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Fate of Unintegrated Viral DNA in *Fv-1* Permissive and Resistant Mouse Cells Infected with Murine Leukemia Virus

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We have found that levels of unintegrated linear viral DNA were nearly identical in several *Fv-1* resistant cell lines, whereas levels of closed circular viral DNA are markedly reduced in these resistant cells, to the same extent as virus production (P. Jolicoeur and E. Rassart, J. Virol. 33:183-195, 1980). To determine the fate of linear viral DNA made in resistant cells we performed pulse-chase experiments, labeling viral DNA with 5-bromodeoxyuridine and following it with a thymidine chase. 5-Bromodeoxyuridine-labeled viral DNA (HH) recovered by banding on cesium chloride gradients was sedimented on neutral sucrose density gradients or separated by the agarose gel-DNA transfer procedure and detected by hybridization with complementary DNA. Levels of linear viral DNA made in *Fv-1*$^{b/b}$ (JLS-V9 and SIM.R) and *Fv-1*$^{n/n}$ (NIH/3T3 and SIM) cells were found to decrease during the chase period at about the same rate in permissive and nonpermissive conditions, indicating that linear viral DNA is not specifically degraded in *Fv-1* resistant cells. Levels of the two species of closed circular viral DNA made in *Fv-1* permissive cells increased relative to the levels of linear DNA during the chase period. This confirmed the precursor-product relationship between linear DNA and the two species of circular DNA. In *Fv-1* resistant cells, this apparent conversion of linear viral DNA into circular forms was not seen, and no supercoiled viral DNA could be detected. To determine whether the transport of linear viral DNA from the cytoplasm into the nucleus was prevented by the *Fv-1* gene product, SIM.R cells were fractionated into cytoplasmic and nuclear fractions, and viral DNA was detected in each fraction by the agarose gel-DNA transfer procedure. Levels of linear viral DNA were nearly identical in both cytoplasmic and nuclear fractions of permissive or resistant cells. Circular viral DNA could be detected in the nuclear fraction of permissive cells, but not in that of resistant cells. A pulse-chase experiment was also performed with SIM.R cells. During the thymidine chase period, linear viral DNA was seen to accumulate in nuclei of both permissive and resistant cells, whereas supercoiled viral DNA accumulated only in nuclei of permissive cells. These results indicate that the *Fv-1* gene product does not interfere with the transport of linear viral DNA into the nucleus. Our data also suggest that the *Fv-1* restriction does not operate through a degradation process. Therefore, the *Fv-1* gene product could either block the circularization of linear viral DNA directly or promote the synthesis of a faulty linear viral DNA whose defect (yet undetected) would prevent its circularization.

In mice and in cultured murine cells, a single autosomal gene (*Fv-1*) determines susceptibility or resistance to infection by many strains of murine leukemia virus (MuLV) (for review, see references 8 and 14). The two main alleles of *Fv-1*, *Fv-1*^{b} and *Fv-1*^{n}, are dominant and confer resistance to replication of N-tropic and B-tropic viruses, respectively (5, 16). In consequence, replication of B-tropic viruses is restricted in *Fv-1*$^{n/n}$ cells and growth on N-tropic viruses is restricted in *Fv-1*$^{b/b}$ cells.

The steps of the virus cycle which might be impaired by the *Fv-1* gene product have been extensively studied. Several biological experiments have indicated that the *Fv-1* gene product operates after penetration of the virus into the cell and during the early phase of the virus cycle (2, 7, 13, 26). Experiments designed to measure virus-specific nucleic acids confirmed these biological results on the early restriction of the *Fv-1* gene product. Indeed, it was found that levels of virus-specific RNA were decreased in *Fv-1* resistant cells (10), and we (11) and others (23) have shown that integration of viral DNA into the cell genome was prevented in *Fv-1* resistant cells, whereas accumulation of unintegrated

viral DNA was not grossly affected by the *Fv-1* gene product. In a more detailed study we have recently reported that accumulation of closed circular viral DNA was markedly reduced in *Fv-1* resistant cells, whereas levels of linear double-stranded viral DNA were much less affected in these cells (12). We postulated four models to explain these results: (i) a block of circularization of linear viral DNA by the *Fv-1* gene product, (ii) synthesis of a faulty linear viral DNA in resistant cells, (iii) a selective degradation of circular viral DNA in resistant cells, and (iv) a block of transport of linear viral DNA into the nucleus, where circularization occurs. In an effort to determine the site of action of the *Fv-1* gene product more precisely, we have directly tested the last hypothesis and have studied the fate of linear viral DNA made in *Fv-1* permissive and nonpermissive cells. We report that the stability of linear viral DNA and its transport into the nucleus are not significantly affected by the *Fv-1* gene product.

MATERIALS AND METHODS

Cells and viruses. The origins of JLS-V9, NIH/3T3, SIM, SIM.R, and BALB/3T3 cells have been given elsewhere (9). Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum (GIBCO). The cloned N-tropic (N-Cl-35) and B-tropic (B-Cl-11) endogenous BALB/c viruses have been described elsewhere (9). The N- and B-tropic Friend leukemia viruses were obtained from Ruy Soeiro (Albert Einstein College of Medicine, Bronx, N.Y.). The virus titers of stocks were 1×10^6 to 8×10^6 PFU/ml as determined on permissive NIH/3T3 or BALB/3T3 cells by the XC plaque assay (17). Moreover, the infectivity of each stock of N- or B-tropic virus was tested at lower dilution by measuring the reverse transcriptase activity of the progeny virus 1, 2, and 3 days after infection of permissive SC-1 cells.

Virus infection and measure of *Fv-1* restriction. Conditions of infection in 257-mm roller bottles have been described elsewhere (12). Briefly, roller bottles were seeded with 4×10^6 cells per bottle and infected 16 to 24 h later with 10 ml of virus suspension in the presence of 8 μg of Polybrene (Aldrich) per ml (27). Cells were exposed to the virus for 6 or 12 h, as indicated in the legend to each figure. To validate our biochemical findings, the *Fv-1* restriction was measured by a biological assay in each experiment on sister rollers (also infected in the presence of 5-bromodeoxyuridine [BUdR] when indicated). The progeny virus was measured by its reverse transcriptase activity 1, 2, and 3 days after infection (10, 12).

