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Cell Cycle Dependence of Foamy Retrovirus Infection

PAUL D. BIENIASZ,[1] ROBIN A. WEISS,[2] AND MYRA O. McCLURE[1]*

Department of GU Medicine and Communicable Diseases, The Jefferiss Research Trust, St. Mary's Hospital Medical School, London W2 1PG,[1] and Virology Laboratory, Institute of Cancer Research, London SW3 6JB,[2] United Kingdom

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In common with oncoviruses but unlike the lentivirus human immunodeficiency virus type 1, foamy (spuma) viruses require host cell proliferation for productive infection. We show that human immunodeficiency virus type 1 replicates in RD-CD4 cells regardless of the growth arrest condition of the cells, while murine leukemia virus is unable to infect growth-arrested RD-CD4 cells or cells progressing through a partial cell cycle that includes S phase but not mitosis. Human foamy virus, like murine leukemia virus, does not productively infect G_1/S or G2 growth-arrested cells. Two other foamy viruses, simian foamy virus type 1, isolated from a macaque, and simian foamy virus type 6, isolated from a chimpanzee, also fail to establish productive infection in G_1/S-arrested cells.

Integration of viral DNA into chromosomal DNA of the host cell is an essential step of the retroviral replication cycle. In the case of lentiviruses, the integration event is preceded by active nuclear import of a preintegration complex consisting of retroviral Gag and Pol proteins and linear viral DNA. This process is ATP dependent but does not require cell proliferation. Thus, lentiviruses (e.g., human immunodeficiency virus [HIV]) can replicate in metabolically active nondividing cells (e.g., terminally differentiated or irradiated macrophages) (8, 10, 20) or cells whose growth has been arrested in G_1 by treatment with DNA synthesis inhibitors (4) or in G_2 by irradiation (11). This distinguishing property of lentiviruses is (in the case of HIV) attributable in part to the presence in the Gag matrix protein of a peptide motif which is homologous to known nuclear localization signals and expression of the *vpr* gene product (3, 6, 9).

It has been established, however, that a prerequisite for oncovirus replication is host cell proliferation (18, 19, 21). Infection of nondividing cells is blocked after reverse transcription but before integration. In proliferating cells integration of the type C retrovirus murine leukemia virus (MLV) correlates temporally with host cell mitosis (12, 16). Furthermore, cells which are permitted to progress through a partial cell cycle in the presence of MLV are efficiently infected only when the partial cell cycle includes mitosis.

To explain these observations, it has been postulated that the preintegration complexes of oncoviruses and HIV nuclear localization signal mutants cannot cross the nuclear membrane (3, 16). Thus, partial breakdown in the structure of the nuclear envelope which occurs during mitosis permits access of the preintegration complex to the nuclear chromatin.

Until now whether members of the third subfamily of retroviruses, the foamy (spuma) viruses, are able to replicate in nondividing cells has not been documented. Mutagenesis of the human foamy virus (HFV) Gag proteins has shown that the nucleocapsid protein contains a nuclear localization signal (17), potentially capable of directing nuclear import of preintegration complexes to the nucleus in growth-arrested cells. In this study we have established growth arrest conditions using aphidicolin treatment and irradiation of CD4-expressing human rhabdomyosarcoma (RD-CD4) cells in order to compare the cell cycle dependence of HFV with that of HIV and MLV.

RD-CD4 cells transduced with a retroviral vector expressing the human CD4 gene (5) were selected for this study because they are highly susceptible to infection with HIV type 1 (HIV-1), amphotropic MLV, and HFV. Moreover, their growth can readily be arrested by treatment with aphidicolin or by irradiation.

