

Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection

(preintegration complex/nuclear import)

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ABSTRACT We have examined components of the preintegration complex of human immunodeficiency virus type 1 (HIV-1) and have analyzed features which govern the association of these components. HIV-1 nucleoprotein complexes, isolated from nuclear and cytoplasmic extracts of CD4⁺ cells after acute virus infection, contained viral RNA and DNA in association with viral matrix (MA), integrase (IN), and reverse transcriptase (RT) antigens but not capsid (CA) antigens and possessed integration activity *in vitro*. Association of IN but not RT or MA antigens with viral DNA was detergent-stable. Analysis of viral DNA synthesis and nuclear import of viral nucleoprotein complexes in the presence of a reversible RT inhibitor demonstrated that reverse transcription of viral RNA could be completed entirely in the host cell nucleus. Our studies demonstrate structural and functional features of the nucleoprotein (preintegration) complex of HIV-1 which are pertinent to the understanding of early events in the lentiviral life cycle.

In the life cycle of human immunodeficiency virus type 1 (HIV-1), molecular events following virus infection and preceding establishment of the integrated provirus are poorly characterized. Following virus-receptor interaction, viral proteins remain associated with viral nucleic acids in a high molecular weight nucleoprotein (preintegration) complex (1, 2). For retroviruses such as murine leukemia virus (MLV) the viral preintegration complex comprises viral DNA and integrase (IN) and capsid (CA) antigens (1), while preintegration complexes prepared from detergent and RNase-treated cytoplasmic extracts of HIV-1-infected cells comprise viral DNA and IN antigen only (2). The preintegration complex of HIV-1 is transported to the nucleus of the host cell by an active transport process which requires ATP but which is independent of cell division (3). These findings are supported by the demonstration that nondividing monocyte-derived macrophages (4) and CD4⁺ human epithelial cells (5) support HIV-1 provirus establishment.

To define features of the preintegration complex of HIV-1 which account for its active nuclear import characteristics, we have characterized components of the preintegration complex of HIV-1. We present evidence that the preintegration complex of HIV-1 comprises viral IN, matrix (MA), and reverse transcriptase (RT) antigens but not CA antigen in association with viral RNA and DNA. The association of RT antigen with the viral nucleoprotein preintegration complex during its nuclear import accounted in part for the ability of HIV-1 to undergo reverse transcription in the host cell

nucleus. These findings are important for the delineation of critical early events in the life cycle of HIV-1.

MATERIALS AND METHODS

Cell and Virus Stocks. Preparation of MT-4 cell culture stocks and cloned HIV-1 stocks, standardization of virus titers, and conditions for virus infection were as detailed (6). Prior to cell infection, the virus inoculum was treated with DNase (7).

Isolation and Analysis of HIV-1 Nucleoprotein Preintegration Complexes. HIV-1-infected MT-4 cells ($2-3 \times 10^8$) were harvested 15–18 hr postinfection (hpi) and lysed in hypotonic medium (8). Nuclear and cytoplasmic extracts (3) were fractionated on nonionic density gradients (1) and analyzed by immunoprecipitation and polymerase chain reaction (PCR) (3). Integration activity in cell extracts was analyzed as detailed (3, 9).

Immunoprecipitation of HIV-1 Nucleoprotein Complexes. Nuclear and cytoplasmic extracts were dialyzed (Spectrapor 6000, Spectrum Medical Industries) overnight at 4°C against 1 liter of a phosphate-buffered saline solution (pH 6.8) with 1 mM phenylmethanesulfonyl fluoride and 0.2 mM EDTA and then immunoprecipitated overnight at 4°C with polyclonal antiserum to HIV-1 MA or CA (obtained from Michael Phelan, University of California, Santa Barbara) or antiserum to HIV-1 IN (residues 1–16, 23–34, and 276–288; obtained from Duane Grandgenett, St. Louis University Medical School). Complexes were precipitated with protein A-Sepharose (10 mg per sample) and analyzed by PCR.

