JOURNAL OF VIROLOGY, May 1978, p. 306-315
0022-538X/78/0026-0306$02.00/0

Vol. 26, No. 2
Printed in U.S.A.

Abrogation of Fv-1^b Restriction with Murine Leukemia Viruses Inactivated by Heat or by Gamma Irradiation

R. H. BASSIN,* G. DURAN-TROISE, B. I. GERWIN, AND A. REIN
Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Maryland 20014

Received for publication 21 November 1977

Fv-1^b restriction in BALB/3T3 cells is temporarily abrogated following infection with N-tropic murine leukemia virus. The mechanism of this phenomenon was investigated by comparing the inactivation rates for viral infectivity and for the ability of the same virus to abrogate Fv-1 restriction. Inactivation of the abrogating ability of N-tropic murine leukemia virus following graduated doses of gamma radiation proceeded at half the rate of that for viral infectivity. This result indicates that viral RNA must function in abrogating Fv-1^b restriction but that only a portion of the viral genome is required. The inactivation kinetics of N-tropic murine leukemia virus were also determined following incubation of virus at 43°C. Abrogating ability of N-tropic murine leukemia virus was found to be about six times as stable under these conditions as was viral infectivity. Interestingly, virion-associated reverse transcriptase activity was inactivated at the same rate as was viral infectivity, indicating that this enzyme may not need to function during abrogation. Virus heated at 43°C was used to study the kinetics of the abrogation phenomenon itself. Abrogation was shown to be transient, requiring 6 to 9 h after virus infection to become maximally effective and beginning to disappear after about 18 h. The data reported here confirm the idea that abrogation of Fv-1 restriction can be separated experimentally from virus replication, and they raise the possibility that a separate biochemical pathway exists for incoming viral RNA in Fv-1 restrictive cells.

Certain murine leukemia viruses (MuLV's) are inhibited in their ability to replicate in certain mouse cells by a naturally occurring restriction system dependent upon the presence of the Fv-1 mouse gene (16, 24). Studies of Fv-1 restriction thus far have shown that MuLV's are probably inactivated after they adsorb to and penetrate nonpermissive cells (17, 21), but before DNA transcribed from incoming viral RNA is integrated into the host cell genome (19, 32). The exact mechanism of Fv-1 restriction, however, has yet to be elucidated.

Fv-1 restriction characteristically alters the titration patterns of N- and B-tropic MuLV's. Although these virus stocks infect corresponding permissive cells with one-hit kinetics, they give two-hit or multi-hit dose-response curves in Fv-1 nonpermissive cells (6, 9, 29, 33). This is an interesting result, since the two viruses required for productive infection are from cloned stocks and are presumably identical. Results from our laboratory (see below) indicate that the change from a one-hit to a two-hit requirement is the only essential manifestation of Fv-1 restriction, at least in BALB/3T3 cells (7). However, it should be noted that at least two laboratories have reported that Fv-1 restriction is not associated with a two-hit requirement for productive infection of nonpermissive cells (18, 31).

To understand and to characterize Fv-1 restriction more fully, we have initiated a series of experiments designed to examine the nature of the two-hit requirement for MuLV replication in nonpermissive cells. This work has been greatly facilitated by the use of defective murine sarcoma virus (MSV) pseudotypes which have been rendered phenotypically sensitive to Fv-1 restriction following mixed infection with N- or B-tropic MuLV during the "rescue" process (1, 7).

In a previous report we used an N-tropic pseudotype of Moloney MSV, rescued from transformed S$^+$L$^-$ cells by N-tropic MuLV, to study Fv-1^b restriction in BALB/3T3 cells (7). The results indicated that a single infectious N-tropic MuLV particle is able to temporarily abrogate Fv-1^b restriction in a BALB/3T3 cell, while neither B-tropic nor NB-tropic MuLV is capable of abrogating Fv-1^b restriction. An abrogated cell becomes fully permissive to productive infection with a second N-tropic MuLV and/or to transformation with N-tropic MSV. The abrogation

phenomenon becomes maximally effective 3 h after infection with N-tropic MuLV and lasts at least 18 h.

To account for the two-hit curves observed when N-tropic MuLV is assayed in BALB/3T3 cells and also for the multi-hit curves observed when N-tropic MSV is assayed in the same cells in the presence of various nonrestricted helper viruses, it was postulated that the N-tropic MuLV particle that is responsible for abrogating Fv-1 restriction in BALB/3T3 cells does not itself replicate (7). Thus infection with two N-tropic MuLV's would be required for productive infection of a single BALB/3T3 cell. To test this idea, we have examined the ability of N-tropic MuLV to abrogate Fv-1 restriction following partial inactivation of the virus by gamma radiation or by heating at 43°C. The ability of MuLV to abrogate Fv-1 restriction is clearly more resistant to either heat or gamma radiation than is the ability of the same virus to replicate in permissive cells, and stocks of virus can be prepared that are almost totally noninfectious, while still retaining the ability to abrogate Fv-1 restriction. These results confirm the idea that, of the two hits required for replication of N-tropic MuLV in BALB/3T3 cells, one hit represents infection with an N-tropic MuLV which abrogates Fv-1 restriction but need not itself replicate.

MATERIALS AND METHODS

Cells and viruses. The use of BALB/3T3 cells, which exhibit an Fv-1[b] pattern of resistance to infection with MuLV's, and of dually permissive 3T3FL cells as a lawn in infectious center experiments has been described (7).

Stocks of N-, B-, and NB-tropic MuLV were propagated in dually permissive SC-1 cells (15). The N-tropic MuLV pool, designated WN-1802N, was derived from the original BALB/c-S2N isolate, and the B-tropic MuLV pool, designated WN-1802B, was derived from the BALB/c-S2B isolate (16). NB-tropic MuLV was a cloned isolate (pool 2107) of Moloney leukemia virus. Seed stocks of all three viruses were generously provided by J. W. Hartley and W. P. Rowe, National Institute of Allergy and Infectious Diseases. The production of N-, B-, and NB-tropic pseudotypes of MSV by superinfection of S^+L^- cells has been described (7). Such MSV stocks were considered suitable for these studies because they possess MSV infectivity titers in excess of those of the infectious MuLV helper virus, which is also present.

