Fv-1 Host Restriction of Friend Leukemia Virus: Analysis of Unintegrated Proviral DNA

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The murine gene *Fv-1* predominantly controls the outcome of infection by murine ecotropic retroviruses. The inhibition of virus replication by the *Fv-1* gene product has been determined to be at an early stage in virus replication. Mechanistically, its effect appears to be on the accumulation of unintegrated proviral DNA or its integration or both. We investigated the synthesis of unintegrated proviral DNA, using several clones of B-, N-, or NB-tropic Friend murine leukemia virus. Our results indicate that the accumulation of B-tropic proviral DNA in NIH cells may be inhibited at either the level of linear (form III) or covalently closed circular DNA (form I), depending upon the degree of restriction of the clone of virus used. We confirmed that there is an effect of the *Fv-1* gene on the accumulation of form I DNA of either B- or N-tropic Friend murine leukemia virus. However, the decrease in infectious centers effected by the *Fv-1* gene did not correlate quantitatively with the effect on form I proviral DNA produced by N-tropic Friend murine leukemia virus in nonpermissive cells. Lastly, we demonstrated in nonpermissively infected NIH cells that a rapidly migrating doublet of viral DNA is formed.

The murine gene *Fv-1* is an autosomal dominant which exerts a major control over the outcome of infection of mice by most murine retroviruses (3, 14; for a review see references 6 and 12). This gene, whose phenotype can be demonstrated in tissue culture, confers resistance to the replication of retroviruses. The gene has two codominant alleles, *Fv-1ⁿ* and *Fv-1^b*. *Fv-1ⁿ* homozygous animals are permissive for N-tropic, but resistant to B-tropic, murine leukemia viruses (MuLV's). *Fv-1^{b/b}* animals are permissive for B-tropic, but nonpermissive for N-tropic, viruses. Most murine viruses may be classified as either N- or B-tropic, according to the host range they exhibit. However, some laboratory-derived ecotropic viruses exhibit NB-tropism, which is defined as the ability to replicate well in cells of any *Fv-1* type.

The degree of host resistance conferred by the *Fv-1* gene is relative (3); that is, a 50- to 10,000-fold decrease in infectious centers is observed when a given virus infects a permissive versus a restrictive host. The degree of restriction, which depends upon the exact virus and host cell used, may show either single- or two-hit kinetics on the nonpermissive cell type (1, 7, 15, 19).

The replication of virus in a restrictive as compared with a permissive host was studied for both early and late events in the virus cycle. It was shown in our laboratory, as well as else-

where, that early steps, such as adsorption, penetration, and initiation of proviral DNA synthesis, are unimpaired in the restrictive host (5, 8, 11, 23). However, biological studies indicate that the *Fv-1* restriction effect occurs during the early phase of the viral replication cycle (2, 24). Furthermore, our unpublished studies indicate that if an infectious center is established by a restricted virus, there is no restriction of the expression of either viral proteins or RNA. Our conclusion, therefore, is that the *Fv-1* gene product acts at an early phase in viral replication to decrease the efficiency of establishing an infectious center.

We and others have shown that the integration of proviral DNA after exogenous infection is suppressed during *Fv-1* host restriction (8, 23). The studies of Yang et al. (26) and of Jolicoeur and Rassart (9), suggested that the *Fv-1*-mediated inhibition of proviral DNA integration may be due to an inhibition of the production or accumulation or both of form I circular proviral DNA, the presumed precursor to the integrated provirus.

We studied the early forms of nonintegrated proviral DNA produced during acute infection by clones of either B-tropic or N-tropic Friend MuLV (F-MuLV). Our results indicate that, depending upon the clone of B-tropic F-MuLV used, restriction in NIH cells (*Fv-1^{n/n}*) may be

observed at either of two steps of proviral DNA accumulation. That is, highly restricted clones failed to accumulate linear proviral DNA, whereas less restricted virus showed a decrease only in form I circular DNA. Secondly, N-tropic F-MuLV infection of BALB/c cells (*Fv-1^{b/b}*) manifested a decrease in the level of circular DNA accumulated. However, the decrease in the level of circular DNA found in the restrictive infection did not correlate quantitatively with the decrease in the number of infectious centers formed.

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MATERIALS AND METHODS

Cells. NIH, BALB/c, and XC cells were obtained from Janet Hartley and Wallace Rowe. They were maintained in monolayer culture with Joklik modified Eagle medium containing 10% fetal calf serum and nonessential amino acids. DBA/2 cells were the generous gift of Robert Bassin and were maintained in monolayer culture with Dulbecco modified Eagle medium containing 10% fetal calf serum.

Viruses. N-tropic and B-tropic F-MuLV were obtained from Frank Lilly as homogenates of spleens from infected NIH and BALB/c animals, respectively. The virus complex containing both the spleen focus-forming virus, as well as the replicative competent helper F-MuLV, was passed in tissue culture at high dilution in *Fv-1* permissive mouse fibroblast cell lines. Progeny virus was subsequently passed in uninfected fibroblast cells to eliminate the spleen focus-forming virus component. The N and B type lymphatic leukemia virus components (F-MuLV) were then cloned twice by limiting dilution (using XC assay [18]), and the cloned virus was passaged to high titer and frozen in aliquots.

Virus infection. Cloned virus passaged to high titer was used to infect permissive subconfluent mouse fibroblasts. Virus aliquots were quickly thawed, passed through Millex HA 0.20- μ m filter units to remove virus clumps, and titers were determined by direct XC plaque assay. Similarly, processed aliquots were used for experiments. Confluent mouse fibroblast cell lines were trypsinized and seeded at a density of 1×10^6 cells per 100-mm dish or 3×10^6 to 5×10^6 cells per T150 flask (Falcon Plastics, Oxnard, Calif.) in medium containing Polybrene at 5 μ g/ml. At 9 to 12 h later, the cells were infected with appropriate amounts of virus in medium containing Polybrene (5 μ g/ml). At this time, a sample dish was trypsinized, and the cells were counted to ensure an accurate multiplicity of infection (MOI) determination.

