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Intravirion Reverse Transcripts in the Peripheral Blood Plasma of Human Immunodeficiency Virus Type 1-Infected Individuals

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Variable levels of viral DNA have been demonstrated within human immunodeficiency virus type 1 (HIV-1) virions purified from cell cultures. In the present studies, it is demonstrated that DNase-resistant viral DNA is associated with HIV-1 virions purified from the peripheral blood plasma of both symptomatic and asymptomatic HIV-1-infected individuals. The differences in viral DNA copy numbers, detected by quantitative PCR in various regions of the HIV-1 genome, indicated that the intravirion HIV-1 DNA is frequently, but perhaps not totally, the result of partial reverse transcription. These in vivo data suggest that it may be valuable to further investigate the impact of virion-associated viral DNA upon the efficiency of intra- and interhost HIV-1 transmission modes.

Cell-free virions of human immunodeficiency virus type 1 (HIV-1) are believed to play an important role in intra- or interhost transmission (4, 30). HIV-1 virions have been isolated from different physiological fluids of HIV-1-seropositive individuals, including blood, semen, vaginal fluids, breast milk, tears, stool, saliva, and cerebrospinal fluid (4, 11, 13, 14, 18, 25, 37, 39, 41). The titer of HIV-1 virions usually increases in the blood plasma of HIV-1-infected persons early in viral infection and then declines to low levels, presumably because of the host's immune response (30). As infection progresses to the point of overt disease, however, the titer of HIV-1 in the peripheral blood plasma will again increase (9, 10, 17, 30). Moreover, the study by Coombs et al. indicated that plasma viremia was associated with a decline in the $CD4^+$ lymphocyte count in the peripheral blood and the development of symptomatic disease (9). Recently, several groups have demonstrated the presence of virion-associated genomic RNA in the peripheral blood plasma of almost all asymptomatic HIV-1-infected individuals, by a sensitive and quantitative reverse transcriptase-initiated PCR methodology (30, 40). Further, the viral RNA level in the blood plasma was inversely correlated with the efficiency of antiviral treatment (30).

The genetic substance carried by a retrovirus is well characterized and consists of two identical positive strands of RNA. Reverse transcriptase, which is carried by the virion, catalyzes proviral DNA synthesis, with the two genomic RNAs acting as the templates (8, 20). Traditionally, this process was thought to occur in the cytoplasm of newly infected cells (38, 43). However, recent data have indicated that variable quantities of viral DNA, which stem from partial reverse transcription, are carried by HIV-1 virions purified from cell cultures (26, 36, 44). Further, virion-associated HIV-1 DNA synthesis can occur in a very simple reaction system, without detergent, and even in human physiological fluids (44). The infectivity of HIV-1 virions increases, if given prior incubation with deoxynucleoside triphosphates (dNTPs) to initiate endogenous reverse transcription in vitro (44). Therefore, we assumed that virion-associated HIV-1 DNA might have an impact on the efficiency of viral dissemination and transmission in vivo. To assess this hypothesis, virion-associated HIV-1 DNA in the physiological fluids of HIV-1-infected individuals must be investigated, and if present, these viral nucleic acids must be characterized. Although the presence of viral DNA has been demonstrated in cell-free blood plasma of HIV-1-infected individuals, its location remains to be determined (16). Given that the concentrations of HIV-1 virions in many body fluids may be significantly lower than those in many cell culture systems, the strategies to isolate and purify the virion-associated HIV-1 DNA from cell cultures may not be suitable for the isolation of virion-associated HIV-1 DNA from body fluids. Thus, a quick and sensitive assay was required to concentrate and purify the virions from in vivo infections. It has been reported that anti-gp120 and anti-gp41 antibody-coated latex beads can be used to concentrate HIV-1-specific RNA in the peripheral blood plasma from HIV-1-infected individuals (15). We have recently determined that these beads can immunospecifically capture HIV-1 virions but not human T-cell leukemia virus type I (HTLV-I) virions (our unpublished data). Therefore, these antibody-coated beads were used to immunoprecipitate HIV-1 virions from human peripheral blood plasma.

