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Reconstitution of Infectivity and Transcriptase Activity of Homologous and Heterologous Viruses: Vesicular Stomatitis (Indiana Serotype), Chandipura, Vesicular Stomatitis (New Jersey Serotype), and Cocal Viruses

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RNA transcriptase activities have been reconstituted from fractionated components of four rhabdoviruses: vesicular stomatitis virus (VSV) (Indiana serotype), Cocal, VSV (New Jersey), and Chandipura viruses. Heterologous reconstitution of transcription has been observed between template and enzyme fractions of VSV Indiana and Cocal viruses but not to any significant extent between components of the other two viruses with VSV Indiana template or enzyme. Homologous reconstitution of infectivity has been obtained for each virus from their respective parts as well as heterologous reconstitution between Cocal and VSV Indiana components.

Six rhabdoviruses have been demonstrated to possess virion RNA-dependent RNA polymerase (transcriptase) activities (1, 2, 7). These include vesicular stomatitis virus (VSV) Indiana, VSV New Jersey, Cocal, Chandipura, Piry, and Kern Canyon viruses. Apart from Kern Canyon virus (which has not been studied), the transcription process is initiated for all of these viruses by some similar product RNA sequences involving purine nucleotides (7, 14). In contrast, hybridization studies have indicated that apart from VSV Indiana and Cocal viruses there is little exact genome homology between these viruses (12).

It has been shown for VSV Indiana that the viral RNA is not infectious, although subviral structures can be isolated that are infectious (2, 4, 6, 15).

It has further been demonstrated that neither the viral glycoprotein (G) nor the membrane protein (M) are required for RNA transcription (3). Also, when VSV is dissociated by detergent and high salt then resolved by centrifugation (9), an RNA-N protein fraction can be obtained essentially free from solubilized proteins (G, M, and the minor proteins L and NS). Neither of these fractions possess endogeneously templated RNA polymerase activity, although together they can reconstitute the RNA polymerase activity (9). In a recent study, Emerson and Wagner have found that the probable enzyme component in the solubilized proteins is the viral L protein (10).

In the present communication we will present evidence to show that not only transcriptase activity but also infectivity can be restored from separated viral components. Homologous and some heterologous reconstitution of enzyme activity and infectivity has been observed by using components derived from four rhabdoviruses: Cocal, VSV New Jersey, Chandipura, and VSV Indiana.

MATERIALS AND METHODS

Reagents. [α-^{32}P]UTP (specific activity 13 mCi per μmol) was purchased from New England Nuclear Corp., Boston, Mass. [^{3}H]uridine was obtained from I.C.N., Irvine, Calif.

Preparation and purification of unlabeled or ^{3}H-labeled virus. The purification of unlabeled or ^{3}H nucleoside-labeled virus from the supernatant fluids of BHK-21 monolayers infected with cloned preparations of VSV (New Jersey serotype), Chandipura, VSV (Indiana serotype), or Cocal viruses involved polyethylene glycol precipitation followed by equilibrium and velocity gradient centrifugations as described previously (13). Virus obtained from the velocity gradient centrifugation was pelleted and suspended in 0.15 M NaCl, 0.01 M Tris buffer, pH 7.4, by sonication at 1 A for 40 s by using a Raytheon ultrasonic disintegrator. Virus serotypes were checked by homologous and heterologous antisera neutralization of infectivities (5). Labeled virus protein concentrations were adjusted to 3 mg of protein per ml.

Transcription reaction assays. Assays for viral RNA polymerase activities have been described (1). For the enzyme reconstitution experiments, the assay mixture contained the following ingredients per total

volume of 125 μliters: deionized water, 46 μliters; 1 M NaCl, 5 μliters; 1 M Tris buffer, pH 8.0, 8 μliters; 0.05 M ATP, 2 μliters; 0.05 M CTP, 2 μliters; 0.05 M GTP, 2 μliters; 0.05 M UTP, 0.1 μliters; [α-^{32}P]UTP (1.6 mCi per ml), 20 μliters; 1 M $MgCl_2$, 0.9 μliters; 0.1 M dithiothreitol, 2 μliters; 1% triton N101, 2 μliters; dissociated viral pellet (template) fractions, 17.5 μliters; and dissociated viral supernatant (enzyme) fractions, 17.5 μliters. Reaction mixtures were incubated at 31 C, and samples were removed at intervals to determine the acid-insoluble incorporation of ^{32}P label. The final UTP specific activity was 9,700 counts per min per pmol.

