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Interferon Action on Parental Semliki Forest Virus Ribonucleic Acid

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Actinomycin D-treated chick fibroblasts were infected with purified ^{32}P-labeled Semliki forest virus, and ribonucleic acid (RNA) was extracted after 1 or 2 hr. Within 1 hr, viral RNA forms sedimenting in sucrose gradients at 42*S*, 30*S*, and 16*S* were present. The 42*S* form corresponded to the RNA of the virion. The 16*S* form appeared to be a double-stranded template for the formation of new viral RNA, since nascent RNA was associated with it and the molecule could be heat-denatured and subsequently reannealed by slow cooling. Interferon treatment before infection, or puromycin (50 μg/ml) or cycloheximide (200 μg/ml) added at the time of virus infection, had no effect on the formation of the 30*S* RNA but inhibited the production of the 16*S* form. Several findings made it unlikely that these results were due to breakdown of parental RNA and reincorporation of ^{32}P into progeny structures. The results suggested that the mechanism of interferon action involves inhibition of protein synthesis by parental viral RNA, since a specific viral RNA polymerase had previously been demonstrated to be necessary for production of 16*S* RNA. No protein synthesis appears necessary for formation of 30*S* RNA from parental virus RNA.

Although the exact mechanism of action of interferon is still unknown, recent studies have suggested that it inhibits a step between transcription and translation of viral genetic information. Interferon blocks the formation of polysomes carrying vaccinia virus messenger ribonucleic acid (RNA), but not the production of this RNA (9). In vitro studies appeared to show inhibition of viral RNA-ribosome complex formation and of viral RNA translation in ribosomes from interferon-treated cells (1, 10; H. B. Levy, *personal communication*). In addition, during the log phase of virus growth, interferon treatment inhibits the formation of at least two virus specific proteins, the RNA polymerases of Mengo virus (13) and of Semliki forest virus (SFV) (16).

As noted by the authors of the last-mentioned studies, however, inhibition of polymerase production could have been due to depression either of virus protein or of virus RNA synthesis by parental virus early in the replicative cycle, since assays of polymerase activity were performed fairly late in the infectious cycle. Interferon treatment inhibits the formation of all species of progeny virus RNA (8). The present study, conducted early in the eclipse phase of the virus growth cycle in cells infected with radioactive virus, extends these observations by demonstrating that interferon inhibits the formation of the specific product of the SFV RNA polymerase (12), a ribonuclease-resistant RNA form into which parental virus RNA enters after infection. Inhibitors of protein synthesis had a similar effect. The formation by 42*S* parental virus RNA of a ribonuclease-sensitive 30*S* interjacent RNA, a form sedimenting in sucrose gradients between the parental RNA and the replicative RNA form (10), was unaffected by treatment with interferon or inhibitors of protein synthesis.

The results suggested that interferon blocks virus growth by inhibiting production by parental virus of the polymerase necessary for formation of the ribonuclease-resistant templates employed in the production of new viral RNA. This in turn suggested, in agreement with previous observations (1, 9, 10, 13, 16), that the mechanism for interferon action involves the inhibition of virus protein synthesis.

MATERIALS AND METHODS

Cells and viruses. The method of production of primary chick embryo fibroblast (CEF) cultures and of pools of SFV have been described (6).

Interferon. Partially purified interferon was produced by the method of Fantes (4). The pool used in these studies contained 10,000 units of interferon and

