Time Course of Synthesis and Assembly of Influenza Virus Proteins

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The synthesis of viral polypeptides was analyzed in BHK-21-F cells infected with the WSN strain of influenza virus at various times in the growth cycle. The relative amounts of polypeptides P, HA, NP, and NS did not change markedly between early and late times in the growth cycle; however, there was a progressive increase in the relative amount of the M polypeptide at later time points. In cell fractionation experiments, the patterns of newly synthesized polypeptides associated with various cytoplasmic fractions remained similar throughout the growth cycle except for an increase in polypeptide M in all fractions late in the growth cycle. The HA polypeptide was chased out of cytoplasmic membranes completely 6 h after synthesis, whereas the M polypeptide was not chased effectively from such membranes. Marked differences were found in the incorporation into mature virions of polypeptides synthesized at different times in the growth cycle. Polypeptides P and NP synthesized at early times were incorporated preferentially, whereas M was synthesized and incorporated predominantly late in the growth cycle. The fact that the rates of incorporation of polypeptides into virions differed significantly from their rates of synthesis indicates that different polypeptides were assembled into virions by distinct pathways.

It has been established that influenza virus particles contain seven or eight different species of polypeptides (8, 10, 13, 22, 26). The detailed arrangement of most of these polypeptides in the virion has also now been established and the available information summarized in several recent reviews (12, 18, 22a; R. W. Compans and P. W. Choppin, *In H. Fraenkel-Conrat and R. R. Wagner, ed., Comprehensive Virology*, in press). In addition to the structural viral polypeptides, one or two virus-coded nonstructural polypeptides can be found in infected cells (13, 15, 17, 24). Distinct patterns of association of viral polypeptides with different cell fractions have been observed, and evidence has been obtained that there is a control mechanism which determines the amount of individual viral polypeptides synthesized (6, 7, 14, 15, 17, 27). In addition, some evidence has also been obtained for differences in relative rates of synthesis of individual polypeptides at different times in the growth cycle (25), and there is preferential incorporation into influenza B virions of one polypeptide species at early times and another at late times in the growth cycle (P. W. Chop-

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In this paper we describe the viral polypeptides synthesized in BHK-21-F cells infected with the WSN strain of influenza virus at various times during the growth cycle. The association of these polypeptides with cytoplasmic fractions is analyzed at late times during the growth cycle and after long chase periods. The incorporation into mature virus particles of polypeptides synthesized at different times during the viral replication cycle has also been determined.

MATERIALS AND METHODS

Virus and cells. The WSN strain of influenza virus was used as inoculum in all experiments. Virus stocks were grown in MDBK cells as described by Choppin (4). Radioactively labeled virus was grown and purified as described previously (18, 16), using precipitation with polyethylene glycol followed by banding in a potassium tartrate gradient. The BHK-21-F line of baby hamster kidney cells and the MDBK line of bovine kidney cells were grown according to described procedures (4, 11).

Radioactive labeling of infected cells. Confluent monolayers of cells were inoculated at a multiplicity

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of 20 to 50 PFU/cell. After a 2-h adsorption period at 37°C, the inoculum was removed and replaced with reinforced Eagle medium (REM) (1) with 2% calf serum. This medium was removed 10 min before labeling and was replaced with REM with 2% calf serum but without leucine. The medium for labeling, containing [4,5-³H]leucine, consisted of one part REM and three parts REM lacking leucine, with 2% calf serum. After the labeling period, the cells were washed twice with warm REM with 2% calf serum, harvested in 0.25 ml of 1% sodium dodecyl sulfate (SDS) in 0.005 M phosphate buffer, immediately boiled for 1 min, and stored frozen. For pulse-chase experiments, the label was removed. The cells were washed twice, incubated further in REM with 2% calf serum, and harvested as described above. Exact conditions of labeling for each experiment are indicated in figure legends.

