Delay in the Incorporation of Protein into Virus Nucleocapsid in Newcastle Disease Virus Infected Cells

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In cells infected with paramyxovirus progeny 50 S RNA seems to be incorporated immediately into the nucleocapsid (Blair & Robinson, 1970). There has been no report of any attempt to estimate the time interval between the synthesis of a molecule of nucleocapsid protein and its incorporation into the structure of the nucleocapsid. In the experiments described in this paper the problem has been approached with the use of a pulse-chase, using protein precursors. The increase of label in the 200 S peak under chase conditions was used as a measure of the rate of nucleocapsid formation.

Stocks of the BEAUDETTE strain of Newcastle disease virus (NDV) were grown in eggs. Primary chick embryo cell cultures were inoculated as described by Kingsbury (1966), at a multiplicity of 10 to 20 p.f.u./cell and incubated at 37 °C. Lactalbumin hydrolysate (0.5%) in Hanks’s balanced salt solution (BSS) was used as maintenance medium. For radioactive labelling cell monolayers were washed three times with warm BSS and incubated with BSS containing either [14C]-algal hydrolysate (final concentration 3 μCi/ml) or [14C]-leucine (final concentration 0.38 μCi/ml, specific activity 90 mCi/m-mol). In pulse-chase experiments, [14C]-leucine was replaced after a short interval with 100-fold excess of cold leucine dissolved in maintenance medium.

Cell extracts were prepared as follows. Cells were scraped from the glass, washed once with chilled BSS, suspended in standard buffer (0.01 M-triethanolamine-HCl, pH 7.6; 0.01 M-KCl) and disrupted with 15 strokes in a Dounce homogenizer. Homogenates were centrifuged for 10 min at 2000 g. The supernatant fluids were collected and treated with EDTA and sodium deoxycholate (DOC) to final concentrations of 0.01 M and 0.012 M, respectively. If the extracts were not to be fractionated immediately, they were fixed with formaldehyde (pH 7.0, final concentration 4%) and diluted with an equal vol. of standard buffer, in order to lower DOC concentration. No artifacts due to aldehyde fixation of cell extracts (Baltimore & Huang, 1968) have been observed (see below).

Cell extracts were centrifuged in 15 to 30% (w/w) sucrose gradients in a 3 x 23 bucket rotor of a Superspeed 50 ultracentrifuge (MSE, England). Fractions were collected and u.v. extinction at 254 nm was measured with a spectrophotometer fitted with a flow cell (Uvicord I, LKB, Sweden), linear recorder PSR 1-08 (USSR) and either a Perspex peristaltic pump (LKB, Sweden) or a membrane pump MK-1 (USSR).

For buoyant density analysis the fractions of sucrose gradients were dialysed overnight against standard buffer containing 4% formaldehyde (Spirin, Belitsina & Lerman, 1965) and analysed in a pre-formed CsCl gradient. The material to be analysed was used for preparation of light (1.17 g/ml) CsCl solution. The heavy (1.54 g/ml) solution did not contain the analysed material. The gradients were centrifuged in a 3 x 5 rotor of the Superspeed 50 ultracentrifuge at 35000 rev/min for 16 h. The bottom of the tube was punctured and 20 drop fractions were collected: 5 drops of every fifth fraction were taken separately for refractometric determination of density (Ifft, Voet & Vinograd, 1961) with a correction for formaldehyde content. For the determination of radioactivity, acid-insoluble material was precipitated with 5% TCA in the presence of carrier protein (0.2 mg/fraction), washed with
Fig. 1. Rate zonal sedimentation of the extracts of cells infected with NDV after different times of labelling with [14C]-algal hydrolysate. The cells were labelled from 6 to 7 (a), 6 to 8 (b) and 6 to 9 (c) h after infection. The amount of extract layered on the gradient was in (a) 4 times as much as in (b) or (c) as measured by $E_{260}$ nm. Sedimentation was for 1.5 h at 27000 rev/min and 6 °C.

TCA and ethanol on nitrocellulose filters, dried and immersed into scintillation fluid (PPO 5 g, POPOP 0.3 g, toluene 1 l). Samples were counted in a TriCarb spectrometer (Packard, USA).

The sedimentation analysis of EDTA-DOC-treated extracts of NDV-infected cells labelled with [14C]-algal hydrolysate always revealed a 200 S peak. The sedimentation coefficient was calculated from the position of formaldehyde-fixed chick embryo 80 S ribosomes added to the material to be analysed. The 200 S peak increased with the duration of labelling (Fig. 1). No such peak was revealed in the extracts of uninfected cells. Moreover, when the extract of labelled uninfected cells was mixed with an extract of unlabelled NDV-infected cells (prior to EDTA-DOC treatment and formaldehyde fixation) no radioactivity was detected in the 200 S zone (Fig. 2a). This result seems to eliminate the possibility of attachment of labelled soluble proteins to nucleocapsid by formaldehyde. In the same experiment the infected cells were labelled from 6 to 7.5 h after infection and the 200 S material was analysed in a CsCl gradient (Fig. 2b, c). The buoyant density of the labelled material (1.31 g/ml) was characteristic of paramyxovirus nucleocapsid in CsCl (Compans & Choppin, 1967; Hosaka, 1968).