Isolation of viral DNA. At appropriate times after infection with virus, cells were trypsinized, rinsed with serum containing medium, centrifuged into a pellet, and suspended in 10 mM Tris-hydrochloride (pH 7.5)–10 mM EDTA at $<2.0 \times 10^6$ cells per ml. Cells were heated at 65°C (personal communication, Ann Skalka)

for 15 min, then lysed in 1.0% sodium dodecyl sulfate at 65°C for 20 min. NaCl was added to 1 M (4), and the samples were kept at 4°C overnight. Samples were centrifuged at 12,000 rpm in a Sorvall SS 34 rotor, and the supernatants were extracted with phenol and chloroform-isoamyl alcohol (24:1) and precipitated with 2 volumes of ethanol at –20°C.

Agarose gel electrophoresis and blot hybridization. DNA samples were separated by horizontal electrophoresis in 0.8% agarose slab gels submerged 2 to 3 mm under the running buffer (0.089 M Tris–0.082 M boric acid–0.002 M EDTA). The gel was stained with 1 μ g of ethidium bromide per ml in 0.5 M ammonium acetate for Polaroid photography under UV illumination, and the DNA was transferred to nitrocellulose by the Southern transfer procedure (22). Pretreatment with 0.15 M HCl was used for partial depurination of DNA to enhance the transfer of the larger fragments from the gel (25). Viral DNA transferred to nitrocellulose paper was detected by molecular hybridization to ³²P-labeled F-MuLV DNA prepared by nick translation (17) of F-MuLV DNA cloned into the plasmid vector pBR322, a generous gift of A. Oliff (13). The specific activities routinely obtained were 0.5×10^8 to 2.0×10^8 cpm/ μ g of DNA. Hybridization of the nitrocellulose-bound viral DNA to the [³²P]DNA probe was performed in 10% dextran sulfate (25), 5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 0.01 M phosphate buffer (pH 7.0), and 100 μ g of salmon sperm DNA per ml at 42 to 44°C for 12 to 16 h. This was followed by extensive sequential washings at 65°C in 3 $\times$ SSC, 1 $\times$ SSC, 0.4 $\times$ SSC, and 0.2 $\times$ SSC containing 0.2% sodium dodecyl sulfate. Filters were air dried and exposed at –70°C to Kodak X-Omat R film, using a Picker MAX-1 intensifying screen.

Quantitation of viral DNA. The band intensities of the autoradiograms were measured with a double-beam recording microdensitometer (MKIII C; Joyce, Loeb and Co., Ltd., England). Several autoradiographic exposures of each hybridized blot were obtained so that a single wedge setting on the microdensitometer could be used to compare adjacent lanes for bands representing both forms I and III DNA. The background exhibited on the microdensitometry tracing was then carefully considered, and the peak area representing either form III or form I was cut from the tracing and weighed on an analytical balance (Mettler). It was demonstrated that, within a well-defined range, the microdensitometer tracing method gave a linear response to different amounts of hybridized viral DNA, similar to the standardization procedures described by Keshet et al. (10). Therefore, only those peak weights obtained from a single autoradiogram which fell within this defined range were compared.

Restriction endonucleases. Restriction enzymes were obtained from Bethesda Research Laboratories, Gaithersburg, Md. Two to five units of enzyme per μ g of DNA was used with the reaction conditions provided by the supplier. The extent of reaction was monitored with either lambda DNA or adenovirus type 2 DNA.

RESULTS

Quantitation of nonintegrated proviral

DNA from acutely infected cells. Acute infection by retroviruses initially results in the formation of free nonintegrated proviral DNA and subsequently in the integration of this DNA into the host genome. Previous studies have demonstrated two predominant forms of free DNA in the infected cells: a double-stranded linear DNA (form III), which gives rise to a covalently closed circular supercoiled DNA (form I), which in turn is presumed to be the precursor of the integrated provirus (21). Because relatively few copies of viral DNA are synthesized per cell, our initial studies were directed at optimizing the recovery of these major nonintegrated DNA forms. A very poor recovery of closed circular proviral DNA was obtained when the Hirt extraction procedure (4) was applied to 5×10^7 permissively infected cells growing in roller bottles. Our suspicions were that this low yield of circular DNA could be due to extraction artifacts. Accordingly, we modified both the extraction procedure and the infected cell number. The modification of the Hirt procedure involves lysing and treating the infected cells at 65°C before salt precipitation of chromosomal DNA. Using this method, we were able to isolate significant amounts of both form I and form III DNA from as few as 1×10^6 cells. However, scaling up the procedure to process larger numbers of cells grown in roller bottles still resulted in a poor recovery of form I DNA. We attribute these results either to mechanical entrapment or nuclease degradation of unintegrated proviral DNA due to a greater cell concentration during extraction.

