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Infective Substructures of Sendai Virus from Infected Ehrlich Ascites Tumor Cells

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Increase of infectivity for embryonated eggs was observed in Ehrlich ascites tumor cells after intraperitoneal inoculation of Sendai virus into tumor-bearing mice. Virus-induced actinomycin-resistant ribonucleic acid consisting of 14*S*, 18*S*, 22*S*, 35*S*, and 48*S* was synthesized, and S antigen was produced in infected cells. The infectivity was suggested to be due to viral ribonucleoprotein for the following reasons: (i) the infectivity was unaffected by V antiserum but was abolished by whole hyperimmune serum, (ii) the infectivity was resistant to ribonuclease, (iii) virus particles were found neither in cells nor on red blood cell stroma treated with cellular extracts, (iv) structures similar to Sendai virus ribonucleoprotein with a maximal length of 10,500 A were observed in cellular extracts.

The formation of giant polynuclear cells and the production of noninfectious hemagglutinins by Ehrlich ascites carcinoma cells infected with Sendai virus have been described (10–13). In similar experiments, using the 960 strain of Sendai virus, which does not induce the fusion reaction of cells (4), we found an increase in both hemagglutinins and infectivity for embryonated eggs. As whole virus particles were never seen in ultrathin sections of infected Ehrlich tumor cells, either in the cytoplasm or at the cell surface (B. V. Guschin et al., *in press*), it seemed possible that the virus activity could be due to virus substructures.

The experiments described in this paper were designed to explore this possibility.

MATERIALS AND METHODS

Virus growth and titration in Ehrlich ascites tumor cells. The 960 strain of Sendai virus was used as allantoic fluid preparations harvested 72 hr after inoculation of 10-day-old chick embryos with 10^3 EID_{50} and subsequent incubation at 35 C.

Mice were inoculated intraperitoneally with 4×10^7 hyperdiploid Ehrlich ascites tumor cells. Seven days later, when the number of tumor cells was about 5×10^8/ml, the mice received intraperitoneally 2×10^{10} EID_{50} of Sendai virus; 5, 24, 48, 72, and 96 hr after virus inoculation, samples of cells were removed, washed three times with Earle's saline, and disrupted either by osmotic shock or by grinding with glass. The cell disruption by osmotic shock was performed by resuspension of cells in buffered distilled water, *p*H 7.0, at a final concentration of 1.5×10^7 cells/ml. The disrupted cells were centrifuged at 3,000 rev/min for 15 min, the supernatant fluid was then centrifuged at 10,000 × *g* for 10 min, the resulting supernatant fluid was centrifuged at 40,000 × *g* for 40 min, and the final supernatant fluid was centrifuged at 108,000 × *g* for 2.5 hr. The pellets were resuspended in 1 ml of balanced salt solution (BSS) and titrated. Hemagglutinin (HA) titers were determined with 0.5% suspensions of chicken or guinea pig erythrocytes (RBC). Complement-fixing antigen (CF antigen) titration was performed by a standard method with the use of hyperimmune guinea pig serum, and infectivity was determined by inoculation of 10-day-old embryonated eggs with serial 10-fold dilutions of the material. The data obtained were recorded on the basis of yield per 10^7 cells.

Immune sera. Hyperimmune antiserum was obtained by immunization of guinea pigs with Sendai virus grown in chick allantois. Its titer in a hemagglutination-inhibition (HI) test with purified V antigen was 1:1,280, in a CF test with V antigen, < 1:10, and with S antigen, 1:80. Immune V antiserum was obtained by the method of Lief and Henle (9) with the modification of separation of V antibodies on a cellulose immunosorbent (15). Its titer in an HI test with V-antigen was 1:160 and in a CF test with S antigen, < 1:10.

