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Early Synthesis of Semliki Forest Virus-Specific Proteins in Infected Chicken Cells

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Cells preinfected with fowl plague virus followed by treatment with actinomycin D are a suitable system for studying early protein synthesis in cells infected with Semliki forest virus. One and one-half hours after superinfection, three new nonstructural proteins (NVP) were detected: NVP 145, NVP 112, and NVP 65. They appeared in parallel with a low incorporation of mannose at the beginning of the infectious cycle. Behavior on chasing suggested a precursor relationship of NVP 112 to the envelope glycoproteins. Two kinds of NVP 65 are described, both of which are varieties of NVP 68 with an incomplete mannose content. One type, detected early after infection, was converted into NVP 68 by supplementary glycosylation. The second, late type was stable. It contains fucose and resembles the NVP 65 observed after impairment of glycosylation. The mechanism of NVP 68 glycosylation is discussed. The presence of the complete carbohydrate moiety is crucial for the cleavage of NVP 68 into the envelope proteins E_2 and E_3 and, thus, for virus maturation. Only the complete form of NVP 68 was precipitated by envelope-specific antisera. A large production of NVP 78 is a further feature of the early events in infected cells. It is not related to the structural proteins.

The structural proteins of Semliki forest virus (SFV) are very probably translated polycistronically from a messenger that has been shown to be a 26*S* RNA found on the polysomes of infected cells (24). This mRNA encodes for one nonstructural protein with a molecular weight of about 130,000 (NVP 130) containing the polypeptides of all four structural proteins: the core protein C and the envelope glycoproteins E_1, E_2, and E_3 (10, 11). Its molecular weight equals the sum of the molecular weights of the viral structural proteins in their nonglycosylated form (2). These proteins are derived from the primary gene product by a sequence of events, including proteolytic cleavage and glycosylation (14). In this process a series of precursors intermediate in size are formed, nearly all of which are short lived and can be detected only under appropriate conditions, in which cleavage is retarded. One of them, NVP 68, is normally found in relatively large amounts. It has been shown to be the precursor of E_2 and E_3 (21).

Besides the structural proteins and the precursor NVP 68, a second precursor of the envelope proteins, NVP 97, has been detected in infected cells (11, 14). Furthermore, a protein, NVP 78, which appears in relatively large amounts at the beginning of the infectious cycle (7), and two larger proteins of presumably viral origin, NVP 165 and NVP 105, which could be demonstrated after inhibition of proteolytic activity (14), have been described.

It is not known how far NVPs 78, 105, and/or 165 could be encoded by the second 42*S* mRNA, which has been observed in smaller amounts on the polysomes (25). The 42*S* mRNA of the related Sindbis virus has been shown to contain a second ribosomal binding site that is not identical with that of the 26*S* mRNA (1), suggesting the possibility that some information may be translated from this larger mRNA.

Analyzing SFV-specific proteins synthesized early after infection, we observed new precursor proteins (7), which from their properties can now be integrated among the already known ones. It will be shown that correct glycosylation of the glycoprotein precursors is an important condition for their cleavage.

MATERIALS AND METHODS

Virus strains and tissue culture. Primary chicken embryo (CE) cells, 22 h after seeding, were used throughout. SFV strain Osterrieth (15) was investigated. For preinfection of cells, fowl plague virus (FPV), strain Rostock, was used.

Virus multiplication. Cultures on plastic petri dishes, 5 or 9 cm in diameter, were infected at a multiplicity of 10 to 50 PFU/cell at 37 C. Time of addition of the inoculum is considered as zero time. Cells were maintained in Earle basal salts solution

supplemented with 11 mM glucose and 2 mM glutamine (modified Earle basal salts solution). For multiplication of SFV, the culture medium also contained 0.2 μg of actinomycin D per ml.

Pretreatment of cells with FPV and actinomycin D (7). For suppresion of host-specific protein synthesis, CE cells were infected with FPV in modified Earle basal salts solution. Exactly 1 h and 15 min postinfection (p.i.), actinomycin D was added at a concentration of 2 μg/ml. At 4.5 h p.i. the pretreated cells were superinfected with SFV, as described above.

Radioactive labeling. The culture medium for all labeling experiments contained 0.02 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid instead of sodium bicarbonate. [^{14}C]protein hydrolysate or a mixture of tritiated amino acids consisting of equal amounts in radioactivity of [^{3}H]leucine, [^{3}H]lysine, and [^{3}H]tyrosine was used. Details are given in the figure legends.

Inhibition of proteolytic cleavage by incorporation of amino acid analogues. A mixture of amino acid analogues, according to Jacobson and Baltimore (4), was used. The L-acetidine-2-carboxylic acid, canavanine, DL-ethionine, and DL-*p*-fluorophenylalanine used are analogues for proline, arginine, methionine, and phenylalanine, respectively, and should not interfere with the tritiated amino acids used.

PAGE and estimation of molecular weights. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate was described earlier (6, 7) and, in principle, is according to Lämmli (12). Migration in all patterns is from left to right. Values of apparent molecular weights were estimated by the method of Shapiro et al. (20), using 8.75% gels. For extrapolations the molecular weight values used were: SFV-specific core protein C, 32,000; envelope glycoproteins E_1 plus E_2, 50,000; NVP 68, 68,000; NVP 94, 94,000. The values agree well with the nonlabeled standards used: chymotrypsinogen A (Boehringer, Mannheim), 25,000; ovalbumin (Boehringer), 45,000; bovine serum albumin (Boehringer), 68,000; and phosphorylase a (Sigma), 95,000. The values obtained for NVP 68 seem to be too high, due to its high carbohydrate content (16, 23). Deviations are also possible in the molecular-weight range exceeding 100,000. Nonstructural proteins are abbreviated as NVP.

Trichloroacetic acid-insoluble radioactivity. Estimations of trichloroacetic acid-insoluble radioactivity were made according to known procedures (17).

Antisera. An antiserum specific for the envelope glycoproteins of SFV was prepared. Purified virus preparations, grown in BHK-21 cells, were emulsified with an equal volume of Freund complete adjuvant and injected subcutaneously in rabbits. After two boosters in incomplete adjuvant, a potent serum was obtained which, however, also precipitated some core protein. The antiserum was therefore reacted overnight with a preparation of purified viral cores, obtained by the method of Kääriäinen et al. (5). The resulting precipitate was removed by centrifugation for 30 min at 100,000 × *g*.

