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Properties of Canine Herpesvirus DNA[1] (38072)

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Discrepant values have been reported for the base composition of canine herpesvirus (CHV) DNA as determined from its buoyant density in CsCl gradients (1, 5, 11). Aurelian (1) reported that DNA extracted from partially purified CHV had a density of 1.725 g/cm^3 and a guanine plus cytosine (G + C) content of 65–67%. In contrast, Plummer *et al.* (11) reported a density of 1.692 g/cm^3 with a corresponding G + C content of 33%. Because preliminary data obtained in our laboratory (5) on thymidine-$^{14}CH_3$-DNA extracted from CHV-infected dog kidney cell homogenates suggested a buoyant density range of 1.70–1.72 as interpolated from somewhat broad peaks of thymidine-$^{14}CH_3$-DNA, further studies were done using several modifications of a previously described method (1) that included purification of CHV, rigid temperature control during isopycnic centrifugation, an extended centrifugation period, and a reduction in the original quantities of DNA added to CsCl gradient tubes. Such modifications gave consistent results in repeated experiments that indicated a buoyant density of 1.691 g/cm^3. The low G + C content of the CHV-DNA (31%) as calculated from its buoyant density, was substantiated by additional experiments where the thermal denaturation profile (melting temperature, Tm) was determined. In this report we present recent data on the buoyant density, melting temperature, G + C mole fraction, and an estimate of the molecular weight of the DNA isolated from purified CHV.

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Methods. Essential properties of the F205 strain of CHV and its growth in primary and secondary dog kidney cells (DKC) have been described (6). The virus was allowed to adsorb at room temperature for 2 hr onto monolayer DKC cultures grown in glass or plastic bottles. Medium containing either 5 μCi/ml of thymidine-$^{14}CH_3$ or 10 μCi/ml of thymidine-C^3H_3 (New England Nuclear Corp., Boston, Mass., specific activities were 40 mCi/mmole and 20 Ci/mmole, respectively) then was added. Uninfected DKC and similar cultures infected with CHV or pseudorabies virus were incubated at 36°C for 16–20 hr when essentially all cells of infected cultures had evidence of cytopathic effect. Attached cells were scraped from the surfaces and washed 4 times with 0.15 *M* phosphate buffered saline (PBS), pH 7.3, to remove unspecifically-bound radioactive label. Washed cells were stored frozen as stock in PBS at −70°C until used. To obtain purified virus, stock infected cells were thawed rapidly and sonified for 1 min in a Branson Sonifier Cell Disruptor. The homogenate was centrifuged at 2500 rpm using a SS-34 rotor in a Sorval PRC2 centrifuge. Supernatant solutions were layered onto preformed, linear sucrose gradients (20%–55% (w/w) in 0.05 *M* Tris buffer, pH 7.3) and centrifuged for 90 min at 4°C using the SW 27 rotor at 24,000 rpm in a Spinco L265B ultracentrifuge. The bands of CHV corresponding to maximum infectivity and cpm were diluted with tris buffer and ultracentrifuged at 100,000 $\times g$ for 90 min to sediment the virus. The pellet was sonified for 20–30 sec in Tris buffer to resuspend the virus. $MgCl_2$ was added to a final concentration of

0.01 *M*, 30 μg/ml DNase, and 30 μg/ml RNase were added, and the mixture was incubated for 2 hr at 37°C. Thereafter this suspension was cooled to room temperature and adjusted to 0.05 *M* with EDTA pH 7.3 and again layered over preformed sucrose gradients (20%–55% in Tris–EDTA) and virus was obtained as described above. The resultant virus pellet was sonified for 20 sec in Tris buffer containing .02 *M* EDTA. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%; preincubated pronase then was added to give a concentration of 100 μg/ml, and the mixture was incubated for a further 2 hr at 37°C. After cooling to room temperature 1 volume of freshly prepared, Tris–EDTA-washed phenol (redistilled) was added, the tubes were gently inverted 20 times and the phases separated by slow centrifugation. The buffer layer was reextracted with 1 volume of phenol and then extracted twice with 2 volumes diethyl ether, or dialysed directly against 3 changes of 1 liter of 0.1 SSC (0.015 *M* NaCl, 0.0015 *M* Na-citrate pH 7.3) for 24 hr. Radioactively labeled DNA from uninfected dog kidney cells was isolated by extracting the cell-free homogenates with phenol. The dialysed product was treated with 30 μg/ml RNase at 37°C for 2 hr, reextracted with phenol and again dialysed against 0.1 SSC. Pseudorabies virus (obtained originally in June, 1962, from T. Ben-Porat) was cultured simultaneously in separate monolayers of DKC and its DNA was labeled with thymidine-C^3H_3. DNA content of all preparations was determined by a micro modification of the diphenylamine method (3), using calf thymus DNA as a standard; counts per min also were recorded. About 0.5–1.0 μg DNA was mixed with CsCl so that the starting density of the CsCl solution was 1.700 g/cm³. The CsCl (Calbiochem Corp.) was dissolved in 0.1 SSC, pH 7.3, and centrifuged at 20,000 rpm in the PRC-2 Sorval centrifuge for 30 min and filtered through a 0.45 μm Millipore filter to remove microcrystals and small fibers. Isopycnic centrifugation was done in a SW 39 rotor at 33,000 rpm for 72 hr at 20°C in the Spinco L265B ultracentrifuge. Thereafter 4 drop fractions were collected through the bottom of the tubes with a 23 gauge needle. The refractive index of a 5 μl aliquot of each fraction was measured immediately with a Bausch and Lomb Abbe-3L Refractometer. Refractive indices were then converted to density (8). Radioactivity was quantitated by placing aliquots of each fraction on filter papers (3 × 7 cm) which were dried under a heat lamp and counted in toluene containing 2,5-diphenyloxazole using a Beckman liquid scintillation counting system.