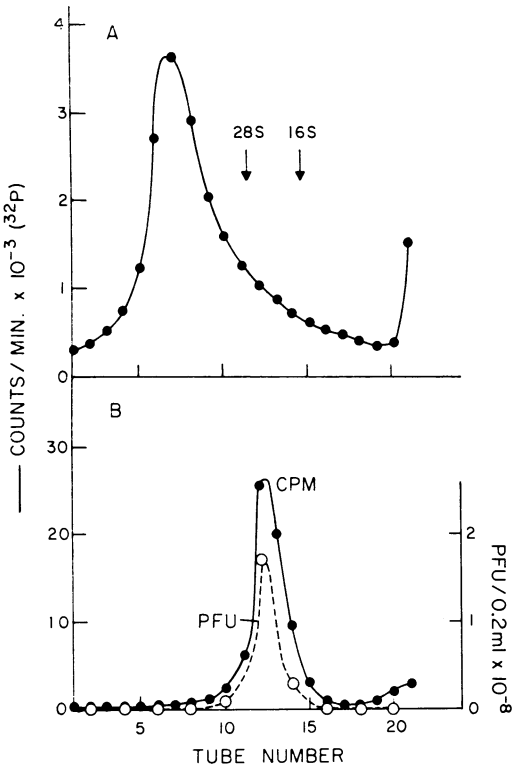

FIG. 1. *Purified Semliki forest virus (SFV) and viral RNA. SFV was purified as described in Materials and Methods. The final step (B) was sedimentation in a 6 to 30% sucrose gradient. Fractions were assayed for radioactivity and plaque-forming units (PFU) of infectivity. RNA was extracted from purified virus with phenol and 1% sodium dodecyl sulfate (A). The resulting preparation was sedimented in a 6 to 30% sucrose gradient at 100,000 × g for 3 hr, and the fractions were analyzed for acid-precipitable radioactivity. In all figures, the bottom of the gradient is to the left, and the designations 28S and 16S refer to the optical density (260 mμ) peaks of ribosomal RNA present in or added to the extract.*

179 μg of protein/ml; 1 unit of interferon inhibited SFV plaque development by 50%.

Preparation of purified virus. Actinomycin-treated cells were infected at a virus-cell multiplicity of 40:1 as previously described (6). One hour after warming to 37 C, 7 mc of ^{32}P was added to 50 ml of phosphate-free medium; 2 ml of this medium was added to each of 25 plastic petri dishes, containing about 2.5×10^7 cells each. After 7 hr of incubation at 37 C, the fluids were harvested. Virus was purified by the method of Cheng (2) with the addition of a terminal purification in a 6 to 30% sucrose gradient [0.1 M KCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris), and 0.001 M ethylenediaminetetraacetic acid (EDTA), *p*H 7.8] for 1 hr at 30,000 × *g* in an SW 39 rotor. The specific activity of the virus preparation was 1 count per min per 1,000 to 3,000 plaque-forming units.

Sucrose density gradient analyses. Analyses were carried out in a 6 to 30% gradient as previously described (5); the fractions were analyzed for acid-precipitable counts by the method of Dalgarno et al. (3).

RNA extraction. CEF cultures were infected with ^{32}P-labeled virus at a multiplicity of 80:1. After 1 or 2 hr, the cells were washed five times with 0.01 M phosphate-buffered 0.85% saline (*p*H 7.2) and once with 0.1 M NaCl-0.01 M sodium acetate (*p*H 5.1). The monolayers were removed from plates and suspended in the acetate buffer. Cells were disrupted with 1% sodium dodecyl sulfate (SDS) and twice extracted with an equal volume of acetate buffer-saturated phenol at 60 C. The aqueous layer was removed and extracted three times with ether; the ether was removed by bubbling nitrogen through the solution. Samples (0.1 to 0.4 ml) of these preparations were employed in sucrose density gradient analysis studies. All counting was performed in a Tri-Carb liquid scintillation counter (model 3003; Packard Instrument Co., Inc., Downers Grove, Ill.) as previously described (5). In some experiments, RNA was extracted with 5% SDS only (6, 15).

Chemicals. Puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Cycloheximide was from the Sigma Chemical Co., St. Louis, Mo. Actinomycin D was graciously donated by Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. ^{32}P was purchased as carrier-free $H_3{}^{32}PO_4$ from the New England Nuclear Corp., Boston, Mass.; and uridine-5-^3H (20 c/mmole) was from Schwarz Bio Research Inc., Orangeburg, N.Y.

RESULTS

Nature of radioactive virus products. In experiments employing radiolabeled parental virus, it is difficult to prove rigorously that a radiolabeled macromolecule which differs from the input virus is not that of progeny formed from breakdown of the parental macromolecule and reincorporation of its radiolabeled components or from ^{32}P contaminating the input virus. The following data tend to make these difficulties unlikely in this study.

