JOURNAL OF VIROLOGY, June 1973, p. 915-925

Vol. 11, No. 6
Printed in U.S.A.

Complementary RNA Species Isolated from Vesicular Stomatitis (HR Strain) Defective Virions

POLLY ROY, P. REPIK, E. HEFTI, AND D. H. L. BISHOP

Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey 08903

Received for publication 13 February 1973

The wild-type strain of vesicular stomatitis virus (VSV) contains in its complete virion (VSV-1, B particles) a minus strand RNA. The principle defective particle of the wild-type strain (VSV-111, T particles) contains a shorter minus strand, homologous to part of the VSV-1 genome. Neither virion contains any detectable complementary (plus) strand RNA. In contrast, a preparation of a heat-resistant (HR) strain of VSV containing defective virions was found to contain both plus (21%) and minus strand RNA, present in several distinct size classes. It was found that the RNA in the HR virion preparation was at least 94% single-stranded and principally (96%) in ribonucleoprotein complexes. On extraction the plus and minus strand RNA species partially annealed to give a population of double- and multistranded RNA species. A small amount of RNA polymerase activity was associated with the HR defective virus preparation.

Vesicular stomatitis virus (VSV) in a bullet-shaped rhabdovirus which contains a single-stranded RNA in the wild-type, infectious virion (VSV-1, B particle). The virus particle contains three principle proteins, an outer glycoprotein (G), a membrane protein (M), and a nucleoprotein (N) which is associated with the virion RNA in a ribonucleoprotein complex. Minor amounts of other proteins are also present (19). It has been shown that the virus particle contains an RNA polymerase which is able to transcribe the virion RNA repetitively, sequentially, and completely to form a series of complementary RNA species (1-3, 6-8, 17). Since the VSV messenger RNA in infected cells is also complementary to the virion genome (12, 13), presumably the function of the polymerase is to supply an infected cell with these mRNA species by transcription of the virion RNA. By convention we describe messenger RNA as plus strands, so that the virion RNA is thereby designated as a minus strand.

In experiments designed to continue to test the genome homology of various VSV defective virion RNA species to that of the complete particle (17), we have employed the heat-resistant (HR) strain of VSV described by A. Holloway to obtain new defective virion types (11). In such experiments we have found that populations of VSV HR strain virus containing defective virions possess both plus and minus strands. The presence and characterization of these RNA species and their associations in the virus population are described in this paper.

MATERIALS AND METHODS

Virus stocks purification of virions. The HR strain (11) of VSV was obtained from M. E. Reichmann (University of Illinois). The wild-type strain of VSV was obtained from R. W. Simpson (Rutgers University). The purification of ^{3}H-uridine-labeled VSV through polyethylene glycol (PEG) precipitation and successive equilibrium and velocity sucrose gradient centrifugations has been described (1, 17). The preparation of wild-type complete virions (VSV-1, B particles) involved infecting cells with a dilution of a twice-cloned preparation of wild-type virus. The preparation of wild-type virion populations containing both VSV-1 and defective T particles (VSV-III) involved infecting cells at 37 C with a preparation of virus which had been previously passed at high multiplicity of infection. The preparation of HR virus utilized in these studies involved infecting cells with an inoculum of HR virus which had been passed three times at high multiplicity of infection.

Virions labeled with ^{3}H-cytidine or ^{3}H-adenosine were prepared as described previously (1, 3, 6); virions labeled by ^{32}P-phosphoric acid were grown in Eagle medium (4) containing 100 μCi of phosphoric acid per ml.

Transcription reaction conditions, purification of free transcription product RNA. A 10-fold stan-

dard reaction mixture containing ^{32}P-α-CTP to label product RNA species was primed by ^{3}H-uridine-labeled VSV-1 and incubated at 31 C for 5 h as described previously (1). After addition of sodium dodecyl sulfate (SDS) and 50 μg of unlabeled *Escherichia coli* bulk RNA, the reaction mixture was extracted for RNA by phenol, the aqueous phase was passed through Sephadex G-50 to remove triphosphates, and the RNA in the void fractions was precipitated by ethanol (7). Since the labeled RNA contained both ^{3}H-VSV-1 virion (minus) strand and ^{32}P complementary (plus) strand nucleic acid, the total RNA was subsequently chromatographed through a column of 4% agarose in 0.4 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA and 0.1% SDS (17). The void fractions contained all the ^{3}H label as well as a portion (about 5%) of the ^{32}P. The included fractions contained the rest of the ^{32}P-labeled RNA which was then collected by alcohol precipitation. This free product RNA contained no detectable ^{3}H label and on self-annealing exhibited no increase in ribonuclease resistance, indicative of the sole presence of VSV product, plus-strand RNA. The extraction of RNA from virus preparations has been described previously (4).

Melting, annealing, and polyacrylamide gel electrophoresis of RNA. Melting experiments on RNA samples suspended in 0.01 M sodium phosphate-0.005 M EDTA (pH 7.0) were accomplished by heating an RNA sample at 100 C for 90 s and immediately freezing it by plunging in a dry-ice—ethanol bath (16). Annealing experiments were performed in the presence of 0.4 M NaCl by incubating the RNA sample at 60 C for 60 min or longer (7). Polyacrylamide gel electrophoresis of RNA samples was performed as described previously (4, 6).

Ribonuclease resistance of RNA. The resistance of an RNA sample to digestion by ribonucleases was determined by incubating the RNA in 1 ml of 0.4 M NaCl-0.01 M Tris-hydrochloride buffer-0.005 M EDTA (pH 7.4) for 30 min at 37 C with 10 μg of ribonuclease T_1 and 10 μg of ribonuclease A. The residual trichloroacetic acid-insoluble radioactivity was determined and is expressed as a percentage of the label in the original sample. Limited ribonuclease digestion to remove single-stranded RNA from multistranded species was performed as described previously (7).

RESULTS

Properties of the defective virions of VSV HR strain. A preparation of VSV HR strain (previously passed three times at high multiplicity of infection) was used to infect monolayer cultures of BHK cells in the presence of ^{3}H-cytidine. After purification of the virus through PEG precipitation and equilibrium sucrose gradient centrifugation, the virus preparation was loaded and resolved by velocity sucrose gradient centrifugation (Fig. 1). No discernible band of complete virions (VSV-1, B particles) was detected, although a defective virus band having a slower sedimenting shoulder was observed and collected. A sample of the defective virus pool was incubated at 37 C for 30 min in 1 ml of 0.15 M NaCl-0.01 M Tris-hydrochloride buffer (pH 7.4) with or without 100 μg of ribonuclease A and then subjected to centrifugation in a similar gradient of sucrose. No difference in the distribution of trichloroacetic acid-soluble or insoluble radioactivity was found for either sample or by comparison with the result shown in Fig. 1. When a sample of the labeled virus was incubated in 0.3 M KOH at 37 C for 18 h, all the radioactivity was rendered acid soluble. These results indicated that the ^{3}H label in the virions was not present in DNA nor was it, in its unextracted state, sensitive to digestion by ribonuclease, and presumably it was present within the viral particles.

