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A Replication-Defective Variant of Moloney Murine Leukemia Virus

I. Biological Characterization

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We have studied the virus produced by a clone, termed 8A, that was isolated from a culture of murine sarcoma virus-transformed mouse cells after superinfection with Moloney murine leukemia virus (MuLV-M). Clone 8A produced high levels of type C virus particles, but only a low titer of infectious murine sarcoma virus and almost no infectious MuLV. When fresh cultures of mouse cells were infected with undiluted clone 8A culture fluids, they released no detectable progeny virus for several weeks after infection. Fully infectious MuLV was then produced in these cultures. This virus was indistinguishable from MuLV-M by nucleic acid hybridization tests and in its insensitivity to Fv-1 restriction. It also induced thymic lymphomas in BALB/c mice. To explain these results, we propose that clone 8A is infected with a replication-defective variant of MuLV-M. Particles produced by clone 8A, containing this defective genome, can establish an infection in fresh cells but cannot produce progeny virus at detectable levels. Several weeks after infection, the defect in the viral genome is corrected by back-mutation or by recombination with endogenous viral genomes, resulting in the formation of fully infectious progeny MuLV. The progeny MuLV's that arose in two different experiments were found to be genetically different from each other. This is consistent with the hypothesis that, in each experiment, the progeny virus is formed as a result of an independent genetic event. DNA was isolated from clone 8A cells and assayed for infectivity by the calcium phosphate transfection technique. No detectable MuLV was produced by cells treated with this DNA. This finding, along with positive results obtained in control experiments, indicates that clone 8A cells do not contain a normal MuLV provirus.

The study of virus mutants is frequently a powerful approach in the analysis of viral functions. However, this approach has not been widely used with the mammalian type C viruses; although several temperature-sensitive mutants of these viruses have been described (43, 45, 48, 53), the number and variety of these reagents are still somewhat limited.

The present report deals with a clone of mouse cells isolated from a culture that had been infected with Moloney murine leukemia virus (MuLV-M). This clone, 8A, produces virus particles that exhibit an extremely low specific infectivity. Several properties of clone 8A suggest that it is infected with a variant of MuLV-M that does not perform one or more functions required for virus replication. This non-conditionally defective variant thus appears to represent a new type of reagent for genetic studies on MuLV.

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MATERIALS AND METHODS

Cells and viruses. The origins of 3T3FL cells (5, 18) and of the S^+L^- clones D56 and C243-3, which are murine sarcoma virus (MSV)-transformed derivatives of 3T3FL cells (5) and contain the m1 and m3 genomes (15), respectively, have been described previously. The continuous mouse cell lines BALB/3T3 (2), C3H 10T 1/2 (40), NIH/3T3 (31), BALB × NIH (44), and SC-1 (23) have also been described (39), as has the XC line of rat cells (49). Normal rat kidney cells (11) were obtained from G. Todaro of the National Cancer Institute; SIRC rabbit cells were obtained from the American Type Culture Collection.

Seed stocks of N-tropic (WN1802N) and B-tropic (WN1802B) MuLV were obtained from J. Hartley of the National Institute of Allergy and Infectious Diseases (25). The IC isolate of MuLV-M (13) was obtained from P. Fischinger of the National Cancer Institute. MuLV's were grown in 3T3FL cells. All virus

preparations were 24-h harvests, clarified by low-speed centrifugation or filtration and stored at −70°C.

Cell culture and cloning. Cells were grown in McCoy 5a medium or, where noted, Eagle minimum essential medium, supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), penicillin, and streptomycin. Falcon tissue culture vessels were used for all experiments. Cells were subcultured by trypsinization, except for the transformed lines C243 and 8A, which were mechanically dislodged from the culture vessel at each passage. Cells were cloned in Falcon Microtest II tissue culture wells (20) by seeding cell suspensions at ≤0.4 cells per well.

Virus infectivity assays. All infections were performed on cells pretreated with 20 μg of DEAE-dextran per ml. MuLV was assayed as described previously by the S^+L^- focus assay on D56 cells (6) or, where indicated, the XC test (18, 41). The results of the S^+L^- assay are given as focus-inducing units (FIU) per milliliter.

MSV was assayed on 3T3FL cells as described (5); optimal amounts of MuLV-M were added to the MSV assay plates at the time of infection with MSV (16, 22).

In some experiments (viz., MuLV growth curves, dilution end-point assays, and transfection tests), infected cultures were monitored for virus production for several passages after infection. In these experiments, the cells were allowed to reach confluence and were then subcultured at a split ratio of 1:20 to 1:100 at each passage. Harvests were taken at 24 h, when the cells were 50 to 90% confluent. In each of these experiments, a control culture was mock infected and then passaged and assayed in parallel with the experimental cultures. In no case did these control cultures show any evidence of virus production; these negative data have been omitted from the presentation of the results for simplicity.