The thermal denaturation temperature (Tm) of CHV-DNA was measured by the method of Mandel and Marmur (7). Larger quantities of purified virus were prepared for these measurements, and the unlabeled DNA was purified as described before. About 10 μg of CHV-DNA in 0.1 SSC ($A_{260\,nm}$ 0.20–.30) were used to determine the Tm in a Beckman model DBG spectrophotometer equipped with a temperature controlled circulating water bath. The standard conditions of Mandel and Marmur (7) were employed. Commercially available calf thymus DNA was used as control DNA for this procedure. The molecular weight of CHV-DNA was estimated by velocity sedimentation using the method of Burgi and Hershey (2) with the modifications of Smith and McAuslan (14) to prevent shear damage to DNA molecules. For molecular weight estimations, thymidine-$^{14}CH_3$-labeled DNA from T_2-phage cultured in *E. coli*-B, and thymidine-C^3H_3-labeled DNA obtained from pseudorabies virus were used as reference markers.

Results. Results of isopycnic centrifugation in CsCl gradients are illustrated in Fig. 1; buoyant density, Tm, guanine plus cytosine mole content, and molecular weight of CHV-DNA are presented in Table I. The profile of Fig. 1 represents a single isopycnic centrifugation run of a mixture of the three DNA's shown. Similar results were obtained when the buoyant density of each DNA was determined separately. The 2 density markers, pseudorabies virus DNA and DKC-DNA, banded at a density of 1.731 g/cm³ and 1.697 g/cm³, respectively; CHV-DNA reproducibly banded at a CsCl density of 1.691 g/cm³ and did not have

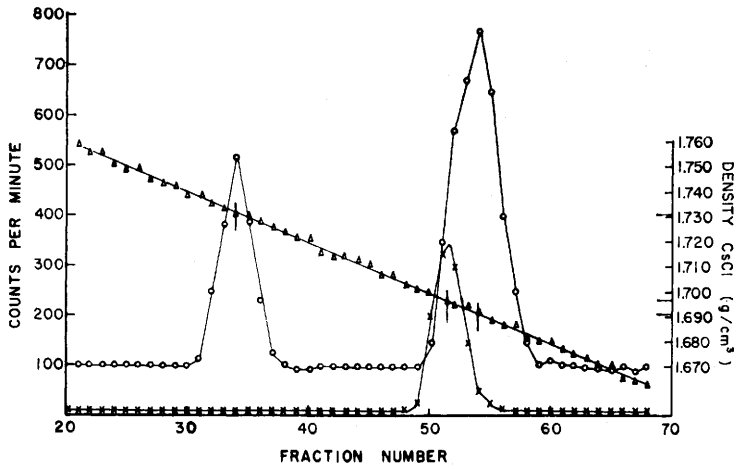

FIG. 1. Analysis of 3 DNAs by isopycnic centrifugation in CsCl. About 0.5 μg of each type of isolated DNA was placed into a single centrifuge tube and mixed with CsCl in 0.1 SSC pH 7.3 (starting density was 1.700 g/cm³). Open circles—thymidine-C^3H_3-DNA of pseudorabies virus (peak on left 1.731 g/cm³) and thymidine-C^3H_3-DNA of CHV (peak on right 1.691 g/cm³); crosses—thymidine-$^{14}CH_3$-DNA of dog kidney cells (1.697 g/cm³); open triangles—CsCl density in g/cm³. Specific methods are described in text.

a peak in the 1.720 g/ml density region of the gradients. Thymidine-$^{14}CH_3$-labeled CHV-DNA also had a peak at 1.691 g/ml. Host cell DNA labeled with either thymidine-$^{14}CH_3$, or thymidine-C^3H_3 banded at a density of 1.697. Therefore, in this study, isotope effects in the newly synthesized DNA did not significantly affect the buoyant density determinations.