Western Blot Analysis of Gradient-Fractionated Nucleoprotein Complexes. Density-gradient fractions containing HIV-1 nucleoprotein complexes were dialyzed against PBS, lyophilized, resolved in SDS/polyacrylamide gradient gels, blotted to poly(vinylidene difluoride) filters (Immobilon-P, Millipore), and probed with monoclonal antibodies 4C9 and 9G5 to HIV-1 MA—both of which recognize MA p17 protein but not p55^{gag} protein (10)—or with pooled HIV-1-positive human serum. Blots were then incubated with alkaline phosphatase-conjugated anti-mouse or anti-human IgG and visualized with 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Composition of the Preintegration Complex of HIV-1. A prime consideration was to maintain the integrity of the viral

Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; BHAP, bis(heteroaryl)piperazine; IN, integrase; CA, capsid; MA, matrix; RT, reverse transcriptase; MLV, murine leukemia virus; hpi, hour(s) postinfection.

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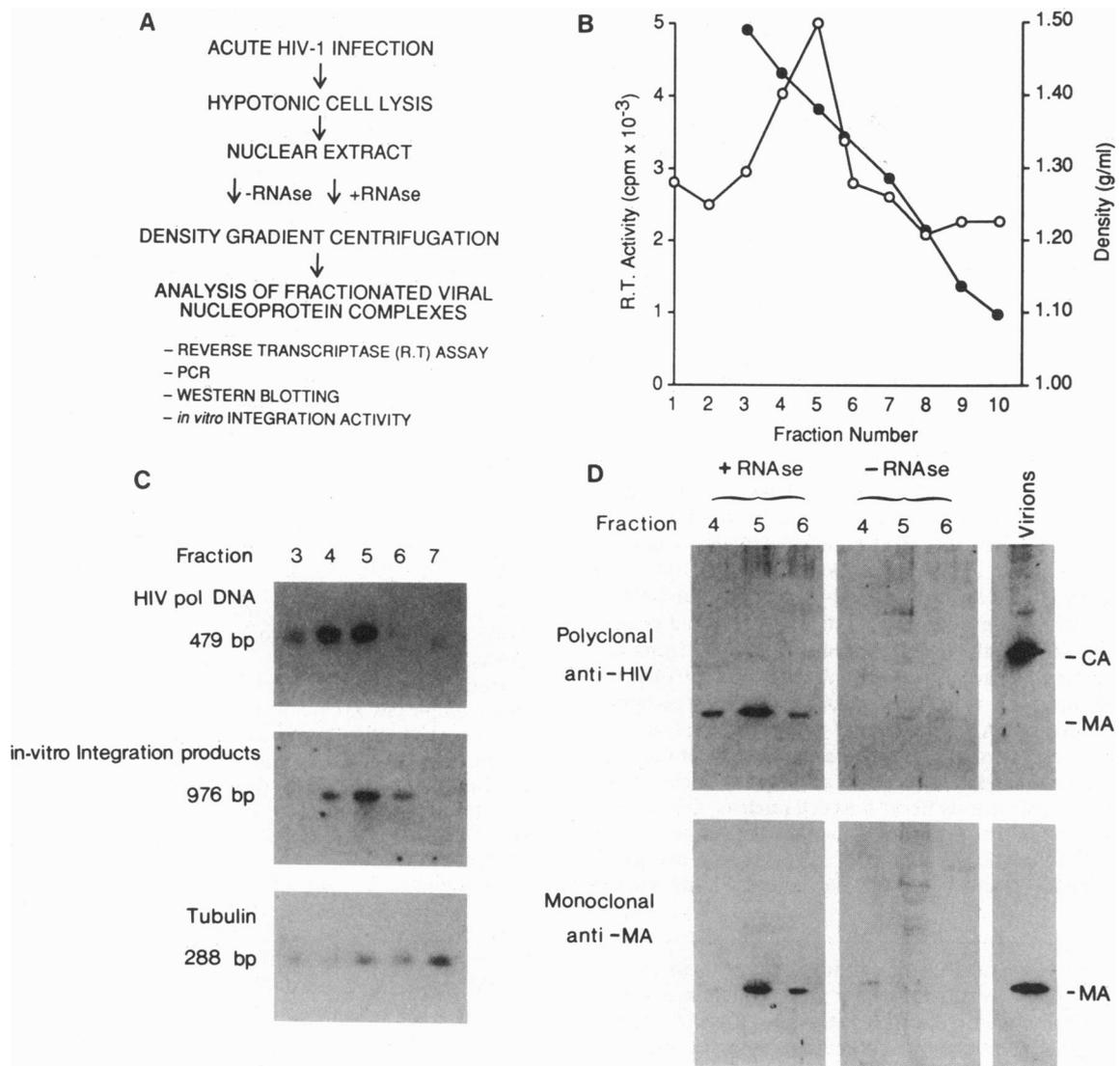


