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Linear, Single-Stranded Deoxyribonucleic Acid Isolated from Kilham Rat Virus

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Kilham rat virus (KRV) was grown in a rat nephroma cell line and was purified by two isopycnic centrifugations in cesium chloride. The virus contains single-stranded deoxyribonucleic acid (DNA) with a molecular weight of approximately 1.6×10^6. The DNA was extracted from the virion by both phenol extraction and by 2% sodium dodecyl sulfate at 50 C. KRV DNA, extracted by both procedures, was observed in an electron microscope by using a cytochrome *c* or diethylaminoethyldextran monolayer. The DNA was also exposed to exonuclease I, an enzyme which hydrolyzes specifically linear, single-stranded DNA. Hydrolysis of 70 to 80% of the DNA was observed. Both the enzymatic and the electron microscope studies support the conclusion that extracted KRV DNA is a single-stranded, linear molecule. The length of the DNA was measured in the electron microscope and determined to be 1.505 ± 0.206 μm.

Kilham rat virus (KRV) is a parvovirus (20 nm in diameter) originally isolated from a rat sarcoma (6). The virion has a molecular weight of 6.6×10^6 and contains three major proteins and a deoxyribonucleic acid (DNA) core (12, 13). The core DNA has been demonstrated by a variety of techniques to be a single-stranded molecule with a molecular weight of approximately 1.6×10^6 (11, 12). Other members of the parvovirus group, the related H-1 virus, isolated from human transplantable tumors and the minute virus of mice are also reported to contain single-stranded DNA of molecular weight 1.5×10^6 to 2.5×10^6 (1, 15). Similar to these animal viruses in size and DNA content is the bacteriophage ϕX174. ϕX174 is also about 20 nm in diameter and contains a core of single-stranded DNA with a molecular weight of approximately 1.7×10^6. The bacteriophage DNA has been shown by enzymatic and sedimentation observations to have a circular configuration (3, 4). The following studies were undertaken to determine if KRV like ϕX174 contained circular DNA or if the KRV DNA was a linear molecule. Our results, based on electron microscope studies and on enzymatic degradation of the DNA, indicate that KRV DNA is a linear, single-stranded molecule. It is the first documented report of the existence of a single-stranded, linear viral DNA.

MATERIALS AND METHODS

Virus growth and purification. KRV strain 308 was grown in a rat nephroma cell line and purified as previously described (12). The final steps in purification include two successive isopycnic centrifugations in cesium chloride.

Extraction of the DNA. DNA was extracted from KRV by two methods. The first procedure was a phenol extraction of the virion. Purified KRV in 0.05 M tris(hydroxymethyl)aminomethane (Tris) at *p*H 9.0, 0.03 M ethylenediaminetetraacetic acid (EDTA), and 0.15 M NaCl was rotated for 20 min following the addition of an equal volume of redistilled phenol. After centrifugation, the aqueous phase containing the DNA was extracted with ether to remove residual phenol. Traces of ether were removed by an N_2 stream. The DNA solution (1 ml) was then dialyzed at 4 C for 16 hr against 4 liters of buffer containing 0.02 M Tris at *p*H 9.0 and 0.001 M EDTA.

Virion DNA was also extracted by the addition of sodium lauryl sulfate (SLS) to a final concentration of 2%. The suspension was heated at 50 C for 20 min and chilled, and 1 g of $CsCl_2$ per ml of solution was added. After centrifugation at $7,000 \times g$ and 4 C the clear DNA-CsCl solution was easily separated from the SLS pellicle. The DNA was centrifuged to equilibrium in a cesium chloride gradient (density 1.71) for 48 hr at 40,000 rev/min in an SW-40 rotor of a Spinco model L2 centrifuge. The KRV DNA was located at a density of 1.715 (12) and dialyzed against 0.02 M Tris buffer (*p*H 7.5) containing 0.001 M EDTA.

Radioactive DNA, labeled with ^{3}H-thymidine or ^{14}C-thymidine, was prepared as previously described (12) and extracted by the two procedures above.

Preparation and assay conditions for exonuclease I. Exonuclease I (hydroxyapatite fraction) was purified 600-fold from *Escherichia coli* 1100, a strain which lacks DNA endonuclease I. The purification procedure and assay conditions were essentially as described by Lehman (8) and Lehman and Nussbaum (9). Each assay mixture (0.3 ml) contained 20 μmoles of glycine buffer (*p*H 9.5), 2 μmoles of $MgCl_2$, 0.05 μM 2-mercaptoethanol, and approximately 1 μg of KRV DNA containing ^{3}H-thymidine. Incubations were carried out as stated at 37 C for various indicated time periods.

