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Virus-Specific Protein Synthesis in Cells Infected by Infectious Pancreatic Necrosis Virus

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A study of virus-specific protein synthesis in infectious pancreatic necrosis virus-infected RTG-2 cells was undertaken to find a relationship between the coding capacity of the virus genome (two segments of double-stranded RNA of 2.5×10^6 and 2.3×10^6 molecular weight) and the sizes and relative amounts of polypeptides in the virion and in infected cells. The time course of virus-specific protein synthesis was followed by pulse labeling infected UV-irradiated cells with [^{35}S]methionine and analyzing the labeled proteins by polyacrylamide gel electrophoresis followed by autoradiography. Three size classes of virus-specific polypeptides were synthesized, in the same relative proportion, throughout the infectious cycle, beginning 3 h postinfection. Their designation and molecular weight was as follows: α_1, 100,000; α_2, 90,000; β_1, 59,000; β_2, 56,000; γ_1, 32,000; γ_2, 30,000; and γ_3, 28,000. Experiments using amino acid analogues, protease inhibitors, $ZnCl_2$, and supraoptimal temperatures showed that polypeptides of the β and γ families did not arise from the α polypeptides by post-translational cleavage. Slow cleavage late in the infectious cycle could be demonstrated, since during a 12-h period radioactivity was chased from β_1 via β_3 to β_4 (molecular weight, 50,000) and β_5 (molecular weight, 49,000). During the chase most of γ_2 was degraded, whereas radioactivity could not be chased from the remaining virus-specific polypeptides. Purified virus contained polypeptides α_1, α_2, β_4, β_5, and γ_1. The β polypeptides made up over 60% of the virion proteins. The results suggest that infectious pancreatic necrosis virus possesses a unique mechanism for synthesis of three size-classes of proteins using mRNA transcripts from two high-molecular-weight double-stranded RNA genome segments.

Infectious pancreatic necrosis (IPN) virus is the causal agent of a contagious, high-mortality disease of young, hatchery-reared salmonids. It replicates in a variety of fish cell lines at temperatures below 24°C (20). A single cycle of replication takes 16 to 20 h at 22°C, resulting in a characteristic cytopathogenic effect (CPE) (20). Virus multiplication takes place in the cytoplasm, and studies with metabolic inhibitors indicate that the virus nucleic acid is RNA (20). Electron microscopic observations of purified virus reveal structures of naked icosahedrons, similar in size and shape to reovirus but lacking the characteristic double capsid of the latter (6, 14).

Considerable controversy existed, until recently, concerning the size and shape of the virus genome, that is, whether it was single-stranded, unsegmented (14), or double-stranded (ds), segmented RNA (6). Recent experiments, utilizing high-resolution polyacrylamide gel electrophoresis followed by autoradiography, indicated that IPN virus contains two segments of dsRNA with molecular weights of 2.5×10^6 and 2.3×10^6 (7a). The virus genome was resistant to RNase and in sucrose gradient centrifugations showed a single boundary with a relative sedimentation constant of 14*S* (6, 7a); upon denaturation in 99% formamide, the sedimentation constant increased to 24*S*, as measured in formamide-sucrose gradients, and the RNA became sensitive to RNase (7a).

On the basis of the nature of the virus genome, and the size and shape of purified virions, IPN virus appears to belong to the family Reoviridae (6). However, since it contains only a single (large) size class of dsRNA, the virus cannot be included in any of the established genera (13).

Previous publications indicated that the structural proteins of the virion fall into three size classes, although the actual number of virion polypeptides reported from different laboratories ranges from 3 to 7 (6, 18). The question arises: how can a large-size-class dsRNA ge-

nome mediate the synthesis of three different size classes of proteins? We sought an answer to this question by analyzing the synthesis of virus-specific proteins in infected cells at various times after infection.

Little is known about the intracellular synthesis of virus-specific macromolecules due to a number of attributes characteristic of IPN virus replication. Some of these attributes are as follows. (i) Virus yield is very low (about 0.1 to 0.2 mg of virus per 10^9 cells) compared to reovirus (30 mg/10^9 cells). (ii) IPN virus replicates only in certain fish cell lines, which can only be propagated as cell monolayers (28); thus, (iii) scaling up virus production by using suspension cultures is not feasible. IPN virus infection fails to depress host cell protein synthesis (22). Consequently, studies on the synthesis of virus-specific proteins are impeded by a high background level of cellular protein synthesis; (iv) there is no discernible quantitative difference when the rate of protein synthesis in IPN virus-infected cells is compared to that in uninfected cells (22). There is a quantitative difference between the rate of RNA synthesis in infected cells as compared with that in uninfected cells (15, 22), but it is not known how much of the RNA is virus specific. Virus replication is very sensitive to actinomycin D (Act D) (e.g., virus yield is reduced 90 to 95% in the presence of 0.1 μg of Act D per ml [15]; in contrast, reovirus replication is not inhibited by even 0.5 μg of the drug per ml), and Act D therefore cannot be used to inhibit host RNA synthesis selectively.

The purpose of the study reported here was twofold. First, it was essential to develop a method whereby host cell protein synthesis could be reduced without inhibiting virus-specific protein synthesis. Second, we wished to determine the pattern of virus-specific protein synthesis in infected cells at various times after infection. Answers were sought to such questions as: Are there early versus late proteins? Can the synthesis of all capsid proteins be demonstrated? Are there noncapsid polypeptides in the infected cell? Is there any precursor-product relationship between the various virus-specific proteins? Is there rapid post-translational cleavage of virus-specific polypeptides in the infected cell? Is there any cleavage associated with virus maturation? It was especially important to examine the relationship between the virus genome and virus-specific polypeptides.

The results reported here demonstrate that the host cell protein synthesis can be suppressed, without inhibiting virus replication, by irradiating infected cells with UV light 2 to 3 h after infection. It was found that three size classes of virus-specific polypeptides were synthesized in the same relative proportion throughout the infection cycle. Studies with amino acid analogues, protease inhibitors, $ZnCl_2$, and supraoptimal temperatures indicated that the medium- and small-size polypeptides did not arise from the large size class one via post-translational cleavage. Furthermore, the relative molar ratios of the three size classes of polypeptides indicated that the frequency of their synthesis was inversely related to their molecular weights. This type of protein synthesis might be expected to occur by the translation of three size classes of monocistronic mRNA's. So far, however, only a single large species (24*S*) of virus-specific single-stranded RNA has been found in IPN virus-infected cells (2), a size of single-stranded RNA that forms when the 14*S* viral dsRNA genome is denatured (7a).

These results seem to indicate that IPN virus has a unique mechanism for synthesizing three size classes of proteins using mRNA transcripts from two high-molecular-weight dsRNA genome segments.

(Portions of these results were presented at the Annual Meeting of the American Society of Microbiology, 1976 [P. Dobos, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S165, p. 232].)

MATERIALS AND METHODS

Cells. Rainbow trout gonad (RTG-2) cells and fathead minnow cells were obtained from the American Type Culture Collection. Atlantic salmon cells were kindly provided by B. Nicholson, University of Maine (23). The cells were grown at 22°C as monolayers in plastic tissue culture vessels (Corning Plastics Inc.), using Eagle minimum essential medium (MEM) with Earle salts, supplemented with 10% (vol/vol) fetal calf serum and 40 μg of gentamycin per ml.

Virus. IPN virus (ATCC VR299) was propagated in RTG-2 cell monolayers at a multiplicity of infection (MOI) of 0.01 PFU/cell. Infected cultures were incubated at 22°C until extensive CPE was observed, usually 2 to 3 days. The cells were scraped into the tissue culture medium, the culture vessels were placed in crushed ice, and the cells were sonicated for 30 s with a Biosonik III sonicator at its maximum setting. This crude virus preparation (5×10^7 to 5×10^8 PFU/ml) was dispensed in 1-ml samples and stored at −20°C. Plaque titration of the virus was performed under atmospheric conditions using a gum tragacanth overlay, as described previously (7).

Preparation of radioactively labeled virus. To prepare virus labeled in the protein moiety, the medium from cell monolayers was replaced with diluted MEM (1 volume of MEM without serum + 9 volumes of Earle balanced salt solution [EBSS]) containing either a ^{14}C-labeled amino acid mixture at a final concentration of 1 μCi/ml or [^{35}S]methionine at

5 μCi/ml. IPN virus was added at a low-input MOI (as above), and the cultures were incubated at 22°C until advanced CPE was evident. Occasionally the cells were infected at a high-input MOI by removing the growth medium and adding enough stock virus to cover the monolayer. After an adsorption time of 1 to 2 h at 22°C, the inoculum was decanted, the cells were overlayed with diluted MEM containing the desired radioactive precursor, and the cultures were incubated as above.

Virus purification was achieved by a modification of the method of Cohen et al. (6) as described previously (7a). Briefly, sonicated, infected cultures were centrifuged in the cold at 78,000 × *g* for 2 h in a preparative ultracentrifuge (Beckman L2-65b with an SW27 rotor). The crude virus pellet was resuspended by sonication in TNE buffer (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH 7.1) and extracted three times with a one-quarter volume of Freon 113, using a Dounce homogenizer. The aqueous phase containing the Freon-extracted virus was layered onto a column of 10% (vol/vol) sucrose solution in TNE buffer and sedimented at 78,000 × *g* for 3 h. The virus pellet was resuspended by sonication in TNE buffer, CsCl was added to a density of 1.33 g/cm^3, and the virus was isopycnically banded at 100,000 × *g* for 16 h (SW50.1 rotor). After centrifugation, fractions were collected, and the acid-precipitable radioactivity of a small portion of each fraction (usually 5 to 10 μl) was determined as described previously (7a). The buoyant densities of representative fractions were calculated from their refractive index measurements (11). Fractions containing the virus were pooled, diluted by TNE buffer, and recentrifuged through another 10% sucrose column in TNE buffer as described above. The resulting purified virus pellet was resuspended in the appropriate buffer, depending on the analysis to be made subsequently.