Samples of HR virus-defective particles were incubated with SDS prior to dilution into 1 ml of 0.4 M NaCl-0.01 M Tris-hydrochloride (pH 7.4) containing ribonucleases A and T_1. It was found that the HR defective virions were relatively insensitive to subsequent digestion by ribonucleases at low pretreatment conditions of SDS, in contrast to similar experiments with our wild-type VSV virus particles (either VSV-1 or VSV-III) (Fig. 2). Only above 5% SDS pretreatment was most (94%) of the virion nucleic acid sensitive to subsequent ribonuclease digestion. These results suggest that the

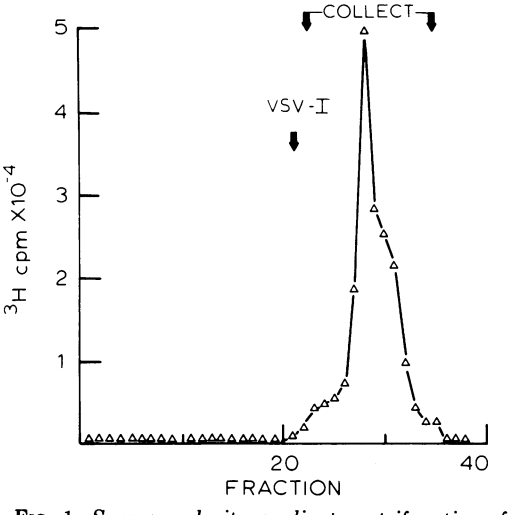

FIG. 1. *Sucrose velocity gradient centrifugation of VSV HR defective particles. A preparation of ^{3}H-cytidine-labeled VSV HR defective particles was purified as described in Materials and Methods. The virus was loaded over a linear gradient of 30 to 15% sucrose and centrifuged for 35 min at 35,000 rpm at 3 C in a Spinco SW41 rotor as described previously (17). The position of the complete VSV-1 B particles is given. The indicated fractions were collected and used in subsequent studies.*

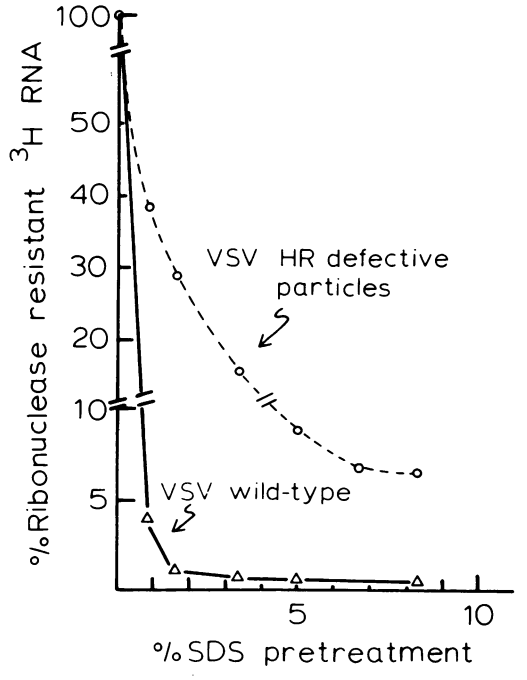

FIG. 2. *Effect of SDS pretreatment on the ribonuclease resistance of VSV virion RNA. A preparation of ^{3}H-cytidine-labeled VSV HR defective virions, or ^{3}H-cytidine-labeled wild-type VSV-1 was mixed with various concentrations of SDS at room temperature for one min and then diluted 50-fold into 1 ml of 0.4 M NaCl-0.01 M Tris-hydrochloride buffer (pH 7.4) containing 10 μg of ribonuclease A and 10 μg of ribonuclease T_1. After incubation at 37 C for 30 min, the residual acid-insoluble radioactivity was determined.*

HR strain of VSV differs structurally from our wild-type virus, and this is presumably related to its heat-resistant properties (11).

These results also indicated that the majority (94%) of the virion RNA was single stranded, although whether the residual 6% of RNA was unreleased from the virions, double-stranded, or annealed rapidly during the extraction procedure could not be determined by these preliminary experiments.

A preparation of the defective HR virions was mixed with 10% SDS and then loaded and subjected directly to electrophoresis in a swollen 2.2% polyacrylamide gel. Several radioactive bands of RNA were observed (Fig. 3). If the same quantity of labeled virus was pretreated with 10% SDS, subjected to ribonuclease digestion, and then resolved by gel electrophoresis, a smaller quantity of one of the bands was recovered in an amount representing approximately 5% of the initial label.

Pretreatment of the defective virions with 1% SDS prior to electrophoresis resulted in RNA bands which were better resolved by comparison to the previous 10% SDS pretreatment (see Fig. 3 legend). However, if after electrophoresis the individual gel slices containing labeled RNA were incubated with ribonucleases and the residual acid-insoluble radioactivity was determined, it was found that there were two regions of ribonuclease-resistant RNA remaining. Of the major discernible RNA peaks from the 1% SDS-treated virus [designated (a), (b), (c) and (d) containing, respectively, 9%, 55%, 16%, and 15% of the virion label (Fig. 3)] all the peak (c) was ribonuclease resistant whereas all of the peaks (d), (a), and most of (b) were ribonuclease sensitive. The slower edge of the peak (b) was partially ribonuclease resistant. From the amounts and size of the ribonuclease-resistant species found, in comparison to those seen by pretreatment by ribonucleases prior to electrophoresis reported above, these results indicated that double-stranded RNA species were generated after extraction of the viral RNA and either before, during, or after the electrophoresis. Alternatively, the results could be interpreted to indicate that residual protein remained attached to the RNA species despite the SDS pretreatment and electrophoresis in gels containing 0.1% SDS.

To determine if the ribonuclease-resistant RNA was due to newly-formed double-stranded RNA species containing plus and minus strands or RNA hairpins or RNA-protein complexes, some of the virion RNA was extracted by SDS-phenol in the presence of 0.4% NaCl for further analysis.

