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Genetic Analysis of the Zinc Finger in the Moloney Murine Leukemia Virus Nucleocapsid Domain: Replacement of Zinc-Coordinating Residues with Other Zinc-Coordinating Residues Yields Noninfectious Particles Containing Genomic RNA

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The effect of changing zinc (Zn^{2+})-coordinating residues in the nucleocapsid protein of Moloney murine leukemia virus was investigated by introducing a His-34-to-Cys or Cys-39-to-His mutation into the putative Zn^{2+} finger. Mutant virions contained normal levels of properly processed Gag and Env proteins and wild-type levels of full-length viral RNA. However, the specific infectivity of the mutants was $\sim 4 \times 10^{-4}$ that of wild-type particles. They were probably noninfectious because of the inability of the particles to synthesize cDNA transcripts, since full-length viral DNA could not be detected in Hirt supernatants of NIH 3T3 cells infected with the CCCC or CCHH virus. These mutants will provide an extremely valuable tool for analysis of the role of retroviral Zn^{2+} fingers in infection processes, independent of viral RNA recognition and packaging.

In all retroviruses except spumaretroviruses (19), the nucleocapsid (NC) (18) domain of the Gag polyprotein contains one or two copies of the sequence motif Cys-X_2-Cys-X_4-His-X_4-Cys. The conserved Cys and His residues in this motif bind a Zn^{2+} ion in the virus particle in a near tetrahedral coordination complex (5, 6, 33). After virus assembly and release, the viral protease (PR) cleaves the Gag polyprotein, and the NC protein (retaining the coordinated Zn^{2+} ions) binds the entire genomic RNA of the virus in a sequence-independent fashion (32, 34).

Previous studies on the function of the conserved motif (termed the Cys array or Zn^{2+} finger) have used site-directed mutagenesis to change the Cys and His residues to other amino acids, which would be unable to coordinate Zn^{2+}. In general, the resulting mutants assemble into virus particles with full efficiency, but the majority of these particles fail to package viral RNA. These findings indicate that the Zn^{2+} fingers function in RNA packaging during virus assembly (2, 9, 13, 14, 20, 28).

There is also strong evidence that the Zn^{2+} fingers perform other functions for the virus in addition to their role in RNA packaging. In particular, quantitative studies on mutant virus preparations have shown that the deficiency in genomic RNA in these particles is not sufficient to account for their lack of infectivity (13, 14, 20).

In this work, we have characterized two novel mutants of Moloney murine leukemia virus (Mo-MuLV). This retrovirus contains only one Zn^{2+} finger in its NC protein; in the two mutants, H34C (His-34 changed to Cys) and C39H (Cys-39 changed to His), we have replaced Zn^{2+}-binding residues with other Zn^{2+}-binding residues (Fig. 1A). We anticipated that these mutant proteins (unlike those of previously analyzed mutants) would retain the ability to coordinate Zn^{2+}; studies by others have shown that Zn^{2+} fingers of the classes tested here retain the ability to bind Zn^{2+} with essentially equivalent dissociation constants (17). Our results show that the new mutants can package genomic RNA with full efficiency. Remarkably, however, they are almost totally noninfectious. Infection by these particles is blocked before or during reverse transcription. There was a recent report of a mutant, similar to those described here, in which amino acid 23 in the human immunodeficiency virus type 1 (HIV-1) NC protein was changed from a His to a Cys residue. This created a virus with a CCCC type of Zn^{2+} finger in the first position of the HIV-1 NC protein. The phenotype of this mutant appears to be similar to the phenotypes of the mutants studied in this work (8).

The findings of this work show unequivocally that the Zn^{2+} finger is required for a step or steps in the viral life cycle in addition to its role in RNA packaging. Further, these new mutants should facilitate the identification of the additional function(s), since the particles contain normal amounts of genomic RNA. Finally, the results show that the additional function(s) is exquisitely sensitive to changes in the chemical or physical properties of the Zn^{2+} finger.

To examine effects of introducing eukaryotic-type Zn^{2+} fingers (steroid hormone receptor [CCCC] or classical [CCHH] [30]) on the retrovirus life cycle, mutations were introduced into the NC protein of Mo-MuLV to replace the CCHC Zn^{2+} finger. The mutant and wild-type Zn^{2+} fingers that were examined are depicted in Fig. 1A.