Radioactive labeling of infected cells for polyacrylamide gel electrophoresis. Confluent RTG-2 cell monolayers in plastic tissue culture plates (6 cm in diameter) were infected with IPN virus at 22°C at an MOI of 10 to 20 PFU/cell. After allowing 1 h for adsorption, the virus inoculum was replaced with growth medium and the cultures were incubated for an additional 2 to 3 h. At this time the medium was removed, the monolayers were rinsed three times with EBSS, and the uncovered plates were irradiated with UV light from a distance of 25 cm for 15 to 20 s using a GE G8T5 germicidal lamp (44 ergs/s per mm^2 at 25 cm). The medium was replaced and incubation was continued at 22°C. At various times postinfection, the growth medium was replaced with EBSS containing 5 μCi of [^{35}S]methionine per ml. At the end of the labeling period, the cell layers were washed with fresh EBSS, and the monolayers were dissolved in 0.5 ml of electrophoresis sample buffer (ESB; 0.05 M Tris-hydrochloride, pH 6.8, 1% [wt/vol] sodium dodecyl sulfate [SDS], 2 mM EDTA, 1% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, and a trace of bromophenol blue tracking dye). The preparation was forced through a small-gauge hypodermic needle to shear the DNA, heated for 2 min in a boiling-water bath, and stored in screw-capped vials at −20°C until used.

In pulse-chase experiments the cells were washed three times, after the labeling period, with EBSS and further incubated in complete growth medium to which a 1,000-fold excess of unlabeled methionine was added. At the end of the chase the cultures were treated as above.

Quantitative determination of protein synthesis in infected and uninfected RTG-2 cell monolayers using tritiated leucine. Tissue culture trays (Linbro Chemical Co., New Haven, Conn.) containing 24 flat-bottomed wells of 16 mm in diameter were used. Confluent RTG-2 cells were infected (or mock infected) at a high-input MOI with IPN virus. After an adsorption period of 1 h, the virus inoculum was removed and 1 ml of MEM diluted 10-fold with EBSS containing 5 μCi of [^{3}H]leucine per ml was added to each monolayers. At various times after infection, the incorporation of radioactive leucine was stopped by removing the medium and washing the cell layers three times with cold EBSS. The monolayer was washed three times with cold 10% trichloroacetic acid and three times with cold ethanol and allowed to air dry for 3 h. Then 0.2 ml of 0.5 N NaOH was added to each well and, after an additional hour at room temperature, 0.1 ml of each sample was transferred into scintillation vials containing 10 ml of toluene-Triton X-100 scintillation fluid [to 2 liters of toluene containing 12 g of 2,5-diphenyloxazole (PPO), and 0.6 g of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP), 1 liter of anhydrous Triton X-100 was added] and counted in a Beckman model LS150 liquid scintillation counter.

Polyacrylamide gel electrophoresis of proteins. Radioactively labeled proteins were analyzed (i) in cylindrical gels using the SDS-phosphate gel system of Maizel (19) and (ii) in slab gels by the discontinuous SDS-gel system as described by Laemmli (16). In the SDS-phosphate gel system, cylindrical gels (10 by 0.8 cm) contained 7.5% acrylamide and 0.2% bisacrylamide or 10% acrylamide and 0.266% bisacrylamide in 0.1 M phosphate buffer, pH 7.2, containing 0.1% (wt/vol) SDS and 0.02 M EDTA. Polymerization was achieved with 0.05% *N,N,N',N''*-tetramethylethylenediamine (TEMED) and 0.5% ammonium persulfate. The electrophoresis buffer consisted of 0.1 M phosphate buffer, pH 7.2, 0.1% SDS, and 0.02 M EDTA. Occasionally, 2 or 6 M urea was incorporated into the gels to improve resolution. The gels were prerun at 4 mA/gel for 30 min, after which the samples (50 to 100 μl) in ESB were layered onto the gel column and electrophoresis was continued at 4 mA/gel until the bromophenol blue marking dye reached the bottom, usually 16 h. After the run the gels were stained with 0.1% (wt/vol) Coomassie brilliant blue in acetic acid-methanol-water (5:40:55, vol/vol/vol), destained in the latter solvent, and cut into four longitudinal slices with a gel slicer. The middle two slices were dried under vacuum (1), and placed in the dark on Kodak X-ray film (PR/R-2 Royal X-O mat) for autoradiography (2 to 10 days depending on the amount of radioactivity present in the gels). Quantitation of the amount of radioactivity in the labeled protein bands was accomplished by one of two ways: (i) the developed films were scanned with a chromoscan recording microdensitometer (Joyce-Loebl, Ltd.), or (ii) the bands contain-

ing the radioactive polypeptides were sliced out of the dry gel and digested in tightly capped scintillation vials with 0.1 ml of 30% H_2O_2 for 16 h at 60°C. The samples were cooled to −20°C for 1 h, 10 ml of Triton-toluene scintillation cocktail was added to each vial, and the radioactivity was measured in a liquid scintillation counter.

In the discontinuous SDS-gel system, described by Laemmli (16), the gels were formed as slabs (0.1 by 10 by 12 cm) between glass plates. The general apparatus and methodology for electrophoresis followed the description of Studier (26); 10 and 12% resolving gels and 5% spacer gels were prepared from a stock solution in which the ratio of acrylamide to bisacrylamide was 30:0.8. Five to twenty microliters of labeled cell lysate or virus in ESB (containing 10,000 to 100,000 cpm) was loaded into the wells with a microsyringe. Electrophoresis was for 3 to 4 h at 120 V. The gel slabs were stained, destained, dried, and autoradiographed according to the method described above for SDS-phosphate gels.

The molecular weights of IPN virus-specific proteins were determined by comparing their electrophoretic mobilities in polyacrylamide gels with proteins of known molecular weight. The following marker proteins were used: reovirus proteins as characterized by Smith et al. (25); encephalomyocarditis virus proteins as characterized by Dobos and Martin (8); bovine serum albumin (67,000 daltons), gamma globulin H chain (50,000 daltons); ovalbumin (45,000 daltons); gamma globulin L chains (25,000 daltons); and tobacco mosaic virus protein (17,500 daltons).

Media and reagents. Fetal calf serum (virus and mycoplasma screened), MEM, and EBSS were purchased from Grand Island Biological Corp. The following materials were obtained from New England Nuclear Corp.: PPO, POPOP, Triton X-100, [^{35}S]methionine (400 Ci/mmol), [4,5-^{3}H(N)]leucine (40 Ci/mmole); [U-^{14}C]-labeled amino acid mixture (0.1 mCi/ml). Recrystallized acrylamide, bisacrylamide, and TEMED were obtained from Bio-Rad Laboratories Ltd. The protease inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK), phenyl methyl sulphonyl fluoride (PMSF), phenyl boronic acid, and Act D were purchased from Sigma Chemical Co., as were the amino acid analogues DL-*p*-fluorophenylalanine, canavanine sulfate, L-azetidine-2-carboxylic acid, and ethionine. SDS, specially pure, was obtained from British Drug Houses.

RESULTS

Structural polypeptides of IPN virus. When Freon-extracted IPN virus that had been labeled with ^{14}C-amino acids and [^{3}H]leucine-labeled reovirus were isopycnically banded in a CsCl gradient, the acid-precipitable radioactivity was distributed into three different density zones (Fig. 1). Reovirus and IPN virus banded at densities of 1.36 and 1.33 g/cm^3, respectively, whereas both reovirus and IPN top components had a buoyant density of 1.29 g/cm^3. All of the IPN virus infectivity was associated with the

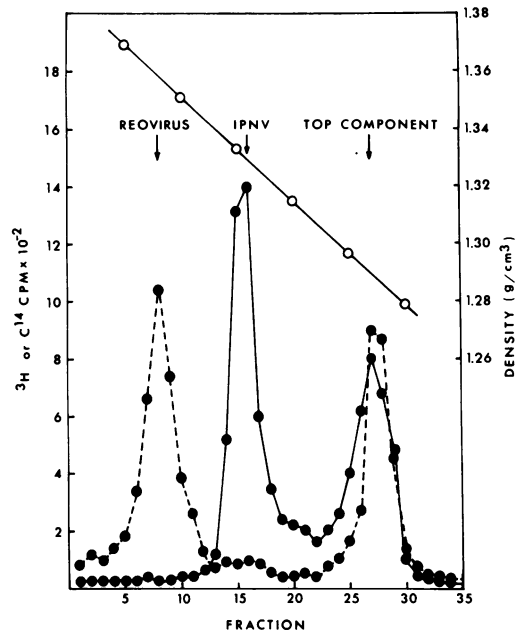

FIG. 1. *Isopycnic sedimentation of IPN virus in CsCl. Freon-extracted, semipurified IPN virus, labeled with a mixture of ^{14}C-amino acids, was mixed with ^{3}H-amino acid-labeled reovirus in TNE buffer. The mean density was adjusted to 1.33 g/cm^3 by adding an equal volume of a 1.64-g/cm^3 density solution of CsCl in the same buffer. The preparation was centrifuged at 5°C for 18 h at 45,000 rpm using the SW50.1 rotor in a Beckman L2-65B preparative ultracentrifuge. At the end of the run the gradient was fractionated from the bottom (0.2 ml/fraction), and the acid-precipitable radioactivity of a small portion of each fraction (usually 10 μl) was determined as described in Materials and Methods. Symbols: ^{14}C-amino acid-labeled IPN virus (●——●); ^{3}H-amino acid-labeled reovirus (●----●). The buoyant densities of representative fractions were calculated from their refractive index measurements (11).*

1.33-g/cm^3 density band.

