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Infectious, Linear, Unintegrated DNA of Moloney Murine Leukemia Virus

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A closed circular, double-stranded infectious DNA of Moloney leukemia virus has been described previously. The present report characterizes a second type of infectious, unintegrated viral DNA which is linear, largely double stranded, and of mass comparable to that of the closed circular viral DNA. The linear form is of nonpermuted sequence, and *Sal*I endonuclease cleaves at one site 45% from one end.

Shortly after infection by RNA tumor viruses, several nonintegrated forms of the viral DNA can be detected in the cell by using molecular hybridization (2, 4, 7, 13). These forms can be readily extracted by the Hirt procedure (8) and fractionated by ethidium bromide-cesium chloride (EtBr-CsCl) isopycnic centrifugation. The lower band in such an EtBr-CsCl gradient contains a closed circular, double-stranded form of viral DNA (3, 7). In the case of Moloney murine leukemia virus (M-MuLV), which is studied here, this closed circular DNA has a molecular weight of 5.5×10^6 (2). The upper band contains a complex mixture of viral DNA molecules (4). The present report characterizes one component of this complex mixture of non-closed-circular molecules.

Analysis of upper-band DNA, using sodium iodide (NaI) isopycnic centrifugation, revealed that this non-closed-circular class of molecules contains a mixture of M-MuLV DNAs that are single-stranded, partially double-stranded, and completely double-stranded (4, 11). Most of these molecules appear to be aborted in the course of their maturation into complete genomes, since most of them contain DNA strands of subgenomic length (4).

Infectious virus particles result from the application of nonintegrated viral DNA to susceptible cells by the Graham-Van der Eb transfection procedure (5, 6). The closed circular DNA is the source of some of this infectivity (11). Unexpectedly, the bulk of the infectivity is derived from the upper-band DNA, which contains the heterogeneous mixture of apparently incomplete viral genomes (11).

One small, homogeneous component of this mixture is responsible for the infectivity. In a previous report, we demonstrated that this non-closed-circular, infectious component sediments homogeneously at a rate of 18S (11). Such a sedimentation constant is consistent with several linear and circular configurations. In addition, these infectious molecules band a bit more densely than double-stranded DNA in a NaI gradient, indicating that they contain a small region of single-strandedness (11).

The present report demonstrates that the infectious molecules are linear DNA of nonpermuted sequence and of virtually complete genome length. These molecules do not arise as a consequence of random degradation during their preparation and may represent an immediate precursor to the closed circular, double-stranded form of the viral DNA.

MATERIALS AND METHODS

Preparation of viral DNA. A large roller bottle culture of JLS-V9 cells was infected with M-MuLV and then subjected to the Hirt extraction procedure 10 to 15 h postinfection. The deproteinized nucleic acids of the Hirt supernatant fraction were fractionated by EtBr-CsCl centrifugation as described previously (3).

Analysis of viral DNA by electrophoresis in cylindrical agarose gels. Agarose gels were cast in Perspex cylinders (0.6 cm ID by 7.5 cm) in a buffer containing 50 mM sodium acetate, 1 mM EDTA, and 40 mM Tris (pH 8.3). The percent agarose content and the running conditions are described in the text. The electrophoresis tanks carried the same buffer as described above. After electrophoresis, gels were stained with 0.5 μg of EtBr per ml for 20 min (10) and photographed under short-wavelength UV illumination, using Polaroid type 55 positive-negative film. When desired, the resulting negatives were scanned in a Canalco GII densitometer. Gels to be analyzed by hybridization with a ^{125}I-labeled RNA probe were cut into 1-mm slices, and the sliced gels were exposed to short-wavelength UV light to localize marker bands.

