Proc. Nat. Acad. Sci. USA
Vol. 72, No. 12, pp. 4910–4913, December 1975
Biochemistry

Infectious viral DNA of murine leukemia virus

(Moloney murine leukemia virus/provirus/transfection/closed-circular DNA)

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Communicated by David Baltimore, October 8, 1975

ABSTRACT **A fraction of the unintegrated viral DNA that appears early after infection of mouse cells by Moloney leukemia virus is infectious. The infectivity could be demonstrated by an XC plaque assay of the cells exposed to DNA co-precipitated with calcium phosphate. The number of plaques deriving from closed-circular, supercoiled DNA was proportional to the concentration of added DNA, indicating that a single DNA molecule of about 5.5×10^6 daltons carries all the viral information. Nonsupercoiled viral DNA is also infectious; these molecules appear to be largely double-stranded and 5 to 6×10^6 daltons in mass.**

Following infection by RNA tumor viruses, various forms of unintegrated viral DNA appear and can be detected by molecular hybridization (1–3). Some of these forms, including the closed-circular, supercoiled form, are probable precursors to the integrated viral genome. In this report both the closed-circular, supercoiled DNA and a fraction of the nonsupercoiled DNA are shown to be infectious. The DNA infectivity assay we describe is simple and quantitative, and the number of infectious events, also referred to as "transfections," can be directly determined by counting the plaques produced in an XC assay (4). The demonstration of the biological activity of a fraction of the newly synthesized viral DNA proves that these forms contain the complete viral genetic information and thus makes stronger the hypothesis that these are intermediates in the synthesis of the integrated provirus.

MATERIALS AND METHODS

Infection. Roller bottle cultures of JLS-V9 cells, a murine line derived from Balb/c mice (5), containing about 10^8 cells per 257-mm bottle, were infected with 20 ml of a stock of cloned Moloney murine leukemia virus (M-MuLV) in the presence of 8 μg/ml Polybrene (6). The virus titers were between 1 and 2×10^7 plaque-forming units/ml as assayed by the XC plaque assay (4).

Extraction of Viral DNA. Nine hours after the start of infection, cells were extracted by the Hirt procedure (7). The supernatant fraction was twice extracted with $CHCl_3$–isoamyl alcohol followed by ethanol precipitation. This nucleic acid will be referred to as the Hirt-soluble nucleic acid. Unless otherwise noted, the nucleic acid was resuspended in 10 mM Tris·HCl, pH 7.5, 5 mM EDTA, and LiCl was added to a concentration of 2 M in order to precipitate single-stranded, high-molecular-weight RNA. The nucleic acid remaining in the supernatant was ethanol precipitated and treated with RNase A at 50 μg/ml in 1 mM Tris·HCl, pH 7.5, 1 mM EDTA for 1 hr at 37°. Closed-circular, supercoiled DNA and nonsupercoiled DNA were then separated by centrifugation in an ethidium bromide–cesium chloride (EtdBr–CsCl) isopycnic gradient as described previously (3).

Infectious DNA Assay. Transfection was performed by the method described by Graham and Van Der Eb (8, 9) in which DNA is made infectious by co-precipitation with calcium phosphate. DNA was taken up in 10 mM Tris·HCl, pH 7.5, 5 mM EDTA and diluted at least 20-fold into a buffer containing, per liter, 8.0 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4 \cdot 2H_2O$, 1.0 g of dextrose, and 5 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.05. DNA concentrations were adjusted to 20–30 μg/ml by the addition of salmon sperm DNA (Sigma). Calcium chloride was then added to a final concentration of 125 mM and incubations were carried out for 20 min at room temperature. In initial experiments cultures of NIH/3T3 cells plated on the previous day at 5×10^5 cells per dish were treated with 0.5 ml aliquots of the DNA–calcium phosphate precipitates. After 20 min at room temperature, 4.5 ml of Dulbecco's modified Eagle's medium containing 10% calf serum was added. In later experiments, the DNA–calcium phosphate precipitates were added directly to cultures of cells containing 4.5 ml of growth medium. As reported (10), this simpler technique gives identical results. The cultures were then incubated for 5 hr at which time the medium was changed. After an additional 3 hr the cells were trypsinized and seeded into 10 cm dishes at a density of 1.5×10^5 cells per dish. The procedure for the XC assay (4) was then followed. Plates were UV irradiated after 3–4 days and 6×10^6 XC cells were added. Plaques were counted 2 days later.

