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Construction and Characterization of a Temperature-Sensitive Human Immunodeficiency Virus Type 1 Reverse Transcriptase Mutant

MINGJUN HUANG,† RALF ZENSEN, MICHAEL CHO, AND MALCOLM A. MARTIN*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

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A temperature-sensitive (*ts*) human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) mutant was generated by charged-cluster-to-alanine mutagenesis. The mutant virus, containing three charged residues within the RT finger domain changed to alanine (K64A, K66A, and D67A), replicated normally at 34.5 but not 39.5°C. Quantitating virus particle production by p24 antigen capture or virion-associated RT activity and virus infectivity by the MAGI cell assay, we found that (i) mutant virions produced at the permissive temperature were indistinguishable from wild-type virus in assays performed at the nonpermissive temperature, suggesting that the *ts* mutation did not impair early steps in the virus replication cycle and that the mutant RT enzyme was not *ts*; and (ii) virus particle production in cells transfected with the *ts* mutant at the nonpermissive temperature was comparable to that of wild-type virus. However, the particle-associated RT activity and infectivity of mutant virions produced at the nonpermissive temperature were greatly reduced when assays were conducted at the permissive temperature. These results are consistent with an irreversible *ts* event affecting RT that occurs during virus particle production. Radioimmunoprecipitation analyses revealed that both p66 and p51 RT subunits were absent from mutant virions generated at 39.5°C. The presence of normal levels of HIV-1 integrase in mutant particles produced at the nonpermissive temperature was inconsistent with defective Gag-Pol synthesis or Gag-Pol incorporation into progeny virions. Furthermore, wild-type levels of the mutant Pr160$^{gag\text{-}pol}$ were detected in virions produced at the nonpermissive temperature when the HIV-1 protease was inactivated by site-specific mutagenesis. Taken together, these results are most consistent with a *ts* defect affecting the degradation or aberrant processing of the mutated RT during its processing/maturation within nascent particles.

The conversion of viral genomic RNA into unintegrated double-stranded linear DNA molecules during the early phase of the productive virus infection is the signature of *Retroviridae*. This reaction is catalyzed by reverse transcriptase (RT), encoded by the highly conserved *pol* gene found in retroviruses and eukaryotic retrotransposable elements. In the case of human immunodeficiency virus type 1 (HIV-1), the mature RT is a heterodimer (13), consisting of 66- and 51-kDa subunits that are colinear at their N termini. The larger subunit contains DNA polymerase and RNase H domains, whereas p51 lacks the C-terminal RNase H domain. Retroviral RTs possess two enzymatic activities: (i) a DNA polymerase that can use RNA or DNA templates and (ii) RNase H, which degrades genomic RNA present in DNA-RNA intermediates.

Crystal structures of unligated HIV-1 RT (44) or RT complexed with the non-nucleoside inhibitor Nevirapine (28) or an 18/19-mer oligonucleotide (23) have been reported. The structure of the polymerase domain of the 66-kDa component within the heterodimer has been likened to a right hand, possessing finger, palm, thumb, and connection domains. Although the same subdomains are present in p51, they differ in arrangement: both the DNA binding groove and polymerase active site of p66 are missing from p51.

As is the case for other retroviruses, the *pol* gene of HIV-1 encodes a high-molecular-weight Gag-Pol precursor (Pr160$^{gag\text{-}pol}$) that is incorporated into nascent particles as a result of its noncovalent association with the Gag precursor polyprotein Pr55^{gag} (40, 47, 54). Processing of Pr160$^{gag\text{-}pol}$ by the HIV-1-encoded protease (PR) during virus budding from the cell membrane gives rise to the mature, virion-associated PR and integrase (IN) proteins as well as the p66 and p51 forms of the RT heterodimer (25). Subsequent to adsorption, fusion, and entry into a newly infected cell, the particles are partially uncoated and commence synthesizing DNA copies of their genomic RNAs as they traverse the cytosol. Thus, the generation of full-length linear viral DNAs by RT during the early phase of infection reflects the successful completion of multiple biosynthetic, metabolic, and enzymatic reactions in both the virus-producing and newly infected cells.

The identification and characterization of RT mutants has proven to be invaluable for integrating structural and functional properties of this critical HIV-1 protein. Both in vitro mutagenesis of the HIV-1 RT and studies of drug-resistant RT variants have delineated several functionally important domains, the majority of which involve its DNA-polymerizing activity (2–9, 11, 16, 22, 24, 26, 30, 32–34, 36, 43). We have attempted to create conditional RT mutants affected in one or more of the myriad of processes and functions that must be completed during a productive virus infection. The charged-cluster-to-alanine mutagenesis approach, previously used to generate temperature-sensitive (*ts*) mutants of cellular and viral genes (12, 19, 50, 55), was employed to alter exposed regions of the HIV-1 RT. Such mutants may exhibit impaired interaction with other cellular or viral proteins or be unable to

* Corresponding author. Mailing address: Laboratory of Molecular Microbiology, NIAID, NIH, 4 Center Dr., Building 4, Room 315, Bethesda, MD 20892-0460. Phone: (301) 496-4012. Fax: (301) 402-0226. E-mail: malm@NIH.gov.

† Present address: Laboratory of Antiviral Drug Mechanisms, SAIC Frederick, NCI-FCRDC, Frederick, MD 21702.

stably associate with the viral RNA template or the DNA product of the reverse transcription reaction.

