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Replication of Nondefective Parvoviruses: Lack of a Virion-Associated DNA Polymerase

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We have examined four of the nondefective parvoviruses for an associated DNA polymerase. Virions were purified from neuraminidase-treated infected-cell lysates by isopycnic centrifugation in CsCl or from infected cell material by $CaCl_2$ precipitation and centrifugation through sucrose into CsCl. Preparations of bovine parvovirus or Kilham rat virus obtained by the former procedure contained DNA polymerase activity but were not free of contaminating cellular proteins. The latter method produced viral preparations free of contaminating cellular proteins, and no DNA polymerase activity was detected in light infectious particles of H-1, LuIII, bovine parvovirus, or Kilham rat virus. Examination of levels of each cellular DNA polymerase in these preparations from each step of both purification procedures revealed that DNA polymerase β had a greater tendency to copurify with bovine parvovirus and Kilham rat virus than did DNA polymerases α or γ. Disruption of infectious virions obtained by the second purification method with detergents and sonic treatment did not result in the detection of a DNA polymerase activity. The biological activity and purity of each of the four different viruses obtained by the latter procedure were determined by hemagglutination and infectivity assays, polyacrylamide gel electrophoresis, and electron microscopy. In each case, the virions banding at a density of 1.39 to 1.41 g/cm^2 in CsCl were infectious and contained only the virion structural proteins. DNA polymerase activity was not detected in any of these preparations, and we have concluded that a virion-associated DNA polymerase is not required for productive infection with the nondefective parvoviruses.

The small, icosahedral members of the family Parvoviridae contain a linear, single-stranded DNA genome of 1.35×10^6 to 1.70×10^6 daltons, a size sufficient to code for only the virion structural proteins (22). Two infectious particle types, heavy (banding at 1.46 to 1.48 g/cm^2 in CsCl) and light (banding at 1.39 to 1.42 g/cm^2 in CsCl), are produced during infection. The light particles have a higher specific infectivity and hemagglutinating activity than the heavy particles. The heavy particles are considered to be precursors of the light infectious particles (19, 21).

Due to their limited coding capacity, parvoviruses must rely heavily on the replicative system of the host cell. The proposed mechanism for replication of parvovirus DNA contains several steps (5), and it is possible that one or more DNA polymerases of different specificities may be involved. Our experiments to date have implicated cellular DNA polymerases α and γ in the replication of bovine parvovirus (BPV) DNA (2, 12a).

It is possible, however, that some of the required enzymes may be viral in origin. Salzman (14) reported that Kilham rat virus (KRV) contained a virion-associated DNA polymerase, which, when purified from the virions and characterized further (16), corresponded in molecular weight to one of the virion structural proteins. However, Rhode (13) was not able to demonstrate a similar activity in H-1 and KRV purified by the method of Salzman or by a detergent procedure.

Because of the potential importance of such an enzyme in the replication process of the paroviruses, we felt that it was necessary to explain this discrepancy, and, therefore, we examined four of the nondefective parvoviruses, BPV, H-1, LuIII, and KRV, for a virion-associated DNA polymerase activity. We have determined that, although at least one of the cellular DNA polymerases tends to copurify with the viruses through several purification steps, highly purified light infectious virus free of contaminating cellular material did not have DNA polymerase activity.

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MATERIALS AND METHODS

Cell cultures and virus propagation. Primary

bovine fetal spleen (BFS) cell cultures were prepared and maintained as described previously (12). Chimpanzee liver (CL) cell line was obtained from Flow Laboratories, Inc. and maintained by standard methods. H-1 virus was purchased from the American Type Culture Collection and LuIII was supplied by K. Soike. Two different strains of KRV and the cells in which they are propagated were used for these studies. L. Salzman supplied KRV strain 308 and rat nephroma (RN) cells. A second strain of KRV, strain 171 (23), and normal rat kidney (NRK) cells were provided by G. Lavelle. Virus for purification studies was prepared by infecting parasynchronous cell cultures in roller bottles with 1 to 5 PFU of plaque-purified virus per cell. Hemagglutination and infectivity assays were performed as previously described (3, 4, 12).

