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Murine Leukemia Virus: Detection of Unintegrated Double-Stranded DNA Forms of the Provirus

(Moloney murine leukemia virus/proviral genome/supercoiled DNA)

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ABSTRACT **Infection of JLS V-9 (mouse bone marrow) cells with Moloney murine leukemia virus leads to appearance of virus-specific DNA in the low molecular weight DNA fraction from infected cells. Most of this DNA is double-stranded. Two discrete forms of double-stranded DNA, both of molecular weight 6 × 10^6, have been characterized. One of these forms is closed-circular, supercoiled DNA; the other form is a mixture of nicked-circular and linear DNA.**

The RNA tumor viruses are able to transfer their genetic information from the single-stranded RNA form found in their virions to a double-stranded DNA genome covalently integrated into the chromosome of the infected host (1, 2). A series of intermediate forms must exist between the virion RNA and the integrated viral DNA genome. The present report describes two forms of double-stranded viral DNA that are putative intermediates in this flow of viral genetic information.

The double-stranded DNA molecules described here are found in a free, unintegrated state 6 hr after infection of mouse bone marrow cells (JLS V-9) by Moloney leukemia virus (M-MuLV). These DNA molecules are detected by their homology either to ^{125}I-labeled RNA of Moloney leukemia virus or to cDNA synthesized by virions of M-MuLV. They are not found in uninfected cells and are probable precursors to the integrated viral DNA genome.

While the present research was in progress, its results were in part anticipated by reports from the laboratory of Varmus and Bishop (3, 4). Their experiments were performed with avian Rous sarcoma virus. Together with the present results, they indicate a basic similarity between the nucleic acid metabolism of avian and murine RNA tumor viruses.

MATERIALS AND METHODS

Virus. Virus stocks were prepared from roller bottle cultures of NIH/3T3 cells chronically infected with a cloned line of Moloney leukemia virus (5) which were continuously perfused with fresh medium. Newly formed virus particles were withdrawn within 10 min of their synthesis from the culture and were pumped into storage vessels at 4°. Virus stocks produced under these conditions contained at least 3 × 10^7 plaque-forming units (PFU)/ml by the XC-cell assay (6).

Infection. Infections were performed with roller bottle cultures of JLS V-9 cells (7). The cultures were grown to subconfluence, 1 × 10^8 cells per 257-mm roller bottle, prior to infection. Cultures approaching confluence were not used since we found that the efficiency of infection dropped markedly as cultures became confluent.

Roller bottle cultures were exposed for 2 hr to 15 ml of a virus stock of 1 × 10^7 PFU/ml. Thirty ml of fresh medium was then added. The virus titer was determined on NIH/3T3 cells by the UV-XC assay (6). The effective multiplicity of infection of JLS V-9 cells in roller bottles was significantly higher because virtually every cell in the culture was infected under these conditions, as indicated below.

The efficiency of infection of the roller bottle JLS V-9 cultures was determined by an infectious center assay (8). JLS V-9 cells were infected at a multiplicity of 1, and 2 hr later were dispersed with trypsin and replated into new cultures of 100–800 cells per 5-cm dish. After 3–4 days, 2 × 10^6 XC cells were introduced, and the following day cultures were examined microscopically for a halo of syncytia around the colonies. Virtually all JLS V-9 clones were surrounded by XC-cell syncytia, indicating that the effective multiplicity of infection was greater than 2 per cell.

Extraction of Viral DNA from Infected Cells. Infected cells were extracted by the procedure of Hirt (9). The Hirt supernatant fraction was extracted twice with $CHCl_3$–isoamyl alcohol, and the nucleic acids were then precipitated with 2 volumes of ethanol. Nucleic acid to be analyzed directly by hybridization was treated with 0.3 M NaOH, 5 mM EDTA at 100° for 20 min, followed by neutralization with HOAc and another ethanol precipitation. Nucleic acid prepared for fractionation was precipitated directly without alkaline hydrolysis.

