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Effect of Mutations in the Nucleocapsid Protein (NCp7) upon Pr160$^{gag\text{-}pol}$ and tRNA^{Lys} Incorporation into Human Immunodeficiency Virus Type 1

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COS-7 cells were transfected with DNAs containing mutations in the NCp7 sequences of human immunodeficiency virus. Selective incorporation into the virus of tRNA^{Lys} was measured by two-dimensional polyacrylamide gel electrophoresis, and Pr160$^{gag\text{-}pol}$ incorporation into the virus was detected in Western blots of viral protein. Mutations tested included cysteine and histidine mutations in either of the Cys-His boxes, as well as mutations in the N- and C-terminal flanking regions and in the linker region between the two Cys-His boxes. Of 10 mutations tested, only 2 inhibited tRNA^{Lys} incorporation: a P31L mutation in the linker region and a deletion which removed both Cys-His boxes and the linker region (ΔK14-T50). The P31L mutation prevents the incorporation of Pr160$^{gag\text{-}pol}$ into the virus. Cotransfection of COS cells with both P31L DNA and a plasmid coding only for unprocessed Pr160$^{gag\text{-}pol}$ resulted in the viral incorporation of Pr160$^{gag\text{-}pol}$ and the rescue of selective packaging of tRNA^{Lys} into the virion. In the ΔK14-T50 mutant, Pr160$^{gag\text{-}pol}$ is incorporated into the virus. Selective tRNA^{Lys} packaging is not rescued by cotransfection with a plasmid coding for Pr160$^{gag\text{-}pol}$ but is rescued by cotransfection with DNA coding for wild-type Pr55^{gag}. Since Pr55^{gag} does not by itself selectively package tRNA^{Lys}, the ΔK14-T50 mutation may be affecting tRNA^{Lys} binding to a cytoplasmic Pr55^{gag}/Pr160$^{gag\text{-}pol}$ complex.

tRNA$_3^{Lys}$ is the primer tRNA for the synthesis of minus-strand DNA produced by reverse transcription in human immunodeficiency virus type 1 (HIV-1) (38). During the transient production of HIV from COS cells transfected with HIV proviral DNA, the virus selectively packages the major tRNA^{Lys} isoacceptors, tRNA$_{1,2}^{Lys}$ and tRNA$_3^{Lys}$ (20). This packaging occurs independently of the incorporation of genomic RNA or the processing of precursor proteins Pr55^{gag} and Pr160$^{gag\text{-}pol}$ (20, 26) but is dependent upon the presence of precursor protein Pr160$^{gag\text{-}pol}$ (26). Viral particles consisting of Pr55^{gag} alone do not selectively package tRNA^{Lys}, while Pr55^{gag} particles containing unprocessed Pr160$^{gag\text{-}pol}$ do show selective incorporation of tRNA^{Lys}.

Because reverse transcriptase (RT) sequences interact with tRNA$_3^{Lys}$ during reverse transcription, RT sequences within Pr160$^{gag\text{-}pol}$ seem a likely binding site for tRNA^{Lys}. RT sequences within the Gag-Pol precursor have been shown to be important for packaging of primer tRNA in both avian retrovirus (32) and HIV-1 (26); i.e., deletion of part or all of the RT sequences prevents primer tRNA packaging. However, an investigation of smaller mutations in RT sequences which prevented tRNA^{Lys} packaging showed that the inhibition of tRNA^{Lys} packaging was correlated with the inhibition of packaging of the mutant Pr160$^{gag\text{-}pol}$ precursors, and any additional effect of these mutations upon the binding of tRNA^{Lys} to the precursor was not determined (27).

Since mature HIV-1 nucleocapsid (NCp7) has been shown to facilitate tRNA$_3^{Lys}$ annealing to the viral genomic RNA in vitro (2, 13, 36) and since genomic placement of tRNA$_3^{Lys}$ in vivo does not require processing of Pr160$^{gag\text{-}pol}$ (19), the nucleocapsid sequence within Pr160$^{gag\text{-}pol}$ is another candidate tRNA^{Lys} binding site in the precursor. With the exception of spumaviruses, all known retroviruses contain NC, a small protein which is rich in proline and basic amino acid residues and whose sequences are always found C terminal to the matrix and capsid domains in the Gag and Gag-Pol polyproteins. (For reviews on viral nucleocapsid protein and RNA packaging, see references 3, 11, and 39). NC binds to the viral genomic RNA in the core by sequence-independent interactions (21). Different retroviruses contain within the NC domain one or two basic amino acid subdomains, referred to alternatively as the Cys-His boxes (because they contain the CCHC motif, Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys) (10) or Zn^{2+} fingers because of their ability to bind Zn^{2+}. The positions of the cysteine and histidine residues are conserved, as is a glycine immediately N-terminal to the histidine. An aromatic residue is also usually found in either the first or second variable position in the Cys-His boxes. The positions of the two Cys-His boxes in HIV-1 define other subdomains of NCp7. As defined by Berkowitz et al. (3), from N to C terminus, these may be termed the N-terminal subdomain, box 1, the linker subdomain, box 2, and the C-terminal domain.

