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Synthesis and Cleavage of Enterovirus Polypeptides in Mammalian Cells

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Evidence is presented that the entire enterovirus ribonucleic acid genome is translated into a single giant polypeptide of well over 200,000 daltons molecular weight. This giant protein was detected repeatedly in coxsackievirus B1-infected cells, even in the absence of amino acid analogues. The enzymes which cleave this giant protein into smaller structural and enzymatic proteins accumulate with increasing time after infection. It appears that they are either virus-coded proteolytic enzymes or else host enzymes which are activated or released from an intracellular site as a result of virus infection. These cleavage enzymes do not cause grossly apparent cleavage of host-cell proteins.

The early work of Maizel, Summers, and coworkers (8, 9, 12) showed that poliovirus infection of HeLa cells induces the synthesis of numerous discrete protein molecules. Recently, it was shown in three different laboratories that many (perhaps all) of these proteins are derived by specific cleavage of giant "polycistronic" polypeptides into smaller structural and functional products (2–5, 11). The present study represents an investigation of this phenomenon with several enteroviruses in a number of different cells. It is shown here that this cleavage phenomenon is widespread, if not universal, among the enteroviruses, that the cleavage points are specific for each virus and are the same in many different host cells, that all of the proteins of these viruses are derived by cleavage from a single polypeptide chain which must be coded by the entire viral genome, that the cleavage enzyme activity accumulates with time after infection, and that the cleavage enzyme does not cause apparent degradation of host cell proteins even by the later stages of infection.

MATERIALS AND METHODS

All cells were grown on Eagle's minimal essential medium (MEM) and labeled in MEM containing ^{3}H- or ^{14}C-labeled phenylalanine, valine, and tyrosine in place of the same unlabeled amino acids. All virus pools were grown on HeLa cells. Type 1 poliovirus (Mahoney), type 2 poliovirus (MEF 1), type 1 coxsackievirus (Conn 5), and type 5 coxsackievirus were all originally obtained from J. T. Syverton. Mengovirus was originally provided by John Colter. Virions were purified by ammonium sulfate precipitation, dialysis, differential ultracentrifugation, density gradient banding in cesium chloride, and further dialysis to remove CsCl.

Cells were infected at effective multiplicities of several hundred plaque-forming units (PFU) per cell. Times after infection are computed from the beginning of virus adsorption.

Electrophoretic separations were carried out in 5% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), by a modification of the techniques developed by Maizel and his colleagues (7, 10, 12). The electrophoretic tray buffer consisted of 0.1 M tris (hydroxymethyl)aminomethane (Tris)-acetate buffer (*p*H 9.0), with sodium acetate added to a concentration of 0.05 M to raise the anion concentration, 0.1% SDS, and 0.01% ethylenediaminetetraacetate (EDTA). Mercaptoethanol (0.1%) and 0.01% mercaptoethylamine were added immediately before use. The polyacrylamide gel was polymerized in 0.1 M Tris-acetate buffer (*p*H 9.0) containing 0.1% SDS. Gel polymerizing components in this buffer were 5% acrylamide, 0.167% bis acrylamide, 0.07% ammonium persulfate, and 0.035% *N,N,N',N'*-tetramethylethylenediamine. Gels were prerun for several hours. Samples were loaded onto gel columns (23 by 0.6 cm) after mixing appropriate amounts of ^{14}C-labeled and ^{3}H-labeled proteins that were to be compared. Samples were applied under the electrophoresis buffer in sample buffer plus 20% glycerol in volumes of 0.3 ml or less. Sample buffer consisted of 0.01 M Tris-acetate buffer (*p*H 9.0), 0.1% SDS, 0.001% EDTA, 0.5 M urea, and 0.1% freshly added mercaptoethanol. Protein samples were exhaustively dialyzed against large volumes of this buffer and then were heated in this buffer for about 30 sec before application to the top of the electrophoresis column. Electrophoretic separations were carried out at a constant voltage of 3 v/cm for about 16 hr. The gels were crushed sequentially on the linear fractionator designed by Maizel (7) and

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collected in scintillation vials in a stream of water. The water was evaporated at 60 C, and a special scintillation fluid (designed for maximal gel rehydration and recovery of label) was added to each vial. This scintillation fluid contained 4 g of 2,5-diphenyloxazole, 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, 27 ml of a homogeneous 5.5:1 mixture of Amersham/Searles NCS and water, and sufficient toluene to make a final volume of 1 liter. This NCS-water-based scintillation fluid, when added to thoroughly dried gel particles, allows recovery of more than 98% of the counts/min in proteins added to the gel column if the scintillation vials are incubated at 50 C for 10 to 20 hr after scintillation fluid is added (to facilitate gel swelling before counting). The vials were counted in Beckman scintillation counters under conditions appropriate for dual label counting, and the data tapes were processed by a computer (Computer Data Co., model 3600) for cross-talk correction and automatic graph plotting. (The figures in this paper are photographs of the computer plots.)

Virus-directed proteins were labeled by exposure of infected cells to Eagle's MEM containing 2% dialyzed calf serum plus ^{3}H- or ^{14}C-labeled valine, tyrosine, and phenylalanine. The specific activities of the ^{3}H-amino acids were 1-phenylalanine, 6,300 mc/mmole; 1-tyrosine, 35,100 mc/mmole; and 1-valine, 2,400 mc/mmole. The specific activities of the ^{14}C-amino acids were: 1-phenylalanine, 459 mc/mmole; 1-tyrosine, 305 mc/mmole; and 1-valine, 200 mc/mmole. Protein concentration was determined by the method of Lowry et al. (6), and proteins were dissolved in 10-fold diluted sample buffer minus mercaptoethanol when such determinations were to be carried out.

