Endogenous Reverse Transcription of Human Immunodeficiency Virus Type 1 in Physiological Microenvironments: an Important Stage for Viral Infection of Nondividing Cells

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Endogenous reverse transcription (ERT) of retroviruses has long been considered a somewhat artificial process which only mimics reverse transcription occurring in target cells, as detergents or amphipathic peptides have classically been used to make the envelopes of retroviruses in these reaction systems permeable. Recently, several studies suggested that ERT of human immunodeficiency virus type 1 (HIV-1) might occur without detergent treatment. However, this phenomenon could be due to damage of the retroviral envelope during the process of virion purification or freezing and thawing. In this report, intravirion HIV-1 ERT, without detergent-induced permeabilization, is demonstrated to occur in the natural microenvironments of HIV-1 virions and is not caused by artificial processes. Therefore, this stage of the viral life cycle was termed natural ERT (NERT). The efficiency of NERT in HIV-1 virions was markedly augmented by several physiological substances in the extracellular milieu, such as polyamines and deoxyribonucleoside triphosphates. In addition, HIV-1 virions in seminal plasma samples harbored dramatically higher levels of full-length or nearly full-length reverse transcripts than virions isolated from peripheral blood plasma samples of HIV-1-seropositive men. When HIV-1 virions were incubated with seminal plasma samples, infectivity in initially nondividing cells was also significantly enhanced. Thus, we suggest that HIV-1 virions are actively altered by the extracellular microenvironment and that NERT may play an important role in viral infection of nondividing cells.

Since the discovery in 1970 of the retroviral enzyme reverse transcriptase (RT) (6, 61), this virally encoded protein has been demonstrated to be critically important toward the understanding of retroviral pathogenesis. RT and related enzymes are encoded by the *pol* gene of all retroviruses. RT functions as an RNA-dependent DNA polymerase, which is used to produce a double-stranded DNA moiety from one (or both) of the two identical positive-strand RNA genomes, carried within all retroviral virions. RT also has DNA-dependent DNA polymerization and RNA hydrolysis (RNase H) activities (15). Traditionally, RT activity is measured either within the virions made permeable by low levels of detergents (endogenous RT activity), to allow ingress of deoxyribonucleoside triphosphates (dNTPs) into virions (7, 31, 32, 61), or after total disruption of virion particles with high concentrations of nonionic detergents (e.g., Nonidet P-40 and Triton X-100) (exogenous RT activity) (51). The endogenous RT reactions have been key in the evaluation of the kinetics and molecular intermediates of reverse transcription in various retroviruses (7, 8, 32). It was demonstrated, in initial studies of endogenous RT reactions using human immunodeficiency virus type 1 (HIV-1), that a narrow concentration range of nonionic detergents was helpful in making the virions permeable and allowing full-length (or nearly full-length) viral DNA synthesis (8, 66). Melittin, a 26-amino-acid amphipathic peptide, was also demonstrated to make retroviral envelopes permeable to dNTPs (7, 8, 66). Nevertheless, a significant level of endogenous RT activity was also detected in nonpermeable HIV-1 virions by several groups (8, 20, 66, 73).

Retroviral reverse transcription occurs as an ordered, stepwise process, and the full-length viral DNA synthesis is completed after virions infect their target cells (15, 63). However, it now appears that analogous to several RT-dependent DNA viruses (e.g., hepatitis B virus), retroviruses can initiate reverse transcription prior to infection of target cells. Recently, data from several laboratories have demonstrated that reverse transcripts of retroviruses can be demonstrated within cell-free virions, rather than solely after infection of target cells (3, 40, 62, 69, 73). This has extended earlier findings of viral DNA within other retroviral particles (12, 19, 21). Virions obtained from HIV-1-infected cell cultures and the blood plasma samples of HIV-1-infected individuals were found to harbor strong-stop negative-strand DNA in approximately 1 in 10^2 to 1 in 10^3 virion particles. Lower levels of more-complete viral DNA were also demonstrated (69, 70, 73). When virus-producing cells were treated with zidovudine (AZT), the virion-associated DNA elongation was inhibited (3). Nevertheless, the biological significance of intravirion reverse transcripts remains to be clarified.

A preliminary study demonstrated that virions from AZT-treated cells showed no differences in infectivity, with a proliferating human T-cell line as a target, compared with virions from nontreated infected cells (3). Nevertheless, it was demonstrated that without detergent, the addition of dNTPs to isolated HIV-1 virions could stimulate intravirion RT activity, leading to higher levels of virions carrying strong-stop negative-strand moieties and more-complete negative-strand intermediates (73). Similar results were obtained if a murine leukemia virus was treated with dNTPs at high concentrations (71). In addition, several human physiological fluids, including seminal and blood plasma, could drive intravirion HIV-1 DNA

synthesis (73). This was hypothesized to be due to dNTPs within these fluids, which can support intravirion DNA synthesis. Most importantly, it has been demonstrated that increasing the levels of intravirion HIV-1 DNA by highly concentrated dNTPs (5 mM) led to increased viral infectivity in replicating T-lymphocytic cell lines (73). HIV-1 virions harboring solely RNA may still be infectious; however, infectivity titers appear to increase significantly upon initiation of ERT (73). These data, which demonstrate that dNTP concentrations may have profound effects on intravirion reverse transcription, suggest that the physiological microenvironment and extracellular milieus within infected individuals may enhance ERT activity and, therefore, alter HIV-1 replication.

In contrast to many other type C retroviruses, HIV-1, and probably other lentiviruses, can infect nondividing cells, partially through their ability to transport their preintegration complex into the nuclei of these nonproliferating cells, utilizing the nuclear localization signals in the p17 matrix protein, and possibly using another targeting sequence in Vpr (10, 11, 33, 38, 54). Quiescent T lymphocytes and tissue-bound macrophages have been shown to be the major cellular reservoirs for HIV-1 in vivo (11, 23). In addition, sexual transmission of HIV-1 may largely depend upon the successful infection of nondividing macrophages and/or Langerhans cells in the mucosal regions (45, 52, 74). However, it usually takes a relatively long time to complete full-length viral DNA synthesis in these cells. The low concentrations of dNTPs in the cytoplasm or other cellular factors may have contributed to these relatively slow kinetics (16, 30, 43, 47).

Note that in both mature macrophages and quiescent T lymphocytes, initiation of viral DNA synthesis was efficient, but the kinetics of elongation was delayed (47, 68). The addition of exogenous nucleosides to cell cultures increased RT elongation in macrophages but not in quiescent T lymphocytes (43, 47). Hydroxyurea, which inhibits ribonucleotide reductase and leads to reduced dNTP pools, has also been demonstrated to inhibit HIV-1 replication by decreasing processivity of intracellular HIV-1 reverse transcription (39). It is instructive to investigate the influence of intravirion reverse transcription upon the kinetics of viral replication in these cells, as this aspect may be critical toward the understanding of HIV-1 pathogenesis and intra- or interhost transmission.

In this report, we have analyzed intravirion HIV-1 reverse transcription in the physiological microenvironments of virions and identified specific physiological substances in the extracellular milieus which can enhance intravirion HIV-1 reverse transcription. As viral infectivity could be positively altered by intravirion reverse transcription, we conclude that this molecular process may play an important role for cell-free HIV-1 virion infection of nondividing cells.

MATERIALS AND METHODS

Samples from humans. All samples from humans were obtained with full written consent of the subjects in these studies, via protocols approved by the University's Institutional Review Board.

Exogenous reverse transcription. Exogenous reverse transcription assays were performed, as previously described with some modifications (51). Briefly, HIV-1_{NL4-3} virions (10 ng of HIV-1 p24 antigen), isolated from the supernatants of virus-producing T-lymphocytic cells (CEM cells), were disrupted with 1% Triton X-100 in 1 M KCl. The RT reaction mixture included 40 mM Tris-HCl (pH 7.3), 100 mM KCl, 5 mM MgCl$_2$, 1 μg of poly(rA)-oligo(dT) (Pharmacia), and 2.5 μM (total) [^{3}H]dTTP-dTTP (ratio, 1:4). Spermine and spermidine (pH 7.3), at their physiological concentrations in seminal plasma (59) (3 and 0.1 mM, respectively), dithiothreitol (2 mM), or distilled water was added to the reaction mixtures. Every 10 min, separate reactions were stopped, using 10 mM EDTA and heat inactivation (95°C). The trichloroacetic acid-insoluble ^{3}H was placed on filter paper and measured in a liquid scintillation counter.

ERT. Large quantities of HIV-1_{AAV} virions were purified from the supernatants of virus-producing T-lymphocytic cells (HUT78) by equilibrium density

centrifugation, as previously described (69, 73). Each aliquot of viruses (0.5 μg of HIV-1 p24 antigen) was mixed with an endogenous reverse transcription (ERT) reaction buffer system, composed of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl$_2$, 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont, Wilmington, Del.), and dNTPs at various concentrations, with or without spermine (3 mM; Sigma) and spermidine (0.1 mM; Sigma). For controls, melittin (15 μg/ml; Sigma), an amphipathic short peptide which makes HIV-1 virions permeable (7, 8, 66) was added to the reaction buffer. AZT triphosphate (2 μM; Moravek, Inc.) was also added to selected reaction mixtures to specifically inhibit intravirion HIV-1 DNA synthesis. In addition, seminal plasma from an HIV-1-seronegative man was mixed with purified HIV-1_{AAV} virions in the presence of 50 μCi of [α-^{32}P]dCTP. The mixtures were incubated at 37°C for 12 h or as indicated in Fig. 1. The reactions were stopped by adding 10 mM EDTA, 1% sodium dodecyl sulfate, and 50 μg of proteinase K per ml. After 1 h of incubation at 60°C, the viral DNA was extracted by phenol-chloroform (1:1) and then precipitated with ethanol. The excess free [α-^{32}P]dCTP was further eliminated by passing the sample through a quick-spin G-50 column (Boehringer Mannheim, Indianapolis, Ind.). The [α-^{32}P]dCTP-labelled viral DNA was then electrophoresed on alkaline-denatured agarose gels. The gels were dried and visualized by autoradiography.

To confirm that intravirion ERT of HIV-1 was not due to envelope damage by an artificial process (e.g., ultracentrifugation of virions), intravirion ERT was initiated prior to virion isolation. After growth in fresh, conditioned medium for 2 days, the virus-producing T-lymphocytic cells (CEM cells) were pelleted at 300 × g for 10 min. The HIV-1_{NL4-3} virion-containing supernatant was then aliquoted. dNTPs at various concentrations, with or without spermine (3 mM) and spermidine (0.1 mM), were added to the samples of supernatants. The concentration of MgCl$_2$ was adjusted to 1 mM. The ERT reaction was then allowed to progress at 37°C for 20 h. The virions were immunocaptured, using anti-gp120 and anti-gp41 antibody-coated latex beads (34, 69, 70). The virion-bead complexes were treated with DNase, and viral DNA was then extracted and amplified by PCR, with SK38-SK39 primer pairs (see below). Analysis by Southern blotting was performed, as previously described (69, 73).

Quantitation of dNTPs. The enzymatic assay developed by Sherman and Fyfe was adapted to detect dNTPs in human physiological fluids (55). In this assay, templates and primers for the polymerization reaction were used to detect and quantitate dNTP levels.