Culture medium used in all experiments as well as for cell growth and virus production consisted of McCoys 5A supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.) and antibiotics.

Virus assays. The ability of N-tropic MuLV stocks to abrogate Fv-1 restriction in BALB/3T3 cells was quantitated by a novel assay procedure using an N-tropic pseudotype of MSV and a technique that permitted the separation of MuLV abrogating activity from its ability to replicate. The basic infectious center technique employed in this assay has been described previously (7). Briefly, 500,000 BALB/3T3 cells seeded in flasks 24 h previously were infected with the N-tropic MuLV to be tested. After 3 to 6 h, at which time abrogation of Fv-1 restriction is maximal, the cells were treated a second time with DEAE-dextran and infected again, this time with a constant amount of N-tropic MSV. Sufficient NB-tropic MuLV helper virus (multiplicity of infection [MOI] = 0.5 focus-inducing unit [FIU] per cell) was also added to permit the replication of any MSV particle able to infect a BALB/3T3 cell successfully. The number of MSV-producing cells was then scored in an infectious center test on a lawn of permissive 3T3FL cells. Under these conditions, the number of MSV-producing cells is proportional only to the amount of abrogating N-tropic MuLV (7). In this way any change in the abrogating ability of an N-tropic MuLV stock after treatment with agents such as heat or gamma radiation could be measured independently of the ability of the virus to replicate. Each infectious center assay included a control flask infected with the N-tropic MSV pseudotype and NB-tropic MuLV but not with N-tropic MuLV. This flask contained a small number of MSV-producing cells, presumably due to the abrogating activity of the N-tropic MuLV normally present in the MSV stocks (7). This background number (usually 4 to 10% of the total MSV added) was subtracted from all calculations except those in Fig. 4. The background number in each of these experiments did not differ significantly from the number of cells estimated to have been doubly infected with both MSV and N-tropic MuLV helper virus present in the original inoculum. Abrogating activity was considered significant only if the numbers of foci in the infectious center plates were at least twice this background level.

MuLV infectivity titers of all virus pools, including heated and irradiated samples, were determined by the S^+L^- focus assay method (3). MSV titers were determined by monolayer focus assays using 3T3FL cells and optimal amounts of NB-tropic MuLV helper virus as described previously (2, 14). All virus infections were carried out in cells pretreated with 20 μg of DEAE-dextran per ml. In the case of dual infections involving virus adsorptions at different times, each infection was preceded by a DEAE-dextran treatment.

Reverse transcriptase assays. Virion-associated reverse transcriptase activity in response to poly(rA)·$(dT)_{12-18}$ was measured in disrupted high-speed pellets from clarified tissue culture fluids as described previously (11). Polymerase reaction mixtures contained, in addition to the viral sample, 50 mM Tris-hydrochloride (pH 7.8), 0.5 mM $Mn(OAc)_2$, 20 mM each of KCl and dithiothreitol, 5 μg of poly(rA)·$(dT)_{12-18}$ (1:1 hybrid; Collaborative Research, Inc., Waltham, Mass.) per ml, and 2.2 μM [^{3}H]TTP (40,000 cpm/pmol, Schwarz/Mann, Inc.). Assays were incubated for 1 h at 37°C. Samples were diluted in disruption buffer to achieve linearity of [^{3}H]dTMP incorporation with enzyme concentration.

Irradiation of virus. Virus samples to be gamma irradiated consisted of filtered tissue culture fluids

harvested from chronically infected SC-1 cells. The culture medium had been changed approximately 3 h before harvest. Following bulk storage at −70°C for 2 months, the virus was thawed and aliquoted in 1-ml glass ampoules, which were then sealed and placed at −70°C once again. Approximately 2 weeks later, the ampoules, kept frozen in dry ice, were moved to the cobalt-60 source located at the National Bureau of Standards, Gaithersburg, Md. The characteristics of this irradiation equipment have been described (28). (Approximately 80% of the radiation from this source is in the 1.17 to 1.33 MeV range.) Virus was gamma irradiated while in a frozen state as follows. The ampoules were placed in a small thermos bottle together with a mixture of dry ice and ethanol, and the thermos bottle was placed inside a water-tight canister. The canister was then lowered into a pool of water which covers the radioactive source and was positioned so that the virus samples were at the center of the radiation. The dose rate on the day of the experiment described in Fig. 1 was computed to be 440.5 rads/s in water. The activity of unirradiated virus was calculated from results obtained with a mock-irradiated control sample.

Thermal inactivation of virus. Virus-containing tissue culture fluids, previously harvested, filtered, and stored at −70°C, were thawed and aliquoted in either small plastic tubes or glass vials. To inactivate the virus, the tubes were placed for varying periods of time in a water bath that was maintained at 43°C and kept covered throughout the procedure. After inactivation, virus was stored at −70°C until used. Incubation at 37°C was accomplished by a similar procedure, while that at 4°C was carried out in a refrigerator.

RESULTS

The rate of inactivation of virus particles following exposure to ionizing radiations such as X-rays and gamma rays, as well as to UV light, has been measured under carefully controlled conditions for a variety of viruses (12). The amount of genetic material that is responsible for given viral-encoded functions such as induction of cell transformation or production of infectious progeny can be estimated by comparing the relative inactivation rates for particular viral properties following stepwise irradiation. In this way viral functions may be differentiated from one another.

We employed gamma irradiation from a cobalt-60 source to measure and compare the radiosensitivity of two functions of N-tropic MuLV: the ability to replicate, as indicated by induction of foci in S^+L^- cells, and the ability to abrogate Fv-1 restriction, as measured by enhancement of N-tropic MSV replication in BALB/3T3 cells in the presence of NB-tropic MuLV helper virus (7).

