Synthesis of Full-length Viral DNA in CD4-positive Membrane Vesicles Exposed to HIV-1

A MODEL FOR STUDIES OF EARLY STAGES OF THE HIV-1 LIFE CYCLE

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CD4-positive membrane vesicles (MV) were isolated under isotonic conditions from human T lymphoblastoid cells MT-2 and CEM and tested for their ability to support reverse transcription of viral RNA upon exposure to human immunodeficiency virus, type 1 (HIV-1). MV contained cytoplasmas as confirmed by the presence of mitochondrial DNA but were devoid of chromosomal DNA. Virus binding and vesicle lysis assays revealed that 4–19% (depending upon virus dose) of MV-bound HIV-1 entered the vesicles. HIV-1 internalized in MV was able to initiate and complete viral DNA synthesis as determined by the detection of products of reverse transcription using polymerase chain reaction amplification of viral DNA using regions present in early (strong stop) transcripts and full-length double-stranded molecules. Viral DNA was undetectable in MV exposed to HIV-1 at 0 °C, in MV exposed to UV-inactivated virus at 37 °C, or after exposure to intact virus at 37 °C in the presence of reverse transcriptase inhibitors 2',3'-dideoxyctydine and a tetrahydroimidazo[4,5,1-](1,4)-benzodiazepin-2-(1H)-thione derivative, indicating that viral DNA detected in HIV-1-exposed MV was synthesized de novo. Kinetic studies revealed that HIV-1 DNA synthesis in MV was very rapid; full-length viral DNA was detected within 15 min of exposure at 37 °C, and the DNA levels increased 90-fold after 1 h and declined thereafter. Strong stop viral DNA was 10-fold more abundant than full-length DNA after 1 h at 37 °C, indicating that 10% of input viral genomes are fully transcribed in MV within this time frame. This system preserves the critical features of intact CD4-bearing cells to permit studies of HIV-1 entry, uncoating, and reverse transcription of viral RNA.

Human immunodeficiency virus, type 1 (HIV-1) enters T cells and macrophages by binding to surface CD4 receptors and fusion with plasma membranes (1–7). Following entry and uncoating, the viral RNA is used as a template by viral reverse transcriptase (RT) for synthesis of double-stranded DNA that then migrates into the nucleus and integrates into host chromosomal DNA (8). Reverse transcription of viral RNA is a critical process in the retroviral life cycle and a major target for anti-HIV-1 therapy (9). Several approaches have been employed to study the enzymatic activity and products of RT. The exogenous RT assay measures incorporation of deoxynucleoside 5'-triphosphates (dNTPs) into polymers using an exogenous homopolymeric template and has been extensively used for HIV-1 infectivity studies and for standardization of virus preparations (10–13). The endogenous reverse transcription reaction utilizes detergent-permeabilized virions, exogenous dNTPs, and viral genomic RNA as a template (14, 15). In contrast to the exogenous RT assay, the endogenous reaction products include genomic length minus strand and discontinuous plus strand viral DNA (16–18), and thus the reaction closely models the reverse transcription reaction that occurs after viral entry into cells (19). In the last several years the process of reverse transcription has also been assessed in infected cells using polymerase chain reaction (PCR) amplification and primers that amplify initial products before the first strand transfer (strong stop DNA), intermediate products synthesized before the second strand transfer, and full-length double-stranded viral DNA (20). Here we present a new model system utilizing CD4-carrying membrane vesicles derived from T cell lines to study reverse transcription by taking advantage of the natural property of HIV-1 to bind to the cellular surface receptor molecule, CD4, and fusing its membrane with that of the cell (4–7). The major utility of this system is that it permits the evaluation of the endogenous reverse transcription process as a function of virus entry into native cytoplasm without the background of subsequent cycles of reverse transcription resulting from the progression of viral infection in intact cells (21–23).

MATERIALS AND METHODS

Cells and Viruses—The CD4-bearing T cell lines MT-2, SupT1, and CEM were obtained from the AIDS Research and Reference Reagents Program, National Institutes of Health, Bethesda, MD and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The HIV-1 molecular clone N1T-A was described previously (24). Virus cultures were initiated by transfection of N1T-A DNA into SupT1 cells, and viral stocks for the experiments described herein were prepared using a procedure which minimizes carry-over viral DNA (25). Briefly, 6–7 days after transfection cells were washed twice, suspended in fresh medium, and cultured for 24 h at a density of 1 × 10⁶ cells/ml (25). Culture supernatants were then collected and filtered through a 0.45-μm pore filter (Millipore Corp., Marlborough, MA), and virus was concentrated by centrifugation. Virus stocks were tested for infectivity and standardized by their p24 core antigen content as described previously (22, 24).