DNA polymerase assays. Virus was pelleted out of clarified tissue culture fluid by centrifugation for 1 h at 100,000 × *g*. DNA polymerase activity in response to poly(rA) · $(dT)_{12-18}$ (Collaborative Research, Inc., Waltham, Mass.) was measured by adding portions of viral pellets, disrupted in a buffer consisting of 50 mM Tris-hydrochloride (pH 7.8), 1 mM dithiothreitol, 0.5 M KCl, 1% (vol/vol) Triton X-100, and 20% (vol/vol) glycerol, to reaction mixtures that contained 50 mM Tris-hydrochloride (pH 7.8), 20 mM KCl, 20 mM dithiothreitol, 0.5 mM $Mn(OAc)_2$, 2.2 μM [*methyl*-^{3}H] TTP (40,000 cpm/pmol), and 5 μg of poly(rA) · $(dT)_{12-18}$ (1:1 hybrid) per ml. Reactions were incubated at 37°C for 1 h, and the radioactivity incorporated into trichloroacetic acid-precipitable material was measured. All measurements were made at virus dilutions where incorporation was proportional to input virus concentration.

Nucleic acid hybridization. (i) Synthesis of [^{3}H]cDNA. Virus was purified from clarified culture fluids as described (17a). [^{3}H]DNA complementary to the viral RNA of MuLV-M or to that of N-tropic MuLV was synthesized by purified virus, by using the endogenous reverse-transcriptase reaction in the presence of 60 μg of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml, as described by Benveniste and Scolnick (8). [^{3}H]cDNA was extracted once with 1 volume of phenol plus 1 volume of chloroform-isoamyl alcohol (24:1) and once with chloroform-isoamyl alcohol alone. The aqueous phase was precipitated with 2.5 volumes of ethanol, dissolved in a minimal volume of 1 mM Tris-hydrochloride (pH 7.2)–0.1 M NaCl, and applied to a column of Sephadex G-50 (0.9 by 15 cm) equilibrated in the same buffer. The void volume was collected, pooled, made 0.3 M in NaOH, and hydrolyzed for 24 h at 37°C. The sample was then extensively dialyzed against 1 mM Tris-hydrochloride (pH 7.2) and stored at −70°C.

(ii) Preparation of cellular RNA. Cytoplasmic extracts were prepared by lysing cells in a buffer containing 10 mM Tris-hydrochloride (pH 7.8), 0.15 M NaCl, 1.5 mM $Mg(OAc)_2$, and 1% (vol/vol) Triton X-100; then nuclei were removed by centrifugation at 2,000 × *g* for 15 min. The cytoplasmic fraction was made 0.5% (wt/vol) in sodium dodecyl sulfate and extracted three to five times with 1 volume of phenol plus 1 volume of chloroform-isoamyl alcohol (24:1). The aqueous phase was then reextracted once or twice with chloroform-isoamyl alcohol alone. Nucleic acids were precipitated with 2.5 volumes of ethanol. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min, dissolved in a minimum volume of 4% Sarkosyl–0.1 M Tris (pH 8.0), and then purified through a discontinuous CsCl gradient as described by Glisin et al. (19). RNA was recovered by dissolving the resulting pellet in 20 mM NaOAc (pH 5.0) containing 0.5% sodium dodecyl sulfate and was precipitated with 2.5 volumes of ethanol at −20°C, collected by centrifugation, and dissolved in water.

(iii) Hybridization reactions. Hybridization reactions were performed essentially as described by Benveniste and Scolnick (8). Each 0.05 ml of reaction mixture contained 2,000 to 4,000 cpm of the specified [^{3}H]cDNA and 2.0 μg of carrier calf thymus DNA in addition to the specified RNA, in a solution that was 20 mM in Tris-hydrochloride (pH 7.2), 0.6 M in NaCl, 0.1 mM in EDTA, and 0.1% (wt/vol) in sodium dodecyl sulfate. These reaction mixtures were incubated at 66°C for 72 h and were analyzed by S1 nuclease digestion (8).

Transfection. (i) Extraction of DNA. Cells were removed from culture vessels and pelleted. The volume of each pellet was estimated in a graduated 15-ml conical centrifuge tube. The cells were then suspended in McCoy 5a medium containing 15% serum and 10% dimethyl sulfoxide and were stored at −70°C before extraction. After thawing, they were rinsed in calcium-magnesium-free phosphate-buffered saline, suspended in 1× SSC (0.15 M NaCl, 15 mM sodium citrate), and treated with 100 μg of proteinase K (EM Laboratories, Inc., Elmsford, N.Y.) per ml in the presence of 0.5% sodium dodecyl sulfate. They were then extracted repeatedly with chloroform-isoamyl alcohol (24:1). One-half volume of buffer-saturated phenol–*m*-cresol–8-quinlinol (800:110:1) was added to the chloroform at least once during the extraction of each sample. After extraction, the nucleic acids were precipitated with ethanol, spooled onto a glass rod, and dissolved in 0.1× SSC. They were then treated with RNase A (Worthington Biochemicals Corp., Freehold, N.J.; 100 μg/ml) at 37°C for 30 min and extracted,

precipitated, spooled, and redissolved as described above. The DNA was stored at 4°C until use. All DNA preparations had absorbance at 260 nm/absorbance at 280 nm ratios of 1.88 to 1.94.