RESULTS

Virus-directed synthesis in different mammalian cells. We have examined the patterns of protein synthesis induced by several enteroviruses in a wide array of different mammalian cells in culture. In an earlier short report (2), it was shown that electrophoretic patterns of virus-induced proteins are very similar in several different cell types. Subsequently, we examined patterns of protein synthesis directed by several enteroviruses in a wide variety of different cells in culture (Table 1). In every case, virus-directed synthesis was specific for the virus type employed; for a given virus, the electrophoretic patterns were nearly identical, regardless of the host-cell type. Figure 1A shows some typical examples of this when comparing mengovirus proteins synthesized in those cell types which are highly sensitive (maximal virus yielders). Figure 1B compares poliovirus proteins synthesized in HeLa cells which are very susceptible and in diploid human fibroblasts which are sensitive to poliovirus, but which give lower virus yields. Even though type 1 poliovirus grew more slowly in diploid human skin-muscle fibroblasts than in HeLa cells (and gave only about 10% as much progeny virus yield per cell as did HeLa cells), the virus proteins made late after infection were of the same molecular weight and were produced in approximately the same relative ratio to each other (Fig. 1B). Figure 1C shows that a rabbit kidney cell line which yields only 1% as much mengovirus as L-cells synthesized the same virus proteins as L-cells. Figure 1D shows the same phenomenon in a marsupial cell line which yields less than 0.1% as much virus as L-cells. In this case, the amount of virus-directed synthesis was so low that the small amount of remaining host-directed synthesis partially obscured the gel patterns. Figure 1E shows that a bovine kidney cell line which yields less than 0.01% as much mengovirus as L-cells did not synthesize virus proteins at a sufficient rate to be detectable above residual host background synthesis. After scores of experiments of this kind on many different cell types, we have concluded that, whenever enterovirus-directed protein synthesis can be observed

TABLE 1. *Cell lines and primary cell cultures examined with regard to viral protein patterns induced by mengovirus infection*[a]

Cell line	Primary cell culture origin
L-cell	Mouse fibroblast
HeLa	Human cervical carcinoma
HGS-17	Human diploid fibroblasts
MBK	Bovine kidney epithelial cells
MDCK	Canine kidney epithelial cells
WI-38	Diploid human fibroblasts
WI SH	Diploid human amnion epithelial cells
HEp-2	Human carcinoma
CV 1	African green monkey kidney cell line
BHK 21	Baby hamster kidney cells
MA 111	Rabbit kidney epithelial cells
PtK 1	Marsupial cell (kangaroo rat)
Ad-7H	Adenovirus type 7 transformed hamster tumor cells
Ad-3H	Adenovirus type 3 transformed hamster tumor cells
Ad-7M	Adenovirus type 7 transformed monkey tumor cells
SV40-H	SV40 transformed hamster tumor cells
Py-H	Polyoma transformed hamster tumor cells
Rous-H	Rous sarcoma virus transformed hamster tumor cells
	Primary human amnion epithelial cells
	Primary mouse embryo cells
	Primary chick embryo cells
	Primary mouse kidney epithelial cells

[a] All of these cell lines and cell cultures have shown similar patterns.