BUdR labeling. Viral DNA was labeled with BUdR by infecting cells with N- or B-tropic virus suspensions containing 8 μg of Polybrene per ml and 5 μg of BUdR (Sigma) per ml for 5 to 12 h in the dark. Samples were protected from light until final analysis.

Cell fractionation and extraction of viral DNA. The technique for the extraction of unintegrated viral DNA from cells has been described (11). The Hirt procedure (6) was followed. Briefly, cells were lysed in situ by the addition of a solution containing 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M EDTA, and 0.6% sodium dodecyl sulfate (10 ml per roller bottle). This cell lysate was collected, brought to 1 M NaCl, left at 4°C for 12 to 15 h, and centrifuged. The pellet was discarded, and the supernatant (Hirt supernatant), containing unintegrated viral DNA, was first extracted with a mixture (1:1) of phenol-$CHCl_3$–4% amyl alcohol before precipitation with 2 volumes of ethanol at −20°C. The procedure for fractionation of cytoplasm and nuclei has been described (10). For extraction of viral DNA from cytoplasm, the cytoplasm was brought to 0.5% (final concentration) sodium dodecyl sulfate and extracted three times with phenol before ethanol precipitation. For Hirt extraction of unintegrated viral DNA from the nuclei, nuclei were suspended in 0.01 M EDTA–0.01 M Tris-hydrochloride (pH 7.5) and lysed with 0.6% (final concentration) sodium dodecyl sulfate at room temperature for 30 min. This lysate was then processed as described above.

CsCl density gradients. BUdR-substituted viral DNA was fractionated by equilibrium banding in density gradients of CsCl. Samples of DNA extracted from the Hirt supernatant were resuspended in 6 ml of 0.01 M Tris-hydrochloride (pH 7.5)–0.01 M EDTA and added to 8.2 g of CsCl (Beckman). Centrifugation was for 48 to 60 h at 33,000 rpm at 20°C in a Beckman type 50 or 75 rotor.

Sucrose gradient sedimentation. Samples were layered over 15 to 30% (wt/vol) sucrose gradients containing 0.01 M Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 5 mM EDTA, and 0.2% sodium dodecyl sulfate. The gradients were centrifuged at 23,000 rpm for 16 h at 20°C in an SW41 Spinco rotor. After centrifugation, fractions of 0.4 ml were collected from the bottom of the tube.

Agarose slab gel electrophoresis and transfer. DNA samples were layered onto separate channels of a vertical slab gel (20 by 20 by 0.3 cm) cast with 1% agarose (Sigma). DNA was separated by electrophoresis in 40 mM Tris (pH 8.3)–50 mM sodium acetate–1 mM EDTA for 18 to 22 h at 35 mA per gel. After electrophoresis, gels were stained with 0.5 μg of ethidium bromide per ml in electrophoresis buffer and photographed with Polaroid film, using UV illumination (21). The DNA which had been resolved by electrophoresis was then denatured and neutralized in situ and transferred onto nitrocellulose sheets (0.45-μm pore size, Millipore) with 20× SSC (1× SSC is 0.15 M sodium chloride–0.015 M sodium citrate) as described previously (22).

Synthesis and purification of viral cDNA. ^{3}H-labeled complementary DNA (cDNA) was prepared from banded B-tropic Friend virions as described previously (12). Its specific activity was 2×10^7 cpm/μg. B-Cl-11 [^{32}P]cDNA was synthesized as described previously (12). Its specific activity varied between 2×10^8 and 4×10^8 cpm/μg. In each case, calf thymus oligonucleotides (2 mg/ml) were used as primers (25).

Liquid hybridization procedure. Viral DNA in each fraction from the sucrose gradients was detected by molecular hybridization after acid depurination

(15). Hybridization of [^{3}H]cDNA to virus-specific DNA was measured by S1 nuclease digestion as described previously (12).

Hybridization to DNA on nitrocellulose filters. After transfer of the DNA to nitrocellulose filters, the filters were baked at 80°C for 2 h. Filters were then soaked for 10 to 12 h at room temperature in 25 to 100 ml of 3× SSC solution containing 50% formamide and 100 μg of calf thymus DNA per ml and supplemented with Denhardt solution (0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin) as described previously (18). The wet filters were then incubated at 39°C for 30 to 48 h in plastic bags with 3 ml of the same solution containing 3×10^6 to 6×10^6 cpm of [^{32}P]cDNA. After annealing, filters were washed in 2× SSC for 15 min, in 0.1× SSC–0.1% sodium dodecyl sulfate solution for 1 h at 50°C, and then in 0.1× SSC at 20°C for 5 min (19). Filters were air dried and exposed at −20°C to Kodak-RP-Royal X-Omat film, using a Du Pont Cronex Lightning Plus intensifying screen (24).

RESULTS

BUdR labeling of viral DNA and efficiency of thymidine chase. With most retroviruses only a few copies of viral DNA are synthesized per infected cell (for review, see reference 29). Therefore, labeling this viral DNA with radioactive precursors has been impractical (28). However, it was discovered that viral DNA synthesized in duck embryo fibroblasts infected with avian sarcoma viruses could be density labeled with BUdR (28). For pulse-chase experiments, it was also found that thymidine could block the incorporation of BUdR into viral DNA efficiently (20).

In experiments designed to fractionate unintegrated viral DNA from endogenous viral sequences, we have previously found that viral DNA synthesized in mouse cells infected with MuLV could also be efficiently labeled with BUdR (12). To determine whether the incorporation of BUdR into MuLV viral DNA could be blocked by thymidine during the chase period of a pulse-chase experiment, JLS-V9 cells were first incubated with medium containing BUdR for 3.5 h and then infected with a B-tropic Friend virus suspension containing either BUdR (5 μg/ml) or thymidine (10 μg/ml). Uninfected cells were incubated with BUdR and then with thymidine. After 5.5 h of infection, cells were lysed by the Hirt procedure, and DNA, purified from the Hirt supernatant, was centrifuged in CsCl gradients. Viral DNA was detected by hybridization to [^{3}H]cDNA. Virus-specific DNA extracted from uninfected cells banded at two densities in CsCl gradients corresponding to unsubstituted DNA (LL) (1.70 to 1.71 g/cm^3) and to DNA substituted with BUdR in one strand (HL) (1.74 to 1.75 g/cm^3) (Fig. 1A). These two species most likely represented endogenous viral sequences. Most virus-specific DNA extracted from cells infected in the presence of BUdR banded at a density of 1.78 to 1.80 g/cm^3, as expected for DNA molecules substituted with BUdR in both strands (HH). However, when cells preincubated in BUdR-containing medium were infected in the presence of thymidine, most of the virus-specific DNA banded at a density of 1.70 to 1.71 g/cm^3 (Fig. 1B) and was therefore unsubstituted (LL). This result indicated that the addition of thymidine had blocked BUdR incorporation into viral DNA completely. This experiment showed that viral DNA synthesized in MuLV-infected mouse cells could be pulse-labeled with BUdR and chased efficiently.