When labeled IPN virus and top component were analyzed by SDS-acrylamide gel electrophoresis, followed by autoradiography, the polypeptide pattern shown in Fig. 2 was obtained. Three size classes of polypeptides can be detected in the infectious virus in different proportions (large = α, medium = β, and small = γ). The polypeptide composition of the top component is different from that of the full particle, since it contains only traces of α proteins and the γ polypeptide is missing. Furthermore, there are at least two polypeptides of β size, one that is also present in full particles, and another with a slightly lower electrophoretic mobility, which may itself be made up of two components.

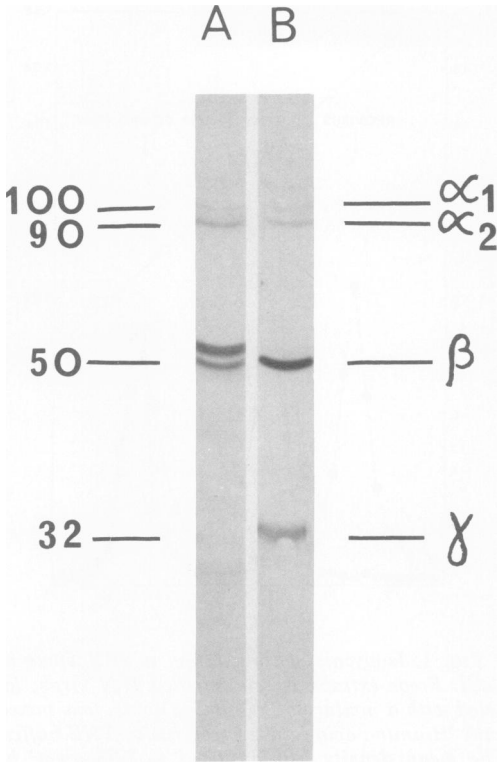

FIG. 2. *Polypeptide composition of IPN virus top component (A) and IPN virus (B). Purified IPN virus and top component labeled with a mixture of ^{14}C-amino acids were dissociated by heating in SDS (1%), 2-mercaptoethanol (2%), urea (5 M) for 2 min at 100°C, and the polypeptides were separated by electrophoresis in 7.5% SDS-phosphate acrylamide gels. After electrophoresis the gels were sliced longitudinally into four strips with a gel slicer, and the slices were dried under vacuum and placed in the dark on Kodak X-ray film for autoradiography for 7 days. The direction of electrophoresis was from top to bottom. The molecular weights were determined in both 7.5 and 10% gels by using the 15 marker proteins listed in Materials and Methods. Number indicate molecular weight × 10^3.*

Estimates of the molecular weights and relative contribution of the polypeptides of each species to the total protein content of the virion are detailed in Table 1. Since [^{35}S]methionine was the amino acid precursor used in most of these experiments concerning polypeptide synthesis, the efficiency of incorporation of this isotope into the structural polypeptides was compared with that of the ^{14}C-labeled amino acid mixture and with the efficiency of staining with Coomassie brilliant blue. The results given in Table 1 show that [^{35}S]methionine was not incorporated into the virion proteins in a ratio significantly different from that in which they bound the dye or incorporated a mixture of ^{14}C-labeled amino acids. It seemed, therefore, that [^{35}S]methionine was a reasonable choice of precursor to study the biosynthesis of viral proteins, especially since this isotope was available at very high specific activities.

Intracellular protein synthesis in virus-infected cells. It has been shown previously (22) that there is no quantitative difference in the incorporation of ^{3}H labeled-amino acids into protein between uninfected and IPN virus-infected cells. We sought to determine if there were any qualitative changes in the polypeptide pattern of RTG-2 cells at various times after infection with IPN virus.

To achieve some degree of synchrony of infection, RTG-2 cell monolayers were infected with IPN virus at a high MOI (50 to 60 PFU/cell), and at various times after infection the cultures were pulse labeled with [^{35}S]methionine for 60 min. The medium was removed, and cells were rinsed with BSS, lysed in ESB, heated, and analyzed by acrylamide gel electrophoresis in SDS-phosphate gels, followed by autoradiography. The labeled polypeptide pattern of uninfected cells, infected pulse-labeled cells, and purified virus is shown in Fig. 3. The data indicate that IPN virus infection did not bring about an inhibition of host cell protein synthesis, for even at 11 h after infection all of the cellular proteins (Fig. 3a) became labeled. At these latter times, the amount of labeled precursor was incorporated at reduced rates, because by this time the cells were dying and characteristic CPE was evident. Polypeptides γ_1 and γ_2 were readily distinguished in infected

TABLE 1. *Estimates of the molecular weights and the relative abundance of the polypeptides of IPN virus*

Polypeptide	Mol wt (× 10^{-3})[a]	Total dye bound (%)	^{14}C-labeled amino acid content (%)	[^{35}S]-methionine content (%)
α_1	100	4	6	5
α_2	90	4	4	3
β	50	63	62	63
γ	32	29	28	29

[a] The molecular weights were estimated by the method of Shapiro et al. (24) using the 15 marker proteins listed in Materials and Methods. Polyacrylamide gels stained with Coomassie brilliant blue were scanned using a Joyce-Loebl microdensitometer. The amount of radioactivity in labeled virus gel profiles (see Fig. 2) was determined by slicing out the bands from the dried gels, dissolving them in 30% H_2O_2 at 60°C in tightly capped scintillation vials, and counting the ratioactivity in a liquid scintillation counter.

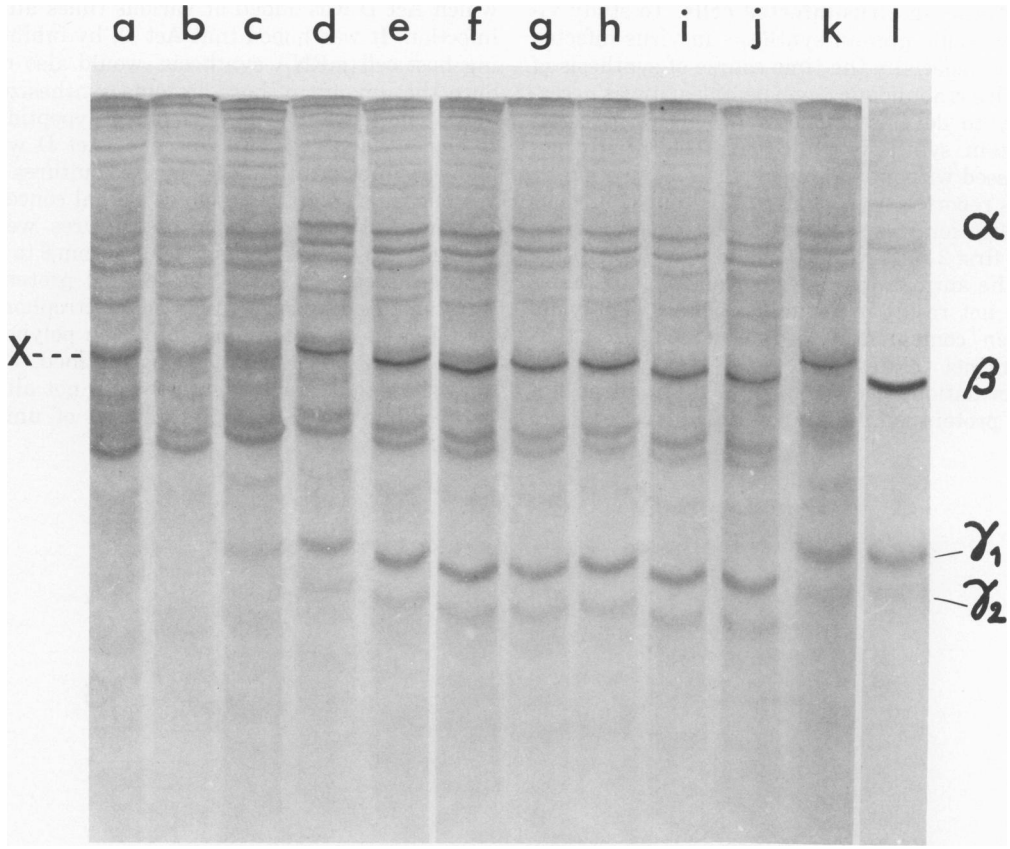

FIG. 3. *Time course of protein synthesis in IPN virus-infected RTG-2 cells. Mock- and virus-infected cell monolayers were labeled with [35S]methionine for 1 h at different times after infection. The cells were solubilized by heating in ESB, and the polypeptides were analyzed in 7.5% SDS-phosphate acrylamide gels together with [35S]methionine-labeled virus purified in a CsCl gradient. The gels were processed for autoradiography as described in Fig. 2. Gel a shows the labeled polypeptide profile of uninfected cells. Gels b to k represent infected-gel profiles of cultures pulse labeled for 1 h at the following times after infection: b, 2 to 3 h; c, 3 to 4 h; d, 4 to 5 h; e, 5 to 6 h; f, 6 to 7 h; g, 7 to 8 h; h, 8 to 9 h; i, 9 to 10 h; j, 10 to 11 h; k, 11 to 12 h. Gel l represents purified labeled virus. X is the position of one of the major cellular polypeptides.*

cells as early as 3 h after infection since no polypeptides of comparable size became labeled in uninfected cells under the experimental conditions used (Fig. 3a and c). These polypeptides were synthesized at a uniform rate from 5 to 10 h postinfection. By 11 h the rate of synthesis of all intracellular polypeptides began to decrease.

