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NOTES

Replication-Defective Ecotropic Murine Leukemia Viruses: Detection and Quantitation of Infectivity Using Helper-Dependent XC Plaque Formation

ALAN REIN* AND ROBERT H. BASSIN
Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Maryland 20014

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Clones 8A and NP-N, which appear to be infected with replication-defective variants of murine leukemia virus, produce particles which do not form plaques in the XC test. These particles formed XC plaques when amphotropic murine leukemia virus, which is XC negative, was added to the assay plates. This phenomenon can be used as a quantitative infectivity assay for these replication-defective murine leukemia viruses.

Precise methods for the quantitation of infectivity in preparations of murine leukemia viruses (MuLV's) have been of great value in analyzing the biology of these viruses, particularly in cell culture systems (1, 14). In general, these methods depend upon the ability of the infectious particles to replicate themselves.

Assay techniques have also been devised for the replication-defective sarcoma viruses (4, 16). These assays are feasible because (i) the sarcoma viruses can be "rescued," i.e., produced in infectious form, in cells dually infected with a sarcoma virus and a nondefective leukemia virus; and (ii) the rescued sarcoma virus particles can be specifically detected, even in the presence of leukemia virus, by their ability to transform the assay cells.

We have recently described a clone which appears to be infected with a replication-defective variant of Moloney MuLV (MuLV-M) (13). We now report an infectivity assay for this variant and for other replication-defective ecotropic MuLV's. This procedure relies upon the fact that some isolates of MuLV, such as the "wild-mouse" amphotropic MuLV(MuLV-amph), replicate well in mouse cells, but, unlike nondefective ecotropic MuLV's, do not induce fusion in XC cells (2, 3, 6, 7, 10, 12, 17). We find that particles of replication-defective ecotropic MuLV, which do not themselves initiate XC plaque formation, give rise to XC plaques in cells which are coinfected with XC-negative, nondefective MuLV. Thus, the assay uses fusion of XC cells as a marker by which the defective virus can be specifically detected in the presence of a nondefective virus, in analogy to the use of transformation to detect the sarcoma viruses.

The defective variant of MuLV-M which we have studied is present in a clone of mouse cells termed "8A." Clone 8A was isolated from a murine sarcoma virus (MSV) transformed derivative of mouse 3T3FL cells which had been superinfected with MuLV-M. Clone 8A cultures release virus particles which appear to be almost totally noninfectious in standard MuLV assays, such as the S+L− focus assay or the XC test. However, at least some of these particles are clearly "infectious," in the sense that they can penetrate new host cells and establish an infection, because cultures infected with undiluted clone 8A supernatant and passaged for several weeks ultimately produce fully infectious MuLV-M. It was argued (13) that these particles contain a defective genome and are consequently unable to produce progeny viruses which can efficiently infect new host cells. This inability to undergo successive rounds of replication within a short period after infection is presumably why the particles do not register in standard MuLV infectivity assays. It was suggested that several weeks after infection, a mutational or recombinational event in the infected cells corrects the defect in the viral genome, allowing the production of fully infectious, nondefective MuLV (13).

MuLV-amph is one of several XC-negative, nondefective MuLV isolates which have been described recently (2, 6, 12). To test for an interaction between clone 8A particles and MuLV-amph, we infected dishes of normal mouse cells with a mixture of these two virus

stocks and then treated the dishes as in the standard UV-XC assay for ecotropic MuLV (14). Control dishes were inoculated with either clone 8A supernatant or MuLV-amph alone. As is shown in Fig. 1a and b, dishes infected with clone 8A particles or MuLV-amph alone contained no XC plaques. In contrast, the dish simultaneously infected with clone 8A particles and MuLV-amph contained over 100 plaques (Fig. 1c). The plaques were composed of numerous syncytia; their morphology is typical of the XC plaques formed by standard ecotropic MuLV (9, 14).

To investigate the role of the clone 8A particles in the formation of these plaques, we infected a series of plates with serial dilutions of clone 8A supernatant, along with a constant amount (0.25 focus-inducing unit [1] per cell) of MuLV-amph. As is shown in Fig. 2, the average number of plaques per plate was directly proportional to the amount of clone 8A supernatant added to the plate. Each plaque is therefore initiated by a single particle present in clone 8A supernatant, in concert with MuLV-amph; consequently, this test can be used as a quantitative infectivity assay for the clone 8A particles.