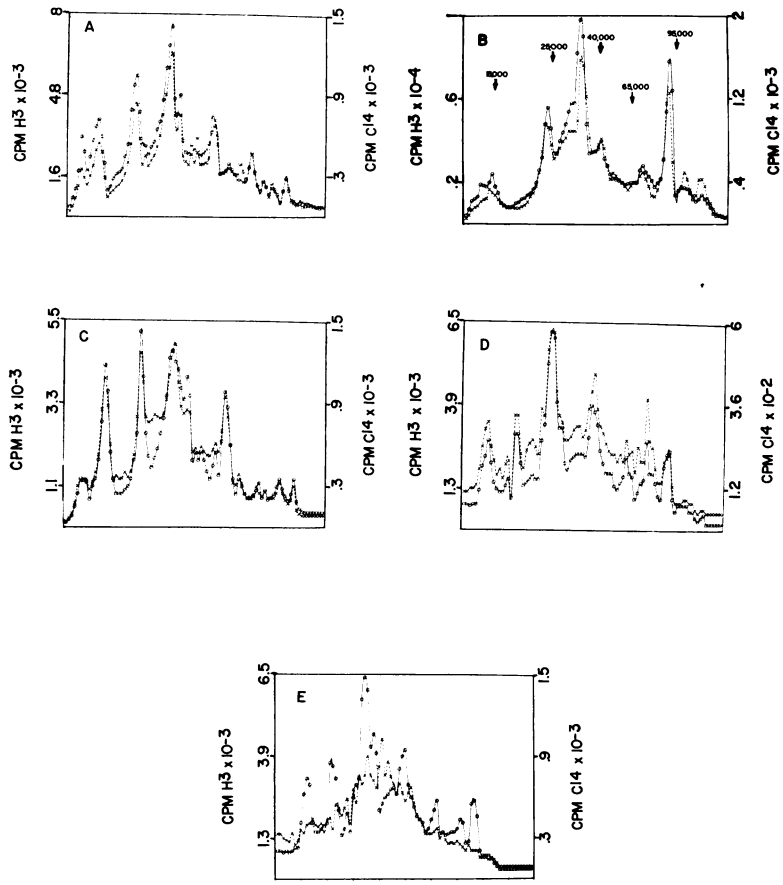

FIG. 1. *Acrylamide gel electropherograms of enterovirus proteins synthesized by different cells infected by the same virus. (A) Symbols: ○, human epithelial cell line (WISH) labeled with ^{3}H-amino acids between 5.5 and 6.5 hr after mengovirus infection; ×, L-cell mouse fibroblast line labeled with ^{14}C-amino acids between 5.5 and 6.5 hr after mengovirus infection. (B) Symbols: ×, diploid human fibroblast cell line (HGS-17) labeled with ^{3}H-amino acids between 6.0 and 7.0 hr after infection with type 1 poliovirus; ○, HeLa cell line labeled with ^{14}C-amino acids between 4.5 and 5.5 hr after type 1 poliovirus infection. (C) Symbols: ○, rabbit kidney cell line (MA 111) labeled with ^{3}H-amino acids between 6.5 and 7.5 hr after mengovirus infection and addition of actinomycin D (5 μg/ml); ×, L-cell line labeled with ^{14}C-amino acids between 5.5 and 6.5 hr after mengovirus infection. (D) Symbols: ○, L-cell line labeled with ^{3}H-amino acids between 6.0 and 7.0 hr after mengovirus infection; ×, marsupial cell line (PtK 1) labeled with ^{14}C-amino acids between 7.0 and 8.0 hr after infection with mengovirus and treatment with actinomycin D (5 μg/ml); (E) Symbols: ○, L-cell line labeled with ^{3}H-amino acids between 6.0 and 7.0 hr after mengovirus infection; ×, bovine kidney cell line (MBK) labeled with ^{3}H-amino acids between 7.0 and 8.0 hr after infection with mengovirus and treatment with actinomycin D (5 μg/ml). In all cases, the electropherograms show the gel origin (cathode) on the right.*

at all, giant virus proteins are synthesized and enzymatically cleaved into the same molecular species, even in relatively resistant cells in which the rate of viral protein synthesis is barely adequate to be detectable. It is, of course, possible that, in cells such as the bovine kidney line in which virus-directed protein synthesis is too low to be discerned, virus proteins are synthesized aberrantly or are cleaved at the wrong points to yield aberrant polypeptides, or both occur.

Figure 2 shows further examples of the virus-specific nature of the proteins induced in enterovirus-infected cells. It should be pointed out that differences in electrophoretic separation occur between individual gels. Therefore, all of our conclusions are based on single-gel comparisons of proteins labeled with different isotopes. In an earlier report (3), we showed some differences between the proteins induced by different viruses. Fig. 2A shows that the virion proteins of type 1 poliovirus did not correspond to the protein peaks synthesized in HeLa cells infected by type 2 virus,

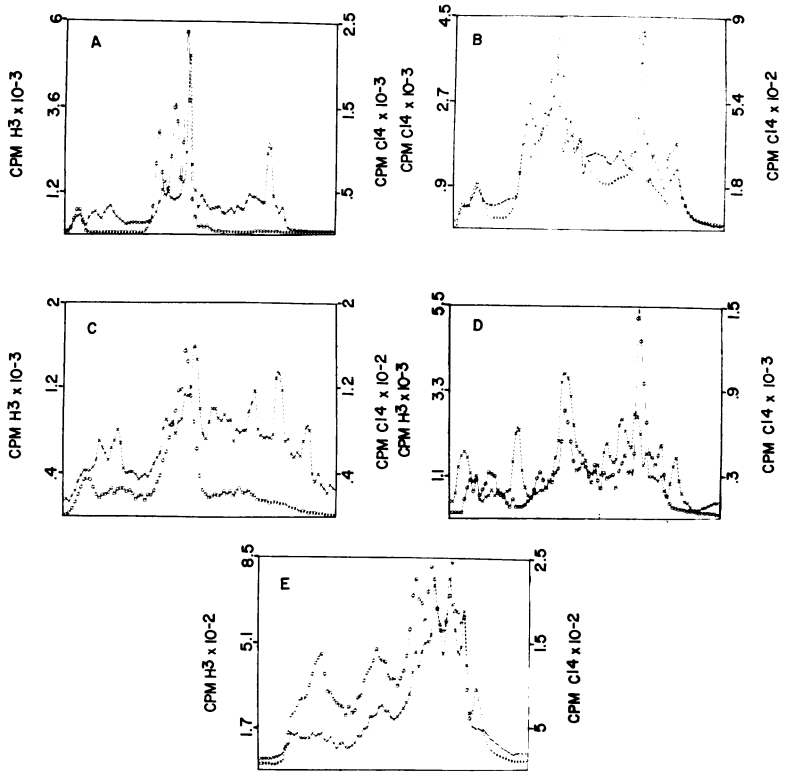

FIG. 2. *Electrophoretic comparisons of different enterovirus virion proteins and of virus-directed proteins in infected cells. (A) ^{3}H-virion proteins of purified type 1 poliovirus labeled between 4 and 6 hr after infection (○) compared to the ^{14}C-labeled proteins induced in cells infected by type 2 poliovirus (×) between 4 and 5 hr after infection. (B) HeLa cells were labeled with ^{3}H-amino acids between 4.5 and 5.5 hr after type 1 poliovirus infection (○). HeLa cells were labeled with ^{14}C-amino acids between 4.5 and 5.5 hr after type 2 poliovirus infection (×). (C) ^{3}H-virion proteins of purified coxsackievirus B5 between 4 and 6 hr after infection (○) compared to the ^{14}C-labeled proteins induced in cells infected by type 1 poliovirus (×) between 4 and 5 hr after infection. (D) HeLa cells were labeled with ^{3}H-amino acids between 4.5 and 5.5 hr after type 1 poliovirus infection (○). HeLa cells were labeled with ^{14}C-amino acids between 4.0 and 4.5 hr after infection with coxsackievirus B5 (×). (E) HeLa cells were pulse-labeled for 2 min with ^{3}H-amino acids at 5.0 hr after infection with mengovirus (○). HeLa cells were pulse-labeled for 2 min with ^{14}C-amino acids 4.5 hr after infection with coxsackievirus B1 (×).*

