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POLYPEPTIDES INDUCED IN CHICK EMBRYO CELLS BY KEMEROVO VIRUS

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Summary. — Fifteen polypeptides induced by Kemerovo virus were detected in chick embryo cells (M_r 140, 98, 89, 72, 65, 62, 57, 54, 50, 47, 43, 41, 39, 31 kD, and 30 kD). Nine of them, namely the 140, 98, 65, 62, 57, 54, 50, 47 kD, and 41 kD polypeptides were also found in the partially purified virus. However, the latter contained also considerable amount of host cell proteins, predominantly the 205 kD, 45 kD, and 37 kD polypeptides. In the electron microscope the spherical viral particles exhibited a poorly defined surface structure of a diameter of 70—75 nm.

Key words: *Kemerovo virus*; *polypeptides*; *virus purification*

Introduction

The viruses belonging to the Kemerovo serogroup of Orbivirus genus, such as Nugget or Mill Door/79 virus, are difficult to purify probably due to their strong association with cellular material (Gorman *et al.*, 1984). The R-10 strain of Kemerovo virus (KV) (Libíková *et al.*, 1964) seems to possess similar properties when partially purified from infected cells (Slávik and Nermut, 1970; Rosenbergová and Slávik, 1975; Slávik *et al.*, 1984). The viral structural polypeptides can be found among the virus-induced polypeptides in infected cells (Spence *et al.*, 1985) and also among the polypeptides synthesized in a cell free translation system (Eley *et al.*, 1985; Spence *et al.*, 1986). The aim of our work was to specify the polypeptides induced by KV in chick embryo cells (CEC) and to compare them with those found in the partially purified virus.

Materials and Methods

Virus. The R-10 strain of KV (Libíková *et al.*, 1964) underwent 3 passages in CEC cultures 8 passages in the yolk sac of chick embryos and 5 passages in the brains of suckling rats. Lyophilized brain suspension (Slávik *et al.*, 1984) served as stock virus. For experimental purpose a high titre virus stock was prepared in CEC cultures as follows: primary CEC cultures were seeded in Petri dishes in amount of 5.5×10^5 cells per cm^2 in Parker medium 199 containing 10% calf serum and 7 mmol/l Tris-HCl pH 7.6. After 18—24 hr incubation at 37 °C the monolayers were infected at MOI of 0.01 PFU per cell for 1 hr at room temperature and further incubated in fresh medium without serum for 18—24 hr at 37 °C (Slávik *et al.*, 1984). Cells were

then scraped off into growth medium and disrupted with 30 strokes in a Dounce homogenizer (Bellco). The crude suspension served as a high titre virus stock.

Plaque titration was performed in primary CEC cultures grown in 60 mm Petri dishes for 48 hr at 37 °C in basal Eagle's medium (BEM) supplemented with 10% calf serum. Tenfold dilutions of virus samples in PBS pH 7.2 were applied to duplicate cultures. After 1 hr absorption at room temperature, the inoculum was removed and cells were overlaid with 4.5 ml 0.9% agar (Bacto Agar Special Noble, Difco) in Eagle's medium per dish. After 64 hr at 37 °C the cultures were stained with 0.01% Bacto Neutral Red (Difco) in 0.9% agar in physiological saline for 4—6 hr at room temperature and the plaques of lysed cells were counted.

Labelling of virus-induced polypeptides. CEC monolayers grown in 60 mm Petri dishes were infected at MOI of about 100 PFU per cell at room temperature. After 1 hr the inoculum was removed, the cells were washed and fresh BEM was added. After various incubation intervals at 37 °C the cultures were pre-incubated for 30 min in a medium containing actinomycine D (0.5 μg per ml) and a reduced (5%) amount of methionine. Thereafter, 1.85 MBq of ^{35}S-methionine (Rotop, G.R.D.) was added per dish and labelling proceeded for 1 or 6 hr, respectively. At appropriate intervals after inection the cells were scraped off and pelleted for 5 min at 2,500 g. The cells were washed twice with PBS, lysed in 250 μl of loading buffer (0.062 mol/l Tris-HCl pH 6.8, 0.005% bromphenol blue, 2% sodium dodecyl sulphate, 5% 2-mercaptoethanol and 10% glycerol) by heating in a boiling water bath for 3 min and then cooled in an ice bath. The samples were stored at — 20 °C..

