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Comparison of the Ribonucleic Acid Polymerases of Two Rhabdoviruses, Kern Canyon Virus and Vesicular Stomatitis Virus

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A ribonucleic acid (RNA)-dependent RNA polymerase has been demonstrated in Kern Canyon virus (KCV) particles. The RNA product of the KCV polymerase hybridizes to KCV viral RNA. The properties of this viral enzyme have been characterized and compared with those of vesicular stomatitis virus (VSV). RNA polymerases from both viruses require similar conditions of temperature, *p*H, and detergent and magnesium concentrations for maximal synthesis of RNA. The RNA polymerase contained in the virion of KCV was more dependent on the presence of a sulfhydryl agent than was the VSV enzyme. Under optimal conditions, the specific activity of the VSV polymerase is about twenty-five times as great as that of KCV.

The rhabdovirus vesicular stomatitis virus (VSV) has been found to possess a virion-bound ribonucleic acid (RNA)-dependent RNA polymerase (1). This polymerase transcribes at least 94% of the genome (Bishop, *submitted for publication*) and synthesizes RNA which is complementary to the viral genome (1, 1*a*). Since no evidence for in vitro replication of the viral genome has been obtained, the VSV polymerase should be described as a transcriptase. Recently, it has been shown that messenger RNA isolated from polyribosomes of infected cells will hybridize to the viral RNA (6, 9). The fact that both VSV messenger RNA and in vitro-synthesized RNA hybridize to viral RNA suggests that the product of the viral transcriptase is messenger RNA.

To determine whether viruses possessing a virion-bound RNA polymerase are widely spread in nature, we have examined another rhabdovirus, Kern Canyon virus (KCV). This virus is morphologically similar to VSV but antigenically quite distinct (7, 10).

The purpose of this paper is to report the demonstration of an RNA-dependent RNA polymerase in Kern Canyon virions and to compare the optimal conditions for demonstrating the RNA polymerase activity of both KCV and VSV.

MATERIALS AND METHODS

Viruses and cells. BHK-21- cell monolayers (8) were grown at 37 C in Blake bottles by using 100 ml of Eagle's medium (4) containing 5,000 units of penicillin and 5 mg of streptomycin and supplemented with twice the normal content of amino acids and vitamins, 0.44% sodium bicarbonate, and 10% fetal calf serum (13). Infectivity assays were performed with BHK-13S cells with a suspension agarose-plaquing technique (12). VSV, Indiana serotype, was obtained from R. Simpson, Rutgers University. KCV was obtained from A. Chappell (Center for Disease Control, Atlanta, Ga.) in the form of a fourth suckling mouse brain passage. It was passed once in suckling mouse brain and then plaque purified three times.

Preparation of virus. Confluent monolayers of BHK-21 cells were washed once to remove serum and infected with KCV or VSV (multiplicity of infection = 1 or 0.01, respectively) by adding a 10-ml suspension of virus in Eagle's medium and allowing the virus to adsorb for 30 min. The cells were then overlaid with 100 ml of fresh, supplemented Eagle's medium (but containing only 2% fetal calf serum for the propagation of VSV or 0.1% bovine serum albumin for KCV growth). When present, ^{3}H-uridine was added to give a concentration of 10 μCi per ml. The infected cells were incubated at 32 C for 24 hr, after which the supernatant fluids were decanted and replaced with fresh medium. At 48 hr postinfection, the second batch of medium was harvested. The titers obtained for VSV were in the range of 2×10^9 plaque-forming

units (PFU) per ml, whereas that for KCV was around 5×10^8 PFU per ml.

One to two liters of virus containing supernatant fluids were clarified by centrifugation at 1,000 × *g* and 4 C for 15 min. Calf serum was added to the KCV harvests [2% (v/v) final concentration] as a coprecipitant for the virus during the subsequent addition of ammonium sulfate (300 g per liter). The ammonium sulfate was added slowly with stirring, and the mixture was maintained at *p*H 7.5 by addition of 1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 8, and held at 4 C for at least 30 min. The precipitated proteins were then recovered by centrifugation at 2,000 × *g* for 30 min and 4 C and dissolved in a minimal volume of 0.1 M NaCl, 0.01 M Tris-hydrochloride buffer, 0.01 M ethylenediaminetetraacetic acid (EDTA), *p*H 7.5 (NTE); the material from each harvest was combined. Virus was recovered from this solution by sedimentation (80,000 × *g* for 90 min and 5 C in an SW27 Spinco rotor) onto a 2-ml cushion of 100% glycerol. The visible virus band was collected by pipette from above the glycerol cushion. Usually the cushion surface was washed with a small volume of buffer to recover residual virus. Glycerol and ammonium sulfate were removed by passing the virus suspension through a 2 by 6 cm column of Sephadex G 50 (coarse grade) employing NTE buffer for elution. The virus suspension was layered over a 10 to 30% linear gradient of sucrose (prepared in NTE buffer) and centrifuged in the SW27 Spinco rotor for 50 min at 80,000 × *g* and 5 C. The visible virus band was collected by pipette, diluted twofold with 0.1 M NaCl, 0.01 M Tris-hydrochloride buffer (*p*H 7.5), and concentrated by centrifugation over a glycerol pad as described above. The final virus suspension usually contained 1 to 2 mg of protein per ml.