Isolation of nucleic acid and sucrose density centrifugation. Ascites tumor cells obtained from mice on the 7th day after tumor inoculation were washed with Earle's saline and infected with Sendai virus at a multiplicity of infection of about 100 EID_{50} per cell. Adsorption was performed for 30 min at room temperature; the cells were then sedimented at 1,000 rev/min, warm medium 199 was added to give a cell concentration of 10^8/ml, and the cells were incubated at 37 C. The bottles with cells were shaken gently during incubation. Actinomycin D (2 μg/ml) was added just after infection, and 2 μc/ml of ^{3}H-uridine (specific activity, 3.8 mc/mole) was added 2 hr after

infection. After 4 hr, the cells were sedimented, washed twice with Earle's saline, resuspended in tris(hydroxymethyl)aminomethane (Tris)-chloride buffer containing 0.001 M EDTA and 0.1 NaCl, pH 7.4, and ribonucleic acid (RNA) was extracted with buffer-saturated phenol, *p*H 6.0, at 4 C. The nuclei in the intermediate layer were washed twice with phenol at 4 C, and nuclear RNA was extracted with phenol at 65 C for 3 min. Sodium dodecyl sulfate (0.5%) was added to the RNA solution, which was deproteinized twice with phenol and precipitated twice with ethyl alcohol. RNA dissolved in Tris-chloride buffer was layered on a 5 to 20% sucrose gradient prepared in the same buffer and was centrifuged in a Superspeed 25 rotor at 20,000 rev/min for 14 hr. The fractions were collected from the bottom of the tubes and tested for optical density and radioactivity in a Packard Tri-carb liquid scintillation counter.

Electron microscopy. A drop of resuspended pellet, obtained by centrifugation of allantoic virus or disrupted cells at 40,000 × *g*, was placed on a carbon-Formvar grid prepared by the method of Choppin and Stoeckenius (5), excess fluid was removed, and the material on the grid was treated with 2% phosphotungstic acid adjusted to *p*H 7.0. The grids were examined in a JEM-6C electron microscope.

RESULTS

Virus growth in Ehrlich ascites tumor cells. The results shown in Fig. 1 represent the average data of three (for HA and CF antigen) and six (for EID_{50}) experiments with cells disrupted by grinding with glass. It is seen that HA, CF antigen, and EID_{50} titers increase in Ehrlich tumor cells after virus inoculation. The maximal titers of HA and CF antigen were observed on the second day; EID_{50} titers continued to increase up to the fourth day after virus inoculation. Similar titers of HA and EID_{50} were observed when the cells were disrupted by osmotic shock.

The cellular hemagglutinins differed from allantoic HA by their ability to agglutinate guinea pig RBC in higher titers than chicken RBC (Table 1), whereas the allantoic HA agglutinated both kinds of RBC in the same titer.

The specific nature of Ehrlich cellular HA was established in HI tests with V antiserum, which reacted with both V antigen and cellular HA at the same dilution (1:160).

When disrupted cells were sedimented by sequential differential centrifugation at 10,000, 40,000, and 108,000 × *g*, as described under Materials and Methods, the largest portion of cellular HA and infectivity was shown to sediment at 10,000 and 40,000 × *g*; these pellets resuspended in BSS were used for further investigations. When disrupted cells and resuspended pellets were allowed to adsorb at 4 C on stroma of chicken RBC (0.05 ml of stromal pellet per 10 ml), viruslike particles were never observed in the electron microscope (Fig. 2a). Allantoic virus diluted appropriately to the same HA titer as cellular virus was used as a control. In this case, viruslike particles were regularly seen on RBC stroma (Fig. 2b).

