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Fv-1 Restriction and Its Effects on Murine Leukemia Virus Integration In Vivo and In Vitro

PETER M. PRYCIAK[1] AND HAROLD E. VARMUS[1,2]*

Department of Biochemistry and Biophysics[1] and Department of Microbiology and Immunology,[2] University of California, San Francisco, San Francisco, California 94143-0502

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We have investigated the mechanisms by which alleles at the mouse *Fv-1* locus restrict replication of murine leukemia viruses. Inhibition of productive infection is closely paralleled by reduced accumulation of integrated proviral DNA as well as by reduced levels of linear viral DNA in a cytoplasmic fraction. Nevertheless, viral DNA is present at nearly normal levels in a nuclear fraction, and total amounts of viral DNA are only mildly affected in restrictive infections, suggesting a block in integration to account for reduced levels of proviral DNA. However, integrase (IN)-dependent trimming of 3′ ends of viral DNA occurs normally in vivo during restrictive infections, demonstrating that not all IN-mediated events are prevented in vivo. Furthermore, viral integration complexes present in nuclear extracts of infected restrictive cells are fully competent to integrate their DNA into a heterologous target in vitro. Thus, the *Fv-1*-dependent activity that restricts integration in vivo may be lost in vitro; alternatively, *Fv-1* restriction may prevent a step required for integration in vivo that is bypassed in vitro.

Fv-1 is a normal mouse gene, alleles of which encode the ability to inhibit the replication of certain classes of mouse retroviruses, the murine leukemia viruses (MLVs) (for reviews, see references 28 and 50). The two common alleles of *Fv-1*, *Fv-1*^{n} and *Fv-1*^{b}, are so called because of their presence in the prototypical mouse strains NIH and BALB, respectively. MLV strains are grouped according to their susceptibility to *Fv-1* alleles: *Fv-1*^{n} inhibits replication of B-tropic MLVs, and *Fv-1*^{b} inhibits replication of N-tropic MLVs. This inhibition, known as *Fv-1* restriction, is dominant; *Fv-1*$^{n/b}$ heterozygotes inhibit replication of both N- and B-tropic MLV replication. Virtually all inbred strains of laboratory mice carry one of these two *Fv-1* alleles, but wild mice, *Mus* species other than *M. musculus*, and cell lines derived from them may be permissive for both classes of MLV and hence are called *Fv-1*$^{-/-}$ (28, 35).

The determinants of viral tropism lie within the *gag*-encoded capsid (CA) protein (formerly called p30^{gag}), and a swap of two adjacent amino acids in CA between N and B sequences can completely reverse viral tropism (1, 11). Nearly all isolates of ecotropic MLVs from laboratory strains of mice are either N or B tropic (28), although some isolates from wild mice or other species of mice are insensitive to both alleles of *Fv-1* (37, 49). In addition, many common laboratory strains of MLV, such as Moloney MLV (MoMLV), have acquired insensitivity to *Fv-1* restriction, presumably through mutation during multiple passages in culture (14, 24). Such MLV strains that are not restricted by either allele of *Fv-1* are termed NB tropic and appear to be missing determinants for restriction, since sensitivity to restriction is dominant (32, 44).

Studies of *Fv-1* restriction in cell culture demonstrated that restriction operates after entry of virus into the cell but before or during integration of viral DNA into the host cell genome (7, 27, 29–31, 48, 51). Restriction is not absolute; usually, between 10- and 1,000-fold fewer cells are productively infected in a restrictive host than in a permissive host. The stage of the viral replication cycle that is inhibited is somewhat variable and apparently dependent on the particular host-virus combination. In some cases, restriction can be accounted for by an inhibition of viral DNA synthesis (51). In most cases, however, normal or nearly normal levels of linear viral DNA are synthesized during infection of restrictive cells, yet the acquisition of integrated proviruses (usually monitored by virus production from the recently infected cells) is severely decreased (7, 30, 31, 51). Transfection experiments also show that virus production from integrated genomes is not inhibited in restrictive hosts (9, 25).

Thus, *Fv-1* restriction provides an intriguing example of host cell participation in the early stages of the retroviral life cycle. Earlier studies also found that, while linear viral DNA appears in normal amounts, formation of circular viral DNA is inhibited in restrictive hosts (7, 30, 31, 51). At the time, it was generally believed that circular viral DNA (particularly the two-long-terminal-repeat [LTR] circle) was the precursor to integrated DNA and therefore that *Fv-1* restriction of integration proceeded by inhibition of precursor circle formation. It is now clear that linear viral DNA, not circular DNA, is the normal precursor to integrated DNA (4, 10, 15, 20, 33, 39); thus, decreased circular DNA formation is more likely a reflection of the same inhibitory action that prevents integration in restrictive cells.

The recent development of in vitro assays for retroviral integration (3–5, 10, 19, 20, 33) provides an opportunity to learn more about the contribution of the *Fv-1* gene product (the identity of which remains unknown) to the control of MLV replication. Intriguingly, the CA protein has been found to remain associated after infection with MLV DNA-containing integration complexes (2). We report here our efforts to duplicate *Fv-1*-mediated inhibition of MLV integration in a cell-free system by using extracts of infected restrictive or permissive cells and assaying for integration of MLV DNA into an exogenously-provided target DNA in vitro. We have found that the cell-free assay fails to duplicate *Fv-1* restriction; instead, extracts from infected restrictive cells contain fully functional preintegration nucleoprotein complexes. Thus, either the restricting component

* Corresponding author.

present in vivo is inactivated in our in vitro system or restriction does not operate by directly inhibiting integration activity in vivo. Importantly, we found that a separate process also mediated by the viral integrase (IN) protein, the removal of two bases from the 3′ ends of linear viral DNA, occurs normally in vivo during infection of restrictive cells; this shows that not all integration functions are inhibited in vivo. In addition, we present some novel observations on the accumulation of linear viral DNA in restrictive infections.

