

A Mutant of Human Immunodeficiency Virus with Reduced RNA Packaging and Abnormal Particle Morphology

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A deletion of 30 bases was created in the noncoding region of the human immunodeficiency virus type 1 genome that extends between the 5' splice donor and the start of the *gag* gene. Viral particles produced after transfection of this mutant provirus had a normal protein pattern but a reduced RNA content. The infectivity of the mutant virus was also markedly reduced but not completely abolished. Electron-microscopic examination of the mutant virions revealed major abnormalities of the nucleoid structure, mostly related to the dense material characteristic of mature particles, suggesting that the presence of RNA is essential to the normal structure of the nucleoid.

The association of viral RNA with virion structural proteins is an essential step in the assembly of infectious retroviral particles. This process is termed encapsidation or packaging. The mechanisms by which the viral genome specifically associates with structural proteins to form particles are not fully understood. However, regions of the genome of some retroviruses that can act in *cis* as signals for encapsidation of full-length genomic RNA molecules, a process that is believed to involve the recognition of such elements by a viral protein, have been identified (2, 3, 10, 12, 16, 17). In murine retroviruses, the main encapsidation signal lies between the 5' splice donor, just 3' of the U5 region, and the start of the *gag* open reading frame (2, 11, 12). Mutant retroviruses whose genomes lack the packaging signal make normal viral proteins and assemble viral particles that do not contain viral RNA (2, 10, 12). Such mutant retroviruses have been used to package defective recombinant retroviral genomes into vector particles, which can in turn be directed to target cells, where the recombinant genomes subsequently undergo reverse transcription and are integrated and expressed (5, 10, 12).

We report here the identification of a region of the human immunodeficiency virus type 1 (HIV-1) genome that plays a role in the efficiency of packaging viral RNA into HIV particles. HIV-1 mutants in which this region of the genome has been deleted display a markedly reduced infectivity but make normal viral proteins. The viral RNA content of purified viral particles is also markedly reduced. Overall, our results are in agreement of those of Lever et al. (9), who used a similar mutant. However, our electron microscopy studies of mutant particles revealed major abnormalities, suggesting that the presence of the viral RNA is essential for the morphogenesis of the mature viral core.

We assumed that, as observed for other retroviruses, the principal determinant of HIV-1 RNA encapsidation would be located in the noncoding region of the genome that lies between the major 5' splice donor and the start of the *gag*

open reading frame. Although the corresponding region in HIV is much smaller, we deleted 30 bases from this region in an infectious molecular clone of HIV-1, pNL4-3 (1). The deletion, extending from bases 301 to 331, was created by site-directed mutagenesis as previously described (19). The structure of the resulting plasmid, pNLW1, is shown in Fig. 1. Human SW480 colon carcinoma cells, in which HIV-1 infection does not spread but in which high levels of viral proteins and particles can be produced following introduction of DNA (1), were transfected by the calcium phosphate coprecipitation technique with plasmids pNL4-3 and pNLW1 (10 µg of DNA per subconfluent 25-cm² flask). Forty-eight hours later, the cells were lysed for analysis of viral proteins by immunoblotting as previously described (18) with the serum of an HIV-1-infected individual. Comparable amounts of normal viral proteins were found in the wild-type and mutant-transfected cells (Fig. 2). Supernatants from the same cultures were monitored for the production of viral particles by reverse transcriptase (RT) assay as described elsewhere (18). Wild-type and mutant plasmids produced similar amounts of particles (data not shown). The supernatants from the same transfection experiments were centrifuged at 10⁵ × g, and the pellet was evaluated for the presence of viral proteins by immunoblotting. The protein profiles of the mutant and wild-type viral particles were again undistinguishable (Fig. 2).

The infectivity of the mutated virus was assessed by exposure of CD4-positive human A3.01 lymphoid cells (6) to filtered supernatant samples from the SW480 cultures harvested 48 h after transfection. The supernatant from pNL4-3-transfected cells, which contained a comparable amount of viral particles as measured by reverse transcriptase activity (approximately 3 × 10⁵ cpm per inoculum), was used as a positive control. In A3.01 cultures infected with the wild-type pNL4-3 virus, a peak of RT activity was observed on day 12 (Fig. 3A), a sign of a spreading HIV-1 infection. In contrast, no productive infection was detected in cultures infected with supernatant from pNLW1-transfected cells.