The radiopurity of the input virus is indicated in Fig. 1B. Only one radioactive component was present in the final gradient step of the purification scheme employed. This corresponded to the peak of virus infectivity. Extraction of viral RNA also yielded a single peak of radioactivity, at about 42*S*, similar to that previously reported (6) for the RNA of SFV (Fig. 1A). Therefore, all input label was contained in virus.

When ^{32}P-SFV was incubated with actinomycin D-treated CEF at a multiplicity of 80:1 for 1 hr, about 10% of the label could not be removed by repeated washing. Of the cell-associated radioactivity, about 70% was extracted by the phenol-SDS method. Of this extracted radioactivity, a maximum of 10% was present as acid-soluble material in any experiment; about 10% was

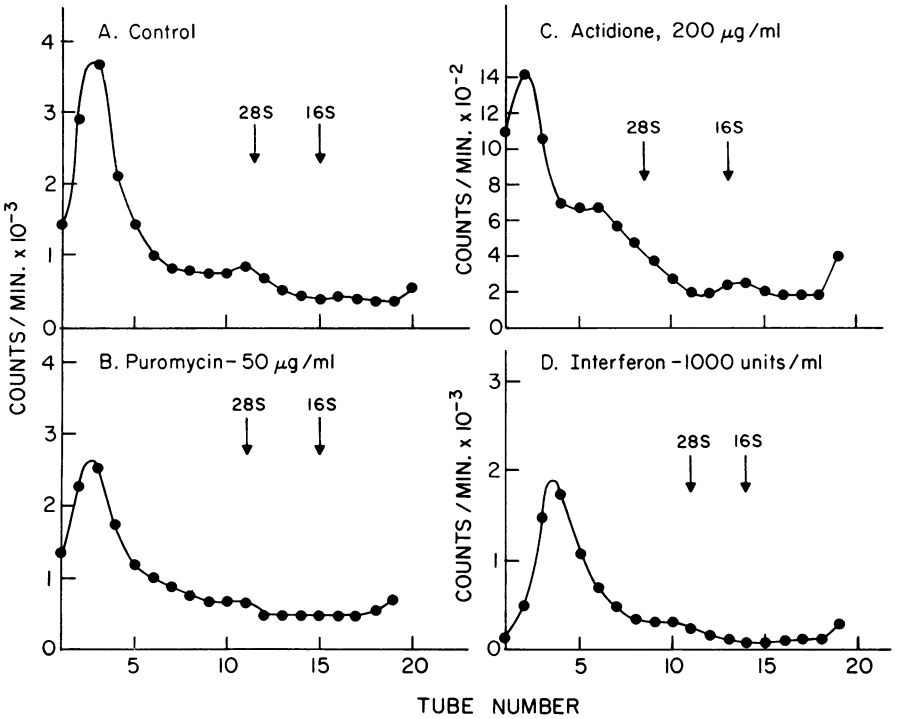

FIG. 2. *Sucrose density gradient analysis of RNA extracted from cells infected with ^{32}P-labeled Semliki forest virus (SFV). Actinomycin D-treated (0.5 μg/ml) monolayers containing about 5 × 10^7 chick embryo fibroblasts were infected with ^{32}P-SFV at a virus-cell multiplicity of 80:1. After 1 hr, RNA was extracted with phenol-SDS, and 0.1 ml of the extract was analyzed in a 6 to 30% sucrose density gradient by sedimentation (3 hr at 100,00 × g in an SW 39 rotor). Cycloheximide (C) and puromycin (B) were added with the virus. Cells were treated with interferon (D) for 6 hr before the addition of actinomycin D.*

usually present as acid-precipitable counts in low-molecular-weight products seen at the top of the gradient; at least 20% was present in the 35*S* to 16*S* region, the location of the 30*S* and 16*S* interjacent and replicative forms, respectively; and about 50% was present as 42*S* RNA, the position of parental virus RNA (Fig. 2 and 3). Previously published studies suggested that the latter arises from uneclipsed virus particles, since free 42*S* infectious RNA was found to pass rapidly into the virus replicative forms (5). Relatively few counts were broken down into acid-soluble molecules, and a very large fraction of the cell-associated counts was present in viral RNA forms. Incorporation into progeny of radioactivity released by breakdown of parental RNA would have to be remarkably efficient to explain these findings.

