Role of Lysine in the Replication of Reovirus

I. Synthesis of Complete and Empty Virions

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Lysine is essential for the replication of infectious reovirus. Omission of lysine from the extracellular medium not only permitted the continued synthesis of structural viral proteins and viral double-stranded ribonucleic acid (RNA), but also caused an enhanced formation of viral structures which were separable by isopycnic sedimentation of CsCl into a top band consisting of empty particles with a buoyant density of 1.29 g/cm³ and essentially free of viral RNA, and two lower bands which were difficult to resolve and had an average buoyant density of 1.37 g/cm³. The lower bands contained most of the viral nucleic acid. The above effects were reversed when lysine was restored early after infection. In contrast, a single band with a buoyant density of 1.38 g/cm³ was obtained from lysine-plus infected cells.

The requirement for the amino acid arginine in the replication of several animal virions has been reported (2, 11). For both the adenovirus and the herpes simplex virus, arginine was found to be essential for viral maturation (1, 6). No other amino acid has been determined to play such a vital role as that exhibited by arginine. The present report concerns the role of another amino acid lysine in the replication of reovirus and its effects on the synthesis of viral structural proteins, viral ribonucleic acid (RNA), and the formation of complete virions.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown as monolayers in Eagles basal medium (EBM) supplemented with 10% fetal calf serum. Stock cultures of reovirus type 2 (D-5 Jones) were prepared in HeLa cells. The procedures for reovirus infection of cells have been described (4). Virus titration was performed by the immunofluorescent assay technique (9). The titer of the stock virus was 1.8×10^8 to 2.5×10^8 infectious units (IU) per ml.

Purification of virion. For purification of reovirus, the infected cells were harvested after 30 hr at 37°C. The cells were frozen and thawed twice, and virion and cell debris were concentrated by centrifugation at $78,000 \times g$ for 2.5 hr. The pellet was then resuspended in Dulbecco's phosphate buffered saline (pH 7.2) without Ca²⁺ (D-PBS). The virion-cell preparation was extracted with fluorocarbon, Genetron 113 (Allied Chemical Co.), and the virion-containing aqueous layer was recovered. The virion was concentrated by overlaying the fluorocarbon-treated suspension on to a cushion of CsCl (1.39 g/cm³) and centrifuged at $64,000 \times g$ in SW25 rotor for 1 hr. The band containing reovirus was collected and rebanded in CsCl (1.37 g/cm³; 33,000 rev/min for 24

hr). The band of virion was collected and dialyzed against D-PBS. The virion had a density in CsCl solution of 1.38 g/cm³.

Preparation of radioactive virus. The preparation of structural proteins of reovirus labeled with either a reconstituted mixture of ³H-labeled amino acids or ³H-leucine has been described (5). Reovirus preparations dually labeled with ³H-leucine and ¹⁴C-uridine were prepared in HeLa cell cultures by the procedures described for the preparation of singly labeled virion (5). Briefly, monolayers of the HeLa cells were infected with reovirus at a multiplicity of 10 IU per cell. At the end of the adsorption period the cultures were washed, and fresh EBM without leucine, supplemented with 0.1% fetal calf serum, ³H-leucine (1 μc/ml), and ¹⁴C-uridine (0.1 μc/ml), was introduced. After 30 hr of incubation virion yields were harvested and purified.

Solubilization of virus. Radioactive-labeled purified virus preparations were degraded by treatment with 2% sodium dodecylsulfate (SDS), 1% 2-mercaptoethanol (ME), and 8 M urea for 2 to 3 hr at room temperature. The samples, dialyzed for 2 to 3 hr against 0.1% SDS, 0.1% ME, and 8 M urea in 0.01 M phosphate buffer (pH 7.2), were then applied to polyacrylamide gels.

Polyacrylamide-gel electrophoresis. Gels were made as described previously (10) and consisted of 10% acrylamide, 0.26% N,N'-bis-methylene acrylamide, 0.1% SDS, and 0.1 M phosphate buffer (pH 7.2). Radioactive samples of virus protein in 0.05 to 0.2 ml of buffer containing 10 to 15% sucrose were electrophoresed for 24 hr at 4 mamp per gel at 20°C. After electrophoresis the gels were frozen, sliced, solubilized in H₂O₂, and counted (8).

