Size and Composition of Marek's Disease Virus Deoxyribonucleic Acid

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Deoxyribonucleic acid (DNA) extracted from purified nucleocapsids of Marek's disease herpesvirus (MDV) was cosedimented with T4 and with herpes simplex virus (HSV) DNA in neutral sucrose density gradients and with T4 DNA in alkaline sucrose density gradients. These experiments indicated that the intact MDV DNA had a sedimentation constant of 56S corresponding to a molecular weight of 1.2×10^8 daltons. In the alkaline gradients, the largest and most prominent band contains a DNA sedimenting at 70S corresponding to 6.0×10^7 daltons in molecular weight. The DNA is therefore double-stranded and not cross-linked. Isopycnic sedimentation of the MDV DNA molecules with SPO1, *Micrococcus lysodeikticus*, and HSV DNA gave a density of 1.705 g/cm³ corresponding to 46 guanine plus cytosine moles per cent. Lastly, in hybridization tests the DNA hybridized with RNA of infected cells but not with that of uninfected cells supporting the conclusion that it is viral.

In recent years a herpesvirus (6, 7, 11) has been associated with Marek's disease, a fulminating fatal infection of fowl. The causal relationship of the Marek's disease virus (MDV) to the disease is based on several lines of evidence as follows. (i) Virus can be invariably recovered from infected birds. (ii) Virus recovered from feather follicles of infected chickens is highly infectious in the cell-free form (5, 12). (iii) Chickens surviving infection have antibody against the virus (3, 14). (iv) Preinoculation with a turkey herpesvirus, avirulent for chickens, prevents tumor formation upon challenge with pathogenic MDV (13).

Preliminary studies from our laboratories (9) have shown that the virus contains deoxyribonucleic acid (DNA). The purpose of this communication is to report on the properties of this DNA.

MATERIALS AND METHODS

Solutions. Virus buffer consisted of 0.15 M NaCl and 0.02 M tris(hydroxymethyl)aminomethane (Tris; pH 7.5). Neutral DNA buffer consisted of 1.0 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), and 0.05 M Tris (pH 7.5). Alkaline DNA buffer consisted of 0.9 M NaCl, 0.1 M NaOH, and 0.001 M EDTA. Reticulocyte standard buffer (RSB) consisted of 0.01 M NaCl, 0.0015 M MgCl₂, and 0.01 M Tris (pH 7.5). TES buffer consisted of 0.02 M Tris (pH 7.5), 0.001 M EDTA, and 0.15% Sarkosyl (NL-97; Geigy Industrial Chemicals, Ardsley, N.Y.). Standard saline citrate (1× SSC) consisted of 0.15 M NaCl and 0.015 M sodium citrate.

Cells. Duck eggs were obtained from Ridgeway Hatcheries, Inc., LaRue, Ohio. Duck egg cell cultures were prepared according to Solomon et al. (17) and grown in medium consisting of mixture 199, F10, and 4% calf serum all obtained from Grand Island Biological Co., Grand Island, N.Y. HEp-2 cells were originally obtained from Flow Laboratories, Rockville, Md., and were grown in minimal essential medium (MEM) supplemented with 10% calf serum both obtained from Microbiological Associates, Inc., Bethesda, Md.

Virus and infection of cells. Duck embryo fibroblasts were grown in roller bottle cultures as described previously (17). Nearly confluent monolayer cultures were seeded with JN strain of MDV-infected duck embryo cells at a ratio of 1 infected cell per 10 uninfected. The infection of HEp-2 cells with the F prototype of subtype 1 (8) of herpes simplex virus (HSV) was performed as described previously (15).

Labeling of cells with radioactive isotopes: DNA. After 2 days of incubation, infected duck embryo fibroblast cultures were replenished with fresh media consisting of mixture 199 lacking thymine and supplemented with 1% dialyzed calf serum and sufficient thymidine-methyl-³H to yield 10 μCi per ml of media (Schwarz-Mann, Orangeburg, N.Y.). The cells were then incubated an additional 48 hr before harvesting. HEp-2 cells infected with F virus were labeled between 4 and 20 hr postinfection with 0.1 μCi of ¹⁴C-thymidine (Schwarz-Mann) per ml of medium consisting of mixture 199 lacking thymine and supplemented with 1% dialyzed calf serum.