In a parallel experiment, A3.01 cells were cocultivated with SW480 cells 48 h after transfection with either wild-type or mutant proviral clones. In this case, both cocultures

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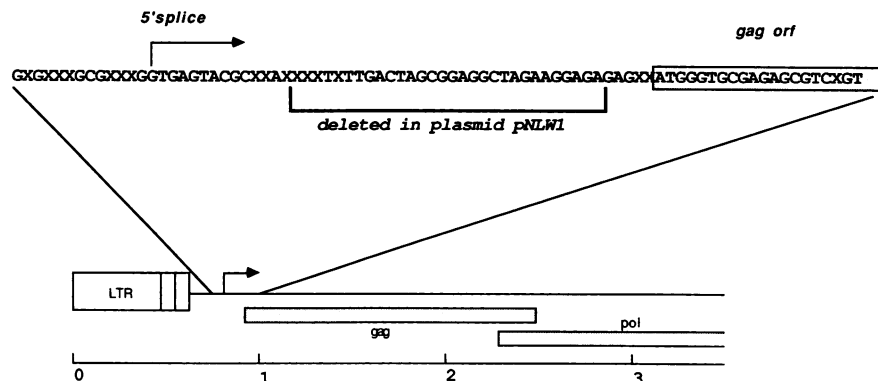


FIG. 1. Location of the deletion in mutant clone pNLW1. The sequence in the upper part of the figure displays the letters A, T, G, and C for bases that are conserved in all sequenced HIV-1 genomes and X for variable bases. LTR, Long terminal repeat.

yielded a spreading infection, but the peak of RT activity in the pNLW1 culture was delayed 5 days compared with the wild-type transfection (Fig. 3B). To further quantify the replicative defect of the mutant virus, A3.01 cells were infected with serial 10-fold dilutions of filtered supernatant from the previously described cocultures harvested at the peak of viral production. These coculture supernatants contained significantly higher levels of RT activity than those taken from the SW480 cells 48 h after transfection. The infectivity of supernatants containing mutant NLW1 particles was found to be about 3 orders of magnitude lower than that of a wild-type supernatant containing a comparable amount of viral particles, on the basis of RT counts (Fig. 3C). This lower infectivity was retained upon further passage in A3.01 cells (data not shown).

We anticipated that the defect in pNLW1 particles would involve the packaging of viral RNA into virions. Therefore, we harvested particles from a further passage in A3.01 cells of the virus produced during the coculture experiment illustrated in Fig. 3B. These particles, which retained the infectivity defect illustrated in Fig. 3C, were pelleted at $10^5 \times g$ and lysed in 0.5% Nonidet P-40, and RNA corresponding to equivalent amounts of virions on the basis of RT activity was immobilized on a nitrocellulose membrane. The membrane was then hybridized to a 6.5-kilobase probe spanning most of the genome of HIV-1 (4). The amount of viral RNA detected in mutant virions was reduced by a factor of approximately 4 to 10 compared with the amount detected in wild-type virions (Fig. 4). This observation, taken together with the reduced infectivity and the similar amount of viral proteins produced, suggests that the pNLW1 mutation affected the encapsidation of viral RNA into HIV-1 particles.