RNA-ribonucleoprotein complexes of VSV HR defective virions. Although experiments reported above suggested that the label in the virus preparation was within the virions, it did not determine if the RNA was completely present in ribonucleoprotein complexes or whether some was free RNA within the virions. To determine the amount of RNA in virion ribonucleoprotein complexes, a preparation of ^{3}H-adenosine-labeled, HR defective virus was centrifuged on gradients of sucrose either directly or after pretreatment by Triton-N101, sodium deoxycholate, and urea (to liberate) ribonucleoprotein-RNA complexes) or by SDS (to liberate "free" RNA). The resulting profiles of radioactivity are shown in Fig. 4. Approximately 4% of the total label was recovered from the Triton-dissociated virus as "free" RNA (sedimenting similarly to the RNA obtained from SDS-treated virions). It can be concluded therefore that no more than 4% of the label was present as intravirion-free RNA.

Homology of the RNA extracted from defective virions of VSV HR strain. RNA was

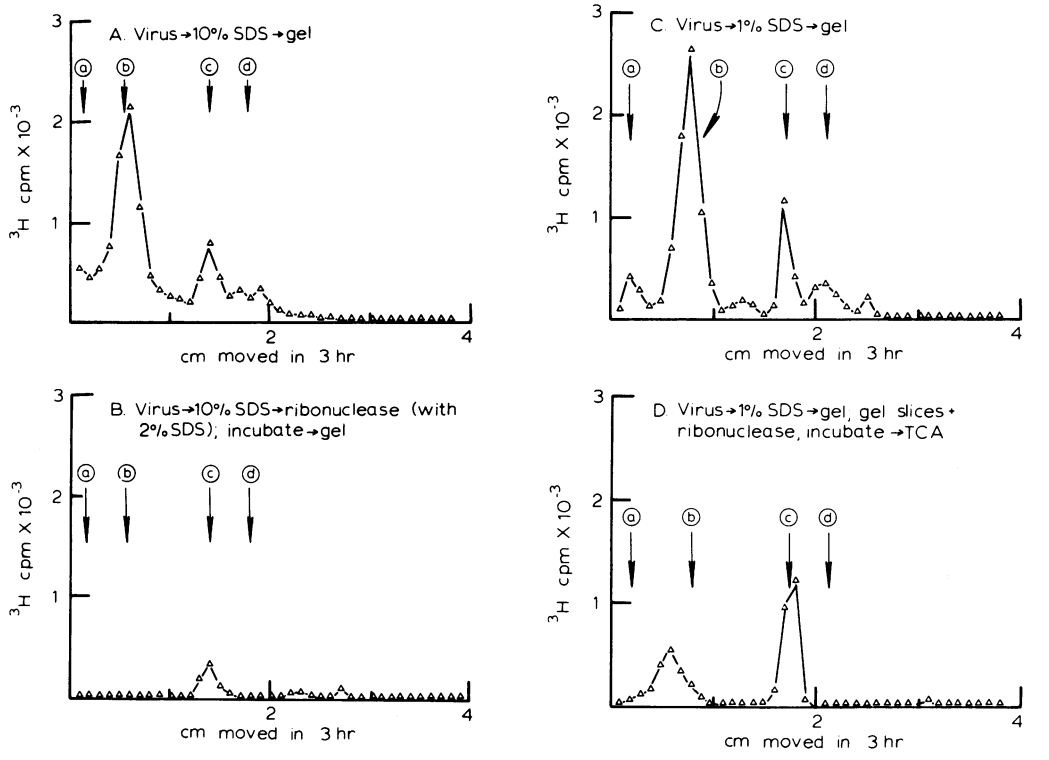

FIG. 3. *Polyacrylamide gel electrophoresis of RNA released from VSV HR defective virions. A sample of ^{3}H-cytidine-labeled VSV HR defective virions was mixed with 10% SDS at room temperature for 1 min and then (A) loaded and resolved by electrophoresis in a 2.2% swollen polyacrylamide gel for 3 h as described previously (4). The gel was sliced and dissolved in 30% hydrogen peroxide, and the distribution of label was determined (6). Alternatively (B), after addition of SDS, the sample was diluted fivefold into 80 μliters of 0.4 M NaCl-0.01 M Tris-hydrochloride (pH 7.4), containing 1 μg of ribonuclease A and 1 μg of ribonuclease T_1. After incubation at 37 C for 30 min the products were resolved by similar polyacrylamide gel electrophoresis. Another sample of the original virus preparation (C) was treated with 1% SDS prior to electrophoresis. A fourth sample of virus (D) was treated as described for (C), but after electrophoresis the individual gel slices were mixed with 1 ml of 0.4 M NaCl-0.01 M Tris-hydrochloride buffer (pH 7.4), containing 10 μg of ribonuclease A_0 and 10 μg of ribonuclease T_1, incubated at 37 C for 30 min, and the acid-insoluble residues (including the gel slice) were collected on a membrane filter. After drying, the filter and contents were dissolved in 0.4 ml of 30% hydrogen peroxide prior to determining the remaining ^{3}H label. The position of four main bands of RNA [(a), (b), (c), and (d)] are indicated. Other RNA bands [between (b) and (c) or after (d)] were also observed (see C) but were poorly resolved from the other species. The present and subsequent identification of these four RNA bands does not imply homogeneity or identity from one experiment to another. Also note that the RNA samples which were loaded in "high-salt" condition (A and B) moved slower and were more poorly resolved than the samples which were loaded under lower salt conditions.*

extracted from the population of HR-defective virions, and its ribonuclease resistance was determined with or without an annealing pretreatment (Table 1). It was found that 20% of the RNA was ribonuclease resistant prior to self-annealing and 38% after self-annealing. A sample of the RNA was melted at 100 C and diluted to various RNA concentrations, and the ribonuclease resistance was again determined before and after annealing (Table 1). It was found that after melting, 92% of the RNA was ribonuclease sensitive and that reannealing was concentration and time dependent, indicating that both plus and minus strands were present.

To determine if the plus strands were complementary to our wild-type VSV-1 virion RNA (minus strands), the melted and diluted HR-defective virion RNA was annealed to an excess of unlabeled VSV-1 virion RNA (Table 1). Approximately 15% of the melted ^{3}H-RNA subsequently annealed to the VSV-1 RNA and was thereby rendered ribonuclease resistant (Table 1, line 10–line 14). These results confirmed the presence of VSV plus strand RNA in the HR-defective virion population. It was also calculated from these results that about 21% of the total label was in the form of plus strand RNA (15 + 13/2, see Table 1).

