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Nuclear Targeting of Incoming Human Foamy Virus Gag Proteins Involves a Centriolar Step†

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The pathways used in the transport of retroviral genomes to the nucleus are poorly identified. Analyzing the intracellular localization of incoming foamy viruses, we have found that the Gag antigens and the viral genome accumulate in a distinct perinuclear domain identified as the centrosome. Colchicine treatment completely abolished pericentriolar targeting of human foamy virus (HFV) proteins, suggesting a role for microtubules in the transport of the incoming viral proteins to the centrioles. Finally, we demonstrate that, similarly to human immunodeficiency virus DNA, HFV DNA can enter the nucleus of G_1/S-phase-arrested cells, although no viral gene expression can be observed. Recent observations have demonstrated that foamy viruses have several features not shared by other retroviruses. The intracellular route of the incoming Gag antigens may constitute a new specificity of this class of viruses.

Foamy viruses, or spumaviruses, are retroviruses which induce persistent infections in their hosts but cause no apparent disease (28, 34). Several features are unique to these viruses, including the formation of polymerase RNA and the presence of large amounts of double-stranded DNA in extracellular virions (36). Thus, despite the absence of any pathology associated with this class of viruses, analysis of their biology has brought new concepts to retrovirology (35). Expression of retroviruses takes place in the nuclei of infected cells after the integration of the reverse-transcribed viral genome. Although many stages of the retroviral cycle have been intensively studied, how the viral genome reaches the nuclei of the infected cells is poorly understood (23). Another issue which remains to be clarified for foamy viruses is their ability to productively infect growth-arrested cells. For oncoretroviruses (e.g., human T-cell leukemia virus type 1), productive infection depends on cell proliferation: the breakdown of the nuclear envelope during mitosis is necessary to bring the preintegration complex to the host genome (17). For human immunodeficiency virus type 1 (HIV-1), cell division is not required, and this virus has the ability to productively infect G_1/S- or G_2/M-arrested cells. In these cells, a nuclear localization signal (NLS) present in one of the components of the preintegration complex (the HIV-1 MA protein) directs the viral genome into the nucleus (8, 9). Nevertheless, oncoviruses and lentiviruses are unable to replicate in quiescent T cells, as reverse transcription leads to the formation of incomplete forms of proviral DNA which remain in the cytoplasm (5, 37). However, nondividing neural cells have recently been shown to be productively infected by HIV-based retroviral vectors (20). It was reported that human foamy virus (HFV) does not productively infect artificially G_1/S- or G_2-arrested cells, but nuclear import of the preintegration complex was not studied in these experiments (2). On the other hand, a recent report showed that stationary-phase cultures could be infected by foamy virus-based vectors encoding a neomycin resistance gene, although with a lower efficiency than in the case of dividing cells (25).

Here, we report that as early as 4 h after infection by HFV, most incoming viral antigens are concentrated in a specific subcellular compartment adjacent to the nucleus. The Gag antigens together with the viral RNA genome (which likely constitute the preintegration complex) concentrate around the centrosome of the infected cell. The HFV genome is able to translocate into the nuclei of G_1/S-arrested cells, where, in contrast to findings with HIV-1, no active viral replication or expression is observed. Such interaction between retroviral Gag antigens and the centrosome in the early steps of the viral replication cycle indicates a previously unrecognized step of HFV replication.

MATERIALS AND METHODS

Cells and virus. Mycoplasma-free HFV stocks were grown on U373MG cells, a human neural cell line maintained in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal calf serum. MRC5 (a human diploid fibroblast line), BHK21 (a hamster cell line), and COS7 (a simian cell line) were maintained in the same medium. Virus stocks were titrated by the endpoint dilution method on U373MG cells as described previously (30).

3′-Azidothymidine (zidovudine; AZT) (5 μM), cycloheximide (20 μM), and colchicine (15 μM) were used during the course of infection. To obtain stationary-phase cultures, MRC5 cells were grown to confluence and serum starved for 30 h. In some experiments, cells were blocked in G_1/S by treatment with 5 μM aphidicolin for 24 h before infection and during infection as described elsewhere (2).

Immunofluorescence analysis. Cells were trypsinized and either fixed at different times postinfection in 4% paraformaldehyde at 4°C for 10 min and permeabilized with methanol at 4°C for 5 min or fixed at different times postinfection in methanol at −20°C for 5 min. After fixation, cells were preincubated in phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20 and 1% bovine serum albumin for 15 min at 37°C. The antibodies used included serum from HFV-infected rabbits (11), a mouse polyclonal antiserum against a glutathione *S*-transferase-Bel1 fusion protein made in *Escherichia coli* (27), a rabbit polyclonal antiserum against a glutathione *S*-transferase-Gag fusion protein (provided by N. Morin), a mouse monoclonal antibody against the Bet protein (D11), and a rabbit polyclonal serum against the capsid-nucleocapsid (provided by A. Aguzzi). None of these reagents gave any labelling by either immunofluorescence or Western blotting on uninfected cells (references 11 and 27 and data not shown). A monoclonal antibody against subunit α of tubulin, a monoclonal antibody against a centriolar protein (CTR910, provided by M. Bornens [1]), and a monoclonal antibody against the Golgi complex (CTR433 [13]) were also used. All antibodies were used at a 1/100 dilution. Anti-immunoglobulin G, fluorescein

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isothiocyanate, or Texas red-coupled antibodies were used as second fluorescent conjugates.