Pulse-chase experiment with BUdR-labeled viral DNA made in *Fv-1$^{b/b}$* cells infected with N- or B-tropic viruses. The majority of unintegrated viral DNA molecules synthesized in cells infected with retroviruses have a linear double-stranded structure and sediment at 20S to 22S on neutral sucrose gradients (4, 28). We have shown that levels of this viral DNA species were almost the same in a number of *Fv-1* permissive or resistant newly infected cells (12).

To study the stability of the linear viral DNA species in *Fv-1* permissive and resistant cells and to determine whether the *Fv-1* gene product was favoring its degradation, we have performed pulse-chase experiments by labeling viral DNA with BUdR and following its fate during a chase period with thymidine. The length and levels of viral DNA were first assessed by sedimentation on neutral sucrose gradients and hybridization to [^{3}H]cDNA. JLS-V9 cells (*Fv-1$^{b/b}$*) were infected with N- or B-tropic Friend MuLV in the presence of BUdR (5 μg/ml). We showed before (10, 12) that N-tropic MuLV replication was significantly reduced in these conditions. In this experiment, levels of progeny viruses in N-tropic-MuLV-infected resistant JLS-V9 cells were reduced by 20- to 25-fold compared with levels produced by B-tropic-MuLV-infected permissive cells. Uninfected cells were incubated with BUdR and served as a control. After 11 h in the presence of BUdR, a group of cells from each of the N-tropic-MuLV-infected, B-tropic-MuLV-infected, and uninfected cell cultures was extracted by the Hirt procedure. The medium of the remaining cells was substituted with fresh medium containing thymidine (10 μg/ml). These cells were then incubated for different lengths of time (3, 6.5, and 14 h). After this chase period, cells of each group were also lysed by the Hirt procedure. DNA purified from Hirt supernatant

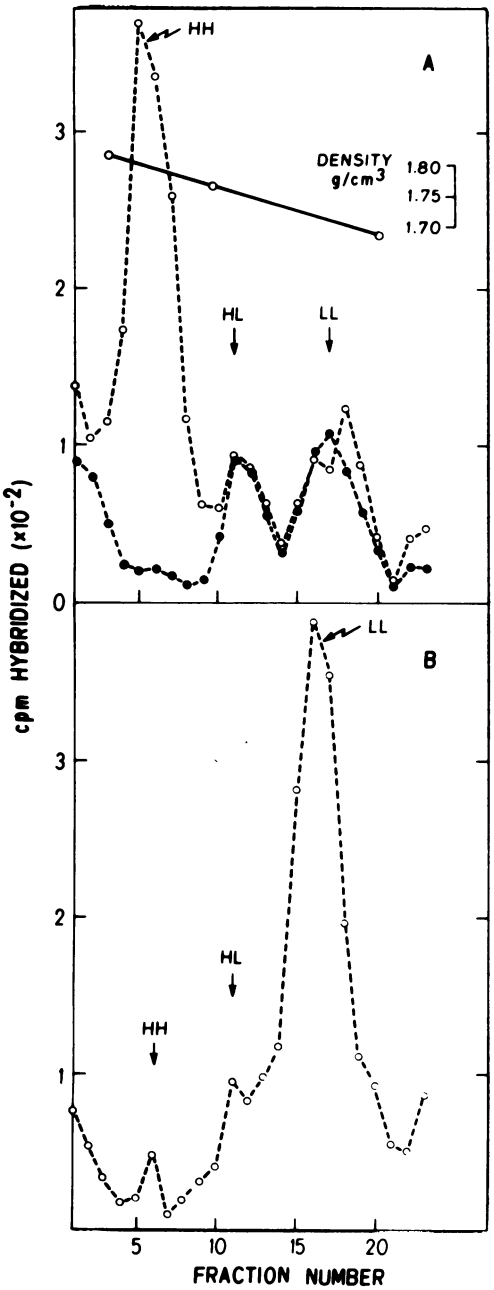

FIG. 1. *BUdR density labeling of viral DNA and efficiency of a thymidine chase. Subconfluent JLS-V9 cells (9.6 × 10⁷ cells) were incubated for 3.5 h in the presence of BUdR (5 μg/ml). Cells were washed once with normal medium and divided into three groups. One group was infected with B-tropic Friend virus (multiplicity of infection, 2) in the presence of BUdR (5 μg/ml) and Polybrene (8 μg/ml). Another group was infected with B-tropic Friend virus in the presence of thymidine (10 μg/ml) and polybrene. A third group was not infected and was incubated in the presence of thymidine (10 μg/ml) and served as control. After 5.5 h in the dark at 37°C, cells were washed with phosphate-buffered saline and extracted by the Hirt procedure. The DNA was extracted from the Hirt supernatant and centrifuged on CsCl gradients. Fractions were collected, and their densities were determined from the refractive indexes before processing for hybridization with B-tropic Friend [³H]cDNA (1,543 cpm) as described in the text. (A) Hirt supernatants DNA from uninfected cells (●) and cells preincubated with BUdR and infected in the presence of BUdR (○). (B) Hirt supernatant DNA from cells preincubated with BUdR and infected in the presence of thymidine.*