Acid precipitation of DNA. After incubation of the KRV DNA with exonuclease I, 0.2 ml of nonradioactive DNA (2.5 mg of salmon sperm DNA/ml) and 0.1 ml of 6% perchloric acid (PCA) were added. After 5 min at 0 C, the resulting precipitate was centrifuged at 12,000 $\times$ *g* for 3 min. The pellet was resuspended in 0.5 ml of 0.2 N NaOH, and the DNA was precipitated a second time in 1.5 ml of water and 0.15 ml of 6% PCA. The pellet was solubilized in an NCS solubilizer (Amersham-Searle, Des Plaines, Ill.) at 50 C for 10 min and the radioactivity was counted in a scintillation fluid (NEN-Liquifluor).

Sedimentation of DNA in alkaline sucrose. After incubation of KRV DNA with exonuclease I, the enzyme activity was halted by the addition of NaOH to a final molarity of 0.2. KRV DNA, labeled with ^{14}C-thymidine, was added to the mixture as an isotope marker. The mixture was then layered on a 5 to 30% linear sucrose gradient containing 1.0 M NaCl, 0.02 M Tris, 0.2 M NaOH, and 0.001 M EDTA (*p*H 12.2). The 11-ml gradient was centrifuged at 35,000 rev/min at 20 C for 18 hr in an SW 41T rotor in a Spinco model L2 centrifuge. Fractions (0.14 ml) were collected into counting vials through a hole punctured in the bottom of the tube. The fractions were assayed for radioisotope content in 10 ml of Triton X-100 scintillation fluid (10) containing 1 ml of water.

Electron microscope studies. DNA isolated from KRV and from the bacteriophage ϕX174, by both procedures described above, was examined under the electron microscope. The DNA (3 to 5 μg/ml) was added to one-tenth volume of either 0.1% solution of cytochrome *c* in water (7) or diethylaminoethyl (DEAE) dextran solution (1% aqueous DEAE dextran, Pharmacia, Sweden; molecular weight, 2 $\times$ $10^6/\eta$ = 0.70; and an equal volume of 1 M Tris-hydrochloride buffer, *p*H 7.4). One drop of either of these mixtures was taken on a Microdiluter (Microbiological Associates Inc., Bethesda, Md., 25 μliter in capacity) and dropped onto a surface of distilled water in a Teflon-coated aluminum tray. The spreading area was restricted to 5 by 5 cm to yield a sufficiently large number of molecules in a small area. The film was picked up on a grid covered by a carbon-coated collodion membrane, washed with 50% and then 90% alcohol, and air dried. DEAE dextran, which is alcohol soluble, relatively small in molecular weight, and free from nuclease contamination, provided fine-grained background and preserved the structural integrity of nucleic acid molecules. The specimens were rotary shadowed with platinum-carbon at an angle of 8° and were examined with a Philips EM 200 electron microscope at direct magnification of between $\times$ 9,000 to 15,000. The magnification was calibrated with grating replicas.

RESULTS

Action of exonuclease I on KRV DNA. Exonuclease I from *E. coli* shows a selectivity for linear, single-stranded DNA attacking the 3'-hydroxyl end of the polydeoxyribonucleotide. It liberates 5'-mononucleotides in a stepwise manner until the terminal dinucleotide, which is not attacked. The enzyme does not hydrolyze circular, single-stranded DNA since circular DNA lacks a free 3'-hydroxyl end. When the enzyme was incubated with radioactive ^{3}H-KRV DNA, there was a rapid hydrolysis of the DNA until 40 to 50% of the DNA had been degraded (Fig. 1). The rate of hydrolysis then fell off and the limit of hydrolysis reached a level at 4 hr of incubation of about 70%. In several preparations tested, it did not exceed 80%. The resistance of a fraction of a preparation of single-stranded DNA to exonuclease I has been previously reported for other DNA molecules (9). When single-stranded, circular ϕX DNA containing ^{3}H-thymidine was similarly tested, less than 8% of the radioisotope became acid soluble in 4 hr. The limited hydrolysis of ϕX DNA is probably due to the presence of

