Posttranslational Cleavage of Virus Polypeptides in Arbovirus-infected Cells

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Recent work with picornaviruses has shown a shift of radioactive label from larger molecular weight virus-coded polypeptides to smaller ones in the interval following a short labelling period (Summers & Maizel, 1968; Holland & Kiehn, 1968). This suggests post-translational cleavage of the larger polypeptides to give specific products. In view of the possibility that most mammalian messenger RNAs may be monocistronic (see Jacobson & Baltimore, 1968) it was of interest to examine mRNA translation in another small ribovirus of an unrelated group, the arboviruses.

All virus growth and protein synthesis experiments were done in BHK21 cells, adapted to suspension culture and grown in modified Eagle's medium in Belco spinner flasks (Capstick et al. 1966). The cells were kindly provided by Dr I. M. Kerr. The Semliki Forest virus stock and its titration in primary chick embryo cell cultures were described by Tan, Sambrook & Bellett (1969). Semliki Forest virus was adsorbed to BHK cells for 1 hr at 15 to 20°, using 15 x 10^6 cells/ml and an input multiplicity of 40 p.f.u./cell. The cells were then diluted to 2 x 10^6/ml and incubated at 35° in modified Eagle's medium containing 2.5 % calf serum and 1 μg./ml. Actinomycin D (donated by Merck, Sharp & Dohme), but lacking tryptose phosphate broth and the amino acids to be used for subsequent labelling. The maximum virus yield was usually 1000 to 3000 p.f.u./cell 8 to 9 hr after infection.

Labelled virus was purified by a modification of the method of Cheng (1961). Infected BHK cells were labelled with 5 μc/ml of either [3H]leucine (1000 c/mole, The Radiochemical Centre, Amersham) or [14C]amino acid mixture (Reconstituted Protein Hydrolysate, Schwarz Bioresearch, Orangeburg, N.Y.) from 3½ to 9 hr after infection. After freezing, thawing and centrifuging at 1000 g for 10 min., the supernatant fluid was treated for 4 hr at 4° with 1 mg./ml. protamine sulphate which was then removed by centrifugation. Finally the supernatant fluid was centrifuged through a 30 to 50% (w/w) sucrose gradient containing 0.1 M-KCl, 0.01 M-tris pH 9 and 0.1 % calf serum for 16 hr at 23,500 rev/min. in a Spinco Model L centrifuge. The radioactive band was located by drying samples on filter papers, acid washing and counting with toluene-based scintillation fluid in a Packard Tri-Carb Scintillation Counter (Tan et al. 1969). Samples from the peak of radioactivity (c. 42 % sucrose) were centrifuged a second time through an identical gradient. The peak of infectivity coincided with the radioactive peak; after negative staining, electron microscopy showed it to contain many uniform particles 60 nm. in diameter resembling well preserved particles of Semliki Forest virus (Acheson & Tamm, 1967).

To examine rates of protein synthesis in infected and uninfected cells, replicate 5 ml. samples of cell suspension were removed and labelled at different times with 0.1 μc/ml. [14C]leucine (305 mc/m-mole, The Radiochemical Centre, Amersham). After 15 min. they were diluted with 5 ml. of ice-cold phosphate buffered saline containing 5 mg./ml. unlabelled leucine, and thereafter kept at 0°. The cells were centrifuged, washed once in normal saline and resuspended in 2 ml. distilled water. One ml. of unlabelled leucine solution (5 mg./ml.), 0.5 ml. N-NaOH, 0.15 ml. carrier bovine serum albumin solution
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(5 mg./ml.) and 2.5 ml. 10% (w/v) trichloracetic acid were added, and after standing for 15 min. the precipitate was centrifuged, washed twice more in 5% (w/v) trichloracetic acid, resuspended in 0.5 ml. 5% trichloracetic acid and 10 ml. dioxane-based scintillation fluid, and counted (Martin & Sonnabend, 1967). Total protein was determined by the method of Lowry et al. (1951) to allow correction for cell sampling errors; in practice, sampling errors were less than 10%.

