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Inhibition of Interjacent Ribonucleic Acid (26S) Synthesis in Cells Infected by Sindbis Virus

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The interrelationship of viral ribonucleic acid (RNA) and protein synthesis in cells infected by Sindbis virus was investigated. When cultures were treated with puromycin early in the course of infection, the synthesis of interjacent RNA (26S) was preferentially inhibited. A similar result was obtained by shifting cells infected by one temperature-sensitive mutant defective in RNA synthesis from the permissive (29 C) to the nonpermissive (41.5 C) temperature. Under both conditions, the viral RNA produced appeared to be fully active biologically. Once underway, the synthesis of viral RNA in wild-type Sindbis infections did not require concomitant protein synthesis.

The synthesis of infectious ribonucleic acid (RNA) in cells infected by a group A arbovirus, Western equine encephalomyelitis virus, has been shown to require concomitant protein synthesis. When cells actively synthesizing viral RNA were treated with puromycin, further production of infectious RNA promptly ceased (15). Similar results were obtained when the synthesis of functional protein was blocked with fluorophenylalanine (17). The most likely explanation for these findings is the lability of some enzyme involved in viral RNA synthesis.

We were surprised, therefore, that a somewhat comparable experiment performed with most temperature-sensitive (TS) mutants of Sindbis virus, a related group A arbovirus, did not yield a similar result. In these studies, mutants defective in viral RNA synthesis (RNA^-) were allowed to infect cultures at a permissive temperature for several hours and then the cultures were shifted to a nonpermissive temperature. This temperature shift should effectively prevent further expression of a gene involved in viral RNA synthesis, thus paralleling the more general effects of an inhibitor of protein synthesis. Most RNA^- mutants tested showed unimpaired synthesis of viral RNA and normal production of virus at the nonpermissive temperature (2, 12; *unpublished results*). Thus, at least one enzyme involved in the synthesis of viral RNA did not appear to be labile.

Therefore, we reinvestigated the interrelationship of viral RNA and protein synthesis in cells infected by wild-type Sindbis virus. This problem is now known to be complicated by the fact that cells infected with group A arboviruses contain at least four types of virus-specific RNA: (i) 42S, ribonuclease-sensitive RNA like that found in the virion; (ii) 20S, ribonuclease-resistant, replicative form RNA; (iii) partially ribonuclease-resistant, replicative intermediate RNA; (iv) 26S, ribonuclease-sensitive RNA that has been given the convenient name "interjacent RNA" (14).

The first three species of RNA find their parallels in infections by a variety of animal and bacterial RNA viruses, and their functions have been reasonably well established. In contrast, the function (if, indeed, there is one) of the interjacent RNA is unknown. Recent evidence indicates that certain conditions allow an interconversion of interjacent and virion RNA (16). Hence, these species may represent different conformational states of the same molecule (14, 16). Elucidation of the conditions that determine the distribution of virus-specific RNA between these two species may be useful in assessing the function of the interjacent RNA. While examining the interrelationship of viral RNA and protein synthesis, we encountered two treatments that result in a preferential inhibition of the synthesis of interjacent RNA.

MATERIALS AND METHODS

Tissue culture and viruses. Primary chick embryo fibroblast cultures were prepared as described by Pfefferkorn and Clifford (10). The isolation and characterization of TS mutants of Sindbis virus have been reported (3). Mutant TS-24 is an RNA^- mutant derived from the HR strain of Sindbis virus, which is wild-type with respect to TS mutations.

Sedimentation analysis was performed on 15 to 30% linear gradients of glycerol dissolved in a buffer con-

taining 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (*p*H 7.5), 0.001 M ethylenediaminetetraacetic acid (EDTA), and 0.25% sodium dodecyl sulfate (SDS). A 0.3-ml sample was layered onto a 4.4-ml gradient and was centrifuged in a Spinco SW-50 rotor at 165,268 × *g* for 2 hr at 23 C. The fractions formed by puncturing the bottom of the tube were treated with 0.3 N trichloroacetic acid in the presence of yeast RNA carrier. The resulting precipitates were collected on membrane filters (Millipore Corp., Bedford, Mass.), washed with 0.3 N trichloroacetic acid, rinsed with ethyl alcohol, and dissolved in 5 ml of Bray's scintillation solution (1) for determination of radioactivity.