The synthesis of polypeptide β seemed to follow the same kind of time course as those of the γ proteins. However, the pattern here was not as clear-cut as in the case of the γ polypeptides because of the presence of a prominent host cell polypeptide (marked X) in this region of the gels. Nevertheless, by 5 h after infection the rate of synthesis of the β polypeptide was sufficiently high to allow it to be distinguished from protein X of the cell. As in the case of the γ proteins, β polypeptide also seemed to be synthesized at a uniform rate between 5 and 10 h after infection.

Polypeptides α_1 and α_2, which constitute a minor part of the virion, are probably produced at a low rate in infected cells for they could not be differentiated from host cell polypeptides under the experimental conditions used.

The β protein of the virion migrated slightly faster in acrylamide gels than the infected-cell-specific polypeptide, which was similarly designated. This is shown more convincingly in subsequent gel profiles (e.g., Fig. 6 and 8).

The γ protein of the virion seemed to be identical to the infected-cell-specific protein γ_1. Polypeptide γ_2, although detectable in the virion protein profile shown in Fig. 3, was usually absent from purified virus preparations (e.g., Fig. 6 and 7).

Effect of Act D and UV light on protein

synthesis in virus-infected cells. To study virus-specific protein synthesis in virus-infected cells, especially the time course of synthesis of the least abundant α polypeptides, it was necessary to develop a method whereby host cell protein synthesis could be preferentially depressed without inhibiting virus replication. It was reported by Nicholson (21) that IPN virus replication was sensitive to Act D only during the first 2 h after infection and that the addition of the antibiotic to infected cells at later times did not result in reduction of the virus yield when compared with infected cultures that were not treated with Act D. In view of this information, we attempted to study intracellular protein synthesis in virus-infected cells to which Act D was added at various times after infection. It was hoped that Act D, by inhibiting host cell mRNA synthesis, would also reduce the amount of host proteins synthesized and would allow the detection of α polypeptides in the infected cells. To this end, Act D was added to infected and mock-infected cultures at various times after infection at a final concentration of 0.5 μg/ml, and the cultures were labeled with [^{33}S]methionine for 4 h from 6 to 10 h after infection. When the labeled proteins were analyzed by acrylamide gel electrophoresis followed by autoradiography, the polypeptide pattern shown in Fig. 4 was obtained. The data show that Act D treatment did not alter appreciably the polypeptide pattern of unin-

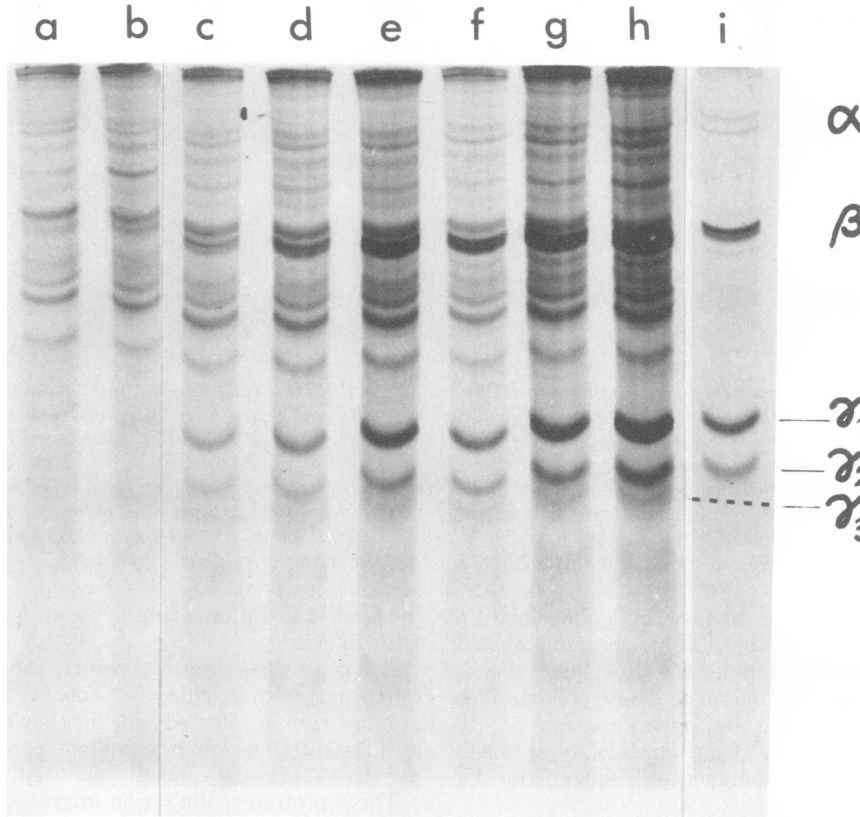

FIG. 4. *Synthesis of polypeptides in uninfected and IPN virus-infected cells treated with Act D (gels a to h) or UV light (gel i) at various times postinfection. Act D was added to uninfected cultures at 0 time (0.5 μg/ml final concentration). IPN virus-infected cultures received Act D at various times after infection. One infected culture received no antibiotic. All (infected and uninfected) cultures were labeled with [^{35}S]methionine from 6 to 10 h postinfection. The cell monolayers were dissolved in ESB, processed, and analyzed in 10% SDS-phosphate acrylamide gels as described in Fig. 3. Autoradiograms a and b represent Act D-treated and untreated uninfected cells. Act D was added to infected cultures at the following hours after infection: c, 1 h; d, 2 h; e, 3 h; f, 4 h; g, 5 h. Autoradiogram h shows the labeled polypeptide pattern of infected cells without Act D treatment. Autoradiogram i shows the labeled polypeptide pattern of infected cells that were UV irradiated at 2 h postinfection as described in Materials and Methods, followed by labeling with [^{35}S]methionine from 6 to 10 h postinfection.*

fected cells (Fig. 4a, b), indicating that RTG-2 cell mRNA's have a relatively long half-life because, even though new mRNA synthesis was blocked by the antibiotic at this concentration (data not shown; 2), mRNA's that were synthesized before the addition of Act D were not degraded but continued to be translated for at least 10 to 11 h after infection. Consequently, the polypeptide gel profiles of Act D-treated and untreated uninfected cells were virtually identical.

When Act D was added to virus-infected cells at 0 or 1 h after infection, the virus yield was greatly reduced compared with control infected cultures that were not treated by the antibiotic (Table 2). The polypeptide gel patterns of these cultures indicated that very little γ_1 and γ_2 proteins were synthesized (Fig. 4e, d). However, when Act D was added at 2, 3, and 4 h after infection, the virus yield was not reduced with respect to control cultures (Table 2) and polypeptides γ_1 and γ_2 were as strongly labeled in these cultures as in those of untreated infected cells (Fig. 4e–h). In these 10% acrylamide gels an additional infected-cell-specific protein, of the small size class, was detected, which was designated polypeptide γ_3. Although this experiment confirmed Nicholson's data on the sensitivity of IPN virus replication to Act D during the early part of the infection cycle, it did not allow the detection of the α polypeptides in infected cells.

TABLE 2. *Effect of Act D and UV irradiation of host cells on the replication of IPN virus*

Time of addition of Act D (h postinfection)[a]	Virus titer at 24 h postinfection (PFU/ml)	Time of UV irradiation of cell monolayers (h postinfection)[b]	Virus titer at 24 h postinfection (PFU/ml)
−1	1.2×10^6	−1	1.5×10^5
0	1.8×10^6	0	5.0×10^5
1	1.4×10^6	1	7.7×10^5
2	4.0×10^7	2	4.0×10^7
3	4.5×10^7	3	1.5×10^7
4	5.0×10^7	4	5.2×10^7
Control (no Act D added)	4.1×10^7	Control (no UV treatment)	5.0×10^7

[a] Replicate cell monolayers were infected with IPN virus at an input multiplicity of approximately 20 PFU/cell. Virus adsorption took place at 20°C for 1 h. The inoculum was removed, and the monolayers were washed three times with BSS and overlaid with fresh MEM containing 5% (vol/vol) fetal calf serum. At various times postinfection, Act D (50 μg/ml) was added to test cultures to give a final concentration of 0.5 μg/ml. The cultures were harvested, sonicated, and assayed for infectivity at 24 h postinfection, a time when extensive CPE was observed in the control cultures.

[b] Replicate RTG-2 cell monolayers were infected with IPN virus as described for the Act D-treated cultures. At various times after infection, the medium was removed, and the rinsed cell layers were UV irradiated as described in Materials and Methods. The virus titer was determined at 24 h after infection.

Clearly, a technique was needed that would either selectively inhibit the translation of cell-specific mRNA's or destroy cellular mRNA's before most of the virus-specific mRNA's would be synthesized. We attempted to use UV irradiation of infected cells to destroy host mRNA, similar to the technique used for the same purpose in T7 phage-infected bacterial cultures (26). The rationale for this technique was as follows. The dsRNA of IPN virus and the virion-associated transcriptase (5) should be more resistant to UV irradiation than host mRNA. Therefore, if infected cells were to be UV irradiated at a time when the addition of Act D no longer inhibited virus replication (2 to 3 h postinfection), host mRNA's would be destroyed but virion transcriptase would continue to function, generating virus-specific mRNA's which in turn would be translated.