The Cys-His boxes in NC have been shown to be involved in the packaging of genomic RNA in both avian and mammalian retroviruses (7, 15, 22, 29, 34, 36, 39, 40, 49). The mature NC protein has been shown to have RNA-unwinding ability (46), a property which may be reflected in its ability to facilitate several steps in the viral life cycle in vitro, such as (i) the binding of primer tRNA to the primer binding site (2, 13, 36), (ii) dimerization of the viral genome (4, 12, 35, 47), and (iii) DNA

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strand transfer (1, 31). In addition, HIV-1 contains two interaction domains (I domains) within the NCp7 sequences which appear to facilitate the assembly of $Pr55^{gag}$ molecules during viral assembly (11). One can therefore imagine that NC sequences might facilitate the selective packaging of $tRNA^{Lys}$ into HIV-1 either by directly binding to $tRNA^{Lys}$ or by facilitating the formation of a $Pr55^{gag}/Pr160^{gag\text{-}pol}$ complex which may be required for either binding to $tRNA^{Lys}$ or carrying $tRNA^{Lys}$ into the virion or both. Therefore, we have investigated the effects of mutations within the various subdomains of the NCp7 sequences upon the selective incorporation of $Pr160^{gag\text{-}pol}$ and $tRNA^{Lys}$ into virus.

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MATERIALS AND METHODS

Plasmid construction. SVC21.BH10 is a simian virus 40-based vector which contains full-length wild-type HIV-1 proviral DNA (23, 45) and was a gift of E. Cohen, University of Montreal. Site-directed mutagenesis was carried out to create the mutations as previously described (16, 29). All mutations (see Fig. 1) were verified by direct sequence analysis of the *Spe*I-*Sal*I fragment after reconstructing the full-length proviral genome, by using Sequenase (United States Biochemical Corp.); pSVGAG-RRE-R and pSVFS5TprotD25G have been described previously (42–44) and were donated by D. Rekosh and M.-L. Hammarskjöld. Nucleocapsid mutants C15S/C18S, C36S, C36S/C39S, and ΔK14-T50 were donated by A. Rein and R. Gorelick (16).

Production of wild-type and mutant HIV-1. Transfection of COS-7 cells with the above plasmids by the calcium phosphate method was as previously described (18). Viruses were isolated from the cell culture medium 63 h posttransfection. The supernatant was first centrifuged in a Beckman GS-6R rotor at 3,000 rpm for 30 min, and the viruses were then pelleted from the resulting supernatant by centrifuging in a Beckman Ti45 rotor at 35,000 rpm for 1 h. The viral pellet was then purified by centrifugation with a Beckman SW41 rotor at 26,500 rpm for 1 h through 15% sucrose onto a 65% sucrose cushion.

For the specific detection of $Pr160^{gag\text{-}pol}$, COS-7 cells were transfected with wild-type and mutant proviral DNAs in presence of the protease inhibitor Saquinovir (Hoffman-La Roche). Thirteen hours posttransfection, the culture medium was removed and replaced with Dulbecco modified Eagle medium (GIBCO-BRL) containing 10% fetal calf serum and 0.8 μM Saquinovir. The viruses were isolated from the culture medium 63 h posttransfection, and viral and cellular lysates were prepared as previously described (27).

RNA isolation. Both cellular and viral RNAs were extracted from pellets by the guanidinium isothiocyanate procedure (9). The RNA pellets were dissolved in 5 mM Tris-HCl, aliquoted, and stored at −70°C.

RNA labeling. The RNA samples were labeled by the ^{32}pCp 3′-end-labeling technique (8). ^{32}pCp was made as follows: 5 mCi of [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol; Dupont) was dried down in a microcentrifuge tube by using N_2. One hundred microliters of reaction solution (50 mM Tris-HCl [pH 9.2], 5 mM $MgCl_2$, 3 mM dithiothreitol, 5% bovine serum albumin, 1 μM 3′-CMP, and 10 U of T4 kinase) was added. The reaction mixture was incubated at 37°C for 3 h, and the conversion of 3′-CMP to ^{32}pCp was monitored by polyethyleneimine thin-layer chromatography in 0.8 M NH_2SO_4, which separates ^{32}pCp from [^{32}P]ATP.

After labeling of the RNA with ^{32}pCp as previously described (8), free ^{32}pCp was removed from the labeled macromolecules either electrophoretically or with G-50 Sephadex (Pharmacia) home-made spin columns, equilibrated with TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA). Before analysis by polyacrylamide gel electrophoresis (PAGE), the samples were heated at 90°C for 2 min.