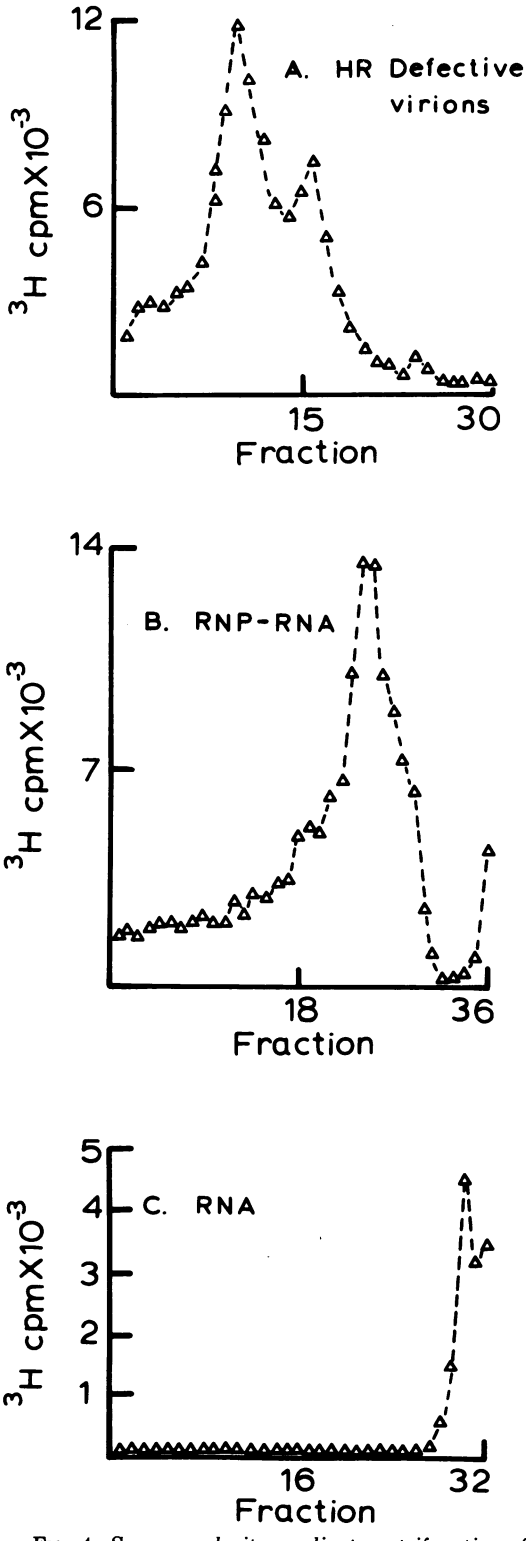

FIG. 4. *Sucrose velocity gradient centrifugation of HR defective virions, RNA-ribonucleoprotein complexes, and SDS-released RNA. A sample of ^{3}H-adenosine-labeled VSV HR defective virions (A) purified as described in Materials and Methods and Fig. 1, was centrifuged as described in Fig. 1 for 90 min. Alternatively (B), the virus preparation was pretreated and dissociated with 0.15 M NaCl-0.01 M Tris-hydrochloride buffer (pH 8.0)-Triton N101 (3 mg/ml)-sodium deoxycholate (1 mg/ml)-urea (0.2 M)-0.01 M mercaptoethanol and incubated at 31 C for 5 min. The mixture was loaded over a linear gradient of 30 to 15% sucrose containing the same ingredients used to dissociate the virus and centrifuged for 90 min at 35,000 rpm and 3 C as above. These conditions of dissociation and centrifugation are sufficient to liberate from the virion particles the RNA-ribonucleoprotein complex containing RNA, the N protein, and some of the minor proteins, L, A, B, and NS (8, and unpublished observations). In (C) the virus was pretreated with 1% SDS and then centrifuged as described for (A).*

To perform the converse experiment, free VSV-1 transcription product (plus strand) RNA was obtained and purified from a wild-type VSV-1 RNA transcription reaction mixture as described in Materials and Methods and annealed to the melted and diluted HR defective virion RNA (Table 1). Since 98% of the total ^{3}H-RNA annealed under these conditions, this result indicated that there was about 80% ^{3}H minus strands in the defective virion population (Table 1, i.e., 98 − 36 + 36/2 = 80%). These results also demonstrated that there was little, if any, cellular or non-VSV RNA in the preparation.

Gel electrophoresis of the RNA extracted from VSV HR defective virions. Samples of the purified RNA were subjected to electrophoresis in 2.2% swollen polyacrylamide gels (Fig. 5). One gel was sliced, dissolved, and counted to determine the distribution of ^{3}H-RNA, the other was sliced, and the individual slices were treated with ribonucleases prior to determining the residual acid-insoluble radioactivity. Relative to the four RNA bands identified in the SDS-disrupted virions, the distribution of label in (a), (b), (c), and (d) (Fig. 5) was 45%, 31%, 17%, and 5%, respectively.

Peak (a). A major amount of label was present in RNA peak (a) recovered near the front of the gel. Part of the label was ribonuclease resistant. Peak (a) was evidently heterogeneous and the result of events occurring during and after extraction of RNA from the virions (compare Fig. 3). Since the ribonuclease resistance of the total extracted RNA was greater than that of SDS-treated virions and both plus and minus strands were present in the RNA population, it could be concluded that annealing had taken place during extraction

TABLE 1. *Presence or absence of plus and minus strand RNA in VSV virion populations*[a]

RNA sample	Pretreatment conditions	Ribonuclease resistance of ^{3}H-RNA (%)
VSV-1 RNA	None or melted	2
VSV-1 RNA	Self annealed	2
VSV-III RNA	None or melted	3
VSV-III RNA	Self annealed	3
VSV HR defective virion RNA	None	20
VSV HR defective virion RNA	Self annealed 1 h	38
VSV HR defective virion RNA	Melted at 4 μg/ml concentration	14
VSV HR defective virion RNA	Melted at 4 μg/ml concentration, self annealed 1 h	38
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration	8
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration, self annealed 1 h	13
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration, self annealed 2 h	22
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration, self annealed 6 h	35
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration, self annealed 16 h	36
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration, annealed 1 h with VSV-1 RNA	28
VSV HR defective virion RNA	Melted at 0.1 μg/ml concentration, annealed 16 hr with VSV-1 transcription product RNA	98

[a] Purified preparations of ^{3}H-cytidine-labeled VSV-1 or VSV-III or VSV HR defective virion RNA were melted or self-annealed as described in Materials and Methods. The self-annealing of VSV-1 and VSV-III were performed at concentrations of 30 μg of RNA per ml. The melting of VSV-1 or VSV-III RNA was performed at 0.1 μg/ml concentration. The melting and self-annealing of VSV HR defective virion RNA was performed as indicated. Purified free VSV-1 transcription product RNA was obtained as described in Materials and Methods.

and purification of the RNA, with the result of generating peak (a).