Preliminary experiments indicated that the optimal UV dose to be used was 44 ergs/s per mm^2 from a distance of 25 cm for 15 to 20 s. The data in Table 2 show that, as with the Act D treatment, IPN virus replication was inhibited if the host cells were UV irradiated at any time earlier than 2 h after infection. After this time the virus yield in UV-irradiated cells was similar to that in control cultures.

To determine whether the effect of UV irradiation of cells could be measured at the level of protein synthesis, the extent of radioactive amino acid incorporation was determined in uninfected, infected, uninfected UV-irradiated, and infected UV-irradiated cultures. One-half of replicate RTG-2 cell monolayers were infected with IPN virus; the other half of the cultures were mock infected with MEM. Two hours after infection the medium was removed, and one-half of the infected and one-half of the uninfected cultures were UV irradiated for 17 s. The cells were overlaid with MEM diluted 10-fold with BSS containing a mixture of ^3H labeled-amino acids, and the cultures were incubated for various times. Incorporation of the label was stopped at hourly intervals in all four series, and the acid-precipitable radioactivity was determined. The data in Fig. 5 show that there was little difference in the extent of amino acid incorporation between infected and uninfected cultures that were not irradiated with UV light. However, UV-irradiated, uninfected cultures stopped incorporating amino acids into protein until about 9 h postinfection. After this time the extent of incorporation of the label increased, indicating the renewed synthesis of active cellular mRNA's. The extent

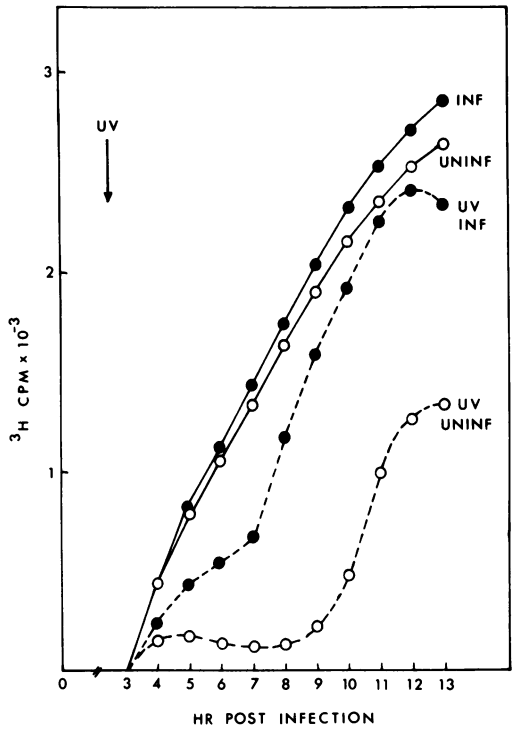

FIG. 5. *Extent of incorporation of ^3H labeled-amino acids into protein by infected (INF) and uninfected (UNINF) RTG-2 cells, with or without UV irradiation treatment. At 3 h postinfection, ^3H labeled-amino acids were added to a series of infected and uninfected cell monolayers; incorporation of the label was stopped at hourly intervals and acid-insoluble radioactivity in each culture was measured as described in Materials and Methods. Another series of infected and uninfected cell cultures was identically treated, except that the cells were irradiated by UV light at 2 h postinfection as described in Materials and Methods.*

of amino acid incorporation in UV-irradiated, infected cells was almost as great as in uninfected, nonirradiated cultures, indicating that an appreciable amount of this protein was virus specific.

Time course of protein synthesis in UV-irradiated, infected cells. It was of interest to analyze the time course of protein synthesis in IPN virus-infected cells under conditions where the rate of host cell-protein synthesis was depressed by UV irradiation. To this end, infected and mock-infected RTG-2 cell monolayers were UV irradiated for 17 s as described above. Both infected and mock-infected cultures were pulse labeled with [^{35}S]methionine for 120 min at 2-h intervals from 2 to 12 h after infection. The protein pattern was analyzed by acrylamide gel electrophoresis and autoradiography. The results (Fig. 6) demonstrated that there was no difficulty in detecting IPN virus-specific polypeptides in infected, UV-treated cells, since host cell protein synthesis was sufficiently depressed to allow easy detection even of polypeptides α_1 and α_2. Both of these proteins seemed to follow the time course of synthesis of polypeptides of the β and γ families. The intracellular β protein seemed to consist of three polypeptides of slightly different electrophoretic mobilities. The upper two seemed to be a doublet and corresponded to the slower-moving β protein in the top-component preparation. The intracellular β polypeptide with the highest electrophoretic mobility was weakly labeled and seemed to correspond to the β polypeptide found in purified virus, which was also present in the top component. In this particular figure it is difficult to discern the three components that make up the intracellular β polypeptide family; in other autoradiograms the separation is more convincing (Fig. 8).

The data in Fig. 6 also indicated that the amount of isotope incorporated into the different virus-specific polypeptides was not distributed in proportion to their molecular weights. Thus there was at all times significantly less precursor incorporated into the α polypeptides than into β proteins, which in turn were less heavily labeled than polypeptides of the γ family.

The disparity in the rates of synthesis of the three size classes of polypeptides in infected cells is further demonstrated by the microdensitometer tracings shown in Fig. 7. The data show the profound effect of UV irradiation in reducing the rate of host cell protein synthesis. In infected cells that were not irradiated with UV light, the α polypeptides could not be detected among the numerous cellular proteins of similar size (Fig. 7a and b). The tracings also show that the cellular protein X has a somewhat lower electrophoretic mobility than polypeptide β. UV irradiation totally eliminated the synthesis of high-molecular-weight host cell proteins, and the rate of synthesis of protein X was greatly reduced. The synthesis of host protein Y was the least affected by UV light, and aside from polypeptide X it was the most prominent labeled host protein in the UV-irradiated cells. Nevertheless, its presence did not hinder the unambiguous detection and quantitation of virus-specific proteins.

Quantitation of the relative amounts of each polypeptide class synthesized at various times after infection showed that there were only minor shifts in the relative rates of synthesis of the β and γ classes as infection progressed (Table 3). There was no evidence for "early" or

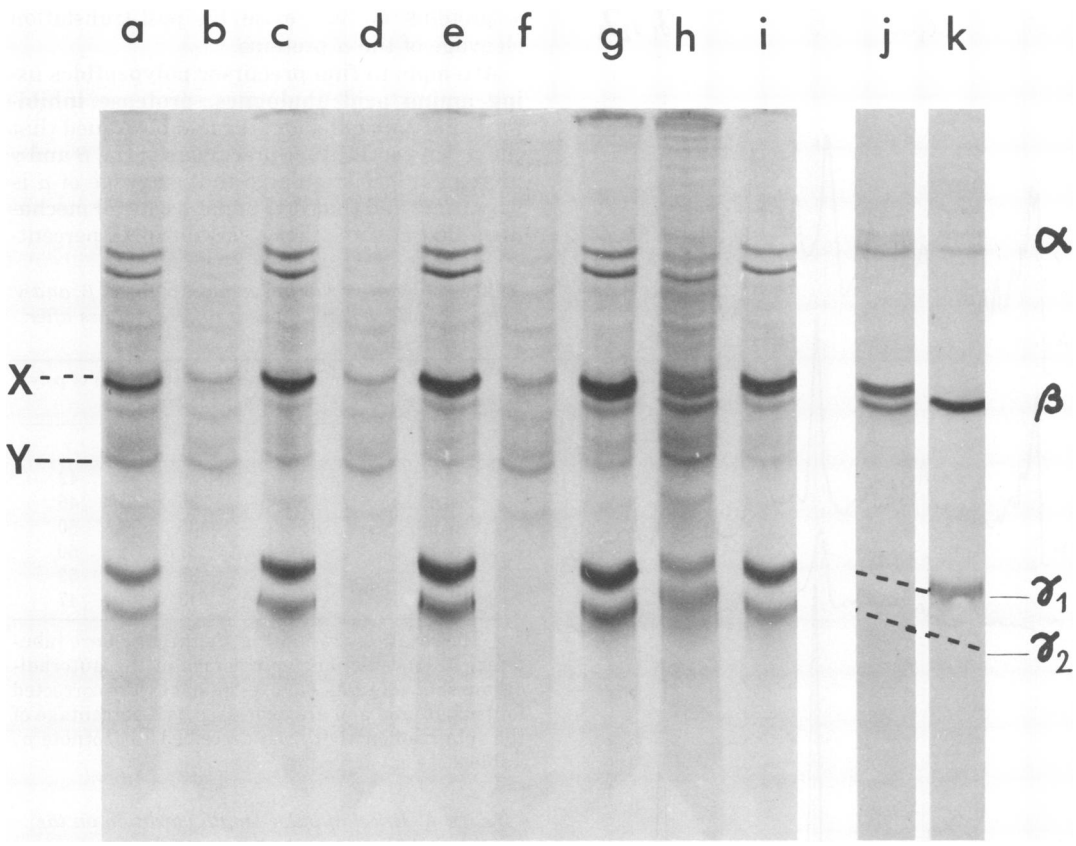

FIG. 6. *Time course of protein synthesis in IPN virus-infected, UV-irradiated RTG-2 cells. Mock-infected and IPN virus-infected cell monolayers were irradiated with UV light at 2 h postinfection as described in Materials and Methods. Both infected and mock-infected cultures were pulse labeled with [35S]methionine for 2 h at different times after infection. Infected cells were processed as described in Fig. 3, followed by acrylamide gel electrophoresis in 7.5% SDS-phosphate gels and autoradiography. Autoradiograms b, d, and f show the polypeptide pattern of UV-irradiated, uninfected cells labeled between 2 and 4, 4 and 6, and 6 and 8 h, respectively. Gel h represents a mixture of proteins from infected and uninfected cells. Gels a, c, e, g, and i show the polypeptide patterns of infected, UV-irradiated cells labeled between 2 and 4, 4 and 6, 6 and 8, 8 and 10, and 10 and 12 h, respectively. Purified [35S]methionine-labeled top component (f) and virus (k) were analyzed in the same manner. X and Y denote two major cell-specific polypeptides.*

"late" polypeptide classes. At early times α_1 and α_2 were synthesized twice as fast as at later times. At all times the γ class polypeptides were synthesized faster than the β proteins, even though the latter represented over 60% of the protein of the virion.