For gel electrophoresis, purified virus or cell extracts (see below) were made up to 20 mg./ml. sodium dodecyl sulphate and 0.1 M-2-mercaptoethanol and heated to 100° for 4 min. After dialysis overnight against 0.01 M-phosphate buffer pH 7.3 containing 1 mg./ml. sodium dodecyl sulphate, 0.05 M-urea and 0.01 M-2-mercaptoethanol, samples were layered over 10% (w/v) polyacrylamide gels 15 cm. in length which had been prerun with 0.2 ml. 0.1 M reduced glutathione and 0.1 ml. dithiothreitol (100 mg./ml.) (Strauss, Burge & Darnell, 1969; Cleland, 1964). Electrophoresis was performed as described by Summers, Maizel & Darnell (1965) but with the inclusion of 0.01% thioglycollic acid in the electrophoresis buffer. Gels were homogenized with water containing 150 µg./ml. bovine serum albumin by forcing them through a nozzle with a motor-driven plunger (Maizel, 1966); samples corresponding to 0.2 cm. gel fractions were collected in scintillation vials and counted with 10 ml. of dioxane-based scintillation fluid. Recovery was 60 to 70% of counts applied to the gel.

Three distinct polypeptides were reproducibly found in the gel electrophoretic pattern produced by the structural proteins of purified Semliki Forest virus; their proportions in the virus were markedly unequal (Fig. 1 A). The results of Friedman (1968 b) for Semliki Forest virus grown in chick embryo cells were similar, while Hay, Skehel & Burke (1968), in the same system as Friedman, reported only peaks 2 and 3. Semliki Forest virus nucleocapsids, labelled with [14C]amino acid mixture and purified from infected chick embryo cell extracts (Tan et al. 1969), were obtained from Mr K. B. Tan. This preparation gave two peaks on electrophoresis, and on co-electrophoresis with [3H]labelled purified virus, the major peak of nucleocapsid protein coincided with peak 3 while the minor peak appeared to coincide with peak 1. Thus peak 2 represented the membrane protein, agreeing with the observations of Friedman (1968 b). Fig. 1 B and 1 C are referred to below.

Protein synthesis in BHK cells was decreased after infection, with or without Actinomycin D, (Fig. 2 A); the method is given above. Friedman (1968 a, b) reasoned, from similar results and from results obtained with infected cells pretreated with interferon, that more than 75% of the protein synthesis occurring 4 hr after Semliki Forest virus infection in chick embryo cells in the presence of Actinomycin D was virus directed. This suppression of host-protein synthesis permitted examination of virus-coded proteins present in infected cells. For this purpose cells were labelled, 5 hr after infection, with radioactive leucine or amino-acid mixture. After 3 min. the cells were centrifuged and resuspended in similar unlabelled medium containing leucine or amino acids at 500 times the concentration used during the labelling period. Samples removed after different periods in unlabelled medium were washed, resuspended in 0.01 M-tris pH 8.5 and 0.001 M-MgCl₂, and disrupted in a Dounce homogenizer. After sedimenting nuclei, the cytoplasm was denatured and electrophoresed as described above. A typical alteration in polypeptide pattern occurred during incubation in unlabelled medium (Fig. 1 B, C). There was an apparent shift of label from peaks N1 and N2 to the peaks corresponding to structural proteins 2 and 3; this is compatible with breakdown of proteins N₁ and N₂ to give the smaller proteins 2 and 3. Similar patterns were seen whether leucine or an amino-acid mixture, labelled with
[\textsuperscript{3}H] or [\textsuperscript{14}C], was used as the precursor, and cytoplasm from intermediate lengths of incubation with unlabelled medium gave intermediate patterns.

