Fv-1 Host Cell Restriction of Friend Leukemia Virus: Microinjection of Unintegrated Viral DNA

JEFFREY CHINSKY, 1 RUY SOEIRO, 1,2,3* AND JOHN KOPCHICK 4

Departments of Cell Biology, 1 Medicine, 2 and Microbiology and Immunology, 3 Albert Einstein College of Medicine, The Bronx, New York 10461, and Merck Institute for Therapeutic Research, Rahway, New Jersey 07065 4

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The murine gene Fv-1 has been shown to exert a major influence over the replication of ecotropic murine leukemia viruses. Studies of the replication of Friend murine leukemia viruses have shown that the restriction of viral replication occurs intracellularly after the initiation of viral DNA synthesis. The precise mechanism of the block imposed by the Fv-1 gene product is not completely understood. Our studies of Fv-1 restrictive infection have shown a variable decrease in the accumulation of intracellular unintegrated form I viral DNA. Analysis by microinjection of the viral DNA formed in nonpermissively infected BALB/c cells indicates that this DNA is infectious. These studies indicate that the form I DNA accumulated by permissive infected BALB/c cells contains the complete viral sequences necessary for the production of viral progeny and therefore suggest that the Fv-1 host restrictive mechanism recognizes viral factors other than form I DNA alone. These results support the possibility that Fv-1 host restriction occurs after formation of infectious viral DNA, perhaps at the integration step itself.

* Corresponding author.

It has been demonstrated that the murine locus Fv-1 encodes a resistance towards infection by ecotropic murine leukemia virus (MuLV) (10). Growth on the restrictive cell type varies from 1/100th to 1/1,000th of that on the permissive cell type, depending upon the exact cell and virus used (for a review, see reference 6).

Although the precise mechanism of action of the Fv-1 gene product is not completely understood, it has been demonstrated that it restricts viral replication intracellularly after the steps of adsorption and penetration (7, 18). After infection of restrictive hosts, the reverse transcription of viral RNA into unintegrated viral DNA can be demonstrated (1, 8, 20); however, the integration and establishment of proviral DNA does not appear to occur (7, 18). By comparison with infection of Fv-1 permissive cells, the results of Fv-1 restrictive infections are not uniform in terms of the patterns of accumulation of unintegrated viral DNA forms. That is, in some restrictive virus-cell combinations, a virtual absence of form I DNA accumulation is found, whereas other virus-cell combinations result either in a small (two- to fivefold) or in no apparent decrease in form I DNA accumulation relative to that observed in permissive infections (1).

In at least one cell line, NIH Swiss (Fv-1/mo), which is nonpermissive for B-tropic MuLV, we have demonstrated restriction of either form III or form I DNA accumulation, depending upon the degree of restriction of the clone of B-tropic MuLV used to infect these cells (1). Therefore, although in most host-restrictive infections an alteration of unintegrated viral DNA occurs, the exact pattern of this restriction varies. Furthermore, the mechanism by which the Fv-1 gene product restricts proviral DNA integration remains unresolved.

We have previously described a clone of N-tropic Friend MuLV (F-MuLV) which consistently demonstrates a 100- to 500-fold restriction for growth on BALB/c cells (Fv-1/mo) in tissue culture assays (1). Analysis of DNA accumulated in acute BALB/c cell infection under restrictive conditions over a range of input multiplicities reveals that only a two- to fivefold reduction in form I DNA is observed when compared with infection of permissive (NIH Swiss Fv-1/mo) cells (Fig. 1). The above results, therefore, are inconsistent with the proposal that Fv-1 restriction operates by preventing the synthesis or accumulation of form I DNA (8, 20).

We have attempted to understand the failure of this virus to establish an infectious center by studies of the unintegrated viral DNA with restriction endonuclease analysis (1). This approach has failed to reveal gross differences between the form III viral DNA accumulated in Fv-1 restrictive versus permissive cells (1; J. Chinsky and R. Soeiro, unpublished result; similar results have been obtained by W. Yang and P. Jolicoeur, personal communication). It therefore appears that grossly normal viral DNA (forms I and III DNA) can be synthesized in this Fv-1 restrictive infection. We therefore tested the biological completeness of this viral DNA by determining its infectivity in both permissive and nonpermissive cells.