Peripheral blood plasma samples were collected from three persons with AIDS (hereafter referred to as AIDS samples) and four asymptomatic HIV-1-infected individuals. The pertinent clinical data for these subjects are summarized in Table 1. Five hundred microliters of blood plasma from each patient was centrifuged at 10,000 × *g* for 15 min to form a pellet of platelets or other large, blood-borne particles (24). The supernatant (400 μl) was then added to 100 μl of anti-gp120 and anti-gp41 antibody-coated latex beads (a generous gift from D. Henrard, Abbott Laboratories). The mixture was incubated at room temperature for 3 h with shaking (200 rpm) on a plate rotator (Baxter Healthcare Co., Miami, Fla.). The virion-bead complexes were then pelleted in a microfuge at 7,000 × *g* for 5 min. The supernatant was removed, and the virion-bead complexes were washed twice with phosphate-buffered saline

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TABLE 1. Pertinent clinical data for HIV-1-infected individuals

Patient	CDC classification disease stage[a]	Total CD4 lymphocyte level (cells/mm^3)	HIV-1 p24 antigen in serum (pg/ml)[b]	Duration of zidovudine treatment (yr)
JD1N	IV	216	0	4
JAW1	IV	8	30	4
M1M1	IV	302	0	2 1/2
FR	II	513	0	1 1/2
DJ	II	436	0	No zidovudine
JL	II	313	0	1
TT	II	300	0	4

[a] CDC, Centers for Disease Control and Prevention.
[b] HIV-1 p24 antigen in the serum was detected by enzyme-linked immunosorbent assay (DuPont, Inc., Wilmington, Del.).

(PBS) and resuspended in 100 μl of TN buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl). Five units of RQ1 RNase-free DNase (Promega, Inc., Madison, Wis.) was added, along with 10 mM $MgCl_2$. The mixture was then incubated at 37°C for 30 min. DNase was washed away by twice pelleting the virion-bead complexes in PBS and was further inactivated by boiling for 10 min. Subsequently, the virion-associated DNA was extracted with a lysing buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1% sodium dodecyl sulfate, proteinase K [100 μg/ml]) prior to phenol-chloroform (1:1) extraction and ethanol precipitation. As controls, blood plasma samples from HIV-1-seronegative individuals were mixed with solubilized HIV-1 proviral DNA (extracted from the ACH-2 cell line [7]) and were then treated with anti-gp120 and anti-gp41 antibody-coated beads prior to DNase treatment under the same conditions. Quantitative PCR amplification was performed for HIV-1 DNA, as previously described (44). To prevent PCR carryover, 0.2 U of uracil-*N*-glycosylase (Perkin-Elmer, Inc., Norwalk, Conn.) was added to the PCR system (50 μl) and the mixture was incubated at 37°C for 30 min prior to amplification. The locations of the primers used in the PCR along the HIV-1 genome are illustrated in Fig. 1.

Figure 2A demonstrates that DNase-resistant HIV-1 DNA was copurified with HIV-1 virions by immunoprecipitation from the peripheral blood plasma of all seven HIV-1-seropositive individuals. However, the solubilized HIV-1 DNA which was artificially added to the blood plasma of HIV-1-seronegative persons was completely eliminated by DNase treatment (Fig. 2A, lanes 8 to 10). Finally, virions from the peripheral blood plasma of three HIV-1-infected individuals were also directly isolated by ultracentrifugation (44) prior to treatment with DNase. This methodology also yielded DNase-resistant virion-associated HIV-1 DNA in each sample evaluated, but in some samples ultracentrifugation led to somewhat lower levels of HIV-1-specific nucleic acids than the levels in anti-gp120 and anti-gp41 antibody-coated bead isolations. This may be due to virion damage resulting from this isolation technique (Fig. 2B and data not illustrated).

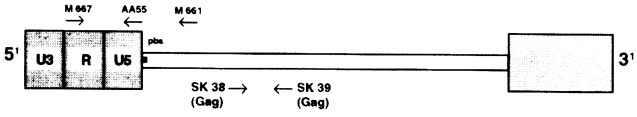

FIG. 1. Map of primer locations for PCR along the HIV-1 genome. The shaded areas at each terminus represent the long terminal repeats, and the solid box at the 5′ end represents the primer binding site (pbs).