Preparation of the partially purified KV. About 5×10^8 CEC in five 150 mm Petri dishes were used for virus production. At 24 hr p.i. the cells were harvested, sedimented at 3,500 g for 30 min at 4 °C. The cell pellet was resuspended in 20 ml of TE-TX-100 buffer (10 mmol/l Tris, 5 mmol/l EDTA-Na_2, 2% Triton X-100), homogenized with 30 strokes in a Dounce homogenizer and centrifuged at 5,000 g for 30 min. The supernatant representing the cytoplasmic extract was treated twice with 1/2 volume of Genetron 113 (Fluka) by manual shaking for 5 min. After centrifugation at 2,500 g for 5 min the virus containing aqueous phase was layered onto 5 ml 40% sucrose (made in 10 mmol/l Tris and 5 mmol/l EDTA-Na_2) and centrifuged in SW-25.1 rotor (Beckman) at 21 000 rev/min for 2 hr. The pellet was resuspended in 16 ml of TE-TX-100 buffer, layered onto 1 ml of 40% sucrose and centrifuged in SW-50.1 rotor (Beckman) at 30 000 rev/min for 45 min. Finally, the pellets were resuspended in 0.5 ml of TE-TX-100, layered onto 4.5 ml of preformed CsCl density gradients ($\rho = 1.25 - 1.40$ g.cm^{-3}) and centrifuged in SW-50.1 rotor at 35 000 rev/min for 2.5 hr. The bands containing virus material ($\rho = 1.32$ g.cm^{-3}) were collected, diluted with TE-TX-100 buffer and the material was pelleted through 1 ml of 40% sucrose as described above .

Polyacrylamide gel electrophoresis (PAGE). Proteins were electrophoretically separated in 18 cm long and 1 mm thick 10% polyacrylamide slab gels using 4.5% stacking gel of 1.5 cm length and a discontinuous Tris-glycine buffer system (Laemmli, 1970). Electrophoresis was run for 8 hr at 180—200 V at room temperature. The gels were fixed and stained in 45% (v/v) methanol, 10% (v/v) acetic acid and 0.4% Coomassie Brilliant Blue R-250. Then, the gels were destained in 45% methanol and 10% acetic acid to visualize the proteins, washed in water, saturated with 1 mol/l sodium salicylate and dried. Autoradiography was performed for about one week at — 70 °C, using X-ray film Medix-Rapid (Foma, Hradec Králové, Č.S.S.R.).

Determination of M_r. The relative molecular weights (M_r) of the polypeptides were calculated by using markers obtained from Sigma (MW-SDS-200). Distancies of protein bands from the start were measured on stained and dried gels, and distancies of radioactive protein bands on the autoradiograms.

Negative staining and electron microscopy. Pellet of KV material obtained before purification by CsCl density gradient was resuspended in PBS and aliquots were negative stained with 2% phosphotungstic acid (PTA) pH 7.0. The samples were examined in Philips EM 300 electron microscope at 80 kV. Primary magnification was 33 000×.

Results

The growth curve of KV in primary CEC cultures was assayed 6—30 hr p.i. at MOI of 0.01 PFU per cell (Fig. 1). The peak virus titre was reached by 18 hr p.i.

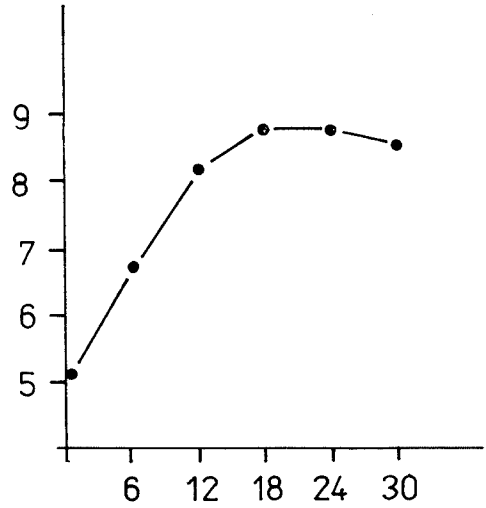

Fig. 1
Multiplication of KV in CEC cultures
Suspensions of disrupted cells were prepared at the indicated time and assayed by plaque titration.
Abscissa: time (hr); ordinate: virus titre (log PFU ml).