Hybridization to DNA contained in gel slices. To

a 1-mm agarose gel slice of about 25-μl volume, a 25-μl solution containing 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 5.5), 20 mM EDTA, and 2,000 to 5,000 cpm of a ^{125}I-labeled MuLV (specific activity, 0.3×10^8 to 1.4×10^8 cpm/μg) RNA probe was added. These samples were overlaid with 0.1 ml of mineral oil and boiled for 5 min. When closed circular DNA was analyzed, boiling was carried out for 45 min to ensure breakage and denaturation of the closed circular molecules. The samples were then incubated for 48 h at 67°C. The samples were then diluted with 0.7 ml of 2× SSC at 67°C and cooled to 45°C; 100 μl of RNase A at a concentration of 0.5 mg/ml was then added. Incubation at 37°C was continued for 1 h followed by the addition of 300 μl of 50% trichloroacetic acid and 40 μl of yeast tRNA carrier at 14 mg/ml. Filtration of the resulting precipitates was performed through two glass-fiber filters. Mineral oil was washed off of the filters with a 1:1 mixture of chloroform and ethanol.

Analysis of infectivity of DNA run on gels. A slice of a cylindrical agarose gel was placed above a glass wool plug in the tip of a 10-ml plastic pipette to which a small dialysis bag had been attached. Salmon sperm DNA (15 μg) was placed in the bag as carrier; the DNA in the gel migrated into the bag as a result of electrophoresis for 6 h at 100 V. The DNA in the bag was used for the transfection procedure described in detail previously (11). The DNA for each gel fraction was used for the transfection of a single culture of NIH/3T3 cells in a 6-cm dish.

Treatment of DNA with the ATP-dependent DNase of *Haemophilus influenzae*. The ATP-dependent DNase was received as a gift from Michael Mann of Johns Hopkins University. Fifteen units of this enzyme was placed in 80 μl of a buffer (10 mM $MgCl_2$, 1 mM ATP, 2 mM mercaptoethanol, 250 mM Tris, pH 8.0) containing 20 μg of the DNA to be tested (1). The mixture was incubated for 30 min at 37°C, and the reaction was terminated by the addition of EDTA to 33 mM. A 5-μl volume of the reaction mixture was withdrawn for agarose gel electrophoresis; the remainder was extracted with phenol, dialyzed extensively against 0.3 M NaCl-1 mM EDTA-10 mM Tris (pH 7.5), precipitated with ethanol, and used for transfection as described previously (11).

Transfer of DNA from agarose gels to cellulose nitrate filter. Non-closed-circular DNA was subjected to electrophoresis on a 1.4% agarose slab gel (10 by 13 cm). Electrophoresis was carried out for 24 h at 25 V in the buffer described above. After electrophoresis, the gel was soaked in 1.5 M NaCl-0.5 M NaOH for 60 min and then neutralized in 3 M NaCl 0.5 M Tris-chloride (pH 7.0) for 90 min. The gel was placed on a piece of Whatman 3 MM paper soaked in 20× SSC. A piece of cellulose nitrate filter soaked in 2× SSC was placed on top of the gel. Two pieces of Whatman 1 MM paper were placed along both sides of the cellulose nitrate filter. Several layers of absorbent filter paper were placed over the cellulose nitrate filter and changed periodically for 24 h. The filter was then rinsed in 2× SSC and baked at 80°C for 2 h. The procedure described above closely follows that of Southern (12).

Hybridization to DNA on a cellulose nitrate filter. A 2.5-ml volume of the ^{125}I-labeled MuLV RNA probe (0.02 μg/ml; specific activity as stated above) in 5× SSC-10 mM EDTA (pH 5.5) was added to the filter. The filter was then placed between two pieces of cellulose acetate. This was sealed with a parafilm gasket and finally placed between two pieces of glass which were then clamped together. After hybridization at 67°C for 24 h, the filter was rinsed in hybridization buffer for 60 min at 67°C and then placed in a solution of 0.05 mg of RNase A per ml for 60 min at 37°C. The filter was dried under a heat lamp and exposed to Kodak No-Screen X-ray film at room temperature for 4 days (see Fig. 3, channels a and b) or 14 days (see Fig. 3, channels c and d).