The guanine plus cytosine mole fraction of CHV-DNA calculated (8) from its buoyant density was 31.6% and the value calculated from Tm (7) was 30.8%. The G + C content of DKC-DNA and pseudorabies virus-DNA as calculated from their buoyant density was 37.8% and 72.5%, respectively; the G + C content of control calf thymus DNA calculated from Tm was 43%. The experimental values of these control marker DNA's corresponded with values reported by others, as shown in Table I.

Three replicate molecular size determinations were done and the molecular weight

TABLE I. Buoyant Density in CsCl, Tm, G + C Mole Fraction and Molecular Weight of DNA from Purified CHV Compared to Marker DNA's.

Source of DNA	Buoyant density in CsCl	Tm	Calculated G + C	Literature G + C	Molecular weight
Canine Herpesvirus	1.691 g/cm³		31.6%	33%[11a] 66[1]	63×10^6
		66.5°C	30.8	—	
Reference controls					
Dog kidney cells	1.697	—	37.8	37.8[11]	—
Pseudorabies virus	1.731	—	72.5	73[4]	76×10^6
Calf thymus	—	71.5	43.0	42[9]	—
T_2-phage	—	—	—	—	130×10^6

[a] Superscripts refer to literature references.

of CHV-DNA was calculated to be 63 × 10^6 daltons relative to 130 × 10^6 daltons for T_2 DNA and 76 × 10^6 for pseudorabies virus DNA.

Discussion. Aurelian (1) used procedures similar to those described here to determine the density of CHV-DNA, whereas Plummer *et al.* (11) used an analytical Spinco model E ultracentrifuge to characterize different DNA species. The latter method offers the advantage that the DNA bands and CsCl gradient are not disturbed during measurements because separate fractions are not collected. On the other hand, the use of radioactive precursors which measure primarily the DNA synthesized after infection should be more sensitive in determining small amounts of new DNA. With partially purified extracts, both methods suffer the disadvantage of having to detect small amounts of viral DNA in the presence of a large excess of cellular DNA. This makes interpretation difficult when the molecular species have similar densities. The buoyant densities of CHV-DNA and DKC-DNA differed by only 0.006 g/cm^3 and, therefore, the host cell DNA would obscure the presence of viral DNA. In this study, longer centrifugation times (72 hr), temperature control during the run and greater purification of the CHV preparations and the CHV-DNA were found to be important in obtaining consistent results.

Isopycnic centrifugation in CsCl gradients in a preparative ultracentrifuge as used here, and determining buoyant density in the analytic ultracentrifuge as done by Plummer *et al.* (11) gave virtually the same values for the buoyant density in CsCl and the corresponding G + C content for CHV-DNA. In addition, in this study, a similar value for the G + C content was obtained by first determining the Tm of the DNA (also see (12)).

By the method used in this study, CHV had a molecular size of 63 × 10^6 daltons. Although the average molecular weight estimate of CHV-DNA was somewhat lower than reported values of some herpesviruses, it did not appear unusual. For example, the DNA weight of infectious bovine rhinotracheitis virus (bovine herpesvirus-1), has been reported to be 54 × 10^6 daltons (12). Indeed, molecular weights of herpesviruses may vary more than presently believed. The molecular size of herpes simplex virus DNA until very recently was accepted as 100 × 10^6 daltons, but data based on several perameters now suggest that 82–85 × 10^6 daltons may be the correct value (10). In this study the molecular weight of pseudorabies virus DNA was estimated to be 76 × 10^6 daltons, which is slightly higher than the reported values of 68–70 × 10^6 (4, 13). Additional data using confirmatory technics such as band-width measurements of CHV-DNA centrifuged to equilibrium in CsCl gradients and measurement of the kinetics of reannealing of CHV-DNA will be required to provide confidence for any molecular size value obtained by a single method.

Summary. The DNA isolated from purified canine herpesvirus had the following biophysical properties: Buoyant density in CsCl gradients of 1.691 g/cm^3; Tm of 66.5°C in 0.1 SSC; guanine plus cytosine content of 31%; and a molecular weight of 63 × 10^6 daltons as estimated by velocity sedimentation in neutral sucrose gradients.

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