Mutational Analysis of the N-Terminal Domain of Moloney Murine Leukemia Virus Capsid Protein

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Retroviral capsid (CA) proteins contain a structurally conserved N-terminal domain (NTD) consisting of a β-hairpin and six to seven α-helices. To examine the role of this domain in Moloney murine leukemia virus (MoMLV) replication, we analyzed 18 insertional mutations in this region. All mutants were noninfectious. Based on the results of this analysis and our previous studies on additional mutations in this domain, we were able to divide the NTD of MoMLV CA into three functional regions. The first functional region included the region near the N terminus that forms the β-hairpin and was shown to control normal maturation of virions. The second region included the helix 4/5 loop and was essential for the formation of spherical cores. The third region encompassed most of the NTD except for the above loop. Mutants of this region assembled imperfect cores, as seen by detailed electron microscopy analyses, yet the resulting particles were efficiently released from cells. The mutants were defective at a stage immediately following entry of the core into cells. Despite possessing functional reverse transcriptase machinery, these mutant virions did not initiate reverse transcription in cells. This block could be due to structural defects in the assembling core or failure of an essential host protein to interact with the mutant CA protein, both of which may prevent correct disassembly upon entry of the virus into cells. Future studies are needed to understand the mechanism of these blocks and to target these regions pharmacologically to inhibit retroviral infection at additional stages.

During retroviral assembly, Gag polyproteins form a spherical core that encloses the viral genome. As the particle matures, the Gag polyprotein is proteolytically processed into at least three major proteins: MA, which associates with the plasma membrane, NC, which associates with the viral genome, and CA, which forms the mature core (52). The immature cores of all retroviruses are spherical. Upon maturation, the cores assume a shape that differs for each genus. Mature cores of gamma-, delta-, and alpharetroviruses are spherical and cores of betaretroviruses are cylindrical, while lentiviral capsids are conical (25). These shapes depend upon the CA protein, as evidenced by cores generated by various human immunodeficiency virus type 1 (HIV-1)/Rous sarcoma virus (RSV) chimeric CA proteins (1). Despite these different shapes assumed by the mature core, the tertiary structure of the CA protein is very similar for viruses across genera (8). CA proteins from N-tropic murine leukemia virus (N-MLV) (38), HIV-1 (18, 23), RSV (33), human T-cell leukemia virus type 1 (11, 32), and equine infectious anemia virus (31) all contain two domains, an elongated, tapered N-terminal domain (NTD) and a globular C-terminal domain (CTD) connected to each other by a flexible linker. High-resolution structural analysis and cryo-electron microscopy of virions, as well as X-ray crystallography of the CA protein, have also shown that CA NTDs are arranged in hexamers, with the monomers contacting each other via the central α-helices 1, 2, and 3 of the NTD (38).

Each hexamer is joined with neighboring hexamers to form a lattice, and this is aided by dimeric contacts between the CTDs (19, 36). The incorporation of CA pentamers is thought to curve and close the planar hexagonal lattice (9, 20, 22), and the location of these pentamers within the lattice determines the final shape of the core (20, 21).

Following a proteolytic cleavage event, the N terminus of most retroviral CA proteins folds into a β-hairpin. This hairpin, together with six α-helices (or seven, for some retroviruses), forms the NTD. The role of the NTD has been examined by extensive deletion and substitution mutagenesis of the HIV-1 CA NTD. Most mutants retained the ability to assemble and release particles. Some of these mutant cores assumed aberrant shapes (16, 46, 53, 57) and showed altered stability when treated with detergents (17, 54). However, these mutations blocked viral replication at an early stage, before reverse transcription. In rare cases, the block was between the stages of reverse transcription and integration (13). Mutations in the β-hairpin of the RSV CA NTD have also been shown to prevent infectivity and reduce viral DNA synthesis at an early stage, yet RSV mutant cores showed no alteration in core sensitivity to detergent (49).

In contrast to the extensive mutational analysis of the NTD of HIV-1 CA, few mutations in the NTD of Moloney MLV (MoMLV) CA have been characterized in detail. Most mutagenesis of MoMLV CA has been performed on the CTD with special focus on the major homology region (2, 30, 59). Previous mutagenesis of the NTD of MoMLV CA consisted of a large deletion of ~80 amino acids, which inhibited particle release (47). In addition, disruption of the proteolytic cleavage between p12 and CA results in noninfectious particles with ill-defined cores (39) and defects in viral DNA synthesis in vivo (45). Using genetic footprinting, we have previously shown

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MATERIALS AND METHODS

Mutagenesis. The mutagenesis of pNCA, an infectious clone of MoMLV (10), leading to 12 of the CA-NTD mutations analyzed here, has been described in detail before (4, 48). To isolate these 12 mutations from the large library of mutations in gag, clones were individually screened using PCR. One primer, complementary to a portion of the insertion (short G, 5′-GGCCGCTGCAAGCTTTCA-3′), and a second primer that hybridized outside of the region of mutagenesis (3263G, 5′-TTCCTTCGGGGTTTCTCGTTT-3′) resulted in PCR products whose lengths depended upon the location of the insertion in each mutant. Insertions located in the NTD of CA resulted in products of ≈650 bp, and the corresponding mutations were sequenced and analyzed further.