was banded in CsCl gradients. Fractions with densities higher than 1.76 g/cm³ were pooled, and BUdR-substituted viral DNA was further analyzed on neutral sucrose gradients by hybridization to [³H]cDNA. B-tropic cDNA could be used to detect both N-tropic and B-tropic-Friend-virus-specific DNA because the two MuLV genomes are highly homologous (12, 23). Figure 2 displays the patterns of virus-specific DNAs detected in these groups. Nearly identical amounts of 20S linear virus-specific DNAs could be detected in cells infected with N- and B-tropic viruses 11 h after infection (Fig. 2A and E), in agreement with our previous results (12). A minor viral DNA species migrating at 25S to 27S was also frequently observed. Preliminary experiments have indicated that it does not represent supercoiled molecules (P. Jolicoeur and E. Rassart, unpublished data) expected to sediment at 28S to 30S (3). When the incorporation of BUdR into viral DNA was blocked by thymidine and the fate of fully substituted (HH) viral DNA studied during this chase period, it could be observed that levels of 20S viral DNA species decreased in B-tropic-MuLV-infected cells (permissive conditions) (Fig. 2B through D). At the same time a new viral DNA species migrating at about 30S was observed from 3 to 6.5 h after the beginning of the chase (Fig. 2B and C). This viral DNA species migrated with the same sedimentation coefficient as supercoiled Moloney MuLV DNA (4), suggesting that some 20S linear viral DNA might have been converted to a closed circular form during the chase period, as shown below.

Under *Fv-1* nonpermissive conditions, levels of 20S linear viral DNA also progressively decreased during the chase, but at a slower rate than in permissive cells (Fig. 2E through H). Indeed, as we have previously estimated (12), the levels of 20S linear viral DNA seen at the end of the chase period in both permissive and resistant conditions (cf. Fig. 2D and H) varied by approximately three- to eightfold. This result indicated that the *Fv-1* restriction was not de-

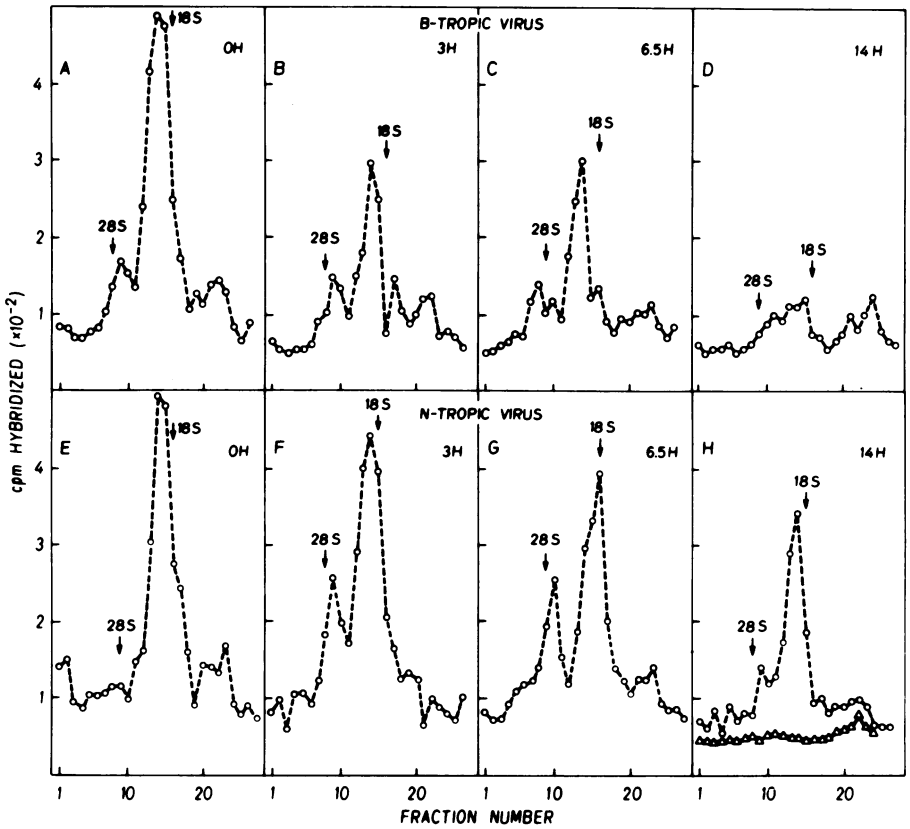

FIG. 2. *Pulse-chase studies on viral DNA from JLS-V9 cells infected with N- or B-tropic Friend MuLV. Subconfluent JLS-V9 cells (2.5 × 10⁶ cells) were infected with N-tropic and B-tropic Friend viruses (multiplicity of infection, 1) in the presence of BUdR (5 μg/ml) and Polybrene (8 μg/ml) for 11 h. One group (3.2 × 10⁷ cells) was not infected and was incubated with BUdR and Polybrene only. After this incubation, 3.2 × 10⁷ cells from the uninfected group and each of the infected groups were extracted by the Hirt procedure (A, E). The remaining cells were subjected to the chase procedure and incubated with medium containing thymidine (10 μg/ml) for 3 (B, F), 6.5 (C, G), and 14 (D, H) h. At each time, 3.2 × 10⁷ cells from each group were extracted by the Hirt procedure. The DNA was extracted from the Hirt supernatant and centrifuged on CsCl gradients. Fractions were collected, and all fractions with densities above 1.76 g/cm³ were pooled and diluted sixfold with water before precipitation with ethanol in the presence of 200 μg of yeast RNA as carrier. Nucleic acids were suspended in 0.01 M Tris (pH 7.5) and centrifuged on 15 to 30% neutral sucrose gradients. Fractions were collected and processed for hybridization with B-tropic Friend [³H]cDNA (1,800 cpm) as described in the text. Five hours after the beginning of infection, the medium of one roller bottle of each infected group was changed and collected daily for 3 days to measure its reverse transcriptase activity. The medium harvested on day 3 after infection from N-tropic-MuLV-infected JLS-V9 cells gave 25,728 cpm, whereas medium from B-tropic-MuLV-infected JLS-V9 cells gave 595,101 cpm. Symbols: △, Hirt supernatant DNA from uninfected JLS-V9 cells; ○, Hirt supernatant from JLS-V9 cells infected with B-tropic (A to D) or N-tropic (E to H) MuLV.*

grading linear viral DNA, but was slightly retarding its processing. No virus-specific DNA migrating at 30S was detected in N-tropic-MuLV-infected *Fv-1* resistant cells. This finding is compatible with our previous experiments in which lower amount of supercoiled viral DNA was found in *Fv-1* resistant cells (12).

