In Vivo Conversion of the Single-Stranded DNA of the Kilham Rat Virus to a Double-Stranded Form

LOIS ANN SALZMAN AND WESLEY WHITE

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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Kilham rat virus (KRV) contains linear, single-stranded DNA in the virion. The fate of radioactive viral DNA was followed after infection of monolayer cells. Within 60 min after infection of cells, 28 to 42% of the parental viral DNA is converted to a new form. This new DNA form is believed to be double stranded and linear on the basis of its sedimentation in neutral and alkaline sucrose gradients, elution from hydroxyapatite columns, its buoyant density in equilibrium CsCl density gradients, and appearance in the electron microscope. The double-stranded linear KRV DNA may be analogous to the replicative form of certain bacteriophages, including ϕ X174, which contain single-stranded circular genomes.

The parvoviruses are a group of small (15-30 nm) icosahedral viruses containing single-stranded DNA. Some of the viruses included in the group are: Kilham rat virus (KRV) isolated from a rat sarcoma (10), H-1 virus isolated from a human transplantable tumor (26), the widespread adeno-associated virus (2, 12; M.D. Hoggan, *Fed. Proc.*, p. 248, 1965), and the minute virus of mice (5). KRV has been found to cause a fatal neonatal disease, physical deformities and mental retardation in newborn rats (9), mice (11) and hamsters (8). The virion contains a molecule of linear, single-stranded DNA with a molecular weight of about 1.6×10^6 (18) and three major proteins (17).

Similar to the animal viruses in size and DNA content is a group of bacteriophages including ϕ X174. ϕ X174 contains single-stranded DNA of molecular weight approximately 1.7×10^6 . This DNA has a circular configuration (22). The replication of ϕ X174 has been shown to involve a double-stranded, circular, replicative intermediate. Very little has been published concerning the replicative forms of the single-stranded, linear DNA of animal virus members of the parvovirus group. The following studies were undertaken to determine if KRV, like ϕ X174, assumed a double-stranded form after infection and if this double-stranded molecule has a linear or circular configuration. Our results, based on the use of an infective

virion containing thymidine-*methyl*- 3H in the parental DNA, indicate that a portion of the virion DNA rapidly becomes double-stranded after infection. However, this double-stranded DNA, on the basis of sedimentation in alkaline sucrose and by observation under the electron microscope, appears to have a linear configuration.

MATERIALS AND METHODS

Virus growth and purification. KRV strain 308 was grown in a rat nephroma cell line and purified as previously described (16). KRV containing 3H -thymidine in the virion DNA was prepared as reported previously (16). The final steps in all purifications involve two successive isopycnic centrifugations in cesium chloride and dialysis against 0.02 M tris (hydroxymethyl) aminomethane (Tris), pH 7.4. The virus was titered for plaque-forming units (PFU) on the rat nephroma cell line. The simian virus 40 (SV40) DNA components I and II were obtained from G. Fareed. They had been purified by sedimentation in ethidium bromide and sucrose gradients. Adenovirus 2 DNA containing ^{32}P was the gift of J. Rose.

Virus infection and extraction of cellular DNA. Monolayer cultures of rat nephroma cells containing approximately 2×10^6 cells in a 15 by 60 mm petri dish (Falcon Plastics, Los Angeles, Calif.) were washed twice with Eagle medium and infected with 0.5 ml of KRV containing 3H -thymidine (6.4×10^4 counts per min per 0.5 ml). The viral multiplicity was 2 PFU per cell. At 30 min after infection, the unadsorbed virus and medium were aspirated from

the petri dishes, and the monolayers were washed with Eagle medium. Eagle medium (5.0 ml), containing 5% fetal calf serum and 0.06% glutamine, was then added to each petri dish. The infected cells were incubated at 37 C in a humidified 5% CO₂-air mixture. At 0.5, 1, 3, 7.5, 11, 17, and 20 hr after infection, the medium was removed from four petri dishes, and the monolayers were washed twice with phosphate-buffered saline (0.05 M PO₄, pH 7.4, containing 0.9% NaCl). Extraction of cellular and viral DNA and salt precipitation of high-molecular-weight DNA was accomplished by a modification of the procedure described by Hirt (7). Lysing solution (0.5 ml) containing 0.6% sodium lauryl sulfate, 0.01 M ethylenediaminetetraacetic acid (EDTA), and 2 mg of pronase per ml was added to each petri dish. The petri dishes were gently rotated for 30 min, and the contents were added to a polyallomer tube containing NaCl (final concentration 1 M). The samples were then allowed to remain at 4 C overnight. The high-molecular-weight DNA was pelleted in a Sorvall centrifuge at a speed of 10,000 × *g*, and the resulting supernatant solution (Hirt supernatant fluid) was used for the following sedimentations.

