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Integration Is Required for Productive Infection of Monocyte-Derived Macrophages by Human Immunodeficiency Virus Type 1

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Certain human immunodeficiency virus type 1 (HIV-1) isolates are able to productively infect nondividing cells of the monocyte/macrophage lineage. We have used a molecular genetic approach to construct two different HIV-1 integrase mutants that were studied in the context of an infectious, macrophage-tropic HIV-1 molecular clone. One mutant, HIV-$1_{\Delta D(35)E}$, containing a 37-residue deletion within the central, catalytic domain of integrase, was noninfectious in both peripheral blood mononuclear cells and monocyte-derived macrophages. The HIV-$1_{\Delta D(35)E}$ mutant, however, exhibited defects in the assembly and/or release of progeny virions in transient transfection assays, as well as defects in entry and/or viral DNA synthesis during the early stages of monocyte-derived macrophage infection. The second mutant, HIV-$1_{D116N/8}$, containing a single Asp-to-Asn substitution at the invariant Asp-116 residue of integrase, was also noninfectious in both peripheral blood mononuclear cells and monocyte-derived macrophages but, in contrast to HIV-$1_{\Delta D(35)E}$, was indistinguishable from wild-type virus in reverse transcriptase production. PCR analysis indicated that HIV-$1_{D116N/8}$ entered monocyte-derived macrophages efficiently and reverse transcribed its RNA but was unable to complete its replication cycle because of a presumed block to integration. These data are consistent with the hypothesis that integration is an obligate step in productive HIV-1 infection of activated peripheral blood mononuclear cells and primary human macrophage cultures.

The growing number of primary human immunodeficiency virus type 1 (HIV-1) isolates containing intact IN coding regions is a strong indication that integration of viral DNA is an obligate step during productive lentivirus infections. Nonetheless, a series of conflicting reports during the past 10 years (2, 11, 22, 28) has raised questions about the requirement for lentiviral DNA integration, particularly when it was appreciated that members of this retroviral subfamily were able to infect nondividing monocyte-derived macrophages (MDM). This last property initially appeared to be in conflict with bromodeoxyuridine density-labeling studies involving Rous sarcoma virus in which cell replication was shown to be necessary for the integration of the newly synthesized viral DNA (13, 31). Further confounding the question of a requirement for integration were experiments reporting that DNA of the prototypical lentivirus, visna virus, was not covalently associated with high-molecular-weight DNA during a spreading infection in sheep choroid plexus cells but, instead, was present in a low-molecular-weight (unintegrated) form (11). The accumulation of significant amounts of unintegrated viral DNA in cells acutely infected with both visna virus (11) and HIV-1 (26) was also viewed by some as consistent with a replication model that utilized unintegrated lentiviral DNA as a template for mRNA synthesis (28).

A molecular-genetic approach, employing *Bal*31 deletion mutagenesis, had been used previously to show that viral DNA integration was an obligate step during infections initiated by the oncoretrovirus Moloney murine leukemia virus (24); when IN coding sequences were interrupted, infectivity was reduced by more than 300-fold. More recently, several groups have reported that mutations introduced into the IN coding sequences of infectious HIV-1 molecular clones result in the loss of infectivity for a variety of human T-cell lines (3, 6, 17, 23, 27, 29, 34). In many of these studies, the interruption of IN had pleiotropic effects involving a variety of steps in the virus life cycle. Small deletions, or even single amino acid substitutions, caused defective processing of precursor polypeptides, alterations in particle morphology, impaired viral DNA synthesis, reduced virion-associated reverse transcriptase (RT) activity, or smaller amounts of IN protein incorporation into released particles (3, 6, 27, 29, 34).