The defects in RNA packaging and infectivity associated with the NLW1 mutation are not absolute. In repeated experiments, viral RNA was consistently detected in association with the mutant particles, perhaps reflecting nonspecific encapsidation or the action of sequences outside of the NLW1 deletion, for example, in the gag coding region or in the U5 region of the long terminal repeat. Such sequences appear to play a role in encapsidation in addition to the ψ region, as described for murine retroviruses (3, 14). Mutant NLW1 virions were able to initiate a spreading infection but only when used at a high concentration. In this regard, the levels of infectious mutant virions contained in 48-h-old supernatants from transfected cells were probably too low to start an infection. By contrast, in the cocultures, freshly

budded virions that were continuously produced next to their CD4-positive target could initiate a productive infection with greater efficiency. The fact that the mutant HIV particles obtained after transfection and coculture retained their low infectivity after further passage in A3.01 cells is an indication of the absence of a rescue of the mutation through genetic recombination with endogenous retroviral sequences, as is often the case for other retroviruses (13, 15). Overall, our observations are in agreement with the findings

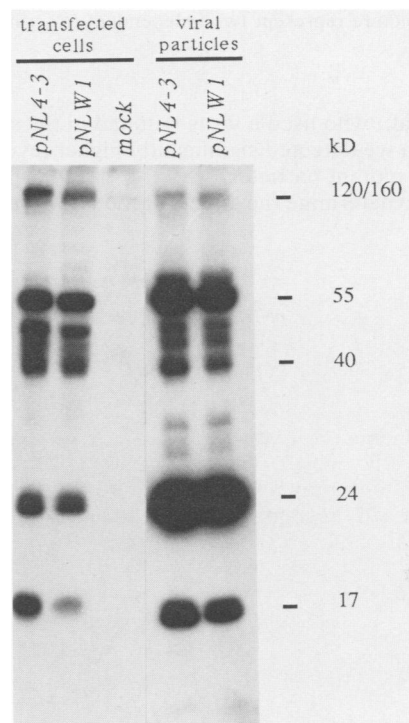


FIG. 2. Immunoblot of viral proteins of wild-type (pNL4-3) and mutant (pNLW1) viruses. SW480 cells (5×10^6) were lysed 48 h after transfection and analyzed by immunoblotting as described elsewhere (16). Viral particles produced by these same transfected cells were pelleted at $10^5 \times g$, lysed, and immunoblotted by the same method. Equal volumes of culture supernatant which contained similar amounts of reverse transcriptase activity were used. kD, Kilodaltons.