Hybridization. Viral DNA was detected using either [^{3}H]-cDNA or ^{125}I-labeled RNA. [^{3}H]cDNA was synthesized by incubation of Moloney leukemia virions with deoxyribonucleoside triphosphates (10). The DNA product was boiled and cooled to room temperature; the single-stranded viral DNA was isolated by hydroxylapatite chromatography. The viral [^{3}H]DNA was then annealed to an excess of 70S virion RNA to an RNA C_0t of 1 (11). The DNA that had annealed to the 70S viral RNA was then purified by hydroxylapatite chromatography and was freed of RNA by alkaline hydrolysis. This

Abbreviations: M-MuLV, Moloney murine leukemia virus; EtBr, ethidium bromide; cDNA, viral DNA synthesized *in vitro;* standard saline-citrate, 0.15 M NaCl–0.015 M Na citrate; PFU, plaque-forming units; C_0t, product of nucleotide concentration (moles) and time (sec) of incubation.

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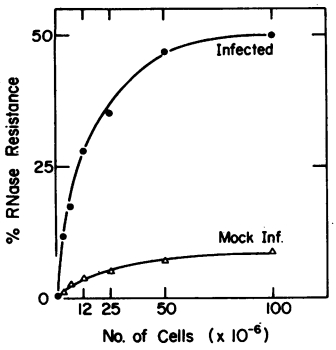

FIG. 1. Detection of virus-specific DNA in the Hirt supernate of infected cells. Cultures of JLS V-9 cells were subjected to the Hirt extraction procedure (9) at 7 hr after adsorption. Nucleic acids prepared from the Hirt supernatant fraction were subjected to alkali hydrolysis and ethanol precipitation. Constant amounts of ^{125}I-labeled RNA (1500 cpm, 1.5 × 10^7 cmp/μg) were incubated for hybridization with extracted DNA from increasing numbers of cells. Hybridization was carried out in 25 μl of 5 × standard saline-citrate (pH 5.5)–5 mM EDTA for 40 hr at 67°. Hybrid formation was assayed by measuring the acid-precipitable radioactivity remaining after incubation of the hybridization mixture at 37° for 60 min in 2 × standard saline-citrate containing 50 μg/ml of RNase A. The RNase resistance of ^{125}I-labeled RNA was determined (1%) and subtracted from the values of RNase resistance obtained after the incubations with extracted DNA.

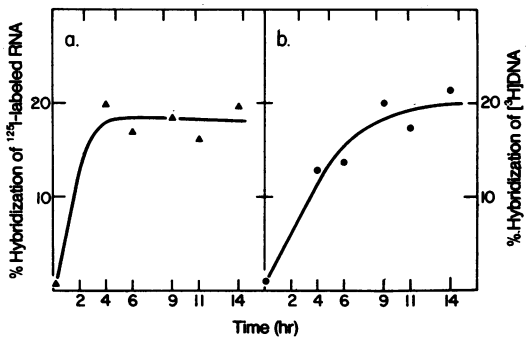

FIG. 2. Kinetics of proviral DNA synthesis. At various times after the beginning of the adsorption period, nucleic acids were extracted from aliquots of 10^7 infected cells by the Hirt procedure (9). After base hydrolysis and ethanol precipitation, samples were resuspended in 25 μl of 5 × standard saline-citrate–5 mM EDTA and incubated at 67° for 40 hr in the presence of 1500 cpm of ^{125}I-labeled RNA (panel a) or [^{3}H]DNA (panel b) of approximately the same specific activity (1.5 × 10^7 cpm/μg). The hybrids were assayed by measuring the amount of ^{125}I-labeled RNA resistant to RNase A (as in Fig. 1) or of cDNA resistant to S1 nuclease (13).

viral DNA probe had a specific activity of 1.5 × 10^7 cpm/μg and a resistance to S1 nuclease of 2%, which did not increase after further incubation. ^{125}I-labeled RNA was prepared by iodination (12) of 70S viral RNA that had previously been purified by chromatography on oligo(dT)–cellulose. After separation of the iodinated RNA from free ^{125}I by exclusion chromatography on Sephadex G-75, the ^{125}I-labeled RNA was precipitated by 2 M NaCl. It had a ribonuclease resistance of 1–2% and a specific activity of 1 to 3 × 10^7 cpm/μg.

Proviral DNA was assayed by incubation with either of the probes in 20 μl of 5 × standard saline-citrate (0.75 M NaCl, 0.075 M Na citrate)–10 mM EDTA (pH 5.5) at 67° for 48 hr. About 1500 cpm of [^{3}H]DNA or ^{125}I-labeled RNA were routinely used per hybridization. Hybridization was quantitated by resistance of [^{3}H]DNA to S1 nuclease (13) or resistance of ^{125}I-labeled RNA to pancreatic RNase at 50 μg/ml incubated for 1 hr at 37°.

