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Synthesis of RNA by Mutants of Vesicular Stomatitis Virus (Indiana Serotype) and the Ability of Wild-Type VSV New Jersey to Complement the VSV Indiana *ts* G I-114 Transcription Defect

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The ability of certain vesicular stomatitis virus (VSV; Indiana serotype) temperature-sensitive (*ts*) mutants to synthesize intracellular viral complementary RNA (vcRNA) at permissive or nonpermissive temperatures for productive infections has been investigated. Mutants belonging to complementation groups II, III, and V synthesize RNA at nonpermissive temperature in amounts essentially equivalent to that obtained at permissive temperatures. Mutant *ts* G I-114 possesses a thermolabile transcriptase and does not synthesize vcRNA at 40°C; however, mutants *ts* O I-5, O I-53, O I-78, and O I-80 possess thermostable transcriptases that are capable of some vcRNA synthesis at 40°C. All five group I mutants are defective in their secondary transcription ability at 40°C. Wild-type VSV New Jersey virus is able to complement the transcription defect of *ts* G I-114 at 40°C. This complementation is inhibited by puromycin, suggesting that a viral gene product of VSV New Jersey (e.g., its transcriptase or a transcriptase component) is involved. Mokola virus is not able to complement the *ts* G I-114 defect, although Mokola does synthesize vcRNA in infected cells (in the presence or absence of cycloheximide).

The vesicular stomatitis virus (VSV) subgroup of rhabdoviruses includes the serotypes VSV Indiana, VSV New Jersey, VSV Argentina, VSV Brazil, and Cocal viruses (4). Their interrelationships have been demonstrated by comparative complement fixation and neutralization of infectivity tests (2, 4, 18). Chandipura and Piry viruses are possibly also related to this subgroup, although they are perhaps more distant relatives (4, 18).

It has been shown that certain temperature-sensitive (*ts*) mutants of VSV Indiana and Cocal viruses can complement each other (15); however, it has been reported that this complementation is not symmetrical and involves only group III and V mutants of VSV Indiana (i.e., mutant groups that correspond to the VSV M and G proteins, respectively; 10). No complementation has been observed between VSV New Jersey mutants and mutants of VSV Indiana or Cocal viruses or between mutants of Chandipura and VSV Indiana viruses (12, 14). These observations have been supported, in part, by the detection of some sequence homology between Cocal and VSV Indiana genomes but little homology, if any, between either virus and VSV New Jersey, Chandipura virus, or Piry virus (16). In addition, it has been shown that transcriptase enzyme components of VSV Indiana and Cocal viruses can transcribe and render infectious RNA-ribonucleoprotein complexes of either virus but not the RNA-ribonucleoprotein complexes of Chandipura or VSV New Jersey viruses, and vice versa (1).

The object of the experiments reported here was to determine if any function relating to the intracellular transcription of VSV Indiana can be complemented by a VSV New Jersey coinfection. Since viral-complementary RNA (vcRNA) of VSV Indiana can be distinguished by hybridization procedures from that of VSV New Jersey, the effect of wild-type VSV New Jersey upon the intracellular synthesis of vcRNA by *ts* mutants of VSV Indiana was determined at permissive or nonpermissive temperatures for the growth of the *ts* mutant.

MATERIALS AND METHODS

Viruses. The following *ts* mutants of VSV Indiana were used: *ts* G I-114, *ts* O II-52, *ts* O III-23, *ts* O IV-100, and *ts* O V-45. The Orsay mutants (O) were provided by Anne Flamand, Université de Paris Sud, Orsay, France. The Glasgow mutant (G) was obtained from P. Marcus, University of Connecticut,

Storrs. Wild-type VSV New Jersey originally came from R. Simpson, Rutgers University, New Brunswick, N.J., and Mokola virus was kindly provided by H. F. Clark of the Wistar Institute, Philadelphia, Pa.

Virus growth condition. All viruses were grown in BHK-21 cell monolayers at 33°C (multiplicity of infection [MOI], approximately 0.01 PFU/cell) using Eagle minimum essential medium containing 5% (vol/vol) fetal calf serum and 20 μCi of [^{3}H]uridine per ml (5, 6). Except for Mokola virus, the viruses were recovered from the cell supernatant fluids 36 h postinfection and purified by polyethylene glycol-NaCl precipitation, followed by brief centrifugation in a gradient of sucrose and eventual agarose column chromatography in Eagle minimum essential medium as described previously (6). Mokola virus was also used to infect cells at an MOI of 0.01 PFU/cell; however, 3 days postinfection the initial unlabeled infected cell supernatant fluids were discarded and replaced with fresh medium containing [^{3}H]uridine. Virus was subsequently harvested 2 days later and purified as described previously (6). We have observed (unpublished data) that 10-fold higher yields of infectious virus are obtained for both rabies and Mokola viruses by this protocol, the reason for which is not known.

Infection of cells, plaque assays, and measurement of vcRNA. Monolayers of 3×10^6 BHK-21 cells were infected with purified, [^{3}H]uridine-labeled virus at input MOIs of between 1 and 1,000 PFU/cell (see Results). After an absorption period of 30 min at 4°C, the inoculum virus was removed, 5 ml of prewarmed Eagle minimum essential medium containing 5% (vol/vol) fetal calf serum was added, and the monolayers were incubated at the desired temperatures. One hour postinfection, the supernatant fluids (containing desorbed virus) were replaced with fresh prewarmed media and the incubations were continued (5, 6). At the indicated times, monolayers were washed with low-salt buffer (0.15 M NaCl, 0.01 M Tris buffer, pH 7.4), and the cells were recovered and extracted for nucleic acids after addition of sodium dodecyl sulfate (1% final concentration), diethylpyrocarbonate (0.1% final concentration), and 1 volume of phenol mixture (500 g of phenol-80 ml of *m*-cresol-0.5 g of 8-hydroxyquinoline-200 ml of chloroform). The total extract was briefly sonicated to shear DNA, which would otherwise interfere with the phase separation obtained during subsequent centrifugation. After a second phenol extraction, nucleic acids were precipitated from the aqueous phase by addition of 2 volumes of ethanol. After a second alcohol precipitation, the nucleic acids were dissolved in 0.2 ml of 0.4 M NaCl, 0.01 M Tris buffer, pH 7.4 (previously saturated with diethylpyrocarbonate), and stored at −20°C. Except for omission of the sonication step, labeled viral RNA was extracted from unused ^{3}H-labeled virus (as well as from the recovered inocula) by a similar procedure. The specific activities of the different viral RNA samples (5, 6) were found to be essentially similar ($\sim 10^6$ cpm/μg of RNA). Other than for Mokola virus, assays of infectious virus in the various virus inocula, or in the supernatant fluids from infected cells, used BHK-21 cell monolayers as described elsewhere (6). Mokola virus was similarly assayed, using, however, a hamster cell line obtained from T. Wiktor of the Wistar Institute.