RNA polymerase assay. The standard reaction mixture contained per 125 μliters: 8 μmoles of Tris-hydrochloride buffer, *p*H 8.2; 13 μmoles of NaCl; 1 μmole of $MgCl_2$; 0.5 μmoles of 2-mercaptoethanol; 0.1 μmole of three unlabeled ribonucleoside triphosphates [(usually adenosine triphosphate (ATP), guanosine triphosphosphate (GTP), and cytidine triphosphate (CTP)]; 0.01 μmole of ^{32}P-α-uridine triphosphate (UTP) or ^{32}P-α-GTP (*see* figure legends); 20 μg of Triton-N101; and 10 to 50 μg of virus protein. In particular experiments, the constituents were varied as indicated. Reaction mixtures were incubated at 31 C in capped tubes (0.8 by 7 cm) and portions were removed at intervals to determine either the amount or rate of incorporation of ^{32}P-uridine monophosphate (UMP) or ^{32}P-guanosine monophosphate (GMP). Incorporation of radioactivity was determined by precipitation of insoluble materials with at least 10 volumes of cold 10% trichloroacetic acid after addition of 100 μliters of a mixture of saturated sodium pyrophosphate and saturated disodium hydrogen phosphate. The insoluble precipitate was collected on a cellulose nitrate membrane filter and washed 10 times with 5-ml volumes of cold 10% trichloroacetic acid. After drying, the filters were placed in a toluene-based cocktail and counted in a scintillation counter. When the rate of RNA polymerase activity was determined, the incorporation of ^{32}P-UMP or ^{32}P-GMP was normalized to a constant ^{3}H recovery (*see below*) to avoid variation in sample volume. All rates of enzyme activity represent the initial 60-min linear incorporation of label after subtracting the value obtained for an unincubated control reaction mixture.

Isotopes, chemicals, and enzymes. ^{3}H-uridine (specific activity 22 mCi/μmole) was purchased from ICN, Irvine, Calif. ^{32}P-α-UTP and ^{32}P-α-GTP (specific activity of 0.1 to 5.0 mCi/μmole) were synthesized and purified as previously described (5). Ammonium sulfate (enzyme grade) was obtained from Mann Research Laboratories, Orangeburg, N.J. Triton-N101 was obtained from Sigma Chemical Co., St. Louis, Mo.; Nonidet P40 was from Shell Chemical Co., New York, N.Y.; and actinomycin D was a gift from Merck & Co.

RESULTS

Purification procedure. The purification procedure involving ammonium sulfate precipitation and ultracentrifugation has been successfully employed by us not only for the purification of KCV and VSV but also for two other rhabdo-

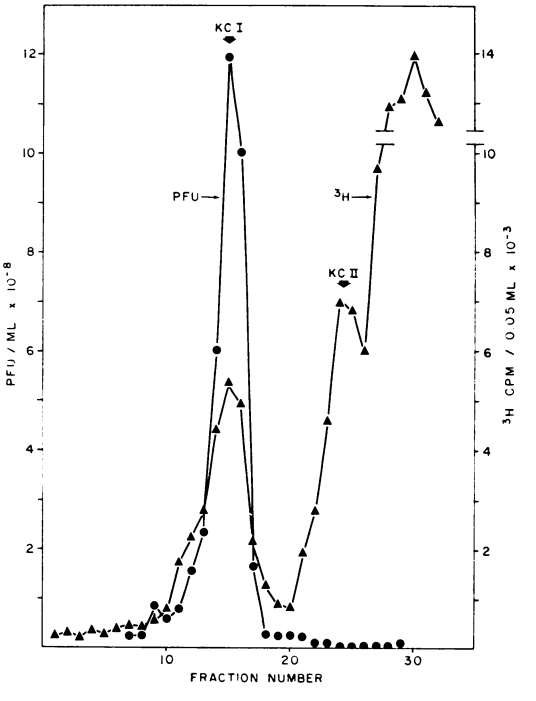

FIG. 1. *Separation of KCV particles by velocity gradient sedimentation. A concentrated preparation of ^{3}H-uridine-labeled KCV obtained as the post-ammonium sulfate pellet was dissolved in NTE buffer and sedimented for 50 min in a sucrose gradient. The gradient was fractionated by bottom puncture. Portions of each fraction were titered for PFU (●) or for content of trichloroacetic acid-insoluble radioactivity (▲).*

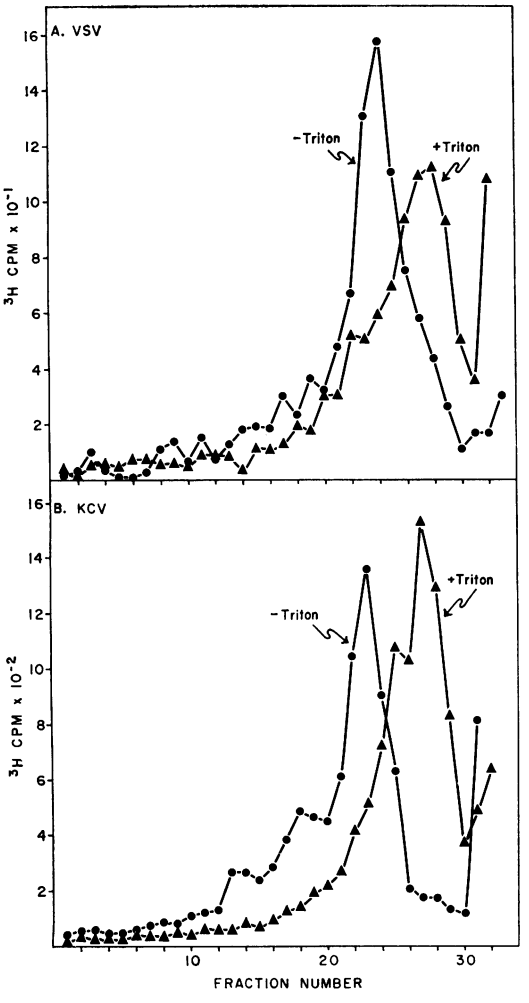

FIG. 2. *Subviral ribonucleoproteins of KCV and VSV. Purified ^{3}H-uridine-labeled KCV or VSV were sedimented for 25 min at 80,000 × g and 5 C in a 10 to 30% sucrose gradient in NTE buffer. Additionally, samples of each virus were pretreated with 0.1% Triton N101, adjusted to 0.02 M EDTA, and similarly sedimented, in parallel, to the untreated samples. The distribution of trichloroacetic acid-insoluble radioactivity was determined as described in Fig. 1. The values obtained for the untreated (●) or treated (▲) preparations of the same virus are superimposed.*

