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In Vitro Synthesis of Double-Stranded DNA from the Kilham Rat Virus Single-Stranded DNA Genome

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Double-stranded, full-length linear DNA was synthesized in vitro by using single-stranded linear DNA as a self-priming template from the parvovirus Kilham rat virus and *Escherichia coli* DNA polymerase "large fragment" as the polymerizing enzyme. To ascertain the order of the synthesis of the cleavage fragments and to assess the accuracy of the in vitro synthesis, restriction endonuclease cleavage sites with known recognition sequences were mapped on the DNA. Comparing the cleavage pattern of the synthesized DNA with that of double-stranded viral DNA isolated from infected cells confirms that the in vitro synthesis produces a faithful copy of the viral single-stranded genome. Electron micrographs of the in vitro product reveal it to be a double-stranded linear molecule.

The parvovirus Kilham rat virus (KRV) is a small DNA virus containing three proteins and one molecule of linear single-stranded DNA (SS-DNA) (15, 17). KRV DNA has a molecular weight of approximately 1.6×10^6. It has been reported to have terminal palindromic sequences at the 5′ and, probably, the 3′ termini (13). The replication scheme of the virion SS-DNA is not completely understood. It is known that at least 40% of the virion DNA is converted to a linear double-stranded DNA (DS-DNA) molecule within an hour after infection (16). Viral linear DS-DNA has been implicated as an intermediate in replication of the virion SS-DNA (9). Concatenated molecules of KRV DS-DNA, including dimers and trimers, have also been isolated from infected cells. Their role, if any, in virus replication is unknown (6; L. A. Salzman and P. Fabisch, J. Gen. Virol., in press). It is, however, difficult to obtain from infected cells enough homogeneous viral DS-DNA for studies on the DS-DNA fine structure to define its role in transcription or for use as a substrate for restriction endonucleases for preparation of a physical map.

To circumvent the problems of isolating homogeneous cellular KRV DS-DNA, we have established a simple procedure for synthesizing DS-DNA in vitro, using as a substrate the purified SS-DNA extracted from the virion and as a polymerizing enzyme the *Escherichia coli* DNA polymerase "large fragment." The virion DNA is self-priming with the DNA polymerase. Using the homogeneous, synthesized DS-DNA, we have obtained restriction enzyme fragments in the order of their synthesis and prepared a linear map of the fragments for KRV DNA.

MATERIALS AND METHODS

Viral SS-DNA. KRV was grown in rat nephroma cells and purified by two isopycnic centrifugations in cesium chloride, as previously described (14). The virions were disrupted in 0.3 M NaOH for 5 min at 23°C and sedimented through a 10-ml gradient (pH 13; 5 to 20% sucrose, 0.4 M NaCl, 0.6 M NaOH, 1 mM EDTA) at 20°C in a Beckman SW41 rotor at 27,000 rpm for 16 h. Viral DNA (16S) was located by using a radioactive simian virus 40 (SV40) component II (16S) DNA marker in a separate gradient. (SV40 DNA was a gift from N. P. Salzman.) The KRV DNA was concentrated by vacuum dialysis and dialyzed against 0.02 M Tris (pH 7.4)-0.001 M EDTA.

Viral DS-DNA. The DS replicative form of KRV DNA was isolated from rat nephroma cells 14 to 16 h after infection with KRV (10 PFU per cell). The cells were lysed in 1% sodium dodecyl sulfate-20 mM Tris (pH 7.4)-10 mM EDTA and incubated with 200 μg of proteinase K (EM Laboratories, Inc., Darmstadt, Germany) per ml at 23°C for 45 min. The high-molecular-weight DNA was precipitated at 4°C with 1 M NaCl using the procedure described by Hirt (7). After centrifugation for 45 min at 4°C and 10,000 × *g*, the supernatant was mixed with benzoylnaphthoyl-DEAE-cellulose at a ratio of 1 g of benzoylnaphthoyl-DEAE-cellulose per 8 mg of DNA. In the presence of 1 M NaCl (8, 18), only completely or partially single-stranded DNA will attach to benzoylnaphthoyl-DEAE-cellulose. The suspension was centrifuged at 23°C and 2,000 × *g* for 5 min. Cesium chloride was added to the supernatant containing DS-DNA to a concentration of 1.72 g/ml, and the solution was centrifuged at 10°C in a fixed-angle Beckman 50 Ti rotor at 35,000 rpm for 72 h. The DNA at a cesium chloride

