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 insertion 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 capsids is very similar for viruses across genera (8). CA shapes assumed by the mature core, the tertiary structure of the capsids is very similar for viruses across genera (8).

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).

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most of the NTD of MoMLV CA to be essential for viral replication (4). We also reported a small region here, in the loop between α-helices 4 and 5 (the helix 4/5 loop), to be important for formation of spherical cores (5). In the current study, we report a detailed biochemical and morphological characterization of 15 additional mutations in the NTD of MoMLV CA. These mutations flank our previously identified domain for the formation of spherical cores yet have an entirely different phenotype. They are assembled into particles that are roughly similar in size and shape to wild-type (WT) particles and are released efficiently from cells but are blocked at an early stage postentry.

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 pCAG, clones were individually screened using PCR. One primer was complementary to a portion of the insertion (short G, 5′-GGGCGGCTGCAAGCCTTCAAG-3′) and a second primer that hybridized outside of the region of mutagenesis (236G6L; 5′-TTCTTCCGGGTTTTCTGTT-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 mutants 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 spanning system (New England Biolabs, Beverly, Mass.). This allows the isolation of a Tn7 transposase-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 restriction endonuclease sites that flank the transposable element are used to remove all but 10 bp of the transposable element. This 10 bp, in addition to the restriction endonuclease sites that flank the transposable element are used to amplify rat mitochondrial DNA, 5′-GTTTAAGGCTAAGCATAGTGGG (26), were used to check the quality of DNA left over from the transfection), and used to infect Rat2 cells (26). Infected cultures supernatants with the RT reaction mix for 1 hour at 37 °C, samples were spotted through a cushion of 25% (wt/vol) sucrose, and the pellets were resuspended 48 h after transfection of 293T cells with proviral DNA 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. Enblock staining using 1% tannic acid in water was followed by washing and dehydration to 100% (BDH). After dehydration to 100%, pellets were embedded in Epon 812 (EMS, Fort Washington, PA). Sections were cut with a diamond knife, mounted on formvar-carbon-coated copper grids (EMS), stained with 1% aqueous uranyl acetate and 0.4% lead citrate, and examined on a JEOL JEM-1200 EXII transmission electron microscope. Exemplary fields were photographed with a 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.

**Virus purification and Western blot analysis.** Culture supernatants were collected 48 h after transfection of 293T cells with proviral DNA and centrifuged through a cushion of 25% (wt/vol) sucrose, and the pellets were resuspended in TNE buffer (10 mM Tris [pH 7.4], 150 mM sodium chloride, 1 mM EDTA [pH 8]) (for the RT assay) or radioimmunoprecipitation buffer (for Western blotting) (55). Details of virus purification, lysis, and Western blot analysis were as described previously (55, 61). Goat anti-CA serum (NCI serum 79/804; gift of S. P. Goff, Columbia University) diluted 500:1 was used for Western blot analysis. Blots were also probed with anti-MA (NCI serum 76/155), anti-p12 (78S-373), anti-NC (80S008), and rabbit anti-p15E from A. Rein (NCI), all diluted 1/5,000. All cell lysate blots were probed with rat antibasophil antibody (Pierce) to determine differences in loading. Amounts of protein were measured using densitometry and ImageQuant software (Molecular Dynamics).

**Transmission electron microscopy of virions.** Culture supernatants (25 ml) were collected 48 h after transfection 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. Enblock staining using 1% tannic acid in water was followed by washing and dehydration to 100% (BDH). After dehydration to 100%, pellets were embedded in Epon 812 (EMS, Fort Washington, PA). Sections were cut with a diamond knife, mounted on formvar-carbon-coated copper grids (EMS), stained with 1% aqueous uranyl acetate and 0.4% lead citrate, and examined on a JEOL JEM-1200 EXII transmission electron microscope. Exemplary fields were photographed with a 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.

**Fusion assay.** 293T cells were transfected with mutant proviral DNA using our standard protocol. At 48 h posttransfection, the cells were irradiated to prevent further growth. Rat XC cells were laid on top of the irradiated 293T cells and then checked for syncytia 24 h later (41). XC cells and the protocol for fusion were kindly provided by Monica Roth (Robert Wood Johnson Medical School, Piscataway, NJ) (27).