With this new method, and using only between 1×10^6 and 5×10^6 cells, we examined the proviral DNA accumulated in several cell lines considered either permissive or restrictive in terms of their *Fv-1* genotype. Our results, shown in Fig. 1A, indicated that distinctly different amounts of nonintegrated proviral DNA are formed in different cell types even if the cells are considered equally permissive at the *Fv-1* gene. Using an NB-tropic F-MuLV, a virus whose host range is not limited by the *Fv-1* gene, we compared the proviral DNA accumulated in NIH (*Fv-1^{n/n}*), BALB/c (*Fv-1^{b/b}*), and DBA/2 (*Fv-1^{n/n}*) cells. Extrachromosomal DNA obtained by the modified Hirt extraction was separated by electrophoresis in agarose, transferred to nitrocellulose (22), and hybridized to a [³²P]DNA probe prepared by nick translation of cloned F-MuLV DNA (13). It is evident that the recovery of both form I and form III from each of the three cell lines was different. Recovery was greatest from NIH (lane 2), least from DBA/2 (lane 3), and intermediate from BALB/c (lane 1) cells. A study comparing N-tropic virus in

FIG. 1. Autoradiograms of filter blot hybridization analysis of Hirt DNA extracts from virally infected cells. Subconfluent mouse fibroblast cell lines (5.0×10^6 cells) were infected with either B-tropic F-MuLV or NB-tropic F-MuLV (MOI of 1.0) in the presence of Polybrene (5 μ g/ml) for 16 h. After infection, cells were extracted by the modified Hirt procedure (see Materials and Methods), and the Hirt supernatant DNA was loaded onto 0.8% agarose gels. Electrophoresis was for 20 h at 50 V. Each lane contains the DNA obtained from the entire extract of 5×10^6 cells. After electrophoresis, the DNA was transferred to a nitrocellulose filter and hybridized to a nick-translated [³²P]DNA (specific activity, 0.5 to 1.0×10^8 cpm/ μ g) probe containing F-MuLV sequences. Autoradiography was performed with X-Omat film and intensifier screens at -70°C. (A) NB-tropic F-MuLV infection of BALB/c cells (lane 1), NIH cells (lane 2), and DBA/2 cells (lane 3). (B) B-tropic F-MuLV infection of BALB/c cells (lane 1), NIH cells (lane 2), and DBA/2 cells (lane 3). The bands of hybridized DNA are indicated at the sides of each autoradiogram and represent form I circular and form III linear viral DNA. The numbers between the autoradiograms represent the locations and sizes (in kilobase pairs) of *Bam*HI-digested adenovirus type 2 DNA fragments migrating in the same gel. The broad bands at the top of the gels represent residual host cell DNA that was not completely eliminated by the Hirt procedure.

NIH versus DBA/2 cells gave similar results (data not shown). The basis for these differences may be at the level of synthesis, degradation, or extractability of proviral DNA from the individual cell lines. The relative plating efficiency of each cell line also differs and must be controlled to ensure identical cell numbers, both at the time of infection and at the time of harvest. These possibilities have not all been explored. We concluded that the differences in total recovery between cell lines would complicate an attempt to compare the effect of the *Fv-1* alleles in these different cell lines and that this fact must be considered in any such analysis.

In addition to visual analysis of the autoradiogram, we compared the amounts of form I and form III DNA by assaying the labeled probe

bound to the nitrocellulose filter. In our hands, the total amount of radioactivity that could be directly ascribed to the DNA bands was very low, usually of the order of 50 to 200 cpm above a background of approximately 50 cpm found in the interband regions. We considered that these small differences of radioactivity would not permit us to make an accurate comparison between different cells.

To quantitate the presence of proviral DNA, microdensitometry tracing of the autoradiogram presented an alternative to scintillation counting. By analyzing the weight of the area under the curve of the microdensitometry tracing, we measured the total amount of annealed radioactive DNA and minimized the background. Furthermore, we elected to compare the ratio of forms I and III DNA in those cells which differed in accumulation or recovery or both of proviral DNA. In this way, an analysis of ratios could be made with a method that would normalize results, regardless of the total proviral DNA recovered. In Fig. 1A, for example, the ratios of form III to form I DNA for the three NB tropic F-MuLV infected cells are very similar; for NIH it is 1.34, for DBA/2 it is 1.39, and for BALB/c it is 2.12. We feel that, within the limits of resolution of the technique, these numbers are not significantly different. Comparison of the ratios of the proviral forms therefore permits the determination of the effect of different *Fv-1* alleles on the synthesis or accumulation or both of these proviral DNA forms by a single virus type.

B-tropic F-MuLV infection of *Fv-1^{n/n}* cells. The degree of restriction of growth of B-tropic F-MuLV in NIH Swiss fibroblast cell lines can vary up to 100-fold depending upon the stock of B-tropic virus examined. We cloned stocks of B-tropic F-MuLV that are restricted either 10^{-2} or 10^{-4} when analyzed by the direct XC plaque assay on NIH versus BALB/c cells. The synthesis of unintegrated viral DNA after infection of various cell lines by the 10^{-2} restricted B-tropic F-MuLV (B^{-2}) was examined by the Southern blot hybridization technique. The results are shown in Fig. 1B. In both permissive BALB/c cells (*Fv-1^{b/b}*) and nonpermissive NIH cells (*Fv-1^{n/n}*) (lanes 1 and 2, respectively), nearly equivalent amounts of linear proviral DNA (form III) were found. However, in the nonpermissive cell, essentially no form I DNA could be detected (lane 2). By comparison with DNA size standards, we estimate the size of the linear proviral DNA in both cell types to be about 8.8 to 9.1 kilobase pairs (kb). Using CsCl-ethidium bromide density gradients, we demonstrated for the permissive cell that the bands migrating between the 6.1- and 4.5-kb

markers represent the two closed circular proviral DNA species (form I) known to accumulate in retrovirus-infected cells (20, 27). By analogy with published data, the slower migrating of the two species of form I should contain two copies of the long terminal repeat sequence (LTR), whereas the faster band should contain only a single copy (20, 27). This clone of B-tropic F-MuLV thus appeared not to be restricted in its production of form III DNA, but accumulated markedly diminished amounts of both form I DNA species. In contrast, in another *Fv-1^{n/n}*-type cell (DBA/2), the accumulation of both form III and form I was diminished relative to the NIH cell (lanes 3 and 1, respectively) as would be predicted from our earlier results (Fig. 1A). In the case of DBA/2 cells, however, both species of form I DNA are evident (Fig. 1B, lane 3).