density of 1.715 g/ml was dialyzed against 0.02 M Tris (pH 7.4)-0.001 M EDTA and precipitated with 2.5 volumes of ethanol at −20°C for 16 h. The DNA was deproteinized by suspension in 4 M guanidine hydrochloride (pH 8.0), layered on top of a 10-ml 5 to 20% sucrose gradient containing 4 M guanidine, and incubated for 16 h at 23°C (9). The gradient was then centrifuged at 40,000 rpm in a Beckman SW41 rotor for 7 h at 20°C. KRV DS-DNA with a sedimentation coefficient of 14.5S was located with the aid of a radioactive SV40 component II 16S marker in another gradient. The fractions containing DS-DNA were dialyzed against 10 mM Tris (pH 7.6)-1 mM EDTA-50 mM NaCl and then precipitated in 2.5 volumes of ethanol at −20°C for 16 h. The precipitate was stored in 0.1× SSC (SSC = 0.14 M NaCl plus 0.014 M sodium citrate) at −20°C. Radioactive KRV DS-DNA was prepared by adding [^{3}H]thymidine (40 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at a concentration of 20 Ci/ml from 11 to 14 h postinfection.

Enzymes. Endonucleases *Eco*RI, *Hpa* II, and *Hin*dIII were obtained from New England Biolabs, Beverly, Mass. *E. coli* DNA polymerase ("enzyme A" according to H. Klenow; "large fragment," according to P. Setlow) was purchased from Boehringer Mannheim Co., Indianapolis, Ind.

DNA polymerase reaction. Incubation mixtures contained (per 200 μl): Tris-hydrochloride (pH 7.4), 20 mM; $MgCl_2$, 2 mM; β-mercaptoethanol, 0.3 mM; KRV SS-DNA, 2 μg; dCTP, dGTP, dTTP, and dATP, 20 nM each; and DNA polymerase "large fragment," 2 U (19). The reaction mixture was incubated at 37°C for the time periods indicated. Enzyme activity was halted by addition of 20 mM EDTA and 0.5 M NaCl. The DNA was precipitated in 2.5 volumes of ethanol at −20°C for 16 h. After centrifugation for 30 min in a Beckman SW60 Ti rotor at 30,000 rpm and −5°C, the supernatant was discarded and the precipitate was suspended for digestion with restriction endonucleases as described below. Radioactive DS-DNA was prepared in the incubation mixture as described above, with the addition of 0.5 μCi each of [^{3}H]dATP, [^{3}H]dGTP, [^{3}H]dCTP, and [^{3}H]dTTP (specific activities, ca. 0.5 mCi/μmol; New England Nuclear Corp.).

Digestion with restriction endonucleases. Viral DNA (1 μg per sample) was dissolved in a 100-μl solution containing 20 mM Tris-hydrochloride (pH 7.5), 7 mM $MgCl_2$, 1 mM β-mercaptoethanol, 50 μg of bovine serum albumin, and 10 to 20 U of restriction enzyme *Hpa* II (4). Digestion reactions with 10 to 20 U of *Eco*RI or *Hin*dIII contained in addition 50 mM NaCl and 100 mM Tris (pH 7.5) (1, 10). Incubations were carried out for 4 h at 37°C. Slab-gel electrophoresis utilized gels (0.3 by 12 by 16 cm) of 2.5% acrylamide-0.5% agarose in an EC 470 apparatus (E-C Apparatus Corp., Philadelphia, Pa.). The buffer used for the gel and for electrophoresis contained 0.04 M Tris (pH 8.0), 0.02 M sodium acetate, 0.002 M EDTA, and 0.014 M NaCl. Electrophoresis lasted for 16 to 18 h at 20°C with a constant voltage of 39 V. The gels were added to a solution containing 1.5×10^{-6} g per ml of ethidium bromide, and the bands were located with UV light. Photographs were taken with a Polaroid camera containing Polaroid type 665 film. After electrophoresis, gels containing radioactive DNA bands were washed successively at room temperature with water for 60 min, three times with dimethyl sulfoxide each time for 60 min, and with 50 ml of dimethyl sulfoxide containing 20% 2,5-diphenyloxazole (PPO) for 16 h. The gel was then rinsed six times in water to remove the PPO precipitate, vacuum dried, and exposed to Kodak XR-2 film at −70°C for 2 to 48 h as required for location of the radioactive bands.

