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# Determination of the Gene Sequence of Poliovirus with Pactamycin

**(translation/HeLa cells/protein precursor molecules/gel electrophoresis/initiation)**

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**ABSTRACT** **By examination of the virus-specific polypeptides formed after the addition of pactamycin, an inhibitor of protein chain initiation, to infected cells, it has been possible to tentatively locate the virus coat proteins at the amino terminus of the large, virus-specific protein precursor, and, therefore, to assign the coat protein cistron to the 5′ end of the RNA.**

The synthesis of structural and nonstructural proteins of poliovirus is directed by the single-stranded RNA genome, which acts as a mRNA in infected cells (1, 2). The functional virus-specific proteins are derived by cleavage of larger precursor protein molecules (3–5), which in turn may be derived from a single large polypeptide, having a molecular weight of greater than 200,000, which might result from the complete translation of the entire virus mRNA (6, 7).

One large molecule [noncapsid viral polypeptide (NCVP) 1a of MW 105,000] is the precursor of the four structural proteins of the virion (7, 8) and contains most, if not all, of the peptides found in these capsid proteins. Another protein (NCVP 2) (MW 70,000) was shown by pulse–chase experiments to be a stable protein that probably serves some nonstructural role in virus replication (3, 8).

Baltimore *et al.* showed that another noncapsid protein, which they called NCVP-X (MW 35,000), was labeled very rapidly during short pulses with radioactive amino acids, and suggested that this molecule might be an initial cleavage product, along with NCVP 1a and NCVP 2, of the putative single large protein made from the virus genome (6).

The total MW of these three proteins (35,000, 70,000, and 105,000) is about 210,000 and, therefore, could represent the majority of the peptide sequences coded for by the entire virus genome. Although genetic recombination studies have provided a crude map of the ordering of genes in the viral RNA (9), no information on the direction or termini of the genome has been available. We have, therefore, attempted to establish the sequence of the virus-specific proteins within the large precursor and, by inference, the sequence of these "genes" in the virus RNA.

Since the growth of the virus-specific polypeptides is probably from the amino terminal amino-acid residue to the carboxyl terminus, and the virus mRNA is read from the 5′ end to the 3′ end (10), it should be possible by differential labeling of growing polypeptide chains to determine which of the large precursor proteins are translated from the initiating end of the virus mRNA.

Abbreviations: MW, molecular weight; NCVP, noncapsid viral polypeptide.

This report is concerned with studies in which the drug pactamycin was used at low concentrations to study the translation of the large, poliovirus-specific precursor proteins. Recent investigations have shown that low concentrations of pactamycin inhibit the initiation of protein synthesis by intact reticulocytes and by cell-free extracts from reticulocytes (11, 12). At higher concentrations, pactamycin also affects polypeptide chain elongation. We have found that in poliovirus-infected HeLa cells, 1 $\mu$M pactamycin primarily inhibits the initiation of new protein chains, but allows the completion of nascent proteins and their release from the ribosomes, thus permitting study of a single round of translation. By pulse labeling the virus-specific nascent proteins at different times after the addition of pactamycin, and thereby progressively restricting incorporation to the carboxy-terminal portion of the large precursor molecule, we have been able to "map" the smaller protein molecules that arise from this protein.

## MATERIALS AND METHODS

*Cells and Virus.* Suspension cultures of HeLa $S_3$ cells were grown in Eagle's minimal essential medium supplemented with 5% fetal-calf serum. Type 1 poliovirus was used throughout the studies. Growth of virus stocks, purification of the virus, infection of suspension cultures at a high multiplicity of infection, and preparation of extracts of infected cells have been described (13).

*Acrylamide Gel Electrophoresis and Assay of Radioactivity.* The procedures used in preparing discontinuous acrylamide gels containing sodium dodecyl sulfate have been described in detail (14), as were the procedures used in gel fractionation, assay of radioactivity in the gel fractions (15), and assay of radioactive material in sucrose gradients (16).

*Source of Materials.* Mixtures of 15 amino acids ($^{14}$C- or $^{3}$H-labeled) ($^{14}$C, 80–400 Ci/mol, $^{3}$H, 0.5–60 Ci/mmol; 1 mCi/ml, New England Nuclear Corp.) were used to label virus proteins. Actinomycin D was a gift from Merck and Co. and pactamycin was supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute.

