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Herpesvirus sylvilagus
I. Polypeptides of Virions and Nucleocapsids

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Herpesvirus sylvilagus was propagated in juvenile cottontail rabbit kidney cells and purified from the cytoplasmic fraction of the infected cells. The purification procedure included zonal centrifugation through a 5 to 30% dextran T-10 gradient, followed by equilibrium centrifugation in a 5 to 50% potassium tartrate gradient. *H. sylvilagus* formed one band after centrifugation through the tartrate gradient at a density of 1.22 g/cm^3. Contamination of the purified virus preparation by cellular proteins was less than 0.2% as determined by the removal of radioactivity from an artificially mixed sample containing [^{35}S]methionine-labeled control cells and nonlabeled infected cells. *H. sylvilagus* nucleocapsids were isolated from infected cell nuclei and purified by sedimentation through a 36% sucrose cushion, followed by equilibrium centrifugation in a 5 to 50% tartrate gradient. Forty-four polypeptides ranging in molecular weight from 18,000 to 230,000 were resolved when [^{35}S]methionine-labeled enveloped *H. sylvilagus* was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Seventeen polypeptides found within the enveloped virus were also identified within the nucleocapsid. Six additional nucleocapsid polypeptides had no counterparts within the enveloped virus. The major polypeptide within both the virus and the nucleocapsid had a molecular weight of 150,000.

According to the proposal suggested at the Third Conference on Oncogenesis and Herpesviruses (27), herpesviruses have been classified into three groups primarily based on their biological characteristics (17). Herpes simplex 1, the prototype of the alpha herpesviruses, is highly cytopathic and possesses a wide host range and a short replication cycle. Beta herpesviruses, typified by human cytomegalovirus, are less cytolytic and possess a restricted host range, a long eclipse phase, and a lengthy growth cycle. Epstein-Barr virus (EBV) is the prototype of the gamma herpesviruses. Gamma herpesviruses have a narrow host range and a predilection for lymphoblastoid cells and have been associated with lymphoproliferative diseases. Molecular analyses of these viruses have been somewhat hindered by the typical low virus yields (2, 25).

Herpesvirus sylvilagus is an antigenically distinct herpesvirus isolated from the blood of cottontail rabbits trapped in central and southern Wisconsin (11, 13). Inoculation of *H. sylvilagus* into healthy cottontail rabbits produced a persistent low-grade viremia, with infectious virus recoverable from the cellular portion of the blood. The major pathological changes seen in experimentally infected cottontail rabbits were generalized lymphoid hyperplasia, lymphocytosis with the destruction of lymph node architecture, and the invasion of abnormal mononuclear cells into the kidney, spleen, and myocardium (12, 14). The disease progression ranges from a benign to an apparent malignant lymphoma and has been likened to the outcome of EBV infections in humans (15).

H. sylvilagus has a narrow host range both in vivo and in vitro. New Zealand white rabbits, Swiss mice, Syrian hamsters, and guinea pigs are not susceptible to *H. sylvilagus* infection. Cell cultures derived from humans, monkeys, hamsters, mice, and chicks are also nonpermissive to *H. sylvilagus* (13). Based on the type of disease produced within its natural host and the narrow host range of the virus, *H. sylvilagus* has been classified as a gamma herpesvirus (17).

H. sylvilagus has been propagated in New Zealand white and adult cottontail rabbit kidney cell cultures, but the low virus yield has precluded in-depth analysis of the virus. We have established a cell line, derived from a 2-week-old cottontail rabbit, which is highly susceptible to *H. sylvilagus* infections. This study describes the purification of *H. sylvilagus* from the cytoplasmic fraction of the infected cells and presents information concerning the purity of the final virus preparation. *H. sylvilagus* nucleocapsids have also been isolated, and the polypep-

tides of the enveloped virus and nucleocapsids have been analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (21).

MATERIALS AND METHODS

Cells. Juvenile cottontail rabbit kidney (JCRK) cell cultures were prepared from a 2-week-old cottontail rabbit. Kidneys were removed and washed extensively with Ca^{2+}- and Mg^{2+}-free Dulbecco phosphate-buffered saline (CMF-PBS). The kidneys were minced into small fragments, washed with CMF-PBS, and dissociated into single cells with 0.25% trypsin in CMF-PBS (9). Approximately 5×10^6 cells were plated into blake bottles containing 100 ml of prewarmed Eagle minimal essential medium (8) supplemented with 0.22% $NaHCO_3$, 100 U of penicillin and 100 μg of streptomycin per ml, and 5% (by volume) heat-inactivated calf serum (5% CS-MEM). The medium was changed at 3-day intervals, and confluent monolayers were attained after 1 week of incubation at 37°C. Cultures were divided (1 to 2) at weekly intervals until passage 9. Thereafter, cultures were divided (1 to 3) and used after passage 20. For large-scale cell production, glass roller bottles were seeded with 5×10^7 cells. Confluent monolayers of 5×10^8 cells were attained after 4 days.