Electron microscopy. DNA from preparations of DNA polymerase-treated virion SS-DNA was mounted for electron microscopy by the aqueous or formamide technique described previously (16). Electron micrographs were taken on Kodak electron image plates at a magnification of × 8,000. Magnification was calibrated with a grating replica (E. F. Fullam, Schenectady, N.Y., catalog no. 1000).

Determination of the molecular weight of KRV restriction enzyme fragments. SV40 DNA of molecular weight 3.6×10^6 was digested with restriction enzymes as reported previously (11). The log of the known molecular weight of each SV40 DNA fragment was plotted as a function of the distance migrated. The molecular weights of the KRV restriction enzyme fragments were calculated from the plotted curve as a function of the distance migrated. SV40 markers included: (i) *Hin*dII and *Hin*dIII digests, yielding 11 fragments of molecular weights 811,000, 540,000, 378,000, 360,000, 306,000, 270,000, 252,000, 198,000, 180,000, 162,000, and 144,000; (ii) *Hin*dIII digest yielding 3 large-molecular-weight fragments with molecular weights of 1,400,000, 1,100,000 and 720,000 plus smaller-molecular-weight fragments that were not used here; and (iii) a *Bam* and *Hpa* II digest yielding 2 fragments of molecular weights 2.0×10^6 and 1.58×10^6 (11). All three SV40 DNA digest markers were subjected to co-electrophoresis with each sample in a slab gel, as described above, to determine the molecular weights of KRV digest fragments.

RESULTS

Digestion of viral DS-DNA isolated from infected cells with the restriction endonucleases *Hpa* II, *Hin*dIII, and *Eco*RI. KRV DS-DNA was isolated from rat nephroma cells 16 h after infection. The DNA was purified by chromatography through benzoylnaphthoyl-DEAE-cellulose, isopycnic centrifugation in cesium chloride, and two velocity sedimentations in sucrose (see above). A 1.0-μg sample of the DNA was digested with each of the following restriction enzymes: *Hpa* II, *Hin*dIII, and *Eco*RI. Electrophoresis was performed in a 2.5% acrylamide-0.5% agarose slab gel with appropriate SV40 DNA markers (see above). The pattern of the fragments from each enzyme digestion is shown in Fig. 1. The log of the molecular weight of each SV40 DNA fragment was plotted versus its measured relative electrophoretic mobility. The molecular weights of the KRV fragments were calculated from the SV40 DNA fragment linear plot, using the mobility of KRV DNA fragments in the same gel. The molecular