Samples of an N-tropic MuLV pool were irradiated for varying periods of time while frozen. After irradiation, virus was stored at −70°C for about 3 weeks before testing. (In several other experiments the duration of this storage time did not appear to affect results significantly.) Virus employed in the abrogation experiments was usually thawed only once after irradiating, just before testing, although refreezing virus samples also did not appear to alter either abrogating activity or infectivity.

The lower curve in Fig. 1 represents the residual infectivity present in samples of N-tropic

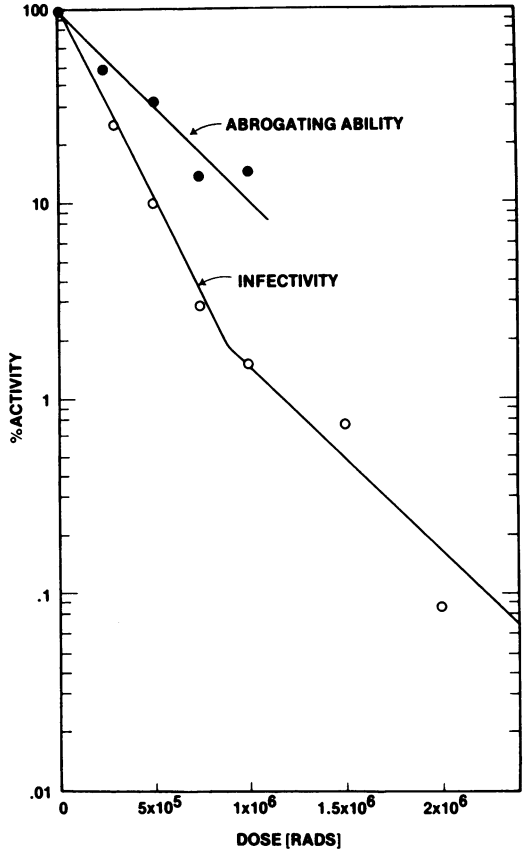

FIG. 1. *Gamma irradiation of N-tropic MuLV. Comparative inactivation rates for viral infectivity and for ability to abrogate Fv-1^b restriction. Frozen samples of N-tropic MuLV (2 × 10^6 FIU/ml) were irradiated for various times using gamma radiation from a cobalt-60 source. Residual viral infectivity* (○) *was measured for each sample by the S^+L^- focus assay for replicating MuLV. The ability of N-tropic MuLV to abrogate Fv-1^b restriction in each sample* (●) *was determined from the enhancement of focus formation by an N-tropic pseudotype of MSV in non-permissive BALB/3T3 cells simultaneously infected with NB-tropic MuLV (MOI = 0.5) (7). The dilution of MSV used to infect each flask of BALB/3T3 cells contained approximately 6,250 focus-forming units of MSV together with 625 FIU of helper N-tropic MuLV. Values for infectivity and abrogating ability are expressed as percent activity of mock-irradiated virus.*

MuLV after various doses of gamma irradiation. N-tropic MuLV was inactivated by gamma irradiation at a rate that gave a 37% survival dose (D_{37}) of about 2.32×10^5 roentgens (corresponding to approximately 2.20×10^5 rads in Fig. 1). The initial portion of the curve appears to be linear on a semi-log plot and to extrapolate to the origin, indicating that a single adsorption event or "hit" is sufficient to inactivate viral infectivity in this system. The size of the radiosensitive area or "target" can be estimated from the inactivation rate for viral infectivity shown in Fig. 1 and corresponds to a target size of roughly 2.85×10^6 daltons, a value consistent with the estimated size (4) for either genomic MuLV RNA (7×10^6 daltons) or, more likely, for one of its two subunits. It seems probable, therefore, that the mechanism of inactivation of the infectivity of N-tropic MuLV in this experiment is by a single ionizing event involving viral RNA.

As shown in Fig. 1, upper curve, the ability of N-tropic MuLV to abrogate Fv-1 restriction was also inactivated by gamma radiation with single-hit kinetics, but at a slower rate than was viral infectivity. The D_{37} for the ability to abrogate Fv-1 restriction was approximately 4.35×10^5 rads, corresponding to an RNA target size of 1.45×10^6 daltons or approximately one half of the estimated size of the RNA necessary for viral replication. These results indicate that some segment of viral RNA, different from or smaller than that required for virus replication, is probably involved in the abrogation of Fv-1 restriction.

It cannot be excluded that complementation or rescue involving gamma-irradiated N-tropic MuLV and either the MSV or the NB-tropic MuLV used in our infectious center assay system might give data that would lead to an underestimation of the actual target size for abrogation. However, our data are in close agreement with a previous study by Niwa et al. (26), who measured the abrogating ability of UV-irradiated virus using an assay system that did not involve coinfection with either NB-tropic MuLV or MSV.

Determinations of the levels of RNA-dependent DNA polymerase in gamma-irradiated virus samples indicated no significant loss of enzyme activity following doses of as much as 2×10^6 rads (data not shown). The fact that viral polymerase was not significantly inactivated following gamma irradiation with 2×10^6 or more rads, while the ability to abrogate was markedly reduced after this amount of irradiation, suggests that viral polymerase by itself is not responsible for the abrogation of Fv-1 restriction.

Heating of virus stocks under controlled conditions represents a second method for differentiating viral properties (12). It has been shown with AKR leukemia virus, for example, that at 43 to 44°C viral infectivity and virion reverse transcriptase activity are inactivated at approximately the same rate (10). Thus, by studying thermal inactivation of MuLV abrogating ability, we hoped to gain added insight into the mechanism of abrogation. In the following experiment, we incubated N-tropic MuLV stocks at 43°C for varying periods of time in a further attempt to compare quantitatively the abrogation phenomenon with viral infectivity under conditions of stepwise inactivation.