FIG. 1. Characterization of gradient-fractionated preintegration complexes of HIV-1. (A) CD4⁺ MT-4 cells were harvested 15 hpi and nuclear extracts were prepared following hypotonic cell lysis. RNase A-treated and untreated extracts were then fractionated by density gradient centrifugation. (B) Gradient fractions of non-RNase-treated extracts were analyzed for RT activity. (C) Viral DNA in each fraction was analyzed by PCR with HIV-1 *pol*-specific primers, and cellular DNA was identified with tubulin primers. Biological activity of viral nucleoprotein complexes was confirmed by their ability to integrate within heterologous DNA. *In vitro* integration products were identified by using nested HIV-1 long terminal repeat (LTR) primers as outlined in Tables 1 and 2. (D) RNase-treated and untreated nuclear extracts were further analyzed by Western blotting with pooled sera from HIV-1-seropositive individuals and with monoclonal antibodies to HIV-1 MA.

Table 1. HIV-1-specific oligodeoxynucleotides used as PCR primers and probes in this study

	Strand*	Coordinates	Sequence (5' to 3')
Primers			
<i>pol</i> I	+	2131-2149	TTCTTCAGAGCAGACCAG
<i>pol</i> J	-	2592-2610	ACTTTTGGGCCATCCATT
LTR U3	+	9194-9214	CAGATATCCACTGACCTTTGG
LTR U3'	-	9126-9143	GTGGTAGATCCACAGATC
LTR U3"	-	9099-9117	CTTGCTCTCGTTGGGAGTG
LTR R	-	9591-9610	GAGGCTTAAGCAGTGGGTTTC
LTR U5	+	9650-9679	GTCTGTTGTGTGACTCTGGT
LTR U5'	+	9668-9688	GTAAGTAGAGATCCCTCAGAC
<i>gag</i>	-	794-815	GCTTAATACTGACGCTCTCGCA
Probes			
R	+	9568-9587	GGGAGCTCTCTGGCTAACT
<i>pol</i>	+	2311-2331	GGAAGCTCTATTAGATACAGG
U5	+	9696-9713	GTCAGTGTGGAAAATCTC

Coordinates are numbered according to ref. 12. Only 3' LTR coordinates are given.

*+, complementary to negative strand; -, complementary to positive strand.

Table 2. Primer sets and probes used for PCR amplification

Primer set	Probe	Viral DNA forms amplified				<i>In vitro</i> integration products
		Early	Intermediate	Late	Circles	
LTR U3/R	R	+	+	+	-	-
<i>pol</i> I/J	<i>pol</i>	-	+	+	-	-
LTR U3/ <i>gag</i>	R	-	-	+	-	-
LTR R/U5	R	-	-	-	+	-
LTR U5/U3' (round 1), LTR U5'/U3" (round 2)	U5	-	-	-	-	+