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Unintegrated viral DNA was isolated by modified Hirt extraction (1, 3) from both nonpermissive BALB/c and permissive NIH cells acutely infected with N-tropic F-MuLV at multiplicities of infection (MOIs) known to exhibit the Fv-1 restrictive phenotype (MOI of 0.5). The extracted DNA was further fractionated by cesium chloride-ethidium bromide gradient centrifugation to separate viral DNA from cellular sequences which are known to contain endogenous, but rarely expressed, viral sequences (2). The DNA was pooled from the region of the cesium chloride-ethidium bromide gradient previously determined to contain form I DNA. As shown in Fig. 2, the circular DNA from N-tropic MuLV-infected permissive NIH cells (lane 1) as well as that from nonpermissive BALB/c cells (lane 2) was isolated free from virtually all cellular as well as most of the viral form III DNA. Because we had established that infection of BALB/c cells results in two- to fivefold less form I DNA as compared with NIH cells, twice as many infected BALB/c cells (versus NIH cells) were used to prepare the viral form I DNA. In
To microinject these cultures, before the addition of the XC cells, contained virus at a titer of 10^9/ml expressing an expected N-tropic pattern, identical to the virus used as the source of DNA. Therefore, the closed circular DNA (Fig. 2) obtained from nonpermissively infected BALB/c cells was infectious, like the same species of DNA obtained from permissively infected cells. In addition, these experiments indicated that there appeared to be no restriction of the production of viral progeny in the otherwise restrictive BALB/c (Fv-1b/b) cells after the microinjection of purified viral DNA obtained either from permissively infected NIH cells (Fig. 2, lane 1) or from nonpermissively infected BALB/c cells (Fig. 2, lane 2).

In a separate experimental protocol (method 2), we attempted to assay for virus production at an earlier time after microinjection. In this experiment, lower amounts of viral DNA (one to three copies per cell) were microinjected. We tested both for viral progeny released from the microinjected cells as well as for the approximate number of individual infectious centers established at both 24 and 55 h postmicroinjection.

Again, 300 cells on a cover slip containing 10^5 cells were microinjected, and the cells were allowed to recover for 24 h. At this time, the medium from the microinjected culture was tested and found to be negative for released viral progeny. The cells were trypsinized from the cover slip, both to prevent any possible cell-to-cell spread of virus and for testing of infectious centers.

Ten percent of the trypsinized cells were plated in dupli-
mic with 10^6 uninfected NIH cells (method 2), allowed to grow to confluence, and tested for foci by the XC assay. Medium taken before the addition of XC cells demonstrated released virus, but at a low titer (<1.0 x 10^4 PFU/ml). A predicted maximum of 30 infectious centers (foci) could result from this sample (10% of 300 microinjected cells). The XC assay revealed two to four foci per sample of cells and suggested, therefore, that only 10% of the microinjected cells had successfully become infectious centers. This was true of both microinjected NIH and BALB/c cells in experiments with DNA obtained from either the permissively infected cells (NIH) or the nonpermissively infected cells (BALB/c). These results appeared to indicate that at 24 h, most of the cells which had been injected by microinjection of viral DNA had not recovered sufficiently from the microinjection procedure to release progeny virus into the medium. This latter fact is a well-recognized phenomenon associated with microinjection techniques (Dennis Stacey, personal communication). However, given sufficient recovery time, some of the microinjected cells (an estimated 10%) were able to go on to become infectious centers.

The remaining 70% of the cells were replaced and allowed to undergo three or four generations of cell division under conditions in which cell-to-cell spread of newly produced virus was minimized. These cells, now at 55 h after microinjection, were trypsinized, counted, divided into portions, mixed with 10^5 uninfected NIH cells, and allowed to grow to confluence for testing for infectious centers. The data in Table 1 show the results of this assay. After normalization for the three or four rounds of replication of the cells and for the fraction of total cells in the sample used for each focus assay, it was calculated that between 5 and 10% of the originally microinjected cells were expressing virus.

These data demonstrate that, although at 24 h after microinjection no released viral progeny could be demonstrated, when these cells were further incubated with uninfected cells, clear infectious centers and released progeny virus could be detected. Our results suggest that only 5 to 10% of microinjected cells under our assay conditions successfully developed as infectious centers. Whether this was due to the efficiency of the microinjection process or the assay or both was not determined by our results. Furthermore, the apparent efficiency of focus induction by form I DNA was the same whether the source of the DNA was a permissive or restrictive infection. This result is not consistent with a model in which only a minority (between 10^{-2} to 5 x 10^{-3}) of form I DNA accumulated in a restrictive infection is complete and the remainder is defective.

These results suggest that the N-tropic F-MuLV viral DNA accumulated in the restrictively injected BALB/c cells is not grossly deficient in structure. Its ability to encode for the production of progeny virus compared with the viral DNA accumulated in permissively infected NIH cells is approximately the same. In addition, N-tropic viral DNA introduced by microinjection does not appear to be restricted for either virus production or the establishment of infectious centers in cells which are clearly Fv-l restrictive towards infection with intact N-tropic virus.