RESULTS

Effect of deletion of lysine in the formation of reovirus. To determine the role of lysine, arginine,

and leucine on the production of reovirus, HeLa cell cultures infected with reovirus type 2 at multiplicities of 10 to 15 IU/cell were maintained under medium from which these individual amino acids were singly omitted. Maximal viral yields were determined at 30 hr after infection. Representative results can be found in Table 1.

Whereas the omission of leucine and arginine caused an 82 to 99% inhibition of virion production, the deletion of lysine resulted in a smaller inhibition of virion yields (55 to 82%).

Kinetics of virion production. Since the omission of lysine may have caused a delay in the attainment of maximum titers, the production of infectious virion under conditions of a single cycle of growth in the presence and absence of lysine was examined.

Monolayers of HeLa cells were infected with approximately 10 IU of reovirus type 2 per cell. After a 2-hr adsorption period at 37 C, the cultures were washed and experimental media was added. At various intervals duplicate cultures were removed and the total amount of virions produced was determined.

Under the conditions used in these experiments, a characteristic one-step growth curve of reovirus type 2 in HeLa cell monolayers maintained in complete medium (L^+) showed an eclipse period of approximately 6 hr (Fig. 1). After a period of exponential increase, maximal virion yields were attained at 21 hr after infection. In cultures deprived of lysine (L^-) the eclipse period was found to be extended to 9 hr, and was probably due to the overall decrease in virion yields throughout the growth cycle. However, maximum yields were again attained at 21 hr after infection. These

results indicated that there was no delay in the formation of infectious virion.

Characterization of virion. Examination of the virion yields obtained from L^- cultures by isopycnic sedimentation of CsCl revealed the presence of two bands (Fig. 2A), a top band (no. 1) with a density of 1.29 g/cm³, and a lower band

FIG. 1. One-step growth curve of reovirus type 2 in HeLa cell monolayers in the presence of complete (O) and lysine-free (●) medium. Total virion formed is plotted as a function of time.

TABLE 1. Effect of the deletion of amino acids on reovirus multiplication

Medium	Titer ^a log	Per cent of inhibition ^b
Complete		
Expt. A	8.5	
Expt. B	7.9	
Minus lysine		
Expt. A	7.8	82
Expt. B	7.6	55
Minus arginine		
Expt. A	5.6	99.9
Expt. B	6.3	97.5
Minus leucine		
Expt. A	7.3	94
Expt. B	7.1	82

^a Infectious units per milliliter. Titration by the immunofluorescent assay technique.

^b Values are expressed as a percentage of the inhibition of the untreated cultures.

FIG. 2. Sedimentation profiles of tritiated leucine-labeled virion preparations from L^- -(A) and L^+ -(B) infected HeLa cell cultures. Virion yields were harvested at 30 hr after infection and purified by treatment with Genetron and isopycnic sedimentation in CsCl. After centrifugation, samples were collected from the bottom of the centrifuge tube and the radioactivity in each fraction was determined. The buoyant densities of representative fractions were calculated from their refractive index measurements.

(no. 2) with a density of 1.37 g/cm^3 . In some experiments the lower band (no. 2) appeared to consist of two bands. However, this was frequently seen as a shoulder in radioactive-labeled virion preparations and was difficult to resolve (Fig. 2A). In contrast, infected cultures maintained in complete medium (L^+) yielded a single band of virions with a density of 1.38 g/cm^3 (Fig. 2B).

Electron microscopy examination of band no. 1 revealed the presence of uniformly empty virion particles (Fig. 3A). These particles possessed only the outer capsid and inner layer structures and had the same dimensions as the complete virions (70 nm diameter). Band no. 2 was found to consist of predominantly complete virion particles (Fig. 3B). In such preparations a number of the particles appeared to have small portions of their

FIG. 3. Negatively stained preparations of reovirus particles from L^- cultures. (A) Top component no. 1; $\times 78,600$. (B) Bottom component no. 2; $\times 134,000$. (C) Partially disrupted particles from the bottom component no. 2; $\times 90,750$.

outer capsid structure removed (Fig. 3C). The possibility that such defective structures were produced as a result of a greater susceptibility of virion samples from L⁻ cultures to mechanical stresses during their preparation for electron microscopy cannot be excluded. The single band obtained from L⁺ cultures was comprised of complete virions.