MATERIALS AND METHODS

Plasmids, viruses, and cells. Plasmids pN20-7 and pB16-5 (11) were obtained from P. Jolicoeur (Montreal, Canada), and pWN41, pWB-5, and pGN104 (1) were obtained from W. K. Yang (Oak Ridge, Tenn.). In order to study the effects of *Fv-1* restriction on integration in vitro at the time when these experiments were initiated, we required a genetic selection of integration products (3). For this purpose, we generated N- and B-tropic viruses carrying the *supF* bacterial amber suppressor tRNA gene (3) in two ways (see Fig. 1): (i) insertion of the *supF* gene into the N- and B-tropic MLV LTRs and (ii) conversion of MoMLV-SupF from NB-tropic to N- and B-tropic versions by changing *gag* sequences. To construct pN-SupF and pB-SupF, the viral *Hin*dIII fragments from pN20-7 and pB16-5 were first cloned into the *Hin*dIII site of a *Pst*I site-deleted derivative of the vector pSP65 (Promega). A *supF*-containing *Pvu*II fragment of plasmid pVSU-II (38), which contains the *supF* gene flanked by both *Eco*RI and *Pvu*II sites, was then cloned into the viral LTRs at the unique *Pst*I sites which had been made blunt with T4 polymerase; the resulting viruses retained their original tropism (see Fig. 1). Plasmids pN-MoF and pB-MoF were constructed by site-directed mutagenesis of the parental plasmid pMoMLV-SupF (3, 40), changing the codons at positions 109 and 110 of CA in *gag* (Gln and Ala) to Gln and Arg (pN-MoF) or Thr and Glu (pB-MoF), which are the tropism-determining amino acids in naturally occurring N- and B-tropic viruses (11); these point mutations did not convert the NB-tropic parent virus to N or B tropism (see Fig. 1). Plasmids pN-ASMF and pB-ASMF were constructed by replacing the *Aat*II-to-*Sal*I fragment in pMoMLV-SupF with the analogous fragments from pN20-7 and pB16-5, respectively; these swaps were sufficient to convert the NB-tropic parent to N or B tropism (see Fig. 1).

Virus-producing cell lines were established by first transfecting SC-1 cells with plasmid DNAs encoding the viral genomes (which, for all except the MoMLV-SupF derivatives, were first cleaved with *Hin*dIII and self-ligated) and then cocultivating pooled transfected cells with fresh SC-1 cells. Virus used for infections was always harvested fresh from these producer lines.

The cell lines NIH 3T3 (*Fv-1*$^{n/n}$), BALB/3T3 (*Fv-1*$^{b/b}$), and SC-1 (phenotypically Fv-1$^-$) and the virus-producing SC-1 derivatives were all maintained in Dulbecco modified Eagle H16 medium supplemented with 10% fetal calf serum.

Viral infections. Cells were plated 48 h before infection at densities of 2×10^6/10-cm-diameter dish for target cells and 1×10^5/10-cm-diameter dish for virus producer cells. At 12 h before infection, the growth medium was replaced on the virus producer cells. Cells were infected in the presence of 8 μg of Polybrene per ml in a volume of 4 ml of virus plus growth medium. Each plate of target cells received 1/4 of the virus harvested from one plate of virus producer cells (for naturally occurring AKV N- and B-tropic viruses and their derivatives [top of Fig. 1]) or 1/10 of the virus harvested from one plate of virus producer cells (for MoMLV-SupF derivatives [bottom of Fig. 1]).

When virus production from the newly infected cells was to be assayed, medium was removed from the cells 12 h after infection, cells were rinsed once with phosphate-buffered saline solution, and fresh growth medium was added; virus was usually harvested 24 to 36 h after infection, except in one experiment (63 h [Fig. 4]). Reverse transcriptase assays were performed with virus pelleted from 5 or 0.5 ml of clarified supernatant, which was resuspended in a 50-μl cocktail containing 50 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.05% Nonidet P-40, 50 μg of poly(rCMP) per ml, 12 μg of oligo(dGMP) per ml, and 20 μCi of [α-^{3}H]dGTP. Incubation was for 2 to 4 h at 37°C. The reaction mixtures were spotted onto DE-81 paper (Whatman, Inc.), which was then washed six times with 0.5 M Na_2HPO_4 and dried, and the radioactivity was counted in a scintillation counter.

Preparation of cell extracts, integration reactions, and analysis of nucleic acids. Infected cells were harvested by trypsinization, and cytoplasmic and nuclear extracts were prepared by digitonin lysis and ball bearing homogenization as described previously (3), except that 125 μl of extract rather than 250 μl was prepared for each 10-cm plate of cells. Separation of the cytoplasmic and nuclear fractions was performed no longer than 5 min after digitonin lysis. Extracts were frozen in liquid nitrogen and stored at −80°C and were thawed immediately before integration assays or preparation of nucleic acids.

Integration reactions were performed essentially as described elsewhere (3, 4, 42) by using 500 ng of φX174 DNA as an integration target for 50 μl of integration extract. Nucleic acids were prepared from extracts or integration reactions as described elsewhere (3).

DNAs were analyzed either with or without restriction enzyme digestion by electrophoresis in an 0.7% agarose gel, blotting to a Hybond-N nylon membrane (Amersham), UV cross-linking, and hybridization with a nick-translated probe. The probe used was either a mixture of AKV virus plasmid clones (either pN20-7 and pB16-5 or pWN41, pWB5, and pGN104) or of MoMLV-derived plasmid clones (pN-ASMF and pB-ASMF) or a *supF Pvu*II fragment from plasmid pVSU-II. Tests for processing of the termini of viral DNA were performed as described in the legend to Fig. 6 and in reference 4.