From this experiment with JLS-V9 cells, it appears that the *Fv-1* gene product does not prevent the formation of linear viral DNA in *Fv-1* resistant cells. After linear viral DNA has been synthesized in these cells, it is not specifically degraded by the *Fv-1* gene product.

Pulse-chase experiments of BUdR-labeled viral DNA made in *Fv-1*$^{n/n}$ cells infected with N- or B-tropic virus. To determine whether the *Fv-1*^{n} allele had the same effect on the stability of linear viral DNA, NIH/3T3 cells (*Fv-1*$^{n/n}$) were infected with N-tropic or B-tropic Friend MuLV in the presence of

BUdR, and BUdR-labeled viral DNA was studied during a thymidine chase (Fig. 3). As in the previous experiment, virus-specific DNA of density superior to 1.76 g/cm^3 was first collected on CsCl gradients but was subsequently separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and detected by hybridization to B-Cl-11 [^{32}P]cDNA. B-Cl-11 [^{32}P]cDNA could be used to detect both of the highly homologous N- and B-tropic Friend MuLVs, because the three genomes share sufficient sequences to allow reasonable detection by this procedure. At the end of the BUdR pulse period, linear viral DNA (form III) could be detected in N- and B-tropic-MuLV-infected cells (Fig. 3a and e), despite a 150-fold reduction of progeny virus in B-tropic-MuLV-infected resistant NIH/3T3 cells. Levels of this viral DNA species were lower in resistant cells than in permissive cells, by a factor of three- to fivefold as estimated previously by endpoint dilution (12). In both groups, amounts of linear viral DNA were relatively stable during the chase period and decreased only slightly (Fig. 3b through d and f through h). In agreement with our previous results (12), both species of form I supercoiled DNA could be detected in higher amounts in N-tropic-MuLV-infected cells (permissive conditions) than in B-tropic-MuLV-infected cells (nonpermissive conditions). Under permissive conditions, levels of form I viral DNA increased between 0 and 6.5 h of the chase period (Fig. 3f and g), suggesting that some linear viral DNA was converted into two species of closed circular DNA species. Between 6 and 14 h of the chase period, levels of both form I DNA species decreased (Fig. 3h), whereas the level of linear viral DNA remained relatively stable. This late decrease of supercoiled viral DNA suggests that both form I species are specifically degraded after this period or, alternatively, that they both integrate into the cell genome. The apparent conversion of linear viral DNA into two species of supercoiled DNA was not observed in *Fv-1* resistant cells (Fig. 3b through d).

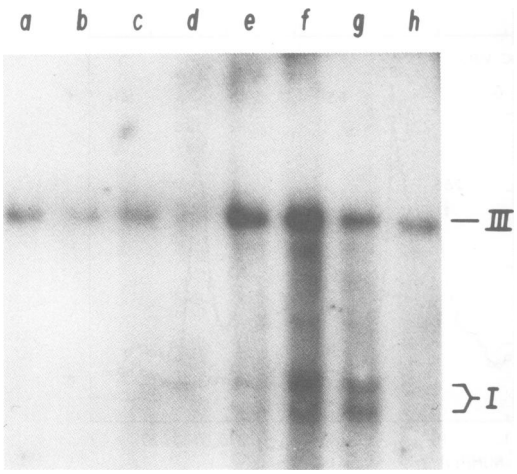

FIG. 3. *Pulse-chase studies on viral DNA from NIH/3T3 cells infected with N- or B-tropic Friend MuLV. Subconfluent NIH/3T3 cells (3.2 × 10^7 cells) were infected with N-tropic and B-tropic Friend MuLV (multiplicity of infection, 1) in the presence of BUdR (5 μg/ml) and Polybrene (8 μg/ml) for 11 h. Medium was then removed, and 4 × 10^6 cells from each group were extracted by the Hirt procedure. The remaining cells were subjected to the chase procedure and incubated with medium containing thymidine (10 μg/ml) for 3, 6, and 14 h. At each time, 4 × 10^6 cells from each group were extracted by the Hirt procedure. The DNA was extracted from the Hirt supernatant and centrifuged on CsCl gradients. DNA in fractions with densities above 1.76 g/cm^3 was subjected to agarose gel electrophoresis with EcoRI-digested bacteriophage lambda DNA as a marker. DNA was transferred to nitrocellulose filters and hybridized with [^{32}P]cDNA, and virus-specific DNA was detected by autoradiography as described in the text. The medium harvested on day 2 after infection from N-tropic-MuLV-infected NIH/3T3 cells gave 337,029 cpm, whereas medium from B-tropic-MuLV-infected cells gave 1,870 cpm, in the reverse transcriptase assay. The gel contains Hirt supernatant DNA (equivalent to one-third of the total extract) from cells infected with B-tropic (a through d) and N-tropic (e through h) Friend MuLV. Cells were infected for 11 h in the presence of BUdR and then incubated for 0 (a, e), 3 (b, f), 6 (c, g) and 14 (d, h) h in the presence of thymidine. Form I and form III DNAs are indicated.*

These observations were extended by infecting SIM cells, another *Fv-1$^{n/n}$* cell line, with two different strains of MuLV. SIM cells were infected with the N- and B-tropic endogenous viruses of the BALB/c mouse in the presence of BUdR for 12 h. After the BUdR pulse, chases of 3, 6, and 12 h were performed by incubating cells with thymidine. BUdR-labeled viral DNA, first selected in CsCl gradients, was analyzed by the agarose gel-filter transfer procedure. As found with other cell lines, levels of linear viral DNA were only slightly lower in B-Cl-11 MuLV-infected resistant SIM cells as compared with permissive cells, despite an 80-fold reduction of progeny virus (Fig. 4). (Notice that 70% of the sample in one group [Fig. 4a] was lost by accident.) During the chase period, levels of linear viral DNA species progressively decreased at a slow rate in both N- and B-tropic-MuLV-infected groups (Fig. 4b through d and f through h). Both species of closed circular viral DNA were detected in N-Cl-35 MuLV-infected permissive cells, but at a much lower level in resistant cells. Relative to linear DNA, levels of these two species of supercoiled DNA increased slightly during the chase period (Fig. 4f through