The demonstration that integration of HIV-1 DNA is necessary during acute infections of nondividing cells such as primary human MDM has been hampered by the unavailability of full-length molecular clones of HIV-1 capable of directing the synthesis of macrophage-tropic virus. Until very recently, the most compelling evidence (33) supporting a requirement for integration in human MDM consisted of Southern blot analyses of chromosomal DNA prepared from cells infected with the HIV-$1_{Ba\text{-}L}$ (9) macrophage-tropic isolate. The results of these analyses, however, did not eliminate the possibility that unintegrated viral DNA was simply trapped in the high-molecular-weight fraction.

In an attempt to definitively answer whether integration is required during productive HIV-1 infections of human MDM, mutations were introduced into the IN coding sequences of two different molecular clones, each capable of directing the synthesis of macrophage-tropic virus. Cloned proviral DNA was obtained by infecting human peripheral blood mononuclear cells (PBMC) with HIV-$1_{AD\text{-}87(M)}$ (21), a derivative of the macrophage-tropic HIV-1_{Ada} strain (10). Unintegrated, circularly permuted viral DNA was cloned from the Hirt su-

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pernatant fraction (12) with an *Eco*RI-digested lambda phage vector (29a). One of the clones obtained was inserted into pBR322 (pAD8-1) and, following transfection of HeLa cells, generated virus that was infectious in human MDM. This clone was subsequently converted to a two-long terminal repeat (two-LTR) linear HIV-1 proviral DNA, designated pAD8. A second wild-type, macrophage-tropic molecular clone used in these studies was a derivative of pNL4-3 (1), into which the 1.7-kbp fragment containing *env* coding sequences located between the *Kpn*I and *Bsm*I sites from the macrophage-tropic pAD8-1 clone was substituted for the corresponding region of pNL4-3. The resulting pNL(AD8) clone generates progeny virus that readily infects human MDM (8).

A primary MDM system requiring no exogenous cytokines was employed to assess the requirement of IN during productive virus infections (18). Elutriated monocytes were allowed to differentiate over a 14-day period in 100-mm^2 bacteriological petri plates (Corning; catalog no. S27050-100) and then were transferred to 24-well tissue culture plates (NUNC; catalog no. 1-48982; 10^6 cells per well) and allowed to adhere overnight prior to infection. The nonproliferating status of differentiated MDM cultures was documented by measuring [^{3}H]thymidine uptake by cultures of adherent primary human macrophages or HeLa cells, plated at initial densities of 3×10^6 and 5×10^4 cells, respectively, in individual wells of a 24-well plate. Cells were labeled for 18 h with 2.5 μCi of [^{3}H]thymidine (6.7 Ci/mmol) in 1 ml of medium at 3, 6, 9, and 12 days after plating. Following labeling, the cells were washed with phosphate-buffered saline, lysed in 0.1 M NaOH, and trichloroacetic acid precipitated before the precipitates were counted. As a negative control, similar amounts of [^{3}H]thymidine were added to wells containing no cells; the empty wells were also washed with phosphate-buffered saline and subjected to NaOH lysis plus trichloroacetic acid precipitation as described above. The MDM cultures incorporated less than 1% of the [^{3}H]thymidine incorporated by the HeLa cells (e.g., 2,600 versus 364,000 cpm). This level of [^{3}H]thymidine incorporation by MDM was essentially the same as that observed for the negative control (e.g., ~2,400 cpm).