Virion RNAs were analyzed by pelleting 450 μl of virus suspension for 5 min. at 30 lb/in^2 in a Beckman Airfuge. The supernatant was removed by aspiration, and the pellet was resuspended in 100 μl of 10 mM Tris (pH 7.4)–10 mM EDTA–200 mM NaCl–0.5% sodium dodecyl sulfate and then extracted twice with phenol-chloroform and twice with ether. The sample was then heated to 65°C for 2 min and mixed with 100 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). This mixture was subjected to serial fivefold dilutions in 10× SSC, and the dilutions were then blotted onto a Hybond-N nylon membrane by using a dot blot apparatus; the blot was then UV cross-linked and hybridized as described above for DNA analysis.

RESULTS

Accumulation of viral DNA in permissive and restrictive infections. Earlier work showed that, in some host-virus combinations, *Fv-1* restriction could be accounted for by inhibition of viral DNA synthesis (51). In other host-virus combinations, however, no defect in accumulation of linear viral DNA synthesis was seen, but formation of circular

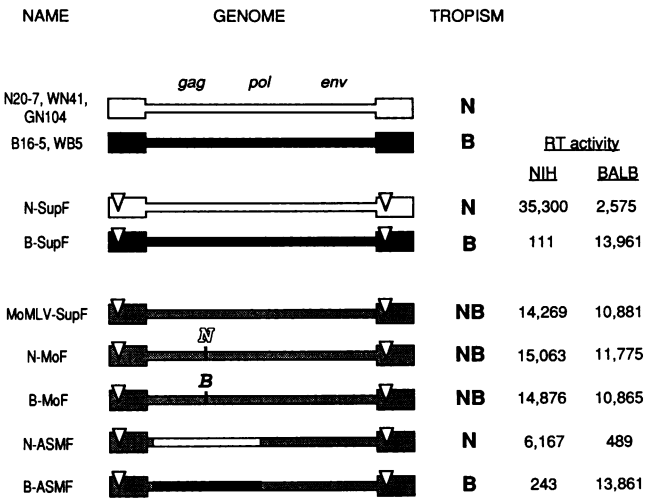

FIG. 1. Viruses used in this study. The names, genome organizations and tropisms of parental and derivative viruses are given. The genomes of prototypical virus clones N20-7 and B16-5 (11) and clones WN41, WB5, and GN104 (1) are schematically indicated as white or black for N- or B-tropic viruses, respectively. N-SupF and B-SupF are derivatives of N20-7 and B16-5, respectively (see Materials and Methods). Examples of reverse transcriptase (RT) activity (given in counts per minute) released 24 h after infection of either NIH 3T3 or BALB/3T3 cells are given to demonstrate that the derivatives retain their tropism. A derivative, MoMLV-SupF (see reference 3), of a prototypical NB-tropic virus (shaded genome) served as the parent for the additional derivatives N-MoF, B-MoF, N-ASMF, and B-ASMF (see Materials and Methods). Examples of RT activity released 24 h after infection of NIH 3T3 or BALB/3T3 cells with these viruses demonstrates that the point mutations (N-MoF and B-MoF) were insufficient to confer N or B tropism, while the larger chimeric swaps (N-ASMF and B-ASMF) were sufficient to confer N or B tropism upon the derivative viruses. Thus, mutations in regions of *gag* other than the 109/110 dinucleotide are probably responsible for the insensitivity of MoMLV to *Fv-1* restriction. Notably, the swaps in the chimeras did not include the IN domain of *pol*; therefore, MoMLV is not resistant to *Fv-1* restriction because of differences acquired in the IN-coding region.

DNA and integration were inhibited (7, 30, 31, 51). We wanted to address specifically, using an in vitro integration assay, the nature of the integration block in situations where linear DNA synthesis is normal in restrictive infections. Therefore, we analyzed the accumulation of viral DNA after infection of permissive and restrictive host cell lines by the viruses depicted in Fig. 1 and described in Materials and Methods. Since our intent was to test integration in vitro of DNAs synthesized in vivo, we fractionated newly infected cells into cytoplasmic and nuclear extracts of the sort that can serve as the source of integration machinery in unrestricted MoMLV infections (3). The appearance of viral DNA in these fractions was then studied.

We observed a novel effect of *Fv-1* on the accumulation of viral DNA: the appearance of linear DNA in the cytoplasmic fraction, but not in the nuclear fraction, was inhibited during infection of restrictive cells (Fig. 2). This effect is shown for three different viruses infecting NIH 3T3 and BALB/3T3 cell lines (Fig. 2A) and for two additional viruses infecting NIH 3T3, BALB/3T3, and SC-1 cell lines (Fig. 2B); it was also observed for the two AKV/MoMLV chimeric viruses (see Fig. 6). Importantly, the degree of effect on cytoplasmic viral DNA correlated well with the degree of restriction of virus production. For example, in the experiment whose results are shown in Fig. 2A, roughly 20-fold differences were seen

