Protein Synthesis in Cells Infected with Bovine Rotavirus

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SUMMARY

Bovine rotavirus was found to multiply efficiently in LLC-MK₂ cells, a continuous line of rhesus monkey kidney, with a growth cycle which was essentially completed within 9 h after infection. The presence of low concentrations of trypsin (10 µg/ml) in the virus inoculum was essential for infectivity. Polyacrylamide gel electrophoresis of infected cell extracts demonstrated the synthesis of at least eight virus-specific polypeptides 6 h post-infection with mol. wt. ranging from 102 × 10³ to 29 × 10³. Six polypeptides (about p102K, p91K, p84K, p45K, p37K and p34K) were identified as structural components of the virion. Two other polypeptides (p54K and p29K) were identified as non-structural components. The synthesis of non-structural polypeptides appeared to precede that of the structural proteins. Pulse–chase experiments showed only one minor post-translation modification of the virus-specified proteins, namely an increase in the mobility of the 29K polypeptide.

Rotaviruses are important agents of neonatal diarrhoea in a variety of animal species, including humans (Flewett & Woode, 1978; Kapikian et al., 1978; Viera de Torres et al., 1978). Since rotaviruses are frequently found in great quantities in the faeces of infected individuals, a great deal of work has been done on the characterization of the virus particles. These studies demonstrated that rotaviruses share a number of characteristics with the reoviruses, including a double-stranded segmented RNA genome (Kalica et al., 1978; Rodger & Holmes, 1979) and a virion composed of two concentric protein shells (Esparza & Gil, 1978). Additionally, the presence of an RNA-dependent RNA polymerase (Cohen, 1977; Hruska et al., 1978) and a poly(A) polymerase (Gorziglia & Esparza, 1981) have been demonstrated in purified rotaviruses.

However, a major drawback in the basic characterization of rotaviruses, particularly in the case of the human virus, has been the difficulties encountered in obtaining efficient in vitro cultivation, and particularly its serial passage in cell cultures (Schoub et al., 1979; Esparza et al., 1980). Thus, some authors have turned to tissue culture-adapted strains of animal rotaviruses in an attempt to study the intracellular event of rotavirus replication (Matsuno et al., 1977; Estes et al., 1979; Matsuno & Mukoyama, 1979). In this communication we report some features of virus protein synthesis of a tissue culture-adapted strain of bovine rotavirus in LLC-MK₂ cells.

All experiments were done with a tissue culture-adapted strain of Nebraska calf diarrhoea rotavirus (Mebus et al., 1971), a generous gift of C. A. Mebus. The seed virus, previously grown in foetal bovine kidney cells, was obtained at a 203 passage level. In our laboratory the virus was multiplied in LLC-MK₂ cells, a continuous line of rhesus monkey kidney. LLC-MK₂ cells were grown at 37 °C in disposable tissue culture flasks (Corning Glass Works, Corning, N.Y., U.S.A.), using Eagle's essential medium supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2-5 µg/ml amphotericin B. Virus stocks were prepared as follows. Confluent cell monolayers were washed three times with phosphate-buffered saline (PBS) and inoculated with a virus suspension containing 10 µg/ml trypsin (Almeida et al., 1978; Esparza et al., 1980). After 1 h adsorption, cells were washed once with PBS, and serum-free medium containing 10 µg/ml.
trypsin was added to the cultures. Infected cells were incubated at 37 °C until complete cytopathic effect (c.p.e.) was observed. Cells were then frozen and thawed once, clarified by low-speed centrifugation and supernatant fluids were stored in 1 ml amounts at −90 °C until used.

For radiolabelling of infected cells, confluent monolayers, grown in 25 cm² tissue culture flasks (Corning Glass Works) were washed three times with PBS and then inoculated with bovine rotavirus at a multiplicity of infection (m.o.i.) of approx. 10³ physical particles/cell. After 1 h adsorption at 37 °C, cells were washed once with PBS, and serum-free medium containing 0.5 µg/ml actinomycin D and lacking leucine was added to the cultures. Cells were then incubated at 37 °C. For radiolabelling, L-4,5-³H-N-leucine (New England Nuclear) with a specific activity of 60 Ci/mmol was used as indicated for each experiment. After labelling, cell cultures were harvested by scraping the cells off the glass into the medium with a rubber policeman, followed by centrifugation at 1000 rev/min for 10 min. Cells were washed once with PBS and dissociated by boiling for 3 min in a mixture containing 2 % SDS, 0.1 M-2-mercaptoethanol, 2 mM-phenylmethane sulphonyl fluoride and 0.1 M-tris-HCl pH 6.8.

