Structural and Nonstructural Proteins of an Arbovirus

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Purified Semliki Forest virus (SFV) contains three structural proteins while its core (nucleocapsid) contains two of these proteins. To identify all of the proteins synthesized under virus direction, cells were infected with SFV in the presence of actinomycin D and guanidine. Cell protein synthesis was markedly and irreversibly inhibited under these conditions; virus growth was reversibly inhibited by guanidine and began when the cells were washed to remove the guanidine. When cells were treated with guanidine for 4 hr after virus infection and then were washed, five major proteins were produced early in infection. Three of these proteins corresponded to virus structural proteins. None of these five proteins was a major protein of uninfected cells or of virus-infected cells which had been incubated with partially purified interferon before infection. Late in infection, three major proteins, the virus structural proteins, were produced.

Arboviruses grow rapidly in the cytoplasm of most animal cells; they have a homogeneous ribonucleic acid (RNA) of relatively small molecular weight (about 2×10^6 daltons) and thus would be expected to contain genetic information for the synthesis of only four to eight viral proteins (11, 19). Temperature-sensitive mutants have been isolated for some viruses of this group (1, 15). As is shown in this study, Semliki Forest virus (arbovirus group A) appears to exercise control over the translation of its genetic information during the course of virus infection, since early in infection five major proteins, three of which correspond to virus structural proteins, are produced; late in infection, only three major proteins are produced. The proteins produced during infection are not major proteins of uninfected cells or of infected cells treated with interferon before infection.

MATERIALS AND METHODS

Cells and viruses. The preparation of primary chick embryo fibroblast (CEF) cultures in this laboratory has been described previously (7). Pools of Semliki Forest Virus (SFV) were also prepared by previously described methods (7). Where high multiplicities of virus were used, virus was concentrated by sedimentation at $78,000 \times g$ for 3 hr.

Virus and interferon assay. These assays were performed according to the methods of Taylor (18). One unit of interferon inhibited SFV replication by 50%.

Preparation of purified virus and viral "cores." Purified radioactive virus was prepared as previously described (6) except that, after protamine sulfate

treatment and sedimentation at $60,000 \times g$ for 3 hr, the virus was subjected to an additional sedimentation at $300,000 \times g$ for 1 hr before being banded in a 15 to 60% sucrose density gradient. This method yields virus preparations which are concentrated in a single sharp band in the sucrose and which contain only virus particles on electron microscopic examination and only viral RNA (6).

To prepare virus cores (nucleocapsids), cells were incubated with $10 \mu\text{C}$ of ^{14}C -amino acids per ml 4 to 5 hr after virus infection. At this time, cell protein synthesis is markedly inhibited in virus-infected cells (4). Cytoplasmic extracts were prepared as previously described. After sedimentation in sucrose density gradients, the fractions containing the 140S peak of radioactivity, which represents the viral cores (5), were again sedimented at $300,000 \times g$ for 2 hr. The sediment of the latter preparation was employed in studies of the 140S virus core proteins.

RNA extraction. A previously described variation of the Scherrer and Darnell (13) method was employed to extract viral RNA.

Sucrose density gradient analysis of RNA. Sucrose density gradient analysis and the assay for acid-precipitable radioactivity in gradient fractions were carried out as previously described (6).

Protein and RNA synthesis. Protein synthesis was studied by the rate of incorporation of ^3H -leucine into a perchloric acid-precipitable fraction; RNA synthesis was studied by the rate of incorporation of ^3H -uridine into the same fraction (4). Total protein was measured by the method of Lowry et al. (10).

Infection procedure. CEF in monolayers containing about 5×10^6 cells were treated for 1 hr with actinomycin D ($2 \mu\text{g}/\text{ml}$). They were then infected at a virus-cell multiplicity of 100 plaque-forming units (PFU) in the presence of guanidine ($3 \text{ mg}/\text{ml}$). After

4 hr, the cells were washed five times and fresh medium was added.

Polyacrylamide gel electrophoresis. Proteins from cytoplasmic extracts of cells labeled with ^{14}C -amino acids, or from purified SFV, or from viral "cores" were solubilized, and electrophoresis was performed on 10% polyacrylamide gels by the methods of Summers et al. (17). Gels were sliced, stained, and prepared for radioautography as previously described (2, 12). For radioautography, films were exposed to gels from periods as long as 14 days; longer exposure failed to reveal additional lines. Films were scanned in a Joyce-Loebl microdensitometer (12).