The amount of vcRNA in cell extracts was determined as described previously (6). In brief, duplicate one-tenth (or smaller) samples of cell nucleic acids were annealed with 5,000 or 10,000 cpm of [^{3}H]RNA, and, post-annealing, the [^{3}H]RNase resistance was determined after digestion with both RNases A and T_1 (6, 16). The net increases, corrected for RNase-resistant core values of viral RNA annealed to uninfected cell extracts (usually about 1 to 3% of the added ^{3}H label), and the self-annealed values in some cases (see figure legends), were used to determine the content of vcRNA present in the total nucleic acid extracts (6).

RESULTS

The initial synthesis of vcRNA in cells infected by VSV Indiana is termed primary transcription (5, 6, 8, 11, 20). This synthesis of RNA involves the virion genome and transcriptase enzymes and is proportional to the input MOI (6), at least up to an MOI of 200 PFU/cell). For wild-type VSV, the presence of cycloheximide and/or puromycin does not inhibit primary transcription, although either, or both, block de novo protein synthesis as well as secondary transcription—presumably by preventing the synthesis of new transcriptase enzymes and inhibiting replication of the genome by denying newly synthesized viral proteins (6).

We have demonstrated previously that certain mutants of all five complementation groups of VSV Indiana perform primary transcription at temperatures nonpermissive for productive infections (5). Mutants belonging to two of these five groups (groups I and IV) do not perform secondary transcription at high temperatures (6). We have so far analyzed intracellular transcription of four group I mutants (*ts* O I-5, *ts* O I-53, *ts* O I-78, and *ts* O I-80) and four group IV mutants (*ts* O IV-62, *ts* O IV-100, *ts* IV-125, and *ts* O IV-194) and obtained similar results (unpublished data). An exception to these observations is shown in the results obtained for the mutant *ts* G I-114 (Fig. 1). For this virus, like that of the wild-type virus, RNA synthesis at permissive temperatures (31°C) could be differentiated into primary and secondary transcription through the use of cycloheximide (Fig. 1). However, little, if any, vcRNA was observed at 40°C either in the presence or absence of cycloheximide. This mutant can therefore be characterized as essentially completely RNA negative. These results contrast the results obtained for the group I Orsay mutants analyzed previously (6). For those viruses RNA synthesis (in the presence or ab-

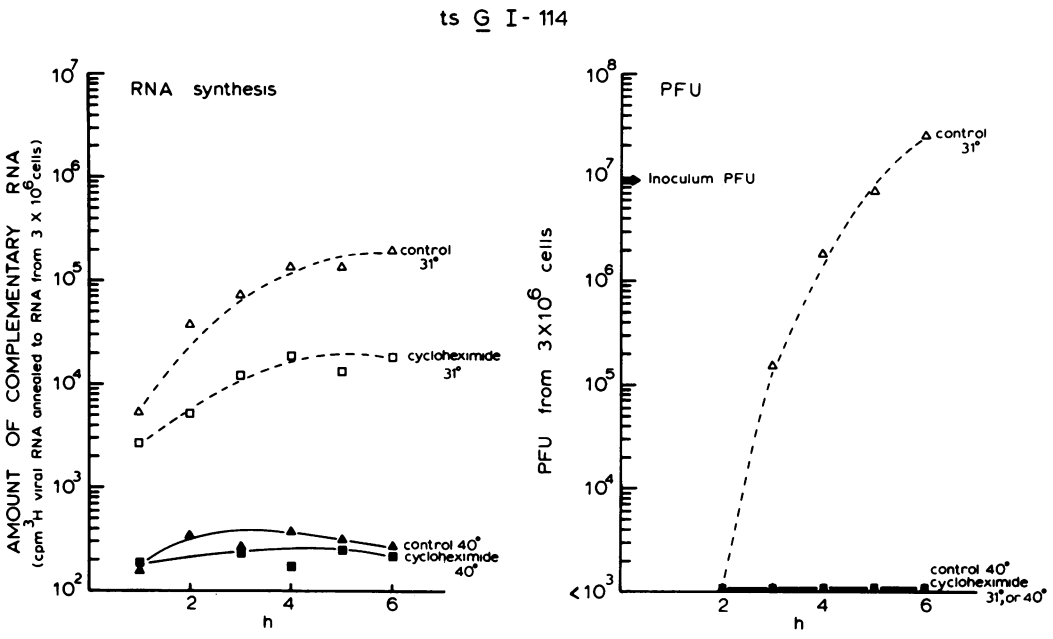

FIG. 1. *vcRNA synthesis by VSV Indiana ts G I-114. Freshly purified [³H]uridine-labeled VSV Indiana ts GI-114 in Eagle medium was applied to confluent monolayers of 3 × 10⁶ BHK-21 cells (MOI = 3), and after an absorption period of 30 min at 4°C the inoculum virus was recovered, the cells were washed with medium, and then 5 ml of fresh Eagle medium containing 5% (vol/vol) fetal calf serum (prewarmed to 31 or 40°C and containing or lacking 100 of μg of cycloheximide per ml) was added. After 1 h at either temperature, the supernatant fluids were replaced to remove desorbed virus. The monolayer and their supernatant fluids were recovered at the indicated times, and for the latter the content of infectious virus, assayed at 32°C, was determined (right panel). As judged by plaque assays conducted at 39.5°C, all progeny were temperature sensitive (data not shown). Residual inoculum virus or infected cells were extracted for nucleic acids. Samples of the infected cell nucleic acids were then annealed to samples of the ³H-labeled viral RNA, and the increase in RNase resistance was used to determine the content of vcRNA per monolayer (references 5 and 6; left panel).*

sence of cycloheximide) at 39.5 or 40°C essentially equalled that obtained at 31°C in the presence of cycloheximide; therefore, these mutants are partial RNA-negative types.

We conclude, therefore, that for *ts* G I-114 the transcriptase enzyme is thermolabile in vivo in that, although functional at permissive temperatures, it is nonfunctional when exposed to a temperature of 40°C. Similar results have been obtained in vitro (19). In addition, we conclude that the transcriptase enzyme of *ts* O I-5 (and the other group I Orsay mutants listed above) is thermostable when exposed to 39.5 or 40°C.

We have performed some preliminary experiments to determine the in vivo cut-off temperature in BHK-21 cells for transcriptase activity of *ts* G I-114, by measuring the ability of the virus to synthesize vcRNA at different temperatures. Some vcRNA can be detected at 39°C in BHK-21 cells (about 10-fold lower than the transcription rate observed at 31°C in the presence of cycloheximide, but 10-fold higher than the minimal levels seen at 40°C in Fig. 1). Whether the cut-off temperature is a function of host cell type has not been investigated.

The ability of certain mutants belonging to groups II, III, and V of VSV Indiana to give productive infections and perform primary and secondary transcription at 31 (or 32) and 40°C has been investigated (Fig. 2). The mutants *ts* O II-52, *ts* O III-23, and *ts* O V-45 all synthesize more vcRNA at 40 or 31°C (or 32°C) in the absence of cycloheximide than in its presence. This agrees in part with the classification of group III, group V, and certain group II mutants as RNA positive (see reference 12). As shown by Flamand and Lafay (7), some group II mutants are RNA positive depending on the MOI, whereas others are RNA negative; the reason for this difference is not known (12, 13).