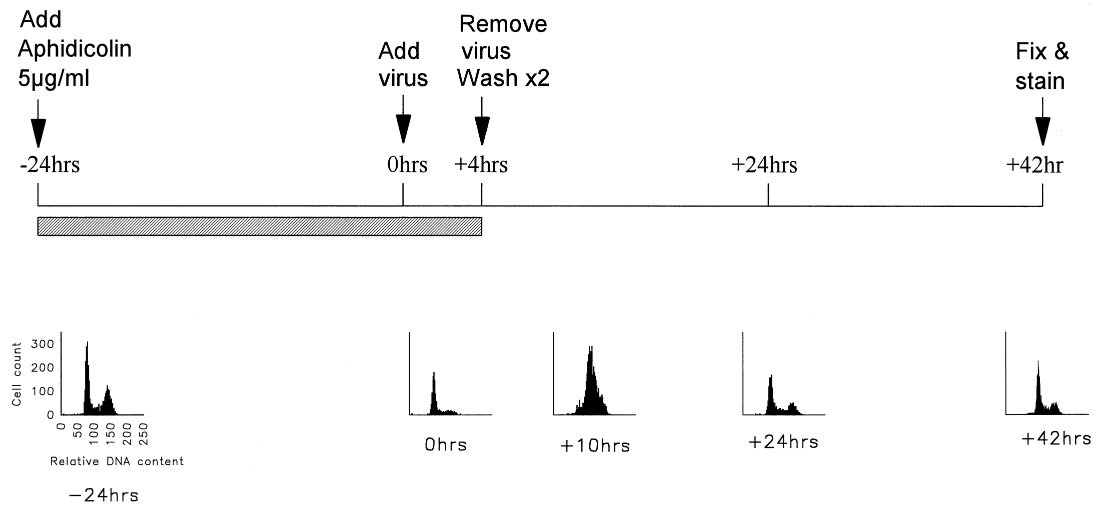
RD-CD4 cells were seeded in 25-cm^2 flasks and subjected to each of five growth arrest-infection protocols (A to E), as described below. Cells were reversibly arrested in G_1/S by inclusion of 5 μg of aphidicolin (a eukaryotic DNA polymerase inhibitor) per ml in the culture medium (15). In some experiments cells were irreversibly arrested in G_2 by irradiation with 9,000 rad from a ^{137}Cs source. The precise timing of growth arrest application is indicated in Fig. 1.

To demonstrate cell synchrony, after fixation in 35% ethanol, cells were simultaneously treated with DNase-free RNase and stained with propidium iodide. DNA content was assessed by fluorescence-activated cell sorter (FACS) analysis to determine the proportion of cells in the G_1, S, and G_2 phases of the cell cycle.

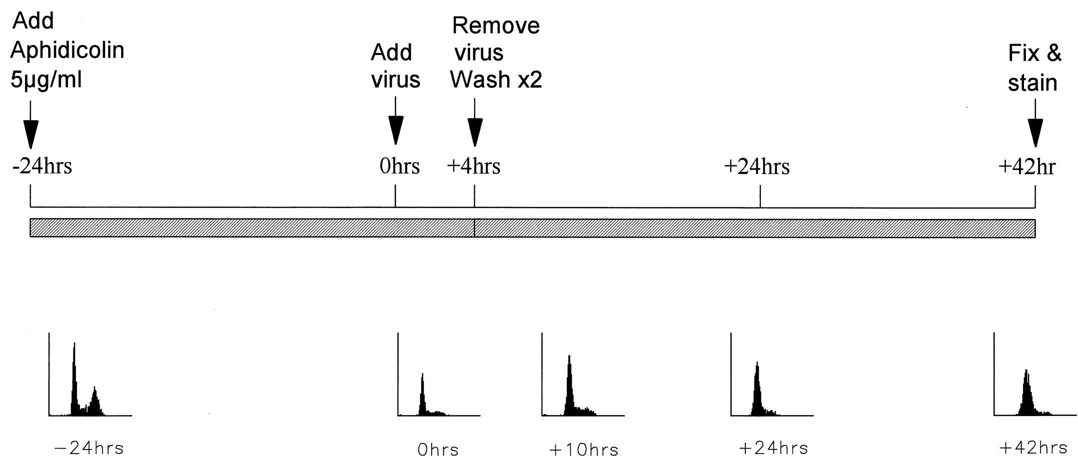
HIV-1_{LAI} was harvested from the supernatant of chronically infected H9 cells. HFV and simian foamy virus type 1 (SFV-1) and type 6 (SFV-6) were harvested from acutely infected BHK-21 cells. SFV-6 is a chimpanzee-derived isolate closely related to HFV; SFV-1 is a rhesus macaque-derived isolate which is phylogenetically divergent from HFV and SFV-6 (2). Supernatant from Am12 packaging cells (13) containing a *lacZ* vector (7) was used as a source of *lacZ*-transducing amphotropic MLV. Foci of infection in cell monolayers were enumerated following immunoperoxidase staining for HIV-1 antigens with HIV-1-positive human sera, for SFV-1 with a pool of sera from foamy virus-positive macaques, and for HFV and SFV-6 with HFV-positive human serum. For immunoperoxidase staining, cell monolayers were fixed for 2 min at −20°C with 1:1 acetone-methanol. Fixed cells were incubated with primary antiserum diluted 1:200 in phosphate-buffered saline (PBS)–1% fetal calf serum for 45 min at room temperature, washed, and incubated with sheep anti-human immunoglobulin G conjugated to horseradish peroxidase for a further 45