Fifteen virus-induced polypeptides in CEC were detected already at 2 to 3 hr p.i. (Fig. 2). M_r of the polypeptides were 140 kD, 98 kD, 89 kD, 72 kD, 65 kD, 62 kD, 57 kD, 54, kD, 50 kD, 47 kD, 43 kD, 41 kD, 39 kD 31 kD and 30 kD. The 57 kD polypeptide was the most efficiently labelled one. A decrease of intensities of virus-induced polypeptide bands was observed between 4 and 7 hr p.i. In the lane 2/7 (Fig. 2) the polypeptides labelled during the whole 6 hr period of virus multiplication are presented; the polypeptides exhibited the same M_r as those found by the 1hr-pulse at early phases of virus replication, but polypeptides 72 kD and 31 kD were not detected. The most labelled cellular polypeptide was that with M_r of 45 kD.

In the partially purified virus 9 polypeptides of M_r 140 kD, 98 kD, 65 kD, 62 kD, 57 kD, 54 kD, 50 kD, 47 kD, and 41 kD were found (Fig. 3), which corresponded to those which had been induced by the virus in CEC cultures. A considerable amount of cellular proteins, predominantly of M_r of 205 kD, 45 kD, and 37 kD was also detected.

When examined by electron microscopy, KV particles had poorly defined surface structure (Fig. 4) with a diameter of 70—75 nm.

Discussion

One-step growth curve of KV, using CEC and a multiplicity of infection of 1—10 PFU per cell reached the plateau of infectious titres at about 8 hr p.i. (Mayer *et al.*, 1964). We used this figure without further examination in our experiments analysing the KV-induced polypeptides in CEC after infection at multiplicity of about 100. Virus-specific proteosynthesis was detected already within 2 hr p.i.; the decrease of its intensity occurring from

4 to 7 hr. p.i. seemed in correlation with the duration of replication cycle of KV in CEC.

The M_r of 15 virus-induced polypeptides ranged from 140 kD to 30 kD. Polypeptide(s) of M_r smaller than 28 kD, e.g. the polypeptide of M_r about 14 kD which might be coded for by the smallest dsRNA segment of KV could not be detected under conditions of PAGE used in our experiments.