Production of viral structural protein. During the course of preparation of radioactive-labeled virions for polyacrylamide gel electrophoretic analysis, it was determined that the deletion of lysine from infected cultures resulted in a greater yield of viral structures which could be banded in CsCl. The total viral protein produced by L⁻ cultures (both top and bottom bands) was 1.7 times greater than that formed in the presence of lysine (Table 2).

Polyacrylamide-gel electrophoresis of viral proteins. Purified ³H-leucine-labeled preparations of the top and bottom bands of virions from L⁻ cultures and the single band from L⁺ cultures were dissociated by treatment with SDS, ME, and urea, and the resulting solubilized protein mixtures were examined by electrophoresis in 10% polyacrylamide gels.

It was previously shown that the solubilized proteins of reoviruses contained three major (I, II, and IIIb) and four minor components (ml, m2, m3, and IIIa) (5). By using the same designations, it was found in the present experiment that the electrophoretic profiles of the solubilized proteins of the virions from L⁺ cultures and from bands no. 1 and 2 of L⁻ cultures were closely similar. The profiles from bands no. 1 and 2 consisted of three major and three to four minor components (Fig. 4A and B). Owing to the gel-slicing technique employed, some of the minor components were poorly resolved.

Distribution of viral protein and nucleic acid. Since electron micrographs of the top band from L⁻ virion preparations suggested that such empty structures may essentially be free of nucleic acid, the presence of viral nucleic acid in such structures was tested. Reovirus type 2 dually labeled with ³H-leucine and ¹⁴C-uridine was prepared in HeLa cell cultures in the absence of lysine as described above. Empty structures were separated from the complete virions by isopycnic sedimentation in CsCl, and the amount of radioactivity was determined. Virion preparations from L⁺ cultures yielded a single peak (density = 1.38 g/cm³) containing both viral protein and viral nucleic acid (Fig. 5A). In contrast, virion preparations from L⁻ cultures exhibited two peaks: the lower band (density = 1.37 g/cm³) containing both ³H-leucine and ¹⁴C-uridine, and the upper band (density = 1.29 g/cm³) containing essentially only

TABLE 2. *Effect of the deletion of lysine on the production of viral protein*

Sample	Protein ^a
	μg
Lysine-plus.....	256
Lysine-minus	
Band no. 1.....	132
Band no. 2.....	324

^a Monolayers of HeLa cell cultures were infected with reovirus type 2 in the presence and in the absence of lysine. At 30 hr after infection, virion yields were harvested, treated with Gene-tron, and banded in CsCl. The total protein of the various bands was determined by the Lowry method.

FIG. 4. *Polyacrylamide-gel electropherograms of dissociated proteins from purified bottom component (A) and top component (B) of L⁻ virion preparations labeled with ³H-leucine. Migration from left to right (anode).*

³H-leucine (Fig. 5B). Calculation of the distribution of radioactivity in the two kinds of viral components from L⁻ cultures indicated that, whereas the lower band had 66% of ³H-leucine and 94% of ¹⁴C-uridine, the upper or empty structures had 34 and 6% of protein and RNA, respectively (Table 3). As will be seen in a report to follow, the RNA of the bottom band is composed of viral double-stranded RNA and the single-stranded adenine-rich polyribotide, as are the virions from L⁺ cultures.

Reversal of lysine-minus effect. Monolayer cultures of HeLa cells were infected with reovirus type 2 in the absence of lysine and at various times after infection, L-lysine at a concentration normally found in Eagle's basal medium (36 μg/ml) was added back. After 30 hr of incubation virion

FIG. 5. Isopycnic sedimentation analyses in CsCl of virion preparations from L^+ cultures (A) and L^- cultures (B). Purified, dually labeled reovirus type 2 samples (^3H -leucine, broken line; ^{14}C -uridine, solid line) were prepared from L^+ and L^- cultures. After centrifugation in CsCl, samples were collected from the bottom of the centrifuge tube, and the radioactivity in each fraction was determined in liquifluor-toluene.