For confocal analysis, observations were made with an MRC-600 (Biorad) confocal scanning laser microscope with the Comos software package. Discrete photon counting allowed a sharp visualization of weak label even at the highest magnification. A multiple-argon-ion laser beam was operated at full power and attenuated with one, two, or three density filters. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 250 ng/ml in PBS.

Electron microscopy. Electron microscopic analysis was performed after fixation with 1.6% glutaraldehyde in PBS for 10 min at room temperature. After dehydration, cells were embedded in Lowicryl K4M as described previously (24). Ultrathin sections were first blocked with 5% bovine serum albumin in PBS for 30 min and then incubated for 1 h with a rabbit polyclonal antibody against HFV MA (1/25, in PBS) or an anti-HFV serum from infected rabbits (1/50, in PBS) and subsequently incubated with goat anti-rabbit immunoglobulin G conjugated to gold particles (5 or 10 nm in diameter), followed by staining with uranyl acetate.

The hybridization solution used to detect the viral genome consisted of 50% deionized formamide, 10% dextran sulfate, 2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 400 μg of competitor *E. coli* DNA/ml, and 3 μg of pHSRV13 probe/ml labelled by nick translation in the presence of biotin-dCTP according to the manufacturer's instructions (Boehringer). Ten-nanometer gold label particles were used to reveal the viral genome. Some grids were pretreated with 0.2 mg of protease (bacterial protease type VI; Sigma Chemical Co.) per ml for 15 min at 37°C in order to increase the accessibility of the viral RNA in the section to the probe and also to suppress eventual nonspecific binding of the probe to proteins of the section.

For detection of both the viral genome and Gag proteins, grids bearing sections were incubated successively over drops of viral-RNA-containing hybridization solution, then over anti-Gag antibody, and finally over a cocktail of gold-labelled antibiotin antibody and goat anti-rabbit immunoglobulins (10 and 5 nm in diameter, respectively).

Viral-DNA analysis. Viral DNA was extracted as described by Hirt from 10^7 infected cells treated or not with AZT (12) and was analyzed by Southern blotting (29). Membranes were hybridized overnight with an [α-^{32}P]dCTP-labelled probe at 42°C in a solution containing 5× SSC, 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, 50% formamide, and 100 μg of denatured salmon sperm DNA/ml. Washes were performed in 2× SSC–0.1% SDS buffer at 60°C for 30 min. The infectious clone pHSRV13 (provided by R. Flügel) was used as a probe.

Enzymatic amplification of the viral DNA was performed on Hirt supernatants with the following primer couples: for the circular DNA with one long terminal repeat (LTR), 1LC5′ (5′-ATG GAA GCT TAT GGA CCT CAG-3′) and 1LC3′ (5′-CTT CAA CAT TAC TTC CTG AAG C-3′); for the circular DNA with two LTRs, 2LC5′ (5′-TTA GCC TTG CTA AGG GAG-3′) and 2LC3′ (5′-T CTG CGG CTA GTA TAA TCA-3′). The PCR program consisted of 30 cycles of denaturation at 94°C for 1 min, followed by annealing at 50°C (Circle2LTR, 324 bp) or 56°C (Circle1LTR, 1,405 bp) for 1 min and by extension at 72°C for 1 min. Before cell infection, viral stocks were treated with DNase as described previously to remove contaminating proviral DNA associated with HFV virions (36–38).

Protein analysis. For Western blotting, 20 μg of total protein extract was loaded onto an SDS-polyacrylamide gel electrophoresis gel (16). The proteins were transferred to a nitrocellulose membrane (Millipore) which was incubated with serum from an infected rabbit (1/400 in PBS) and were subsequently revealed with 3-3′ diaminobenzidine substrate.