Total and ribonuclease-resistant labeled RNA were assayed in SDS extracts of infected cells as described previously (11).

Infectious RNA. Infected cells were washed twice with cold phosphate-buffered saline lacking calcium and magnesium (7), rinsed once with 0.05 M EDTA, and dissolved in 0.05 M EDTA containing 1% SDS and 1% bentonite. An equal volume of phenol saturated with 0.05 M EDTA was added and the mixture was shaken vigorously for 5 min at room temperature. The aqueous phase was extracted with ether and diluted in ice-cold 1 M NaCl containing 0.1 M $MgCl_2$, 0.1% bentonite, and 0.001 M phosphate buffer (*p*H 7.6). Inocula of 0.5 ml were allowed to adsorb to monolayers previously washed twice with cold Hanks' balanced salt solution and were treated with 0.6 M NaCl for 5 min. After 30 min at room temperature, the cultures were drained, overlayered with agar, and incubated at 29 C for 3 days to allow formation of plaques.

RESULTS

Inhibition of protein synthesis early and late in the course of infection. First, we investigated the requirement for protein synthesis to initiate and maintain the synthesis of viral RNA. Cultures were treated with puromycin at 1, 3, 5, and 7 hr after infection in the presence of actinomycin D. Viral RNA synthesis was measured by the incorporation of ^{3}H-uridine added at the same time as the puromycin. Replicate cultures that received no puromycin served as controls. Blocking protein synthesis 1 hr after infection almost completely inhibited subsequent viral RNA synthesis (Fig. 1A). Probably, the amount of viral RNA polymerase synthesized during the first hour of infection was simply insufficient for detectable incorporation of ^{3}H-uridine. However, once underway, viral RNA synthesis was quite insensitive to puromycin (Fig. 1B–D).

We have already presented evidence that the virus-specific RNA synthesized in the presence of puromycin added 5 hr after infection is normal with respect to its sedimentation pattern (13). Moreover, preliminary experiments have shown no significant effect of puromycin on the infectious RNA content of the cells. Thus, the synthesis of viral RNA does not appear to require concomitant protein synthesis. Sindbis virus appears to differ from Western equine encephalomyelitis virus in this respect.

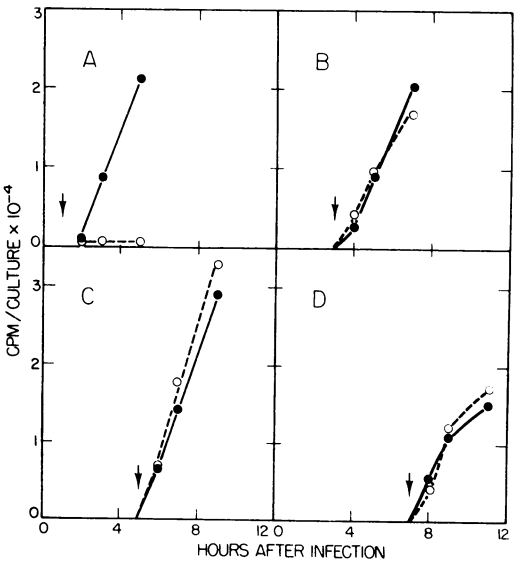

FIG. 1. *Effect of puromycin on the synthesis of virus-specific RNA. Infected cultures were incubated at 37 C in Eagle's medium containing actinomycin D (1 μg/ml) and 3% rabbit serum. At the times indicated (A, B, C, D), one-half of them were treated with 50 μg of puromycin per ml (○), whereas the others served as controls (●). Drug-treated and control cultures were labeled continuously after puromycin addition with ^{3}H-uridine (3.3 mc/mmole; 1 μc/ml) and were removed at specified intervals for determination of labeled RNA.*

Inhibition of protein synthesis during the exponential phase of viral RNA synthesis. Experiments in which protein synthesis was inhibited by puromycin 1.5 hr after infection yielded a paradoxical result that is best described by reference to Fig. 2. Puromycin had little effect on infectious RNA synthesis for the following 2.5 hr, although production of virus-specific RNA, as measured by incorporation of ^{14}C-uridine in the presence of actinomycin D, was markedly reduced. Note that these data do not indicate the production of noninfectious RNA but rather the production of more infectious RNA per virus-specific RNA.