The central region of the HIV-1 IN protein is the most highly conserved part of the molecule (6, 14, 15), containing the principal catalytic domain of the enzyme which mediates 3′ processing, DNA strand transfer, and disintegration activities, as measured by in vitro assays (4, 5, 15, 19, 30, 32). This portion of IN is relatively protease resistant (5) and includes the now well-recognized D,D(35)E motif required for catalysis (5, 15, 30, 32). The first IN mutant was constructed by introducing an in-frame 111-bp deletion (Fig. 1), encompassing the highly conserved D(35)E motif, into the 4.3-kbp *Pst*I-*Eco*RI fragment of pAD8 DNA by oligonucleotide-directed mutagenesis using pIBI30 as previously described (16). The 111-bp deletion within the *pol* gene was confirmed by nucleotide sequencing following reinsertion of the mutagenized fragment into pAD8 DNA; the resulting mutant was named $pAD8_{\Delta D(35)E}$. The second IN mutant consisted of an Asp-to-Asn substitution affecting the invariant D-116 residue within the D(35)E motif (Fig. 1). This substitution was introduced by PCR mutagenesis into the *pol* gene of pNL4-3, as previously described (6). The *Age*I-to-*Pfl*MI fragment (nucleotides 3485 to 5297 [20]) of the IN-defective $HIV\text{-}1_{IN/D116N}$ was substituted for the homologous 1.4-kbp *pol* fragment in pNL(AD8), generating molecular clone $pNL(AD8)_{D116N/8}$. Stocks of the two wild-type and the two IN-defective macrophage-tropic HIVs were prepared by transfecting HeLa cells with pAD8 and pNL(AD8) DNAs and $pAD8_{\Delta D(35)E}$ and $pNL(AD8)_{D116N/8}$ DNAs, respectively.

Virus infectivity in primary lymphocyte and macrophage cultures was monitored by RT assay (7), an indicator of a spreading virus infection. In this experiment, approximately equal amounts of virus (based on virion-associated RT activity present in the supernatants collected from transfected HeLa cells) were used as inocula. As shown in Fig. 2, the two wild-type virus preparations [AD8 and NL(AD8)] readily infected PBMC (panel A) or MDM (panel B). T-cell line-tropic virus strains such as $HIV_{NL4\text{-}3}$ are unable to replicate in this MDM system (8). Figure 2 clearly shows that both of the IN mutant virus preparations failed to initiate spreading infections in either phytohemagglutinin-stimulated primary lymphocytes or MDM during 2 to 4 weeks of observation.

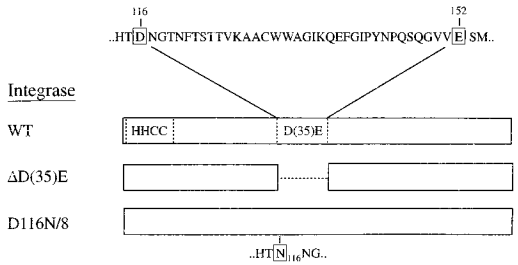

FIG. 1. Structures of wild-type and mutant integrase proteins. The amino acids shown at the top are residues 114 to 154 of the integrase protein encoded by the macrophage-tropic $HIV\text{-}1_{AD8}$ strain (29a). For the wild-type (WT) IN, the approximate locations of the Zn finger-like HHCC domain and the D(35)E sequence of the central, catalytic region are indicated by dashed lines. The mutant ΔD(35)E expresses an IN protein which lacks residues 116 to 152, inclusive, and was constructed by introducing an in-frame deletion of nucleotides 346 to 456 into the IN coding sequence of pAD8. The mutant D116N/8 bears an Asp-to-Asn substitution at residue 116 and was constructed by cloning the 1,813-bp *Age*I-to-*Pfl*MI fragment from the pNL4-3-derived mutant IN/D116N (6) into pNL(AD8) (8).

Because the introduction of *pol* mutations into molecular clones of HIV-1 has been reported to disrupt a number of steps in the virus life cycle (3, 6, 27, 29, 34), it has been difficult to identify mutations that solely impair the integration of viral DNA into the host cell chromosome. Consequently, the *pol* mutations under study here were evaluated for possible defects perturbing two critical points during virus replication: (i) entry into MDM and subsequent reverse transcription of the viral genome and (ii) the assembly and/or release of progeny virus particles. The effects of the *pol* mutations on the latter function(s) were evaluated by measuring the RT activity released into the supernatant medium 48 h after transfection of HeLa cells with plasmid DNA. As shown in Fig. 3A, the deletion eliminating the catalytic domain of IN reduced RT production by more than twofold. In independent experiments, a 2.5- to 5-fold decrease in RT production was observed with $pAD8_{\Delta D(35)E}$ (data not shown). In contrast to these results, the $pNL(AD8)_{D116N/8}$ point mutant released about the same amount of RT activity as its wild-type pNL(AD8) parent (Fig. 3A). This result is in excellent agreement with a previous analysis of this particular *pol* mutation which demonstrated that pelleted $HIV\text{-}1_{IN/D116N}$ particles contained wild-type amounts of p24 Gag, p66 RT, and p32 IN proteins (6).