Virus. An initial lyophilized sample of *H. sylvilagus* obtained through the courtesy of H. C. Hinze (University of Wisconsin, Madison) was reconstituted with sterile water. A virus stock was made by infecting a confluent monolayer of 2×10^7 cells with 1.0 ml of the reconstituted virus. The infected cells were harvested at 7 days postinfection by scraping the cells into the tissue culture fluid and collecting the cells by centrifugation at $800 \times g$ for 15 min. The cell pellet was resuspended into 10 ml of the tissue culture fluid and disrupted by sonication for a total of 1.5 min in three intervals on ice at an amplitude of 6 μm peak to peak in an MSE sonicator. The virus stock was titrated on JCRK cells, using an overlay containing 0.7% ion-agar in 2% CS-MEM. Well-isolated plaques approximately 3 mm in diameter were picked after 5 days. The plaque isolate in 1 ml of 5% CS-MEM was subjected to three cycles of freeze-thawing and titrated again on JCRK cells. The plaque purification was performed a total of three times, with the final plaque used to infect approximately 10^7 cells. The infected cells were harvested at 5 days postinfection, when 80 to 90% of the monolayer demonstrated virus-induced cytopathology. The infected cells were harvested as described above, except the sonicated cells were made 20% (by volume) with sterile glycerol and stored at −70°C.

Radioisotopes and labeling media. [^{35}S]methionine (specific activity, 760 Ci/mmol) and [^{3}H]thymidine (specific activity, 2 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Methionine-containing labeling medium consisted of minimal essential medium with a 10% normal concentration of unlabeled methionine supplemented with 2% dialyzed calf serum and 5 μCi of radioactive methionine per ml. Thymidine labeling medium consisted of minimal essential medium supplemented with 2% dialyzed calf serum and 5 μCi of radioactive thymidine per ml.

Virus purification. Virus was purified from the cytoplasm of the infected cells essentially as described by Spear and Roizman (29). Cells were infected at a multiplicity of infection (MOI) of 10. After 1 h of adsorption at 37°C, the inoculum was removed and the monolayers were replenished with 50 ml of labeling medium. At 36 h postinfection, the infected cells were scraped and collected by centrifugation at $800 \times g$ for 10 min.

The cell pellet was resuspended into 2 volumes of 1 mM phosphate buffer (pH 7.4), held on ice for 10 min, and broken by Dounce homogenization. The extent of cell disruption was monitored by phase-contrast microscopy. Sucrose was added to a final concentration of 0.25 M, and the nuclei were removed by centrifugation at $1,000 \times g$ for 10 min.

The nuclear pellet was saved for nucleocapsid isolation, and the virus within the cytoplasmic fraction was collected by centrifugation at $72,000 \times g$ for 120 min. The cytoplasmic pellet was resuspended with 2 ml of virus buffer (VB [0.02 M Tris-hydrochloride, pH 7.4, 0.15 M NaCl]), and solid urea was added to a final concentration of 0.5 M. The solution was held on ice for 10 min and then clarified by centrifugation at $1,000 \times g$ for 15 min. The supernatant was layered onto 36-ml gradients of 5 to 30% dextran T-10 and centrifuged at $72,000 \times g$ for 60 min. The translucent virus band located midway in the gradient was removed, diluted fivefold in VB, and pelleted at $72,000 \times g$ for 120 min. The partially purified virus was resuspended into 2 ml of VB and layered onto 5 to 50% (wt/vol) potassium tartrate gradients made in VB. The gradients (10 ml) were centrifuged for 80 min at $160,000 \times g$, and the floculent virus band located just below the center of the gradient was removed. The tartrate virus band was diluted fivefold with VB and pelleted. The virus pellet was resuspended into 0.1 ml of VB by brief sonication and stored at −70°C.

Purification of nucleocapsids. Nucleocapsids were isolated from the nuclear pellet of infected cells (30) and purified as described by Cohen et al. (3). The nuclei were washed with 1% Tween 80 and broken by sonication. Solid sodium deoxycholate was added to 0.5%, and the solution was incubated for 60 min at 36°C. $MgCl_2$ was added to 1 mmol, and nucleases were added to 10 μg/ml. After a second 60-min incubation, $MgCl_2$ was added to 20 mmol to precipitate the deoxycholate, which was removed by centrifugation at $2,000 \times g$ for 30 min. The supernatant was layered over 36% sucrose cushions and centrifuged at $160,000 \times g$ for 90 min. The pelleted nucleocapsids were resuspended in VB and centrifuged to equilibrium in 5 to 50% tartrate gradients. The nucleocapsid band was removed, diluted fivefold with VB, pelleted at $160,000 \times g$ for 90 min, and stored at −70°C in 0.1 ml of VB until further use.

SDS-PAGE. Samples to be analyzed by acrylamide gel electrophoresis, using the discontinuous buffer system containing SDS (21), were boiled for 3 min in sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 4% sucrose, 0.005% bromophenyl blue, and 0.05 M Tris-hydrochloride, pH 7.0. The samples were applied to the stacking gel and electrophoresed for 5 h at a constant current of 25 mA until the tracking dye had migrated 14 cm into the separating gel slab. The stacking gel consisted of 1% SDS, 9% acrylamide, and 0.375 M Tris-hydrochloride, pH 8.8. Both gels were polymerized by the addition of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and ammonium persulfate to 0.05

and 0.5%, respectively. The electrophoresis buffer contained 0.19 M glycine, 0.1% SDS, and 0.025 M Tris-hydrochloride, pH 8.3. The gels were stained with 0.01% Coomassie brilliant blue-50% methanol-7% acetic acid, destained in 25% methanol-7% acetic acid, and then dried onto Whatman type 3 filter papers under vacuum at 80°C. Autoradiograms of the gel were made on Kodak NS-5T Safety film.

