Synthesis and Cleavage of Influenza Virus Proteins

J. ETCHISON, M. DOYLE, E. PENHOET, AND J. HOLLAND¹

Department of Biology, University of California, San Diego, La Jolla, California 92037

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The NWS strain of influenza virus grows rapidly in and kills the MDCK dog kidney cell strain. Within 1 to 2 hr, the virus inhibits host cell protein synthesis and for 3 to 4 hr more it directs the synthesis of influenza virus proteins at a rate about twice that of uninfected cell synthesis. The rates of virus ribonucleic acid (RNA) and protein synthesis reach a maximum within the first few hours after infection and then drop. Plaque assays exhibit a linear dose-response, indicating that only one virion is necessary for productive infection. We have confirmed earlier reports regarding the fragmented nature of the RNA genome of purified influenza virions. However, high resolution gel electrophoresis indicated that each size class of viral RNA is heterogeneous, so that there are at least 10 and probably more fragment sizes of RNA in these virions. Repeated attempts to detect infectivity in preparations of extracted viral RNA were completely negative (over a 10^8 -fold loss of infectivity after extraction). Even infection of the "infectious" RNA-treated cells with intact, related, influenza viruses failed to support infectivity of the isolated RNA or to rescue a host range genetic marker of the RNA. Purified influenza virions exhibit only three major protein peaks based on separation according to molecular weights. These three major virion proteins are the only major virion proteins synthesized in infected cells. This is true throughout the infectious cycle from several hours after infection until the cells are dying. However, the molecular weight of these virion proteins differs slightly depending upon the cell type in which the virus is grown. No host membrane proteins are incorporated into the virions as they bud through the cell membrane. Pulse-chase labeling early after infection or prolonged chase experiments indicate that influenza virus proteins are cleaved from one or more precursor polypeptides. In fact, each of the three major peaks seems to be a heterogeneous mixture of polypeptides in various stages of cleavage. Peptide analysis confirms that the three major peaks share common peptides, but the exact precursor product relationships are not clear. There may be one or several precursor proteins. Also there could be overlapping messenger RNA molecules of varying length giving rise to polypeptides of various sizes and overlapping sequences. Late in infection, amino acid labeling shows a preponderance of internal nucleocapsid protein synthesis, indicating that either this protein is much more stable to cleavage in infection or it is made from a more stable messenger. There is no obvious relationship between virion RNA fragments and viral protein sizes, so these fragments may be artifacts.

Influenza virus matures by budding from the plasma membrane of infected cells as the internal nucleocapsid moves to the cell surface within patches of altered plasma membrane (6). The areas of the plasma membrane which bud to form virions are composed entirely of viral proteins with all host protein displaced at these points (10). However, the lipids and glycolipids of the virion envelope are derived from the host cell (3, 13, 16), there is a carbohydrate host antigen covalently bound to viral envelope protein (20), and ad-

ditional carbohydrate chains are present in the glycolipid (16). The genome of influenza virus consists of a number of different size molecules of of single-stranded ribonucleic acid (RNA; references 7, 23, 24). Electrophoretic separation of the virion RNA and of the double-stranded replicative form indicate that there are five, or possibly six, distinct RNA species (24, 25). These are associated with internal protein subunits to form the helical nucleoprotein cylinders of the nucleocapsid (26).

The proteins of influenza virus remain ill-defined. There is clear evidence for two distinct,

¹ Present address: Institut de Biologie Moléculaire, Université de Genève, Genève, Suisse.

virion membrane proteins: the neuraminidase and the hemagglutinin (17, 18, 19), in addition to the internal nucleocapsid protein. There is also evidence that influenza virus induces a new replicase in the infected cell (8, 27). Evidence from this laboratory (10) and from Taylor et al. (32) shows only three major protein peaks in the virion and in virus-infected cells (based on size distribution in gels). Dimmock and Watson observed several additional protein peaks in infected cells (5), and detected additional antigens in the cells after adsorptive elimination of virion-reactive antibody (4). Webster (33) reported that neuraminidase and hemagglutinin preparations which are pure by electrophoretic, immunological, and electron microscopic criteria are composed of subunits of 58,000 and 47,000 daltons molecular weight, respectively, yet these sizes do not correspond to the major protein subunit sizes seen in other laboratories (10, 32). Furthermore, Webster presented data which indicated that these purified proteins were either strongly aggregated, or heterogeneous, or both (33).

The present report shows that influenza virus proteins undergo proteolytic cleavage in infected cells. However, these cleavages are much more complex and difficult to demonstrate in myxovirus-infected cells than in enterovirus-infected cells where they were first observed (9, 11, 12, 14, 30).

MATERIALS AND METHODS

Cells were grown in Eagle minimum essential medium containing 5% calf serum and labeled in minimum essential medium containing 2% dialyzed calf serum plus ^3H - or ^{14}C -labeled amino acids in place of the same unlabeled amino acids.