RESULTS

HFV infection leads to accumulation of input Gag antigens near the nucleus. One of the principal characteristics of foamy viruses is the tendency to give rise to a strong nuclear staining when infected cells are studied by immunofluorescence (7). This has been explained by a transient nuclear concentration of de novo-synthesized Gag precursors which is observed 48 h postinfection. An NLS in the nucleocapsid (NC) domain of Gag precursors was found to be responsible for this subcellular compartmentation (31). However, the fate of incoming Gag proteins in infected cells is unknown. We have infected MRC5 cells with wild-type HFV stocks at high multiplicity to monitor the cellular targeting of incoming viral proteins by indirect immunofluorescence. After 1 h of adsorption at 37°C, cells were washed with PBS to remove the virus. At intervals ranging from 2 to 48 h postinfection, cells were trypsinized and fixed for indirect immunofluorescence assay (IFA) with serum from an HFV-infected rabbit which recognizes the major HFV virion components in immunoprecipitation experiments (11, 26). Two hours after infection, viral antigens were seen in the cytoplasm without specific location (Fig. 1A). However, at later times (from 4 to 6 h postinfection), viral antigens were preferentially observed in a specific area adjacent to the nucleus (Fig. 1B). Similar images were observed with a 1/1,000 dilution of the antibody. Mock-infected cells were strictly and constantly negative with this antibody even at a 1/10 dilution, demonstrating the specificity of this labelling. Positive results were also obtained with rabbit polyclonal sera against MA or CA/NC, but these early steps of HFV infection were constantly negative for Bel1 and Bet staining (data not shown). Later, Bel1, Bet, and Gag fluorescence was observed within the nucleus at 20 h postinfection (Fig. 1C and data not shown) or both in the cytoplasm and nucleus at 48 h postinfection (Fig. 1D), suggesting that at both times de novo expression of the provirus occurred.

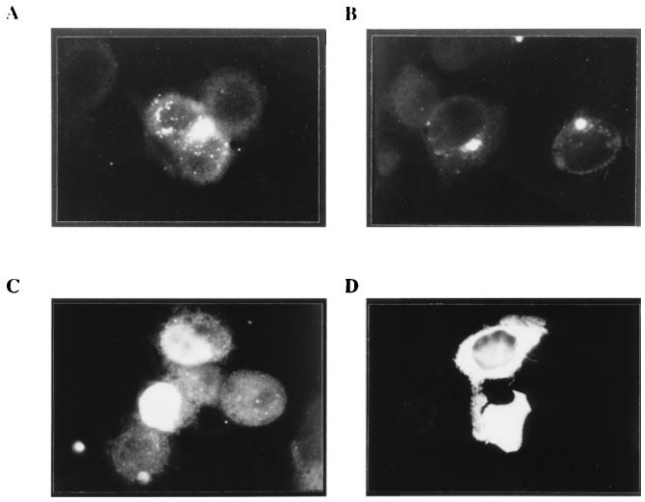

FIG. 1. Analysis of subcellular localization of HFV antigens by indirect immunofluorescence of infected MRC5 cells. Cells were infected with 50 PFU/cell and fixed at 2 h (A), 5 h (B), 20 h (C), and 48 h (D) postinfection. Note the juxtanuclear speckled labelling 5 h postinfection. Cells were stained with polyclonal serum from an HFV-infected rabbit which recognizes major HFV proteins. At 20 h postinfection, viral antigens were found exclusively in the nucleus, and later (48 h), the entire cell was stained.

To demonstrate that this early perinuclear punctate staining was due to incoming virus, cells were treated with 5 μM AZT prior to and during the course of infection. Twenty-four hours postinfection, no cell toxicity was observed as measured by cell viability (data not shown). We first tested the ability of AZT to inhibit HFV reverse transcription by Southern blot analysis of Hirt extracts 10 h postinfection. The 12-kb viral cDNA could be detected in untreated cultures. In AZT-treated cells, a very weak band was detected; this band was likely related to the presence of large amounts of double-stranded viral DNA in virus preparations (36). This demonstrates an inhibition of HFV reverse transcription in these cells (Fig. 2A). Proteins extracted from the same cells were studied by Western blotting (with polyclonal serum from an HFV-infected rabbit which specifically recognizes the major HFV proteins), and Fig. 2B reveals the presence of similar amounts of Gag precursors at 68 and 72 kDa in AZT-treated and untreated cell cultures. This experiment demonstrates that the proteins detected by Western blot or IFA analysis are mostly the structural Gag proteins from the incoming virus. By immunofluorescence, similar juxtanuclear stainings were observed 5 and 10 h postinfection, in either AZT-treated or untreated cells (Fig. 2C). In