In this report, we describe the construction and characterization of a *ts* mutation affecting the HIV-1 RT. When charged residues at positions 64, 66, and 67 within the finger domain of RT were changed to alanine, the resultant virus replicated at the permissive (34.5°C) but not the restrictive (39.5°C) temperature. Further characterization of the *ts* mutant indicated that progeny virions produced at 39.5°C possessed no RT activity, contained no detectable or only background levels of p66 and p51, and were not infectious. In contrast, the mutant (mt4) particles produced at 34.5°C possessed RT activity when assayed at 34.5 or 39.5°C, contained the RT heterodimeric protein, and were infectious in MAGI cells at both temperatures. The *ts* step in the life cycle of mt4 occurs in virus-producing cells subsequent to the incorporation of Pr160$^{gag\text{-}pol}$, very likely during the processing of the Gag-Pol precursor in nascent particles.

MATERIALS AND METHODS

Construction of RT mutants. Recombinant plasmids were constructed and used to transform bacteria by standard procedures. The full-length infectious molecular clone of HIV-1_{LAI} (pLAI [41]) was used in these experiments as the wild-type control and as the source of mutant derivatives. A 2,219-bp *Bcl*I-*Eco*RI fragment (nucleotides 2511 to 4730) from pLAI was subcloned into M13mp18. Oligonucleotide-directed mutagenesis was performed as previously described (29) to construct 33 charged-cluster-to-alanine mutations within the RT coding region of the *pol* gene. We defined a charged cluster as containing at least two charged amino acid residues within a group of five consecutive residues. In most mutants, at least two charged residues within a cluster were changed, although occasionally only one residue was altered. Each set of mutations created a novel *Bst*UI restriction endonuclease site which allowed the presence of the introduced mutations to be readily identified. A 1,057-bp *Bcl*I-*Pfl*MI (nucleotides 2511 to 3568) or a 1,162-bp *Pfl*MI-*Eco*RI (nucleotides 3568 to 4730) fragment, carrying the desired mutation, was excised from the mutagenized DNA and cloned back into pLAI. The presence of putative mutations in viral mutants that failed to replicate following transfection of CEM (12D7) cells and the absence of undesired changes in subcloned *pol* gene segments were confirmed by DNA sequencing.

Cell culture, transfection, and infection. HeLa cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum, 100 U of penicillin G sodium per ml, and 0.1 mg of streptomycin sulfate per ml at 37°C unless otherwise specified. HeLa cells were transfected by the calcium phosphate precipitation method as previously described (14). Forty-eight hours posttransfection, culture supernatants were tested for RT activity and p24 Gag protein as outlined below. Transfected cell supernatants were filtered through a 0.45-μm-pore-size filter for use as virus stocks.

CEM (12D7) cells (45) were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U of penicillin G sodium per ml, and 0.1 mg of streptomycin sulfate per ml. CEM (12D7) cells were transfected using DEAE-dextran or infected with cell-free virus preparations as previously described (14). For infections, an amount of the wild-type HIV-1_{LAI}, prepared at 37°C from transfected HeLa cells, equivalent to 10^6 ^{32}P-RT cpm was used to infect 5×10^6 CEM (12D7) cells. This HIV-1_{LAI} inoculum contained approximately 5 ng of p24 Gag protein; this amount of *ts* mutant virus was also used in infectivity assays.

The CD4-positive long terminal repeat–β-galactosidase-expressing HeLa (MAGI) cell indicator line (27) was obtained from the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and maintained in DMEM supplemented with 5% fetal bovine serum, 100 U of penicillin G sodium per ml, 0.1 mg of streptomycin sulfate per ml, 0.2 mg of G418 sulfate per ml, and 0.1 mg of hygromycin B per ml. Cells were seeded at a density of 4×10^4 per well in a 24-well plate 24 h prior to infection. Samples of virus (5 ng of p24 in 150 μl) were adsorbed to cells in the presence of 3 μg of DEAE-dextran for 3 h at 34.5°C, 2 h at 37°C, or 1 h at 39.5°C prior to the addition of 1 ml of medium. Following incubation for 72 h at 34.5°C, 48 h at 37°C, or 40 h at 39.5°C, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as previously described (27).

RT and p24 assays. The RT assay was performed as reported previously (51), with the addition of 0.8 mM EDTA to the reaction cocktail. Briefly, 10 μl of cell-free supernatant from infected-transfected cells was added to 50 μl of RT cocktail for 3 h at 37°C unless otherwise specified. A 5-μl aliquot was spotted onto DE-81 paper, washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and counted in a liquid scintillation counter.

The amount of p24 Gag protein in cell-free supernatants was determined by enzyme-linked immunosorbent assay (Coulter Immunology), using the instructions and standards supplied by the manufacturer. Samples from transfected CEM (12D7) cultures were diluted 2,000- to 4,000-fold in RPMI 1640 medium containing 10% fetal bovine serum, and samples from transfected HeLa cultures were diluted 5,000- to 10,000-fold in DMEM containing 5% fetal bovine serum so that they were in the linear range of the assay.

Metabolic labeling and radioimmunoprecipitation. The procedure used for metabolic labeling of transfected HeLa cells has been previously described (21), although the incubation temperatures were modified for these experiments. Cells were initially transfected at 37°C for 3 h. The cultures were then incubated at 34.5°C for 54 h or at 39.5°C for 40 h and then labeled for 16 h at 34.5°C or 12 h at 39.5°C.