To detect the four dNTPs, four similar oligonucleotide DNA strands were synthesized as the templates: 5′-TTATTATTATTATTATTAGGCGGTGGAG GCGG-3′ (for dTTP detection), 5′-TTTGTTTGTTTGTTTGTTTGGGCGGT GGAGGCGG-3′ (for dCTP detection), 5′-TTTCTTTCTTTCTTTCTTTCGGC GGTGGAGGCGG-3′ (for dGTP detection), and 5′-AAATAAATAAATAAA TAAATGGCGGTGGAGGCGG-3′ (for dATP detection). An oligonucleotide DNA was also synthesized as a primer for detection of dNTPs: 5′-CCGCCTCC ACCGCC-3′. To make the template-primer duplex, the primer oligonucleotide DNA was annealed to each of the template oligonucleotide DNAs by heating at 95°C for 10 min and then gradually cooling down to room temperature. The template-primer duplex was then isolated from a 15% nondenaturing polyacrylamide gel, and the quantity was determined with a spectrophotometer. The physiological fluids were prepared prior to the enzymatic reaction. For seminal plasma samples, the fluids were chilled at 4°C for 30 min and then the samples were centrifuged at 1,000 × g for 5 min and then at 14,000 × g for 15 min to eliminate the sperm cells and large particles. The supernatants were then passed through 0.22-μm-pore-size filters, and 100 μl was added to 150 μl of 100% methanol. The blood plasma samples were centrifuged at 14,000 × g for 15 min and then passed through 0.22-μm-pore-size filters. One hundred microliters of supernatant was then added to 150 μl of 100% methanol. For preparation of standard dNTP curves, 100-μl samples of dNTPs at various concentrations were added to 150-μl portions of 100% methanol. The fluid-methanol mixtures were then incubated at room temperature for 5 min and centrifuged at 14,000 × g for 5 min. The supernatants were removed and dried. The dry pellets were resuspended in 40-μl portions of distilled H$_2$O. Then, the enzymatic reaction for dNTP detection was followed, with some modifications (55). The reaction cocktail (50 μl) included 40 mM Tris-HCl (pH 7.5), 0.5 mg of bovine serum albumin per ml, 10 mM MgCl$_2$, 10 mM dithiothreitol, 0.05 μg of oligonucleotide template-primer duplex, 0.5 U of Sequenase version 2 (U.S. Biochemical Corp.) 2.5 μM [^{3}H]dATP (for detection of dCTP, dGTP, and dTTP) or dTTP (for detection of dATP), and 5 μl of sample. This mixture was then incubated at 37°C for 1 h. Thirty microliters of this mixture was then placed onto each DE81 filter. The filters were completely dried at room temperature (> 2 h), washed in 5% Na$_2$HPO$_4$ three times for 10 min each time, rinsed in distilled H$_2$O for 3 min, and washed in 95% ethanol for 5 min. The filters were dried and measured in a liquid scintillation counter.

Quantitation of intravirion HIV-1 DNA and RNA by PCR. The intravirion DNA and RNA from the blood plasma samples of HIV-1-infected men were quantitated by DNA PCR and RT-PCR, as previously described (50, 69, 70, 73). To identify the intravirion DNA and RNA in the seminal plasma samples, samples of seminal fluids were obtained from HIV-1-infected men (n = 9) at the same time as the peripheral blood samples were collected. After the seminal fluid samples were placed at 4°C for 30 min, the samples were centrifuged first at 1,000 × g for 10 min and then at 15,000 × g for 15 min to remove cells and cellular debris. By the same procedure described above, HIV-1 virions were then cap-

tured by anti-gp120 and anti-gp41 antibody-coated beads from seminal plasma samples (diluted 1:1 with phosphate-buffered saline [PBS]), and then treated with DNase. Quantitative DNA and RT-PCR were then performed. As described in previous studies (67, 69, 70, 73), the RU5 region (negative-strand strong-stop DNA) was detected with the primer-probe set M667-AA55-SK31; the *gag* region was detected by the primer-probe set SK38-SK39-SK19; and the RU5-PBS-5NC region (named for RU5, primer binding site, and 5′ noncoding region), which amplified nearly full-length reverse transcripts consisting of moieties containing positive-strand DNA after the second template switch and past the primer binding site, was detected with the primer-probe set M667-M661-SK31. Note that absolutely full-length double-stranded provirus cannot be detected by this approach. The ^{32}P-labelled blots were analyzed and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and copy numbers were calculated by comparison to standard curves. DNA PCR analyses for β-globin and mitochondrial gene sequences were performed on all virion isolations from blood and seminal plasma samples to demonstrate the absence of contaminating cellular debris in the intravirion nucleic acid preparations (data not illustrated).

Quantitative PCR analysis of intracellular HIV-1 reverse transcription. HIV-1_{NL4-3} virions in the fresh supernatants of virus-producing cells (CEM cells) were mixed with various reagents, as described above. After incubation at 37°C for 20 h, the mixtures were placed onto a 20% sucrose–TN solution (10 mM Tris-HCl [pH 7.4], 100 mM NaCl) and virions were pelleted in a Ti60 rotor at 90,000 × *g* for 1 h. Peripheral blood lymphocytes (PBL) were isolated via Ficoll-Hypaque centrifugation, with two rounds of overnight adherence to plastic flasks, to remove monocytes/macrophages. The virions (0.5 μg of HIV-1 p24 antigen equivalents) were then resuspended in RPMI 1640 medium and, at 37°C for 4 h, were allowed to infect 8 × 10⁶ quiescent PBL from an HIV-1-seronegative person. For a control, heat-inactivated HIV-1 virions (56°C for 1 h) were also used to infect quiescent PBL. The unbound viruses were washed off, and soluble DNA was eliminated by DNase treatment. The infected cells were then aliquoted into four portions. One portion was immediately added to lysis buffer and frozen, while three other cell aliquots were cultured at 37°C. At 48 h postinfection, the cells were stimulated with phytohemagglutinin (PHA) (5 μg/ml; Sigma) and natural human interleukin 2 (IL-2) (50 U/ml; BRL-GIBCO). Soluble CD4 molecules (20 μg/ml; AIDS Reagent Repository, National Institutes of Health) were added to the cultures, daily, to prevent reinfection of cells by newly expressed HIV-1. At 48, 72, and 96 h postinfection, the cells were harvested. Viral DNA was extracted from the infected cells via a quick-lysis method and amplified by PCR. Analysis by Southern blotting was then performed, as previously described (69, 70, 73).

Virion uptake experiments. HIV-1_{NL4-3} virions (400 ng of HIV-1 p24 antigen equivalents) were incubated with or without dNTPs (100 μM) and/or polyamines (3 mM spermine and 0.1 mM spermidine) at 37°C for 4 h. The aliquots (200 ng of HIV-1 p24 antigen) were then mixed with 10⁶ CEM cells or 10⁷ unstimulated PBL from an HIV-1-seronegative donor and incubated at 37°C for 4 h. The unbound virions were then washed off with PBS (two washes) and subsequently the uninternalized virions were removed by incubation with 0.25% trypsin (Sigma) for 5 min at 37°C. Three more washings, including once with serum-containing medium, were then performed. The levels of HIV-1 p24 antigen in the cell lysates were quantitated by enzyme-linked immunosorbent assays (ELISA) (kits from Dupont).

Viral infectivity assays. Viral infectivity assays were performed to determine the influence of physiological substances upon intravirion HIV-1 reverse transcription and HIV-1 replication. Infectivity was measured in two cellular targets: initially quiescent PBLs and monocytes/macrophages.

(i) Initially quiescent PBLs. HIV-1_{NL4-3} virions in the fresh supernatants of virus-producing cells (CEM cells) were mixed with various reagents. After incubation at 37°C for 20 h, the mixtures were placed onto a 20% sucrose–TN solution and virions were then pelleted in a Ti60 rotor at 90,000 × *g* for 1 h. The virions (10 ng of HIV-1 p24 antigen equivalents) were then resuspended in RPMI 1640 medium and allowed to infect 4 × 10⁶ quiescent PBL from an HIV-1-seronegative person at 37°C for 4 h. The unbound viruses were washed off, via three vigorous washes with PBS. The infected cells (2 × 10⁶) were then cultured, in duplicate, in 2 ml of RPMI 1640 medium plus 10% fetal calf serum (FCS). After overnight incubation, the supernatants (0.5 ml) were collected for HIV-1 p24 antigen detection on day 0. Forty-eight hours postinfection, PHA (5 μg/ml; Sigma) and natural human IL-2 (50 U/ml; BRL-GIBCO) were added to the cultures. On the fifth day postinfection, PHA and IL-2 were washed off by pelleting the infected cells via centrifugation. The cells were then cultured in 2 ml of RPMI 1640 medium plus 20% FCS with IL-2 (10 U/ml). Samples (0.5 ml) of the supernatants were collected at specified time points. The HIV-1 p24 antigen levels were quantitated by ELISA (kits from Dupont).

(ii) Monocytes/macrophages. Isolation of monocytes/macrophages was performed by a previously described protocol (17). Briefly, Ficoll discontinuous gradient-purified peripheral blood mononuclear cells were placed onto 2% gelatin-coated flasks to allow attachment of monocytes/macrophages. After the nonadherent cells were vigorously washed off, monocytes/macrophages were then resuspended with 5 mM EDTA and the purity of the cell population was analyzed (>94% cells were positive by nonspecific esterase staining). In addition, the purity of the monocytes/macrophages was further confirmed by their resistance to infection with a lymphotropic HIV-1 strain (HIV-1_{NL4-3}) at high infec-

tious titers (data not shown). The monocytes/macrophages (4 × 10⁵ cells per well) were then cultured in a mixture of 10% FCS, 10% horse serum, 0.1 mM nonessential amino acids (NEAA) (GIBCO), 10 U of granulocyte-macrophage colony-stimulating factor (Sigma) per ml, and 10 U of macrophage colony-stimulating factor (Sigma) per ml for 7 days. HIV-1_{ADA} virions (monocytotropic strain) were incubated with samples of various physiological fluids at 37°C for 20 h, and then the mixtures were placed onto a 20% sucrose–TN solution. Virions were then pelleted in a Ti60 rotor at 90,000 × *g* for 1 hour. The virions (10 ng of HIV-1 p24 antigen equivalents) were resuspended in Dulbecco's modified Eagle's medium (DMEM) and allowed to infect 4 × 10⁵ monocytes/macrophages at 37°C for 16 h. The unbound viruses were washed off, via three vigorous washes with DMEM. The infected cells were cultured, in duplicate, in 2 ml of DMEM plus 10% FCS, 10% horse serum, and 0.1 mM NEAA. After overnight incubation, the supernatants (0.5 ml) were collected for HIV-1 p24 antigen detection on day 0. The cells were then cultured in 2 ml of DMEM plus 10% FCS, 10% horse serum, and 0.1 mM NEAA. Samples (0.5 ml each) of the supernatants were collected at specified time points. The HIV-1 p24 antigen levels were quantitated by ELISA. Experiments utilized peripheral blood cells and seminal plasma samples from different HIV-1-seronegative donors.

Viral infectivity assays were utilized to determine the influence of AZT treatment of virus-producing cells upon intravirion HIV-1 reverse transcripts and viral infectivity. HIV-1_{NL4-3}-infected CEM cells were washed and cultured in RPMI 1640 medium plus 10% FCS for 2 days. AZT (Sigma) was then added to the medium at various concentrations (0, 2, and 10 μM). After 12 h, the AZT-treated cells were vigorously washed three times with PBS and resuspended in fresh RPMI 1640 medium plus 10% FCS for an additional 12 h. The virions in the supernatants were normalized by HIV-1 p24 antigen level and genomic HIV-1 RNA level, using ELISA and quantitative RT-PCR, respectively. The viruses were then serially diluted (fourfold) and incubated with 2 × 10⁶ unstimulated PBL at 37°C for 4 h. The unbound viruses were washed off, via three washes with PBS. The infected cells (10⁶) were then cultured in duplicate in 2 ml of RPMI 1640 medium plus 10% FCS. After overnight incubation, the supernatants (0.5 ml) were collected for HIV-1 p24 antigen analysis on day 0. Five days postinfection, PHA (5 μg/ml; Sigma) and natural human IL-2 (50 U/ml; BRL-GIBCO) were added. On the eight day postinfection, PHA and IL-2 were washed off by pelleting the infected cells via centrifugation. The cells were then cultured in 2 ml of RPMI 1640 medium plus 10% FCS with IL-2 (10 U/ml). Samples (0.5 ml each) of the supernatants were collected every 3 or 4 days until 28 days poststimulation. The HIV-1 p24 antigen levels were quantitated by ELISA.