RESULTS

Previous experiments showed that infectious, non-closed-circular viral DNA sediments at a rate of 18*S* (11). Unfortunately, this sedimentation rate alone did not allow one to distinguish between nicked circular and linear forms of the viral DNA. The sedimentation rate of 18*S*, however, does allow for the enrichment of this DNA form by rate zonal centrifugation, since the bulk of the non-closed-circular viral DNA sediments more slowly. Therefore, in the experiments described below, the infectious, non-closed-circular DNA was taken from the upper band of an EtBr-CsCl gradient and subjected to centrifugation through a sucrose gradient. The material sedimenting between 16*S* and 20*S* was used for further analysis. This 16*S* to 20*S* material contains all of the infectious, non-closed-circular viral DNA (11).

Agarose gel electrophoresis provides good resolution between the closed circular (form I), nicked circular (form II), and linear (form III) configurations of a DNA genome of viral size. PM2 DNA, whose native genome is a closed circular DNA of 6.4×10^6 daltons (9), serves as a convenient marker. PM2 form I DNA migrates the most rapidly, form III DNA migrates at an intermediate rate, and form II DNA migrates the most slowly under the conditions used here (Fig. 1a, Fig. 2a). M-MuLV DNA forms I and II, detected by hybridization of a virus-specific probe to the DNA of each gel slice, migrate slightly more rapidly than the comparable forms of PM2. This greater electrophoretic mobility is consistent with the lower molecular weight of the M-MuLV genome (5.5×10^6) (2).

M-MuLV DNA was prepared as described above and subjected to agarose gel electrophoresis. The peak of hybridization in Fig. 1a shows that the viral DNA sedimenting from 16*S* to 20*S* migrates more rapidly than PM2 form III DNA, indicating its linear conformation. There is no detectable nicked circular M-

MuLV DNA. This rules out the possibility that non-closed-circular, infectious viral DNA is derived from nicking of closed circular DNA.

As seen in Fig. 1b, the infectious DNA migrates in a parallel agarose gel with the same mobility as the viral DNA detected by hybridization (Fig. 1a). Thus, the major species of open circular 16S to 20S viral DNA, detectable by hybridization, has an electrophoretic mobility identical to that of the infectious form of the viral DNA.

An independent demonstration of the linearity of this form of the viral DNA is made possible by the use of restriction endonuclease cleavage. As mentioned above, uncleaved, closed circular MuLV DNA (form I) and nicked circular MuLV DNA (form II) migrate a bit more rapidly than PM2 DNA forms of like conformation (Fig. 2a). Upon cleavage of a mixture of these M-MuLV DNA forms with *Sal*I endonuclease, isolated from *Streptomyces albus* (J. R. Arrand, P. A. Myers, and R. J. Roberts, unpublished data), one linear fragment of 5.5×10^6 molecular weight is generated (Fig. 2b). The enzyme therefore introduces one break into the viral DNA genome.

*Sal*I endonuclease cleavage of the non-closed-circular, infectious form yields two cleavage products. They are detectable by the agarose gel hybridization technique that we used (Fig. 2c) as well as the newer filter hybridization technique of Southern (12; Fig. 3c). In both cases, two fragments of 2.5×10^6 and 3.0×10^6 molecular weight are detected. The partial digestion effected by the *Sal* endonuclease (Fig. 3c) yields the two fragments plus remaining uncleaved linear DNA. This latter uncleaved DNA runs identically to viral DNA not subjected to the enzyme treatment (Fig. 3b).

The production of two fragments after *Sal*I endonuclease cleavage demonstrates once more the linearity and full length of this form of the genome. In addition, most, if not all, of these linear molecules are of nonpermuted sequence. The *Sal*I endonuclease makes one cut about 45% from an end of the linear molecule.