Indirect radioimmune precipitation. The procedure for indirect radioimmune precipitation has been described recently (7), as have the conditions under which the correct concentrations of the SFV-specific antiserum and that of the anti-rabbit serum are determined, to obtain optimal precipitation. Cells from one 5-cm petri dish were suspended by two consecutive washings at 4 C with 1 ml of a lysis buffer consisting of 0.02 M Tris-hydrochloride, pH 7.5, *N*-tosyl-L-phenylalanylchloromethane (Merck, Darmstadt), 0.2 mM *N*-tosyl-L-lysyl-chloromethane hydrochloride (Merck), and 0.2 mM phenylmethyl sulfonyl fluoride (Serva, Heidelberg). The lysate was centrifuged for 10 min at 10,000 × *g* at 4 C, made 0.5% in sodium deoxycholate, and spun once more for 50 min at 100,000 × *g*. Aliquots (200 μl) in siliconized plastic tubes were reacted with 5 μl of the SVF-specific antiserum overnight at 4 C and then for 4 h with an anti-rabbit immunoglobulin G serum from sheep. The precipitate was washed three times with lysis buffer and dissolved in 25 μl of sample buffer (12) for PAGE.

Materials. Actinomycin D, *N,N,N',N'*-tetramethylenediamine, *N,N'*-methylenebisacrylamide, and sodium dodecyl sulfate were purchased from Serva, Heidelberg, Germany. Nonidet P-40 was a generous gift from the Shell Co., Frankfurt, Germany. L-Acetidine-2-carboxylic acid, DL-ethionine, canavanine, and DL-*p*-fluorophenylalanine were bought from Calbiochem, Buchs, Switzerland. L-[4,5-^{3}H]leucine (38 Ci/mmol), L-[4,5-^{3}H]lysine hydrochloride (13 Ci/mmol) L-[3,5-^{3}H]tyrosine (41 Ci/mmol), [5-^{3}H]uridine (29 Ci/mmol), D-[2-^{3}H]mannose (2 Ci/mmol), and [U-^{14}C]protein hydrolysate (58 Ci/matom) were obtained from the Radiochemical Centre, Amersham, England. All remaining chemicals, reagent grade, were bought from Merck, Darmstadt, Germany.

RESULTS

Kinetics of the early protein synthesis in SFV-infected CE cells preinfected with FPV and treated with actinomycin D. We have shown recently that the host-specific protein synthesis of CE cells can be suppressed by infection with FPV and subsequent inhibition of FPV-specific protein synthesis with actinomycin D. Such pretreated cells can be superinfected with SFV, and it is possible to follow SFV-specific protein synthesis by PAGE already at 1 h p.i. (7). The observed protein profile differs from that of non-pretreated cells labeled about 4 h p.i. in the appearance of several nonstructural proteins (cf. Fig. 4). These are NVP 145, NVP 130, NVP 112, NVP 105, and NVP 65. Furthermore, NVP 78 is pronounced, whereas NVP 97 is missing and NVP 68 is diminished at the cost of NVP 65, as will be shown later. The proteins NVP 145, 112, and 65 have not been described yet by other investigators.

These observed differences become smaller as virus replication proceeds (Fig. 1), with the

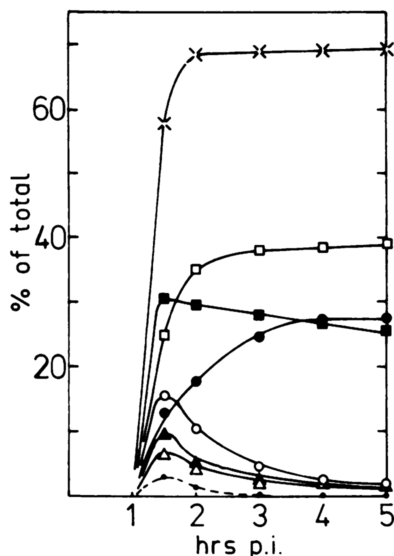

FIG. 1. *Relative distribution of viral polypeptides during the first hours of infection with SFV. (The graph is in place of a series of PAGE patterns and suffers from some difficulties in correction for background activities [cf. Fig. 4]. The values are therefore approximate.) At the times after superinfection with SFV indicated on the abscissa, cells pretreated with FPV and actinomycin D were pulse labeled for 15 min with 25 μCi of tritiated amino acids per 1.5 ml of modified Earle basal salts solution in parallel 5-cm petri dishes. Cells were then processed immediately for PAGE. Aliquots (25 μl) from identical sample volumes (150 μl) were run on 8.75% polyacrylamide gels, sliced, and counted. The areas of the individual peaks were estimated for the plotted polypeptides. From their sum (100%) the contributions of the particular polypeptides were calculated. Symbols: ○, NVP 78; ●, C; △, NVP 105; ▲, NVP 112; - · -, NVP 130; □, E_1 + E_2; ■, NVP 68 + NVP 65; X, sum of NVPs 130, 112, 68 and 65 and E_1 and E_2.*

relative amounts of the viral core protein C and the envelope glycoproteins E_1 plus E_2 increasing in a nearly constant relation. The sum of NVPs 68 and 65 reaches its highest value at the beginning of the infectious cycle and later declines slowly, but remains relatively high throughout the replication cycle. The amounts of all other nonstructural proteins are maximal at about 1.5 h p.i. and become barely detectable after 4 h p.i. The chasing behavior of these nonstructural proteins was studied next (Fig. 2). Radioactivity from the large polypeptides NVP 145, 130, 112, and 105 and from NVP 65 disappears if a 15-min pulse is followed by a 30-min chase. At the same time, radioactivity at the positions of NVP 68 and the envelope glycoproteins E_1 plus E_2 increases. This observation suggests a precursor relationship between at least one of the large NVPs and the envelope glycoproteins. Most likely, NVP 112 could be a precursor of all three envelope glycoproteins. Its molecular weight agrees well with the calculated value of 111,000. Since it disappears quickly on chasing, direct labeling with mannose was unsuccessful. At longer times of exposure, some incorporation of labeled fucose could be demonstrated, however (cf. Fig. 5B). NVP 97 is not detected early after infection. Since this was recently reported to contain the tryptic peptides of the envelope proteins E_1 and E_2 (11), it is possibly the precursor of these two proteins only, whereas NVP 112 would contain E_3 also. The core protein C is not affected by the chase conditions and serves as a suitable quantitative marker.