Statistical analyses. Paired Student's *t* tests were utilized to compare the differences in dNTP concentrations in blood plasma and seminal plasma samples, and the intravirion DNA/RNA ratio differences in the blood and seminal plasma samples of HIV-1-infected men. All tests were two-tailed. A *P* value of 0.05 or less was considered statistically significant.

RESULTS

HIV-1 ERT can occur in the presence of physiological substances. HIV-1 ERT, without detergent or amphipathic peptide, was demonstrated by direct labelling the reverse transcripts in a reaction buffer with [α-^{32}P]dCTP by electrophoresis on denaturing gels. Kinetics studies indicated that the ERT, driven by 100 μM dNTPs, occurs as early as 30 min (Fig. 1, lane 2), and the completion of reverse transcription takes place by 4 h (lane 5). Maximum accumulation of reverse transcripts occurs at 11 h (Fig. 1, lane 7). Thereafter, the products of reverse transcription decrease, possibly because of radiolysis of the labelled DNA or minor DNase contamination in the virus preparation (Fig. 1, lane 8). The direct labelling assays of intravirion HIV-1 reverse transcripts also indicated that at least the negative-strand DNA could be completed (9.7 kb), albeit most reverse transcripts were incomplete (Fig. 1). Note that because of discontinuous positive-strand synthesis for HIV-1 DNA (13), it is unlikely that the approximately 9.7-kb band represents positive-strand moieties. Interestingly, there are several relatively strong blocks to reverse transcription at approximately 1.8, 3.8, and 6.0 kb. As these partial blockages occur prior to the completion of full-length negative-strand DNA (9.7 kb), they are most likely to occur in negative-strand DNA synthesis. However, the approximately 0.6-kb band may represent the initiation of positive-strand synthesis (Fig. 1), starting from the polypurine tract just upstream of the U3 region (65).

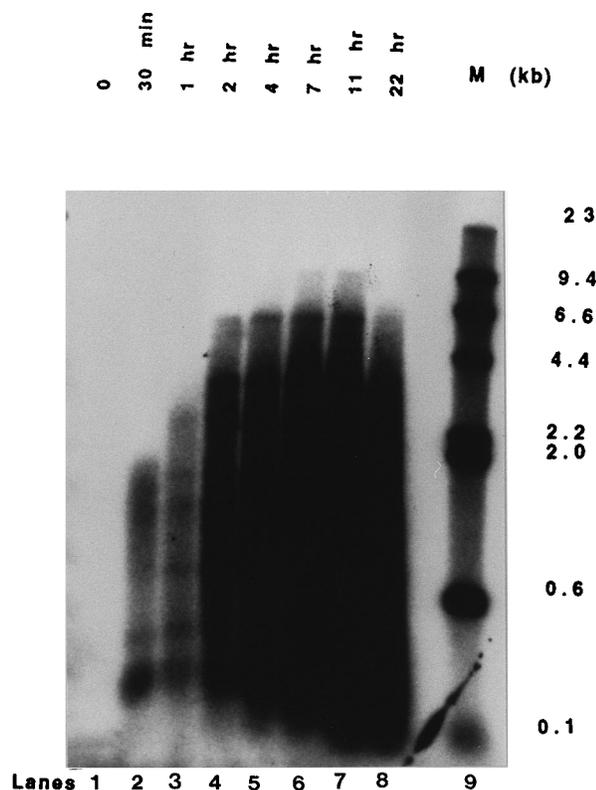Although the direct labelling experiments demonstrated that

FIG. 1. Time course analyses of HIV-1 ERT, without detergents or amphipathic peptides. HIV-1$_{AAV}$ virions were incubated with 100 μM dNTPs and [α-^{32}P]dCTP at 37°C. At various time points, as indicated over lanes 1 to 8, the reaction was stopped, and the ^{32}P-labelled viral DNA was extracted and analyzed by electrophoresis on alkaline-denatured gels. Lane 9 contains molecular size markers (M) (in kilobases), specifically, [α-^{32}P]dCTP-labelled, HindIII-digested λ phage DNA. This autoradiograph is representative of at least three independent experiments.

full-length or nearly full-length viral DNA can be synthesized in the HIV-1 virion without detergent or amphipathic peptide, two issues needed to be clarified. First, the dNTP concentrations in the reaction buffer were as high as those found in cells which are actively replicating (28). The dNTP concentrations in most extracellular milieus would not reach such a high level. Second, the virions utilized in most reactions were isolated by ultracentrifugation followed by equilibrium density centrifugation. During these processes, the viral envelope may have been nonspecifically damaged, such that the dNTPs could enter into the virions. To address these issues, the concentrations of dNTPs in samples of the seminal and blood plasma from the human body were analyzed by a highly sensitive enzymatic technique. The concentrations of dNTPs (nanomolar) in the blood and seminal plasma samples are shown in Table 1. The concentrations of dNTPs in the seminal plasma samples were significantly higher than in the blood plasma samples ($P < 0.01$ by paired Student's t test), as rapid spermatogenesis is accom-

TABLE 1. dNTP concentrations in human physiological fluids

Plasma samples	dNTP concn (nM) (mean ± SD)			
	dATP	dGTP	dCTP	dTTP
Blood ($n = 8$)	16.1 ± 3.2	21.8 ± 0.8	27.3 ± 3.5	26.2 ± 2.0
Semen ($n = 8$)	55.5 ± 3.7	48.7 ± 6.6	89.9 ± 15.6	94.4 ± 9.9

panied by high levels of degeneration of immature and mature sperm, which may release dNTPs into the extracellular milieu (18). Note that dNTP concentrations at these levels could still drive reverse transcription in exogenous and endogenous RT reactions, albeit the progression is slower than with higher levels of dNTPs (see below and data not shown).

Subsequently, to exclude the possibility that the intravirion HIV-1 ERT was induced by viral envelope perturbations during virion purification and freezing plus thawing, ERT was investigated by quantitative DNA PCR. In these experiments, only the fresh supernatants of HIV-1-producing cells were utilized and intravirion ERT was initiated prior to viral isolation by the immunocapture method. Figure 2A demonstrates that HIV-1 intravirion ERT could still progress in the presence of dNTPs in the range of physiological concentrations within seminal plasma samples (lanes 7 and 8). Note that baseline levels of strong-stop and *gag* intravirion reverse transcripts of 1 in 10^3 and 1 in 10^5 particles, respectively, were increased in these experiments to approximately 1 in 1 and 1 in 10^2 particles (not shown). Again, time course studies have shown that the initiation of ERT can occur as early as 30 min, indicating that the progression of ERT does not rely on potential envelope damage, secondary to long incubation periods in vitro. In addition, no difference in ERT of fresh versus properly cryopreserved virions was consistently demonstrated (data not shown).

As polyamines can increase the efficiency of DNA replication and RNA transcription, both in vitro and in vivo, and the concentrations of polyamines reach high levels in seminal plasma samples (59, 60), we postulated that polyamines might enhance HIV-1 reverse transcription. Figure 2B demonstrates that spermine and spermidine, at the physiological concentrations found in seminal plasma samples (59), potently augmented exogenous reverse transcription after complete disruption of HIV-1 virions with detergent. Similar results were observed when purified recombinant HIV-1 RT protein (Intracel, Inc., Cambridge, Mass.), instead of virion-associated RT molecules, was utilized in the exogenous RT reaction (not shown). In the presence of polyamines, the ERT, processed prior to any virion isolation, was also remarkably enhanced (Fig. 2A, lanes 1 to 5). Interestingly, without the addition of exogenous dNTPs into the reaction system, certain levels of nascent intravirion HIV-1 DNA were synthesized upon treatment with polyamines, suggesting that there may be intravirion dNTPs which were packaged within virions from the virus-producing cells (Fig. 2A, lane 1). However, formally proving this assumption is very difficult because current methods for dNTP detection cannot analyze such low levels of dNTPs precisely (data not shown).

To further investigate the influence of dNTPs and polyamines upon HIV-1 ERT, the direct labelling method was utilized. ERT was analyzed by mixing purified HIV-1 virions with dNTPs at various concentrations, with or without polyamines (Fig. 2C). At very high concentrations of dNTPs, the enhancement effect of ERT by polyamines was only slight (compare lanes 8 and 12). However, the enhancement effect was significant when the dNTP concentration was relatively low, indicating polyamines increase the usage of substrates by the RT enzyme (compare lanes 9, 10, and 11 to lanes 13, 14, and 15). Importantly, at the physiological concentrations of dNTPs and polyamines in seminal plasma, the majority of the de novo reverse transcripts were 2 to 3 kb and have passed the first template switch (Fig. 2C, lane 14). We utilized the physiological concentrations of spermine and spermidine found in human seminal fluid (60). Note that the RU5 region in HIV-1 genome, where the strong-stop DNA is reverse transcribed, is 181 bp (65). Importantly, a certain portion of HIV-1 intra-
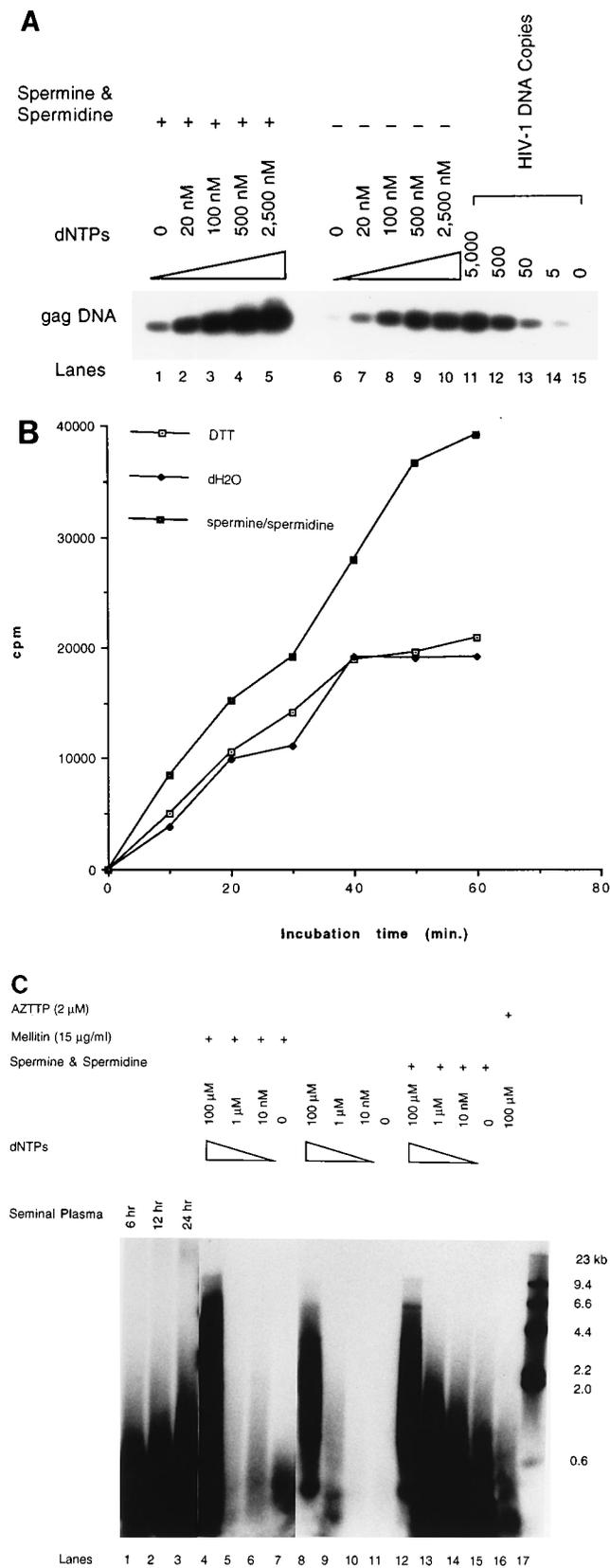
A

B

C

virion reverse transcription almost reach the completion of full-length negative-strand synthesis (*gag* DNA) at low concentrations of dNTPs (Fig. 2A, lanes 2, 3, 7, and 8), which could be detected by PCR but could not be demonstrated by the less sensitive direct labelling method (Fig. 2C).