Three additional mutants (1719, 1897, and 2010) were generated from another insertional mutagenesis in pNCA performed with the GPS-LS linker screening system (New England Biolabs, Beverly, Mass.). This allows the insertion of a 27-nucleotide-based minimal transposable element into the plasmid of interest at random and single locations (5). Plasmids carrying an insertion can be selected on the basis of kanamycin resistance encoded by the transposon. Two PmeI random and single locations (5). Plasmids carrying an insertion can be selected on the basis of kanamycin resistance encoded by the transposon. Two PmeI

3-bp duplication of target sequence generated by the transposition reaction, remove all but 10 bp of the transposable element. This 10 bp, in addition to the 5-bp duplication of target sequence generated by the transposition reaction, results in an insertion of 15 bp or 5 amino acids. Our library contained 2.9 × 10⁹ independent clones, averaging 26 insertional events per nucleotide. Individual clones were screened for mutations in the NTD of CA. PCR primers 1608U (5′-GAGAGGCAGCCTCGCGCGGAG) and 3199L (5′-GGGTTAGACTT GCCTTCAGA) were used to amplify DNA from individual colonies of the library. PCR products were digested with Pmel, and positive clones were further purified, digested, and sequenced to identify the exact position of the insertion.

Transmission electron microscopy of virions. Culture supernatants (25 ml) of cells transfected with WT and mutant proviral DNAs were filtered and layered over a sucrose cushion (5 ml of 20% [wt/vol] sucrose in TNE buffer) and centrifuged for 1 h at 25,000 rpm in a Beckman SW28 rotor at 4°C in Ultra-Clear centrifuge tubes (Beckman). Pellets were resuspended in 1 ml 2.5% glutaraldehyde in 0.1 M Sorenson's buffer and further spun at 16,000 × g for 10 min at 4 °C in an Eppendorf 5415D centrifuge followed by overnight storage at 4°C. Pellets were postfixed with 1% OsO₄ in 0.1 M Sorenson's buffer for 1 h. After dehydration staining (27), grids were placed on microscope grids (Electron Microscope Sciences), stained with 1% uranyl acetate and 0.4% lead citrate, and examined under a JEOL 1200 EXII electron microscope. Electron micrographs were captured using ORCA-HR digital camera (Hamamatsu), and particle diameters were measured using an AMT Image Capture Engine (Advanced Microscopy Techniques, Danvers, MA). Only those particles with diameters between 60 and 150 nm were included in our analysis of particle size and distribution. Objects outside this size range were usually not of a defined shape and were most likely cellular debris.

Analysis of viral DNA synthesized in vivo. Virions used to inoculate Rat2 cells were harvested with Dnase I (Boehringer Mannheim) to remove any plasmid remaining from the transfections (37). At 24 to 48 h after viral inoculation, low-molecular-weight DNA (29) was prepared from these Rat2 cells and analyzed by PCR to detect reverse transcription products. Different primer pairs were used for detection of specific intermediates of reverse transcription. For minus-strand-long-stop DNA [−(ssDNA)]B-LTR2 (5′-AGTCTCTCGATTTACGCTGAGA) and B-LTR1 (5′-CGGTTAGACTTGCCTTCAGA) (37) were used to detect long minus-strand DNA, primers in the gag region, 5′-GGCCGCTGCAAGCTTTCA-3′, and 5′-TTCCTTCGGGGTTTCTCGTTT-3′, were used. Primers flanking the long terminal repeat (LTR) junction, LTR1 (5′-CTCTTTTATTAGCTCCGG) and LTR2 (5′-AGTCTCTCGATTTACGCTGAGA) were used to detect the presence of 1-LTR or 2-LTR circular viral DNA. Primers that amplify rat mitochondrial DNA, 5′-GTTAATGGCTAGCTTAAAAAGC and 5′-GTTATGGGCAACTAAGTGGG (26), were used to check the quality of the low-molecular-weight HIV transcript. Thermocycling conditions were as described elsewhere (48), except for annealing temperatures, which were 57°C for (−)ssDNA and mitochondrial DNA primers, 63°C for gag primers, and 50°C for the LTR primers.