A slight increase in radioactivity during the first minutes of the chase is also observed at the position of NVP 78, which, however, like the core protein C, remains constant thereafter. The questions arise as to whether this protein is a precursor related to the structural proteins and whether there exists another precursor protein that would yield NVP 78 on cleavage.

NVP 78 has never been detected as a struc-

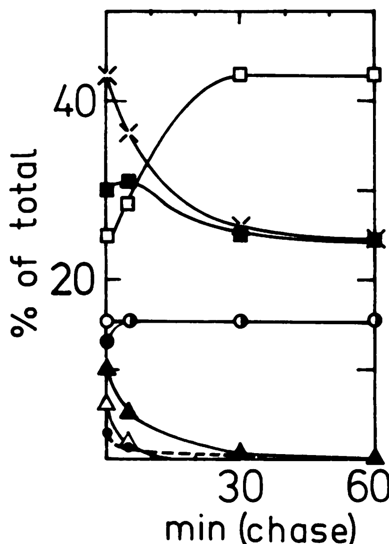

FIG. 2. *Relative distribution of viral polypeptides under chase conditions after a 15-min pulse 1.5 h after superinfection of FPV-pretreated cells with SFV. One and one-half hours after superinfection, cells on parallel dishes were pulse-labeled with tritiated amino acids as described in the legend to Fig. 1. Cells were either immediately processed for PAGE or were processed after a chase period, as indicated on the abscissa. Evaluation of patterns was as in Fig. 1. Symbols: ○, NVP 78; ●, C; △, NVP 105; ▲, NVP 112; – · –, NVP 130; □, E_1 + E_2; ■, NVP 68 + NVP 65; ×, NVP 130 + NVP 112 + NVP 68 + NVP 65.*

tural part of the virion (unpublished data). Its lysine content has been shown to be smaller than that of the core protein (14), and no evidence exists for a carbohydrate content (cf. Fig. 5). Thus, NVP 78 does not seem to be related to any of the structural proteins and rather, seems to be considered an early protein in infection that is stable on chasing.

When proteolytic cleavage was inhibited by the presence of amino acid analogues, the results in Fig. 3 were obtained. PAGE reveals an absolute lack of NVP 78, whereas the struc-

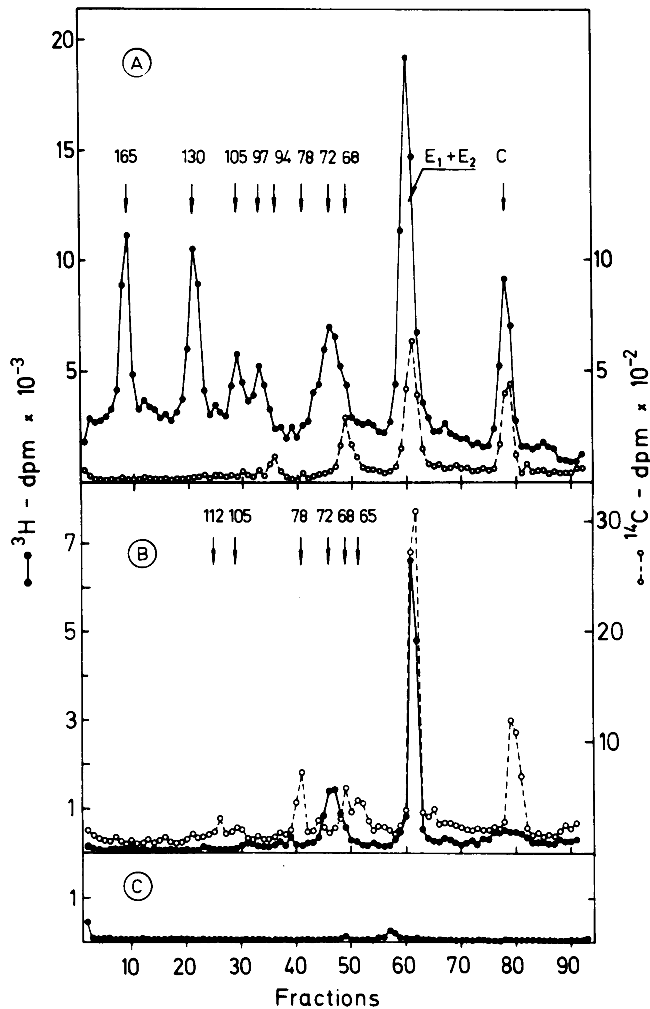

FIG. 3. *Profile of immune-precipitated, early SFV-specific proteins synthesized in the presence of amino acid analogues. Two parallel 5-cm petri dishes with CE cells pretreated with FPV and actinomycin D in modified Earle basal salts solution (BSS) were used. One and one-half hour after superinfection with SFV, the culture medium was changed to 1.5 ml/dish of modified Earle BSS containing the following amino acid analogues: 3.4 mM L-acetidine-2-carboxylic acid, 3.3 mM canavanine, 1.8 mM DL-ethionine, and 2.5 mM DL-p-fluorophenylalanine. Twenty minutes later a 10-min pulse with 50 μCi of tritiated amino acids per dish was started. Cells from one dish were immediately processed for PAGE and subjected to coelectrophoresis with a sample of SFV-infected CE cells labeled 4.5 to 5.5 h p.i. with 0.25 μCi of [^{14}C]protein hydrolysate. The gel was 8% in acrylamide (A). Cells from the second dish were extracted for radioimmune precipitation as described in the text. An aliquot was precipitated with an antiserum raised against the envelope glycoproteins only (B), and another aliquot was precipitated with the preimmune serum from the same rabbit as a control for nonspecific precipitation (C). The washed precipitates were dissolved in sample buffer (12) and run on parallel gels as above. For coelectrophoresis (B) a sample of FPV-pretreated cells superinfected with SFV, labeled with 0.25 μCi of [^{14}C]protein hydrolysate per dish 1.5 to 2.5 h after superinfection, was used. Symbols: ●, tritiated sample; ○, ^{14}C marker.*

tural proteins are still being produced. Five new polypeptides, NVPs 165, 130, 105, 97, and 72, are observed, however.