Seminal plasma also potently stimulated the initiation of HIV-1 ERT in this assay system (Fig. 2C, lanes 1 to 3). This further supports the findings that physiological concentrations of dNTPs and polyamines drive intravirion HIV-1 reverse transcription.

The levels of HIV-1 intravirion reverse transcripts in seminal plasma samples are significantly higher than in blood plasma samples. As the dNTPs and polyamines at their physiological concentrations in the seminal plasma of the human body can enhance intravirion HIV-1 reverse transcription, we hypothesized that the levels of intravirion HIV-1 reverse transcripts in various physiological fluids in vivo may differ. To test this hypothesis, both seminal and blood plasma samples were simultaneously obtained from the same HIV-1-infected men ($n = 9$). By an immunocapture method coupled to DNA PCR and RT-initiated PCR assays, it was demonstrated that, unlike the intravirion HIV-1 DNA in cell cultures and in the blood plasma samples of infected individuals, which is predominantly composed of partial reverse transcripts (Fig. 3A), the intravirion reverse transcripts in seminal plasma samples consisted of relatively high levels of full-length or nearly full-length moieties (Fig. 3B and Table 2). As such, the majority of intravirion HIV-1 DNA were strong-stop negative-strand reverse transcripts in blood plasma samples, while in seminal fluid samples significant levels of the intravirion HIV-1 DNA moieties demonstrated positive-strand synthesis past the second template jump during reverse transcription (Table 2). The intravirion HIV-1 DNA/RNA ratios in the peripheral blood plasma samples were similar to those previously described for HIV-1-infected patients (69, 70). However, for all species of intravirion DNA, the intravirion HIV-1 DNA/RNA ratios in the seminal plasma samples, were dramatically higher than in the blood plasma samples ($P < 0.01$ by paired Student's t tests), further indicating both the remarkable initiation and completion or near completion of intravirion reverse transcription in this physiological fluid (Table 2). For controls, DNA PCR analyses for β-globin and mitochondrial gene sequences were performed on all virion isolations from blood and seminal plasma samples to demonstrate the absence of contaminating cellular debris in the intravirion nucleic acid preparations (data not shown). Further, to confirm that seminal plasma does not

were treated in vitro with various concentrations of dNTPs, with or without polyamines. The virions were then isolated by the immunocapture method, treated with DNase, and analyzed by PCR and Southern blotting for intravirion HIV-1 *gag* DNA. Lanes 1 to 5, dNTPs and polyamines (spermine [3 mM] and spermidine [0.1 mM]); lanes 6 to 10, dNTPs alone; lanes 11 to 15, samples with known numbers of HIV-1 DNA copies by reference to the standard curve of ACH-2 DNA (one proviral copy per cell). (B) Time course studies of the exogenous HIV-1 RT assay were performed, with and without spermine and spermidine (spermine/spermidine) (3 mM and 0.1 mM). Dithiothreitol (DTT) (2 mM) was also used as an antioxidant control. [³H]dTMP incorporation into DNA products (in counts per minute) is illustrated on the *y* axis. This figure is representative of at least three independent experiments. dH₂O, distilled water. (C) HIV-1 ERT measured via a direct labelling method. Purified HIV-1$_{AAV}$ virions were incubated with seminal plasma from an HIV-1-seronegative man or with dNTPs at various concentrations, with (+) or without polyamines, melittin, and AZT triphosphate (AZTTP), as indicated in the figure. The ³²P-labelled viral DNA was resolved on alkaline-denatured agarose gels. Lanes 1 to 3, seminal plasma samples; lanes 4 to 7, dNTPs and melittin; lanes 8 to 11, dNTPs alone; lanes 12 to 15, dNTPs and polyamines; lane 16, dNTPs and AZTTP; lane 17, DNA molecular size markers ([α-³²P]dCTP-labelled, *Hin*dIII-digested λ phage DNA).

FIG. 2. Polyamines enhance both exogenous and endogenous reverse transcription of HIV-1. (A) HIV-1 ERT was analyzed prior to virion isolation and measured by the quantitative DNA PCR method. HIV-1$_{NL4-3}$ virions, obtained from the fresh supernatants of chronically infected CEM T-lymphocytic cells,
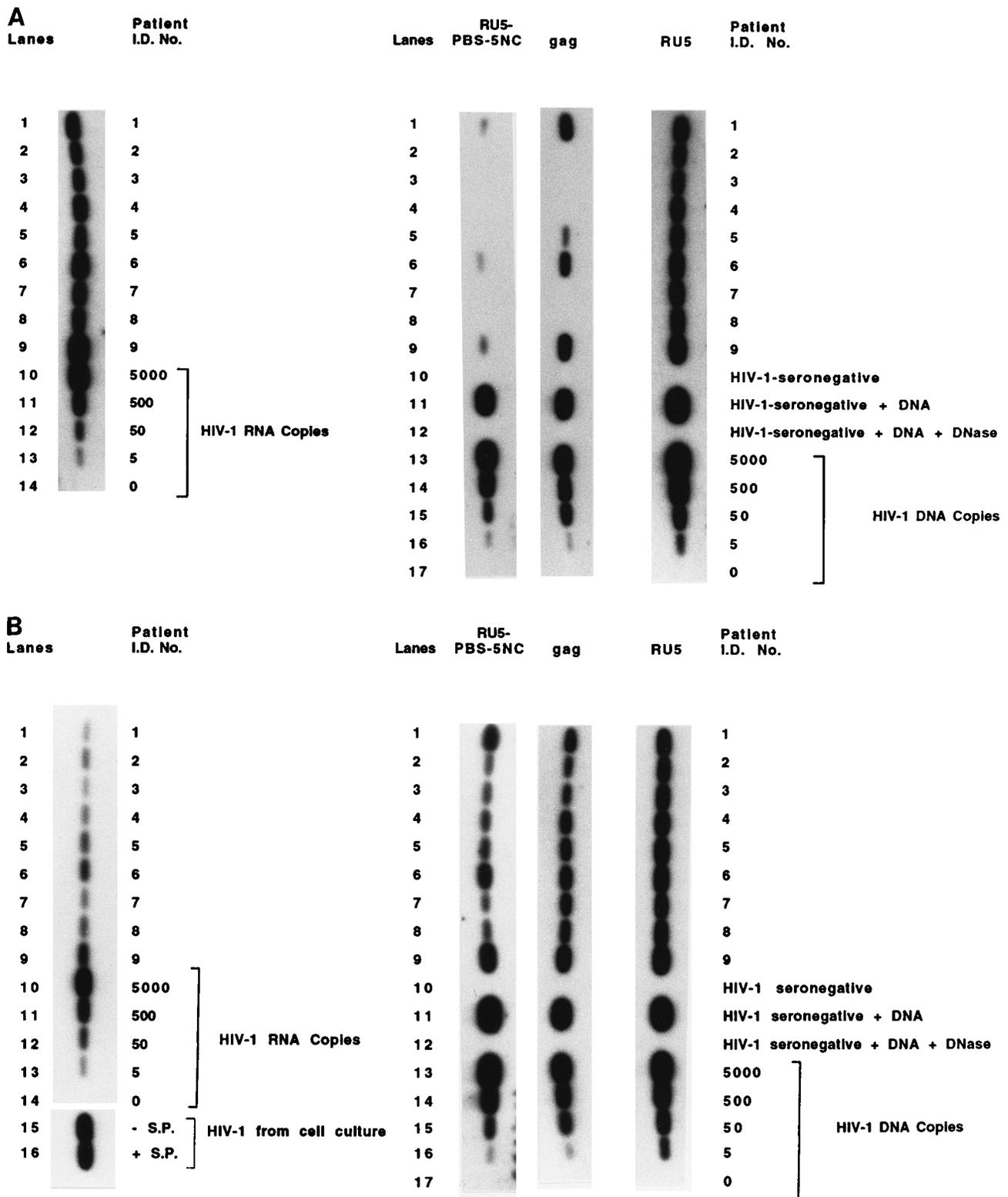
FIG. 3. Quantitative DNA and RT-PCR analyses of intravirion HIV-1-specific DNA and RNA from the blood and seminal plasma samples of HIV-1-infected men. (A) Blood plasma samples from HIV-1-infected men. HIV-1 virions were captured by anti-gp120 and anti-gp41 antibody-coated latex beads from blood plasma samples of HIV-1-seropositive men ($n = 9$) and an HIV-1-seronegative man and then were treated with DNase. Viral nucleic acids were extracted, and intravirion DNA was amplified by PCR with different primer pairs. Viral RNA was treated with DNase, reverse transcribed into cDNA, and then amplified by PCR. The PCR products were analyzed by Southern blot assays. In the three blots on the right, intravirion HIV-1 DNA levels, detected by quantitative DNA PCR, are shown. All samples were diluted fivefold prior to PCR. HIV-1 virions from the peripheral blood samples of HIV-1-infected men (lanes 1 to 9), and blood plasma from an HIV-1-seronegative man (lane 10) were used. The pelleted beads, mixed and isolated from the blood plasma of the HIV-1-seronegative subject, were combined with HIV-1 proviral DNA from ACH-2 cells with and without further DNase treatment (lanes 11 and 12). Lanes 13 to 17 contain samples with known numbers of HIV-1 DNA copies by reference to the

TABLE 2. Intravirion DNA and RNA levels in the blood and seminal plasma samples of HIV-1-infected men

Patient identification no.	No. of CD4-positive lymphocytes in peripheral blood samples (no. of cells/mm^3)	HIV-1 RNA (no. of copies/ml)		HIV-1 DNA/HIV-1 RNA ratio					
				RU5 region (strong stop)		gag region		RU5-PBS-5NC region (full-length)	
		Blood	Semen	Blood	Semen	Blood	Semen	Blood	Semen
1	580	750,000	1,500	0.0028	0.7330	0.00067	0.300	0.000067	0.030
2	1,000	94,000	5,000	0.0060	0.1100	0	0.030	0	0.020
3	500	91,000	1,500	0.0067	0.4333	0	0.120	0	0.008
4	200	505,000	3,100	0.0015	0.2731	0	0.117	0	0.047
5	350	148,000	22,000	0.0056	0.0480	0.00034	0.014	0	0.008
6	400	985,000	28,000	0.0015	0.0528	0.00021	0.015	0.000030	0.019
7	580	296,000	3,500	0.0029	0.1096	0	0.097	0	0.029
8	294	320,000	6,000	0.0033	0.1331	0	0.060	0	0.025
9	<50	2,510,000	75,000	0.0014	0.1212	0.00016	0.073	0.000040	0.060
Mean		633,000	16,200	0.0034	0.2237	0.00015	0.101	0.000015	0.027

inhibit the isolation, reverse transcription, or subsequent amplification of viral RNA from the seminal plasma sample, HIV-1 virions from cell culture with or without seminal plasma were immunocaptured, and the genomic viral RNA was extracted and analyzed by RT-PCR. The addition of seminal plasma did not decrease the yield of viral RNA (Fig. 3B, leftmost blot, lanes 15 and 16).