Exogenous RT assays. Supernatants were harvested from infected cells or cells transfected with proviral DNA and used in exogenous RT assays to determine amounts of virus released from cells. Oligo(dT)·poly(A) primer-template assays were performed in the presence of radiolabeled [α-32P]dCTP and Mn²⁺, as previously described (55). In brief, after incubating the viral supernatants with the RT reaction mix for 1 hour at 37 °C, samples were spotted onto DE81 DEAE cellulose paper (Whatman) and unincorporated label washed away with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Virion-associated RT was analyzed using a Storm 820 PhosphorImager system and then quantified with the ImageQuant software (Molecular Dynamics).
Endogenous RT assays. Supernatant (25 ml) from cells transfected with proviral DNA was pelleted as described above for transmission electron microscopy. Pellets were reuspended in 25 μl of TNE buffer. The exogenous RT assay and Western blotting with anti-CA antibodies were used to determine amounts of virus in each supernatant. To detect the (−)ssDNA intermediate, equal amounts of concentrated virus were incubated in buffer (50 mM NaCl, 6 mM MgCl₂, 1 mM dithiothreitol, 0.03% NP-40, 50 mM Tris, pH 8.0) containing unlabeled deoxyinosine triphosphates (100 μM each) and [32P]dTTP (0.6 μM) at 37 °C for 40 min. Reactions were terminated, purified, and treated with RNase (100 μg/ml) and subjected to electrophoresis in 6% denaturing polyacrylamide gels (55). Gels were dried and exposed to film. To detect later intermediates, the blots were also probed with antibodies to CA protein (Fig. 3B). Note the CA protein appears to migrate differently for some mutants; this is due to differing lengths of insertions, 5 or 12 amino acids.

Overall, the levels of Gag protein were not drastically different for any of the mutations. Also, the Gag protein from all mutants was proteolytically processed to produce CA protein (30 kDa) in amounts similar to that seen with WT Gag. However, the mutants displayed varied persistence of the different intermediates of Gag proteolytic processing. To better define these intermediates, the blots were also probed with antibodies specific to the MA and NC proteins (not shown). The three most-N-terminal mutants located in the β-hairpin showed significantly higher amounts of the Pr65gag precursor, the 57-kDa MA-p12-CA intermediate, the 52-kDa p12-CA-NC intermediate, and the 40-kDa CA-NC intermediate. Two other mutants located nearest the β-hairpin (1794 and 1800) displayed persistence of the same intermediates, but to a lesser extent. Cell-associated Gag proteins from the remaining mutants appear to be processed very similarly to WT Gag, i.e., cell lysates contain mostly the Pr65gag precursor and the CA protein.

We next analyzed the released virions using Western blotting (Fig. 3C). All mutants displayed near-WT amounts of CA protein migrating at 30 kDa, indicating that none of the mutations completely blocked Gag processing. Many of the mutants did exhibit higher amounts of intermediates of proteolytic processing. The 40-kDa CA-NC intermediate was observed in higher amounts in the released virions for the three β-hairpin mutants, as it was in cells. The CA-NC intermediate was also seen in five additional CA mutants located after α-helix 1 and throughout α-helix 2 (1794, 1800, 1823, 1833, and 1848). This confirmed by Western blot analysis with anti-NC and anti-MA antibodies (data not shown; see Materials and Methods). Despite the fact that these mutants have insertions closer to the p12-CA cleavage site, the cleavage between CA and NC appears to be more affected.

Virion morphology using transmission electron microscopy. Virion morphology was analyzed by electron microscopy of pelleted thin-sectioned virions. Representative sections showing multiple particles of each mutant are shown in Fig. 4. Mutant virions were compared to WT and protease minus (PR−) particles that were processed in parallel. All mutants produced particles that were mostly spherical, though some were more regular in size and shape than others. We measured the maximum and minimum diameters of over 50 particles for each mutant and used the average of these two values to plot diameter distributions. This graph of particle diameters is

RESULTS

Location of mutations in the NTD of CA. Genetic footprinting analysis of the MoMLV CA protein has shown that a 12-amino-acid insertion anywhere within the N-terminal 77 amino acids results in nonviable virus, suggesting that this region plays an essential role in the viral life cycle (4). We recently reported that mutants with insertional mutations in the helix 4/5 loop of the NTD are defective in formation of spherical cores (3). We now report our analysis of 15 additional mutations in the NTD of CA that are located on either side of the domain required for spherical core formation. These mutations were selected from our library of mutations in gag based on the size of the PCR product they generated when the insertional sequence was used as a priming site. A mutation was chosen if the size of the PCR product indicated that the insertion was located in the NTD. Twelve of the 15 selected mutants contained 12-amino-acid insertions, and the remaining 3, generated from a separate mutagenesis, contained 5-amino-acid insertions (see Materials and Methods). These insertional mutations were distributed along the NTD, from the β-hairpin up to the N-terminal margin of α-helix 6. The location of each mutation is displayed on the primary sequence (Fig. 1A) and on high-resolution structures of MLV CA NTD monomer (Fig. 1B) and hexamer (Fig. 1C).

Particle release by mutants with insertions in the NTD of CA. We introduced proviral DNA from the 15 selected mutants in the NTD of CA into 293T cells and measured virion release by assaying the overlying medium for reverse transcriptase activity 48 h posttransfection. High-speed sedimentation of viral supernatants showed that reverse transcriptase activity could be recovered in pellets and was therefore associated with particles. The average of four independent experiments is shown in Fig. 2. For purposes of comparison, we included the three mutants (1955, 1968, and 1970) that were previously reported to be defective for spherical core formation and also defective in viral release. Except for these three mutants, all others released near-WT levels of virus (ranging from ~60 to 120% of WT virus). Mutant 1833 consistently showed the lowest levels of reverse transcriptase activity in the supernatants (averaging ~57%), suggesting, at worst, only a modest defect in particle production.