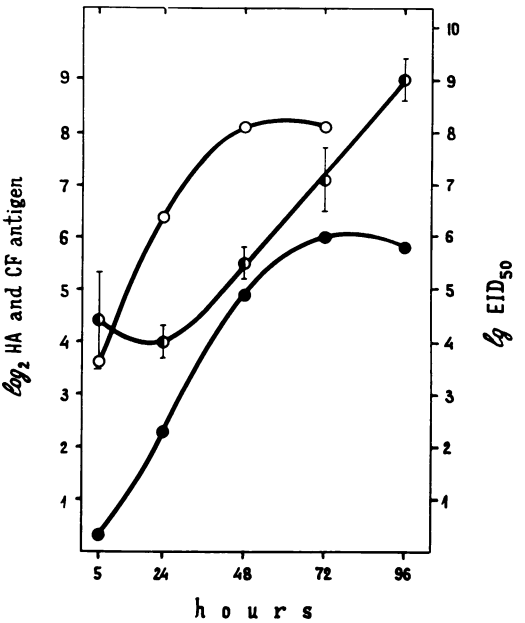

FIG. 1. *Kinetics of hemagglutinin (●), CF antigen (○), and EID_{50} (◐) production in Ehrlich ascites tumor cells after intraperitoneal inoculation of Sendai virus. The samples of cells taken from tumor-bearing mice at different intervals after Sendai virus inoculation were sedimented at 1,000 rev/min, washed three times with Earle's saline, resuspended in 2 ml of BSS, and ground with glass. This material was centrifuged at 3,000 rev/min, and HA (with 0.5% suspension of chicken RBC), CF titer (using hyperimmune serum), and infectivity (for embryonated eggs) were determined in supernatant fluids. The titers counted per 10^7 cells are expressed in log_2 (HA and CF antigen) and in log_{10} (EID_{50}).*

TABLE 1. *HA production in Sendai virus-infected Ehrlich ascites tumor cells as revealed with chicken and guinea pig RBC*

Time after virus inoculation	HA/ml[a]	
	Chicken RBC	Guinea pig RBC
hr		
5	4	16
24	16	256
48	128	1,024
72	256	1,024

[a] HA activity was assayed using a 0.5% suspension of RBC in the disrupted cells as described in Fig. 1.