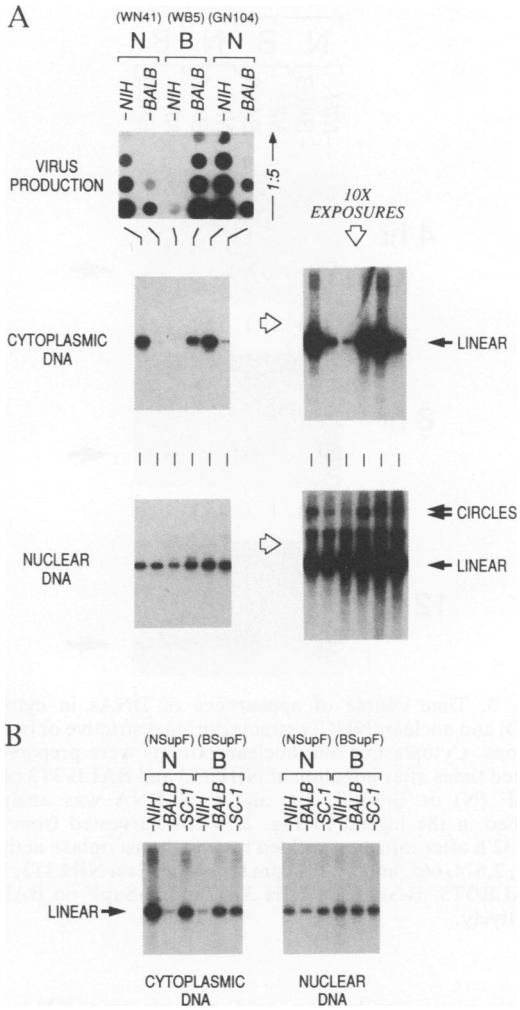

FIG. 2. Accumulation of DNAs in cytoplasmic and nuclear fractions during permissive and restrictive infections. (A) Two N-tropic viruses (WN41 and GN104) and one B-tropic virus (WB5) were used to infect NIH 3T3 and BALB/3T3 cells as indicated. Nuclear and cytoplasmic extracts were prepared 14 h after infection. Virus was harvested 36 h after infection from separated plates infected in parallel, and a dot blot of serial 1:5 dilutions of virion RNA, hybridized with an AKV MLV probe, is shown at the top. DNAs present in the various extracts were analyzed by agarose gel electrophoresis and Southern blotting with an AKV MLV probe. Equal proportions of cytoplasmic and nuclear DNAs were loaded onto a single gel, and the hybridized blot was exposed to film for a short period (left) and a 10-fold-longer period (right). The positions of linear and circular DNAs are indicated (black arrows). (The hybridizing material between linear and circular DNAs in the nuclear fraction corresponds to small amounts of fragmented chromosomal DNA present in the extract.) (B) An N-tropic virus (N-SupF) and a B-tropic virus (B-SupF) were used to infect NIH 3T3, BALB/3T3, and SC-1 cells as indicated. Nuclear and cytoplasmic extracts were prepared 12 h after infection and analyzed for viral DNA as described for panel A. Virus harvested from parallel plates 24 h after infection yielded reverse transcriptase activities of 8,112, 155, 246, and 3,746 cpm for N-SupF on NIH 3T3, N-SupF on BALB/3T3, B-SupF on NIH 3T3, and B-SupF on BALB/3T3, respectively (virus from SC-1 was not assayed). Amounts of DNA loaded on the gels in both panels correspond to approximately 10^6 cell equivalents per lane.

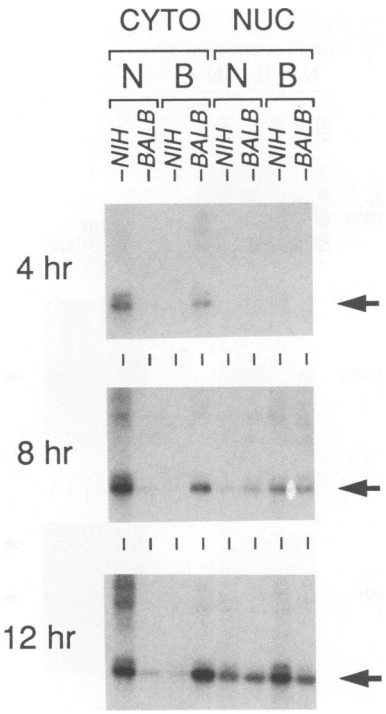

FIG. 3. Time course of appearance of DNAs in cytoplasmic (CYTO) and nuclear (NUC) extracts during restrictive or permissive infections. Cytoplasmic and nuclear extracts were prepared at the indicated times after infection of NIH 3T3 and BALB/3T3 cells with N-SupF (N) or B-SupF (B), and viral DNA was analyzed as described in the legend to Fig. 2. Virus harvested from parallel plates 32 h after infection yielded reverse transcriptase activities of 10,527, 2,674, 665, and 14,275 cpm for N-SupF on NIH 3T3, N-SupF on BALB/3T3, B-SupF on NIH 3T3, and B-SupF on BALB/3T3, respectively.

in both virus production and cytoplasmic DNA during infection of permissive and restrictive hosts; the overexposed panels show that the effect on cytoplasmic DNA was greater than 10-fold. Linear DNA in the nuclear fraction, on the other hand, was decreased only slightly or not at all (Fig. 2). Circular DNA in the nuclear fraction was usually less abundant in restrictive than in permissive hosts (Fig. 2A, bottom right panel), although its appearance even in permissive infections was often difficult to detect. The pattern of decreased amounts of linear DNA in the cytoplasmic fraction was reproducibly observed in over 20 separate experiments and was independent of whether cell lysis was achieved by digitonin, Nonidet P-40, or Dounce homogenization (not shown). In addition, the pattern of DNA accumulation was unaffected when infections were carried out in the presence of cycloheximide (not shown), demonstrating that this restriction-associated phenomenon does not require induction of new protein synthesis in response to infection.