## RESULTS

### The effect of pactamycin on protein synthesis in poliovirus-infected HeLa cells

Fig. 1 shows that poliovirus-infected cells treated with 5 $\mu$M or higher concentrations of pactamycin immediately stop their

synthesis of virus-specific proteins, and that this inhibition occurs without lag. However, 1 μM pactamycin inhibits virus-specific protein synthesis completely only after a lag of 20–30 min. These results are similar to those reported for reticulocytes (11, 12), where 1 μM pactamycin inhibited globin synthesis after a 2-min lag, during which globin chains were being completed and released from the polysomes. Higher concentrations of pactamycin caused a much more rapid inhibition of synthesis, suggesting that these high concentrations also affected chain elongation.

**The effect of pactamycin on polyribosome profiles in poliovirus-infected cells**

Fig. 2 and Table 1 show the distribution of radioactivity in nascent protein chains on poliovirus-specific polyribosomes and in finished proteins that have been released from the polysomes during the 15-min labeling time. It can be seen that higher concentrations of the antibiotic prevent incorporation of amino acids into nascent proteins. Concentrations of about 1 μM permit incorporation into nascent proteins, and also permit the release of completed chains, with a concomitant dissociation of polysomes into single ribosomes (Fig. 2). At this concentration of pactamycin, incorporation into both nascent and released proteins was about 30% that of the control culture (Table 1).

**The effect of 1 μM pactamycin on poliovirus-specific protein synthesis**

The effects of pactamycin, at a concentration that seemed to be inhibiting the synthesis of new protein chains but that allowed the elongation and release of nascent proteins, were studied by measurement of the incorporation of [$^{14}$C]amino-acids during 2-min pulses at different times after the addition of 1 μM pactamycin (Table 2).

As early as 0–2 min after pactamycin addition, the amount of incorporation into polysomes decreased to about 50% of the control (Table 2), and the amount of released protein to about 30% of the control culture. At later times after addition of the antibiotic, the amount of radioactivity in nascent and released proteins falls to very low amounts. In several different experiments like the one shown in Table 2, the time necessary to decrease the amount of amino-acid incorporation to 5–10% of the control was always about 10 min. These results support the hypothesis that pactamycin preferentially inhibits the initiation of new polypeptide chains, but allows the completion of those proteins already begun on the ribosomes.

**Preparation of virus-specific proteins from polyribosomes at various times after the addition of pactamycin**

From the data in Table 2, one can estimate that (at least in the presence of pactamycin) about 10 min are required to complete translation of the virus mRNA. In order to preferentially label the carboxy-terminal portion of the large precursor protein, pactamycin (1 μM) was added to infected cells and, at subsequent times, the cells were labeled for 5 min with radioactive aminoacids. Fig. 3 shows sucrose gradient analysis of these cytoplasmic extracts; there is a progressive decrease in the size and amount of the virus-specific polysomes. Table 3 shows the total acid-precipitable radioactivity in the sucrose gradients shown in Fig. 3. There is a decreasing amount of incorporation into polysomes after the addition of pactamycin, and there is a continued release of proteins to the top fractions of the gradients.