These data demonstrate dramatically higher levels and more-complete partial reverse transcripts within HIV-1 virions in the seminal fluid samples than the blood plasma samples of infected men. As described below, the extracellular milieu of seminal fluids may lead to these alterations in intravirion HIV-1 reverse transcription in vivo.

Intravirion HIV-1 reverse transcription alters the progression of intracellular reverse transcription. To investigate the effects of intravirion ERT upon the efficiency of reverse transcription in the early events of the HIV-1 life cycle, time course studies of intracellular reverse transcription were performed. HIV-1 virions were treated with dNTPs alone, polyamines alone, or dNTPs plus polyamines at their physiological concentrations in human seminal plasma or left untreated. After treatment for 20 h and isolation of the virions from the dNTPs and/or polyamines, the HIV-1 virions were used to infect quiescent PBL. At 48 h postinfection, the PBL were stimulated with PHA and IL-2. At several time points postinfection, the cells were collected and the HIV-1 DNA was analyzed by quantitative PCR. Importantly, the reverse transcripts were mainly full-length in the unstimulated cells infected by dNTP- and/or polyamine-treated HIV-1 virions (Fig. 4), while only partial reverse transcripts could be detected in the PBL infected by untreated HIV-1 virions at 48 h postinfection (compare lanes 8, 12, and 16 to lane 4), consistent with previous data (56, 67, 68). Heat-inactivated HIV-1 virions (a control)

did not lead to an increase of HIV-1 reverse transcripts over time (Fig. 4, lanes 1 and 2), and the baseline levels represented intravirion reverse transcripts in adhering virions. Furthermore, reverse transcription is completed at significantly higher levels after mitogen stimulation of cells infected with dNTP- and/or polyamine-treated HIV-1 virions than in cells infected with untreated virions (Fig. 4, compare lanes 9, 10, 13, 14, 17, and 18 to lanes 5 and 6). These results suggested that there might be negative factors in quiescent cells which irreversibly impair the synthesis machinery for the completion of HIV-1 reverse transcription. Intravirion reverse transcripts may bypass these inhibitory factors in the cytoplasm of nonreplicating cells, although further studies will be required.

As a control, HIV-1 binding and internalization experiments indicated that there were no significant differences in the entry of virions, treated with the various reagents or left untreated, into target cells (Fig. 5). Although the levels of viral internalization are low compared with the input virions, levels of internalized virions were quite consistent throughout these studies. Thus, alterations in binding and penetration of polyamine- and/or dNTP-treated virions do not appear to account for the dramatic differences in intracellular HIV-1 reverse transcription compared with that of untreated virions.

Intravirion reverse transcription alters the infectivity of HIV-1 virions. As intravirion HIV-1 reverse transcription could bypass the negative influences on intracellular reverse transcription in quiescent cells, it is valuable to investigate the effects of intravirion reverse transcription on HIV-1 replication. Intravirion ERT, driven by dNTPs and/or polyamines at physiological concentrations, was shown to dramatically accelerate the replication of HIV-1 in initially quiescent target cells (Fig. 6A). To determine whether the virions in the seminal plasma samples are more infectious than those in the blood

standard curve of HIV-1 DNA from ACH-2 cells (one proviral copy per cell; 10-fold serial dilutions). The leftmost blot shows HIV-1 RNA detected in the blood plasma samples. All samples were diluted 500-fold prior to PCR. Lanes 1 to 9 contain blood samples from subjects evaluated by RT-PCR. Lanes 10 to 14 contain samples with standard copy numbers of in vitro-transcribed HIV-1 gag RNA (10-fold serial dilutions) (73). All samples were also analyzed in reactions lacking the RT step to rule out contamination with HIV-1 DNA (not shown). (B) Seminal plasma samples from HIV-1-infected men. The three blots to the right show HIV-1 DNA detected by quantitative DNA PCR. All samples were diluted fivefold prior to PCR. Seminal plasma samples from HIV-1-infected men (lanes 1 to 9) were used. For controls, seminal plasma from an HIV-1-seronegative man was utilized (lane 10); then the pelleted beads were mixed with HIV-1 proviral DNA from ACH-2 cells with and without further DNase treatment (lanes 11 and 12). Lanes 13 to 17 contain samples with known numbers of HIV-1 DNA copies by reference to the standard curve of HIV-1 DNA from ACH-2 cells. The leftmost blot shows HIV-1 RNA detected in seminal plasma. All samples were diluted 500-fold prior to PCR. Lanes 1 to 9 contain seminal samples from subjects, analyzed by RT-PCR with gag primers or probe. Lanes 10 to 14 contain samples with standard copy numbers of in vitro-transcribed HIV-1 gag RNA (73). To confirm that the seminal plasma had no effect upon virion isolation and HIV-1 RNA analyses, a known quantity of HIV-1$_{NL4-3}$ virions from the supernatants of virus-producing T lymphocytes (CEM cells) was mixed with seminal plasma (+ S.P.) from an HIV-1-seronegative man or left untreated (− S.P.). Virion isolation was immediately performed, and HIV-1 RNA analyses were then conducted, via quantitative RT-PCR, as described above (leftmost blot, lanes 15 and 16). These figures are representative of at least three independent experiments. Patient I.D. No., patient identification number.
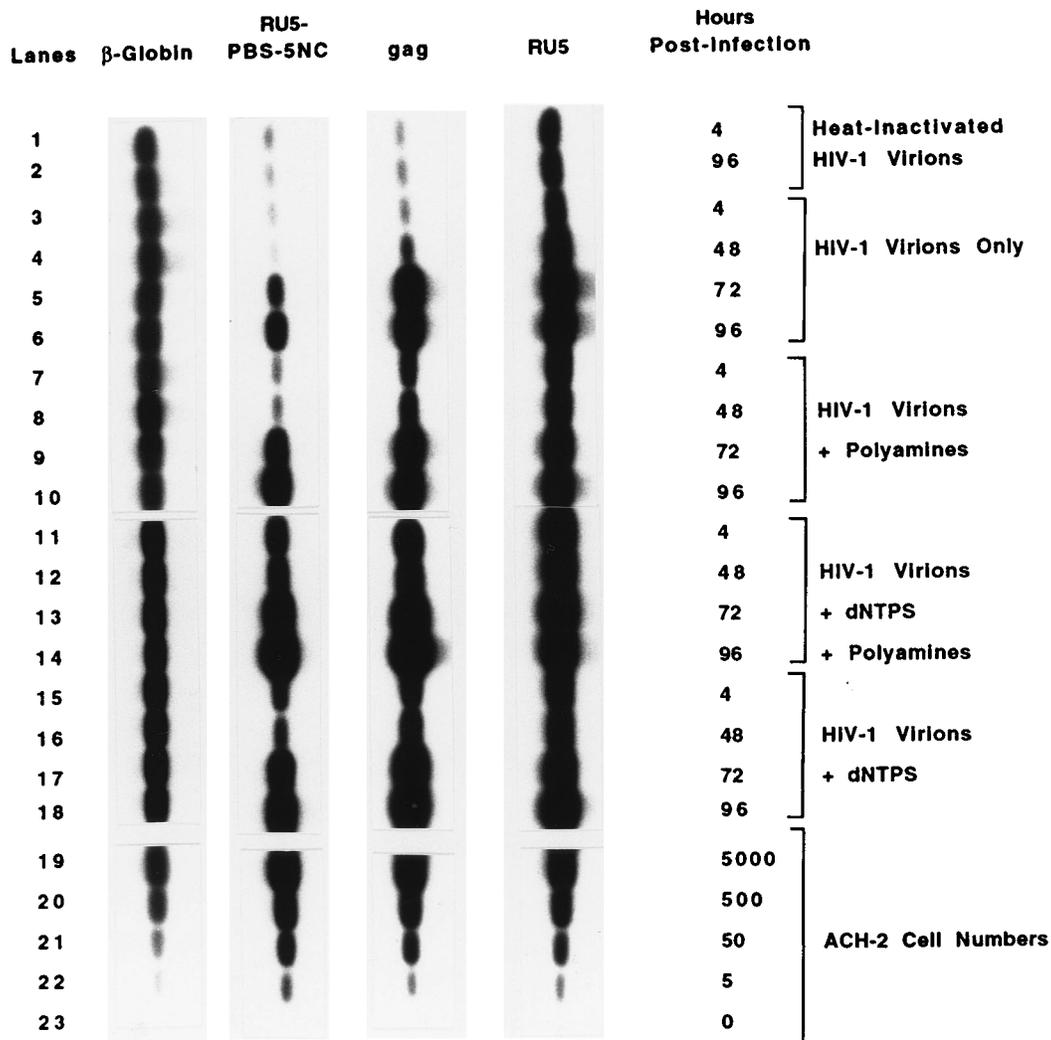
FIG. 4. Time course studies of intracellular reverse transcription in initially quiescent PBL after infection with HIV-1 virions treated with polyamines and/or dNTPs. The HIV-1 DNA moieties (strong stop, *gag*, and full-length) were analyzed by quantitative PCR. HIV-1$_{NL4-3}$ virions were treated with heat inactivation (lanes 1 and 2), conditioned medium only (lanes 3 to 6), the polyamines spermine (3 mM) and spermidine (0.1 mM) alone (lanes 7 to 10), 50 nM dNTPs plus the polyamines spermine (3 mM) and spermidine (0.1 mM) (lanes 11 to 14), or 50 nM dNTPs alone (lanes 15 to 18). Samples containing known numbers of ACH-2 cells by reference to a standard curve of ACH-2 DNA is illustrated on the right (lanes 19 to 23). β-Globin controls for cellular DNA are shown in the leftmost blot. This autoradiograph is representative of at least two independent experiments.

plasma samples because of enhanced ERT, a potentially useful experiment would be to isolate the virions from the seminal and blood plasma samples from an HIV-1-infected individual and infect initially quiescent PBL. However, as the number of infectious virions in the seminal plasma samples of HIV-1-seropositive men, after strict isolation, are quite low, these studies are technically problematic. Thus, an alternative method was adapted. The HIV-1 virions, either lymphotropic or monocytotropic, isolated from cell cultures were incubated with seminal plasma samples from HIV-1-seronegative men to initiate ERT and then reisolated. Infectivity studies with these virions, upon initially quiescent PBL and monocytes/macrophages, were then performed. Figures 6B and C indicated that viral infectivity was indeed enhanced after the virions were incubated with seminal plasma samples. In related experiments, a 16-fold increase in infectivity titers on quiescent PBL of HIV-1 virions treated with physiological levels of dNTPs and polyamines over that of untreated virions was also demonstrated (not shown). Note that no difference in viral repli-

cation was demonstrable in previously stimulated PBL, comparing untreated versus treated HIV-1 virions (Fig. 6D). These results further demonstrated that intravirion ERT occurs within infectious HIV-1 virions and participates in the viral life cycle. As described above, alterations in virion binding and internalization by polyamines and/or dNTPs did not occur (Fig. 5) and should not account for these alterations in HIV-1 infectivity by these agents.