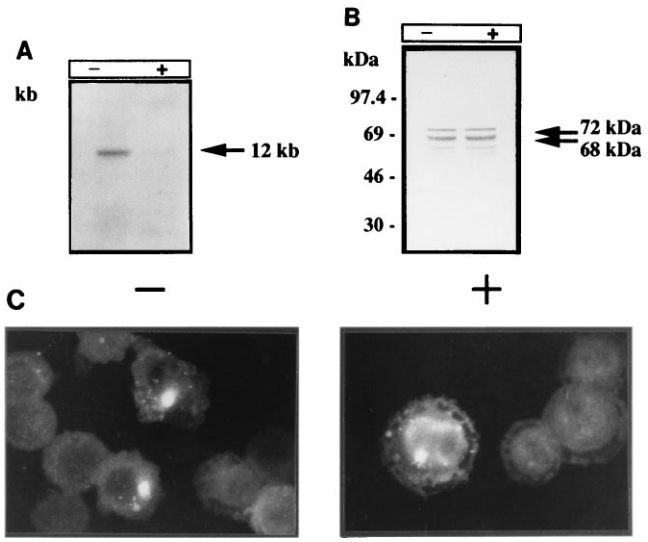

FIG. 2. Effect of AZT (5 μM) on reverse transcription in HFV-infected cells 10 h postinfection. (A) Hirt extracts from AZT-treated (+) or untreated (−) infected MRC5 cells were analyzed by Southern blotting with the HFV genome as a probe. Reverse transcription leading to the formation of the 12-kb viral DNA was inhibited by AZT. (B) Western blot analysis of proteins extracted from the same cells with rabbit anti-HFV serum. The Gag precursors at 72 and 68 kDa were detected. (C) IFA analysis of HFV-infected MRC5 cells with serum from an HFV-infected rabbit in the presence (+) or absence (−) of AZT 5 h after infection.

a related experiment, cells were infected in the presence or absence of 20 μM cycloheximide. Again, similar patterns were detected 5 or 10 h postinfection (data not shown). In contrast to control cells, cells treated with AZT or cycloheximide never gave rise to strong fluorescent staining 48 h postinfection, demonstrating the absence of de novo viral protein synthesis in these cells.

Gag antigens localize around the centrosome. The concentration of Gag antigens in a single restricted cytoplasmic domain near the nucleus raised the issue of the nature of this subcellular compartment. As we hypothesized that it might correspond to the centrosome (also known as the microtubule-organizing center), we performed a double IFA with a monoclonal antibody directed against tubulin α and a polyclonal antibody against HFV MA at 5 h postinfection. Figure 3A and B show the tubulin α network. Note that most of the microtubule cytoskeleton emerges from the centrosome. In Fig. 3C and D, the same cells were stained with anti-MA antibodies. The stainings coincide, implying that Gag antigens aggregate close to the centrosome.

To extend this result, we used monoclonal antibody CTR910, directed against an as-yet-uncharacterized protein of the centriole (1). As shown in Fig. 4A, by double staining and confocal microscopy, CTR910 revealed the centrosome, which was surrounded by viral Gag antigens. Similar results were obtained when a monoclonal antibody against a pericentriolar protein was used (data not shown). As a control, we used an anti-Golgi apparatus monoclonal antibody for which the two stainings are clearly distinct (Fig. 4B). When other cell types (BHK21, U373MG, or COS7) were used in these experiments, identical results were obtained, demonstrating that this phenomenon is not restricted to a specific cell type (data not shown).

Aggregation of Gag antigens around the centrosome was confirmed by immunoelectron microscopy with the rabbit polyclonal anti-whole HFV serum or the rabbit polyclonal antibody against MA. To increase the number of cells presenting these specific punctate images, MRC5 cells were blocked in G_0 by

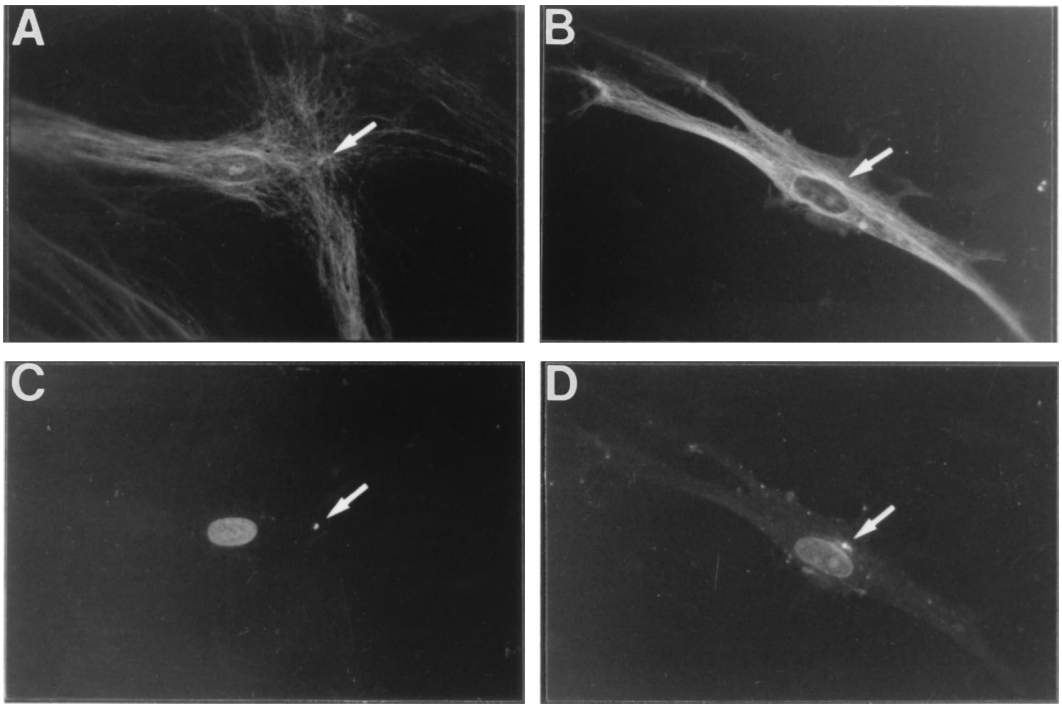

FIG. 3. Analysis of subcellular localization of incoming HFV Gag antigens. (A and B) The microtubule network was revealed with an antitubulin monoclonal antibody. Most of the microtubules emerge from the centrosome (arrows). (C and D) The same cells were revealed with the anti-HFV MA polyclonal antibody. Note the colocalization of the centrosome (arrows) and the Gag antigens.