viruses—rabies and egtved (Bishop et al. *manuscript in preparation*). The recovery of infectious virus at the final stage of purification was about 10 to 20% of the PFU present in the initial supernatant fluids. The best PFU:particle ratio for purified VSV was around 1:500. This value was calculated by relating the PFU per milliliter of a virus suspension to the number of ^{3}H-uridine-labeled viral RNA molecules present. This estimate assumed that each virion has one RNA molecule; the number of RNA molecules was calculated from the counts per minute of ^{3}H label per milliliter, the molecular weight of the viral genome, and the specific activity of the RNA (usually around 3×10^5 counts per min per μg of RNA). Although an 80 to 90% loss of infectious particles would not appear to be satisfactory, the PFU present in various supernatant fluids obtained throughout the purification procedure only accounted for 5 to 10% of the initial PFU. Consequently, the apparent loss of infectious particles does not necessarily correspond to loss of virus, and our final viral preparations could have contained more than 10 to 20% of the initial virus present. That the purified KCV preparation was not contaminated by VSV was verified by plaque titration in BHK-13S and L cells. We have observed that similar plaque titers were obtained with VSV when these two cell lines were employed. However, KCV concentrates which titered in excess of 10^{10} PFU/ml on

TABLE 1. *RNA-dependent RNA polymerase of Kern Canyon virus (KCV) and vesicular stomatitis virus (VSV)*[a]

Reaction conditions	VSV polymerase activity[b]	KCV polymerase activity[b]
Complete	120	5.0
−Cytosine triphosphate	1	0.1
$-Mg^{2+}$	5	0.4
−Triton N101	3	0.3
−2-Mercaptoethanol	112	0.8
−2-Mercaptoethanol + dithioerythritol	129	4.9
−2-Mercaptoethanol + dithiothreitol	128	7.7
+ Deoxyribonuclease (10 μg)	115	4.0
+ Actinomycin D (5 μg)	115	3.7
+ Ribonuclease A (10 μg)	2	0.1

[a] Reaction mixtures containing ^{3}H-uridine-labeled virus were incubated at 28 C with ^{32}P-α-uridine triphosphate (1 mCi per μmole) to label the product species. The constituents of the complete reaction mixture are given in the text; incomplete reaction mixtures were as indicated. In the absence of 2-mercaptoethanol, dithiothreitol or dithioerythritol were added at a concentration of 0.25 μmoles per 125 μliters of reaction mixture. Deoxyribonuclease, actinomycin D, and ribonuclease were included in the reaction mixture at the concentrations indicated. Portions of each reaction were withdrawn at intervals to determine the rate of incorporation.

[b] Expressed as counts per minute × 10^{-5} per hour per milligram of protein.

TABLE 2. *Annealing polymerase product species to homologous viral RNA*[a]

Annealing Mixture	Time (hr)	Ribonuclease resistance (%) ^{32}P-product RNA	Ribonuclease resistance (%) ^{3}H-viral RNA
KCV product (0.1 μg/ml)	0	2	
	0.5	66	
	5.0	95	
KCV product (0.001 μg/ml)	0.5	10	
	5.0	15	
KCV product (0.001 μg/ml) + KCV viral RNA (1.6 μg/ml)	0	4	4
	0.5	40	8
	5.0	85	8
KCV viral RNA (1.6 μg/ml)	5.0		5
VSV product (0.1 μg/ml)	0.5	52	
	5.0	93	
VSV product (0.001 μg/ml)	0.5	8	
	5.0	8	
VSV product (0.001 μg/ml) + VSV viral RNA (1.6 μg/ml)	0	3	3
	0.5	45	3
	5.0	91	3
VSV viral RNA (1.6 μg/ml)	5.0		4

[a] A twofold reaction mixture containing unlabeled Kern Canyon virus (KCV) or vesicular stomatitis virus (VSV) and ^{32}P-α-uridine triphosphate (5 m Ci per μmole) was incubated for 4 hr at 28 C and extracted for product RNA with phenol. The RNA was separated from ribonucleoside triphosphates by passage through Sephadex G-50 and precipitated with ethanol. The product RNA contained both unlabeled viral RNA (of an indeterminate amount) and ^{32}P-labeled product species which had a specific activity 7.6 × 10^6 counts per min per μg (calculated assuming a 25% mole content of ^{32}P-uridine monophosphate). Each RNA sample was dissolved in 200 μliters of 0.01 M sodium phosphate buffer, 0.005 M EDTA (*p*H 7.5) and melted by heating at 100 C for 30 sec. Viral RNA was extracted from ^{3}H-uridine-labeled virus, specific activity 3 × 10^5 counts per min per μg of RNA. Ribonuclease resistance was determined on product RNA or mixtures of product and homologous ^{3}H-viral RNA by incubating a sample with 10 μg of ribonuclease T_1 and 10 μg of ribonuclease A in 1 ml of 0.4 M NaCl for 30 min at 37 C, followed by trichloroacetic acid precipitation. The percentage of ribonuclease resistance was determined by relating the insoluble radioactivity obtained to an unincubated control. The ribonuclease resistance of the product species after annealing at two different ^{32}P-product concentrations (0.1 μg or 0.001 μg per ml) was determined by adjusting the product RNA to 0.4 M NaCl, 0.01 M sodium phosphate buffer, 0.005 M EDTA (*p*H 7.5) and annealing at 65 C for 30 min or 5 hr in a sealed capillary tube (for the 5-μliter high concentration samples) or a capped tube (for the 100-μliter low concentration samples). After annealing, the samples were cooled and the ribonuclease resistance was determined as described above. The ribonuclease resistance was also determined after annealing at low ^{32}P-product concentration with ^{3}H-viral RNA as follows. A 100-μliter mixture of ^{32}P-product (0.001 μg per ml) and ^{3}H-viral RNA (1.6 μg per ml) was adjusted to 0.4 M NaCl, 0.01 M sodium phosphate buffer, 0.005 M EDTA and incubated in capped tubes at 65 C for 30 min or 5 hr and the ribonuclease resistance determined as described above.