* Corresponding author. Mailing address: Department of GU Medicine and Communicable Diseases, The Jefferiss Research Trust, St. Mary's Hospital Medical School, Praed St., London W2 1PG, United Kingdom. Phone: 44-171-725-1539. Fax: 44-171-725-6645.

A



B



C

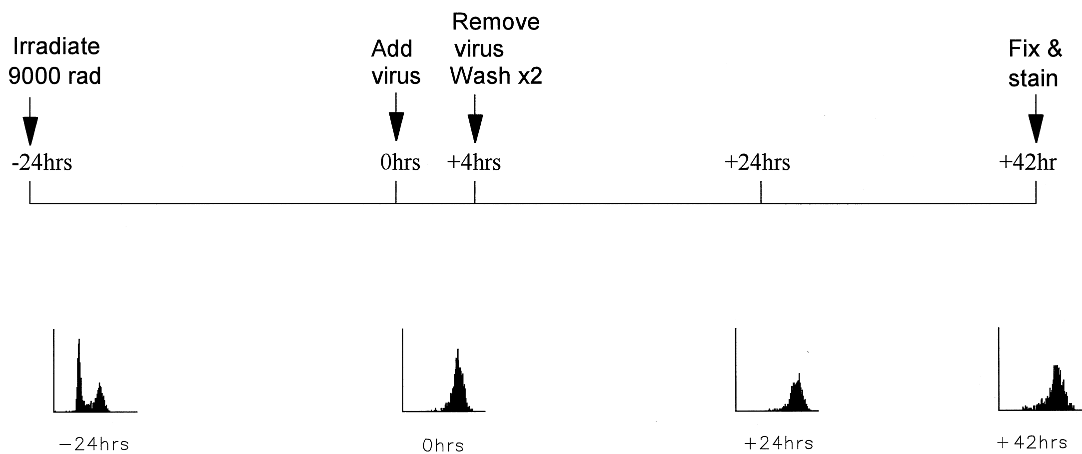
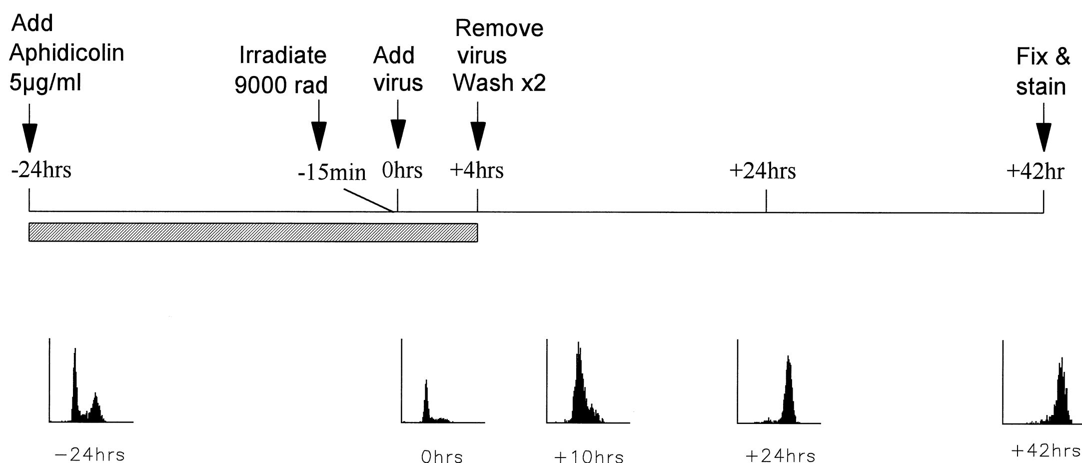