Preparation of Membrane Vesicles—MV were prepared according to standard techniques with modifications to ensure retention of cytoplasm within the vesicles (26, 27). MT-2 or CEM cells were suspended

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1 The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MV, membrane vesicles; RT, reverse transcriptase; PCR, polymerase chain reaction; ddC, 2',3'-dideoxyctydine; TIBO, tetrahydroimidazo[4,5,1-](1,4)-benzodiazepin-2-(1H)-thione; ELISA, enzyme-linked immunoabsorbent assay.
in an isotonic buffer containing 160 mM NaCl, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, and 0.1 mM phenylmethanesulfonyl fluoride, and the cells were disrupted using 20–70 strokes (depending upon cell type) in a Dounce homogenizer, layered onto a discontinuous gradient of 50, 45, 35, and 10% sucrose, and centrifuged in a SW 40 rotor at 100,000 × g for 1 h at 4 °C. MV were collected at the interface between 45 and 35% sucrose (28), washed free of sucrose, resuspended in the isotonic buffer, and tested for their total protein content by the Bradford protein assay (29). To prepare MV containing nucleoside analog inhibitors of reverse transcription, MT-2 cells were preincubated with 2’,3’-dideoxycytidine (ddC) at 10 μM overnight prior to vesicle preparation as described above to allow metabolic processing of ddC to its active phosphorylated form. MV were routinely prepared from 200 × 10⁶ cells, divided into equivalents of 50 × 10⁶ cells, and frozen at −80 °C. MV from 50 × 10⁶ cells contained 210–230 μg of total protein. The content of CD4 in MV preparations was determined using a CD4 ELISA kit according to the manufacturer’s instructions (Intracel, Cambridge, MA) yielding the amount of CD4 per input MV. The number of CD4 molecules/vesicle was calculated using a previously published formula for 1-μm vesicles, N = 4.5 × 10⁵, where the number of CD4 molecules N = fS/70s, where f is the amount of CD4 protein, which we obtained from the ELISA quantitation, S is the surface area of the vesicle, and s is the area of the cross-section of one lipid molecule (0.4 nm²) (30).

**Determination of Optimal HIV-1 Dose for Interaction with MV**—Aliquots of MV equivalent to 50 × 10⁶ cells (217 μg of total protein) were resuspended in RPMI 1640 medium containing 10% fetal bovine serum, incubated with varying amounts of HIV-1/NIT-A for 1 h on ice to permit virus adsorption, and transferred to 37 °C for 1 h to permit virus fusion and entry. Virus-MV mixtures were then centrifuged for 2 min at 11,000 × g to remove unabsorbed virus. MV pellets were treated with 100 μg of trypsin for 30 s in 0.1 ml of isotonic buffer at 37 °C to remove unfused viral particles on the MV surface, and trypsin was inactivated by addition of 0.1 ml of fetal bovine serum and 0.8 ml of isotonic buffer (described above) supplemented with 2 mM CaCl₂. Trypsinized MV were washed, solubilized in p24 ELISA lysis buffer, and tested for the p24 core antigen content. To determine the amount of HIV-1 internalized in MV, trypsized MV equivalent to 50 × 10⁶ cells were lysed in 0.5 ml of a hypotonic buffer containing 10 mM Tris·HCl, pH 7.4, incubated at 0 °C for 30 min, and centrifuged at 11,000 × g for 2 min to sediment membranes. The amount of HIV-1 p24 core antigen in the membrane-free supernatant was determined by p24 ELISA.

**PCR Analysis**—MV samples for PCR analysis (217 μg) were lysed in PCR lysis buffer (31) containing 10 mM Tris·HCl, pH 8.3, 1 mM EDTA, 0.5% Triton X-100, 0.001% sodium dodecyl sulfate, and 300 μg of proteinase K/ml. The samples were incubated at 55 °C for 1 h and then boiled for 15 min. PCR was performed using 2.11 μg of DNA equivalent of 0.5 × 10⁶ cells and previously described primers to detect two regions of viral DNA synthesized at different stages of reverse transcription: the R/U5 region of the long terminal repeat (strong stop primer, 0.2 mM each of the four deoxyribonucleotide triphosphates, 50 mM MgCl₂, 1.5 mM MgCl₂, and 4 units of Taq polymerase (Perkin-Elmer, Norwalk, CT) for 40 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and elongation (72 °C, 1 min). The denaturation time was extended to 10 min during the last cycle. Amplified DNA was analyzed by Southern blot hybridization (35) using 32P-labeled oligonucleotide probes M669 (20) for both strong stop and full length HIV-1 DNA, M10 (33) for mitochondrial DNA, and B34 (32) for β-globin DNA detection.