TABLE 3. Distribution of ^3H -leucine and ^{14}C -uridine in virions produced under minus-lysine conditions^a

Sample	Density gm/cm ³	Protein		RNA	
		Counts/ min of ^3H -leucine	Per cent of ^3H - leucine	Counts/ min of ^{14}C - uridine	Per cent of ^{14}C - uridine
Lysine-minus					
Top band	1.29	7,770	34	160	6
Bottom band	1.37	15,505	66	2,625	94
Lysine-plus					
Virion	1.38	8,135		1,530	

^a HeLa cell cultures were infected with reovirus type 2 in the presence and in the absence of lysine. Tritiated-leucine and ^{14}C -uridine were added at zero time of infection and virion yields were harvested at 30 hr after infection. The virion preparations were purified by treatment with Genetron and isopycnic sedimentation in CsCl. Samples were collected from the bottom of the centrifuge tube, and the radioactivity of trichloroacetic acid-insoluble fractions were counted in liquifluor-toluene. Data were computed from Fig. 4 after isopycnic sedimentation in CsCl.

yields were harvested, purified, and characterized by isopycnic sedimentation analysis in CsCl. It was found that addition of lysine at 2 hr after infection produced a single band of virion with a

TABLE 4. Reversal of the effect of the deletion of lysine on the production of infectious virions^a

Time of addition of L-lysine (hours postinfection)	Titer log ^b	Per cent of inhibition
0	7.8	
2	7.8	
4	7.4	59
6	7.5	43
None	7.3	66

^a Experimental procedure similar to that described in Table 1. The yield of infectious virion was made at 30 hr after infection.

^b Values expressed as infectious units per milliliter.

buoyant density (1.37 g/cm³) similar to that of complete virion. In contrast, when lysine was added at 4 and 8 hr after infection, two viral bands were found possessing similar buoyant density characteristics as those from L^- cultures (top band = 1.29 g/cm³; bottom band = 1.37 g/cm³).

In a corollary experiment, the ability of L^- cell cultures to produce infectious virions upon the addition of L-lysine at different intervals after infection was examined. Again, the addition of L-lysine at 2 hr after infection resulted in normal yields of virion. Addition of the amino acid at 4 and 6 hr did not reverse the inhibitory effect (Table 4).

DISCUSSION

The presence of arginine and histidine in the extracellular medium was found to be essential for the replication of animal virions such as herpes simplex and adenovirus, whereas the omission of lysine did not appreciably affect virion yields (2, 3, 11). Furthermore, in such systems of arginine-deficiency it was found that virion-specific early and structural proteins and viral deoxyribonucleic acid (DNA) were synthesized (1, 6). Restoration of arginine to the infected cells resulted in the rapid assembly of structural proteins and viral DNA to form the mature virions. It was subsequently determined that in the adenovirus system, arginine was required for the synthesis of an internal component known as the P antigen (7). In the absence of arginine no P antigen is formed and consequently virion maturation does not occur.

In the present study, although arginine was determined to be essential for the production of infectious reovirus, lysine was also found to play a vital role. Although the absence of lysine did not completely suppress the production of infectious virions, there was produced in such cell

cultures an additional band of empty particles essentially free of viral nucleic acid. Reversal of this effect occurred only when lysine was added early after infection. It was noted that aside from allowing synthesis of both structural viral proteins and nucleic acids, the absence of lysine also caused an enhanced formation of viral structures. This was reflected in the greater number of virion particles observed electron microscopically in both bands from L⁻ preparations. These results strongly suggest a regulatory role for this basic amino acid in the synthesis of the reovirus. Although the mechanism is at present conjectural, the probable requirement of a hypothetical lysine-containing protein(s) which regulates the assembly of structural viral proteins and viral nucleic acid must be considered and remains to be proved.

In spite of an increase in the formation of viral structures, L⁻ cultures exhibited a concomitant decrease in yields of infectious virions. This decrease could be attributed to the formation of defective or malformed particles as indicated by the electron microscopy observation of a number of particles with partially disrupted capsids present in virion preparations from L⁻ cultures. There is at present insufficient evidence to adequately explain this phenomenon.

Studies employing tritiated lysine have indicated that label is incorporated into the seven electrophoretically identifiable protein components composing the structure of the virion (*unpublished data*). The present evidence does not seem to indicate that any of the structural components are involved in either a regulatory capacity or the formation of defective particles since all of the seven polypeptides are present in the top and bottom components derived from L⁻ cultures. However, the limiting production of one or more of these proteins which may interfere with the normal assembly of the different viral components to form the complete virion cannot be overlooked. The possible utilization of lysine in

other essential metabolic processes is being further investigated.

The production of a large proportion of empty virions in L⁻ cultures deserves further comment. The formation of these particles suggests that the structural viral proteins are able to assemble into structures resembling the complete virion, despite the lack of a complete complement of viral RNA.

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