Transfected CEM (12D7) cells were incubated at 36 ± 0.5°C, and the medium was changed at 2-day intervals up to the time when syncytium formation was first observed (usually around 11 days posttransfection). Cells from 6-ml cultures (approximately 10^7 cells) were pelleted, washed, and plated in 25-cm^2 tissue culture flasks in 2 ml of methionine-free RPMI 1640 medium supplemented with 10% fetal bovine serum. [^{35}S]methionine (500 μCi) was added, and the cells were incubated for 16 h at 34.5°C or for 12 h at 39.5°C. Following the labeling period, the cells were harvested and pelleted by a brief spin in a microcentrifuge. The cell-free supernatants were filtered through a 0.45-μm-pore-size filter, and virion-associated proteins were collected by pelleting for 30 min at 35,000 rpm in an SW50.1 rotor (Beckman).

Pulse-chase experiments were initiated when syncytia became visible in transfected CEM (12D7) cells maintained at 36 ± 0.5°C (approximately on day 10 or 11). Cells from 6 ml of CEM (12D7) cultures were used for each pulse-chase experiment. The remaining steps were performed as described previously (53) except that the incubation temperature was maintained at either 34.5 or 39.5°C. At the conclusion of each pulse and chase period, the CEM (12D7) cells were pelleted and the supernatants were filtered. To obtain virion-associated proteins, 200 μl of cell-free supernatant was pelleted for 99 min at 14,000 rpm in a Tomy high-speed microcentrifuge. The cell-free, virion-free supernatant fractions from the microcentrifugation were also collected after each spin.

Immunoprecipitation was carried out as previously described (52) with the indicated antibodies. AIDS patient sera and a sample of a rabbit anti-HIV-1 RT polyclonal antibody (anti-RT Ab 1) were obtained from National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (human HIV immune globulin, catalog no. 192; rabbit HIV-1 RT antiserum, catalog no. 634). Another anti-HIV-1 RT rabbit polyclonal antibody (anti-RT Ab 2) was purchased from Intracell (Cambridge, Mass.), and anti-HIV-1 IN monoclonal antibody 35 was generously provided by Stephen Hughes. All precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis in 10% acrylamide-AcrylAide (FMC) gels. The gels were fixed in methanol and acetic acid, treated with 1 M salicylic acid, dried at 100°C, and placed on Biomax MR film (Kodak).

Immunoblot analysis of virion-associated proteins. Cell-free supernatants from transfected HeLa cells were pelleted as described above. Equivalent amounts of each virus preparation, as measured by p24 content, were subjected to SDS-PAGE (10% gel) and then transferred to nitrocellulose membranes. Binding of an anti-HIV-1 RT rabbit polyclonal antibody (Intracell) to virion-associated RT subunits was visualized by chemiluminescence using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G as described in the protocol of the manufacturer (Pierce).

RESULTS

Construction and properties of an RT *ts* HIV-1 mutant. The charged-cluster-to-alanine mutagenesis approach (12, 19, 50, 55) was used to generate potential *ts* mutations affecting HIV-1 RT coding sequences. This mutagenesis protocol, outlined in Materials and Methods, involved the substitution of alanine residues in charged regions of the RT coding segment of the pLAI infectious molecular clone, which was derived from the HIV-1_{LAI} isolate (41). pLAI DNAs, containing different mutagenized RT regions, were transfected into HeLa and CEM (12D7) cells, and progeny virus production at 34.5, 37, and 39.5°C was monitored by RT activity released into the medium. None of these RT mutations conferred a *ts* phenotype, although several resulted in mutant viruses that had lost infectivity for CEM (12D7) cells at all temperatures tested (Table 1). One of these (mt1), with alterations affecting amino acids 64 through 67 of RT, failed to establish a spreading infection in CEM (12D7) cells but did generate wild-type levels of progeny virions following transfection of HeLa cells, as determined by p24 antigen capture assay (data not shown). Interestingly, the virion-associated RT activity measured at 39.5°C was approximately fourfold lower than that determined at 34.5°C. Postulating that the mt1 mutation might be located within a potential *ts* region of the HIV-1 RT and that the four-residue alanine

TABLE 1. RT mutants unable to infect CEM (12D7) cells at any temperature[a]

Mutant no.	Amino acid changes
1	K64A, K65A, K66A, D67A
3	K64R, K65A
26	K527A, K528A, E529A
29	D218A, K219A, K220A
32	R72A, K73A
32a	W71F, R72A

[a] Amino acids are numbered with respect to the first residue of the HIV-1 RT protein. Infectivity was monitored by measuring the RT released into the medium from transfected CEM (12D7) cells during a 2-week (39.5 and 37°C) or 3-week (34.5°C) observation period.

substitution might be too incapacitating, three additional mutants (mt2 to mt4) were constructed. As shown in Table 2, a lysine-to-alanine substitution at residue 65 (mt1 and mt3) resulted in loss of infectivity at all temperatures, and replacement of only residues 66 and 67 (mt2) resulted in a virus with a wild-type phenotype. Only mt4, with alanine substitutions at amino acids 64, 66, and 67, exhibited a consistent *ts* phenotype following infection of CEM (12D7) cells.

The infection kinetics of the HIV-1 mutant mt4 and its wild-type HIV-1_{LAI} parent at 34.5, 37, and 39.5°C in transfected CEM (12D7) cells are presented in Fig. 1. The spread of wild-type virus throughout the infected cultures, monitored by both peak RT activity and p24 antigen released into the supernatant medium, was greatest at 37°C, somewhat diminished at 39.5°C, and markedly reduced at 34.5°C. In contrast, mt4 exhibited virtually no infectivity at 39.5°C, as measured by RT activity or p24 production. At the permissive temperature of 34.5°C, mt4 infectivity, as measured by p24 antigen capture, was indistinguishable from that of the wild-type HIV-1_{LAI}. When monitored by release of RT activity into the medium, the spread of mt4 through the CEM (12D7) cell cultures relative to wild-type HIV-1_{LAI} was inversely related to incubation temperature.