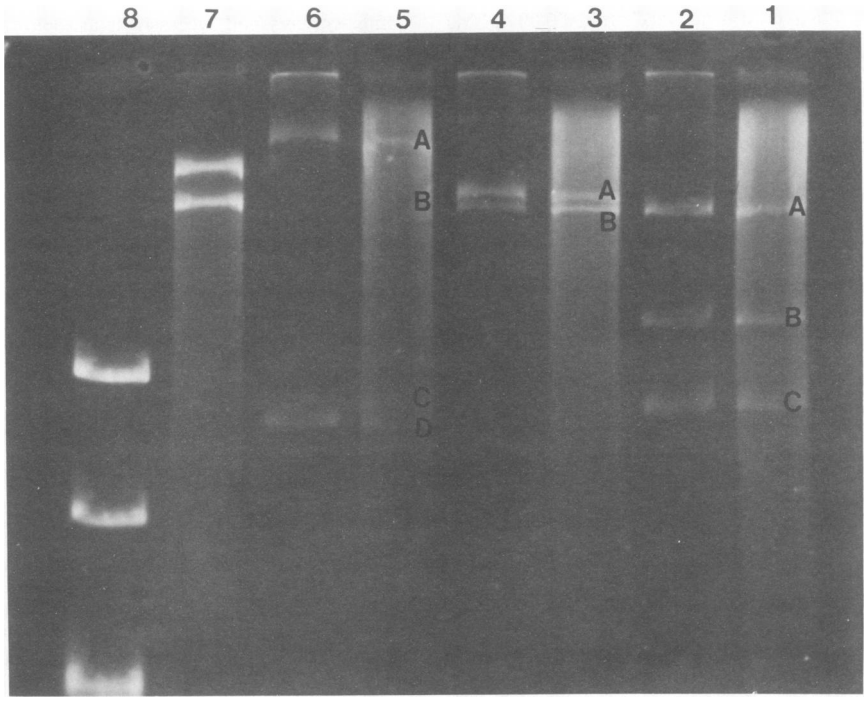

FIG. 1. *Comparison of KRV DS-DNA synthesized in vivo and in vitro and digested with the restriction endonucleases Hpa II, HindIII, and EcoRI. KRV DS-DNA from infected cells digested with Hpa II (1), HindIII (3), and EcoRI (5). In vitro-synthesized DNA digested with Hpa II (2), HindIII (4), and EcoRI (6). The SV40 DNA markers are the (7) Bam and Hpa II digests and (8) HindII and HindIII digests. Electrophoresis in a slab gel was for 16 h at 20°C and a constant voltage of 39 V. Bands are labeled A to D in order of increasing electrophoretic mobility.*

weights of the *Hpa* II fragments (Fig. 1, channel 1), starting with the highest molecular weight, were (A) 1.56×10^6, (B) 0.9×10^6, and (C) 0.74×10^6 and those of the *Hin*dIII fragments (Fig. 1, channel 3) were (A) 1.66×10^6 and (B) 1.56×10^6. The additive molecular weight in each band for each endonuclease was approximately the same as that of intact KRV DS-DNA (molecular weight, 3.2×10^6), strongly suggesting that each band observed represented a unique fragment. Digestion of the DNA with restriction enzyme *Eco*RI (Fig. 1, channel 5) yielded at least four fragments of the following molecular weights: (A) 2.4×10^6, (B) 1.45×10^6, (C) 0.78×10^6, and (D) 0.76×10^6. The additive molecular weights are greater than can be accounted for by the KRV DS-DNA. The B fragment may be due to head-to-tail-linked C and D fragments from a dimer molecule (12). The C fragment is believed to be the 5′ end of a completed double strand in which the hairpin turn has been replicated. The D fragment may contain the unreplicated hairpin turn at the 5′ end of the complementary viral strand (12). This finding will be considered in the Discussion. The DS-DNA preparation has been shown by hybridization to contain a small amount of cellular DNA (3%) as well as concatemers, dimers, and trimers of the viral DS-DNA (12). This contamination or fragmentation of some of the viral DS-DNA may account for the ethidium bromide-reactive material found as a background in the gel. ^{3}H-labeled KRV DS-DNA was also digested as described above, and the gel was developed in dimethyl sulfoxide containing PPO and visualized on X-ray film at −70°C (see Materials and Methods). Only the same bands discussed above could be detected.

Synthesis of KRV DS-DNA from the virion SS-DNA template. To obtain a more homogeneous DS-DNA preparation and to determine the orientation of the restriction enzyme fragments for future work, we worked out a procedure for the replication of the KRV SS-DNA to a DS-DNA molecule. The initial attempts with DNA polymerase I or α were discouraging, producing fragmentation of the viral DNA and bizarre structures. The DNA polymerase I "large fragment," which has lost the 5′→3′ exonuclease activity by proteolytic cleavage of the complete molecule (8), was found to serve as a polymerizing enzyme. Radioactive

deoxynucleoside triphosphates were incorporated into acid-precipitable radioactivity in the presence of 2.0 U of the enzyme and 1 μg of KRV SS-DNA (Fig. 2). The reaction started immediately upon addition of the enzyme. It remained linear for approximately 10 min and then leveled off.