Pulse-length variation and polypeptide synthesis. It has been shown previously that the IPN virus genome is made up of two segments of large-size-class dsRNA of 2.5×10^6 and 2.3×10^6 molecular weight (7a). Since the virion contains RNA transcriptase activity (5), these genome pieces could serve as templates for the production of genome length (large-size-class) mRNA's (24*S*) similar to the large-size-class (25*S*) mRNA's produced in reovirus-infected cells. One of the ways whereby three different size classes of polypeptides can be produced upon the translation of 24*S* mRNA is post-translational cleavage of the primary gene products. A mechanism of this kind operates in picornavirus-infected cells (4, 8, 12). To determine whether this is also the case in IPN virus-infected cells, infected, UV-irradiated cells were pulse labeled for various lengths of time with [35S]methionine beginning 8 h after infection. If a precursor-product relationship exists within the three different size classes of polypeptides, then during a short pulse the majority of radioactivity would be located in the high-molecular-weight precursor proteins, such as the two α polypeptides. If polypeptide β and γ were cleavage products of the α proteins, appreciable amounts of radioactivity would ap-

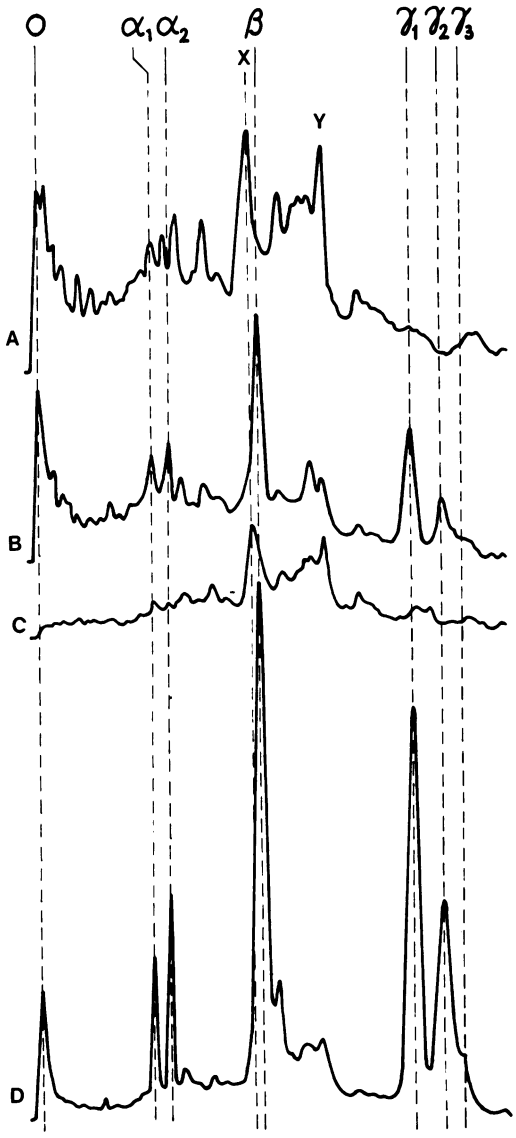

FIG. 7. *Microdensitometer tracings of the autoradiograms shown in Fig. 3 and 6. (A and B) Tracings of Fig. 3a and h, respectively; (B) tracing of Fig. 6f; (C) tracing of Fig. 6g. The letter O marks the origin of the gel; electrophoresis was from left to right. Polypeptides X and Y are cellular proteins, as shown in Fig. 6.*

pear in these putative cleavage products only when the length of the pulse was increased.

Table 4 shows that the relative distribution of radioactivity in the three polypeptide families was independent of the length of the pulse. Furthermore, the relative distribution did not change when a 30-min pulse was followed by a 120-min chase using an excess of unlabeled methionine. These results indicated that the β and γ proteins do not arise by post-translation cleavage of the α proteins.

Attempts to find precursor polypeptides using amino acid analogues, protease inhibitors, and zinc chloride. It could be argued that the α polypeptides are precursors of the β and γ proteins if it is stipulated that cleavage of α is very rapid and that it is under a control mechanism that ensures that a certain small percent-

TABLE 3. *Relative rate of synthesis of the α, β, and γ virus-specific polypeptides at different times after infection*[a]

Time of labeling (h)	Relative rate of synthesis (%) of polypeptide family:		
	α	β	γ
2–4	22	36	42
4–6	18	36	46
6–8	15	35	50
8–10	13	37	50
10–12	12	35	53
12–14	10	43	47

[a] The results shown in Fig. 6a through i are tabulated. Microdensitometer tracings of the autoradiogram shown in Fig. 6a, c, e, g, and i were corrected for background and are expressed as a percentage of the total radioactivity, as described in footnote *a*, Table 4.

TABLE 4. *Effect of pulse length variation on the relative distribution of the three size classes of virus-specific polypeptides (α, β, and γ) in IPN virus-infected, UV-irradiated RTG-2 cells*[a]

Pulse length (min)	Distribution of radioactivity (%) in the three size classes of virus-specific polypeptides		
	α	β	γ
10	15	33	52
20	15	35	49
40	17	37	46
60	13	40	47
120	13	42	45
30-min pulse, 2-h chase	15	39	47

[a] UV-irradiated IPN virus-infected and uninfected RTG-2 cells were pulse labeled with [^{35}S]methionine for various lengths of time beginning at 8 h postinfection. The cells were processed for acrylamide gel electrophoresis and autoradiography as described in Fig. 3. The autoradiograms were scanned using a Mark III Joyce-Loebl Chromoscan equipped with an integrator. Profiles for uninfected control cells were substracted from profiles for infected cells, and the areas under each of the three polypeptide families were expressed as a percentage of the total for each labeling period. The wedges in the Chromoscan were changed according to the intensity of the autoradiogram.

age of the putative precursor protein remains uncleaved. To test this possibility a number of chemicals known to inhibit proteolytic cleavage were used. It has been shown that during picornavirus protein synthesis the cleavage of large precursor polypeptides could be inhibited by adding to the culture medium amino acid analogues (12), protease inhibitors (8), or $ZnCl_2$ (4). It was of interest to determine whether precursor polypeptides could be generated by any of these methods in IPN virus-infected RTG-2 cells. To this end the following experiment was performed.

Virus-infected, UV-irradiated cultures were incubated 6 h after infection for various times (15 to 90 min) with a mixture of canavanine (9×10^{-4} M), *p*-fluorophenylalanine (8×10^{-8} M), and azetidine-2-carboxylic acid (2×10^{-4} M). The cultures were pulse labeled with [^{35}S]methionine for various times ranging from 20 min to 4 h. Similar experiments were carried out in which the concentrations of the analogues were increased stepwise until they completely inhibited protein synthesis in both infected and uninfected cells. The polypeptide pattern was analyzed by acrylamide gel electrophoresis in 12% Laemmli-type analytical slab gels followed by autoradiography. Up to 24 different samples could be analyzed in a single slab, allowing the accurate comparison of labeled polypeptides in different samples. The gel profiles indicated no change in the relative distribution of the three virus-specific polypeptide families, at any of the numerous amino acid analogue concentrations tested, when compared to the gel profiles from infected, untreated control cells.

In another series of experiments, attempts were made to block cleavage with protease inhibitors. The following protease inhibitors were added to a series of infected and uninfected cultures at 6 h after virus infection: tosyl phenylalanyl chloromethyl ketone, tosyl lysyl chloromethyl ketone, phenyl methyl sulfonyl fluoride, and phenyl boronic acid, each at a final concentration of 2×10^{-3} M. The cultures were incubated with the inhibitors for 15 min followed by various lengths of pulses with [^{35}S]methionine (30 min to 4 h). The autoradiograms of the labeled gel patterns from this experiment showed no change in the percentage of distribution of the three polypeptide families with respect to the controls. Similar results were obtained when the protease inhibitor concentrations were increased stepwise. At high concentrations the overall rate of protein synthesis was severely reduced or completely inhibited.

Similarly, no virus-specific precursor proteins were detected when $ZnCl_2$ (4) was added to infected cells at 6 h postinfection in a series of concentrations ranging from 0.1 to 1.5 mM and the cultures were pulse labeled with [^{35}S]methionine for 0.5, 1, 2, and 4 h. At $ZnCl_2$ concentrations higher than 0.75 mM the overall rate of protein synthesis began to decrease, and at concentrations higher than 1.0 mM polypeptide synthesis was completely inhibited in both infected and uninfected cells.

Similar results were obtained when infected cultures were shifted to supraoptimal temperatures of 28, 31, and 35°C followed by pulse labeling for various times (0.5 to 4 h) with [^{35}S]methionine.

It appears, therefore, that polypeptides α_1 and α_2 are not precursors of the β and γ polypeptide families.

