Temperature-sensitive Mutants of Sindbis Virus: Biochemical Correlates of Complementation

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Temperature-sensitive mutants of Sindbis virus fail to grow at a temperature that permits growth of the wild type, but when certain pairs of these mutants, mixed together, infect cells at that temperature, viral growth (i.e., complementation) occurs. The yield from this complementation, however, is of the same order of magnitude as the infectivity in the inoculum. Since in animal virus infections the protein components of the virion probably enter the cell with the viral nucleic acid, it was necessary to demonstrate that the observed complementation required synthesis of new viral protein and nucleic acid rather than some sort of rearrangement of the structural components of the inoculum. To demonstrate that complementation does require new biosynthesis, three biochemical events of normal virus growth have been observed during complementation and correlated with the efficiency of viral growth seen in complementation. These events include: (i) entrance of parental viral ribonucleic acid (RNA) into a double-stranded form; (ii) subsequent synthesis of viral RNA; and (iii) synthesis and subsequent incorporation of viral protein(s) into cell membranes where they were detected by hemadsorption. Although the infecting single-stranded RNA genome of the wild type was converted to a ribonuclease-resistant form, the genome of a mutant (*ts-11*) incapable of RNA synthesis at a nonpermissive temperature was not so converted. However, during complementation with another mutant also defective in viral RNA synthesis, some of the RNA of mutant *ts-11* was converted to a ribonuclease-resistant form, and total synthesis of virus-specific RNA was markedly enhanced. The virus-specific alteration of the cell surface, detected by hemadsorption, was also extensively increased during complementation. These observations support the view that complementation between temperature-sensitive mutants and replication of wild-type virus are similar processes.

We have previously described complementation between certain pairs of temperature-sensitive (*ts*) mutants of Sindbis virus (1, 2). In those reports, complementation was measured by comparing the yield produced by cells infected by two mutants together, at a nonpermissive temperature, with the sum of the yields produced by the same two mutants grown separately.

Under ideal circumstances, a complementing pair of mutants might produce 100 times as much virus as either mutant grown separately, a complementation yield representing 1 to 10% of the titer produced by wild-type virus grown under similar conditions. However, since a high multiplicity of infection was required for suc-

cessful complementation (2), the inoculum used to produce the mixed infection was usually of the same order of magnitude as the complementation yield.

Thus, it could be argued that complementation between *ts* mutants occurred wholly or partly through a reshuffling of viral components introduced into the cell by the inoculum. This explanation could theoretically be tested by determining whether labeled precursors were incorporated into the virus released as a result of complementation. However, the low yields of virus in complementation experiments would complicate its purification.

We have therefore used several biochemical methods to examine the biosynthetic consequences of complementation. (As in the usual complementation test, these biochemical methods were used to compare cells infected at a nonpermissive temperature by single *ts* mutants and

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by pairs of *ts* mutants.) The following important events in viral replication were studied: (i) entrance of the parental viral ribonucleic acid (RNA) into a double-stranded form; (ii) subsequent synthesis of viral RNA; and (iii) synthesis and subsequent incorporation of viral protein(s) into the cellular membrane where they were detected by hemadsorption. In each case, the magnitude of these biochemical changes was compared with the efficiency of complementation as measured by the usual viral titrations.

MATERIALS AND METHODS

Tissue culture and viruses. Methods for the growth and titration of Sindbis virus in primary chick embryo fibroblast cultures have been already described (10).

The *ts* mutants were isolated and characterized as described by Burge and Pfefferkorn (1, 2). These *ts* mutants grow well at 30 C, a permissive temperature, but fail to grow at 40 C, a nonpermissive temperature. All *ts* mutants fall into one of two major categories: (i) those defective in viral RNA synthesis at nonpermissive temperatures (*RNA*⁻), and (ii) those able to make viral RNA but defective in some other maturational step at nonpermissive temperatures (*RNA*⁺). All experiments described here used *RNA*⁻ mutants. When a comparison with the wild-type virus was required, we used the HR strain of Sindbis virus (1) from which the *ts* mutants had been derived. This strain has no *ts* mutations and will be simply called the wild type in this report.

Complementation. This was carried out as described previously (2). Briefly, to test two mutants for the ability to complement, stocks of both mutants were used to infect doubly a monolayer with about 20 plaque-forming units (PFU) of each per cell. This monolayer was incubated at 40 C, and the yield of virus produced between the 4th and the 6th hr of infection was compared with the sum of the yields produced by the two mutants in single infections. The ratios of these two measurements is called the complementation level; for a pair of complementary mutants this level may be from three to 100, depending on several variables, including multiplicity of infection, incubation temperature, and the mutant pair under study (2).