Determination of the sequence of synthesis of the restriction enzyme fragments. We determined the sequence of the synthesis of the restriction enzyme fragments by stopping polymerization of the KRV SS-DNA at various times after exposure to DNA polymerase "large fragment." We then precipitated the DNA in ethanol at −20°C, suspended the precipitate in incubation mixtures for the restriction enzyme being studied, and incubated it at 37°C for 4 h. Incubation of partially replicated KRV SS-DNA with DNA polymerase "large fragment" for 1 min (channel 1) produced an *Hpa* II restriction enzyme fragment which co-electrophoresed with fragment A (Fig. 3). Enzyme incubation for 3 min (channel 2) produced fragments A and C, whereas incubation for 9 min (channels 3 and 4) produced all three fragments. These three fragments co-electrophoresed with the fragments of KRV DS-DNA isolated from infected cells and digested with *Hpa* II under identical conditions (Fig. 1, channel 2). We conclude that *Hpa* II fragment A is the first portion of the KRV SS-DNA replicated and is therefore at the 5′ end of the complementary strand of the viral DNA molecule. *Hpa* II fragment C is the second fragment and *Hpa* II fragment B is the third fragment of the complementary viral strand synthesized. Since no other DNA pieces were detected by ethidium bromide staining of the gel or by radioautography (see Materials and Methods), piece B is believed to contain the 3′ terminus of the complementary viral DNA strand. Material at the top of the gel probably represents some SS-DNA which was not replicated and thus was unable to enter the gel. Between this band and fraction A, at least one faint additional band was found in slots 3 through 5. The band (DS) containing DNA of the highest molecular weight (3.2×10^6) is believed to be undigested, replicated KRV DS-DNA.

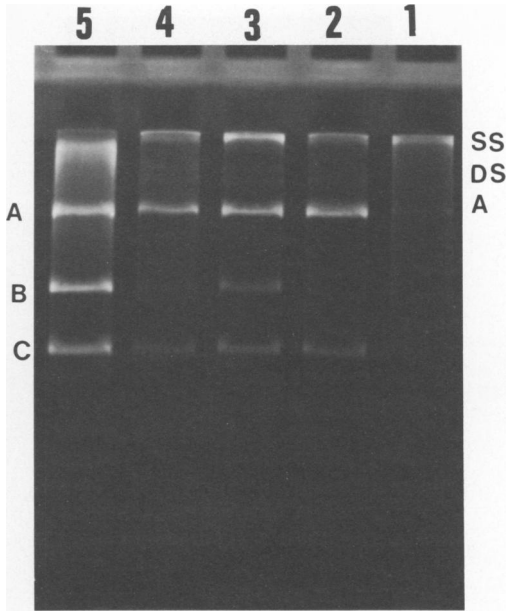

FIG. 3. *In vitro synthesis of KRV DS-DNA by DNA polymerase "large fragment," followed by digestion with restriction endonuclease Hpa II. DNA was synthesized for (1) 1 min, (2) 5 min, (3) 9 min, and (4) 9 min. The DNA was precipitated in ethanol, digested with Hpa II, and subjected to electrophoresis in a slab gel for 16 h at 20°C and a constant voltage of 39 V. KRV DS-DNA from infected cells (5) was digested with HpaII and subjected to electrophoresis as described above. Bands are labeled A to C in order of increasing electrophoretic mobility.*

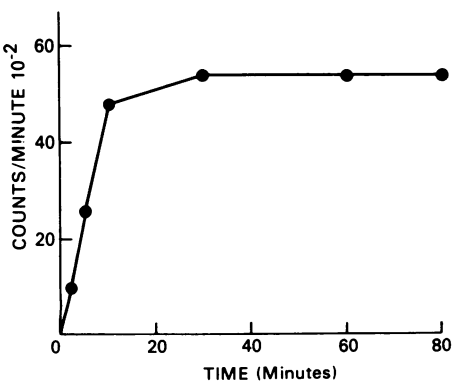

FIG. 2. *Kinetics of incorporation of four ^{3}H-labeled deoxynucleoside triphosphates by DNA polymerase "large fragment" into KRV SS-DNA. At 0, 2, 5, 10, 20, 30, 60, and 80 min of incubation, the amount of radioactivity incorporated into acid-precipitable counts per minute was determined.*