Viral supernatants were collected from transfected 293T cells and used to infect naïve Rat2 cells. As expected from our genetic footprinting analysis (4), all CA mutants were noninfectious when assayed 48 h after infection (Fig. 2) and remained noninfectious even after 10 days of infection. Thus, the near-WT levels of particles that were released by each of the insertional mutations located in the NTD of CA did not lead to productive infection, even after prolonged periods of time.

Analysis of mutant viral proteins in transfected cells and in released virions. Viral protein expression was analyzed by Western blot assays of cell lysates prepared 48 h after cells were transfected with mutant proviral DNA. The blots were probed with antibodies to CA protein (Fig. 3B). Note the CA protein appears to migrate differently for some mutants; this is due to differing lengths of insertions, 5 or 12 amino acids.

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shown just below the particle morphology for each mutant (Fig. 4). For comparison, the diameters of WT particles are shown in each graph. WT and PR\textsuperscript{−}/H11002 particles had an average diameter of 113 and 99 nm, respectively. For the three most-N-terminal mutants located in the β\textsuperscript{9252}-hairpin, the average particle diameters were almost identical to those of WT particles. For all other mutants, the diameters were on average approximately 10 to 20 nm less than that of WT particles, with the smallest mutant particles being 30 nm less than WT. Also, mutants other than those located in the β\textsuperscript{9252}-hairpin displayed a greater variation in particle size, as seen from the broader distribution of particle diameters.

We next examined core morphology. Mature cores contain a dense and evenly stained interior separated from the viral envelope by a well-defined electron-lucent space (Fig. 4 and 5A). Immature cores contain a less-electron-dense center surrounded by a narrow, well-defined electron-dense ring separated from the viral envelope by a space that is not as electron lucent as in the mature particle (Fig. 4 and 5B). Consistent with previous reports, our WT particles contained \( \sim66\% \) ma-

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FIG. 1. Location of insertional mutations in the NTD of MoMLV CA. (A) CA sequence (nucleotides 1715 to 2503), with 1 being the first nucleotide of pNCA, the infectious MoMLV clone. Mutations are denoted with vertical bars, and mutant nucleotide numbers are indicated below (mutants are designated by the nucleotide position at the 5' end of the insertional mutation). Secondary structures are colored as follows: yellow, β-hairpin; purple, α-helix 1; dark blue, α-helix 2; cyan, α-helix 3; green, α-helix 4; light blue, helix 4/5, loop; orange, α-helix 5; pink, α-helix 6. Three mutants marked with an apostrophe (1719, 1897, 2010) contain 5-amino-acid insertions; the remaining mutants contain 12-amino-acid insertions. (B) A ribbon drawing of the CA monomer was taken from the crystal structure of the N-MLV NTD CA hexamer (38). The C\text{α} atom of the amino acid residue that immediately follows each insertion is denoted by a sphere labeled with the nucleotide and residue number for that mutation. (C) N-MLV NTD CA hexamer with mutated positions shown on one monomer as in panel B. The color scheme for the secondary structures follows that of panel A. Drawings were made using MacPyMol (12).

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FIG. 2. Virion release and infectivity assayed by exogenous RT activity in supernatants. Mutant proviral DNAs were introduced into 293T cells. Medium was harvested 48 h posttransfection and assayed for RT activity (black bars). An average of four experiments for all mutants and WT is shown. These supernatants were used to infect naive Rat2 cells. Medium was harvested 48 h postinfection and RT activity measured (gray bars). RT activity of WT virus was set to 100%.
ture cores and ~14% immature cores. Approximately 88% of our PR particles contained immature cores. We were unable to unambiguously assign ~15 to 20% of WT and PR cores to either mature or immature categories. In contrast, for all except the three most-N-terminal mutations, most mutant cores could not be classified as mature or immature. Also within a given mutant, there were obvious differences in core morphology. To better define the morphology of these mutant cores, we created three additional categories of core phenotypes and determined a quantitative estimate for each category by examining 50 to 70 particles for each mutant. The new categories of core were as follows: punctate (irregular cores with numerous, small electron-dense spots, with a clear electron-lucent space separating the core from the envelope, as in Fig. 5C); punctate, with ill-defined boundaries (irregular cores with numerous small electron-dense spots and no clear demarcation between core and envelope, as in Fig. 5D); lacy (a more uniformly electron-dense core, occasional faint speckle seen, with no clear demarcation between core and envelope, as in Fig. 5E). The proportions of immature, mature, and aberrant cores observed for each mutant are represented in a clustered stacked chart (Fig. 5F). For a given mutant, well-defined mature and immature particles are represented in a stack on the left. This left stack is the predominant stack for WT or PR particles but is very small or absent for many mutants, reflecting the relative absence of “normal” cores for these mutants. The stack on the right consists of the cores with aberrant morphology. Going from the N to C terminus, the three mutants in the β-hairpin had two- to fourfold-higher amounts of immature particles than WT, suggesting a defect in maturation. The next four mutants after the β-hairpin, i.e., 1794, 1800, 1823, and 1833, did not display any normal cores despite extensive examination of grids. The majority of these mutant particles contained punctate cores with well-defined or ill-defined boundaries. The remaining mutants also displayed mostly punctate cores and a small number of mature or immature cores. We previously reported three other mutations in the NTD of CA that did not form cores at all and resulted in large patches of Gag protein at the membrane (3). Mutations in the NTD of CA that we are reporting here cause more subtle changes in core morphology without affecting core size or shape too drastically. Yet, these relatively subtle changes in morphology are associated with noninfectious particles.