1D and 2D PAGE. The two-dimensional (2D) PAGE tRNA species-labeled spots 1, 2, and 3 have been previously identified as two species of $tRNA^{Lys}_{1,2}$ and one species of $tRNA^{Lys}_3$, respectively (20). The term $tRNA^{Lys}_{1,2}$ refers to a population of two $tRNA^{Lys}$ species which differ by 1 bp in the anticodon stem (37). Electrophoresis of viral RNA was carried out at 4°C with a Hoeffer SE620 gel electrophoresis apparatus. The gel size was 14 by 32 cm. The first dimension was run in a 10% polyacrylamide–7 M urea gel for approximately 16 h at 800 V, until the bromophenol blue dye was beginning to elute from the bottom of the gel. After autoradiography, the piece of gel containing RNA was cut out and embedded in a second gel (20% polyacrylamide–7 M urea) and run for 30 h (25 W limiting), followed by autoradiography. All electrophoretic runs were carried out in 0.5× TBE (1× TBE is 50 mM Tris, 5 mM boric acid, and 1 mM EDTA-Na_2). The electrophoretic gel patterns shown in this paper show only low-molecular-weight RNA, since the high-molecular-weight viral genomic RNA cannot enter the polyacrylamide gels. Furthermore, these patterns represent only the most abundant tRNA species present, since the high specific activities of the labeled tRNAs used will reveal less abundant species with longer film exposures.

Protein analysis. Western blots were done by the following procedure. Cell culture supernatants were collected 63 h posttransfection and clarified by centrifugation at 3,000 rpm in a Beckman GS-6R rotor for 30 min. Viral particles were pelleted by centrifugation in a Beckman Ti45 rotor at 35,000 rpm for 60 min at 4°C. Viral particles were washed with 1× TNE (1 mM Tris, pH 7.4, 10 mM NaCl, 0.1 mM EDTA), and viral proteins were extracted with 1× radioprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 2 mg of aprotinin per ml, 2 mg of leupeptin per ml, 1 mg of pepstatin A per ml, 100 mg of phenylmethylsulfonyl fluoride per ml) as previously described (6, 25). Western analysis was performed using either 300 μg of cellular protein or 10 μg of viral protein, as determined by the Bradford assay (Bio-Rad). The cellular and viral lysates were analyzed by SDS-PAGE followed by blotting onto nitrocellulose membranes (Gelman Sciences). Detection of protein on the Western blot utilized either HIV patient antisera or monoclonal antibodies specifically reactive with HIV-1 RT which were prepared as previously described (24, 48). Approximately 30 different antibodies recognizing different epitopes throughout HIV-1 RT were pooled for use in the present experiments, in order to maximize recognition of $Pr160^{gag\text{-}pol}$. Detection of $Pr160^{gag\text{-}pol}$ on the nitrocellulose membranes was performed by enhanced chemiluminescence (ECL kit; Amersham Life Sciences) using goat anti-mouse immunoglobulin as a secondary antibody. The sizes of the detected protein bands were estimated with prestained high-molecular-weight protein markers (GIBCO/BRL).

p24 was measured with a commercial kit available for p24 antigen capture (Abbott Laboratories). RT activity was measured by RT assays as previously described (5, 41).

RESULTS

Production of wild-type and mutant HIV-1 viruses from COS-7 cells. COS-7 cells were transfected with either wild-type or mutant HIV proviral DNA, and both cells and virus in the cell culture medium were collected and analyzed 63 h after transfection. The 10 NCp7 mutations investigated are shown in Fig. 1. Table 1 shows the production of wild-type and mutant virions, measured both as the p24 production per milliliter of culture supernatant and as the RT activity per picogram of p24. Western blot analysis of sucrose gradient-purified virus, using patient anti-HIV antisera (Fig. 2A), not only gives further evidence for the production of mutant virus but also shows that some mutant viruses are not able to carry out wild-type processing of $Pr55^{gag}$. This is particularly noticeable in the C15S/C18S, ΔK14-T50, P31L, and K59L viruses. p39 is a processing intermediate of $Pr55^{gag}$ (33). The p66 peptide of RT is not found in the P31L virus, and when the same blot is reprobed with anti-RT (Fig. 2B), the mature p66/p51 RT bands are seen in all mutant virions except P31L virions. These results are supported by the absence of significant RT activity listed for this mutant in Table 1. The reduced amount of RT in this mutant has been reported previously (29). The patterns of protein processing seen for the A30P, R32G, and R7R10K11S mutant viruses in Fig. 2A appear similar to that of the wild-type virus. However, the R32G virus shows the least amount of p66 RT species, relative to the approximately equal amounts of p24 species seen for these virions in Fig. 2A. This is reflected in the reduced amount of RT activity listed for the R32G mutant in Table 1.