**Other Assays**—HIV-1 core antigen p24 was measured using the HIV Ag kit according to the manufacturer’s instructions (Coulter Corp., Hialeah, FL).

**RESULTS AND DISCUSSION**

Isolated MV have been employed to study the mechanisms of membrane transport (26, 36) and virus-receptor interactions (37, 38), including that of HIV-1 (39). Puri and colleagues (30) described MV preparations from HeLa cells that constitutively express transduced CD4. Such MV carried up to 680 CD4 molecules/vesicle and were able to neutralize cell-free HIV-1 and fuse with gp120/gp41-expressing cells (30). Similar studies were reported using MV derived from CEM cells (39). Since HIV-1 fusion with CD4-expressing cells usually leads to virus uncoating and reverse transcription of viral RNA (8), we were interested to determine whether a similar sequence of events, resulting in synthesis of viral DNA, can be reproduced in CD4-bearing MV. MV were prepared from MT-2 or CEM cells as described previously (28) but using an isotonic buffer to ensure the retention of cytoplasm within the MV and thus provide substrate for reverse transcription. As a control for the reaction, MV were prepared similarly but using MT-2 cells preincubated overnight with ddC as an inhibitor of reverse transcription within the vesicles. As determined by CD4 ELISA, purified MV preparations carried 430 pg of CD4/μg of vesicle protein. According to the formula used by Puri et al. (30) and assuming that the largest vesicles were 1 μm in diameter, 28 MV prepared from CEM cells carried up to 193 CD4 molecules/vesicle. This is less than the 680 CD4 molecules on average per one HeLa-CD4 MV (30), probably reflecting the difference in the relative expression of CD4 on CEM and HeLa-CD4 cells, the latter being selected for high expression of the receptor (1). CEM cells have been shown to express on average 40,000–50,000 CD4 molecules on the cell surface (40). Taking 10–20 μm as an average diameter of a CEM cell (41), our MV preparations retain a density of CD4 receptors similar to that of the cells they have been isolated from.

In initial studies, we determined the ability of our MV preparations to bind and internalize cell-free HIV-1. Aliquots of vesicles corresponding to 50 × 10⁶ cells were exposed to the indicated doses of NIT-A virus, and the amount of virus stably associated with the vesicles after incubation for 1 h at 37 °C was determined (Fig. 1). A linear correlation between the amount of virus added and the amount of virus associated with the vesicles after incubation at 37 °C and trypsinization was observed within the range of 7.7–82 ng of p24/217 μg of total MV protein (Fig. 1). These are 6–9 higher virus-vesicle association values than those reported by Benzair et al. (39) for the linear range of HIV-1 bound to plastic-immobilized MV from CEM cells, 3.75–60 pg of p24/μg of MV protein. We attribute the greater efficiency of virus binding by MV in our experiments to the fact that HIV-1-MV interaction was performed in suspension, thus permitting the exposure of an entire MV surface to the virus. In addition, although Benzair et al. (39)
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used 1 µg of MV/well for microtiter plate coating, no information was provided as to the amount of vesicles that actually adsorbed to the plastic surface. The 7.7–82 ng of p24/217 µg of MV protein we observed (Fig. 1) likely represented HIV-1 that is either tightly bound to MV surfaces or internalized and thus resistant to trypsin. Subsequent lysis of these MV in a hypotonic buffer resulted in the release of 4–19% (depending upon virus dose) of MV-associated p24 into a soluble, membrane-free fraction (Fig. 1). This result indicates that a significant fraction of MV-bound HIV-1 internalizes within 1 h of incubation at 37 °C. These results are consistent with similar HIV-1 internalization studies in intact cells (42), and they confirm that only a fraction of HIV-1 in any given viral preparation is capable of fusion with target cell membranes and entry (43). Based on HIV-1 titration experiments, we have chosen for further experiments the dose of 0.25–1 ng of p24 equivalents of HIV-1/µg of MV protein.