Envelope protein function. We proceeded to determine where these released mutant virions might be blocked in the infectious pathway. Some mutations in Gag are associated with defects in proteolytic processing of the envelope protein p15E into the mature p12E protein (61). This cleavage activates the
FIG. 4. Transmission electron microscopy of particles released from cells transfected with proviral DNA from MoMLV WT, PR−, and mutations in NTD of CA. The morphologies of pelleted virions are shown. WT and PR− particles are shown on the top at two different magnifications, followed by CA mutants that are shown in order from the N to C terminus at the higher magnification only. Each mutant is labeled with a nucleotide and amino acid residue corresponding to its location. Each mutant panel shows a representative image of pelleted particles on top and a plot of particle diameters on the bottom. Minimum and maximum diameters were measured for at least 50 particles, and the average diameter (in increments of 10 nm) was plotted relative to its' frequency of occurrence (percentage of total sample), depicted by a solid line. For comparison, the distribution of WT particle diameters is indicated by a dotted line in each plot. Bars, 250 nm and 100 nm, as shown.
FIG. 5. Morphological classification of cores and the prevalence of core subtypes in different mutant populations. Pelleted virions visualized by transmission electron microscopy were grouped into five categories: (A) mature, (B) immature, (C) punctate, (D) punctate with ill-defined boundaries, and (E) lacy. For each mutant, ~50 to 70 particles were examined, and the categories were plotted as a clustered stacked bar graph (F). For each mutant, mature and immature particles are represented in the stack on the left. The stack on the right consists of the cores with aberrant morphologies (C, D, and E).

mature virions most likely entered cells efficiently. This suggests that the envelope proteins were fully functional and the envelope protein processing was defective in virions containing mutants in the NTD of CA, examined their envelope protein function using the XC cell fusion assay (41). XC cells produce large syncytia when cocultivated with cells infected with ecotropic murine leukemia virus (50, 51). Each of our mutants at least half contained CA in the presence of all five mature and immature intermediates of reverse transcription in Hirt DNA. WT1 and WT2 are WT virus particles used as positive controls for the infection. ΔRT is a noninfectious MoMLV mutant with a severely truncated RT and is used as a negative control (4). MA-1491 is a replication-competent insertional mutation in MA (4), also used here as a positive control. WT INAC is heat-inactivated viral supernatant used to inoculate cells to ensure that any PCR amplification seen was due to bona fide products of reverse transcription and not due to carryover of DNA from the transfection. As controls for the PCR, proviral plasmid DNA from four different mutants (1724, 1800, 1941, and 1970) was used as template. The no-DNA control for the PCR contained all PCR reagents except for template DNA. (A) The (−)ssDNA intermediate was detected using primers designed to amplify a 124-bp stretch of the R-U5 region. (B) Viral DNA that includes the gag region was detected using one primer that binds to the insertion and a second primer that binds within the CA region. Since WT provirus and plasmid pNCA do not contain insertions, they lacked the primer-binding site, and no PCR product was generated. The replication-competent mutant MA-1491 and proviral plasmid DNA from the four mutants that contained insertions resulted in PCR products of different sizes, depending on the location of the insertion. (C) Circular viral DNA containing the LTR junction. The arrow indicates the position of the LTR junction product, seen only for WT virus and the replication-competent insertional mutant MA-1491. When proviral plasmid DNA was used in this PCR, the β-lactamase gene located in the region between the two LTRs was amplified (arrowhead). (D) Mitochondrial DNA. Approximately equal amounts of PCR product were seen with each mutant when primers that amplify mitochondrial DNA were used.