Our studies have utilized an N-tropic F-MuLV which was originally employed in vivo to define the Fv-l gene (10). This virus in vitro consistently exhibits a 100- to 500-fold restriction as manifested in standard tissue culture assays. However, analysis of the unintegrated viral DNA flanks of an 100-fold reduction in growth observed in nonpermissive BALB/c (Fv-l^p^-) cells. It has been demonstrated that the virus is unable to establish an integrated proviral state in the nonpermissive host as measured both by solution hybridization studies (7, 18) and by Southern blot analysis (Chinsky and Soeiro, unpublished data). Furthermore, the unintegrated form I DNA appears to be restricted at most by two- to fourfold compared with permissive infections. This small decrease is difficult to correlate directly with the 100- to 500-fold reduction in viral growth manifested in tissue culture assays (1).

Our studies of the production of infectious centers of viral progeny after microinjection of isolated unintegrated form I viral DNA suggest that the viral DNA accumulated in restrictive cells is not as severely deficient with respect to its capacity to encode for viral progeny. However, this same DNA in its native configuration (form I) during a Fv-l restrictive viral infection clearly does not result in the efficient production of viral progeny. It has previously been demonstrated that integrated viral DNA obtained from both Fv-l nonpermissive, as well as permissive, chronically infected host cells is infectious after transfection (4, 5, 14). This suggests that the mechanism of Fv-l restriction of viral progeny production did not act upon already integrated proviral DNA. In an analogous fashion, our data suggest that unintegrated viral form I DNA, observed to accumulate in restrictive cells, is infectious when introduced into recipient cells by microinjection. These results taken together suggest that Fv-l host restriction involves additional factors besides simply the relative amount of form I DNA observed to accumulate in these restrictive cells. The role of viral determinants in the sensitivity to Fv-l host restriction has been amply documented (for review, see reference 6). Presumably, the Fv-l restriction mechanism involves a number of factors of both viral and host origin. Viral DNA produced during the ordinary course of viral replication may be associated with one or more factors (presumably viral proteins) which are recognized by the Fv-l gene product. Microinjection of purified viral DNA appears competent to establish a successful infection in Fv-l resistant cells, pre-

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**Table 1.** XC plaque assay of cells microinjected with viral DNA

<table>
<thead>
<tr>
<th>Source of viral DNA (N-tropic F-MuLV)</th>
<th>Table 1).</th>
<th>No. (%) of XC-positive foci per 60-mm dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH cells (Fv-l^p^-) (permissive)</td>
<td></td>
<td>NIH cells^b^</td>
</tr>
<tr>
<td>BALB/c cells (Fv-l^p^-) (restrictive)</td>
<td></td>
<td>BALB/c cells^b^</td>
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<tr>
<td></td>
<td></td>
<td>57 (4)</td>
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<td></td>
<td></td>
<td>74, 78 (10)</td>
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</table>

^a N-tropic F-MuLV was used to infect both permissive (NIH Swiss) and restrictive (BALB/c) cells. Unintegrated viral DNA was prepared as described in the legend to Fig. 2, and this DNA was used for microinjection experiments (a sample of each DNA is shown in Fig. 2, lanes 1 and 2). Between one and three copies of viral DNA were microinjected per cell, as calculated from the estimation of viral DNA per sample and the known delivery volumes (9). After microinjection, the cells were first allowed to grow for three or four generations. Subsequently, they were trypsinized and analyzed for infectious centers by the XC plaque assay (see the text and reference 13). Values indicate the plaques observed per 60-mm dish, which contained one-third of the total culture of the microinjected cells. The numbers in the parentheses indicate the estimated percentage of microinjected cells which had to be successfully infected to give rise to the number of plaques observed (see the text). |

^b Duplicate plates were used for XC assay of BALB/c cells: the numbers represent plaques per 60-mm dish. |
sumably because viral factors required for host recognition are missing.

We have demonstrated that infectious viral form I DNA is produced in some Fv-1 restrictive cells. This result clearly demonstrates that the viral DNA is biologically complete in that it is able to code for infectious progeny with approximately the same efficiency as that obtained from a permissive infection. These results, therefore, suggest that the accumulation of N-tropic F-MuLV DNA in the nonpermissive BALB/c cells after acute infection is not sufficient to ensure eventual viral progeny production with the same efficiency as is seen in the Fv-1 permissive NIH cell. Perhaps the integration step itself may be rate limiting in the establishment of an infectious center in this Fv-1 restrictive infection.

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LITERATURE CITED