Virus purification procedures. The first procedure used for purification of BPV and KRV was that described by Salzman and Jori (15) (procedure 1) and consisted of neuraminidase treatment of infected cell lysates for 16 h at 37°C followed by centrifugation in CsCl. A modification of the procedure of Tattersall et al. (20) (procedure 2) was used for subsequent purification of all four nondefective parvoviruses. In this procedure, infected cells were homogenized in the presence of phenylmethylsulfonylfluoride (PMSF), and the virus remaining in the supernatant from the following high-speed centrifugation was precipitated with $CaCl_2$, pelleted, released, and layered on a sucrose-CsCl step-gradient which was centrifuged for 24 to 36 h at 27,000 rpm in an SW 27.1 rotor.

DNA polymerase assay. DNA polymerase activity was measured as the incorporation of radioactively labeled substrate into acid-precipitable product. Reaction mixtures in a total volume of 100 μl contained the following components. The polymerase α assay mixture contained 20 mM KPO_4 (pH 7.2), 0.2 mM each dATP, dCTP, dGTP, and [^{3}H]dTTP (80 cpm/pmol), 7.5 mM $MgCl_2$, 50 μg of DNase-activated calf thymus DNA (18), and 0.5 mM dithiothreitol. The polymerase β assay mixture contained 50 mM Tris-hydrochloride (pH 8.5), 0.2 mM each dATP, dCTP, dGTP, and [^{3}H]dTTP (80 cpm/pmol), 7.5 mM $MgCl_2$, 50 μg of activated DNA, 0.2 M NaCl, and 10 mM *N*-ethylmaleimide. The polymerase γ assay mixture contained 50 mM Tris-hydrochloride (pH 7.5), 0.2 mM [^{3}H]dTTP (80 cpm/pmol), 0.5 mM $MnCl_2$, 2.5 μg of poly(rA)·oligo(dT)$_{10}$ (25), 0.5 mM dithiothreitol, and 0.1 M KCl. A unit of enzyme activity is the amount catalyzing the incorporation of 1 pmol of [^{3}H]dTTP per h at 37°C. The reaction rates for all three enzymes were linear for at least 1 h.

Portions of the assay mixtures employing DNA as template were pipetted to Whatman 3MM filter paper disks and processed through four washes of 5% trichloroacetic acid containing 2 mM $Na_4P_2O_7$ to remove unincorporated substrate, rinsed twice with ether, and dried. Because the trichloroacetic acid wash procedure led to variable recovery of homopolymer products, portions of the γ enzyme reaction mixtures were placed on DE81 disks and processed through the $Na_4P_2O_7$ and NH_4HCOOH wash of Wang et al. (24). Each assay is made partially selective by the addition of optimal concentrations of preferred salts and buffers, and the templates used for each enzyme are those with which each polymerase gives maximal activity under these conditions. The only significant cross-reactivity with the above reaction mixtures is the contribution of DNA polymerase β to the α and γ assays. To account for this, 10 mM *N*-ethylmaleimide, to which the β enzyme is resistant (6), is added to tubes containing the standard α and γ reaction mixtures. The values obtained from these are subtracted from those obtained with the standard mixtures for these enzymes. The addition of 10 mM *N*-ethylmaleimide to the β enzyme reaction mixture permits the specific assay for this polymerase in the presence of DNA polymerases α and γ.

Polyacrylamide gel electrophoresis. Slab gels were prepared by the sodium dodecyl sulfate (SDS)-Tris-glycine system of Laemmli (10) as modified by Anderson et al. (1). A 9-cm resolving gel (7.5% acrylamide–0.2% bis-acrylamide) was overlaid with 1 cm of 4% stacking gel. Virus preparations for electrophoresis were prepared by boiling for 2 min in a solution containing 1 mM Tris-hydrochloride (pH 6.8), 2% SDS, 0.6 M 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue.

Virus samples (3 to 20 μl) and marker proteins were applied to different gel wells. Electrophoresis was performed at 4°C at 1 mA per well until the tracking dye entered the resolving gel and then at 5 mA per well until the dye front reached the bottom of the slab.