Light microscopy. Cells grown on glass cover slips were washed with warmed PBS and infected with *H. sylvilagus* at an MOI of 10 PFU per cell. At hours specified above, cover slips were removed, washed with cold PBS, stained with hematoxylin, and counterstained with eosin (16). Photographs were taken at ×950 magnification with a Leitz automatic camera.

Electron microscopy. Negatively stained samples of the potassium tartrate-purified virus and nucleocapsids were prepared by touching a Formvar-coated grid (200 mesh) to the virus suspension. Excess sample was removed by blotting the grids onto filter papers, and the sample was stained with 1% phosphotungstic acid. Excess stain was blotted from the grids, which were allowed to dry before examination.

Thin sections of infected cells were prepared by infecting 5×10^6 cells grown in 25-ml plastic flasks (Falcon Plastics, Oxnard, Calif.) at an MOI of 10. At 48 h postinfection, the infected cells were scraped from the flask and collected by low-speed centrifugation. The infected cell pellet was fixed with 3% glutaraldehyde, washed with Sorensen phosphate buffer (0.067 M, pH 7.4), and postfixed with 1% OsO_4. The samples were dehydrated through ethanol and embedded into Epon 812. Thin sections were stained with uranyl acetate and lead citrate, washed with 0.02% NaOH, and allowed to dry (4). Grids were examined with an electron microscope (EM 301; Philips Electronic Instruments, Inc., Mahwah, N.J.).

Solutions and chemicals. Fresh 30% (wt/vol) solutions of dextran T-10 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were prepared by dissolving the dextran under vacuum at 4°C, followed by autoclaving at 12 lb (5.4 kg) for 12 min and filtering the hot solution through a 25-mm nitrocellulose filter with 0.22-μm pore size (Millipore Corp., Bedford, Mass.). Nuclease stock solution consisted of DNase and RNase at a concentration of 10 mg/ml for each enzyme in 0.01 M Tris-hydrochloride (pH 7.4)-1 mM $MgCl_2$. Ultrapure urea was purchased from Swartz/Mann, Orangeburg, N.Y. Acrylamide and *N*,*N*′-methylenebisacrylamide were obtained from Eastman Kodak Co., Rochester, N.Y. Purified proteins for molecular-weight determination were purchased from Bio-Rad Laboratories, Richmond, Calif., and consisted of (molecular weights given in parentheses): myosin (200,000), β-galactosidase (116,500), phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and α-chymotrypsin (19,500).

RESULTS

In vitro propagation of *H. sylvilagus*. *H. sylvilagus* has been shown to have a narrow host range in vitro (11). Although New Zealand white rabbit kidney cells are permissive to *H. sylvilagus* infection, the yield of virus rarely exceeds 10 PFU per cell (13, 22; unpublished observations). Successive passages of the virus at low multiplicities failed to increase the virus yield. Since *H. sylvilagus* was isolated from cottontail rabbits, and young rabbits seemed to be more susceptible to virus infection than adult rabbits,

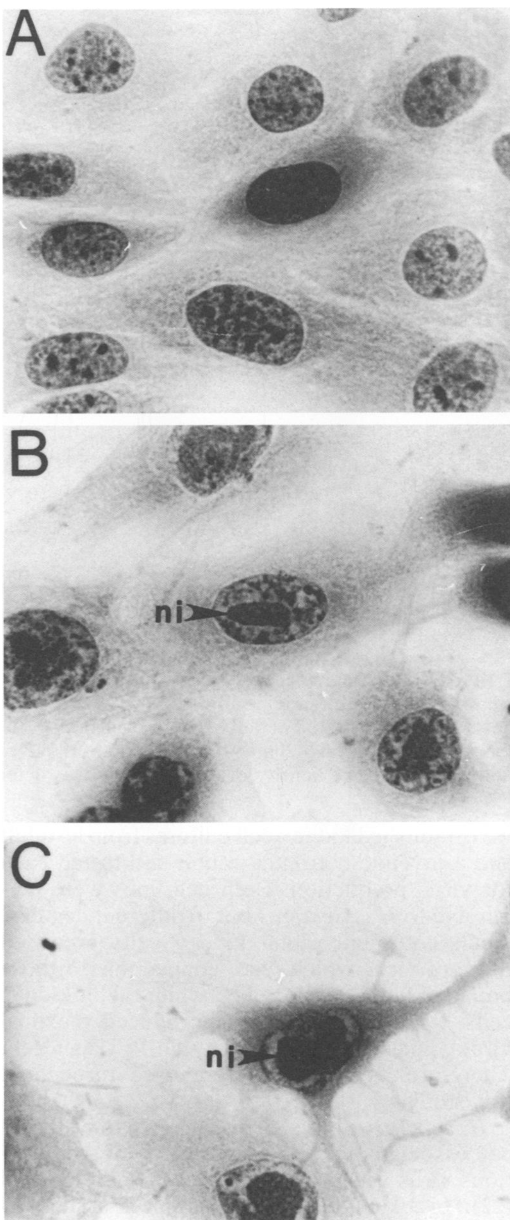

FIG. 1. Photomicrograph of uninfected JCRK cells (A) and JCRK cells infected with *H. sylvilagus* and stained at 24 h postinfection (B) or at 48 h postinfection (C). At 24 h, nuclear inclusion bodies (ni) were evident, and the cytoplasm had begun to contract. By 48 h, the cytoplasm had severely contracted. Cells were stained with hematoxylin and eosin. Magnification, ×950.