The rate of inactivation of N-tropic MuLV infectivity following heating at 43°C is shown in Fig. 2. There was a rapid decrease in infectivity at this temperature, the D_{37} being approximately 15 min. Thermal inactivation of the ability of N-tropic MuLV to abrogate Fv-1 restriction proceeded at a much slower rate than did the inactivation of viral infectivity, and the D_{37} for the ability to abrogate was approximately 88 min. Viral infectivity is therefore approximately six times more sensitive to thermal inactivation at 43°C than is abrogating ability. Both curves in Fig. 2 appear to follow first-order kinetics rather closely over the entire range tested.

The rate of inactivation of viral reverse transcriptase at 43°C is also shown in Fig. 2. Viral reverse transcriptase activity was inactivated at a much faster rate than was the abrogating ability of N-tropic MuLV. This result suggests that abrogation of Fv-1 restriction does not require polymerase-dependent transcription of viral RNA. The rate of thermal inactivation of viral reverse transcriptase seemed to be nearly the same as that observed for viral infectivity over the limited range of activity that could be measured accurately, suggesting that the inactivation of N-tropic MuLV infectivity by heating at 43°C is the result of the inactivation of virion-associated reverse transcriptase or of another molecule with a similar lability (10). On the other hand, the RNA from another N-tropic MuLV isolate, AKR virus, is remarkably stable to heat; 90% of virion RNA migrates as 70S molecules, which are composed of intact 35S subunits, after incubation of this virus at 43 to 44°C for 1 h (10).

Studies on the abrogation of Fv-1^b restriction usually involve infection with large amounts of virus, since it is only under these conditions that significant numbers of cells become permissive to subsequent infection with N-tropic viruses (7). The amount of N-tropic MuLV used in the heat inactivation experiment described in Fig. 2, for example, corresponds to an MOI, before inactivation, of 3.2 FIU per cell. Thus, the possi-

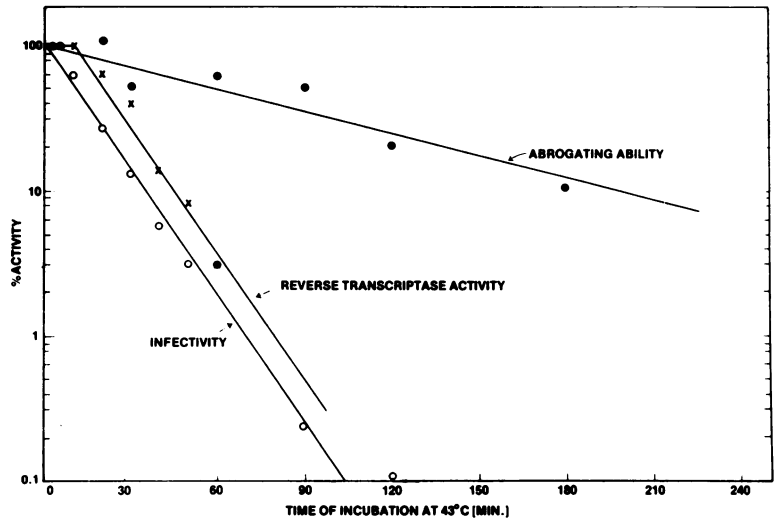

FIG. 2. *Incubation of N-tropic MuLV at 43°C: comparative inactivation of viral infectivity and ability to abrogate Fv-1^b restriction. Samples of N-tropic MuLV (titer = 5 × 10^6 FIU/ml) were incubated in a covered water bath at 43°C for varying periods of time and then refrozen. Residual viral infectivity* (○) *and abrogating activity* (●) *were measured as in Fig. 1, except that 30,000 focus-forming units of N-tropic MSV per ml (together with 20,000 FIU of helper N-tropic MuLV) were used to infect each flask in the abrogation assay. Reverse transcriptase activity of the heated viral samples* (×) *was measured by an exogenous reaction using poly(rA)·(dT)$_{12-18}$ (10). All values are expressed as percent activity of unheated control samples.*

bility exists that complementation or some other interaction occurring among inactivated virus particles might alter the results obtained in these types of experiment, giving inaccurate estimates of the inactivation rates. To test for such interactions, an N-tropic MuLV stock was heated at 43°C for 180 min, and various dilutions of this preparation, together with an unheated control, were tested for abrogating ability. Both the heated and unheated preparations were able to abrogate Fv-1 restriction in essentially all of the BALB/3T3 cell population (Fig. 3). A linear, one-hit portion of each titration curve was observed, indicating, as reported earlier, that infection with a single N-tropic MuLV particle is able to abrogate Fv-1 restriction under these assay conditions (7). Also, the two curves differed by a constant ratio, approximately threefold, at various MOIs. These results seem to rule out complementation as an explanation of the differing inactivation rates seen in Fig. 2, since one would expect multi-hit titration patterns and nonparallel curves if complementation involving two or more noninfectious viruses contributed significantly to abrogation in either case. The inactivation of abrogating ability by first-order kinetics with heated virus, as seen in Fig. 2, would also seem to argue against significant complementation among inactivated viruses. These data show, therefore, that a single N-tropic MuLV particle rendered noninfectious by heating at 43°C is still able to abrogate Fv-1 restriction in BALB/3T3 cells.

Complementation or recombination between inactivated N-tropic MuLV and the MSV or the NB-tropic MuLV required to measure abrogating ability in our assay system could conceivably introduce an error into the estimated rate of heat inactivation of abrogating ability. However, in a separate series of experiments (G. Duran-Troise, unpublished data), we have also observed that the abrogating ability of N-tropic MuLV is resistant to heating at 43°C using a technique similar to that described by Niwa et al. (26), in which MSV and NB-tropic MuLV are not required.

The inactivation rates for both the infectivity and the abrogating ability of N-tropic MuLV were also measured at 37°C and at 4°C (data not shown). At 37°C, the D_{37} values for both functions were the same, approximately 175 min, whereas at 4°C both functions were essentially stable during the 6-h period of the experiment. Data of Yoshikura (33), however, indicate that the ability of N-tropic MuLV to abrogate Fv-1 restriction is no more stable than is its infectivity upon incubation of virus at 50 to 51°C.