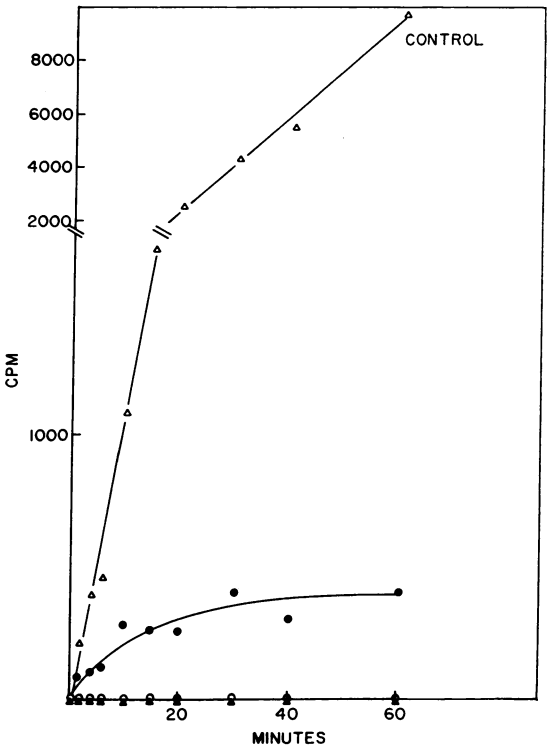


FIG. 1. Inhibition of virus-specific protein synthesis by pactamycin. A culture of HeLa cells (1 × $10^8$ cells) at a concentration of 4 × $10^6$ cells/ml containing 10 μg/ml of actinomycin D was infected with 300 plaque-forming units of poliovirus per cell. At mid-cycle of infection (2.75 hr after infection) the culture was divided into four equal portions; one served as a control and the other three were made 10, 5, and 1 μM with respect to pactamycin at zero time. At zero time, all cultures also received 25 μCi of [$^{14}$C]amino acid mixture. At the indicated times, 0.2 ml of the culture was removed and was assayed for trichloroaceticacid-precipitable radioactivity. ●——●, 1 μM pactamycin; ○——○, 5 μM pactamycin; ▲——▲, 10 μM pactamycin, △——△, no pactamycin.

**Acrylamide gel analysis of released proteins**

The released, completed poliovirus proteins contained in the top few fractions of the gradients shown in Fig. 3 were solubilized and $^3$H-labeled proteins from the control gradient were mixed with released proteins from each of the experimental cultures.

Fig. 4*a* shows that 0.5–5.5 min after the addition of pactamycin, amino acids entered all of the peaks visible in the control sample, but it is also evident that there is a relative decrease in the amount of NCVP 1a.

In contrast to this, it can be seen in Fig. 4*b* that if the treated culture is labeled from 3 to 8 min after pactamycin addition, NCVP 1a is completely absent, but NCVP 2 is present in amounts comparable to those seen in control cultures. Therefore, NCVP 1a appears to be the amino-terminal virus polypeptide, while NCVP 2 is at or near the carboxyl terminus.

## DISCUSSION

Previous reports (11, 12) have shown that selected concentrations of pactamycin inhibit protein synthesis by intact

TABLE 1. *The effect of different concentrations of pactamycin on virus-specific protein synthesis*

| Concentration of pactamycin (μM) | Polysomes | Released protein | Total |
|---|---|---|---|
| Control | 46,488 | 176,642 | 262,188 |
| 50 | 3,704 | 2,093 | 8,256 |
| 6 | 7,268 | 10,673 | 22,563 |
| 3 | 7,328 | 19,651 | 30,693 |
| 1 | 14,313 | 61,920 | 82,950 |
| 0.1 | 27,783 | 85,854 | 124,914 |

The acid-precipitable radioactivity in the polysome region (sample numbers about 1–15) and the top of each gradient (which contained the released, completed, virus-specific proteins of Fig. 2) were totalled, as were the total cpm in each gradient.

TABLE 2. *Poliovirus-specific protein synthesis in the presence of 1 μM pactamycin*

| Time of pulse after pactamycin addition, min | Polysomes | Released protein | Total |
|---|---|---|---|
| Control | 4079 | 14,778 | 20,590 |
| 0–2 | 2379 | 5,533 | 9,113 |
| 2–4 | 1236 | 4,025 | 5,960 |
| 4–6 | 1071 | 2,762 | 5,211 |
| 8–10 | 220 | 1,711 | 1,973 |

Cells were infected as described in Fig. 2, and were divided into five equal cultures at 2.75 hr after infection. Four cultures were made 1 μM with respect to pactamycin, and at the indicated times after the addition of pactamycin the cultures were pulsed for 2 min with 50 μCi of [$^{14}$C]aminoacid mixture; incorporation then stopped by the addition of frozen Earle's solution. Cytoplasmic extracts were prepared and radioactivity in virus proteins was assayed as described in Fig. 2.

reticulocytes and cell-free reticulocyte lysates. The drug acts by blocking the initiation of new globin chains, but allows completion and release of nascent chains. The time required for complete inhibition of protein synthesis by the drug was about 2 min (12); this time reflects the time required to complete and release the nascent globin chains of the poly