Analyses of the ability of VSV New Jersey to complement the in vivo transcription of VSV Indiana *ts* mutants. Since *ts* mutants from groups II, III, and V synthesize considerable quantities of vcRNA at 40°C (Fig. 2), the ability of VSV New Jersey to complement the

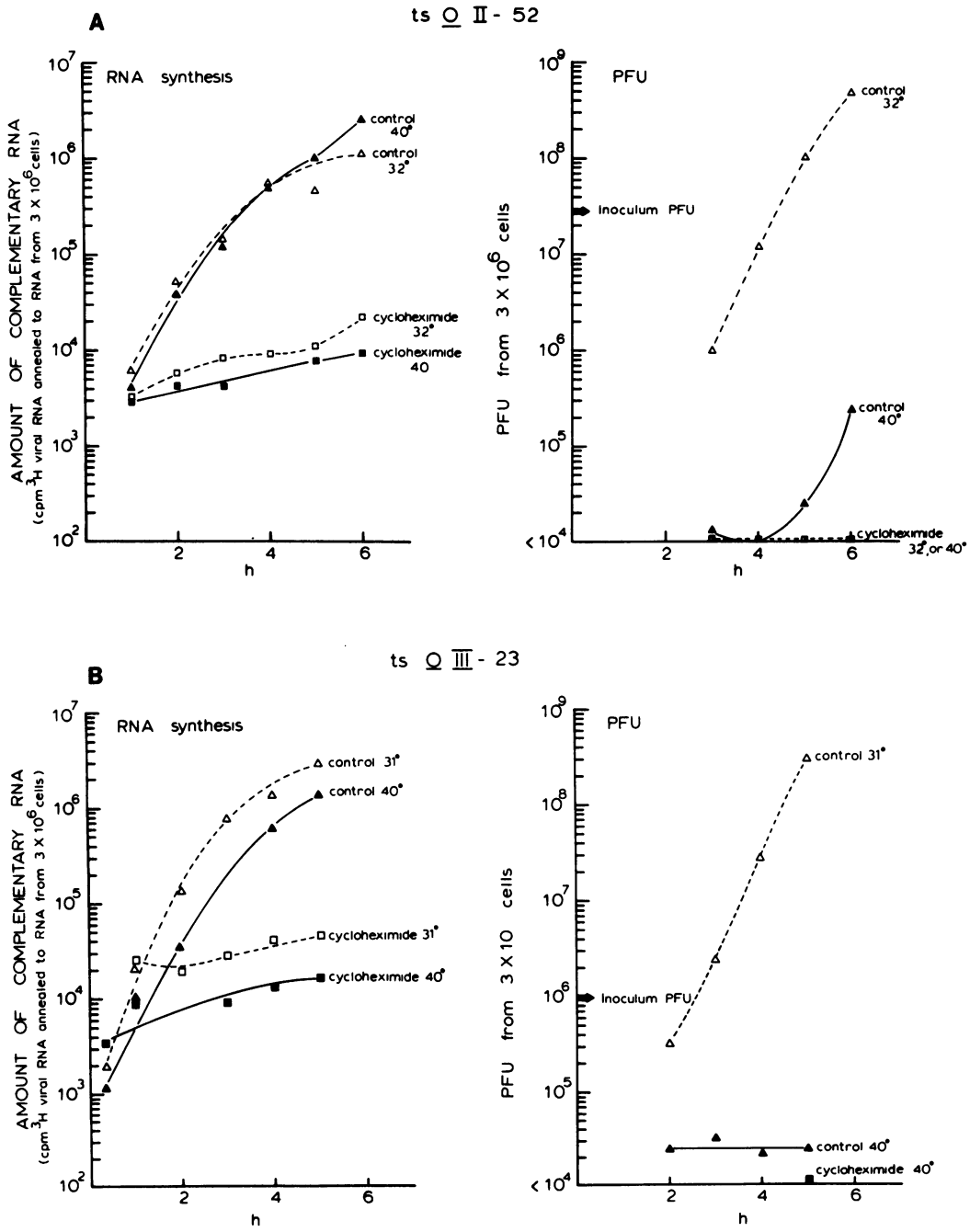

FIG. 2. *vcRNA synthesis by (A) VSV Indiana ts O II-52, (B) VSV Indiana ts O III-23, and (C) VSV Indiana ts O V-45. The protocols used to infect cells and to determine released infectious virus and intracellular vcRNA by these three viruses were the same as those described in Fig. 1. All progeny virus were temperature sensitive as judged by plaque assays conducted at 39.5°C (data not shown). The inoculum PFU added per monolayer are indicated in the right-hand panels.*

overall transcription process of these viruses was not examined. However, the ability of wild-type VSV New Jersey to complement the transcription process of a VSV Indiana group IV mutant (*ts* O IV-100) and the Glasgow group I mutant (*ts* G I-114) was investigated.

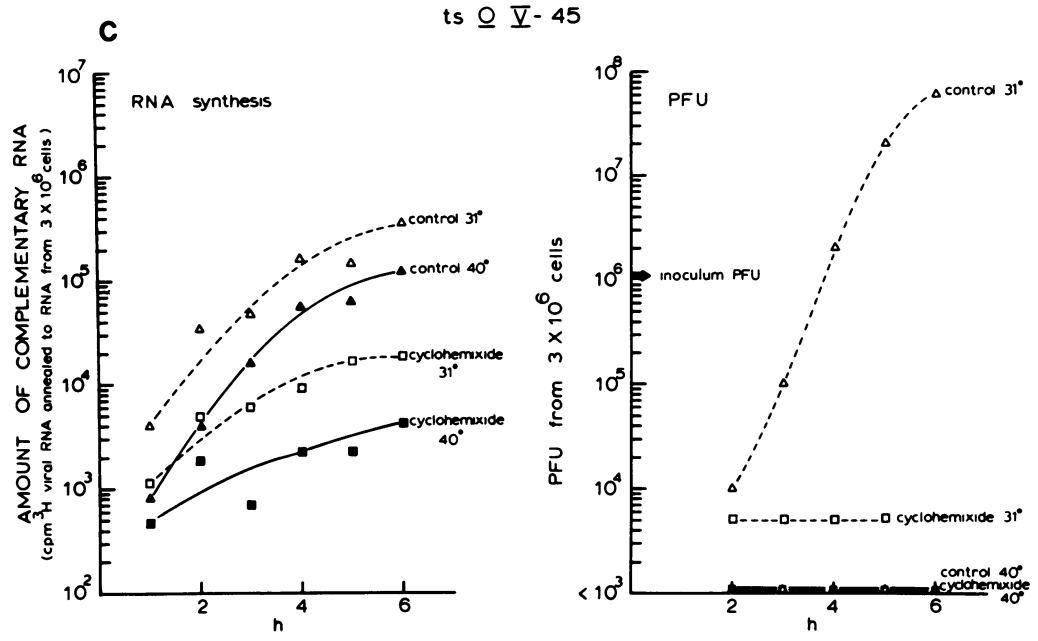

FIG. 2C

The protocol for these experiments was as follows. Freshly purified ^{3}H-labeled viruses (wild-type VSV New Jersey and a VSV Indiana mutant) were used to infect confluent monolayers of BHK-21 cells. Labeled virus was used to confirm that similar quantities of virus adsorbed to each monolayer (5) and to provide a source of radioactive RNA for the annealing experiments. The monolayers were incubated at 31 (or 32) and 39.5°C (or 40°C), and the release of progeny viruses was determined by plaque assays (at 32 or 39.5°C). The content of VSV Indiana and VSV New Jersey vcRNA in the cell extracts was then determined.