(ii) Transfection assay. Infectivity of the DNA preparations was tested by the calcium phosphate method of Graham and van der Eb (21). NIH/3T3 cells were seeded at 8×10^5 cells per 60-mm dish in Eagle minimum essential medium plus 10% fetal calf serum. The following day, DNA was sheared by passing it 10 times through a 23-gauge needle. It was then diluted in phosphate-containing HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)-buffered saline (21) at pH 6.90 to 6.95, since this pH range was found to give the finest calcium phosphate precipitate under our conditions. $CaCl_2$ was added to a final concentration of 0.125 M, and the mixture was allowed to stand at room temperature for 25 min. The medium on the cells was replaced with fresh medium containing 5% serum, and the DNA-calcium phosphate suspension, containing 25 μg of DNA, was added to each culture. Approximately 5 h later, the fluid on the cultures was again replaced with fresh medium containing 10% serum.

When the DNA-treated cultures became confluent, each culture was divided among three 75-cm^2 flasks containing Eagle minimum essential medium plus 10% serum and 2 μg of polybrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml. Upon reaching confluence, the cells in each group of three flasks were pooled. They were then passaged in polybrene-containing medium for at least 6 weeks as described (see virus infectivity assays). They were monitored for MuLV production by UV irradiating portions of the cultures and overlaying them with XC cells (41); cultures that were XC negative were also tested for the production of particle-associated reverse transcriptase.

Electron microscopy. Concentrations of virus particles in tissue culture fluids were determined after negative staining, using the technique of Monroe and Brandt (35).

RESULTS

Isolation of clone 8A. Clone 8A was obtained from a superinfected culture of S^+L^- cells. S^+L^- cells were originally isolated after transformation of 3T3FL cells with Moloney MSV (5). The MSV genome in the C243-3 clone of S^+L^- cells is defective with respect to virus replication, since C243-3 cells release no infectious virus. However, this genome does specify the production of noninfectious virus particles containing several antigens characteristic of murine type C viruses (1, 4, 17). When these cells are superinfected with MuLV, infectious MSV is produced along with MuLV (5), and the cells undergo a striking morphological change. Many of the cells do not survive after superinfection (unpublished data); these changes induced in S^+L^- cells by MuLV infection have been used to develop an assay for MuLV (6).

Clone 8A was isolated as follows. C243-3 cells were superinfected with 2 FIU of MuLV-M per cell. The superinfected culture, consisting of cells only very loosely attached to the substratum, was passaged for 6 weeks and then cloned. Three cell clones were then isolated from the culture; all three have a transformed morphology and pattern of growth. The present report is concerned with the virus particles produced by one of these clones, termed 8A, but one of its sister clones, termed 5B, has been used in some experiments for comparative purposes. Tests of subclones isolated from the clone 8A culture indicate that clone 8A is a homogeneous cell population (data not shown).

Production of noninfectious virus particles by clone 8A. Supernatants of clone 8A cultures, and of appropriate control cultures, were tested for virus production by several methods. The results of one set of assays are shown in Table 1. No infectious MuLV was found in 1 ml of clone 8A supernatant. S^+L^- focus assays for infectious MuLV are usually completely negative, even with undiluted clone 8A supernatant, although a single focus is sometimes seen in these tests. Essentially identical results have been obtained in the XC plaque

TABLE 1. *Virus particle production by clone 8A and related cell lines*

Virus stock[a]	MuLV (FIU/ml)	MSV (FFU/ml)[b]	Particles/ml	Reverse transcriptase (cpm/ml)	Particles/infectious unit[c]	Reverse transcriptase[d]/particle
8A	<1[e]	8×10^1	3×10^8	2.2×10^6	3×10^6	7×10^{-3}
S^+L^-	<1	<1	1×10^8	2.4×10^4	$>1 \times 10^8$	2×10^{-4}
Rescued S^+L^-[f]	9×10^5	2×10^5	1×10^8	7.6×10^6	9×10^1	8×10^{-2}
MuLV-M	6×10^5	<1	8×10^8	1.3×10^7	1×10^3	2×10^{-2}
5B	3×10^6	6×10^4	5×10^8	1.0×10^7	2×10^2	2×10^{-2}

[a] 24-h harvests from parallel cultures, each with approximately 5×10^6 cells per 75-cm^2 flask.
[b] FFU, Focus-forming units.
[c] Infectious unit indicates the sum of MSV and MuLV infectivities.
[d] Counts per minute per milliliter of reverse-transcriptase activity divided by particles per ml.
[e] As discussed in the text, a titer of 1 FIU/ml was occasionally obtained in assays of clone 8A supernatant.
[f] S^+L^- cells were superinfected with 2 FIU of MuLV-M per cell 4 days before harvest.

assay on 3T3FL or SC-1 cells (data not shown). The apparent titer of MuLV, estimated by pooling the results of a number of assays, is approximately 0.5 infectious units per ml of clone 8A supernatant. In contrast, infectious MuLV is never produced by S^+L^- cells, whereas the infectious control stocks contain at least 6×10^5 FIU/ml.

The same supernatants were also tested for infectious MSV. Clone 8A cells produce approximately 10^2 focus-forming units of MSV per ml (Table 1). This MSV titer is roughly 1/1,000 of that found in infectious MSV stocks.

The concentration of physical particles of virus, as opposed to infectious virus, was also determined in each of these supernatants, by direct electron microscopic particle counts. Clone 8A cells produced 10^8 to 10^9 particles per ml, just as did cells producing infectious MuLV-M or MSV-MuLV (Table 1). Thus, the low infectivity found in clone 8A supernatant does not reflect a correspondingly low level of virus particle synthesis; rather, these particles exhibit a specific infectivity that is at least 1,000-fold lower than that observed with infectious virus stocks (Table 1).