In several preliminary experiments infectious DNA was detected using a procedure employing DEAE-dextran (11). This method was abandoned as we have found it to be less effective than the method described above when DNA concentrations exceed 5 μg/ml.

RESULTS

The Hirt-soluble DNA of cells can be resolved into two bands after centrifugation in an EtdBr–CsCl isopycnic gradient. The upper band contains largely linear cell DNA, while most of the lower band consists of closed-circular, supercoiled mitochondrial DNA. Previous work (2, 3) has demonstrated that after oncornavirus infection, viral DNA can be detected in both bands by molecular hybridization. Analysis of the viral DNA in the lower band on alkaline sucrose gradients (2, 3) and by electron microscopic measurement† has shown it to consist of double-stranded closed-circular molecules of 5.3 to 5.7×10^6 daltons.

The supercoiled molecules are attractive candidates for the immediate precursor to the integrated form of the provi-

Abbreviations: M-MuLV, Moloney murine leukemia virus; EtdBr, ethidium bromide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid.

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† A. M. Gianni, J. R. Hutton, D. Smotkin, and R. A. Weinberg, manuscript submitted for publication.

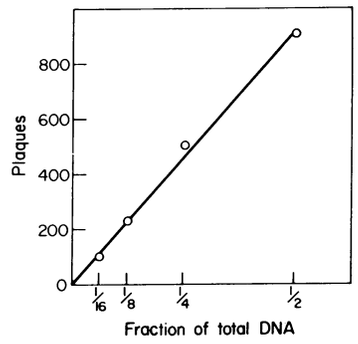

FIG. 1. Kinetics of infection by supercoiled DNA. The Hirt-soluble nucleic acid from 2.5 × 10^9 infected cells was prepared as described in *Materials and Methods* with the omission of the LiCl precipitation of RNA and RNase digestion. This nucleic acid was banded in an EtdBr–CsCl gradient and the lower band of supercoiled DNA was collected, isopropanol extracted, and ethanol precipitated with 60 μg of salmon sperm DNA as carrier. The DNA was dissolved in 10 mM Tris·HCl, pH 7.5, 5 mM EDTA and then diluted into 2 ml of Hepes-buffered saline. One milliliter was diluted by a 2-fold series into Hepes-buffered saline containing 30 μg/ml of salmon sperm DNA carrier. Aliquots (0.5 ml) were then used to transfect each of two plates by the calcium phosphate procedure described in *Materials and Methods*. Results are expressed as the sum of the plaques produced by each of the two transfected plates.

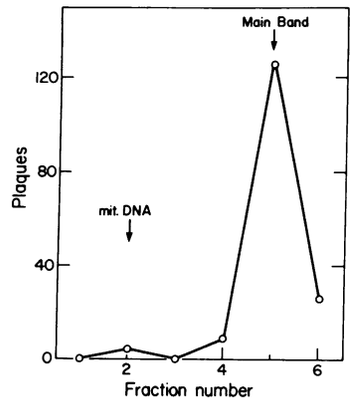

FIG. 2. Analysis of infectious viral DNA by isopycnic banding in EtdBr-CsCl. The Hirt-soluble nucleic acids from 2.5 × 10^9 cells were banded in EtdBr-CsCl as described in *Materials and Methods* without prior LiCl salt precipitation of RNA or RNase treatment. Six fractions of 0.4 ml were taken from a region of the 10 ml gradient where mitochondrial DNA (mit. DNA) and the main band of linear cell DNA could be visualized. Thirty micrograms of salmon sperm DNA was added to each fraction, followed by isopropanol extraction and ethanol precipitation. Half of the nucleic acids from each fraction was diluted into 0.5 ml of Hepes-buffered saline, and, as described in *Materials and Methods*, was used to transfect single cultures of NIH/3T3 cells.

rus, and were the first that were tested for infectivity. The supercoiled DNA from 2.5 × 10^9 infected cells was diluted by a 2-fold series and used in a transfection assay. As is shown in Fig. 1, a total of about 1800 plaque-forming units of infectious DNA was contained in this preparation, and the linearity of the dilution curve indicates that the infectivity follows single-hit kinetics. Thus, a single supercoiled molecule of 5.3 to 5.7 × 10^6 daltons appears to contain all the genetic information necessary for initiating an infection.