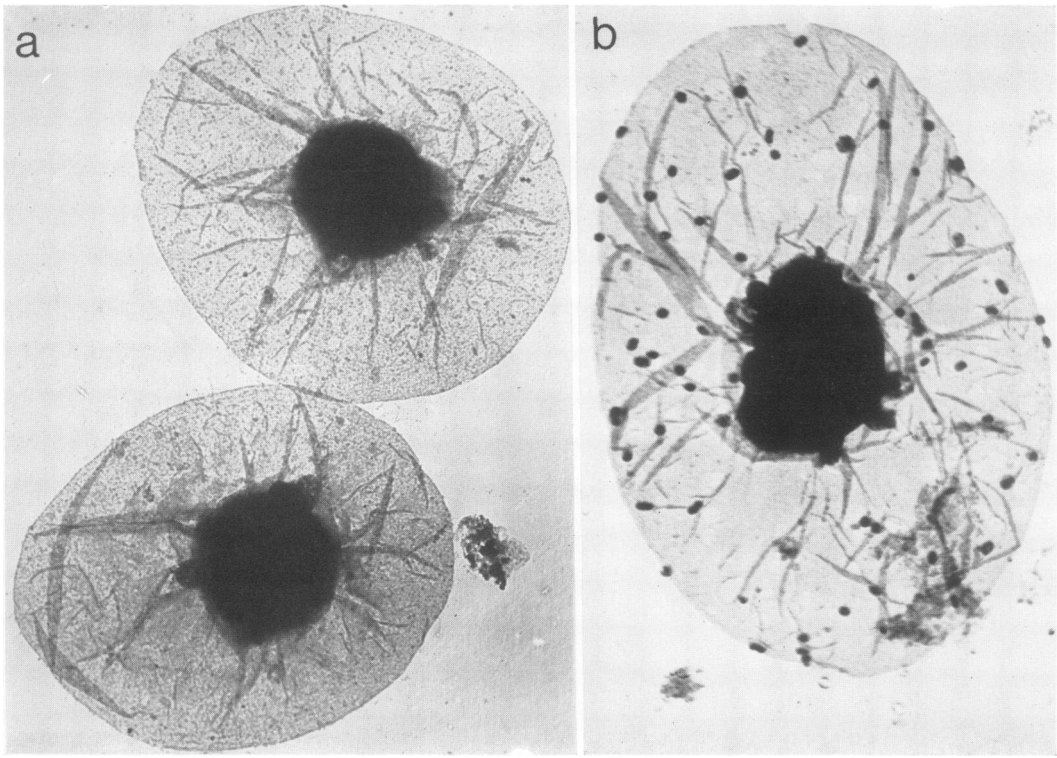

FIG. 2. *Stroma of chicken RBC after 1-hr contact (a) with disrupted Ehrlich cells and (b) with virus grown in chick allantois. The cells were taken from tumor-bearing mice on the 4th day after Sendai virus inoculation and were disrupted as described in Fig. 1. The supernatant fluid contained 128 HA units/ml. The virus grown in chick allantois was diluted with BSS to contain 128 HA units/ml, and 0.05 ml of stroma was added to 10 ml of material. After 1 hr at 4 C, stroma was sedimented, fixed in 1% osmium tetroxide buffered to pH 7.4, and examined under an electron microscope.*

Synthesis of virus-induced RNA in Ehrlich ascites tumor cells. The incorporation of ^{3}H-uridine into RNA of actinomycin-treated uninfected cells was invariably confined to 4*S* RNA. No virus-induced RNA was detected in RNA preparations extracted from whole cells at 4 C (Fig. 3a), whereas the preparations of nuclear RNA extracted at 65 C consisted of several species of RNA. One of the peaks coincided with 18*S* ribosomal RNA presented in nuclei and revealed by optical density (8). Other peaks sedimented in the position of 14*S*, 22*S*, 35*S*, and 48*S* (Fig. 3b). In a number of experiments, two additional peaks sedimented as 28*S* and 10*S* were also found.

Resistance of infectivity to V antiserum. Disrupted cells and allantoic virus were sedimented at 40,000 $\times$ *g*; the pellets were suspended in BSS and used in neutralization tests with immune sera. It was found that V antiserum failed to affect the infectivity of cell extracts but significantly decreased the infectivity of allantoic virus, whereas hyperimmune serum abolished the infectivity of both allantoic virus and cellular extracts (Table 2).

The infectivity in cellular pellets was unaffected after treatment with pancreatic ribonuclease (10 μg/ml) for 1 hr at 37 C.

Electron microscopy. The pellets obtained by centrifugation at 40,000 $\times$ *g* of allantoic fluid from eggs infected with standard virus and from eggs infected with material from disrupted cells were resuspended in BSS and examined in an electron microscope. Typical virus particles were seen in both cases (Fig. 4A and B).

Structures similar to ribonucleoprotein of Sendai virus in mean diameter, diameter of canal, and pitch of spiral were observed in pellets of disrupted cells (Fig. 4C and D). Virus particles were never seen in this material.

The length of ribonucleoprotein structures varied considerably, from 100 A up to 10,500 A. The frequency distribution of lengths is shown in Fig. 5.