FIG. 1. Cell cycle control of RD-CD4 cells. Cells were infected, washed, and fixed for virus detection at the indicated times. Shaded bars indicate the presence of 5 μg of aphidicolin per ml. Histograms show the results of FACS analysis following propidium iodide staining at the indicated times.

D



E

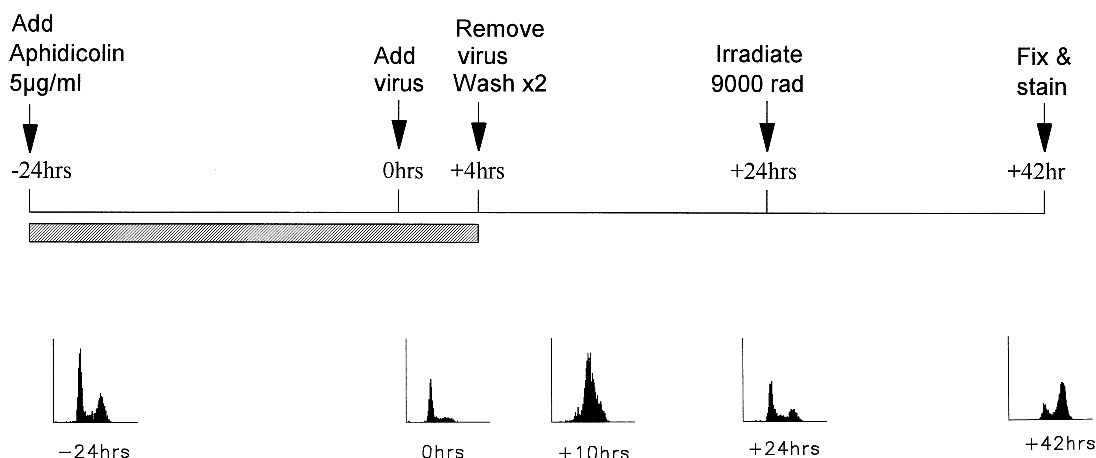


FIG. 1—*Continued.*

min. After further washing twice with PBS–1% fetal calf serum and twice with PBS, infected cells were revealed by incubation with substrate solution (3 mg of 3,3′-diaminobenzidine per ml, 0.1% H_2O_2 in PBS). MLV vector infection was quantified by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining (14).

Protocol A. In protocol A, cells were arrested at the G_1/S junction by culture in the presence of 5 µg of aphidicolin per ml for 24 h. Viruses were added in the presence of aphidicolin, and infection was allowed to proceed for 4 h. The virus inoculum was then removed, and the cells were washed and cultured for 42 h in the absence of aphidicolin before fixation and staining for the presence of virus. During this time the cells progressed from G_1/S through S phase, G_2, and mitosis and reverted to a standard proliferating population (Fig. 1A).

Protocol B. In protocol B, cells were arrested and infected as in protocol A. Following removal of the virus and washing, cells were returned to medium containing aphidicolin. Thus, the G_1/S-arrested state of the cells was maintained throughout the infection period (Fig. 1B).