The vast majority of virus particles present in MuLV stocks prepared from cell cultures are apparently noninfectious. These noninfectious particles also do not appear to affect abrogation, for if noninfectious particles were able to abro-

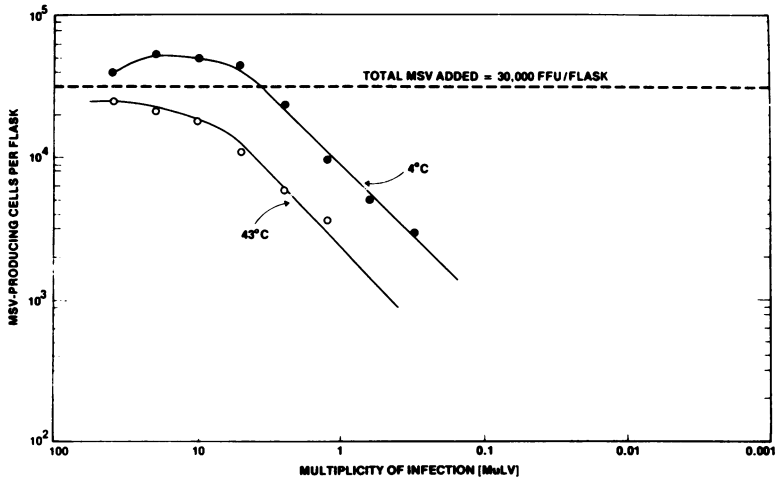

FIG. 3. *Abrogation by heated N-tropic MuLV: lack of evidence for virus-virus interactions. N-tropic MuLV heated for 180 min at 43°C (○) and a control virus sample incubated for 180 min at 4°C (●) were each tested at varying dilutions for the ability to abrogate Fv-1 restriction as described previously, using 30,000 focus-forming units (FFU) of MSV (together with 20,000 FIU of helper N-tropic MuLV) to infect each flask. The total MSV added is indicated by the horizontal dashed line. The titer of the N-tropic MuLV stock was 5 × 10[6] FIU/ml before heating and <10[3] FIU/ml after inactivation at 43°C. The MOI value for each virus stock was calculated on the basis of its infectivity before heat inactivation.*

gate Fv-1 restriction, the two-hit titration patterns normally associated with infection of Fv-1 nonpermissive mouse cells would not be observed. The fact that virus which was experimentally inactivated at 37°C did not abrogate Fv-1 restriction agrees well with the conclusion that noninfectious virions normally present in culture fluids maintained at 37°C do not abrogate Fv-1 restriction. It follows that N-tropic MuLV incubated at 43°C, which still retains its ability to abrogate Fv-1[b] restriction, is inactivated by a second mechanism, different from that responsible for inactivation at 37°C. Also, from the data presented previously (7) as well as from the results shown in the unheated (4°C) curve in Fig. 4, it can be readily calculated that the number of cells rendered permissive to infection with a given dilution of N-tropic MSV corresponds reasonably well to the number of infectious N-tropic MuLV particles known to be present. These results also seem to argue against a significant contribution to abrogation of Fv-1 restriction by the large excess of noninfectious virions normally present in MuLV stocks.

The markedly different inactivation rates of viral infectivity as opposed to abrogating ability after heating at 43°C allows the preparation of pools of virus which are able to completely abrogate Fv-1[b] restriction in populations of BALB/3T3 cells but which are almost totally noninfectious (Fig. 2). Using such heat-inactivated virus, we have been able to define the duration of the abrogation phenomenon and to determine whether abrogation represents a temporary or permanent alteration in the normal cellular Fv-1 restriction pattern. It was previously reported that, when BALB/3T3 cells are infected with infectious stocks of N-tropic MuLV, they become fully permissive to subsequent infection by N-tropic viruses within 3 to 6 h after infection and remain permissive for a period of at least 12 to 15 h (7). However, it was not considered feasible to extend these initial experiments to longer times with infectious virus stocks because of the unknown effects of progeny virus. We have, therefore, performed additional kinetic studies in which the infectious N-tropic MuLV used for abrogation was replaced by inactivated virus.

In the present study, N-tropic MuLV heated at 43°C for 180 min was used to infect flasks of BALB/3T3 cells as usual. At various times, the cells were superinfected with N-tropic MSV (40,500 focus-forming units per flask) and also with NB-tropic MuLV (MOI = 0.5) and subsequently tested for MSV focus formation by the infectious center procedure outlined previously. The results (Fig. 4) agree with earlier data using infectious virus (7). As in the previous study, infection of BALB/3T3 cells with N-tropic MuLV 3 or more h before infection with N-tropic MSV resulted in the abrogation of Fv-1 restriction. When N-tropic MuLV was added together with or after N-tropic MSV, abrogation was much less effective. In the present study, using virus inactivated at 43°C, it can be seen,

