NEW POLIOVIRUS-RELATED POLYPEPTIDES IN THE NUCLEUS-ASSOCIATED FRACTION OF HELA CELL CYTOPLASM.

by

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SUMMARY. Lysates of poliovirus-infected HeLa cells were fractionated by low speed centrifugation into a sediment ("nuclear fraction") which contained the nuclei and part of the cytoplasm (nucleus-associated cytoplasm, NAC) and a supernatant (nucleus-unassociated cytoplasm, NUC). Both the nuclear fraction and NUC promoted the conversion of 14 S precursor particles to poliovirus procapsids. The NAC contained numerous species of poliovirus-related particles. Their analysis revealed the presence of two new viral polypeptides, NACP-1 and NACP-2, which were absent from the NUC; their molecular weight was estimated as 37,000 and 54,000, respectively.

INTRODUCTION

Poliovirus and encephalomyocarditis virus (EMC) display striking similarities with respect to their genetic map, the biogenesis of their proteins, the mechanism of assembly and final composition of the capsid (1-5). It was therefore surprising that no poliovirus-related equivalents had been found to at least some of the EMC-related noncapsidial viral proteins, such as E (M.W. 56,000, migrating between D and ε) and F (M.W. 38,000, migrating between ε and α, 4). A close inspection of the technique revealed that the EMC investigators examined whole-cell extracts, while the poliovirus workers used to free the cytoplasm of nuclei by centrifugation. The cytoplasmic material cosedimenting with the nuclei was thereby routinely, and, as this paper will show, imprudently overlooked.

MATERIALS AND METHODS

1. Labeling procedure.

1×10^9 HeLa cells are suspended in serum-free Eagle medium (6) minus valine and leucine (ED) and complemented with 1 µg/ml actinomycin D (7). Poliovirus of type 1, strain 1a/S3 is added at an input multiplicity of 200 plaque-forming units per cell. The cells are incubated at 37° under constant agitation for 90 minutes, centrifuged, resuspended in 100 ml of ED with 1 µg/ml actinomycin D, and again incubated for 2 hours. [^{3}H]L-valine and [^{3}H]L-Leucine (0,5 µC/ml) are added. The incorporation is

stopped by adding 200 ml of ice-cold ED containing 0.002 M L-leucine and L-valine. The cells are washed twice in the latter medium and resuspended in 5 ml of RSB (i.e., 0.01 M tris buffer of pH 7.2 containing 0.1 M NaCl and 0.0015 M $MgCl_2$).

2. Preparation of cytoplasmic extracts.
The labelled cell suspension after four cycles of freezing at -40° and thawing at 20° is centrifuged for 10 minutes at 800 g. The supernatant is considered to be NUC. The sediment after one washing with RSB constitutes the "nuclear fraction". In order to obtain the NAC, the nuclear fraction is resuspended in 5 ml of RSB containing 0.5 % DOC and 1 % Nonidet (NP40, gift of Shell Oil Co.), incubated for 5 minutes at 37° and centrifuged for 10 minutes at 800 g. This procedure does not rupture the nuclei (8). The supernatant constitutes the NAC.

3. Polyacrylamide gel electrophoresis.
The protein is extracted by the procedure designated as "method 2" in a previous paper (9). The electrophoresis is done in 12.5 % polyacrylamide gels for 16 hours at 4 mA per gel; other technical specifications are as described elsewhere (9).

4. Measurement of radioactivity.
The proteins are precipitated with trichloroacetic acid and collected on glass filters (Millipore type GF/A, 2.1 cm). The filters are washed twice with 5 % trichloroacetic acid, allowed to dry and treated for 90 minutes with 0.2 ml of Soluene 100 (Packard Instruments). The scintillation liquid is then added, which consists of toluene containing 4 g/l of 2,5-diphenyloxazole and 0.8 g/l p-bis-(o-methylstyryl)-benzene. In order to determine their radioactivity profile, gels are cut into 1 mm slices. The slices are wetted with water for 15 minutes and incubated for 12 hours at 37° after adding 0.5 ml of Soluene 100. Ten milliliter of scintillation liquid are added and the mixture is shaken for 3 hours.