and, in Figure 2B, it can be seen that the virus-induced proteins of HeLa cells infected by type 1 and type 2 polioviruses were distinguishably different in molecular weight. Similarly, differences were observed when the capsid proteins and virus-induced proteins of type 1 poliovirus and of coxsackievirus B5 were compared (Fig. 2C and 2D). All of the above results were obtained with 1-hr labeling times, so that the protein patterns show the balance achieved during 1 hr between newly synthesized giant polypeptides and their final cleavage products. When infected cells are pulse-labeled, a preponderance of larger, partially cleaved precursor molecules is observed (3, 5, 11). Figure 2E shows that even these giant precursor molecules differed somewhat in molecular weight when the proteins labeled in a 2-min pulse late during infection with mengovirus and those induced late during coxsackievirus B1 infection were compared. It can be concluded that the giant precursor proteins of each virus type are cleaved into viral polypeptides, the sizes of which are genetically determined by that virus and not by the host cell. However, the host cell greatly influences the rates and amounts of virus-directed protein synthesis. We have consistently observed that cells which give poor yields of mengovirus show only low rates of viral protein synthesis and only small amounts of virus antigen appear in these cells (1). Some examples of this are shown in Fig. 3, in which are plotted the rates of protein synthesis at intervals after infection with mengovirus. The highly susceptible L-cell line and the less susceptible W138 diploid human fibroblast

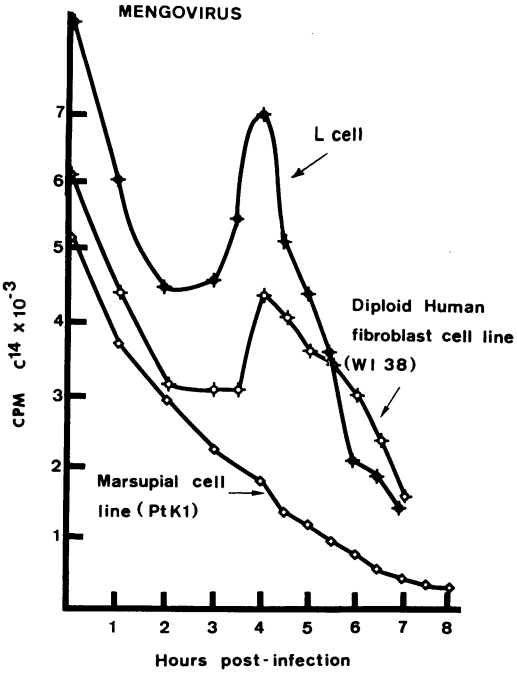

FIG. 3. *Rates of protein synthesis at intervals after mengovirus infection. Identical monolayer cultures containing 2 × 10⁶ uninfected cells or an equal number of cells infected for various times were pulse-labeled for 15 min with ¹⁴C-amino acids (5 μc/ml). Labeled proteins were extracted from cells with 0.1 N NaOH, repeatedly precipitated with hot (95 C) trichloroacetic acid, and redissolved in 0.1 N NaOH. Samples of the final protein solution were mixed with NCS and counted in a scintillation counter.*

line showed definite peaks of virus-directed synthesis beginning several hours after infection. The marsupial cell line PtK1, which is very insusceptible, showed no detectable burst of protein synthesis during the period of low-yield virus synthesis, but the small amount of residual protein synthesis remaining between 7 and 8 hr postinfection did include virus proteins (Fig. 1D).

Synthesis of giant precursor polypeptides. It has generally been observed that the patterns of poliovirus protein synthesis are similar when proteins are labeled late during infection or at earlier times. (Virus-directed synthesis at very early times is difficult to examine because host background synthesis is not yet reduced sufficiently.) Coxsackievirus B1 causes a rapid, profound inhibition of host protein synthesis, so it was employed in an effort to determine how early after infection it might be possible to detect the giant viral protein precursors by using short pulse-labeling periods. Surprisingly, large precursor proteins of coxsackievirus B1 were the predominant species of proteins synthesized as early as 1.5 hr after infection, when actinomycin D was used to help depress host synthesis. Figure 4A shows that, at 1.6 hr after high multiplicity infection, the major protein labeled by a short pulse has a molecular weight of approximately 100,000 daltons. This would account for approximately half of the genetic information in the ribonucleic acid genome. Since the main peak was a very broad one, it may consist of two polypeptides of slightly different molecular weight; thus, it is possible that the predominant proteins seen at this time arise by slightly unequal cleavage of a single polypeptide which corresponds to the entire information content of the genome. Figure 4B shows that nearly identical results were obtained by pulse-labeling at 2.5 hr after coxsackievirus B1 infection. However, by 3.5 hr postinfection (and later), pulse-labeling for the same period of time revealed a number of viral peaks of lower molecular weight (Fig. 4C). This result was obtained consistently at later times after infection, and it suggests that an increase of cleavage enzyme activity occurs with time (whether by synthesis of a virus-coded enzyme or by induction, activation, or release of host enzyme).

For some reason, poliovirus infection does not show such an accumulation of predominantly large polypeptides in a pulse-label at early times in infection, but shows a broad mixture of small molecular weight proteins as well (3, 5, 11). However, Jacobson and Baltimore have demonstrated that the incorporation of amino acid analogues during pulse-labeling seems to retard cleavage and allows recovery of larger precursor polypeptides of poliovirus (5). We have confirmed this at earlier times during infection. However, we found that at later times after poliovirus infection the largest of the giant precursor polypeptides cannot be detected at all (even in the presence of analogues), or else they are present as only a very small proportion of the total pulse-labeled protein. Figure 5A shows that at 5 hr postinfection a 5-min pulse in the presence of analogue produced mainly precursor proteins of molecular weights of 85,000 daltons or smaller. Only traces of labeled polypeptide above 100,000 could be observed. However, at 3.5 hr (Fig. 5B) or earlier, the same labeling procedure in the presence of analogues resulted in the appearance of distinct amounts of giant precursor polypeptides of molecular weights from 100,000 to well over 200,000 daltons. When analogues were present from early in infection, as well as during the pulse-label, the largest precursor polypeptides were found to be present in even larger proportions (Fig. 5C). When such proteins were run in a gel lacking reducing agents and compared to 7*S* gamma globulin (IgG) molecules, there was a major virus protein peak clearly larger than the globulin molecule having a

molecular weight of 160,000 (Fig. 5D). An even larger precursor protein was usually observed as a shoulder or small peak on the peak described above, and its molecular weight is somewhere between 200,000 and 230,000 daltons. In this area of the gels, accurate molecular weight determination was not possible. Nevertheless, it is clear that the very largest precursor polypeptide is of sufficient size to account for all or nearly all of the genetic information in the virus genome.