RESULTS

Analysis of the unintegrated proviral DNA required a technique that would separate this DNA from the bulk of cellular DNA. Preliminary experiments indicated that the Hirt extraction (9) would remove 98–99% of the high-molecular-weight cellular DNA while allowing recovery of 80% of exogenously added simian virus 40 DNA. This procedure was superior to cell fractionation techniques for the enrichment of small-molecular-weight, unintegrated DNA.

Two probes were used for the detection of viral DNA. Viral [^{3}H]DNA was synthesized *in vitro* by incubation of M-MuLV virions in the presence of deoxyribonucleoside triphosphates. This [^{3}H]DNA was prepared so that it would hybridize solely to the DNA of the same strand orientation as virion RNA. In addition, virion RNA was labeled *in vitro* with ^{125}I to yield a probe for viral DNA of opposite strand orientation to the virion RNA.

The JLS V-9 cells used for infection are a line of Balb/c mouse cells and thus derive from a mouse strain whose DNA is known to contain a considerable amount of endogenous viral DNA sequences (14–16). These endogenous sequences exist in the cell prior to infection by an exogenous tumor virus. To determine whether newly made proviral DNA can be detected over the background of nuclear DNA contaminating the Hirt supernatant, we studied the amount of virus-specific DNA sequences present in the Hirt supernatant fraction of uninfected and infected cells (Fig. 1). By hybridizing Hirt supernatant DNA from various amounts of infected or uninfected cells to [^{3}H]cDNA, a much larger amount of virus-specific DNA was evident in the infected cell supernatant, indicating that the leakage of nuclear DNA into the Hirt supernatant does not mask the provirus.

The experiment shown in Fig. 1 also indicates that under conditions of hybridization, the probe can be saturated by DNA extracted from the infected cell. Both the ^{125}I-labeled RNA and [^{3}H]DNA probes demonstrate a plateau of hybridization at 50–70% of input radioactivity, depending upon the specific preparation of RNA or DNA. Therefore, when the amounts of proviral DNA were to be quantitated, care was taken to introduce an amount of infected cell extract sufficient to saturate only 25% of the ^{125}I-labeled RNA or [^{3}H]DNA probe. Under such conditions, the amount of radioactivity protected from nuclease is proportional to the amount of Hirt-extracted DNA added to the hybridization.

Both [^{3}H]cDNA and ^{125}I-labeled RNA were then used to quantitate the amounts of the two strands of the proviral DNA present in the Hirt supernatant at various times after infection. The ^{125}I-labeled RNA probe should react with that strand of the proviral DNA initially made by the RNA-directed DNA polymerase, while [^{3}H]cDNA should hybridize to the second strand of proviral DNA. Hirt extracts were prepared from cells from 0 to 12 hr after infection. Aliquots from each extract were hybridized to either ^{125}I-labeled RNA or [^{3}H]DNA under the subsaturating conditions discussed above. Both strands of the viral DNA appear to reach a plateau within 6 hr of infection (Fig. 2). It is our repeated observation, how-