Table 1 also shows the reverse-transcriptase activity of each of these supernatants. The level of enzyme activity per virus particle is comparable to that found in fully infectious virus stocks. Thus, the low infectivity of the particles produced by clone 8A does not seem attributable to a deficiency of reverse-transcriptase activity.

Since the S^+L^- isolate of MSV is defective for virus replication (5), the production of infectious MSV by clone 8A cells (Table 1) strongly suggests that this clone is, as expected, infected with MuLV. Further evidence for this conclusion is the high reverse-transcriptase activity found in clone 8A particles; the level of enzyme activity per virus particle was at least 30 times greater than that detected in the noninfectious particles produced by S^+L^- cells that had not been superinfected (Table 1). Recent reports indicate that reverse transcriptase is encoded by leukemia viruses but not by defective MSV (36, 46, 50).

In other experiments (not shown), the particles in clone 8A supernatant did not replicate on SIRC cells, which are susceptible to infection by xenotropic MuLV (data not shown) (7). In addition, when clone 8A supernatant was harvested and assayed for MSV and MuLV at 32.5°C, rather than at 37°C, results similar to those shown in Table 1 were obtained. Thus, the particles produced by clone 8A cells do not appear to be xenotropic MuLV or temperature-sensitive mutants of MSV or MuLV.

Growth kinetics of MuLV in clone 8A supernatant. Since clone 8A cells are apparently infected with MuLV, it was highly remarkable that standard infectivity assays (i.e., S^+L^- focus assays and XC tests) detected almost no MuLV in clone 8A supernatants. One possible explanation for this virtual lack of detectable infectious MuLV might be that clone 8A cells release an unusual MuLV, e.g., one that replicates too slowly to be detected in standard assays.

To test for the presence of an unusual MuLV in clone 8A supernatant, we infected 3T3FL cells with undiluted clone 8A supernatant and passaged the infected culture for several months. Supernatants were periodically harvested from this culture and tested for infectious MuLV and for particle-associated reverse-transcriptase activity. A parallel control culture was infected with 10 FIU of MuLV-M. (As noted above, an additional culture was mock infected. No evidence of virus production in the mock-infected culture was seen in this experiment, nor in any other experiment involving long-term passage of infected cells.) The culture infected with MuLV-M began producing detectable levels of MuLV within the first 1 to 3 days after infection (Fig. 1A). The levels of virus particles (as measured by polymerase activity per milliliter) and of infectious MuLV in the supernatant of this culture then rose together to plateaus of approximately 10^6 cpm/ml and 10^6 FIU/ml, respectively. In contrast, the culture infected with clone 8A supernatant produced no virus detectable in either assay for the first 7 weeks after infection. Virus production then began, and the levels of polymerase and infectious MuLV thereupon rose as rapidly as in the MuLV-M-infected culture and reached nearly the same plateau values. The virus obtained from this culture will be termed progeny isolate 1 of MuLV-8A.

These results show that clone 8A supernatant does contain particles (hereafter termed MuLV-8A) capable of giving rise to progeny MuLV. However, these MuLV-8A particles are not normal MuLV; they generate progeny only after an extended lag. The production of progeny MuLV in the experiment shown in Fig. 1A did not date from the time of infection, but from some second event that occurred 6 to 7 weeks after infection. Similar results have been obtained in several other experiments, although the length of the lag is variable.

The progeny MuLV produced by cells infected with MuLV-8A can register in the S^+L^- focus assay (Fig. 1A). The foci of infection that are scored in this assay are quite sizable, and presumably represent several cycles of productive infection occurring in the 5- to 6-day assay interval. Progeny isolate 1 of MuLV-8A thus seems qualitatively different from any of the particles