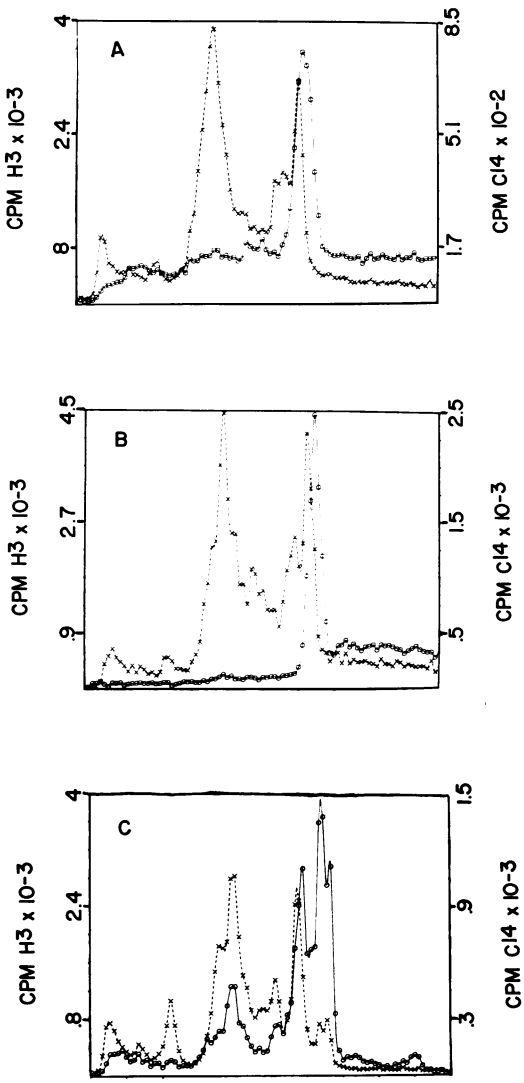

FIG. 4. *Giant precursor polypeptides in HeLa cells with a short pulse at various times after coxsackievirus B1 infection. In all three graphs of this figure, the ^{14}C label (×) was in proteins labeled with ^{14}C-amino acids between 3.5 and 4.5 hr after infection of HeLa cells with coxsackievirus B1. After labeling with ^{14}C, the infected cells were chased for 2 hr with unlabeled Eagle's medium containing twice the usual level of all essential amino acids. The ^{3}H label (○) was in proteins pulse-labeled for 5 min with ^{3}H-amino acids at different times after infection. (A) Symbols: ○, ^{3}H pulse-label at 1.6 hr after coxsackievirus B1 infection (5 μg of actinomycin D per ml added to the cells 2 hr before infection). (B) Symbol: ○, same as (A) but pulse-label at 2.5 hr postinfection. (C) Symbol: ○, same as (A), but pulse-label at 3.5 hr postinfection.*

The same phenomenon was demonstrated more strikingly and much more convincingly in the case of cells labeled in the presence of analogues early after coxsackievirus B1 infection. Figure 6A shows that coxsackievirus B1 proteins pulse-labeled at 3 hr postinfection in the presence of analogues showed two major classes of giant precursor molecules. One of these is about 100,000 daltons molecular weight and the other is between 170,000 and 190,000 daltons. Again, a larger protein was seen as a shoulder. This protein is between 200,000 and 230,000 molecular weight, and it must also represent a translation product of the entire virus genome. It was, of course, important to determine whether this "entire genome polypeptide" is merely an artifact caused by analogue incorporation. However, this is not the case because we frequently observed significant peaks of the largest polypeptides even in the absence of analogues, when we pulse-labeled cells very early after infection with coxsackievirus B1. Figure 6B shows an example of these "polycistronic polypeptides" from cells pulse-labeled without analogues at 2.5 hr after coxsackievirus B1 infection. These largest proteins were usually not observed in the absence of analogues (Fig. 4A and 4B), but they have appeared in about 30% of the early pulse-labels in coxsackievirus B1 infection. Without analogues they were usually not detectable at all in poliovirus-infected cells, but their occasional presence in coxsackievirus B1-infected cells confirms that they are naturally occurring precursors. Usually they probably have a very short half-life before cleavage (or else they are generally cleaved as a nascent polypeptide on the ribosome).

Molecular weights were determined with labeled purified reference proteins for internal control, and there was an excellent linear relationship between distance of migration and log molecular weight as described by Shapiro et al. (10). Figure 7 shows that the relationship holds very well above a molecular weight of 17,000, but these gels are not reliable for sizing polypeptides of 15,000 daltons or smaller.

