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Noninfectious Human Immunodeficiency Virus Type 1 Mutants Deficient in Genomic RNA

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All retroviruses contain, in the nucleocapsid domain of the Gag protein, one or two copies of the sequence Cys-X_2-Cys-X_4-His-X_4-Cys. We have generated a series of mutants in the two copies of this motif present in human immunodeficiency virus type 1. These mutants encoded virus particles that were apparently composed of the normal complement of viral proteins but contained only 2 to 20% of the normal level of genomic RNA. No infectivity could be detected in the mutant particles, while 10^5 infectious U were present in an equivalent amount of wild-type particles. Thus, the mutants have another defect in addition to the inefficiency with which they encapsidate genomic RNA. Our results show that both copies of the motif are required for normal RNA packaging and for infectivity. Mutants of this type may have important applications, including nonhazardous materials for research, immunogens in vaccine and immunotherapy studies, and diagnostic reagents.

All retroviruses encode a polyprotein, the Gag precursor, which is ultimately cleaved into several of the major structural proteins of the virus particle. One of these cleavage products, the nucleocapsid (11) protein, contains one or two copies of a conserved motif termed the cysteine array or Cys-His box (10, 16). This 14-residue sequence has a completely invariant spacing of cysteine and histidine residues and is thus reminiscent of the zinc finger motif found in many DNA-binding proteins (3, 4). The cysteine array appears to be involved in packaging of viral RNA during virus assembly, since avian and murine type-C retroviruses with mutations in the cysteine array produce RNA-deficient, noninfectious virus particles (5, 6, 13–15).

It seemed important to test the generality of these findings by studying cysteine array mutants in more distantly related retroviruses. We have therefore generated cysteine array mutants in an infectious proviral clone of human immunodeficiency virus type 1 (HIV-1). As described in the current report, these mutants give rise to particles which package reduced amounts of HIV genomic RNA and have no detectable infectivity. These intact, noninfectious HIV-1 particles may have a number of important applications.

MATERIALS AND METHODS

Cells and plasmids. HeLa cells were a gift of George N. Pavlakis and Barbara K. Felber, Advanced Bioscience Laboratories, Inc., National Cancer Institute-Frederick Cancer Research Facility (NCI-FCRF), while H9 cells were obtained from Robert C. Gallo, NCI (17). The pNL4-3 plasmid, containing an infectious proviral clone of HIV-1 (1), was a gift of Malcolm Martin, National Institute of Allergy and Infectious Diseases. Sequence positions in this clone refer to GenBank locus HIVNL43. The Bluescript KS^+ plasmid was from Stratagene (La Jolla, Calif.).

Mutagenesis. Oligonucleotide-directed mutagenesis was performed as previously described (6) by using the 4,278-base-pair *Spe*I-*Sal*I fragment (positions 1507 and 5785 in pNL4-3, respectively) in Bluescript KS^+. All mutations were verified by direct sequence analysis (19) of the entire *Spe*I-*Sal*I fragment after reconstructing the full-length viral genome, using Sequenase (United States Biochemical Corp., Cleveland, Ohio). Mutations are designated as in C36S, in which residue 36 of the nucleocapsid protein, $p7^{NC}$ (11), has been changed from cysteine to serine.

Transfection. Plasmids were transfected onto 90% confluent HeLa cell monolayers by the calcium phosphate method (7). Cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. Twenty-four-hour harvests of culture supernatants were taken 2 to 5 days after transfection, clarified, and analyzed for virus.

Reverse transcriptase assays. Samples were analyzed for reverse transcriptase (RT) activity as follows. Virus was precipitated from 0.5 ml of clarified supernatant with 0.25 ml of 50% polyethylene glycol (8,000 M_W) in 0.5 M NaCl for 24 h at 4°C. Precipitated virus was collected by centrifugation, and the pellets were suspended in 25 μl of 50 mM Tris buffer, pH 7.5, with 100 mM NaCl and 1 mM EDTA. A 5-μl portion of the dissolved material was incubated for 2 h at 37°C with 20 μl of 62.5 mM Tris buffer, pH 8.3–125 mM NaCl–7.5 mM MgCl–0.25% (vol/vol) Nonidet P-40–12.5 mM dithiothreitol–5 μg of oligo$(dT)_{12-18}$ per ml–50 μg of poly(rA) per ml–156 μCi of [^{3}H]TTP (Dupont, NEN Research Products, Boston, Mass.) per ml. The reaction was stopped by the addition of 200 μl of 4.5 mM $Na_2H_2P_2O_7$, 15 mM NaCl, 0.1 mg of bovine serum albumin per ml, and 30 μl of 60% (wt/vol) trichloroacetic acid. The precipitates were incubated for 30 min on ice before filtration through fiberglass filters. The filters were then dried and counted in Aquassure scintillation cocktail (Dupont, NEN Research Products).