The CCCC Zn^{2+} finger was made by introducing an H34C point mutation by site-directed mutagenesis using the Muta-Gene phagemid in vitro mutagenesis kit, version 2, as described by the manufacturer (Bio-Rad, Richmond, Calif.). A mutagenic oligonucleotide with the sequence 5′-GCAAAGA AAAGGGGTGCTGGGC-3′, designated AR1240 (synthesized by Marilyn Powers, DNA Support Laboratory to ABL-Basic Research Program, SAIC-Frederick, National Cancer Institute-Frederick Cancer Research and Development Center [NCI-FCRDC] on an Applied Biosystems, Inc., model 380B DNA synthesizer) was used. The sequence of AR1240 corre-

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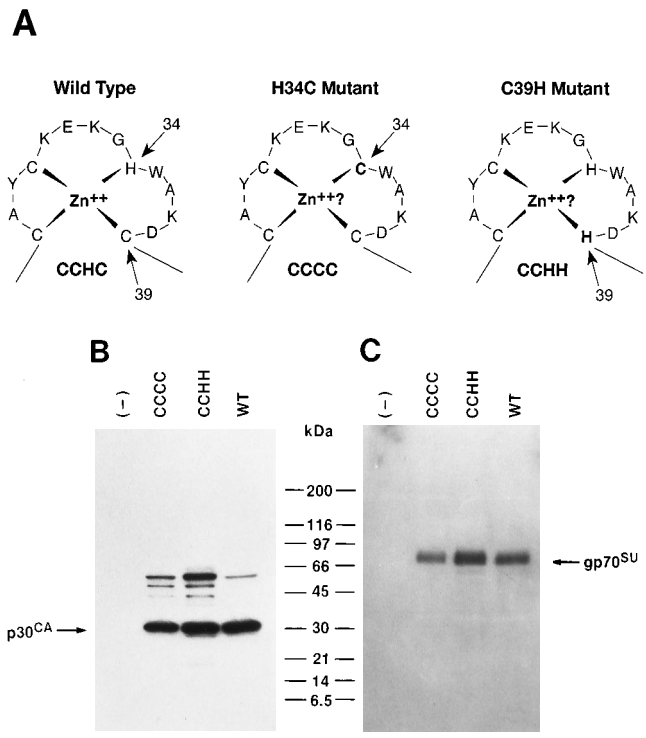

FIG. 1. (A) Schematic representations of the retroviral (CCHC type), H34C mutant (CCCC type), and C39H mutant (CCHH type) putative Zn^{2+} fingers that were introduced into the Mo-MuLV NC protein. The individual point mutations are indicated in boldface type. The numbers represent the positions of the amino acid residues in the Mo-MuLV NC protein that were changed. Zn^{++}? indicates a putative Zn^{2+} binding motif. Binding of Zn^{2+} by these mutant sequences has not yet been determined. (B) $p30^{CA}$ immunoblot analysis of transiently expressed mutant and wild-type (WT) virus particles from 293T cells. Samples were adjusted for equal RT levels (1.8×10^6 cpm), pelleted, fractionated, and treated as described previously (12), using goat antiserum to $p30^{CA}$. Positions of marker proteins and of $p30^{CA}$ are indicated. (−), samples from 293T cells transfected with sheared salmon sperm DNA. (C) Immunoblot analysis using antiserum to $gp70^{SU}$. The conditions are the same as for panel B.

sponds to nucleotides (nt) 2140 through 2161 in the Mo-MuLV genome (29). This oligonucleotide introduces changes (underlined) at nt 2154 (C to T) and nt 2155 (A to G). The full-length proviral clone, designated pDR56, was reconstructed by using pRR88 (an infectious clone of wild-type Mo-MuLV in pGC cos3*neo*) as described previously (13) and used to produce virus particles with a CCCC type of Zn^{2+} finger.