Cell fractionation. The procedure of Caligiuri and Tamm (3) was used to fractionate cytoplasmic extracts in a discontinuous sucrose gradient into six fractions which are numbered 2 through 7. Fractions 2 and 3 contain smooth cytoplasmic membranes; fraction 4 contains membranes of intermediate density; 5 and 6 contain rough microsomal membranes; and fractions 6 and 7 contain free polysomes (3, 6). After collection, fractions 2 plus 3, 4, and 5 plus 6 were diluted to 35 ml with reticulocyte standard buffer and pelleted at 23,000 rpm for 5 h in a Spinco SW27 rotor. Pellets were resuspended in 0.2 ml of 1% SDS in 0.005 M sodium phosphate, pH 7.2, and portions were used for polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Samples in 1% SDS and 1% β -mercaptoethanol in 0.005 M sodium phosphate, pH 7.2, were boiled for 1 min at 100°C (20). Ten percent acrylamide, 0.27% *N,N'*-methylenebisacrylamide gels containing SDS, were used in all experiments and prepared as described by Caligiuri et al. (2). The slicing and processing of gels for the determination of radioactivity by liquid scintillation was done as described previously (7).

Protein determination. The method of Lowry et al. (19) was used. Bovine serum albumin served as a standard.

Chemicals and isotopes. [4,5-³H]Leucine and ¹⁴C-labeled reconstituted protein hydrolysate were obtained from Schwarz-Mann, Orangeburg, N. Y.; polyethylene glycol was obtained from Amend Drug and Chemical Corp.

RESULTS

Synthesis of viral polypeptides in infected cells. Previous studies have indicated that the time of inhibition of cellular protein synthesis by influenza virus, and the time that synthesis of virus-specific polypeptides becomes clearly detectable, varies with the cell type (17, 24, 30). In addition to structural polypeptides, a virus-specific, nonstructural polypeptide designated NS has been found which migrates slightly faster than the smallest structural polypeptide M (17). A second nonstructural polypeptide

with a molecular weight of 11,000 has been observed in chicken embryo fibroblasts infected with fowl plague virus (24). To detect changes in the relative amounts of viral polypeptides synthesized during the growth cycle, a series of WSN-infected BHK-21-F cells were pulse-labeled for 15 min with [³H]leucine (Fig. 1). At 2 h after infection (Fig. 1A), the background of cellular protein synthesis was still high, particularly in the low-molecular-weight region, and newly synthesized virus-specific polypeptides could not be clearly identified. A small peak of the ribonucleoprotein subunit NP may be discernible above background. However, by 2.5 h postinfection (Fig. 1B), the background of host cell proteins was markedly reduced, and the viral polypeptides P, HA, NP, and NS could be clearly identified. At later times, 3.5 h (Fig. 1C), 7 h (Fig. 1D), and 10 h (Fig. 1E), after infection, host-cell protein synthesis had almost completely subsided, and the viral polypeptides were clearly resolved. At these times, we could resolve all structural polypeptides with the possible exception of the neuraminidase polypeptide NA, which may be obscured by the large peak of NP. In addition, the nonstructural polypeptide NS was detected at all times and was clearly resolved from the structural M polypeptide. The relative amounts of most polypeptides synthesized at different times were fairly constant, although a progressive increase of M could be observed at later times. The relative amounts of polypeptides, compared with NS = 1.0, at 3.5 h were: HA, 0.66; NP, 1.41; and M, 0.38. At 7 h they were: HA, 0.60; NP, 1.42; and M, 0.67. At 10 h they were: HA, 0.63; NP, 1.16; and M, 0.78. In another experiment we also resolved M and NS at 2.5 h (ratio of M to NS was 0.28); thus at 2.5 or 3.5 h, we were able to resolve all polypeptides which were detected at later times in the growth cycle.