In the next series of experiments we attempted to determine the increase of radioactivity in the 200 S peak under chase conditions. NDV-infected cells were labelled 6.5 h after infection for 20 min with [14C]-leucine. The label was removed and the cells were incubated with maintenance medium containing 100-fold excess (110 μg/ml) of unlabelled leucine. The overall incorporation of label into protein was stopped practically at once, under such conditions (Table 1). The amount of radioactivity in the 200 S peak increased during the chase: it reached a constant level 45 min after the addition of unlabelled leucine (Fig. 3). Similar results were obtained in several experiments. The absolute amount of radioactivity in the 200 S
Fig. 2. Sedimentation and buoyant density analysis of the extracts of uninfected and NDV infected cells. (a) Uninfected cells were labelled with [14C]-algal hydrolysate for 1.5 h. Cell extract was mixed with an equal amount of extract of unlabelled NDV-infected cells (7.5 h after infection) prior to EDTA-DOC treatment and aldehyde fixation. Sedimentation was for 1.5 h at 27000 rev/min in a 15 to 30 % sucrose gradient. (b) NDV-infected cells labelled from 6 to 7.5 h after infection; sedimentation as in (a). (c) Material of 200 S zone from sucrose gradient analysed in CsCl. ●—●, radioactivity; ○—○, density.

Table 1. Depression of [14C]-leucine incorporation with the excess of unlabelled leucine

<table>
<thead>
<tr>
<th>Time of incubation with the label (min)</th>
<th>Incorporation of [14C] into acid-insoluble fraction (ct/min/10^7 cells)</th>
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<tbody>
<tr>
<td>2</td>
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<tr>
<td>4</td>
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<td>14</td>
<td>7919</td>
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Unlabelled leucine (100-fold excess) was added after 8 min of incubation with the label.
* Mean of two parallel samples.

peak varied with the time after infection chosen for the labelling, but the constant level was always reached after 40 to 45 min of incubation with an excess of unlabelled leucine.

The results presented above indicate that there exists a considerable delay between the synthesis of a molecule of nucleocapsid protein and its incorporation into the structure of the nucleocapsid. If one assumes that half of the whole amount of labelled nucleocapsid protein is synthesized by the middle of the labelling period and that half of it is packed into the nucleocapsid during the first 20 min of chase, the duration of delay may be estimated as approximately 30 min. It should be noted that the amount of 200 S-associated radioactivity in the culture fluid is negligible: the termination of increase of the 200 S peak seems therefore to be caused by the exhaustion of labelled nucleocapsid protein and not by the release of virus from the cell. It is also possible that the increase of the 200 S peak could result from release of nucleocapsid from some material pelleted at 2000 g, e.g. large membranous
Fig. 3. Incorporation of labelled protein into 200 S nucleocapsid under chase conditions. NDV-infected cells were labelled 6.5 h after infection with [14C]-leucine for 20 min and incubated for different intervals with 100-fold excess of unlabelled leucine. (a), (b), (c), (d), (e), (f), duration of chase 0, 15, 30, 45, 60, 75 min respectively. Equal amounts of extract analysed in sucrose gradient as in Fig. 1.

structures. This does not seem very likely as results similar to those presented above have been obtained when direct lysis of whole cells with DOC or DOC-NP-40 mixture was performed. However, the possibility of such release from some detergent-resistant material, as well as the binding of nucleocapsid to such material, cannot be absolutely ruled out.

The data reported by Inuma et al. (1971), on a much longer delay between the labelling of virus protein and its appearance in mature NDV, does not contradict ours. Virus maturation is not very efficient in paramyxovirus-infected chick embryo cells (Blair & Robinson, 1970), and the label may appear in the virus particle long after it had been incorporated into nucleocapsid.

In cells treated with cycloheximide after infection with Sendai virus, 50 S RNA is effectively incorporated into nucleocapsid 2 h or more after protein synthesis stops (Robinson, 1971). The apparent discrepancy of this observation with our data may be due to the inhibition of 50 S RNA synthesis by cycloheximide (Robinson, 1971; Kaverin & Varich, 1971), which leads in turn to the suppression of nucleocapsid formation; a longer time is therefore needed for the exhaustion of nucleocapsid protein pool.

In paramyxovirus-infected chick embryo cells, newly synthesized 50 S RNA appears in the nucleocapsid almost immediately (Blair & Robinson, 1970), so there should be a vast excess of free nucleocapsid protein over free 50 S RNA. A considerable delay in the incor-
poration of protein into nucleocapsid observed in our experiments is in agreement with this assumption.

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REFERENCES


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