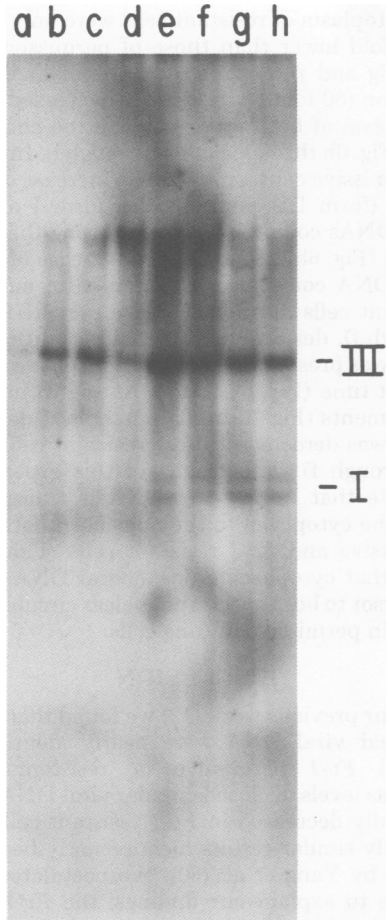

FIG. 4. *Pulse-chase studies on viral DNA from SIM cells infected with N-Cl-35 or B-Cl-11 MuLV. Subconfluent SIM cells (1.2 × 10^6 cells) were infected with N-Cl-35 and B-Cl-11 viruses (multiplicity of infection, 1) in the presence of BUdR (5 μg/ml) and Polybrene (8 μg/ml) for 12 h. Medium was then removed, and 1.6 × 10^7 cells from each group were extracted by the Hirt procedure. The remaining cells were subjected to the chase procedure and incubated with medium containing thymidine (10 μg/ml) for 3, 6, and 12 h. At each time, 1.6 × 10^7 cells from each group were extracted by the Hirt procedure. The DNA banding in CsCl gradients at a density above 1.76 g/cm^3 was processed for the agarose gel electrophoresis-DNA transfer procedure, and virus-specific DNA was detected by [^{32}P]cDNA hybridization as described in the legend to Fig. 3. The medium harvested on day 2 after infection from N-Cl-35 MuLV-infected SIM cells gave 69,583 cpm, whereas medium from B-Cl-11 MuLV-infected cells gave 832 cpm, in the reverse transcriptase assay. The gel contains Hirt supernatant DNA (equivalent to one-third of the total extract) from cells infected with B-Cl-11 (a through d) and N-Cl-35 (e through h) MuLV. Cells were infected for 12 h in the presence of BUdR and then incubated for 0 (a, e), 3 (b, f), 6 (c, g) and 12 (d, h) h in the presence of thymidine. Seventy percent of the sample in (a) was lost by accident. Form I and form III DNAs are indicated.*

h), suggesting that some linear viral DNA molecules were converted to supercoiled DNA molecules in these cells.

These experiments with two different *Fv-1*$^{n/n}$ cell lines confirmed that the *Fv-1* gene product does not specifically degrade linear viral DNA in resistant cells.

Analysis of viral DNA from cytoplasm and nucleus of *Fv-1*$^{b/b}$ cells infected with N- or B-tropic viruses. The previous experiments were all performed on total cell Hirt extracts and did not elucidate the localization of linear viral DNA in different compartments of *Fv-1* permissive or resistant MuLV-infected cells. It has been shown indeed that circularization of viral DNA occurs in the nuclei of avian (20, 28) and murine (1) cells.

To investigate whether the *Fv-1* gene product was interfering with the accumulation of linear viral DNA in nuclei of *Fv-1* resistant cells, SIM.R cells (*Fv-1*$^{b/b}$) were infected with N- and B-tropic MuLV's and fractionated into cytoplasm and nuclei. We have shown before that accumulation of supercoiled viral DNA was markedly reduced in these resistant cells (12). Because a high background of endogenous sequences was encountered with extraction of viral DNA from purified nuclei, cells were infected in the presence of BUdR. BUdR-labeled viral DNA extracted from nuclei was preselected on CsCl gradients before analysis by the agarose gel-DNA transfer procedure. The results of this experiment are shown in Fig. 5. Nearly identical levels of linear viral DNA (form III) were detected in the cytoplasm of SIM.R cells infected with N- or B-tropic MuLV. By endpoint dilution we estimated the difference between both groups to be less than twofold (Fig. 5a through e). Levels of linear viral DNA were also almost identical in nuclei of both groups despite a biological restriction of 70-fold. However, as shown in previous experiments, levels of both form I DNA species were markedly reduced in nuclei of *Fv-1* resistant cells (Fig. 5f and g). This result suggests that the *Fv-1* restriction does not impair accumulation of linear viral DNA in nuclei, where circularization occurs during a normal virus cycle.

To follow the transport and accumulation of linear viral DNA in nuclei of *Fv-1* permissive and resistant cells in more detail, a pulse-chase experiment was performed. SIM.R cells were infected with N- or B-tropic MuLV in the presence of BUdR, and the fate of BUdR-labeled