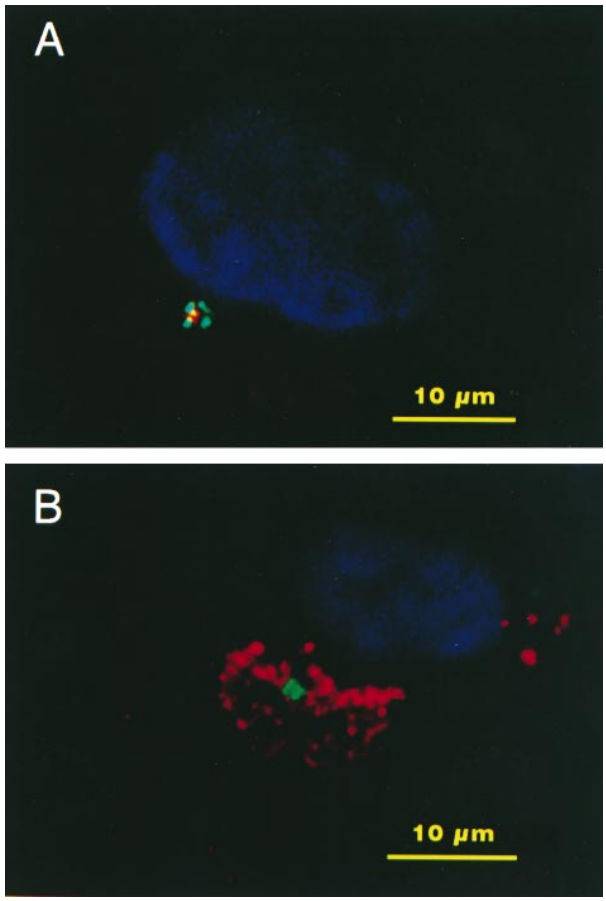

FIG. 4. Confocal microscopy analysis of HFV-infected MRC5 cells. (A) Double staining of MRC5 cells with an anti-centriolar protein monoclonal antibody (CTR910, in red) and anti-HFV MA polyclonal antibody (in green). Note the juxtaposition of the two stainings. (B) Cells were labelled with a monoclonal antibody directed against the Golgi apparatus (in red) and with the anti-HFV MA polyclonal antibody (in green).

confluence and serum starvation. This leads to a situation where 80 to 90% of the cells present this specific staining by immunofluorescence and where these images persist for 24 h postinfection (see below). Electron microscopy again showed that viral Gag antigens (identified by the presence of gold particles) were located around the centrosome (Fig. 5A and B). Note that gold particles do not appear to be specifically associated with microtubules. The increase of gold particles in the immediate vicinity of the centrosome was estimated to be on the order of 8 (with rabbit anti-whole HFV) and 19 (with anti-Gag serum), demonstrating the specificity of labelling (Table 1).

In the virion, the Gag proteins are assembled in a core which contains the viral genome and other viral components (e.g., the reverse transcriptase and the integrase). After uncoating, the core is released into the cytoplasm. The Gag antigens detected here may still be associated with the viral genome and thus represent the precursor of the preintegration complex. Indeed, an HFV biotinylated probe revealed that the viral genome was also localized around this organelle (Fig. 5C). Unlike Gag staining, which could sometimes be found on centrioles, gold particles revealing the viral genome were never observed on this organelle but always in its immediate proximity, probably in the pericentriolar material. The increase of gold particles in the immediate vicinity of the centrosome was much higher (70-fold) than previously, demonstrating the high specificity of labelling (Table 1).

Double staining experiments were done with anti-MA antibodies (5 nm) and the HFV biotinylated probe (10 nm). Figure 5D reveals that both Gag antigens and the viral genome localize around the centrosome. To exclude nonspecific fixation of the nucleic probe on viral antigens that may occur for other viruses, thin sections were treated with protease prior to hybridization (21). Here again, similar results were obtained (data not shown). Identical results were obtained either in cycling cells 5 h postinfection or in AZT-treated cells, implying that the genetic material present in the centrosome is incoming RNA. These observations demonstrate that the HFV preintegration complex aggregates around the centrosome in the early stages of the retroviral cycle.