A *ts* defect is observed during the production of mt4 particles at the nonpermissive temperature. To ascertain whether the observed loss of mt4 infectivity at 39.5°C reflected impaired synthesis, processing, assembly, or release of progeny virions at the restrictive temperature, HeLa cells, which lack surface CD4 and therefore support only the postintegration steps of the virus life cycle, were transfected with wild-type or mt4 mutant viral DNA at various temperatures, and the properties of the particles released into the medium were examined (Table 3). The RT activity associated with mt4 particles produced at 34.5°C was readily detected when assayed at 34.5, 37, and 39.5°C, although the levels measured were consistently two- to threefold lower than wild-type virus levels. In contrast, the RT activity associated with mt4 produced at 39.5°C was 34- to 57-fold lower than that of the wild-type HIV-1_{LAI}, irrespective of the temperature at which the RT assay was conducted. Thus, mutant particles produced at the nonpermissive temperature displayed greatly reduced RT activity. However, equivalent numbers of mt4 and HIV-1_{LAI} progeny virions, as measured by p24 antigen levels in the culture supernatants, were released from the transfected HeLa cells at the permissive and nonpermissive temperatures (Table 3).

TABLE 2. Replication of RT mutants in CEM (12D7) cells

Provirus	Amino acid at residue[a]:				Replication[b] at:		
	64	65	66	67	34.5°C	37°C	39.5°C
Wild type	K	K	K	D	Yes	Yes	Yes
mt1	A	A	A	A	No	No	No
mt2	K	K	A	A	Yes	Yes	Yes
mt3	R	A	K	D	No	No	No
mt4	A	K	A	A	Yes	Yes	No

[a] Numbered with respect to the first residue of the RT protein.
[b] Monitored by measuring RT released from transfected CEM (12D7) cells during a 2-week (39.5 and 37°C) or 3-week (34.5°C) observation period.

A similar phenotype was observed in the single-cycle MAGI cell infectivity assay. HIV-1 mutant mt4 produced at 34.5°C displayed infectivities indistinguishable from those of wild-type virus at all assay temperatures, whereas mutant particles generated at 39.5°C exhibited only background levels of β-galactosidase activity compared to HIV-1_{LAI} (Table 4). Because reverse transcription of the viral genome is a required step for infectivity in MAGI cells, these results indicate that the RT incorporated into mt4 at the permissive temperature is fully capable of directing the synthesis of viral DNA in infected cells at the nonpermissive temperature.

mt4 particles produced at the nonpermissive temperature lack RT protein. When it became apparent that the *ts* phenotype of HIV-1 mutant mt4 was a property of progeny virions produced at 39.5°C but not at 34.5°C, the synthesis, processing, and incorporation of RT and other *pol* gene products were examined at both temperatures to ascertain which step(s) in the virus life cycle was temperature dependent. Cultures of CEM (12D7) cells, transfected for several days with wild-type or mt4 viral DNA at the semipermissive temperature of 36.5°C, were metabolically labeled with [^{35}S]methionine for 16 or 12 h at 34.5 or 39.5°C, respectively. Cell-associated proteins were immunoprecipitated with serum from an AIDS patient (Fig. 2A); virion-associated proteins were immunoprecipitated with the same patient serum (Fig. 2A), two anti-HIV-1 RT polyclonal antibodies (Fig. 2B), or an anti-HIV-1 IN monoclonal antibody (Fig. 2C). All immunoprecipitated proteins were then resolved by SDS-PAGE.

The electropherogram of virion-associated proteins, present in particles produced at 39.5°C and immunoprecipitated with AIDS patient serum, revealed the presence of multiple HIV-1 structural proteins in both the wild-type and mutant virus preparations (Fig. 2A). Notably missing or present at near background levels in samples of mt4 produced at the nonpermissive temperature was the 66-kDa subunit of RT. To confirm the absence of particle-associated RT protein, the same samples of HIV-1_{LAI} and mt4, produced at 39.5°C but immunoprecipitated with two different polyclonal antibodies directed against HIV-1 RT, were similarly analyzed. As shown in Fig. 2B, p66 was immunoprecipitated from wild-type virions but not from the mutant particles with both anti-RT antibodies. Anti-RT Ab 2 also immunoprecipitated the 51-kDa RT subunit from the wild-type virus sample but significantly reduced amounts of p51 from the preparation of mutant virions (Fig. 2B, right).

The Pr160*gag-pol* precursor is synthesized and incorporated into mt4 particles produced at the nonpermissive temperature. Several explanations can be entertained to account for the absence of the heterodimeric RT protein in mutant virions generated at the nonpermissive temperature. It is possible that the Gag-Pol precursor polyprotein is not synthesized at 39.5°C or is so unstable that none of the proteins encoded by the *pol* gene are assembled into progeny virions. From the data presented thus far, this cannot be the case. The HIV-1 Pr160*gag-pol* precursor becomes incorporated into nascent virions and is processed into the mature PR, RT, and IN proteins during or immediately following release of particles from productively infected cells. The presence of fully active protease in mt4

