Composition and Size of Shope Fibroma Virus Deoxyribonucleic Acid

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Deoxyribonucleic acid (DNA) extracted from purified virions of Shope fibroma virus (SFV) (by using DNA from *Micrococcus lysodeikticus* as marker) had a buoyant density of 1.6996 ± 0.0003 g/ml, hence a guanine plus cytosine (G + C) content of $40.4 \pm 0.3\%$, which is close to the G + C content of the DNA of susceptible rabbit cells ($40.9 \pm 0.4\%$) and different from that of vaccinia virus DNA ($35.5 \pm 0.4\%$). For the determination of the molecular weight of DNA, SFV and vaccinia purified virions, treated with Pronase and detergent, were cosedimented in sucrose density gradients. Results showed that SFV-DNA has a molecular weight of about 153×10^6 daltons. By electron microscopy, only one molecule corresponding to this value was observed (its length was $80.3 \mu\text{m}$). The others had a median size of $49.8 \mu\text{m} \pm 0.9$.

The poxvirus group contains the largest viruses present in vertebrates and invertebrates. They have deoxyribonucleic acid (DNA) of very high molecular weight (160×10^6 to 200×10^6 daltons). These viruses develop in the cytoplasm. Some poxviruses are able to induce tumors in their natural host (4). Among them, Shope fibroma virus (SFV) induces benign tumors in adult rabbits and malignant tumors in newborn rabbits (7). We have developed methods of purifying SFV and extracting the SFV DNA (9). More recently, we have shown by hybridization that there was no homology between SFV DNA and the DNA of sensitive rabbit cells (10).

The purpose of this paper is to report the composition and size of the SFV DNA, calculated by sensitive methods.

MATERIALS AND METHODS

Cells. RK13 continuous cell lines derived from young rabbit kidney (1) were grown in minimum essential medium (MEM) supplemented with 10% calf serum. They were tested for the presence of mycoplasma at regular intervals.

Virus and infection of cells. Nearly confluent monolayer cultures were seeded with SFV, OA strain, at a ratio of 10 focus-forming units per cell and maintained in MEM supplemented with 2% calf serum (Merieux).

Labeling of cells with radioactive isotopes. After 1 day of incubation, uninfected cells were labeled for 72 hr, either with $0.1 \mu\text{Ci}$ of ^3H -thymidine (specific activity 10 Ci/mmole) per ml of medium or $0.02 \mu\text{Ci}$ of ^{14}C -thymidine (specific activity $45 \mu\text{Ci/mmole}$) per ml of medium (CEA, Saclay, France).

Labeling and purification of viruses. Cells infected with SFV were labeled between the 5th and the 24th hour postinfection with $0.5 \mu\text{Ci}$ of ^3H -thymidine (26 Ci/mmole) or $0.1 \mu\text{Ci}$ of ^{14}C -thymidine ($45 \mu\text{Ci/mmole}$) per ml of medium, and cells infected with vaccinia virus were labeled between the 1st and 72nd hour postinfection with $0.5 \mu\text{Ci}$ of ^3H -thymidine (15 Ci/mmole) per ml of medium. The purification of SFV was done as previously described (9). Briefly, infected cells were disrupted by ultrasonic treatment (3 min) with a Branson apparatus (20 kHz at full power). After removal of cell debris, SFV was concentrated, and then sedimented three times through a 35% (w/v) sucrose cushion in an SW25-1 rotor at 14,000 rev/min for 1 hr. The pellet was incubated with ribonuclease and deoxyribonuclease ($50 \mu\text{g}$ of each enzyme per ml for 15 min at 37°C) and centrifuged on a linear sucrose gradient (25 to 40% , w/v) for 35 min at 14,000 rev/min in an SW50-1 rotor. Vaccinia virus, obtained from KB-infected cells from the laboratory of A. Kirn (Strasbourg, France), was purified by a similar method except that the nuclease treatment was omitted.