Pulse-chase experiments in whole cells. On the basis of different electrophoretic mobilities in polyacrylamide gels, the nonstructural polypeptide NS and the viral membrane protein M appeared as distinct polypeptides, and they have also been shown to be distinct by peptide mapping (17). The NS polypeptide has also been demonstrated in nuclei (15, 17) and polysomes (6, 21) of infected cells. Gregoriades (9) recently reported that a polypeptide that is present in the nuclei and polysomes of infected cells can be extracted with acidic chloroform-methanol, and it was shown to possess tryptic peptides very similar to the M polypeptide which had been extracted from virions by the same procedure. This raised the possibility that the lower-molecular-weight polypeptide NS

FIG. 1. Polyacrylamide gel electrophoresis of polypeptides synthesized in BHK-21-F cells infected with the WSN strain of influenza virus. At 2 h (A), 2.5 h (B), 3.5 h (C), 7 h (D), and 10 h (E) after infection, the cells were labeled for 15 min with medium containing 20 μ Ci of [4,5- 3 H] leucine per ml. After removal of the label, the cells were washed with REM plus 2% calf serum, and 0.25 ml of 1% SDS in 0.005 M phosphate buffer was added. The cells were scraped off the plate, immediately boiled for 1 min, and stored frozen. 14 C-amino acid-labeled marker virus was included. Migration in these and all subsequent gels is from left to right. The positions of the viral polypeptides are indicated by arrows in pattern B, and designations of viral polypeptides are those proposed at Influenza Workshop I (12).

might be converted into the membrane protein M by post-translational modification. Short chase periods showed no evidence for such a conversion (17). To test whether such an inter-conversion could be demonstrated in longer time intervals, we labeled infected BHK-21-F cells with [3 H]leucine for 15 min at 4 h after infection. One sample was prepared for polyacrylamide gel electrophoresis directly after the pulse, and others were chased for 6 h with REM, with 10 μ g of cycloheximide per ml added to one lot of cells. After the pulse (Fig. 2A), all viral polypeptides were resolved with the exception of the cleavage products of the hemagglutinin polypeptide HA. Under the chase conditions, HA was cleaved into HA₁ and HA₂, but otherwise, the patterns were nearly identical. In particular, the relative amounts of polypeptides M and NS remained unaltered. These results indicate that no precursor-product relationship existed between polypeptides NS and M.

Association of viral polypeptides with cytoplasmic fractions. Previous studies on subcel-

lular fractions of influenza virus-infected cells have established that the virus-specific polypeptides associate specifically with cellular components (6, 7, 14, 15, 17, 27, 28, 29). The polypeptides of the viral envelope were found mainly in the smooth membrane structures, the nucleoprotein subunit NP was found in a fraction of intermediate density, and the rough membranes and polysomes contained predominantly the nonstructural polypeptide NS. To further analyze the process of assembly, we wished to determine the distribution of viral polypeptides in cytoplasmic fractions after long chase periods and also at late times during infection. The polypeptides associated with cytoplasmic fractions derived from infected BHK-21-F cells, pulsed for 15 min at 4 h after infection, were compared to polypeptides in fractions from cells in which the pulse was followed by a 6-h chase period (Fig. 3). After the pulse, a large peak of the uncleaved hemagglutinin polypeptide HA was present in the combined smooth membrane containing fractions 2

FIG. 2. Polyacrylamide gel electrophoresis of polypeptides from BHK-21-F cells infected with influenza virus. The cells were labeled for 15 min with $[4,5\text{-}^3\text{H}]\text{leucine}$ ($20\text{ }\mu\text{Ci/ml}$) at 4 h after infection. (A) The cells were prepared for electrophoresis directly after the pulse; (B) the cells were chased with leucine-containing medium for 6 h; (C) $10\text{ }\mu\text{g}$ of cycloheximide per ml was added during a similar 6-h chase. WSN virion polypeptides with ^{14}C -labeled amino acids are present as markers.

and 3 (Fig. 3A), and peaks of HA were also present in the intermediate and the rough membrane fractions (Fig. 3B and C). In contrast, cell fractions were essentially devoid of the HA polypeptide after the 6-h chase period (Fig. 3D-F). The small amounts of radioactivity in the region of HA_1 and HA_2 further indicated that the disappearance of HA was not explained merely by cleavage into HA_1 and HA_2 . This supports the conclusion that the HA polypeptides associated with cytoplasmic membranes may be precursors of virion polypeptides.