Interferon. A partially purified pool of chick interferon prepared by the method of Fantes was employed (3). This pool contained 10,000 units of interferon per ml and 179 μg of protein per ml. It was previously characterized and found to have all of the usually described properties of interferon (6). The preparation was donated by Karl Fantes, Glaxo Laboratories Ltd., Stoke Poges, England.

Reagents. Actinomycin D was donated by Merck Sharp and Dohme (Rahway, N.J.). ^3H -leucine (38 mc/mg) and ^3H -uridine (2 c/mmole) were purchased from New England Nuclear Corp., Boston, Mass., and from Schwarz BioResearch Inc., Orangeburg, N.Y., respectively. The purified ^{14}C -amino acid mixture (54 mc/matom of carbon) employed was prepared by the Radiochemical Centre, Amersham, England, from *Chlorella* protein hydrolysate.

RESULTS

Proteins of SFV and its core. Electrophoresis in polyacrylamide gels was performed on solubilized proteins from purified SFV and its 140S core labeled with ^{14}C -amino acids. Microdensitometer tracings on radioautograms of the dried gels are shown in Fig. 1. The virus (Fig. 1A) contained three proteins, marked 1, 2, and 3. Protein 3 appeared to be a relatively minor component in the virus but was present in all preparations. The 140S core (nucleocapsid) contained two components, proteins 1 and 3 (Fig. 1B). These results indicated that protein 2 is associated with the virus envelope.

Effect of guanidine treatment on SFV-infected cells. It has previously been shown that, by 4 hr after infection, over 80% of the protein produced in actinomycin D-treated and virus-infected cells is viral in origin (4). Very early in infection, however, it is difficult to identify viral proteins in the presence of a background of cell proteins still being produced, even in cells treated with actinomycin D for several hours before infection. Results obtained with guanidine in poliovirus-infected cells (17) suggested that this agent might also be a useful virus-inhibitory substance in the study of protein synthesis by SFV. In cells infected with SFV in the presence of actinomycin D (2 $\mu\text{g}/\text{ml}$) and guanidine (3 mg/ml), little or

FIG. 1. Analysis of proteins from Semliki Forest virus and its core (nucleocapsid). Densitometer tracings were prepared from radioautograms of dried gels. (A) Radioactive proteins from purified virus grown in the presence of 2 μC of the ^{14}C -amino acid mixture per ml; protein (15,000 counts/min) was applied and the film was exposed for 8 days. (B) Radioactive proteins from purified virus core prepared by labeling cells with 10 μC of the ^{14}C -amino acid mixture per ml from 4 to 5 hr after virus infection; protein (12,000 counts/min) was applied and films were exposed for 9 days. The arrows indicate the cathodal (left) and anodal (right) ends of the gels. All densitometer tracings shown in this study are direct photographs of the original tracings.

no virus growth was seen for 7 hr after infection (Fig. 2); by this time, the end of the log phase of virus growth had been reached in controls infected with SFV and treated with only actinomycin D (6). When cells which had been infected and treated with both actinomycin D and guanidine were washed 4 hr after infection in order to remove the guanidine, virus growth took place. By 7 hr after washing, the virus titers had reached levels comparable to those in infected cells treated with actinomycin D only (Fig. 2). These results suggested that guanidine could be used as a reversible virus inhibitor. Treatment with virus, actinomycin D, and guanidine for 4 hr might inhibit a large percentage of the cell protein synthesis irreversibly; virus protein synthesis would, under these conditions, make up the bulk of the residual protein synthesis early in virus infection.

To determine whether any viral RNA was produced in the presence of guanidine, actinomycin D-treated cells were infected with SFV in the presence or absence of guanidine. In chick cells treated with actinomycin before infection with SFV, almost all of the RNA produced was

viral. After 3 hr, the cells were incubated with 10 μ c of 3 H-uridine per ml for 1 hr and the RNA was extracted and analyzed on sucrose density gradients.