Upon infection of restrictive cells, the reduction in viral DNA in cytoplasmic but not nuclear fractions is visible at the earliest times when viral DNA appears in those fractions (Fig. 3). The absence of DNA in both cytoplasmic and nuclear fractions of restrictive host cells at the earliest time point (4 h) demonstrates that the decrease in cytoplasmic DNA is not due to a more rapid migration of DNA to the nuclear fraction. The time course experiment also shows that the effect of *Fv-1* on total DNA, measured by summation of the cytoplasmic and nuclear DNAs, depends on the time after infection. At the earliest time point, when no nuclear DNA is seen, total DNA is severely decreased in restrictive infections. Eventually, however, at later time points the level of total DNA in restrictive cells is within two- to fivefold of that in permissive cells. A particularly clear example is seen in Fig. 2A. A comparison of GN104 infection of NIH 3T3 and BALB/3T3 cells shows that the nuclear DNA levels in the two infections are roughly equal to the cytoplasmic DNA level in the permissive infection; thus, total DNA is decreased by about 2-fold in the restrictive case, even though restriction (as measured by virus production) was roughly 25-fold.

Restriction of integration in vivo. To confirm that the accumulation of integrated proviruses is inhibited in restrictive infections, we took advantage of the foreign insert (the *supF* gene) present in our N-SupF and B-SupF viruses. Hybridization to high-molecular-weight genomic DNA with the *supF* insert as a probe avoids the problem of background hybridization to endogenous MLV sequences that compromised previous measurements of integration in *Fv-1* restriction experiments (29, 48). The efficiency of integration in vivo was compared for permissive and restrictive infections by first preparing high-molecular-weight DNA from the nuclei of newly infected cells by Hirt fractionation (23). This DNA was then cleaved with a restriction enzyme that releases the *supF* insert from integrated proviruses and hybridized to a *supF* probe after electrophoresis and blotting (Fig. 4). The degree of *Fv-1* restriction of productive infection was well correlated with impaired accumulation of integrated proviruses, since the expected 215-bp *supF* fragment was released from the Hirt pellet DNA after infection of permissive but not restrictive hosts (Fig. 4C). Hybridization of uncut DNAs with an MLV probe showed that the Hirt pellet fractions were not significantly contaminated with differential amounts of unintegrated DNA and that they contained relatively equal amounts of genomic DNA (Fig. 4A).

Integration in vitro. The experiments described above show that levels of linear viral DNA in the nuclear fractions of infected restrictive cells are nearly normal but that integration is inhibited. Therefore, we compared the abilities of nuclear extracts from permissive and restrictive infections to integrate their DNA in vitro into an exogenously provided target. In the in vitro integration assay, DNA-containing nucleoprotein complexes present in cell extracts insert linear viral DNA into an added circular DNA target (ϕX174 DNA). Upon restriction enzyme digestion, the integration products give rise to a large new cleavage product, which is visualized by hybridization with an MLV probe after electrophoresis and blotting (4, 42). We observed that viral complexes in nuclear extracts from restrictive infections were as competent to integrate their DNA as those from permissive infections (Fig. 5), as indicated by the generation of a 13.2-kb product (*Hin*dIII-*Sal*I digest) or a 9.2-kb product (*Bam*HI digest). The mild variability in the final amount of product reflects the initial amount of viral DNA (as indicated by the 1.1- or 3.0-kb band in the *Hin*dIII-*Sal*I or *Bam*HI digest, respectively); thus, the specific activities were roughly equivalent for complexes present after infection of permissive and restrictive cells. Control experiments demonstrated that the integration product bands were not present before incubation or when the reactions took place in the absence of target DNA or in the presence of EDTA (not shown).

Thus, the preintegration complexes present after infection of restrictive cells are not irreversibly disabled; instead, they can be extracted from the cells in functional form. Furthermore, linear DNA in extracts from restrictive infections is

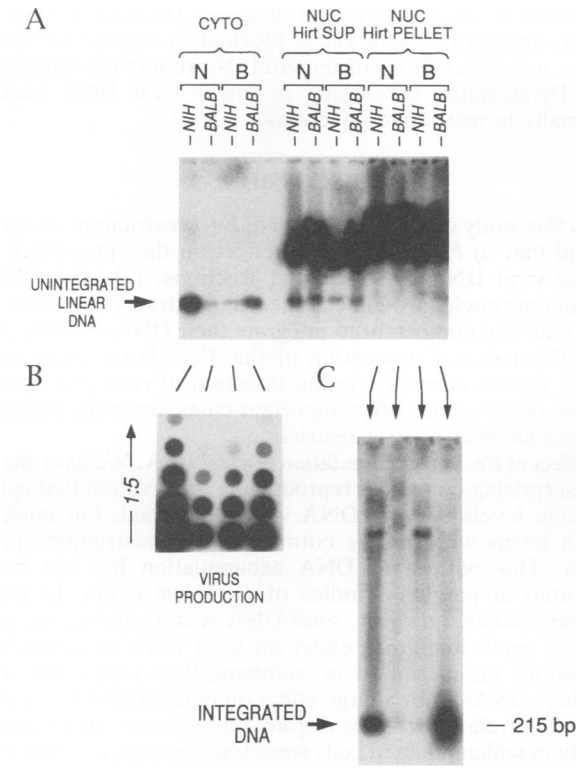

FIG. 4. Restriction is correlated with a decrease in integrated proviruses. NIH 3T3 and BALB/3T3 cells infected with N-SupF (N) or B-SupF (B) were harvested after 36 h. Upon harvest, a cytoplasmic (CYTO) fraction was prepared, and then the nuclear (NUC) pellet was resuspended and fractionated into Hirt supernatant (SUP) and pellet fractions. (A) The uncut DNAs were hybridized with an AKV MLV probe to demonstrate the absence of detectable unintegrated MLV DNA in the Hirt pellet fraction. The strong signal above the position of linear DNA in Hirt supernatant and pellet samples is due to hybridization to endogenous MLV-related sequences in the mouse cell chromosomal DNA. The amounts of DNA loaded on this gel were five times greater for the cytoplasmic DNA than for the nuclear Hirt DNAs (2.5×10^6 cells per lane for cytoplasmic and 5×10^5 cells per lane for nuclear Hirt DNAs). (B) Virus harvested from parallel plates 63 h after infection was analyzed by dot blot hybridization to serial 1:5 dilutions of virion RNA. (C) The nuclear Hirt pellet DNAs were digested with *Eco*RI to release the 215-bp *supF* fragment from integrated proviruses, and the resulting Southern blot was hybridized with a labelled *supF* probe. Additional blots showed the amounts of integrated proviruses in the Hirt pellet fractions from permissive cells to be roughly comparable to the amounts of unintegrated linear DNA in the Hirt supernatant fractions (not shown).