Velocity sedimentation of DNA in neutral and alkaline sucrose. A 0.2-ml amount of the Hirt supernatant fluid, prepared as described above, was layered on top of an 11.5-ml linear gradient of 10 to 30% sucrose. Neutral sucrose gradients containing 0.15% Sarkosyl, 0.01 M NaCl, and 0.05 M Tris (pH 7.5) were centrifuged for 16 hr at 29,000 rev/min and 10 C in a type 41T rotor in a Spinco L2 ultracentrifuge. Alkaline sucrose gradients containing 0.15% Sarkosyl, 0.3 N NaOH, 0.7 N NaCl, 0.0025 M EDTA (21), and 0.01 M Tris were centrifuged at 35,000 rev/min and 10 C for 16 hr. Before addition to the alkaline sucrose gradients, samples were made 0.2 N NaOH and allowed to stand at room temperature for 10 min. After centrifugation, fractions were collected from the gradient through a needle hole punctured in the bottom of the tube. Drops were collected directly into scintillation vials. Water (1 ml) plus a Triton X-100 scintillation fluid (10 ml) (16) was then added to each vial. Alkaline gradients were neutralized by the addition of 0.1 ml of glacial acetic acid to each vial. Radioisotope content was determined in a Beckman LS-250 liquid scintillation system.

Electron microscopy. DNA was mounted for microscopy by using the aqueous or formamide techniques described by Davis et al. (6). Electron micrographs were taken on Kodak electron image plates at magnifications of 6,000 to 12,000. Magnification was calibrated with a grating replica (E. F. Fullam, Cat. no. 1000), and contour lengths were measured by using a Dietzgen (no. 1718) map measurer.

Density of viral DNA from infected cells. Hirt supernatant fluids extracted at various times after infection of cells were centrifuged to equilibrium in a CsCl solution density 1.715 g/cm³ (0.001 M EDTA and 0.01 M Tris, pH 7.6). Adenovirus 2-³²P (density 1.716) (14) was added as a reference DNA standard. Centrifugations were performed at 25 C and 40,000 rev/min for 48 hr in a Spinco model 65 rotor. Fractions (0.2 ml) were collected from the gra-

dients. Portions of each fraction were used to determine its radioisotope content and density. Density determinations were made using a Bausch & Lomb Abbe-3L refractometer, Palo Alto, Calif. Radioisotope determination was made in a Triton-toluene scintillation fluid as described above.

Hydroxyapatite chromatography. Hydroxyapatite (Bio Gel HTP, DNA grade, Bio-Rad Laboratories, Richmond, Calif.) was suspended in water (1:20) and added to a jacketed condenser maintained at 60 C. The hydroxyapatite column (2 by 1 cm) was washed with five to ten column volumes of water and eluted successively with 5 to 10 ml of 0.14 M and 0.4 M PO₄ (pH 6.8). The eluates were collected in 1-ml fractions, and a portion from each fraction was assayed for radioactivity by using the Triton-toluene scintillation fluid described above.

RESULTS

Sedimentation properties in neutral sucrose. Rat nephroma cells were infected with KRV containing thymidine-*methy*-³H in the virion DNA. By 30 min after infection, at least 70% of the added radioisotope and the virus PFU were removed from the medium. A portion of the cells were lysed at 30 min after infection. The high-molecular-weight cellular DNA was removed from the lysate by using the Hirt procedure, as described above. A 0.2-ml portion of the Hirt supernatant fluid was then added to a sucrose gradient, pH 7.4, as described in Materials and Methods. DNA extracted from KRV and containing ¹⁴C-thymidine was used as a marker in the same gradient tube. SV40 DNA component I (21s) and II (16s) (4) were centrifuged at the same time under the same conditions in a separate tube. The sedimentation profile of the Hirt supernatant fluid extracted at 30 min after infection is shown in Fig. 1. The radioactive parental DNA sediments in a profile similar to the reference single-stranded, virion DNA. No radioactivity was found in a faster sedimenting DNA form. The sedimentation coefficient of the single-stranded DNA was calculated from the sedimentation distances in the sucrose gradients as described by Bergi and Hershey (3). The sedimentation coefficient is approximately 6.7s. This is the sedimentation coefficient which would be expected under the low salt (0.01 M NaCl) conditions used here (20). If the same material is sedimented in a sucrose gradient (pH 7.4) as above containing 1.0 M NaCl, the sedimentation coefficient of the majority of the radioactive DNA is approximately 24s. This second value is in agreement with the sedimentation coefficient in 1.0 M NaCl of the DNA from the parvovirus, the adenovirus-associated virus (15).