Effect of VSV New Jersey upon the growth and intracellular RNA transcription of the VSV Indiana mutant, *ts* O IV-100. Wild-type VSV New Jersey (15 PFU/cell) and mutant VSV Indiana (*ts* O IV-100, 70 PFU/cell) were used in single infections or coinfections of BHK-21 cells, and the yields of progeny virions was determined. At both 32 and 39.5°C the yields obtained from the mixed infections were notably lower than the yields obtained from either single-virus infection at 32°C or the VSV New Jersey single-virus infection at 39.5°C (Fig. 3).

For the infections involving just the VSV Indiana mutant, the progeny yield at 32°C was temperature sensitive as judged by plaque assays conducted at 39.5°C. Similar results were obtained for the virus released from the VSV Indiana-infected cells maintained at 39.5°C, although it was not determined whether in the latter case the released virus represented desorbed infecting virions or progeny viruses (due to the leakiness of the mutant).

In our hands, plaque assays of VSV New Jersey virus (inoculum or progeny virions) at 39.5°C usually give approximately one-third the number of plaques obtained at 32°C (data not shown). The results obtained from the 39.5°C coinfection assayed at 39.5°C suggest, therefore, that the progeny from the coinfection were predominantly VSV New Jersey virions, whereas for the 32°C coinfection there were evidently both progeny VSV Indiana and VSV New Jersey virions. This was supported by the observation that the plaque sizes of the 39.5°C mixed-infection progeny were of one type, whereas those of the 32°C mixed infection were of mixed sizes (in the plaque system we use, VSV Indiana gives larger plaques than VSV New Jersey).

Since VSV Indiana vcRNA can be distinguished by hybridization procedures from that of VSV New Jersey (16), the nucleic acids extracted from the various infections were annealed either to the ^{3}H-labeled VSV Indiana viral RNA or to ^{3}H-labeled VSV New Jersey viral RNA (Fig. 4).

It was found that less VSV New Jersey vcRNA was present in the 32 or 39.5°C mixed infections than in the VSV New Jersey single infections at either temperature (Fig. 3B). By contrast, essentially similar (or slightly larger) amounts of VSV Indiana vcRNA were detected in the 32°C mixed-infection extracts to those in the 32°C single-infection extracts (Fig. 3C).

Somewhat less VSV Indiana vcRNA was found in the 39.5°C mixed-infection extracts by comparison to those of the VSV Indiana single-virus infections at 39.5°C (Fig. 3C). Annealing VSV Indiana RNA to the cell extracts obtained from VSV New Jersey infections detected no complementary RNA. It was concluded from these results that no measurable increase in vcRNA induced by the VSV Indiana group IV mutant could be detected upon coinfection with VSV New Jersey at nonpermissive temperatures for the mutant's growth. Since this was a negative result, and since we have no thermolabile group IV transcription mutant, we cannot rule out the possibility that VSV New Jersey could complement the primary transcription capability of VSV Indiana group IV mutants.

Effect of VSV New Jersey upon the growth and intracellular transcription of the VSV Indiana mutant *ts* G I-114. A similar experiment was performed using VSV New Jersey and the VSV Indiana group I mutant *ts* G I-114. This mutant was chosen not only as a representative of the group I mutants, but because it possesses a thermolabile transcriptase (Fig. 1).

When VSV New Jersey (70 PFU/cell) and VSV Indiana *ts* G I-114 (25 PFU/cell) were used in single or mixed infections at 31°C, productive infections were established, as shown by the titers of the released progeny virus (Fig. 4A). The VSV Indiana progeny were temperature