Interpretation of all of the above results is predicated upon the assumption that this gel technique separates single polypeptide chains according to molecular weight, as is illustrated in Fig. 7. Therefore, it was essential to show that these giant precursor proteins are not aggregates of smaller protein chains and that they are really precursors from which the smaller chains are derived. We have employed a wide variety of different electro-

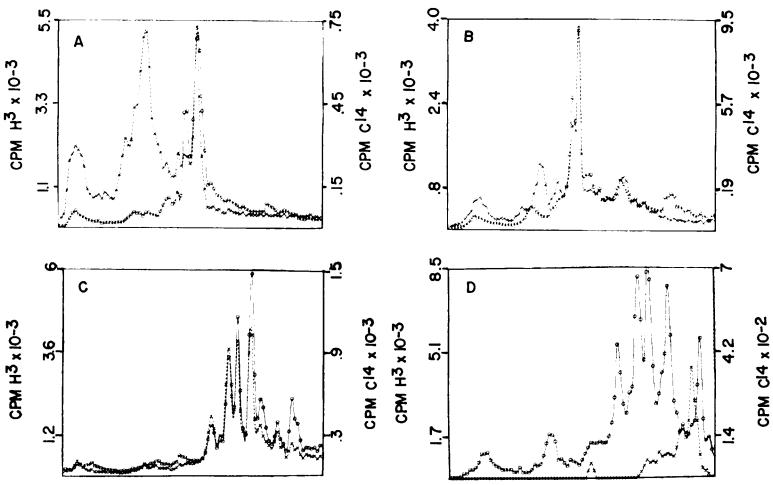

FIG. 5. *Giant poliovirus precursor proteins synthesized in the presence of amino acid analogues. When analogues were employed during protein labeling, they were added along with labeled amino acids at the concentrations utilized by Jacobson and Baltimore (5; 2.5 mM FPA, 3.3 mM canavanine, 1.8 mM ethionine, and 5.4 mM azetidine-2-carboxylic acid). (A) HeLa cells were pulse-labeled with ^{3}H-amino acids in the presence of analogues for 4 min at 5 hr after infection with type 1 poliovirus (○). HeLa cells were labeled with ^{14}C-amino acids and chased as in Fig. 4 (×). (B) HeLa cells were pulse-labeled with ^{3}H-amino acids in the presence of analogues for 4 min at 3.5 hr after infection with type 1 poliovirus (○). HeLa cells were pulse-labeled with ^{14}C-amino acids for 4 min at 3.5 hr after infection with type 1 poliovirus (no analogues present ×). Proteins in this gel were better separated and migrated farther because of longer electrophoresis at higher voltage than usual. (C) Same as 5B, with the following exceptions. For the ^{3}H-labeled proteins, analogues were added at one-fourth the normal concentration from 1.5 hr after infection until 3.5 hr postinfection and then ^{3}H-amino acids and normal analogue concentrations were added for a 4-min pulse-label; ^{14}C-labeled proteins were labeled under identical conditions except cells were not preincubated in analogues. (D) ^{3}H-labeled poliovirus precursor proteins labeled in infected HeLa cells as in 5B (○), compared to ^{14}C-labeled purified bovine gamma globulin molecules (molecular weight, 160,000 for entire molecule of two light and two heavy chains). Mercaptoethanol and other reducing agents were omitted during sample preparation and electrophoresis to keep chain dissociation to a minimum.*

phoretic conditions and buffers to determine whether, under any circumstances, it is possible to disaggregate these giant polypeptides into smaller subunits. We tried high, neutral, and low *p*H buffers, alkylation of the proteins, and strong reducing conditions in the gel buffers. We tried high temperature runs in the gels and employment of several protein denaturing agents simultaneously. Figure 8A shows the results of one experiment in which 8 M urea, 1% SDS, 0.5% deoxycholate, and 0.1% dithiothreitol were present in the gel and buffers during electrophoresis at *p*H 9.0. The giant precursor proteins were not eliminated under these conditions. Similar results were obtained at *p*H 11.0. We conclude that the giant proteins are not aggregates of smaller proteins.

Next we accounted for the fate of pulse-labeled protein counts during a chase in the presence of cold amino acids. Table 2 shows that nearly all of the labeled polypeptide resulting from a 3-min pulse in infected or uninfected cells remained inside the cells. Also, there was no significant increase in labeled protein during the chase; in fact, some of the labeled polypeptide was lost from the acid-precipitable fraction during a short chase. It is, therefore, extremely unlikely that any of the smaller virus proteins that appear during a chase or during longer term labeling are synthesized de novo from labeled amino acids in a slowly exchanging special pool. This conclusion is further strengthened by the data in Fig. 8B and 8C. Incubation of cells at 4 C almost totally inhibited protein synthesis but allowed the turnover of the free amino acid pool. In Fig. 8B, it can be seen that pool exchange with cold amino acids at 4 C immediately after a 3-min pulse-label did not inhibit the appearance of the smaller typical poliovirus proteins during a subsequent chase. Figure 8C shows that they also appeared during a chase in the presence of puromycin, so it must be concluded that this "chase" phenomenon is due to the cleavage of the larger proteins rather than to delayed synthesis of some sort.

Since viral precursor proteins are cleaved by enzymes which appear to increase in activity as infection progresses, they must either be virus-coded proteins or cellular proteins that are activated or