This technique, use of restriction enzyme digestion of partially replicated and completely replicated KRV SS-DNA, was also applied to the fragments obtained with endonucleases *Hin*dIII and *Eco*RI. As seen in Fig. 4 (*Hin*dIII digest), at 1 min (channel 1) and 3 min (channel 2) after enzyme addition, synthesis of the complementary viral strand had not proceeded far enough for cleavage by *Hin*dIII. At 5 min (channel 3) and 7 min (channel 4) after DNA polymerase "large fragment" addition, fragment A (molecular weight, 1.66×10^6) could be seen. At 9 min (channel 5) after enzyme incubation, the

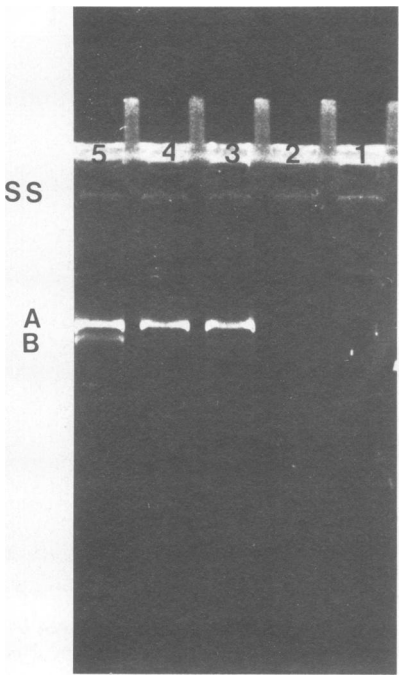

FIG. 4. *In vitro synthesis of KRV DS-DNA and digestion with restriction endonuclease HindIII. DS-DNA was synthesized for (1) 1 min, (2) 3 min, (3) 5 min, (4) 7 min, and (5) 9 min after addition of DNA polymerase "large fragment."*

second fragment, B (molecular weight, 1.56×10^6), could be detected, although it was not present in as great a quantity at this time as the A fragment. Thus, the A fragment contains the 5′ terminus and the B fragment contains the 3′ terminus of the complementary viral strand.

The pattern of fragments obtained by *Eco*RI digestion of the partially and fully replicated KRV SS-DNA molecule was surprising. After 1 min of enzyme incubation (channel 1), only the unreplicated KRV SS-DNA was seen at the top of the gel (Fig. 5). After DNA polymerase "large fragment" incubation for 3 min (channel 2), fragment D (molecular weight, 0.76×10^6) was seen. After incubation for 9 min (channel 3) and 30 min (channel 4), two fragments were seen. The second fragment A has a molecular weight of approximately 2.4×10^6. Together the two fragments would account for the total molecular weight of the virion. The fragmentation pattern, however, varied from that found with *Eco*RI digestion of the KRV DS-DNA from infected cells (Fig. 1).

In vitro completely replicated KRV DNA retained the 5′ hairpin configuration covalently linked to the newly replicated strand as shown in the following experiment. ^{3}H-labeled, in vitro-synthesized DS-DNA was prepared, denatured in 0.25 M NaOH, and cosedimented with SV40 [^{14}C]DNA component II ($17s_{20,w}$) in a 5 to 20% sucrose gradient (pH 13), as described above. The in vitro-synthesized KRV DS-DNA was located in a single sharp peak, with a sedimentation coefficient of 20 to 21S. Under the conditions used, this is the sedimentation coefficient of a linear single-stranded dimer of KRV DNA with twice the molecular weight of KRV virion DNA (6). Virion DNA has a sedimentation coefficient of 16S under these conditions (6).