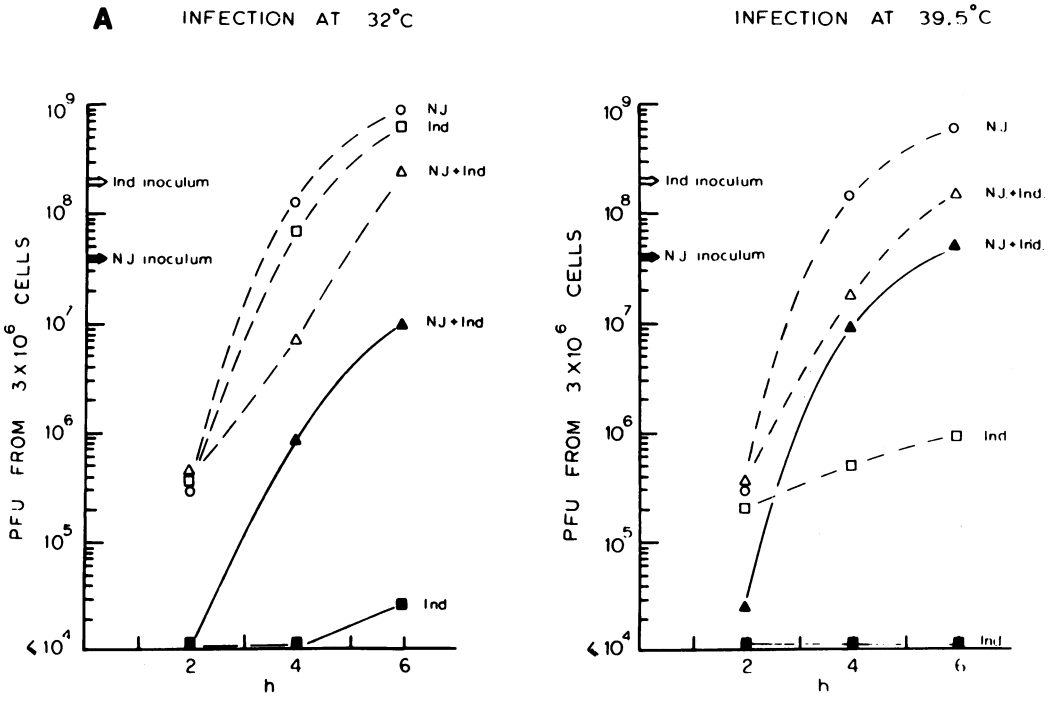

FIG. 3. *vcRNA synthesis and virus release from cells infected with wild-type VSV New Jersey and/or VSV Indiana ts O IV-100. Confluent monolayers of 3 × 10⁶ BHK-21 cells were infected with either wild-type VSV New Jersey (N.J: MOI = 15) or VSV Indiana ts O IV-100 (Ind: MOI = 70), or a combination of both viruses (N.J. & Ind.: MOI = 15 and 70, respectively), and (A) the release of infectious virus was determined by assays at 32°C (broken lines) or at 39.5°C (continuous lines). The amount of VSV New Jersey vcRNA (B) or VSV Indiana vcRNA (C) was determined as described in the text. Since both infecting viruses were labeled and the infecting virion genomes remained cell associated, self-annealing the extracted nucleic acids led to RNase resistance of the cell-associated [³H]viral RNA (5, 6). Consequently, for the calculations of vcRNA of each virus type, the ³H resistances of the self-annealed cellular nucleic acids were subtracted from the total ³H resistances obtained upon annealing with the added [³H]viral RNAs (VSV Indiana or VSV New Jersey) to unambiguously determine the individual vcRNA synthesis. Self-annealed ³H resistances were usually of the order of 10³ cpm/cell extract, so that levels equal or lower than 10³-cpm equivalents should not be considered as significant. However, the amounts of vcRNA of VSV Indiana and VSV New Jersey that were obtained (at 32 or 40°C) were at levels much larger than 10³-cpm equivalents, either for their respective single or double infections. Note that annealing VSV Indiana [³H]RNA to VSV New Jersey single-virus-infected cell nucleic acids (or the reverse, data not shown) gave essentially no detectable net increase in [³H]RNase resistance (16).*

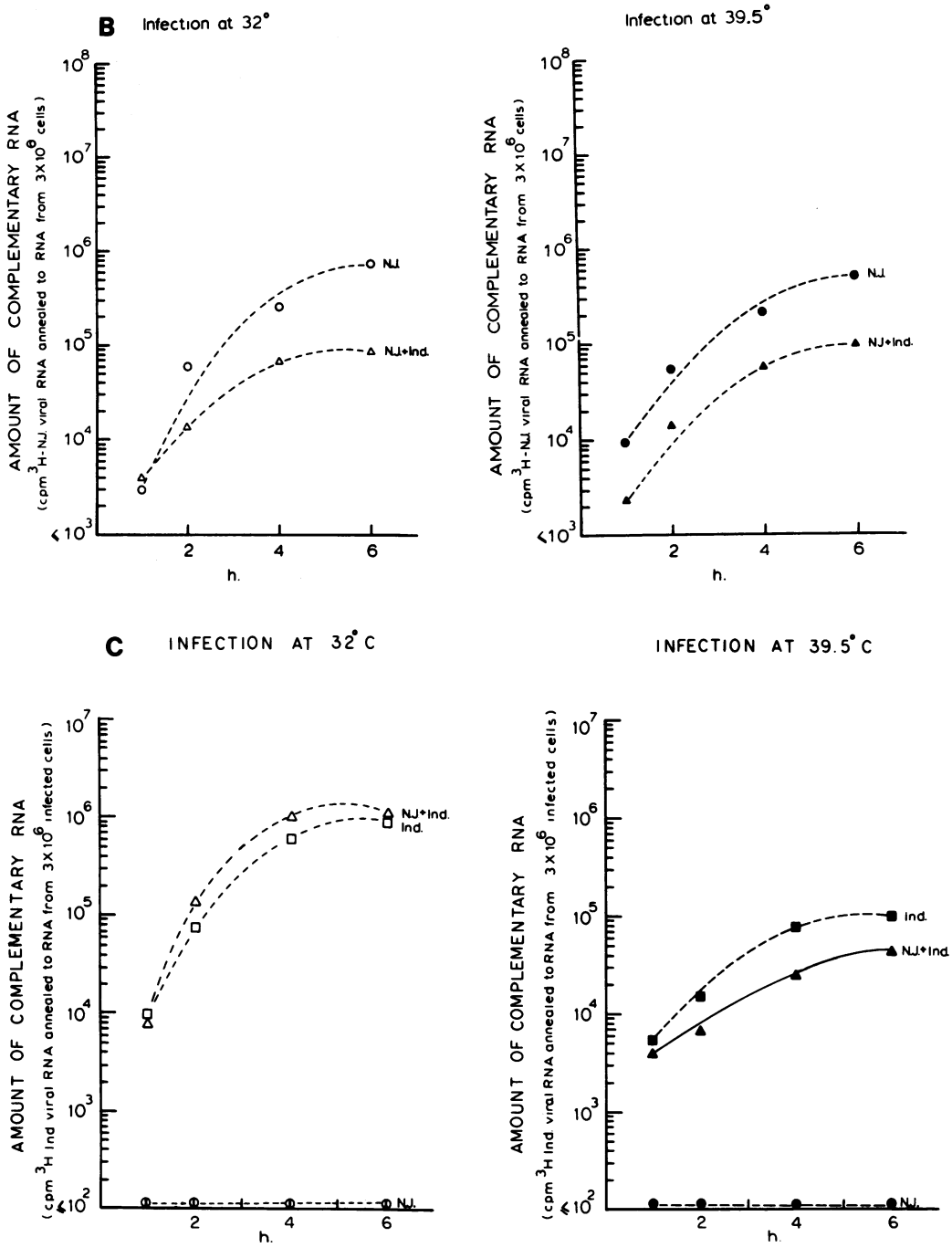

FIG. 3B and C

sensitive, as determined by the 39.5°C plaque assays. No progeny virions from the 40°C single-virus infections with the mutant virus were detected. However, progeny virions were detected for the 40°C infections of either VSV New Jersey or the coinfection of VSV New Jersey with the mutant VSV Indiana (again, based on plaque sizes, VSV New Jersey progeny).

When the infected cell nucleic acids were annealed to either VSV Indiana or VSV New Jersey viral RNAs, it was observed that more VSV Indiana vcRNA was obtained for the 40°C

coinfection than for the single infection at 40°C with the VSV Indiana mutant (Fig. 4C). The amount of VSV Indiana vcRNA observed in the coinfection was one-tenth that observed for the 31°C VSV Indiana infection and one-half that obtained for the 31°C coinfection (Fig. 4B).

The accumulations of VSV New Jersey vcRNA substantially exceeded the accumulations of VSV Indiana RNA either in the single- or the mixed-virus infections, presumably due to the different MOIs used for the two viruses. However, in most cases smaller quantities of VSV Indiana vcRNA (or VSV New Jersey RNA) were obtained in the coinfections at 31°C than in the single-virus infections at 31°C (Fig. 4B and C).

Effect of puromycin on the ability of VSV New Jersey to complement the transcription inability of VSV Indiana *ts* G I-114 at 40°C. To determine if the stimulation of VSV Indiana *ts* G I-114 complementary RNA synthesis at 40°C by VSV New Jersey was due to some function supplied by the latter, the effects of puromycin upon the RNA syntheses were determined. Confluent monolayers of BHK-21 cells were infected with VSV New Jersey (300 PFU/cell) and VSV Indiana *ts* G I-114 (1,000 PFU/cell), and after absorption the cells were incubated in the presence or absence of 50 μg of puromycin per ml of growth medium. From the progeny virus assays of the 31 and 40°C infections, it was evident that, in the absence of puromycin, productive infections were established and the progeny from the *ts* G I-114 infection at 31°C were indeed temperature sensitive (data not shown). No progeny were detected for the infections incubated at either temperature in the presence of puromycin (data not shown).