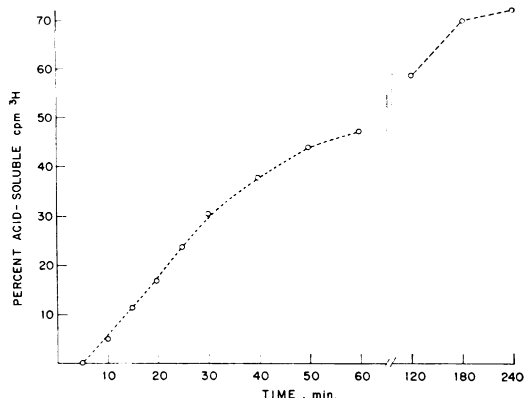

FIG. 1. *Extent of hydrolysis of KRV DNA by exonuclease I. A series of identical reaction mixtures (0.3 ml) was set up, each containing 20 μmoles of glycine buffer (pH 9.5), 2 μmoles of $MgCl_2$, 0.5 μmole of 2-mercaptoethanol, 1 μg of ^{3}H-thymidine KRV DNA, and 2 μg of exonuclease I. Reaction mixtures were incubated at 37 C and samples were removed at the times indicated. "Carrier" DNA was added and the DNA was precipitated by perchloric acid as described in the Materials and Methods section. The percentage of hydrolysis was taken as $(1 - T/T_0) \times 100$ where T and T_0 are the counts per minute found in the precipitate in the presence and absence of enzyme, respectively.*

linear molecules. Breakage of a small amount of the circular ϕX DNA during preparation is often reported (3). Some linear ϕX DNA molecules were observed in the electron microscope studies.

Sedimentation of exonuclease I-treated KRV DNA in alkaline sucrose gradients. Since exonuclease I attacks linear, single-stranded DNA in a stepwise manner to the terminal diphosphate, during hydrolysis the DNA should undergo a change in length detectable by sedimentation in a sucrose gradient. ^{3}H-KRV DNA was hydrolyzed by exonuclease I for 0, 30, or 60 min, the *p*H of the incubation mixture was raised to 12.2 by the addition of NaOH, and the mixture was layered on top of a 5 to 30% alkaline sucrose gradient. After centrifugation, the gradient fractions were counted for radioisotope content. As seen in Fig. 2, the ^{3}H-DNA and the ^{14}C-DNA marker sediment together in a sharp peak of radioactivity after 0 min of incubation. After 30 min of incubation with the enzyme, there is a decrease in the peak of radioisotope attributed to intact KRV DNA. The DNA becomes more heterogeneous in length as shown by a broadening of the peak of radioactivity. Radioactive material of low molecular weight, probably mononucleotides and dinucleotides, are found at the top of the gradient. The ^{14}C-DNA used as a marker is still a sharp, homogeneous peak. Incubation of the ^{3}H-DNA with exonuclease I for 60 min resulted in a further decrease in the amount of unhydrolyzed DNA, an increase in DNA heterogeneity, and an increase in small nucleotides at the top of the gradient. In this time, approximately 50% of the ^{3}H-radioisotope in the DNA no longer sediments with the ^{14}C-DNA marker. The ^{3}H-DNA sedimenting with the untreated ^{14}C-viral DNA after 60 min of incubation probably has not yet been attacked by the enzyme.

Electron microscope studies. KRV-DNA was extracted by both of the procedures described above and visualized in the electron microscope in a cytochrome *c* or DEAE dextran monolayer. The single strands have a tendency to be tangled and to stick together in aggregates. As seen in Fig. 3, the DNA is a linear molecule. None of the preparations observed contained any circular DNA molecules. When the bacteriophage ϕX174 DNA was prepared in the same manner and visualized under the electron microscope, most (80 to 90%) of the molecules were circular in configuration.

In one of the preparations of KRV DNA examined, the lengths of 87 molecules were measured and plotted in a histogram (Fig. 4). The average length of the DNA is 1.505 μm with a standard deviation of $\pm$ 0.206 μm.