In vitro KRV DS-DNA was also synthesized in the presence of [^{3}H]dNTP and digested with each of three restriction endonucleases mentioned above. The [^{3}H]DNA was subjected to electrophoresis, developed in dimethyl sulfoxide containing PPO, and visualized on X-ray film (see Materials and Methods). The digestion fragments described above were seen, but no additional bands of radioactivity with molecular weights greater than 4×10^4 were detected.

Electron microscopy. Electron micrographs of viral linear SS- and DS-DNA of unit virus length are shown in Fig. 6. The SS-DNA in an aqueous preparation (Fig. 6A) is shown as discrete "bushes" or collapsed molecules. By 5 min after enzyme addition (Fig. 6B) DS-linear DNA

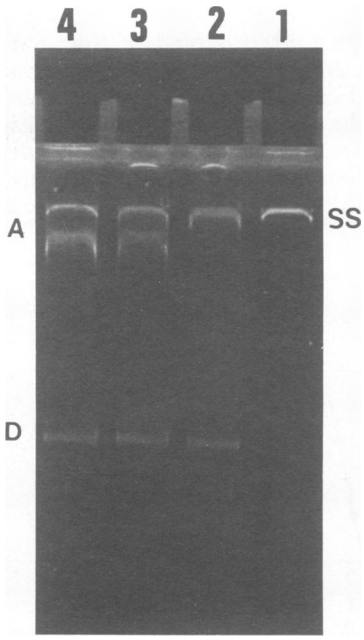

FIG. 5. *In vitro synthesis of KRV DS-DNA and digestion with restriction endonuclease EcoRI. DS-DNA was synthesized for (1) 1 min, (2) 3 min, (3) 9 min, and (4) 30 min after addition of DNA polymerase "large fragment."*

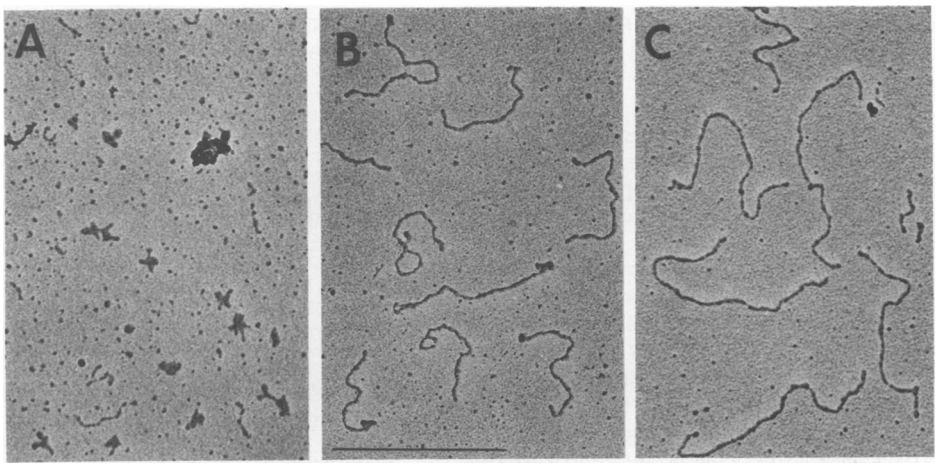

FIG. 6. *Electron micrographs of KRV DNA molecules present in the E. coli polymerase reaction mixture after (A) 0 min, (B) 5 min, and (C) 15 min of incubation at 37°C. Samples were mounted for microscopy by the Kleinschmidt aqueous procedure and rotary shadowed with platinum-palladium. Bar represents 1 μm.*

is shown attached to the bushes of SS-DNA. At 15 min after enzyme addition, all of the molecules are DS and linear and of unit length (1.50 ± 0.15 μm) (2). The DNA seen in Fig. 6B, if spread in formamide, confirmed the partial DS nature of over 90% of the molecules. Occasional branched molecules and aggregates were seen in the preparations.