By 60 min after infection, a change in the sedimentation profile can be seen (Fig. 2). A peak of radioactivity is observed as in the 30-min sample in the region of single-stranded, linear virion DNA (fraction 35). In addition, a second peak (fraction 25) is found of faster sedimenting radioactive DNA. This faster sedimenting DNA has a sedimentation coefficient calculated as above, of approximately 14.5s. It accounts for 28 to 42% of the radioactivity in the Hirt supernatant fluid. The percentage of parental DNA in the 14.5s peak varied in different experiments. A sedimentation profile similar to Fig. 2 is found for the other time periods observed, 3, 7.5, 11, 17,

and 20 hr. The 14.5s peak was present in all of these time periods. The fraction of radioisotope in the 14.5 to 6.7s remained about one-third. The pellet precipitated by the Hirt procedure was examined and contained from 15 to 30% of the total recovered radioisotope. The total recovered radioisotope which includes the radioisotope in the Hirt supernatant fluid and pellet is 63 to 83% of the radioactive input DNA.

Sedimentation in alkaline sucrose. Hirt supernatant solutions prepared as described above were also sedimented in alkaline sucrose gradients to determine the characteristics of the DNA under denaturing conditions.

FIG. 1. Velocity sedimentation in a 10 to 30% neutral sucrose gradient of Hirt supernatant fluid taken 30 min after infection from a rat nephroma cell monolayer infected with KRV containing ^3H -thymidine (●—●). A ^{14}C -thymidine KRV DNA reference marker (○—○) was sedimented with the sample. SV40 component I (21s) and component II (16s) were sedimented in a similar but separate gradient at the same time. Sedimentation was at 29,000 rev/min for 16 hr in an SW41T rotor as described in Materials and Methods. Sedimentation is from right to left.

FIG. 2. Velocity sedimentation in a 10 to 30% neutral sucrose gradient of Hirt supernatant fluid taken 60 min after infection from a rat nephroma cell monolayer infected with KRV containing ^3H -thymidine in the parental DNA. SV40 DNA components I and II were used as sedimentation markers. The samples were sedimented as described in Fig. 1.

Component II DNA of SV40 was used as a standardization marker. When SV40 DNA component II is sedimented in alkaline gradients it is resolved into two peaks, the 18s peak of single-stranded, circular DNA and the 16s peak of single-stranded, linear DNA (1). As seen in Fig. 3, sedimentation of the Hirt supernatant fluid from cells 60 min after infection with KRV revealed that the parental KRV DNA sedimented with the linear strand (16s) of SV40 DNA. A single peak of KRV DNA is seen in the alkaline sucrose gradient. The faster sedimenting peak observed in the neutral sucrose gradient is not present. After denaturation by the alkali, the 14.5s DNA sedimented with the KRV DNA. Extracted single-stranded KRV DNA has previously been reported to have a sedimentation coefficient of 16s in alkaline sucrose gradients (16).

Electron microscopy. Further characterization of the fast sedimenting 14.5s peak of radioactivity observed in the neutral sucrose gradient was accomplished with the aid of the electron microscope. The Hirt supernatant solution was added to a neutral sucrose gradient as described above. Fractions were collected in small tubes and portions were counted for radioisotope content. The peak fraction of the fast sedimenting DNA was dialysed against 0.02 M Tris, pH 7.0, to remove sucrose and Sarkosyl and was then condensed. It was then mounted for microscopy by using both the formamide and aqueous

techniques (6). Figure 4 is an aqueous preparation of the DNA in the peak fraction from the fast sedimenting 14.5s material. The radioactive parental DNA is present as a double-stranded curved filament. This is characteristic of double-stranded DNA. Single-stranded DNA would be condensed into bushes because of random base-base interactions (6). No bushes were observed in the preparations examined. The aqueous technique is useful for obtaining length measurements of double-stranded DNA. An insufficient number of DNA molecules (25) were present to make a statistically accurate measurement of the lengths. The average length of the limited number of the molecules (19) was approximately $1.40 \pm 0.15 \mu\text{m}$. There were four molecules of approximately 0.7 to 0.8 μm and one molecule of longer length, about 2.2 μm . Fewer molecules were seen in the formamide preparations, but they were all small, double-stranded DNA molecules of the same approximate length (1.3–1.5 μm) as above.