Protocol C. In protocol C, 24 h prior to infection cells were irradiated with 9,000 rads. The cells became arrested in G_2 during infection and subsequent culture for 42 h in the absence of aphidicolin (Fig. 1C).

Protocol D. In protocol D, cells were arrested in G_1/S as in protocols A and B. Immediately prior to infection cells were irradiated so that after infection (in the presence of aphidicolin) and washing, the cells progressed through S phase and became rearrested in G_2 (Fig. 1D).

Protocol E. In protocol E, cells were arrested, infected, and washed as in protocol A. However, 20 h after release from the aphidicolin block, when most of the cells had passed through mitosis, they were irradiated. Thus, the cells accumulated in G_2 during the following 18 h and therefore progressed through one complete cell cycle and part of a second after exposure to virus (Fig. 1E).

Aphidicolin (5 µg/ml) reversibly arrested RD-CD4 cells in G_1/S, while gamma irradiation (9,000 rad) produced a population of cells arrested in G_2. Trypan blue exclusion (not shown) confirmed that >95% viability following either treatment was maintained, but mitosis and DNA synthesis were blocked, as shown by propidium iodide staining and FACS analysis.

The end point titer of each virus serially diluted 10-fold on

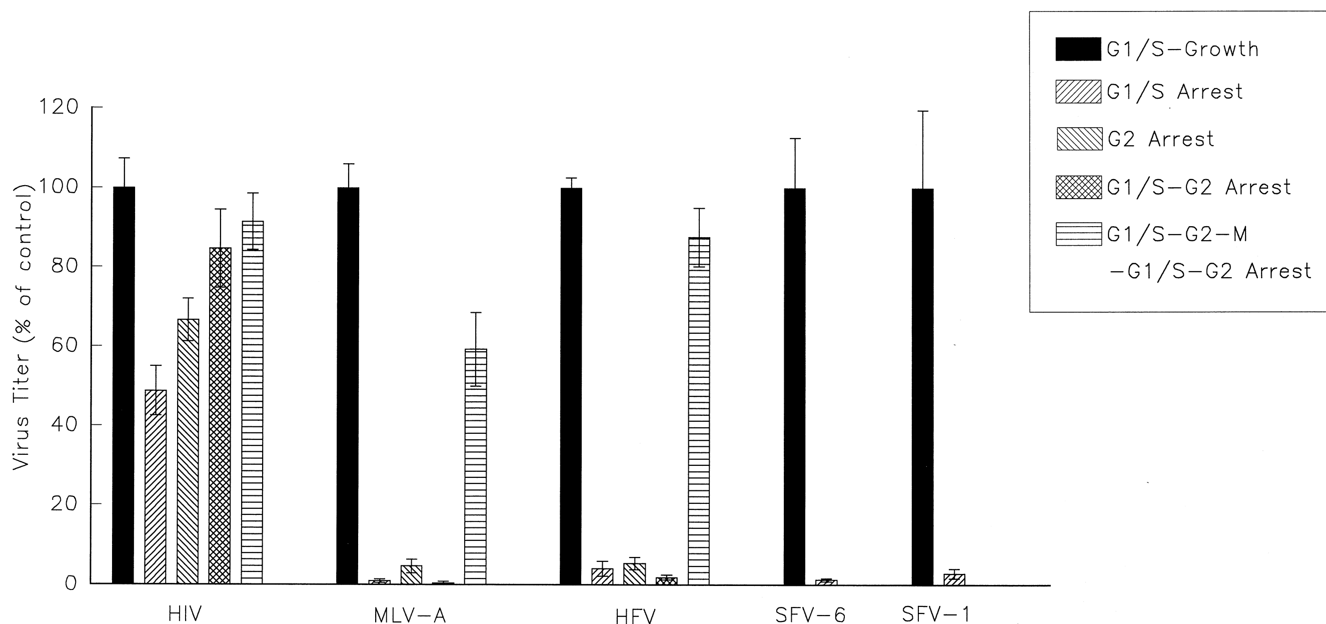


FIG. 2. Viral titers ± standard deviations expressed as a percentage of titers observed on proliferating cells.

cells subjected to each of the five growth arrest and infection protocols is shown in Table 1. Data normalized to 100% are shown in Fig. 2.