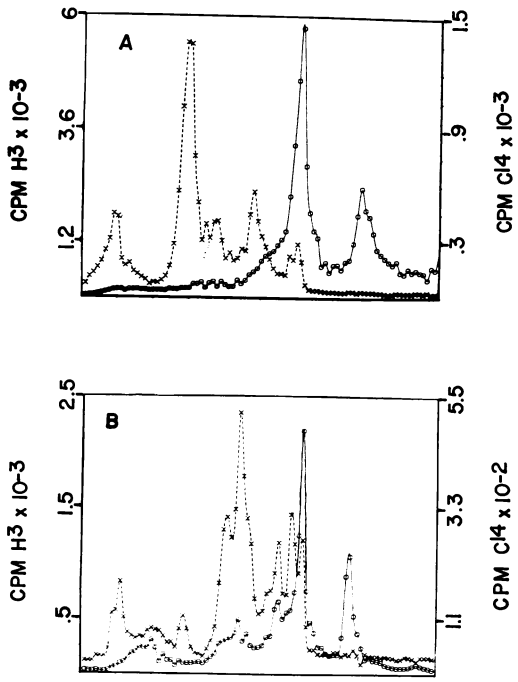

FIG. 6. *Giant coxsackievirus B1 precursor proteins synthesized in the presence and absence of the amino acid analogues. Analogue concentrations were the same as those in Fig. 5. (A) HeLa cells were pulse-labeled with ^{3}H-amino acids in the presence of analogues for 4 min at 3.0 hr after infection with coxsackievirus B1 (○). HeLa cells were labeled with ^{14}C-amino acids for 1 hr from 3.7 to 4.7 hr after coxsackievirus B1 infection (×). The labeled medium was removed, and unlabeled Eagle's medium was added for an additional 2-hr chase period. (B) HeLa cells were pulse-labeled with ^{3}H-amino acids for 4 min at 2.5 hr after infection with coxsackievirus B1 (○). No analogues were employed. Coxsackievirus B1-infected HeLa cells were labeled with ^{14}C and chased as in Fig. 6A (×).*

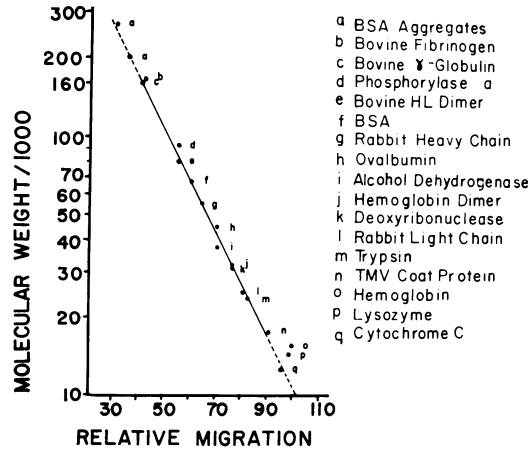

FIG. 7. *Migration of purified labeled proteins in sodium dodecyl sulfate-acrylamide gels as a logarithmic function of molecular weight. Purified proteins were labeled with ^{3}H- or ^{14}C-dimethyl sulfate, and their migration distances were plotted relative to each other by use of ^{3}H-^{14}C dual label techniques, in which a number of proteins were run at the same time in a single gel.*

released (e.g., from lysosomes) by virus infection. In either case, it would be of interest to know whether they cause gross cleavage of host cell proteins late in infection. Figure 9A shows that normal cell proteins which had been labeled before infection were not grossly degraded by 8 hrs after subsequent coxsackievirus B1 infection (by which time all cells were dead or dying). Thus, if the cleavage enzymes act on host cell proteins at all, they must cleave only a small percentage of them.

Finally, we wish to confirm the report of Jacobson and Baltimore (5), in which they observed a slight effect of amino acid analogues on the relative size distribution of pulse-labeled proteins in normal HeLa cells. Figure 9B shows that cells labeled in the presence of analogues exhibited a larger proportion of high molecular weight proteins than smaller molecular weight proteins. This could be due to some trivial cause such as selective inhibition of synthesis of smaller proteins, but it could be evidence for an important cleavage function operating to process many normal cell proteins immediately after their synthesis.

DISCUSSION

Jacobson and Baltimore (5) first suggested the possibility that the entire genome of poliovirus might be translated into a single giant precursor polypeptide. The present demonstration of a specific protein of greater than 200,000 daltons in poliovirus- and coxsackievirus-infected cells indicates strongly that they were correct. The presence of this protein in coxsackievirus B1 infection even in the absence of analogues shows that it is a normal virus precursor polypeptide. However, it is cleaved so rapidly that it is very difficult to observe, except in pulse-labeled cells at quite early times after infection when cleavage is slower.

Our failure to observe detectable degradation of host cell proteins, even late in infection when all cells were dying, suggests that the enzymes responsible for clipping precursor proteins either are rather specific proteases or else are confined to certain limited areas of the cytoplasm where they can degrade only a small proportion of total cellular proteins.

The accumulation of cleavage enzyme activity with increasing time after infection is consistent