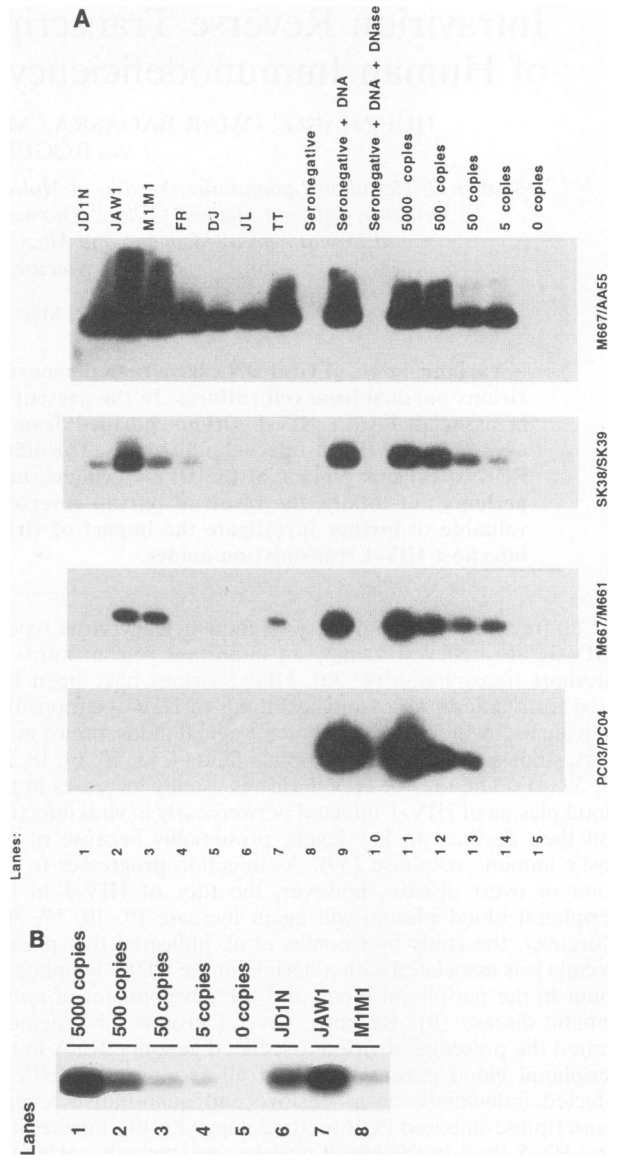

FIG. 2. (A) Copurification of DNase-resistant viral DNA associated with HIV-1 virions by immunoprecipitation. HIV-1 virions were captured by anti-gp120 and anti-gp41 antibody-coated latex beads from blood plasma of various HIV-1-seropositive individuals and HIV-1-seronegative persons and were then treated with DNase. Viral DNA was then extracted and amplified by PCR, with different primer pairs (Fig. 1). The PCR products were analyzed by Southern blot assays (44). Lanes 1 to 7, HIV-1 virions from peripheral blood of HIV-1-infected individuals; lane 8, HIV-1-seronegative blood plasma; lane 9, HIV-1-seronegative sample and total cellular DNA from ACH-2 cells; lane 10, HIV-1-seronegative sample with DNA from ACH-2 cells and DNase treatment; lanes 11 to 15, standard curve of ACH-2 cells (10-fold serial dilutions). (B) Ultracentrifugation of HIV-1 virions from blood plasma of infected individuals. HIV-1 virions were isolated by ultracentrifugation, as previously described (44). Viral DNA was extracted from three samples, treated with DNase, and amplified by PCR with primer pair M667/AA55 (lanes 6 to 8). A standard curve of 10-fold serial dilutions of ACH-2 DNA is also included (lanes 1 to 5).