The ratio of linear DNA to the combined two species of closed circular DNA was obtained by means of comparison of the areas under the curves described by microdensitometry. In the case of B virus infection of BALB/c cells, the form III/form I ratio equaled 0.76, whereas B virus infection of DBA/2 cells resulted in a ratio of 1.38. No ratio could be determined for NIH cells because no measurable form I was accumulated (Fig. 1B, lane 2). By direct XC assay, this clone of B-tropic virus was restricted 100- to 500-fold on NIH cells, but only 50-fold on DBA/2 cells. In agreement with published results from other laboratories, *Fv-1* host restriction of this B-tropic F-MuLV in NIH cells is associated with a drastic inhibition of the accumulation of form I (9, 26). However, in the less restrictive DBA/2 cells, clear-cut accumulation of closed circular DNA was found, and at a ratio to linear forms only slightly different from that found in the permissive infection. For reference, on this autoradiogram, a nonrestricted NB-tropic virus infection of DBA/2 cells resulted in a ratio of 1.67 (Fig. 1A). These results suggest that the decreased accumulation of form I DNA found in B-tropic infection of restrictive DBA/2 cells (ratio, 1.38) relative to the permissive infection of BALB/c cells (ratio, 0.76) may not completely explain the 50-fold decrease in infectious centers observed in the XC assay.

The synthesis of proviral DNA in NIH Swiss cells was further examined with a clone of B-tropic F-MuLV that exhibits 10,000-fold restriction (B^{-4}) when examined by direct XC assay. The results are shown in Fig. 2. Linear proviral DNA species (form III) were drastically reduced in the nonpermissive NIH cells (Fig. 2A, lane 3; Fig. 2B, lane 7) compared to permissive BALB/c cells (Fig. 2A, lane 2; Fig. 2B, lane 6) in two different experiments with two different input

FIG. 2. Autoradiograms of filter blot hybridization analysis of cells infected with highly restricted B-tropic F-MuLV. Subconfluent mouse fibroblast cell lines (1.5×10^6 to 2×10^6 cells) were infected with either B- or N-tropic F-MuLV for 24 h. Hirt supernatant DNA was subjected to electrophoresis in 0.8% agarose for (A) 11.5 h at 50 V or (B) 16 h at 50 V. DNA, transferred to nitrocellulose, was hybridized to 32 P-labeled F-MuLV DNA. (A) Lane 1, N-tropic F-MuLV infection of NIH cells (MOI of 0.4); lane 2, B-tropic F-MuLV infection of BALB/c cells (MOI of 0.4); lane 3, B-tropic F-MuLV infection of NIH cells (MOI of 0.4). (B) Lanes 1 to 5, N-tropic F-MuLV infection of: NIH cells (MOI of 1.0), DBA/2 cells (MOI of 1.0), BALB/c cells (MOI of 1.0), BALB/c cells (MOI of 2.0), BALB/c cells (MOI of 5.0), respectively; lane 6, B-tropic F-MuLV infection of BALB/c cells (MOI of 1.0); lane 7, B-tropic F-MuLV infection of NIH cells (MOI of 1.0).

multiplicities. Other sets of permissive and non-permissive infections are presented in this figure for comparison of the amounts of form III DNA synthesized; these will be discussed later. This result, demonstrating the absence of form I and reduced amounts of form III, is clearly different from that which was observed for the 100-fold-restricted B virus (B^{-2}), which exhibited little to no decrease in linear DNA (form III). Independent clones of B-tropic virus can therefore exhibit different types of restriction of their proviral DNA in NIH Swiss cells. This highly restricted viral clone (B^{-4}) did not appear to be able to accumulate linear proviral DNA to the same extent as the less restricted clone (B^{-2}). These data may explain the different degrees of restriction exhibited by these two B-tropic clones.

The effect of the multiplicity of infection (MOI) on the synthesis of proviral DNA in Fv-1 permissive cells compared with nonpermissive cells was examined. B-tropic F-MuLV which exhibits 10^{-2} restriction in NIH cells (B^{-2}) was used to infect NIH and BALB/c cells at MOIs ranging from 0.25 to 1.0. The results are shown in Fig. 3. As expected, the total amount of proviral DNA increased relative to the MOI. At each MOI, the amount of form III DNA ob-

served in the nonpermissive cells was equivalent to that observed in the permissive cells. However, regardless of the MOI, the amount of form I DNA in the nonpermissive cells was greatly reduced. It is unclear whether the lack of detection of form I in the nonpermissive host at an MOI of 0.25 (lane 1) is due simply to insufficient numbers of infected cells used in this analysis or whether this form is completely absent at this MOI.

Two rapidly migrating bands of DNA are clearly observed at the higher MOI in the non-permissive cells (lanes 3 and 4). These bands are seen to migrate more rapidly than the form I DNAs found in the permissive BALB/c cells (lanes 5 to 8). Because these bands in the non-permissive cells are present at low concentration, we have thus far been unable to demonstrate, as we have in permissive infections, that they are, in fact, form I DNA either by CsCl-ethidium bromide density gradient or by restriction enzyme criteria (data not shown). In the non-permissive cell, these presumed form I bands were present at a ratio of 1:10 to the amount of form III present. If the proviral DNA synthesized in the nonpermissive cell had a lower molecular