Labeling of cells with radioactive isotopes: ribonucleic acid (RNA). Infected and uninfected duck embryo fibroblast cultures were washed with MEM

supplemented with 1% dialyzed calf serum and then overlaid for 1 hr with MEM supplemented with 1% dialyzed calf serum and 10 μ Ci of 3 H-uridine (Schwarz-Mann) per ml of medium.

Purification of MDV and HSV nucleocapsids for extraction of DNA. The purification of MDV and HSV nucleocapsids was done as follows. Cells were harvested and washed with phosphate-buffered saline. MDV-infected duck embryo fibroblasts were disrupted by Dounce homogenization after 10 min of swelling in RSB. HSV-infected HEp-2 cells were disrupted by exposure to 0.5% Nonidet P-40 (Shell Oil Co.) in RSB. Nuclei were removed by centrifugation at $2,000 \times g$ for 5 min. Cytoplasm from MDV infected cells was made 0.5% with respect to Nonidet P-40. The HSV- and MDV-infected cytoplasm were then cleared of debris by centrifugation at $12,000 \times g$ for 5 min, layered onto 37-ml linear sucrose density gradients (12 to 52%, w/w) prepared in virus buffer, and centrifuged for 1 hr at 26,000 rev/min in the Spinco SW27 rotor at 5 C. Under these conditions the nucleocapsids, stripped of their envelopes by the detergent treatment, form two discrete bands. The bands were aspirated with a syringe by puncturing the side of the tube. T4 phage labeled with 14 C-uridine was prepared for this study by Robert Haskelkorn; SP01 DNA was obtained from Kenneth Bott. *Micrococcus lysodeikticus* DNA was obtained from Sigma Chemical Co., St. Louis, Mo.

Molecular weight determination of MDV DNA. The molecular weight of MDV DNA was determined by the procedure of Burgi and Hershey (4). For sedimentation of DNA in neutral sucrose gradients we followed one of two procedures. In procedure 1, samples of MDV and HSV or T4 were suspended in neutral DNA buffer containing 2% Sarkosyl and 0.5% sodium dodecyl sulfate (SDS) and gently rolled at 60 C with phenol and chloroform. This procedure was necessary to release T4 DNA from virions. In the second procedure applied to MDV alone or in mixture with HSV, the DNA was released from the nucleocapsids by exposure to 0.5% SDS and 2% Sarkosyl at 55 C after suspension of the nucleocapsids in neutral DNA buffer. The DNA mixtures were poured onto 12-ml linear sucrose gradients (10 to 30% w/w) prepared in neutral DNA buffer, containing 0.15% Sarkosyl, and centrifuged at 40,000 rev/min at 20 C in the Spinco SW41 rotor. Fractions containing 0.3 ml were collected from the top of the tube. The DNA in the fractions was then precipitated with trichloroacetic acid using herring sperm DNA as a carrier. For sedimentation of DNA in alkaline sucrose gradients, a sample of MDV DNA was first centrifuged on a neutral sucrose gradient as described above, and the fractions containing the high molecular weight DNA band (bottom component) were pooled, diluted, and mixed with T4 phage DNA. The DNA was then denatured by the addition of 0.1 N NaOH and gently poured onto a 10 to 30% (w/w) sucrose gradient made up in alkaline DNA buffer containing 0.15% Sarkosyl. The length of centrifugation is given in the legends to the appropriate figures.

Determination of buoyant density of MDV DNA. Samples of MDV DNA were centrifuged on 10 to

30% (w/w) neutral sucrose gradients as described in the previous section, and the fractions containing the bottom component DNA and the material banding near the top of the gradient (top component) were each pooled separately and dialyzed first against TES buffer overnight. The fractions were then dialyzed against a CsCl solution made up in TES buffer until the refractive index of the equilibrated solution corresponded to a density of 1.726 g/cm³. Samples of bottom component and top component DNA were centrifuged to equilibrium (44,000 rev/min for 20 hr at 25 C) in a four-place Spinco analytical rotor. Various combinations of SP01 DNA, HSV DNA, and *Micrococcus lysodeikticus* DNA were used as density markers with the unknown samples. Ultraviolet absorption photographs were scanned with a Joyce Loebel microdensitometer.