BHK-13S cells did not produce plaques on L cells at dilutions as low as 10^{-2}.

When ^{3}H-uridine-labeled KCV preparations were centrifuged through a sucrose gradient, two light-scattering bands were observed (KC-I and KC-II). The lower band (KC-I) contained all of the virus infectivity as well as some ^{3}H-uridine label (Fig. 1). Electron microscopy, employing phosphotungstic acid-negative staining, showed that the KC-I band contained bullet-shaped particles (10), whereas the KC-II band contained truncated parts of bullet-shaped particles as well as rounded particles. Separation of VSV populations into an infective and one or more defective particles by sucrose gradient velocity centrifugation has been observed by numerous investigators. Only infectious virus was utilized in the following experiments.

Subviral ribonucleoproteins of KCV and VSV. Detergent treatment is a prerequisite to demonstrate the activity of VSV transcriptase (1). We, therefore, investigated the effect of the nonionic detergent, Triton N101, on the dissociation of the virions of VSV and KCV as revealed by sucrose gradient velocity sedimentation. It has been demonstrated that treatment of VSV (2) or rabies virus (14) with deoxycholate, a weakly anionic detergent, causes dissociation of the virion into a slower sedimenting ribonucleoprotein and separate immunizing components. Treatment of VSV with 0.2% Nonidet P40 (a nonionic detergent) also has been shown to dissociate the virus, but this treatment alone resulted in aggregation of the subviral ribonucleoproteins into complexes which sedimented faster than untreated virions (2). We have found that treatment of KCV or VSV with 0.1% Triton N101 and subsequent sedimentation in linear sucrose gradients containing 0.01 M EDTA results in the dissociation of the virion into a slowly sedimenting subviral ribonucleoprotein (Fig. 2). The subviral ribonucleoprotein of KCV and VSV are evidently similar as judged by their sedimentation properties. In an experiment in which a purified suspension of rabies virus, labeled with ^{3}H-uridine and ^{32}P-phosphate, was treated with detergent and sedimented as above, only 5% of the ^{32}P counts

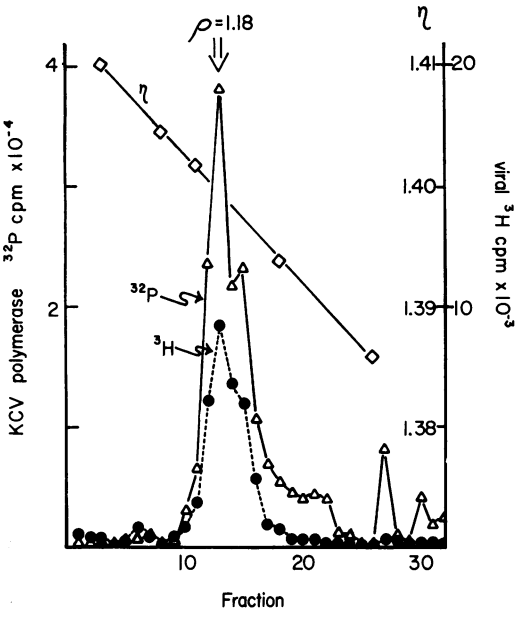

FIG. 3. *Isopycnic centrifugation of KCV virus preparations. A preparation of purified unlabeled KCV was mixed with a small amount of ³H-uridine-labeled KCV and centrifuged to equilibrium at 4 C and 80,000 × g for 18 hr in a 30 to 45% (w/v) linear gradient of sucrose containing 0.1 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.5) in an SW27 Spinco rotor. The gradient was fractionated and the distribution of trichloroacetic acid-insoluble ³H label was obtained by trichloroacetic acid precipitation of 20-μliter samples of each fraction (●). Density of the various fractions indicated was determined from refractive index determinations (◇). Samples (20-μliter) of each fraction were incubated with ³²P-α-UTP (5 mCi per μmole) in standard reaction mixtures to determine the KCV RNA-dependent RNA polymerase activity (△). The reaction mixtures were held at 28 C for 7 hr prior to trichloroacetic acid precipitation.*

remained coincident with the ³H; the rest was recovered at the top of the gradient. Additionally, detergent treatment has been found to strip glycoprotein from purified rabies virus, yielding a ribonucleoprotein core particle (E. Gyorgy and F. Sokol, Bacteriol. Proc., p. 216, 1971).

RNA-dependent RNA polymerase activity of KCV and VSV. Table 1 compares the RNA polymerase activity of a purified preparation of KCV to that of VSV of equivalent age and purity. Both RNA polymerases show an absolute requirement for all four ribonucleoside triphosphates, magnesium ions, and detergent. VSV transcriptase was not severely affected by the absence of 2-mercaptoethanol, whereas the RNA polymerase of KCV was substantially inhibited by its omission. Either dithiothreitol or its isomer dithioerythritol could substitute for the 2-mercaptoethanol; in fact, dithiothreitol was more stimulatory than 2-mercaptoethanol in the KCV-catalyzed reaction mixture. The activites of both polymerases were not restricted to any significant extent by the inclusion of deoxyribonuclease or actinomycin D. Ribonuclease almost totally inhibited the demonstration of RNA polymerization.

Two questions must be answered when determining the nature of the synthesized product. First, will the product hybridize to viral RNA? Second, does all of the product hybridize to added viral RNA, or is some of it unable to anneal because it is identical in base sequence to the RNA contained in the virion?