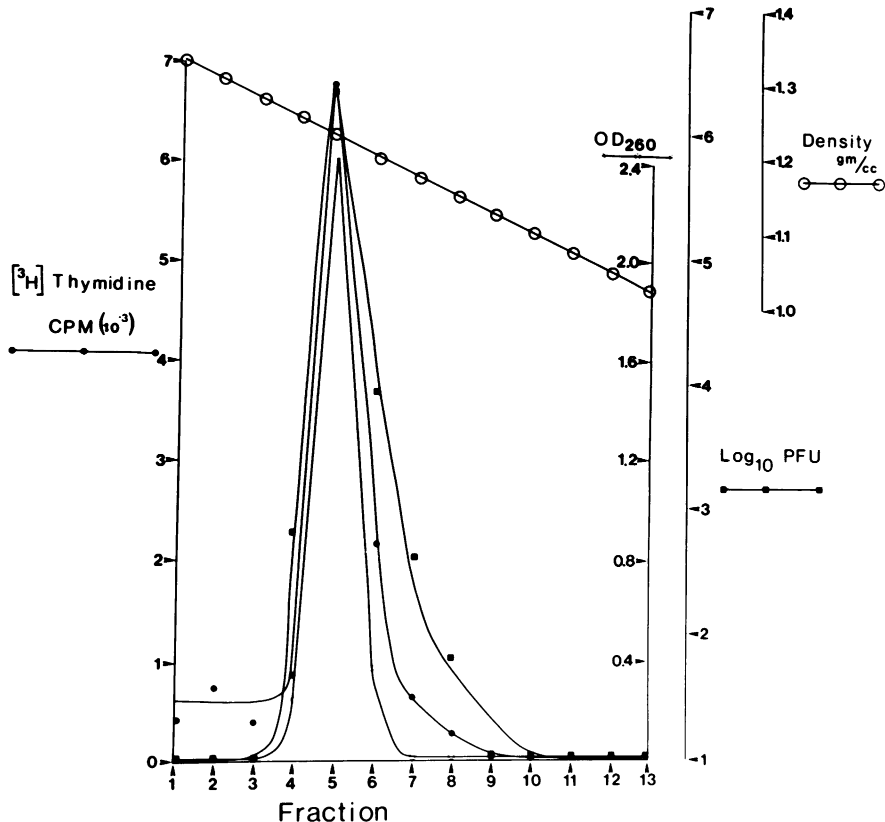

FIG. 2. Potassium tartrate equilibrium density gradient of [^{3}H]thymidine-labeled *H. sylvilagus* labeled by incorporating [^{3}H]thymidine into the maintenance medium and purified through the dextran T-10 gradient as described in the text. The dextran T-10–purified virus was centrifuged to equilibrium in potassium tartrate, and the gradient fractionated into 1.0-ml aliquots. Each fraction was assayed for radioactivity, optical density at 260 nm, and PFU. The density of each fraction was determined with a refractometer.

we established kidney cell cultures from an adult and a juvenile cottontail rabbit and tested each for virus production. Both cell lines were permissive to *H. sylvilagus* but to different degrees. Adult cottontail rabbit kidney cells produced titers of virus which were comparable to those obtained with New Zealand white rabbit kidney cells, whereas JCRK cells produced maximal virus yields of approximately 50 PFU per cell. Therefore, JCRK cells were used throughout this study.

H. sylvilagus has also been shown to be highly cell associated, with as little as 1% of the infectious virus found free in the tissue culture fluid (22). In JCRK cells, maximum cell-associated virus is obtained between 24 and 48 h postinfection. At this time, internuclear inclusion bodies are evident in all of the cells, and the cytoplasms of most cells have begun to contract (Fig. 1B). As the infection progresses, the cytoplasm continues to contract, making extraction of the nuclei by Dounce homogenization exceedingly difficult (Fig. 1C). The infected cultures were harvested at 36 h postinfection, when intercellular virus attained maximum titer, and before extensive cytoplasmic contraction had ensued.

Purification of enveloped *H. sylvilagus* and assessment of virus purity. To minimize contamination of enveloped virus with nucleocapsids, the cytoplasmic fraction of infected cells was used to obtain enveloped virus (29). When the virus from seven independent virus trials was centrifuged through the tartrate gradients, the virus from each trial banded at 1.217 ± 0.005 g/cm^3. The location of the virus within the tartrate gradient was unaffected by prolonged (5 h) centrifugation or by recentrifugation of the virus through a total of three tartrate gradients. When [^{3}H]thymidine-labeled virus was purified, and the tartrate gradient fractionated coinciding peaks of radioactivity, optical density at 260 nm and PFU values were found (Fig. 2). This peak was shown to contain exclusively enveloped virus by electron microscopy (Fig. 3).