Peak (b). Prior to electrophoresis it has been shown that 20% of the total label was ribonuclease resistant (Table 1, presumably 10% plus and 10% minus strands). Since there was only 21% of the total label present as plus strands in the preparation and 31% of the label after electrophoresis was in peak (b), these observations lead us to conclude that peak (b) was mostly single-stranded minus strands.

Prior to phenol-SDS extraction and RNA purification, 55% of the label was present in peak (b); therefore 24% was involved in the annealing which occurred on RNA extraction. It was also noted that peak (b) was much broader than the faster moving peak (c), indicating that there was a mixture of RNA species therein. This was also evident from the fact that the slower moving region of peak (b) was partially ribonuclease resistant.

Peak (c). The maximum ribonuclease resistance of the initial virion RNA was found to be 6%. In the extracted RNA, peak (c) contained 17% of the total label. These results suggest, therefore, that single-stranded plus and minus strands annealed during extraction to give peak (c). From the electrophoretic mobility it was evident that the minus strand component of peak (c) was smaller than that of peak (b). The amount of the total label in peak (c) was essentially the same as that observed in the SDS-disrupted virions (see Fig. 3).

Peak (d). Very little of the RNA initially identified as peak (d) (Fig. 3) remained in the extracted RNA (Fig. 5). Whether the (d) RNA was plus or minus strand, or both, could not be determined by quantitative comparisons of Fig. 3 and 5.

When the ^{3}H-cytidine-labeled VSV HR defective virion RNA was subjected to coelectrophoresis with purified ^{32}P-labeled VSV-1 and VSV-III RNA species, it was found that peak (b) migrated faster than VSV-1 RNA and peak (c) migrated slower than VSV-III RNA (see Fig. 5). It also appeared that peak (b) migrated slightly faster than the VSV-II RNA species which we and L. Prevec previously reported (6, 14). A preparation of ^{3}H-adenosine-labeled VSV HR defective virions after extraction by 10% SDS and phenol gave essentially the same distribution of radioactivity on gel electrophoresis (see Fig. 6) as that shown in Fig. 5 and the same postextraction ribonuclease resistance as that shown in Table 1.

Are there more than one type of plus strands? Since both plus and minus strands were present in the initial virion population, the question therefore arose as to whether there was more than one size class of plus strands. For example if there was only one size of plus strands but two sizes of minus strands (one identical in size to the plus strand and the other

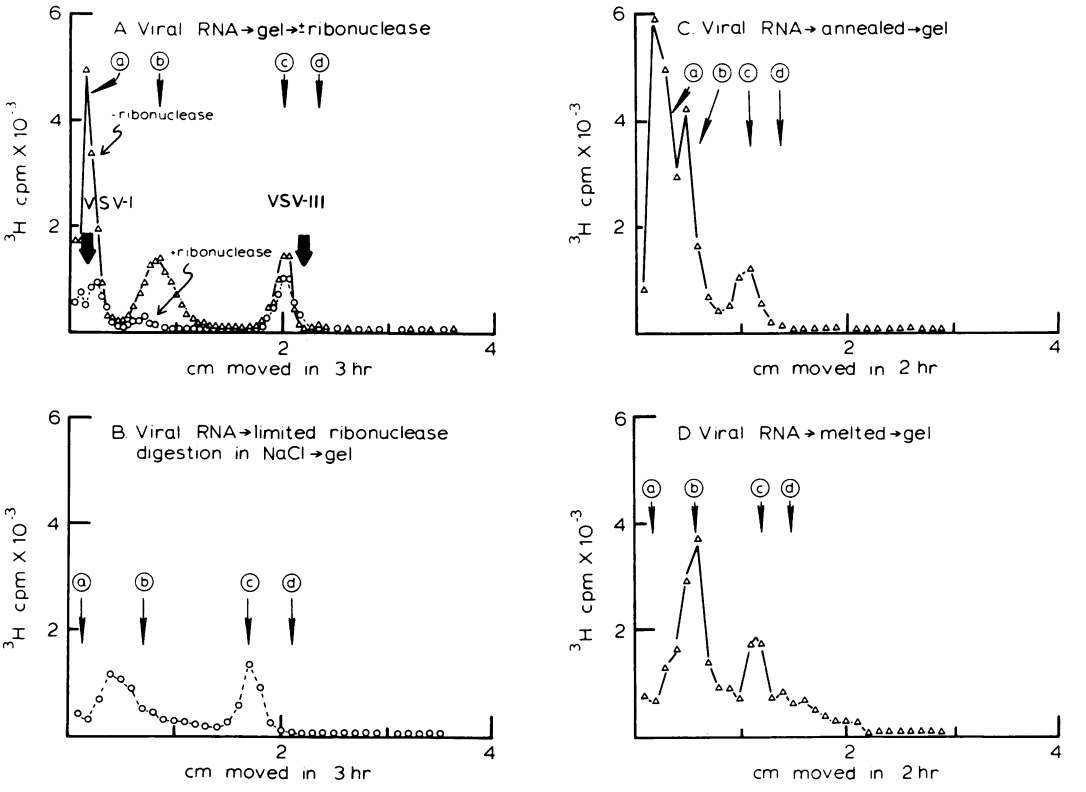

FIG. 5. *Polyacrylamide gel electrophoresis of RNA extracted from VSV HR-defective virions. RNA was extracted from ^{3}H-cytidine-labeled VSV HR defective virions as described previously (4). Samples of RNA were subjected to electrophoresis for 3 h in swollen polyacrylamide gels (A) and then either used to determine the distribution of label or incubated with ribonucleases to determine the distribution of ribonuclease-resistant acid-insoluble label (see Fig. 3D). Another sample of RNA (B) was incubated at room temperature in 0.4 M NaCl-0.01 M Tris-hydrochloride buffer (pH 7.4), containing 0.1 μg of ribonuclease T_1 per ml, for 10 min and then mixed with SDS (1% final concentration) and resolved by polyacrylamide gel electrophoresis as described in Fig. 3. A third sample (C) of RNA was annealed at 60 C for 60 min in 0.4 M NaCl at a concentration of 4 μg/ml and then resolved by polyacrylamide gel electrophoresis for 2 h. A fourth sample (D) of RNA was melted as described in Materials and Methods prior to electrophoresis for 2 h. The positions of the four RNA bands identified in Fig. 3 are indicated. In similar coelectrophoresis of the ^{3}H-cytidine-labeled RNA with ^{32}P-labeled VSV-1 and VSV-III purified RNA, the positions of the ^{32}P species relative to the ^{3}H species [(a), (b), (c), and (d)] was determined and is indicated with the large arrows in panel A.*