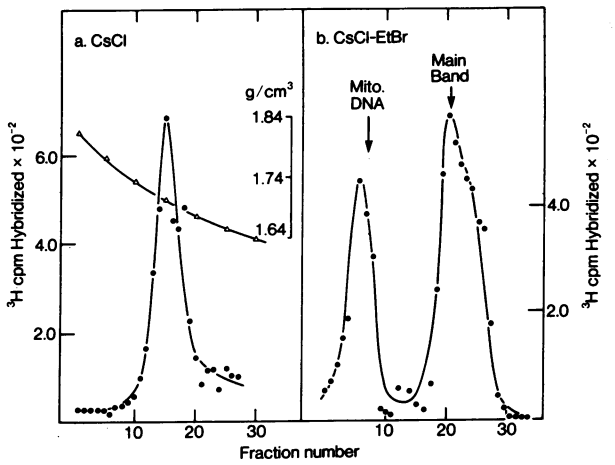

FIG. 3. Isopycnic centrifugation of proviral DNA in CsCl and in CsCl containing ethidium bromide. (a) Centrifugation in CsCl. Seven hours after adsorption, nucleic acids were extracted from 10^9 cells fractionated according to the Hirt procedure. The nucleic acids were resuspended in 8 ml of 10 mM Tris·HCl (pH 7.6)–5 mM EDTA, and solid CsCl was then added to a final density of 1.71 g/cm³. The sample was centrifuged for 50 hr at 40,000 rpm at 15° in the no. 65 Spinco rotor. Fractions of 0.3 ml were collected, NaOH was added to 0.3 M, and the samples were boiled for 20 min. The samples were neutralized with 3 M acetic acid and the nucleic acids were precipitated with ethanol after addition of 40 μg of yeast tRNA carrier. Each pellet was resuspended in 20 μl of 5 × standard saline-citrate (pH 5.5)–5 mM EDTA containing 1500 cpm of [³H]cDNA and incubated in a sealed capillary for 48 hr at 67°. The samples were then diluted into 0.5 ml of 250 mM potassium acetate (pH 4.5), 40 mM $ZnSO_4$, and 20 μg/ml of denatured calf thymus DNA, and analyzed for resistance to digestion to S1 nuclease by incubation for 60 min at 45°. (b) Centrifugation in CsCl containing ethidium bromide. Nucleic acids from infected cells were prepared as above and resuspended in 8 ml of 10 mM Tris·HCl (pH 7.6)–5 mM EDTA. CsCl was then added to a density of 1.51 g/cm³, and ethidium bromide was added to 250 μg/ml (22). The sample was centrifuged for 48 hr at 40,000 rpm in the no. 65 Spinco rotor. Sixty-five fractions of 0.15 ml were collected and processed as in panel a.

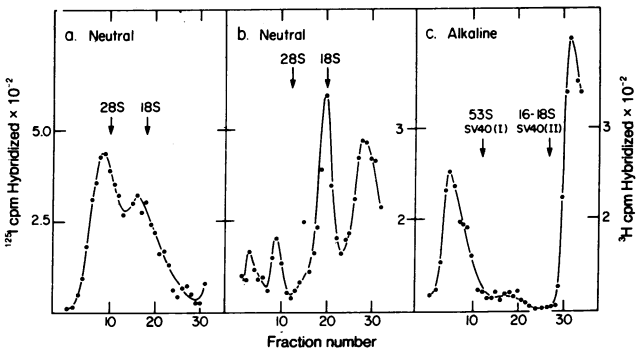

FIG. 4. Sedimentation analysis of closed-circular proviral DNA by centrifugation in neutral and alkaline sucrose gradients. (a) Centrifugation of closed-circular DNA in neutral sucrose. DNA was prepared from the lower band of a CsCl–EtBr gradient as described in the legend of Fig. 3b. Fractions containing closed-circular DNA were pooled, precipitated with ethanol, and resuspended in 10 mM Tris·HCl (pH 7.6), 0.1 M NaCl, 5 mM EDTA, and 0.2% sodium dodecyl sulfate. The sample was layered over a 15–30% (w/v) sucrose gradient containing the same buffer as the sample and centrifuged 23,000 rpm for 16 hr at 20° in the SW41 Spinco rotor. 18S and 28S ribosomal RNA markers were run in a parallel gradient. Fractions of 0.35 ml were collected and processed as in the legend of Fig. 3, with 1500 cpm of ^{125}I-labeled RNA as a probe. Ribonuclease-resistant hybrids were assayed after digestion in 2 × standard saline-citrate containing 50 μg/ml of ribonuclease A for 1 hr at 37°. (b) Centrifugation of closed-circular DNA after prolonged storage. Closed-circular DNA was prepared identically to part a. The DNA was stored for 1 month at −20° and then analyzed by centrifugation as above. Detection of hybrids was performed as in Fig. 3, using 1500 cpm of cDNA per sample. (c) Centrifugation of closed-circular DNA on an alkaline sucrose gradient. Closed-circular DNA was prepared as above and centrifuged through a 15–30% (w/v) sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl, 5 mM EDTA, and 0.1% Sarcosyl. Centrifugation was performed for 15 hr at 23,000 rpm, 20° in the Spinco SW41 rotor. [³H]cDNA probe was used as in panel b to detect viral sequences. Simian virus 40 (SV 40) [³H]DNA containing form I and form II DNAs were run as markers on the parallel gradient.

ever, that the DNA complementary to ^{125}I-labeled RNA reaches its maximum (Fig. 2a) prior to the DNA complementary to [³H]DNA probe (Fig. 2b). This is consistent with the first strand of the proviral DNA being synthesized 1–2 hr earlier than the second strand, although further experimentation will be necessary to clarify this point.