A possible explanation of this paradox lay in the observation that the interjacent RNA found in cells infected by other group A arboviruses has little or no infectivity (14). This is also true of Sindbis virus infections. Whereas interjacent RNA was the major species of virus-specific RNA in Sindbis-infected cells, nearly all of the infectivity was found with the 42*S* peak (Fig. 3).

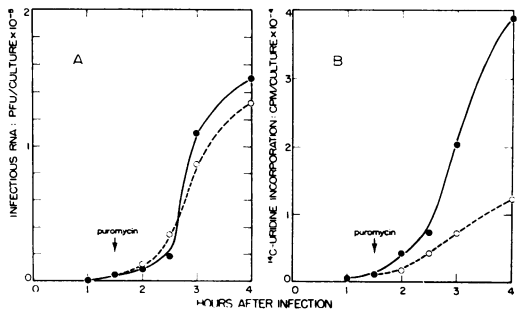

FIG. 2. *Effect of puromycin on the synthesis of virus-specific RNA. Infected cultures were incubated as described in the legend to Fig. 1. A. Synthesis of infectious RNA after the addition of puromycin 1.5 hr after infection. Infectious RNA was assayed as described in Materials and Methods. B. Incorporation of ^{14}C-uridine into virus-specific RNA. Drug-treated and control cultures were labeled continuously from 1.5 hr after infection with ^{14}C-uridine (2 μc/ml; 1 μg/ml) and were removed at the indicated intervals for determination of labeled RNA. Symbols: ○, puromycin-treated; ●, control.*

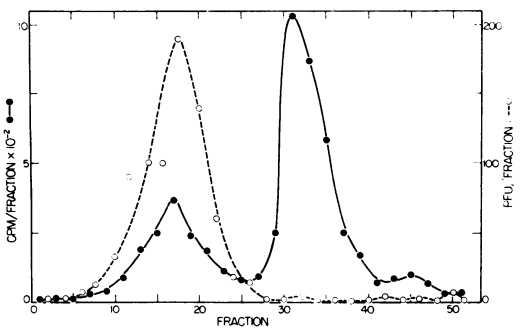

FIG. 3. *Association of infectious RNA with labeled RNA. Infected cultures were incubated as described in the legend to Fig. 1. At 4 hr after infection, the cells were labeled with ^{3}H-uridine (20 c/mmole; 10 μc/ml) for 1 hr. The SDS-extract of the infected cells was layered on 15 to 30% glycerol-SDS gradients and was centrifuged in a Spinco SW39 rotor at 117,851 × g for 3.5 hr at 23 C. Fractions of 3 drops were collected and analyzed, alternately, for infectious RNA (○) and labeled RNA (●) as described in Materials and Methods. Sedimentation was from right to left.*

The relevance of this pattern of viral RNA synthesis can be seen in Fig. 4, which records a gradient analysis of labeled virus-specific RNA extracted from puromycin-treated and control cells. These data confirm that puromycin added 1.5 hr after infection inhibits ^{14}C-uridine incorporation into virus-specific RNA. However, the inhibition is confined to the noninfectious interjacent RNA, thus explaining the lack of effect of puromycin on the appearance of infectious RNA.

One could argue that since protein synthesis has been nonspecifically inhibited, the nucleocapsid and envelope proteins were not available for virus assembly. Therefore, virion RNA accumulated within the drug-treated cells and masked a decreased rate of 42S RNA synthesis. However, in control cultures, only 6.2% of the total RNA labeled during this interval appeared in virus. This could account for less than one-third of the virion RNA present in the puromycin-treated cells. Thus, the rate of 42S RNA synthesis, if decreased at all, was only slightly affected, whereas the synthesis of interjacent RNA was truly inhibited to a much greater extent.