Density of viral DNA from infected cells. The buoyant densities of the radioactive DNA found in the Hirt supernatant fluid were determined in a CsCl equilibrium density gradient. In the DNA extracted from cells 60 min after infection, two peaks of radioactivity were found. One peak of radioactivity was found at a density of 1.715, the reported density for single-stranded KRV DNA (16). A second smaller peak was found at a density of 1.702. Adenovirus-2 DNA containing ^{32}P was centrifuged to equilibrium under similar conditions and was found to band at a density of 1.715. This is close to the reported CsCl density of adenovirus DNA, which is 1.716 (14).

Elution from hydroxyapatite columns. The Hirt supernatant fluid extract from cells 60 min after infection with KRV was diluted and added to a hydroxyapatite column (2 by 1 cm). Fractions were collected and assayed for radioisotope content (Fig. 5). No radioisotope was detected in the eluate after addition of the sample or water. Addition to the column of 0.14 M PO_4 , which causes the elution of single-stranded DNA (13), resulted in the elution of a peak of radioactive material. This peak consisted of about 66% of the radioactivity recovered. Addition of 0.4 M PO_4 buffer to the hydroxyapatite column, which causes the elution of double-stranded DNA (14), resulted in the elution of another peak of radioactive material. This second peak accounted for about 34% of the radioisotope recovered from the column.

FIG. 3. Velocity sedimentation in a 10 to 30% alkaline sucrose gradient of the fast sedimenting peak fraction, no. 25 shown in Fig. 2. SV40 component II containing ^{14}C -thymidine sedimented in a circular (18s) and linear (16s) form. Sedimentation was at 35,000 rev/min for 16 hr in an SW41T rotor as described in Materials and Methods. Sedimentation is from right to left. SV40 DNA (○—○), KRV DNA 14.5 S (●—●).

FIG. 4. Double-stranded KRV DNA. The DNA was taken from the peak fraction no. 25 shown in Fig. 2. It was mounted for electron microscopy as described by Davis, Simon, and Davidson (aqueous technique) and stained with uranylacetate. Magnification is 3.8×10^4 -fold. Preparation of the sample and photograph taken by C. Garon.

DISCUSSION

KRV grown in a rat nephroma cell line has a one-cycle growth curve of about 23 hr (19), and the above experiments were limited to this time period. Radioactive parental virion DNA, extracted from rat nephroma cells 30 min after infection, sediments in a single peak of radioactivity in both neutral and alkaline sucrose gradients. The sedimentation coefficients of this radioactive material correspond to the values found for sedimentation of reference virion DNA. In neutral sucrose gradients, KRV DNA has a sedimentation coefficient of 24 when the sucrose gradient contains 1.0 M NaCl, and 6.7 when the sucrose

gradient contains 0.01 M NaCl. These ratios of sedimentation coefficients under conditions of varying salt molarity, are in agreement with those reported by Studier (23) for sedimentation of denatured and native DNA of the bacteriophage 21 hy. Single-stranded DNA, being a polyelectrolyte, contracts as the salt concentration increases, and the sedimentation coefficient is higher in solutions of higher ionic strength. The sedimentation of native DNA is virtually unaffected by ionic strength. We used conditions of low ionic strength in our neutral sucrose gradients so that any new DNA forms would sediment faster than the parental DNA.