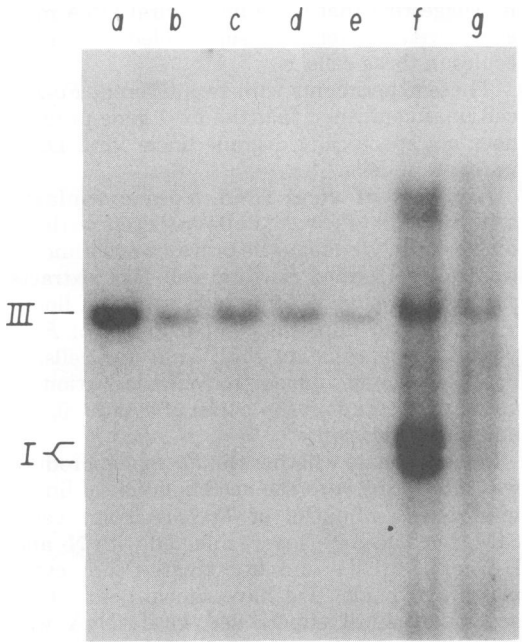

FIG. 5. *Agarose gel electrophoresis of pulse-labeled viral DNA from cytoplasm and nucleus of SIM.R cells infected with N-Cl-35 or B-Cl-11 MuLV. Subconfluent SIM.R cells (8 × 10^7 cells) were infected with N-Cl-35 or B-Cl-11 virus (multiplicity of infection, 0.8) in the presence of BUdR (5 μg/ml) and Polybrene (8 μg/ml) for 12 h. After incubation, cells were trypsinized and separated into cytoplasmic and nuclear fractions as described in the text. Nuclei were treated by the Hirt procedure, and DNA was extracted from the Hirt supernatant. This DNA was centrifuged on CsCl gradients as described in the legend to Fig. 1. Fractions with densities above 1.76 g/cm^3 were pooled and diluted sixfold with water before precipitation with ethanol in the presence of 100 μg of yeast RNA as carrier. The cytoplasmic fractions were brought to 0.2% sodium dodecyl sulfate, phenol extracted, and ethanol precipitated. Nucleic acids of cytoplasm and nuclei were electrophoresed through 1% agarose gels, and DNA was transferred to nitrocellulose filters and hybridized with [^{32}P]cDNA as described in the legend to Fig. 3. The medium harvested on day 2 after infection from B-Cl-11 MuLV-infected SIM.R cells gave 28,534 cpm, whereas medium from N-Cl-35 MuLV-infected cells gave 423 cpm, in the reverse transcriptase assay. The gel contains cytoplasmic extract (30 μl is equivalent to 1/10 of the total extract) in amounts of 30 (a) and 10 (b) μl from B-Cl-11 MuLV-infected cells and 70 (c), 50 (d), and 30 (e) μl from N-Cl-35 MuLV-infected cells and nuclear extract (equivalent to 1/2 of the total extract) from B-Cl-11 (f) or N-Cl-35 (g) MuLV-infected cells. Form I and form III DNAs are indicated.*

viral DNA was followed in the cytoplasm and nuclei during a thymidine chase. At the beginning of the chase, levels of linear viral DNA in the cytoplasm of resistant cells were only three- to sixfold lower than those of permissive cells (Fig. 6g and j), despite a drastic biological restriction (80-fold). These levels decreased in the cytoplasm of both groups during the chase period (Fig. 6h through i and k through l). In nuclei of permissive cells a progressive increase of both linear (form III) and circular (form I and II) viral DNAs could be observed during the chase period (Fig. 6b through c). An increase of linear viral DNA could also be observed in nuclei of resistant cells during the chase period (Fig. 6e through f), despite the fact that very little viral DNA was present in the cytoplasm of these cells at that time (Fig. 6k and l). As shown in other experiments (Fig. 2 through 5), no circular viral DNA was detected in these resistant cells (Fig. 6d through f). The results of this experiment indicate that linear viral DNA is transported from the cytoplasm to the nucleus in both *Fv-1* permissive and *Fv-1* resistant cells. They also show that cytoplasmic linear viral DNA is the precursor to both species of nuclear circular viral DNA in permissive murine cells.

DISCUSSION

In our previous work (12) we found that levels of linear viral DNA were nearly identical in several *Fv-1* permissive or resistant cells, whereas levels of closed circular viral DNA were markedly decreased in *Fv-1* resistant cells. Essentially similar results have recently been obtained by Yang et al. (30). We postulated four models to explain our findings; the *Fv-1* gene product could: (i) block the circularization of precursor linear viral DNA into the closed circular form, (ii) favor by its action the accumulation of faulty linear viral DNA unable to circularize, (iii) degrade supercoiled viral DNA soon after its synthesis, or (iv) block the transport of linear viral DNA into the nucleus, where it is circularized. In the present study, we have directly tested the last hypothesis to determine whether the *Fv-1* gene product blocks the transport of linear viral DNA into the nucleus. We also studied the fate of linear viral DNA made under nonpermissive conditions to determine whether its degradation was accelerated. Our findings suggest that the *Fv-1* gene product does not interfere at these two steps.

The stability of linear viral DNA in permissive and resistant cells has been studied in pulse-chase experiments, labeling viral DNA with BUdR and incubating cells with thymidine for the chase period. Under our conditions, during the thymidine chase, levels of linear viral DNA are independent of its synthesis but would depend upon its degradation, its conversion into