NVP 72 shares a common antigenic determinant with the envelope glycoproteins, since it is precipitated by an antiserum raised against the envelope components of the virion (Fig. 3, lower graph). It could be a completely glycosylated form of NVP 68 which, due to incorporation of amino acid analogues, is not cleaved (cf. Fig. 9).

Among the four larger polypeptides, shown in Fig. 3, NVP 97 has been reported to be an envelope precursor (11, 14) and NVP 130 the precursor of all structural proteins (14). The significance of NVP 165, the molecular weight of which has been estimated only approximately and seems to be even larger, and that of NVPs 145 and 105 is not known. Since in untreated cells NVP 145 and NVP 105 are the largest proteins of unknown function, it is possible that one of them could be a precursor of NVP 78. These two proteins disappear on chasing as quickly as NVP 78 increases (Fig. 2). After treatment with analogues of amino acids, NVP 78 and, similarly, NVP 145 are missing, but NVP 165 is detected (Fig. 3). It seems reasonable to assume that NVP 78 could be related to NVP 145, and possibly this, in turn, could be related to NVP 165. If NVP 145 would be cleaved to yield NVP 78, a second protein with a molecular weight exceeding 60,000 should result, which, however, would be concealed in the electrophoretic patterns by NVP 68/65. The possible relationships must, therefore, still be further proved.

Formation of the envelope precursor NVP 68 early after infection. PAGE reveals two peaks in the region of NVP 68 in patterns of FPV-pretreated CE cells early after infection with SFV. The additional new peak corresponds to a protein with an apparent molecular weight of 65,000 (NVP 65), which cannot be detected later than 4 h p.i. The amount of NVP 65 is relatively greater the shorter the applied pulse with radioactive amino acids and the earlier after superinfection with SFV the pulse is started. Furthermore, the amount of NVP 65 changes during a chase (Fig. 4). If labeled by applying a 15-min pulse, it disappears nearly completely during a 30-min chase, whereas radioactivities increase at the positions of NVP 68 and the envelope glycoproteins E_1 plus E_2. Although radioactivity disappears from all nonstructural proteins larger than 80,000 in molecular weight at the same time, this decrease can account for only about 50% of the observed increase (cf. Fig. 2). The rest is contributed at the cost of NVP 65.

That NVP 68 is derived from NVP 65 can be shown by the inhibition of this conversion, if the chase medium contains 10 mM 2-deoxy-D-glucose (DG), a drug that has been shown to inhibit glycosylation (6). Under these conditions only one single peak corresponding to NVP 65 is observed after the chase, whereas radioactivity disappears from NVP 68 and the nonstructural proteins larger than 80,000 daltons. At the position of E_1 plus E_2, accordingly, a diminished increase is found (data not shown).

This is evidence for a glycosylation mechanism underlying the conversion of NVP 65 into NVP 68. It can be shown that both forms of the precursor incorporate labeled mannose (Fig. 5A). This experiment is, however, hampered by the fact that, for demonstration of NVP 65, relatively short pulses are needed. Attempts to determine the incorporation of other labeled sugars into the two forms were therefore unsuccessful because of the low incorporation of label (data not shown). If the exposure to radioactive sugars is extended, results like those in Fig. 5B are obtained. Incorporation of fucose label into NVP 65 can be demonstrated in this way, and the results resemble those obtained after labeling infected cells without pretreatments with tritiated mannose (Fig. 5C). Not sufficiently glycosylated forms of NVP 68 are obtained, as described earlier (6). This labeled NVP 65 is, however, stable under chase conditions and will be described later (see below).

Enhancement of [^{3}H]mannose incorporation during an infectious cycle. The above observations suggest an increase of glycosylating activity in the infected cells caused by the infection. The rates of [^{3}H]mannose incorporation under single-cycle conditions were therefore measured (Fig. 6). The experiment was performed with non-pretreated CE cells. The rate of incorporation is at the level of noninfected cells during the first 2 h, is then strongly enhanced as the replication proceeds, and starting about 6 h p.i. declines again. The obtained curve is nearly parallel with a growth curve. Similar results are obtained if incorporation of labeled DG is measured (18). An increase in the incorporation rate in the noninfected cells was not observed. The confluent cells used, 22 h after seeding, are apparently in a resting state, with a constant rate of mannose consumption.

For convenience, the incorporation rates of [^{3}H]uridine have been measured simultaneously, since maximal uridine incorporation has been shown to coincide with maximal incorporation of labeled amino acids (7). Thus, mannose incorporation reaches maximal values