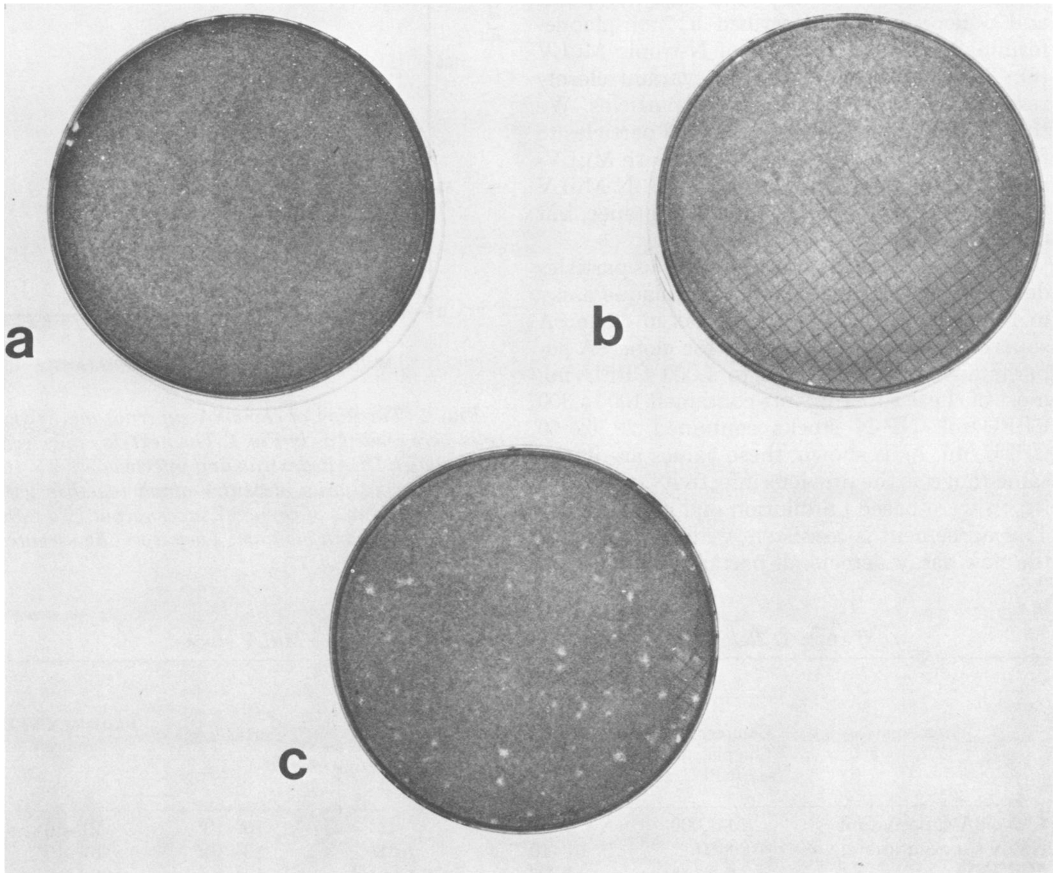

FIG. 1. *XC plaque formation after dual infection with clone 8A supernatant and MuLV-amph. 3T3FL cells (8×10^4) were seeded in 60-mm Falcon tissue culture dishes in growth medium (McCoy 5A medium with 10% fetal calf serum). The next day they were pretreated with 20 μg of DEAE-dextran per ml and infected with 1.0 ml of growth medium containing (a) 2×10^4 focus-inducing units (1) of MuLV-amph (isolate 1504A [2, 6, 12] obtained from E. Scolnick [National Cancer Institute] and propagated in SC-1 cells [5]); (b) 0.5 ml of clone 8A supernatant; or (c) 2×10^4 focus-inducing units of MuLV-amph plus 0.25 ml of clone 8A supernatant. The plates were then overlaid with growth medium. Six days later the medium was removed, the cells were irradiated with 1,800 ergs/mm^2 with a germicidal UV lamp, and 10^6 XC cells were added in Eagle minimum essential medium containing 10% fetal calf serum. This medium was also supplemented with 10^{-6} M hydrocortisone, which enhances syncytium formation in the XC test (L. Boone, personal communication). Three days later the cells were fixed with methanol and stained with Giemsa. Unless specified here, all reagents and procedures were as described (13).*