Cylindrical gels were prepared by the neutral SDS-phosphate system of Maizel (11). Virus samples for electrophoresis were disrupted in 0.01 M $NaPO_4$ (pH 7.2), 1.5% SDS, and 1.5% 2-mercaptoethanol at 100°C for 2 min. The solution was then adjusted to 10% glycerol and 0.01% bromophenol blue. The resolving gel was 7.5% acrylamide, overlaid with a 2.5% acrylamide stacking gel. Electrophoresis was at room temperature at 5 mA per tube.

After electrophoresis, both slab and cylindrical gels were fixed in 20% trichloroacetic acid for 1 h followed by staining in 0.2% Coomassie brilliant blue R in 50% methanol and 7% acetic acid for 3 h. Destaining was done in 10% methanol–7% acetic acid overnight. The destained gels were scanned at 590 nm with a Gilford 240 spectrophotometer equipped with a linear gel transport. Molecular weights of viral proteins were determined by the method of Chrambach and Radbard (7). The following were used as molecular weight markers: phosphorylase a, 100,000; transferin (human), 76,600; bovine serum albumin, 68,000; catalase, 55,000; and ovalbumin, 45,000.

RESULTS

Copurification of DNA polymerase activities with KRV and BPV. In preliminary experiments, preparations of KRV and BPV obtained by the method of Salzman and Jori (15) were found to contain DNA polymerase activity. To determine the nature of this enzyme (copurifying cellular enzyme or virion-associated enzyme), DNA polymerase α, β, and γ activities were measured at each step of the purification procedure. Both viruses were purified from 5 × 10^7 to 10^8 infected cells after a single cycle infection in parasynchronous cells. The results are shown in Table 1. The activities of all three

TABLE 1. *DNA polymerase activities[a] during purification of BPV and KRV by procedure 1[b]*

Purification step	Enzyme units (pmol/h)					
	KRV[c]			BPV		
	Alpha	Beta	Gamma	Alpha	Beta	Gamma
1. Infected cell lysate[d]	5,839	4,347	515	2,175	50	389
2. Lysate plus neuraminidase (16 h at 37°C)	1,770	2,525	448	611	78	434
3. 1,000 × *g* supernatant	207	572	185	218	86	301
4. Virus band (d = 1.39–1.41 g/cm^2); first CsCl gradient	79	153	120	26	59	7
5. Virus band (d = 1.39–1.41 g/cm^2); second CsCl gradient	65	162	29	17	51	10

[a] DNA polymerase reaction mixtures contained in a total volume of 100 μl the components listed in the text and 50 μl of sample from each purification step. The reaction mixtures were incubated for 1 h.
[b] Salzman and Jori (15).
[c] Strain 308, supplied by L. Salzman.
[d] A total of 10^8 infected cells.

DNA polymerases in the KRV preparation and of the α and γ enzymes in the BPV preparation decreased greatly by purification step 5 (second CsCl gradient) when compared with the starting infected-cell lysate. On the contrary, DNA polymerase β increased in activity during intermediate purification steps, exhibiting a greater tendency to copurify than the other DNA polymerases.

The data presented in Table 1 are representative of several purification trials and suggest that neither of these viruses is free from contaminating cellular DNA polymerase activity at the final step in the purification procedure. In both cases, the greatest amount of residual enzyme activity was DNA polymerase β.

Both of the virus bands from step 5 were subjected to electrophoresis on 7.5% gels in a cylindrical tube with a neutral SDS-phosphate buffer system. Three protein bands with molecular weights similar to values reported by Salzman and White (17) for KRV and by Johnson and Hoggan (9) for BPV were visualized. However, when these same samples were subjected to electrophoresis on a high-pH SDS-discontinuous 7.5% gel slab in a Tris-glycine buffer system, several additional bands were detected (Fig. 1). Although not directly determined, these bands may represent contaminating cellular DNA polymerases. The 92,000-molecular-weight protein of a molecular weight higher than the largest KRV polypeptide (Fig. 1A) and the prominent 77,000- (Fig. 1A) or 72,000-molecular-weight protein (Fig. 1B) located between the KRV and BPV structural proteins can be removed by further purification of these viruses either on sucrose or by procedure 2 (described below). These proteins apparently are not virion structural proteins.