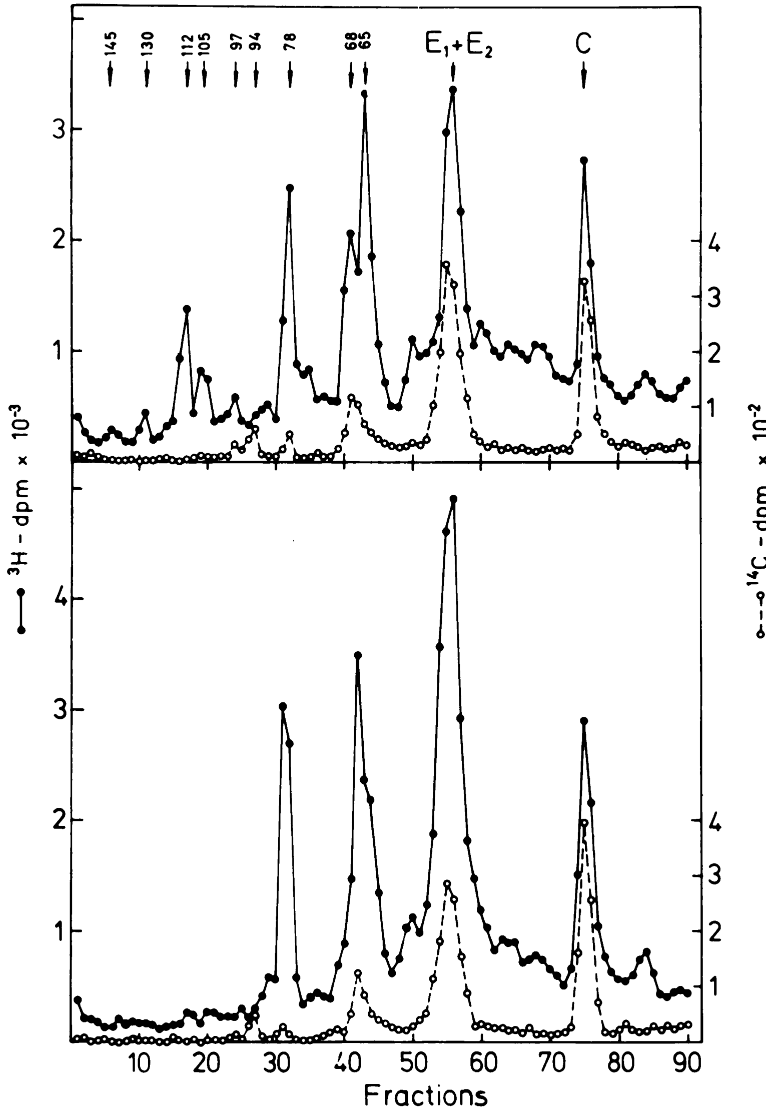

FIG. 4. *Pulse-chase experiment 1.5 h after superinfection of FPV-pretreated cells with SFV. Two parallel cultures of CE cells in 5-cm petri dishes were pretreated with FPV and actinomycin D. One and one-half hours after superinfection, with SFV, a 15-min pulse with 25 μCi of tritiated amino acids in modified Earle basal salts solution (BSS) was started. One culture was then immediately processed for PAGE (upper); the second one was processed after a 30-min chase (lower). Samples were subjected to coelectrophoresis with a marker sample, consisting of SFV-infected cells without pretreatment exposed to 0.25 μCi of [^{14}C]protein hydrolysate/dish in 1.5 ml of modified Earle BSS 4.5 to 5.5 h p.i. Symbols: ●, tritiated sample; ○, ^{14}C marker.*

when most of the proteins have already been synthesized. An important parameter, however, is the pool size of available activated mannose, which could be diminished. This question is still under investigation.

NVP 65 detected late after infection. The results obtained in SFV-infected cells pretreated with FPV and actinomycin D indicate the formation of NVP 65 as a normal intermediate early after infection. This convertible form is designated as cNVP 65. A similar protein, cNVP 65 was also observed under different conditions, namely, in non-pretreated infected cells given 1- or 3-min pulses with labeled amino acids 4.5 h p.i. It disappears again after a chase of about 30 min (unpublished data). Similarly, cNVP 65 is observed 4.5 h p.i. if cells maintained in a culture medium lacking sugars

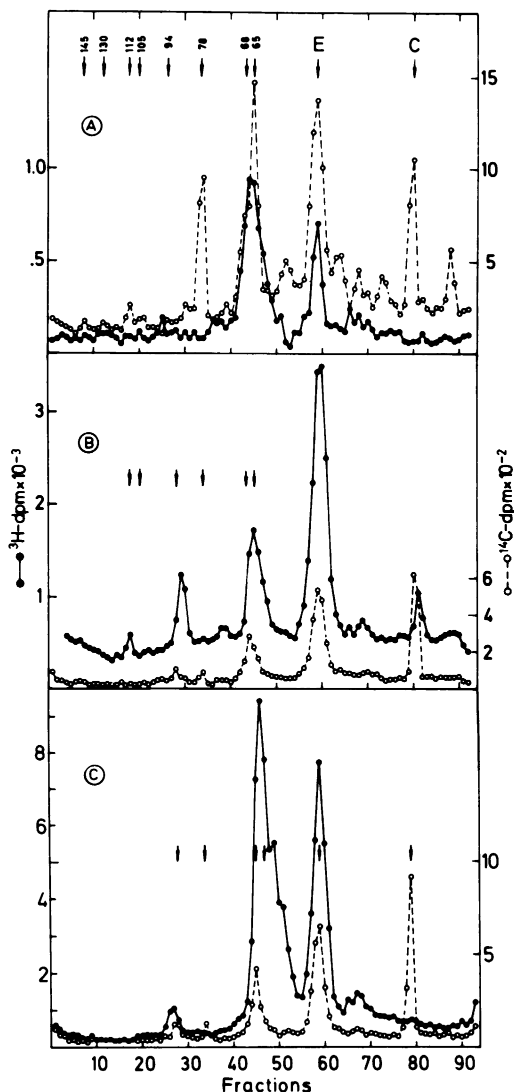

FIG. 5. *Incorporation of ³H-labeled sugars into NVP 65 and NVP 68 early after infection. CE cells pretreated with FPV and actinomycin D were exposed 1.5 h after superinfection with SFV to 0.25 μCi of [¹⁴C]protein hydrolysate and 25 μCi of [³H]mannose in 1.5 ml of modified Earle basal salts solution (BSS). One hour later they were processed for PAGE and run on a 8.75% gel (A). Cells of a parallel dish were labeled for 6 h with 50 μCi of [³H]fucose per ml, starting 1 h after superinfection with SFV, and subjected to coelectrophoresis with a sample of ¹⁴C-labeled infected cells without pretreatments (B). For comparison, SFV-infected CE cells without pretreatments were labeled for 6 h with 25 μCi of [³H]mannose in 1.5 ml of Earle BSS containing 5 mM fructose instead of glucose and supplemented with 2 mM glutamine, starting 3 h after infection. The sample was subjected to coelectrophoresis (C) with a marker sample as in (B). Symbols: ●, ³H-labeled sugar; ○, ¹⁴C-labeled amino acids.*