In cells infected in the absence of guanidine, the previously described (7), complex sedimentation pattern of viral RNA was seen (Fig. 3). In the presence of guanidine, very little or no isotope was incorporated into acid-precipitable RNA. With the low levels of radioactivity found in the extracts from guanidine-treated cells, it was difficult to determine whether any viral RNA at all was produced in these cells. When guanidine inhibition was reversed by washing the cells, virus RNA production started; by 3 hr after washing, the sedimentation pattern of the cytoplasmic RNA resembled that of the cells infected in the absence of guanidine (shown in Fig. 3).

As previously shown, when cells treated with guanidine at the time of infection were washed, virus growth was initiated and proceeded normally (Fig. 2). This finding is also suggested by the data shown in Table 1, concerning the synthesis of protein in virus-infected, guanidine-treated cells. In actinomycin D-treated cells in the presence of guanidine and virus, protein synthesis was less than 3% of uninfected controls. When these cells were washed to remove the guanidine, protein synthesis rose to 10% of the controls. The inhibitory effect of actinomycin D or virus or both on chick cell protein synthesis

has been shown elsewhere (4). Guanidine alone did not have a permanent effect on the cells.

Nature of proteins produced in guanidine-treated, SFV-infected cells. The nature of the

FIG. 3. Effect of guanidine on Semliki Forest virus RNA synthesis. CEF were treated with 2 μ g of actinomycin D per ml for 1 hr. The cells were then infected with Semliki Forest virus at a virus-cell multiplicity of 200 in the presence or absence of guanidine (3 mg/ml). After 3 hr, 10 μ c of 3 H-uridine per ml was added to the incubation mixture. After 1 hr of additional incubation, the cells were washed and RNA was extracted. The extracts were layered over a 6 to 30% sucrose gradient and were sedimented for 3 hr at 100,000 $\times$ g. Fractions were collected and analyzed for acid-precipitable RNA. Ribosomal RNA optical density markers are designated as 28S and 16S. Bottom of the gradient is to the left.

FIG. 2. Reversible inhibition of Semliki Forest virus growth by guanidine. Chick cells were infected with virus at a multiplicity of 20:1 in the presence of actinomycin D (2 μ g/ml). After 1 hr, the cells were washed and virus was allowed to grow until 7 hr after infection. Cultures were then assayed for virus yield. Identical cultures were infected in the presence of guanidine hydrochloride (3 mg/ml). After 4 and 7 hr, sets of cultures were assayed for virus yields; another set was washed after 4 hr to remove guanidine, placed in guanidine-free medium until 11 hr after infection, and then assayed.

TABLE 1. Inhibition of chick cell protein synthesis by virus infection in the presence of guanidine and actinomycin D

Treatment ^a	Washed	Specific activity (counts per min per μ g of protein)	Per cent control specific activity
None.....		150	100
Virus, actinomycin D, guanidine.....	No	4.1	2.7
Virus, actinomycin D, guanidine.....	Yes	14.4	9.6

^a Chick cells were treated with 2 μ g of actinomycin D per ml for 1 hr. They were then infected with virus at a multiplicity of 100 PFU in the presence of guanidine (3 mg/ml). After 4 hr, cells were washed and incubated for 2 hr in Eagle's medium. Monolayers were labeled for 10 min with 2 μ c of 3 H-leucine per ml in leucine-free medium. Cells were then washed and scraped into 2.5% perchloric acid. The precipitates were collected, acid-washed, dissolved in 0.3 N NaOH, and then analyzed for protein and radioactivity.

proteins produced in virus-infected CEF 1 to 2 hr after the reversal of guanidine inhibition is shown in Fig. 4A. Five bands containing radioactive protein could be identified in polyacrylamide gels of cytoplasmic extracts of infected CEF. The five radioactive bands did not correspond to any of the prominent, stained bands of cell