A possible defect affecting the reverse transcription of the $HIV\text{-}1_{\Delta D(35)E}$ and $HIV\text{-}1_{D116N/8}$ viral genomes following entry into MDM was investigated by subjecting cells, harvested at 0 and 24 h postinfection, to DNA PCR analysis. The primers chosen to amplify the 3′ region of *pol* in $HIV\text{-}1_{AD8}$, $HIV\text{-}1_{NL(AD8)}$, and $HIV\text{-}1_{D116N/8}$ would be expected to generate a 580-bp product, whereas $HIV\text{-}1_{\Delta D(35)E}$ would produce a smaller, 469-bp fragment. As shown in Fig. 3B, PCR products of the predicted sizes were synthesized in the infected MDM

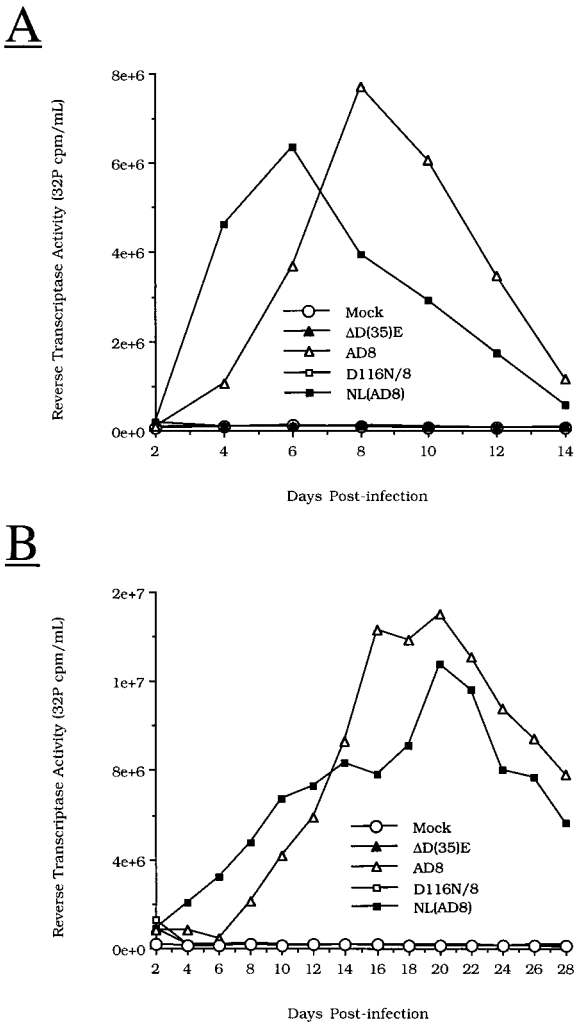

FIG. 2. Infectivity of wild-type and integrase mutant viruses. (A) Infection of primary human PBMC. Ficoll-Hypaque-purified PBMC (10^6/ml) were stimulated with 250 ng of phytohemagglutinin-P (Burroughs Wellcome; catalog no. HA17) for 96 h in RPMI 1640–10% fetal bovine serum. Cells were then washed once with phosphate-buffered saline, resuspended at 10^6/ml in RPMI 1640–10% fetal bovine serum, supplemented with 64 NIH-BRMP U of interleukin-2 (Pharmacia; catalog no. 6011) per ml, and infected with HIV-$1_{\Delta D(35)E}$, HIV-1_{AD8}, HIV-$1_{D116N/8}$, or HIV-$1_{NL(AD8)}$ at a multiplicity of infection of 10^{-3} or mock infected (mock). (B) Infection of primary human MDM at the same multiplicity as for panel A and with the same inocula. Infection and culture of MDM were carried out as described in the text.