Reverse transcription in infected cells. We proceeded to examine if the mutant virions entering cells were able to perform reverse transcription. Cells were infected with WT and mutant virions harvested from transfected cells, using equal amounts of virus as judged by their exogenous reverse transcriptase activity. Twenty hours after infection, low-molecular-weight Hirt DNA was isolated from cells (29). The Hirt DNA fraction typically contains various intermediates of viral DNA synthesis as well as full-length viral DNA. This Hirt DNA was analyzed by PCR using primers that amplify specific intermediates of viral DNA synthesis, viz. (−)ssDNA, one of the earliest intermediates of reverse transcription, as well as later products, such as long plus strand and plus strand transfer. We also attempted to amplify the LTR junction present in full-length double-stranded DNA that has been transported into the nucleus but fails to integrate and circularizes instead (7). We were able to detect these stages of viral DNA synthesis in Hirt DNA prepared from cells infected with WT virus (Fig. 6A to C). In contrast, none of these intermediates was detected in cells infected with any of the mutant viruses (Fig. 6A to C). Occasionally, when using PCR to detect the (−)ssDNA intermediate for mutant 1719, we observed a faint band, suggesting that mutant 1719 might be slightly better than the other CA mutants at initiating reverse transcription (Fig. 6A). Analysis of serially diluted samples indicated that the mutants were at least 100 to 1,000 times less efficient than WT (data not shown), with 1719 closer to 100 times less efficient, at best. The absence of PCR products was not due to low yield or poor quality of Hirt DNA, since primers that amplified mitochondrial DNA resulted in approximately equal amounts of product for each sample (Fig. 6D). Mitochondrial DNA, which is 16.5 kb in size and circular, copurifies with viral DNA in Hirt DNA.
small fraction of the unlabeled endogenous reaction as template and two sets of primers, one to specifically amplify the long minus-strand DNA and another for the plus-strand DNA. A third primer set was used to confirm the presence of (−)ssDNA (Fig. 7B). Not surprisingly, (−)ssDNA was amplified for all mutants, corroborating our results shown in Fig. 7A. A faint band was seen in the no-incubation control, most likely due to efficient synthesis of short products at lower temperatures or during brief periods of warming. For all mutants, we were also able to detect both long minus-strand and long plus-strand viral DNA intermediates (Figs. 7C and D). We performed these PCRs with 10 serial dilutions of the unlabeled endogenous RT reaction mixture as template (8 of the 15 mutants were chosen at random to determine relative RT activities). We found that the endogenous RT activity for the majority of mutants was nearly equivalent to WT (data not shown). To ensure that amplified products were derived from reverse-transcribed DNA and not contaminating plasmid DNA, a negative control containing WT virus and the RT reaction mixture that was not incubated at 37°C was included. No PCR product was detected in this negative control, indicating that the PCR products were generated from reverse-transcribed DNA and not from residual plasmid DNA remaining after extensive DNase treatment of viral inocula. The results of the endogenous RT assays indicate that virions containing mutations in the NTD of CA have functional RT machinery. The genomic RNA, the tRNA primer, and the reverse transcriptase that are encapsidated in mutant virions are together able to generate viral DNA in vitro in the endogenous reaction. Yet, for reasons on which we can only speculate at this stage, these mutants are unable to reverse transcribe in cells.

**DISCUSSION**

Our current analysis of the MoMLV CA, along with our previous studies (3, 4), has allowed us to divide the NTD into three functional regions: (i) the β-hairpin at the N terminus, which appears to be essential for correct core maturation, (ii) the helix 4/5 loop, which is essential for formation of spherical cores during immature assembly, and (iii) the region including α-helices 2 to 5, except for the above-mentioned loop, which appears to be essential for proper core assembly and for early stages following viral entry into cells.

The possible mechanisms for phenotypes of these mutants are more obvious in some cases than others. An extensive morphological analysis of particles from mutations located in the first region, i.e., the region forming the β-hairpin, showed mostly immature cores, a phenotype similar to protease-defective mutants. This was corroborated by Western blot analysis of released virions, which showed a persistence of several intermediates of Gag processing. It has been suggested that proteolytic cleavage at the N terminus of CA triggers a refolding of the protein, leading to condensation of the entire core structure (56). It is likely that insertions within the β-hairpin prevent the proper cleavage and/or refolding of the N terminus, leading to persistence of immature or aberrant particles. We also observed that when cleavage at the N terminus of CA was inhibited, cleavage between CA and NC was also inefficient (Fig. 3; note the greater levels of p40, the CA-NC inter-

preparations. From this we concluded that virions with mutations in the NTD of CA were severely defective in initiating reverse transcription upon entry into cells.

**Endogenous RT assay.** Judging from the near-normal levels of exogenous RT activity for all the mutants (Fig. 2), it was unlikely that the insertions in CA had a direct effect on the RT enzyme itself. Yet, no viral DNA was synthesized in infected cells. We therefore proceeded to characterize the functionality of the RT machinery by other means. When concentrated MoMLV virions are permeabilized by small amounts of non-ionic detergent and then incubated with deoxyribonucleotides, viral DNA is synthesized in vitro by a process called the endogenous RT reaction (55). We performed this reaction using equal amounts of pelleted mutant and WT virions (as determined by Western blot analysis using anti-CA antibodies). To detect (−)ssDNA, the earliest intermediate of reverse transcription, radiolabeled (32P) dTTP was included in the reaction mixture. All mutants synthesized the 145-nt (−)ssDNA at amounts comparable to WT virus (Fig. 7A). Longer DNA products were seen as a smear at the top of the gel, since this gel was unable to resolve them. To detect these longer products of reverse transcription, PCR was performed, using a