We next tested whether the HIV-1-MV interaction at 37 °C includes reverse transcription of viral RNA (Fig. 2). MV were isolated from untreated or ddC-treated MT-2 cells and exposed to 1 ng of p24 HIV-1 equivalents/µg MV protein for 1 h at 37 °C, and total vesicle lysates were tested for the presence of full-length HIV-1 DNA by PCR. MV exposed to UV-irradiated HIV-1 served as control for carry-over viral DNA present in some viral preparations (44, 45). Lysates of 8E5 cells that carry one copy of viral DNA/cell (46) were used to construct a standard HIV-1 DNA curve assayed in parallel. As shown in Fig. 2, full-length viral DNA was detected in MV from untreated MT-2 cells exposed to N1T-A but not in those from cells treated with ddC or exposed to inactivated N1T-A. The number of viral copies was about 100/2.17 µg of MV protein or vesicles derived from 0.5 × 10⁶ cells. The DNA detected in MV exposed to N1T-A was probably newly synthesized by RT within vesicles, because N1T-A virus preparation was free of carry-over viral DNA and MV containing ddC did not permit DNA synthesis (Fig. 2). These results are consistent with HIV-1 binding and entry studies shown in Fig. 1, and they indicate that HIV-1 entry into MV triggers the series of steps that in intact cells culminates in the synthesis of full-length double-stranded viral DNA. These steps include virus uncoating and activation of reverse transcription (8). Thus MV recapitulate the cellular environment for the major early steps of the HIV-1 life cycle including binding, entry, uncoating, and reverse transcription of virion RNA.

We next tested the kinetics and extent of viral DNA synthesis in MV (Figs. 3 and 4). In these experiments MV were exposed to HIV-1 at 0.25 ng of viral p24/µg of MV protein, incubated on ice for 1 h to allow virus binding, and transferred to 37 °C. Samples for PCR analysis were collected at the end of 0 °C incubation and at indicated times at 37 °C. CEM cells were infected at multiplicity of infection of 1 (1 pg p24/cell) and incubated and analyzed in parallel. Full-length HIV-1 DNA was detected in MV exposed to N1T-A virus and incubated at 37 °C but not in MV exposed to N1T-A at 0 °C (Fig. 3), confirming that our N1T-A virus preparations were free of carry-over viral DNA. Viral DNA was detected in MV within 15 min of incubation at 37 °C at the level of about 3 copies/2.17 µg of vesicle protein (Fig. 4). HIV-1 DNA levels increased about 90-fold, to 270 copies/2.17 µg of vesicle protein, after 1 h of incubation at 37 °C and then declined, with DNA still detectable in MV 7 h after exposure to HIV-1 (Figs. 3 and 4). In contrast, full-length viral DNA was detected in HIV-1-infected CEM cells at low levels 5 h after infection, but then it increased to 450 copies/15,000 cells at 7 h and about 1 copy/cell at 48 h, as described previously for other T lymphoid cells (21, 34). β-Globin DNA was detected in cellular lysates but not in MV lysates, indicating that MV contain no nuclear DNA contamination detectable by PCR. Mitochondrial DNA was detected both in MV and in CEM lysates, demonstrating that MV prepared in isotonic buffer by mechanical disruption have cytoplasmic contents (Fig. 3). These data demonstrate that viral DNA synthesis in MV exposed to HIV-1 is very rapid compared with intact cells, with the bulk of the synthesis completed by 1 h at 37 °C.

To determine whether our system permits analysis of distinct steps of viral reverse transcription described during retroviral infection in intact cells (reviewed in Ref. 47) and to confirm that viral DNA detected in MV exposed to HIV-1 at 37 °C (Fig. 2) is synthesized de novo, we repeated previous experiments with the modifications described below and analyzed viral DNA by PCR using primers to detect either strong stop or full-length double-stranded viral DNA forms (Fig. 5). Strong stop DNA represents the R/U5 region of the viral long terminal repeat, which is synthesized first during reverse transcription, prior to the first template switching. Since strong
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Stop DNA is initially present in excess over full-length DNA, comparison of the two permits determination of the progress and completion of the reverse transcription process (20, 47). In the experiment shown in Fig. 5, MV were exposed to HIV-1 at 0.25 ng of viral p24/µg of MV protein, incubated on ice for 1 h to allow virus binding, and transferred to 37 °C. A TIBO derivative, R82150, an inhibitor of HIV-1 replication that specifically inhibits HIV-1 RT and does not require phosphorylation for its anti-HIV-1 activity (48), was added to the designated systems during the 37 °C incubation at a final concentration of 2.5 µM. Samples for PCR analysis were collected after 1 h at 0 °C and at the indicated times at 37 °C. In this experiment, HIV-1 DNA was detected 1 h after exposure of MV to HIV-1 (Fig. 5), although low levels of viral DNA could be detected at the 15-min time point upon longer exposure (not shown), confirming that viral DNA synthesis is very rapid in MV. The levels of strong stop DNA at this time were about 10-fold higher than those of full-length DNA (Fig. 5), indicating that within 1 h at 37 °C about 10% of reverse transcription events in MV result in the formation of a full-length double-stranded DNA and the rest yield incomplete viral DNA forms. No viral DNA was synthesized at 0 or 37 °C in the presence of RT inhibitor TIBO, confirming that viral DNA detected in MV under standard conditions at 37 °C is newly synthesized. These results demonstrate that the MV system can be used for the analysis of the discrete steps of reverse transcription occurring after HIV-1 entry into the cytoplasm, as well as for the evaluation of different inhibitors of the RT reaction.