Dissociation of viruses into template (pellet) and solubilized protein (supernatant) fractions. The procedure for dissociating and reconstituting these viruses followed the procedure described by Emerson and Wagner (9) with particular modifications as described below.

High-salt solubilizer buffer (HSS) contained 0.01 M Tris buffer, pH 8.0, 1% glycerol, 0.001 M $MgCl_2$, 0.001 M 2-mercaptoethanol, 0.5% triton N101, and NaCl at either 0.8 M (0.8 M HSS), 1.25 M (1.25 M HSS), or 2.5 M (2.5 M HSS). The Tris, glycerol, $MgCl_2$, and NaCl components were autoclaved prior to preparing these solutions.

Eighty microliters of virus preparation was mixed with 40 μliters of 2.5 M HSS, 500 μliters of 0.8 M HSS, and 10 μliters of 0.1 M dithiothreitol. The mixture was loaded over a 20-μliter pad of 100% sterile glycerol in a Spinco SW 50L, 0.7-ml centrifuge tube (3/16-inch diameter by 53/32-inch length) and centrifuged at 42,000 rpm and 3 C for 90 min. The supernatant fluids containing solubilized viral proteins were carefully removed, leaving the interface and the glycerol pad intact. A band of presumed subviral structures was visible at the interface. The supernatant was loaded into a vacuum dialysis bag and dialyzed for 40 min at 4 C under a water pressure vacuum against 0.01 M Tris buffer, pH 8.0, 0.002 M $MgCl_2$, 0.001 M 2-mercaptoethanol, 1% glycerol, and 0.02 M NaCl. Prior to use, the dialysis tubing was boiled in deionized water for 1 h. After dialysis the conductivity of the protein solution was determined and the NaCl concentration was adjusted to 0.1 M by addition of 4 M NaCl. The volume was adjusted to 350 μliters. This solution of solubilized viral proteins was stored at 4 C until use.

The interface and pellet fractions from the ultracentrifugation were mixed with 600 μliters of 1.25 M HSS and loaded over a second 20-μliter cushion of sterile glycerol and centrifuged as before. The supernatant fluids from the second centrifugation were discarded, and the interface and glycerol pad were suspended with 0.001 M Tris buffer, pH 8.0, to give a total volume of 350 μliters. The salt concentration of this final suspension (pellet fraction) was determined from its conductivity to be 0.1 M. All operations were performed at 4 C, and viral components were transferred with sterile Pasteur pipettes and stored at 4 C in plastic tubes.

The procedure described above was reproducible for all preparations of VSV Indiana and gave solubilized protein supernatant or viral pellet fractions with no or very little detectable RNA polymerase activity when assayed as described above, but excellent reconstitution when combined (see figures). Similar results were obtained for VSV New Jersey and Chandipura viruses, although the pellet fractions still contained some infectivity and some endogeneously templated enzyme activity (see text).

For Cocal virus, no reconstitution was achieved for combinations of both components isolated by this method. However, Cocal virus preparations could be dissociated into potentially active constituents by two dissociation procedures. To prepare Cocal template fractions, the procedure described above was used, and the pellet obtained possessed no endogeneously templated enzyme activity (vide infra). To prepare Cocal solubilized enzyme preparations, the concentration of triton N101 in the HSS was lowered to 0.1%. At this concentration of triton, the pellet fractions possessed 10% of the specific enzyme activity of the initial virus preparation (expressed in terms of the pmol [^{32}P]UMP incorporated per 10^4 counts per min of ^{3}H label of the virus or template fractions). The supernatant fraction, although possessing no endogenous templated enzyme activity, was able to reconstitute activity with the pellet fractions obtained by either procedure. It appeared, therefore, that Cocal enzyme prepared by high triton dissociation was irreversibly inactivated. The reason for this inactivation is not known.

Infectivity assays of virus or reconstituted viral components. Plaque assays of VSV in BHK-21 cell monolayers followed standard procedures. Mixtures of viral pellet and supernatant fractions were incubated at room temperature for 10 min and then were diluted in 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0, containing 500 μg of DEAE-dextran per ml (6). Confluent monolayers of BHK-21 cells in 9-cm diameter petri plates were washed once with Eagle medium (8) and drained, and 0.2 ml of sample was added. After adsorption at room temperature for 30 min, the plates were washed twice with 5 ml of Eagle medium to remove the excess DEAE-dextran and were overlaid with 1% nutrient agar containing Eagle medium and 10% calf serum. Plaques were scored 36 h later after incubation at 33 C.