Further purification of KRV and BPV by step gradient centrifugation. Because purification procedure 1 did not provide virus free of contaminating cellular proteins, it was impossible to determine whether KRV or BPV had an essential associated DNA polymerase. Therefore, purification procedure 2 which incorporates a sucrose-CsCl step gradient was adopted (Table 2). By step 4 of this procedure, for both viruses, a substantial reduction in α and γ enzyme levels was observed, whereas levels of β remained high. Virus purified beyond step 6 had no detectable DNA polymerase α, β, or γ activity. To test for the presence of a virion-associated DNA polymerase, preparations of both viruses from step 8 were treated with detergent to disrupt the capsids and assayed as described by Salzman and McKerlie (16). No DNA polymerase activity was detected.

Eichler et al. (8) recently reported that $CaCl_2$ has a deleterious effect on DNA polymerase α. The levels of DNA polymerases α and β were greatly reduced by the addition of 25 mM $CaCl_2$ (Table 2, step 5). The addition of PMSF (Table 2, step 2) reduced the level of polymerase α by at least twofold during the purification of both viruses. Because this protease inhibitor and $CaCl_2$ might also affect the activity of a viral DNA polymerase, both of these chemicals were omitted from the purification procedure of KRV (Table 3). When the virus was purified by this method, there was a steady decrease in contaminating cellular enzymes as cellular debris was removed from the virus preparation. Although there were substantial levels of all three enzymes in the 12,000 × *g* supernatant, they did not sediment with the virus but remained in the sucrose layer of the sucrose-CsCl step gradient.

KRV and BPV from step 8 of purification procedure 2 were subjected to electrophoresis on a high-pH SDS-discontinuous 7.5% slab gel (Fig. 2). In KRV preparations three distinct proteins with molecular weights corresponding

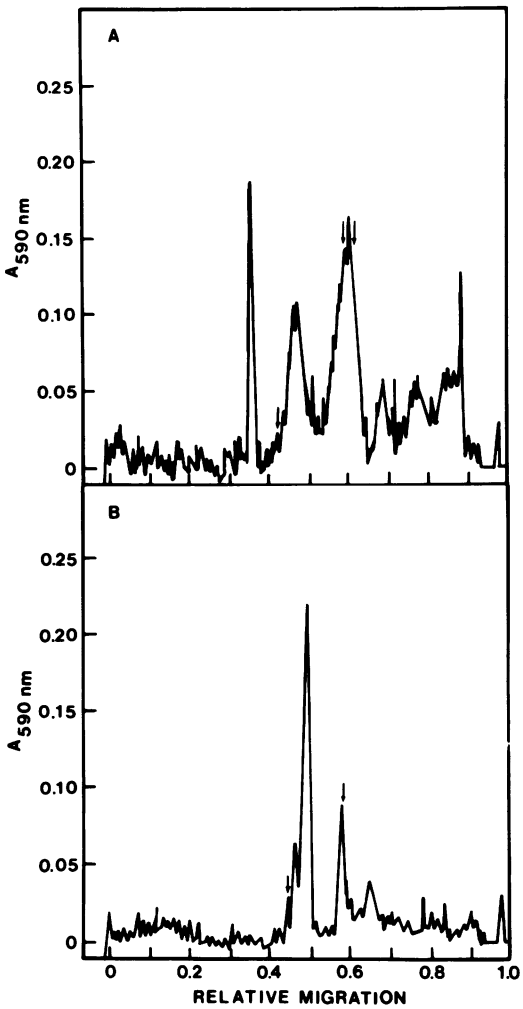

FIG. 1. *Densitometric tracing of Coomassie brilliant blue-stained polyacrylamide slab gel electrophoretogram of (A) KRV and (B) BPV purified to step 5 of procedure 1. Arrows indicate the position of virion proteins as determined by coelectrophoresis of KRV and BPV purified by procedure 2 (Fig. 2). Virion protein molecular weights are (A) KRV, 83,000, 61,000, and 57,000 and (B) BPV, 81,200 and 60,400. A_{590}, Absorbance at 590 nm.*

to 83,000, 61,000, and 57,500 were observed. In preparations of BPV, two distinct protein bands with molecular weights corresponding to 81,200 and 60,400 were evident. The 72,000- or 77,000-molecular-weight proteins seen in virus purified by procedure 1 (Fig. 1) were observed in electrophoretograms of only the first four steps of purification procedure 2.