Selective packaging of $tRNA^{Lys}$. Wild-type HIV produced in COS cells selectively packages $tRNA^{Lys}$, i.e., while $tRNA^{Lys}$ represents 5 to 6% of cytoplasmic tRNA, it represents 50 to 60% of low-molecular-weight viral RNA (26). The ability of the mutant virions to selectively package $tRNA^{Lys}$ was examined by 2D PAGE. Table 1 shows that of the mutant virions tested, only two, the P31L and ΔK14-T50 mutants, did not selectively package $tRNA^{Lys}$. Figure 3 shows the 2D PAGE patterns of viral tRNA extracted from wild-type and representative mutant viruses. The three viral $tRNA^{Lys}$ species (spot 1, $tRNA^{Lys}_1$; spot 2, $tRNA^{Lys}_2$; spot 3, $tRNA^{Lys}_3$ have been previously identified (20). It can be seen in this figure that both P31L (panel D) and ΔK14-T50 (panel G) viruses do not show selective $tRNA^{Lys}$ packaging, while virions containing mutations in Cys-His boxes 1 and 2 (C15S/C18S [panel B] and C36S/C39S [panel C]) both show wild-type selective incorpo-

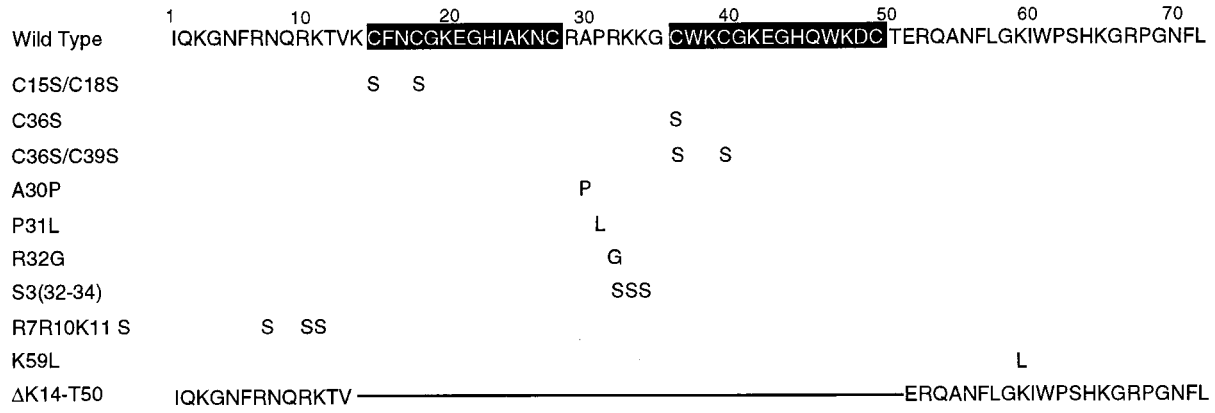

FIG. 1. Schematic representations of wild-type and mutant NCp7 proteins. The two Cys-His boxes are portrayed in black, and their positions define other subdomains of this protein such as the N and C subdomains and the 7-amino-acid linker subdomain between the two boxes.

ration of $tRNA^{Lys}$, even though these mutants have previously been reported to show a significant reduction in genomic RNA packaging (16).

$Pr160^{gag\text{-}pol}$ incorporation in $tRNA^{Lys}$-deficient mutant viruses, P31L and ΔK14-T50 mutants. We determined directly if the inhibition of the selective packaging of $tRNA^{Lys}$ was due to the inhibition of $Pr160^{gag\text{-}pol}$ packaging. Cells were transfected with wild-type or mutant proviral DNA in the presence of the protease inhibitor Saquinovir (Hoffman-La Roche), in order to increase our ability to detect the presence of the unprocessed precursor in the virus. Figure 4 shows Western blots of cellular (panel B) or viral (panel A) proteins probed with a mixture of monoclonal antibodies to RT. For the lanes within each panel, equal amounts of cellular or purified viral protein (measured with the Bradford reaction) were used. Lane wild type represents the $Pr160^{gag\text{-}pol}$ detected in infected-cell lysates (panel B) or in the wild-type virus produced from these cells (panel A). Lane Pr− shows the RT sequence-containing protein in protease-negative virus or cells infected with this virus in panels A and B, respectively. The major band in lane Pr− not only serves as a marker for the position of uncleaved $Pr160^{gag\text{-}pol}$ but also demonstrates that when the viral protease is completely inactive, no proteolysis of $Pr160^{gag\text{-}pol}$ is seen in the cytoplasm. It can be seen in Fig. 4A that in the wild-type virus, in addition to $Pr160^{gag\text{-}pol}$, two major processed RT sequence-containing fragments are observed, at approximately 140 and 48 kDa. The smaller fragment migrates slightly faster than p51 RT (data not shown). These two fragments are also seen in the ΔK14-T50 virus. This virus also contains a protein species migrating slightly faster than full-length $Pr160^{gag\text{-}pol}$, which is most likely the $Pr160^{gag\text{-}pol}$ protein containing the ΔK14-T50 deletion.