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in slab gels by the high resolution method of Laemmli (1970), using 10 % running and 3.3 % stacking polyacrylamide gels in tris-glycine buffer pH 8.6. After electrophoresis, gels were fixed in 7 % acetic acid-30 % isopropanol and the radioactivity detected by the quantitative fluorographic technique described by Bonner & Laskey (1974) and Laskey & Mills (1975). The mol. wt. of virus-specific proteins were determined by comparison with known protein standards (low mol. wt. calibration kit; Pharmacia) run under identical conditions.

Bovine rotavirus was found to multiply efficiently in LLC-MK₂ cells, with a growth cycle which was essentially completed within 9 h after infection. The proteins synthesized in LLC-MK₂ cells with bovine rotavirus at different h post-infection were studied by SDS–PAGE (Fig. 1 a). The polypeptide pattern of uninfected cells (Fig. 1 a, lane A) was modified by rotavirus infection. Presumptive virus-specific polypeptides could be detected as early as 4 to 5.5 h post-infection, and they were more evident in cells labelled between 5-5 and 7 h post-infection. It was relatively easy to identify at least eight polypeptides as being virus-induced, based on the criteria of characteristic electrophoretic mobility and relative rate of synthesis at different times after infection. The mol. wt. of the rotavirus-induced polypeptides were estimated by electrophoresis on 10 % SDS–PAGE, in the presence of standard proteins. The results indicated that rotavirus-induced proteins ranged in mol. wt. from approx. 102 × 10³ to 29 × 10³. However, the mol. wt. calculated for the largest polypeptide was only an approximation since its migration was less than that of the protein marker with the highest mol. wt. used. The apparent mol. wt. of each virus-induced polypeptide (i.e. about p102K, p91K, p84K, p54K, p45K, p37K, p34K and p29K) will be used in this paper.

To further study the identity of the polypeptides synthesized in rotavirus-infected cells, we compared them with the polypeptides present in purified rotavirus particles. Double-capsid particles of bovine rotavirus possess six structural polypeptides (E. Novo & J. Esparza, unpublished results), which coincide with six equivalent polypeptides present in the infected cell extracts (Fig. 1 b). In the figure, the band corresponding to p102K is not clearly visible in the purified particles; however, further studies (E. Novo & J. Esparza, unpublished results) have indicated that it is indeed a structural component of the virion. Thus, at least six of the rotavirus-induced polypeptides are structural components of the virion (about p102K, p91K, p84K, p45K, p37K and p34K), while two of them (p54K and p29K) are non-structural proteins. Other bands identified in the gels from the infected cell extracts were considered to be residual host cell proteins.

Possible post-translational modifications of rotavirus-induced polypeptides were studied by pulse–chase experiments. In these experiments cells were exposed to 100 µCi/ml ³H-leucine at
Fig. 1. Analysis by SDS-PAGE of rotavirus polypeptides. (a) Polypeptides made in LLC-MK₂ cells at different times after infection. Cells were pulse labelled with 10 µCi/ml ³H-leucine and solubilized cell extracts were analysed in 10% SDS-PAGE. Lanes A and I contain extracts from mock-infected cells, labelled from 1 to 2.5 h and 10 to 11.5 h post-infection respectively. The other lanes contain extracts from rotavirus-infected cells, labelled at different h post-infection as follows: B, 1 to 2.5; C, 2.5 to 4; D, 4 to 5.5; E, 5.5 to 7; F, 7 to 8.5; G, 8.5 to 10; and H, 10 to 11.5. The arrows indicate each one of the eight polypeptides identified as virus-induced. (b) Gel patterns of infected cell polypeptides synthesized from 7 to 8.5 h post-infection (lane A) and from ³H-leucine-labelled double-capsid rotavirus particles (lane B). (c) Post-translational modification of rotavirus-induced polypeptides: A, 5 min pulse; B, 30 min chase.
Fig. 2. Rate of synthesis of the different polypeptides observed in rotavirus-infected cells. (a) Virus structural proteins; (b) non-structural virus proteins; (c) residual host cell proteins. The relative molar amount of synthesis for each polypeptide was calculated as described by Honess & Roizman (1973).

7 h post-infection for a period of 5 min, with some of the cultures being chased for 10, 20, 30 or 60 min in maintenance medium containing an excess amount of non-radioactive leucine; pulsed and pulse-chased cultures were then solubilized and subjected to SDS–PAGE. The only post-translational modification observed was an increase in the mobility of p29K (Fig. 1 c). This was a reproducible observation which, in experiments done with shorter periods of chase, was found to be essentially complete within 20 min. Thus, most of the virus-specified proteins seem to behave as primary products of translation.