Treatment with colchicine blocks centrosome targeting of Gag proteins. The association between input Gag proteins and the centrosome in infected cells early postinfection raises the issue of a possible interaction between these proteins and microtubules, the principal components emerging from the centrosome. One pathway by which the HFV preintegration complex may direct the viral genome from the plasma membrane towards the nucleus is by interacting with the microtubule network as described for the intracytoplasmic transport of several cellular organelles or viruses (15, 33). To test this hypothesis, cells were treated with colchicine, a microtubule-depolymerizing drug which inhibits the dynamic formation of microtubules by fixing monomers of tubulin. MRC5 cells were infected in the presence of 15 μM colchicine and then fixed for IFA. Stainings performed with a rabbit polyclonal anti-MA serum 5 h postinfection never detected any viral antigen concentrations near the nucleus. However, it was possible to observe a diffuse fluorescence in the cytoplasm, in contrast to uninfected cells, which were strictly negative. Western blot analysis with proteins extracted from untreated or colchicine-treated cells revealed a similar pattern, suggesting that viral entry was not inhibited by this treatment (data not shown). Thus, an intact microtubule network appears to be required for Gag targeting of the centrosome.

Infection of stationary-phase cell cultures. The ability or inability of foamy viruses to productively infect growth-arrested cells has not yet been fully elucidated. We wondered whether the NLS of the HFV NC domain might perform a role similar to that of the HIV MA NLS or whether the HFV NLS is implicated only in the late steps of viral replication as previously suggested (31). To test this hypothesis, we established G_0 growth arrest culture conditions and examined cells by IFA at 2, 6, 48, and 72 h postinfection. In this experiment, we could not detect viral proteins inside the nucleus at different times postinfection (data not shown). These observations confirm those by others who failed to detect viral replication in growth-arrested cells (2).

The observation that centriolar accumulation of HFV Gag is greatly prolonged in noncycling cells suggested that the nuclear import of the preintegration complex necessitates progression in the cell cycle. To address this issue, we investigated the formation of circular forms of HFV DNA. These circular forms of the retroviral genome containing one or two LTRs are produced only after the transport of the viral cDNA into the nucleus and thus are a convenient marker of nuclear import for lentiviruses and also for oncoviruses (4). Twenty-four hours postinfection, total cellular DNA was extracted from serum-starved cultures and also from MRC5 cells arrested in G_1/S by aphidicolin or from cycling cells. This DNA was analyzed by PCR with specific primers revealing the presence of circular forms of viral DNA (Fig. 6A and B). Cell cycle synchrony was