In contrast, the nonglycosylated envelope protein M did not appear to follow the same pathway. This polypeptide was not chased efficiently from smooth membranes, and a major peak was also found in the intermediate fraction (Fig. 3E) after the 6-h chase. There was an increase in the NP polypeptide in the smooth membrane fraction after the long chase, but there were otherwise no marked changes in the distribution of NP or NS.

We also determined the distribution in cell fractions of newly synthesized polypeptides at late time points during the growth cycle. Cytoplasmic fractions from BHK-21-F cells pulse-labeled for 15 min at 7 or 10 h postinfection were analyzed (Fig. 4). The polypeptides in the smooth membrane and intermediate fractions were similar at the two time points and resembled the fractions obtained 4 h after infection (Fig. 3A-C). The rough membrane and poly-

some-containing fractions also showed polypeptide NS as the major labeled component at 7 and 10 h after infection (Fig. 4C and F). It is apparent, however, that increasing amounts of polypeptide M were present in these fractions at the later times. The fact that this polypeptide was synthesized in increased amounts at late times in the growth cycle therefore appears to be reflected in its presence in all membrane fractions, suggesting that this polypeptide may possess affinity for various intracellular membrane structures.

Incorporation into virions of polypeptides synthesized during different periods of the growth cycle. To study the incorporation into virions of structural polypeptides synthesized at different times in the growth cycle, infected MDBK cells were pulsed with $[^3\text{H}]\text{leucine}$ for 1 h at different times. The cells were then chased with cold maintenance medium, and virus was harvested at 24 h after infection, purified, and prepared for polyacrylamide gel electrophoresis as described. The distribution of the polypeptides of virions grown in MDBK cells labeled at 3 to 4 h, 5 to 6 h, 7 to 8 h, 9 to 10 h, and 11 to 12 h is shown in Fig. 5A-E, respectively. As can be seen, all structural polypeptides contained in influenza virions were resolved at all time points. However, significant differences in the relative amounts of the individual polypeptides are evident if the distribution in the five gel patterns is compared. The highest molecular

weight polypeptide P was presented as a prominent peak in virus preparations derived from cells that were labeled from 3 to 4 h after infection. At later labeling periods, the relative amount of this polypeptide incorporated into virions decreased sharply, until it reached the limit of detectability at the latest time point tested. Another striking change in incorporation occurred with the membrane polypeptide M. Although already present as a large peak in the virus labeled from 3 to 4 h, a marked increase in relative amount was observed at subsequent time points. Similar experiments were performed with BHK-21-F cells, and essentially the same changes were observed in virion polypep-

tide distribution between early and late labeling periods, demonstrating that this pattern of incorporation is a general phenomenon and not peculiar to one host cell.

Quantitative measurements of the percentage of label in the different patterns are summarized in Table 1. The glycoproteins HA, NA, HA₁, and HA₂ were grouped as one class, as it is difficult to resolve them completely. A difference of over 10-fold can be seen in both cell lines for the percentage of polypeptide P present under different labeling conditions. The percentage of the ribonucleoprotein subunit NP also decreased at later labeling periods, but to a lesser extent. In contrast, the percentage of

FIG. 3. Polyacrylamide gel electrophoresis of polypeptides from WSN-infected BHK-21-F cell fractions obtained after labeling for 15 min with $[4,5-^3\text{H}]\text{leucine}$ ($10 \mu\text{Ci/ml}$) at 4 h after infection (O----O). The solid line indicates the polypeptides of marker virus labeled with ^{14}C -amino acids marker virus. (A, B, and C) Distribution after the pulse; (D, E, and F) corresponding pattern after a 6-h chase with leucine-containing medium. (A and D) Combined smooth membrane fractions 2 and 3; (B and E) fraction 4; (C and F) fractions 5 and 6.