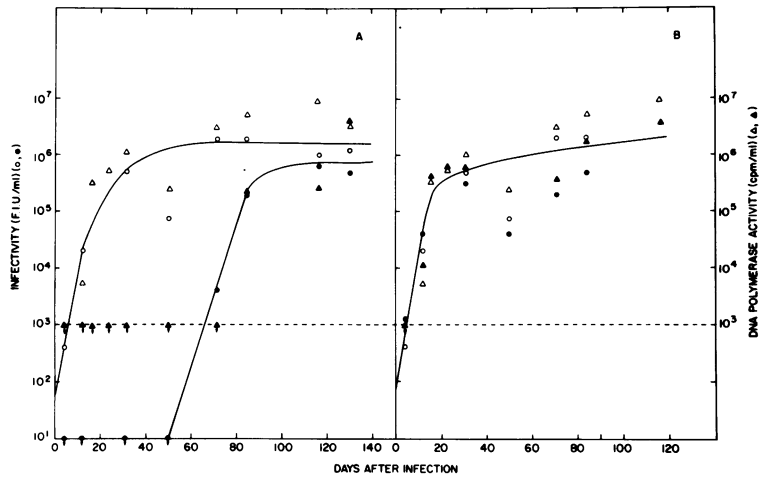

FIG. 1. *Kinetics of MuLV production in cells infected with clone 8A supernatant or with a progeny isolate of MuLV-8A. (A) 10^5 3T3FL cells were seeded in 75-cm² flasks. The next day, they were infected with 1 ml of undiluted clone 8A supernatant or 10 FIU of MuLV-M. The cultures were then passaged, and their supernatants were monitored for infectious MuLV and for particle-associated reverse transcriptase. Symbols: ----, background in the reverse transcriptase assay; ●, infectivity in culture treated with clone 8A supernatant; ▲, polymerase in culture treated with clone 8A supernatant; ○, infectivity in MuLV-M-infected culture; △, polymerase in MuLV-M-infected culture. (B) 10^5 3T3FL cells were seeded in 75-cm² flasks. The next day, they were infected with 10 FIU of MuLV-M or 20 FIU of a progeny isolate of MuLV-8A (obtained from undiluted clone 8A supernatant in an end-point dilution experiment [see text] on 3T3FL cells). The cultures were then passaged and monitored as described. Symbols: ●, progeny isolate of MuLV-8A infectivity; ▲, progeny isolate of MuLV-8A polymerase; ○, MuLV-M infectivity; △, MuLV-M polymerase.*

present in clone 8A supernatant itself, since it can apparently multiply without an extended lag.

Further evidence that progeny isolates of MuLV-8A multiply without a lag is shown in Fig. 1B. In this experiment, the replication kinetics of another progeny isolate were examined directly; as can be seen, these kinetics are virtually indistinguishable from the MuLV-M control.

In principle, the "second event" (Fig. 1A) that initiates the formation of infectious progeny MuLV in cells infected by MuLV-8A could either be physiological, like the induction of endogenous viruses, or genetic, such as a mutation in a viral genome. However, if it were a physiological event, then the progeny virus particles would be genetically identical to the input parental virus. They should therefore exhibit the same properties as the parental virus particles. The fact that the progeny isolates of MuLV-8A differ from MuLV-8A itself, in that they multiply without a lag, shows that this is not the case.

The simplest explanation of these findings appears to be that the particles in clone 8A supernatant can infect cells but are genetically incapable of replicating themselves. The second event, which occurs long after infection and initiates the formation of infectious progeny MuLV in cells infected by these particles, is a mutational or recombinational event that corrects the defect in the viral genome. Fully infectious MuLV is then produced; it spreads through the culture and is detected as a progeny isolate of MuLV-8A.

Dilution end-point experiments were performed to determine the concentration in clone 8A supernatant of particles able to give rise to progeny MuLV upon extended passage of infected cells. 3T3FL or normal rat kidney cell cultures were infected with serial dilutions of clone 8A supernatant, passaged for 2 months, and tested for virus production by the reverse-transcriptase assay and the S^+L^- focus assay. Although cultures infected with undiluted clone 8A supernatant reproducibly produced MuLV, those inoculated with low dilutions did not. The results of these tests (not shown) indicated that clone 8A supernatant only contains a low level ($<10^2$/ml) of particles that can produce detectable progeny MuLV within 2 months after infection of 3T3FL or normal rat kidney cells. It is not known whether longer passage of the infected cells would give a higher infectivity titer.

Genetic difference between two progeny isolates of MuLV-8A. Further experiments showed that not all progeny isolates of MuLV-8A are identical. Figure 2A shows the results of a second experiment, perfomed according to the

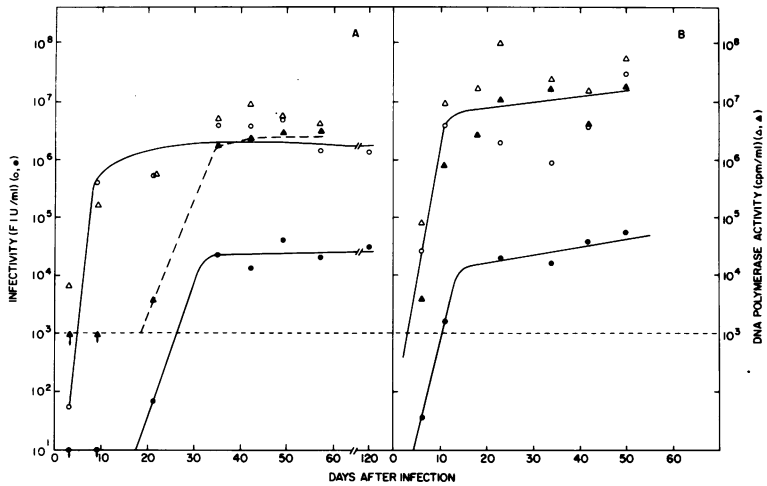

FIG. 2. *Origin and growth properties of progeny isolate 2 of MuLV-8A. (A) Protocol was exactly as in Fig. 1A, except that 3 × 10⁵ cells were seeded in each flask for the initial infections. Symbols: ●, infectivity in culture treated with clone 8A supernatant; ▲, polymerase in culture treated with clone 8A supernatant; ○, infectivity in MuLV-M-infected culture; △, polymerase in MuLV-M-infected culture. (B) 3T3FL cells (2 × 10⁵) were seeded in 75-cm² flasks. The next day, they were infected with 30 FIU of progeny isolate 2 of MuLV-8A or 30 FIU of MuLV-M. The cultures were then passaged and monitored as described. Symbols: ●, progeny isolate 2 infectivity; ▲, progeny isolate 2 polymerase; ○, MuLV-M infectivity; △, MuLV-M polymerase.*

same protocol as that shown in Fig. 1A. The progeny of MuLV-8A were first detected in this experiment after a lag of approximately 2 weeks. The level of reverse transcriptase in supernatants of this culture then rose to approximately 2×10^6 cpm/ml, or roughly the same level as was found in the MuLV-M-infected control culture. However, the infectivity of the progeny of MuLV-8A formed in this experiment (hereafter termed progeny isolate 2 of MuLV-8A) only attained a level of 1×10^4 to 2×10^4 FIU/ml; thus, progeny isolate 2 of MuLV-8A is characterized by a ratio of infectivity to enzyme activity which is approximately 50- to 100-fold lower than that of progeny isolate 1 of MuLV-8A, or of standard MuLV-M (Fig. 1 and 2).