Pulse-chase experiments. Additional attempts were made to detect precursor product relationships between virus-specific polypeptides using pulse-chase experiments. Radioactivity could not be chased from intracellular virus-specific polypeptides by incubating infected cultures for 2 h with medium containing a large excess of unlabeled methionine (Table 4), indicating that rapid post-translational cleavage does not take place during virus-specific protein synthesis. However, it was of interest to find out whether slow "maturation"-type cleavage occurs, the kind found in reovirus- and adenovirus-infected cells (3, 29). To investigate this possibility, infected, UV-irradiated cells were pulse labeled with [^{35}S]methionine at 6 h after infection for 1 h (short pulse) or 4 h (long pulse), followed by a "chase" with medium containing an excess of unlabeled methionine for 12 h. Because at this time CPE was extensive and some cells detached from the culture vessel, the medium from each monolayer was subjected to ultracentrifugation to sediment cells and extracellular virus. The resulting pellet was solubilized with ESB and added back to the corresponding cell culture that was similarly solubilized. Gel electrophoresis was performed using 12% polyacrylamide slab gels, followed by autoradiography.

Figure 8 shows that UV irradiation severely depressed the rate of protein synthesis in uninfected cells (Fig. 8a, b). Infected cells pulse labeled for 1 h revealed the two α polypeptides; polypeptide β, which appeared as a doublet in previous SDS-phosphate gels, was clearly resolved into two components, β_1 and β_2 (Fig. 8c). The three small-size-class polypeptides γ_1, γ_2 and γ_3 were well resolved, although in this particular gel the order of the three polypeptides was inverted, a phenomenon that occurs occasionally as reported by other (27) and is

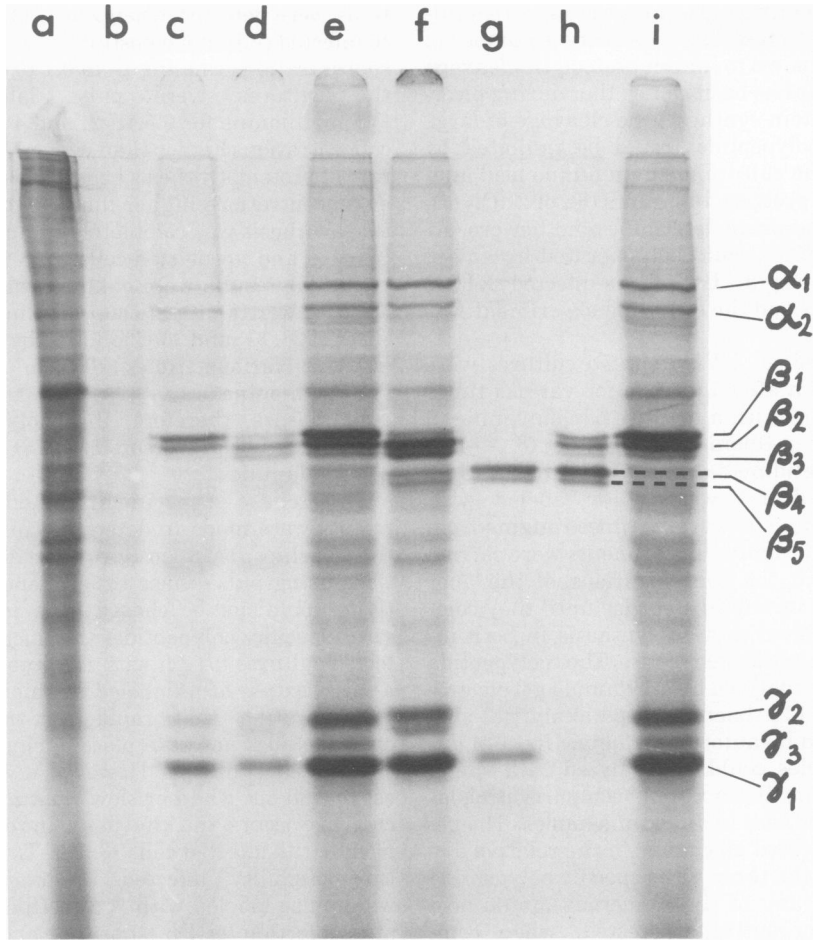

FIG. 8. *Autoradiograms of labeled polypeptides in uninfected, infected, and UV-irradiated cells, purified virus, and top component analyzed on 12% Laemmli-type slab gels. Slot a represents the polypeptide profiles of uninfected RTG-2 cells labeled with [^{35}S]methionine for 4 h; slot b, uninfected cells similarly labeled after UV irradiation as described in Materials and Methods; slot c, infected cells UV irradiated at 2 h postinfection and labeled for 1 h at 6 h after infection; slot d, infected cells UV irradiated at 2 h postinfection and labeled for 1 h at 6 h postinfection and chased for 12 h using an excess of unlabeled methionine; slot e, infected cells UV-irradiated at 2 h postinfection and labeled from 6 to 10 h after infection; slot f, infected cells UV irradiated at 2 h postinfection labeled from 6 to 10 h after infection, followed by a 12-h chase with an excess of unlabeled methionine; slot g, [^{35}S]methionine-labeled purified virus; slot h, [^{35}S]methionine-labeled top component; slot i is identical to slot e. Note the inversion of the order of the γ polypeptides which was probably due to the quality of SDS used in the experiment, as described by others (27).*

said to be due to the quality of SDS used in the gels. Figure 8d shows the gel profile of infected cultures pulse labeled for 1 h and chased for 12 h. Little, if any, radioactivity was lost from the two α polypeptides, but a considerable amount of radioactivity disappeared from β_1, with a concomitant appearance of a new, labeled polypeptide, β_3. The polypeptides of the γ family seemed to have lost some radioactivity during the chase period, but no new polypeptide of the small size class was detected in these gels. This "trimming" of the β_1 polypeptide during a 12-h chase is more convincingly demonstrated in Fig. 8e and f, which show a gel pattern found after a 4-h pulse and a 4-h pulse followed by a 12-h chase.

During a 4-h pulse polypeptides β_1 and β_2 became highly labeled, and the cleavage product of β_1 was also detectable (β_3) because of the long labeling period used (4 h). Figure 8f shows that the two α polypeptides lost little radioactivity during the "chase," but most of the radioactivity disappeared from β_1 and, at the same time, β_3 became heavily labeled and

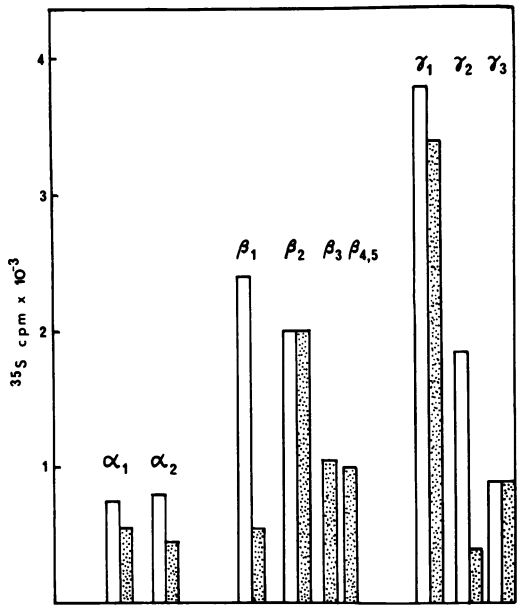

FIG. 9. *Histogram of radioactivity measured in virus-specific polypeptides of the infected, UV-irradiated cells labeled from 6 to 10 h postinfection (Fig. 8e) (clear bars) and similarly treated cells chased, after labeling, for 12 h with an excess of unlabeled methionine (stippled bars) (Fig. 8f). Using the autoradiograms as templates, the virus-specific, labeled polypeptides were sliced out from the dried gel slab and processed for counting as described in Table 1.*

two new middle-size-class polypeptides appeared, designated β_4 and β_5. These two proteins had electrophoretic mobilities identical to that of the β polypeptide of purified virus (Fig. 8g). It appears that polypeptide β_2 did not change during the case period. The pattern of the γ protein family was similar to that observed in the 1-h pulse, 12-h chase experiment (Fig. 8d).

The labeled gel profiles of IPN virus top component and of an infected cell culture pulse labeled for 4 h are shown in Fig. 8h and i. The top component contained no α and γ polypeptides; however, it contained polypeptides β_4 and β_5, also found in the virion, as well as polypeptides β_1 and β_2, which were only encountered in infected cells. The ratio of $\beta_1 + \beta_2$ to $\beta_4 + \beta_5$ varied in different top-component preparations (Fig. 2 and Fig. 3 versus Fig. 8).

The data presented in Fig. 8 indicate that cleavage was taking place during virus morphogenesis. This involved the trimming of polypeptide β_1 via β_3 to β_4 and β_5. The difference in molecular weight between β_1 and $\beta_4 + \beta_5$ was less than 10,000.

A quantitative presentation of these results is shown in Fig. 9. To construct the histogram, the virus-specific bands were sliced out from the dried gel slab, processed, and counted in a scintillation counter as described in Materials and Methods.

The α polypeptides lost about 20% of their radioactivity during the 12-h chase period. On the other hand, approximately 75% of the radioactivity could be chased from β_1, and this amount of radioactivity was found in β_3 and $\beta_{4,5}$. No counts could be chased from β_2. With respect to the small-size-class proteins, the amount of radioactivity did not increase after the chase in any of the three γ polypeptides, indicating that they are not cleavage products of larger proteins. About 80% of the radioactivity was lost from γ_2 and approximately 10% was lost from γ_1, whereas polypeptide γ_3 remained unchanged during the chase period.

Figure 9 also shows that the amount of radioactivity incorporated into the three size classes of polypeptides was inversely proportional to their molecular weights. This was also the case when a shorter (60-min) pulse was used (data not shown). The relative distribution of radioactivity among the three size classes of polypeptides also indicates that the α proteins are not precursors of the β and γ polypeptides.