Influenza virus employed was strain NWS (mouse neurotropic), which had been subjected to passage twice in mouse brain and twice in BHK-21 hamster cells to enhance its mammalian cell virulence before working stocks were prepared in 10-day embryonated chicken eggs. The PR8 strain and the J305 A₂ Asian influenza virus strain were grown in 10-day embryonated eggs by inoculation of 100 to 1,000 infectious particles. Eggs were incubated at 38 C and harvested between 32 and 42 hr postinfection.

All plaque assays were carried out on monolayers of MDCK dog kidney cell strain. Cells in culture were infected as monolayers at an effective multiplicity of approximately 50 plaque-forming units (PFU) per cell. Virus attachment was allowed to proceed for 30 min. All times of infection shown below are computed from the time at which virus attachment was begun.

Influenza virions were purified by differential centrifugation, agarose column chromatography, and banding in density gradients of sodium-potassium tartrate or sucrose followed by dialysis. In some cases, adsorption-elution from red blood cells and 60% ammonium sulfate precipitation at 4 C were also

employed, but these were not necessary to prepare virus of high purity.

Electrophoretic separation of labeled proteins was carried out on 5% polyacrylamide gels as described in detail elsewhere (14, 15). This is a high pH adaptation of the 0.1% sodium dodecyl sulfate acrylamide gel technique of Maizel and his colleagues (22, 31). Tris(hydroxymethyl)aminomethane-acetate buffer, pH 9.0, was employed to aid in solubilizing proteins resistant to sodium dodecyl sulfate denaturation at neutral pH. Preparative gel electrophoresis of viral proteins for peptide analysis was carried out under identical conditions, except that the proteins were eluted off the end of a gel tube (11 by 0.6 cm) into an elution chamber designed by Robert Bryan of this department. The chamber is within a small Plexiglas block that fits over the end of the gel tube. Buffer flowing into the chamber through a small orifice carries eluted proteins through plastic tubing out the opposite end of the chamber from the orifice and into a fraction collector. Gravity flow is employed and flow rate is adjusted by varying the hydrostatic pressure. The separated protein peaks were mixed with 2.0 mg of ovalbumin, reduced with 0.1 M dithiothreitol, and then alkylated with 0.02 M iodoacetamide for 20 min at 20 C, pH 8.6. The alkylated proteins were then precipitated with 10% trichloroacetic acid. The pellets were washed at least five times with 100% ethanol and then once with ether. The final pellet was dried by blowing off residual ether, and the dried pellet was suspended in 1 ml of 0.15 M ammonium bicarbonate, pH 8.4, containing 100 μg of TPCK trypsin (Worthington). The pellet was incubated in a 37 C water bath for 5 to 10 hr, and then 100 μg of fresh TPCK trypsin was added and incubation was continued for an additional 10 to 15 hr. The digested peptides were evaporated to dryness, suspended in water, and evaporated. The residue was dissolved in starting buffer for chromatography, and insoluble particulates were removed by centrifugation at $2,000 \times g$.

Peptide chromatography was carried out on high pressure columns of Aminex A5 (Biorad) resin, by a modification of the technique of Schroeder et al. (28). A column (30 by 0.6 cm) was employed with an elution pressure between 400 and 500 psi in a water-jacketed column at 50 C. The packed column was washed with starting buffer (0.2 M pyridine acetate, pH 3.0) and then loaded with 1 to 2 ml of sample under 50 psi at 50 C. The peptides were eluted with a continuous nonlinear gradient from starting buffer at pH 3.0 to finishing buffer at pH 5.0 (2 M pyridine acetate). The mixing chamber contained 175 ml of starting buffer, and the reservoir chamber contained 75 ml of finishing buffer. From the start of trypsin digestion, the entire procedure could be carried out with over 90% recovery of protein label.

Proteins to be employed for peptide analysis were labeled with radioisotopic arginine or lysine from Schwarz BioResearch (^3H -lysine, 15 Ci/mm; ^{14}C -lysine, 300 mCi/mm; ^3H -arginine, 22 Ci/mm; ^{14}C -arginine, 312 mCi/mm). Proteins labeled for analytical gel electrophoresis were labeled with ^3H - or ^{14}C -phenylalanine, -valine, and -tyrosine as described elsewhere (15).