5. Sucrose gradient centrifugation.
Gradients of 15-30 % sucrose (in RSB) in a Sw-25 swinging-bucket rotor were used. The temperature during the runs was kept constant at 7° C. The contents of the tubes were collected as fractions of 0.8 ml.

6. Location of viral proteins.
To each sample of ^{3}H viral proteins to be electrophoresed, a sample of ^{14}C virion proteins was added and co-electrophoresed; the ^{14}C peaks located VP-1, 2 and 3. In some cases, protein of ^{14}C top component was added to locate VP-0.

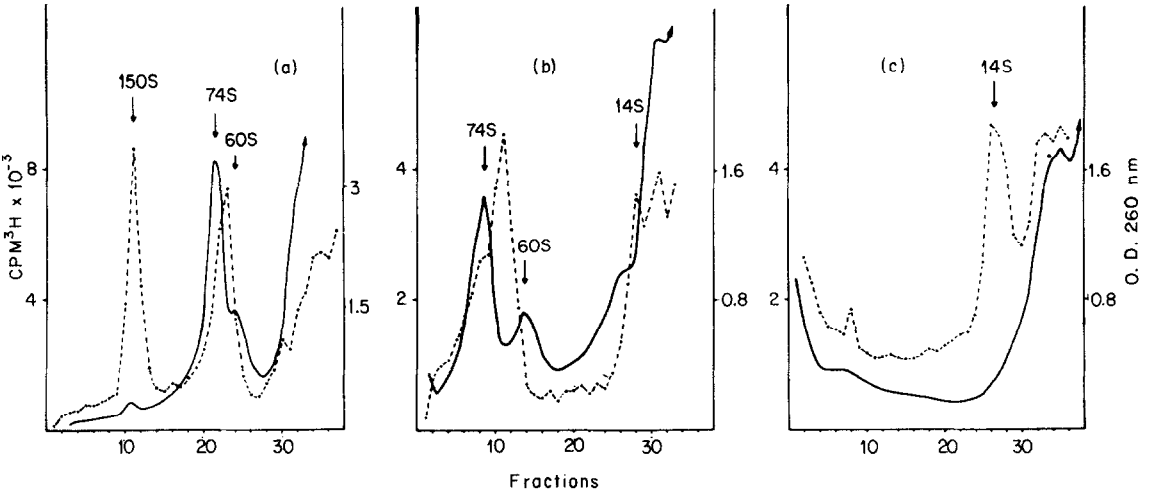

Fig. 1. : Preparation of viral precursor particles.
HeLa cells were infected with poliovirus and labelled for 1 hour with [^{3}H] leucine and [^{3}H] valine as described under Materials and Methods. The cytoplasm after removing the nuclei by centrifugation was centrifuged in a sucrose density gradient at 25,000 rpm for 4 1/2 hours (a), 13 hours (b) or 26 hours (c).
Full line : optical density at 260 nm
Dashed line : radioactivity.

The noncapsidial proteins NCVP-1 and 2 were located by addition of protein of an extract of NUC prepared from HeLa cells which were labelled for 10 minutes with [^{14}C] algal protein hydrolysate 3 1/2 hours postinfection. NCVP-1 and NCVP-2 were distinguished on the basis of the fact that NCVP-1 is converted into capsidial proteins, while NCVP-2 is stable (2, 10). The above 10 minutes pulse was followed by a 20 minutes chase and one polypeptide, which was therefore considered as NCVP-1 lost most of its radioactivity. The other remained stable and was identified as NCVP-2. The relative position of the two NCVP's was in agreement with the literature data.

RESULTS AND DISCUSSION

Infected HeLa cells in suspension were labelled for one hour using 0.5 µC/ml of [^{3}H] L-valine and of [^{3}H] L-leucine (see Materials and Methods 1) and the contents were analyzed by centrifugation in sucrose density gradients at 24,000 rpm for various lenghts of time; Figure 1 shows the optical density and radioactivity profiles. Centrifugations for 4 1/2 and 13 hours allowed a good separation of the virions (150 S, Fig. 1a) and procapsids (70 S as determined by comparison with 74 S HeLa cell ribosomes, Fig. 1b). Well-separated 14 S particles could be harvested from the gradient after 26 hours of centrifugation (Fig. 1c). The conversion of 14 S to 70 S particles was ob-

