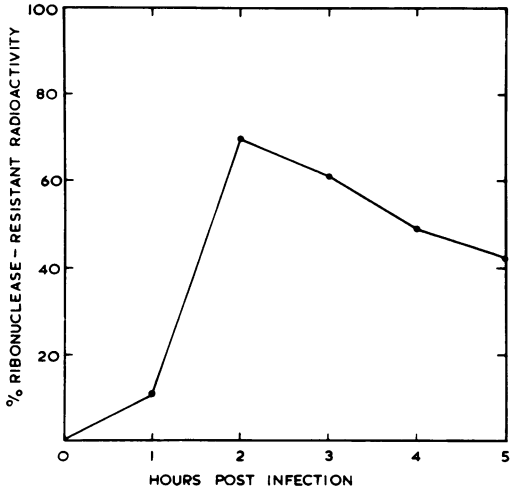

FIG. 1. *Transcription of the influenza genome in infected chicken embryo fibroblasts. Cells were infected with virus containing ^{32}P-labeled RNA, and total intracellular RNA was extracted at various times postinfection. RNA preparations were annealed, treated with RNase, and precipitated with ice-cold 5% trichloroacetic acid to determine the proportion of labeled trichloroacetic acid-insoluble RNA in a double-stranded form.*

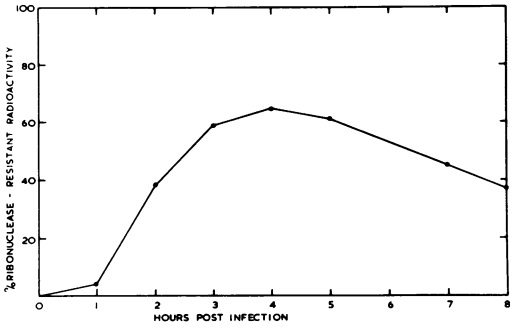

FIG. 2. *Transcription of the influenza genome in infected L cells. Cells were infected with virus containing ^{32}P-labeled RNA, and total intracellular RNA was extracted at various times postinfection. RNA preparations were annealed, treated with RNase, and precipitated with ice-cold 5% trichloroacetic acid to determine the proportion of labeled trichloroacetic acid-insoluble RNA in a double-stranded form.*

ability to form RNase-resistant hybrids with added unlabeled virion RNA.

L cells infected with fowl plague virus were labeled with [^{3}H]uridine for periods of 1 h at various times after infection. RNA was extracted and annealed with unlabeled virion RNA (40 μg). Table 2 shows the results of this experiment and of the same experiment carried out in chick cells.

Small amounts of cRNA can be detected in L cells in the presence of a large excess of labeled cellular RNA.

The proportion of cRNA is similar to that found in productively infected chick cells and shows a similar decrease at later times postinfection.

Having established the presence of cRNA in L cells, annealing methods can be used to identify intracellular virion RNA by self-annealing in the absence of added virion RNA. This procedure will only produce RNase-resistant material if virion RNA is present in the cells. Cellular RNA will not self-anneal, as transcription is asymmetric within any gene.

The self-annealing of RNA extracted from L cells at various times postinfection is shown in Table 3. The equivalent data from chick cells are included for comparison. The RNase resistance of the L-cell sample was always less than 1%, whereas a maximum value of 15% was found in infected chick cells.

TABLE 2. *Annealing of infected cell RNAs with unlabeled virion RNA*

Hours post-infection	RNase-resistant radioactivity (%)	
	Chick cells[a]	L cells[b]
1		12.9
2		8.2
2.5	4.7	
3		8.2
4	8.4	7.8
5	9.4	7.1
6	3.7	7.6
7	3.3	4.3
8	3.2	4.8

[a] Data taken from reference 1; 10^4 counts/min per sample.

[b] 5×10^3 counts/min per sample.

TABLE 3. *Self-annealing of infected cell RNAs*

Hours post-infection	RNase-resistant radioactivity (%)	
	Chick cells[a]	L cells[b]
1		0.9
2		0.8
2.5	4.2	
3		0
4	10.9	0.6
5	15.3	0
6	7.0	0
7	6.5	0.2
8	6.1	0.2

[a] Data taken from reference 1; 10^4 counts/min per sample.

[b] 5×10^3 counts/min per sample.

DISCUSSION

Several differences are apparent when the process of influenza infection in L cells is compared to that in chick cells. Firstly, the infecting virus genome is considerably less stable in L cells than in chick cells, as indicated by the decrease in trichloroacetic acid-insoluble radioactivity shown in Table 1. This decreased stability could be explained in a number of ways, including the following. (i) L cells possess RNases, not found in chick cells, which are capable of degrading influenza virus RNA, or (ii) influenza virus RNA exists in a less protected form in L cells, perhaps associated with less protein. Whatever the cause of this decreased stability, it does not prevent transcription of the influenza genome. Figure 2 shows that a maximum of about 65% of the infecting genome can be made RNase resistant on annealing, so that transcription of at least this proportion of the genome has occurred. However, comparison of the data obtained in the two cell types (Fig. 1 and 2) shows that the rate of transcription is apparently decreased in L cells. This may result from either a decreased rate of synthesis or an increased rate of degradation of cRNA. The reduced rate of transcription accounts for the reduced rate of protein synthesis observed in L cells (7).

In one important respect Fig. 1 and 2 do not differ markedly. Thus, in both figures the maximum annealing obtained was very similar. This indicates that the extent of transcription of the influenza genome in L cells is not significantly less than in chick cells. Consequently, there is no evidence to suggest switch-off of synthesis of any class of mRNA in L cells.

Table 3 shows the results of a typical experiment designed to detect synthesis of intracellular virion RNA by self-annealing of infected cell RNAs. Whereas chick cell RNAs showed considerable self-annealing, that obtained for L cells RNAs was essentially zero after 4 h of infection. Prior to this, small ($<1\%$) amounts of annealing were detectable. This probably represents annealing of labeled cRNA with input unlabeled virion RNA. This suggestion is reinforced by the disappearance of this annealing with increasing time postinfection, coincident with the degradation of input virion RNA shown in Table 1. If intracellular virion RNA synthesis were occurring, it would be expected to be at a maximum at later times. At such times self-annealing of RNA extracted from L cells is at a minimum. I conclude from these results that RNA of the sense found in influenza virus particles is not synthesized in L cells, and that this is the prime cause of the lack of production of infectious virus by these cells. The molecular basis of this deficiency remains to be elucidated.

Finally, it may be noted that abortively infected L cells may well prove useful for the study of transcription of influenza virus mRNA in a system where virion RNA synthesis is absent.

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