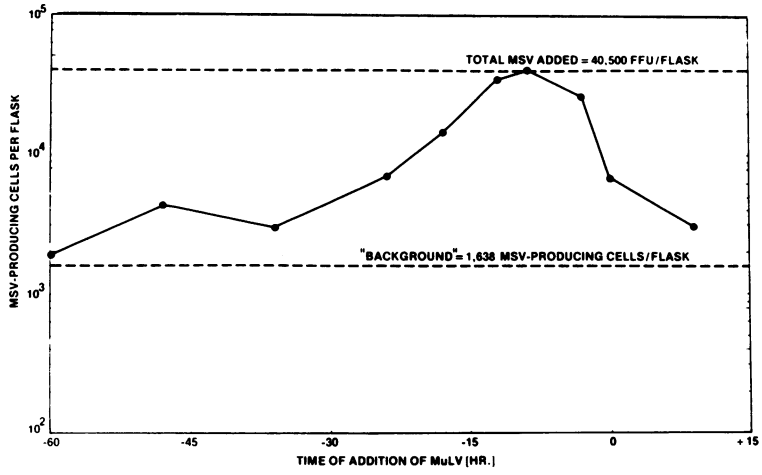

FIG. 4. *Kinetics of abrogation: time curve using heat-inactivated N-tropic MuLV. BALB/3T3 cells were infected with N-tropic MuLV heated at 43°C for 180 min. The cells were also infected with a mixture of N-tropic MSV (40,500 focus-forming units [FFU] per flask containing 27,000 FIU of helper N-tropic MuLV) and NB-tropic MuLV (MOI = 0.5) before, after, or at the same time as infection with N-tropic MuLV. Cells were plated as infectious centers onto 3T3FL lawns, and MSV foci were scored 4 days later by the usual procedures. Cells infected with N-tropic MuLV more than 24 h before the addition of MSV were subjected to one or more additional trypsinizations before being infected the second time. The upper and lower limits of sensitivity of the assay are defined by the amount of N-tropic MSV added to each flask and by the number of infectious centers produced by control cells infected with N-tropic MSV and NB-tropic MuLV (but not infected with heated N-tropic MuLV), respectively. These two values are indicated by horizontal dashed lines (- - -). The time of addition of MSV is defined as 0 h, and the time of addition of MuLV is expressed relative to this event.*

additionally, that abrogation is maximally effective for only about 18 h. The cells then appear less and less permissive to N-tropic MuLV and regain most of their Fv-1 restriction after 36 h (Fig. 4).

When B-tropic or NB-tropic MSV pseudotypes were substituted for N-tropic MSV in parallel control experiments, no significant effects resulting from variations in the time of addition of heated N-tropic MuLV were noted. Thus the transitory nature of abrogation noted in Fig. 4 is related to Fv-1 restriction itself and not to some other effect such as viral interference or a nonspecific change in host cell susceptibility.

The conclusion from the data presented in Fig. 4, that abrogation is a transient phenomenon, fits well with other data which show that clones of BALB/3T3 cells derived from cells infected with a single particle of N-tropic MuLV retain their Fv-1 restriction when tested approximately 1 month after infection (R. H. Bassin, G. Duran-Troise, B. Gerwin, A. Rein, and B. Barlow, in press).

DISCUSSION

The experiments described here were designed to contribute to an understanding of the mechanism of Fv-1 restriction by analyzing the two-hit requirement for replication of N-tropic MuLV in nonpermissive BALB/3T3 cells. Our previous studies, which employed tropic pseudotypes of MSV to quantitate Fv-1 restriction, showed that infection with a single N-tropic MuLV particle renders a cell temporarily permissive to subsequent infection with a second N-tropic MuLV (or N-tropic MSV) particle (7). This phenomenon was termed abrogation of Fv-1 restriction. Our initial data suggested that abrogating MuLV does not itself replicate and that this behavior is responsible for the two-hit requirement for productive infection of Fv-1^b cells with N-tropic MuLV. This model predicts that there is a functional difference between N-tropic MuLV which abrogates Fv-1 restriction and N-tropic MuLV which is able to replicate. The experiments presented here were primarily designed to test whether such a difference between abrogating and replicating MuLV could be confirmed experimentally by quantitative measurements of these virus functions following stepwise inactivation by either of two physical agents, heat or gamma radiation.

Inactivation of viruses upon irradiation with graduated doses of gamma radiation has been used extensively as a means of measuring and comparing the amount of genetic information responsible for the expression of virus functions (12, 22). Studies of this type depend upon the fact that the primary mechanism of inactivation of a given viral function by gamma rays consists

of a single ionization event within the target molecule, which is usually assumed to be viral nucleic acid (12). The inactivation curve for the infectivity of N-tropic MuLV by gamma radiation, shown in Fig. 1, indicates that the target for viral infectivity has a molecular weight of approximately 2.85×10^6 and that there is only one such molecule per virion. This estimate for target size agrees well with previous studies on gamma inactivation of Gross leukemia virus (22) and Rous sarcoma virus (13) and is reasonably close to the accepted genomic size of MuLV, 7×10^6 daltons, or of one of its two subunits. It seems very likely, therefore, that under our experimental conditions N-tropic MuLV is inactivated after irradiation by a single hit within its genomic RNA.

The target size for abrogation of Fv-1 restriction by N-tropic MSV, as estimated from gamma radiation studies (Fig. 1, upper curve), is approximately one half as large (molecular weight = 1.45×10^6) as that for viral infectivity. Thus, although abrogation of Fv-1 restriction also appears to require the ability of RNA to function, less of the RNA is required than that necessary for viral infectivity. This finding is in agreement with previous biological studies that indicate that abrogation can occur in the absence of viral replication (7). Niwa et al. (26) have reported abrogation of Fv-1^b and Fv-1^n restriction by UV-irradiated MuLV's and have estimated the target size for abrogation to be about one third that of viral infectivity. Yoshikura (33) has also reported that one of the two N-tropic MuLV's necessary for productive infection of Fv-1 nonpermissive cells is UV resistant. Inactivation of RNA tumor viruses by UV irradiation may not be entirely due to damage to viral RNA (5, 25, 27), however, and it is possible that UV and gamma irradiation data may not be directly comparable.

A second method, thermal inactivation, was used in a further attempt to characterize viral functions required for abrogation of Fv-1 restriction. As was the case with gamma irradiation, inactivation of virus infectivity at 43°C was much faster (D_{37} = 15 min) than was the ability of the same virus to abrogate Fv-1 restriction (D_{37} = 88 min). The inactivation curves for both abrogation and virus infectivity suggest first-order reactions with one (or a few) heat-sensitive molecules present per virion (Fig. 2).