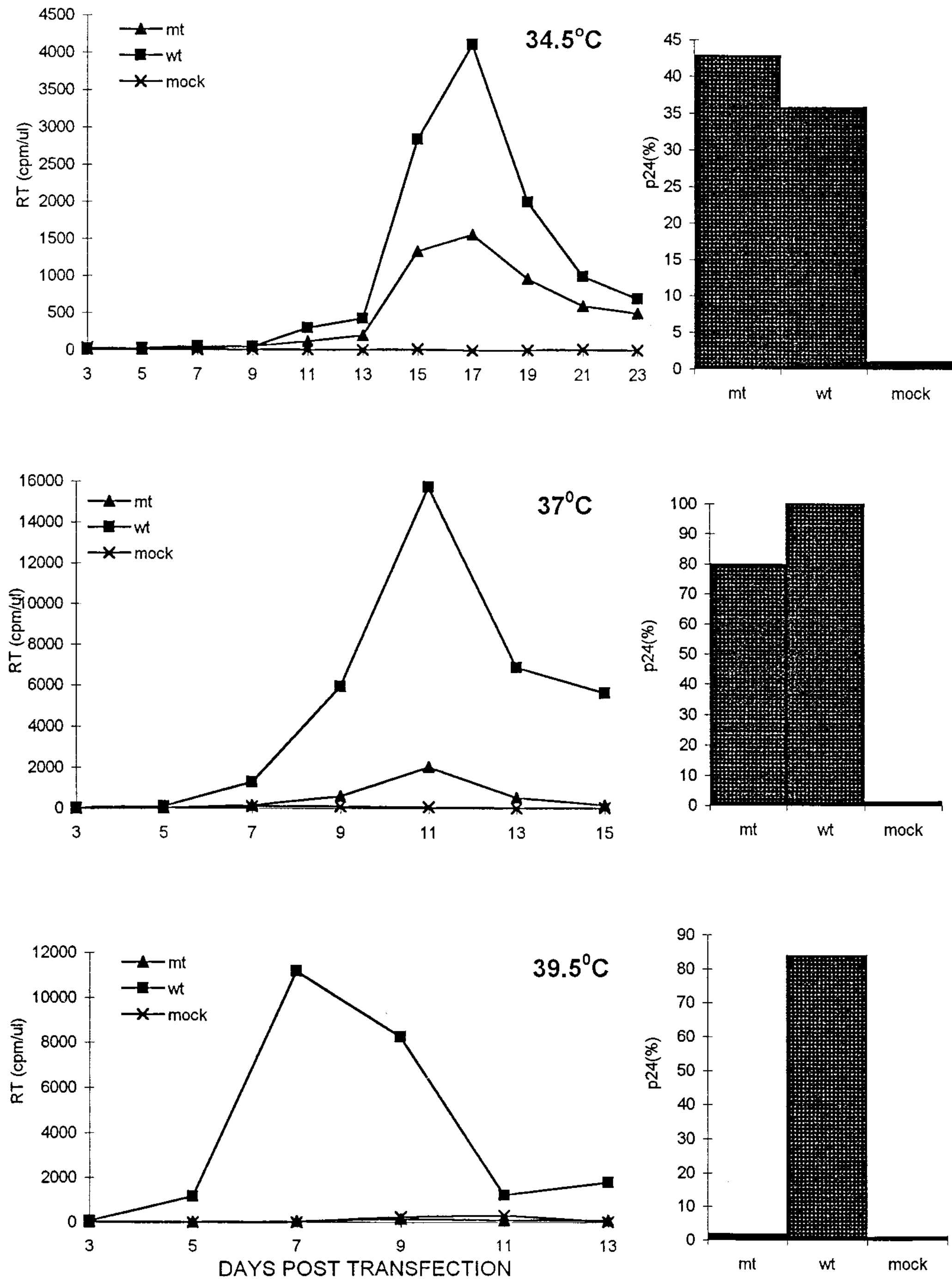

FIG. 1. Replication kinetics of mt4 (mt) and its wild-type HIV-1_{LAI} parent (wt) in CEM (12D7) cells. CEM cells were transfected with the indicated proviral DNAs as described in Materials and Methods. Cells were incubated at 34.5°C (top), 37°C (middle), or 39.5°C (bottom) and were split 1:3 (37 and 39.5°C) or 1:2 (34.5°C) every 2 days. RT activity in the supernatant was monitored at the indicated times (left panels). Release of $p24^{gag}$ into the medium was also determined at the peak of RT production (right panels). The amount of $p24^{gag}$ detected in duplicate samples of supernatant medium collected from cells transfected with the wild-type HIV-1_{LAI} proviral DNA at 37°C was arbitrarily set to 100%.

particles produced at the nonpermissive temperature can be inferred from the presence of several virion-associated Gag cleavage products, including p24 (Fig. 2A). The AIDS patient antiserum used in that experiment also immunoprecipitated a 32-kDa protein from both wild-type and mutant virus particles with an electrophoretic mobility consistent with HIV-1 IN, another product of the processed $Pr160^{gag\text{-}pol}$. Confirmation that this was indeed the IN protein was obtained by immunoprecipitating mt4, generated at 39.5°C, with an anti-HIV-1 IN monoclonal antibody (Fig. 2C); a prominent 32-kDa band was observed in samples of both wild-type and mutant virus particles.

The presence of the protease and IN proteins but not the RT heterodimer in mutant virions produced at the nonpermissive

TABLE 3. RT and p24*gag* released from transfected HeLa cells

Provirus	Culture temp (°C)	RT activity (cpm/μl) assayed at[a]:			Relative p24*gag* level (%)[b]
		34.5°C	37°C	39.5°C	
mt4	34.5	10,778	15,984	10,118	50
Wild type	34.5	26,277	34,090	28,632	53
mt4	37	6,185	9,238	5,978	93
Wild type	37	34,444	43,100	35,414	100
mt4	39.5	449	680	406	60
Wild type	39.5	17,498	23,128	23,128	65

[a] After incubation of transfected cells at the indicated culture temperatures for 48 h, cell-free supernatants were collected and monitored for RT activity at the indicated assay temperatures. Each value is the average of duplicate experiments.