**FIG. 7.** Endogenous RT activity of WT and mutant virions. (A) 32P-labeled viral DNA products made by WT and mutant virus particles in the endogenous RT reaction. 32P-labeled AvilIII size markers are shown on the right. An arrow indicates the position of the 145-bp (−)ss DNA. (B to D) PCR for detection of other intermediates of reverse transcription. An endogenous RT reaction identical to that shown in panel A, but unlabeled, was set up for WT and mutant virus particles. The reverse-transcribed DNA that was generated in the reaction was amplified using appropriate primer sets. Primers were designed to detect specific intermediates of viral DNA synthesis, such as minus-strand strong stop-DNA (B), long minus-strand DNA using primers that amplify a 309-bp region in gag (C), and plus-strand DNA to confirm plus-strand jumping and elongation (D). For negative controls, a reaction mix containing WT virus that did not undergo the incubation for endogenous RT reaction was used. The mock control underwent identical reactions, except no proviral DNA was used in the transfection. Proviral pNCA plasmid DNA was used as an additional positive control for the PCR. DNA size markers are shown on the left.
mediated). This was similar to the phenotype of a missense mutation in the first residue of MoMLV CA that also blocks cleavage between p12 and CA (39, 45). Mutants for the second region—the helix 4/5 loop—assemble large planar lattices of Gag on the cell surface but do not make spherical capsids or particles. This is consistent with the mutations allowing a lattice of hexamers to be generated, without incorporating the necessary pentamers that add curvature to a planar lattice (3, 9, 57). This loop lies in the outer margin of the CA NTD hexamer, and it is possible that insertions at this location might disrupt the pentamer-hexamer interaction or pentamer formation. The timing and requirement for pentamer incorporation during assembly remains unclear. Mutants with mutations located in the fairly extensive third region, between α-helices 2 and 5, assembled into particles of approximately normal size, which were efficiently released. Their core morphologies, however, did not resemble either immature or mature cores, and the mutants were defective at a stage immediately following viral entry into cells. Despite a functioning reverse transcriptase machinery in the virions, these mutants did not reverse transcribe their genome in cells.

The aberrant core morphologies seen in mutants in the third region of the NTD might be due to the mutations affecting CA or Gag conformation, processing, and protein-protein interactions. A common feature of many of these mutant cores was the lack of the clearly defined space that normally separates a mature core from the viral membrane. It is likely that in WT virus this clear space is formed upon complete proteolytic processing of Gag, allowing the CA protein to condense around the genome (60). Many of our mutants showed incomplete proteolytic processing of Gag. The intermediates of processing may prevent proper packing of proteins, resulting in a core with ill-defined margins, variegated densities on the surface, and a lack of clear space between the core and the envelope. The different types of abnormal cores that were observed might reflect differential processing and assembly of variably processed intermediates. The most severe morphological defects were seen with mutants located within the 18-helix bundle of the NTD. This is perhaps not surprising, as this bundle is formed by interactions between α-helices 1, 2, and 3, which are structurally conserved among retroviral CA NTDs and stabilize the center of the hexameric ring (38). Destabilization of this central feature of the hexamer might also contribute to the aberrant shapes and sizes of the relevant mutant cores. We currently lack high-resolution structural data to accurately define how the NTD hexamers interact with each other to form a lattice. It is possible that some of our insertional mutants affect correct lattice formation, resulting in aberrant cores. The mutant cores might also be explained by the mutations disrupting the NTD-CTD interactions that are important for proper core assembly. Structural data, hydrogen-deuterium exchange studies, and chemical cross-linking applied to in vitro-assembled HIV-1 tubes imply that the NTD and CTD are in close contact (34, 35). Furthermore, second-site suppressor mutations in the RSV CTD also suggest an NTD-CTD interaction (6). Defining such NTD-CTD interactions in MoMLV CA will need further studies.

With some exceptions, our findings are consistent with data obtained from mutagenesis of the NTD of HIV-1 CA. In general, mutations in the HIV-1 CA NTD result in efficient release of particles that often contain abnormal cores, are noninfectious, and are usually blocked at an early stage following entry, all of which are features shared with our MoMLV mutants. Here we further elaborate the similarities and differences between phenotypes of mutations in specific regions of MoMLV and HIV-1 CA NTD. Alanine-scanning mutations in the β-hairpin of HIV-1 CA NTD produced particles with mature (conical) cores and moderately reduced infectivity (56). Our insertion mutations in the β-hairpin of MoMLV NTD CA contained mostly immature cores and had severely reduced infectivity. It is possible that this difference in severity of phenotypes might be related to the difference between point mutations and 12-amino-acid insertions.