DNA-RNA hybridization: preparation of nucleic acids. MDV DNA for hybridization was obtained by centrifugation on linear 10 to 30% neutral sucrose gradients of DNA extracted from naked nucleocapsids as described in the preceding section. The fractions containing the high molecular weight (100×10^6 to 120×10^6) native MDV DNA were dialyzed against $1.5 \times$ SSC, and digested with 50 μ g of heat-inactivated pancreatic ribonuclease (Schwarz-Mann) per ml. SDS was added to 0.5% and the protein extracted by the hot phenol SDS method (16). The DNA was precipitated with 2 volumes of absolute ethanol, washed with ethanol, and dissolved in $0.1 \times$ SSC.

Labeled RNA was obtained from infected and uninfected duck embryo cells as follows. Cytoplasm cleared of nuclei and debris after treatment with Nonidet P-40 was extracted by the hot phenol-SDS method (16). The RNA was precipitated with ethanol, dissolved in RSB, and digested with electrophoretically purified deoxyribonuclease (Schwarz-Mann). It was then reextracted with phenol and chloroform and precipitated with ethanol. This final RNA precipitate was dissolved in $5 \times$ SSC.

DNA-RNA hybridization: preparation of DNA discs. DNA in $0.1 \times$ SSC was alkali denatured by addition of NaOH to 0.10 N. After 10 min at 0 C the solution was diluted with $0.1 \times$ SSC and brought back to neutrality with HCl. Nitrocellulose filters (type HAWP, 25 mm, 0.45 μ m pore size; Millipore Corp., Bedford, Mass.) presoaked in $0.1 \times$ SSC and containing 100 μ g of DNA were prepared by filtration, dried at 60 C for 1 hr, and baked at 80 C for 5 hr. Microhybridization discs containing approximately 10 μ g of DNA were prepared from the larger discs by cutting out 5-mm discs with a leather punch.

DNA-RNA hybridization: conditions. Microhybridization discs loaded with DNA and blank control discs were incubated with RNA for 36 hr at 53 C in a solution of $5 \times$ SSC and 30% formamide (Matheson, Coleman and Bell, Norwood, Ohio) in a total volume of 0.15 ml.

After incubation, the filters were washed three times with $2 \times$ SSC, soaked in $2 \times$ SSC for 30 min, digested with heat-inactivated pancreatic ribonuclease (50 μ g/ml) in $2 \times$ SSC for 1 hr, again washed and soaked with $2 \times$ SSC, washed with 70% ethanol, and finally dried for counting.

RESULTS

Partial purification of MDV nucleocapsids. Lysates of MDV-infected cells treated with Nonidet P-40 yielded two bands on centrifugation through a linear sucrose density gradients (12 to 52%, w/w). Electron microscopic observation of negatively stained preparations of top and bottom band material suggested that the top band contained mostly empty nucleocapsids, whereas the bottom band contained mostly full nucleocapsids (Fig. 1) interspersed with membrane fragments and apparent breakdown products of viral nucleocapsids.

Centrifugation of viral DNA in neutral sucrose density gradients. The DNA extracted from MDV nucleocapsids and T4 virions or from mixtures of MDV and HSV nucleocapsids was centrifuged on neutral sucrose density gradients as described in Materials and Methods. Figure 2 shows the distribution of the various DNA in the gradients. The data indicate the following.

(i) T4 and HSV DNA both yielded a single band near the mid-point of the gradient (Fig. 2A, B). MDV DNA obtained by coextraction of MDV and T4 viruses with hot phenol yielded two bands, one broad in width near the top of the

gradient (top component) and one narrow band (bottom component) only slightly above the T4 DNA band (Fig. 2A). The formation of two bands is not an artifact due to phenol extraction since MDV DNA obtained by dissolution of the virion with SDS yielded on cosedimentation with T4 DNA the same two bands. The bimodal distribution of MDV DNA is not an artifact of the extraction with SDS and Sarkosyl, since HSV nucleocapsids extracted at the same time yielded DNA which formed only one band on sedimentation through sucrose density gradients.

(ii) T4 DNA used as a sedimentation marker in this study was intact double-stranded DNA by the following criteria. T4 DNA yielded a single discrete band in neutral sucrose with sedimentation velocity identical to that described by Berns and Silverman (2). Alkali denaturation of the T4 DNA yielded a single discrete band on alkali sucrose or neutral sucrose gradients with a sedimentation velocity identical to that of T4 single strands prepared by lysing T4 phage in alkali directly on the top of the gradients.