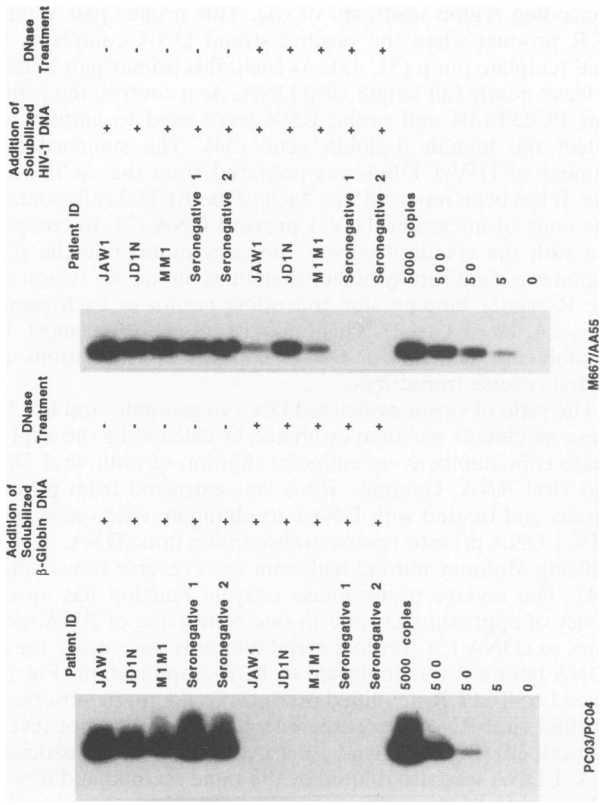

FIG. 3. DNase treatment of solubilized cellular and HIV-1 DNAs attached to the surface of HIV-1 virions. Solubilized HIV-1 DNA and cellular DNA, containing β-globin and isolated from ACH-2 cells, were mixed with the blood plasma from HIV-1-seropositive and -seronegative individuals. The HIV-1 virions were captured by anti-gp120 and anti-gp41 antibody-coated latex beads. Some mixtures were then treated with DNase, while others were not treated with DNase. The DNA was extracted and amplified by PCR with primer pairs M667/AA55 (upper panel) and PC03/PC04 (lower panel), respectively. The PCR products were analyzed by Southern blot assays. The symbols in the DNase columns represent those experiments with mixtures not treated with DNase (−). The symbols in the β-globin DNA column represent cellular DNA, containing the β-globin gene, mixed with each sample, by the addition of ACH-2 cellular DNA (+). Standard curves of 10-fold serial dilutions of ACH-2 DNA are included at the far right of the upper and lower panels (5,000 to 0 proviral DNA copies).

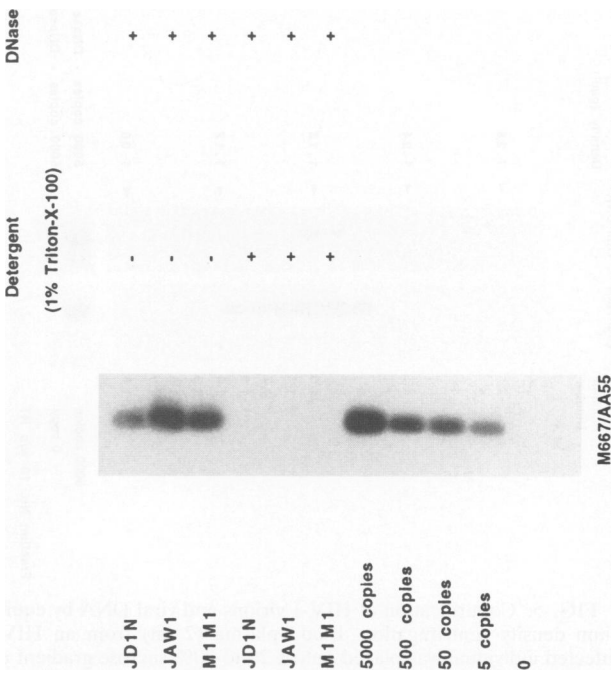

FIG. 4. DNase treatment of detergent-permeabilized HIV-1 virions. HIV-1 virions were captured by anti-gp120 and anti-gp41 antibody-coated latex beads from the blood plasma of three individuals with AIDS. Triton X-100 (1%) was added to the virion-bead complexes prior to DNase treatment (lanes JD1N, JAW1, and M1M1). Viral DNA was extracted and amplified by PCR with primer pair M667/AA55. The PCR products were analyzed by Southern blot assays. A standard curve of 10-fold serial dilutions of ACH-2 DNA (5,000 to 0 proviral DNA copies) is also included.

Solubilized HIV-1 proviral DNA and cellular DNA, containing β-globin DNA and isolated from ACH-2 cells, were then added to the blood plasma of three individuals with AIDS and two HIV-1-seronegative persons. The amount of HIV-1 DNA decreased in the three AIDS samples mixed with ACH-2 DNA, but did not completely disappear, after DNase treatment. This indicated that solubilized HIV-1 DNA which attached to the surface of virions could be eliminated by DNase, while HIV-1 DNA within the virions was not digested (Fig. 3, upper panel; compare DNase-treated lanes with untreated lanes). The HIV-1 DNA, from ACH-2 cells, mixed with plasma from two HIV-1-seronegative individuals was completely digested by DNase treatment (Fig. 3, upper panel). In addition, since there is no β-globin DNA within HIV-1 virions, all the solubilized β-globin DNA which attached to the virion surface was digested by DNase (Fig. 3, lower panel). This HIV-1-specific DNA is unlikely to be due to internalized viral cores released from degenerated cells, since the isolation procedure utilized anti-gp120 and anti-gp41 antibodies, which would not immunoprecipitate viral cores.