The degree of hybridization of both the [³H]DNA and ^{125}I-labeled RNA probes is comparable in Fig. 2a and b. Since these probes were of comparable specific activity, these data would indicate that by 6 hr after infection the amounts of the two strands of the proviral DNA are comparable and suggest that the majority of the proviral DNA is in the form of double-stranded DNA molecules.

Having determined an optimal time for the isolation of proviral DNA from the Hirt supernatant fraction, we then performed experiments to further characterize this DNA. Centrifugation of the DNA in CsCl (Fig. 3a) under conditions of isopycnic banding resulted in one band of detectable proviral DNA at a density of 1.7133. This density is consistent with a base composition of 54% G+C (17), a value close to the reported base composition of the virion RNA (52%; ref. 18). This value of the proviral DNA base composition is only tentative, since we have not excluded the possibility of small amounts of single-stranded virus-specific DNA in this preparation.

The proviral DNA was additionally fractionated on an ethidium bromide CsCl (EtBr–CsCl) gradient to determine whether any of the proviral DNA might be in the form of supercoiled circles (19, 20). In the presence of EtBr, a substantial proportion of the proviral DNA bands at a density indicative of closed double-stranded circular DNA (Fig. 3b). The relative positions of the light and densely banding forms of the proviral DNA would suggest a degree of supercoiling comparable to that of mitochondrial DNA.

The viral DNA found in the denser band in the EtBr–CsCl gradient was further characterized by sedimentation in sucrose gradients both at neutral and alkaline pH. In the neutral sucrose gradient (Fig. 4a), the DNA from the dense band in the EtBr–CsCl gradients sediments at a rate of about 30 S with a smaller, variable shoulder at 19–20 S. After storage for 1 month at −20°, little material continues to sediment at 30 S. Instead, a prominent peak is now observed at about 19 S. In an alkaline sucrose gradient (Fig. 4c), half of the viral DNA is seen to sediment very rapidly with a sedimentation coeffi-

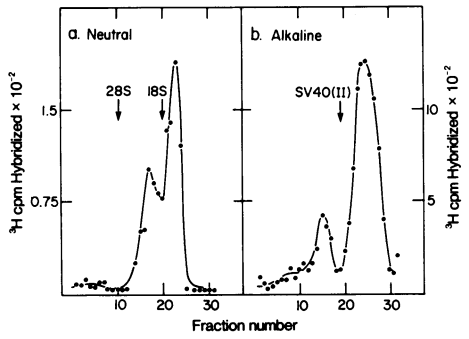

FIG. 5. Sedimentation analysis of linear and nicked-circular DNA by centrifugation in neutral and alkaline sucrose gradients. (a) Sedimentation in a neutral sucrose gradient. DNA was prepared from the upper, less-dense band of a CsCl–EtBr gradient (Fig. 3b) and processed by the procedures of Fig. 4a, except that 1500 cpm of [^{3}H]cDNA was used as a probe for viral sequences in each sample. (b) Sedimentation in an alkaline sucrose gradient. DNA was prepared from the upper, less-dense band of a CsCl–EtBr gradient (Fig. 3b) and processed by the procedures of Fig. 4c, except that the centrifugation was performed for 18 hr at 30,000 rpm.

cient of 70 S. The radioactivity detected at the top of this gradient is possibly an artifact deriving from inhibition of the S1 nuclease by slowly sedimenting material.

The rapid sedimentation of this material in the neutral and alkaline sucrose gradients and the EtBr–CsCl banding behavior together indicate double-stranded, closed-circular DNA molecules containing no alkali-labile linkages and of molecular weight 6×10^6. After storage, the acquisition of one or more single-stranded breaks (Fig. 4b) has converted this molecule into a more slowly sedimenting, nicked-circular form of equal molecular weight. Both these molecules carry an amount of genetic information comparable to one 35S subunit of virion RNA.

The DNA found in the less-dense band of the EtBr–CsCl gradient (Fig. 3b) was also analyzed by sedimentation under neutral and alkaline conditions. As seen in Fig. 5, this virus-specific DNA contains two components in both the neutral and alkaline sucrose gradients. The rapidly sedimenting component behaves like double-stranded linear or nicked-circular DNA of 6×10^6 daltons. The slowly sedimenting material appears from its sedimentation rate to contain DNA fragments of about 5×10^5 daltons.