Table 1 shows the recovery of virus from ^{35}S-labeled control cells. The purification process

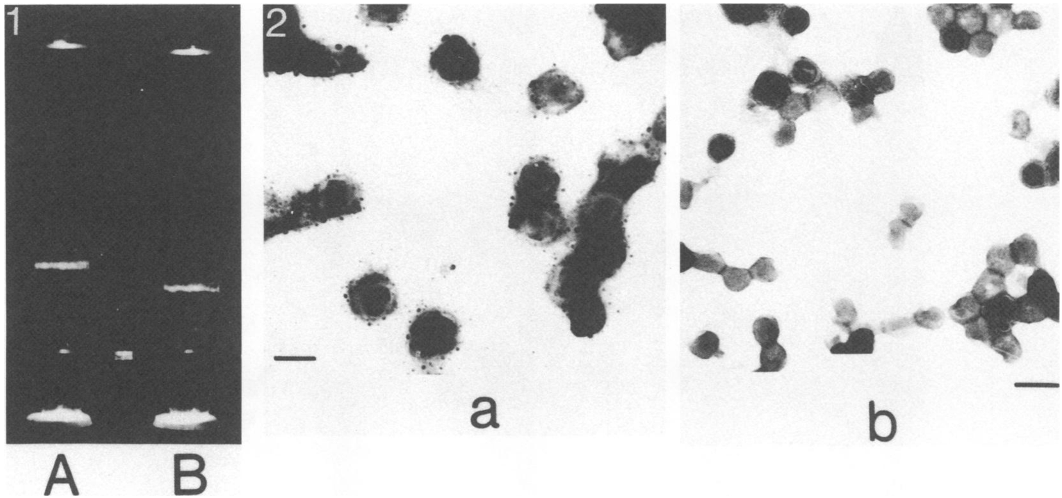

FIG. 3. (1) Isopycnic banding of *H. sylvilagus* in 5 to 50% potassium tartrate gradient. (A) After centrifugation at 160,000 × *g* for 90 min, virus formed one band located at a density of 1.22 g/cm^3. (B) In a parallel centrifuge tube, the nucleocapsids formed one band at a density of 1.27 g/cm^3. (2) Electron micrographs of material recovered from the potassium tartrate band and stained with phosphotungstic acid. (a) Virion; (b) nucleocapsids. Bar, 100 nm.

resulted in the recovery of 3.7% of the ^{35}S label from infected cells while removing over 99.88% of the ^{35}S label associated with host cell proteins. Therefore, 97.6% of the radioactivity contained within the final virus preparation is directly attributable to the virus, with 2.4% of the radioactivity associated with cellular contamination. A typical yield for 10^8 infected cells is 5.6 × 10^9 PFU.

Nucleocapsids. Herpesviruses encapsidate their genome within the nucleus and acquire an envelope as the particle migrates through the nuclear membrane (25). Figure 4A shows the intranuclear form of *H. sylvilagus*. The electron-dense core is encapsidated to form the nucleocapsid. Figure 4B shows the nucleocapsid acquiring an envelope near the nuclear membrane. Since the nucleus contains predominately non-enveloped nucleocapsids, the nuclear pellet was used to isolate nucleocapsids. The nucleocapsids formed a single discrete band upon isopycnic centrifugation in potassium tartrate. The nucleocapsids isolated from three separate trials banded at a density of 1.267 ± 0.005 g/cm^3 in the potassium tartrate density gradient. Figure 3 shows the separation of enveloped virions (A)

TABLE 1. Purification of *H. sylvilagus*

Step	Expt				
	I[a]		II[b]		
	cpm × 10^{-3}	% Recovery	cpm × 10^{-3}	% Removal	PFU
Complete cell	9,000.0	100.00	7,246.8	0.00	5.6 × 10^9
Cytoplasmic fraction	5,800.5	64.45	4,674.2	35.50	ND[c]
1,000 × *g* 10-min supernatant	1,894.5	21.05	658.4	90.09	ND
Dextran T-10 band	769.5	8.6	49.0	99.32	ND
Tartrate band	329.8	3.7	8.4	99.88	1.5 × 10^8

[a] A total of 10^8 JCRK cells were infected with *H. sylvilagus* at an MOI of 10 PFU per cell. At 36 h postinfection, cells were harvested and the virus was purified as described in the text. At various steps during the purification process, samples were removed and precipitated with 10% cold trichloroacetic acid. The precipitates were collected onto Whatman GF/A glass fiber filters, washed twice with 5% cold trichloroacetic acid and once with 67% ethanol, and dried, and radioactivity was determined with a toluene-based liquid scintillation spectrometer.

[b] A total of 10^8 JCRK cells infected with *H. sylvilagus* at an MOI of 10 PFU per cell were mixed with 10^8 uninfected [^{35}S]methionine-labeled JCRK cells. The virus within the mixture was purified, and trichloroacetic acid-precipitated ^{35}S counts per minute were determined at various steps as described above.

[c] ND, Not done.