Actinomycin D-resistant ³H-uridine incorporation. Measurement was in a conventional way. Cells were exposed to 1 μ g of actinomycin D per ml (Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.) throughout the infection. At a specified time, fresh prewarmed medium containing actinomycin and ³H-uridine (20 c/mmole, 0.03 mc/ml; Schwarz Bio Research Inc., Orangeburg, N.Y.) was added; 2 hr later the monolayers were chilled, rinsed twice with phosphate-buffered saline (4) lacking calcium and magnesium and containing 0.001 M ethylenediaminetetraacetic acid (EDTA), and dissolved at room temperature in 2 ml of the same solution containing 1% sodium dodecyl sulfate (SDS). A sample of this solution was precipitated with 0.3 N trichloroacetic acid with 200 μ g of yeast RNA as carrier. The rest was

stored frozen at -60 C for later sedimentation analysis.

Sedimentation analysis. It was carried out on 15 to 30% linear gradients of glycerol dissolved in a buffer containing 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane, pH 7.5, 0.001 M EDTA, and 0.2% SDS. A 0.24-ml sample was layered onto a 4.5-ml gradient and centrifuged in a Spinco SW-39 rotor at 38,000 rev/min for 2.75 hr at 25 C. Fractions were collected by puncturing the tube and collecting drops. All fractions were precipitated with 0.3 N trichloroacetic acid with 200 μ g of yeast RNA as carrier, collected on membrane filters (Millipore Corp., Bedford, Mass.), rinsed with ethyl alcohol, and dissolved in 2 ml of polyether scintillation solution for determination of radioactivity. When a sedimentation marker was required, a small amount of chick fibroblast monolayer previously labeled for 3 days with ¹⁴C-uridine and dissolved in SDS was added to the sample. A correction was made for ¹⁴C counts in the ³H channel.

Fate of the infecting viral RNA. This was traced by use of virus labeled with ³H-uridine (20 c/mmole; Schwarz Bio Research). To prepare this virus, cultures were infected at 28 C in a medium containing 0.25 μ g of actinomycin D per ml and 125 μ Ci of ³H-uridine per ml. The low temperature allowed *ts* mutants and the wild type to be labeled under identical conditions. The presence of actinomycin assured that most of the ³H-uridine would be incorporated into virus-specific RNA. After 16 hr of incubation, the virus in the medium was partially purified by one cycle of differential centrifugation. The resulting preparation was radiochemically homogeneous by several criteria including sucrose gradient centrifugation, resistance of the radioactivity to ribonuclease, and ultracentrifugal homogeneity of the labeled RNA extracted from the virus. The virus (about 3×10^{-3} counts per min per PFU) was suspended in medium containing 100 μ g of uridine per ml to eliminate incorporation of any residual ³H-uridine from the growth medium.

This labeled virus was allowed to adsorb to monolayer cultures for 1 hr at 4 C. The cultures were then rinsed and incubated at the desired temperature. At intervals, these cultures were examined for conversion of the infecting viral RNA to a ribonuclease-resistant form by use of a modification of the method described by Lodish and Zinder (8). Rinsed cultures were dissolved in 3.5 ml of a solution containing 0.07 M NaCl, 0.001 M KCl, 0.001 M EDTA, and 0.1% SDS. The resulting SDS extract was centrifuged at $2,000 \times g$, and samples of the supernatant fluid, which contained nearly all of the radioactivity, were diluted 10-fold into water and buffer (8) containing 25 μ g of pancreatic ribonuclease per ml. These solutions were incubated at room temperature for 1 hr; then the ribonuclease-resistant material was precipitated with 0.3 N trichloroacetic acid, and its radioactivity was determined in a scintillation counter. In each case, the ribonuclease-resistant radioactivity from the sample diluted into water was subtracted from the value obtained in buffer as recommended by Lodish and Zinder (5). Only a negligible fraction of the infecting viral

RNA was ribonuclease-resistant immediately after adsorption. Under conditions that permitted viral replication, the infecting viral RNA was partially converted to a form that was ribonuclease-resistant only at high salt concentrations and had the sedimentation properties reported for double-stranded arbovirus RNA (6, 11). [The characterization of the labeled virus and details of the intracellular conversion of its RNA to a double-stranded form will be presented elsewhere (Pfefferkorn, Burge, and Coady, *Virology*, *in press*).]