In addition, when the parental virus was labeled with both ^{32}P and ^{3}H, the ^{32}P-^{3}H count ratio of the parental virus RNA was 4.2. The ^{32}P-^{3}H ratio of the 30*S* to 15*S* fractions, which contained the viral replicative forms (*see* below), was 4.3. Because of the various metabolic pools into which the labeled RNA fragments from virus RNA breakdown would have to enter, the isotopes might not be reincorporated into new structures in the same ratio as they appear in the parental structure.

Infection of chick cells with radioactive SFV gave rise to viral RNA with a prominent 42*S* peak (Fig. 2 and 4). Labeling viral progeny RNA with isotope for any length of time during the first 2 hr of infection gave rise to an entirely different pattern of radioactive viral RNA, similar to that seen in the tritiated products in Fig. 4.

These findings make it likely that the ^{32}P-labeled replicative and interjacent RNA forms seen in these studies contained intact parental virus RNA, and that they were not produced by breakdown of parental RNA and incorporation of the radiolabel into progeny RNA.

Effect of interferon and inhibitors of protein synthesis on replicative RNA formation. CEF monolayers were incubated for 6 hr with 1,000 units of interferon per ml; then, along with controls, they were treated with 0.5 μg of actinomycin D per ml for 2 hr. This latter treatment inhibited cell RNA

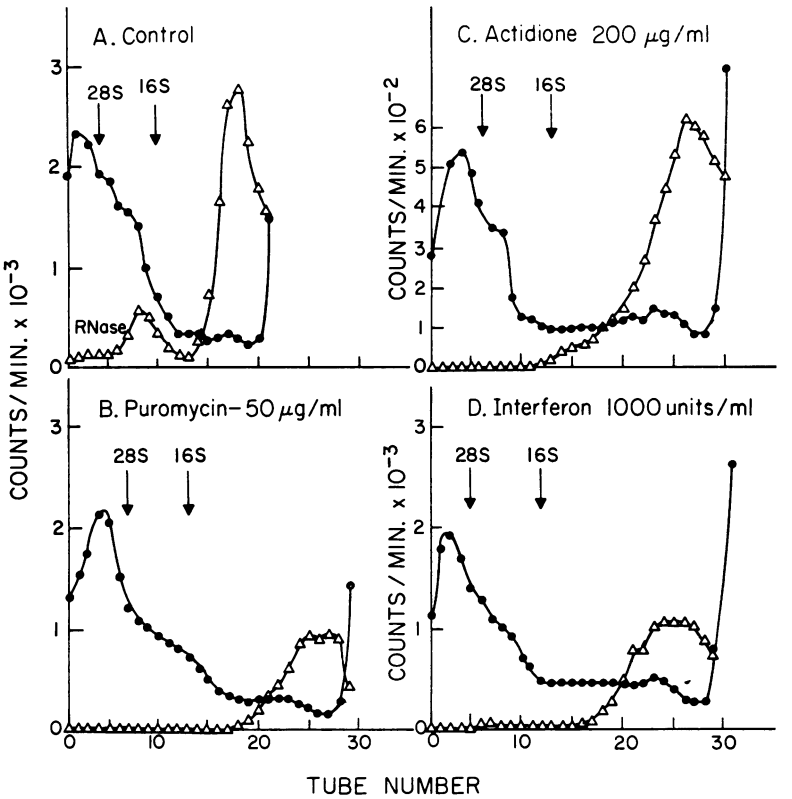

FIG. 3. *Sucrose density gradient analysis of RNA extracted from cells infected with ^{32}P-labeled Semliki forest virus. Monolayers were prepared, treated, and infected as in Fig. 2. RNA was extracted with 5% SDS (6); 0.4 ml of the extract was layered over a 6 to 30% sucrose gradient and sedimented (3 hr at 200,000 × g in an SW50 rotor). Extracts were treated with ribonuclease (1 μg/ml, 10 min, 37 C, 0.1 M NaCl) before sedimentation analysis.*