**Analysis of viral DNA synthesized in vivo.** Virions used to inoculate Rat2 cells were collected by centrifugation of 293T cells (Boehringer Mannheim) to remove any plasmid DNA remaining from the transfections (37). At 24 to 48 h after viral inoculation, low-molecular-weight DNA (29) was prepared from these 293T 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-strand DNA (−)ssDNA), B-LTR2 (5′-AGTCCTCCGATTG ACTGAG) and B-ss-as (5′-CCTGATGCAATCACCTCAG) (37) were used. To detect long minus-strand DNA, primers in the gag region, 5′-GGGCCGTTGCCGCGTTCCTCAAGCCTTCAAG-3′ and 5′TTCTTCCGGGGTTTTCTGTT-3′, were used. Primers flanking the long terminal repeat (LTR) junction, LTR1 (5′-CCTTCTTATTTAGCTCGG) and B-LTR2 (5′-AGTCCTCCGATTGACTGAG) were used to detect the presence of 1-LTR or 2-LTR circular viral DNA. Primers that amplify rat mitochondrial DNA, 5′-GTTAATGTCATGATATAAAAGGC and 5′-GTTTACGGCTAACAGCATGATGTCG (26), were used to check the quality of the low-molecular-weight Hirt DNA. 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 59°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(rA)) primer-template assays were performed in the presence of radiolabeled [α-32P]dATP and MnCl2, 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 resuspended 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 deoxyinosinic acid triphosphates (100 μM each) and [³²P]dTTP (0.6 μM) at 37 °C for 40 min. Reactions were terminated, purified, 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 products of reverse transcription, the endogenous reverse transcription reactions were set up as above, except no [³²P]dTTP was included and the incubation was extended to 7 h with higher concentrations of deoxyinosinic acid triphosphates (2 mM each) to allow for synthesis of longer products (55). Detection of these unlabeled RT products was performed by PCR, using either 2 μl or 0.2 μl of the endogenous RT reaction mixture as template in a 25-μl-total-volume PCR mixture. PCR products were analyzed on agarose gels stained with ethidium bromide. An RT deletion mutant (∆RT) that resulted in translation of less than 100 amino acids of RT (4) was included as an additional control to ensure that products were generated from authentic reverse transcription products rather than from contaminating plasmid DNA.

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 naive 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 inreleased 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. 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 Pr65gagg 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 Pr65gagg 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 once again 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 was 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
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 particles had an average diameter of 113 and 99 nm, respectively. For the three most-N-terminal mutants located in the β-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-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 ~66% ma-
ture cores and ~14% immature cores. Approximately 88% of our PR<sup>-</sup> particles contained immature cores. We were unable to unambiguously assign ~15 to 20% of WT and PR<sup>-</sup> 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<sup>-</sup> 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.

FIG. 3. Proteolytic processing of mutant Gag proteins expressed in cells and in virions. (A) Organization of Pr65<sup> gag</sup>, shown on top, with Gag cleavage products containing CA underneath. (B) Viral protein expression in 293T cells transfected with mutant proviral DNA. Western blot analysis using anti-CA polyclonal serum detected CA and other Gag intermediates 48 h after transfection. Arrows indicate the positions of the Gag precursor (Pr65<sup> gag</sup>), p55 (MA-p12-CA), p42 (p12-CA), and p40 (CA-NC). Open arrowheads, mutant CA proteins; closed arrowheads, WT CA proteins. Blots were reprobed with antibodies to MA and NC proteins to characterize the intermediates of proteolytic processing. The lower blot in panel B is the same blot, probed with antitubulin antibody. (C) Virions released into the medium following transfection with proviral DNAs were pelleted and lysed, and the proteins were analyzed by Western blotting using anti-CA antibodies.
FIG. 4. Transmission electron microscopy of particles released from cells transfected with proviral DNA from MoMLV WT, PR<sup>-</sup>, and mutations in NTD of CA. The morphologies of pelleted virions are shown. WT and PR<sup>-</sup> 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.
membrane fusion capability of the envelope protein and is required for MoMLV infectivity (40, 44). To determine if envelope protein processing was defective in virions containing mutations in the NTD of CA, we examined their envelope proteins using Western blotting. All 15 mutations permitted normal levels of envelope protein expression in cells, as well as its incorporation into virions and subsequent cleavage (data not shown). We proceeded to test these mutants for envelope protein function using the XC cell fusion assay (41). XC cells are rat tumor cells induced by avian sarcoma virus, and they produce large syncytia when cocultivated with cells infected with ecotropic murine leukemia virus (50, 51). Each of our mutants in the NTD of CA produced syncytia that were equivalent to WT, both in size and number (data not shown), with 1719 closer to 100 times less efficient, at best. The 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 mitochon-
drial 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-

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).

FIG. 6. Mutant virions did not synthesize viral DNA upon entry into cells. Cells inoculated with mutant virus particles were lysed to make Hirt DNA. PCR was used to detect the presence of three 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.
Endogenous RT reaction. 32P-labeled viral DNA products made by WT and mutant virus particles in the reverse transcription upon entry into cells. From this we concluded that virions with mutations in the NTD of CA were severely defective in initiating reverse transcription. 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 genome 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-
mediate). 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, variagated 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 insertional 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 insertional 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.
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 insertional mutation, and lack of such a factor may lead to the defects in morphology of the assembling core. Alternatively, the insertional 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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