By 60 min after infection, the extracted

FIG. 5. Hydroxyapatite chromatography of a Hirt supernatant fluid prepared 60 min after infection of cells with KRV containing ^3H -thymidine. The sample was diluted 1:20 with water and was applied to a 2 by 1 cm column of hydroxyapatite. The column was washed with water. Single-stranded DNA was eluted with 0.14 M PO_4 (pH 6.8) containing 0.4% sodium dodecyl sulfate (SDS). Double-stranded DNA was eluted with 0.4 M PO_4 (pH 6.8) containing 0.4% SDS. The eluates were collected in 1-ml fractions, and a portion from each fraction was counted in a Triton-toluene scintillation fluid.

parental DNA of the virus is found to contain two peaks of radioactivity in neutral sucrose gradients. One peak is composed of the 6.7s, parental, single-stranded DNA. A second, fast sedimenting peak of radioactivity has a sedimentation coefficient of approximately 14.5s. The double-stranded, linear form of SV40 DNA is reported to have a similar sedimentation coefficient of $14 \pm 3\%$ (1). The fast sedimenting KRV DNA accounts for 28 to 42% of the radioactive DNA in the sample. The efficiency of infection by KRV in cultures of rat embryo cells has been reported to be related to the synthetic activity of the cells (25). The maximum efficiency of infection (number of cells synthesizing virus within the first replication cycle) as seen with fluorescent-antibody technique was approximately 30% (24). In the above experiments the proportion of 14.5s DNA molecules, 28 to 42%, may indicate the proportion of cells sustaining an active infection. The large amount of single-stranded, parental DNA present after infection may indicate abortive infections in cells or cells that are infected but not in the stage of the cell cycle to initiate virus replication. The Hirt supernatant solution from cells 60 min after infection was also denatured by alkali and sedimented in alkaline sucrose with SV40 DNA, component II. The KRV DNA from the Hirt supernatant solution sedimented peak

with the 16s linear strand of SV40 DNA. The trailing found with the KRV DNA peak may indicate some heterogeneity resulting from some intermediate DNA forms or break-down products. In the alkaline sucrose gradient, the KRV DNA strands are the same length and molecular weight as the SV40 DNA strands, so the fast sedimenting KRV DNA is probably linear. The sedimentation profiles of the Hirt supernatant fluids from the other time periods used (3, 7.5, 11, 17, 20 hr), were similar to the 60-min sample. The proportion of parental DNA in the fast sedimenting 14.5s form seems to remain constant during the remainder of the virus growth cycle.

A sample from the 14.5s peak of parental DNA was examined under the electron microscope. On the basis of a small number of measurements in aqueous and formamide preparations, the DNA was found to be linear and double stranded. The average length was approximately $1.4 \pm 0.15 \mu\text{m}$. It is not known, however, if the molecules measured in the electron microscope are the radioactive derivatives of the parental KRV DNA. The possibility exists that cellular DNA broken to pieces of this size sedimented with and obscured the small number of radioactive virion DNA molecules. The fact that the DNA is uniform and small is expected for either viral or degraded cellular DNA since the material was obtained as a cut from a sucrose gradient. SV40 DNA component III is reported to have a distinct maximum in length measurements at 1.4 to $1.52 \mu\text{m}$ (1, 17). The 14.5s peak of radioactive parental KRV DNA resembles component III of SV40 DNA. They both have a sedimentation coefficient of approximately 14s in neutral sucrose, 16s when denatured and sedimented in alkaline sucrose, and a similar length when measured in the electron microscope. SV40 has a reported molecular weight of 3.2×10^6 (4). The 14.5s component of KRV DNA therefore would be twice the molecular weight of the single-stranded KRV virion DNA.

A great deal of information has accumulated about the replication of the bacteriophage ϕX174 (17). The single-stranded, circular phage DNA is converted in vivo into a double-stranded, circular form which Sinsheimer has called the "replicative form" or RF. The RF is synthesized immediately after infection, and the RF molecule containing the parental DNA replicates semiconservatively. It precedes and is required for production of progeny, single-stranded DNA. In the above experiments, some of the parental KRV DNA has been shown to be converted to a double-

stranded form. It is synthesized soon after infection (within 60 min) preceding the production of progeny viral DNA which does not start until 7 to 8 hr after infection (19). Preliminary experiments have been carried out in which DNA is pulse-labeled with ^3H -thymidine at various times after infection of cells with KRV. The results indicate that a small double-stranded DNA molecule with some of the above characteristics is synthesized within 3 hr after infection. This pulse-labeled DNA can be hybridized to KRV DNA but not to cellular DNA, suggesting the synthesis of a strand complementary to the single-stranded virion DNA. Further experiments are necessary to show whether or not this double-stranded KRV DNA form is a replicative form involved in the production of progeny KRV DNA molecules.

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