preintegration complex during its isolation from acutely infected cells. The HIV-1 core complex, unlike that of other lentiviruses such as equine infectious anemia virus, is highly unstable at low detergent concentrations (11). Thus, gentle hypotonic cell-lysis conditions (Fig. 1A) were adopted in order to maintain the integrity of the HIV-1 nucleoprotein complex and to prevent dissociation of its components during isolation. Following acute HIV-1 infection, the viral nucleoprotein complex was analyzed as outlined in Fig. 1A. In both nuclear extracts (Fig. 1B) and cytoplasmic extracts (data not shown), RT activity was detected in gradient fractions with a density of 1.36 g/ml. In addition, viral DNA content (as determined by PCR with HIV-1 *pol* primers) and integration activity (Fig. 1C) were greatest in those fractions containing RT activity. When gradient fractions of nuclear extracts containing viral DNA, RT, and *in vitro* integration activity (fractions 4–6) were analyzed by Western blotting using HIV-1-positive human serum (Fig. 1D Upper) or monoclonal antibodies to HIV-1 MA (Fig. 1D Lower), MA antigen was detected. MA antigen in extracts prepared in the presence of RNase was far more evident (Fig. 1D Upper), suggesting that MA antigen was masked conformationally while associated with RNA. In contrast to previous studies with MLV (1), CA antigen was not detected in HIV-1 nucleoprotein complexes from acutely infected cells (Fig. 1D Upper). It is highly unlikely that the association of RT activity and MA antigen with viral nucleic acids was due to simple contamination of nuclear and cytoplasmic extracts with intact virions: (i) the absence of CA antigen in HIV-1 nucleoprotein complexes suggests that these complexes are not residual virus particles; (ii) intact virions separate at a higher density during gradient centrifugation; and (iii) viral preintegration complexes could not be identified in nuclear and cytoplasmic extracts from uninfected cells mixed with intact HIV-1 virions (data not shown).

Association of HIV-1 MA and IN Antigens with Viral Nucleic Acids. Association of MA with viral nucleic acids following acute HIV-1 infection was examined by immunoprecipitation of nuclear extracts with polyclonal CA, MA, and IN antibodies (Fig. 2). Analysis of immunoprecipitates by PCR with HIV-1 *pol*-specific primers demonstrated the association of viral DNA with both MA and IN antigens in nuclear extracts of HIV-1-infected cells (Fig. 2A). Treatment of nuclear extracts with detergent prior to immunoprecipitation resulted in disassociation of MA antigen with viral DNA (Fig. 2A Upper) but did not disrupt association of IN antigen and viral DNA (Fig. 2A Lower). In addition, antibodies to both HIV-1 MA and IN antigens were capable of immunoprecipitating genomic viral RNA (identified by reverse transcription-PCR of RNA) from nuclear extracts of acutely infected cells (Fig. 2B). At 0 hpi (just after addition of HIV-1), a low level of amplification product (representing early reverse transcription products encapsidated within the virion; refs. 13 and 14) was evident after reverse transcription-PCR of nuclear RNA, both in reaction mixtures containing RT and in those lacking the enzyme. At 8 and 24 hpi, viral RNA could be immunoprecipitated with MA (Fig. 2B Upper) and IN (Fig. 2B

Lower) antibodies. This RNA did not represent *de novo* synthesized transcripts following provirus establishment, since doubly spliced *tat* RNA species—the first HIV-1-specific RNAs produced in an infected cell (18)—were not detectable by PCR at 8 hpi and were present at only low levels by 24 hpi (data not shown). The ability to immunoprecipitate viral RNA with antibodies to MA was eliminated when nuclear extracts were treated with detergent (0.5% Triton X-100) (Fig. 2B), suggesting a weak association of MA with viral RNA and DNA within the context of the maturing preintegration complex. In agreement with data in Fig. 1,