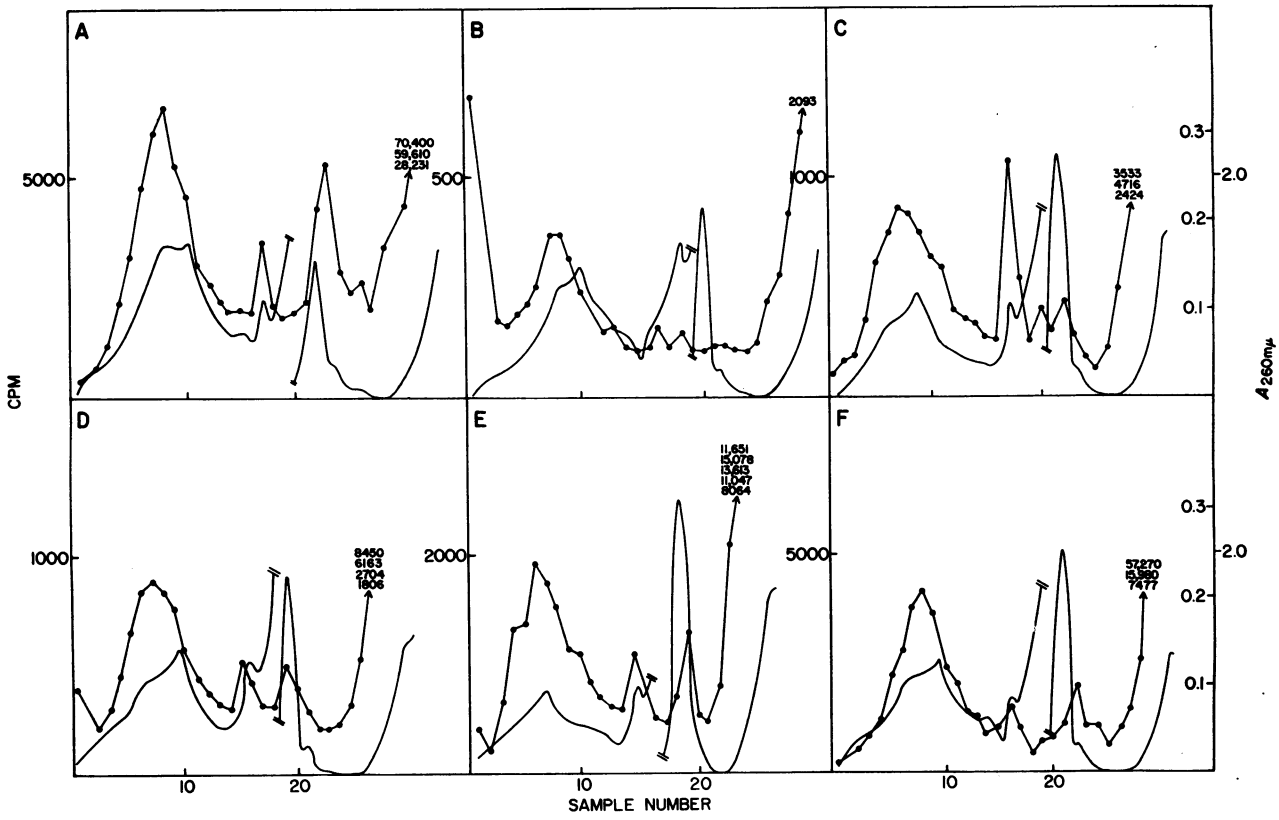


FIG. 2. The effect of different concentrations of pactamycin on poliovirus-specific polysomes. A culture of HeLa cells ($1.2 \times 10^8$) at $2 \times 10^6$ cells/ml plus actinomycin D (10 μg/ml) was infected with a multiplicity of infection of 200 plaque-forming units for 2.75 hr, at which time the cells were divided into six equal cultures. One served as control, and pactamycin was added to the remaining cultures at the indicated concentrations. 60 μCi of [$^{14}$C]amino acid mixture was immediately added to each culture, and 15 min later all six cultures were poured over a frozen slurry of Earle's balanced salt solution (EBS) to stop incorporation. The cells were washed once with EBS, cytoplasmic extracts were prepared, the extracts were made 1% with respect to Brij 58 and deoxycholate, and they were layered over linear 7–52% (w/w) sucrose–RSB (RSB = 0.01 M NaCl–1.5 mM $Mg^{++}$–0.01 M Tris, pH 7.4) gradients. The gradients were contrifuged at 15,000 rpm and 5°C for 16 hr in a Spinco SW27 rotor. Fractions of each gradient were collected and their absorbance at 260 nm was recorded by pumping the gradients through a Gilford recording spectrophotometer. Each fraction was precipitated with trichloroacetic acid and radioactivity was assayed. *A*. Control, *B*. 50 μM pactamycin, *C*. 6 μM pactamycin, *D*. 3 μM pactamycin, *E*. 1 μM pactamycin, *F*. 0.1 μM pactamycin. Note change in cpm scales. $A_{260}$, solid line; cpm, ●——●.