When the relative intracellular distribution and molar ratio are calculated for the three polypeptide families (Table 5), the results show that assuming an equal rate of chain growth for all molecular species, the frequencies of translation were inversely proportional to the molecular weights. Consequently, during a given time period, shorter mRNA's were translated more often than longer ones.

DISCUSSION

Polyacrylamide gel electrophoresis of IPN virus revealed: (i) two large-size-class polypeptides, α_1 and α_2, which represented less than 10% of the virion protein; (ii) a middle-size-class polypeptide, β, which was the most abundant virion protein and may be made up of two components (Fig. 8g); and (iii) a small-size-class polypeptide, γ. Polypeptide γ_2 was present in some virus preparations (Fig. 3). With respect to the relative distribution and molecular weights of the polypeptides of the virion, my results are in good agreement with those of Cohen et al. (6) but differ from those of Loh et al. (18), who reported seven structural proteins in the virion. Cohen et al. (6) designated the large-size-class polypeptide G, the middle size M, and the small size P. Since these designations may give the impression that IPN virus contains glycoproteins (G), membrane proteins

TABLE 5. *Molecular weights, percent distributions, and molar ratios of IPN virus-specific polypeptides labeled with [^{35}S]methionine*

Polypeptide	Mol wt[a]	% of intracellular virus-specific radioactivity[b]		Molar ratio[c]	% Radioactivity	
		4-h pulse	4-h pulse, 12-h chase		Virion	Top component
α_1	100,000 ± 10,000	5.0	5.2		5.0	A[d]
α_2	90,000 ± 10,000	5.5	4.2		3.0	A
$\alpha_{(1-2)}$		(10.5)[e]	(10.1)	0.06	(8.0)	
β_1	59,000 ± 5,000	18.6	5.4		A	17.7
β_2	56,000 ± 5,000	15.0	19.3		A	24.4
β_3	54,000 ± 5,000	5.8	10.4		A	A
$\beta_4 + \beta_5$	50,000 ± 3,000	A	11.7		63.0	58.0
$\beta_{(1-5)}$		(39.5)	(44.3)	0.44	(63.0)	(100.0)
γ_1	32,000 ± 2,000	27.9	30.8		29.0	A
γ_2	30,000 ± 2,000	14.2	4.6		A	A
γ_3	28,000 ± 2,000	6.2	10.4		A	A
$\gamma_{(1-3)}$		(48.4)	(45.8)	1.00	(29.0)	

[a] Values given are the means of 14 different determinations using a number of marker polypeptides of known molecular weights, as described in Materials and Methods.

[b] The percent distribution of radioactivity was the same when infected cells were pulse labeled for 1 h.

[c] Molar ratios were calculated by dividing the percentage of radioactivity associated with each polypeptide by its molecular weight and normalized to γ_1.

[d] A, Polypeptide absent.

[e] Numbers in parentheses indicate the combined percentage of radioactivity of all members of each polypeptide family (that is α, β, and γ).

(M), or phosphoproteins (P), I preferred to use the more "neutral" nomenclature of α, β, and γ.

Contrary to the identical polypeptide pattern found in reovirus and reovirus top component, the protein compositions of IPN virus and IPN top component differ from one another. IPN top component contains only the β protein of the virion and two other slightly larger polypeptides also found in infected cells (Fig. 8h, i). In both reovirus and IPN virus the middle-size-class protein (μ_2 versus β) is the most abundant polypeptide.

The study of the time course of protein synthesis in infected cells (Fig. 3) confirmed previous observations that IPN virus infection does not "shut off" host cell protein synthesis (22). Although the high resolution obtained by autoradiography allowed the detection of the more abundant β and γ polypeptides in infected cells, minor proteins, such as the α polypeptides, were lost in the high level of background cellular protein synthesis.

Experiments using Act D confirmed the results of Nicholson (21) demonstrating the sensitivity of IPN virus replication to the antibiotic during the early part of infection (0 to 2 h postinfection). These are unusual findings since other animal viruses with dsRNA genomes are not sensitive to Act D at the concentration used here (0.5 μg/ml). Similar sensitivity to Act D has been described for influenza virus (9). This may indicate that during the early part of the infectious cycle some host-specific mRNA has to be transcribed in order for the virus to replicate. It would be interesting to see whether IPN virus could replicate in enucleated cells.

UV irradiation of infected cell monolayers (at times when virus replication was no longer sensitive to Act D; Table 2) allowed the detection of the synthesis of even the minor α polypeptides. The results show that the synthesis of all IPN virus capsid polypeptides can be demonstrated in infected cells (Fig. 6). As is the case in reovirus-infected cells (29), the three size classes of IPN virus-specific polypeptides are formed synchronously: there is no evidence that some begin to be formed earlier than any others, although during the first 2 h after infection (when virus replication is sensitive to UV irradiation) it was not possible to test this point. Two of the three size classes of polypeptides (β and γ) were produced at all times at more or less constant rates between 5 and 12 h postinfection. Notable exceptions were the α polypeptides, which made up a larger percentage of virus-specific proteins at early times (2 to 4 h) than at later times (10 to 12 h) (Table 3). An opposite trend was observed in reovirus-infected cells with the large-size-class λ polypeptides (29). No specifically "early" or "late" proteins were detected in IPN virus-infected cells. Polypeptides β_2 and γ_3 are nonstructural viral proteins (Fig. 8). γ_2 was present in some virus preparations (Fig. 3) but not in others (Fig. 1

and 9); therefore, its designation needs further study.

The slow cleavage of β_1 during maturation via β_3 to β_4 and β_5 (Fig. 8) is similar to the trimming of μ_1 polypeptide during reovirus morphogenesis to produce a slightly shorter polypeptide, μ_2, which is the major virion capside protein (29) just as $\beta_{4,5}$ is the major capsid protein of IPN virus. Since the amount of radioactivity present in β_1, β_3, and $\beta_{4,5}$ in Fig. 8f (4-h pulse–12-h chase) is equal to the amount of radioactivity found in polypeptide β_1 before the chase (Fig. 8e and 9), and since β_2 does not chase, I conclude that proteins β_4 and β_5 are cleavage products of β_1. Peptide map comparisons of the β polypeptides (currently under study) should confirm this conclusion. In recent analyses using Laemmli-type slab gels, attempts to separate the β polypeptide of the virion into two components failed; therefore, it may be that virion protein β_5 (Fig. 8g) is a degradation product of β_4.

The molecular weight determinations of virus-specific polypeptides revealed that the sum of the molecular weights of β and γ polypeptides was equal to that of an α protein. It was, therefore, important to find out whether the middle- and small-size polypeptides were cleavage products of the α proteins. If that were the case, then the two α polypeptides would be the primary gene products of the two viral genome segments and the β and γ polypeptides would be generated by post-translational cleavage of these large proteins, a mechanism well documented in picornavirus-specific protein synthesis. All attempts to block the "supposed" post-translational cleavage and allow the accumulation of high-molecular-weight precursor-polypeptides have failed. Furthermore, the relative distribution of radioactivity among the three virus-specific polypeptide families remained unchanged when infected cells were pulse labeled for only 10 min. In these experiments, if post-translation cleavage was operative, the longer α polypeptides would have contained more radioactivity than the shorter β proteins, which in turn would have contained about twice as much label as the γ polypeptides, assuming random distribution of the isotope. This was clearly not the case (Table 4). Furthermore, radioactivity could not be chased from the α polypeptides during a 12-h chase period (Fig. 8 and 9), even though the cleavage of β_1 to $\beta_{4,5}$ could easily be observed. Taken together, these results indicate that the β and γ polypeptides are not cleavage products of the α proteins. This conclusion was further strengthened when the relative molar ratios of the three polypeptide families were calculated (Table 5). The frequency of translation of the three size classes of polypeptides was inversely related to their molecular weights, indicating that they are the products of three different cistrons.

The coding capacity of the two dsRNA genome segments (2.5×10^6 and 2.3×10^6 daltons) is, at the most, a protein with a molecular weight of 266,000, assuming complete genome length mRNA transcripts and little or no untranslated regions on these mRNA's. The cumulative molecular weight of virus-specific polypeptides α_1, α_2, β_1, β_2, γ_1, γ_2, and γ_3 is 390,000, clearly more than the genome could code for. This indicates that some of these polypeptides may be degradation products of others. For example, polypeptide α_2 may arise by premature termination of translation of α_1 or by proteolytic degradation of α_1 after the cells have been lysed for electrophoresis. Other variations are also possible, involving the β and γ polypeptides. Peptide map comparisons of the different polypeptides could clarify this point (P. Dobos and D. Rowe, manuscript in preparation).

It is interesting to speculate as to how two large dsRNA genome segments could mediate the production of three different size classes of polypeptides. For example, α_1 could be a primary gene product of one of the genome segments via the translation of a large-genome-length mRNA. Indeed, a 24S virus-specific mRNA was reported recently in IPN virus-infected fathead minnow cells (2). Assuming that the amino acid sequence of α_1 and α_2 is the same, it would leave the second genome segment to code for the β and γ polypeptides. This would have to occur by a mechanism different from that found in reovirus-infected cells (29). For example, a genome length (24S) mRNA could be transcribed that would have multiple initiation and termination sites for translation similar to the mRNA of RNA phages (17). Alternatively, subgenomic size mRNA's could be transcribed, as they are in vesicular stomatitis virus-infected cells (10). Whatever the case, IPN virus-specific RNA synthesis probably will follow a unique mechanism.

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