Table 2 summarizes the results obtained by annealing the KCV or VSV reaction products to homologous viral RNA. The reaction products

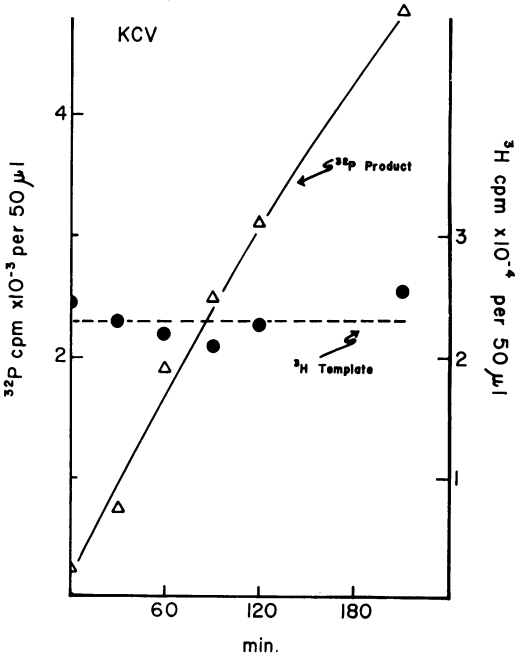

FIG. 4. *Time course of RNA synthesis and template conservation. ³H-uridine-labeled KCV was incubated at 32 C under standard conditions in a 10-fold standard reaction mixture containing ³²P-α-UTP (specific activity 1 mCi per μmole) to label the product species. Portions were removed at intervals to ascertain the conservation of acid-soluble ³H label during the time-course (●) and the ³²P-UMP incorporation into product by the KCV RNA-dependent RNA polymerase (△).*

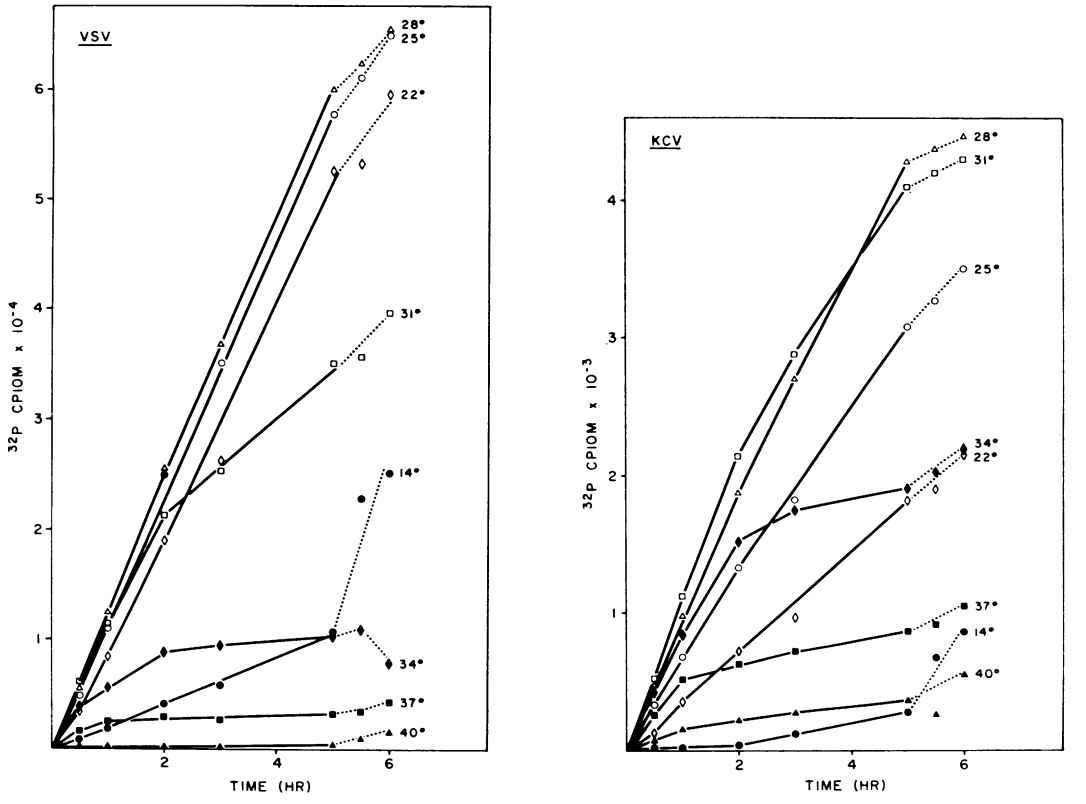

FIG. 5. *Kinetic analysis of product synthesis by KCV and VSV polymerases. A 100-fold reaction mixture was prepared containing ^{32}P-α-GTP (specific activity 0.1 mCi per μmole). The mixture was divided and each half was mixed with ^{3}H-uridine-labeled KCV or VSV. Portions (625 μliter) were incubated at 10 different temperatures and the incorporations of ^{32}P-GMP into RNA were determined by trichloroacetic acid precipitation of 50-μliter samples at the intervals shown. The ^{32}P counts incorporated were normalized to a constant ^{3}H recovery and are plotted (continuous lines) with respect to the time of sample subtraction. After 5 hr of incubation, every sample was transferred to the 31 C water bath, and two additional samples were withdrawn at 30-min intervals (broken lines).*

were derived from reactions employing ^{32}P-α-UTP to label the product species, and unlabeled virus was the source of polymerase and viral RNA template. Consequently, the crude reaction products contained unlabeled viral RNA (of an indeterminable quantity) as well as ^{32}P-labeled product species (0.1 μg per ml). The concentration of product species was estimated from the specific activity of the ^{32}P-α-UTP utilized, assuming a 25% mole content of UMP in the product. The reaction product prepared from a KCV-containing reaction mixture contained only 2% ribonuclease resistance after melting. Annealing at a product concentration of 0.1 μg/ml for 30 min resulted in an increase in ribonuclease resistance to 66%; annealing for 5 hr led to 95% ribonuclease resistance. These data can be interpreted as evidence for either the self-annealing of ^{32}P product RNA or its annealing to unlabeled template RNA present in the reaction mixture. No increase in resistance to ribonuclease could be shown when ^{3}H viral RNA was added (*data not shown*). Since RNA hybridization is concentration-dependent (11), a reduction in the concentration of RNA extracted from the RNA polymerase reaction should provide data which would resolve these alternatives.