To determine whether this low infectivity/reverse transcriptase ratio is a stable characteristic of progeny isolate 2 of MuLV-8A, we also examined the virus produced when this isolate was propagated in a fresh culture of 3T3FL cells. When fresh cells were infected with this MuLV stock, they produced infectious virus without a significant lag (Fig. 2); significantly, these progeny again showed an infectivity/enzyme ratio that was 50- to 100-fold lower than that of MuLV-M. Passage of progeny isolate 2 to fresh cells has given a ratio in this range in several independent experiments. Furthermore, the infectivity value obtained in the S^+L^- focus assay has been confirmed by a dilution endpoint assay (data not shown). Thus, the infectivity/enzyme ratio is a heritable property by which progeny isolate 2 of MuLV-8A differs from progeny isolate 1. The existence of this genetic difference between two progeny isolates of MuLV-8A conforms well with the hypothesis presented above, i.e., that each isolate is formed as a result of an independent alteration in the parental viral genome.

We have measured the infectivity/reverse transcriptase ratios of a total of six progeny isolates of MuLV-8A. In addition to progeny isolate 2, one other isolate showed a somewhat lower ratio than the others or MuLV-M (data not shown).

Comparison of progeny isolates of MuLV-8A with MuLV-M and with endogenous MuLV's. As noted above, the progeny isolates of MuLV-8A appear to be genetically different from MuLV-8A itself. This difference between parent and progeny could arise either by mutation (54) or by recombination with endogenous viral genomes (14, 26, 27, 47, 51, 52). A third possibility is that the progeny isolates are actually nondefective endogenous viruses and that infection with clone 8A particles somehow activates their production. We therefore tested the progeny isolates to determine their relative similarities to the known ecotropic endogenous MuLV's and to MuLV-8A and its presumed infectious parent, MuLV-M.

One experimental approach that can distinguish between different types of MuLV is nucleic acid hybridization. As shown by several laboratories (10, 12, 34), the known ecotropic MuLV's

can be divided into at least two broad classes, based on the degree of hybridization between virus-specific RNAs and cDNA probes. One class includes the Rauscher and Moloney isolates of MuLV; the other class consists of the Gross-AKR group of N- and B-tropic endogenous MuLV's, which show nearly complete homology with each other (32). We therefore prepared [^{3}H]cDNA probes from MuLV-M and from N-tropic MuLV and measured the degree to which RNA from infected cells could protect these probes from S1 nuclease digestion. RNA from MuLV-M-infected cells protected MuLV-M cDNA to a level of 60 to 70%, whereas RNA from cells infected with B-tropic MuLV only gave ~25% protection of this probe (Fig. 3A). Thus, as expected, the MuLV-M cDNA can be used to distinguish between MuLV-M-specific cellular RNA and B-tropic MuLV-specific cellular RNA.

The results obtained with RNA from clone 8A cells and from cells producing progeny isolate 1 or progeny isolate 2 of MuLV-8A are also shown in Fig. 3A. These RNAs all reacted with the MuLV-M probe to nearly the same extent as the homologous MuLV-M cellular RNA. Thus, the virus-specific RNA in these cells is very similar to MuLV-M cellular RNA and clearly different from B-tropic MuLV cellular RNA, by this test with the MuLV-M probe. RNA from clone 8A cells actually protected the cDNA slightly less than RNA from cells producing MuLV-M or the progeny isolates of MuLV-8A. The significance of this difference is now under study.

The same cellular RNA preparations were also tested for their ability to hybridize with a cDNA probe made from N-tropic MuLV. The data (Fig. 3B) are a mirror image of those presented in Fig. 3A. This probe was protected to a level of 52% by B-tropic MuLV cellular RNA, but only 15 to 22% by RNA from either MuLV-M-infected cells, clone 8A cells, or cells producing progeny isolates 1 or 2 of MuLV-8A. These two sets of results therefore indicate that the progeny isolates of MuLV-8A, like the viral genome in clone 8A cells, exhibit considerably more base sequence homology with MuLV-M than with the known ecotropic endogenous viruses.