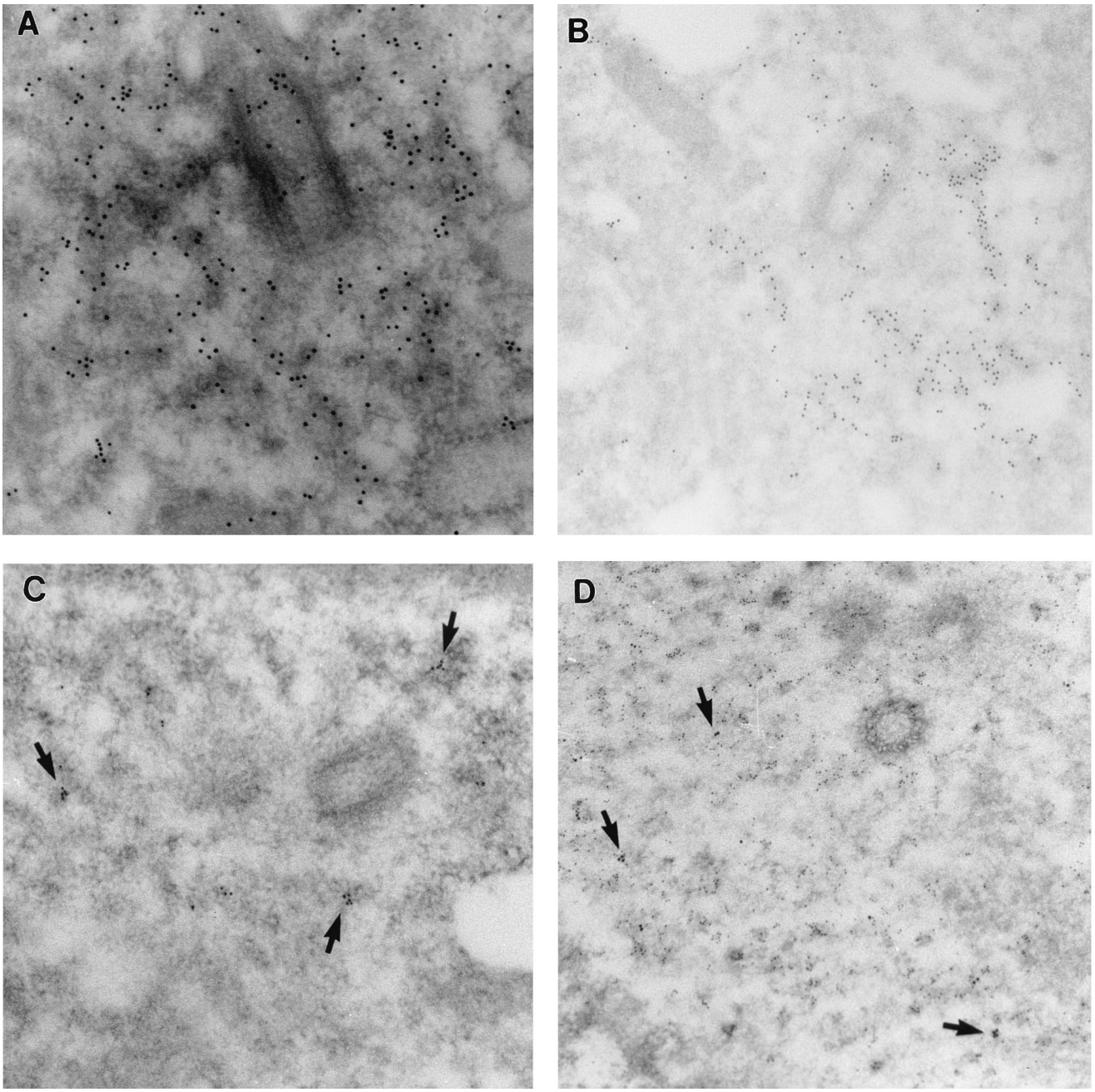

FIG. 5. Electron microscopy analysis of HFV genome and proteins. (A) Immunostaining of thin section of HFV-infected cells with rabbit polyclonal anti-HFV serum. Gold particles are located over the pericentriolar material, which is adjacent to the microtubules, and over the centriole. The microtubules are devoid of labelling. Magnification, ×60,000. (B) Detection of viral antigens with rabbit polyclonal anti-MA serum. Once again, gold particles are associated with the centriole and perinuclear material. Magnification, ×39,000. (C) Detection of viral genomes by in situ hybridization with a biotinylated HFV probe and 10-nm gold particles (arrows). Gold particles are much less abundant than in panels A and B. They are present over the pericentriolar material but not over its enclosed microtubules. Magnification, ×39,000. (D) Concomitant detection of viral genomes and viral proteins. The viral RNA molecules are revealed by in situ hybridization with the specific probe and 10-nm gold particles (arrows), whereas the Gag proteins are revealed by immunogold labelling with the specific antibody and 5-nm gold particles. Both 5- and 10-nm gold particles are observed over the moderately electron-opaque material which surrounds the centrioles and encloses the microtubules. Magnification, ×43,000. The results of quantitative analysis of this data are shown in Table 1.

controlled by the propidium iodide assay followed by fluorescence-activated cell sorter analysis as described previously (2) (data not shown). As expected, AZT-treated cells were negative by all HFV PCRs. Colchicine-treated cultures were positive but fainter than cycling cells, implying that the preintegration complex can still enter the nucleus, although less efficiently. As shown in Fig. 6C, circular forms of HFV cDNA with one and two LTRs are formed in G_1/S-arrested cultures or cycling cells but not in G_0-arrested cells. These results demonstrate that the viral genome is able to enter the nuclei of G_1/S-arrested cells but not of G_0-arrested cells. Note that G_1/S-arrested cells showed a nuclear staining by IFA which progressively decreased and finally disappeared during the course of the study. The fact that no viral gene expression occurred in

TABLE 1. Cellular distribution of viral RNA or DNA and viral proteins

DNA or antibody	No. of gold particles/μm^2 in[a]:	
	Centrosome	Cytoplasm
HFV probe	14 ± 4	≤0.2
Anti-Gag MA	179 ± 52	9 ± 5
Anti-whole HFV	141 ± 33	18 ± 5

[a] Estimated on eight representative sections.

these cells is consistent with previous reports on HFV replication (2).

DISCUSSION

This report demonstrates that input HFV Gag antigens associated with the viral genome aggregate around the microtubule-organizing center prior to nuclear import. While the viral genome cannot enter the nuclei of G_0-arrested cells, nuclear import, but not HFV gene expression, is observed in G_1/S-arrested cells.

To our knowledge, the association of the HFV Gag antigens with the centrosome is unprecedented among retroviruses. Preliminary evidence suggests that no similar situation is encountered in HIV infection, implying that this specific intracellular route of incoming Gag proteins may be another unique feature of foamy viruses, together with the existence of a specific polymerase mRNA and the formation of a spliced pregenome (27, 35, 36). This specific localization of the Gag antigens and the viral genome (likely constituting the preintegration complex) around the centrosome could either contribute to an association of foamy virus DNA with the mitotic spindle or be a step prior to nuclear import. Our results on nuclear import in G_1/S-arrested cells strongly favor the latter hypothesis. The mechanism of Gag core targeting of the centrosome, as well as the nature of interactions between the pericentriolar material and the viral proteins, remains to be determined.