Thus, the ability of N-tropic MuLV to abrogate Fv-1 restriction may be clearly differentiated from the ability of the same virus to replicate, as based on these quantitative inactivation studies using either radiation or heat. Abrogation and virus replication, therefore, represent separate functions of N-tropic MuLV. This conclusion confirms our earlier infectious center experiments, in which it was shown that the two N-tropic MuLV's necessary for productive infection of a BALB/3T3 cell differ in their biological properties. This two-hit requirement, which is the only essential manifestation of Fv-1 restriction in BALB/3T3 cells, very likely reflects the need for both functions, abrogation and replication, to be supplied by separate virions and implies that a virus which abrogates Fv-1 restriction does not itself replicate (7). The data presented here show that a virus need not be fully infectious in order to abrogate Fv-1 restriction, although it is still not known whether abrogation and virus replication are always mutually exclusive functions.

The nature of the viral function responsible for abrogation remains unknown, but some information can be derived from the studies presented here on residual virion reverse transcriptase activity present in gamma-irradiated and heat-inactivated virus samples. Reverse transcriptase activity was unaffected by up to 2×10^6 rads of gamma radiation, and it seems likely that introduction into cells of this enzyme or, presumably, other virion-associated proteins during infection with even large amounts of virus is not sufficient to abrogate Fv-1^b restriction. The lack of any observable "cooperative" effect when large amounts of partially inactivated virus were used to abrogate restriction (Fig. 3) also indicates that incoming virion-associated proteins are not involved in this process. This conclusion is also supported by preliminary experiments using noninfectious N-tropic MuLV particles synthesized in the presence of actinomycin D. These particles (Act D virions) contain normal levels of the virus structural proteins as well as functional reverse transcriptase (10, 23), but lack the ability to abrogate Fv-1 restriction (J. Levin and R. H. Bassin, unpublished data).

Although virion reverse transcriptase did not appear to be inactivated significantly by gamma irradiation, measurements of reverse transcriptase activity in heated virions show that the rate of inactivation at 43°C of this enzyme is rapid, closely approximating the rate of inactivation of virus infectivity (Fig. 2). In agreement with previous data (10), inactivation of N-tropic MuLV at 43°C thus appears to be primarily the result of an inactivation of viral reverse transcriptase. Of particular interest with regard to Fv-1 restriction is the observation that abrogating ability and reverse transcriptase activity are inactivated at very different rates at 43°C. This suggests that abrogation can proceed not only in the absence of viral replication but also in the absence of reverse transcriptase activity. The significance of this observation is discussed below.

(It cannot be excluded, however, that a small thermostable fraction of reverse transcriptase is still present in each virion after incubation at 43°C.)

Heating at 43°C is a useful method of separating viral infectivity from abrogating ability not only because the method of inactivation is simple and readily adaptable to large volumes of material, but also because the rates of inactivation of the two functions differ to a greater degree than they do following gamma irradiation. The kinetics are such that by heating a stock of N-tropic MuLV containing 3×10^6 to 5×10^6 FIU/ml at 43°C for 180 min, for example, one can obtain a stock of virus which contains only a few hundred FIU of infectivity but which retains sufficient activity to abrogate an entire population of cells (Fig. 2 and 3). Virus heated at 43°C for 180 min was used, for example, to define the transient nature of the abrogation phenomenon in an experiment where it was desirable to minimize virus replication (Fig. 4).

Finally, abrogation is a phenomenon that depends upon the ability to function of about one half of the viral RNA necessary for replication, as indicated by the gamma irradiation data (Fig. 1). On the other hand, virus particles heated at 43°C can abrogate but contain very little reverse transcriptase activity (Fig. 2). This indicates that the function of RNA in abrogation is not dependent on reverse transcriptase and, therefore, would not appear to involve synthesis of viral DNA. Kinetic studies show that abrogation is maximal for a period between 3 and 18 h after infection with N-tropic MuLV and then is greatly diminished (Fig. 4). We consider the above data to be compatible with the idea that the RNA of N-tropic abrogating MuLV is translated within 3 h after infection of a BALB/3T3 cell. One of the resulting products temporarily inactivates or complexes with the Fv-1^b restriction substance, rendering a BALB/3T3 cell permissive to infection with a second N-tropic MuLV. It is of interest that translation of the incoming RNA of avian myeloblastosis virus has already been reported (8). Moreover, the existence of one such viral product which interacts with Fv-1 restriction substance has been proposed on the basis of phenotypic mixing experiments (1, 20, 30). This product, which has not been identified, is present in MuLV virions, where it serves as a target for the Fv-1 restriction substance. Intracellular synthesis of large amounts of the target or of some other viral product might be expected to overcome Fv-1 restriction by the process which we have termed abrogation.

ACKNOWLEDGMENTS

We thank J. C. Humphreys, Center for Radiation Research, National Bureau of Standards, for his advice and assistance with the radiation experiments. The excellent technical assistance of Carol Gaskins and Brenda Wallace is also gratefully acknowledged.

These studies were supported by a contract from the Virus Cancer Program of the National Cancer Institute.