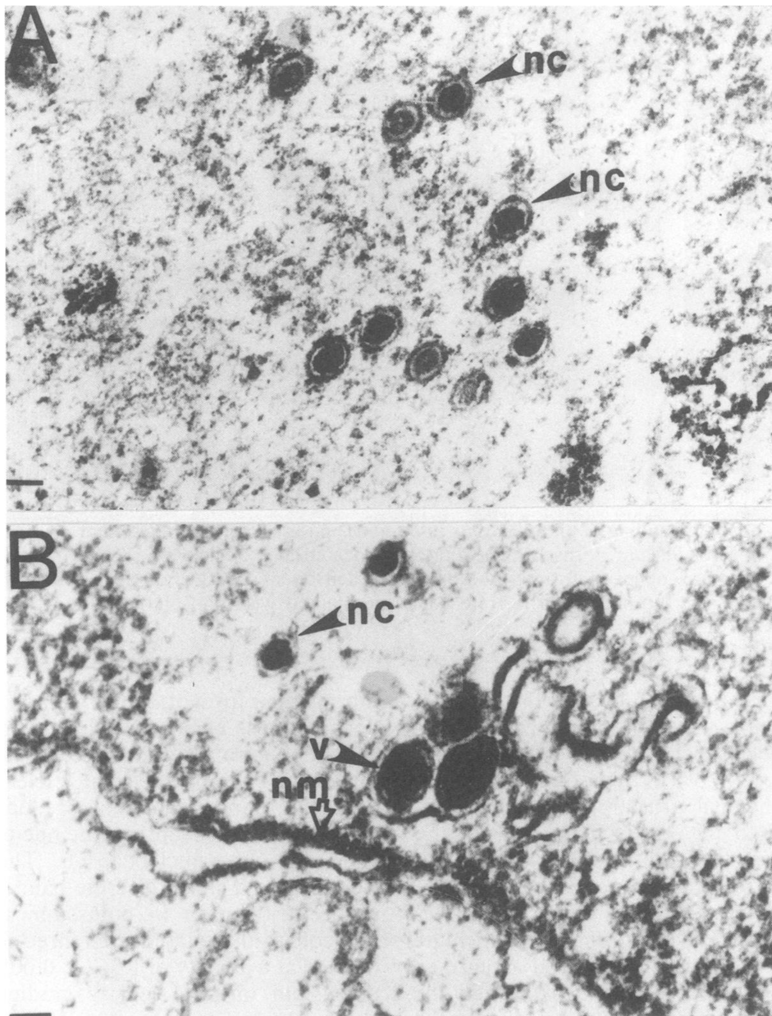

FIG. 4. Electron micrograph of a thin section through an *H. sylvilagus*-infected JCRK cell nucleus (A) and the nuclear periphery (B). Nucleocapsids (nc) are evident within the nucleus. Near the nuclear membrane (nm), the nucleocapsids become enveloped to form the complete virion (v). Bar, 100 nm.

and nucleocapsids (B) attained by isopycnic centrifugation in potassium tartrate. The nucleocapsid preparation contained predominately nonenveloped particles (greater than 95%) with an average diameter of 72 nm.

Polypeptides of enveloped *H. sylvilagus*. A total of 44 polypeptides ranging in molecular weight from 18,000 to 230,000 were resolved when [^{35}S]methionine-labeled *H. sylvilagus* was purified and analyzed by SDS-PAGE (Fig. 5). Virus polypeptide 5 (Vp5) was found to be the major protein component of the enveloped virus with respect to both the amount of Coomassie brilliant blue stain and ^{35}S label. Twofold dilutions of the purified virus were resolved by SDS-PAGE to reduce the band width of Vp5. The results indicated that Vp5 is a single polypeptide. The molecular weight of the virus polypeptides and the relative amount of ^{35}S radioactivity located within the major virus polypeptides as determined from the autoradiogram with an automatic recording microdensitometer are shown in Table 2. The molecular weights of polypeptides larger than 200,000 are estimated by assuming the linear relationship between log (molecular weight) and relative distance of migration extending beyond the largest reference protein (31).

Comparison of polypeptides between enveloped virus and nucleocapsids. Figure 6 shows the autoradiogram of ^{35}S-labeled enveloped virus and nucleocapsids along with the densitometric tracings of each sample. Nucleocapsid polypeptides (Ncps) which had identical migrational

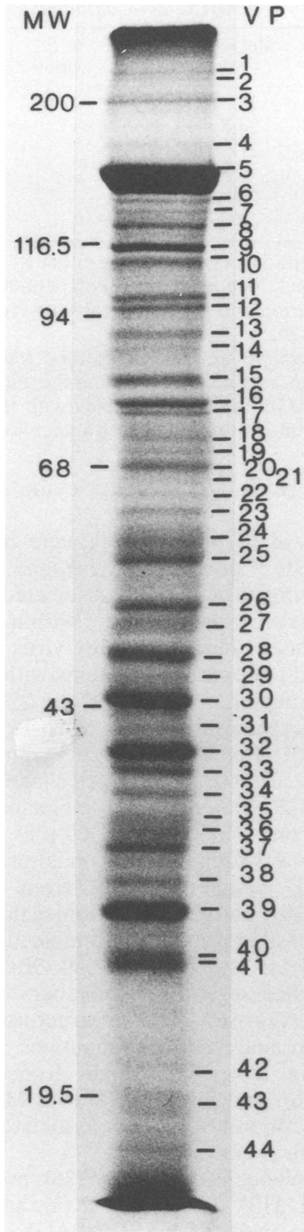

FIG. 5. Polypeptides of enveloped *H. sylvilagus*. [^{35}S]methionine-labeled *H. sylvilagus* was purified and analyzed by SDS-PAGE as described in the text. The autoradiogram shows 44 viral proteins which are numbered in order of decreasing mobility. The location of the molecular weight reference proteins is shown on the left. Molecular weight is expressed $\times 10^3$.