RESULTS

Growth of NWS strain of influenza virus in MDCK cell strain. The NWS strain of influenza virus used here is highly virulent for the MDCK cell strain. High-multiplicity infection causes complete cytopathic effects and cell monolayer destruction within 9 to 20 hr, and very low multiplicities destroy the cells between 20 and 30 hr after infection. Confluent, "contact-inhibited," monolayers are affected more slowly than are light, incomplete monolayers. This cell strain does not exhibit detectable Von Magnus-type autoinhibition of virus growth during high-multiplicity infection on first passage. Final yields are approximately the same whether the cells are infected at high or low multiplicity. Yields vary (according to the density of the monolayer and the physiological state of the cells) from several PFU per cell to 50 PFU per cell. Maximum yields were reached by 10 hr postinfection after high-multiplicity infection or 24 hr after low-multiplicity infection. In a typical high-multiplicity infection of a light, semiconfluent monolayer of approximately 10^7 cells, the 12-hr yields were 3×10^8 PFU, 25,000 hemagglutinin units on human group O red blood cells, and 1,700 neuraminidase units on fetuin substrate. The PFU yields give a low estimate of infectious virion production since egg infectivity assays are 5 to 10 times as sensitive as this plaque assay. Figure 1 shows the production of viral hemagglutinating particles with increasing time after infection of the MDCK cell strain. Hemagglutinin production continues between 12 and 24 hr, even though viral

FIG. 1. Production of viral particles (hemagglutinin) at times after infection of MDCK cells with NWS strain influenza virus. Cells (10^7) were infected at high multiplicity, washed twice, and incubated in 50 ml of fresh medium at 37 C. One-milliliter samples of medium were withdrawn at the times indicated and were diluted and assayed for hemagglutination by using human group O red blood cells.

infectivity reaches a peak by 12 hr. This presumably is due to the continuing production of infective particles being counterbalanced by thermal inactivation of accumulated virus.

Figure 2 shows the kinetics of viral RNA production in MDCK cells infected with a high multiplicity of NWS influenza virus. Actinomycin was present only in the ^3H -uridine, pulse-labeling medium. The cells were not pretreated with actinomycin because influenza virus replication is actinomycin sensitive during the first 4 hr (2). The biphasic nature of RNA production seen in Fig. 2 is typical of many experiments, but in a few experiments only one continuous period of maximal synthetic rate was observed. It is interesting that the first period of maximal synthesis corresponds to the actinomycin-sensitive period, and the second occurs during the period of actinomycin insensitivity.

Figure 3 shows the kinetics of protein synthesis following high-multiplicity influenza virus infection. Note the early stimulation of protein synthesis. We have repeatedly observed that influenza virus stimulates protein synthesis to twice and sometimes even three times the control rate of protein synthesis within 30 min to 1 hr after infection. This remarkable rate of synthesis continues for several hours, and most of the protein

FIG. 2. Rates of viral RNA production in MDCK cells infected with NWS strain influenza virus. Matched identical monolayers of approximately 2×10^6 MDCK cells from the same seed pool were infected with a high multiplicity of influenza virus and incubated at 37 C. Ten minutes before the indicated times after infection, monolayers received 5 μg of actinomycin D per ml and 10 μCi of ^3H -uridine per ml in Eagle minimum essential medium. After 20 min, labeled monolayers were dissolved in 0.1% sodium dodecyl sulfate-tris(hydroxymethyl)aminomethane-hydrochloride solution (pH 9.0), and their RNA was precipitated and repeatedly washed with 10% trichloroacetic acid before counting. Results are shown as per cent of counts observed in noninfected, similarly treated controls.

being made is viral protein, as is shown below. The time by which host protein synthesis is completely shut-off and replaced by virus synthesis usually varies between 2 and 3 hr postinfection, but as early as 1.5 hr most protein synthesis is usually virus-directed.

Lack of infectious RNA in influenza virus. During the last decade, numerous laboratories have tried without success to obtain infectious RNA from influenza and related viruses. Isolated claims for success have not been repeatable. Because the NWS-MDCK system is a sensitive and quantitatively reproducible cell culture system, we have made a major effort to detect even traces

FIG. 3. Rates of protein synthesis in MDCK cells infected with influenza virus. Conditions same as for Fig. 2, except that cells were labeled 20 min in Eagle minimum essential medium in which ^3H -phenylalanine, -valine, and -tyrosine replaced the same unlabeled amino acids. Protein-specific activities were determined after overnight dialysis of each dissolved monolayer against a large volume of electrophoresis of sample buffer, pH 9.0. Results are expressed as per cent of uninfected (mock infected) control in which 450 ^3H counts per min per μg of protein were incorporated at all times after infection.

of infectivity in preparations of RNA from influenza viruses and vesicular stomatitis virus. Enterovirus RNA preparations were used as positive controls. All results with influenza virus and vesicular stomatitis virus were negative, although enterovirus RNA extracted together with the myxovirus RNA was highly infectious. Typical results are shown in Table 1. Poliovirus RNA was about 0.1% as infectious as whole virus, whereas influenza virus and vesicular stomatitis virus showed no infectivity (less than one-hundred millionth the infectivity of whole virus). Clearly influenza virus might lack infectivity because it requires several genome fragments to infect (24, 25). However, vesicular stomatitis virus RNA is not fragmented (29), and its lack of infectivity is probably due to the necessity for a virion replicase to make minus-strand messenger RNA for replication (1). If influenza virus RNA is noninfectious because it requires enzyme(s) provided by the virion, or because it carries different genetic messages on different RNA fragments, then genetic marker rescue might be possible. We therefore tested the infectivity of the host range marker of NWS strain RNA exposed to cells already infected by whole PR8 strain virus. MDCK cells (in which PR8 grows very poorly) and chick embryo fibroblasts (in which PR8 replicates well) were infected for 30 min with about 50 PFU of PR8 whole virus per cell. They were then exposed to RNA from 2×10^{10} PFU of NWS strain and incubated at 30 C for 24 hr. The yields were harvested and assayed on MDCK cells (NWS strain gives large plaques and PR8 strain gives no plaques). No plaques resulting from complementation or recombination were obtained in several such marker rescue experiments. Since these closely related type A₀ influenza strains can recombine genetic characteristics in double infections with whole virus, this failure to rescue