FIG. 4. Analysis of proteins from Semliki Forest virus-infected cells and from uninfected cells. Densitometer tracings were prepared from radioautograms after 4 (A and B) or 6 (C and D) days of exposure of films to dried gels to which 20,000 counts/min of ^{14}C -labeled proteins had been applied. (A) Radioactive proteins from the cytoplasmic extract of virus-infected cells, labeled by addition of $10\ \mu\text{C}$ of ^{14}C -amino acid per ml 1 to 2 hr after washing. Infection was at a virus-cell multiplicity of 100 and in the presence of actinomycin D ($2\ \mu\text{g}/\text{ml}$) and guanidine ($3\ \text{mg}/\text{ml}$). After the 1-hr labeling period, cells were incubated for 5 min with ^{14}C -amino acids. (B) Cytoplasmic extract of cells, labeled by addition of $10\ \mu\text{C}$ of ^{14}C -amino acids per ml from 4.5 to 5.5 hr after infection. The cells had been treated with $2\ \mu\text{g}$ of actinomycin D per ml for 2 hr before infection. (C) Uninfected chick cells labeled for 10 min ($10\ \mu\text{C}$ of ^{14}C -amino acids per ml). (D) As in (A) except that the cells were treated with 1,000 units of chick interferon per ml for 14 hr before infection. Arrows indicate anodal (right) and cathodal ends of each gel. A variable amount of radioactivity was present at the cathodal end of all gels run with chick cytoplasmic extracts. The significance of this radioactive material in this and previous studies (12) is unknown.

protein (R. M. Friedman, *in preparation*). Proteins 1, 2, and 3 were identical in position to the three proteins found in the purified virus. Proteins A and B were not present in the virus but were probably viral in nature, since these radioactive bands were not prominent in uninfected and untreated CEF (Fig. 4C). Also, treatment with partially purified chick interferon before infection prevented the appearance of all five proteins (Fig. 4D). A variety of radioactive proteins was present in uninfected, untreated cells (Fig. 4C). The major radioactive bands corresponded to the bands seen in the stained gels. Treatment of uninfected CEF with actinomycin D and guanidine for 4 hr decreased protein synthesis but did not qualitatively alter the pattern of radioactive proteins present. The tracing from interferon-treated, virus-infected cells (Fig. 4D) was qualitatively similar to the tracing from uninfected cells (Fig. 4C). Interferon treatment inhibits translation of virus genetic information but does not affect cell protein synthesis (8).

Virus protein synthesis during the log phase of virus growth, 4.5 to 5.5 hr after infection (6), was also investigated. During this growth phase, guanidine treatment was unnecessary since more than 80% of the protein synthesized at this time was viral (4). As shown in Fig. 4B, at 4.5 to 5.5 hr after infection, three major proteins were produced, virus structural proteins 1, 2, and 3.

DISCUSSION

The results of this investigation suggest that the RNA of SFV contains genetic information for the production of at least five proteins. A total of five proteins might be expected from the size of the RNA (19). Of the five proteins identified, proteins A and B appear to be nonstructural. Of the three structural proteins, 1 and 3 are associated with the core or nucleocapsid, and 2 is associated with the outer membrane or envelope which the virus picks up as it emerges from the cell by budding (5). Protein 3 may be of some interest, because it is a major component in the infected cell but a minor component in the virus.

The viral nature of proteins A and B is suggested by the findings that these proteins were not prominent in uninfected CEF and that interferon treatment inhibited the formation of these proteins. In addition, electrophoretograms of proteins from SFV-infected L cells, treated and infected exactly as were the CEF in this study, were almost identical to those shown in Fig. 4A (R. M. Friedman, *unpublished data*). It is unlikely that SFV infection in both chick and mouse cells would cause almost identical cell proteins to become the major cell proteins produced.

Proteins A and B, the nonstructural proteins, were produced in very small amounts, if at all, late in infection, suggesting that some genetic regulation takes place late in infection. This may involve a regulatory function of one of the structural proteins, as has been shown in the RNA bacteriophages (9).

Some differences were found between the structural proteins of SFV and those previously reported for a related virus, Sindbis (16). With Sindbis virus, only two structural proteins were identified, one associated with the viral core and one associated with the envelope. No structural protein corresponding to protein 3 in the present study was found. It may be that the resolution of the autoradiographic technique employed in this study permitted the identification of an additional structural protein. A more likely possibility is that this difference is related to the morphological differences already noted between SFV and Sindbis virus (14). In Sindbis virus infection, it has been shown that virus structural proteins are the major proteins which are synthesized late in infection in actinomycin D-treated, infected chick cells (16). In this instance, the findings with SFV closely resemble those seen with Sindbis virus.

An inhibitory effect of guanidine on arbovirus replication has not been previously reported and arboviruses are usually considered to be guanidine-resistant. It should be noted, however, that the dose of guanidine employed in these studies was about ten times that usually employed to inhibit poliovirus replication (17).

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