Hemadsorption. The capacity of monolayer cultures to adsorb goose red blood cells was determined by a modification of the hemagglutination test for Sindbis virus (3). To allow quantitative estimation of hemadsorption, the red cells were labeled with ^{51}Cr as in a modification of the usual hematological procedure (5). A 7.5% suspension of gander red cells in barbiturate buffer containing glucose and gelatin (3) was incubated with $\text{Na}_2^{51}\text{CrO}_4$ ($0.125 \mu\text{C}/\text{ml}$) for 1 hr at room temperature. The labeled red cells were then washed twice with the same buffer and once with a 1:1 mixture of the borate and pyrophosphate buffers (final pH 5.8) used in hemagglutination titration (3, 10). The cells were finally suspended at a 3% concentration in the borate-pyrophosphate mixture and used within the next few hours.

Monolayers to be assayed for hemadsorption were chilled in an ice bath, drained and rinsed twice with borate-pyrophosphate buffer. A 0.3-ml volume of the ^{51}Cr -labeled red cell suspension (about 7×10^{-4} counts per min per cell) was then pipetted onto the monolayer, which was held at 4 C with occasional rocking to redistribute the red cells. After 45 min, the monolayers were drained and rinsed gently four times with 2.5-ml volumes of cold borate-pyrophosphate buffer.

The monolayer, together with any adsorbed red cells, was dissolved in 1 ml of 0.3 N NH_4OH , added to 10 ml of polyether scintillation solution (7), and counted in a scintillation counter. Quenching by the dissolved monolayer was a constant factor in all samples. Hemoglobin from cultures positive for hemadsorption did not quench significantly. The hemadsorption appeared to be a virus-specific reaction, for it was reduced to the control level by prior treatment of the infected monolayer with rabbit anti-Sindbis virus serum that had been exhaustively adsorbed with uninfected cells.

RESULTS

Complementation between RNA^- mutants. Complementation between the RNA^- *ts* mutant *ts-6* and other RNA^- mutants is quite efficient, in terms of both the complementation level achieved and the absolute titer of virus produced (9a). Hence, if complementation did involve an increase in viral RNA and protein synthesis, these increases should be easily detectable. Table 1 records the results of a complementation test involving mutants *ts-6*, -11 and -24. In each case, the single infections by mutants produced only about 0.01% the infectivity

TABLE 1. *Complementation as measured by infectivity^a*

Inoculum	Virus produced between 4 and 6 hr (PFU/ml)	Complementation level ^b	Per cent of wild-type yield
Wild-type	1.9×10^9		100.0
<i>ts-6</i>	32.0×10^4		0.016
<i>ts-11</i>	2.0×10^4		0.001
<i>ts-24</i>	10.0×10^4		0.005
<i>ts-6</i> $\times$ <i>ts-11</i>	12.0×10^7	353	6.3
<i>ts-6</i> $\times$ <i>ts-24</i>	8.0×10^7	190	4.2

^a Complementation was carried out as described in Materials and Methods. Data in Tables 1 and 2 were obtained in the same experiment.

^b The complementation level is the titer produced by the double infection divided by the sum of the titers produced by the two mutants in single infections.

produced by the wild type. The complementing pairs, however, produced about 5% of that yield. These results are similar to those presented elsewhere (2). With the exception of the study of the fate of mutant viral RNA, which required the use of labeled parental virus, all of the biochemical assays of complementation were done with replicate cultures infected and incubated with those described in Table 1. Thus, the biochemical results can be directly compared with this bioassay of complementation.

Fate of parental viral RNA during complementation. The replication of a single-stranded RNA virus probably requires the formation of a virus-determined enzyme that directs synthesis of new "minus" strands, complementary to the viral RNA "plus" strand (8, 9, 12). Newly synthesized "minus" strands are found together with parental "plus" strands in a double-stranded structure that can be identified by its resistance to ribonuclease.

The RNA of wild-type Sindbis virus labeled with ^3H -uridine was partially converted to a ribonuclease-resistant form very early in the course of infections at 30 or at 40 C. This form had a sedimentation constant of about 20S and several of the other properties expected for double-stranded RNA (Pfefferkorn, Burge, and Coady, *in press*).