homologous, but larger than it), then one might expect to obtain after annealing two types of complex, a double-stranded complex (of one plus and one minus strand) and a larger, partially double-stranded complex (of one plus and more than one minus or vice versa). However, if in the initial virus preparation there were two types of minus strands and two types of respective homologous plus strands, then one would minimally obtain two double-stranded complexes in the extracted and annealed RNA and various multistranded complexes, depending on the homology of the plus strands to each other and the relative amounts of each species, etc. To distinguish between these possibilities, limited ribonuclease digestion (7) of the extracted RNA was undertaken to remove single-stranded RNA species from the complexes and reveal the size classes of the residual double-stranded backbones (Fig. 5). It was found that at least two types of double-stranded RNA were recovered after this treatment, thus demonstrating the presence of at least two types of plus strand RNA in the original virion preparation. Since the slower moving double-stranded RNA was in all probability the ribonuclease-resistant core of peaks (a) and (b), this result also suggested that peak (a) was probably multistranded.

The effect of self-annealing or melting the extracted RNA prior to gel electrophoresis is also shown in Fig. 5. Although these gels were only subjected to electrophoresis for 2 h, it can be seen that the peak (a) was essentially abolished by the melting treatment whereas peaks

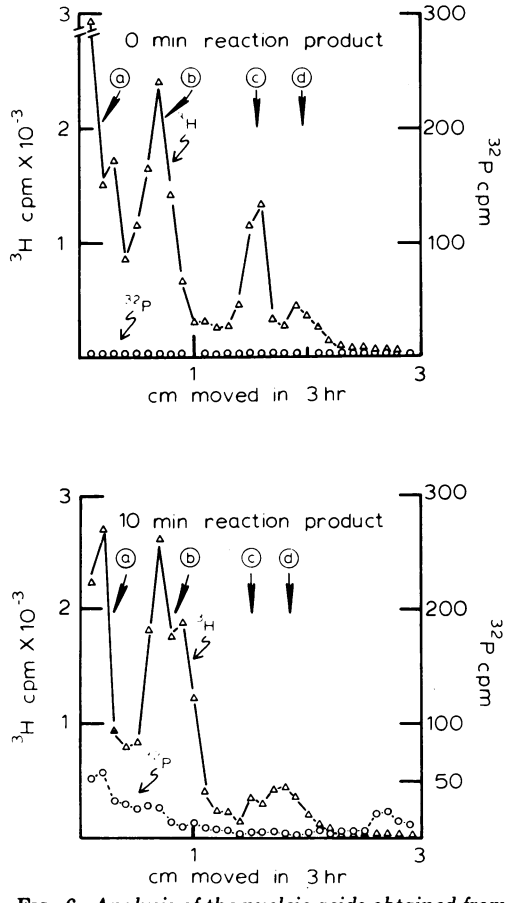

FIG. 6. *Analysis of the nucleic acids obtained from an RNA polymerase reaction primed by VSV HR defective virions. A standard reaction mixture containing ^{32}P-α-CTP to label product RNA synthesis was templated by ^{3}H-adenosine-labeled VSV HR defective virions and incubated at 31 C for 10 min. The total RNA was purified from triphosphates and resolved by polyacrylamide gel electrophoresis to determine the distribution of label. Most of the ^{32}P-RNA (90%) was recovered as free, quickly moving species retrieved between 4 and 5 cm of the gel (not shown). An unincubated (0 min) control reaction is also shown.*

(b) and (d) were augmented by the heat dissociation, in agreement with previous experiments that (b) and (d) were single stranded. Under these conditions of melting, the ^{3}H-ribonuclease resistance of the whole RNA only decreased to 15% (compare Table 1 where the melting was also performed under more dilute conditions). This quantity of ribonuclease resistance compares reasonably well with the percentage of label recovered in peak (c) of the melted sample (Fig. 5), and it was presumed that either (c) did not completely melt or that during the treatment (or subsequent electrophoresis), its constituents rapidly reannealed. When the melted RNA was subsequently reannealed, a profile identical to that shown in Fig. 5C was obtained.

In summary, these experiments demonstrated that the defective virions of the HR strain of VSV contained separate but complementary RNA species. Most (80%) of the RNA was minus strand (like the wild-type virion RNA), but the rest (about 20%) was complementary, VSV plus strand. There were at least two species of plus and also two species of minus strands present [note though that both (b) and (d) were broad bands in the gels]. On phenol-SDS extraction of the virion RNA, much of the complementary RNA species annealed together to give double- and multistranded complexes, presumably due to their high concentration and the presence of 0.4 M NaCl during the extraction procedure. Since the mobilities of the phenol-SDS-purified RNA species were similar to those found by SDS treatment of the virions, it was concluded that the observed electrophoretic mobilities of the SDS-treated virion RNA reflected the size and nature of the RNA species alone and that there was no remaining protein attached.

These experiments did not define the nature of the plus strand RNA in the virion population, other than indicate that there were two or more size classes. However, from the amount of total plus strand (20%) by comparison to the lowest observed ribonuclease resistance of the virion RNA (Fig. 2), it could be concluded that the majority (i.e., 20 − 6/2 = 17%, Fig. 1, Table 1) of the plus strand RNA was separate, that is not hydrogen bonded to minus strands. Although separate, the plus strands were, however, encapsulated in the virion particles and possibly in the form of RNA-ribonucleoprotein complexes since none of the virus particle radioactivity was sensitive to ribonuclease in the absence of pretreatment with SDS, and only 4% of the virion RNA was "free" (Fig. 4).