Search for virion-associated DNA polymerase activity in four parvoviruses. Four parvoviruses, KRV and BPV (as described above), H-1, and LuIII, were purified by procedure 2 and assayed for DNA polymerase activity at each step of the purification process. The data clearly show that this purification procedure was effective in removing contaminating DNA polymerase activity from these viruses regardless of the host cell in which the virus was propagated (Table 4; Fig. 3). However, it can be seen that contaminating polymerase activity was not as rapidly removed from KRV and BPV preparations during the initial purification steps as compared to H-1 and LuIII (Fig. 3). This may be due to inherent differences of the viruses or to the host cells in which they were propagated.

The biological activity and purity of each of the viruses purified to step 8 were determined by hemagglutination, infectivity, polyacrylamide gel electrophoresis, and electron microscopy. The hemagglutinating activity of the virus preparations ranged from 250,000 hemagglutinating units for LuIII to 8,190,000 hemagglutinating units for KRV (Table 4). Virus infectivity ranged from 1.0×10^9 PFU for LuIII virus to 1.36×10^{10} PFU for KRV recovered from 10^8 infected cells (Table 4). Electrophoretic analysis of the light infectious virions (1.39 to 1.41 g/cm^2) showed that the four parvoviruses have polypeptides with molecular weights of approximately 81,000 and 61,000 (Table 4). Furthermore, LuIII and KRV had an additional polypeptide with a molecular weight of approximately 58,000. Coelectrophoresis of the virion preparations revealed that minor differences existed between the molecular weights of the structural proteins. The 81,000-molecular-weight protein was 13 to 17% of the total viral protein (Table 4). The relative percent of the 61,000- and 58,000-molecular-weight proteins for LuIII and KRV varied from preparation to preparation. Electron microscopic examination of each virus preparation revealed 20- to 24-nm icosahedral virions free of contaminating cellular material.

Each of the parvoviruses purified to step 8 of purification procedure 2 and characterized as described above was assayed in two ways for DNA polymerase activity. First, undisrupted virus was assayed in α, β, and γ polymerase reaction mixtures. Secondly, virus disrupted by detergent treatment was assayed for viral polymerase by the method described by Salzman and McKerlie (16). DNA polymerase activity was not detected by either procedure. Therefore, we conclude that KRV, BPV, H-1, and LuIII do not contain an essential virion-associated DNA polymerase required for productive infection.

DISCUSSION

We have reported here that KRV and BPV preparations contained DNA polymerase activity when purified by the method of Salzman and

TABLE 2. *DNA polymerase activities[a] during purification of BPV and KRV by procedure 2[b]*

Purification step	Enzyme units (pmol/h)					
	KRV[c]			BPV		
	Alpha	Beta	Gamma	Alpha	Beta	Gamma
1. Infected cell lysate[d]	97,386	9,173	803	87,135	9,728	648
2. Lysate plus PMSF[e]	40,461	4,744	780	27,917	4,005	628
3. Homogenate	30,518	9,314	788	13,562	8,035	656
4. 27,000 × *g* supernatant	15,411	8,742	459	17,611	7,882	405
5. Supernatant plus $CaCl_2$[f]	544	502	407	347	396	412
6. Suspended $CaCl_2$ pellet	0	340	0	0	189	0
7. 12,000 × *g* supernatant	0	0	0	0	0	0
8. Virus band (d = 1.39–1.41 g/cm^2)[g]; sucrose-CsCl step gradient	0	0	0	0	0	0

[a] DNA polymerase reaction mixtures contained in a total volume of 100 μl the components listed in the text and 50 μl of sample from each purification step. The reaction mixtures were incubated for 1 h.
[b] Tattersall et al. (20).
[c] Strain 308, supplied by L. Salzman.
[d] A total of 10^8 infected cells.
[e] Final concentration, 1 mM.
[f] Final concentration, 25 mM.
[g] A 50-μl amount of virus purified to this step contained 12 to 24 μg of protein and for KRV, 256,000 hemagglutinin units and 6.8×10^8 PFU and for BPV, 512,000 hemagglutinin units and 5×10^8 PFU.