FIG. 3. Autoradiogram of filter blot hybridization analysis of effect of MOI by B-tropic F-MuLV. Subconfluent NIH Swiss (lanes 1 to 4) and BALB/c cells (lanes 5 to 8) were infected with B-tropic F-MuLV at various MOIs for 16 h and subjected to modified Hirt extraction as described in the legend to Fig. 1. Hirt supernatant DNA was subjected to electrophoresis in 0.8% agarose at 60 V for 16 h. After electrophoresis, DNA was transferred by the Southern blot procedure to nitrocellulose and hybridized to 32 P-labeled F-MuLV DNA. Autoradiography was performed as in Fig. 1. Lanes 1 to 4: B-tropic F-MuLV infection of NIH Swiss cells at MOIs of 0.25 (lane 1), 0.50 (lane 2), 1.0 (lane 3), and 2.0 (lane 4). Lanes 5 to 8: B-tropic F-MuLV infection of BALB/c cells at MOIs of 0.25 (lane 5), 0.50 (lane 6), 1.0 (lane 7), and 2.0 (lane 8). The hybridized bands of viral DNA representing form III linear and form I circular DNA are indicated on the sides of the autoradiogram.

weight than that in the permissive cell, circularization would result in a form I DNA which would migrate more rapidly than its permissive equivalent. A small difference in the size of the linear proviral DNA (form III) synthesized in the nonpermissive cell would not be detected by direct visual examination of our gels because of the limited resolving power of 0.8% agarose gels for DNA species that are greater than 8.0 kb in size. We therefore digested the proviral DNA obtained from nonpermissive versus permissive cells with the restriction endonuclease *EcoRI*. This enzyme cuts the viral genome only once, producing two bands of 5.7 and 3.4 kb (unpublished data and reference 13). If a difference in the size of the linear DNA from the nonpermissive cells were present, it might be detected as an altered migration of one of the viral bands resulting from *EcoRI* digestion. The results of this analysis are shown in Fig. 4. The pattern of digestion obtained from proviral DNA extracts from nonpermissive cells was similar to that obtained from proviral DNA extracted from the permissive cells. Preliminary studies using *HindIII*, an enzyme which appears to cut twice, confirmed these results. Therefore, it does not appear that the basis for the restriction in the nonpermissive cells is a major deletion or incomplete synthesis of the form III DNA accumulated in these cells. The basis for the altered migration pattern of the doublet DNA (presumed circular species) is not yet understood and is under investigation.

Additionally, we tested whether extensive methylation of form III in different host cells could be associated with differential accumulation of circular DNA. The results (data not shown) of *HpaII* digestion of linear DNA accumulated in either a permissive or a nonpermissive host reveal no resistant fragments of proviral material. Since resistance to this enzyme correlates with methylation of the internal cytosine in the *HpaII* recognition sequence (16), these results suggest that extensive methylation of proviral DNA does not occur in either cell type, but they do not exclude limited site-specific methyl base modification.

In summary, independent clones of B-tropic virus exhibited wide variation in the restriction by NIH Swiss cells when examined by the direct XC plaque assay. The degree of restriction appears to correlate not only with the ability to accumulate normal amounts of proviral DNA but also with the types of DNA made. B-tropic F-MuLV that exhibits a high degree of restriction in NIH cells (B^{-}) appeared to accumulate severely reduced levels of linear, and essentially no circular, forms of proviral DNA. Viruses which are less restricted (B^{-2}) were able to ac-

FIG. 4. *EcoRI* restriction enzyme analysis of form III DNA. Subconfluent cell lines (5×10^6 cells) were infected with B-tropic F-MuLV (MOI of 1.0) in the presence of Polybrene ($5 \mu\text{g}/\text{ml}$) for 23 h and subjected to modified Hirt extraction. Viral DNA samples were treated with endonuclease *EcoRI* ($5 \text{ U}/\mu\text{g}$ of DNA) at 38°C for 3 h and then subjected to electrophoresis on 0.8% agarose to 60 V for 17 h. After electrophoresis, DNA was transferred to nitrocellulose and hybridized to ^{32}P -labeled F-MuLV DNA. Autoradiography was performed as in Fig. 1. Undigested DNA from B-tropic F-MuLV infection of BALB/c cells (lane 1) and NIH cells (lane 2) was run in parallel with digested DNA from BALB/c cells (lane 3) and NIH cells (lane 4). The numbers at the side of the autoradiogram represent the molecular size (in kilobase pairs) estimated from restriction fragments of adenovirus type 2 DNA and lambda DNA that were run in parallel. In lane 4, the band migrating at 9.0 kb represents endogenous cellular sequences found in genomic DNA of uninfected as well as infected cells. In this gel, these sequences result from the contaminating chromosomal DNA that is found in all Hirt extracts. In lane 3, the bands migrating to 9.0 kb represent these same cellular sequences as well as form III (DNA) derived from form I after a single cut by endonuclease *EcoRI*.

cumulate normal amounts of linear DNA, but accumulated markedly reduced levels of abnormally migrating presumptive circular DNA. These results suggest that, in NIH cells, restriction of B-tropic virus replication may occur at either the accumulation of linear or circular DNA. Whether only one or both of these effects may be correlated with the *Fv-1* phenotype is at this time not resolved.

Furthermore, in DBA/2 cells, B-tropic F-MuLV was restricted to a lesser degree than that observed with NIH cells. However, the ratio of the accumulation of circular forms relative to

linear appears equivalent to that observed with a permissive infection (NB-tropic virus) in those same cells (Fig. 1). Therefore, the degree of biological restriction observed in tissue culture assays with DBA/2 cells did not correlate well with a decreased accumulation of form I proviral DNA.