TABLE 1. Comparative infectivity of the RNA species from poliovirus, influenza virus, and vesicular stomatitis virus (VSV)

Viral source of RNA	Cell type used for plaque assay	PFU/ml of infectious virions ^a	PFU of infectious RNA	Comparative efficiency of RNA/virion
Poliovirus (type 1)	HeLa cells	5×10^9	3×10^6	0.6×10^{-3}
VSV (Ogden)	HeLa cells	1.6×10^{10}	$<1 \times 10^2$	$<10^{-8}$
Influenza (NWS)	MDCK	2.0×10^{10}	$<1 \times 10^2$	$<10^{-8}$

^a Before phenol extraction. Virions were suspended in 0.15 M NaCl, 10^{-2} M ethylenediaminetetraacetic acid, 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) before extraction. They were extracted with an equal volume of hot (60 C) phenol for 3 min at 60 C and spun at $2,000 \times g$ for 5 min; the supernatant was extracted four times with ether to remove phenol. RNA was absorbed to 2×10^6 cells in 0.2-ml portions of 2 M MgSO_4 on triplicate monolayers with appropriate dilutions. Similar negative results were obtained when 0.1% sodium dodecyl sulfate was added to phenol before RNA extraction, or when RNA uptake was facilitated by use of diethylaminoethyl dextran or polyornithine instead of high salt concentrations.

RNA markers suggests that the RNA of these viruses cannot function biologically in the isolated state. It seems likely that this RNA must be combined with protein as in the nucleoprotein to function genetically. Or perhaps these viral RNAs are always degraded during extraction.

Attempts to detect RNA replicase in influenza virions. Because there is an RNA replicase induced in influenza virus-infected cells (8, 27) and because purified virions show the same proteins as do infected cell extracts (10, 32), we have attempted to detect replicase activity in purified influenza virions. All attempts have been negative so far, whether we employed intact virions, virions disrupted with varying concentrations of deoxycholate, Brij-58, Triton X-100, or sodium dodecyl sulfate detergents, virions sonically treated in varying concentrations of ammonium sulfate or detergent, or virions absorbed to actinomycin D-pretreated cells at very high multiplicity. Infected MDCK cells contained levels of polymerase activity similar to that previously reported for other systems (8, 27). If there is a virion-associated replicase, very specific conditions may be required to activate it.

Dose-response in the plaque assay. Because of the fragmented viral RNA and the total lack of infectious RNA in influenza virions, we carefully examined the dose-response curve for plaquing NWS on MDCK cells. Figure 4 shows that the plaque response is strictly a linear function of the amount of input virus. It is clear, therefore, that a single influenza virion is sufficient to infect a cell. No cooperation among particles is necessary, although cooperation may occur at higher multiplicities of infection.

Size of RNA molecules in influenza virions. We used very long (23 cm) 3% acrylamide gels

FIG. 4. Dose-response in the plaque assay of strain NWS influenza virus on MDCK cells. Twofold dilutions of a stock suspension of influenza virus were assayed (each in triplicate) on monolayers of approximately 2×10^6 MDCK cells.

FIG. 5. Acrylamide gel electrophoresis of influenza virion RNA and ribosomal RNA species on long (23 cm) 3% gels. PR8 virions were labeled by injecting each of 25 ten-day embryonated eggs with 1 mCi of ^{32}P -orthophosphate and 10^3 infectious particles of NWS virus. Eggs were harvested at 38 hr, and the virions were purified. The NWS strain virions were labeled by exposing 10^8 MDCK cells to 2 mCi of ^3H -uridine in minimum essential medium between 0.5 and 20 hr after infection with NWS strain. Phenol extraction was carried out with an equal volume of phenol and of electrophoresis sample buffer (pH 9.0) with 0.1% sodium dodecyl sulfate. Phenol was removed by dialysis for 4 hr at 35 C against sample buffer. Electrophoresis was exactly as described for protein gels, except that 3% acrylamide was used and the samples were subjected to 100 v for 19 to 20 hr. The gels were crushed on a divider as for proteins.