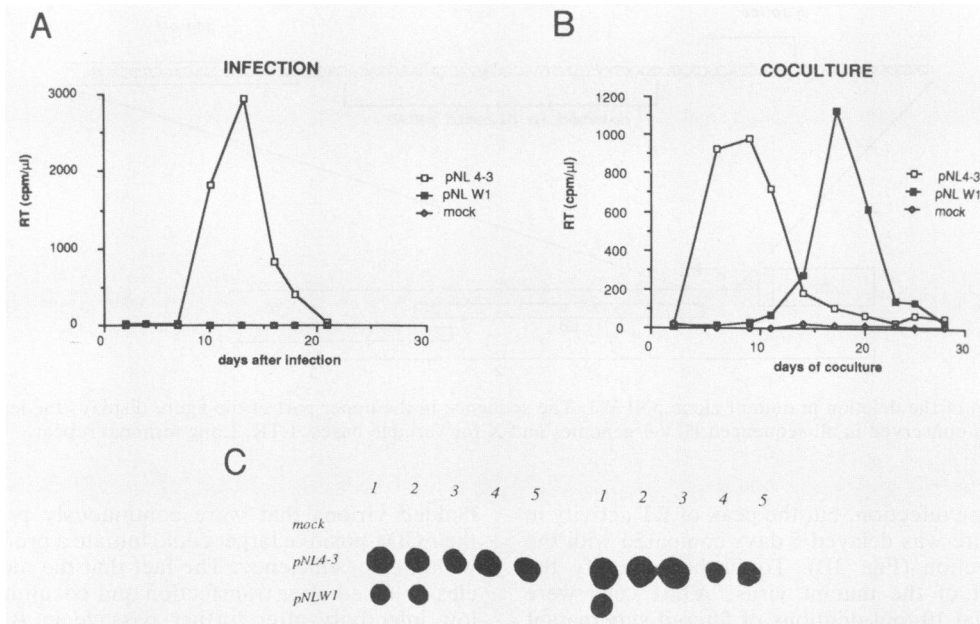


FIG. 3. Infectivity of the mutant NLW1 virus. (A) Infection of A3.01 cells with filtered supernatants harvested 48 h after transfection of SW480 cells with wild-type (pNL4-3) and mutant (pNLW1) plasmids. Reverse transcriptase activity was monitored during the course of the infection as described previously (18) and revealed by scintillation counting. (B) Coculture of A3.01 cells with transfected SW480 cells, starting 48 h after transfection. (C) Infectivity of the virus produced at the peaks of reverse transcriptase activity shown in panel B. A volume of supernatant (approximately 1 ml) containing an equal amount of reverse transcriptase activity for each virus was serially diluted 10-fold and used to infect A3.01 cells (5×10^5 cells for each dilution). RT activity in those cultures was measured at day 10 as previously described (18) and revealed by autoradiography. The numbers above the autoradiograms represent the logarithms of the dilutions. The right and left parts of the picture represent two independent experiments on the same supernatants.

of Lever et al., who used a virus mutated in the same region (9), although we detected significantly higher levels of residual RNA in mutant particles.

In order to determine the effects of the NLW1 mutation on

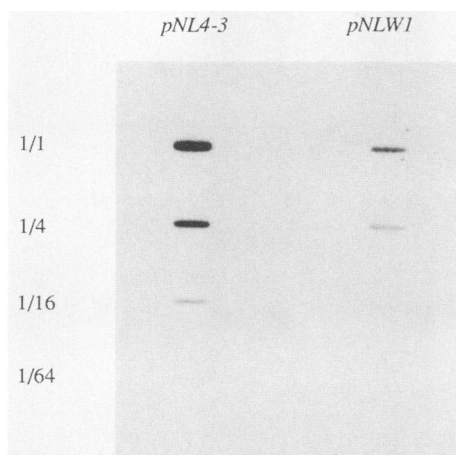


FIG. 4. Viral RNA content of the mutant virus. Viral particles harvested during an acute infection of A3.01 cells with wild-type (pNL4-3) or mutant (pNLW1) virus were pelleted at $10^5 \times g$ and lysed in 0.5% Nonidet P-40. Serial fourfold dilutions of the lysates (indicated at the left) were slot blotted onto a nitrocellulose membrane. The membrane was then hybridized with a 6.5-kilobase HIV-1 DNA probe (4), washed, and autoradiographed.

particle morphology, infected cells were examined by transmission electron microscopy. A3.01 cells infected with either wild-type or mutant virus were examined in parallel. While budding, the wild-type and mutant virions were morphologically indistinguishable (Fig. 5A and C). Over 75% of the free wild-type virions displayed the morphology of the typical mature HIV-1 particles, with a conical nucleoid having a dense, broad end (Fig. 5B). Although examples of this morphology were seen for virions of the mutant infection, the majority of free virions (over 75%) were aberrant in appearance (Fig. 5D to F). Most common were virions containing nucleoids free of dense material (empty). The dense material was either displaced to a lateral position under the membrane or totally absent from the virion. Nucleoids with dense material either in a midposition or in the tapered end of the nucleoid were also observed. Larger-than-normal mutant virions with pleomorphic nucleoids were also often encountered. Aberrant particles, some similar in appearance to forms described here, have been seen as minor components of cultures of HIV-1 (7, 8), as was the case in the wild-type control infections. However, they never reach the proportions seen in the mutant infections. Therefore, this mutation, which clearly interferes with RNA packaging without affecting production or processing of viral proteins, appears to affect the normal nucleoid morphogenesis, especially in relation to the dense material that is characteristic of the mature nucleoid. This observation suggests that normal packaging of viral RNA may be required for the orderly arrangement of the nucleoid components.