synthesis by more than 95%. The cells were then infected for 1 or 2 hr with ^{32}P-SFV at a multiplicity of infection of 80:1. The virus was suspended in 0.5 ml of Eagle's medium containing no additive, or puromycin (50 μg/ml), or cycloheximide (200 μg/ml). This concentration of puromycin depressed protein synthesis in SFV-infected CEF cells by more than 98% (7); cycloheximide inhibited protein synthesis in SFV-infected CEF cells by about 95%. After the 1 or 2 hr of infection, RNA was extracted and analyzed by sucrose density gradient sedimentation. Only the results obtained with cells infected for 1 hr are shown, but the results with cells infected for 2 hr were very similar, both qualitatively and quantitatively.

When RNA was layered over sucrose gradients and sedimented by centrifugation for 3 hr at 100,000 × *g*, the profiles of radioactivity from the untreated controls and from cells treated with interferon, puromycin, or cycloheximide appeared to be similar (Fig. 2). Sedimentation at 200,000 × *g* for 3 hr, combined with ribonuclease treatment before sedimentation (Fig. 3), revealed some differences between the groups. All preparations had prominent ribonuclease-sensitive peaks at about 30*S*. The control also had a ribonuclease-resistant peak sedimenting at about 16*S*, but the interferon-treated cells and the cells treated with puromycin or cylcoheximide lacked the 16*S* ribonuclease-resistant peak. All of the radioactivity was rendered acid-soluble by 14 hr of treatment with 0.3 M KOH at 37 C.

Actinomycin D treatment inhibits the development of the antiviral action of interferon (17). As shown in Table 1, incubation of cells with actinomycin D *before* treatment with interferon prevented the inhibitory action of interferon on the passage of parental virus into the ribonuclease-resistant form. In this experiment, some ribonuclease-resistant RNA was found in the cells treated with interferon before actinomycin D was added. The amount of this RNA was much less than that seen in controls not receiving interferon or receiving interferon after incubation with actinomycin D.

The 30*S* RNA would appear to be the interja-

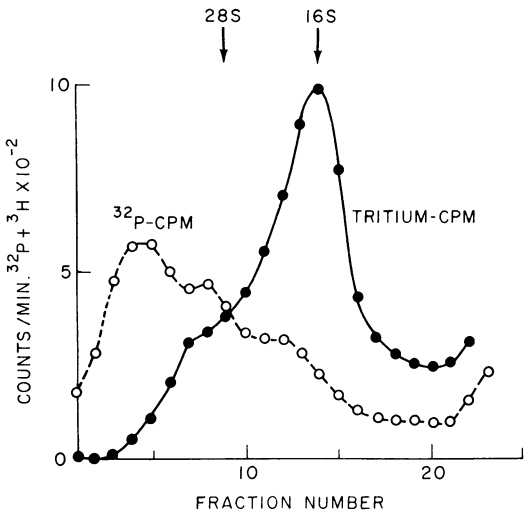

FIG. 4. *Pulse label with tritium in cells infected with ^{32}P-Semliki forest virus (^{32}P-SFV). Cells were prepared and infected with ^{32}P-SFV as described in Fig. 2. After 1 hr of infection, 100 μc of uridine-5-^{3}H was added for 2 min. RNA was extracted with phenol and 1% SDS and subjected to sucrose density gradient analysis (6 to 30% sucrose) at 100,000 × g for 3 hr in an SW 39 rotor. ^{32}P and ^{3}H were simultaneously counted in a liquid scintillation spectrometer.*

TABLE 1. *Actinomycin D effect on interferon inhibition of ribonuclease-resistant RNA formation*[a]

Treatment	Ribonuclease-resistant RNA
Actinomycin D only	470
Interferon, followed by actinomycin D	70
Actinomycin D followed by interferon	380

[a] Chick embryo fibroblasts (5×10^{7}) were treated with 0.5 μg (per ml) of actinomycin D for 2 hr and then with 1,000 units of interferon for 6 hr. The cells were infected at a virus-cell multiplicity of 80:1 with ^{32}P-SFV for 1 hr. RNA was extracted, treated for 10 min at 37 C with 1 μg (per ml) of ribonuclease, and subjected to sucrose density gradient analysis. The total number of acid-precipitable counts in the 16*S* region of the gradient, minus a background of 10 counts per min, is reported. Controls were treated with actinomycin D only, or with actinomycin D after 6 hr of interferon treatment.

cent RNA form previously described for SFV (6). The function of this RNA is not known.