RESULTS

The reconstitution of transcriptase enzyme activity of VSV (Indiana serotype) and Cocal viruses. It was shown by Emerson and Wagner that transcriptase enzyme activity can be reconstituted from viral template and solubilized protein components derived from VSV Indiana (9). We confirmed this result and found that all of the initial viral enzyme specific activity (expressed as the pmole [^{32}P]UMP incorporated per 10^4 counts per min of ^{3}H-labeled viral RNA) can be recovered when freshly prepared virus is used (Fig. 1). In this experiment, no detectable enzyme activity was associated with either component when assayed alone.

We were also able to reconstitute enzyme activity with Cocal virus components, although the recovered specific activity was only 8% of

the initial viral specific activity (Fig. 1).

Reconstitution of enzyme activity between these two viruses with solubilized enzyme from one virus and template from another has also been obtained. In this case the Cocal enzyme gave a reconstituted activity with VSV template fractions which was approximately 1% the initial VSV enzyme-VSV template reaction. In contrast, the VSV enzyme gave a reconstituted activity with Cocal template fractions which was 10-fold greater than the Cocal homologous enzyme-template fractions.

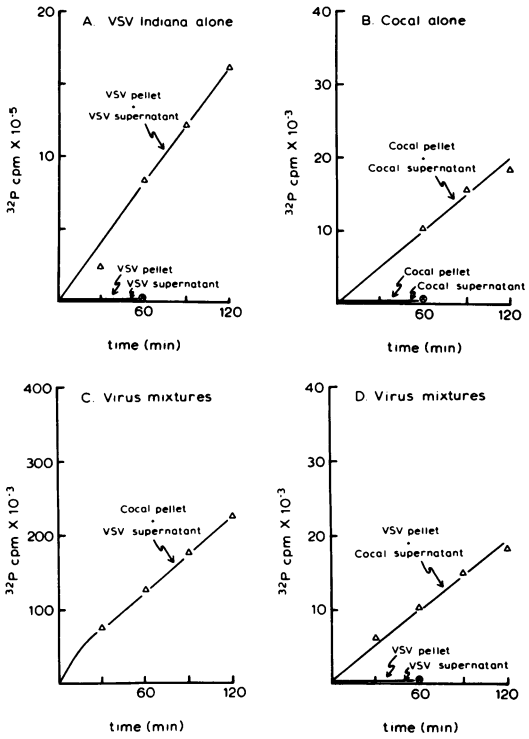

FIG. 1. *Reconstitution of transcriptase activity by dissociated components of VSV (Indiana serotype) and Cocal viruses. Purified preparations of [^{3}H]uridine-labeled VSV Indiana and Cocal viruses were dissociated as described in Materials and Methods. Samples of each fraction were assayed for transcriptase activity either separately or in the indicated combinations. The initial VSV Indiana virus preparation possessed a specific enzyme activity of 118 pmol [^{32}P]UMP incorporated per h per 10^4 ^{3}H counts per min. The observed reconstituted VSV template plus VSV supernatant specific activity was equivalent to 130 pmol [^{32}P]UMP incorporated per h per 10^4 ^{3}H counts per min. The initial Cocal virus preparation possessed a specific enzyme activity of 94 pmol [^{32}P]UMP incorporated per h per 10^4 ^{3}H counts per min. The observed reconstituted Cocal template plus Cocal supernatant specific activity was equivalent to 7.5 pmol [^{32}P]UMP incorporated per h per 10^4 ^{3}H counts per min.*

The reconstitution of transcriptase enzyme activity of VSV (New Jersey serotype) and Chandipura viruses in comparison with VSV Indiana virus. Essentially complete recovery of transcriptase activity has been obtained for both Chandipura and VSV New Jersey from their respective parts. Although for each virus there was no detectable endogenously templated enzyme activity associated with the supernatant fractions, there was some residual activity associated with the pellet fractions of both viruses as well as for another preparation of VSV Indiana prepared on the same day (Fig. 2). Notwithstanding that activity, the addition of their respective supernatant fractions restored full transcriptase functions.

A small amount of heterologous reconstitution of transcription was obtained with enzyme from one virus and template from another for the three viruses: VSV Indiana, VSV New Jersey, and Chandipura viruses (Cocal virus combinations were not assayed). This heterologous reconstitution was at most only 50% higher than the respective residual endogenous activities of the template fractions alone and was not, percentage-wise, as significant as that obtained between Cocal and VSV Indiana parts (Fig. 1). Because the reconstituted incorporation levels obtained from VSV and Cocal combinations were low (see Fig. 1 ordinates), the supernatant fractions of Chandipura and VSV New Jersey were used with the VSV Indiana template used in Fig. 1. No reconstituted activity was obtained for these heterologous mixtures, although homologous reconstitution was given with the VSV Indiana supernatant.