Assays for viral proteins. Virus was pelleted at 25,000 rpm for 2.5 h in a rotor at 4°C (model SW28.1; Beckman Instruments, Inc., Fullerton, Calif.). Competition radioimmunoassays for the capsid protein, $p24^{CA}$, and the surface glycoprotein, $gp120^{SU}$ (11), were performed on viral pellets as described previously (18). For protein immunoblotting, samples were adjusted for equal amounts of RT activity and fractionated on a 10 to 20% sodium dodecyl sulfate-polyacrylamide gradient gel. The proteins were transferred onto Immobilon-P paper (Millipore Corp., Bedford, Mass.) as previously described (8, 9). Mouse monoclonal antibody to

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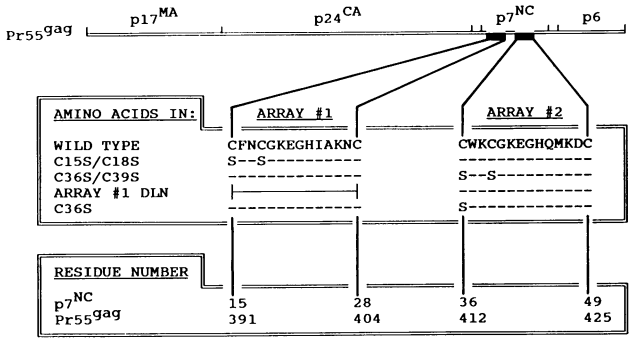

FIG. 1. Mutants of HIV-1 nucleocapsid ($p7^{NC}$). The top line shows the cleavage pattern of $Pr55^{gag}$ (the Gag precursor polyprotein) and the positions of the two cysteine arrays within $p7^{NC}$ (9). Beneath this line are shown the amino acid sequences of the two arrays in the pNL4-3 clone (1) of HIV-1 and in the mutants described here.

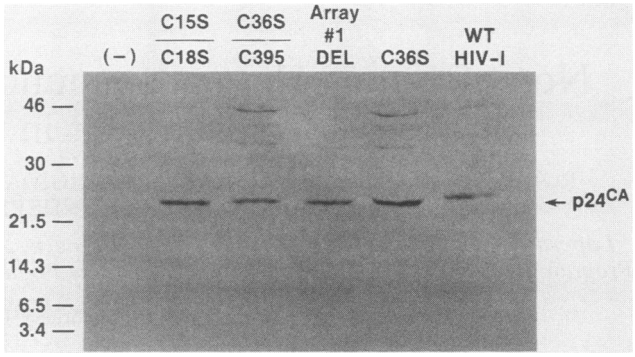

FIG. 2. Protein immunoblotting analysis of mutant and wild-type virus particles. Particles were analyzed for $p24^{CA}$ as described in Materials and Methods. (−), HeLa cells transfected with calf thymus DNA. Molecular masses of marker proteins are indicated on the left.

$p24^{CA}$ (a gift from P. M. Watson, Program Resources, Inc., NCI-FCRF) was used at a dilution of 1:200. The antibody-antigen complex was visualized by using goat anti-mouse immunoglobulin G (light plus heavy chains)-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) and the 4-chloro-1-naphthol Membrane Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).

RNA blot analysis. RNA was isolated and treated as described previously (2, 6). Samples were adjusted for equal amounts of RT activity before electrophoresis of viral RNA. HIV-1 genomic RNA was visualized by probing with a nick-translated, ^{32}P-labeled pNL4-3 plasmid (12).

Infectivity assays. H9 cells (2×10^6) (17) were incubated with 1 ml of HeLa supernatant in the presence of 2 μg of Polybrene per ml at 37°C for 12 h. The H9 cells were then diluted and maintained at a cell density of 2×10^5 cells per ml in a total of 10 ml of RPMI 1640 media supplemented with 5% fetal calf serum and 2 μg of Polybrene per ml. Samples were periodically collected from the H9 cultures, the cultures were refed, and the fluids were assayed for RT activity. Parallel tests were also performed by using human peripheral blood lymphocytes instead of H9 cells, with virtually identical results (data not shown).