AZT treatment of HIV-1-producing cells can decrease the intravirion DNA levels and thus inhibit viral infectivity upon initially quiescent cells. Finally, we attempted to further explore the biological significance of intravirion HIV-1 DNA identified in the supernatants of cell cultures. These relatively low levels of intravirion HIV-1 DNA may be due to intravirion reverse transcription utilizing dNTPs, either packaged from the virus-producing cells during virion budding or in the supernatant after release from damaged cells. It seems unlikely that HIV-1 intravirion DNA is due to the partial RT activity of the p160 Gag-Pol fusion protein inside the virus-producing
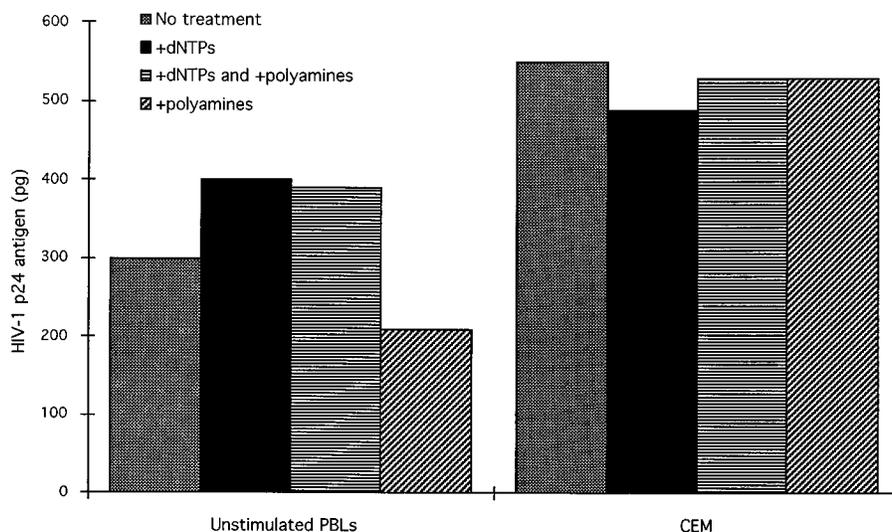
FIG. 5. HIV-1 virion binding and internalization. HIV-1$_{NL4-3}$ virions (200 ng of HIV-1 p24 antigen equivalents) were treated with a variety of reagents (dNTPs [100 μM] and/or the polyamines spermine [3.0 mM] and spermidine [0.1 mM]) or left untreated and then were utilized to infect CEM T-lymphocytic cells or quiescent human PBL. After 4 h, the unbound virions were vigorously washed off the cell surfaces and further eliminated by treatment with trypsin. The internalized virions were quantitated by measuring HIV-1 p24 antigen in cell lysates. These results represent at least three independent experiments. Standard deviations were less than 20% between experiments.

cells. It has been shown that, when maturation of HIV-1 RT (p66-p51) during or after virion budding was inhibited by mutations in the protease gene, the intraviral DNA levels decreased (3). Nevertheless, when the virus-producing cells were treated with AZT, the virion-associated DNA elongation was inhibited. However, viral infectivity in replicating cells was not altered by inhibiting intravirion HIV-1 DNA elongation by this treatment. As the kinetic advantage of relatively low levels of intravirion reverse transcripts versus larger quantities of genomic RNA could not be observed in the replicating cells (3), we asked whether the infectivity upon the initially quiescent cells could be altered by the same treatment. After AZT (2 or 10 μM) was incubated with virus-producing cells for 12 h, which were then vigorously washed, the cells were allowed to produce virions for 12 h. The virions were then immunocaptured, and intravirion DNA was then analyzed. As demonstrated in Fig. 7A (compare lanes 1 to lanes 5 in the three right blots), the intravirion HIV-1 DNA levels decreased in the virions produced from the AZT-treated cells. The strong-stop DNA and the viral DNA in *gag* and the RU5-PBS-5NC regions decreased significantly. When the virions were allowed to infect the initially quiescent PBL, both the infectious titers and the kinetics of viral replication were decreased (Fig. 7B and Table 3). As the AZT had been vigorously washed from virus-producing cells and the fresh supernatants which harbor the virions were serially diluted before the quiescent target cells were infected, it is highly unlikely that there is residual AZT in the virion preparation. To further confirm that there was no residual active AZT in the virion preparation which might affect the viral replication in the target cells, the undiluted virion preparations were heat inactivated and then added to a cell culture infected with HIV-1. No inhibitory effect upon HIV-1 replication was observed (data not shown). In addition direct treatment of HIV-1 virions with dNTPs plus polyamines and AZT triphosphate (AZTTP) ablated the effects of the agents which drive ERT (Fig. 2C) and, thus, also dramatically decreased virion infectivity on initially quiescent cells (unpublished data).

Overall, these data indicated that the intravirion HIV-1 re-

verse transcripts isolated from cell cultures could play an important role in infection of initially quiescent cells. Treating virus-producing cells with AZT to decrease the level of intravirion reverse transcripts impairs the viral infectivity upon initially quiescent cells.

DISCUSSION

HIV-1 virions: sites for reverse transcription. In this study, HIV-1 virions were demonstrated to be biochemically active particles. Complicated biochemical reactions occur within these retroviruses, including DNA polymerization and RNase H activity, which could be influenced by the physiological microenvironments. Some of the physiological substances in the extracellular milieu can potently enhance intravirion reverse transcription. Moreover, intravirion HIV-1 reverse transcription is important for the establishment of infection in nondividing cells and therefore participates in the viral life cycle. It is notable that critical microenvironments for HIV-1 may include not only the physiological fluids such as seminal plasma, blood plasma, and fluids in rectal and cervical mucosa but also the extracellular fluids in certain tissues, which may harbor highly concentrated dNTPs and polyamines from deteriorated cells and enhance ERT. The viral infectivity would, thus, be increased by enhanced ERT. As such, ERT might affect viral transmission locally (by fluids within certain tissues) or at a distance for intrahost transmission (e.g., by blood) or interhost transmission (e.g., seminal fluid or fluids in the female genital tract). On the basis of these findings, we termed this stage of the viral life cycle, natural ERT (NERT). Because retroviral virions were classically thought to be quiescent particles and ERT merely served as a model for understanding the reverse transcription occurring inside target cells, the term we propose here would be helpful to clarify our understanding of HIV-1 virions, ERT, and the retroviral life cycle.

This report also demonstrates that the HIV-1 virion itself, as a member of retrotransposon family, could be considered an intact transcription unit in which the transition of its genetic substance can be completed without assistance from either
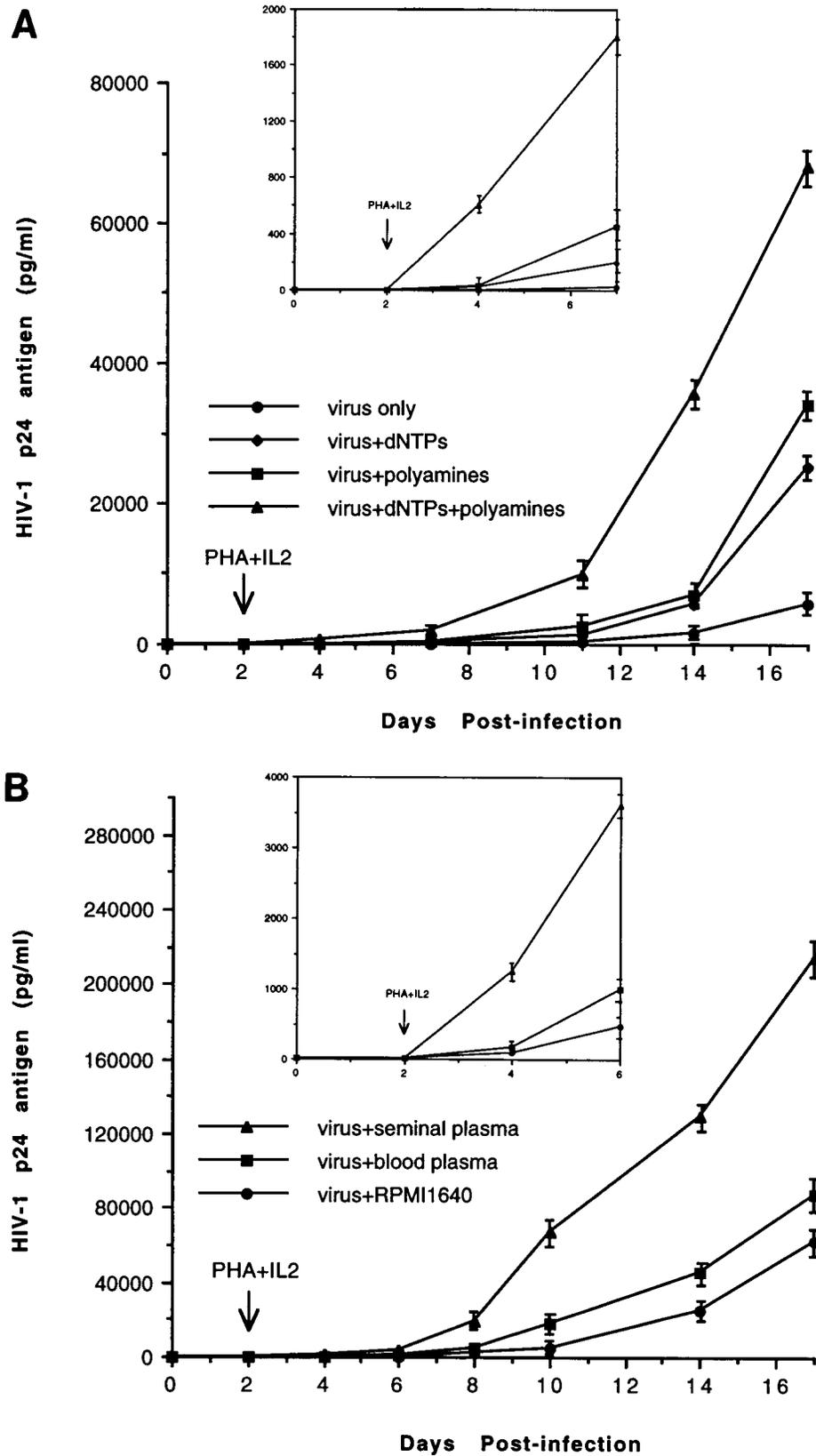
FIG. 6. Effects of intravirion ERT upon HIV-1 infectivity. The HIV-1$_{NL4-3}$ virions from the fresh supernatants of virus-producing cells (CEM cells) were mixed with various reagents or physiological fluids and then allowed to infect quiescent PBL (A and B). In addition, HIV-1$_{ADA}$ virions (macrophage-tropic viral strain) from the fresh supernatants of virus-producing peripheral blood mononuclear cells were mixed with various physiological fluids and then allowed to infect isolated monocytes/macrophages (C). Finally, HIV-1 virions, both treated and untreated, were used to infect previously stimulated and proliferating PBL (D). The kinetics of HIV-1 replication was measured by HIV-1 p24 antigen detection in the culture supernatants in an ELISA (kit from Dupont). (A) HIV-1$_{NL4-3}$ virions were treated with conditioned medium only, 50 nM dNTPs, 50 nM dNTPs plus the polyamines spermine (3 mM) and spermidine (0.1 mM), or the polyamines spermine (3 mM) and spermidine (0.1 mM) alone. The inset shows HIV-1 expression over the first 7 days postinfection to further illustrate kinetic differences in growth between virions