to examine the size distribution of RNA strands in purified influenza virions. Figure 5 confirms the results of Pons and Hirst (24, 25) in demonstrating size heterogeneity. However, the size distribution of PR8 virion RNA (grown in eggs) is different from that of NWS virion RNA (grown in MDCK cells). Moreover, our preparations are even more heterogeneous than the preparations of Pons and Hirst in which five pieces of single-stranded RNA and of replicative form were seen. Purified NWS virions from chick embryo fibroblasts gave a slightly different pattern. We conclude that the size of influenza virus RNA can vary with host cell, passage history, conditions of infection, etc. The smallest size class seen clearly in the PR8 virion RNA was not present in the NWS virion RNA. The smaller

FIG. 6. Acrylamide gel patterns of proteins of influenza virions, of virus-infected cells, and of deoxycholate-solubilized virions. A, Comparison of proteins from 3H -labeled purified virions ($\times$), and of viral proteins synthesized in infected cells labeled with ^{14}C amino acids between 8.0 and 9.0 hr after infection ($\circ$). B, Protein patterns of purified 3H -labeled virions as in A, except that virions were dissolved in 0.15 M NaCl, 10^{-3} M ethylenediaminetetraacetic acid, 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) containing 0.5% sodium deoxycholate to dissolve the viral envelope. The nucleocapsid internal component was then layered on top of the same solution containing 10% sucrose, and spun at $105,000 \times g$ for 2 hr. The pellet was gently washed in the above solution and again pelleted at $105,000 \times g$ for 2 hr. The final pellet was dissolved in sample buffer and analyzed by gel electrophoresis in sodium dodecyl sulfate as for A. C,

pieces of RNA may be artifacts generated by scission of one or more larger pieces. Note also that the molecular weight of the largest pieces of RNA from both strains of virion is about 1.0 million to 1.3 million daltons as estimated by using internal marker ribosomal RNA from HeLa cells of 0.17×10^6 daltons (18S) and 1.9×10^6 daltons (28S; see reference 21). This is somewhat larger than was estimated by Pons and Hirst with their preparations of RNA.

Size of influenza virus proteins in virions and infected cells. Figure 6A shows a typical comparison of the proteins of purified NWS virions and of virus-directed proteins synthesized by infected cells. It can be seen that they are nearly identical: most, if not all, of the protein synthesized in infected cells is the same protein that goes into the virion. The size of the three major viral proteins is about 30,000 daltons (peak A); 62,000 daltons (peak B); and 85,000 daltons (peak C), estimated by using internal marker standards as described previously (15). Figure 6B shows that only peak B remains sedimentable after solubilization of virion envelope protein by sodium deoxycholate. Thus, it appears that the internal nucleoprotein is composed of the 62,000-dalton protein subunits of peak B, and peaks A and C are the major subunit proteins of the lipoprotein membrane derived by budding from the host cell plasma membrane. Figure 6C shows that the proteins of an A₂ Asian influenza strain are similar to those of NWS (although peak C predominates). We have already shown that no host proteins (or only insignificant amounts) are present in the purified virion (10). Therefore, peaks A and C, the two major peaks of "membrane protein," must completely displace the hundreds of host proteins which normally make up the plasma membrane (15). They probably do this in local areas of the plasma membrane where nucleation points initiate the aggregation of more and more viral membrane protein until the altered spot on the cell surface has grown to a size critical for enclosing a nucleoprotein core and budding to the outside of the cell. Figure 7 shows NWS viral proteins altering only discrete patches of plasma membrane in infected chick cells. In all electron micrographs, the viral proteins are generally seen as altered patches of various sizes along the cell membrane. It appears that viral proteins preferentially aggregate with other viral proteins on the membrane to form these patches rather than distributing themselves

3H -labeled purified virion proteins of A₂ Asian influenza virus (strain JAP 305). The anode is on the left in all electrophoregrams shown here.

FIG. 7. Electron micrograph of surface membrane of cells infected with NWS influenza virus. Fourteen-day-old chick chorioallantoic membrane and budding viruses 13 hr after infection with 10^3 egg infectious doses of influenza strain NWS. The membranes were fixed in gluteraldehyde followed by OsO_4 , and were dehydrated in ethanol containing 1% phosphotungstate. The Epon-embedded membranes were further stained in section with uranyl acetate and lead citrate. The budding virus and its earlier stage to the right has material associated with it that stains differently from the cell membrane. $\times 192,500$. Electron micrograph courtesy of Garth L. Nicolson.

homogeneously among all the host membrane proteins. It is not obvious visually, however, that viral proteins are totally displacing host proteins in these patches on the plasma membrane, although prelabeling experiments show that this is occurring (10).

Nature of viral proteins synthesized at various times after infection. We looked for evidence of regulation of viral protein synthesis during early and late phases. Figure 8 shows a sequence of patterns of protein synthesis at increasing times after infection of MDCK cells with NWS. There is considerable change from the normal pattern of cellular protein synthesis by 1 hr after infection, and by 4 hr only the three major peaks of viral protein are being synthesized. These three peaks continue to be synthesized at a declining rate (see Fig. 3) until the cell dies. Later in infection peak B, the polypeptide subunit of the nucleoprotein, is synthesized at a somewhat faster rate relative to peaks A and C than it is earlier. Early in infection a polypeptide peak of about 100,000 daltons is also present (the tallest peak in Fig. 8B 1 hr after infection). This is

seen early after infection of all cell types. It may be a viral precursor protein.