TABLE 3. *DNA polymerase activities[a] during purification of KRV[b] without $CaCl_2$[c]*

Purification step	Enzyme units (pmol/h)		
	Alpha	Beta	Gamma
1. Infected cell lysate[d]	3,365	1,200	371
2. Homogenate	3,015	1,292	314
3. 27,000 × *g* supernatant	1,008	275	92
4. 12,000 × *g* supernatant	197	53	61
5. Virus band (d = 1.39–1.41 g/cm^2)[e]; sucrose-CsCl step gradient	0	0	0

[a] DNA polymerase reaction mixtures contained in a total volume of 100 μl the components listed in the text and 50 μl of sample from each purification step. The reaction mixtures were incubated for 1 h.
[b] Strain 308, supplied by L. Salzman.
[c] Modification of the method of Tattersall et al. (20).
[d] A total of 10^8 infected cells.
[e] A 50-μl amount of virus purified to this step contained 12 μg of protein, 204, 800 hemagglutinin units, and 6×10^8 PFU.

Jori (15). Although these findings were consistent with previous reports for KRV purified by this method (14, 16), attempts by others to repeat the finding of a virion-associated polymerase were not successful. Rhode (13) reported that H-1 and KRV purified by the method of Salzman and by a detergent method were free of DNA polymerase activity. Similarly, highly purified minute virus of mice had no detectable DNA polymerase activity (D. C. Ward, personal communication). In contrast to the previous report for KRV (14, 16), we found that the DNA polymerase activity could be separated from both KRV and BPV preparations by sedimentation through sucrose or by an alternative purification procedure. In addition, we examined two strains of KRV which were propagated in two different cell types. No essential differences in the patterns of cellular DNA polymerases were observed during purification of the KRV strains from these cell types.

Although the absolute values of the DNA polymerase activities varied in different purification trials for each of the parvoviruses examined, virus purified to step 8 of procedure 2 was free of detectable DNA polymerase activity and contaminating cellular protein. Because virus purified in this manner retained infectivity, we conclude that a virion-associated DNA polymerase is not required for productive infection by the nondefective parvoviruses.

Analysis of the preparation at each purification step for contaminating cellular DNA polymerase α, β, and γ activities has provided information concerning the tendency for a DNA polymerase to copurify with KRV and BPV. In purification procedure 1, the major contaminating protein as detected by gel electrophoresis of both viruses had a molecular weight of 72,000 or 77,000 (Fig. 1). In addition to detection of this protein in the final virus band from procedure 1, it was also seen in electrophoretograms of samples from the first 4 steps of procedure 2 and occasionally as a minor protein in virus purified to step 8. It cannot be directly determined from the experiments described here whether this protein is a contaminating cellular DNA polym-