The linearity of this form of the M-MuLV viral DNA is also suggested by the susceptibility of this molecule to degradation by the ATP-dependent DNase of *H. influenzae*. This enzyme is reported to degrade single- and double-stranded linear DNA molecules without degrading single- or double-stranded circular molecules (1). Nicked circular molecules and double-stranded circular molecules with single-stranded gaps are resistant to this enzyme treatment (1).

Control experiments confirmed that this en-

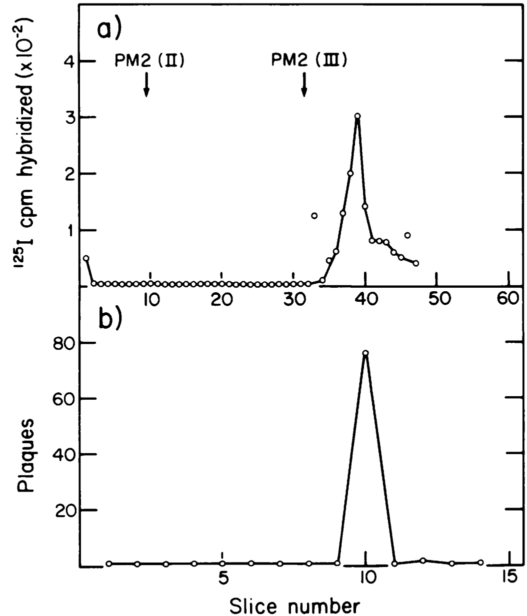

FIG. 1. *Agarose gel electrophoresis of non-closed-circular viral DNA. Hirt-extracted DNA from the upper band of an EtBr-CsCl gradient was purified further by layering over a 15 to 30% sucrose gradient. DNA migrating between 16S and 20S was precipitated with ethanol. PM2 DNA (forms II and III) was added as an internal marker. Two aliquots of DNA containing 40 μg of salmon sperm DNA as a carrier were subjected to electrophoresis on parallel 1.4% agarose gels for 14.5 h at 4 V/cm. (a) One gel was cut into 1-mm slices and used for molecular hybridization. Viral DNA was detected by incubation of each gel slice with a ^{125}I-labeled RNA probe. (b) The parallel gel was cut into 4-mm slices, which were used for transfection.*

zyme degrades linear, double-stranded, lambda DNA (form III) while leaving the nicked circular form of the phage DNA unaffected. A mixture of 80% λ form III and 20% λ form II DNAs had its infectivity reduced from 4.6×10^4 to 1.1×10^4 PFU/μg upon treatment with this enzyme. The infectivity that was resistant to this enzyme was due to the form II DNA. Heating of the DNAs to 67°C for 2 min, which converts all of form II DNA to form III DNA, caused a 100-fold reduction in infectivity if the DNA was treated with the ATP-dependent DNase before titering. In addition, agarose gel electrophoresis analysis indicated that the enzyme, as reported, specifically degrades linear double-stranded DNA while leaving nicked circular DNA unaffected (data not shown).

When the infectious, upper-band DNA is treated with this enzyme, 85% of the infectivity is destroyed. A similar portion of simian virus