LITERATURE CITED

1. **Bassin, R. H., G. Duran-Troise, B. I. Gerwin, S. Gisselbrecht, and A. Rein.** 1975. Murine sarcoma virus pseudotypes acquire a determinant specifying N or B tropism from leukaemia virus during rescue. Nature (London) **256**:223–225.
2. **Bassin, R. H., N. Tuttle, and P. J. Fischinger.** 1970. Isolation of murine sarcoma virus-transformed mouse cells which are negative for leukemia virus from agar suspension cultures. Int. J. Cancer **6**:95–107.
3. **Bassin, R. H., N. Tuttle, and P. J. Fischinger.** 1971. Rapid cell culture assay technique for murine leukaemia viruses. Nature (London) **229**:564–566.
4. **Beemon, K. L., A. J. Faras, A. T. Haase, P. H. Duesberg, and J. E. Maisel.** 1976. Genomic complexities of murine leukemia and sarcoma, reticuloendotheliosis, and visna viruses. J. Virol. **17**:525–537.
5. **Bister, K., H. E. Varmus, E. Stavnezer, E. Hunter, and P. K. Vogt.** 1977. Biological and biochemical studies on the inactivation of avian oncoviruses by ultraviolet irradiation. Virology **77**:689–704.
6. **Declève, A., O. Niwa, E. Gelmann, and H. S. Kaplan.** 1975. Replication kinetics of N- and B-tropic murine leukemia viruses on permissive and nonpermissive cells *in vitro*. Virology **65**:320–332.
7. **Duran-Troise, G., R. H. Bassin, A. Rein, and B. I. Gerwin.** 1977. Loss of Fv-1 restriction in BALB/3T3 cells following infection with a single N-tropic murine leukemia virus particle. Cell **10**:479–488.
8. **Gallis, G. M., R. N. Eisenman, and H. Diggelman.** 1976. Synthesis of the precursor to avian RNA tumor virus internal structural proteins early after infection. Virology **74**:302–313.
9. **Gautsch, J. W., and H. Meier.** 1976. A short term quantitative XC assay for murine leukemia virus. Virology **72**:509–513.
10. **Gerwin, B. I., and J. G. Levin.** 1977. Interactions of murine leukemia virus core components: characterization of reverse transcriptase packaged in the absence of 70S genomic RNA. J. Virol. **24**:478–488.
11. **Gerwin, B. I., S. G. Smith, and P. T. Peebles.** 1975. Two active forms of RD-114 virus DNA polymerase in infected cells. Cell **6**:45–52.
12. **Ginoza, W.** 1968. Inactivation of viruses by ionizing radiation and by heat, p. 139–209. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. IV. Academic Press, New York.
13. **Golde, A., R. Latarjet, and P. Vigier.** 1962. Interférence isotypique *in vitro* par le virus de Rous inactivé par les rayons X. C.R. Acad. Sci. (Paris) **255**:2846–2848.
14. **Hartley, J. W., and W. P. Rowe.** 1966. Production of altered cell foci in tissue culture by defective Moloney sarcoma virus particles. Proc. Natl. Acad. Sci. U.S.A. **55**:780–786.
15. **Hartley, J. W., and W. P. Rowe.** 1975. Clonal cell lines from a feral mouse embryo which lack host range restriction for murine leukemia viruses. Virology **65**:128–134.
16. **Hartley, J. W., W. P. Rowe, and R. J. Huebner.** 1970. Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. J. Virol. **5**:221–225.
17. **Huang, A. S., P. Besmer, L. Chu, and D. Baltimore.** 1973. Growth of pseudotypes of vesicular stomatitis virus with N-tropic murine leukemia virus coats in cells resistant to N-tropic viruses. J. Virol. **12**:659–662.
18. **Jolicoeur, P., and D. Baltimore.** 1975. Effect of the Fv-1 locus on the titration of murine leukemia viruses. J.

Virol. 16:1593–1598.
19. **Jolicoeur, P., and D. Baltimore.** 1976. Effect of Fv-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia viruses. Proc. Natl. Acad. Sci. U.S.A. 73:2236–2240.
20. **Kashmiri, S. V. S., A. Rein, R. H. Bassin, B. I. Gerwin, and S. Gisselbrecht.** 1977. Donation of N- or B-tropic phenotype to NB-tropic murine leukemia virus during mixed infections. J. Virol. 22:626–633.
21. **Krontiris, T. G., R. Soeiro, and B. N. Fields.** 1973. Host restriction of Friend leukemia virus. Role of the viral outer coat. Proc. Natl. Acad. Sci. U.S.A. 70:2549–2553.
22. **Latarjet, R.** 1970. Inactivation du virus leucémogène de Gross par les radiations UV, X et γ. Int. J. Cancer 6:31–39.
23. **Levin, J. G., and M. J. Rosenak.** 1976. Synthesis of murine leukemia virus proteins associated with virions assembled in actinomycin D-treated cells: evidence for persistence of viral messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 73:1154–1158.
24. **Lilly, F., and T. Pincus.** 1973. Genetic control of murine viral leukemogenesis. Adv. Cancer Res. 17:231–277.
25. **Lovinger, G. G., H. P. Ling, R. V. Gilden, and M. Hatanaka.** 1975. Effect of UV light on RNA-directed DNA polymerase activity of murine oncornaviruses. J. Virol. 15:1273–1275.
26. **Niwa, O., A. Declève, and H. S. Kaplan.** 1976. Conversion of restrictive mouse cells to permissiveness during sequential and mixed double infection by murine leukemia viruses. Virology 74:140–153.
27. **Owada, M., S. Ihara, K. Toyoshima, Y. Kozai, and Y. Sugino.** 1976. Ultraviolet inactivation of avian sarcoma viruses: biological and biochemical analysis. Virology 69:710–718.
28. **Petree, B., and P. Lamperti.** 1967. A comparison of absorbed dose determinations in graphite by cavity ionization measurements and by calorimetry. J. Res. Nat. Bur. Stand. 71C:19–27.
29. **Pincus, T., J. W. Hartley, and W. P. Rowe.** 1975. A major genetic locus affecting resistance to infection with murine leukemia viruses. IV. Dose response relationships in Fv-1 sensitive and resistant cell cultures. Virology 65:333–342.
30. **Rein, A., S. V. S. Kashmiri, R. H. Bassin, B. I. Gerwin, and G. Duran-Troise.** 1976. Phenotypic mixing between N- and B-tropic murine leukemia viruses: infectious particles with dual sensitivity to Fv-1 restriction. Cell 7:373–379.
31. **Schuh, V., M. E. Blackstein, and A. A. Axelrad.** 1976. Inherited resistance to N- and B-tropic murine leukemia viruses in vitro: titration patterns in strains SIM and SIM.R congenic at the Fv-1 locus. J. Virol. 18:473–480.
32. **Sveda, M. M., and R. Soeiro.** 1976. Host restriction of Friend leukemia virus: synthesis and integration of the provirus. Proc. Natl. Acad. Sci. U.S.A. 73:2356–2360.
33. **Yoshikura, H.** 1973. Ultraviolet inactivation of murine leukemia virus and its assay in permissive and nonpermissive cells. Int. J. Cancer 11:739–746.