We attempted to correlate complementation with the fate of labeled viral RNA in single and mixed infections. Figure 1 records the conversion of labeled input RNA of mutant *ts-11* to a ribonuclease-resistant form in single infection at 30 C and in single and mixed infection with mutant *ts-6* at 40 C. In single infections, conversion of the RNA of mutant *ts-11* to a ribonuclease-resistant form was normal at 30 C

FIG. 1. Intracellular conversion of labeled infecting viral RNA to a ribonuclease-resistant form early in the course of infection. Labeled virus was prepared and the fate of its RNA was traced as described in the Materials and Methods. Symbols: Δ , ^3H -labeled wild-type infection at 40 C; $\bullet$, ^3H -labeled mutant *ts-11* infection at 40 C; $\blacktriangle$, ^3H -labeled mutant *ts-11* infection at 30 C; $\circ$, ^3H -labeled mutant *ts-11* and unlabeled mutant *ts-6* mixed infection at 40 C.

but completely defective at 40 C. Since the wild type showed normal conversion of its RNA to a ribonuclease-resistant form at 40 C, mutant *ts-11* appears to be defective in this initial step of viral replication at the nonpermissive temperature. This defect was partially relieved in the mixed infection with unlabeled mutant *ts-6*, another *RNA*⁻ mutant. The efficiency of conversion of mutant viral RNA to a ribonuclease-resistant form, about 5% of the value obtained for the mutant at 30 C or the wild type at 40 C, was comparable to the efficiency of complementation between these two mutants (Table 1).

Viral RNA synthesis during complementation. We next sought to correlate complementation with net synthesis of viral RNA. Since actinomycin D was used to suppress cellular RNA synthesis, incorporation of ^3H -uridine was largely into viral-specific RNA. Table 2 shows that cultures infected by any of the three *RNA*⁻ mutants at a nonpermissive temperature incorporated no more ^3H -uridine than did the uninfected control. This, of course, is the definition of an *RNA*⁻ mutant. However, the cultures infected by the mutants together showed a

substantial increase in incorporation of ^3H -uridine to values 8 to 10% of the wild-type level. These values are only slightly greater than the complementation measured by virus production (Table 1).

TABLE 2. Complementation as measured by hemadsorption and ^3H -uridine incorporation^a

Inoculum	Hemadsorption ^b	^3H -uridine incorporation ^b
Uninfected	103	4.9×10^3
Wild-type	12,481	325.0×10^3
<i>ts-6</i>	112	5.2×10^3
<i>ts-11</i>	99	5.1×10^3
<i>ts-24</i>	125	6.8×10^3
<i>ts-6</i> $\times$ <i>ts-11</i>	5,419	25.5×10^3
<i>ts-6</i> $\times$ <i>ts-24</i>	4,680	32.5×10^3

^a Data in Tables 1 and 2 were obtained in the same complementation experiment.

^b Measurement of hemadsorption and ^3H -uridine incorporation is described in Materials and Methods. Both are expressed in counts per minute per monolayer.

Sedimentation properties of the RNA labeled during complementation. Virus-specific RNA produced during arbovirus infections is known to sediment in two major peaks, at 42S and 26S (6, 11). Both RNA species are ribonuclease sensitive. A third, ribonuclease-resistant peak, sedimenting at 20S, may be resolved by digesting away the ribonuclease-sensitive species (6, 11). Since the physiological significance of these RNA's, particularly the 26S species, is not certain, it was of interest to examine the sedimentation properties of viral RNA produced during complementation between *ts RNA*⁻ mutants.

Samples of SDS-dissolved monolayers from the complementation described in Tables 1 and 2 were analyzed on linear gradients of glycerol-SDS. Figure 2 shows the pattern seen in the wild-type infection at 40 C. No attempt was made to resolve the minor 20S ribonuclease-resistant fraction.

In Fig. 3, the ^3H -uridine incorporated by the *RNA*⁻ mutants in single infections (in the presence of actinomycin D) can be compared to the ^3H -uridine incorporated by an uninfected monolayer. In all samples, most of the labeled RNA appeared in a slowly sedimenting band of about 4S. A small fraction of the radioactivity sedimented more rapidly than the 16S marker, but there was no indication of any preferential synthesis of the virus-specific 42 and 26S RNA. Thus, the *RNA*⁻ mutants in single infections at 40 C stimulate a negligible amount of virus-specific RNA synthesis.

FIG. 2. Sedimentation analysis of ^3H -uridine incorporated into a monolayer infected with wild-type virus. A monolayer was infected with a high multiplicity of virus in the presence of $1\text{ }\mu\text{g}$ of actinomycin D/ml. At 4 hr after infection, ^3H -uridine (0.03 mc/ml , 20 c/mmole) was added and after a 2-hr labeling period the monolayer was dissolved in SDS buffer. A 0.24-ml sample with added ^{14}C -labeled chick cell RNA for sedimentation markers was centrifuged in a Spinco SW-39 rotor at $38,000\text{ rev/min}$ for 2.75 hr at $25\text{ }^\circ\text{C}$. Symbols: ●, ^3H -labeled virus-specific RNA; ○, cellular RNA. This sample and those analyzed in Fig. 3 and 4 were obtained from the complementation experiment recorded in Tables 1 and 2.