A second property by which MuLV-M can be distinguished from the known ecotropic endogenous viruses is its lack of susceptibility to Fv-1 restriction. MuLV-M infects N-type cells (e.g., NIH or C3H mouse cells) and B-type cells (e.g., BALB/c mouse cells) with equal efficiency and is therefore NB-tropic. In contrast, the known ecotropic endogenous MuLV's are all sensitive to Fv-1 restriction in either N-type or B-type cells and are classed as B-tropic or N-tropic (25). Recent results suggest that viral tropism is determined by a virion component (possibly p30

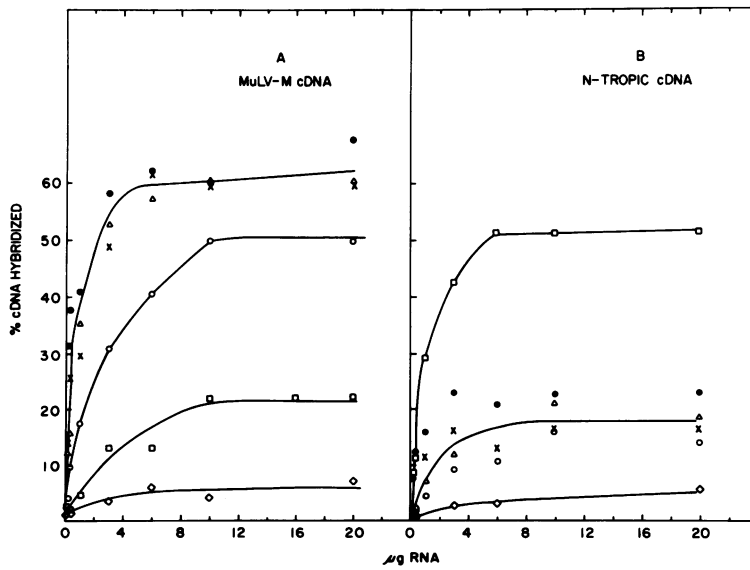

FIG. 3. *Hybridization of cellular RNA with [^{3}H]cDNA's. Cellular RNAs were extracted and tested as described in the text for hybridization with (A) MuLV-M cDNA and (B) N-tropic MuLV cDNA. RNA from: ●, 3T3FL cells producing MuLV-M; ×, 3T3FL cells producing progeny isolate 1 of MuLV-8A; △, 3T3FL cells producing progeny isolate 2 of MuLV-8A; ○, clone 8A cells; □, SC-1 cells producing B-tropic MuLV; ◇, uninfected 3T3FL cells.*

or a related protein [30, 42]) that is synthesized under the direction of viral genes (3, 33, 39). We therefore determined the infectivity of progeny isolates 1 and 2 of MuLV-8A on C3H and BALB/c cells by the XC test. Each of these isolates showed equal infectivity on the two cell lines (Table 2). In other experiments (data not shown), the isolates were also found to infect BALB × NIH (dually restrictive) hybrid cells as efficiently as they did 3T3FL (dually permissive [18]) cells. Thus, these two progeny isolates of MuLV-8A are, like MuLV-M, NB tropic.

In summary, tests of viral host range and of base sequence homology with standard virus isolates have failed to detect any differences between the progeny isolates of MuLV-8A and MuLV-M itself. Control experiments showed that these techniques can distinguish ecotropic endogenous MuLV's from MuLV-M. Thus, most or all of the genetic information of the progeny isolates is apparently derived from MuLV-M; these isolates may arise by back-mutation or by incorporation of a relatively small amount of information from endogenous viral genes.

Apparent lack of normal MuLV provirus in clone 8A cellular DNA. As discussed above, the replication kinetics of MuLV-8A suggest that the particles in clone 8A supernatant are genetically incapable of directing the synthesis of infectious progeny MuLV and that the progeny isolates of MuLV-8A arise as a result of an alteration in the viral genome in cells infected by MuLV-8A. In turn, it seems likely that the genetic defect in the particles produced by clone 8A cells reflects a defect in the proviral DNA that is presumably present in these cells.

Techniques have recently been developed for demonstrating infectivity of the DNA provirus present in the DNA of cells infected by RNA tumor viruses (21, 28). It thus seemed possible to test the hypothesis presented here. If clone 8A cells lack a normal MuLV provirus (i.e., a provirus able to direct the synthesis of infectious MuLV without an extended lag), then we should be unable to detect infectivity in clone 8A cellular DNA. In contrast, DNA isolated from cells producing a progeny isolate of MuLV-8A should be infectious.

Accordingly, cellular DNA was extracted from clone 8A cells and from cells producing progeny isolate 1 of MuLV-8A. To test the extraction procedures, we divided our pool of clone 8A cells into two portions. A suspension of MuLV-M-infected cells was added to one of the portions; the resulting mixture contained 80% (vol/vol) clone 8A cells and 20% MuLV-M-infected cells. The DNA from this mixture was extracted in parallel with that from the unmixed portion of clone 8A cells. Each of the DNA preparations was then tested for infectivity, as described above.

MuLV was not produced in any of the six cultures treated with 25 μg of clone 8A cellular DNA (Table 3). In contrast, MuLV was produced in five out of six cultures treated with DNA isolated from the cell mixture. Since each of the latter cultures received roughly 5 μg of DNA from MuLV-M-infected cells plus 20 μg of clone 8A cellular DNA, this positive result

TABLE 3. *Failure to detect a competent MuLV provirus in clone 8A cells by transfection*[a]

Source of DNA	Expt[b]	Infected cultures/total cultures
Clone 8A	1	0/3
	2	0/3
80% clone 8A + 20% MuLV-M	1	2/3
	2	3/3
Progeny isolate 1 of MuLV-8A	1	3/3
	2	2/3
3T3FL	1	0/3
	2	0/3

[a] Method as described in the text.