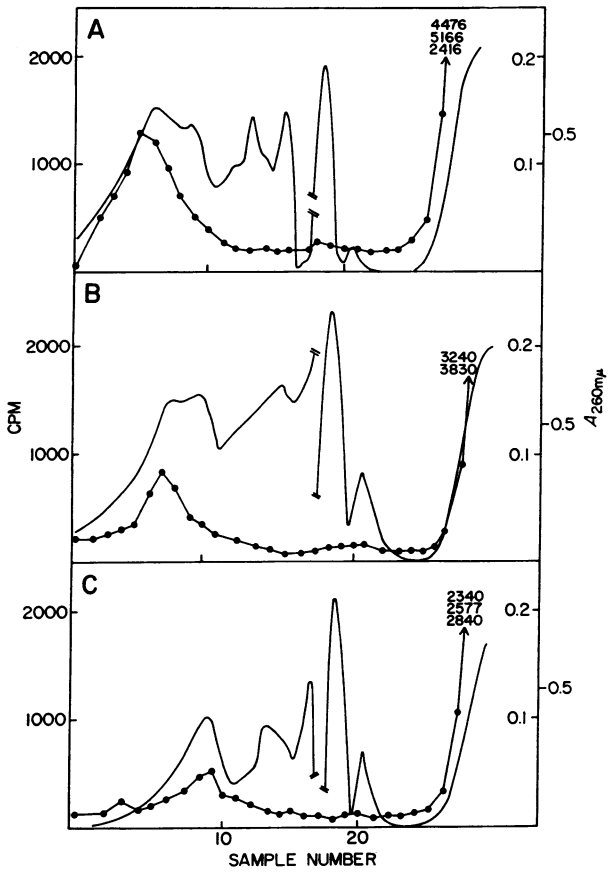


FIG. 3. 5-min label of poliovirus proteins after addition of 1 $\mu$M pactamycin. Cells were infected as in Fig. 2, and at mid-cycle of the infection the culture was split into three aliquots, each containing $2 \times 10^7$ cells. The control culture was labeled for 5 min with 300 $\mu$Ci of [$^3$H]amino acid mixture and stopped. The two other cultures were made 1 $\mu$M in pactamycin; one culture was labeled from 0.5 to 5.5 min after the addition of pactamycin and the second was labeled from 3 to 8 min after pactamycin with 50 $\mu$Ci of [$^{14}$C]amino acid mixture. Cytoplasmic extracts were prepared from each culture and were analyzed on 7–52% sucrose–RSB gradients.

0.1 ml of each gradient fraction was assayed for acid-precipitable radioactivity, and the remainder of each fraction was saved for acrylamide gel analysis (See Fig. 4). $A_{260}$, solid line; cpm, ●——●.