Size of influenza virus proteins synthesized in different cell types. When a single NWS strain virus inoculum was used to infect different cell types, unexpected differences in the molecular weights of the viral proteins synthesized were observed. The most striking differences were seen with peaks B and C in MDCK cells as compared to the same peaks in chick embryo cells (Fig. 9A). Identical results are seen in the proteins of purified virions grown in these two cells (Fig. 9B). Figure 9C shows that peak C of viral protein grown in L cells is several thousand daltons lower in molecular weight than when it is grown in MDCK cells. These minor differences are reproducible even when the labels are reversed, and similar differences are apparent among influenza virus proteins produced in other cell types also. Many of these are very poor hosts for virus replication. A comparative biochemical study of different cell types as hosts for influenza virus replication will be published elsewhere (Etchison and Holland, *manuscript in preparation*).

FIG. 8. Patterns of protein synthesized in MDCK cells before, and at increasing times after, infection with strain NWS influenza virus at high multiplicity. Cells were labeled for 20 min with ^3H -amino acids in each case. (Labeling was begun 10 min before and completed 10 min after the indicated time point). A, Uninfected MDCK cells; B, cells infected 1 hr; C, cells infected 2.0 hr; D, cells infected 4.0 hr; E, cells infected 6.25 hr; F, cells infected 8.0 hr.

FIG. 9. Comparison of proteins of the same virus inoculum grown in different cells. All cells were infected with a high multiplicity of NWS strain. A, Viral proteins synthesized in primary chick embryo fibroblasts labeled with ^3H -amino acids between 5.0 and 6.0 hr after infection ($\circ$); viral proteins labeled with ^{14}C in MDCK cells between 4.5 and 5.5 hr after infection ($\times$). B, Purified virions labeled with ^3H -amino acids in chick embryo cells between 4.0 and 4.3 hr after infection ($\circ$); purified virions labeled with ^{14}C in MDCK cells between 4.0 and 5.0 hr after infection ($\times$). C, Viral proteins labeled with ^3H -amino acids in L cells between 4.0 and 6.0 hr after infection ($\circ$); viral proteins labeled with ^{14}C -amino acids in MDCK between 4.0 and 6.0 hr after infection ($\times$).

Peptide analysis of tryptic digests of influenza virus proteins. The size differences among viral proteins grown in different cells led us to examine their tryptic digests to determine whether they really were the same proteins or whether the

peaks might be composed of different proteins when grown in different cells. Figures 10A, 10B, and 10G show that each major viral peak exhibits the same peptide pattern, regardless of the cell which produced it. The patterns of lysine peptides of peak A are nearly identical, whether they came from HeLa cells or dog kidney cells. Note also that the arginine peptides of peak B from MDCK (dog kidney) cells correspond to those of peak B from chick cells, despite the apparent size difference noted above. This is true also of the lysine peptides of peak B from both cell types. This is surprising since the size difference would indicate that some peptides should be missing in the smaller polypeptide chains. It is possible that some differences do exist among those peptides which did not chromatograph (break-through peak at the left margin of the graphs), but it is more likely that the lower-molecular-weight peaks (A and B) of chick cells are composite peaks containing some molecules as large as those of MDCK cells and others which have been cleaved. In fact, peaks A, B, and C in all cells probably represent proteins in various stages of proteolytic cleavage, with the position of the peak representing an average size. All of these peaks are broader than is seen with single, pure proteins, and their breadth varies from preparation to preparation. Note the great breadth of peak A from chick cells in Fig. 9A. It is obviously multicomponent, yet it contains the same peptides as do sharp A peaks from MDCK cells. Peak B always has a shoulder of varying size on its low-molecular-weight side and, on long gel separations, peak C is seen to be complex and too broad for a single protein. Thus, all of the peaks may be complex. Some may migrate as broad bands because of covalently linked polysaccharides.