[b] The same DNA preparations were used for experiments 1 and 2.

TABLE 2. *Tropism of progeny isolates of MuLV-8A*[a]

Virus	PFU/ml on:		N/B[b]
	C3H cells	BALB/3T3 cells	
Progeny isolate 1 of MuLV-8A	3×10^4	5×10^4	0.6
Progeny isolate 2 of MuLV-8A	2×10^3	7×10^3	0.3
B-tropic MuLV standard	$<1 \times 10^{2c}$	2×10^5	<0.0005
N-tropic MuLV standard	8×10^4	8×10^{2c}	100
MuLV-M	1×10^7	2×10^7	0.5

[a] MuLV stocks were assayed on C3H (N-type) or BALB/3T3 (B-type) cells by the XC test.

[b] Ratio of infectivity on C3H (N-type) cells/infectivity on BALB/3T3 (B-type) cells.

[c] Plaques × dilution factor was used to compute these values, without regard for the "hitness" of these titrations.

shows that an inoculum of 25 μg should be sufficient for detection of a normal provirus, if any, in clone 8A cellular DNA. In addition, since the infectious DNA in MuLV-M-infected cells could be detected even when these cells were extracted together with a fourfold excess of clone 8A cells, it seems likely that the apparent lack of infectious DNA in clone 8A cellular DNA is not due to nucleases or inhibitors in these cells, nor to improper handling of the DNA.

Table 3 also shows that the DNA from cells producing progeny isolate 1 of MuLV-8A did give rise to infectious MuLV. Thus, in agreement with the predictions given above, these results suggest that clone 8A cells lack a normal MuLV provirus, whereas cells producing progeny isolate 1 of MuLV-8A do contain such a provirus.

In vivo effects of clone 8A particles and of progeny isolate 1 of MuLV-8A. It was also of interest to determine whether the particles produced by clone 8A cells, or progeny isolate 1 of MuLV-8A, are leukemogenic. In experiments performed by C. D. Scher (Sidney Farber Cancer Center, Boston, Mass.), these virus stocks were injected into day-old BALB/c mice. Control mice were inoculated with MuLV-M. Progeny isolate 1 of MuLV-8A induced thymic lymphomas (Table 4). Tumors appeared in these mice and in those infected with MuLV-M within 4 to 5 months of inoculation. In contrast, no pathological changes were found in mice held for 7 months after injection of undiluted clone 8A supernatant.

These results show that the relative lack of infectivity of clone 8A particles in standard in vitro assays is reflected by an apparent inability to cause neoplastic disease under the test conditions used here. In addition, progeny isolate 1 of MuLV-8A was found to resemble MuLV-M with respect to pathogenicity as well as host range and overall nucleic acid sequence.

TABLE 4. *Induction of leukemia by progeny isolate 1 of MuLV-8A*[a]

Virus	Polymerase units injected	No. of mice with leukemia/ no. of mice inoculated
Progeny isolate 1 of MuLV-8A	3.5×10^6[b]	9/11
8A	3.4×10^5	0/8
MuLV-M	3.4×10^5	5/8

[a] Day-old BALB/c mice were inoculated intraperitoneally with 0.1 ml of tissue culture supernatant. The mice were held for 7 months and then were sacrificed and examined for enlarged thymus or spleen.

[b] Counts per minute per 0.1 ml of tissue culture supernatant.

DISCUSSION

Virus production by clone 8A, which was isolated from a mouse cell culture after infection with MuLV-M, has been analyzed. On the basis of (i) the unusual replication kinetics of the particles produced by clone 8A, (ii) the genesis from these particles of heritably different progeny MuLV's, and (iii) the failure of transfection assays to detect a normal MuLV provirus in clone 8A cellular DNA, it is proposed that clone 8A cells are infected with a replication-defective variant of MuLV-M.

Several types of biological and biochemical experiments (not shown) have been performed on clone 8A particles, but no defect(s) responsible for the reduced infectivity of the particles has yet been identified. Further studies of the particles are now in progress.

Replication-defective variants are probably present at relatively high frequency in other MuLV preparations, as well as in our MuLV-M stock. In a careful study, Hopkins and Jolicoeur (29) have described a non-plaque-forming variant of N-tropic MuLV. The growth of this variant closely resembles that of MuLV-8A, and it seems likely that this virus fails to form XC plaques simply because it is unable to produce infectious progeny without an extended lag period. Similar clones have also been isolated in our laboratory from cultures producing B-tropic (33) or N-tropic MuLV (Gisselbrecht et al., manuscript in preparation). Finally, MuLV with analogous growth kinetics is apparently produced by C3H cells after treatment with 5-iododeoxyuridine (37).

Replication-defective variants should be powerful tools in many types of experiments on MuLV. Thus, they were recently used to demonstrate the donation of N- or B-tropism to NB-tropic MuLV (33). Furthermore, the fact that infectious progeny isolates can be obtained from these variants should be particularly helpful in attempts to correlate biochemical abnormalities with biological defects.

We have recently found that clone 8A cells and cells producing the amphotropic isolate of MuLV (9, 24, 38) show a form of complementation that can be detected by the XC test. This phenomenon has been used to devise a quantitative plaque assay for replication-defective ecotropic MuLV (manuscript in preparation). This new technique should greatly facilitate the isolation and analysis of defective variants of MuLV.

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