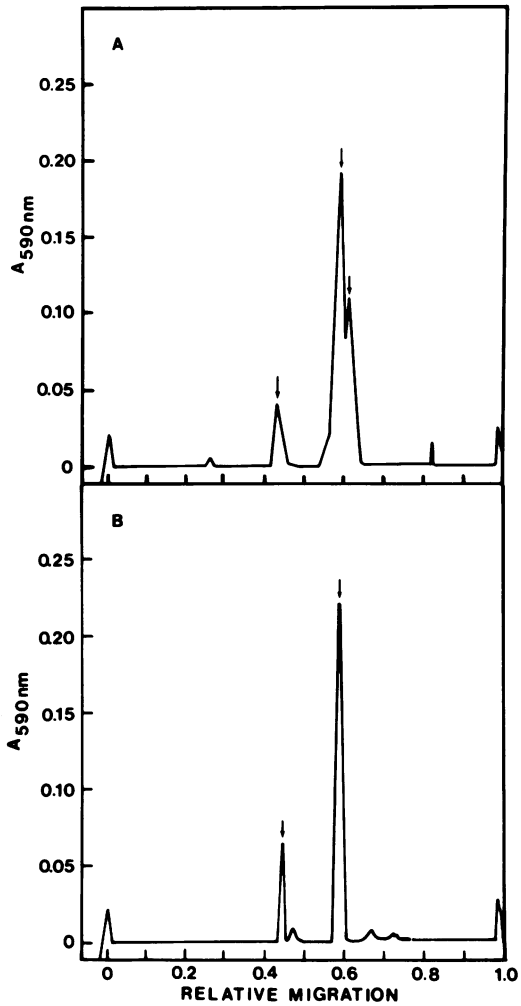

FIG. 2. *Densitometric tracing of Coomassie brilliant blue-stained polyacrylamide gel electrophoretogram of (A) KRV and (B) BPV purified to step 8 of procedure 2. Arrows indicate the position of virion proteins. Virion protein molecular weights are (A) KRV, 83,000, 61,000, and 57,500 and (B) BPV, 81,200 and 60,400. A_{590}, Absorbance at 590 nm.*

erase. Our data show, however, that DNA polymerase β copurifies more extensively with these viruses than does polymerase α or γ (Tables 1 and 2). However, it is unlikely that the contaminating protein (72,000 or 77,000 molecular weight) described above is polymerase β because the reported molecular weight for po-

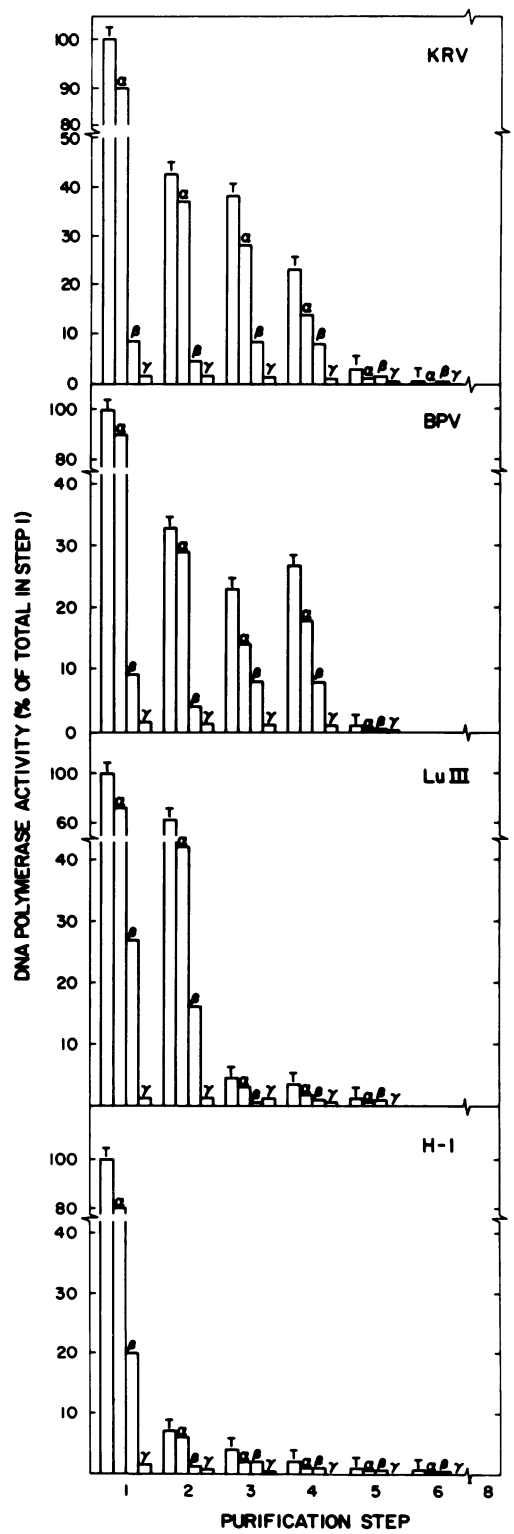

FIG. 3. *DNA polymerase activities at each step during purification by procedure 2 of KRV, BPV, LuIII, and H-1. Activity is expressed as percentage of total activity found in step 1 of the purification procedure. T = sum of α, β, and γ activities.*