rates with polypeptides present within the enveloped virus were identified by the number corresponding to the viral polypeptide. A total of 17 Ncps, ranging in molecular weight from 24,500 to 200,000, were identified within the enveloped virus and are listed in Table 2. Six Ncps possessed migrational rates differing from enveloped virus polypeptides analyzed within the same gel. The Ncps which did not correspond to any virus polypeptide were identified by the number associated with the nearest virus polypeptide, followed by a letter indicating that these Ncps were not found within the complete virus. Thus, Ncp9a was slightly larger than Vp9, and Ncp39a and Ncp41a were slightly smaller than Vp39 and Vp41, respectively. The three Ncps 23a, 24a, and 25a had the same appearance within the gel, with respect to relative location and amount of ^{35}S label incorporation, as Vp23, Vp24, and Vp25, except the three Ncps banded above the similar group of polypeptides within the enveloped virus. The relative amounts of ^{35}S label located within the major Ncps were calculated from the densitometric tracing of the autoradiogram and are listed in Table 2. Although the relative amount of Vp5 located within the virus was greater than the amount of ^{35}S label found within the corresponding nucleocapsid protein, Vp5 was the major protein component within the nucleocapsid. The total molecular weight of the 44 virus structural proteins was 3.4×10^6. The total molecular weight of the 17 Ncps which corresponded to polypeptides within the complete virus was 1.3×10^6.

DISCUSSION

The purpose of the present study was to analyze the structural polypeptides of *H. sylvilagus*. In the course of the study, it was found that JCRK cells were highly permissive to virus infection. Previous investigations into the relationship of age to virus infection have shown that juvenile cottontail rabbits are more susceptible to virus infection than are adult cottontail rabbits (12). A total of 27% of the juvenile (3-week to 3-month-old) rabbits and 10% of the adult cottontail rabbits showed virus-induced lymphocytosis after experimental inoculation with *H. sylvilagus*. The effect of age upon susceptibility to *H. sylvilagus* in vivo is also seen in vitro. Cottontail rabbit kidney cell cultures are permissive to *H. sylvilagus* infection, but JCRK cells produce at least fivefold more infectious virions per cell than do adult cottontail rabbit kidney cells. The effect of *H. sylvilagus* infection in JCRK cells is rapid virus multiplication, with progeny virus seen as early as 9 h postinfection. Virus-induced cytopathic effects are seen at this time as cell rounding due to cytoplasmic contraction. Type A intranuclear inclusion bodies are first evident by 12 h postinfection and develop to include all infected cells by 24 h postinfection. By 48 h, most of the cells have become detached, with those cells remaining

TABLE 2. Polypeptides of *H. sylvilagus* and nucleocapsids

Polypeptide no.	Mol wt ($\times 10^3$)	Location[a]	% ^{35}S virion	Incorporated nucleocapsid[b]
1	230	V		
2	225	V		
3	200	S		
4	165	V		
5	150	S	10.9	7.2
6	130	V		
7	128	V		
8	120	S		
9	115	V	3.1	
9a	117	N		4.7
10	107	V		
11	98	S		
12	96	S		
13	88	S		
14	86	S		
15	80	S		3.6
16	77	V		
17	75	V	2.7	
18	72	S		
19	70	V		
20	68	V		
21	66	V		
22	65	V		
23	63	S		2.3
24	59	V		
25	56	V		
23a	64	N		
24a	62	N		
25a	60	N		
26	52	V		
27	51	V		
28	47	S		
29	46.5	V	3.8	
30	44	S	4.8	5.7
31	41	V		
32	40	S	4.2	
33	37.5	V		
34	36	S		
35	35	V		
36	34	V		
37	32	V		
38	31.5	V		
39	29	S	5.5	4.5
39a	28.5	N		4.0
40	26.5	S		
41	26	S	2.0	
41a	24.5	N		
42	21	V		
43	20	V		
44	18	V		

[a] Polypeptides were identified either as virion (V) or nucleocapsid (N) components. Polypeptides present in both the virion and the nucleocapsid were designated as shared (S).

[b] The relative amount of isotope located within major bands was determined from the autoradiograph of SDS-PAGE–separated samples with the aid of an automatic integrator attached to a microdensitometer.

attached showing extensive cytoplasmic contraction.

A total of 44 polypeptides were resolved by SDS-PAGE of purified *H. sylvilagus*. This is the highest number of proteins associated with purified herpesviruses to date. Within the alpha herpesviruses, herpes simplex virus 1 (HSV-1) was found to contain 24 proteins with molecular weights ranging from 25,000 to 275,000 (27). Equine herpesvirus (EHV) consists of 28 proteins with molecular weights ranging from 16,000 to 276,000 (18). Varicella-zoster virus contains 33 proteins with molecular weights ranging from 16,000 to 240,000 (28). Pseudorabies virus was found to contain 20 proteins with molecular weights ranging from 20,000 to 230,000 (19), and murine cytomegalovirus contains at least 33 proteins with molecular weights ranging from 11,500 to 255,000 (20). EBV and Marek's disease virus are members of the gamma herpesviruses whose structural proteins have been analyzed. EBV contains 33 proteins ranging in molecular weight from 28,000 to 290,000 (6), and initial work with Marek's disease virus found 8 proteins associated with the purified virus (2).