When the KCV product was diluted 100-fold and annealed, the ribonuclease resistance of the product was only 15% after 5 hr. After the addition of an excess of ^{3}H-viral RNA to the diluted reaction product (0.001 μg/ml), the ribonuclease resistance increased to a maximum of 85% after annealing for 5 hr. This result indicates that the initial KCV reaction product contained a minimum of 78% [85% − (15%/2)] of the ^{32}P product complementary in composition to the viral RNA. It should be noted that the ^{3}H-viral RNA did not

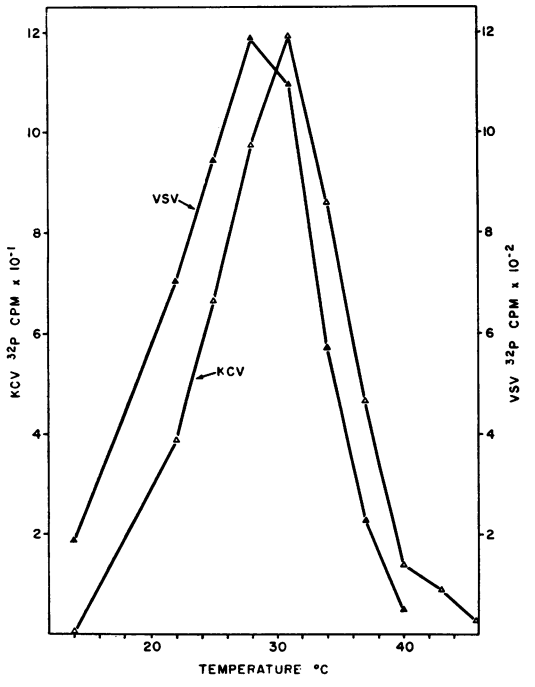

FIG. 6. *VSV and KCV polymerase activity as a function of temperature. The initial linear 60-min rate of incorporation of* ^{32}*P-GMP into product by VSV* (▲) *or KCV* (△) *polymerases was determined for the reactions incubated at 10 different temperatures (Fig. 5).*

self-anneal and that during annealing with KCV product RNA the ribonuclease resistance of ^{3}H-viral RNA increased from 4 to 8%. In an identical experiment with VSV reaction product, a minimum value of 87% [(91% − (8%/2)] was obtained for the amount of product present which was complementary in composition to the viral RNA.

To check the specificity of the annealing reaction, ribosomal RNA extracted from BHK-21 cells was mixed with the KCV and VSV reaction products. Ribonuclease resistance values of 5 and 6% were obtained, respectively, after 5 hr of annealing. Both ^{32}P-UMP and ^{32}P-GMP (Fig. 5) were incorporated into trichloroacetic acid-insoluble RNA by both virion-bound RNA polymerases.

Figure 3 demonstrates that the RNA polymerase of KCV is an integral part of the virion. The RNA polymerase activity and ^{3}H-uridine-labeled virus show coincidental distribution after isopycnic banding in a sucrose density gradient. A similar association of polymerase activity with the viral particles has been demonstrated by Baltimore et al. (1) for VSV.

The time course of RNA synthesis catalyzed by KCV was examined next (Fig. 4). Incorporation of ^{32}P-UMP into product species was linear through at least 3 hr. The ^{3}H-uridine-labeled RNA contained in the KCV particle remained trichloroacetic acid-insoluble during the course of the reaction, suggesting that the viral genome was not degraded to nucleotides.

Optimal conditions for the RNA polymerase activities of KCV and VSV. The following parameters for obtaining optimal in vitro activity of the viral RNA polymerases were investigated: temperature, *p*H, divalent cation requirement, and the concentrations of magnesium ion, monovalent cation, detergent, and sulfhydryl agent.

Kinetic analyses more extensive than shown in Fig. 4 were performed with KCV and VSV RNA polymerases with regard to temperature optimization in the following fashion. A 100-fold reaction

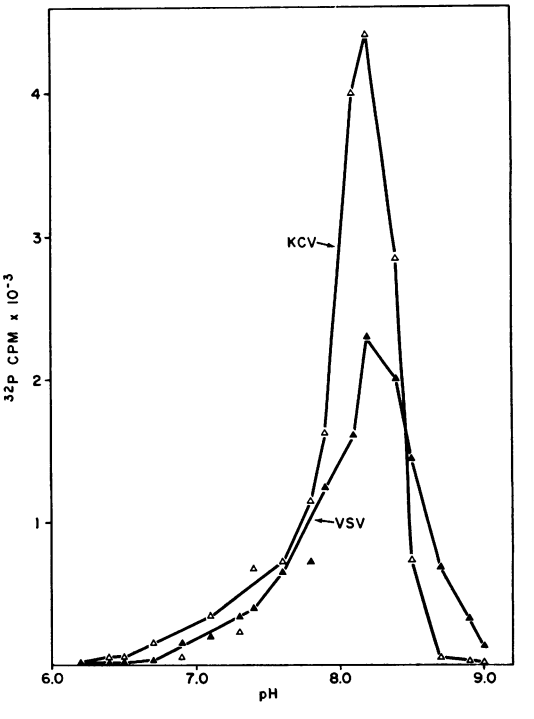

FIG. 7. *pH optimum of VSV or KCV polymerases The rate of* ^{32}*P-UMP incorporation by VSV* (▲) *or KCV* (△) *polymerases was determined in reactions templated by* ^{3}*H-labeled virus containing* ^{32}*P-α-UTP (specific activity 0.1 mCi per μmole) and Tris-hydrochloride buffer solutions at pH values correct at the incubation temperature (28 C) and concentration. Portions were withdrawn at intervals to determine the rate of incorporation.*