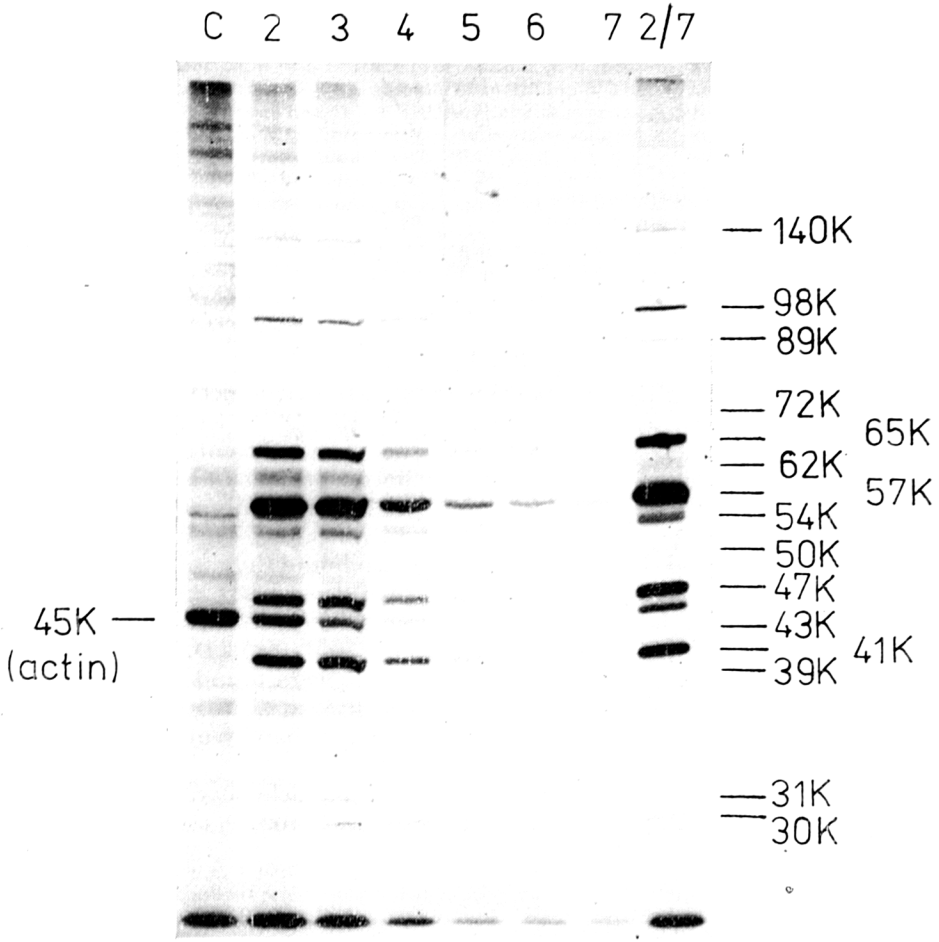

Fig. 2

Autoradiogram of a 10% polyacrylamide gel electrophoresis showing virus-induced polypeptides Labelled with ^{35}S-methionine (0.925 MBq/ml) for different intervals. Lanes: C, mock-infected cells labelled 1—2 hr p.i.; 2, 3, 4, 5, 6, 7, infected cells labelled from 1-2, 2-3, 3-4, 4-5, 5-6, 6-7 hr p.i., respectively; 2/7, infected cells labelled from 1 to 7 hr p.i.

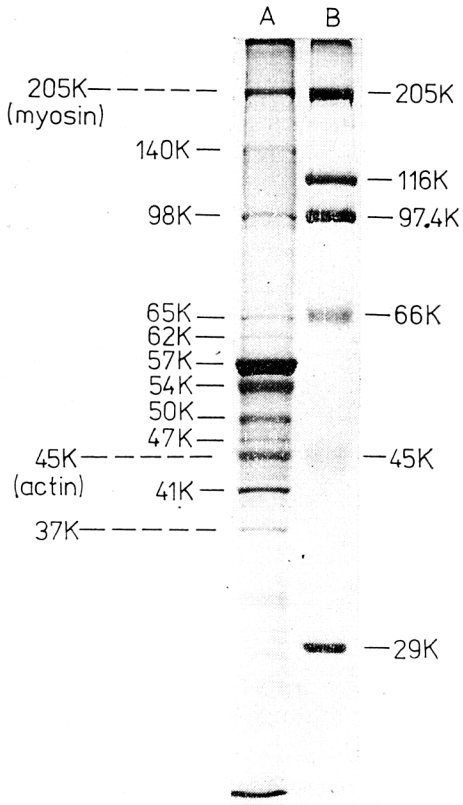

Fig. 3
Electrophoresis of partially purified KV in 10% polyacrylamide gel. Lanes: A, partially purified KV; B, M_r markers. Detection: Coomassie Brilliant Blue R-250.

To explain the synthesis of more than ten (thirteen) polypeptides induced by Mill Door/79 or FT 363 viruses (other viruses of the Kemerovo serogroup) in infected cells, the posttranslational modification of polypeptides, premature termination of translation and the presence of more than one open reading frame in viral mRNAs were suggested (Spence *et al.*, 1985; Eley *et al.*, 1986). Mill Door/79 virus induced three polypeptides of M_r lower than 30 kD (27 kD, 22 kD, and 20 kD), and the FT 363 virus two polypeptides of that size (23 kD and 22 kD).

The purification procedure of KV yielded only a partially purified virus. Nine virus-specific polypeptides were identified according to their M_r; these polypeptides were easily distinguishable from the others and seemed to represent a majority of those found in partially purified virus. Cellular proteins of M_r 205 kD, 45 kD (probably myosin and actin) and 37 kD seem to be substantial contaminants of KV after the purification procedure.

Viral particles with diameters of 70—75 nm with poorly defined surface structure were found in KV material not purified by CsCl density gradient. Presence of a diffuse or structureless outer capsid layer was reported to be

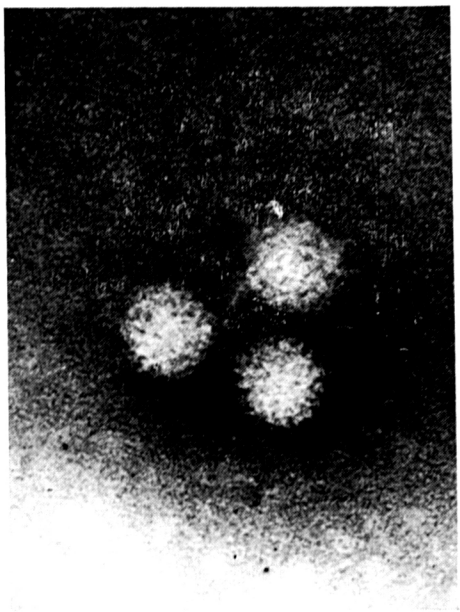

Fig. 4
Electron micrograph of KV particles negatively stained with 2% phosphotungstic acid, magn. × 198 000.

a general feature of the Orbivirus genus (Verwoerd *et al.*, 1979). In this respect, our findings seem to be comparable with virion morphology of other orbiviruses.

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