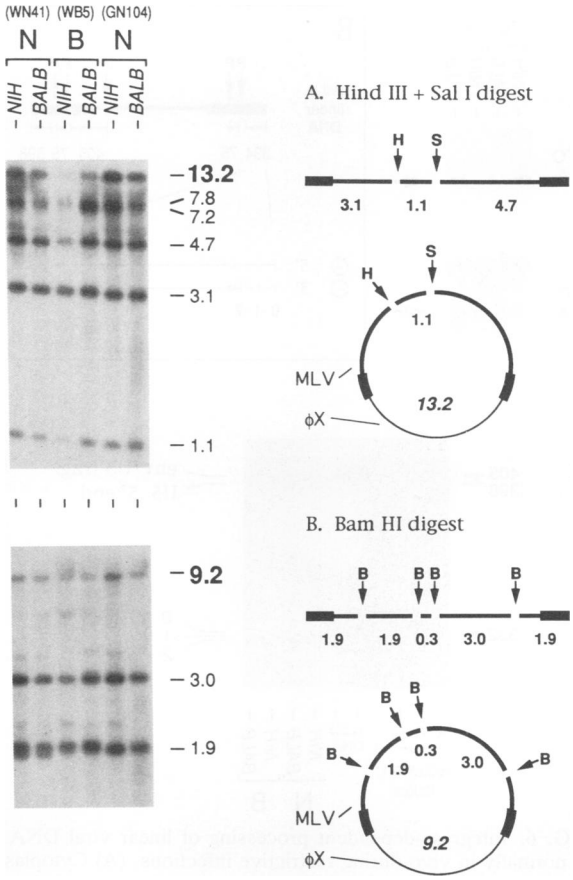

FIG. 5. Restriction does not operate during integration in vitro. Nuclear extracts from infections of permissive and restrictive cells, containing similar levels of linear viral DNA (uncleaved DNAs in these same extracts are shown in Fig. 2A), were tested for integration activity in vitro. Extracts were incubated with φX174 DNA as an integration target and then analyzed by digestion with *Hin*dIII and *Sal*I (A) or with *Bam*HI (B) followed by Southern blotting and hybridization with an AKV MLV probe. Integration of MLV DNA into φX174 DNA gives unique 13.2-kb (top) or 9.2-kb (bottom) digestion products (as diagrammed on the right), which are seen to be approximately equal for extracts from infections of permissive and restrictive cells. The relative amounts of viral DNA in the extracts are indicated by internal digestion products of 1.1 kb (top) or 3.0 kb (bottom) in size, which are generated from both unintegrated and integrated DNAs. The bands at approximately 7.8 and 7.2 kb in the *Hin*dIII-*Sal*I digest (top) correspond in size to either one- or two-LTR circles (expected in small amounts in these nuclear extracts [Fig. 2A]) or intramolecular recombinants (autointegrants [17, 36, 47]); the absence of a significant two-LTR circle digestion product in the *Bam*HI digest (expected size = 3.8 kb) suggests that the observed signal results primarily from autointegrants.

found associated with nucleoprotein complexes of normal size, as measured by sucrose gradient sedimentation (not shown), and normal amounts of CA protein appear in cytoplasmic and nuclear fractions in restrictive infections (not shown). It is possible that some restricting component that is associated with the integration machinery in vivo becomes disassociated or inactivated upon preparation of the in vitro integration extracts. Alternatively, restriction in vivo may not operate by directly inhibiting integration activity (see Discussion).

Processing of linear viral DNA in permissive and restrictive infections. To determine whether activities ascribed to the viral IN other than integration into the chromosome are restricted by *Fv-1* in vivo, we analyzed the IN-mediated removal of two bases from the 3′ ends of linear DNA (4, 10, 20, 33, 45). The exact 3′ terminus of the U3 end of viral DNA was determined by restriction enzyme cleavage at a point close to the end (Fig. 6B) followed by denaturing polyacrylamide gel electrophoresis and hybridization with an LTR probe equivalent to the plus strand of viral DNA (4, 20). We found that the U3 end of linear DNA in the nuclear fraction of infected restrictive cells had a normally processed 3′ end, which was recessed two bases from the 5′ end (332-base fragment [Fig. 6C]). Note that this blot also documents the integrity of the 5′ end of U5 (398-base fragment). Other experiments indicated that the 3′ end was also properly