(iii) The ratio of distances sedimented of the discrete homogeneous bottom band of MDV DNA to T4 DNA is 0.975 (range, 0.97 to 0.98)

FIG. 1. Electron micrographs of negatively stained MDV from a sucrose density gradient. This and similar preparations were used to extract DNA for buoyant density determinations. The bar indicates 1 μ m in the low magnification micrographs. The two insets are higher magnification pictures of nucleocapsids (100 nm in diameter) such as those seen in the low magnification micrograph.

FIG. 2. Sedimentation of native ^3H -MDV DNA with native ^{14}C -T4 DNA (Fig. 2A) or with ^{14}C -HSV DNA (Fig. 2B) through a linear 10 to 30% neutral sucrose gradient. Centrifugation was for 3.5 hr at 40,000 rev/min at 20 C in a Spinco SW41 rotor. ^3H -labeled DNA, solid line; ^{14}C -labeled DNA, dashed line. Direction of sedimentation is to the right.

This corresponds to an S_{suc} for MDV DNA of 56S (10). Using the equation of Burgi and Hershey (4, 10), the ratio of molecular weight of MDV DNA to T4 DNA is 0.93 ± 0.01 . Based on the determination of the molecular weight of T4 DNA as 1.3×10^8 daltons by molecular autoradiography (10), the molecular weight of MDV DNA is estimated to be 1.2×10^8 daltons. Centrifugation of HSV DNA with T4 DNA yielded a ratio of distances sedimented of HSV DNA to T4 DNA of 0.95 ± 0.01 , corresponding to a molecular weight of 1.1×10^8 daltons. This value for the molecular weight of HSV DNA is in good agreement with the value of 10^8 published by Becker et al. (1) using the Kleinschmidt technique. Cosedimentation of HSV DNA with MDV DNA on neutral sucrose demonstrated that MDV DNA sedimented slightly faster than HSV DNA (Fig. 2B). We conclude that the largest species of DNA contained in the MDV nucleocapsid preparation is on the same order of magnitude but slightly larger than HSV DNA.

Sedimentation of MDV DNA in alkaline sucrose density gradients. The bottom band of MDV DNA obtained by centrifugation of MDV DNA in a neutral sucrose gradient was cosedimented with T4 DNA in alkaline sucrose gradients as described above. The results shown in Fig. 3 indicate the following. (i) T4 DNA yielded a single band with an S value of 73 corresponding to a molecular weight of 6.5×10^7 daltons (18). (ii) The MDV DNA cosedimented with T4 DNA was of heterogeneous size. The largest and predominant fragment had an S value of 70, corresponding to a molecular weight half that of the bottom band obtained in neutral sucrose solutions (6.0×10^7 daltons). (iii) It seems likely that the heterogeneous slower sedimenting DNA species result from

FIG. 3. Sedimentation of alkali-denatured ^3H -MDV DNA bottom band (solid line) with alkali-denatured ^{14}C -T4 DNA (dashed line). A mixture of ^3H -MDV bottom band DNA and native T4 DNA was denatured and poured onto 10 to 30% alkaline sucrose gradient as described in Materials and Methods. Centrifugation was for 3.5 hr at 40,000 rev/min at 20 C in a Spinco SW41 rotor. Direction of sedimentation is to the right.

random single-stranded breaks. We cannot exclude the possibility of some nonrandom single-strand breaks.

Buoyant density of MDV DNA. This experiment was done with MDV DNA from either the top or bottom band obtained after neutral sucrose density centrifugation. The MDV DNA was centrifuged with SP01 DNA and either *M. lysodeikticus* DNA (Fig. 4) or HSV DNA (Fig. 5) in the model E Spinco centrifuge. The results summarized in Fig. 3 and 4 for bottom component yield a buoyant density of 1.705 g/cm^3 , after correction for the nonlinearity of the CsCl gradient (19). This corresponds to a guanine plus cytosine content of 46 moles per cent. Since both top and bottom component DNA have the same buoyant density, we conclude that they are both MDV DNA.