Transcriptase enzyme activity associated with defective virions of VSV HR strain. The wild-type strain of VSV has been shown to contain an RNA transcriptase (2) in the complete VSV-1 (B particles) but not (7, 17) in the defective VSV-111 particles (T particles). A defective particle with an intermediate size (VSV-11) was found to contain some transcriptase enzyme activity (7). Consequently, it was of interest to determine if the defective particles of VSV HR strain contained transcriptase or other RNA polymerase enzyme activity, and if so how much by comparison with wild-type B particles. Using ^{32}P-α-CTP as the precursor

nucleotide for RNA synthesis, we have previously shown (5) that the transcriptase of wild-type VSV-1 incorporates approximately 34,000 pmol of CMP per h per mg of virion protein (depending on the age of the virus preparation). We can report that the mixed population of VSV HR defective virions used in these studies incorporated approximately 460 pmol of CMP per h per mg of virion protein. Since this figure represented about 1.4% of the activity attainable with wild-type VSV-1, it is possible that the observed enzyme activity could be associated with contaminating VSV HR B particles as was found for preparations of VSV-111 particles (17). When the virion RNA was dissociated by 1% SDS and subjected to coelectrophoresis with ^{32}P-labeled VSV-1 and VSV-III RNA in an unswollen 2.1% polyacrylamide gel (to obtain better resolution of the ^{3}H-RNA species), it was found that about 3% of the total label was in a small peak which migrated with ^{32}P-VSV RNA. As a maximum of 3% of the label was present where we would expect to recover ^{3}H-VSV-1 HR (B particle) RNA, this result indicated that there was no more than that amount of VSV-1 HR virions present in the population.

If the defective virions of VSV HR contained transcriptase enzyme activity, then a simple demonstration of such activity can be obtained by running a short-term reaction and looking at the association of product (labeled by ^{32}P) with the defective virion template RNA (labeled by ^{3}H). It has been shown previously that, during the first few minutes of such short-term reactions using VSV-1, the product is principally associated with the template RNA (7). A reaction primed by the ^{3}H-VSV HR defective virions was incubated with ^{32}P-α-CTP for 10 min at 31 C, and the RNA was purified from precursor triphosphates and resolved by gel electrophoresis. The distribution of labels obtained after electrophoresis of the 10-min reaction product, by comparison with an unincubated control reaction, is shown in Fig. 6. It was observed that a little of the ^{32}P was recovered near the gel front, and the majority (90%) was recovered as quickly migrating, free RNA species (mostly very small and found between 4 and 5 cm of the gel, not shown in Fig. 6). The distribution of ^{3}H-RNA species in the 10-min reaction product showed two differences by comparison to the unincubated sample. Peak (b) in the reaction product nucleic acids had a faster moving shoulder, and peak (c) was diminished. The significance of these observations is obscured by the fact that a population of particles was being investigated. Nor was it possible to conclude whether the product RNA at the front of the gel was associated with a small amount of ^{3}H-VSV-1 (HR strain) or the defective particle RNA.

When self-annealing experiments were performed on the total reaction product nucleic acid, the ribonuclease resistance of the product RNA increased from 11% to 21%. Addition of an excess of VSV-1 RNA did not increase this amount of product ribonuclease resistance.

The proteins of VSV HR defective particles. We can report that, after resolution by SDS-polyacrylamide gel electrophoresis, the virus preparation was found to contain the three major VSV virion proteins, G, N, and M (19), as well as the minor proteins, L, B, and NS (the latter being the Mudd and Summers protein described by us previously [8] as M-S). The proportions of each protein were similar to those described in a previous communication for the complete VSV-1 virions (8). The presence or absence of the minor protein A, described previously (8), was not ascertained.

Analysis for polyadenosine tracts in the VSV HR defective virion RNA. Samples of the ^{3}H-cytidine-labeled or ^{3}H-adenosine-labeled VSV HR defective virion RNA were melted and then subjected to combined ribonuclease A and T_1 digestion in low-salt buffer, and the digest was resolved by polyacrylamide gel electrophoresis with a ^{32}P-labeled *E. coli* 4*S* RNA (10) (Fig. 7). No polyadenosine tracts (^{3}H-adenosine nucleotides migrating slower than the 4*S* marker) were detected (less than 0.1% of the total label). Since VSV mRNA contains approximately 10 to 15% of its adenosine in polyadenosine tracts (10), we conclude that little or no VSV mRNA was present in these VSV HR defective virion RNA preparations. We can confirm the observations of Ehrenfeld and Summers (10) that VSV-1 virion RNA does not contain polyadenosine, whereas VSV mRNA does possess polyadenosine tracts. We can also report that the VSV-III defective virion RNA does not contain polyadenosine tracts. The complete transcription product of VSV-1 (when labeled by ^{32}P-α-ATP) also has no detectable quantity of polyadenosine (unpublished observations).

DISCUSSION

Analysis of the RNA isolated from a population of defective virions of VSV HR strain leads us to conclude that the RNA therein is a mixture of sizes and complementary species. In none of our previous studies (7) nor in subsequent analyses (see reference 18) have we been

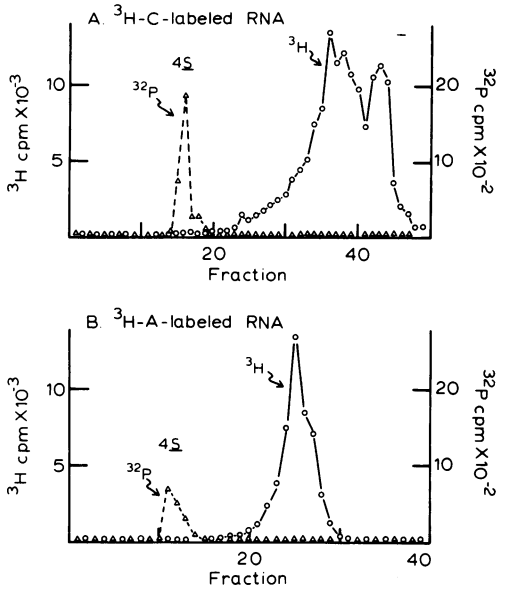

FIG. 7. *Analysis of VSV HR defective virion RNA species for polyadenosine tracts. Samples of the ^{3}H-cytidine-labeled VSV HR defective virion RNA (A) or the ^{3}H-adenosine-labeled VSV HR defective virion RNA (B) were heated at 100 C for 30 s in 150 μliters of 0.01 M sodium phosphate-0.003 M EDTA (pH 7.0). After freezing, the samples were mixed with 1.5 μg of ribonuclease A and 1.5 μg of ribonuclease T_1 and incubated at 37 C for 30 min. The solutions were then adjusted to 1% SDS and 0.4 M NaCl and extracted by phenol to remove the nucleases. Two 8% polyacrylamide gels were loaded with a sample of ^{32}P E. coli 4S RNA in 50% glycerol and subjected to electrophoresis for 12 min. The aqueous phase from phenol extractions was adjusted to 15% glycerol and then loaded on either of the two gels. Electrophoresis was continued for 2 h, the gels were sliced and dissolved, and the distribution of labels was determined. The low-salt nuclease digestion conditions were chosen to maximize the RNA digestion by minimizing reannealing between complementary RNA species.*