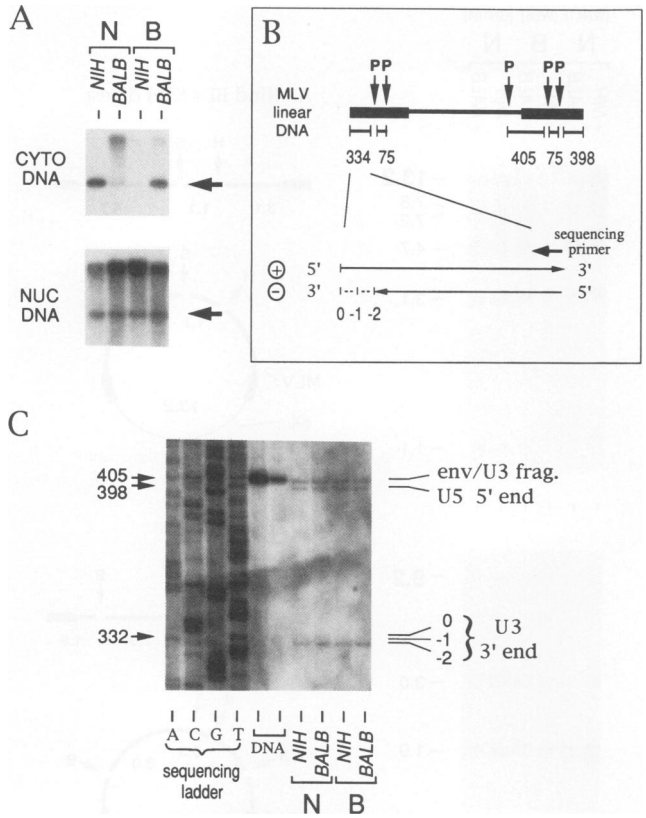

FIG. 6. Integrase-dependent processing of linear viral DNA occurs normally in vivo during restrictive infections. (A) Cytoplasmic (CYTO) and nuclear (NUC) extracts were prepared 18 h after infection of NIH 3T3 and BALB/3T3 cells with N-ASMF (N) or B-ASMF (B) virus. Virus harvested from parallel plates 36 h after infection showed the following relative amounts of virus: 100, 10, 4, and 100 for N-ASMF on NIH 3T3, N-ASMF on BALB/3T3, B-ASMF on NIH 3T3, and B-ASMF on BALB/3T3, respectively (as measured by dot blot hybridization of virion RNAs [not shown] in a manner similar to that shown for Fig. 2A and 4). Linear DNA (visualized by hybridization with a probe made from pN-ASMF and pB-ASMF MLV plasmid clones) present in cytoplasmic and nuclear fractions is indicated (arrows). The hybridization signal above the indicated position of linear DNA is due to fragmented chromosomal DNA contaminating the fractions; this region did not hybridize upon reprobing of the blot with a *supF* probe, as did the indicated linear species (not shown). (B) Diagram of the products of *Pvu*II digestion of linear DNA that are detectable with the probe used. An enlarged view of the U3, or left, end is shown. Proper cleavage of the 3′ end at position −2 (4, 10, 20, 45) reduces the 334-base fragment of minus-strand DNA to 332 bases in length. (C) Full-length linear viral DNAs in the nuclear fractions shown in panel A were agarose gel purified from approximately 10^7 cell equivalents each, digested with *Pvu*II, denatured, and electrophoresed on a 6% polyacrylamide–7 M urea gel (4). The gel was electroblotted, and the blot was hybridized with a minus-strand-specific M13 clone containing an *Xba*I-*Xba*I circle junction MoMLV LTR fragment as described previously (4). Also run in the same gel were samples of pMoMLV-SupF plasmid DNA that had been digested with *Pvu*II and *Sac*I (lanes DNA) and products of sequencing reactions using the M13 DNA described above as a template and a primer that has a 5′ end equivalent to that formed by the *Pvu*II digestion (primer D in reference 4). The blot shows that in all nuclear extracts, the U3 3′ ends have been properly processed to the 332-base −2 position; the arrow indicating the 332-base fragment points to the A nucleotide in the sequence 5′...CATT...3′ present at the terminus of linear viral DNA, which can be read from the sequencing ladder. Also indicated are the 398- and 405-base *Pvu*II digestion products representing the U5 5′ end and *env*/U3 fragments which come from the right LTR, as diagrammed in panel B.

recessed at the U5 terminus of linear DNA (not shown). Thus, although integration is blocked in restrictive infections, not all functions of the viral IN product are inhibited; the IN-mediated processing of linear viral DNA occurs normally in restrictive infections.

DISCUSSION

In this study of the mechanism of *Fv-1* restriction, we have found that (i) *Fv-1* has a novel effect on the appearance of linear viral DNA in subcellular fractions, (ii) viral DNA-containing nucleoprotein complexes in extracts from restrictive cells are competent to integrate their DNA in vitro, and (iii) IN-mediated processing of the 3′ ends of linear viral DNA occurs normally during infection of restrictive cells. These observations offer important clues about the molecular mechanisms of *Fv-1* restriction.

Effect of *Fv-1* on accumulation of viral DNA. We have made the surprising but highly reproducible observation that cytoplasmic levels of viral DNA were decreased, but nuclear DNA levels were nearly normal, in *Fv-1*-restrictive infections. This pattern of DNA accumulation has not been reported in previous studies of *Fv-1* restriction. In most earlier studies, however, total DNA was analyzed; we saw only a small inhibitory effect on total DNA in restrictive infections (as measured by summing the cytoplasmic and nuclear DNAs), but a large effect on cytoplasmic DNA that is well correlated with the degree of restriction. In one other study in which infected cells were fractionated, a 3- to 6-fold reduction in cytoplasmic viral DNA was observed, with a 60- to 70-fold restriction of viral replication (31); differences in fractionation procedures may account for the more dramatic effects on cytoplasmic DNA seen here.