FIG. 4. Polyacrylamide gel electrophoresis of polypeptides from WSN-infected BHK-21-F cell fractions after a 15-min pulse of $[4,5\text{-}^3\text{H}]\text{leucine}$ ($10\text{ }\mu\text{Ci/ml}$) at 7 h (A, B, C) and 10 h (D, E, F). WSN virion proteins labeled with ^{14}C -amino acids were included as markers (not shown), and the positions of the viral polypeptides are indicated. (A and D) Fractions 2 and 3; (B and E) fraction 4; (C and F) fractions 5 and 6.

radioactivity in the membrane protein M was significantly higher if the infected cells were labeled later in the growth cycle. The percentage of label in the viral glycoproteins remained relatively constant during the various labeling periods.

Rate of incorporation of polypeptides into virions. To compare rates of synthesis and incorporation of the individual structural polypeptides into virions, the amount of total protein applied to each polyacrylamide gel was standardized by protein determination. The relative rates of incorporation in MDBK cells are shown in Fig. 6. The incorporation of polypeptide P rose most rapidly, reaching a maximum when the cells were labeled from 7 to 8 h

after infection, and declined at the later time points. The nucleoprotein subunit NP and the glycoproteins were incorporated at an intermediate rate, with maximal incorporation occurring at 9 to 10 h. The membrane polypeptide M on the other hand showed progressively increasing incorporation, reaching maximum at the latest labeling period tested. These results indicate marked differences in the rates of incorporation into virions of the three nonglycosylated viral polypeptides synthesized during different intervals in the growth cycle.

DISCUSSION

The present results indicate that the relative rates of synthesis of influenza viral polypeptides in BHK-21-F cells were similar at different times in the replicative cycle, with the exception of the M polypeptide which was synthesized in increasing amounts as the infection progressed. Since the overall rate of protein synthesis in BHK-21-F cells is unchanged up to 11 h postinfection (17), these results further indicate that the absolute rates of synthesis of individual viral polypeptides, with the exception of M, do not vary markedly from 3.5 to 10 h postinfection. A progressive increase in the rate of synthesis of polypeptide M, relative to other viral polypeptides, was also observed by Skehel (24) in fowl plague virus-infected chicken embryo fibroblasts; however, other features described for that system differ from the present results. There were no qualitative differences between polypeptide species detected early and late in the replicative cycle in the present system, whereas only polypeptide P, NP, and NS were detected in fowl plague virus-infected chicken fibroblasts until 3 h postinfection. In addition, the rate of synthesis of NS declined rapidly at later times in fowl plague virus-infected chicken fibroblasts, but no such decline was observed in the present system. The lack of correspondence in these observations indicates that aspects of control of polypeptide synthesis observed in a particular virus-cell system may not be essential features of influenza virus replication in general.

Previous studies have provided evidence for association of influenza viral polypeptides with distinct cytoplasmic membrane fractions (6, 7, 14, 27). The present results indicate that the association of envelope polypeptides with smooth membranes, NP with cytoplasmic fraction 4 and NS with polysome-containing fractions, is not altered at later times in the growth cycle. The fact that the HA glycoprotein can be chased completely from cytoplasmic fractions

FIG. 5. Polyacrylamide gel electrophoresis of polypeptides of WSN virions grown in MDBK cells and labeled with $[4,5\text{-}^3\text{H}]\text{leucine}$ ($10\text{ }\mu\text{Ci/ml}$) from 3 to 4 h (A), 5 to 6 h (B), 7 to 8 h (C), 9 to 10 h (D), and 11 to 12 h (E) after infection. Following the labeling period, the cells were washed and incubated further in unlabeled medium until virus was harvested and purified at 24 h after infection.