N-tropic F-MuLV infection of BALB/c cells: analysis of nonintegrated proviral DNA. Several clones of our stock N-tropic F-MuLV were tested and all consistently exhibited between 100- and 500-fold restriction on nonpermissive BALB/c cells when examined by the direct XC plaque assay. To compare the site(s) of *Fv-1* restriction, we examined the proviral DNA species of N-tropic F-MuLV accumulated in NIH versus BALB/c cells. The results represent infection at a range of MOI from 0.25 to 1.0 at 16 to 17 h postinfection (Fig. 5). Visual evaluation of the autoradiogram of the blot hybridization suggests that at each MOI, the amount of form III species accumulated was slightly less in the nonpermissive, when compared with the permissive, infection. In other experiments, the amounts of form III were identical. However, the amount of form I DNA in the BALB/c cell

(lanes 1 to 4) was reduced at each MOI, when compared with the permissive cell (lanes 5 to 8). Microdensitometry tracings were taken for each lane, and the areas under the peak for each DNA form were weighed for quantitation (Table 1). Two points are evident: (i) at each MOI the amount of form III in permissive versus nonpermissive cells was nearly equivalent, and (ii) the amounts of form I found in the nonpermissive cell were reduced. The ratios of form III to form I indicate that only a two- to threefold decrease in the amount of circular species occurred in the restrictive infection. A series of similar experiments, in which the proviral DNA forms were examined at between 6 and 20 h after infection, all confirmed these findings. Within 6 h after infection, we documented the accumulation of linear DNA in excess of circular DNA, that is, a high ratio of form III to form I. With time this ratio approached that seen at 12 to 16 h (Table 1). In the nonpermissive infection, a parallel time-dependent change in accumulation of DNA species occurred. But the same relative decrease in accumulation of circular DNA seen at 16 h

FIG. 5. Autoradiogram of filter blot hybridization analysis of effect of MOI by N-tropic F-MuLV. Subconfluent BALB/c (lanes 1 to 4) and NIH Swiss cells (lanes 5 to 8) were infected with N-tropic F-MuLV at various MOIs for 16 h and subjected to modified Hirt extraction as described in the legend for Fig. 1. Hirt supernatant DNA was subjected to electrophoresis in 0.8% agarose at 60 V for 16 h. After electrophoresis, DNA was transferred by Southern blot procedure to nitrocellulose and hybridized to 32 P-labeled F-MuLV DNA. Autoradiography was performed as in Fig. 1. Lanes 1 to 4: N-tropic F-MuLV infection of BALB/c cells at MOIs of 0.25 (lane 1), 0.50 (lane 2), 0.75 (lane 3), and 1.0 (lane 4). Lanes 5 to 8: N-tropic F-MuLV infection of NIH Swiss cells at MOIs of 0.25 (lane 5), 0.50 (lane 6), 0.75 (lane 7), and 1.0 (lane 8). Lane 9 represents DNA obtained from B-tropic F-MuLV infection of NIH Swiss cells at an MOI of 5.0 and is shown for comparison of the electrophoretic migration of form I (Fig. 3).

TABLE 1. Effect of MOI on proviral DNA in N-tropic F-MuLV infection

Host cell	MOI	Form III ^a	Form I ^a	Ratio (III/I)
(A) NIH Swiss	0.25	0.016	0.015	1.07
	0.50	0.031	0.030	1.03
	0.75	0.040	0.050	0.80
	1.00	0.057	0.0655	0.87
(B) BALB/c	0.25	0.0285	0.011	2.59
	0.50	0.045	0.019	2.37
	0.75	0.065	0.027	2.41
	1.00	0.092	0.030	3.07
(C) NIH Swiss	0.25	0.053	0.055	0.96
	0.50	0.076	0.066	1.15
	0.75	0.049	0.021	2.30
	1.00	0.059	0.022	2.68
(D) BALB/c	1.00	0.061	0.023	2.65
	0.50	0.034	0.024	1.42
	0.75	0.051	0.032	1.59
	1.00	0.074	0.045	1.64
(E) NIH Swiss	1.00	0.123	0.062	1.98
	2.0	0.115	0.030	3.83
	5.0	0.199	0.048	4.15

^a Numbers indicate weight in grams of peak areas cut from the microdensitometry tracings of autoradiograms. All the measurements in each lettered group were made using a single wedge setting on the microdensitometer. (A) and (B) represent different autoradiographic exposures to the same hybridized Southern blot. (A) and (B): 5×10^6 cells were infected in the presence of Polybrene (5 μ g/ml) for 16 h. The autoradiogram can be seen in Fig. 5. (C): 2×10^6 cells were infected in the presence of Polybrene (5 μ g/ml) for 16 h. (D): 1.5×10^6 cells were infected in the presence of Polybrene (5 μ g/ml) for 24 h. The autoradiogram can be seen in Fig. 2B.

was observed at each time point in the nonpermissive infection. That is, although both the absolute amount and the type of proviral DNA species accumulated in cells varied with time, there was a consistent finding (indicated in the ratio of form III to form I) of a decreased accumulation of only two- to fourfold of circular DNA in BALB/c cells infected with N-tropic F-MuLV relative to that seen in the permissive infection. These data are consistent with the decreased accumulation of form I reported by other investigators (9, 26). However, they are inconsistent with the degree of reduction of form I noted by Jolicoeur and Rassart (9). Yang et al. (26) suggested a multiplicity-dependent effect on the formation of form I DNA in nonpermissive cells. Our data suggest that the decrease in form I DNA accumulation is independent of multiplicity, since the ratio of form III to form I was constant over a wide range of viral input.

The phenomenon of the *Fv-1* gene restrictive effect has, however, been shown in many laboratories to follow two-hit kinetics. That is, titration of either infectious centers or of progeny virus production in nonpermissive cells decreases geometrically rather than linearly with virus dilution. In contrast to the permissive cell infections, these findings have been interpreted to indicate that a nonpermissive cell must be infected by at least two infectious viruses to establish an infectious center. By contrast, the permissive cell requires only a single "hit" (15). However, single-hit restriction curves, apparently due to the *Fv-1* host gene, have been reported in many laboratories (reviewed by Jolicoeur, 6).