Further experiments were designed to establish that the virus emerging from the transfected cells was of identical genotype to that of the donor viral DNA. This would establish the fidelity of the transfection process and rule out the possibility that an activated endogeneous virus is being detected in this procedure. A culture of NIH/3T3 cells was treated with a preparation of supercoiled viral DNA and was subsequently found to be virus-producing as tested by the XC assay (4). When tested for host range (Table 1), the virus from this culture was not restricted in growth on either NIH/3T3 or Balb/3T3 cells. Thus it had the same tropism for N and B cells as M-MuLV, in contrast to the tropism of known endogenous viruses. Also, analysis of the products of T1 RNase digestion of the 70S RNAs of M-MuLV and of the virus derived from the DNA-treated culture revealed identical fingerprint maps, indicating that the nucleotide sequences of the two viruses are indistinguishable‡.

Having established that the supercoiled form of viral DNA is infectious, we investigated whether infectivity could also be detected in the nonsupercoiled DNA. As shown in Fig. 2, infectious DNA was detected in both the upper and lower bands when each fraction of an EtdBr–CsCl gradient was used in a transfection assay. Unexpectedly, the amount of infectivity in the nonsupercoiled DNA was much larger than the amount in the supercoiled DNA. Five independent experiments have shown less than 10% of the total infectivity to band in the region of supercoiled DNA, though by hybridization as much as 30% of the total viral DNA is frequently found to be supercoiled. The finding of little infectivity in the supercoiled DNA is unlikely to be explained by the conversion of these molecules to an open-circular form during extraction, since supercoiled simian virus 40 DNA added as an internal marker and carried through the extraction and centrifugation procedures was undegraded. The causes of the apparently low specific infectivity of the supercoiled DNA are not understood.

The infectivity of the nonsupercoiled DNA could derive either from newly synthesized DNA of M-MuLV, or from the DNA of an endogenous integrated viral genome released into the Hirt-soluble fraction by fragmentation of cellular DNA during the extraction procedure. As illustrated in the first two lines in Table 2, infectivity can be detected only in the Hirt-soluble DNA from newly infected cells and not in the Hirt-soluble DNA from uninfected cells. None of the infectivity detectable in newly infected cells can be attributed to the DNA of endogenous viruses.

The infectivity of this newly formed viral DNA could derive from unintegrated molecules or from M-MuLV DNA which had first integrated into the cellular chromosomal DNA and had subsequently appeared as part of the frag-

Table 1. Host range of the virus derived by DNA infection

Virus	Titer on NIH/3T3	Titer on Balb/3T3	Ratio of titer on NIH/3T3 to titer on Balb/3T3
Virus derived by DNA infection	1.6 × 10^6	5.4 × 10^5	3
M-MuLV	1.1 × 10^7	5.4 × 10^6	2
N-tropic virus*	2.0 × 10^5	3 × 10^3	66
B-tropic virus*	<20	7.4 × 10^3	<0.003

Titers were determined by XC plaque assay.

* The N-tropic virus and the B-tropic virus were obtained from Drs. J. W. Hartley and W. P. Rowe (12).

‡ K. Beemon and D. Smotkin, manuscript in preparation.

Table 2. Comparison of the infectivities in the Hirt-soluble, nonsupercoiled DNA from uninfected, newly infected, and chronically infected cells

Cells from which DNA was extracted	Number of XC plaques from the following amounts of DNA (μg/ml) co-precipitated with calcium phosphate: 20	40	80	160
Uninfected JLS-V9 cells	0	0	0	0
JLS-V9 cells infected for 9 hr	55	80	16	0
Chronically infected NIH/3T3 cells	1	0	0	0

DNA was extracted as described in *Materials and Methods* with the omission of the LiCl precipitation and the RNase digestion. From the upper bands of EtdBr–CsCl gradients 0.58 mg of DNA was recovered from 2.5×10^9 uninfected JLS-V9 cells, 0.8 mg of DNA was recovered from 2.5×10^9 newly infected JLS-V9 cells, and 3.3 mg was recovered from 7×10^9 NIH/3T3 cells chronically infected with M-MuLV. Aliquots (0.5 ml) of a calcium phosphate precipitate of various amounts of the three samples of DNA were used in the DNA infectivity assay. Results are expressed as the number of XC plaques derived from treating single plates of NIH/3T3 cells with the 0.5 ml aliquots.

mented cell DNA released as Hirt-soluble nucleic acid. To determine how much infectivity could be due to already integrated M-MuLV provirus, we measured the infectivity of Hirt-soluble, nonsupercoiled DNA from a line of NIH/3T3 cells chronically infected with M-MuLV. The cells in this line contain an integrated viral genome. As is shown in the third line of Table 2, much less infectivity was found in this DNA than in the DNA from newly infected cells. Thus, the infectious nonsupercoiled DNA must have been unintegrated prior to its extraction.