Extraction and purification of SFV DNA. The DNA was extracted from purified SFV with four successive treatments: (i) 2-mercaptoethanol at a concentration of $25 \mu\text{l}$ per ml in standard saline citrate (0.15 M sodium chloride, 0.015 M sodium citrate, $\text{pH } 7.4$; $1 \times \text{SSC}$); (ii) sodium dodecyl sulfate (SDS) (0.3%) for 1 hr; (iii) Pronase ($500 \mu\text{g/ml}$) overnight at 37°C , and (iv) phenol. Then the DNA was dialyzed against $0.1 \times \text{SSC}$ (one-tenth dilution SSC). Control of the purity of SFV DNA so extracted was made by the methods of Jungwirth (12). Briefly, the DNA was denatured at 100°C for 10 min. Then cesium chloride (CsCl) was added to obtain a final concentration of 0.5 M , and the DNA was renatured

by incubation at 60 C for 12 hr. The density was then increased to 1.700 g/ml with CsCl, and the DNA was centrifuged for 60 hr at 30,000 rev/min at 25 C either in a Spinco SW65 rotor or in a Spinco 65 angular rotor, as described by Flamm (5).

Determination of buoyant density of SFV DNA. Samples of DNA were centrifuged to equilibrium (40,000 rev/min for 24 hr at 25 C) in a Spinco analytical AnD rotor. *Micrococcus lysodeikticus* DNA was used as a buoyant density marker of 1.731 g/ml. Ultraviolet absorption photographs were scanned with a Joyce-Loebel microdensitometer.

Molecular weight determination of SFV DNA: (i) zonal centrifugation in sucrose gradient. Purified ^3H -thymidine-labeled vaccinia virus was mixed with purified ^{14}C -thymidine-labeled SFV virions. These viral particles were incubated on top of a sucrose gradient (10 to 30%, w/w) overnight at 37 C, either with 2-mercaptoethanol (25 μl /ml), SDS (0.2%), and Pronase (500 μg /ml) according to the method previously described (9), or with sodium deoxycholate (DOC; 1%) and Pronase (500 μg /ml) according to the method used for isolating vaccinia DNA (15). Then the mixture was centrifuged for 4 hr at 25,000 rev/min in a Spinco SW65 rotor at 25 C. Fractions were collected from the bottom of the gradient and the DNA was precipitated onto filters with 5% trichloroacetic acid. These were dried and assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. The molecular weight value of SFV DNA was calculated from McCrea's value (14) for vaccinia DNA following the formula of Burgi and Hershey (2): $D_1/D_2 = (M_1/M_2)^{0.35}$.

(ii) Length measurements in electron microscopy of DNA molecules. DNA molecules which were not treated with phenol were diluted twice in a 0.06% solution of cytochrome *c* prepared in 2 M ammonium acetate. They were spread on a solution of 0.25 M ammonium acetate, picked up on collodion carbon-coated grids, and dried in absolute ethanol. Grids

were shadowed with uranium oxide (shadowing angle of 10°) while rotating at approximately 60 rev/min (13). They were examined in an AEI EM6B electron microscope and photographed at an instrumental magnification of about 5,000. This magnification was verified with a carbon grating replica. Adjacent sections of each molecule were photographed, printed at a magnification of 3, and then joined together. The outline of each individual DNA molecule was traced to measure its length by using a calibrated map measurer. Conversion of DNA length to molecular weight units was made by multiplying the strand length in micrometers by the factor M/L calculated according to Freifelder's formula (6): $(M/L \text{ in daltons})/\mu\text{m} = 1.54 \times 10^6 (A + T)\% + 2.42 \times 10^6 (G + C)\%$.

RESULTS

Homogeneity of viral DNA preparation. The homogeneity of SFV DNA was studied on linear sucrose gradients. After centrifugation, the majority of the labeled molecules sedimented in a nearly homogeneous peak, and some others were located at the upper part of the gradient (Fig. 1).