Our N-tropic F-MuLV appears to be restricted by single-hit kinetics as measured by the direct XC assay. These kinetics may also be demonstrated by progeny yield. NIH and BALB/c cells were infected at an MOI of either 2.0 or 0.2. The progeny yield from such permissively or nonpermissively infected cells was measured on NIH

cells at 24 through 96 h postinfection. The results, shown in Table 2, were (i) at 24 h in permissive cells there was the expected 10-fold difference in yield, comparing an MOI of 0.2 with one of 2.0; (ii) at both 24 and 48 h postinfection, there was a 100-fold decrease in yield, comparing permissive with nonpermissive infection, and this result occurred at either MOI; (iii) at 72 and 96 h, there was only a 10-fold restriction in yield, a result presumably due to cell-to-cell spread, a phenomenon previously found by us (Soeiro, unpublished data) and others (7). Similar experiments studying infectious centers produced by N-tropic F-MuLV infection of NIH versus BALB/c cells have also indicated such single-hit kinetics. Our results, in terms of the ratio of form III to form I in an *Fv-1* restrictive infection, must therefore be interpreted in the light of apparent single-hit kinetics.

To examine proviral DNA formed under more extreme conditions of multiplicity, we compared the N-tropic F-MuLV infection of BALB/c versus NIH cells at an MOI of 5.0. At this MOI, at least 96% of the cells were infected by two or more infectious particles. It would be expected, according to the two-hit model, that at this MOI restriction would be completely overcome and the patterns of proviral DNA synthesis might be identical. For comparison, we also infected a much larger number of BALB/c cells at an MOI of 0.5, so that the amounts of total linear DNA produced by N-tropic F-MuLV would be approximately equal to that found by infection of fewer cells at a higher MOI. The results of this experiment depicted as microdensitometry tracings from the autoradiogram are shown in Fig. 6. Figure 6A represents the two circular forms (form I) and the linear form III of permissively infected NIH cells. Figure 6B represents similar tracings taken from BALB/c cells infected at an MOI of 5.0. Figure 6C is the tracing obtained from a greater number of BALB/c cells infected at an MOI of 0.5. The ratio of weights of form

TABLE 2. Effect of MOI on progeny yield from permissive versus restrictive infection^a

MOI	Progeny yield at h postinfection of:							
	24		48		72		96	
	N	B	N	B	N	B	N	B
2.0	4×10^4	3×10^2	1.2×10^7	1.2×10^5	0.9×10^6	1.3×10^5	1.4×10^6	2×10^5
0.2	3×10^3	ND ^b	8×10^6	4×10^4	9×10^5	5×10^4	3×10^6	3×10^5

^a Subconfluent mouse fibroblast cell lines (1.0×10^6 cells) were infected with N-tropic F-MuLV in the presence of Polybrene (5 μ g/ml) at an MOI of 2.0 or 0.2. After a 3.5-h adsorption period, cells were washed and given fresh medium. At the times indicated, the medium was replaced, and the titers of the progeny virus in the removed media were determined on permissive (NIH) cells by direct XC plaque assay. The results obtained from media removed from infected NIH Swiss cells (N) or BALB/c cells (B) are given as the number of macroscopic PFU per milliliter.

^b ND, Not detected.

FIG. 6. Microdensitometry tracings of filter blot hybridization analyses of infection by N-tropic F-MuLV. Subconfluent cell lines were infected for 24 h in the presence of Polybrene (5 $\mu\text{g}/\text{ml}$) and subjected to modified Hirt extraction (see Materials and Methods). After electrophoresis through 0.8% agarose for 16 h at 50 V, the DNA was transferred by the Southern blot procedure to nitrocellulose and hybridized to ^{32}P -labeled F-MuLV DNA. Autoradiography was performed as described in the legend to Fig. 1, and the exposed film was analyzed lane by lane with a double-beam recording microdensitometer. After careful consideration of background, the peak areas were cut out and weighed; thus, the areas under the curves are given in gram units. (A) N-tropic F-MuLV infection of 1.0×10^6 NIH Swiss cells, MOI of 5.0. (B) N-tropic F-MuLV infection of 1.0×10^6 BALB/c cells, MOI of 5.0. (C) N-tropic F-MuLV infection of 4.0×10^6 BALB/c cells, MOI of 0.5. The peaks representing form I DNA and form III DNA are indicated.

III to form I is included in the figure. We can conclude from comparing both the total amount of linear DNA formed and the ratio of linear to circular forms that, even at a very high MOI, at which restriction is expected to be overcome, there is a two- to threefold reduction in the accumulation of form I in nonpermissive cells. Furthermore, even at an MOI of 0.5, by comparison with 5.0, the ratio of linear to circular forms in nonpermissive cells remains the same.

These results confirm that, in a nonpermissive infection, accumulation of form I is reduced. But they also show that the degree of reduction is much less than that expected by the 100-fold restriction of viral progeny yield. Furthermore, at MOIs of 5.0 and 0.5, the ratios of form III to form I are essentially identical, a result inconsistent with a two-hit restriction phenomenon. We conclude that decreased accumulation of form I does occur in restrictive infection with N-tropic F-MuLV but that the decrease is inconsistent with the degree of restriction observed in tissue culture assays. In addition, with our virus and cell combination, the ratio of form III to form I in BALB/c cells did not reflect a two-hit effect on the decrease of circular DNA.

