JOURNAL OF VIROLOGY, June 1995, p. 3675–3682 Vol. 69, No. 6
0022-538X/95/$04.00+0

Nascent Human Immunodeficiency Virus Type 1 Reverse Transcription Occurs within an Enveloped Particle

HUI ZHANG,[1]† YING ZHANG,[1] TIMOTHY SPICER,[1] DENIS HENRARD,[2] AND BERNARD J. POIESZ[1]*

Departments of Medicine and Microbiology, State University of New York Health Science Center at Syracuse, Syracuse, New York 13210,[1] and Abbott Laboratories, Abbott Park, Illinois 60064[2]

Received 28 November 1994/Accepted 18 March 1995

Although a small amount of viral DNA has been shown to be enclosed within human immunodeficiency virus type 1 (HIV-1) virions, the majority of full-length viral DNA is formed after this virus infects target cells. Hence, we undertook investigations to identify the physical characteristics of the HIV-1 replication unit during the early events of infection. In these studies, nascent viral DNA synthesis was found to occur between 15 and 30 min after purified, DNase-treated HIV-1 virions were added to HUT 78 cells. At 1 h postinfection, a large amount of strong-stop viral DNA and some first-strand viral DNA had been synthesized. Several lines of evidence, including purification, nuclease digestion, and immunoprecipitation, indicated that these nascent viral DNAs were located within particles containing components such as reverse transcriptase and p24^{gag} and gp120^{env} proteins and having physical characteristics similar to those of intact virions.

Classically, it has been taught that retroviruses contain two copies of single, plus-strand RNA that are each reverse transcribed into double-stranded DNA within the cytoplasm of target cells (39, 42). This process involves several temporally discrete steps, including the initial synthesis of strong-stop DNA and the subsequent digestion of the complementary R-U5 plus-strand RNA by RNase H, allowing for the negative-strand strong-stop DNA to jump and anneal to the complementary 3′ R of the plus-strand viral RNA and thereby initiate first-strand DNA synthesis (39, 42). Recently, however, we and others have demonstrated both in vitro and in vivo that cell-free human immunodeficiency virus type 1 (HIV-1) virions contain small amounts of incomplete and complete viral DNA transcripts (7, 23, 38, 44, 45). Furthermore, nascent HIV-1 DNA synthesis can occur in purified virions incubated in either a buffer containing Mg^{2+} and deoxynucleoside triphosphates (dNTPs) or human physiological fluids (45). Virions thus treated show a 1,000-fold increase in their infectivity of human $CD4^+$ T lymphocytes (45).

Although a small amount of viral DNA has been shown to be enclosed within HIV-1 virions (genomic RNA/strong-stop DNA ratios have ranged from 10^2:1 to 10^3:1 [44, 45]), the majority of full-length viral DNA is formed after the virus infects target cells (7, 9, 19, 36, 40). While under certain conditions reverse transcription may occur within the nuclear compartment of target cells, most DNA synthesis is associated with the cytoplasm (7, 9, 10, 18, 19, 36, 40). At 6 h postinfection, after it has been reverse transcribed and prior to its integration into the host genome, full-length HIV-1 double-stranded DNA has been demonstrated to exist as a cytoplasmic nucleoprotein complex ranging in size from 160 to 320S. This complex has been shown to contain various combinations of the viral integrase, reverse transcriptase (RT), protease, and p17 matrix proteins but not viral p24^{gag} and gp41^{env} and gp120^{env} proteins (7, 9, 10, 18). However, the physical moiety in which initial HIV-1 DNA replication occurs has not been well characterized and may be quite different from the aforementioned preintegration complex. Using primer pairs and probes and DNA- and RNA-dependent PCRs, which are specific for various steps of HIV-1 reverse transcription, coupled with physical separation techniques and other methods of HIV-1 analysis, we have attempted to further define the early events of HIV-1 replication.

* Corresponding author. Mailing address: Department of Medicine, SUNY-HSC, 750 East Adams St., Syracuse, NY 13210. Phone: (315) 464-8231. Fax: (315) 464-8255.

† Present address: Dorrance H. Hamilton Laboratories, Department of Medicine, Thomas Jefferson University, Philadelphia, PA 19107.

MATERIALS AND METHODS

Cell culture and virus purification. HIV-1_{AAV} is a type B HIV-1 isolate utilized in our laboratory to study the biology of HIV (3, 21, 32, 41, 45). It was grown in the $CD4^+$ cell line HUT 78 (13). Supernatants from centrifuged cell-free conditioned media from these HIV-1-producing cells, HUT 78/HIV-1_{AAV}, were filtered (0.22-μm-pore size) and centrifuged at 45,000 × *g* for 1 h with an SW27 rotor (Beckman, Inc., Palo Alto, Calif.) at 4°C. The viral pellet was resuspended in 1 ml of TN buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl). The preparation was treated with 80 U of RQ1 DNase and 10 U of RNase I (Promega, Madison, Wis.) at 37°C for 1 h. The sample was then centrifuged at 45,000 × *g* for 1 h. The viral pellet was resuspended in 0.5 ml of TN buffer, and virions were further purified by rate-zonal sedimentation and equilibrium density centrifugation. Human T-cell leukemia virus (HTLV-I) (31) cultured in HUT 102 cells (13, 31) was similarly purified.

Viral infection. To determine the kinetics of HIV-1 viral DNA synthesis, purified DNase- and RNase-treated HIV-1 virions (60 ng of HIV-1 p24) were mixed with 1.2×10^6 HUT 78 cells in 100 μl of RPMI 1640 medium for 15 min on ice. The volume was then increased to 1 ml, and the cells were incubated at 37°C. At successive time intervals (0 min to 6 h), the cells were removed, and unbound virions were removed by washing with Hanks' balanced salt solution three times. The cells were then pelleted, mixed with quick-lysis buffer (45) on ice for 10 min, and then analyzed by RT-directed PCR (RT-PCR) and PCRs for HIV-1 RNA and DNA species, respectively.

In larger-scale infections, purified DNase- and RNase-treated cell-free virions (3 μg of HIV-1 p24) were mixed with 5×10^7 HUT 78 cells in 1 ml of RPMI 1640 medium for 15 min on ice and then at 37°C for 1 h. The unbound virions were removed, and the cells were washed and pelleted as described above. The cell membranes were lysed by incubation in a buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.02% [vol/vol] Nonidet P-40) on ice for 10 min with gentle shaking. Cellular nuclei were removed by centrifugation at 900 × *g* at 4°C for 3 min. The supernatant was filtered (0.45-μm-pore size), and the filtrate, termed the "cytoplasmic extract," was placed on ice for subsequent studies as outlined in Fig. 1.