For the single-virus infections, the synthesis of VSV New Jersey vcRNA at 40°C indicated (data not shown) the capacity of that virus to perform primary transcription (i.e., the RNA synthesis in the presence of puromycin) and secondary transcription (i.e., greater quantities of VSV New Jersey RNA synthesis in the absence of puromycin).

As before, from the coinfection, elevated amounts of VSV Indiana vcRNA were obtained at 40°C by comparison to that from single-virus infection. However, no elevated quantities of VSV Indiana vcRNA were obtained when puromycin was included in the overlay medium of the coinfections incubated at 40°C (Fig. 5), even though in this experiment high MOIs for both viruses were used.

To determine if preformed VSV New Jersey

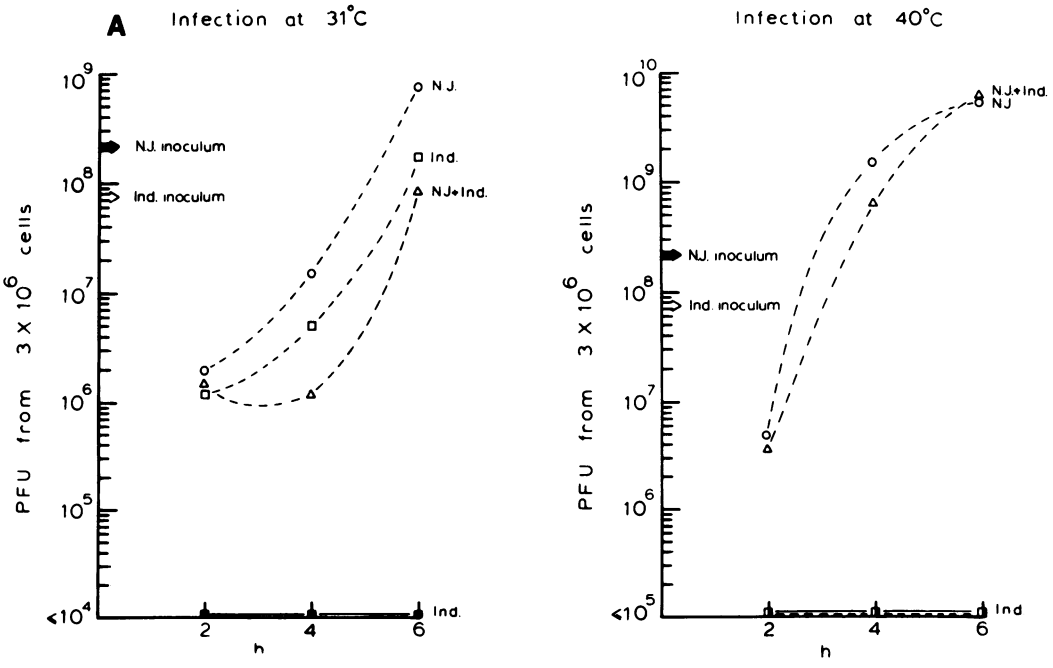

FIG. 4. *vcRNA synthesis and virus release from cells infected with wild-type VSV New Jersey and/or VSV Indiana ts G I-114. The protocols used were similar to those described in Fig. 1 and 3. The release of infectious virus (A) assayed at 32°C (broken lines) or 39.5°C (continuous lines) and net synthesis of VSV Indiana vcRNA (left-hand panels of B and C) or VSV New Jersey (right-hand panels of B and C) for the infections conducted at 31°C (B) or 40°C (C) are given.*