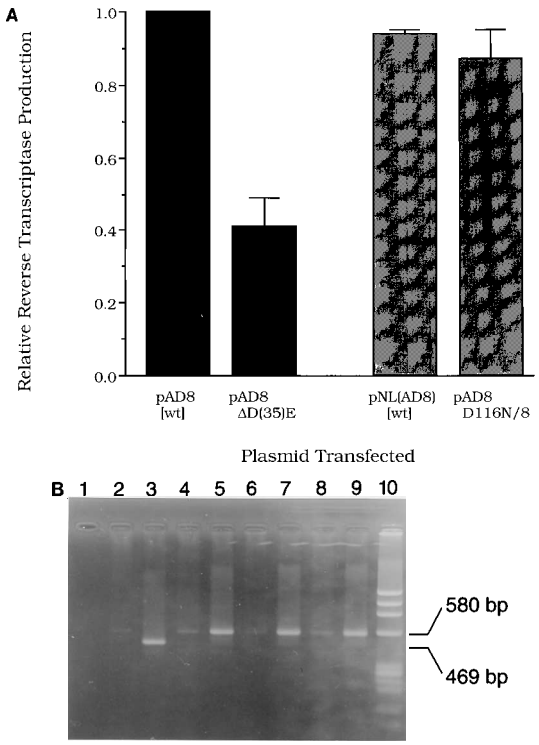

FIG. 3. (A) Relative production of wild-type (wt) and mutant integrase viruses by transfected HeLa cells. Confluent HeLa monolayers propagated in 25-cm^2 flasks were transfected with 10 μg of proviral DNA by calcium phosphate coprecipitation (Stratagene; catalog no. 200285). As a control for transfection efficiency, 0.1 μg of pXGM5 (25) was included in each transfection mixture. The supernatants were harvested 48 h posttransfection, passed through 0.45-μm-pore-size filters, and assayed for RT activity (7) and human growth hormone (Nichols Institute; catalog no. 40-2205) levels. Aliquots of virus were stored at −70°C. Data are expressed as the fraction of RT activity produced relative to that for cells transfected with pAD8. The values plotted represent the simple averages of three independent transfections, ± the standard errors, and have been normalized relative to values for human growth hormone expression. (B) Viral DNA production in primary MDM cultures. Infected MDM cultures were harvested at 0 and 24 h postinfection. At the time of harvest, the cultures were washed twice with phosphate-buffered saline, treated with 50 U of DNase I (Promega; catalog no. M6101) at 37°C for 15 min, washed twice again with phosphate-buffered saline, and treated with trypsin-EDTA (Gibco; catalog no. 610-5300AG) at 37°C for 30 min. The cells were then washed twice with phosphate-buffered saline and lysed in 10 mM Tris–1 mM EDTA–0.001% Triton X-100–0.0001% sodium dodecyl sulfate–proteinase K (100 μg/ml; Boehringer Mannheim Biochemicals; catalog no. 161 519) at 56°C for 1 h. Proteinase K was inactivated by incubation of the lysates at 95°C for 10 min. The lysates were frozen on dry ice and stored at −20°C. For PCR analysis, 25-μl aliquots of each lysate (equivalent to 1.5×10^5 cells) were amplified in a 50-μl reaction volume adjusted to 10 mM Tris (pH 8.3)–50 mM KCl–2 mM $MgCl_2$–200 μM (each) dATP, dCTP, dGTP, and dTTP–0.02% gelatin–1.25 U of *Taq* polymerase (Perkin-Elmer). The primers used for amplification had the following sequences: ΔIN+, 5′-TAGCCAGCTGTGATAAATGTC-3′ (NL4-3 positions 4339 to 4359 [20]); and ΔIN−, 5′-CCCTGTAATAAACCCGAAAAT-3′ (NL4-3 positions 4894 to 4914 [20]). Amplification was carried out according to the following protocol: preheating at 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 60°C for 30 s, and 72°C for 2 min. Lanes: 1, lysis buffer alone; 2 and 3, HIV-$1_{\Delta D(35)E}$-infected macrophages at 0 (lane 2) and 24 (lane 3) h postinfection; 4 and 5, HIV-1_{AD8}-infected macrophages at 0 (lane 4) and 24 (lane 5) h postinfection; 6 and 7, HIV-$1_{D116N/8}$-infected macrophages at 0 (lane 6) and 24 (lane 7) h postinfection; 8 and 9, HIV-$1_{NL(AD8)}$-infected macrophages at 0 (lane 8) and 24 (lane 9) h postinfection; 10, ϕX-174–*Hae*III molecular weight standards. The positions of PCR products are indicated on the right.