[b] p24 levels were determined from the same cell-free supernatants used for the RT assays. The value for p24*gag* released from cells transfected with wild-type virus at 37°C was arbitrarily set at 100%. Each value represents an average of duplicate experiments.

temperature suggested that RT was either aberrantly processed or proteolytically degraded after the incorporation of the Pr160*gag-pol* precursor into budding virions. This possibility was first examined by pulse-labeling mt4-infected CEM (12D7) cells, maintained at 34.5 or 39.5°C, for 30 min followed by a 4-h chase. Figure 3 shows that cell-associated Pr160*gag-pol* was synthesized at both temperatures and that increasing levels of p66 RT and p32 IN appeared in the progeny virions released from cells at 34.5°C. At 39.5°C, however, little if any p66 was associated with virus particles, even after only 30 min of chase, nor was free, non-virion-associated p66 shed into the supernatant medium at the restrictive temperature (data not shown).

To definitively demonstrate that the Gag-Pol precursor encoded by mt4 was incorporated into nascent particles at 39.5°C, the PR coding sequence was inactivated in the context of both the wild type and the temperature-sensitive mutant, and progeny virions released from HeLa cells were examined for the presence of unprocessed Pr160*gag-pol*. A preliminary experiment revealed that HeLa cells, labeled with [^{35}S]methionine for 12 h, 40 h following transfection with cloned mt4 DNA at the nonpermissive temperature, released virus particles containing little or no p66 and p51 (Fig. 4A). An Asp-to-Asn substitution was then introduced at residue 25 of PR into both HIV-1_{LAI} and mt4 cloned DNAs, which were then transfected into HeLa cells maintained at 39.5°C. This mutation in the HIV-1 PR had previously been shown to eliminate HIV-1 PR activity (35). As expected for HIV-1 PR$^-$ mutants, the cell-associated and virion-associated samples contained unprocessed Pr55*gag* (Fig. 4B). More importantly, both the HIV-1_{LAI} and mt4 progeny virions contained nearly equivalent amounts of the Pr160*gag-pol* (Fig. 4B, right), confirming that the Gag-Pol precursor encoded by mt4 was, in fact, incorporated into virus particles at 39.5°C.

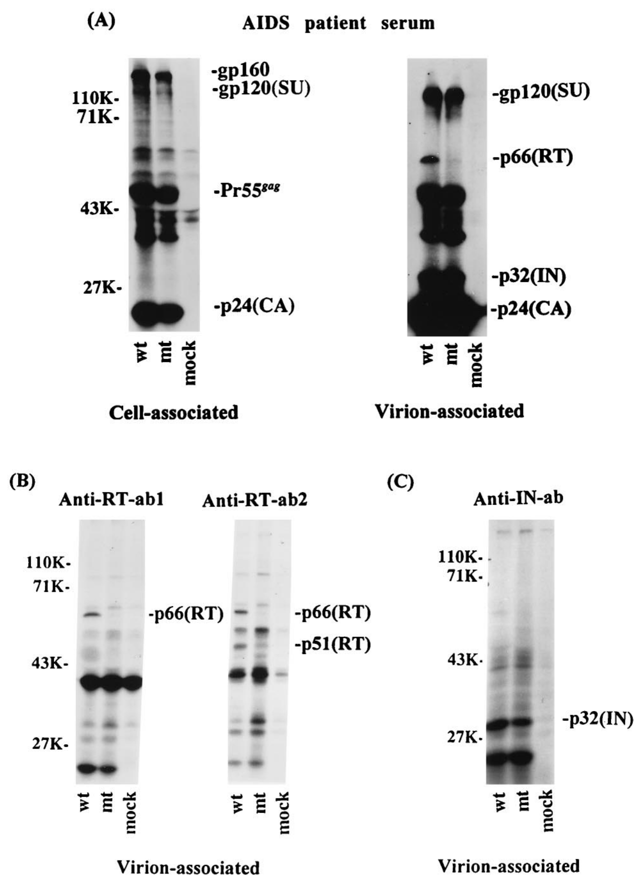

FIG. 2. Radioimmunoprecipitation analysis of HIV-1 proteins produced in transfected CEM (12D7) cells at the nonpermissive temperature. CEM cells were transfected with the indicated DNAs (wt, HIV-1_{LAI} proviral DNA; mt, mt4 proviral DNA; mock, pUC19) and were maintained at 36 ± 0.5°C. About 11 days posttransfection, the cells were shifted to 39.5°C and metabolically labeled at this temperature with [^{35}S]Met for 12 h as described in Materials and Methods. (A) Cell-associated or virion-associated HIV-1 proteins were immunoprecipitated with serum from an AIDS patient. Virion-associated proteins were also subjected to immunoprecipitation with anti-HIV-1 RT Ab 1 and Ab 2 (B) or with an anti-HIV-1 IN monoclonal antibody (C). All immunoprecipitated proteins were resolved on SDS–10% polyacrylamide-AcrylAide gels. The positions of the gp160 envelope glycoprotein precursor, the gp120 surface (SU) envelope glycoprotein, the 66- and 51-kDa RT subunits, the 55-kDa Gag precursor [Pr55*gag*], the 32-kDa IN [p32(IN)], and the 24-kDa capsid protein [p24(CA)] are indicated at the right; the positions of molecular weight standards are shown on the left.