Only the 48-kDa fragment is detected in the P31L virus in the presence of Saquinovir. This species could be derived from

TABLE 1. Characteristics of HIV-1 nucleocapsid mutants

Source	p24 (pg/ml of medium)	RT (cpm/ng of p24)	Selective $tRNA_3^{Lys}$ incorporation
Wild type	980	117,820	+
Mutants			
C15S/C18S	3,600	62,375	+
C36S	5,600	58,768	+
C36S/C39S	7,200	63,214	+
ΔK14-T50	8,950	59,111	−
A30P	4,630	62,783	+
P31L	8,140	1,711	−
R32G	5,030	33,856	+
S3(32-34)	6,120	85,576	+
R7R10K11S	1,200	80,342	+
K59L	24,800	141,998	+
Cotransfection with pSVgag-RRE			
ΔK14-T50	4,650	37,592	+
P31L	2,300	1,890	−
Cotransfection with pSVFS5TprotD25G			
ΔK14-T50	5,800	58,687	−
P31L	4,700	17,641	+

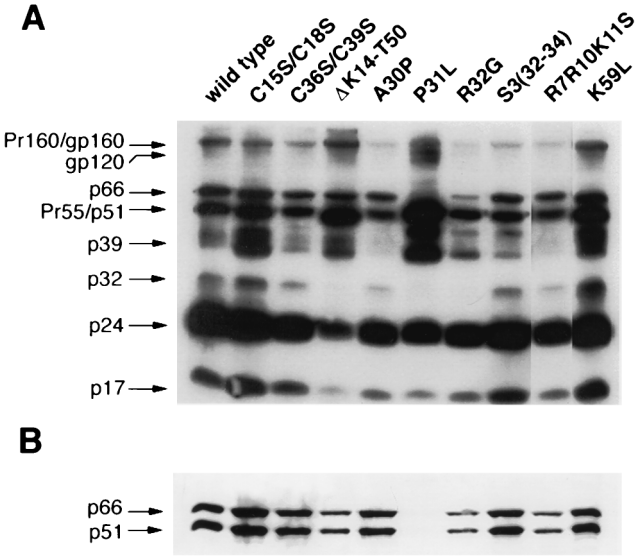

FIG. 2. Western blot analysis of wild-type and mutant viral proteins. Pelleted viruses were lysed with RIPA buffer, and aliquots of total viral protein were resolved by SDS–12% (wt/vol) PAGE and blotted onto nitrocellulose membranes. Protein detection utilized either HIV patient antisera (A) or monoclonal antibodies specifically reactive with HIV-1 RT (B) and was performed by enhanced chemiluminescence, using as a secondary antibody either protein A conjugated to horseradish peroxidase or goat anti-mouse immunoglobulin conjugated to horseradish peroxidase, respectively.