This phenomenon is not a consequence of the particular mechanism of inhibition of protein synthesis, since the same preferential inhibition of interjacent RNA synthesis was seen in cells treated with cycloheximide (10 μg/ml) 1.5 hr after infection.

Preferential inhibition of interjacent RNA synthesis: temperature-shift experiments. The preceding studies showed that inhibition of protein synthesis early in the course of infection produced a distorted pattern of viral RNA synthesis with abnormally low levels of interjacent RNA. We hypothesized that this did not result from the general inhibition of protein synthesis but rather

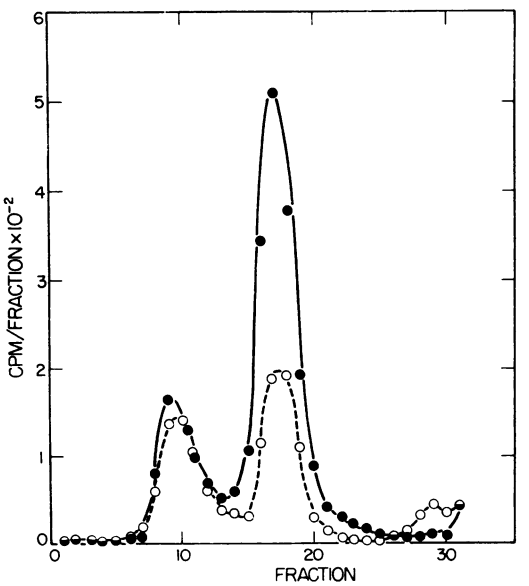

FIG. 4. *Sedimentation analysis of virus-specific RNA from puromycin-treated and control cells infected with Sindbis virus. A portion of each 4-hr sample from Fig. 2B was sedimented as described in the legend to Fig. 3 and the acid-insoluble radioactivity of each fraction was determined. Symbols: ○, puromycin-treated; ●, control.*

from the lack of a specific gene function. If this were true, a temperature-shift experiment with a TS mutant defective in that function should yield a similar effect. The results with puromycin indicated that an early function was involved; thus, we examined a series of RNA$^-$ mutants. Most of these showed no significant difference between the shifted and the unshifted cultures. However, one of our RNA$^-$ mutants proved to have a similar and, indeed, an even more extremely distorted pattern of viral RNA synthesis.

Cells infected with mutant TS-24 were incubated at 29 C and were shifted from the permissive to the nonpermissive temperature of 41.5 C at 4.25 hr after infection. By this time, viral RNA synthesis had just begun. The monolayers were pulse-labeled with ^{3}H-uridine for 40 min at 4.75, 5.75, and 6.75 hr, respectively. The labeled RNA was analyzed by sedimentation in 15 to 30% glycerol-SDS gradients. The synthesis of interjacent RNA was preferentially inhibited under these shift-up conditions (Fig. 5). The control 4:1 ratio of 26*S* to 42*S* RNA evident in the 29 C cultures decreased to 0.94, 0.70, and 0.64 progressively in the shifted cells.

Cultures infected with the HR strain of Sindbis virus and treated in an identical manner did not show this effect (Fig. 6). Although again there was a general, nonspecific increase in ^{3}H-uridine incorporation in the shifted cultures, the sedimentation pattern of the virus-specific RNA remained about the same.

We also measured ribonuclease-resistant virus-specific RNA in shifted and unshifted cultures. Table 1 shows no difference between the control and experimental groups under any conditions. These data contradict our earlier report (12), and we have no clear explanation for the varying results. In the present case, however, the alteration in viral RNA synthesis in cells infected by mutant TS-24 and shifted to the nonpermissive temperature seems to be specific for the interjacent RNA.