Comparison of tryptic peptides indicates that proteolytic cleavage is taking place after the synthesis of influenza virus proteins. Figure 10C shows that peaks A and C contain many identical lysine peptides. Figure 10D shows that nearly all the peptides of peak B are also present in peak C. It appears likely that peak C is a precursor polypeptide (or mixture of polypeptides) which undergo(es) cleavage to give rise to peaks A and B. Actually, peaks A and B themselves undergo cleavage also, because small polypeptides of heterogeneous size are frequently seen in the 5,000- to 20,000-dalton area of the gels. When these were separated, digested with trypsin, and analyzed, their peptides proved to be almost identical to those of peak A. Figure 10E shows a comparison with peak B tryptic peptides. Note that all of the peptides of peak B are present in the lower-molecular-weight material. It seems that influenza virus proteins are arising from and

FIG. 10. Peptide analysis of individual protein peaks of influenza virus. Peaks A, B, C refer to the three major polypeptide peaks assigned labels in Fig. 6A. Solid line, ^{14}C -labeled tryptic peptides; dashed line, ^3H -labeled tryptic peptides. The conditions for purification, tryptic digestion, and chromatographic analysis on Aminex A5 resin are given in Materials and Methods.

subject to proteolytic cleavage, but the exact precursor-product relationships are not yet clear. The observed conservation of most of the tryptic peptides during this process suggests that an en-

zyme with trypsin-like specificity is involved in the *in vivo* cleavage reactions. Figure 10F shows the total arginine and lysine peptides of unfractionated virus-directed protein in infected cells.

The pattern is about as complex as would be expected with only a few major proteins.

Pulse and chase experiments. White and his colleagues (32) and we have observed that pulse-chase experiments usually show little evidence for a precursor-product relationship among the influenza virus proteins such as is easily demonstrated with the enterovirus proteins (9, 11, 12, 14, 29). Pulse labeling usually shows about the same viral protein pattern, whether it is followed by a chase or not. However, we have found that a pulse label given early after infection reveals an instability of peak C, especially if it is followed by a prolonged chase period. Figure 11A compares influenza virus proteins pulse-labeled early after infection (2.5 hr) with those seen after a prolonged chase in BHK-21 cells. Much of the pulse label is on the high-molecular-weight side of peak C (which looks composite here), but much of peak C disappears, and only peaks A and B are prominent after a 4-hr chase. What remains of peak C after the chase is lower

in molecular weight (about 85,000 daltons) than the main crest of peak C in the unchased, early-pulsed protein. Because peak C contains less than a third of the total counts after a pulse early after infection, and because a part of peak C remains even after a long chase, it was not possible in numerous experiments to show that the label lost from peak C is converted quantitatively into peaks A and B. The altered ratios of peak C to A and B and peptide analysis suggest that this is occurring, but the apparent size heterogeneity of all three major proteins makes precursor-product relationships difficult to establish with certainty.

Figure 11B shows that later in infection in MDCK cells (at 4 hr) a pulse label gives about the same pattern as a chase, even though amino acid analogues were added to the labeling medium in an effort to retard cleavage as with enteroviruses (9). Peak C, which is quite large after an early pulse, becomes extensively degraded and nearly eliminated during a 24-hr chase period (Fig. 12). Peak B has a slightly lower apparent molecular weight after this prolonged chase, and the material still remaining in peak C after the chase is broad and obviously heterogeneous in size. These data indicate that peak C is an unstable, high-molecular-weight protein or mixture of proteins, and its peptide homology with peaks A and B suggests that it contains a precursor of the latter, or is a mixture of precursors. However, better separation techniques and the use of more effective amino acid analogues for pulse-labeling may be necessary to prove the

FIG. 11. Amino acid pulse-chase early in infection. A, Influenza virus proteins pulse-labeled with ^3H for 10 min in BHK-21 cells infected 2.5 hr ($\circ$); influenza virus proteins in BHK-21 cells labeled with ^{14}C ($\times$) for 20 min at 2.5 hr after infection and then chased in an excess of cold amino acids for 4 additional hr. B, Same as A, except that MDCK cells were pulse-labeled beginning 4.0 hr after infection, and amino acid analogues were present during ^3H pulse-labeling.

FIG. 12. Pulse label early in infection with influenza virus, compared to a pulse label followed by a prolonged chase. MDCK cells were pulse-labeled for 10 min with ^3H - or ^{14}C -amino acids starting 3.5 hr after high-multiplicity infection with NWS strain. The ^3H -labeled proteins were immediately dissolved in electrophoresis sample buffer for analysis ($\times$). The ^{14}C -labeled cells were incubated in unlabeled Eagle medium at 37 C for an additional 20-hr chase period before they were dissolved in sample buffer and analyzed ($\circ$).

precursor-product relationships suggested here. It is not clear why it is so difficult to prove or disprove a precursor-product relationship with short pulse labels during myxovirus infection, but it might be due to a very high rate of cleavage of precursors. Even with the enteroviruses, in which cleavage is easily demonstrated by pulse-labeling, it is necessary to employ short pulses very early after infection, or the largest precursor proteins cannot be observed without analogues (14).