Hybridization tests. RNA extracted from MDV-infected and uninfected duck embryo cells labeled with ^3H -uridine were hybridized with MDV DNA by procedures described above. The results sum-

FIG. 4. Buoyant density determination of MDV DNA bottom component centrifuged with F strain HSV DNA and SP01 DNA in a model E analytical ultracentrifuge as in Materials and Methods. The ultraviolet absorption photograph was scanned with a Joyce Loebel microdensitometer.

marized in Table 1 show that MDV DNA hybridized with RNA from infected cells but not from uninfected cells. On the basis of the hybridization of the DNA with transcription products from infected cells and of the absence of hybridization with transcription products in uninfected cells, we conclude that the DNA extracted from partially purified nucleocapsids is viral DNA and not host DNA.

DISCUSSION

The properties of the DNA of MDV may be summarized as follows.

(i) The molecular weight estimate of the DNA molecules in the bottom component of MDV DNA is based on cosedimentation with T4 and HSV DNA in linear neutral sucrose density gradients. On the basis of the sedimentation properties of the T4 DNA we have estimated that MDV DNA has a molecular weight of 1.20×10^8 daltons. In the same series of experiments HSV DNA exhibits a molecular weight of 1.1×10^8 daltons; this value agrees very well with the value

FIG. 5. Buoyant density determination of MDV bottom component DNA cocentrifuged with *Micrococcus lysodeikticus* DNA and SP01 DNA in a model E analytical ultracentrifuge as in Materials and Methods. The ultraviolet absorption photograph was scanned with a Joyce Loebel microdensitometer.

TABLE 1. Hybridization of RNA from infected and uninfected duck embryo fibroblast cells with DNA from Marek's disease virus (MDV)^a

Labeled RNA	Input (counts/min)	Net bound (counts/min)	Per cent of input bound
Infected cells.....	416,000	734	0.176
Uninfected cells....	380,000	26	0.007

^a Hybridization was carried out as described in Materials and Methods. Five-millimeter discs, each containing 10 μ g of MDV DNA, were hybridized with ³H-uridine-labeled RNA from cells infected with MDV and from uninfected cells. Background counts per minute bound to filters equaled 0.0072% of input counts per minute.

of 10^8 daltons published previously (1). These experiments indicate that MDV DNA is of the same order of magnitude and approximately 10% larger than that of HSV DNA. With respect to DNA fragments in the top component it is noteworthy that in the analytical centrifuge this DNA sediments with the same buoyant density as the bottom band, indicating that it is MDV DNA. One hypothesis that could explain the appearance of low molecular weight MDV DNA is that a fraction of the nucleocapsids were partially decomposed exposing viral DNA to enzymatic or physical degradation.

(ii) The results of the sedimentation of MDV DNA in alkaline gradients indicate that the DNA is double-stranded and most likely not cross-linked.

(iii) Isopycnic sedimentation of MDV DNA with SP01, with HSV, and *M. lysodeikticus* DNA as markers in the analytical centrifuge has yielded a buoyant density value for the MDV DNA of 1.705 g/cm³. This value is lower than that reported previously (9) from our laboratories, and we cannot account for this difference. It is clear, however, that the DNA obtained by the procedure used in this study was extracted from much more highly purified viral particles and the DNA itself was more uniform in its physical properties than that obtained in the previous studies. To ascertain that we were in fact dealing with viral DNA rather than some hypothetical species of cellular DNA, hybridization tests were done on the DNA extracted from virions with RNA from infected and uninfected cells. The hybridization tests show that transcription products of the DNA extracted from virus particles are present in infected cells but not in uninfected cells. On the basis of the hybridization test, the size distribution, buoyant density, and the source of DNA, we are concluding that the DNA in this study is MDV DNA and is not cellular.

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ADDENDUM IN PROOF

Since the submission of this paper for publication, several independent absolute determinations of the molecular weight of T4 DNA have been performed. The results reviewed by Freifelder (J. Mol. Biol. **54**: 567-577, 1970) indicate that the molecular weight of T4 DNA may be lower than previously determined, i.e., 1.1×10^8 daltons. Using this value for the molecular weight of T4 DNA and the revised exponent (Freifelder, J. Mol. Biol. **54**:569-577, 1970) for the Burgi and Hershey relationship, the molecular weight of MDV DNA is 1.0×10^8 daltons.

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