In both scales of infection, some aliquots of target cells were not subjected to lysis with detergent. Rather, they were maintained in culture for up to 30 days

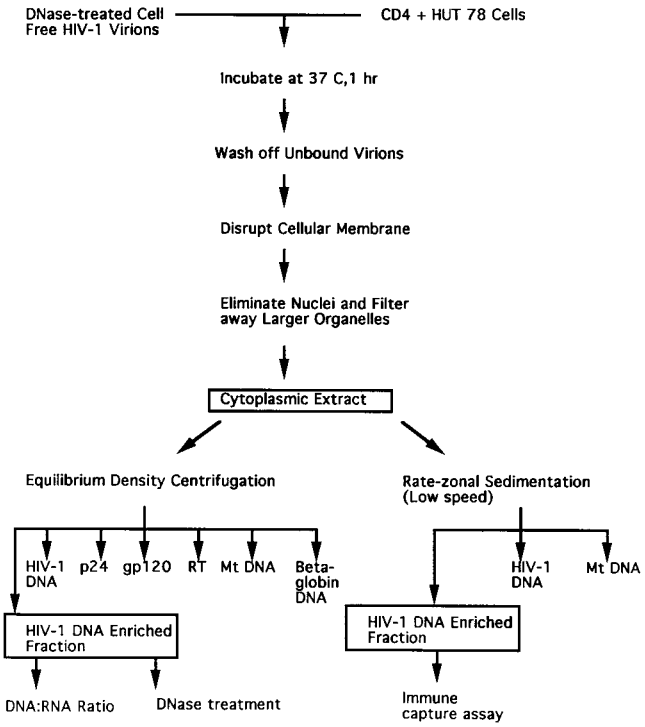

FIG. 1. Outline of experimental procedures for investigating intracellular HIV-1 reverse transcription. Mt, mitochondrial.

and monitored for HIV-1 infection and expression by HIV-1 PCR and RT-PCR and HIV-1 $p24^{gag}$ protein enzyme-linked immunosorbent assay (ELISA).

Rate-zonal sedimentation. Viral suspensions or cytoplasmic extracts were centrifuged on 12-ml 5 to 20% continuous sucrose gradients at 45,000 × *g* for 55 min at 4°C in an SW41 rotor (Beckman). For different purposes, the velocity and the time of centrifugation were altered. Fractions (0.67 ml) were collected from the bottom. Their sedimentation coefficients were calculated (26), and they were analyzed for HIV-1 nucleic acid.

Equilibrium density centrifugation. Equilibrium density gradients were made by layering 10 ml of a 20 to 65% sucrose gradient on top of 0.5 ml of 70% sucrose. Viral suspensions or cytoplasmic extracts were then centrifuged at 150,000 × *g* for 20 h at 4°C in an SW41 rotor. Fractions (0.67 ml) were collected from the bottom. Buoyant density was determined with a refractometer. Fractions were analyzed for HIV-1 proteins and nucleic acids.

Detection of HIV-1 proteins. HIV-1 RT was measured as the rate of incorporation (picomoles per hour) of [^{3}H]dGMP into a poly(rC)-oligo(dG) template or primer, as previously described (31). HIV-1 $p24^{gag}$ and $gp120^{env}$ antigens were detected with commercial antigen-capture ELISA kits (Cellular Products, Inc., Buffalo, N.Y., and American Biotechniques Co., Boston, Mass., respectively) (3, 28, 41).

Nucleic acid analyses by PCR. Samples (50 μl) were boiled and subjected to quick lysis as described previously (45). RNA was detected with RT-PCR (44, 45), while DNA was detected with a conventional PCR (44, 45). The primer pair-probe systems M667 and AA55/SK31, SK38 and SK39/SK19, and M667 and M661/SK31 were utilized to detect HIV-1 strong-stop (R-U5), first-strand (*gag*), and full-length DNA, respectively (21, 42, 44, 45). The primer pair-probe systems SK110 and SK111/SK112, PC03 and PC04/RS06, and MT05 and MT06/MT07 were used to detect HTLV-I, human β-globin, and mitochondrial DNA, respectively (20, 44, 45). Amplified products were detected by liquid or Southern blot hybridization (44, 45).

The DNA standard curves utilized for quantification were derived from lysed 8E5 cells, which contain one integrated defective copy of HIV-1 double-stranded proviral DNA (11). It should be pointed out that each provirus contains one copy of *gag* but two copies of the long terminal repeat and, hence, two copies of strong-stop DNA. The number of HIV-1 integrants within the 8E5 cells was verified by performing Southern blot hybridization with the ^{32}P-labeled nick-translated *FspI*-to-*NaeI* full-length HIV-1 fragment from pNL4-3 (supplied by the National Institutes of Health AIDS Research and Reference Reagent Program) (2). The mean HIV-1 *gag* and strong-stop copy numbers per 8E5 cell were also verified by serially diluting the cells in a constant number of normal HIV-1-negative peripheral blood mononuclear cells in replicates and performing PCR with both the SK38/SK39 and M667/AA55 systems and calculating the Poisson distribution that best fit the data (37). The RNA standards were derived from synthetic RNA produced in vitro from DNA amplified with modified M667/AA55 or SK38/SK39 primers (45). The copy numbers of these RNA standards were calculated by measuring the A_{260} and A_{280} and again verifying this number by performing RT-PCR on replicates of serial dilutions and calculating the Poisson distribution. The DNA or RNA copy numbers of test samples were calculated by performing densitometric analyses of these samples versus the standard curve and by serially diluting them and calculating the Poisson distribution (37). All quantitations were ultimately repeated 10 times and were done with various input amounts of test samples.

DNase treatment. Samples (50 μl) were taken from purified virions, the nascent viral DNA-enriched fraction, or control preparations (e.g., HIV-1 plasmid) and added to 10 U of RQ1 DNase with 20 mM $MgCl_2$ at 37°C for 60 min. The reaction was stopped by boiling for 30 min, and viral DNA was extracted with quick-lysis buffer.

Immune capture assay. Anti-HIV-1 gp120 and anti-HIV-1 gp41 antibody-coated, carboxylated latex microbeads (16) were washed once with phosphate-buffered saline (PBS [pH 7.2]) and were resuspended in PBS at room temperature. Fifty microliters of test sample was diluted with 150 μl of PBS and incubated at room temperature for 3 h with a 50-μl suspension of the microbeads. The microbeads were pelleted in a microcentrifuge at 5,000 rpm for 10 min. The supernatant was removed, and 50 μl of fresh microbeads was added to it while the pellet was resuspended in 250 μl of fresh PBS. Nucleic acid was extracted from 50 μl of either the pellet or the supernatant and analyzed via PCR or RT-PCR.

Thin-section electron microscopy. HIV-1 virions were incubated with HUT 78 cells for 1 h at 37°C. The cells were washed and pelleted as described above. After centrifugation, the cell pellet was resuspended in 2 ml of TNE buffer (10 mM Tris-HCl [pH 7.2], 100 mM NaCl, 1 mM EDTA) and fixed with 2.5% glutaraldehyde for 10 min. The cells were pelleted again and washed with TNE buffer and pelleted. A small drop of 2% agar (45°C) was added, and the mixture was cooled and cut into small pieces. The cells were then postfixed with 1% osmium tetroxide for 1 h, washed with PBS, and dehydrated in graded ethanol. The ethanol was replaced with propylene oxide three times, and ultimately the sample was embedded in plastic.