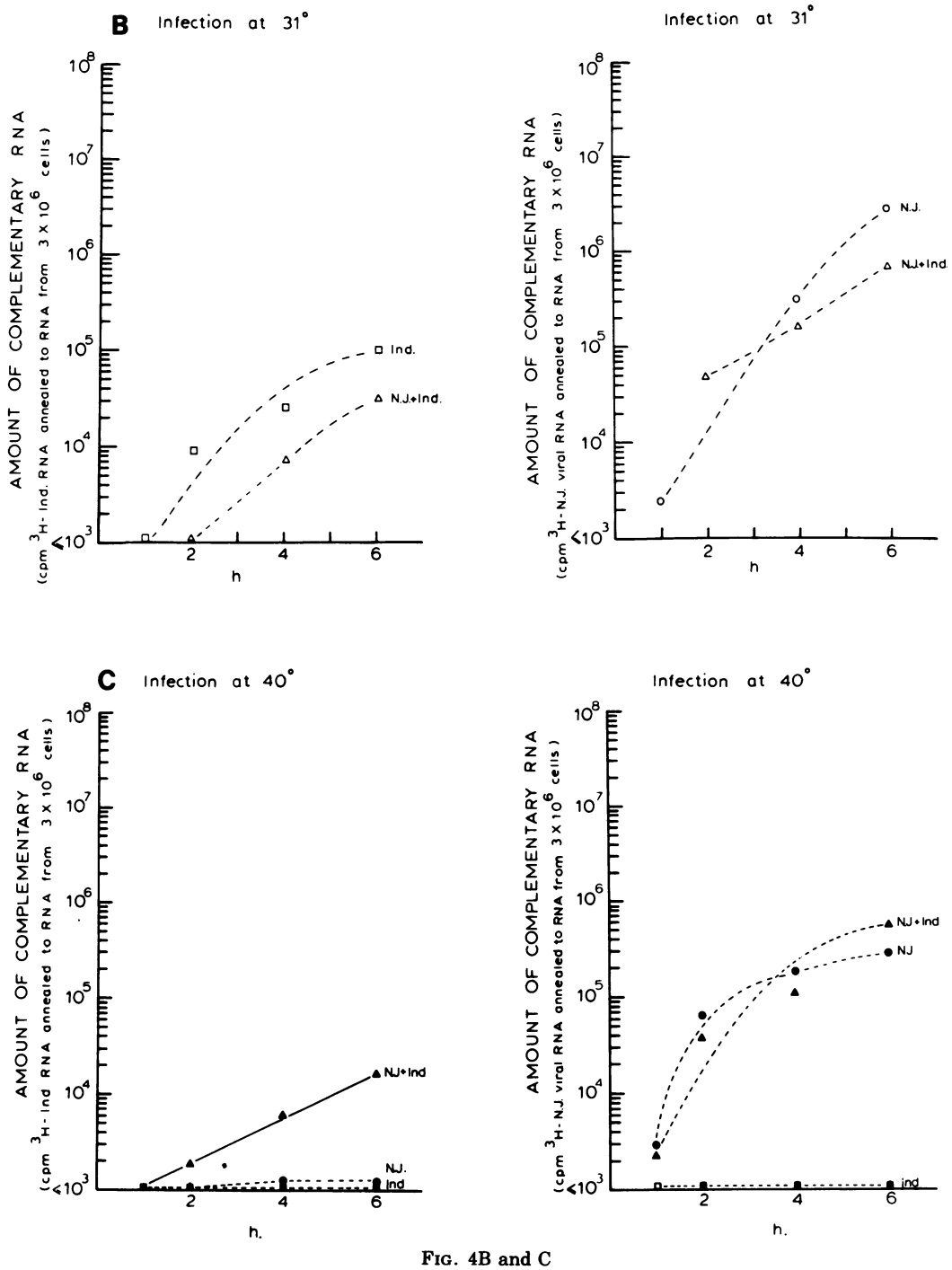

FIG. 4B and C

gene products could increase the intracellular synthesis of VSV Indiana *ts* G I-114 vcRNA at 40°C, this experiment was repeated, except that VSV New Jersey was allowed to first establish productive infections at 40°C for 4 h before addition of the VSV Indiana *ts* G I-114 inoculum. The monolayers were then incubated in the presence or absence of puromycin. As before, VSV Indiana vcRNA was obtained in the absence of puromycin but not in its presence (data not shown).

These results suggest, therefore, that de novo

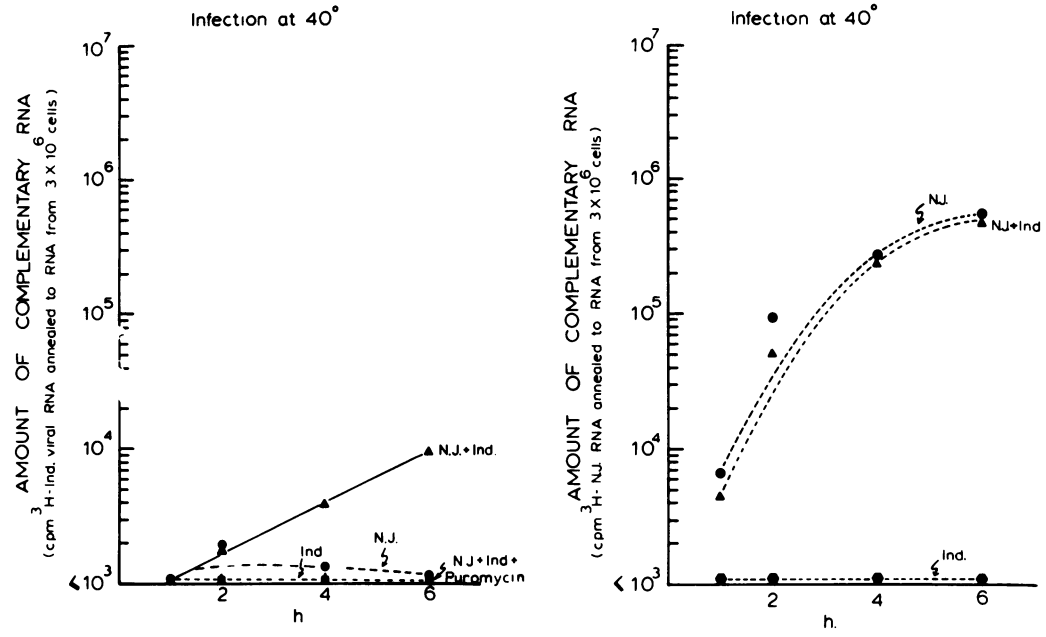

FIG. 5. *vcRNA synthesis in cells infected with wild-type VSV New Jersey and/or VSV Indiana ts G I-114 in the presence or absence of puromycin. The experimental protocols were essentially the same as those described in Fig. 4, with the exception that all infections were conducted in the presence or absence of 50 μg of puromycin per ml of medium. The results obtained for the 31°C infections in the absence of puromycin (data not shown) were similar to those given in Fig. 4A and B. The progeny virus yields of the 40°C infections were also similar to those presented in Fig. 4A. No progeny virus were obtained for any infection incubated in the presence of puromycin (data not shown). VSV Indiana RNA synthesis at 31°C in the presence of puromycin was comparable to that shown in Fig. 1 for cycloheximide-treated cells. No VSV Indiana RNA was detected at 40°C (in the presence or absence of puromycin). VSV New Jersey vcRNA in the presence of puromycin (single or double infections at 31 or 40°C) was approximately one-tenth that observed in the absence of the drug (data not shown).*

VSV New Jersey gene products are required for VSV Indiana RNA synthesis by *ts* G I-114 at 40°C.

Effect of a heterologous rhabdovirus, Mokola, on the synthesis of vcRNA by VSV Indiana *ts* G I-114. The results presented in the previous two sections strongly suggested that a gene product of the serologically related VSV New Jersey is able to stimulate the synthesis of vcRNA for VSV Indiana *ts* G I-114 at 40°C. Although the results of the preceding section suggest that de novo New Jersey proteins are required, an additional experiment was undertaken to determine if the effect could also be obtained with a heterologous rhabdovirus type. Mokola virus was selected since it is serologically related to the rabies subgroup of rhabdoviruses and quite distinct from the VSV sugroup (17, 18).

The protocol used was similar to that used with VSV New Jersey (Fig. 4), except that the time course was extended since the Mokola growth cycle (like that of rabies virus) is somewhat longer than that of VSV New Jersey (vide infra). Monolayers of confluent BHK-21 cells were infected with either VSV Indiana *ts* G I-114 (10 PFU/cell) or Mokola virus (10 PFU/cell) and incubated at 32 or 40°C. Mokola vcRNA was synthesized in the single- or dual-virus infections in comparable amounts for the two incubation temperatures, again less in the dual-virus infection than in the Mokola single-virus infection (Fig. 6).

When, in a separate experiment, the Mokola single-virus infection was conducted in the presence of cycloheximide, some Mokola vcRNA was observed (Fig. 6), indicating that either Mokola virus possesses a virion transcriptase or a preexisting cellular function is involved in transcription of the Mokola genome.

VSV Indiana vcRNA was observed only for the 32°C infections involving VSV Indiana; no VSV vcRNA was obtained for either the 40°C single- or dual-virus infections (Fig. 6).