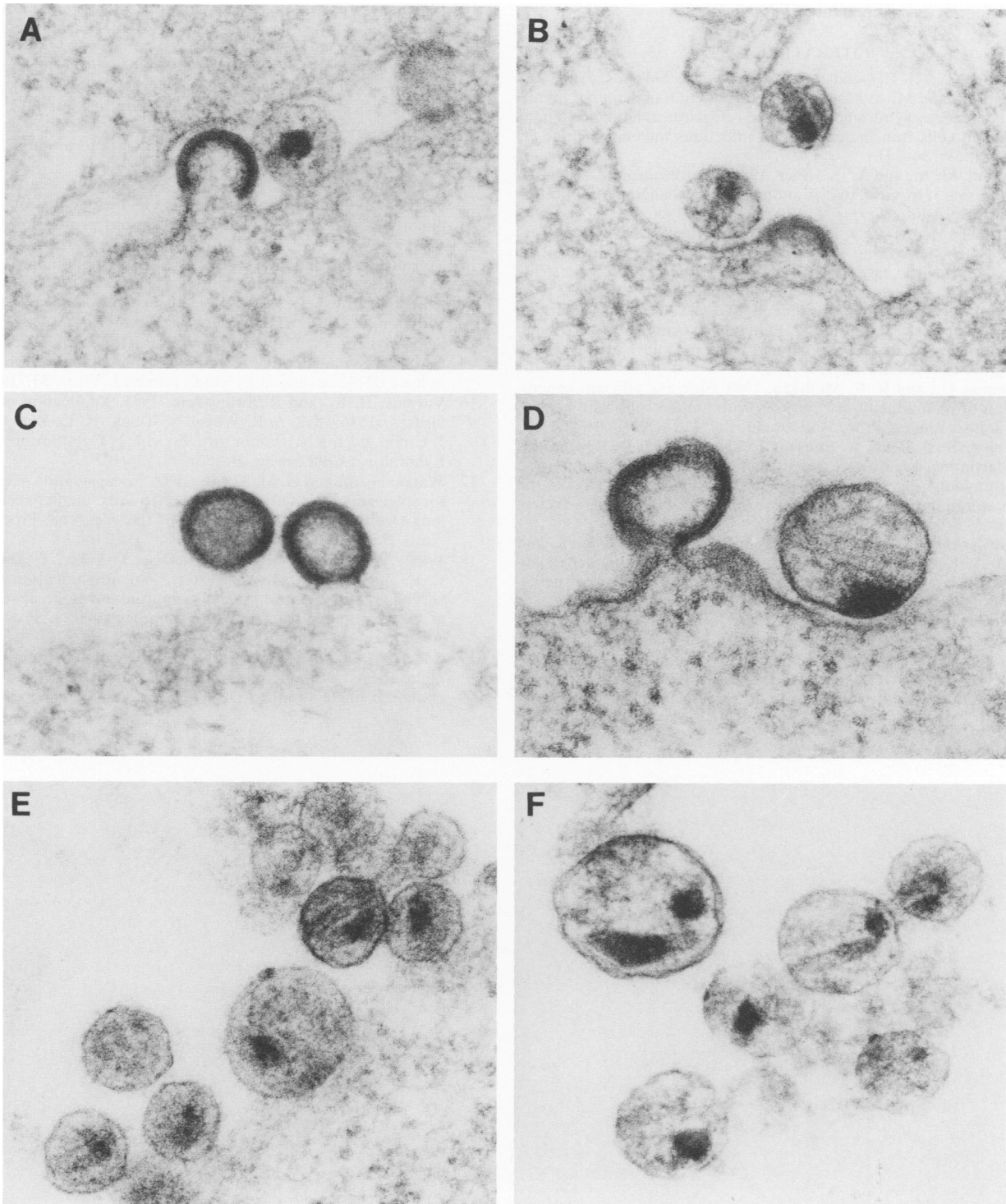


FIG. 5. Electron micrographs of wild-type and mutant viral particles. (A and B) Infection with wild-type virus. Transmission electron micrographs of early- and late-budding particles and of mature particles with conical nucleoids sectioned in different planes. (C through F) Infection with mutant virus. Late-budding and free immature particles (C) and a double-budding particle (D) are shown. Aberrant particles varying in size, shape, and nucleoid morphology (D through F) can be seen. One large particle has a long and empty nucleoid and eccentric dense material (D), others have misplaced dense material (E and F), and others have absent or inapparent nucleoids (E and F). Magnification, $\times 110,000$ in all panels.

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