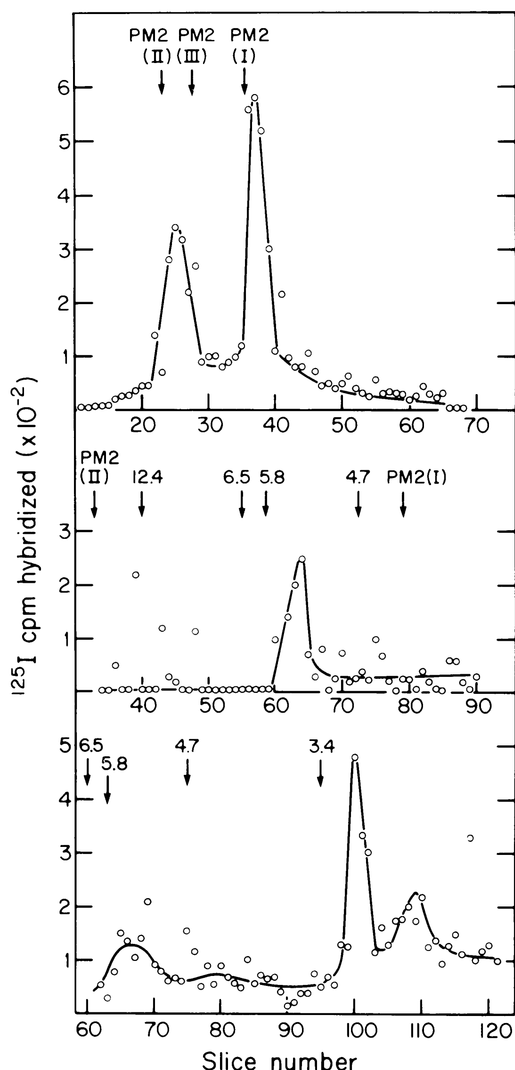

FIG. 2. *SalI endonuclease cleavage of closed circular and non-closed-circular viral DNA. (a) A mixture of form I M-MuLV DNA and II M-MuLV DNA was subjected to electrophoresis on a 0.6% agarose gel for 4 h at 4 V/cm, together with marker PM2 DNA (forms I, II, and III). (b) One-half of the amount used in (a) was treated with SalI endonuclease in 10 mM Tris-chloride (pH 7.4)-10 mM $MgCl_2$-1 mM dithiothreitol at 37°C for 1 h. Adenovirus 2 DNA (1 μg) was added as an internal control for the completion of digestion and for markers for molecular weight determinations. The DNA was treated with chloroform-isoamyl alcohol and precipitated with ethanol. The precipitate was solubilized in 10 mM Tris (pH 7.4)-1 mM EDTA buffer and subjected to electrophoresis on 1% agarose gels for 19 h at 2.5 V/cm. PM2 DNA was run on a parallel gel. (c) Non-closed-circular DNA, prepared as described in the legend of Fig. 1, was digested with SalI endonuclease and treated as described in (b). In all three experiments, viral DNA was detected in the gel slices by using a ^{125}I-labeled RNA probe. The marker arrows indicate the three forms of PM2 DNA and linear double-stranded DNA markers whose mass is shown in millions of daltons.*

form III DNA, added as an internal marker, was also degraded. Simian virus 40 form II DNA was unaffected. This enzyme sensitivity of the infectious DNA rules out once more a nicked circular conformation of the upper-band DNA.

DISCUSSION

Three different lines of evidence (ATP-dependent DNase sensitivity, agarose gel mobility, and restriction endonuclease cleavage) have together indicated that the infectious, non-closed-circular viral DNA is a linear, largely double-stranded molecule of complete genome length. No nicked circular (form II) DNA is found in these preparations. The sensitivity of this form of infectious DNA to ATP-dependent DNase rules out any significant contribution of nicked circular (form II) DNA to the infectivity of the non-closed-circular viral DNA. The reduction in infectivity of this DNA was proportional to the degree of degradation of simian virus 40 linear (form III) DNA, under conditions in which nicked circular DNA was unaffected. The absence of nicked circular viral molecules and the nonpermuted sequence indicated by restriction endonuclease cleavage together exclude the possibility that linear molecules are degradation products arising during extraction.

These linear molecules contain a small amount of single strandedness, as evidenced by their banding in NaI isopycnic gradients (11). Nevertheless, they behave very much like linear, double-stranded molecules of complete genome length (Fig. 1). We assume that the single-stranded regions occur in short, specific sites on these molecules and do not significantly affect the mobility of these molecules during agarose gel electrophoresis.

These molecules represent the bulk of the infectious, nonintegrated DNA found in the newly infected cell. They apparently have a higher specific infectivity than the closed circular form (11). We cannot explain the apparent inability of these molecules to mature further into functional, integrated genomes.