Aggregation controls. It is essential to demonstrate that the electrophoretic separations employed here are not permitting aggregation to occur and generate artifactual peaks. For example, if peak C were merely an aggregate of peak A and B material, all of the results here would be due to an artifact. Therefore, the possibility of reversible aggregation has been carefully

examined. Figures 13A and B show that reversible aggregation is not occurring. When peaks A and B were each collected after preparative electrophoretic separation, heated at 70°C for 1 min in pH 9.0 buffer, dialyzed against sample buffer, and electrophoresed on a second gel column, they again appeared almost exclusively at the original molecular weight position in the gel. The same results were obtained with peak C. There was no evidence of aggregation even when small amounts of labeled, electrophoretically purified peak A protein were mixed with several milligrams of purified unlabeled influenza virus and dialyzed before being run on gels. All three peaks (A, B, C) from labeled virus have been separated by sizing on a Sepharose 4B column using a buffer containing 6 M guanidine, 0.01 M ethylenediaminetetraacetic acid, 0.1 M Cleland reagent. Again, when each peak was dialyzed against sample buffer and analyzed by gel electrophoresis, it migrated mainly as a single peak at the correct molecular weight position. Finally, dissolution of the labeled viral proteins at 70°C in phenol-acetic acid-water (1:1:1), a powerful lipoprotein-solubilizing mixture, and dialysis against sample buffer again showed the same three peak proteins upon gel electrophoresis. We conclude that aggregation is not a problem with these proteins in this pH 9.0 sodium dodecyl sulfate-gel system, although minor amounts of aggregation can never be ruled out.

DISCUSSION

It was obviously important to determine how the RNA genome of influenza virus is related to the proteins it encodes. The protein subunit pattern demonstrated here is unexpectedly simple, particularly so since the peptide analyses show peptide homologies among the three major peaks. Very early in infection (Fig. 8B, 8C), a large heterogeneous peak of protein of about 100,000 molecular weight is seen in the area later occupied by peak C. This could be a precursor protein representing the entire genetic information of influenza virus. If so, the largest fragments of virion RNA seen in Fig. 5A and B are more than sufficient to code for all virus protein synthesis, since 10^6 daltons of RNA could direct synthesis of a 100,000-dalton precursor protein. However, the presence of several precursor proteins or of minor peaks of protein coded by monocistronic messenger cannot be ruled out. In fact, the apparent increase in relative rate of synthesis of peak B as compared to peaks A and C late in infection (Fig. 8D, 8E, 8F) is hard to explain if there is only one precursor. However, the stoichiometric relationships be-

FIG. 13. Evidence that reversible aggregation of influenza virus protein subunits is not causing artifactual peaks. Individual preparations of each major viral peak were obtained by preparative gel electrophoresis in pH 9.0 sample buffer with 0.1% sodium dodecyl sulfate. The purified samples of each peak were then dialyzed against sample buffer and run on analytical gel columns to detect reaggregate or disaggregate tendencies. A, Reelectrophoresis of peak A protein labeled with ^3H -amino acids (O), coelectrophoresed with ^{14}C -labeled ovalbumin (molecular weight 45,000) and ^{14}C -labeled bovine serum albumin (molecular weight 67,000) as internal markers (X). B, Reelectrophoresis of peak B protein labeled with ^3H -amino acids. No internal marker proteins.

tween these peaks constantly changes with increasing time after infection; this could be due to increasing rates of cleavage of proteins, or to decreasing size of a polycistronic messenger, or both, as infection progresses. The A₂ Asian influenza virion has a great predominance of peak C over the other two peaks (Fig. 6C). This has been observed in all preparations of virions from this strain, thus its cleavage rates must be lower than for other influenza virus strains. We are attempting to purify influenza virus neuraminidase, replicase, and hemagglutinin and to determine their subunit size. The size of the nucleoprotein subunit is clearly about 62,000 daltons in the undigested state (Fig. 6B). An accurate assignment of subunit sizes for these other proteins would be very useful in understanding this virus and its replication.

The size relationships and peptide overlaps of the three major protein peaks are difficult to interpret if each is a discrete protein species. Peak A protein and peak C protein are very similar in peptide patterns and both share the peptides of peak B in addition to others, but it is not clear how peak C can be cleaved to give peak A and peak B, particularly since all of these show size heterogeneity. Perhaps there are several primary gene products which undergo cleavage, or one precursor protein may undergo ambiguous cleavages at alternative sites of cleavage. Better protein separation should help to resolve this. Another possible explanation for the size heterogeneity and peptide homologies reported here could be the presence of overlapping messenger RNA molecules of different lengths leading to polypeptide sequence overlap in polypeptides of varying size. Both mechanisms could be operating to give the above results.

Whatever else is found regarding the relationship between RNA and protein, the data in Fig. 5 indicate that there is no clear distinct size grouping for the RNA pieces of the virions. The size of each "piece" seems to be variable for different strains, and heterogeneity occurs in each size class for a given strain. It is likely that the sizes of virion RNA are somewhat fortuitous. It is quite possible that the small pieces of RNA are artifacts. Figure 10F suggests that the total amount of polypeptide encoded by influenza virus is not very great, so that one large molecule of RNA might be capable of encoding all the protein of influenza virus. However, much more work will be necessary to determine whether only one or several pieces of influenza virion RNA are required for infection, and to clarify the relationship between viral RNA and protein.

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