These results demonstrate that reverse transcription was initiated and completed in MV within 15–30 min of incubation with HIV-1 and that the DNA persisted within the MV for 7 h of incubation at 37 °C (Figs. 2–5). The decline in viral DNA levels in MV observed at later incubation times at 37 °C (Figs. 3 and 4) may result from DNA degradation or gradual lysis of the vesicles. That the viral DNA detected in MV was newly synthesized after HIV-1 internalization was demonstrated by several controls, including absence of viral DNA in MV exposed to UV-inactivated virus (Fig. 2) or in vesicles exposed to intact virus either at 0 °C (Figs. 3 and 5) or at 37 °C after treatment with RT inhibitors ddC and TIBO (Figs. 3 and 5). The sharp increase in strong stop and full-length viral DNA levels between 15 min and 1 h incubation at 37 °C (Figs. 3 and 5) is also consistent with intravesicular DNA synthesis rather than the presence of carry-over viral DNA, in particular since we have shown previously that HIV-1 entry is completed within 15 min of incubation at 37 °C (4).

The rapid onset and completion of synthesis of viral DNA in MV exposed to HIV-1 were unexpected. Previous studies demonstrated the synthesis of complete viral DNA upon exposure to DNase-treated HIV-1 virions no earlier than 4 h postinfection in peripheral blood lymphocytes (49), 6 h postinfection in HUT-78 T lymphoid cells (50), 8 h postinfection in monocyte-derived macrophages in one study (51), and 36–48 h postinfection in another study (49). Previous studies from this laboratory on the kinetics of HIV-1 DNA synthesis in T cell lines revealed an intermediate viral DNA form at 2 h postinfection (34). The range in the earliest time of detection of viral DNA may reflect significant biological differences among systems used, but it also likely reflects the ease of DNA detection by PCR. We and other authors detect roughly five full-length DNA copies using plasmid DNA in aqueous solution as substrate (Fig. 3) but no fewer than 50–100 copies using cell lysates as standard (Fig. 2) in this study. With this in mind, it is possible that it is easier to amplify viral DNA by PCR using MV lysate as substrate than by using cell lysates that contain interfering cellular DNA. Using a quantitative assay for HIV-1-cell membrane fusion, we have shown previously (4) that the half-time of fusion is 3–4 min at 37 °C. Taken together, these results indicate that the linked processes of virion binding, fusion, uncoating, and reverse transcription (revealed in the MV system described here) are extremely rapid and are largely completed within 15–30 min.

We have developed a convenient model system for evaluation of the early phases of the HIV-1 life cycle; this model preserves the critical features of the virus-cell interaction and operates within physiological limits relative to what is observed in cells in culture. One limitation that should be noted is the finite supply of phosphorylated nucleotides required for reverse transcription, which may limit the number of RT reactions possible in this system. Another is the instability of the vesicles after more than 5 h of incubation at 37 °C (not shown) likely resulting in the leakage out of DNA products. With these limitations in mind two significant advantages of the analysis of HIV-1-MV interactions are the rapidity of the steps in virus replication, up to and including reverse transcription; and the abil-

![Fig. 4. Quantitation of viral DNA in MV exposed to HIV-1. The autoradiogram shown in Fig. 3 was subjected to densitometry and the number of full length DNA copies/2.17 µg of vesicle protein (MV) or 15,000 infected CEM cells (CEM) was calculated based on the reference HIV-1 DNA copies panel and after adjustment for the volume of MV or CEM cell extracts used in each amplification.](image)

![Fig. 5. Synthesis of strong stop and full-length viral DNA in MV exposed to HIV-1. CEM cells or their MV were exposed to HIV-1 in the absence or presence of 2.5 × 10^{-6} M TIBO and were tested at the designated times for the presence of strong stop or full-length HIV-1 DNA as described under “Materials and Methods.” The DNA standard curve was prepared as described in Fig. 3.](image)
ity to isolate MV from cells under various treatments or stimuli, exemplified here by ddC and TIBO. We believe that MV present a versatile system for the evaluation of native HIV-1-cellular interactions which take place early in the virus life cycle and that such systems can be employed conveniently to assess potential inhibitors of virus-cell fusion, virus uncoating, and reverse transcription in close analogy to their activity inside intact cells.

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