DISCUSSION

Fv-1-mediated host restriction of B-tropic MuLV affects the accumulation of nonintegrated proviral DNA in *Fv-1^{n/n}* cells. The results previously reported by other investigators (9, 26) have shown that, in NIH cells, there is a block in the accumulation of form III DNA (and therefore no form I is found) after infection with WN1802B. Other *Fv-1^{n/n}* cells (DBA/2) infected with this same virus produce amounts of linear DNA equivalent to that formed in permissive cells, but levels of form I are reduced. It has been suggested that the differences observed between these cell types could be due to the presence of a second gene in NIH cells which modifies the *Fv-1* effect (9, 26). N-tropic MuLV (WN1802N) infection of *Fv-1^{b/b}* cells resulted also in the accumulation of normal amounts of linear but decreased amounts of circular DNA. Since previous results from this lab and others (8, 23) have indicated that *Fv-1* restriction occurs at or before proviral DNA integration, it was concluded that the block in accumulation of circular proviral DNA is the primary effect of the *Fv-1* gene.

We have demonstrated that individual clones of B-tropic F-MuLV can exhibit different degrees of restriction by NIH cells. Cloned virus stock exhibiting a low level of restriction (10^{-2}) by XC assay were shown to accumulate normal levels of form III, but reduced levels of form I, DNA. However, a second clone, showing high (10^{-4}) restriction, was unable to accumulate even normal levels of form III DNA in these cells.

These data suggest that NIH cells are able to restrict B-tropic virus at several points in the early proviral DNA phase of replication. These effects could be due to a single pleiotropic *Fv-1*ⁿ allele capable of interacting at different steps during proviral DNA accumulation. Alternatively, NIH cells may carry a second gene which modifies the *Fv-1*ⁿ allele. Our studies with two clones of one virus that appear blocked at different steps leading to integration do not resolve this question. They do, however, show that these two clones are recognized differentially by the same cell, suggesting the possibility of more than a single recognition feature in host cell restriction. There might be two sites on the virus recognized by a single *Fv-1* gene product or, alternatively, two gene products. This conclusion is underscored by the fact that both B-tropic clones are restricted to the same degree (50-fold) in DBA/2 cells (*Fv-1*^{n/n}). This suggests that DBA/2 cells may only be able to recognize a single feature shared by both viruses. These patterns of proviral DNA accumulation may be due to the presence of a second restricting gene product(s) in NIH cells which is not present in DBA/2 cells or to an increased efficiency of a single *Fv-1*ⁿ gene product in the NIH cells.

Our studies of the restriction of N-tropic virus in BALB/c cells have revealed a decreased accumulation of form I DNA in agreement with studies from other laboratories. However, we observed that the reduction of form I was only of the order of two- to threefold when compared with the amount of proviral DNA obtained from permissively infected cells. Our results are clearly different from the estimated 25- to 50-fold reduction observed by Jolicoeur and co-workers (9) and do not show a multiplicity dependence as observed by Yang et al. (26). A possible reason for the differences observed, in addition to modifications of technical procedures, may reside in the virus stocks used in these studies. The titration pattern observed by Yang and co-workers is clearly two hit in non-permissive cells, whereas our results demonstrated a one-hit restriction curve, that is, a lack of multiplicity effects on virus yield or on the proviral DNA pattern.

Several features of *Fv-1* restriction have been described, including "hitness," "refractoriness,"

"expression," and "prozone" effects (15). These are well discussed by Jolicoeur (6), whose conclusion is that both refractoriness and two-hit kinetics appear to be part of the *Fv-1* gene effect.

Our findings are that decreases in the accumulation of form I (two- to threefold) are inconsistent with the degree of restriction (100-fold) and that the decrease in form I accumulation is not multiplicity dependent (Fig. 6). These data suggest that for our cells and virus the decrease in form I may be associated with refractoriness, that is, a non-multiplicity-dependent *Fv-1*-mediated restriction effect. (The word "refractoriness" is used to imply a decreased efficiency of formation of infectious centers in nonpermissive host cells.)

The data of Yang et al., which show a two-hit effect on the accumulation of form I DNA, suggest that a decrease in circular DNA is the site of *Fv-1* restriction. Our data, which did not show such a multiplicity-dependent effect on form I accumulation, suggest that *Fv-1* restriction may not be due simply to effects on circular DNA accumulation. An alternative model for the site(s) of *Fv-1* gene effect is suggested by our data. If the efficiency of integration varies geometrically with the concentration of form I, then small decreases in accumulation of form I might result in large differences in the establishment of infectious centers. Therefore, in addition to a small decrease in form I accumulation, there might be a large effect on the integration process itself. This second effect might be considered secondary, but could result in a geometric outcome of a linear process. There is no evidence against such a model that we are aware of. Also, it must be considered that the biological significance of the accumulated proviral DNA species found after the first few hours of infection is yet to be completely defined and may represent an easily measurable but dead-end feature of an acute infection.

Lastly, we have attempted to assay mechanisms which might result in decreased accumulation of form I DNA in the restrictive cell. Our data, which show no residual *HpaII*-resistant proviral fragments, suggest that extensive methylation of the DNA at cytosine residues in either a permissive or restrictive infection is not involved in this effect. Also, no large deletions in the length of linear DNA could be found by preliminary restriction analysis of the form III DNA accumulated in restrictive cells. However, these studies do not exclude the possibility that very small, especially terminal, fragments may be missing or base modified. The easily recognized alteration in the electrophoretic migration of putative form I in Fig. 3 and 5 is as yet unexplained. It may represent a more highly

supercoiled structure than is normally accumulated. Whether this structure is a result of an interaction of proviral DNA with the *Fv-1* gene product must be determined. An understanding of this result may define mechanistically how the *Fv-1* gene effect is mediated. Finally, the mechanism(s) for the decreased accumulation of form I has not been determined and may result from either changes in synthesis or degradation or both of circles formed in the restricting cell.

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