DISCUSSION

The demonstration by others (3, 4) and the present report indicate that proviral DNA exists as a free molecule for a substantial length of time prior to its integration into the cellular DNA. The kinetics of its synthesis, migration to the nucleus, and the subsequent increase in integrated viral sequences (4) strongly indicate its physiological role as precursor to the integrated viral genome. Unpublished experiments in which we have saturated proviral DNA with ^{125}I-labeled viral RNA suggest that about one proviral DNA molecule is made per infected cell.

The most striking of the various molecular forms of the proviral DNA is the closed-circular, double-stranded form demonstrated here and in the work of Varmus *et al.* (4). Much of the material found in the lower band of an EtBr–CsCl isopycnic gradient behaves like a molecule of 6×10^6 daltons having about the same density of supercoiling as mitochondrial DNA. This form of the proviral DNA has no alkali-labile linkages, as shown by its rapid sedimentation in alkaline conditions.

Table 1 summarizes the sedimentation properties of this supercoiled form and other forms of the proviral DNA. As expected, partial or complete nicking of the supercoiled form (Fig. 4a and b) generates a circular form sedimenting at two-thirds the initial rate. The upper band of the EtBr–CsCl isopycnic gradient may contain much of this nicked-circular form (form II) along with linear double-stranded forms (form III). These forms are not clearly resolved by the centrifugation techniques used here (Fig. 5a). In addition, a very large proportion of the proviral DNA from this upper band consists of low-molecular-weight fragments. Their size can only be estimated from the alkaline velocity gradient, which suggests a molecular weight of about 5×10^5. Guntaka *et al.* (3) have also observed similar low-molecular-weight fragments. The clarification of the roles of forms II and III and these fragments also awaits detailed kinetic studies. The forms may be precursors or degradation products of the supercoiled form I, or even side-products resulting from aborted syntheses.

The present results tend to exclude the existence of large amounts of free single-stranded proviral DNA. No single-stranded DNA of whole genome size (3×10^6) is detectable upon neutral sedimentation analysis (Fig. 5a). Also, the kinetics of accumulation of the two strands of the proviral DNA indicate a comparable concentration of both strands of the proviral DNA by 6 hr after adsorption, which suggests a relatively small amount of single-stranded material. None of these analyses deals with DNA·RNA hybrids, which might also be expected but would band too densely for detection under the conditions found here.

Studies on the kinetics of synthesis of proviral DNA indicate that by 6 hr after adsorption, the growth cycle of Mo-

TABLE 1. *Predicted and observed sedimentation rates of molecular forms of a genome of 6×10^6 double-stranded molecular weight*

	Predicted sedimentation rate	Ref.	Observed
Form I, neutral pH	28S, 30S	23, 27	30S
Form I, alkaline pH	64S	28	68–70S
Form II, neutral pH	21S, 22S	23, 27	21S
Form III, neutral pH	18.5S	24	
Form II, alkaline pH	20S and 23S	24–27	22S
Form III, alkaline pH	20.6S	24	
Single-stranded, 3×10^6 daltons	37S	24	Not observed

Form I is closed-circular double-stranded DNA; form II is nicked-circular double-stranded DNA; form III is linear double-stranded DNA. Sedimentation values were predicted from the formulae or data given in the cited references.

loney leukemia virus becomes insensitive to disruption by the DNA analogue, bromodeoxyuridine (D. Smotkin and R. Weinberg, unpublished observations). Such evidence would indicate that the plateau in the level of proviral DNA seen by this time (Fig. 2) is largely a reflection of the cessation of the synthesis of new proviral DNA. Much later, the level of free proviral DNA might be expected to decline as the DNA becomes converted to forms no longer extractable by the Hirt procedure (2, 9).

The intracellular location of the proviral DNA is not indicated by the extractability of this DNA with the Hirt procedure, which is specific only for relatively small molecules of DNA, irrespective of their intracellular origin (9). A technique for preparation of proviral DNA by the phenol fractionation procedure, known to yield only cytoplasmic nucleic acid (21), yielded an equivalent amount of proviral DNA (unpublished results). Most compelling, however, is the evidence derived from infection of enucleated avian cells, which indicates that proviral synthesis of Rous sarcoma virus is a cytoplasmic event (4).

The biological roles of the various forms of proviral DNA remain to be proven. The steps in the pathway from RNA-directed DNA synthesis to supercoiled DNA are still largely unexplained. One can now say, however, that the supercoiled proviral DNA fulfills the characteristics of a molecule postulated by Campbell as the topologically acceptable form of the integrating λ genome (22).

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