TABLE 4. *Properties of purified parvoviruses tested for DNA polymerase activity*[a]

Virus	Host cell	Virus band (g/cm^2)	HAU ($\times 10^{-5}$)[b]	PFU ($\times 10^{-9}$)[c]	Virion polypeptides		
					Mean (mol wt $\times 10^{-3}$)	n[d]	Wt %[e]
BPV	BFS	1.39–1.41	10.2	2.0	81.2 ± 0.5	4	16
					60.4 ± 1.0	4	84
H-1	CL	1.39–1.41	20.4	2.4	80.9 ± 0.8	4	17
					59.8 ± 1.3	4	83
LuIII	CL	1.39–1.41	2.5	1.0	80.9 ± 0.8	4	13
					61.2 ± 0.8	4	70
					58.3 ± 0.8	4	17
KRV[f]	NRK	1.39–1.41	81.9	12.0	82.5 ± 0.5	4	13
					61.2 ± 0.5	4	75
					58.2 ± 1.0	4	12
KRV[g]	RN	1.39–1.41	5.1	13.6	83.0 ± 2.0	2	ND
					61.0 ± 0.5	2	ND
					57.5 ± 0.5	2	ND

[a] Purified by procedure 2.
[b] Total hemagglutinin units (HAU) recovered from 10^8 infected cells.
[c] Total PFU recovered from 10^8 infected cells.
[d] Number of determinations from which mean was derived.
[e] Weight percent of total protein as determined from relative areas of densitometric tracings of Coomassie brilliant blue-stained gels.
[f] Strain 171, supplied by G. Lavelle.
[g] Strain 308, supplied by L. Salzman. ND, Not determined.

lymerase β is 45,000 (25). Copurification of polymerase β with these viruses might be explained by the basic nature of the protein allowing for ionic interactions with the negatively charged virus during purification. It is possible that the copurifying protein is a virion-modified cellular DNA polymerase or a proteolytic enzyme involved in the maturation process of the capsid proteins.

Evidence for the possession of a virion-associated DNA polymerase with the nondefective parvoviruses could be provided by the demonstration that polymerase activity could be detected only after disruption of the virus particles. The parvoviruses are very stable to disruptive treatments; however, we have adopted the procedure of Salzman and McKerlie (16) which employs both nonionic (Nonidet P-40) and anionic (deoxycholate) detergents and sonic treatment to disrupt the virions. We have tested all four nondefective parvoviruses used in this study for virion-associated DNA polymerase activity after capsid disruption with the optimized reaction mixture described by Salzman and McKerlie (16). In our hands, DNA polymerase activity was not detected even when eightfold-greater specific activity of the labeled substrate was used in the reaction mixture. The amount of virus protein used per reaction was 12 to 24 μg, well above the 9 μg of protein per assay needed to detect enzyme activity in previous studies (14, 16).

We were concerned that the $CaCl_2$ and PMSF employed in the purification procedure of Tattersall et al. (20) had a deleterious effect on any virion-associated polymerase. We therefore purified KRV without $CaCl_2$ or PMSF by using differential centrifugation and a sucrose-CsCl step gradient. Although high levels of all three cellular DNA polymerases were present in the virus preparation after high-speed centrifugation, they remained in the sucrose fraction of the gradient, whereas the virions banded to their buoyant density in the CsCl layer. The virions were again disrupted and assayed as described above, and no DNA polymerase activity was detected.

The experimental results reported here demonstrate that the nondefective parvoviruses lack an essential virion-associated DNA polymerase. Our preliminary evidence suggests that these viruses use host cell DNA polymerases for viral DNA synthesis (12a). However, the possibility exists that virion-coded or virion-modified enzymes are used in this process. We are characterizing the enzymes associated with parvovirus

replication complexes to distinguish between these possibilities.

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