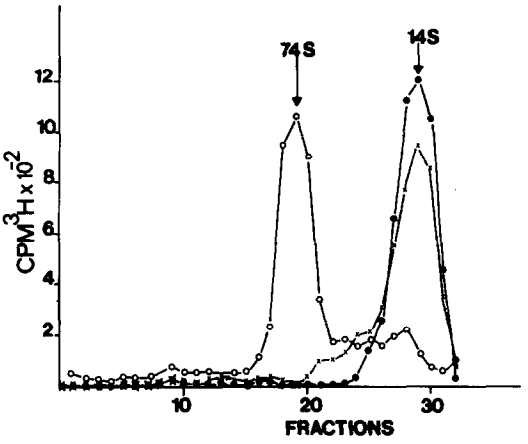

Fig. 2. : Assembly of 14 S into 74 S particles.

To 0.5 ml of a suspension of [^{3}H] labelled 14 S particles in RSB, one of the ingredients listed below was added and the mixture was incubated at 37° C under constant agitation. The reaction was stopped after 10 minutes by addition of 1 ml of ice-cold RSB and transfer to an ice bath. The mixture was then centrifuged at 23,000 rpm for 12 1/2 hours.
Solid circles : 1 ml of RSB
Open circles : 1 ml of RSB containing the nuclear fraction (see Materials and Methods) of 5 x 10^7 HeLa cells collected 3 1/2 hours after infection with 200 plaque-forming units of poliovirus.
Crosses : 1 ml of RSB containing the nuclear fraction of 5 x 10^7 uninfected HeLa cells.

served both in vivo by pulse-chase experiments and in vitro by adding NUC of infected HeLa cells to labelled 14 S particles (10). In our hands, unfractionated cytoplasm in RSB promoted the conversion of 90 % of the 14 S particles in 10 minutes at 37°. When the nuclear fraction was used instead, the same conversion occurred. Figure 2 shows the results of incubating 14 S particles with the nuclear fraction of infected cells. After 10 minutes, the conversion to 74 S particles was almost complete; to the contrary, no conversion occurred when the 14 S particles were incubated alone or with the nuclear fraction of uninfected cells. These results show the presence of viral material in the nuclear fraction of infected cells.

After labeling infected cells for one hour with [^{3}H] L-leucine, the NAC and NUC were examined for the presence of virus-related particles by centrifugation in a sucrose density gradient. Somewhat unexpectedly, 60 % of the total radioactivity was found in the NAC, and the sedimentation profile of the NAC (Fig. 3c and d) offered a more complex picture than that of the NUC. In the NUC, only the familiar 150 S, 70 S and 14 S particles were

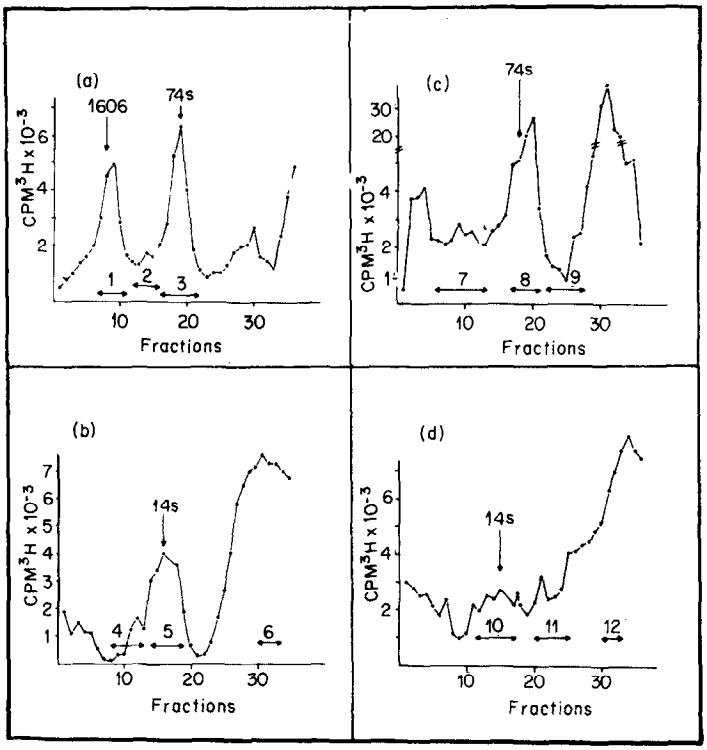

Fig. 3. : Poliovirus-related particles in the NUC and NAC of infected HeLa cells.