Data indicating that MuLV-amph acts by complementing the replication defect of the clone 8A particles will be presented elsewhere; in the present report we will refer to the new assay as a "complementation plaque" assay and to the ecotropic virus particles which form plaques in the assay as "complementation-plaque-forming units" (CPFU). We have also found (data not shown) that another XC-negative, nondefective MuLV, termed Moloney clone 83 (17), can be substituted for MuLV-amph in the complementation plaque assay.

It was of interest to determine whether viruses other than the clone 8A particles could register in the complementation plaque assay. Hopkins and Jolicoeur have described a "non-plaque-forming" variant ("NP-N") of N-tropic MuLV (8). The growth pattern of this variant closely resembles that of the clone 8A particles. We therefore tested the ability of NP-N particles to form XC plaques in cells coinfected with MuLV-amph. It was found that stocks of NP-N MuLV do give rise to XC plaques in the presence, but not in the absence, of MuLV-amph.

Table 1 shows the titers of infectious particles detected by the complementation-plaque assay in NP-N MuLV stocks, as well as in clone 8A supernatants. Titers observed for clone 8A supernatant ranged from 40 to 1,000 CPFU/ml; most of these supernatants contained 100 to 300 CPFU/ml. NP-N stocks contained 20 to 60 CPFU/ml. As is shown, these values are in the same range as the previous infectivity estimates, which were based on dilution end point assays. This agreement is consistent with the idea that the new assay detects all particles that are ca-

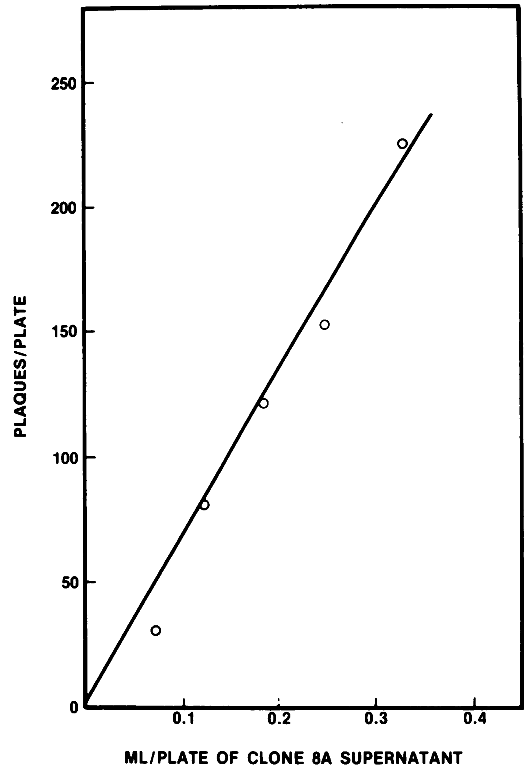

FIG. 2. *Titration of clone 8A supernatant. 3T3FL cells were seeded as in Fig. 1. The next day they were treated with DEAE-dextran and infected with 2×10^4 focus-inducing units of MuLV-amph together with different dilutions of clone 8A supernatant in a total of 1.0 ml of growth medium. They were then treated as described in Fig. 1.*

TABLE 1. *Relative infectivities of standard and defective MuLV stocks*[a]

Virus isolate	Infectivity as estimated by: Complementation plaque assay (CPFU/ml)	Dilution end point assay (infectious units/ml)	Standard XC assay (PFU/ml)	Particles/ml	Particles/CPFU (approx)
Clone 8A supernatant	40–1,000	$<10^2$ [b]	<1	10^8–10^9	10^5–10^7
NP-N supernatant	20–60	10^1–10^2 [c]	<1	10^8–10^9	10^6–10^8
MuLV-M	2.6×10^6	ND[d]	2.1×10^6	10^8–10^9	10^2–10^3

[a] Complementation plaque assays were performed as described in the legend to Fig. 1. Standard XC assays were performed on parallel plates in the same manner, except that MuLV-amph was omitted from the inocula. Data for particles per ml are taken from electron microscopic particle counts published in reference 13 in the case of clone 8A supernatant and MuLV-M, and are inferred from polymerase activity in the case of NP-N (reference 8 and data not shown). Polymerase measurements of clone 8A supernatant and MuLV-M (13) were also consistent with these values.