The nucleocapsids of EHV (26), pseudorabies virus (30), HSV-1 and HSV-2 (3), and EBV (7) have been analyzed by SDS-PAGE. The major nucleocapsid protein of these viruses has a molecular weight of approximately 150,000, a result compatible with the theory that all herpesviruses contain a similar nucleocapsid protein. The molecular weight of the nucleocapsid proteins of EHV, pseudorabies virus, and HSV-1 and HSV-2 ranges from 12,000 to 155,000, whereas the molecular weight of EBV nucleocapsid proteins ranges from over 20,000 to 200,000. EHV nucleocapsids have been shown to contain five major and eight minor proteins, pseudorabies virus nucleocapsids contain one major and seven minor proteins, HSV-1 and HSV-2 nucleo-

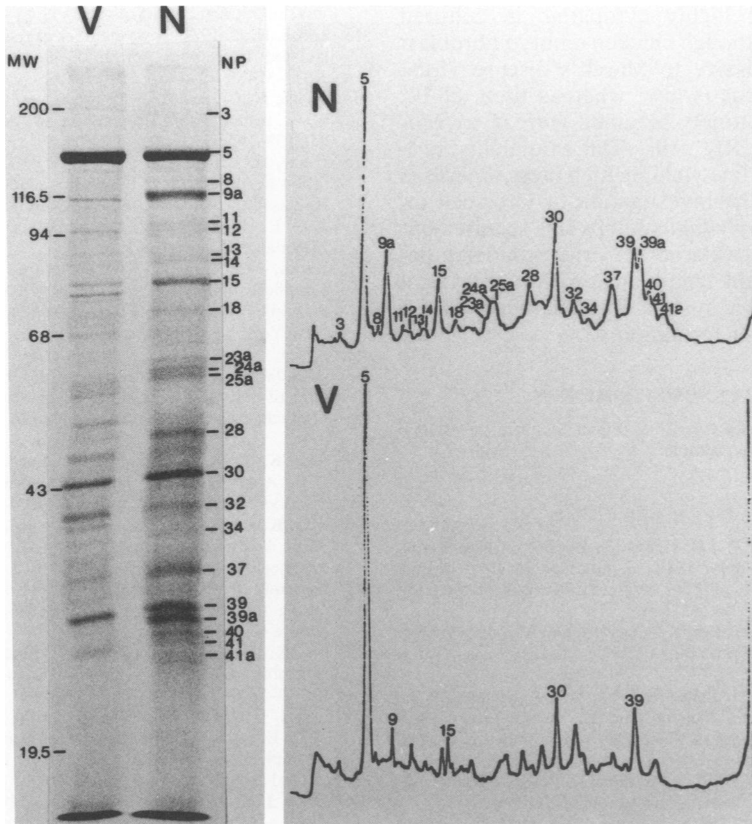

FIG. 6. Comparison of polypeptides of enveloped *H. sylvilagus* and the nucleocapsids. [^{35}S]methionine-labeled enveloped *H. sylvilagus* (V) and nucleocapsids (N) were purified, and the polypeptides were resolved by SDS-PAGE. The location within the gel of the molecular weight reference proteins is shown on the left (molecular weight is expressed $\times 10^3$). Nucleocapsid polypeptides (NP) are listed on the right of the autoradiogram. The densitometric tracings of the autoradiogram is shown on the right of the figure.

capsids contain seven proteins, and EBV nucleocapsids contain seven major and nine minor proteins. The major protein of *H. sylvilagus* nucleocapsids is Vp5 and has a molecular weight of 150,000. A total of 5 major and 12 minor proteins were found to be shared between the nucleocapsid and the enveloped virion (Table 2).

The resolution of polypeptides within the nucleocapsid which do not correspond to polypeptides within the mature, enveloped virus suggests either processing or selective removal of nucleocapsid proteins during maturation. Specifically, Ncp41a and Ncp39a seem to be removed from the nucleocapsid, whereas Ncp9a and the triplet of nucleocapsid proteins (Ncp23a, Ncp24a, and Ncp25a) seem to be processed to a lower-molecular-weight species found within the enveloped virion. Processing of nucleocapsid proteins during virus maturation has been suggested to occur with both HSV (10) and EHV (18). Such processing events could account for the relative enrichment of Vp5 in the enveloped virion. The removal of Ncp9a and Ncp39a, which together account for 8.7% of the ^{35}S label within the nucleocapsid proteins, could account for the increase in the relative amount of Vp5 within the enveloped virion.

Gamma herpesviruses have been shown, or are strongly implicated, in tumor formation. EBV, the most widely studied gamma herpesvirus, lacks a permissive tissue culture system. Complete virions are produced by the rare activation of transformed lymphocytes (5). Although chemical inducers are available which greatly increase the amount of virus DNA produced within the transformed lymphocyte, there is little effect upon the production of complete virions (1, 23, 24). Marek's disease virus, which causes a lymphoproliferative disease in chickens, can be propagated in chicken embryo fibroblasts. In chicken embryo fibroblast cells, Marek's disease virus is cell associated and cytopathic. *H. sylvilagus* induces a lymphoproliferative disease in cottontail rabbits and is cell

associated and highly cytopathic in cultured JCRK cells. Although chicken embryo fibroblast cells are permissive to Marek's disease virus, the yield of virus is low, whereas titers of 10^8 PFU/ml are routinely obtained with *H. sylvilagus*-infected JCRK cells. The availability of a permissive cell line yielding high titers of virus is mandatory to the investigation of virus-cell interactions. *H. sylvilagus* fulfills this requirement and, being a gamma herpesvirus producing benign to malignant lymphomas within its natural host, provides a system for the study of the process of tumor formation.

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