Isopycnic centrifugation of cellular DNA and SFV DNA. The physicochemical characteristics of DNA from RK13 cells and SFV DNA were studied in CsCl gradients in a preparative centrifuge. ^{14}C -thymidine-labeled cellular DNA and ^3H -thymidine-labeled SFV DNA centrifuged in swinging bucket rotor had the same buoyant density. Denaturation and renaturation of the two DNA species (12) increased the buoyant density of cellular DNA and did not modify that of viral DNA. These two peaks were separated better when the DNA was centrifuged in an angular rotor (Fig. 2) than in a swinging bucket rotor. As a result of this process, no

FIG. 1. Sucrose gradient (10 to 30%, w/w) centrifugation of ^3H -thymidine SFV DNA in an SW65 rotor at 25,000 rev/min for 4 hr. Fractions collected were treated ($\blacktriangle$) or not treated ($\bullet$) with deoxyribonuclease (DNase).

cellular DNA contaminated the viral DNA used in the present experiments.

Buoyant density of SFV DNA. The buoyant densities of the cellular DNA, SFV DNA, and vaccinia virus DNA were determined more accurately with the analytical centrifuge in CsCl gradients, *M. lysodeikticus* DNA being used as control. Results are summarized in Table 1. The buoyant density values for cellular DNA and SFV DNA were very close. After renaturation at 60 C for 12 hr, the density of RK13 DNA increased by 0.012 g/ml, whereas SFV DNA recovered its original density, increasing only 0.002 g/ml. The G + C content values calculated from the buoyant densities (16) were 40.9 ± 0.4 for

cellular DNA and 40.4 ± 0.3 for SFV DNA, while that of vaccinia virus was found to be 35.5 ± 0.4 .

The densitometric tracings of the buoyant density determination of SFV DNA permitted calculation of molecular weight according to Hearst and Vinograd (8) with a range of 4×10^6 to 8×10^6 daltons for different purifications of DNA. These values correspond to small fragments. The per cent G + C allowed us to calculate the M/L factor for electron microscopy, a value of 1.89×10^6 daltons/ μ m.

Molecular weight of SFV DNA: (i) **Zonal centrifugation in sucrose gradient.** The molecular weight of SFV DNA was determined by using vaccinia DNA as control. As described in

FIG. 2. Isopycnic centrifugation of ³H-thymidine SFV DNA (▲---▲) with ¹⁴C-thymidine RK13 DNA (△—△) in CsCl for 60 hr at 30,000 rev/min in an SW65 rotor in a preparative centrifuge.

TABLE 1. Buoyant densities of DNA species in equilibrium density gradient centrifugation in cesium chloride in an analytical ultracentrifuge

DNA from	State of DNA	Buoyant density ^a (g/ml)	Per cent G + C ^a
<i>Micrococcus lysodeikticus</i> RK13 cells	Native	1.731	57
	Native	1.7000 ± 0.0003^e (6) ^d	40.9 ± 0.4 (6)
	Denatured ^b and renatured	1.7126 (2)	
SFV	Native	1.6996 ± 0.0003 (40) ^e	40.4 ± 0.3 (40)
	Denatured ^b and renatured	1.7017 (2)	
	Native	1.6948 ± 0.0004 (2)	35.5 ± 0.4

^a Numbers in parentheses indicate the number of experiments.

^b Heat-denatured.

^c $\pm$, Type error on the average ($\sigma/\sqrt{n}$; σ = type error of the distribution of the results, n = number of experiments, and $\sqrt{n}$ = the square root of n).

^d Six values correspond to three experiments.

^e Forty values correspond to nine experiments.

Materials and Methods, when purified vaccinia and SFV were incubated with DOC and Pronase on top of sucrose gradients, the DNA molecules of each virus were localized in one peak, in which 12% of the ^{14}C radioactive input of SFV and 64% of the ^3H radioactive input of vaccinia virions were recovered (Fig. 3a).

When these viruses were incubated with 2-mercaptoethanol, SDS, and Pronase, DNA molecules were localized in two peaks, one bigger than the other, corresponding to 20% for SFV and 63% for vaccinia virus of the total radioactivity input (Fig. 3b). For SFV DNA, in these two types of experiments, the rest of the radioactivity was mostly found at the upper part of the gradient.