Reconstitution of infectivity by dissociated components of VSV (Indiana serotype) and Cocal viruses. Samples of the dissociated fractions obtained from VSV and Cocal viruses were used for infectivity determinations either separately or in combinations (Table 1). Considerable reconstitution of infectivity was obtained for VSV components, representing an increase of more than 3,000-fold.

Cocal reconstitution of infectivity was at least six times that of the Cocal pellet fraction alone (although at the dilutions plated no plaques were obtained for the Cocal pellet fraction). Cocal pellet reconstitution (if any) by VSV enzyme was low. In contrast, VSV pellet reconstitution by Cocal enzyme was more than 70-fold greater than the individual component infectivities.

The reconstituted VSV Indiana infectivity obtained from VSV pellet and supernatant fractions represented 0.006% of the initial amount of infectious virus used for the dissociation procedures.

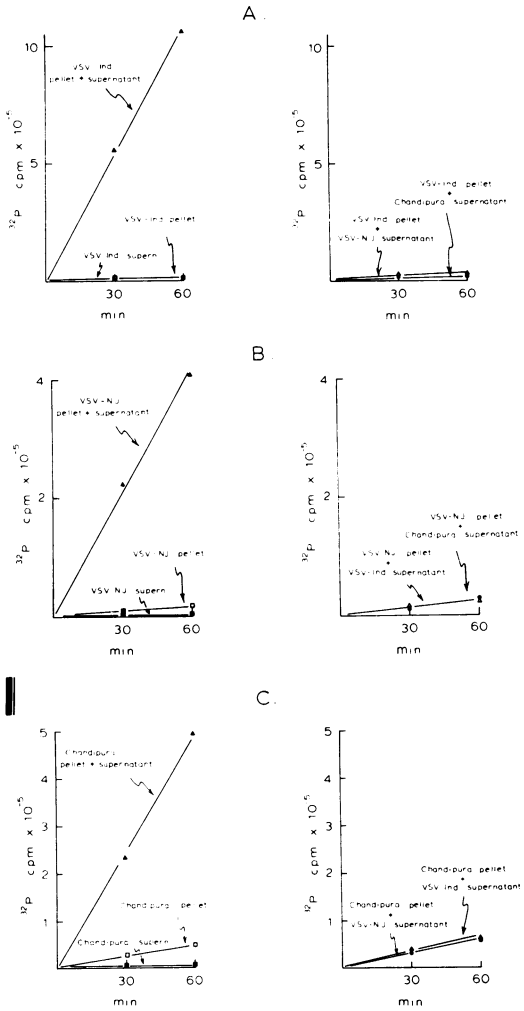

FIG. 2. *Reconstitution of transcriptase activity by dissociated components of VSV (Indiana serotype), VSV (New Jersey serotype), and Chandipura viruses. Purified preparations of [^{3}H]uridine-labeled VSV Indiana, VSV New Jersey, and Cocal viruses were dissociated as described in Materials and Methods. Samples of each fraction were assayed for transcriptase activity either separately or in the indicated combinations. The initial specific activities of the virus preparations of VSV Indiana, VSV New Jersey, and Chandipura were 95, 54, and 47 pmol [^{32}P]UMP incorporated per h per 10^4 ^{3}H counts per min of viral RNA, respectively. The specific activities of the homologous reconstituted activities of VSV Indiana (A), VSV New Jersey (B), and Chandipura (C) viruses were 92, 60, and 50 pmol [^{32}P]UMP incorporated per h per 10^4 ^{3}H counts per min of RNA, respectively.*

Reconstitution of infectivity by dissociated components of VSV (New Jersey or Indiana serotypes) and Chandipura virus. Samples of the dissociated fractions obtained from VSV (New Jersey), VSV (Indiana), and Chandipura viruses were used separately or in combination to determine the presence of homologous or heterologous reconstitution of infectivities. In each case, homologous reconstitution of infectivity was obtained, but no significant heterologous reconstitution was observed (Table 2).