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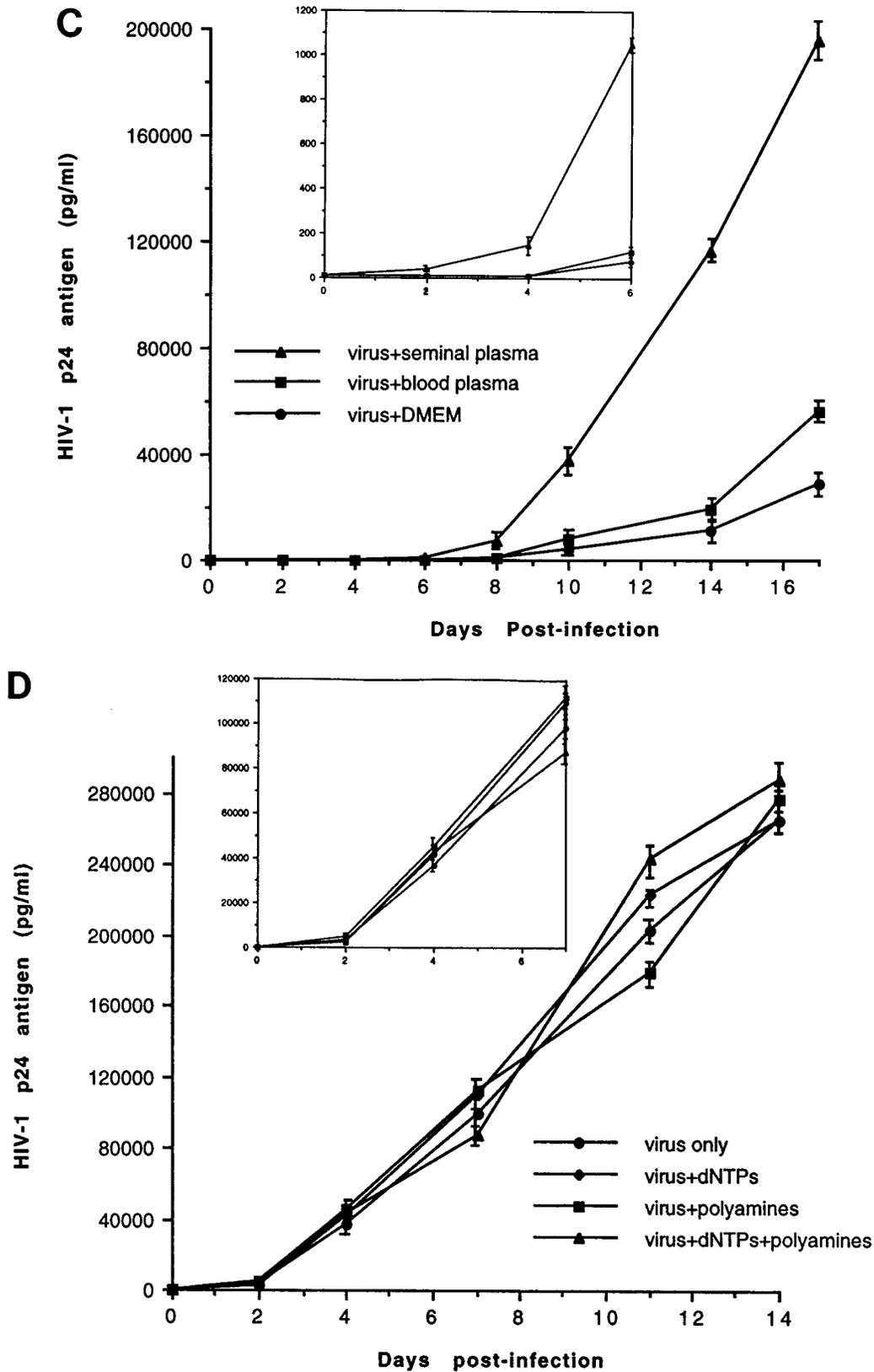
treated with dNTPs and/or polyamines and untreated virions. (B) HIV-1$_{NL4-3}$ virions were treated with conditioned medium only, seminal plasma from an HIV-1-seronegative man, or blood plasma from an HIV-1-seronegative man (virion-physiological fluids [vol/vol], 1:4). The inset illustrates HIV-1 growth over the first days postinfection. (C) HIV-1$_{ADA}$ virions were treated with conditioned medium only, seminal plasma from an HIV-1-seronegative man, or blood plasma from an HIV-1-seronegative man (virion-physiological fluid [vol/vol], 1:4). The inset illustrates HIV-1 replication over the first week postinfection of macrophages. (D) HIV-1$_{NL4-3}$ virions were either treated or not treated, as described above, and used to infect PHA- and IL-2-stimulated PBL. The inset illustrates HIV-1 replication over the first week postinfection. These graphs are representative of at least two independent experiments.
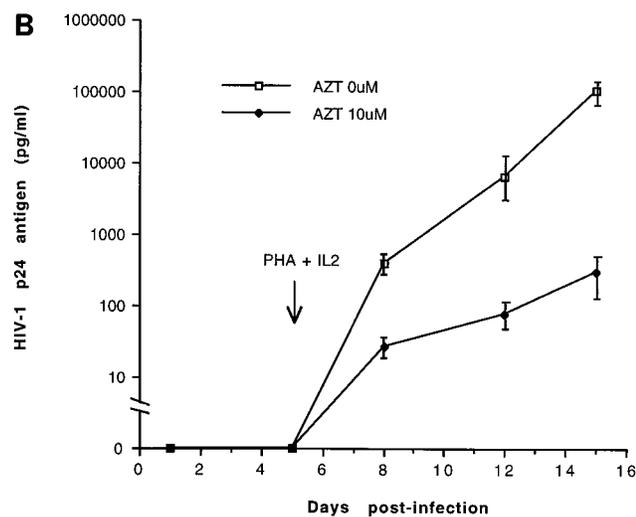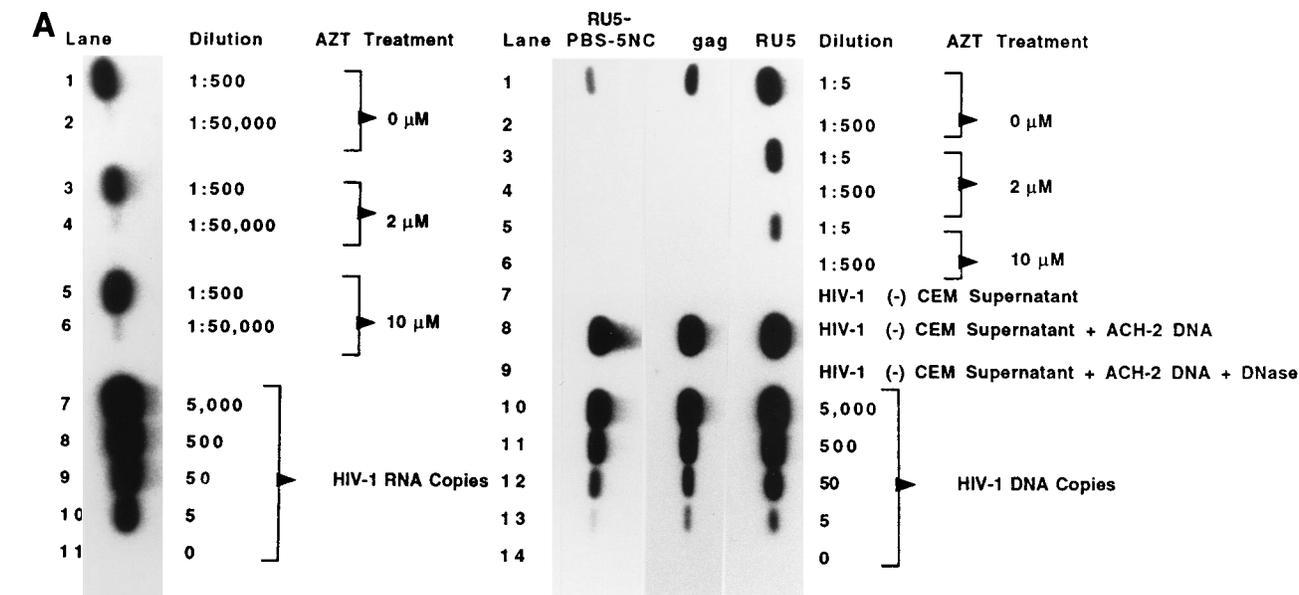
FIG. 7. Influence of AZT treatment of HIV-1-producing cells on intravirion reverse transcripts and viral infectivity. Kinetic studies of HIV-1 replication in initially quiescent PBL. Virus-producing T-lymphocytic cells (CEM cells) were treated with AZT (2 or 10 μM) or not treated. After the AZT was vigorously washed off, the cells were allowed to express HIV-1 viruses for 12 h. The HIV-1$_{NL4-3}$ virions in the supernatant were then analyzed by DNA PCR and/or RT-PCR. (A) Quantitative DNA PCR and RT-PCR for intravirion HIV-1 DNA and RNA. In the three blots to the right, DNA PCR was used. Lanes 1 to 6, HIV-1 virions from cells treated with various concentrations of AZT; lanes 7 to 9, uninfected (−) CEM cell supernatants alone (lane 7) or mixed with ACH-2 DNA with or without subsequent treatment with DNase (lanes 8 and 9); lanes 10 to 14, samples with known numbers of HIV-1 DNA copies by reference to the ACH-2 DNA standard curve. The leftmost blot shows HIV-1 RNA levels in virion preparations, assayed by RT-PCR. Lanes 1 to 6, HIV-1 virions from cells treated with various concentrations of AZT; lanes 7 to 11, samples containing standard copy numbers of in vitro-transcribed HIV-1 *gag* RNA. (B) HIV-1$_{NL4-3}$ virions (0.2 ng of HIV-1 p24 antigen equivalents), which were diluted 256-fold from the supernatants of virus-producing cells, either treated with AZT (10 μM) or not treated, were allowed to infect unstimulated human PBL. At 5 days postinfection, the cells were stimulated with PHA and IL-2. The replication of viruses in the cultures was monitored by HIV-1 p24 antigen detection via ELISA. These data are representative of at least two independent experiments, performed in duplicate.

target cells or nonphysiological substances. It has been well documented that the nucleoprotein complex is necessary for the completion of reverse transcription (27). For RT-dependent DNA viruses (e.g., hepatitis B virus and cauliflower mosaic virus), the nucleoprotein complex with mature RT enzyme is formed before viral budding (42, 58). Therefore, reverse transcripts can be completed (at least negative strand) before viral release. However, because of the stepwise function of the retroviral protease, which occurs during or after virion release from virus-producing cells (4, 53), reverse transcription for lentiviruses or type C retroviruses is unlikely to occur in the virus-producing cells. When the mature RT enzyme is released from its precursor, the nucleoprotein complexed with a mature RT enzyme could be found only in the cell-free virions. The three-dimensional structure in the virion has been shown to support full-length viral DNA synthesis efficiently (15). Traditional ERT with detergent or melittin and ERT without detergent or melittin (Fig. 1) have confirmed this capability of HIV-1 virions (8, 66). Once the substrates (dNTPs) are available for packaging into virions from host cells or from the

microenvironment, potentially through pores in the viral envelope, ERT should be initiated or enhanced by physiological substances.

The relationship between the viral structural proteins and reverse transcription remains largely unknown. Given that the space within the virion is quite small and a complicated, time-consuming polymerization process occurs within it, the viral structural proteins could directly, or indirectly by supplying a three-dimensional structure, participate in progression of reverse transcription, especially the template switches. It has been shown that, in a reconstructed in vitro system, HIV-1 NCp7 can facilitate the first template switch during reverse transcription (48). It has also been demonstrated that some mutations in the HIV-1 capsid protein (p24) will not affect the budding of the virion but will impair viral infectivity (22). Thus, it is instructive to further investigate the correlation between these mutations and HIV-1 NERT. Conversely, as the composition of a virion is relatively simple, NERT supplies an ideal system to study the natural relationship of nucleic acids and retroviral proteins.