TABLE 4. MAGI cell assay of wild-type and mt4 mutant viruses

Virus stock	Preparation temp (°C)	No. of blue cells assayed at[a]:		
		34.5°C	37°C	39.5°C
mt4	34.5	395	23,900	3,900
Wild type	34.5	583	23,200	4,010
mt4	39.5	2	13	10
Wild type	39.5	513	5,910	5,350

[a] MAGI cells were infected with similar amounts (approximately 5 ng of p24*gag*) of the indicated virus preparations. Only cells containing dark blue nuclei were counted as positive; each value is the average from two independent experiments.

DISCUSSION

The charged cluster of amino acids (KKKD) mutated by alanine substitution in this study (residues 64 to 67) is situated in the finger domain of the 66-kDa subunit of HIV-1 RT, within a loop connecting beta sheets β3 and β4 (23, 28, 44). Three of these residues are also included within a highly conserved retroviral RT motif (IKKK), consisting of a highly hydrophobic amino acid (I or V) followed by at least two basic amino acids. For lentiviruses and the Mason-Pfizer monkey virus, three lysines follow an initial isoleucine. From structural analyses, it has been proposed that this region of RT is involved in the interaction of the enzyme with the primer (8).

Several mutations affecting HIV-1 RT residues 64 to 67 have been previously reported. For example, the substitution of

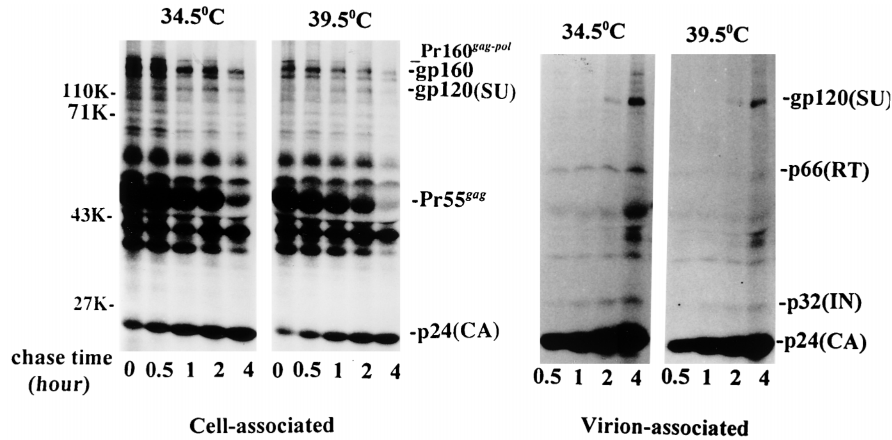

FIG. 3. Pulse-chase analysis of mt4 viral protein expression and release from transfected CEM (12D7) cells at permissive and nonpermissive temperatures. CEM (12D7) cells were transfected with mt4 proviral DNA and maintained at 36 ± 0.5°C for approximately 11 days. The cells were then split, pulse-labeled with [^{35}S]Met for 30 min, and chased in unlabeled medium for the indicated times at either 34.5 or 39.5°C. At each time point, equal aliquots were removed and separated into cell-associated and virion-associated fractions (the cell-associated fraction was collected at the end of the pulse period since a preliminary experiment indicated that negligible amounts of viral proteins had been released into the medium at that time). Lysates from each fraction were immunoprecipitated with AIDS patient sera and then resolved on SDS–10% polyacrylamide-AcrylAide gels. The positions of the HIV-1 marker proteins (described in the legend to Fig. 2) and molecular weight standards are shown.

arginine for lysine 65 results in >95% loss of RNA-dependent DNA-polymerizing activity, as measured in bacterial lysates containing 66-kDa homodimeric forms of RT (5, 6); this mutation had no detectable effect on RNase activity. In contrast, mutagenesis of lysines 64 and 66 (K to R) had only modest or no significant effects, respectively, on either polymerizing or RNase H activity (5, 6, 26). More recent analyses of a lysine-to-arginine substitution at codon 65 (K65R) of the HIV-1 RT, associated with the emergence of resistance to 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI), and the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (15, 17, 18, 56), revealed no loss of infectivity of the resultant virus when assayed in MT-4 cells or activated peripheral blood mononuclear cells. As noted in Table 2, any RT mutant containing an alanine-for-lysine substitution at residue 65 (viz., mt1 or mt3) was replication incompetent in CEM (12D7) cells. These results suggest

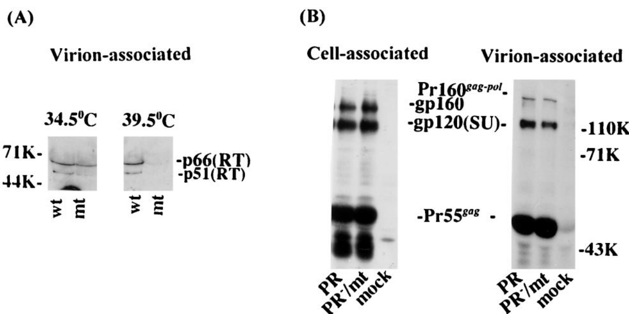

FIG. 4. Analysis of wild-type and *ts* mutant viral proteins produced in transfected HeLa cells. HeLa cells were transfected with the indicated plasmids (wt, HIV-1$_{LAI}$ proviral DNA; mt, mt4 proviral DNA; PR$^-$, PR-defective proviral DNA; PR$^-$/mt, mt4 proviral DNA with a mutated PR; mock, pUC19). (A) Forty-eight hours posttransfection, viral particles were harvested from the cells incubated at either 34.5 or 39.5°C. Virion-associated proteins were analyzed by immunoblotting using the anti-HIV-1 RT polyclonal antibody described in Materials and Methods. (B) Forty-eight hours posttransfection, the HeLa cells were metabolically labeled with [^{35}S]Met at 39.5°C for 12 h. Cell-associated or virion-associated proteins were immunoprecipitated with an AIDS patient serum and resolved on SDS–10% polyacrylamide-AcrylAide gels. The markers used are described in the legend to Fig. 2.

that for the establishment of a spreading virus infection, a positively charged amino acid must be present at this position of the HIV-1 RT; an uncharged alanine substitution causes loss of infectivity.