In further studies, detergent (1% Triton X-100; Sigma Chemical Co., St. Louis, Mo.) was added to the virion-bead complexes of three AIDS samples before DNase treatment. The virion-associated DNA became sensitive to the DNase, which further indicated that the viral DNA is enclosed by a membranous viral envelope (Fig. 4).

To verify that the HIV-1 DNA is virion-associated, equilibrium density centrifugation was employed to copurify the viral DNA and HIV-1 virions (26). After large particles, such as platelets, were removed by pelleting at 10,000 × *g* for 15 min, 2-ml aliquots of blood plasma from the three individuals with AIDS were placed onto the top of a 20-to-70% continuous sucrose gradient and centrifuged at 40,000 rpm (150,000 × *g*) for 20 h at 4°C in a Beckman Ti-50 rotor. Gradients were distributed from the bottom into 18 fractions. Buoyant density was determined with a refractometer. The fraction of buoyant density (1.16 g/ml) in which HIV-1 virions are located (32, 44) was diluted with TN buffer to the buoyant density of 1.08 g/ml. This diluted fraction was then placed onto the top of another 20-to-70% sucrose gradient and was centrifuged under the same conditions, as described above. The final gradient was fractionated into 18 tubes. Five units of RNase-free DNase was added to 50-μl volumes from each fraction, with 10 mM $MgCl_2$ (final concentration). After incubation at 37°C for 1 h, the mixture was boiled for 10 min to inactivate DNase. Nucleic acids were then extracted, as described above. Figure 5 illustrates the copurification of HIV-1 virions (marked by genomic

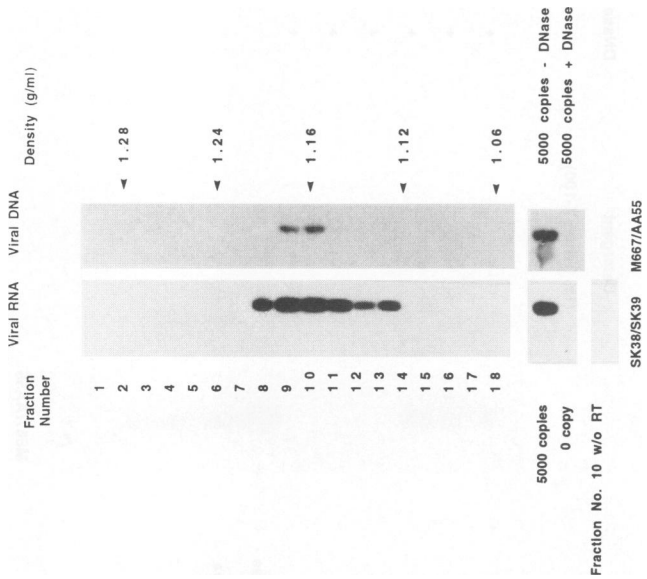

FIG. 5. Copurification of HIV-1 virions and viral DNA by equilibrium density centrifugation. Blood plasma (2 ml) from an HIV-1-infected individual was placed onto a 20-to-70% sucrose gradient and centrifuged at 150,000 × *g* for 20 h. The gradient was fractionated into 18 tubes. The fraction at a density of 1.16 g/ml was diluted and placed onto a second 20-to-70% sucrose gradient for centrifugation under the same conditions. This gradient was also divided into 18 fractions. All the fractions were treated with DNase, which was then inactivated. Viral DNA and RNA were extracted from each fraction. Viral DNA was directly amplified by PCR with primer pair M667/AA55 (upper panel). As controls, solubilized HIV-1 proviral DNA (5,000 copies) was treated with DNase or left untreated (far right two lanes of upper panel). Viral RNA was first treated with DNase and then reverse transcribed into cDNA in vitro utilizing Moloney murine leukemia virus reverse transcriptase. The cDNA was then amplified by PCR with primer pair SK38/SK39 (lower panel). The PCR products were analyzed by Southern blot assays. Fraction 10 is also shown in the last lane of the lower row, in which a reaction omitting reverse transcriptase (RT) was performed. A 5,000-copy-number in vitro-transcribed HIV-1 genomic RNA standard was utilized, as previously described (44), as a positive control for the lower panel of reactions.