Sections were made with an MT-2 ultramicrotome, transferred to grids, and poststained with lead citrate. The grids were examined with a Siemens Elmiskop 102 electron microscope (Karlsruhe, Germany).

RESULTS

Kinetics of HIV-1 reverse transcription. As previously published (44, 45), HIV-1 cell-free virions were found to contain small amounts of HIV-1 DNA (Fig. 2 and Table 1). For every 10^6 RNA molecules, there were 10^3 strong-stop and 10^1 first-strand DNA copies (Table 1). When HUT 78 cells were infected with cell-free HIV-1, nascent strong-stop and first-strand viral DNA syntheses were observed within 15 to 30 and 30 to 60 min, respectively. Full-length viral DNA could not be detected until 2 h (data not shown). Nascent viral DNA synthesis, including that of full-length DNA, peaked by 6 h (Fig. 2 and data not shown). As previously published (3, 40, 41, 45), the amounts of cell-free HIV-1 utilized in both the small- and large-scale infections resulted in sustained, productively infected cultures of HUT 78 cells, as measured by DNA- and RNA-directed PCR and HIV-1 $p24^{gag}$ ELISA (data not shown). Because we were interested in studying the physical characteristics of the early HIV-1 replication unit, all subsequent analyses were performed at 1 h postinfection.

Characterization of the HIV-1 replication unit. Before embarking on the examination of cytoplasmic extracts of HUT 78 cells at 1 h postinfection with HIV-1, it was important to demonstrate that the treatment used to lyse the cellular membranes (0.02% Nonidet P-40 in TN buffer at 4°C for 10 min) would not significantly disrupt whole virions or their subunits. Indeed, as shown in Fig. 3, HIV-1 virions were stable after such treatment, whereas harsher treatments with increased amounts of detergent for greater periods of time generated either core particles or the complete solubilization of the virions.

The cytoplasmic extract of HUT 78 cells at 1 h postinfection with cell-free HIV-1 was found to contain at least 70% of the nascent viral DNA, with the remaining DNA being found in the nuclear compartment or bound to the 0.45-μm-pore-size

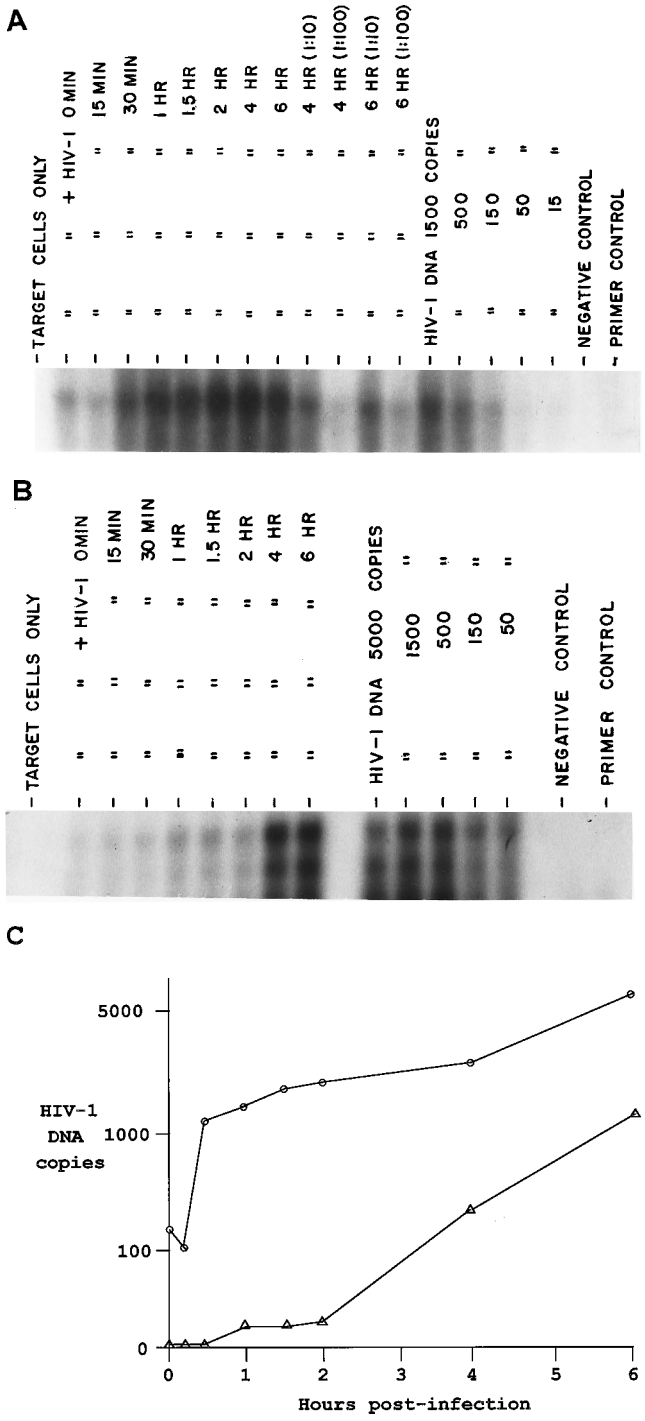

FIG. 2. Time course study of nascent HIV-1 DNA synthesis. Each aliquot of target HUT 78 cells (1.2×10^6) was infected with the same amount of purified, DNase-treated HIV-1_{AAV}. At each time point (shown as individual lanes on the gels), HIV-1 DNA was extracted and analyzed by PCR. (A) Viral DNA was amplified with the strong-stop (R-U5) primer pair M667/AA55. (B) Viral DNA was amplified with the first-strand (*gag*) primer pair SK38/SK39. The amplified products were analyzed by liquid hybridization and subsequent electrophoresis on an 8% acrylamide gel followed by autoradiography. Negative controls included DNA from uninfected HUT 78 target cells only, an HIV-negative volunteer blood donor, and a primer-only control to which no nucleic acid had been added. Positive controls included DNA from a dilution series of HIV-1-infected 8E5 cells. All bands shown on the gel are the result of positive hybridizations. (C) Comparison of HIV-1 strong-stop (○) and first-strand (△) copy numbers in HUT 78 cells at different time points postinfection with HIV-1. Copy numbers were derived by densitometric analysis of the autoradiographs in panels A and B and additional dilution of each sample in panels A and B (data not shown) compared with linear curves generated by similar analyses of the positive controls. As can be seen, even the virions at 0 min contain small amounts of HIV-1 DNA, and, as expected, strong-stop DNA synthesis is initiated prior to first-strand DNA synthesis. By 1 h, a significant amount of strong-stop DNA synthesis and a small amount of first-strand nascent DNA synthesis have occurred.