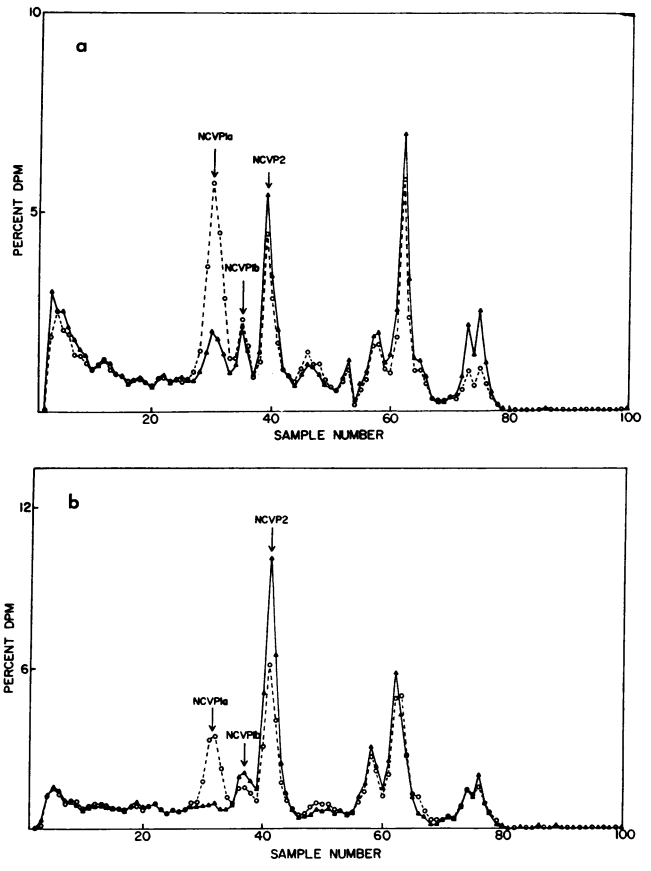


FIG. 4. Acrylamide gel analysis of completed virus proteins in pactamycin-treated cells. The fractions from the top portions of the gradients shown in Fig. 3 were concentrated by acid precipitation, solubilized in sodium dodecyl sulfate buffer, and applied to 20-cm acrylamide gel columns that contained 7.5% acrylamide in their upper half and 15% acrylamide in their lower half: portions of the solubilized $^3$H-labeled proteins from the control culture were mixed with $^{14}$C-labeled proteins from the pactamycin-treated cultures before electrophoresis. These gels were run with a discontinuous buffer system (14) and were assayed for radioactivity after fractionation.

The raw data from a Beckman l quid scintillation spectrometer was put into an IBM computer (1800), which corrected the channels for spill and plotted the data as percent of total disintegrations per minute (% dpm). $^{14}$C(pactamycin treated), ●——●; $^3$H (control), O——O.

somes. This inhibition was correlated with a conversion of the reticulocyte polysomes to monomeric ribosomes, and a release of completed globin molecules. A concentration of 1 $\mu$M pactamycin maximally inhibited initiation in whole cells, while minimally affecting chain elongation; higher concentrations increasingly inhibited elongation and peptide release (12).

It has not been possible to define precisely the mechanism of action of pactamycin on the large poliovirus polysomes, since an *in vitro* system comparable with the reticulocyte system is not available, and the synthesis and release of the polypeptide product produced by the large poliovirus mRNA cannot be easily followed. Rather, the evidence to date (3–8) suggests that the poliovirus mRNA has one initiation site and is translated into one large polypeptide precursor (MW > 200,000) that is cleaved during translation to produce several other large virus proteins, one of which (NCVP 1a) is further cleaved to produce the virion proteins. After addition of

TABLE 3. *5-min pulses and labeling of polivirus proteins in the presence of pactamycin*

| Time of pulse after pactamycin addition, min | Polysomes | Released protein | Total |
|---|---|---|---|
| Control | 7488 | 14,025 | 24,851 |
| 0.50–5.50 | 4036 | 8,291 | 14,890 |
| 3–8 | 2644 | 9,141 | 13,880 |

This data is derived by summation of the acid-precipitable radioactivity in the various regions of the gradients shown in Fig. 3.

pactamycin, and before complete inhibition of protein synthesis, there is continued incorporation of labeled amino acids into acid-precipitable material that occurs over a 20–30 min interval (Fig. 1). During this time, polyribosomes begin to break down (beginning with the larger complexes), nascent peptide is released to gradient supernatants, and 74S monomeric ribosomes accumulate. These results are in agreement with those of Stewart-Blair *et al.* (12), with the exception of the time required to completely inhibit protein synthesis. Since it took about 2 min to stop globin synthesis completely (12), and the MW of the globin mRNA is about $2 \times 10^5$ (17), it was not unexpected that the poliovirus mRNA, with a MW of $2.6 \times 10^6$ (18), took 20 min or longer to be completely translated in the presence of pactamycin.

The acrylamide gel patterns of completed and released poliovirus proteins synthesized in the presence of pactamycin show that the capsid precursor molecule, NCVP 1a, decreases and finally disappears as one delays the addition of labeled amino acids for longer times after pactamycin addition. This finding strongly suggests that this protein is on the amino terminus of the putative large precursor protein, and that the coat protein "cistron" is at the 5′ end of the virus genome. Since NCVP 2, a stable protein of unknown function, shows little or no decrease, even at the latest times of addition of labeled amino acid, we suggest that this "gene" is at or near the 3′ end of the genome. Since the relationship of the several other noncapsid, virus-specific proteins (13) to translation and virus replication is not clear, further models of cleavage and gene order must await future studies.

Since NCVP 1a is the precursor protein to the four capsid proteins (6, 8), and since, based on its MW, it probably represents about 30% of the virus genome, one could conceivably order the four coat proteins within the larger molecule by the use of pactamycin as reported above. These studies, using different labeling times after pactamycin addition, are now in progress.

## NOTE ADDED IN PROOF

Conclusions similar to these concerning gene order in picornaviruses have been derived from experimentation with pactamycin-heated, poliovirus-infected Hela cells (R. Taber, D. Rekosh, and D. Baltimore, personal communication), and from studies on the kinetics of *in vivo* synthesis and maturation of encephalomyocarditis virus (R. Rueckert, personal communication).

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