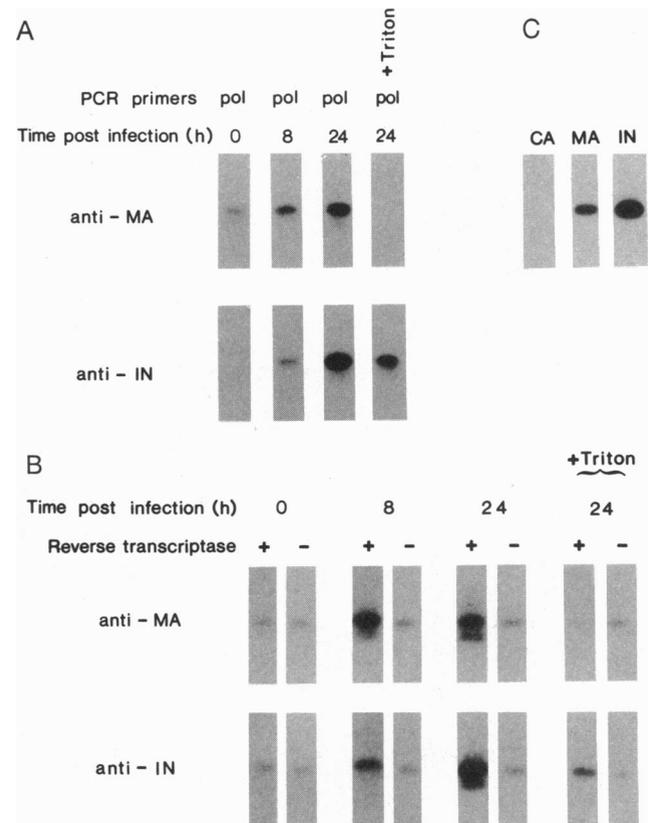


FIG. 2. HIV-1 MA and IN antibodies, but not CA antibody, immunoprecipitate viral DNA and RNA from nuclear extracts of HIV-1-infected CD4⁺ cells. At the indicated times after HIV-1 infection, nuclear extracts were prepared from hypotonic cell lysates and immunoprecipitated with polyclonal antibodies to CA, MA, or IN. Purified immunoprecipitates were then analyzed for the presence of viral DNA (A and C) or RNA (B) by PCR or reverse transcription-PCR, respectively, with HIV-1 *pol*-specific primers (Table 1). In Triton X-100-treated samples, nuclear extracts were first incubated in the presence of the detergent (0.5%, vol/vol) and then immunoprecipitated with MA or IN antibodies. Immunoprecipitation of viral DNA (identified by PCR with HIV-1 *pol*-specific primers) in nuclear extracts of CD4⁺ cells 24 hpi is compared for antibodies to HIV-1 MA, CA, and IN (C).

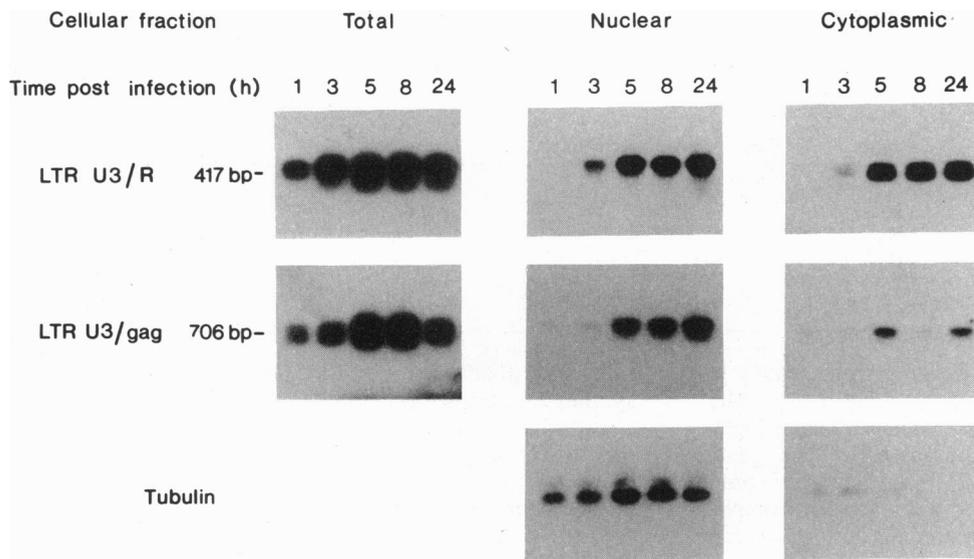


FIG. 3. Distribution of viral nucleic acids in nuclear and cytoplasmic extracts of acutely infected cells. At the indicated times post infection, cellular DNA from whole, nuclear, or cytoplasmic extracts was analyzed by PCR for the presence of early (LTR U3/R primers) and late (LTR U3/gag primers) products of HIV reverse transcription.