Infectious RNA from cells infected by TS-24. Our first clue in understanding the effect of early puromycin addition on wild-type infections was an increase in the plaque-forming units/counts per minute ratio, which indicated that the viral RNA had greater than normal infectivity. If shifting cultures infected by mutant TS-24 to a nonpermissive temperature were a true parallel of the puromycin treatment, the shifted cultures should also show increased infectivity of virus-specific RNA.

This prediction was studied in an experiment that compared cultures infected by wild-type virus or mutant TS-24. Half of the cultures were shifted to 41.5 C at 4.25 hr after infection, whereas the others remained at 29 C. All cultures were incubated with medium containing ^{3}H-uridine and were harvested for titration of infectious RNA and determination of incorporated radioactivity (Table 2). There was no significant difference in the infectivity of virus-specific RNA extracted from the cells infected by the wild type at either temperature or from the cells infected by TS-24 at 29 C. However, the mutant-infected culture shifted to 41.5 C showed an increase of about threefold. This approximates the value predicted by the sedimentation data of Fig. 4 and

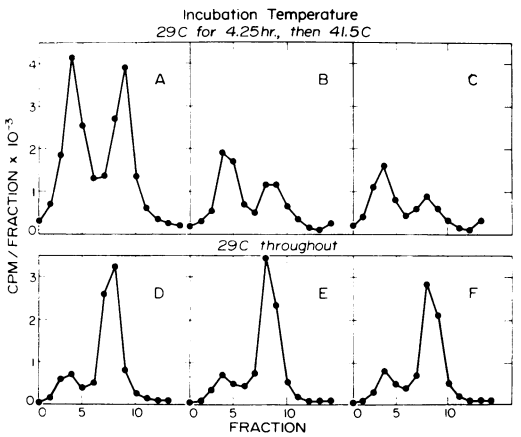

FIG. 5. *Sedimentation analysis of virus-specific RNA from cells infected with mutant TS-24. Cultures with 5 PFU adsorbed per cell were incubated at 29 C in Eagle's medium containing 1 μg of actinomycin D/ml and 3% rabbit serum. At 4.25 hr after infection, the monolayers were washed and incubated in fresh, prewarmed medium at either 29 or 41.5 C. Cells were pulse-labeled with ^{3}H-uridine (20 c/mmole; 20 μc/ml) for 40 min at 41.5 or 29 C, respectively, beginning 4.75 (A, D), 5.75 (B, E), and 6.75 hr (C, F) after infection. Sedimentation was from right to left.*

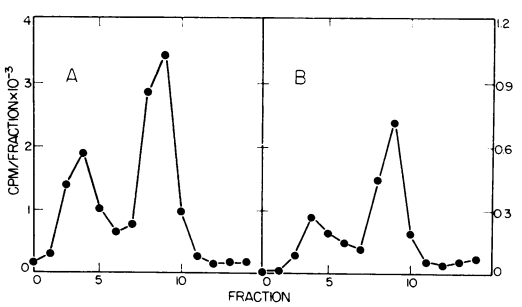

FIG. 6. *Representative sedimentation analysis of virus-specific RNA from cells infected with wild-type Sindbis virus. Procedure was identical to that described for Fig. 5. A. Virus-specific RNA labeled at 41.5 C. B. Virus-specific RNA labeled at 29 C.*

TABLE 1. *Per cent of ^{3}H-uridine incorporated into ribonuclease-resistant RNA under shift-up conditions*

Incubation temp	Cells infected with	Interval after shift-up		
		30–70 min	90–130 min	150–190 min
29 C	HR	7.1	4.6	4.2
	TS-24	6.7	5.1	4.9
29 C for 4.25 hr, then 41.5 C	HR	4.3	5.2	4.9
	TS-24	5.3	5.0	5.0

TABLE 2. *Infectivity of virus-specific RNA*[a]

Incubation temp	Wild-type virus	Mutant TS-24
29 C	0.6	0.7
29 C for 4.25 hr, then 41.5 C	0.7	2.0

[a] Cultures with 5 plaque-forming units adsorbed per cell were incubated at 29 C in medium containing 1 μg of actinomycin D/ml and 3% rabbit serum. The cells were labeled with ^{3}H-uridine (20 c/mmole; 5 μc/ml) for 2.25 hr and then were incubated in fresh, prewarmed, radioactive medium at either 29 or 41.5 C for an additional 70 min. Infectious RNA and acid-insoluble radioactivity were measured as described in Materials and Methods. Values are expressed as plaque-forming units/counts per minute.

serves as strong evidence that the 42S RNA made under shift-up conditions is biologically active.