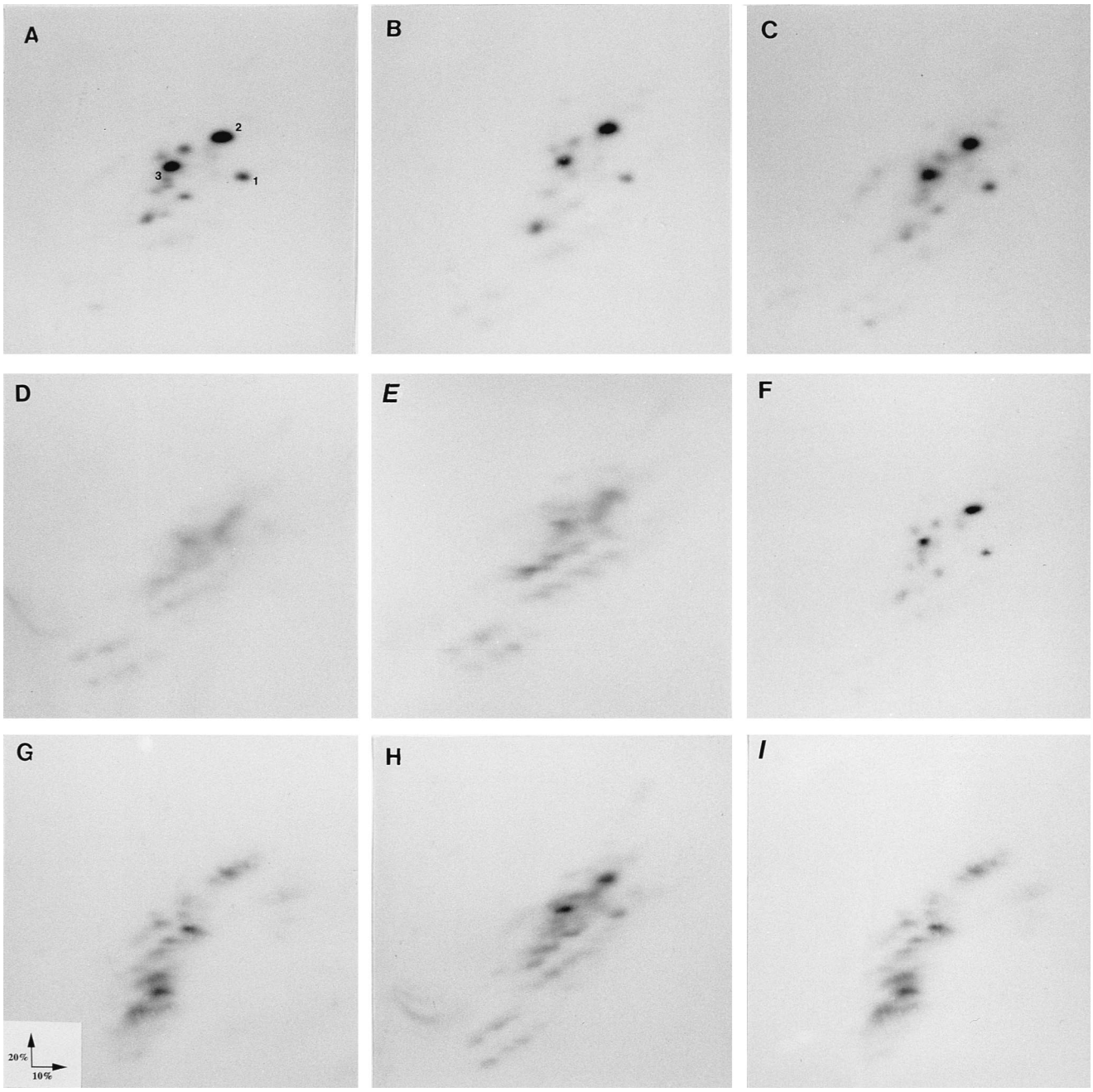

FIG. 3. 2D PAGE patterns of viral tRNA extracted from wild-type and representative mutant viruses. (A) Wild type; (B) C15S/18S; (C) C36S/C39S; (D) P31L; (E) cotransfection with P31L and pSVGAG-RRE; (F) cotransfection with P31L and pSVFS5TprotD25G; (G) ΔK14-T50; (H) cotransfection with ΔK14-T50 and pSVGAG-RRE; (I) cotransfection with ΔK14-T50 and pSVFS5TprotD25G. Spot 3 in panel A is $tRNA_3^{Lys}$, while spots 1 and 2 are $tRNA_{1,2}^{Lys}$.

the incorporation of either the full length $Pr160^{gag\text{-}pol}$ or the 48-kDa species found in the cytoplasm (Fig. 4B). Because of its size, it is unlikely that this species has RT activity. In the absence of protease inhibitor, neither this species nor p66 and p51 RTs are detected in the P31L virus (Fig. 2B), which correlates with the virus's very low RT activity (Table 1). It can be seen in Fig. 4B that the ratio of full-length $Pr160^{gag\text{-}pol}$ to processed proteins is much lower in the cells infected with the P31L virus than in those infected with either wild-type or ΔK14-T50 virus, indicating more rapid degradation of $Pr160^{gag\text{-}pol}$ in the cells infected with the P31L mutant. This degradation in the presence of a protease inhibitor probably indicates that the small amount of $Pr160^{gag\text{-}pol}$ normally present in the cytoplasm is degraded in the absence of the drug, which would account for the absence of RT protein (Fig. 2B) and RT activity (Table 1) in this mutant virus.

The absence of p66 and p51 RT in P31L virus (Figure 2B) implies the absence of viral protease, and yet the P31L virus contains processed proteins such as p24 (Fig. 2A and 5A). This might mean that a small amount of $Pr160^{gag\text{-}pol}$, undetectable in Fig. 2B, does get packaged. However, we have observed in a variety of cell types, including COS cells, cytoplasmic viral

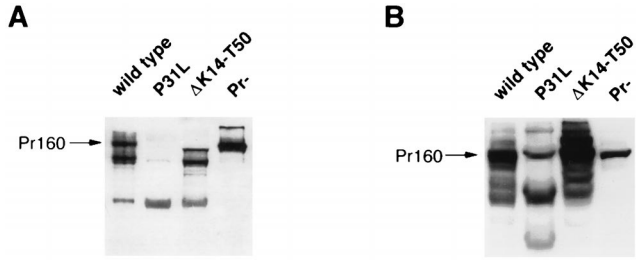

FIG. 4. Western blot analysis of RT-containing proteins in transfected cells or virions grown in the presence of the protease inhibitor Saquinovir. Viruses produced from transfected cells grown in the presence of 0.8 μM Saquinovir were pelleted from culture medium and lysed with RIPA buffer, and aliquots of either the viral protein (A) or cellular protein (B) were resolved by SDS–10% and 8% (wt/vol) PAGE, respectively, and blotted onto nitrocellulose membranes. Detection of protein utilized monoclonal antibodies specifically reactive with HIV-1 RT sequences and was performed by enhanced chemiluminescence, using goat anti-mouse immunoglobulin as a secondary antibody.

protease activity (see, for example, Fig. 4B, lane wild type), and this observation has been reported elsewhere as well (44). Therefore, we do not know if the p24 present in the P31L virus indicates the presence of a small amount of protease activity incorporated as part of $Pr160^{gag\text{-}pol}$ or if cytoplasmic p24 was incorporated into the virus during its assembly.