Previous studies using molecular hybridization have shown that the unintegrated nonsupercoiled viral DNA is heterogeneous both in its size (3) and in the extent of its double-strandedness (13). In order to determine whether all or just a subfraction of the nonsupercoiled viral DNA is infectious, this DNA was fractionated by two techniques. Nonsupercoiled DNA from infected cells was centrifuged in an isopycnic sodium iodide gradient. This type of gradient has been used to separate DNAs of different base composition (14) and to resolve single- and double-stranded DNAs (15). Viral DNA bands slightly denser than mouse cell DNA in CsCl (3) and would be expected to band more densely in NaI as well (14). As shown in Fig. 3, incubation of an aliquot of each fraction of a NaI gradient with radioactive M-MuLV probe (^{125}I-RNA; ref. 3) reveals that the viral DNA is heterogeneous and consists of single-stranded, partially single-stranded, and completely double-stranded molecules, in agreement with the results reported earlier (13). When the remaining portion of each gradient fraction was tested for biological activity, infectious DNA was detected only in a region just denser than double-stranded mouse cell DNA. This is a position expected for viral DNA that is largely double-stranded. The banding position of the infectious DNA is in fact slightly denser than the least dense viral-specific DNA detectable by hybridization, suggesting that the infectious DNA may contain a small proportion of single-stranded regions.

Nonsupercoiled DNA was also fractionated by centrifugation on a neutral sucrose gradient. It has been previously demonstrated by hybridization (3) that part of the nonsupercoiled viral DNA sediments as a double-stranded molecule of about 6×10^6 daltons and the rest sediments more slowly. As is shown in Fig. 4, infectivity was detectable only in a single peak of DNA sedimenting at 18 S, a rate consistent with a double-stranded molecule of 5 to 6×10^6 daltons. On the basis of these results, we conclude that only a subfraction of the nonsupercoiled DNA is infectious and that this infectious DNA is a largely double-stranded copy of the viral 35S RNA. In addition, the sedimentation of the infectious DNA as a homogeneous species in a neutral sucrose gradient provides further evidence that it is not derived from an integrated genome.

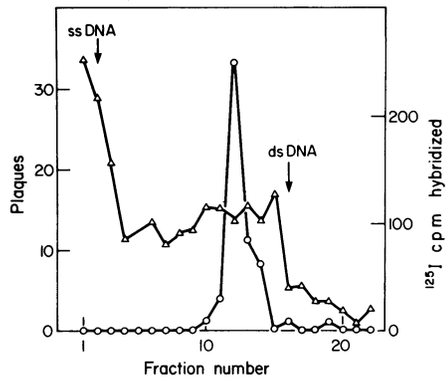

FIG. 3. Analysis of infectious nonsupercoiled viral DNA by isopycnic banding in NaI. Hirt-soluble DNA from 1.0×10^9 infected cells was prepared as described in *Materials and Methods* and banded in an EtdBr–CsCl gradient. The upper band DNA was collected and then centrifuged in a 10 ml gradient to which NaI was added to give a density of 1.546 g/cm^3. Centrifugation was carried out for 48 hr at 45,000 rpm in a Spinco 65 rotor. Fractions (0.3 ml) were collected and to each was added 30 μg of salmon sperm DNA. The fractions were split into halves. The DNA in one half was denatured by boiling and then hybridized to 2000 cpm of ^{125}I-RNA in 20 μl of 0.75 M NaCl, 0.075 M Na citrate, 10 mM EDTA, pH 5.5. After incubation for 48 hr, the amount of ^{125}I-RNA hybridized was measured by the cpm resistant to RNase A digestion (△). The remaining half of each fraction was diluted into 0.5 ml of Hepes-buffered saline and used to transfect single cultures as described in *Materials and Methods* (○). ^{3}H-Labeled native double-stranded (ds) and denatured single-stranded (ss) DNAs from mouse cells were run as internal markers.

DISCUSSION

The infectivity of the DNA of oncornaviruses was established by Hill and Hillova (16–18). The infectious viral DNA which they characterized was almost certainly the integrated form of the provirus (18). The detection of unintegrated viral DNA by molecular hybridization shortly after infection (2, 3) raised the possibility that this DNA, a probable precursor to the integrated form, was also infectious. The present work establishes that at least two forms of this DNA are infectious, the supercoiled DNA and one form of nonsupercoiled DNA. When analyzed by electron microscopy† and by alkaline sucrose gradient sedimentation (2, 3), the supercoiled form has a double-strand molecular weight of 5.3 to 5.7×10^6. As demonstrated in this report, the infectious nonsupercoiled form has a roughly similar molecular weight, is largely double-stranded, and is not likely to be derived from the supercoiled form by degradation during the extraction procedure. Whether the infectious nonsupercoiled DNA is of an open-circular or a linear conformation is not determined by the studies reported here.