RNA and a density of 1.16 g/ml) and DNase-resistant "minus-strand strong-stop" viral DNA, in this "double-banding" process, by which two independent sucrose gradients were sequentially utilized to purify the virions. A similar copurification of virion and DNase-resistant viral DNA, in two other samples from HIV-1-infected individuals, was also observed (data not illustrated). On the basis of the experiments described above, it was concluded that viral DNA is enclosed in HIV-1 virions in the blood plasma of HIV-1-seropositive individuals.

To characterize the intravirion HIV-1 DNA, aliquots of virion-associated DNA from seven HIV-1-seropositive individuals were amplified and detected by PCR with different primer pairs and probes (Fig. 1). Primer pair M667/AA55 and probe SK31 were used to amplify and detect the RU5 (strong-stop DNA) region (31, 43). This region of minus-strand strong-stop HIV-1 DNA relates to the synthesis of viral DNA prior to the first template switch, during reverse transcription. Primer pair SK38/SK39 and probe SK19 were used to amplify and detect the *gag* region, where negative strand DNA synthesis is near completion (31). Primer pair M667/M661 and probe SK31 were used to amplify and detect the region which includes the R region, U5 region, primer binding site, and a portion of the noncoding region upstream of *gag*. This primer pair yields a PCR product when the positive-strand DNA completes the final template jump (31, 43). As such, this primer pair detects, at least, nearly full-length viral DNA. As a control, the primer pair PC03/PC04 and probe RS06 were used to amplify and detect the human β-globin gene (34). The standard copy number of HIV-1 DNA was prepared from the ACH-2 cell line. It has been reported that each of the ACH-2 cells contains one copy of integrated HIV-1 proviral DNA (7). In comparison with the standard curves, the copy number in the RU5 region was significantly higher than those in the *gag* region and the R-primer binding site noncoding region in each sample (Fig. 2A, lanes 1 to 7). This finding suggested that most, but possibly not all, HIV-1 DNAs copurified with virions are partial reverse transcripts.

The ratio of virion-associated DNA to genomic viral RNA in these specimens was then estimated by calculating the approximate copy numbers via endpoint dilution of both viral DNA and viral RNA. Genomic RNA was extracted from purified virions and treated with DNase to eliminate virion-associated HIV-1 DNA prior to reverse transcription into cDNA, in vitro, utilizing Moloney murine leukemia virus reverse transcriptase (44). The reverse transcriptase enzyme reaction has an efficiency of approximately one-to-one conversion of RNA molecules to cDNA (5). Tenfold serial dilutions were made for the cDNA before it was subjected to PCR amplification (Fig. 6A, lanes 1 to 8). PCR-amplified products could not be observed if purified viral RNAs were treated with DNase and not reverse transcribed (Fig. 6A, lane 9). In addition, virion-associated HIV-1 DNA was also diluted by the same method and directly subjected to PCR amplification (Fig. 6B). Table 2 summarizes the approximate ratios of HIV-1 DNA, in various regions of the viral genome, to genomic HIV-1-specific RNA (*gag* region) in two individuals with AIDS (JAW1 and M1M1) and an asymptomatic HIV-1-seropositive person (FR). The ratios of DNA to RNA for two individuals (M1M1 and FR) are similar to those found in the virions purified from cell cultures (10^{-3} to 10^{-5}) (44).

It should be noted that the largest quantity of virion-associated HIV-1 DNA was observed in an individual with late-stage AIDS (JAW1), with a very low total CD4-positive lymphocyte level in the blood (8 cells per mm^3), who died 40 days later. Although the level of virions in the sample was quite high, the ratio of the viral DNA to genomic RNA was also significantly high. It is possible that the reverse transcriptase carried by the virus in the blood plasma of patients with late-stage AIDS may be more efficient in carrying out reverse transcription. It will be important to further evaluate a possible correlation between the progression of disease in HIV-1 infection and the relative amounts of viral DNA carried by HIV-1 virions.

Partial reverse transcription may occur in the cytoplasm of virus-producing cells because of the inefficient reverse transcriptase activity of immature Gag-Pol fusion proteins (26). The resultant viral DNA would then be packaged into virions along with the genomic RNA. Nevertheless, several groups have indicated that endogenous reverse transcription of HIV-1 can occur without detergents (3, 42, 44). The concentration of dNTPs in human blood plasma may not be high enough to support complete reverse transcription, leading to partial reverse transcription within virions.