TABLE 3. Effect of intravirion DNA level upon viral infectivity

HIV-1 viral input	HIV-1 p24 antigen expression[a] after treatment with AZT					
	Day 0			Day 33 (postinfection)		
	No AZT	2 μM AZT	10 μM AZT	No AZT	2 μM AZT	10 μM AZT
1:1 (50 ng of HIV-1 p24)	−	−	−	++++	++++	++++
1:4	−	−	−	++++	++++	++++
1:16	ND	ND	ND	++++	++++	++++
1:64	ND	ND	ND	++++	++++	+++
1:256	−	−	−	++++	++++	++
1:1,024	ND	ND	ND	++++	−	−
1:4,096	−	−	−	++++	−	−
1:16,384	−	−	−	−	−	−

[a] Symbols: −, <30 pg/ml; +, 30 to 60 pg/ml; ++, 60 to 120 pg/ml; +++, 120 to 240 pg/ml; ++++, >240 pg/ml. ND, not determined.

Compared with intracellular reverse transcription in replicating cells, it seems that the efficiency of NERT is relatively low. In target cells, full-length viral DNA (9.7 kb) can be clearly detected after 4 to 6 h (24, 36, 57), while the product of NERT is a smear on the gel after 4 to 6 h (Fig. 1), even after native gel electrophoresis followed by Southern blotting (our unpublished data). PCR analysis of the intravirion DNA levels in several regions also indicated that most of the products are partial reverse transcripts. This discrepancy may be due to poorly described temporary blocks, which prevent the elongation of reverse transcripts in virions. In addition, as indicated in Fig. 2A and C, the concentrations of dNTPs and polyamines play an important role in altering the molecular size of reverse transcripts. To further clarify the molecular structures of the intravirion HIV-1 reverse transcripts, a number of strand-specific riboprobes have been developed, and the analyses, either by conventional Southern blotting or modified RNase protection assays for detection of reverse transcripts, are now under way.

The in vitro and in vivo data, presented in this study, demonstrate that making the retroviral envelope permeable to dNTPs does not depend upon an artificial process. The mechanism(s) involved with this process remains to be precisely determined. It has been shown that the composition of HIV-1 envelope is different from that of cellular membranes, although the HIV-1 envelope is initially derived from cellular membranes (1). Further, membrane-associated HIV-1-specific proteins (e.g., gp41 and p17) may make the retroviral envelope permeable to dNTPs (2). It has also been demonstrated that the C terminus of HIV-1 gp41 can bind to the cellular membrane, and two amphipathic domains in this region, termed lentivirus lytic peptides, may induce pore formation in the cellular membrane and make the membrane permeable to several compounds (2, 46, 64). When synthesized lentivirus lytic peptides were mixed with biological membranes, pore formation was observed. Electrical conduction experiments indicated that when the density of the peptides on the membrane reached the same density as that of gp41 in the viral envelope, the pore size can reach 1 nm, through which even a short peptide may pass (14). On the basis of those observations, it is reasonable to assume that these amphipathic domains at the C terminus of gp41 may bind to the viral envelope and cause pore formation and make viral envelopes permeable to dNTPs. Our preliminary data support this hypothesis (unpublished data).

Polyamines enhance HIV-1 reverse transcription. In addition to the substrates for reverse transcription (e.g., dNTPs), NERT can be greatly enhanced by polyamines. Polyamines have low molecular weights (202 for spermine and 145 for spermidine) and have been demonstrated to potently interact with various nucleic acids to stabilize specific DNA conformations and neutralize DNA (59, 60). Their concentrations in cells are cell cycle dependent (37), and the key enzyme in synthesis of polyamines, ornithine decarboxylase, is under the control of E2F1, a cell-cycle-dependent factor (26). Further, polyamines may enhance RNA transcription and lead to chromosomal DNA compaction (59, 60). Note that there are several lines of evidence which suggest that polyamines affect the retroviral life cycle. Polyamines can alter +1 ribosomal frameshifting for Ty1 retrotransposition in *Saccharomyces cerevisiae* (5). In addition, in vitro dimerization of HIV-1 RNA has been demonstrated to be enhanced by spermidine (41).

Polyamines obviated the requirement for further dNTP treatment of the virions to stimulate NERT (Fig. 2A and C). The high concentrations of polyamines in seminal fluid may supply a perfect microenvironment for HIV-1 virions to complete intravirion reverse transcription, which is compatible with high concentrations of polyamines at the S stage of the cell cycle being essential for cellular DNA replication (37, 59, 60). As polyamines have multiple biochemical functions in cells, it remains formally possible that polyamines increase HIV-1 NERT by yet other mechanisms, such as optimizing the RT reaction by increasing the dimer formation of the genomic viral RNA, increasing the efficiency of template switching, or accelerating the protease function in virions. However, it is unlikely that polyamines can make the viral envelope permeable, because the patterns of enhancement of ERT by melittin and polyamines are different. In contrast to melittin making the viral envelope permeable, which will dramatically increase reverse transcription at high concentrations of dNTPs, polyamines seem to increase usage of the dNTPs by the reverse transcriptase at a wide range of dNTP concentrations (Fig. 2C). Moreover, there have been no reports that polyamines may make biological membranes permeable. We can conclude, from data shown in Fig. 2B, that the enhancement of NERT by polyamines was partially due to direct stimulation of reverse transcription. The altered enzymatic kinetics of HIV-1 reverse transcriptase by polyamines and the mechanism(s) involved are now under investigation.

Efficient use of the synthesis machinery for reverse transcription may account for the increase in HIV-1 infectivity. Although it has been recognized that the nucleoprotein complex is necessary to complete reverse transcription (27), it still remains to be proven precisely what the synthesis machinery for full-length reverse transcription in target cells is. It has been demonstrated that the de novo initiation of reverse transcription of HIV-1 in target cells could be carried out in an enveloped viral particle, presumably the whole virion on the cellular surface or in the endosome (72). However, it is still not

known whether the production of full-length DNA occurs within this particle. In addition, the full-length viral DNA of murine leukemia virus has been shown to be associated with a 160S nucleoprotein complex which harbors the viral capsid protein, at 20 h postinfection (9). On the basis of these findings, it has been hypothesized that viral DNA is synthesized in the viral core-like nucleoprotein complex (63). However, it should be noted that full-length viral DNA is the end product of reverse transcription. The viral components associated with it may be the remnants of the synthesis machinery, which have been passively degraded or actively dissociated at that time. Conversely, p24, the capsid protein of HIV-1, could not be identified in the full-length viral DNA-associated preintegration complex (25, 29, 35). As full-length viral DNA associated with the preintegration complex is merely the end product of HIV-1 reverse transcription, it should not be also concluded that p24 is unnecessary for efficient reverse transcription. The progression from a complicated virion to a relatively simple preintegration complex of HIV-1 reveals that it is likely that the synthesis machinery may be gradually degraded and/or dissociated in the target cells, especially if it stays in the target cell for long periods of time. This dissociation and/or degradation may alter reverse transcripts with two consequences. If reverse transcription has been completed, the structural alterations of the synthesis machinery, because of either degradation or dissociation of its components, may augment the preintegration complexes' migration into the nucleus and subsequent integration into chromosomal DNA. However, if reverse transcription is not initiated or is initiated but not completed, degradation or dissociation of the synthesis machinery will irreversibly abort reverse transcription. Therefore, these virions which already harbor the full-length or partial reverse transcripts might not depend upon the fully intact synthesis machinery in target cells and thus could have more of a chance to complete full-length viral DNA synthesis.

The relatively slow progression of intracellular HIV-1 reverse transcription in quiescent cells could be a major negative event to overcome in the viral life cycle, as the gradual degradation or dissociation of synthesis machinery could impair its support for full-length viral DNA synthesis. Which step(s) of reverse transcription depends upon the intact synthesis machinery is still to be determined. However, we noted that although most reverse transcripts in the virions treated with polyamines, with or without dNTPs, are only 2 to 3 kb (Fig. 2C), (even though PCR can detect some full-length viral DNA synthesis at certain levels [Fig. 2A]), a large number of full-length viral DNA moieties can be completed after the cells were stimulated with mitogens. Further, the infectivity of these virions on quiescent cells could also be enhanced (Fig. 6A). These results suggest that completion of the first template switch may largely decrease the reliance upon the synthesis machinery for full-length DNA expression in target cells.

Our data also indicated that the virions which harbor intravirion reverse transcripts in the supernatants of cell cultures have higher infectivity in initially quiescent cells than the virions which solely harbor genomic HIV-1 RNA (Fig. 7B and Table 3). As discussed above, these virions, which harbor solely genomic RNA, would gradually lose the synthesis machinery in quiescent target cells, which may be necessary for the initiation or completion of reverse transcription. However, the virions which harbor some partial or full-length intravirion reverse transcripts would not rely totally upon the synthesis machinery in the target cells and subsequently could establish infection after the cells were stimulated by mitogen. When target cells are replicating at the time of viral infection, the advantage of the small levels of intravirion reverse transcripts could not be

shown. Note that the virions which harbor only genomic RNA are infectious (3). Only when a high level of reverse transcription was completed within the virions, in the presence of highly concentrated dNTPs (5 mM), would the advantage of the intravirion reverse transcripts on viral infectivity in proliferating cells be demonstrable (73).

Enhancement of viral infectivity in seminal plasma may facilitate the sexual transmission of HIV-1. At this stage in the AIDS pandemic, little is known regarding the molecular mechanisms involved in sexual transmission of HIV-1. Both host and viral factors may be operative. Our data indicate, first, that specific molecular moieties in human seminal plasma can alter the characteristics of HIV-1 virions and, therefore, their infectivity. It remains to be proven whether increased HIV-1 infectivity is correlated with the frequency of sexual transmission. However, it has been shown that if small amounts of simian immunodeficiency virus SIV_{mac251} virions are mixed with human seminal plasma samples and then inoculated into the vaginas of female rhesus macaques, the monkeys are more likely to become infected than those female rhesus macaques receiving only SIV_{mac251} virions (44). Although certain factors, such as those in seminal plasma that neutralize the acidity in the vagina, may contribute to this difference, enhanced NERT in seminal fluid could increase the transmission rate by increasing viral infectivity. To test this hypothesis, an animal model is required. Nevertheless, the present data at least suggest a scenario for HIV-1 transmission in vivo. HIV-1 virions, which would have gained increased infectivity in the seminal plasma, could directly enter into an individual's bloodstream or submucosa through trauma during homosexual or heterosexual activity and/or ulcers secondary to sexually transmitted diseases (49). These virions would be more infectious for critical target cells, such as monocytes/macrophages or quiescent T lymphocytes, than HIV-1 virions in which NERT was not augmented by the microenvironment.

In summary, we have demonstrated that ERT can occur in the HIV-1 virion in the presence of physiological substances found in extracellular milieus. Intravirion reverse transcription appears to enhance viral infectivity for initially nondividing cells. This may be due to virions harboring reverse transcripts which could bypass certain negative factors which inhibit the efficiency of intracellular reverse transcription. Data presented in this study strongly suggest a causal relationship between intravirion HIV-1 reverse transcription and viral infectivity, although further studies are necessary to absolutely prove causality. Further studies exploring the alterations of the HIV-1 virion by extracellular and/or intracellular milieus in the human host and investigating the molecular structure(s) of intravirion reverse transcripts and the viral DNA synthesis machinery within virions and in target cells will be required for the dissection of molecular mechanisms involved with inter- and intrahost spread of HIV-1.

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