Nature of the 16S RNA. The ribonuclease resistance of the 16*S* material suggested that it was similar to the replicative form described for many single-stranded RNA viruses, including SFV (11, 14). A 2-min pulse with uridine-5-^{3}H showed tritium counts predominantly in the 16*S* region (Fig. 4), indicating association of nascent RNA with structures sedimenting in this area of the gradient.

TABLE 2. *Denaturation and annealing of ribonuclease-resistant Semliki forest virus RNA*

Treatment of RNA	Ribonuclease-resistant RNA (counts/min)	
	^{3}H-labeled	^{32}P-labeled
Ribonuclease[a]	580	101
Denaturation, ribonuclease	37	25
Denaturation, annealing, ribonuclease	304	97

[a] Samples from the 16*S* peaks of Fig. 4 were dialyzed against 0.01 M Tris-0.003 M EDTA (pH 7.2) (portions were then denatured by treatment at 102 C for 4 min, and immediately cooled in ice water. Annealing took place in sealed tubes slowly cooled from 90 to 20 C. Annealing was carried out in 0.1 M NaCl. Ribonuclease (1 μg/ml) treatment was at 37 C for 10 min in 0.1 M NaCl. After ribonuclease treatment, RNA was precipitated with 2.5% perchloric acid in the presence of 2 mg of yeast RNA. Precipitates were collected on membrane filters, washed with 2.5% perchloric acid, and counted in a liquid scintillation counter.

When the ^{32}P- and ^{3}H-labeled 16*S* RNA (Fig. 4, fractions 12–14) was dialyzed against 0.01 M Tris and 0.003 M EDTA and then denatured by boiling for 4 min, most of the ribonuclease-resistant counts became ribonuclease-sensitive (Table 2). Annealing of the denatured RNA by slow cooling from 90 to 20 C over a 6-hr period partially restored the ribonuclease resistance.

DISCUSSION

Both interferon and inhibitors of protein synthesis had no effect on the formation of the interjacent 30*S* RNA form. The nature of this form is unknown, but it has been found to have the same base composition as does the 42*S* form present in the virion or in SFV-infected cells (5, 15). The interjacent RNA has little or no infectivity (6, 15). Also, thermal denaturation of both 42*S* RNA and the ribonuclease-resistant 16*S* RNA gives rise to the interjacent RNA (R. M. Friedman, *unpublished data*). The production of interjacent RNA may be related to a conformational change in the 42*S* viral RNA, similar to those proposed by Spirin (18), the interjacent RNA being a functioning form of viral RNA produced during the course of infection. It is also possible that interjacent

forms could arise from a break in the parental RNA structure.

The results also indicate that interferon treatment, like treatment with cycloheximide or puromycin, inhibits the passage of parental virus RNA into the 16S ribonuclease-resistant form. Previous reports and the present study indicate that this form is a replicative template on which new viral RNA is synthesized, since very short pulses of radioactive RNA precursors are always associated with this form (6). The physical properties of the ribonuclease-resistant form, and its base composition, also suggest that it is indeed a replicative form of virus RNA consisting of either a base-paired double strand or a base-paired double strand plus nascent chains of new viral RNA (5; R. M. Friedman, *unpublished data*).

Synthesis of ribonuclease-resistant viral RNA almost certainly involves production of a new protein, the viral RNA polymerase. Since the ribonuclease-resistant RNA is the only demonstrated product of this enzyme (12), the inhibition by interferon of 16S RNA formation from parental RNA suggests that interferon inhibits the formation of this enzyme by parental RNA. This is consistent with the idea that virus protein synthesis in general is inhibited by interferon.

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