able to detect complementary RNA in purified wild-type VSV-1 or the defective particles VSV-II or VSV-III (see Table 1). Usually infection of BHK cells at high multiplicity results in the production of one, or maybe two, defective virions in addition to the VSV-1 particle. In these studies at least two defective virions were present (Fig. 1 and 4), although which species contained plus strand RNA has not yet been determined. None of the HR defective virion RNA species appeared to be equivalent to the VSV-II or VSV-III RNA we have observed previously (Fig. 5), although their exact homology to VSV-1, II, or III, or each other, will involve initial purification of the various RNA species and subsequent hybridization to VSV-I transcription product RNA. The RNA species of wild-type VSV-III migrates between the HR defective virion RNA species (c) and (d) (Fig. 5).

We do not at present understand the mechanism of defective virion production in an infected cell nor what controls virion evolution, replication, and encapulsation. Genetic, physiological, and host factors may all have roles to play in defective virion production.

Form of the plus strand RNA in HR defective virions. From reasons presented in the text we have concluded that the majority of plus and minus strands are not hydrogen bonded together in the virions. Nor are the majority of the plus strands free RNA molecules, since Triton and deoxycholate treatment of virions leaves the majority (96%) of the RNA in ribonucleoprotein-RNA complexes. We conclude therefore that the plus strand RNA is present in these RNA-ribonucleoprotein complexes.

We envisage three possible sources of the plus strand RNA: mRNA, transcription product, or defective RNA replicative forms. We define mRNA species as those VSV transcription products which have additional polyadenosine nucleotide sequences (10). We have found no polyadenosine sequences in the complete in vitro transcription product of VSV-I (unpublished observations). This result allows us to operationally distinguish VSV mRNA from VSV transcription product RNA. Analyses of ribonuclease digests of adenosine-labeled VSV HR defective virion RNA and resolution on high concentration polyacrylamide gels (10) has indicated that there is no detectable polyadenosine in the RNA population (Fig. 7), and this result suggests that there is no VSV mRNA in the preparation (10).

We conclude therefore that either the plus strand RNA is transcription product or replicative forms of the defective RNA species. Even though we cannot positively ascribe RNA polymerase activity to the HR defective virions, since the observed activity could be due to contaminating HR VSV-1 virions, this result does not rule out the possibility that the defective virions, prior to their release from the cells, contained some RNA polymerase activity. This activity could be a transcriptase or even an RNA replicase. To distinguish between these possibilities will require further more detailed analysis of the small-molecular-weight RNA product formed by the RNA polymerase activity of the HR defective virion preparation, and these analyses will involve selective hybridization of the free product to purified plus or minus RNA strands of the defective virion preparation

as well as to VSV-1 and its transcription product. Such experiments are being undertaken currently.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10692 from the National Institute of Allergy and Infectious Diseases and by Damon Runyon grant DRG 1169.

We thank Roger Van Deroef for excellent technical assistance.

LITERATURE CITED

1. Aaslestad, H. G., H. F. Clark, D. H. L. Bishop, and H. Koprowski. 1971. Comparison of the ribonucleic acid polymerases of two rhabdoviruses, Kern Canyon virus and vesicular stomatitis virus. J. Virol. 7:726–735.
2. Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. U.S.A. 66:572–576.
3. Bishop, D. H. L. 1971. Complete transcription by the transcriptase of vesicular stomatitis virus. J. Virol. 7:486–490.
4. Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. J. Mol. Biol. 26:373–387.
5. Bishop, D. H. L., J. F. Obijeski, and R. W. Simpson. 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for in vitro activity of the ribonucleic acid-dependent ribonucleic acid polymerase. J. Virol. 8:66–73.
6. Bishop, D. H. L., and P. Roy. 1971. Kinetics of RNA synthesis by vesicular stomatitis virus particles. J. Mol. Biol. 57:513–527.
7. Bishop, D. H. L., and P. Roy. 1971. Properties of the product synthesized by vesicular stomatitis virus particles. J. Mol. Biol. 58:799–814.
8. Bishop, D. H. L., and P. Roy. 1972. Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. J. Virol. 10:234–243.
9. Eagle, H. 1959. Amino acid metabolism in mammalian cell culture. Science 130:432–437.
10. Ehrenfeld, E., and D. F. Summers. 1972. Adenylate-rich sequences in vesicular stomatitis virus messenger ribonucleic acid. J. Virol. 10:683–688.
11. Holloway, A. F., P. K. Y. Wong, and D. V. Cormack. 1970. Isolation and characterization of temperature-sensitive mutants of vesicular stomatitis virus. Virology 42:917–926.
12. Huang, A. S., D. Baltimore, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. Virology 42:946–957.
13. Mudd, J. A., and D. F. Summers. 1970. Polysomal ribonucleic acid of vesicular stomatitis virus infected HeLa cells. Virology 42:958–968.
14. Petric, M., and L. Prevec. 1970. Vesicular stomatitis virus—a new interfering particle, intracellular structures, and specific RNA. Virology 41:615–630.
15. Reichmann, M. E., C. R. Pringle, and E. A. C. Follett. 1971. Defective particles in BHK cells infected with temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. 8:154–160.
16. Roy, P., and D. H. L. Bishop. 1970. Isolation and properties of poliovirus minus strand ribonucleic acid. J. Virol. 6:604–609.
17. Roy, P., and D. H. L. Bishop. 1972. The genome homology of vesicular stomatitis virus and defective T particles and evidence for the sequential transcription of the virion ribonucleic acid. J. Virol. 9:946–955.
18. Schaffer, F. L., A. J. Hackett, and M. E. Soergel. 1968. Vesicular stomatitis virus RNA: complementarity between infected cells RNA and RNA's from infectious and autointerfering fractions. Biochem. Biophys. Res. Commun. 31:685–692.
19. Wagner, R. R., L. Prevec, F. Brown, D. F. Summers, F. Sokol, and R. MacLoed. 1972. Classification of rhabdovirus proteins: a proposal. J. Virol. 10:1228–1230.