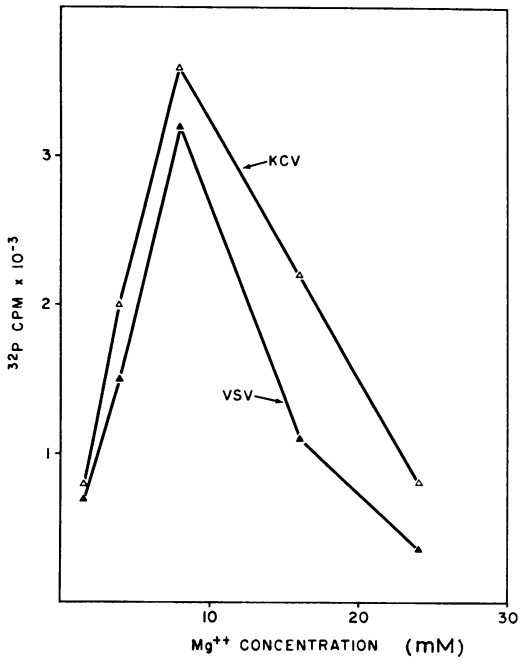

FIG. 8. *Optimum concentration of $MgCl_2$. The rate of incorporation of ^{32}P-UMP by VSV (▲) or KCV (△) polymerases into product was determined in reaction mixtures containing ^{32}P-α-UTP (specific activity 0.1 mCi per μmole) and indicated concentrations of $MgCl_2$.*

mixture was prepared containing ^{32}P-α-GTP to label the product species. The mixture was divided into two equal portions and mixed with ^{3}H-uridine-labeled virus (KCV or VSV); 625 μliter volumes were pipetted into two series of 10 tubes. The reaction mixtures were incubated simultaneously at 10 different temperatures in water baths regulated to within 0.5 C as indicated in Fig. 5 and 6. Samples of 50 μliters were removed from each tube at the indicated times, and the incorporation of ^{32}P-GMP was determined. After 5 hr of incubation, all reaction mixtures were placed at 31 C, and two further samples were taken at 30-min intervals. The ^{32}P-GMP counts incorporated into product RNA were normalized to a constant ^{3}H recovery since virion RNA template was apparently conserved during the reaction (Fig. 4). The data were plotted as the counts incorporated with respect to time (Fig. 5) or the initial 60-min rate (Fig. 6). Figure 6 shows that the VSV enzyme was more active at lower temperatures (14 to 22 C) than that of KCV, and the optimal temperature for initial enzyme activity was 28 and 31 C, respectively.

Figure 5 additionally shows that the initial rate of RNA synthesis for all samples incubated at or above 34 C was not maintained through the 5 hr of incubation. It is of interest to note that the 14 C samples of both KCV and VSV show increased activity after stepping up to 31 C. Their new rate of activity paralleled that of the corresponding reactions incubated at optimum temperatures. Apart from the 34 C reaction of VSV, all of the reactions involving a step-down to 31 C showed some increase in activity, even those held for 5 hr at 40 and 43 C (*not shown in Fig. 5*).

The *p*H optimum for RNA synthesis was tested by using Tris-hydrochloride-buffered solutions at *p*H values determined at the working temperature and concentration utilized in the experiment. The initial 60-min polymerase activity rate is plotted as a function of *p*H in Fig. 7. (The rate of enzyme activity is also plotted in Fig. 8–10.) The enzymes of both KCV and VSV had similar *p*H optima of 8.2.

The requirement of magnesium as divalent cation for assaying the RNA-dependent RNA polymerase activity (1) was tested by omitting

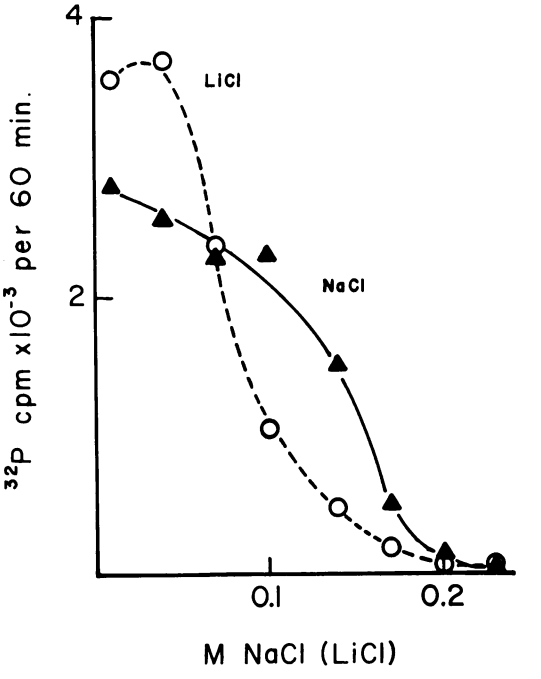

FIG. 9. *Monovalent cation effect on VSV polymerase. The rate of incorporation of ^{32}P-UMP by VSV polymerase into product was determined in reaction mixtures containing ^{32}P-α-UTP (specific activity 0.1 mCi per μmole) and various amounts of NaCl (▲) or LiCl (○).*

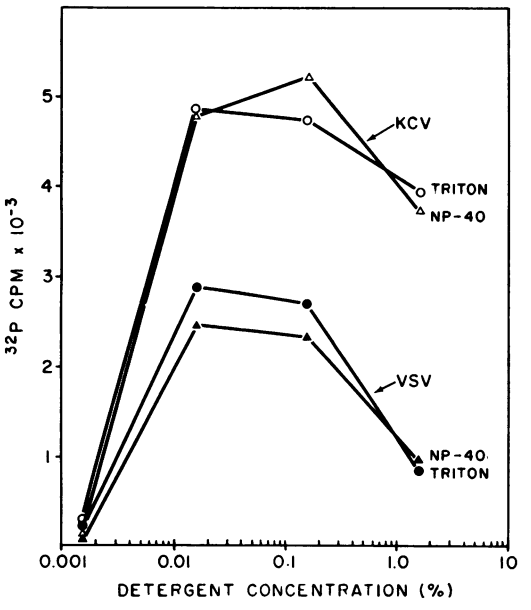

FIG. 10. *Effect of detergent concentration on VSV and KCV polymerase activity. The rate of incorporation of ^{32}P-UMP by VSV polymerase into product when activated by various amounts of either Triton N101 (●) or Nonidet P40 (▲) was determined in a reaction mixture containing ^{32}P-α-UTP (specific activity 0.1 mCi per μmole). A similar determination of the Triton N101 (○) and Nonidet P40 (△) activation of the KCV polymerase was made.*

the magnesium ion and substituting the chloride salts of manganese, calcium, nickel, zinc, and iron. Apart from some stimulation by $ZnCl_2$ at a concentration of 8 mM in the KCV reaction mixture, no substitute for the magnesium cation was found. Manganese ion at 1 mM failed to stimulate the VSV polymerase. The optimum concentration of $MgCl_2$ was found to be 8 mM for RNA synthesis by both rhabdoviruses (Fig. 8).