by 24 h postinoculation. In this and other experiments, no impairment in viral DNA synthesis was ever detected in human MDM cultures infected with HIV-$1_{D116N/8}$. This was not the case for HIV-$1_{\Delta D(35)E}$-infected MDM, which invariably contained smaller amounts of viral DNA 24 h following infection than the wild-type virus. In other experiments of this type, PCR analyses indicated that cells exposed to HIV-$1_{\Delta D(35)E}$ produced significantly less viral DNA than those infected with the wild type at 1, 2, 4, and 14 days after infection (data not shown). This result presumably reflects the effect(s) of impaired entry and/or reverse transcription of HIV-$1_{\Delta D(35)E}$ progeny particles.

Other investigators have mutagenized the highly conserved Asp residue at position 116 within the D(35)E motif of IN, and have reported conflicting results with respect to its effect on virus production. Shin et al. (27) reported that an Ala substitution at this position in HIV-1_{HXB2} severely impaired processing of the $Pr55^{Gag}$ precursor and was associated with the release of morphologically abnormal particles containing virtually no detectable RT activity. In contrast, two other groups have reported that Ala at this position does not impair particle

production (3, 34). A different substitution at the same residue (D116E) also resulted in no alterations of virus assembly (29). We have also recently reported that the D116N substitution present in the T-cell line-tropic strain HIV-1_{NL4-3} caused no deleterious effects on virion morphology or virus production (6).

Our evaluation of viral DNA synthesis within 24 h of MDM infection with HIV-$1_{D116N/8}$ revealed no discernable difference from infection with wild-type virus (Fig. 3B). In a previous study, we reported that T cells productively infected with virus containing the identical D116N mutation accumulated large amounts of unintegrated circular DNA relative to total viral DNA, as determined by analysis with primers that amplified sequences unique to two-LTR-containing circular DNA (6). Increased amounts of circular DNA following infection of HeLa-CD4-LTR-β-gal cells with a D116A IN mutant of HIV-1_{HXB2} have also been recently reported (34). Although it has been suggested that the unintegrated circular viral DNA, accumulating in HeLa-CD4-LTR-β-gal cells following infection with IN mutants such as HIV-$1_{D116N/8}$, may direct the synthesis of Tat mRNA and Tat protein (thereby generating significant numbers of blue colonies) (34), this process, even if it occurs in acutely infected MDM, fails to sustain a detectable spreading viral infection (Fig. 2B).

Despite the recent retraction of a report describing the infectivity of an IN-defective simian immunodeficiency virus (22), the question of whether integrated lentivirus DNA is required for productive infection continues to be raised (2). The loss of infectivity for primary macrophage cultures as a consequence of a single amino acid substitution within the catalytic domain of IN, which has no demonstrable effect on virus entry, reverse transcription, assembly, or release, is strong evidence that impaired integration is solely responsible for the observed replication defect. The failure of HIV-$1_{D116N/8}$ to infect MDM is in agreement with a similar, recently published study (34) and provides molecular genetic evidence that a functional HIV-1 IN protein is required for the productive infection of both activated PBMC and nondividing primary human macrophage cultures.

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