TABLE 1. Comparison of the strong-stop RNA/DNA ratio in purified HIV-1 virions versus nascent viral DNA-associated particles in cytoplasmic extracts of target cells

Particle	Copy no.[a]		RNA/DNA ratio
	RNA	DNA	
Purified virions	10^6	10^3	10^3:1
Nascent viral DNA-associated particles from HUT 78 cytoplasmic extract at 1 h postinfection[b]	10^3	10^4	10^{-1}:1

[a] M667/AA55 were used as a primer pair.
[b] Fraction 9 in Fig. 4.

filter (data not shown). Furthermore, when the cytoplasmic extract was subjected to rate-zonal sedimentation, most of the viral DNA was found to be associated with a particle having a sedimentation coefficient greater than 330S (data not shown). With the controls, we demonstrated that solubilized viral DNA, obtained either by phenol and chloroform extraction followed by ethanol precipitation of the cytoplasmic extract or by restriction endonuclease digestion of HIV-1 plasmid DNA (10 kb), had a much smaller sedimentation coefficient.

When subjected to equilibrium density centrifugation, the nascent viral DNA in the filtered cytoplasmic extract was found to be associated with a particle having a buoyant density similar to an intact virion, namely 1.19 g/ml (Fig. 4). Again, as controls, the solubilized nascent HIV-1 DNA from the infected cytoplasmic extract, the excised plasmid HIV-1 DNA (10 kb), and the HIV-1 plasmid DNA that had been mixed with an uninfected HUT 78 cell cytoplasmic extract were all shown to sediment higher in the gradient than the intact particles found in the infected cytoplasmic extract (data not shown). This observation does not mean that the controls described above had a lower buoyant density than intact virions but rather that, given their lower mass, there was insufficient centrifugation time for them to reach equilibrium. Furthermore, the lack of detectable human β-globin DNA in the equilibrium density gradient fractions containing HIV-1 nascent DNA obviates nuclear DNA contamination (Fig. 4). However, the fact that human mitochondrial DNA cofractionated with the nascent viral DNA (Fig. 4) raised the possibility that the newly synthesized viral DNA might be associated with mitochondria. This hypothesis was negated when we subjected the cytoplasmic extract to rate-zonal sedimentation and were able to clearly separate the nascent viral DNA from the mitochondria (Fig. 5).

As anticipated from the earlier kinetic studies (Fig. 2), the number of copies of strong-stop DNA at 1 h postinfection far exceeded the number of first-strand DNA copies (Fig. 4), indicating that we, indeed, had chosen a time point at which the jump from strong-stop to first-strand DNA synthesis had recently been initiated. The ratio of viral RNA to DNA in the strong-stop region significantly decreased in these cytoplasmic extract particles compared with that of the extracellular HIV-1 virions (Table 1), an observation consistent with the premise

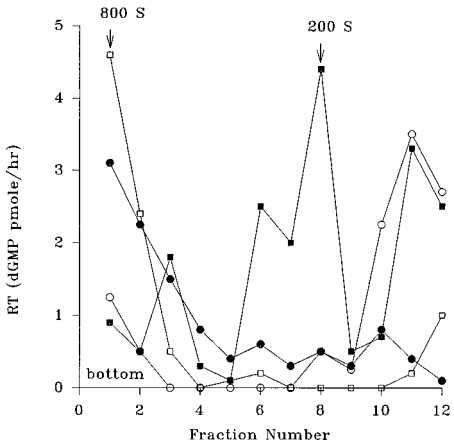

FIG. 3. Size comparison of HIV-1 particles treated with different concentrations of the detergent Nonidet P-40. HIV-1 virions treated with detergent were centrifuged on 5 to 20% sucrose gradients at 45,000 × *g* for 55 min at 4°C. RT activity was analyzed in each fraction. The 800S and 200S size ranges are shown. □, virions without any treatment; ●, virions treated with 0.02% Nonidet P-40 in TN buffer at 4°C for 10 min; ■, virions treated with 0.5% Nonidet P-40 in H_2O at 4°C for 30 min; ○, virions treated with 1.5% Nonidet P-40 at 37°C for 1 h.

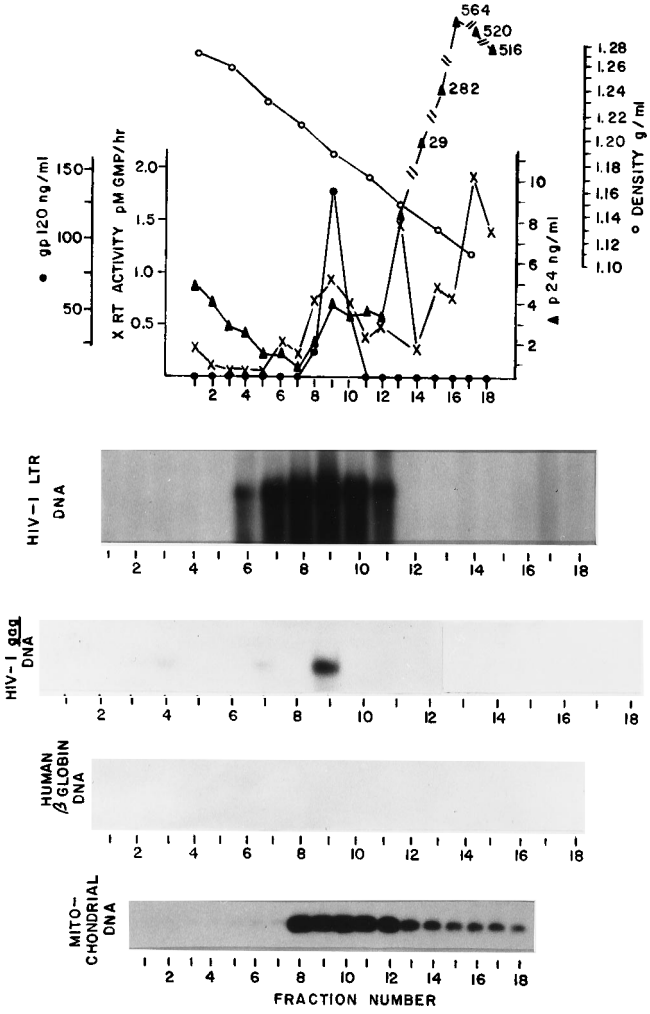

FIG. 4. Copurification of nascent HIV-1 DNA and viral proteins in an equilibrium density gradient. The cytoplasmic extract from HUT 78 cells at 1 h postinfection by HIV-1 was centrifuged at 150,000 × *g* for 20 h on a continuous sucrose gradient. The density of each fraction was determined with a refractometer, and each fraction was analyzed for HIV-1 RT, $p24^{gag}$ and $gp120^{env}$ proteins, HIV-1 strong-stop (R-U5) or first-strand (*gag*) DNA, and human mitochondrial and nuclear β-globin DNA.

that either virions containing strong-stop DNA preferentially bind to or enter the target cells or, more likely, that, coincident with strong-stop DNA synthesis, significant RNase H activity had resulted in a decrement of complementary R-U5 RNA content within the particles in the 1-h postinfection cytoplasmic extract.