RESULTS

Mutagenesis. To investigate the role of the cysteine arrays in HIV-1 replication, we generated a series of mutants in the HIV-1 molecular clone, pNL4-3, by oligonucleotide-directed mutagenesis. As indicated in Fig. 1, these mutants included replacements of one or two cysteine codons with serine codons in either of the two cysteine arrays and also the complete elimination of the proximal array.

Virion production by cysteine array mutants. The mutants were initially tested for their ability to generate virus particles by using RT assays and competition radioimmunoassays for $p24^{CA}$ and $gp120^{SU}$ (18) as quantitative measures of virus production. The mutants were transfected into HeLa cells, and supernatants were collected as described above. As shown in Table 1, all of the mutants produced virus particles at approximately the same levels as wild type did. In addition, the samples all exhibited similar ratios of RT activity, $p24^{CA}$ content, and $gp120^{SU}$ content.

We also analyzed the particles by protein immunoblotting

TABLE 1. Characteristics of HIV-1 nucleocapsid mutants

Mutant	RT (cpm/ml)[a]	$p24^{CA}$ (pg/ml)[b]	$gp120^{SU}$ (pg/ml)[b]
Calf thymus DNA[c]	0	0	0
C15S/C18S	125,900	710	338
C36S/C39S	70,200	361	404
Array one deletion	44,200	260	153
C36S	67,200	383	244
Wild type	122,700	779	557

[a] cpm of [^{3}H]TTP incorporated per ml of culture fluid.
[b] Picograms of $p24^{CA}$ or $gp120^{SU}$ per milliliter of culture fluid, determined by competition radioimmunoassay (18).
[c] Cells transfected with carrier DNA alone. A background of 420 cpm/ml has been subtracted from the RT values shown.

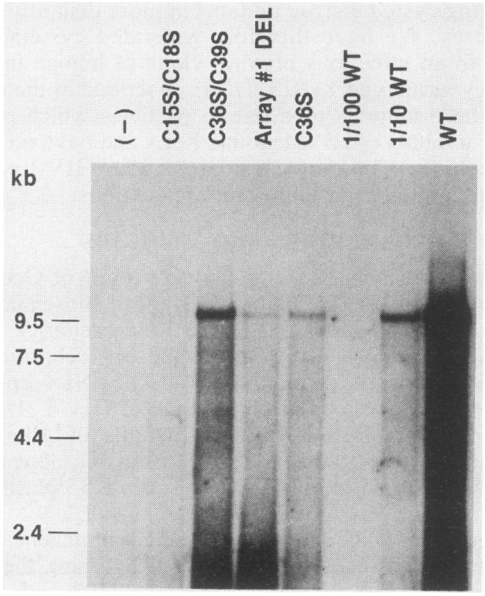

FIG. 3. RNA blot analysis of mutant and wild-type virus particles. Particles were analyzed for HIV-1 genomic RNA as described previously by using nick-translated (12), ^{32}P-labeled pNL4-3 plasmid as a probe. Dilutions (10- and 100-fold) of the wild-type sample were also tested. Sizes of marker proteins are indicated on the left.