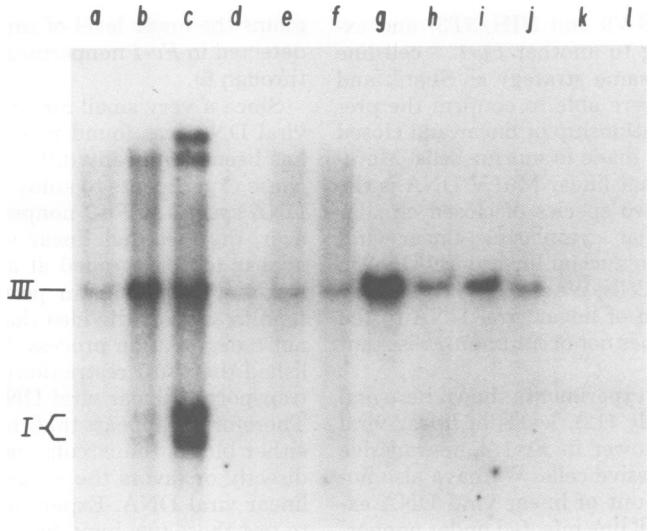

FIG. 6. *Pulse-chase studies on viral DNA from the nuclei and cytoplasm of SIM.R cells infected with N-Cl-35 or B-Cl-11 MuLV. Subconfluent SIM.R cells (2.4 × 10^6 cells) were infected with N-Cl-35 or B-Cl-11 virus (multiplicity of infection, 0.8) in the presence of BUdR (5 μg/ml) and Polybrene (8 μg/ml) for 6 h. After this incubation, medium was removed and 3.6 × 10^7 cells of each group were trypsinized and separated into cytoplasmic and nuclear fractions. The remaining cells were subjected to the chase procedure and incubated with medium containing thymidine (10 μg/ml) for 3 and 6 h. At each time, 3.6 × 10^7 cells of each group were trypsinized and separated into cytoplasmic and nuclear fractions. DNA was extracted from cytoplasm and from the Hirt supernatant of the nuclei and centrifuged on CsCl gradients, as described in the legend to Fig. 1. Fractions of density above 1.76 g/cm^3 were pooled and electrophoresed through 1% agarose gels. Viral DNA was detected with [^{32}P]cDNA as described in the legend to Fig. 3. The medium harvested on day 2 after infection from B-Cl-11 MuLV-infected SIM.R cells gave 40,991 cpm, whereas medium from N-Cl-35 MuLV-infected cells gave 814 cpm, in the reverse transcriptase assay. The gel contains nuclear extract (equivalent to one-third of the total extract) from B-Cl-11 (a through c) or N-Cl-35 (d through f) MuLV-infected cells and cytoplasmic extract (equivalent to one-third of the total extract) from B-Cl-11 (g through i) and N-Cl-35 (j through l) MuLV-infected cells. Cells were infected for 6 h in the presence of BUdR and then incubated for 0 (a, d, g, j), 3 (b, e, h, k), and 6 (c, f, i, l) h in the presence of thymidine. Form I and form III DNAs are indicated.*

the supercoiled form, or its integration into the cell genome if indeed the immediate precursor to integrated provirus is the linear molecule. A higher rate of degradation, conversion, or integration would then reduce levels of linear DNA. Our experiments done with three different cell lines (JLS-V9, NIH/3T3, and SIM) (Fig. 2 through 4) and the result obtained with another cell line (SIM.R) during cell fractionation (Fig. 6) all indicate that levels of linear viral DNA do not decrease in *Fv-1* resistant cells at a faster rate than in *Fv-1* permissive cells. Considering the fact that little integration would occur during the first 3 or 6 h of the chase period and that only a small proportion of linear DNA seems to be converted to circular DNA in the same time (Fig. 2B and C, 3f and g, and 4f and g), it is likely that the relatively equal rates of disappearance of linear viral DNA found in *Fv-1* permissive and resistant JLS-V9, SIM, NIH/3T3, and SIM.R cells during thymidine chase reflect similar degradation rates.

If linear viral DNA made in the cytoplasm of resistant cells was not transported into the nucleus, where circularization occurs (1, 20), the reduced levels of supercoiled molecules found in these cells could be explained. In an attempt to investigate this possibility, we measured levels of linear and circular viral DNA in the cytoplasm and nuclei of *Fv-1* permissive and resistant cells. We confirmed that the two circular viral DNA species are found exclusively in nuclei of infected murine cells (1). We also showed that nearly equal levels of linear viral DNA are measured and accumulated into the nuclei of permissive and resistant cells. These results suggest that the *Fv-1* gene product does not significantly impair the transport of linear viral DNA into the nucleus.

Our previous experiments have shown that closed circular viral DNA was reduced in *Fv-1* resistant JLS-V9, NIH/3T3, and SIM.R cells to the same extent as virus production (12). In the present study we confirmed these results with

two cell lines (JLS-V9 and NIH/3T3) and extended this finding to another *Fv-1*$^{n/n}$ cell line (SIM). Using the same strategy as Shank and Varmus (20), we were able to confirm the precursor-product relationship of linear and closed circular viral DNA made in murine cells. Moreover, we showed that linear MuLV DNA is the precursor to the two species of closed circular viral DNA and that cytoplasmic linear viral DNA is precursor to nuclear linear viral DNA in permissive murine cells. We also directly showed that the conversion of linear viral DNA to the supercoiled form does not occur in *Fv-1* resistant cells.

In several of the experiments shown here and presented previously (12), levels of linear viral DNA are slightly lower in *Fv-1* nonpermissive cells than in permissive cells. We have also noticed that the amount of linear viral DNA extracted from one cell line infected under nonpermissive conditions might vary somewhat (two- to threefold) (Fig. 5 and 6). Because this inhibition of linear viral DNA is much less than the biological restriction observed by measuring progeny viruses and because it is not observed reproducibly in all cell lines (12, 30), it might not be caused by the *Fv-1* gene product. It could reflect the action of another gene acting with *Fv-1*, as we have previously suggested (12). It could also reflect variations in recovery from the Hirt supernatant or the difficulty of obtaining the same effective multiplicity of infection with two different virus stocks (N- and B-tropic) and one cell line. However, we have pointed out the advantages of this approach to the study of *Fv-1* restriction (12). The alternative method, infecting two different cell lines, *Fv-1*^{b} and *Fv-1*^{n}, with a single virus stock (N- or B-tropic), could yield results showing differences in replication of MuLV unrelated to *Fv-1*.

The basic feature of the *Fv-1* gene is its ability to block the replication of certain classes of natural MuLV. For reasons which are yet unclear, one-hit and two-hit titration patterns on *Fv-1* nonpermissive cells have been obtained (for a full discussion of this problem, see reference 8). The molecular basis of these titration patterns has not yet been elucidated and was not studied here. In our previous experiments (12) and in the experiments presented here, we have concentrated on the molecular mechanisms responsible for the biological restriction observed. In each experiment, the *Fv-1* biological restriction was measured by the reverse transcriptase activity of the progeny virus and was 25- to 150-fold, as compared with the permissive conditions. The only quantitative difference we have found to explain this biological restriction remains the lower level of supercoiled viral DNA detected in *Fv-1* nonpermissive cells (12) (Fig. 3 through 6).

Since a very small amount of closed circular viral DNA was found in *Fv-1* resistant cells, it has been technically difficult to perform pulse-chase experiments to study the fate of this viral DNA species in *Fv-1* nonpermissive cells. However, the fact that linear viral DNA does not appear to be degraded at a faster rate in *Fv-1* resistant cells than in permissive cells would tend to support the idea that *Fv-1* restriction is not a degradation process. We have also established that *Fv-1* restriction is not a blockage of transport of linear viral DNA into the nucleus. Therefore, it appears that the *Fv-1* gene product either blocks the circularization of linear DNA directly or favors the accumulation of a faulty linear viral DNA. Experiments are in progress to test these two hypotheses.

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