Growth arrest of target cells had a limited effect on HIV-1 infectivity. The titers obtained on G_1/S- and G_2-arrested cells were, respectively, 49 and 67% of that observed on proliferating cells (Fig. 2). HFV shared similar properties with MLV in that infection was highly cell cycle dependent. In the case of MLV, titers were reduced 100- and 21-fold on G_1/S- and G_2-arrested cells, respectively. HFV was 24- and 19-fold less infectious on G_1/S- and G_2-arrested cells, respectively. The infectivity of SFV-1 and SFV-6 on G_1/S-arrested cells was also assessed. The SFV-6 titer was reduced 76-fold and the SFV-1 titer was reduced 34-fold on G_1/S-arrested cells compared with those on proliferating cells.

Foamy viruses require host cell proliferation for productive infection. We have shown that HFV is unable to productively infect G_1/S or G_2 growth-arrested RD-CD4 cells. We also find that two other foamy viruses (SFV-1 and SFV-6) are unable to productively infect G_1/S-arrested cells, suggesting that cell cycle dependence is a common property of foamy viruses. HIV-1 is able to replicate in RD-CD4 cells, regardless of the growth arrest condition of the cells. In contrast, HFV and MLV were not able to infect growth-arrested RD-CD4 cells or cells progressing through a partial cell cycle that included S phase but not mitosis.

TABLE 1. Infectivity of different viruses on proliferating and growth-arrested cells

Virus	Virus titer on RD-CD4 cells in protocol:				
	A (G_1/S→ growth)	**B** (G_1/S arrest)	**C** (G_2 arrest)	**D** (G_1/ S→G_2 arrest)	**E** (G_1S→ G_2→M→ G_1/S→G_2 arrest)
HIV	7.5×10^3	3.7×10^3	5.0×10^3	6.4×10^3	6.9×10^3
MLV-A	1.1×10^4	1.1×10^2	5.0×10^2	0.5×10^2	6.2×10^3
HFV	2.1×10^4	8.8×10^2	1.2×10^3	4.0×10^2	1.9×10^4
SFV-1	1.4×10^4	4.0×10^2	ND[a]	ND	ND
SFV-6	1.8×10^4	2.4×10^2	ND	ND	ND

[a] ND, not done.

Productive infection was observed only if target cells were allowed to pass through mitosis. Although HFV, like HIV, contains a nuclear localization signal in one of its core proteins, it is in a location (the nucleocapsid) different from that of HIV (matrix). This sequence probably serves a function other than mediating HFV infection of nondividing cells.

Foamy viruses are found in the central nervous system in infected primates and are expressed in nonproliferating cells (1, 14). The in vivo tissue tropism thus seems at odds with our observations of the experimental infection of cells in vitro which is dependent on a quantal mitosis for viral replication. However, little is known of the natural mode of foamy virus infection and whether cells may be in a proliferative state. Moreover, the residual infection in arrested cell cultures might permit infection of and eventual integration in nondividing cells in vivo over the long course of persistent infection. It will be of interest to test the cell cycle dependence of other cell types, such as macrophages, glia, and neuronal cells, for foamy virus infection.

Our demonstration that foamy viruses are dependent upon mitosis for productive infection of RD-CD4 cells suggests that the dissolution of the nuclear membrane during mitosis is the permissive event for foamy virus integration and expression, as for type C retroviruses (16). However, this remains to be shown experimentally. The cell cycle dependence of foamy virus infection indicates that vectors based on foamy virus genomes and packaging proteins may not be suitable for targeting nonproliferating cells. Nonetheless, the neurotropism of foamy viruses is worth testing in animal models with foamy virus vectors.

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