The linear molecules may well be the immediate precursors of the closed circular viral DNA. The existence of the infectious linear molecules suggests that, after the initial synthesis of the minus-strand DNA by reverse transcription, the second plus-strand of the

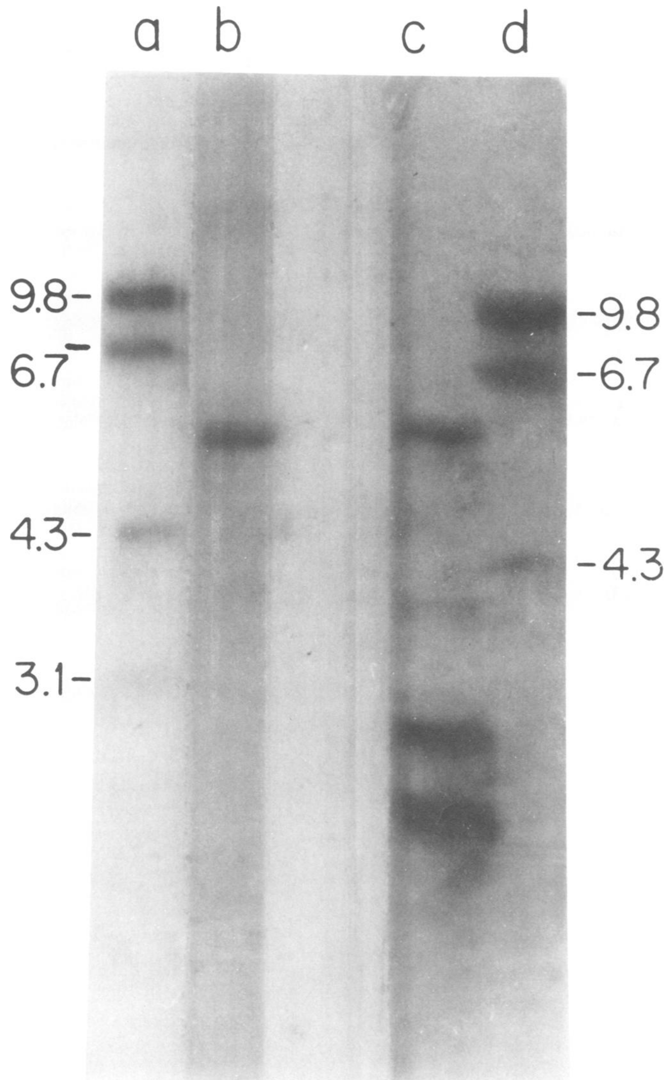

FIG. 3. *Autoradiography of cellulose nitrate filter after DNA hybridization to a ${}^{125}I$-labeled RNA probe. Both undigested and SalI endonuclease-treated non-closed-circular DNA were subjected to electrophoresis on 1.4% agarose slab gels along with BamI endonuclease-cleaved, ${}^{32}P$-labeled adenovirus 2 DNA in separate channels. Transfer of DNA to cellulose nitrate filters and hybridization to a ${}^{125}I$-labeled RNA probe were performed as described in the text. Channels a and b were exposed to Kodak X-ray film for 4 days and channels c and d were exposed for 14 days, in both cases at room temperature. Channels a and d contain BamI endonuclease-digested, ${}^{32}P$-labeled adenovirus 2 DNA as molecular weight markers (R. J. Roberts, manuscript in preparation). Channel b contains 0.04 ng of undigested non-closed-circular MuLV DNA prepared as described in the legend of Fig. 1. Channel c contains 0.02 ng of the same DNA digested with SalI endonuclease as described in the legend of Fig. 2 (part b).*

viral genome is largely completed before the subsequent circularization of the viral DNA. Such circularization may depend on the small regions of single strandedness present in these molecules, perhaps at their ends.

A similar, if not identical, infectious linear form of reticuloendotheliosis DNA has been reported recently by E. Fritsch and H. Temin (Cold Spring Harbor RNA Tumor Virus Meeting, Cold Spring Harbor, N. Y., 1975).

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