[b] Reference 13.

[c] Reference 8.

[d] ND, Not determined. However, virus cloning experiments have consistently shown that XC tests, S+L− focus assays, and dilution end point assays give virtually identical infectivity titers for standard ecotropic MuLV stocks.

pable of penetrating into cells and establishing an infection. Further data supporting this conclusion, based on other dilution end point experiments, will be presented elsewhere.

A normal, "infectious" stock of MuLV-M was also tested in the complementation plaque assay. Unlike the defective variants, this virus gives essentially the same titer in the new assay as in the standard XC test (Table 1). (Some stocks of normal N-tropic and B-tropic MuLV have shown two- to threefold higher titers in the complementation plaque assay than in the standard XC assay [data not shown].)

Clone 8A was originally isolated from an MuLV-M-infected culture which was transformed by the S+L− isolate of Moloney MSV (13). Thus, the levels of infectious particles detected by the complementation-plaque assay can be compared with the levels of infectious MSV produced by clone 8A cells. In general, when cells are dually infected by the S+L− isolate of MSV and by MuLV, they release MSV and MuLV at roughly equal titers (11). Clone 8A appeared at first to be an exception to this generalization, because it releases about 10^2 focus-forming units of MSV per ml, but virtually no MuLV detectable in standard infectivity assays (13). The present results show that clone 8A supernatant does contain roughly equal numbers of infectious particles containing the MSV and the defective MuLV genomes: the MSV is detectable in the standard transformation assay, but the defective MuLV particles are only detectable if their replication defect is complemented (or by passaging infected cells for extended periods [13]).

Because the complementation plaque assay appears to offer a way of measuring the number of particles which contain an ecotropic MuLV genome and can successfully infect fresh cells, it was also of interest to compare these resuslts with the concentrations of physical virus particles present in the respective MuLV stocks. Previously reported data (summarized in Table 1) indicate that clone 8A supernatant, NP-N MuLV, and standard MuLV-M stocks all contain approximately 10^8 to 10^9 virions per ml. Thus, in a normal stock of MuLV, 1 in 10^2 to 10^3 particles is capable of successfully infecting a new host cell (13, 15). In contrast, particles produced under the direction of defective MuLV genomes have a much lower probability, i.e., 1 in 10^5 or less, of infecting a new cell (Table 1).

Clone 8A particles were originally described as "replication-defective," because they were unable to undergo a complete replicative cycle (i.e., infect a cell and reproduce themselves, yielding progeny which could infect another cell) at a rate which was detectable by standard assay techniques. We now propose that the defectiveness of clone 8A particles and of NP-N MuLV is not absolute and can be fully accounted for by quantitative considerations. The results obtained with the complementation plaque assay (Table 1) imply that these MuLV variants direct the synthesis of virus particles which can only infect fresh cells with an extremely low probability (i.e., 10^{-5} or less). Presumably, cells newly infected with these variants will in turn produce progeny particles with a very low probability of infecting other cells. Thus, although it may be possible for these variant MuLV's to spread from cell to cell, this spread might be so inefficient as to be undetectable by the XC test, the S+L− focus assay, or even more extended measurements of virus growth.

The complementation plaque assay described here measures a type of biological activity which is not detectable in standard infectivity assays. It may be possible to extend the assay to other experimental systems, including the detection of ecotropic MuLV genomes which are absolutely, rather than relatively, replication-defective. These might include variant MuLV's produced by chemical or physical inactivation of standard virus preparations, or fragments of proviral DNA produced in vivo or in vitro.

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