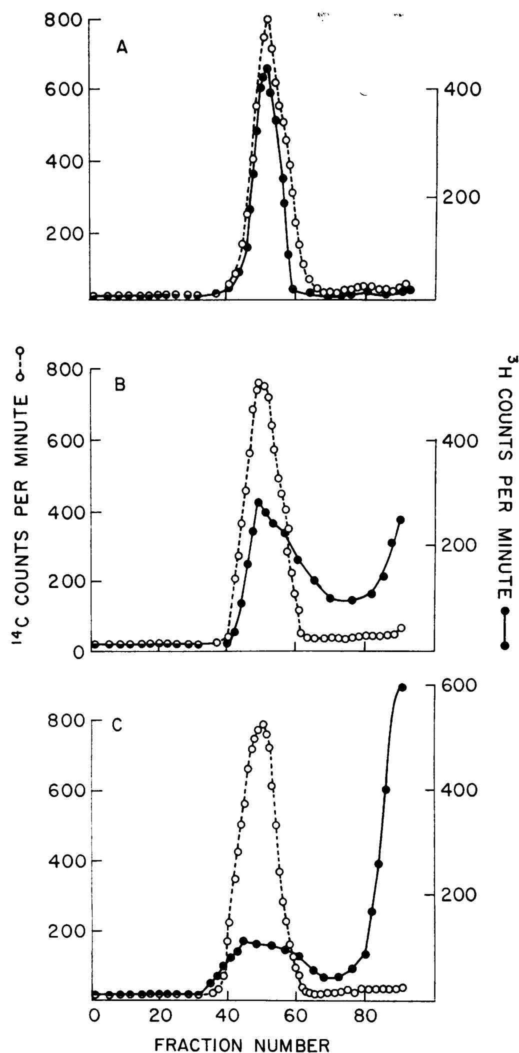

FIG. 2. *Alkaline sucrose gradient sedimentation of ^{3}H-thymidine, after treatment with exonuclease I. KRV DNA was incubated in 0.3-ml reaction mixtures each containing 2 µg of exonuclease I, 20 µmoles of glycine buffer (pH 9.5), 2 µmoles of $MgCl_2$, 0.5 µmole of 2-mercaptoethanol for 0 (A), 30 (B), or 60 (C) min. ^{14}C-thymidine-labeled KRV-DNA was then added as a marker. The pH was adjusted to 12.2, and the mixture was layered on a 5 to 30% sucrose gradient (pH 12.2), 1.0 M NaCl, 0.001 M ethylenediaminetetraacetic acid and centrifuged for 18 hr at 35,000 rev/min. Fractions were collected and assayed for radioactivity. Sedimentation is from right to left.*

DISCUSSION

Our present studies based on susceptibility of the DNA to exonuclease I and its appearance in the electron microscope indicate that the DNA extracted from KRV is a linear, single-stranded

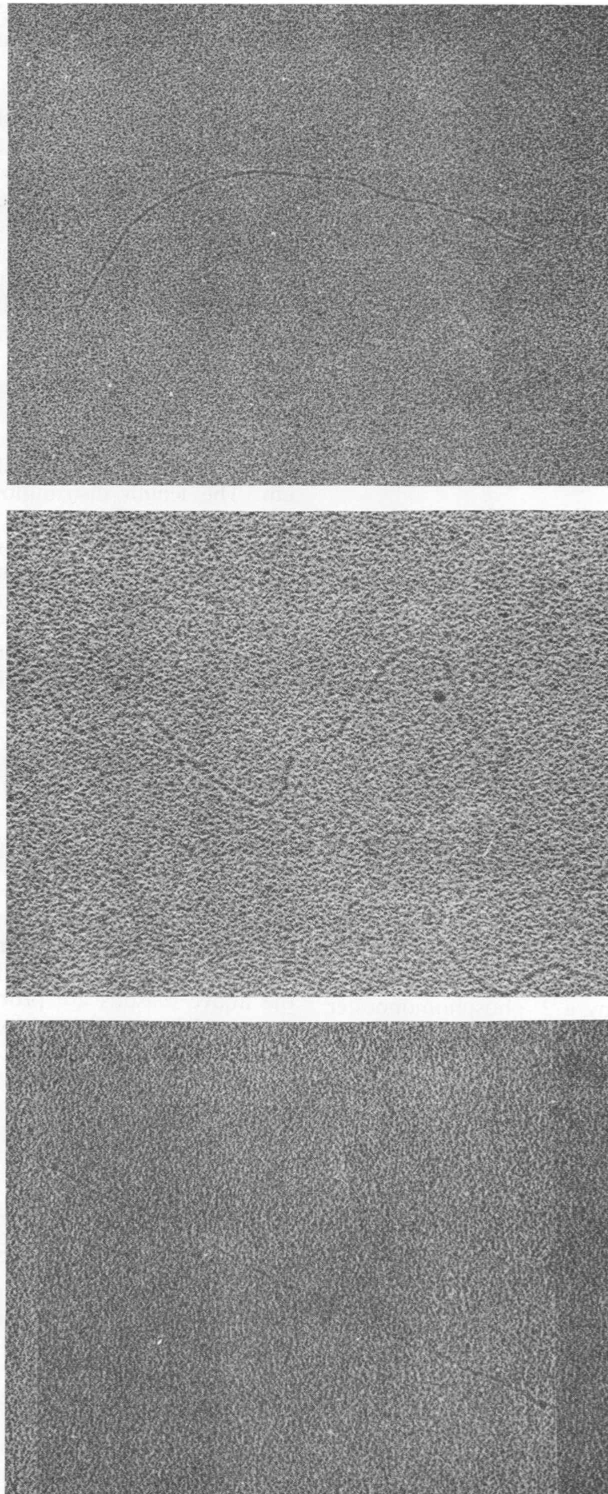

FIG. 3. *Electron micrograph of three KRV DNA molecules. Micrographs taken by T. Kakefuda.* × *72,000. DNA shown was embedded in a diethylaminoethyl dextran film.*