The results presented in Fig. 4 indicate that RT activity, HIV-1 p24, and gp120 were copurified with the nascent viral DNA-containing particle. To rule out the possibility that these particle-associated HIV proteins were due to an unknown preexisting packaged exogenous or endogenous retrovirus in the HUT 78 cells, a cytoplasmic extract from uninfected HUT 78 cells was processed via a similar equilibrium density centrifugation gradient. No RT activity or p24 or gp120 proteins were detected around the 1.19-g/ml fraction in this control gradient, confirming the absence of particle-associated RT activity and HIV-1 proteins in HUT 78 cells (Fig. 6A). However, it should be noted that the uninfected HUT 78 cells do contain some soluble RT activity in the less-dense fractions of the gradient, consistent with the endogenous RT activity we have repeatedly observed in this cell line (Fig. 6A) (unpublished data).

As another control, to evaluate whether soluble HIV-1 proteins could bind to subcellular organelles in the HUT 78 cell extract and thereby give the appearance of being particle associated, HIV-1 virions that had been completely solubilized in vitro were added to an uninfected HUT 78 cytoplasmic extract and centrifuged in another equilibrium density centrifugation gradient (Fig. 6B). None of the viral proteins were detected in the fractions around 1.19 g/ml. Rather, as expected, all were in the soluble portion of the gradient.

To further establish that the particles containing the nascent viral DNA were enveloped and contained HIV-1 envelope proteins, we performed two additional experiments. First, we demonstrated that the nascent DNA inside these particles was protected from DNase, while plasmid DNA, even at a much higher level of input, was not (Fig. 7). Incubation of the nascent DNA-containing particles in 1.5% Nonidet P-40 for 1 h did render the DNA susceptible to degradation by DNase (data not shown). Next, we demonstrated that the nascent DNA from cytoplasmic extracts and DNA inside intact cell-free virions could be immunoprecipitated with beads coated with anti-HIV $gp41^{env}$ and $gp120^{env}$ antibodies (Fig. 8). In contrast, neither solubilized HIV-1 plasmid DNA, plus or minus uninfected HUT 78 cytoplasmic extract (Fig. 8), nor

TABLE 2. Comparison of viral protein levels in purified HIV-1 virions versus nascent HIV-1 DNA-associated particles found in target cell cytoplasmic extracts

Particle	Amt (ng/ml) of:		gp120/p24 ratio
	gp120	p24	
Purified virions	56	288	0.2:1
Nascent viral DNA-associated particles from HUT 78 cytoplasmic extract at 1 h postinfection[a]	138	4	34:1

[a] Fraction 9 in Fig. 4.

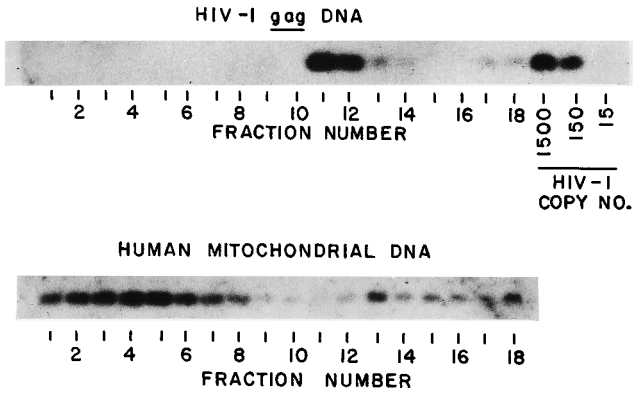

FIG. 5. Comparison of the migration of nascent HIV-1 DNA versus human mitochondrial DNA in a rate-zonal sedimentation gradient. The cytoplasmic extract from HUT 78 cells at 1 h postinfection with HIV-1 was centrifuged at 45,000 × *g* for 55 min on a 5 to 20% continuous sucrose gradient. Fractions were collected from the bottom, and DNA was extracted and analyzed by PCR for HIV-1 *gag* or human mitochondrial sequences. A serial dilution of HIV-1 DNA standards was also analyzed. As can be seen, the larger mitochondria were easily separated from the HIV-1 DNA-containing particles.

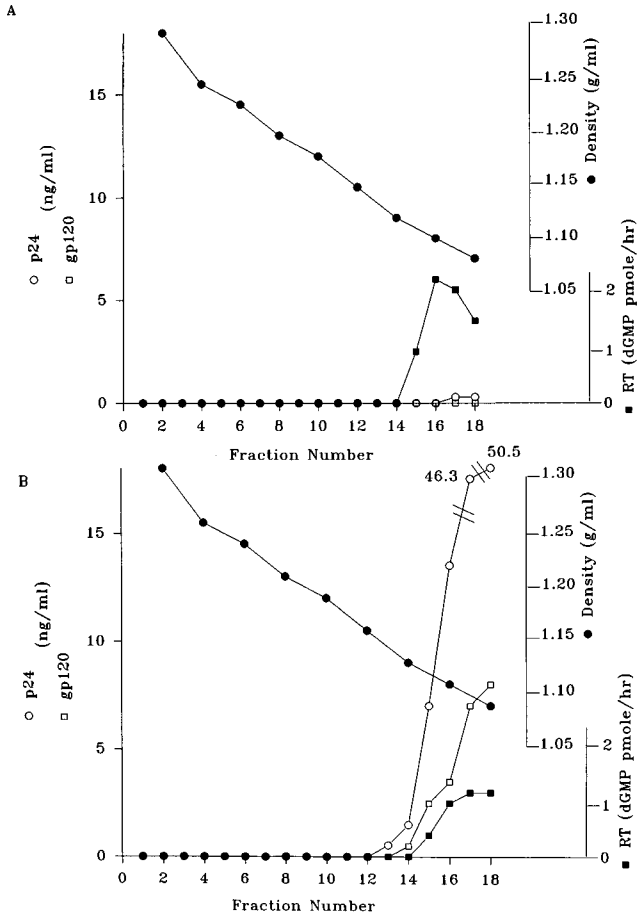

FIG. 6. Equilibrium density centrifugation of cytoplasmic extracts from uninfected HUT 78 cells without (A) or with (B) added soluble HIV-1 components. Cytoplasmic extracts from 5×10^7 uninfected HUT 78 cells were prepared as described in Materials and Methods. HIV-1 virions were disrupted with 1.5% Nonidet P-40 at 37°C for 1 h and added to the cytoplasmic extract utilized in panel B. Both extracts were centrifuged on separate 20 to 70% sucrose gradients as described in the legend to Fig. 4. Each fraction was analyzed for density, RT activity, and HIV-1 p24 and gp120 content.

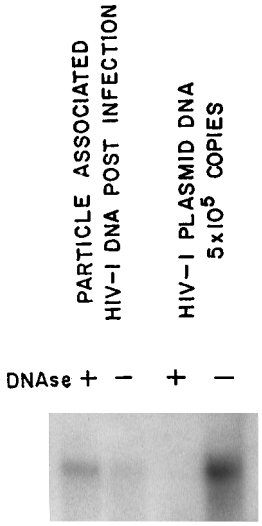

FIG. 7. DNase treatment of particle-associated nascent HIV-1 DNA. Fifty microliters of fraction 9 of the gradient in Fig. 4 was treated with (+) or without (−) DNase. As a control, 50 μl of HIV-1 plasmid DNA (copy number of 5×10^5) was treated with or without DNase. The DNase digestion was stopped by boiling for 30 min. The mixture was extracted for DNA analysis. PCR amplification was performed with primer pair SK38/SK39, and liquid hybridization was performed with the probe SK19.