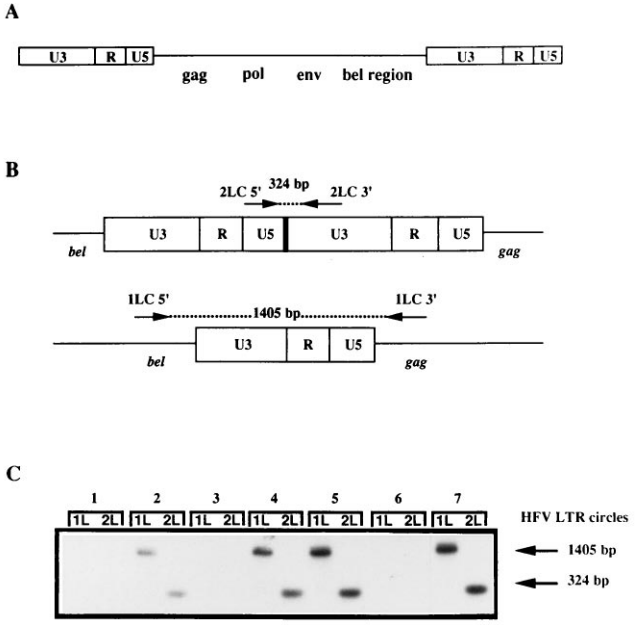

FIG. 6. Analysis of HFV genome nuclear import by the detection of circular viral DNA in infected cells. (A) Schematic representation of the HFV linear DNA genome. (B) Circular forms of HFV DNA with one or two LTRs. The primers used for PCR analysis as well as the size of the amplified fragment are depicted. (C) Enzymatic amplification of Hirt extracts of infected cells. Lanes: 1, DNase-treated HFV stock; 2, colchicine-treated cells; 3, stationary-phase cells (G_0); 4, aphidicolin-treated cells (G_1/S-arrested cells); 5, untreated cycling cells; 6, AZT-treated cycling cells; 7, cycling cells.

The cytoskeleton has been said to play a significant role in the replication of RNA and DNA viruses (18). An integral microtubule network is an essential factor in the replication of adenovirus types 2 and 5 and reovirus type 3 (6, 19). Herpes simplex virus type 1 also uses the microtubule network for axonal transport (15). A role for microtubules (the principal components of the centrosome) in the HFV replication cycle is suggested by inhibition of Gag antigen targeting of the centrosome after colchicine treatment. However, at the viral multiplicity used, other cellular pathways can be used to reach the nucleus. Indeed, by PCR we were able to detect the presence of circular DNA with one or two LTRs in cells treated with colchicine (although in a much smaller amount than in untreated cells) (Fig. 6C, lane 2). This may, however, reflect the entry of the preintegration complex in mitosis-blocked cells.

It has been proposed that the presence of newly synthesized Gag precursors in the nuclei of infected cells might play a role in the late phases of infection (e.g., packaging of the RNA genome or positive regulation of genomic-RNA expression). Our results also suggest a role for Gag antigens in the early phases of infection. Similar dual effects have been described for HIV-1 Gag proteins, in particular for MA. The NLS present in this protein directs the preintegration complex to the nucleus in growth-arrested cells, but this protein is also important for assembly and release of viral particles at the plasma membrane (8, 9). For lentiviruses, uncleaved precursors are included in the virus during assembly at the plasma membrane and are subsequently partially processed within the virion by a virally encoded aspartic protease (32). However, as seen in Western blot analysis after HFV infection (Fig. 2B), the majority of incoming HFV Gag antigens are uncleaved (72-kDa) and intermediate (68-kDa) precursors, which is consistent with the data of Konvalinka et al. on infected cells (14). Similarly, it has recently been shown that efficient proteolytic cleavage of the HFV Gag precursors takes place during the early stages of HFV infection and not, like in other retroviruses, just after viral budding (10). We suggest that after uncoating, the HFV Gag proteins associated with the viral genome use the microtubule network to reach the centrosome. During this transport, Gag precursors may be processed by the viral protease into mature proteins, revealing the NLS of the NC protein, which may subsequently direct the viral genome into the nucleus.

It has been reported that HFV productive infection is cell cycle dependent (2). Conversely, others have shown evidence that viral expression can occur in stationary-phase cells, although to a lesser extent than in dividing cells (22, 25). These apparently contradictory results may be due to the presence of large amounts of full-length viral DNA carried by HFV virions (35, 36). Although foamy viruses are clearly distinct from other retroviruses, the absence of HFV cDNA circles in G_0-arrested stationary-phase cultures strongly suggests that formation of these viral forms depends on the accessibility of the nuclear compartment. We conclude that, like lentiviruses, the HFV genome is able to translocate into the nuclei of artificially G_1/S-arrested cells but not of G_0-arrested cells (3, 4). This result distinguishes these two retroviral genera from all other retroviruses, which need the breakdown of the nuclear envelope (17). However, no viral gene expression was observed in any of these growth-arrested cells compared to cycling cells. Our observations therefore favor the hypothesis that the block

observed in HFV replication in G_1/S-arrested cultures takes place after nuclear entry of the viral cDNA (25).

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