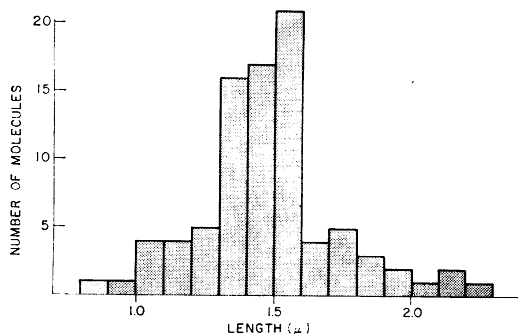

FIG. 4. *Histogram showing size distribution of DNA molecules extracted from KRV. Eighty-seven molecules were measured. DNA was extracted by 2% sodium lauryl sulfate as described in Materials and Methods and embedded in a diethylaminoethyl dextran film.*

molecule. The DNA extracted from KRV was progressively degraded by exonuclease I as shown by an increase in acid-soluble radioactivity and an increase in heterogeneity of the lengths of the DNA molecules. Within 4 hr of incubation with exonuclease I, 70 to 80% of the KRV DNA was hydrolyzed. When circular, single-stranded φX174 DNA was incubated with exonuclease I, under identical conditions, less than 8% of the DNA became acid soluble. The enzyme preparation itself does not appear to cause extensive hydrolysis of circular, single-stranded DNA.

The limit of hydrolysis and the observed change in hydrolysis rate of KRV DNA by exonuclease I are similar to results reported with the enzyme using single-stranded calf thymus DNA as a substrate (9). In these studies, Lehman and Nussbaum (9) suggested that some DNA chains might be terminated by a 3′-phosphomonoester and thus unavailable for enzyme hydrolysis. They found a phosphatase in very small quantities contaminating the nuclease. If present, the phosphatase could remove the 3′-phosphomonoester, thus making the DNA chains accessible to exonuclease I at a limited rate. Single-stranded DNA is also known to form inter- and intrastrand hydrogen bonds readily. When these hydrogen-bonded regions are located at or very near the 3′-hydroxyl end of the DNA, they can result in branch chains or aggregates, which were observed in the electron microscope. Part or all of the polynucleotide could be unsusceptible to exonuclease I under these conditions. If some of the hydrogen bonds were broken during the incubation, the DNA would again be available to nuclease activity at a limited rate.

Further support for the linearity of the DNA extracted from KRV comes from the observations in the electron microscope. Extraction of the DNA by both procedures described above, followed by observation in the electron microscope, revealed only linear molecules. No circular molecules were seen in the several hundred molecules examined. Extraction of φX174 DNA and visualization in the electron microscope by the same procedures revealed a majority (80 to 90%) of the DNA molecules in a circular configuration. Thus, the extraction procedures and the preparation of the sample for the electron microscope did not result in extensive shear or enzymatic breakdown of single-stranded, circular DNA.

KRV DNA (87 molecules) from one preparation were measured to determine the length of the molecule. The average length was found to be 1.505 μm with a standard deviation of ± 0.206 μm. The length distribution of single-stranded DNA is generally reported to be more heterogeneous than that found with double-stranded molecules (2, 5, 14). The significance of the length measurements depends upon the assumption that the mass per unit length of each polynucleotide filament seen in the electron microscope is constant. However, minor variations in conditions influence this measurement especially with single-stranded DNA molecules. Ionic strength, cytochrome *c*, and surface forces during spreading and drying on a support film all influence the length of the DNA molecules. The nucleotide bases in single-stranded DNA are not rigidly held and can stack, stretching and contracting in a nonuniform manner along the DNA molecule. This can result in microkinks and acute angles in the molecules, making tracing with the map-tracing apparatus more difficult. All or some of the above reasons are probably responsible for the broad length distribution of ± 13%.

It is difficult to exclude completely the possibility that KRV-DNA was converted from a circular to a linear DNA molecule by shear or enzymatic hydrolysis during extraction and treatment. If this were the case, however, one might expect to find at least a few circular molecules in the several hundred molecules observed in several preparations in the electron microscope. No circular molecules were seen. One might also expect that the circular, single-stranded DNA of φX 174 would also have been hydrolyzed during the same procedures. Extensive cleavage of circular φX174 DNA was not detectable, however, by either susceptibility to exonuclease I or in the electron microscope. It will be interesting to determine if the linear configuration is a general characteristic of the single-stranded DNA extracted from other parvoviruses.

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