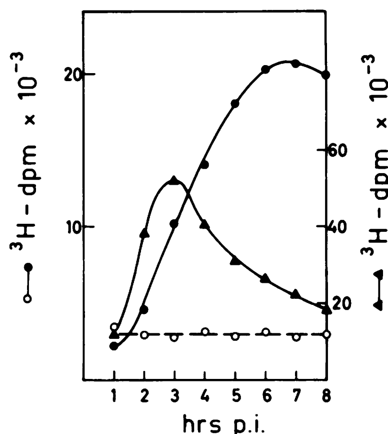

FIG. 6. *Enhancement of [³H]mannose incorporation into SFV-infected CE cells during an infectious cycle. Parallel 9-cm petri dishes with CE cells were infected with SFV and maintained in modified Earle basal salts solution (BSS). At the times indicated on the abscissa, cells were exposed to 2.5 μCi of [³H]mannose in Earle BSS for 30 min and then immediately processed for assay of trichloroacetic acid-insoluble radioactivity. A noninfected control was run every time. In parallel dishes a similar pulse was made with 2.5 μCi of [³H]uridine in the presence of 1 μg of nonlabeled uridine per ml. Symbols: ●, trichloroacetic acid-insoluble [³H]mannose radioactivity of infected cells, and ○, of control cells; ▲, [³H]uridine incorporated into infected cells.*

are labeled (6). In this case formation of NVP 68 at the cost of NVP 65 is possible by a chase in the presence of glucose, during which glycosylation occurs. However, glycosylation is nearly completed only if the chase conditions are applied 15 min or less after the pulse has been started; otherwise a stable form of NVP 65 results. In Fig. 7 the results of a pulse-chase experiment are shown in which the cells were exposed to labeled amino acids for 30 min, followed by chase periods of different lengths. It can be seen that radioactivity disappears from the position of NVP 68, although a small amount remains at the position of NVP 65. This stable form is designated as sNVP 65. It can be labeled with fucose as well as with mannose (Fig. 5B, C).

A similarly stable sNVP 65 is observed under conditions of impaired glycosylation caused by the action of DG or D-glucosamine, and it has been also shown to contain fucose (6). Its stability under chase conditions is shown in Fig. 8. The two parallel horizontal lines indicate that no breakdown or degradation of proteins into a trichloroacetic acid-soluble form takes place. Recently, sNVP 65 has been shown to remain stable under these conditions, as revealed by PAGE (6).

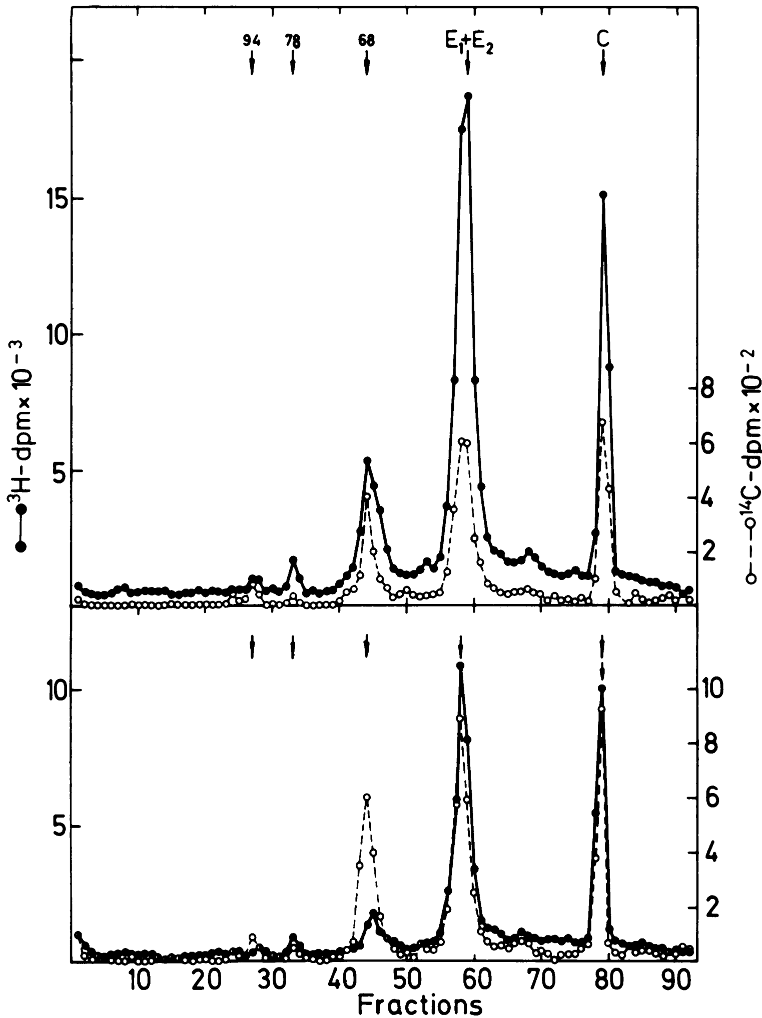

FIG. 7. *Pulse-chase experiment in non-pretreated CE cells. Two parallel cultures of SFV-infected CE cells were labeled 4 h p.i. with 25 μCi of tritiated amino acids in 1.5 ml of modified Earle basal salts solution per dish for 30 min. After a chase period of 20 min, the cells of one dish were processed for PAGE (upper); those of the second dish were processed after a chase of 3 h (lower). Samples were subjected to coelectrophoresis with a sample of marker cells labeled under identical conditions with [^{14}C]protein hydrolysate without chase. Symbols: ●, tritiated sample; ○, ^{14}C-labeled amino acids.*

Thus, depending on the conditions, two kinds of NVP 65 occur in SFV-infected cells. One kind, which is apparently a normal intermediate, is short lived and converted into NVP 68. The second kind, which is stable, contains fucose, and cannot be converted, is apparently not utilized for virus maturation.

Specificity of NVP 68 cleavage and antigenic relations between E_2, NVP 68, and NVP 65. It is considered that the splitting of NVP 68 is very specific and is one of the most critical steps for virus maturation. If extracts from cells labeled 4.5 h p.i. with tritiated amino acids are subjected to an indirect immune precipitation, using an antiserum specific for only the envelope glycoproteins, PAGE yields the results shown in Fig. 9A and B. Radioactivity is found at the position of NVP 68, as well as at the position of the envelope glycoproteins and faintly at the position of NVP 97. However, the maxima of the sample and the simultaneously run extract of ^{14}C-labeled, infected cells do not coincide. The result indicates precipitation of only a part of the region attributed to NVP 68. The apparent molecular weight of the precipitated protein is about 69,000.