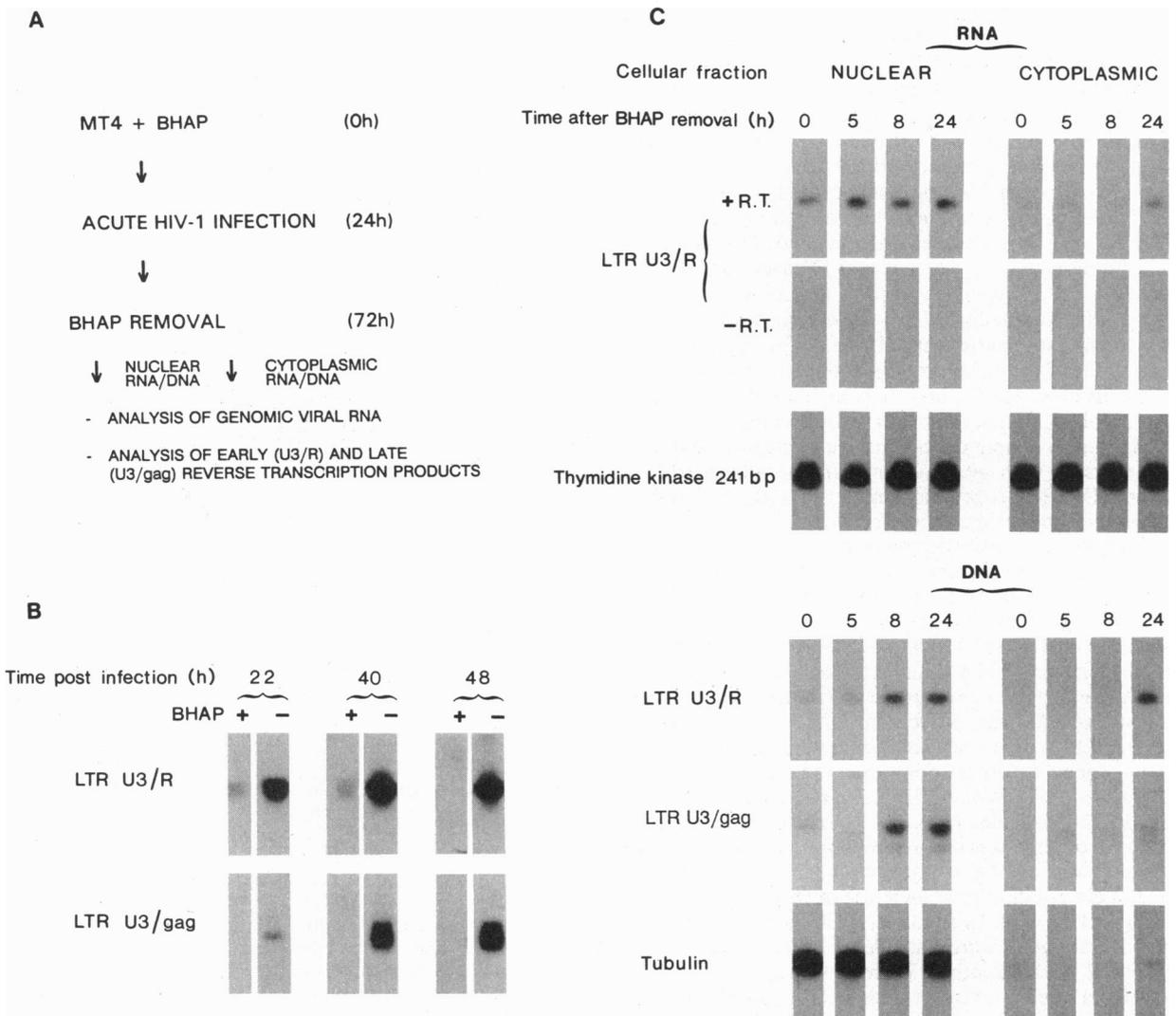


FIG. 4. Subcellular location of viral DNA synthesis before and after removal of a reversible RT inhibitor. Experimental outline is illustrated in Fig. 1 and in A. CD4⁺ MT-4 cells were pretreated with the BHAP nonnucleoside RT inhibitor for 24 hr and then infected with HIV-1 in the presence of BHAP. Virus-infected cells were maintained an additional 48 hr. Synthesis of early (LTR U3/R) and late (LTR U3/gag) reverse transcription products in cultures maintained with (+) and without (-) BHAP is shown in B. Forty-eight hours after HIV-1 infection, BHAP was removed and at the indicated times, the distribution of viral RNA (C Upper) and DNA (C Lower) was analyzed in nuclear and cytoplasmic cell extracts by reverse transcription-PCR.

polyclonal CA antibodies failed to immunoprecipitate viral DNA from cell extracts prepared 24 hpi (Fig. 2C) or at earlier times after infection (data not shown).