In contrast to in vitro data, which suggests that 3′-azido-3′-deoxythymidine (zidovudine) can eliminate viral DNA within cell-free virions purified from cell culture (36), virion-associated viral DNA was found in all the patients, regardless of zidovudine treatment. Since zidovudine-resistant viral strains

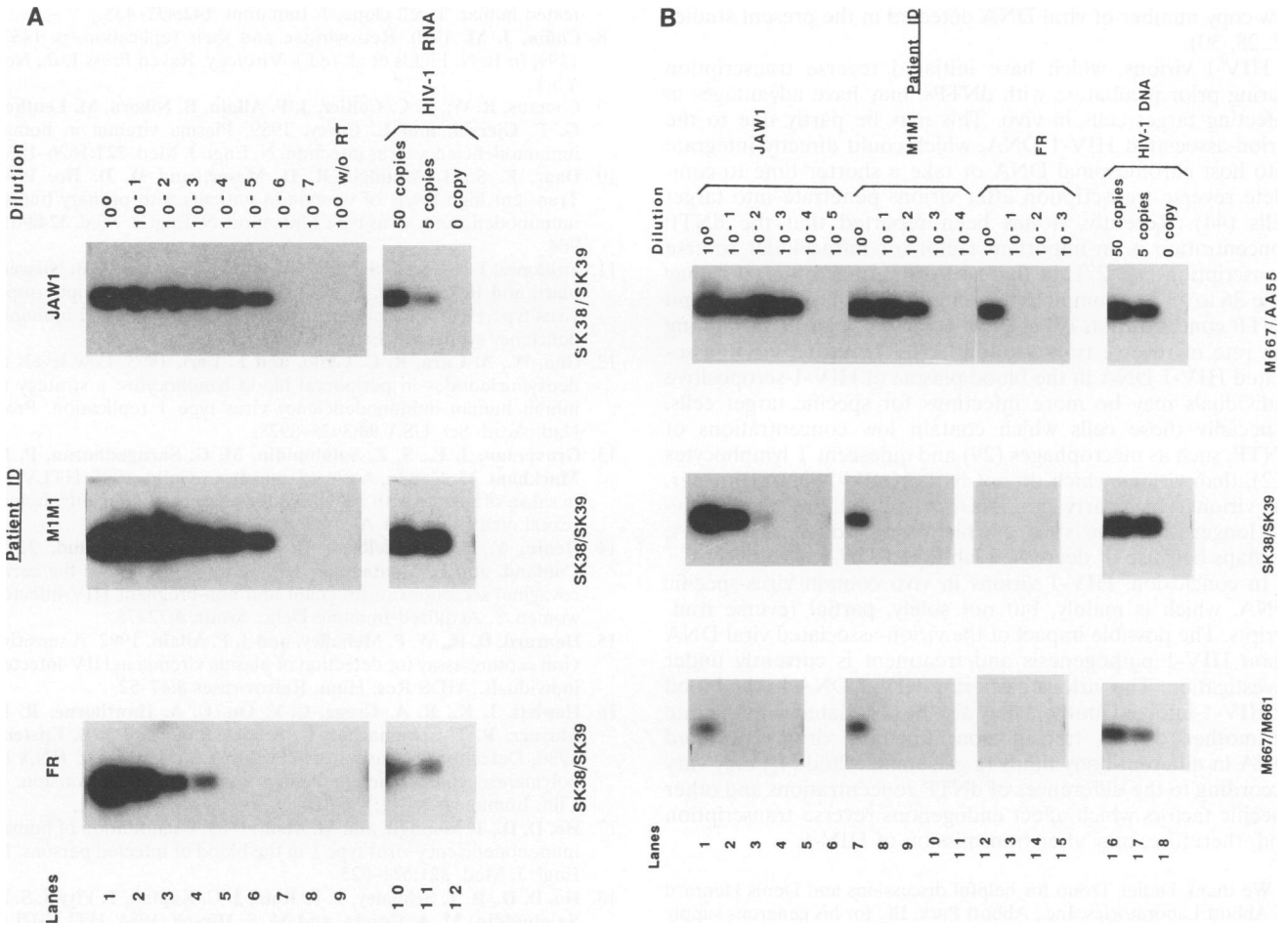

FIG. 6. Quantitation of viral DNA and genomic viral RNA in HIV-1 virions purified from blood plasma of HIV-1-infected individuals. HIV-1 virions were captured by anti-gp120 and anti-gp41 antibody-coated latex beads from the blood plasma of three HIV-1-seropositive persons (JAW1, M1M1, and FR) and treated with DNase. Viral DNA and RNA were then extracted with phenol-chloroform (1:1) and precipitated with ethanol, as previously described (44). (A) Viral RNA was treated with DNase and then reverse transcribed into cDNA in vitro, by Moloney murine leukemia virus reverse transcriptase (RT), with SK39 as the primer. The cDNA was then serially diluted (10-fold) and amplified by PCR with primer pair SK38/SK39. The PCR products were analyzed by Southern blot assays. (B) Viral DNA was serially diluted (10-fold) and amplified by PCR with different primer pairs. The PCR products were analyzed by Southern blotting. The copy numbers of viral DNA and RNA were approximated by endpoint dilution.

can readily develop in the majority of patients who have received zidovudine for more than 6 months (21–23, 33), it is highly possible that zidovudine-resistant strains had already developed in these patients, who had received zidovudine treatment for several years. Therefore, the virion-associated viral DNA could be synthesized in the presence of 3′-azido-3′-deoxythymidine 5′-triphosphate. To clarify this possibility, virion-associated HIV-1 DNA in the blood plasma of infected individuals must be further examined, longitudinally, before and shortly after zidovudine treatment.

TABLE 2. Virion-associated HIV-1 DNA and RNA copies in plasma of infected individuals

Patient	HIV-1 RNA (no. of copies/ml)	HIV-1 DNA (no. of copies/ml)			HIV-1 DNA/RNA ratio		
		Strong stop	*gag*	Full length	Strong stop	*gag*	Full length
JAW1	10^6	10^4	10^3	10^1	$1:10^2$	$1:10^3$	$1:10^5$
M1M1	10^6	10^3	10^1	10^1	$1:10^3$	$1:10^5$	$1:10^5$
FR	10^4	10^1	0	0	$1:10^3$		