The single-hit kinetics of the infectivity of the unintegrated supercoiled form indicate that a single molecule of 5.3 to

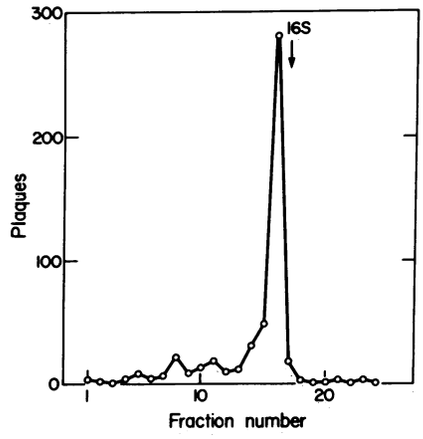

FIG. 4. Sedimentation analysis of infectious nonsupercoiled DNA on a neutral sucrose gradient. Hirt-soluble DNA from 7 × 10^8 infected cells was prepared and banded in an EtdBr–CsCl gradient as described in *Materials and Methods*. The upper band DNA was ethanol precipitated, and redissolved in 0.1 M NaCl, 0.01 M Tris, pH 7.5, 0.001 M EDTA. This sample containing 250 μg of DNA was layered over a 15–30% (weight/weight) sucrose gradient prepared in the same buffer as the sample and centrifuged at 26,000 rpm for 14.5 hr at 20° in a Spinco SW27 rotor. Thirty micrograms of salmon sperm DNA was added to each fraction of 1.5 ml. Each fraction was ethanol precipitated, redissolved in 50 μl of 10 mM Tris·HCl, pH 7.5, 5 mM EDTA. Twenty-five microliters was diluted into 0.5 ml of Hepes-buffered saline and then used to transfect one plate of NIH/3T3 cells as described in *Materials and Methods*. Linear form III simian virus 40 DNA, produced by cleavage with R_I endonuclease, was run on the same gradient as a 16S marker. Sedimentation is from right to left.

5.7 × 10^6 daltons is capable of initiating an infection. A similar conclusion was drawn in an earlier study (19) in which the infectivity of the integrated genomes of avian tumor viruses was shown to be of a single-hit nature and to reside in molecules sheared down to a minimal size of about 6 × 10^6 daltons. These results, indicating a biological complexity of 6 × 10^6 daltons of double-stranded DNA, confirm the nucleotide sequence analyses of RNA tumor viruses which indicate a chemical complexity of the virion RNA of 3 × 10^6 (20, 21).

The infectious DNA assay we describe makes use of the calcium phosphate procedure of Graham and Van Der Eb (8–10) and is a sensitive method for detecting biologically active oncornavirus DNA. Quantitation of viral DNA by molecular hybridization indicates a specific infectivity of about 10^5 plaque-forming units/μg of viral DNA, or one infectious unit per 10^6 genome equivalents. This sensitivity for M-MuLV DNA is roughly comparable to that for simian virus 40 DNA.

The reproducibility of the transfection assay has permitted a preliminary characterization of the restriction enzyme sensitivity of the viral genome. The presence of sites for cleavage by *Hpa* I (22), *Hpa* II (22), and *Bam* (23) restriction endonucleases was indicated by the inactivation of infectivity after treatment by these enzymes. However, treatment with *Escherichia coli* R_I endonuclease (24) resulted in only a slight reduction of biological activity. The suggested absence of R_I sites on the proviral genome was confirmed by the finding that after R_I treatment and rebanding in EtdBr–CsCl, supercoiled viral DNA could be recovered intact†. Mitochondrial DNA, which had been cleaved, was removed, leaving the proviral DNA virtually pure.

While this work was in progress, similar studies were reported by E. Fritsch and H. M. Temin at the Cold Spring Harbor RNA Tumor Virus Meeting of 1975.

This work was supported by Grant VC-140 from the American Cancer Society and Grants CA-14051 and CA-17537 from the National Institutes of Health. D.S. was a Predoctoral Fellow of the National Science Foundation. S.R. was an Eleanor Roosevelt Fellow of the American Cancer Society. R.A.W. is a Cancer Research Scholar of the American Cancer Society, Massachusetts Division. We thank P. A. Sharp for useful discussions.

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