What accounts for the differential effects on cytoplasmic and nuclear DNAs, and how do these effects relate to the restriction mechanism? There are several possible explanations, two of which will be considered here. Perhaps the simplest explanation is that viral DNA synthesis is slowed, but not stopped, upon entry into a restrictive cell. If migration of the viral nucleoprotein complex to the nucleus were to occur at a normal rate, independent of the completion of DNA synthesis, then the slower DNA synthesis would appear to occur primarily in the later compartment, the nucleus. In this model, the linear DNA eventually appearing in the nuclear fraction would nevertheless be blocked from integrating by *Fv-1*. A potentially related observation was made with quiescent cells infected with spleen necrosis virus, where, upon release from quiescence, DNA appeared in the nuclear fraction without a prior appearance in the cytoplasm (18).

An alternative, and perhaps extreme, model posits that the DNA detected in the nuclear fraction does not normally participate in integration and that the DNA in the cytoplasmic fraction is instead the relevant precursor to integrated proviruses. Although this model does not account for the reduced amount of cytoplasmic DNA, it would explain the correlation of cytoplasmic DNA levels, rather than nuclear DNA levels, with integration and virus production. Thus, if integration occurs rapidly upon entry into the nuclear compartment, those integration complexes that successfully integrate their DNA might never be detected as unintegrated DNA in the nucleus, in contrast to those that fail to integrate. While it is true that complexes in nuclear extracts are competent to integrate their DNA in vitro, the integration assay may bypass a condition that normally prohibits the unintegrated DNA detected in the nuclear fraction from integrating in vivo.

Effect of *Fv-1* on integration. If viral DNA in the nuclear fraction does participate in integration in permissive cells, then there must be some mechanism for prevention of integration in vivo in restrictive cells. Integration in vitro, however, is not inhibited by using nucleoprotein complexes present in extracts of infected restrictive cells. The most trivial resolution to this apparent paradox is that there is an *Fv-1*-dependent apparatus that directly inhibits integration activity in vivo but is inactivated or is disassociated from the integration complexes upon preparation of the in vitro extracts. Such an explanation would be difficult to disprove, but an exhaustive survey of conditions for preparation of extracts might uncover a method that preserves restriction activity.

Alternatively, *Fv-1* may not inhibit integration activity directly in vivo. It is possible that *Fv-1* blocks a step that is required for integration in vivo but not in vitro. For instance, the integration machinery in vivo might require transit to particular locations within the nucleus in order to come into contact with chromosomal DNA, and this proper intranuclear localization could be prevented in restrictive infections. Possibly relevant in this regard is the decreased level of circular viral DNA in restrictive infections. Some circular DNAs are in fact intramolecular recombinants, or "autointegrants" (47), but a significant fraction probably represents products of host cell activities (such as ligation and homologous recombination) that are independent of viral integration activities (12, 13, 43, 46, 47). If these host activities were located in the same intranuclear compartment as chromosomal target DNA, mislocalization would prevent both integration and host-mediated circular DNA formation. Indeed, fractionation studies imply differential localization or association of circular and linear DNAs, since release of circular DNAs from the nuclear fraction requires more drastic disruption than does release of linear DNA (unpublished observations).

It is also possible that the linear DNA seen in our nuclear fraction in restrictive infections is not actually in the nucleus but is instead attached to the outside of the nuclear membrane or perhaps associated with the cytoskeleton. The inability to migrate into the nucleus would therefore prevent both integration and circle formation. Biochemical fractionation has previously been misleading about subcellular localization (16, 22). In addition, large cytoplasmic structures, such as the actin-based cytoskeleton, most likely partition with the nuclear fraction (6, 34). Localization of MLV DNA by in situ hybridization (41) rather than by biochemical fractionation would be helpful in resolving whether viral DNA migrates to the nucleus in restrictive infections.

Infection of permissive cells with MLV (and other retroviruses) in the presence of either aphidicolin (a cellular DNA synthesis inhibitor) or cycloheximide (a protein synthesis inhibitor) results in a phenotype similar to that of *Fv-1* restriction—namely, normal levels of linear viral DNA are synthesized, but formation of circular DNA and integration are inhibited (8, 21, 26, 52). Recent experiments demonstrate that the aphidicolin effect (and by analogy probably also the cycloheximide effect) can be explained by a requirement for the cell to proceed through mitosis in order for integration of MLV DNA to take place (44a). This requirement might reflect poor nuclear localization of MLV DNA in the absence of nuclear envelope breakdown during mitosis. We have found that, upon fractionation of cells infected with MLV in the presence of aphidicolin or cycloheximide, linear viral DNA appears normally in the nuclear fraction (unpublished observations); however, the biochemical fractionation may be misleading. Furthermore, integration of DNA synthesized in such drug-treated cells occurs normally in vitro when extracts prepared from these cells are used, even though circle formation and integration in vivo are prevented (unpublished observations); these observations mirror those of *Fv-1* restriction and provide another example in which integration may be prevented in vivo without inhibition of integration activity per se.

Our observations limit the number of potential mechanisms by which *Fv-1* can prevent integration in vivo. The isolation of fully functional integration complexes from infected restrictive cells (Fig. 5) shows that the integration complexes are not irreversibly or drastically damaged in the cell. Thus, mechanisms that involve large-scale proteolytic cleavage or disassembly of the components of the integration complex can be disregarded. Potential mechanisms are further limited by the important observation that the 3′-end processing reaction occurs normally during infection of restrictive cells (Fig. 6). Thus, the *Fv-1*-dependent host cell apparatus cannot simply inhibit all activities of the IN protein in vivo. Instead, IN-mediated 3′ end processing occurs normally, yet integration is prevented. It could be argued that the early stage at which this processing occurs (4, 45) precedes the action of *Fv-1* against IN activity at later stages. However, the effect on cytoplasmic DNA suggests that the *Fv-1*-dependent action occurs very early, before complete synthesis of full-length DNA. Viable models for the mechanism of *Fv-1* restriction will need to incorporate the present observations.

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