Sedimentation patterns for labeled RNA from cells infected by complementing pairs of RNA^- mutants appear in Fig. 4. These patterns were similar to those seen in the wild-type infection; the additional radioactivity incorporated in mixed infections (Table 2) all appeared in the 26S and 42S regions. Proportionately more 42S RNA was seen in the mixed infection $ts-6 \times ts-24$ than in the wild-type infection. The significance of this observation is unknown.

Synthesis of viral protein during complementation. The synthesis of the viral protein active in hemagglutination was measured by determining the capacity of the infected cells to adsorb goose red cells. To obtain quantitative results, we used ^{51}Cr -labeled red cells and measured the radioactivity that could not be washed from the monolayer.

The ability of virus-infected monolayers to adsorb ^{51}Cr -labeled red cells was a very sensitive indicator of viral growth. As Fig. 5 shows, this alteration in cell surface was detectable at least 1 hr before a linear rate of virus release was

FIG. 3. Sedimentation analysis of ^3H -uridine incorporated into an uninfected monolayer and into monolayers infected with single RNA^- ts mutants. Actinomycin D, $1\text{ }\mu\text{g/ml}$, was present in all cultures. See legend of Fig. 2 for conditions of sedimentation.

achieved. The number of red cells adsorbed per monolayer continued to increase until the 4th hr of infection. At this point, the ratio of labeled red cells to monolayer cells was about 3, and saturation of the available surface probably prevented the adsorption of more red cells onto monolayers incubated for longer times.

On the basis of this kinetic experiment, we measured hemadsorption at 6 hr after infection in complementation experiments. Table 2 shows that cultures infected singly with the mutants at the nonpermissive temperature had only the minimal hemadsorbing capacity of uninfected cultures. In contrast, mixed infection by complementing pairs of mutants induced a hemadsorbing capacity that was about 50% that induced by wild-type infection, indicating extensive synthesis of at least one viral protein during complementation.

DISCUSSION

In complementation between bacteriophage conditional-lethal mutants, the viral coat of the infecting phage particle remains outside the cell and could not possibly be used in the formation

FIG. 4. Sedimentation analysis of ^3H -uridine incorporated into monolayers treated with actinomycin D, 1 $\mu\text{g}/\text{ml}$, and infected in a mixed manner with pairs of *ts* mutants. See legend of Fig. 2 for conditions of sedimentation.

of new infectious particles. In animal virus infections, however, all viral components probably enter the cell, and might conceivably be used in the assembly of new particles. It is therefore important to demonstrate that complementation with *ts* mutants of an animal virus involves new synthetic events and that these events are similar to, or reconcilable with, the events observed in a wild-type infection.

In this report, we have shown that complementation between *ts* mutants is accompanied by three biochemical events: (i) an increase in the amount of mutant viral RNA that is converted to a ribonuclease-resistant form; (ii) an increase in the synthesis of virus-specific RNA; and (iii) an increase in the amount of the viral hemagglutinating protein in the cell membrane, reflected by an increase in hemadsorption. Each of these increases is commensurate with, though not precisely proportional to, the increase in titer of viral infectivity.

FIG. 5. Kinetics of appearance of hemadsorption capacity and viral infectivity. A series of monolayers was infected with wild-type Sindbis virus (about 20 PFU/cell) and incubated at 36 C. At intervals, monolayers were withdrawn; the infectivity in the medium was assayed (○) and the hemadsorbing capacity of the monolayer (●) was measured as described in Materials and Methods.

The only serious discrepancy lies in the measure of hemadsorption. Here the cell infected by the mixed mutants showed about 50% of the hemadsorbing capacity of the cells infected by the wild-type virus, although the virus yield from mixed infections was only 5% of the wild-type yield. It must be remembered, however, that only a finite number of red cells can be adsorbed to a monolayer of fixed surface area, regardless of the concentration of hemadsorbing protein in cell membranes. Once this saturation point is reached, the number of red cells adsorbed to the monolayer is no longer proportional to the amount of hemadsorbing protein in the membrane (Fig. 1). In the wild-type infection here compared to complementation, this point has been reached, and thus it is not possible to determine the relative amounts of hemadsorbing protein produced in the two situations.

Using the three criteria listed above, we conclude that complementation requires new biosynthetic activity and involves the same sequence of events that characterize the growth of wild-type Sindbis virus.

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