HeLa cells were infected and labelled for 1 hour with [^{3}H] L-leucine and their contents were fractionated as described under Materials and Methods. The NUC and NAC fractions were centrifuged in a sucrose gradient at 24,000 rpm.
a and b : NUC, centrifuged for 4 1/2 hours (a) or 32 hrs (b)
c and d : NAC, centrifuged for 4 1/2 hours (c) or 32 hrs (d)
Double-headed arrows : pools of fractions used for the determination of the polypeptide pattern (Figg. 4 and 5).

present (Fig. 3a and b). Fractions from the two sucrose gradients were pooled as shown in Fig. 3. The contents were analyzed by extraction and electrophoresis of the protein as described under Materials and Methods. Figures 4 and 5 show the electropherograms. In the fractions of the NUC, only the polypeptides NCVP-1, NCVP-2, VP-0, 1, 2 and 3 were found (10; VP-4 was excluded from the analysis; they were identified as described under Materials and Methods 6). In addition to these, the NAC material yielded two hitherto unreported polypeptides, which will be referred to as NACP-1 and NACP-2.

NACP-1 migrates between NCVP-2 and VP-0 and its molecular weight was estimated by interpollation as 54,000. NACP-2 migrates between VP-0 and VP-

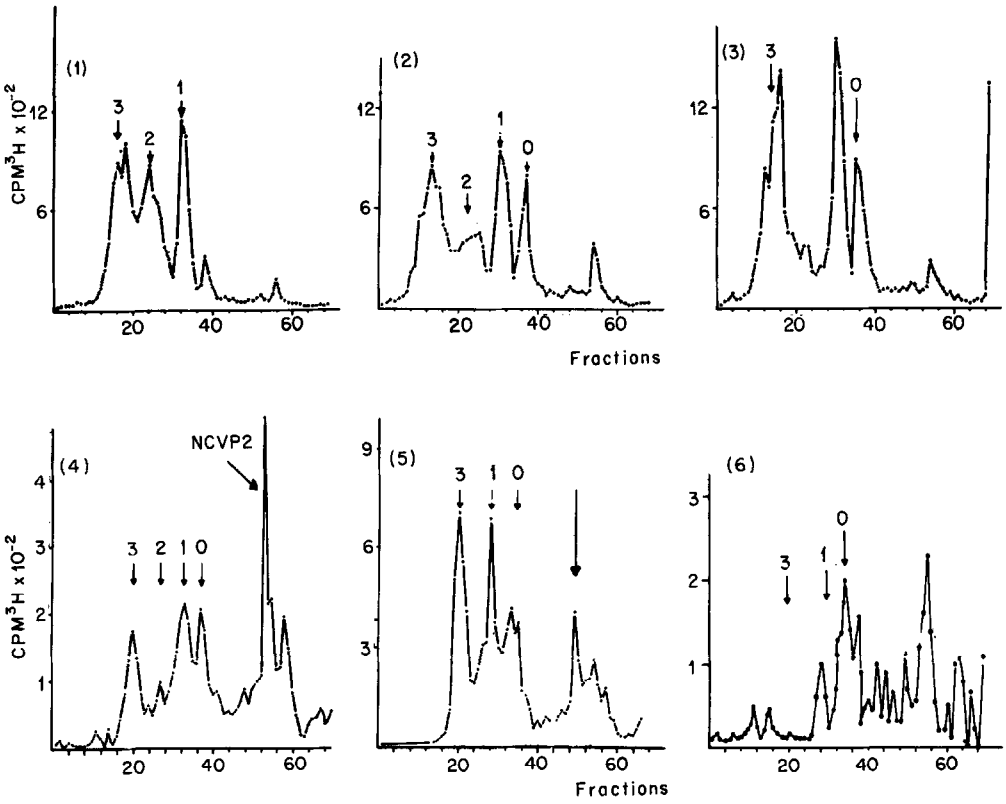

Fig. 4. : Polypeptide pattern of [^{3}H] particles from the NUC of infected HeLa cells.