DISCUSSION

Our results show that Sindbis viral RNA synthesis, once it has achieved its maximal rate, is not dependent on concomitant protein synthesis. However, inhibition of protein synthesis early in the course of infection by antibiotic treatment or temperature shift distorts the subsequent distribution of virus-specific RNA between the virion and interjacent forms.

When interjacent RNA was first found in cells infected by various group A arboviruses, it seemed likely that this component was merely a degradation product of the more rapidly sedimenting virion RNA. However, the use of a variety of extraction procedures of proven reliability has established that the interjacent RNA is not an artifact. Its probable nature was disclosed by the elegant experiments of Sreevalsan et al. (16), who examined certain extreme physical conditions that permitted the interconversion of the interjacent and virion RNA. The suggestion that these two types of viral RNA are simply different physical forms of the same molecule is only the first step in defining the function, if indeed there is one, of the interjacent RNA. Note that every species of virus-specific RNA found in infected cells need not play a significant role; the replicative form is thought now to be a useless by-product. However, the fact that the interjacent RNA represents a substantial fraction of the viral RNA synthetic activity throughout the infection indicates that it does have some function in the production of virus (8, 11, 14). Sonnabend et al. (14) suggested that the interjacent RNA acts as messenger. Alternatively, it may aid in the assembly of the viral nucleocapsid or have some unidentified regulatory function.

Currently, little is known of the biosynthesis of interjacent RNA. Even the sequence of appearance of interjacent and virion RNA is uncertain. This question cannot be answered easily by the use of pulse-labeling techniques, since short pulses preferentially label the replicative intermediate, much of which sediments in the same region as the interjacent RNA.

The conditions used by Sreevalsan et al. (16) for in vitro interconversion of interjacent and virion RNA are far removed from those normally existing within cells. Thus, if these two species of RNA are alternative physical forms of the same molecule, their natural intracellular interconversion is probably mediated by some other molecule, presumably a protein, which could act catalytically or stoichiometrically. A relative deficiency of this hypothetical "conversion protein" could explain our results. Suppose that the initial product of viral RNA synthesis is virion RNA, part of which is subsequently converted to interjacent RNA. At 1.5 hr after infection, the cells might contain sufficient RNA synthetic enzymes to maintain normal RNA synthesis, but insufficient "conversion protein." Our results of inhibiting protein synthesis with puromycin early in the course of infection would thus be explained. Similarly, if the TS defect of mutant TS-24 lay in this "conversion protein," the temperature-shift experiments would parallel those seen with early inhibition of protein synthesis. This speculation has one interesting implication: mutant TS-24 is phenotypically RNA$^-$; that is, it exhibits an almost total defect in viral RNA synthesis at the nonpermissive temperature. Hence, it is defective in a function essential for viral RNA synthesis, and this line of reasoning points to an essential role for interjacent RNA.

There are, of course, several presumptions inherent in this scheme. Although mutant TS-24 is phenotypically RNA$^-$, it may also have other TS

mutations in late functions that actually determine the conformation of the viral RNA. We have genetic evidence for three late functions (4, 5). Two of these have been associated with specific virus-determined proteins (6). Mutant TS-24 complements representatives of each of these three complementation groups and, thus, probably is not defective in any function that we have identified. Moreover, mutants with lesions in these late functions can show, if anything, a relative deficiency in the synthesis of 42*S* RNA (18). Another case which may also affect late as well as early proteins is treatment of infected cells with low doses of interferon (9). Here, too, the synthesis of virion RNA is inhibited to a greater extent than that of interjacent RNA. Nevertheless, other late functions may well not have been discovered as yet, and mutant TS-24 may possess a second mutation affecting one of these.

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