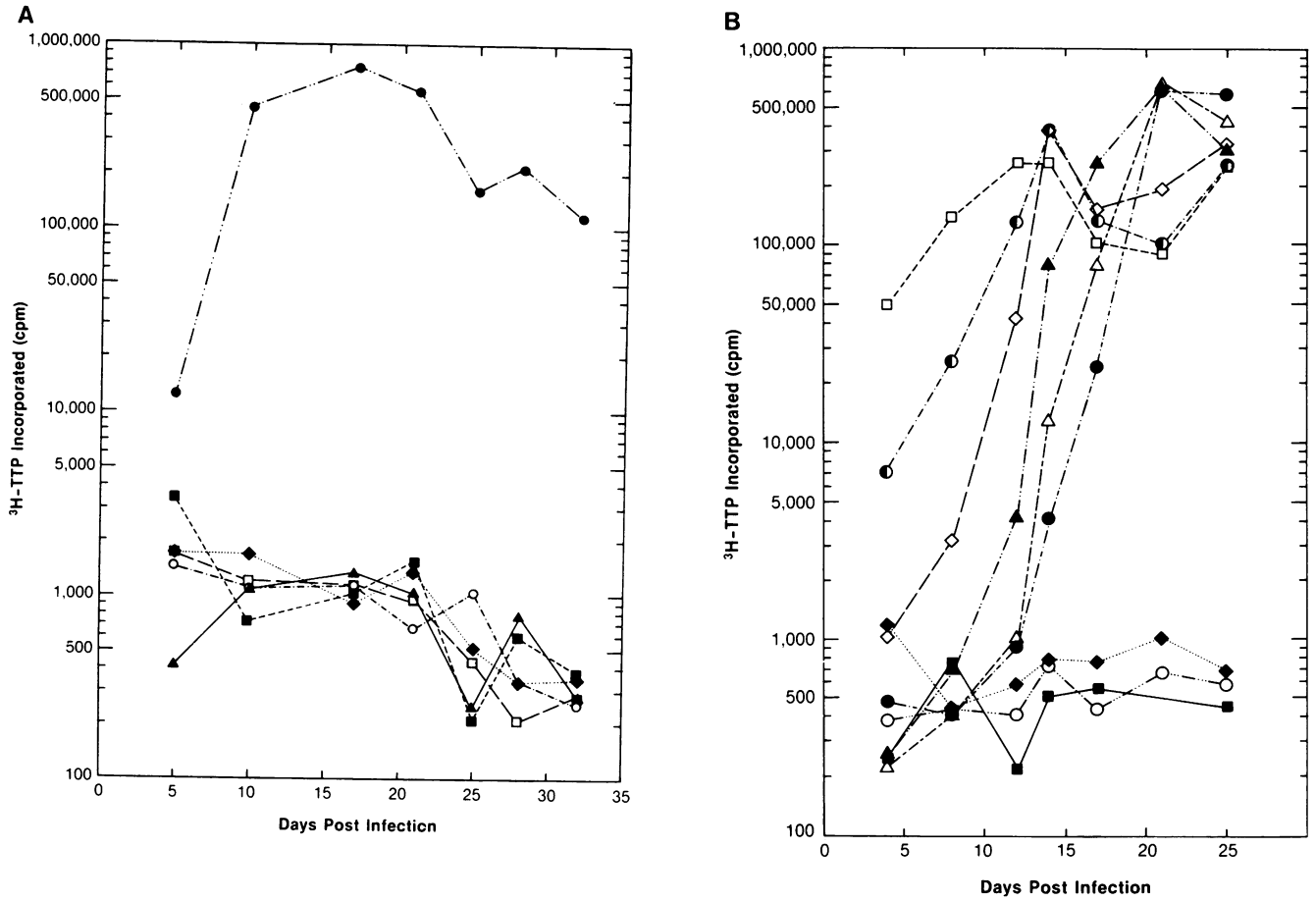

FIG. 4. Infectivity assays of mutant and wild-type virus particles. (A) Infectivity assays of mutants. HeLa supernatants were adjusted for equal amounts of RT activity. H9 cultures were infected and analyzed as described in Materials and Methods. Symbols: ●, wild type; ■, C15S/C18S; □, C36S/C39S; ○, array one deletion; ◆, C36S; ▲, calf thymus DNA negative control. (B) Dilution endpoint assay of wild-type HIV-1. HeLa supernatants containing wild type (□) and the C15S/C18S mutant (◆) viruses were adjusted for equal RT activity. Supernatant containing wild-type virus was also serially diluted 10 (◐)-, 100 (◇)-, 1,000 (▲)-, 10,000 (△)-, 100,000 (●)-, and 1,000,000 (○)-fold; ■, calf thymus DNA negative control. H9 cells were infected and tested for RT activity as described in Materials and Methods.

by using a monoclonal antiserum to $p24^{CA}$. Samples were first adjusted to equal levels of RT activity and then tested by immunoblotting as described in Materials and Methods. As shown in Fig. 2, the mutants were also indistinguishable from the wild type by this assay. The fact that the $p24^{CA}$ epitope recognized by the antibody is present on a 24-kilodalton protein indicates that the Gag precursor is processed normally by the viral protease in the mutant particles. The mutants also gave patterns identical to those of the wild type when subjected to protein immunoblotting with HIV-1-infected human patient antiserum (data not shown). Mutant virus particles were also examined in the electron microscope and were morphologically similar to wild-type particles (data not shown).