1-6 : pools shown in Fig. 3a and 3b.
Identification of polypeptides and other techniques as described under Materials and Methods 3, 4 and 6.

and its molecular weight must therefore approximate 37,000. It was abundant in all three species of particles derived from the NAC and exclusively in these. In view of the similarities in molecular weight, we propose tentatively that NACP-1 and NACP-2 are homologous to the EMC-related noncapsidial proteins E and F, respectively.

At least two of the noncapsidial poliovirus-related proteins, NACP-1 and 2 are exclusively found in one fraction of the cytoplasm. This invites the speculation that different stages of the biosynthetic sequence are located in different cell compartments. This intracellular compartmentation in turn may hold the key to the cellular regulation of poliovirus development exemplified by its dependence on the mitotic stage of the host cell (11).

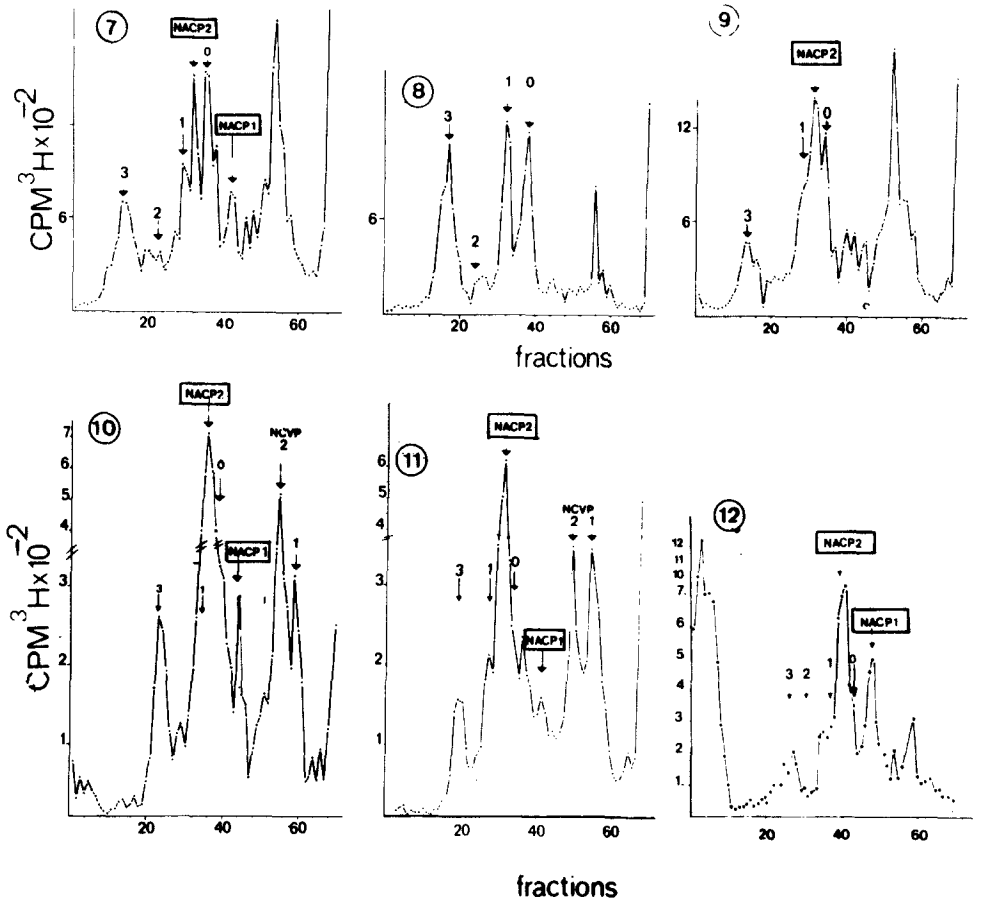

Fig. 5. Polypeptide pattern of [^{3}H] particles from the NAC of infected HeLa cells.

7-12 : pools shown in Fig. 3c and 3d.
The two polypeptides not found in NUC extracts are indicated by NACP-1 and NACP-2 in boxes.

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