HTLV-I virions (data not shown) could be precipitated by the beads. Interestingly, the ratio of gp120 to p24 in nascent viral DNA-associated particles was higher than that of the original purified HIV-1 virions (Table 2).

Electron microscopy studies. Because the data presented above indicated that the nascent viral DNA at 1 h postinfection of HUT 78 cells by HIV-1 was located in a particle similar to an intact virion containing *env* and *gag* proteins, we examined these cells by thin-section electron microscopy. Sections prepared from uninfected HUT 78 cells failed to show any virus-like particles (data not shown). In contrast, HUT 78 cells 1 h postinfection contained apparent HIV-1 virions with bullet-shaped cores either bound to the cell surface or present within endosomelike vesicles (Fig. 9).

DISCUSSION

Significant nascent viral DNA synthesis occurs within 1 h postinfection of HUT 78 cells by DNase-treated HIV-1 virions. Peak viral DNA synthesis occurs by 6 h. At the earlier time point, considerably more strong-stop DNA is synthesized than first-strand *gag* DNA, and there is a relative decrease in the amount of complementary viral RNA sequences, thus confirming that the DNA detected at 1 h primarily represents the intermediate products of reverse transcription. Our studies indicate that this nascent DNA is synthesized in particles that have a sedimentation coefficient and buoyant density very similar to those of intact cell-free virions and contain at least RT and $p24^{gag}$ and $gp120^{env}$ proteins. These particles contain neither human nuclear DNA nor mitochondrial DNA. That the nascent viral DNA is enclosed within these particles is evidenced by the fact that it is not degraded by DNase treatment and that it could be immunoprecipitated by latex beads coated with anti-$gp120^{env}$ and anti-$gp41^{env}$ antibodies. On the basis of the data presented above and electron microscopic examination of HUT 78 cells at 1 h postinfection by HIV-1, we conclude that the nascent DNA is synthesized

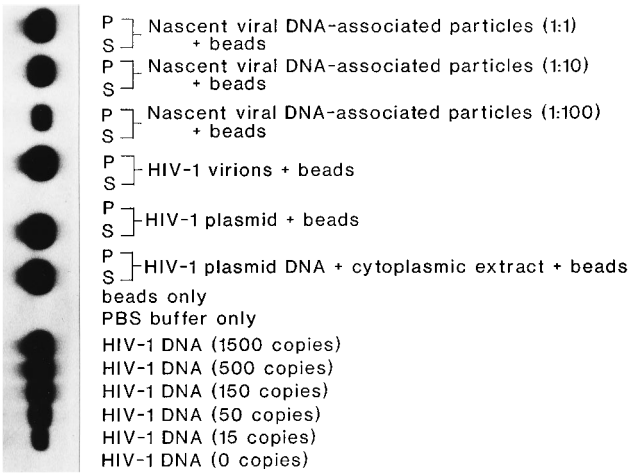

FIG. 8. Immunocapture of nascent viral DNA-associated particles by anti-HIV-1 gp120 and gp41 antibodies. Fraction 11 in Fig. 5 was diluted in PBS and mixed with latex beads coated with anti-HIV-1 gp120 and gp41 antibodies. The immune complexes were pelleted via low-speed centrifugation. The supernatant (S) was removed and normalized with fresh unreacted beads, while the pellet (P) was suspended in an equal volume of PBS. Nucleic acid was extracted from both fractions, PCR was performed with primer pair M667/AA55, and Southern blot hybridization was performed with the ^{32}P-labelled SK31 probe. Results obtained with various dilutions of the nascent viral DNA-associated particles were compared with those obtained with whole HIV-1 virions plus beads, HIV-1 plasmid DNA (plus or minus the cytoplasmic extract from uninfected HUT 78 cells) plus beads, the beads alone, PBS alone, and an HIV-1 DNA standard.

in virion-derived particles either bound to the cell surface or present in intracellular vesicles resembling endosomes. If the endosomes are the site of initial HIV-1 reverse transcription, then our data would be dependent on the detergent conditions used herein lysing the endosome, a point that we have not proven, but one that is plausible given that the endosomal membrane is primarily an inverted cellular membrane.

The nascent viral DNA-associated particles have an average buoyant density of 1.19 g/ml, compared with 1.16 g/ml seen with purified extracellular HIV-1 virions (reference 45 and data not shown). This would suggest that the former may have relatively less percent lipid content than the latter. The differences in the gp120/p24 ratio observed between extracellular HIV-1 virions and particles containing the nascent viral DNA may be attributable to several possibilities. First, the virions which are bound to the target cells may possess a higher quantity of gp120 than the average extracellular virion. It is to be noted that extracellular virions may shed gp120, especially during purification (14, 22, 27). Also, the efficiency of assembly of gp120 into virions may be much lower than that of p24. It has been reported that the *gag* proteins are sufficient to form a noninfectious viruslike particle which has a morphology and density similar to those of the wild-type virus (33, 34). Because gp120 functions by binding to CD4 on the surface of the target cell, those virions that have more gp120 molecules on their surface would more efficiently bind to and infect target cells. Second, p24 may be readily dissociated from HIV-1 virions during entry into the target cells. It has been hypothesized that in HIV-1, the *env* glycoproteins, but not p24^{gag}, are included in the matrix formed by p17 and the ribonucleoprotein complex (6). Reports (7, 9, 10) that at 6 h postinfection, the full-length linear viral DNA in HIV-1-infected cells is associated with only