DISCUSSION

This report describes a method for the replication of SS-DNA extracted from purified KRV virions. From analyses of the restriction enzyme fragments and observation with an electron microscope, we believe that the synthesis of the DS molecule proceeds from the 5′ end of the DNA strand complementary to the viral strand and proceeds in an orderly progression to complete replication of the DS molecule. The completed in vitro-synthesized molecule, when digested by restriction enzymes *Hpa* II and *Hin*dIII, produced fragments which, when subjected to electrophoresis in an acrylamide agarose gel, comigrated with each of the fragments obtained from digests of in vivo-synthesized viral DS-DNA. The order of synthesis of the fragments was determined by interrupting the synthesis of the complementary DNA molecule before completion of the strand. A map of the digest fragments for the three restriction endonucleases used in the order that they are synthesized and with the percentage of the viral genome that they occupy is shown in Fig. 7. Electron micrographs of the SS-DNA and partially synthesized DS-DNA support the concept of an orderly progression from an SS to a completely DS molecule. If this is the case, this

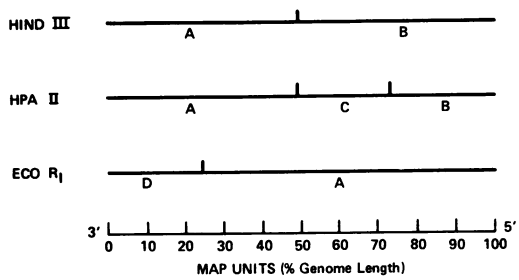

FIG. 7. *Diagrammatic representation of the map order of fragments on the KRV viral genome. The 3′ and 5′ ends of the viral genome and its subdivision into map units are indicated at the bottom of the figure. The map is derived from the gels shown in Fig. 3 through 5.*

procedure would be useful for the production of large amounts of homogeneous DS-DNA and for determining the order of synthesis of many restriction enzyme fragments.

Little is known about the actual mechanism of replication of the parvovirus SS-DNA. Current information suggests the involvement of a DS-KRV intermediate (6, 9, 16). One possible model for synthesis of the DS-DNA is a 3′-terminal palindromic sequence on the SS-DNA which folds back to form a short duplex region or hairpin. This duplex region would serve as a primer for the synthesis of the complementary strand. A series of steps has also been proposed for the replication of the hairpin region after it has served its "primer" function (3, 20, 21).

The analysis of restriction enzyme fragments produced by digestion of in vitro-synthesized viral DS-DNA and viral DS-DNA from infected cells has generated additional support for the

involvement of a self-priming mechanism in parvovirus DS-DNA replication (2, 13). The DNA polymerase "large fragment" used here for replication of the KRV SS-DNA is limited in its rate of synthesis by the number of 5′ primer termini present at the onset of the reaction (19). The immediate onset of DNA replication (Fig. 2) suggests that the viral SS-DNA contains these primer termini and is, therefore, self-priming. Additional support for a self-priming terminus on KRV SS-DNA comes from the analyses of the restriction enzyme *Eco*RI digest of KRV DNA. *Eco*RI digestion of DS-DNA from infectious cells yielded at least four fragments. Two of these fragments, C and D (Fig. 1) (1, 8), differ from each other in molecular weight by only 50 to 70 DNA base pairs. This is the approximate length of the 3′-terminal palindromic sequence in KRV DNA (13). Rhode (12) found a similar *Eco*RI cleavage pattern in the DS-DNA isolated from cells infected with the parvovirus H-1. He suggested that the smaller fragment, D, contains the fold-back or hairpin region and that in the larger fragment, C, the fold-back region has been extended and replicated. This would make fragment C longer than D by the length of the fold-back region. If this hypothesis is correct, then in the in vitro-replicated KRV DNA the hairpin region is present in fragment D and either is near to or serves as the origin of replication. Further support for the presence of a 5′ hairpin terminus serving as a self-primer for the replication of the DS molecule comes from our studies involving the sedimentation of the denatured in vitro-replicated molecule in alkaline sucrose. The molecule sediments as a linear single-stranded dimer with a sedimentation coefficient of 20 to 21S. A likely explanation for the presence of the dimer is that the virion DNA is covalently linked to the complementary replicated strand by the 5′ hairpin terminus. Further replication of such a molecule would have to involve the unfolding and replication of this hairpin terminus. The conditions required for studying the unfolding of this 5′ terminus of the viral complementary strand were not used in our incubations and will be studied further.

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