Possible causes of this change in gel pattern can be listed as follows. (1) Differential rates of loss to the nuclear fraction of the various labelled proteins. This possibility was excluded by the finding that a similar change in gel pattern was seen when whole infected

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Fig. A. Electrophoresis pattern of purified Semliki Forest virus particles labelled with [\textsuperscript{3}H]leucine. The sample was electrophoresed for 14 hr at 8 mA. B, C. Co-electrophoresis patterns of cytoplasm labelled for 3 min. with [\textsuperscript{14}C]leucine 5 hr after infection with Semliki Forest virus (●—●), and purified Semliki Forest virus labelled with [\textsuperscript{3}H]leucine (○—○). The [\textsuperscript{14}C] sample examined in B was taken immediately after the 3 min. labelling period; that examined in C was taken after a further 55 min. incubation in unlabelled medium: 4330 \textsuperscript{14}C counts/min. were applied to the gel in B while 7760 counts/min. were applied in C, and the samples were electrophoresed for 16 hr at 7.5 mA/gel.
cells were examined instead of the cytoplasmic fraction. (2) Non-specific (random) proteolysis of the larger proteins to smaller polypeptides. As no new peaks appeared during the period of incubation with unlabelled medium, this possibility can also be excluded. (3) Preferential proteolysis of the larger proteins to acid-soluble fragments which were subsequently lost during dialysis. This would produce an apparent shift of radioactivity to the more stable protein species. To test this possibility, overall stability of the labelled proteins during a typical ‘pulse-chase’ experiment was examined. Concurrently with samples taken for gel electrophoresis, 1 ml. samples were removed after varying lengths of ‘chase’, cells were washed and resuspended in 2 ml. distilled water, and total cell-associated acid-insoluble radioactivity was counted as described above. A gradual decline in radioactivity (to 79.5% after 55 min.) was seen during the chase (Fig. 2B). This is far less than that necessary to account for the marked decrease in peaks N1 and N2 relative to peak 2 if the change in gel pattern had been due solely to non-specific breakdown of N1 and N2 to acid-soluble fragments. Release of mature virus into the medium may have accounted for some of the slight decline in cell-associated radioactivity during the chase (Scheele & Pfefferkorn, 1969). The large excess of unlabelled amino acids should have prevented reutilization of any label broken down to free amino acids. (4) Specific cleavage of the larger proteins to produce smaller virus proteins. We believe this to be the best interpretation of the data presented above.

Thus at least five virus-coded polypeptides can be resolved by polyacrylamide gel electrophoresis from BHK-21 cells 5 hr after infection with Semliki Forest virus; two of these correspond with the two major structural proteins found in purified virus. After a 3 min. labelling period, subsequent cleavage of non-structural virus-coded polypeptides, presumably at highly specific cleavage loci, appears to contribute to the smaller structural proteins of the virus.

Fig. 2. A. Rates of total protein synthesis in BHK cells: comparison of infected and uninfected cells in the presence (●—●), or absence (○—○), of 1 μg./ml. actinomycin D. The ordinate represents acid insoluble [14C]leucine incorporated in a 15 min. pulse (counts/min./mg. of total cell protein) expressed as a percentage of the value for the uninfected control at each time point. B. Stability of cell associated protein, newly synthesized during a 3 min. labelling period with [14C]leucine, 5 hr after infection with Semliki Forest virus. The ordinate represents the total acid insoluble counts/min. present in the cells obtained from 1 ml. samples of cell suspension, and the abscissa represents the period of incubation in unlabelled medium.
Similar work with Semliki Forest virus in chick embryo cells (Friedman, 1968b; Hay et al. 1968) and with Sindbis virus in chick embryo cells and BHK21 cells (Strauss et al. 1969) made use of relatively long labelling periods. As considerable cleavage occurred in our system after 10 min. incubation with unlabelled medium, the gel pattern from a labelling period of \( \frac{1}{2} \) hr or more would approximate to that of a 'chased' cytoplasm; this is borne out by our unpublished observations, and by the work of Summers & Maizel (1968) with poliovirus. Strauss et al. (1969) suggested metabolic instability of Sindbis-coded polypeptides, possibly analogous to the situation with poliovirus. That this is so with Semliki Forest virus is shown by the results reported in this paper.

Department of Microbiology  
John Curtin School of Medical Research  
Australian National University  
Canberra, A.C.T., Australia

REFERENCES


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