Mutant mt4 particles produced at the nonpermissive temperature contain little or no detectable p66-p51 heterodimeric RT protein. This property is very similar to that described for an RT variant that emerged following exposure to (alkylamino)piperidine bis(heteroaryl)piperizines in vitro. This G190E RT revertant exhibited marked reductions in both particle-associated RT activity and heterodimeric RT protein levels, the latter measured by immunoblotting with a polyclonal antibody to the HIV-1 RT (39). One could argue that our inability to detect the 66- and 51-kDa RT subunits in mt4 particles produced at 39°C merely reflected the failure of the antibodies used to bind to the mutant RT proteins. This seems highly unlikely because the same results were obtained with a variety of antibody preparations (AIDS patient serum, two different rabbit anti-HIV-1 RT polyclonal antibodies, and two different anti-HIV-1 RT monoclonal antibodies [data not shown]) in both immunoprecipitation and immunoblotting assays.

Because the 66- or 51-kDa RT subunits have unique conformations within the precursor homodimer and mature RT heterodimer, the *ts* mutant mt4 described in this study may be useful in delineating the role(s) of each subunit during its assembly into a stable and functional viral polymerase. At present, we can only speculate about the mechanism responsible for the absence, and presumed degradation, of the two RT protein subunits in mt4 produced at the nonpermissive temperature. Nonetheless, several aspects of this temperature-dependent phenomenon are unambiguous. First, Pr160$^{gag-pol}$ containing the *ts* RT mutation is incorporated into progeny virions at 39°C. Second, as long as the mutant RT remains a component of the Gag-Pol precursor, it is stably maintained within virus particles produced under nonpermissive conditions. Third, the mutant p66-p51 heterodimeric RT, formed at 34.5°C, is biochemically and functionally stable at 39°C. This last property, in conjunction with the results of a pulse-chase experiment (Fig. 3) showing that cell-associated Pr160$^{gag-pol}$ is converted to detectable 66-kDa particle-associated RT within

a 30-min chase period at 34.5 but not 39°C, indicates that the mutant p66-p51 heterodimer is very rapidly degraded.

Extracts from bacteria expressing the HIV-1 RT contain the p66-p66 homodimer, which is in equilibrium with p66 monomer, as well as a p66-p51 heterodimer. The latter is a bacterial protease cleavage product of either monomeric or dimeric p66 or both (10, 20, 31, 37, 38, 42, 46, 48). All of these structures possess unique physical and chemical properties (42). For example, the dissociation constant for the p66-p51 heterodimer has been reported to be 10^{-9} M or lower, compared to 10^{-5} M for the p66-p66 homodimer. Furthermore, because the site that must be cleaved to generate p51 is situated within a relatively inaccessible region of the N-terminal β sheet of RNase H, it has been proposed that the p66-p66 homodimer assumes a structurally asymmetric, and possibly less stable, conformation, thereby exposing the C-terminal RNase domain on one of the subunits for digestion by PR (28, 49). During virus replication, p66-p51 heterodimers arise as a result of cleavage of HIV-1 $Pr160^{gag\text{-}pol}$ by the viral PR. It is very likely that other intermediates are also generated during the processing reaction and that the presence of the *ts* mutation further destabilizes one of these RT intermediates, rendering it less resistant to degradation by the HIV-1 PR and/or cellular PRs at the nonpermissive temperature.

The charged-cluster-to-alanine mutagenesis strategy would be expected to alter residues located on solvent-exposed protein surfaces and possibly interfere with electrostatic interactions with other molecules. In the case of the human growth hormone, this mutagenesis approach resulted in the identification of residues involved in hormone binding (1). In general, charged-cluster-to-alanine mutagenesis has also resulted in high frequencies of *ts* cellular and viral mutants (12, 19, 50). For example, 9 of 26 substitutions affecting the poliovirus RNA-dependent RNA polymerase resulted in conditional mutants defective in RNA synthesis (12). This is to be contrasted with the results reported in this study, where only 1 of 33 HIV-1 RT mutants was *ts*, and a previous report describing a single conditional HIV-1 IN mutant of the 24 constructed (55). It is not clear why so few *ts* mutants of HIV-1 *pol* gene products have been obtained by this mutagenesis strategy. However, it is interesting to note that the charged-cluster-to-alanine *ts* HIV-1 mutants affected a step(s) involving virion assembly, not the catalytic functions of RT or IN. Instead, the conditional phenotype exhibited by HIV-1 mutant mt4 described in this report most likely reflects the altered interaction of the RT precursor and RT subunits with other virion components and results in the degradation of an RT intermediate subsequent to its cleavage from the Gag-Pol precursor. Less can be said about the nature of the defect in the previously reported *ts* HIV-1 IN mutant (55). In contrast to our results, the IN protein was detected in virus particles produced at the nonpermissive temperature. Since neither mutation impairs enzymatic activity, one could speculate that, like the *ts* RT mutant, the mutated virion-associated IN protein may be unable to assemble into functionally active IN oligomers, form stable associations with viral and cellular DNA targets, or associate with other viral and cellular proteins during the early phase of productive infection.

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