Completion of HIV-1 Reverse Transcription in the Nucleus. The association of RT with viral nucleic acids was demonstrated in viral nucleoprotein complexes purified from nuclei of acutely infected cells. To examine the biological significance of this association, we examined whether completion of HIV-1 DNA synthesis occurred in nuclear or cytoplasmic compartments of acutely infected cells (Fig. 3). Synthesis of both early and late (7) reverse transcription products of HIV (amplified by LTR U3/R and LTR U3/gag primers, respectively; Tables 1 and 2) in whole cell extracts was evident as early as 1 hpi, and both the abundance and rate of synthesis of early and late reverse transcription products were equivalent (Fig. 3). However, late products of reverse transcription were identified predominantly in nuclear extracts and were observed in greatly reduced amounts in cytoplasmic extracts of acutely infected cells. These results suggested that maturation of the viral preintegration complex coincided with transport of this complex from the cytoplasm to the nucleus of acutely infected cells.

Viral DNA Synthesis Is Not Required for Nuclear Import of HIV-1 Preintegration Complexes. To investigate the relationship between reverse transcription of genomic viral RNA and nuclear import of viral nucleoprotein complexes, reverse transcription was first arrested by infecting CD4⁺ cells in the presence of a reversible nonnucleoside RT inhibitor the bis(heteroaryl)piperazine (BHAP) compound U-87201 (15). Subcellular distribution of viral nucleic acids before and at various times after removal of the reverse transcription block was then analyzed. At the point of BHAP removal, genomic viral RNA was identified almost exclusively in the nucleus (Fig. 4C Upper). The renewal of the synthesis of early and late reverse transcription products was clearly evident by 8 hr after BHAP removal in nuclear extracts but not in cytoplasmic extracts (Fig. 4C Lower). By 24 hr after BHAP removal, both genomic viral RNA (Fig. 4C Upper) and viral cDNAs (Fig. 4C Lower) could be detected in cytoplasmic extracts. Taken together, these results indicate that nuclear import and integrity of viral preintegration complexes do not require the synthesis of viral DNA, supporting the tenet that both initiation and completion of reverse transcription can proceed entirely within the nucleus.

DISCUSSION

These studies address what viral components are contained within the preintegration complex of HIV-1 and what features govern the association of these components. We provide evidence that MA and IN antigens but not CA antigen are associated with viral DNA and RNA, in both the cytoplasmic and the nuclear compartments of acutely infected cells. Other studies have suggested that IN may be the sole component of the preintegration complex of HIV-1 (2). A possible explanation for the discrepancy is that in those studies, infected cells were lysed in the presence of detergent (0.5% Triton X-100), conditions that, as shown here, disrupt MA association with viral nucleic acids prior to analysis. A functional role for MA antigen is suggested by the presence of several putative nuclear localization sequences between residues 18 and 32 and between residues 110 and 114 (12), which, if functional, could influence nuclear import characteristics of the viral preintegration complex.

We provide evidence that HIV-1 can complete reverse transcription in the host cell nucleus, which suggests that DNA synthesis and nuclear import of viral nucleic acids proceed concurrently. Previous studies with MLV and HIV-1 have clearly demonstrated that reverse transcription can proceed to completion in the cytoplasm and that viral preintegration complexes possessing full *in vitro* integration activity can be isolated from that cellular compartment (2, 16). It is also possible that these cytoplasmic complexes contain incomplete products of reverse transcription and that, as demonstrated in an avian system, DNA synthesis is completed *in vitro* after complex isolation (17). It remains to be determined whether concurrent synthesis and nuclear import of HIV-1 DNA are characteristic of lentivirus replication or of retrovirus replication in general.

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