Rescue of $tRNA^{Lys}$-deficient mutant virus by cotransfection with wild-type $Pr55^{gag}$ or $Pr160^{gag\text{-}pol}$. $Pr55^{gag}$ is capable of particle assembly in the absence of other viral proteins (14, 17, 28, 43), while in COS cells, $Pr160^{gag\text{-}pol}$ does not form particles (22, 30, 43). When these two precursors are expressed in the same cell from different plasmids, the $Pr55^{gag}$ particles package the $Pr160^{gag\text{-}pol}$ (30, 43, 44). We have used this cotransfection system to examine how P31L and ΔK14-T50 create deficiencies in $tRNA^{Lys}$ packaging.

P31L. The P31L mutant virion does not show evidence for the presence of viral RT, either by Western blot or RT activity. This lack of RT protein is due to the ability of the P31L mutation to inhibit the incorporation of $Pr160^{gag\text{-}pol}$ into the virus. The data in Figure 4A support this conclusion. This was further demonstrated by cotransfecting COS-7 cells with the P31L DNA and with either pSVGAG-RRE, which codes only for $Pr55^{gag}$, or pSVFS5TprotD25G, which codes only for unprocessed $Pr160^{gag\text{-}pol}$ (obtained from D. Rekosh [42, 43]). The protein patterns of the resulting mutant virus produced are shown in Fig. 5, lanes 3 and 5. Panel A uses human anti-HIV sera as the probe, while panel B uses a mixture of monoclonal RT antibodies (24, 48). P31L DNA cotransfected with DNA coding for $Pr55^{gag}$ shows no evidence of RT in the virus and no selective incorporation of $tRNA^{Lys}$ (Fig. 3E). P31L DNA cotransfected with DNA coding for $Pr160^{gag\text{-}pol}$ shows the presence of this unprocessed precursor in the virus, and selective $tRNA^{Lys}$ packaging is rescued (Fig. 3F).

ΔK14-T50. Table 1 and Fig. 2 indicate that that mature p66/p51 RT is found in the ΔK14-T50 virus, and Fig. 4 demonstrates that in the presence of a protease inhibitor, mutant $Pr160^{gag\text{-}pol}$ is incorporated into the virion. COS-7 cells were transfected with the ΔK14-T50 DNA and with either pSVGAG-RRE or pSVFS5TprotD25G. The protein patterns of the resulting mutant virus are shown in Fig. 5, lanes 2 and 4. ΔK14-T50 DNA cotransfected with DNA coding only for unprocessed $Pr160^{gag\text{-}pol}$ shows the presence of this unprocessed precursor in the virus, but selective $tRNA^{Lys}$ packaging is not rescued (Fig. 3I). On the other hand, when ΔK14-T50 DNA is cotransfected with DNA coding for $Pr55^{gag}$, selective $tRNA^{Lys}$ packaging is rescued (Fig. 3H).

DISCUSSION

The major objective of this work is to determine whether the nucleocapsid protein sequence in HIV-1 plays any role in the viral packaging of $Pr160^{gag\text{-}pol}$ and $tRNA^{Lys}$. Two mutations ΔK14-T50 and P31L, both inhibit $tRNA^{Lys}$ packaging. For P31L, this is most likely due to the inability of the mutant virus to incorporate the mutant $Pr160^{gag\text{-}pol}$. It has been shown that this precursor molecule is essential for $tRNA^{Lys}$ packaging (26), and when wild-type $Pr160^{gag\text{-}pol}$ is introduced into the cell via cotransfection, both $Pr160^{gag\text{-}pol}$ and $tRNA^{Lys}$ are incorporated into the mutant $Pr55^{gag}$ particles.

In the presence of Saquinovir, the P31L mutation causes more extensive cytoplasmic processing of $Pr160^{gag\text{-}pol}$ than is found for either wild-type or ΔK14-T50 virions, implying that in the absence of protease inhibitor, a rapid breakdown of $Pr160^{gag\text{-}pol}$ in the cytoplasm is responsible for the absence of RT in the virus. Other mutations involving the linker subdomain do not affect $Pr160^{gag\text{-}pol}$ packaging (A30P, R32G, and S3 [32–34]), and it is likely that the replacement of the proline 31 by leucine produces a more drastic conformation change in the protein. This may in itself make the protein more susceptible to proteolysis, but it is also possible that the mutation prevents association of $Pr160^{gag\text{-}pol}$ with $Pr55^{gag}$, which may also contribute to the protein's vulnerability to protease. Further studies on the ability of a P31L, protease-negative $Pr160^{gag\text{-}pol}$ to be incorporated into the virus should distinguish between these two possibilities.