TABLE 1. Quantitative differences in incorporation into virions of polypeptides synthesized during different intervals postinfection

Cell type	Labeling interval (h) ^a	Label in polypeptide (%)			
		P	NP	Glyco-proteins	M
MDBK	3-4	6.7	31.0	27.1	35.0
	5-6	3.1	29.7	29.3	37.7
	7-8	1.1	18.9	27.2	52.6
	9-10	0.6	11.8	28.9	58.5
	11-12	0.4	10.8	22.6	66.0
BHK	2-4	5.6	32.6	20.4	41.2
	4-6	2.0	21.2	23.5	51.1
	6-8	1.1	19.4	18.8	60.5
	8-15	0.5	14.4	17.2	67.8
WSN marker virus ^b		2.5	24.1	31.3	42.1

^a Infected cells were labeled with $[^3\text{H}]\text{leucine}$ ($10\text{ }\mu\text{Ci/ml}$), and virions were purified and analyzed as in Fig. 5.

^b Virus was grown in MDBK cells and labeled from 2 h until harvest (8).

provides further support for the suggestion that these glycoproteins in intracellular membranes are precursors of the components in plasma membranes and viral envelopes. On the other hand, the failure to chase the M polypeptide from cytoplasmic membranes in a 6-h period,

and the previous evidence that M is associated with plasma membranes rapidly after synthesis (17), suggests that M is incorporated into plasma membranes by a different pathway, possibly being inserted into the plasma membrane directly after cytoplasmic synthesis. The failure to effectively chase the NP polypeptide from cytoplasmic fractions in 6 h also reflects the fact that this component was synthesized in amounts far in excess of those incorporated into virions.

With the improved resolution of the M and NS polypeptides obtained in the present study, distinct peaks of M were detected in the cytoplasmic fractions which contain predominantly NS. This was particularly true at later time points, reflecting the higher level of synthesis of M at these times. Thus, the M protein appears to bind to various membrane fractions. Cohen et al. (5) have demonstrated that the vesicular stomatitis virus membrane protein adsorbs efficiently to membranes *in vitro*, raising the possibility that association of such proteins with membrane fractions may be artifacts of cell fractionation. If the finding of M associated with cytoplasmic membranes were due to adsorption from a pool following cell disruption, it is likely that this pool would be utilized for virus assembly during a 6-h chase. Thus, the present finding of a stable association of M with cytoplasmic membranes during a 6-h chase supports

FIG. 6. Rates of synthesis and incorporation into WSN virions of polypeptides P, NP, M, and combined glycoproteins (GP). Virions were grown in cells pulse-labeled for 1-h periods, purified, and analyzed by gel electrophoresis, as described for Fig. 5. The total protein applied to each gel was determined, and the total radioactivity in each peak was calculated and normalized to equivalent amounts of protein. The values plotted represent the mean values of three experiments.

the conclusion that M polypeptides are associated with membranes *in vivo*.

Marked differences are found in the kinetics of incorporation of viral polypeptides into mature virus particles. P and NP synthesized early in the growth cycle are incorporated preferentially into virions, compared with M which is synthesized and incorporated maximally at late times. In a previous study using the WSN strain (23), no differences were detected in the polypeptide patterns of virions labeled at different time intervals in the growth cycle. However, cells were infected at low multiplicity and infection was therefore not synchronous. In agreement with the present findings, there is preferential incorporation into influenza B virions of NP synthesized at early times and M synthesized at late times in the growth cycle (P. W. Choppin, S. G. Lazarowitz, and A. R. Goldberg, *In R. D. Barry and B. W. J. Mahy, ed., Negative Strand Viruses*, in press.). The marked differences in incorporation into virions of polypeptides synthesized at various times in the growth cycle which have been observed in the present system, which differ from the rates of polypeptide synthesis, indicate that processes other than polypeptide synthesis control the rate at which each polypeptide species is assembled into virions.

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