The rate of synthesis of each of the virus-induced polypeptides was also analysed as a function of the time post-infection. For this purpose, a densitometer scan of the fluorogram shown in Fig. 1(a) was done, and the area under each peak was determined by weight measurements. The relative molar amount of synthesis for each polypeptide was calculated as described by Honess & Roizman (1973), and the results are presented in Fig. 2. The figure is divided into three panels to separate structural (Fig. 2a), non-structural (Fig. 2b) and host cell (Fig. 2c) polypeptides. Not all polypeptides were synthesized with the same efficiency. For instance, p37K and p45K were synthesized in larger quantities than other structural
polypeptides, not necessarily reflecting their proportion in the mature virion. Another interesting observation is that synthesis of the major structural polypeptides (p37K and p45K) produces peaks at approx. 8 to 9 h post-infection, while the synthesis of the two non-structural polypeptides (p54K and p29K) shows a maximum at 6 to 8 h after inoculation. Host cell protein synthesis decreases after infection, even though a band with a mol. wt. close to 52K was very refractory to inhibition.

The experiments described in the present paper indicate that infection of LLC-MK₂ cells by bovine rotavirus provides a useful system for the study of the basic biology of this group of viruses. It is important to mention that, as reported by others (Babiuk et al., 1977; Almeida et al., 1978; Clark et al., 1979), the presence of low concentrations of trypsin in the virus inoculum was found to be essential for infectivity. In all the experiments described in this paper, cells were infected with virus stocks prepared with 10 μg/ml trypsin in the maintenance medium, although no trypsin was added to the medium thereafter. The virus produced in the absence of trypsin could not be serially passed in LLC-MK₂ cells, unless trypsin was added to a final concentration of 1 to 10 μg/ml. The presence of trypsin was essential only at the time of inoculation because trypsin acts directly on the rotavirus and not on the host cell (Barnett et al., 1979).

The synthesis of virus-specific polypeptides was studied in the presence of 0.5 μg/ml actinomycin D. However, no major differences were found in the gel patterns when actinomycin D was omitted from the maintenance medium. Under those conditions, bovine rotavirus induced some inhibition of host cell protein synthesis that allowed us to identify at least eight virus-specified polypeptides, with mol. wt. ranging from 102 x 10³ to 29 x 10³. Control experiments indicated that the low levels of trypsin present in the virus inoculum, which could have remained in the cultures after the PBS wash, did not alter the polypeptide gel patterns, suggesting that no significant proteolytic cleavage occurred in our experiments. Not all rotavirus-specific polypeptides were synthesized in equimolar amounts, and the results shown in Fig. 2 suggest that the synthesis of the two major non-structural polypeptides (54K and 29K) precedes that of the major structural polypeptides. Our attempts to label virus glycoproteins with ³H-glucosamide or ³H-mannose resulted in very little incorporation of the isotope in the cultures, which was distributed in most of the virus polypeptides shown in the gels, a situation also reported for reovirus (Krystal et al., 1976). This is indicative of metabolic processing of the precursor and its incorporation into amino acids.

Since there have been several previous reports on the polypeptide composition of bovine rotavirus particles (Newman et al., 1975; Bridger & Woode, 1976, Rodger et al., 1977; Matsuno & Mukoyama, 1979; Thouless, 1979) and on its synthesis in infected cells (Matsuno & Mukoyama, 1979; Thouless, 1979), we made efforts to compare our results with those reported by other authors. This proved to be a very difficult task, mainly due to large variations in the mol. wt. reported. However, the general pattern obtained for the structural polypeptides of the tissue culture-adapted rotavirus strains was comparable. An indication of the variations in the reported mol. wt. can be seen with the major structural component, described by us as the 45K polypeptide, and estimated by others in the mol. wt. range of 32 x 10³ to 42 x 10³. Another major difference with previously published reports is the number of virus polypeptides detected in infected cells. Other workers (Matsuno & Mukoyama, 1979; Thouless, 1979) have reported potential gene products for all the genome segments; however, further studies are necessary to prove that all detected polypeptides are in fact virus-specific, and that they represent primary gene products. Some of these ambiguities could be solved by the use of two-dimensional electrophoresis, to obtain a better separation of closely migrating polypeptides, and by immunoprecipitation techniques to select out virus polypeptides from host cell proteins (Cross & Fields, 1976). Other differences observed could be due to use of different cell and/or virus strains and to the methodology used. For instance, Matsuno &
Mukoyama (1979) reported the absence of post-translational modification of rotavirus polypeptides, a process difficult to detect, at least in the experiment shown by the authors. Differences in the reported number of virus structural polypeptides have also occurred: Rodger et al. (1977) reported as many as nine species, whereas our results, qualitatively similar to those of Cohen et al. (1979), indicate the existence of six structural polypeptides, an observation further confirmed by examination of purified rotavirus enzymically labelled with $^{125}$I (E. Novo & J. Esparza, unpublished results).

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