DISCUSSION

It has been shown that reconstitution of transcriptase enzyme activity for VSV (Indiana serotype) can be achieved from dissociated viral components (9). This has been confirmed in this study and extended to VSV New Jersey, Chandipura, and Cocal viruses, which are other viruses in the VSV subgroup of rhabdoviruses (5). Heterologous reconstitution of transcriptase enzyme activity has been obtained with components of Cocal and VSV Indiana but not to any significant extent between VSV Indiana, Chandipura, and VSV New Jersey viral components. Although the viral genome sequences indicate little existing exact nucleotide homology between these viruses (12), virus infectivity neutralization studies have shown that antigenically some homology exists between VSV Indiana and Cocal viruses (5), whereas complement-fixation studies have categorized all these viruses in the VSV subgroup of rhabdoviruses. Similar RNA transcription initiation sequences have been observed for these viruses (7), suggesting, with the other facts, that some func-

TABLE 1. *Reconstitution of infectivity by dissociated components of VSV (Indiana serotype) and Cocal viruses*[a]

Component mixtures	PFU
VSV pellet	less than 2×10^2*
VSV supernatant	less than 20*
VSV pellet and VSV supernatant	6×10^5
Cocal pellet	less than 2×10^2*
Cocal supernatant	less than 17*
Cocal pellet and Cocal supernatant	1.2×10^3
VSV pellet and Cocal supernatant	1.5×10^4
Cocal pellet and VSV supernatant	4×10^2

[a] Purified preparations of VSV or Cocal viruses (each 9.6×10^9 PFU per 80 μliters) were dissociated as described in Materials and Methods to give 0.35-ml volumes of pellet or supernatant fractions. Samples of each component were used separately or in combination to determine the content or reconstitution of infectivity. The PFU observed is expressed as the total obtainable for the complete 0.35-ml fraction (or 0.70-ml combinations). In four instances (*), no PFU were observed at the lowest dilutions plated.

TABLE 2. *Reconstitution of infectivity by dissociated components of VSV (Indiana serotype), VSV (New Jersey serotype), and Chandipura viruses*[a]

Component mixtures	PFU
VSV Indiana pellet	4×10^4
VSV Indiana supernatant	less than 1×10^{2}*
VSV Indiana pellet and VSV Indiana supernatant	2×10^6
VSV New Jersey pellet	3×10^4
VSV New Jersey supernatant	less than 10^{2}*
VSV New Jersey pellet and VSV New Jersey supernatant	4×10^5
Chandipura pellet	10^4
Chandipura supernatant	less than 10^{2}*
Chandipura pellet and Chandipura supernatant	2×10^5
VSV Indiana pellet and VSV New Jersey supernatant	2×10^4
VSV Indiana pellet and Chandipura supernatant	3×10^4
VSV New Jersey pellet and VSV Indiana supernatant	4×10^4
VSV New Jersey pellet and Chandipura supernatant	6×10^4
Chandipura pellet and VSV Indiana supernatant	10^4
Chandipura pellet and VSV New Jersey supernatant	2×10^4

[a] Purified preparations of VSV Indiana, VSV New Jersey, and Chandipura viruses were dissociated, and their components were assayed separately or in combinations as described in Materials and Methods and Table 1. The PFU observed is expressed as the total obtainable for the complete 0.35-ml fraction (or 0.70-ml combination). In three instances, no plaques were observed at the lowest dilutions plated (*). Homologous reconstitution of infectivity was significantly greater than the component infectivities. Heterologous reconstitution of infectivity was not significantly different from the template infectivities, but, because these were much greater than in the comparable experiments recorded in Table 1, small quantities of heterologous reconstitution (less than 10^4) would have been missed.

tional homology remains within this subgroup of rhabdoviruses.

The studies presented here have shown that not only transcription reconstitution but also infectivity reconstitution can be obtained for both homologous and, for the VSV Indiana and Cocal viruses, heterologous combinations of viral components. This result indicates that that heterologous reconstitution of transcription has potential biological consequences. It is clear, however, from the results presented that VSV Indiana infectivity can be most efficiently reconstituted from VSV parts, although Cocal enzyme fractions can be substituted. Although transcription reconstitution did not a priori imply that there would be a reconstitution of infectivity, we have found that such was the case. However, in making quantitative comparisons between the amounts of reconstituted transcription and amounts of reconstituted infectivity (e.g., from Fig. 1 and Table 1), there are a variety of other factors that would render such comparisons invalid (e.g., fidelity of transcription, its completeness, etc.). Difficulties in reconstituting high levels of Cocal infectivity parallel the difficulties we have experienced in obtaining reconstitution of Cocal transcriptase activity (see Fig. 1 legend).

The results obtained for heterologous reconstitution between VSV Indiana and Cocal viruses parallel the genetic complementation results obtained recently by Pringle and Wunner (11) for these two viruses.

The ability to reconstitute transcriptase activity as well as infectivity between other rhabdoviruses is currently being investigated.

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