The effect of varying the concentration of NaCl or LiCl on the activity of the VSV polymerase reaction was also tested (Fig. 9). It was found that the enzyme was active up to 0.1 M NaCl, whereas LiCl inhibited the activity at concentrations above 0.05 M.

The effect of various concentrations of Triton N101 or Nonidet P40 on the RNA polymerase activities of KCV or VSV are shown in Fig. 10. A concentration of 0.01 to 0.1% was required to demonstrate enzyme activity for both viruses, whereas concentrations as great as 1.0% of either detergent inhibited enzyme activity.

It has been shown that KCV polymerase requires the presence of a sulfhydryl group in the reaction mixture (Table 1). The VSV RNA polymerase was far less stringent in its sulfhydryl requirement. Of the three compounds tested (Table 1), dithiothreitol was the most stimulating for the KCV enzyme. The optimal concentration of dithiothreitol was ascertained (Fig. 11) to be in the range of 0.001 M to 0.04 M. Higher concentrations inhibit the reaction.

A linear response between enzyme activity and the amount of virus present per reaction mixture was observed for both KCV and VSV between 2 and 50 μg of protein.

DISCUSSION

We have demonstrated RNA-dependent RNA polymerase activity in a second rhabdovirus: Kern Canyon virus. In our best preparations, KCV has but 4% of the RNA polymerase activity per mg of protein that we can obtain with VSV (Table 1). This could reflect the amount of enzyme present per virion, the number of virions with active enzyme, the inherent activity of the RNA polymerase, or the conditions of the assay itself. If polymerase activity can vary between viruses over several orders of magnitude, it will be extremely difficult to demonstrate polymerase in some viruses that do in fact possess such an enzyme. The recent demonstration of an RNA polymerase in Newcastle disease virus, having a specific activity only 3% of that of VSV, is pertinent to this (5*a*). Thus far we have not been able to detect an RNA polymerase activity for two species of rabies virus that we have examined.

Data summarized in Fig. 5–10 reveal that there is considerable similarity between the conditions needed for demonstrating the RNA

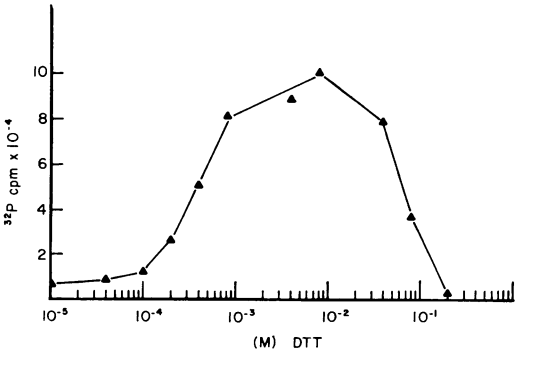

FIG. 11. *Optimum concentration of dithiothreitol (DTT) for KCV polymerase. The incorporation of ^{32}P-UMP into product by reactions templated by KCV and containing ^{32}P-α-UTP (specific activity 5 mCi per μmole) and various concentrations of DTT was determined by trichloroacetic acid precipitation. The ^{32}P-UMP incorporated per reaction mixture after 2 hr of incubation is given.*

polymerase of KCV and that of VSV. Enzymes present in both viruses synthesize RNA at a maximum rate when incubated in the presence of 0.01 to 0.10% nonionic detergent, at 30 C, and in a reaction mixture containing 8 mM $MgCl_2$, 50 mM NaCl, and Tris-hydrochloride buffer at *p*H 8.2. A significant difference between the two RNA polymerases was observed with respect to their requirement for a sulfhydryl compound. Although the KCV enzyme was essentially inactive when assayed in a reaction mixture lacking a sulfhydryl agent, under similar conditions, the VSV RNA polymerase maintained about 90% of its activity (Table 1). Also the KCV RNA polymerase was stimulated by the replacement of 2-mercaptoethanol by dithiothreitol, whereas no such stimulation was observed in the VSV-catalyzed reaction.

Figure 5 shows that KCV and VSV are only able to synthesize RNA to a limited degree at 37 C, confirming a similar observation of Baltimore et al. (1). It is possible that the step-up effect—14 to 31 C (Fig. 5)—allows transcription of regions of the genome which cannot be transcribed by the enzyme at lower temperatures. Similarly, the slight increase in RNA synthesis in step-down reactions might reflect a similar situation. This hypothesis will require further experimentation. The demonstration that the temperature optima of both VSV and KCV were around 30 C could reflect either an inherent property of the viral polymerase or the fact that the viruses were grown at 32 C. It is quite possible that the optimal temperature for the viral polymerase assay varies with the conditions of virus propagation in the host cell. It is important, therefore, that the temperature optimum should be determined by each investigator.

Finally, there is the question of homology between the VSV and KCV genetic system. Antigenically the two viruses are distinct (10). Also, the plaque morphology of VSV and KCV and the time-course of the infectious process in BHK-21 cells are quite different (Clark, *unpublished results*). In addition, VSV is cytolytic to BHK-21 cells as well as other cell lines. KCV can be propagated only in BHK-21 cells and is less cytolytic. Whether the genomes of KCV and VSV (or other rhabdoviruses) have any pimrary structural homology can now be tested by homologous and heterologous annealing, i.e., annealing the product of VSV RNA polymerase to VSV and other rhabdoviral RNA species (KCV, egtved, rabies, etc.). The results of these experiments and a biophysical analysis of the RNA genomes of four rhabdoviruses, VSV, KCV, rabies, and egtved, will be communicated in a subsequent publication.

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