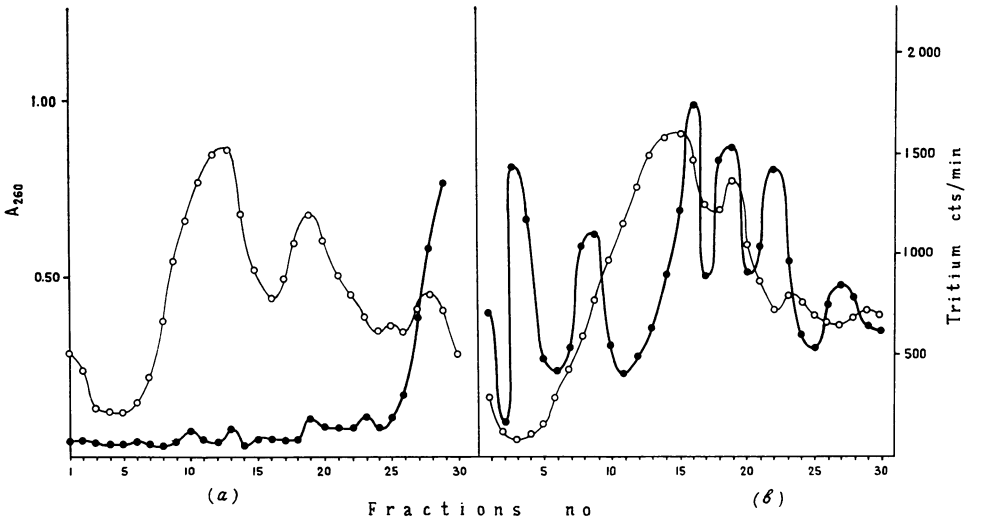

FIG. 3. *Distribution of radioactivity* (●) *and* A_{260} (○) *after sucrose density gradient centrifugation of nucleic acid from Sendai virus-infected Ehrlich ascites cells (a) extracted at 4 C and (b) extracted from cell nuclei at 65 C. Cell RNA was prepared as described in Materials and Methods, dissolved in 0.5 ml of Tris-chloride buffer containing 0.01 M Tris, 0.1 M NaCl, and 0.001 M ethylenediaminetetraacetate (pH 7.4), and layered over a 17-ml linear density gradient consisting of sucrose (5 to 20%) and containing the buffer described above. The RNA was centrifuged in the Superspeed 25 rotor at 20,000 rev/min and 4 C for 14 hr. Fractions were collected from the bottom of the tubes and used for determination of* A_{260} *and radioactivity.*

TABLE 2. *Effect of Sendai virus antisera on the infectivity of allantoic virus and extracts of Ehrlich infected cells*

Sample	EID_{50}/ml	
	Allantoic virus	Cellular extracts
Virus without serum.....	$10^{8.0}$	$10^{6.0}$
Virus + V antiserum....	$10^{5.5}$	$10^{6.0}$
Virus + whole serum....	$10^{4.0}$	$<10^{1.0}$

[a] The pellets of infected cellular extracts obtained by centrifugation at 40,000 × *g* were resuspended in BSS, and 10-fold dilutions were mixed with an equal volume of 1:2 V anti-serum and 1:10 whole serum before being inoculated into embryonated eggs.

DISCUSSION

The data presented in this paper provide evidence that the infectivity in Sendai virus-infected Ehrlich ascites tumor cells is due to the ribonucleoprotein of Sendai virus. It seems unlikely that the infectivity is associated with residual traces of virions because (i) viruslike particles were not seen either in disrupted cells concentrated by centrifugation or on the stroma of erythrocytes treated with cell extracts; (ii) viruslike particles were not detected in ultrathin sections of infected cells [the formation of inclusions containing ribonucleoprotein-like structures was observed in the cell cytoplasm in such sections (Guschin et al., *in press*)]; (iii) structures very similar to Sendai virus ribonucleoprotein were found in cell extracts, (iv) the infectivity was unaffected by V antiserum and was abolished by whole hyperimmune serum which contained antibodies to S antigen of Sendai virus. The infectivity could scarcely be due to free RNA because of its resistance to ribonuclease.

Ribonucleoprotein of Sendai virus released from infected ascites cells induces the production of mature viral particles in embryonated eggs. Its maximal length, 10,500 A, is in a good agreement with the data of Hosaka et al. (7), who found that Sendai virion ribonucleoprotein with a length of 10,000 A contains the whole viral genome. These authors suggested that ribonucleoprotein structures with a length of 20,000 to 40,000 A and greater observed in preparations from virions represent aggregated ribonucleoprotein structures.