RNA content of mutant virus particles. We also tested the mutant virus particles for the presence of genomic RNA by using RNA blot hybridization with an HIV-1 proviral probe. The samples were first assayed for RT activity so that equal levels of virus particles could be compared with respect to RNA content. Serial dilutions of the wild-type sample were also tested, in order to quantitate the hybridization analysis. As shown in Fig. 3, each mutant contained a detectable level of genomic RNA but significantly less than the wild-type control. We estimate the genomic RNA contents of the mutant particles to be: C15S/C18S, ~2%; array one deletion, ~3%; C36S, ~6%; and C36S/C39S; ~20%, taking wild type as 100%.

Infectivity of mutant virus particles. To test the mutant particles for infectivity, we incubated H9 cells with the same virus preparations used above and analyzed the culture fluids for RT activity (Fig. 4A). H9 cultures exposed to wild-type virus showed a rapid rise in RT activity. However, no RT activity was observed in the H9 cultures incubated with the mutant particles, even after passage for 1 month. Similarly, when cultures of human peripheral blood lymphocytes instead of H9 cells were used, progeny virus was rapidly obtained with the wild-type virus but not with the mutant particles (data not shown). We also attempted to propagate the mutant particles by direct cocultivation of the transfected HeLa cells with H9 cells or peripheral blood lymphocytes. No progeny virus was obtained from any of the mutants in these experiments, while the expected increase in RT activity occurred within a few days of coculti-

vation with wild-type-transfected HeLa cells (data not shown).

A quantitative assessment of the relative infectivities of mutant and wild-type virus particles was performed by an endpoint dilution assay (Fig. 4B). Infectious virus was detected in a 1:100,000 dilution of wild-type stock, while undiluted C15S/C18S mutant stock (adjusted to the same RT activity as wild type) failed to show any infectious virus (Fig. 4B). We conclude that the mutants are at least 100,000 times less infectious than an equivalent amount of wild-type virus.

DISCUSSION

The results presented here show that in HIV-1, as in murine and avian retroviruses (5, 6, 13–15), mutants with subtle disruptions of the cysteine arrays in the nucleocapsid protein assemble into virus particles. These particles appear to contain the normal complement of Gag, Pol, and Env proteins (Table 1; Fig. 2), but the level of genomic RNA in the particles is reduced (Fig. 3). These findings thus extend to HIV the conclusion that the cysteine arrays play a crucial role in encapsidation of retroviral RNA during virus assembly.

Since HIV possesses two cysteine arrays, it seemed possible that they are redundant, i.e., that one complete array would be sufficient for wild-type function. However, our experiments show that this is not the case: mutations in either array are sufficient to reduce the packaging of viral RNA and to eliminate any detectable infectivity in the particles. It is interesting to note that alterations in the first array (C15S/C18S and the array one deletion) reduce RNA encapsidation somewhat more than mutations in the second array (C36S and C36S/C39S). This difference is particularly dramatic when C15S/C18S and C36S/C39S are compared, since these two mutations are both changes of the first two cysteines in the respective arrays to serines; they differ by approximately 7- to 10-fold with respect to genomic RNA content (see Fig. 3). In a previous study on avian retroviruses, Meric et al. (14) found that a deletion of the first array reduced infectivity more drastically than a deletion of the second array, while the two mutants exhibited similar phenotypes with respect to RNA packaging.

It is striking that while the mutants described here contain 2 to 20% as much genomic RNA as wild-type virus does (Fig. 3), their specific infectivity is <0.001% that of wild-type virus (Fig. 4B). Similar discrepancies between RNA content and infectivity have been noted in earlier studies of nucleocapsid mutants of avian and murine retroviruses (6, 13–15); in the case of murine leukemia virus, some mutants packaged easily detectable levels of Kirsten sarcoma virus RNA in the near-total absence of infectious Kirsten sarcoma virus (6). These observations strongly suggest that these cysteine array mutants are defective in some other function vital for infection, in addition to their inability to package viral RNA efficiently. Experiments to identify this additional defect and to generate new mutants with more severe packaging defects are now under way.

Because of their near-normal structure, noninfectious HIV-1 mutants of the type described here may have important applications as nonhazardous reagents for use in research and as immunogens in vaccine and immunotherapy studies. In addition, the extreme conservation of the cysteine array among retroviruses may present possibilities for developing universal retroviral diagnostic reagents, while the exquisite sensitivity of the virus to changes in the array suggests that the array is a potential target for the development of antiviral drugs.

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