It is of note that, except for the reports of Hewlett et al. (16) and Bagnarelli et al. (2), HIV-1 viral DNA in blood plasma has seldom been identified, even though virion-associated RNA in the blood plasma of HIV-1-positive individuals was widely studied with quantitative reverse transcriptase-initiated PCR assays (1, 2, 19, 28, 30, 35, 40). The reasons may be as follows. (i) Almost all the studies used primer pairs solely in the *gag* region for PCR amplification. As shown in Fig. 2, the viral DNA copy number in the *gag* region is significantly lower than that in the RU5 region. (ii) The majority of the studies (2, 19, 35, 40) selectively extracted viral RNA utilizing Chomczynski and Sacchi's acid guanidinium thiocyanate-phenol chloroform method (6) or the guanidinium thiocyanate-cesium chloride method (35). By those methods, viral DNA is eliminated from the preparation for PCR. (iii) The detection methods for PCR products are frequently not sensitive enough to measure the

low copy number of viral DNA detected in the present studies (2, 28, 30).

HIV-1 virions, which have initiated reverse transcription during prior incubation with dNTPs, may have advantages in infecting target cells in vivo. This may be partly due to the virion-associated HIV-1 DNA, which could directly integrate into host chromosomal DNA or take a shorter time to complete reverse transcription after virions penetrate into target cells (44). Recently, it has been reported that the dNTP concentration is an important factor for intracellular reverse transcription (12, 27). In the case of the macrophage, it may take 36 to 48 h to complete full-length viral DNA synthesis and dNTP concentration is believed to play a key role in limiting the rate of reverse transcription (29). Therefore, virion-associated HIV-1 DNA in the blood plasma of HIV-1-seropositive individuals may be more infectious for specific target cells, especially those cells which contain low concentrations of dNTP, such as macrophages (29) and quiescent T lymphocytes (12), than virions which do not harbor HIV-1 DNA. Further, the virions which carry viral DNA may maintain their infectivity longer than the virions which carry solely viral RNA, perhaps because of decreased lability of DNA versus RNA.

In conclusion, HIV-1 virions in vivo contain virus-specific DNA, which is mainly, but not solely, partial reverse transcripts. The possible impact of the virion-associated viral DNA upon HIV-1 pathogenesis and treatment is currently under investigation. The virions harboring HIV-1 DNA in the blood of HIV-1-infected mothers may also be correlated with the rate of mother-to-fetus transmission. Further, virion-associated DNA in different body fluids (e.g., seminal fluid [4]) may vary according to the differences of dNTP concentrations and other specific factors which affect endogenous reverse transcription and, therefore, may alter transmission of HIV-1.

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