Compans and Choppin (6) also determined the length of simian virus 5 ribonucleoprotein and concluded that the whole viral genome is 10,200 A long. However, these authors did not study the infectivity of their virus ribonucleoprotein.

The synthesis of virus-induced RNA consisting of the same components (18*S*, 22*S*, 35*S*, and 48*S*) as RNA induced in sensitive cells by Newcastle disease (2) and Sendai virions (1) was detected

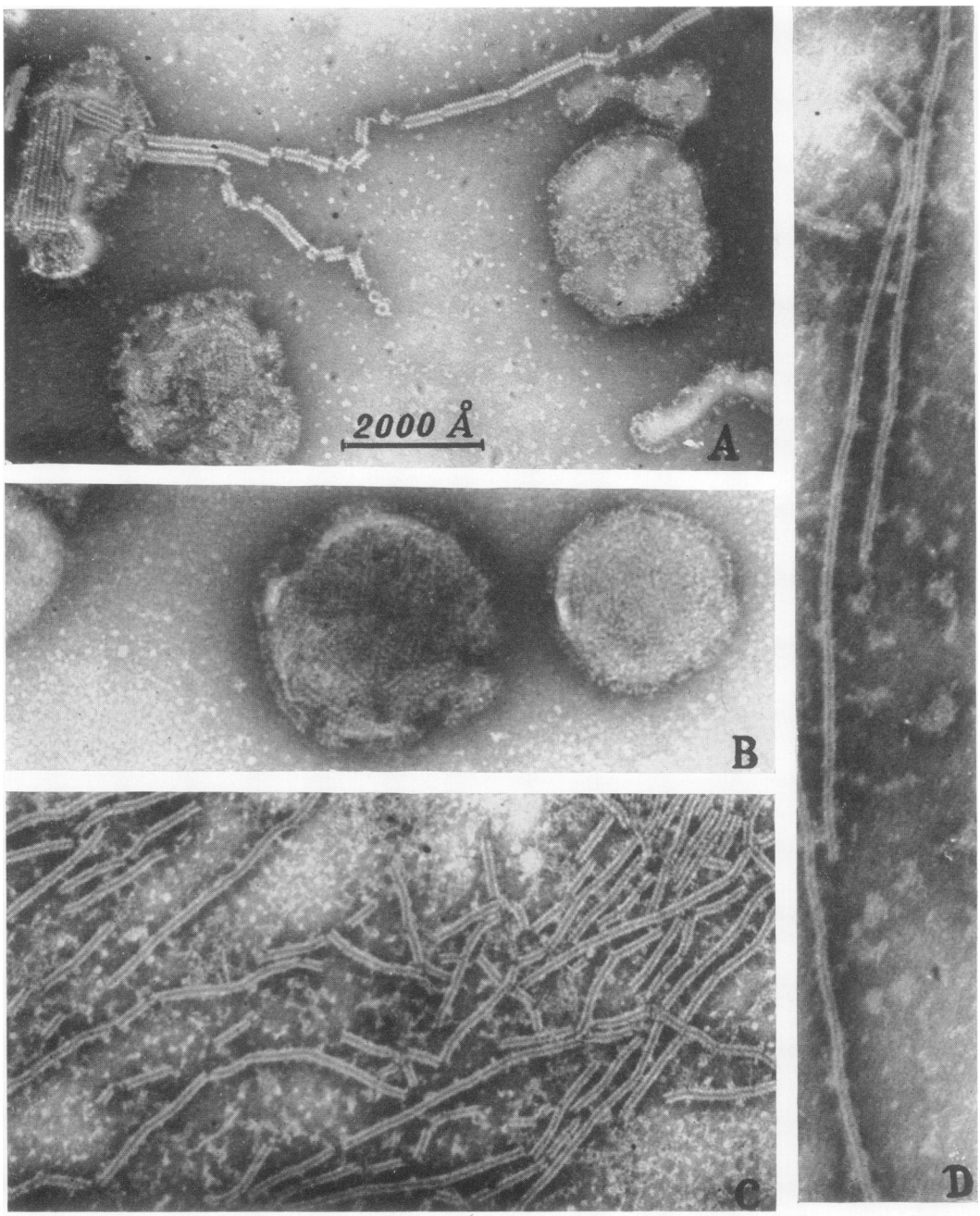

FIG. 4. *Electron micrographs of (a) virions from allantoic fluid of eggs inoculated with allantoic virus, (b) virions from allantoic fluid of eggs inoculated with material from disrupted cells of tumor-bearing Sendai virus-infected mice, (c, d) ribonucleoprotein structures in disrupted cells. Allantoic virus and cells disrupted by osmotic shock were centrifuged at 40,000 × g, and the pellets were suspended in 1 ml of BSS for examination in the electron microscope.*

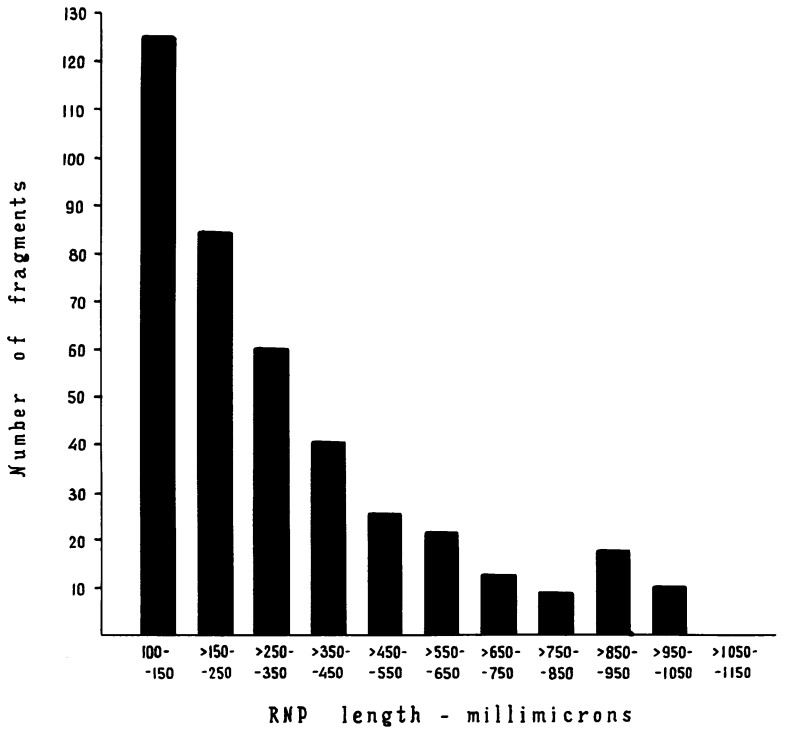

FIG. 5. *Frequency distribution of ribonucleoprotein (RNP) lengths in disrupted cells prepared as described in Fig. 4.*

in infected asciites tumor cells. The nuclear localization of vrus-induced RNA synthesis is in agreement with nuclear localization of parental Sendai virus RNA in Ehrlich tumor cells (3). The 14*S* component has not been previously described for paramyxoviruses; it is partly resistant to ribonuclease and appears earlier than other RNA species (A. G. Bukrinskaya, *in press*).

S antigen and hemagglutinins were also produced. Cellular HA resembled hemagglutinin subunits obtained after ether treatment of myxoviruses (14) in their ability to agglutinate guinea pig RBC. It is not clear why the main portion of the infectivity and hemagglutinins in cell extracts is sedimented at 10,000 and 40,000 $\times$ *g*. It may be explained by adsorption of virus substructures on cellular components.

The above data suggest that the components of Sendai virus are produced in virus-infected Ehrlich ascites tumor cells, but, for unknown reasons, they do not assemble to form mature virus particles.

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