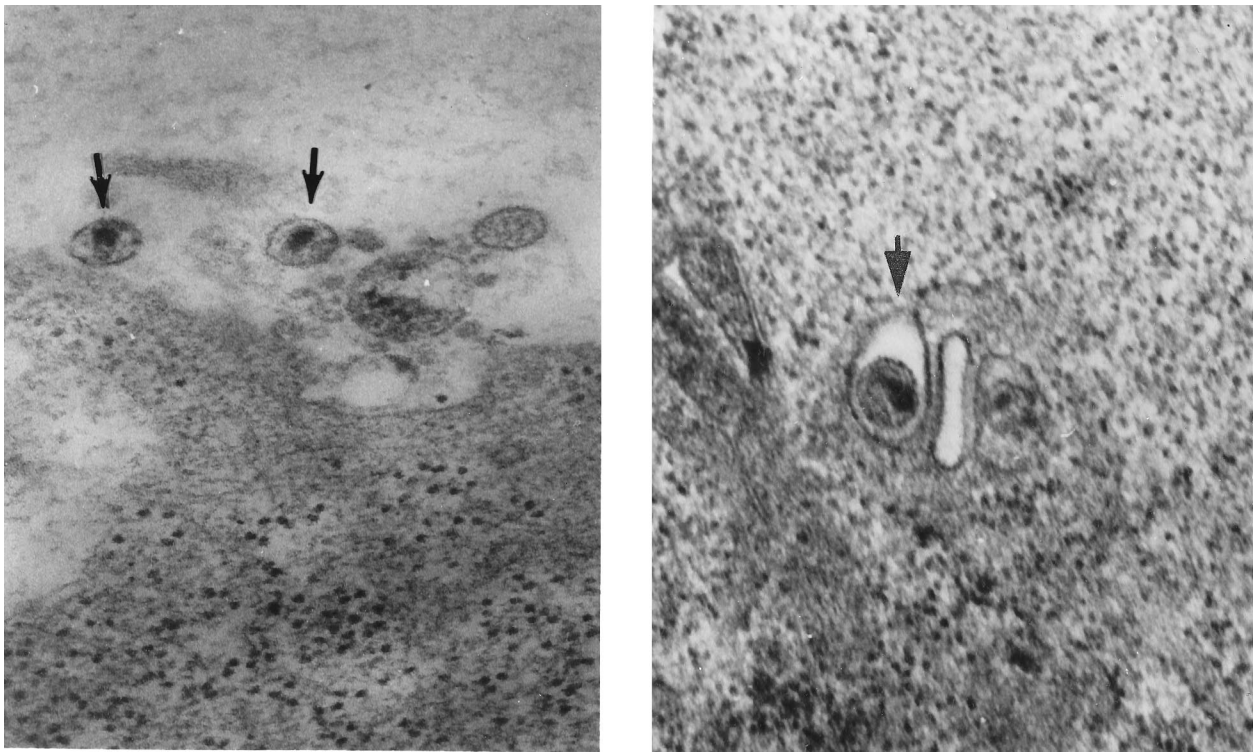

FIG. 9. Electron micrographs of HUT 78 cells 1 h postinfection with HIV-1. Magnification, ×100,000. (Left) extracellular virions (arrows) adjacent to and on the surface of HUT 78 cells. (Right) HIV-1 virion in the endosomelike vesicle (arrow) of a HUT 78 cell.

the integrase and matrix protein but not with p24 support the hypothesis mentioned above. Conversely, others have found that the full-length linear murine leukemia virus DNA is associated with both integrase and capsid proteins (5). Third, we used nonionic detergent (0.02% Nonidet P-40) to disrupt cellular membranes. As is the case with intact virions, this amount of detergent might preferentially solubilize some p24 from the nascent DNA-associated particle (6, 17).

It has been demonstrated that HIV-1 enters a target cell via two mechanisms: direct fusion (15, 25, 36) or endocytosis (15, 24, 29). However, no convincing evidence has been presented to indicate which is the preferred mechanism in vivo. We have shown that endogenous reverse transcription can occur within intact HIV-1 virions given suitable divalent cation and dNTP concentrations (44, 45). The concentration of dNTPs in the cytoplasm should be much higher than that in the extracellular space (12). When a virion attaches to a target cellular membrane, it is conceivable that the dNTPs in the cytoplasm could diffuse through altered cellular membranes, thereby driving de novo viral DNA synthesis. Similarly, it is possible that conditions suitable for reverse transcription exist in the endosomes of HUT 78 cells.

Although RT and RNase H are the predominant enzymes involved in HIV-1 DNA synthesis, viral structural proteins clearly are involved as well (30). Because we are observing nascent DNA synthesis in a particle similar to an intact virion, it is reasonable to assume that components of this structure may be critical for optimal reverse transcription, especially for efficient template switching.

It has been reported that only partial reverse transcripts can be found in quiescent lymphocytes after HIV-1 infection (7, 42, 43). If the same lymphocytes were then stimulated to proliferate by a mitogen, full-length viral DNA was subsequently synthesized (43). These observations are compatible with ours, because they indicated that the genomic viral RNA molecules that are persistent in these lymphocytes were in a physical state that protected them from cellular nucleases and maintained an intact replication unit. Moreover, it has been observed that, although viral particles exist both on the surface and in the cytoplasm of sperm from HIV-1-positive patients or sperm to which HIV-1 has been added in vitro, viral DNA but not RNA can be detected in sperm (4, 35). This phenomenon is compatible with the hypothesis that virions are able to bind to and enter human sperm and undergo the early stages of reverse transcription but are unable to subsequently integrate into the condensed spermatic chromosomes.

The data presented above have several clinical implications, one of which is that in vivo HIV-1 strong-stop DNA copies may be in greater abundance than HIV-1 *gag*, *pol*, or *env* sequences, which are the usual targets of HIV-1 detection (1, 8, 21, 40). Hence, viral detection strategies directed toward strong-stop DNA may prove to be more sensitive. Another point raised by our data is that if the site of nascent HIV-1 DNA synthesis is inside either an extracellular or an intracellular virus particle, then effective antiviral drug therapy would require the accumulation of the active moieties in these virus particles and not just in the cytoplasm of the cell. We are currently conducting experiments with in vitro and in vivo specimens to explore these implications.

ACKNOWLEDGMENTS

We thank Robert Dougherty, Dipak Dube, and Peter Hahn for helpful discussions, Alix Robinson for guidance regarding electron microscopy, and Cheryl Brady and Jayne Love for help in preparing the manuscript.

This work was supported by PHS contracts AI27658 and HB67021.