From the value of 160×10^6 daltons for the molecular weight of vaccinia virus DNA (15), the molecular weight of SFV DNA was calculated from four experiments to be 153×10^6 daltons for the bigger peak, while the small peak represented molecules of 50×10^6 daltons.

(ii) **Electron microscopy of SFV DNA.** DNA

molecules examined by electron microscopy were obtained after treatment of purified virions with 2-mercaptoethanol, SDS, and Pronase.

Sixty-five molecules were examined. All of them were linear with two distinct ends (no circular molecule was observed). Figure 4 shows the contour of one of these molecules. A histogram of strand length measurements of these 65 molecules is given in Fig. 5. The length average is $49.8 \pm 0.9 \mu\text{m}$ corresponding to a molecular weight of 94.1×10^6 daltons by using the factor 1.89. But if one considers the longest molecule, which is $80.3 \mu\text{m}$, the corresponding molecular weight is 151.6×10^6 daltons.

DISCUSSION

The present study describes determination of the molecular weight (by using zonal centrifugation in sucrose gradients and electron microscopy) and the buoyant density of SFV DNA.

Molecular weight of SFV DNA. The results show that the molecular weight of SFV DNA, determined by sucrose gradients, has an average

FIG. 3. Sucrose gradient (10 to 30%, w/w) centrifugation for 4 hr at 25,000 rev/min in an SW65 rotor at 20 C of viral DNA species liberated from the mixture of purified ^3H -thymidine vaccinia virus and ^{14}C -thymidine SFV incubated overnight at 37 C either with DOC and Pronase (a) or with 2-mercaptoethanol, SDS, and Pronase (b).

FIG. 4. Electron micrograph of a SFV DNA molecule. Thick arrows indicate the two ends of the molecule. Thin arrows (A, B, C, D) designate the continuation of the molecule. Bar = 1 μ m.

value of 153×10^6 daltons, which is very close to 160×10^6 daltons for vaccinia virus DNA. Profiles of sedimentation of SFV DNA and vaccinia DNA in sucrose gradients are closely similar, whatever the treatment of virions before centrifugation has been. But in the case of SFV DNA, for both techniques, one population of very small molecules (about 70%) is localized at the upper

part of the gradient. The significance of these small molecules, the molecular weight of which is about 3×10^6 daltons, is not yet clearly established.

By electron microscopy, 64 of 65 molecules measured had a length average of 49.8 μ m, and only one molecule has a length of 80.3 μ m corresponding to the molecular weight of 153×10^6

FIG. 5. Distribution of lengths of molecules observed in electron micrographs similar to those shown in Fig. 4.

daltons determined by the sucrose gradient. That this molecule is an end-to-end complex of two shorter molecules cannot be excluded, but the other possibility to explain the presence of the shorter molecules could be the breakage of the longer molecules during isolation and preparation of DNA for electron microscopy. It is perhaps for the same reason that DNA molecules with a molecular weight of 50×10^6 daltons were located in one small peak near the principal peak in sucrose gradients (Fig. 3b).

Buoyant density of SFV DNA. SFV DNA has a buoyant density of 1.6996 g/ml, hence a G + C content of 40.4%. These two values are very close to those of rabbit cell DNA (1.700 g/ml and 40.9%) and DNA of other mammalian cells (whose G + C values extend from 40 to 42%), whereas the same value for DNA of the other poxviruses is significantly lower than the value of cellular DNA. For instance, the G + C content of vaccinia virus DNA is $35.5 \pm 0.4\%$ and that of the rabbit pox DNA is 36% (11). In comparison with G + C contents of different families of some viruses (3) (papova, adenovirus, herpes

virus, poxvirus) only SV40 DNA has the same property as the SFV DNA.

This similarity between SFV DNA and host cell DNA could indicate a relationship, though our hybridization experiments were negative (10). But these results merit further study.

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