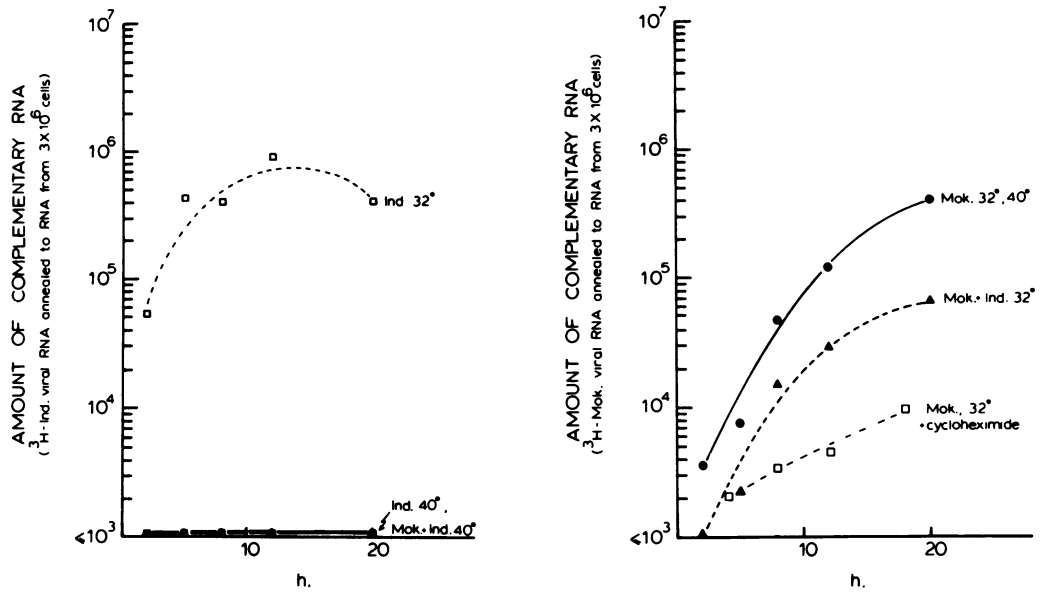

FIG. 6. *vcRNA synthesis in cells infected with Mokola virus and/or VSV Indiana ts G I-114. The protocols used for the infection at 32 or 40°C of confluent BHK-21 cells with VSV Indiana ts G I-114 (Ind. MOI = 10) and/or Mokola (MOK.: MOI = 10) were similar to those described in Fig. 3. No progeny virus assays were performed. However, intracellular vcRNA of VSV (only at 32°C) and vcRNA of Mokola (at 32 or 40°C in the single or mixed infections) were detected as determined in Fig. 3. In a separate experiment, a single-virus infection at 32°C with Mokola virus incubated in the presence or absence of 100 μg of cycloheximide per ml gave Mokola vcRNA in the amounts indicated in the right-hand panel.*

These results suggest, therefore, that Mokola virus cannot complement the transcription inability of VSV Indiana *ts* G I-114 at 40°C.

DISCUSSION

The abilities of various VSV Indiana mutants to synthesize RNA at nonpermissive temperatures have been reviewed by Pringle (12). The results obtained in this investigation confirm the widely accepted conclusions that mutants of groups V, III, and II (i.e., *ts* O II-52 at an MOI of 10) are not substantially inhibited in their capacities to synthesize RNA at temperatures nonpermissive for productive infections.

All group IV mutants that we, or others, have investigated (reviewed by Pringle [12]) are limited, but not totally inhibited, in their capacities to synthesize RNA at 40°C. These mutants presumably possess a thermostable transcriptase, and until a clear thermolabile transcriptase is demonstrated for a group IV mutant we prefer to reserve judgement on whether the group IV gene product is a transcriptase component. Lafay (10) has presented evidence that the group III product corresponds to the viral M protein and that the group V product corresponds to the viral G protein. Hunt and associates (9) have shown that the group I gene product corresponds to the viral L protein. Which of the remaining complementation groups corresponds to the viral N or NS proteins is not known.

Group I mutants exhibit two patterns of behavior. For *ts* O I-5, limited quantities of vcRNA are obtained at 40°C either in the presence or absence of cycloheximide (5, 6). Most group I mutants we have examined fall into this category. An exception is mutant *ts* G I-114, for which essentially no vcRNA is obtained at 40°C either in the presence or absence of cycloheximide (Fig. 1). The results obtained suggest that *ts* G I-114 possesses a thermolabile virion transcriptase component. This agrees with the result of Hunt and associates (9) that the viral L protein is the group I gene product. The other group I mutants we have studied indicate that many group I mutants synthesize thermostable transcriptase components at permissive temperatures, and at nonpermissive temperatures they either synthesize a protein that is transcriptase incorrectly folded or do not synthesize it, or synthesize an incompetent transcriptase component.

Using a very sensitive hybridization procedure, no group I mutant we have so far examined (including *ts* O I-5) replicates viral RNA

(negative strands) at nonpermissive temperatures (unpublished data). The same result has been obtained for group IV mutants. If the group I gene product is both a transcriptase component and a replicase component, then why don't these mutants replicate some viral RNA at 40°C? It is quite possible that certain mutants may be more defective in their replication function than their transcription function; also, it is conceivable that the replicase cannot be derived from the virion transcriptase and that de novo proteins are required.

The question for which this series of investigations has sought an answer is whether transcription-defective VSV Indiana mutants can be complemented by gene products VSV New Jersey. VSV New Jersey is a virus antigenically related to VSV Indiana but, by RNA homology studies, quite distinct (4, 16). Unlike the studies by Pringle and associates (12–15) in which a similar question was asked (using the criterion of complementation of mutants of the two viruses to give productive infections), this study has restricted the question to investigating the intracellular vcRNA syntheses obtained during coinfections by wild-type VSV New Jersey and mutant VSV Indiana at nonpermissive and permissive temperatures.

When VSV New Jersey was used to coinfect cells at 39.5°C with VSV Indiana *ts* O IV-100, no enhancement of VSV Indiana vcRNA was observed. We cannot rule out the possibility that VSV New Jersey is able to aid the primary transcription capability of the VSV Indiana mutant, nor do we know if any VSV Indiana viral RNA replication (i.e., minus-strand synthesis) occurred during the coinfection.

In the equivalent experiment with VSV New Jersey and VSV Indiana *ts* G I-114, an increase in VSV Indiana vcRNA was obtained at 40°C when VSV New Jersey was present. None was obtained when puromycin was present. These results suggest that a VSV New Jersey de novo gene product can aid in the transcription of the VSV Indiana mutant. We have not determined if the VSV New Jersey virus aids in the replication of VSV Indiana RNA, or if the product made represents all species of functional mRNA or complete plus strands.

Although the experimental protocol was designed to look at coinfections involving essentially similar MOIs for both viruses, the experimental design involved using freshly prepared virus, so that PFU assays of inocula and progeny were performed at the end of the infections. This design was adopted to minimize the proglems of loss of infectivity of virus stocks upon storage during the titration period. We have found (5, 6) that viruses purified in the manner described here and used immediately have high infectivity-to-particle ratios (1:3 to 1:6). As can be seen, it turned out that rather widely different MOIs for the two viruses were often used. In addition to this problem (and any complication that it might pose upon comparisons of results between experiments), it should be borne in mind that some *ts* viruses and, of course, the wild-type virus, are more effective at establishing productive infections at permissive temperatures than other *ts* viruses. This is evident in Fig. 2. It is noteworthy, however, that in most cases the dual-virus infections at 31°C (or 32°C) frequently led to reduced virus or vcRNA yields of both viruses. The reasons for these observations are not known. However, although sought for, we have not been able to demonstrate any defective virus production under the conditions of the experiments reported here.

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