REFERENCES

1. **Abbott, M. A., B. J. Poiesz, B. C. Byrne, S. Kwok, J. J. Sninsky, and G. D. Ehrlich.** 1988. Enzymatic gene amplification: qualitative and quantitative methods for detecting proviral DNA amplified in vitro. J. Infect. Dis. **158:** 1158–1168.
2. **Adachi, A., H. E. Gendelmann, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
3. **Amin, R. M., M. T. Dean, L. E. Zaumetzer, and B. J. Poiesz.** 1991. Virucidal efficacy of various lens cleaning and disinfecting solutions on HIV-1 contaminated contact lenses. AIDS Res. Hum. Retroviruses **7:**403–408.
4. **Bagasra, O., H. Farzadegan, T. Seshamma, J. W. Oakes, and R. J. Pomerantz.** 1994. Human immunodeficiency virus type 1 infection of sperm *in vivo*. AIDS **8:**1669–1674.
5. **Bowerman, B., P. O. Brown, J. M. Bishop, and H. E. Varmus.** 1989. A nucleoprotein complex mediates the integration of retroviral DNA. Genes Dev. **3:**469–478.
6. **Bukrinskaya, A. G., and N. K. Sharova.** 1990. Unusual features of protein interaction in human immunodeficiency virus (HIV) virions. Arch. Virol. **110:**287–293.
7. **Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson.** 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. USA **90:**6125–6129.
8. **Dyster, L. M., L. Abbott, V. Bryz-Gornia, B. J. Poiesz, and L. D. Papsidero.** 1994. Microplate-based DNA hybridization assays for detection of human retroviral gene sequences. J. Clin. Microbiol. **32:**547–550.
9. **Farnet, C. M., and W. A. Haseltine.** 1990. Integration of human immunodeficiency virus type 1 DNA in vitro. Proc. Natl. Acad. Sci. USA **87:**4164–4168.
10. **Farnet, C. M., and W. A. Haseltine.** 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J. Virol. **65:**1910–1915.
11. **Folks, T. M., D. Powell, M. Lightfoot, S. Koenig, A. S. Fauci, S. Benn, A. Rabson, D. Daugherty, H. E. Gendelmann, M. D. Hoggan, S. Venkatesan, and M. A. Martin.** 1986. Biological and biochemical characterization of a cloned Leu-3-cell surviving infection with the acquired immune deficiency syndrome retrovirus. J. Exp. Med. **164:**280–290.
12. **Furman, P. A., J. A. Fyfe, H. S. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry.** 1986. Phosphorylation of 3′-azido-3′ deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **83:**8333–8337.
13. **Gazdar, A. F., D. N. Carney, P. A. Bunn, E. K. Russell, E. S. Jaffe, G. P. Schecter, and J. G. Guccion.** 1980. Mitogen requirements for the in vitro propagation of cutaneous T-cell lymphomas. Blood **55:**409–417.
14. **Gelderblom, H. R., E. H. S. Hausmann, M. Ozel, G. Pauli, and M. A. Koch.** 1987. Fine structure of LAV/HTLV-III and immunolocalization of structure proteins. Virology **156:**171–176.
15. **Grew, C., A. Beck, and H. R. Gelderblom.** 1990. HIV: early virus-cell interactions. J. Acquired Immune Defic. Syndr. **3:**965–974.
16. **Henrard, D. R., W. F. Mehaffey, and J. P. Allain.** 1992. A sensitive viral capture assay for detection of plasma viremia in HIV-infected individuals. AIDS Res. Hum. Retroviruses **8:**47–52.
17. **Kaplan, A. H., P. Krogstad, D. J. Kempf, D. W. Norbeck, and R. Swanstrom.** 1994. Human immunodeficiency virus type 1 virions composed of unprocessed Gag and Gag-Pol precursors are capable of reverse transcribing viral genomic RNA. Antimicrob. Agents Chemother. **38:**2929–2933.
18. **Karageorgos, L., P. Li, and C. Burrell.** 1993. Characterization of HIV replication complexes early after cell-to-cell infection. AIDS Res. Hum. Retroviruses **9:**817–823.
19. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.
20. **Kwok, S., D. Kellog, G. Ehrlich, B. Poiesz, S. Bhagavati, and J. J. Sninsky.** 1988. Characterization of a sequence of human T cell leukemia virus type I from a patient with chronic progressive myelopathy. J. Infect. Dis. **158:**1193–1197.
21. **Kwok, S., D. H. Mack, K. B. Mullis, B. Poiesz, G. Ehrlich, D. Blair, A. Friedman-Kien, and J. J. Sninsky.** 1987. Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection. J. Virol. **61:**1690–1694.
22. **Layne, S. P., M. J. Merges, M. Dembo, J. L. Spouge, S. R. Conley, J. P. Moore, J. L. Raina, H. Renz, H. R. Gelderblom, and P. L. Nara.** 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology **189:**695–714.
23. **Lori, F., F. di Marzo Veronese, A. L. De Vico, P. Lusso, M. S. Reitz, Jr., and R. C. Gallo.** 1992. Viral DNA carried by human immunodeficiency virus type 1 virions. J. Virol. **66:**5067–5074.
24. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss,**

and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
25. **McClure, M., M. Marsh, and R. Weiss.** 1988. HIV infection of CD4 bearing cells occurs by a pH-independent mechanism. EMBO J. **239:**88–92.
26. **McEwen, C. R.** 1967. Tables for estimating sedimentation through linear concentration gradients of sucrose solution. Anal. Biochem. **20:**114–149.
27. **McKeating, J. A., A. McKnight, and J. P. Moore.** 1991. Differential loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: effects on infectivity and neutralization. J. Virol. **65:**852–860.
28. **Oh, S. K., W. W. Cruikshank, J. Raina, G. C. Blanchard, W. H. Adler, J. Walker, and H. Koenfeld.** 1992. Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients. J. Acquired Immune Defic. Syndr. **5:**251–256.
29. **Pauza, C. D., and T. M. Price.** 1988. Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. J. Cell Biol. **1107:**959–968.
30. **Peliska, J. A., S. Balasubramaniar, D. P. Giedrov, and S. J. Benkovic.** 1994. Recombinant HIV-1 nucleocapsid protein accelerates HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions and modulates RNase H activity. Biochemistry **33:**13817–13823.
31. **Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA **77:**7415–7419.
32. **Preston, B. D., B. J. Poiesz, and L. A. Loeb.** 1988. Fidelity of HIV-1 reverse transcriptase. Science **242:**1168–1171.
33. **Ross, E. K., T. R. Fuerst, J. M. Orenstein, T. O'Neill, M. A. Martin, and S. Venkatesan.** 1991. Maturation of human immunodeficiency virus particles assembled from the *gag* precursor protein requires in situ processing by *gag-pol* protease. AIDS Res. Hum. Retroviruses **7:**475–483.
34. **Sadaie, M. R., V. S. Kalyananman, R. Mukopadhayaya, E. Tschachler, R. C. Gallo, and F. Wong-Staal.** 1992. Biological characterization of noninfectious HIV-1 particles lacking the envelope protein. Virology **187:**604–611.
35. **Scofield, V. L., B. Rao, S. Broder, C. Kennedy, M. Wallace, B. Graham, and B. J. Poiesz.** 1994. HIV interaction with sperm. AIDS **8:**1733–1736.
36. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
37. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. J. Immunol. **126:**1614–1619.
38. **Trono, D.** 1992. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. J. Virol. **66:**4893–4900.
39. **Varmus, H. E., S. Heasley, K. J. Kung, H. Opperman, V. C. Smith, J. M. Bishop, and P. R. Shunk.** 1978. Kinetics of synthesis, structure, and purification of avian sarcoma virus-specific DNA in the cytoplasm of acutely infected cells. J. Mol. Biol. **120:**55–82.
40. **Wells, K. H., B. C. Byrne, and B. J. Poiesz.** 1990. Detection, prevention and treatment of retroviral infections. Semin. Oncol. **17:**295–320.
41. **Wells, K. H., J. Latino, J. Gavalchin, and B. J. Poiesz.** 1991. Inactivation of human immunodeficiency virus type 1 by ozone in vitro. Blood **78:**1882–1890.
42. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals labile, latent viral structure. Cell **61:**213–222.
43. **Zack, J. A., A. M. Haislip, P. Krogstad, and I. S. Y. Chen.** 1992. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J. Virol. **66:**1717–1725.
44. **Zhang, H., O. Bagasra, M. Niikura, B. J. Poiesz, and R. J. Pomerantz.** 1994. Intravirion reverse transcripts in the peripheral blood plasma of human immunodeficiency virus type 1-infected individuals. J. Virol. **68:**7591–7597.
45. **Zhang, H., Y. Zhang, T. P. Spicer, L. Z. Abbott, M. Abbott, and B. J. Poiesz.** 1993. Reverse transcription takes place within extracellular HIV-1 virions: potential biological significance. AIDS Res. Hum. Retroviruses **9:**1287–1296.