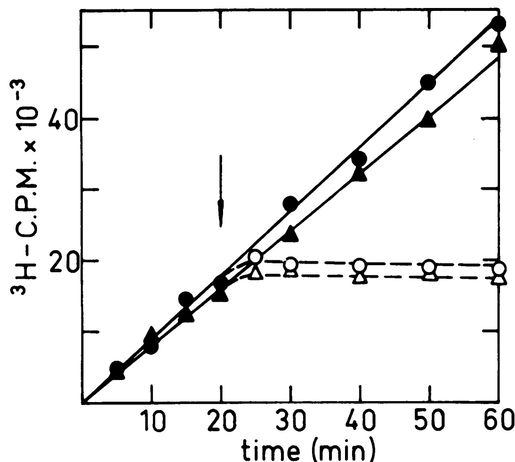

FIG. 8. *Stability of insufficiently glycosylated viral proteins under chase conditions. CE cells in parallel 9-cm petri dishes were used. Immediately after infection they were exposed to 0.25 mM DG in modified Earle basal salts solution (BSS) or held without the drug. At 4.5 h p.i. pulses of different lengths (values of the abscissa) were started by medium change. The pulse medium was modified Earle BSS containing 16.6 μCi of tritiated amino acids and 1 μg of each of the respective nonlabeled amino acids per ml. Cells were then immediately processed for estimates of trichloroacetic acid-insoluble radioactivity. In some of the cultures, the pulse medium was replaced after 20 min by a chase medium (arrow). Cells were then harvested at the times indicated on the abscissa and processed as above. Symbols: closed symbols, pulse experiment; open symbols, after chase; circles, nontreated controls; triangles, DG-treated cells.*

NVP 68 always forms a broad peak in PAGE patterns, and this is thought to be due to microheterogeneity of the carbohydrate chains. Only the mostly completed forms are precipitated. sNVP 65, present in small amounts (Fig. 7), reacts only poorly or not at all with the antibodies. This is also true for the convertible form, cNVP 65, present early after infection (Fig. 9C, D). Thus, the envelope glycoprotein E_2, and possibly E_3, must have a very specific conformation, depending on the completeness of the carbohydrate side chains, or the carbohydrate moiety determines antigenicity.

DISCUSSION

In CE cells pretreated with FPV and actinomycin D we have investigated the synthesis of SFV-specific proteins as early as 1.5 h after superinfection with SFV. Some nonstructural proteins appear characteristically at this early stage of infection. Besides some already known precursor proteins (NVP 130, NVP 105, and NVP 68), three new proteins are detected: NVP 145, NVP 112, and NVP 65. The significance of NVP 145 is not known; NVP 112 could be a precursor of all three envelope glycoproteins, E_1, E_2, and E_3, in their glycosylated forms. NVP 65 is a form of NVP 68 with an incomplete carbohydrate moiety. It is short lived and is converted into NVP 68 by addition of further sugar residues, mainly of mannose, which is present in reduced amounts in NVP 65. This glycosylation step can be inhibited by DG.

The short-lived, convertible cNVP 65 can also be demonstrated later in the infectious cycle: either by application of short pulses or by labeling of cells maintained in a sugar-free culture medium. After short pulses, cNVP 65 disappears during a chase of about 30 min. When demonstrated in cells under conditions of sugar starvation, it can be completely chased after addition of glucose only if the chase conditions are applied 15 min or less after the pulse has been started.

A second kind, sNVP 65, apparently a side product, could be demonstrated after pulses followed by long chase periods or after inhibition of glycosylation by DG or glucosamine. This second kind is stable. Like the convertible form cNVP 65, it is poor in mannose content compared with NVP 68, but it contains fucose.

The following mechanism for NVP 68 formation is considered. Glycoprotein precursors are synthesized primarily without, or at least with little, carbohydrate (8, 13, 22), and as soon as they are completed by addition of sugars they are cleaved. If the supplementary glycosylation is rendered difficult, e.g., by inhibition of glycosylation using DG, or if sufficient amounts of activated sugars are not available (presumably due to an exhaustion of the GTP pools), the growth of the mannose-rich carbohydrate side chains ceases. By addition of a terminal-position fucose molecule, the glycoprotein becomes stable: it is not degraded but cannot be completed in its carbohydrate moiety. This mechanism could be in effect at the level of NVP 68/65 as well as at the level of NVP 97/94.

The proteolytic cleavage of NVP 68 seems to be very specific with regard to its complete carbohydrate moiety, since sNVP 65 is remarkably stable under chase conditions. The specificity of the NVP 97 cleavage seems to be less, since a slow decrease of radioactivity at the position of the sufficiently glycosylated form NVP 94 has been observed, followed by the appearance of the insufficiently glycosylated envelope glycoprotein E_0 (6).

It is assumed that conformation of the glycoprotein precursors is an important condition for