A deletion of HIV-1 CA NTD α-helices 2 and 3 and their flanking turns resulted in efficient particle release, though the particles were not infectious (58). Along similar lines, most point mutations and small insertions spanning HIV-1 α-helices 1 to 4 also produced noninfectious particles that lacked conical cores and showed defects in reverse transcription in vivo (42, 43, 46, 53, 57). These HIV-1 mutant cores were often poorly defined, had unusual shapes, and were eccentrically placed within the particle. Particles were frequently enlarged and contained multiple cores. Our insertion mutations in equivalent regions of MoMLV CA NTD had similar phenotypes, though certain phenotypes seen with mutations in the NTD of HIV-1 CA were not seen in our MoMLV mutations.

The first such discordant phenotypes consisted of particles of heterogenous sizes and particles with multiple cores. Small deletion mutations in HIV-1 α-helix 1 and in the CypA binding loop resulted in particles with sizes ranging from ~75 to 315 nm (14). The MoMLV mutants that we examined had diameters ranging from 60 to 150 nm, similar to WT virions. Mutations in the MoMLV equivalent of the CypA binding loop (the helix 4/5 loop) did not produce any spherical cores or particles. Also, none of our mutants produced particles containing more than one core, nor did they contain cores with varied shapes, as seen for the HIV-1 α-helix 1 mutant (N18A/N21A) (57). We were unable to directly examine the role of MoMLV α-helix 1 because our analysis did not include mutations in that location.

A second phenotype that is different among HIV-1 CA NTD mutants and MoMLV mutants consists of blocks to replication after reverse transcription, viz. at nuclear import and integration into host cell DNA and integration in vitro. Substitution mutations located in HIV-1 α-helices 1 and 2, a double substitution in α-helix 4 (Q63A/Q67A), and substitution mutations in the CypA binding loop all show such blocks (13). None of our MoMLV α-helix 2 or α-helix 4 mutants showed such a nuclear transport or integration defect—they were all blocked before reverse transcription. Mutants of the helix 4/5 loop in MoMLV do not assemble cores at all and so are completely blocked for viral release. It is possible that such differences between the roles of HIV-1 and MoMLV CA NTDs reflect fundamental differences in uncoating and nuclear transport in the two viruses.

Another possible explanation for the irregular core morphologies seen with our MoMLV CA NTD mutants may be due to alterations in core stability as manifested by differing velocity sedimentation profiles. For example, HIV-1 mutants P38A (α-helix 2) and Q63/Q67A (α-helix 4) showed unstable cores, whereas mutant E45A (α-helix 2) had a hyperstable core.
(15, 17). Such altered core stabilities were seen with mutants that contained conical cores as well as those with aberrant cores (17, 57). Some of these mutant cores (those containing substitution mutations in hydrophobic residues of HIV-1 α-helices 1 and 2) demonstrate very little dissociation of CA protein and increased dissociation of RT, from the core (54). This defect in core disassembly might account for reduced viral DNA synthesis in infected cells. Together, these findings suggest that optimal core stability might be critical for uncoating and initiation of reverse transcription. An assay for measuring core stabilities has been difficult to establish for MoMLV cores in our hands; hence, it was not possible to test our mutants for their core stability phenotypes and thus characterize defects in uncoating.

The morphology of mutant cores might reflect a lack of a specific host cell factor(s) incorporated in WT cores as they assemble (24). Such host factors might bind to a specific site in the CA NTD that is disrupted by the insertion mutation, and lack of such a factor may lead to the defects in morphology of the assembling core. Alternatively, the insertion mutations may disrupt the ability of host cell factors to bind to the incoming CA NTD for uncoating the core upon entry into cells. Lack of such host factor activity may inhibit subsequent reverse transcription of the genome and viral trafficking through the cytoplasm. Identification of such host factors participating first in core assembly and later in uncoating will need further studies.

In summary, this study considerably extends previous work demonstrating that the N-terminal portion of the MoMLV CA protein critically contributes to the formation of functional virus cores and plays an essential role in core disassembly and early stages following entry of the core into the cytoplasm. Our analysis of the MoMLV CA has allowed us to divide the NTD into three functional regions. The first functional region includes the β-hairpin and controls normal maturation of virions. The second region lies in the helix 4/5 loop and is essential for the formation of spherical cores. The third region encompasses most of the NTD except for the helix 4/5 loop. Mutants in region three assemble into imperfect core structures, and the resulting particles are efficiently released. The mutants are defective at a stage immediately following entry of the capsid into cells. Despite possessing functional reverse transcriptase machinery, these mutant virions do not reverse transcribe in cells. Many of these findings are consistent with what is known from mutational analyses of HIV-1 and RSV CA. We have not yet elucidated the exact mechanism of this block and postulate either a structural inability of these capsids to correctly assemble and subsequently uncoat or an inability to interact with a host cell factor(s) that is necessary for precise assembly and uncoating. Future studies are needed to understand the mechanisms of these blocks and to explore the possibility of targeting these regions pharmacologically to inhibit additional steps in retroviral infection.

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