Unlike the P31L virus, the ΔK14-T50 mutant virus does incorporate $Pr160^{gag\text{-}pol}$, so the cause of poor $tRNA^{Lys}$ incor-

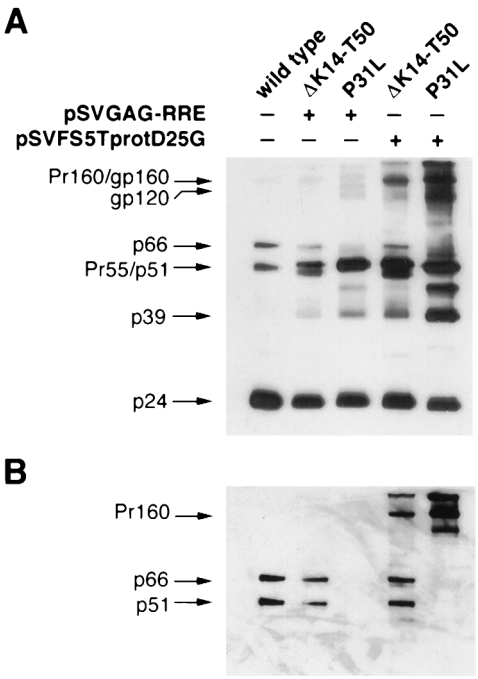

FIG. 5. Western blot analysis of protein from virions produced from cotransfected cells. COS-7 cells were cotransfected with mutant proviral DNA (P31L or ΔK14-T50) and DNA coding for either $Pr55^{gag}$ (pSVGAG-RRE) or $Pr160^{gag\text{-}pol}$ (pSVFS5TprotD25G). Pelleted viruses were lysed with RIPA buffer, and aliquots of total viral protein were resolved by SDS–15% (wt/vol) PAGE and blotted onto nitrocellulose membranes. Protein detection utilized either HIV patient antisera (A) or monoclonal antibodies specifically reactive with HIV-1 RT (B) and was performed by enhanced chemiluminescence, using as a secondary antibody either protein A conjugated to horseradish peroxidase or goat anti-mouse immunoglobulin conjugated to horseradish peroxidase, respectively.

poration into HIV-1 is not known. As expected, cotransfection of the mutant DNA with DNA coding for wild-type $Pr160^{gag\text{-}pol}$ does not rescue $tRNA^{Lys}$ incorporation even though the presence of this unprocessed precursor protein in the virus can be detected. On the other hand, cotransfection of DNA coding for wild-type $Pr55^{gag}$ will rescue $tRNA^{Lys}$ packaging in this mutant. This may indicate that both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ are required as a complex for binding to $tRNA^{Lys}$ in the cytoplasm, and the double-mutant precursor protein complex cannot do so. Although a $Pr55^{gag}/Pr160^{gag\text{-}pol}$ interaction has not yet been directly detected, indirect evidence for such an interaction exists. For example, in HIV, the viral incorporation of $Pr160^{gag\text{-}pol}$ is dependent upon the myristylation of the $Pr55^{gag}$ precursor protein (29, 42). $Pr160^{gag\text{-}pol}$ incorporation is also inhibited by a deletion of the major homology region in the CA domain of this precursor, but incorporation is maintained if the deletion is present in both $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ (44). The ΔK14-T50 mutation may allow sufficient $Pr55^{gag}/Pr160^{gag\text{-}pol}$ interaction for $Pr160^{gag\text{-}pol}$ incorporation into the virus but not allow $tRNA^{Lys}$ binding to the complex.

Alternatively, the inhibition of $tRNA^{Lys}$ packaging could also be due to the fact that the ΔK14-T50 virus produced is likely to have an abnormal structure. In the NCp7 sequences, there are two interaction (I) subdomains which overlap the Cys-His box domains and which are therefore eliminated in the ΔK14-T50 mutation. Mutation of these subdomains results in the production of virus with a lower density than that of the wild type (11). What alteration in viral structure is reflected by this lower density is not yet known, but this might have an effect on $tRNA^{Lys}$ packaging.

Genomic RNA packaging has been previously characterized for many of the mutant virions studied here. Gorelick et al. (16), using transfected HeLa cells, estimated that the genomic RNA contents of some of these mutant particles, relative to those of the wild type, were as follows: C15S/C18S mutant, ~2%; C36S mutant, ~6%; and C36S/C39S mutant, ~20%. Ottmann et al. (29), using transfected COS-7 cells, estimated the genomic RNA contents of some of these mutant particles, relative to those of the wild type, and found little change in the P31L mutation but 5 to 10 times less genomic RNA than in the wild type for the A30P, R32G, and S3 (32–34) mutant virions. This is in contrast to the finding of Poon et al. (34), who measured little if any change in genomic RNA incorporation for an R32A mutation in the virus. They also found that the R7A or K11A mutation significantly reduced genomic RNA packaging (to 52 or 25% of the wild-type level, respectively). We also have unpublished data indicating that the percentage of wild-type genomic RNA packaging for the following mutant virions is as indicated: R7R10K11S mutant, ~30%; K59L mutant, ~40%; and ΔK14-T50 mutant, ~2%. It is clear that there is no correlation between the genomic RNA and $tRNA^{Lys}$ packaging.

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