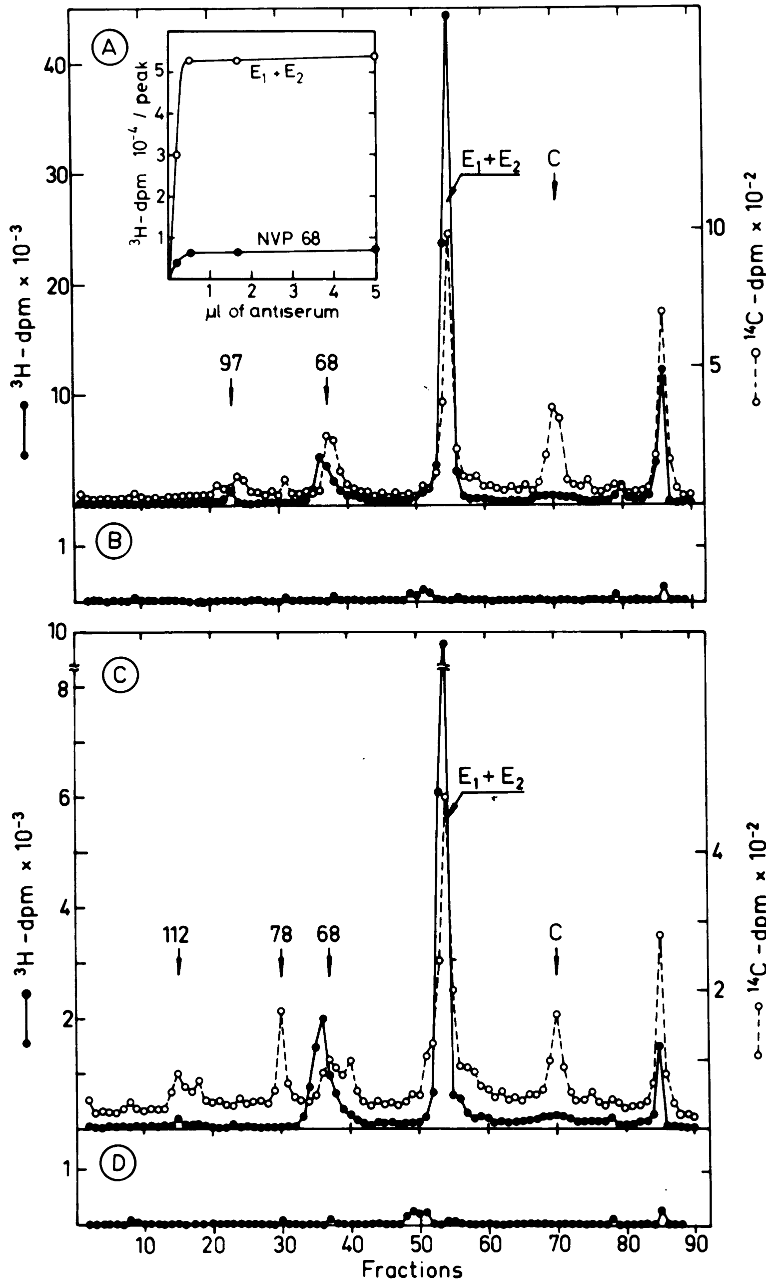

FIG. 9. *Indirect radioimmune precipitation of NVP 68. Two parallel dishes of SFV-infected cells maintained in modified Earle basal salts solution were pulse-labeled 4.5 to 5.5 h p.i.: cells of one dish with 16.6 µCi of tritiated amino acids per ml; cells of the parallel dish with 0.25 µCi of [^{14}C]protein hydrolysate per ml. Similarly, two parallel dishes of CE cells, pretreated with FPV and actinomycin D, were labeled 1.5 to 2.0 h after superinfection. In both cases the ^{14}C-labeled cells were processed immediately for PAGE as markers for coelectrophoresis. The ^{3}H-labeled cells were extracted (see text), and aliquots of the obtained lysates were reacted with antisera, as described in the legend to Fig. 3. The dissolved precipitates were subjected to coelectrophoresis with the prepared ^{14}C-labeled markers. (A) ^{14}C-labeled marker and ^{3}H-labeled immune precipitate, both from nonpretreated, SFV-infected CE cells. (B) Control obtained after reaction of infected, labeled cells with a preimmune serum. (C) ^{14}C-labeled marker and ^{3}H-labeled immune precipitation, both from FPV-pretreated, SFV-superinfected cells. (D) Control obtained after reaction of superinfected cells with a preimmune serum. Symbols: ●, ^{3}H-labeled immune precipitate; ○, ^{14}C marker. The insert in (A) is a control for the completion of precipitation of the envelope glycoproteins. Aliquots (200 µl) of the same lysate used in (A) were reacted with different volumes of the antiserum (abscissa) in a total volume of 5 µl, obtained by dilution of the antiserum with a preimmune serum. After PAGE the peak areas were integrated (ordinate) and plotted against the volume of the antiserum. A 5-µl portion of the undiluted antiserum as used in the precipitation reactions of Fig. 3 and 9.*

their accessibility to proteolytic enzymes. Consistently, the conformation of the resulting envelope glycoprotein is very uniform, giving rise to a viral membrane of high order (3). In no case could an insufficiently glycosylated envelope glycoprotein, such as E_0, be detected in labeled virus preparations.

The necessity of a completed glycosylation and, consequently, the uniformity of conformation of the envelope glycoproteins also explains the high antigenic specificity. Antibodies raised against the envelope glycoproteins of the virion precipitate only the high-molecular-weight portion among the NVP 68 microheterogeneic variety. The cleavable precursor has a somewhat higher molecular weight, about 69,000. NVP 65, in its convertible as well as stable form, is not precipitated or only in diminished amounts.

It seems to be the first time that the relation between the sugar content of a glycoprotein (and perhaps the conformation caused thereby) and its antigenic properties could be demonstrated so clearly, indicating that the carbohydrates of a glycoprotein are important for its antigenicity.

The observation that glycosylation activities of cells are enhanced by infection is interesting. The extent and efficiency of this glycosylating system, above all of mannosylation, are responsible for the amount of infectious virus produced. This is in excellent agreement with our earlier observation (8, 9) that viral titers can be manipulated nearly at pleasure using methods lacking sugars or containing inhibitors such as DG.

In the early stages after infection with SFV, the production of relatively large amounts of NVP 78 was also observed. It seems to be rapidly formed from a precursor, presumably NVP 165 (or NVP 145). This is concluded from pulse-chase experiments and the PAGE pattern obtained after labeling infected cells in the presence of amino acid analogues. Since NVP 78 is stable under further chase conditions, since its formation is inhibited in the presence of amino acid analogues at concentrations that do not influence significantly the production of the structural proteins, and since it is not detectable in the virion nor precipitated by antisera, it is considered not to be related to the structural proteins in any way and, rather, to be a virus-specific protein needed early in infection.

It is worthwhile to consider what type of a nonstructural protein is needed in SFV-infected cells. Since the host-specific protein synthesis is largely suppressed, NVP 78 is unlikely to be an elicitated host-specific protein. Besides the RNA polymerase, no nonstructural proteins seem to be needed by cytocidal viruses, unlike, for example, the oncornaviruses. Thus, it seems to be reasonable to assume that NVP 78 could be at least one of the still unknown proteins of the viral RNA polymerase. This would also be in agreement with the observed preferential synthesis of NVP 78 in the first hours of infection. Furthermore, in Sindbis virus-infected cells a similar protein of about 80,000 daltons, which differs in its electrophoretic mobility from NVP 78, has been detected early after infection (7). The production of two elicited host-specific proteins by these closely related viruses seems to be unlikely, but two similar virus-specific proteins needed early in infection are being produced.

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