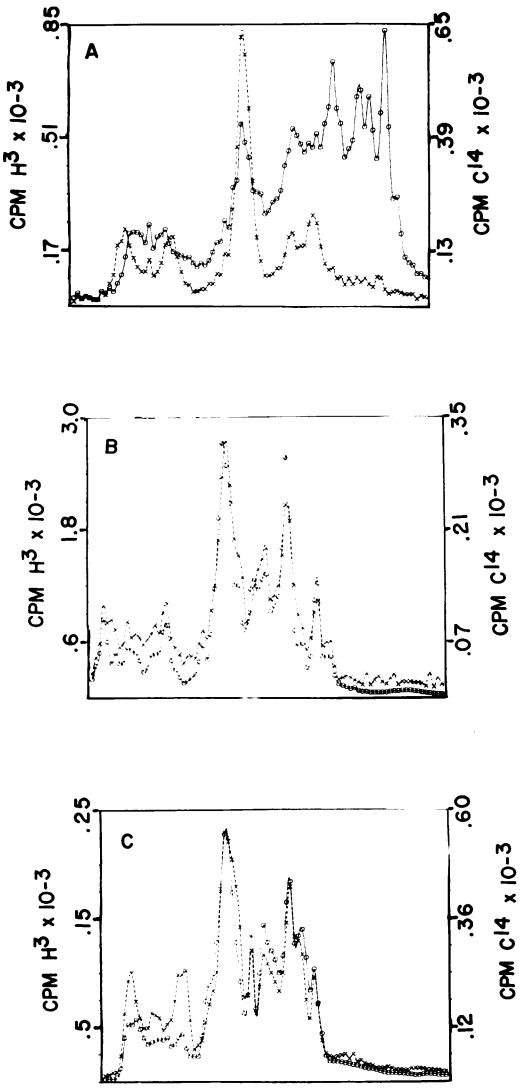

FIG. 8. *Control experiments to detect the possibility that giant precursor proteins might be artifacts of technique. (A) Gel electrophoresis of giant precursor proteins under strongly denaturing conditions. Instead of the usual conditions of electrophoresis, the samples for this run were dissolved in a solution containing 8 M urea, 1% SDS, 0.5% sodium deoxycholate, and 0.1% dithiothreitol in addition to the usual components. The gel column and the electrophoresis vessel buffer contained (in addition to the usual components) 8 M urea and 1% SDS. (○) HeLa cells were pulse-labeled for 5 min with ^{3}H-amino acids at 4 hr after infection with coxsackievirus B1. (×) Coxsackievirus B1-infected HeLa cells were labeled and chased as in Fig. 6A. (B) Chase of labeled precursor proteins into smaller viral proteins after the radiolabeled amino acid pool had been exchanged with cold amino acids at 4 C immediately after a pulse-label of the giant proteins. (○) Poliovirus infected HeLa cells were pulse-labeled for 3 min at 4.5 hr after infection with ^{3}H-amino acids; the cells were immediately chilled to 0 to 4 C, washed, and incubated at 0 to 4 C for 5 min containing five times the normal concentration of amino acids to displace labeled amino acids from the pool. They were then chased in the presence of cold amino acids for 3 hr at 37 C. (×) Poliovirus-infected HeLa cells were labeled with ^{14}C-amino acids between 4.5 and 5.0 hr and were then chased in the presence of cold amino acids for 3 hr at 37 C. (C) Chase of labeled precursor proteins into smaller viral proteins in the presence of puromycin. HeLa cells were pulse-labeled for 2 min with ^{3}H-amino acids at 3.0 hr after infection with coxsackievirus B1. They were then washed and chased for 3 hr at 37 C in Eagle's medium (○) containing 100 μg of puromycin per ml (to prevent further synthesis of large proteins) or (×) without puromycin.*

TABLE 2. *Amount of labeled amino acid in proteins during a chase at various times after pulse-labeling*

Length of chase after pulse-labeling[a]	Counts/min in trichloroacetic acid-precipitable material
min	
0	61,000[b]
5	56,000
10	52,000
20	51,000

[a] HeLa cells infected for 4 hr with coxsackievirus B1 were pulse-labeled for 3 min with ^{3}H-amino acids. They were immediately washed and chased with Eagle's medium containing twice the normal level of amino acids at the indicated times after the pulse; samples of 2×10^{6} cells were dissolved in 0.1 N NaOH and repeatedly precipitated with trichloroacetic acid.

[b] Over 99% of the protein label is found inside cells after such a pulse (both in normal cells and in infected cells), i.e., less than 1% of labeled protein leaks into the medium. With long periods of labeling late in infection, considerable quantities of labeled virus and virus protein are released from cells.

with virus-coded production of enzyme or with virus-induced activation or release of formerly inactive or sequestered enzyme. It is important to determine whether the cleavage enzyme is virus-coded or host-coded. It is clear that the virus does not act by activating or derepressing host genes to produce more cleavage enzyme, because cleavage (and the entire course of virus infection) proceeds normally even when actinomycin D prevents host-gene function from before the start of infection (Fig. 4). Thus, the viruses either are activating preformed host enzymes in some manner or are coding the production of new enzyme. In a subsequent paper, we will report studies of the specific-

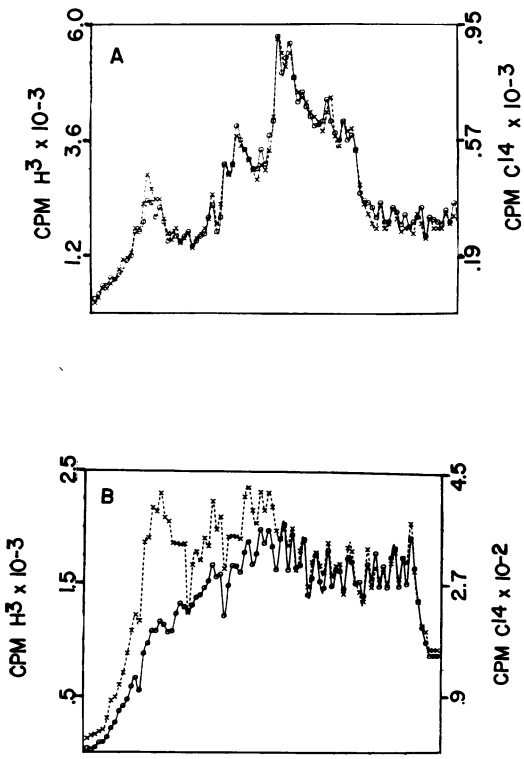

FIG. 9. *Examination of the possibility of extensive cleavage of cell polypeptides as a result of infection or as a normal process. (A) Failure of enterovirus infection to cause detectable breakdown of prelabeled host cell proteins.* (○) *HeLa cells were labeled with* ^{3}H*-amino acids for 1 hr. They were then washed and infected with coxsackievirus B1 for 8.0 hr at which time the cells were dissolved in sample buffer.* (×) *HeLa cells were labeled with* ^{14}C*-amino acids for 1 hr and were washed and chased in Eagle's medium for an additional 3 hr. (B) Effect of amino acid analogues on the size of proteins formed during a pulse-label in normal uninfected HeLa cells.* (○) *HeLa cells were pulse-labeled with* ^{3}H*-amino acids for 4 min in the presence of amino analogues (see Fig. 5 for analogue levels).* (×) *HeLa cells were labeled for 30 min with* ^{14}C*-amino acids in the absence of any analogues.*

ity of cleavage enzymes in vivo and preliminary studies of in vitro clipping of giant precursor proteins by cell-free extracts of infected cells (Doyle, Kiehn, and Holland, *in preparation*).

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