The CCHH Zn^{2+} finger was made by introducing a C39H point mutation in the gene coding for the NC protein. Two oligonucleotides, 62-mer sense strand (5′-TCGATCGCGACC AGTGTGCCTACTGCAAAGAAAAGGGGCACTGGG CTAAAGATCATTCCCAAG-3′; designated 4658-016) and 26-mer antisense strand (5′-TGAGCTCCTGATCCCTCAAA GTGGAT-3′; designated 4658-015) (Operon Technologies, Inc., Alameda, Calif.), were used to PCR amplify the region between the *Pvu*I and *Sst*I sites in the Mo-MuLV genome (nt 2117 and 2554, respectively [29]). 4658-016 contains a *Pvu*I site at its 5′ end corresponding to the *Pvu*I site at nt 2117. 4658-016 also contains the rest of the Mo-MuLV sequence from the *Pvu*I site through nt 2177. Near the 3′ end, 4658-016 has T-to-C and G-to-A changes (underlined; nt 2169 and 2170, respectively) that introduce the C39H mutation into the gene coding for the NC Zn^{2+} finger. 4658-015 contains sequences from nt 2535 through and including the *Sst*I site at nt 2554 (29). The PCR product made from pRR88 with these oligonucleotides was digested with *Pvu*I and *Sst*I, and the resulting 438-bp fragment was ligated with a 560-bp *Xho*I-*Pvu*I fragment (nt 1560 to 2117) into the *Xho*I and *Sst*I sites of the pGEM-7Zf(+) vector (Promega Corp., Madison, Wis.). The resulting plasmid (pDR58) was cut with *Xho*I and *Sst*I, and the 998-bp *Xho*I-*Sst*I fragment was ligated into the corresponding sites of pUCMRI-45. The latter clone (described in reference 13) contained the 2,145-bp *Xho*I-*Sal*I fragment (nt 1560 to 3705 [29]). Full-length proviral clones were constructed as described previously (13). The full-length clone with the C39H (CCHH Zn^{2+} finger) mutation is designated pDR57. By making mutations as described above, none of the residues in the loops of the finger were altered.

To determine whether pDR56 and pDR57 contained only the mutations that were introduced by the mutagenesis procedures, the plasmids were sequenced from the *Pvu*I site to the *Sst*I site (nt 2117 to 2554 [29]) as described previously (12). No mutations other than those introduced were found. The integrity of the remainder of pDR56 and pDR57 was tested for the presence of other mutations by cotransfecting the plasmids with pRB60 into Rat-1 cells. pRB60 contains a 438-bp *Pvu*I-*Sst*I fragment (nt 2117 to 2554 [29]) from pRR88 that was cloned into the homologous sites of pBluescript KS+ (Stratagene, La Jolla, Calif.). pRB60 was cotransfected with either pDR56 or pDR57 by using Lipofectamine (Life Technologies, Gaithersburg, Md.) as described previously (12). For a 75-cm^2 flask, 1.6 μg of pDR56 or pDR57 was cotransfected with 6.2 μg of pDR60. Rat-1 cells (obtained from Don Blair, NCI-FCRDC) were cultured at 37°C in 7% CO_2 and maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Cultures were monitored for increases in reverse transcriptase (RT) activities, indicating reversion of the mutant virus to a wild-type replicating species, through homologous recombination. Both plasmids appeared to recombine with the wild-type sequences, since RT levels increased to wild-type levels in cultures transfected with pRB60 and not in those without pRB60. These results taken together indicate that there were no mutations in pDR56 and pDR57 other than those originally introduced.

The mutant and wild-type viruses were transiently expressed from the proviral clones pDR56, pDR57, and pRR88 transfected into 293T cells. Cells were transfected by the calcium phosphate coprecipitation method (15) by using a calcium phosphate mammalian cell transfection kit from 5′→3′, Inc. (Boulder, Colo.). The 293T line (obtained from Nancy Rice, ABL-Basic Research Program, NCI-FCRDC) is a derivative of 293 cells that expresses the simian virus 40 large T antigen. These cells were maintained under conditions identical to those described above for the Rat-1 cells. Virus was harvested as described previously (13) and quantitated by determining RT activities. RT assays were performed on clarified supernatants as described previously (14), with the following changes. The virus was collected by centrifugation at 60,000 rpm in a model SW60Ti ultracentrifuge rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 1 h at 4°C from 4.2 ml of clarified supernatant. The viral pellet was resuspended in 100 μl of 50 mM Tris buffer (pH 7.5)–100 mM NaCl–1 mM EDTA. The assay was performed in 62.5 mM Tris buffer (pH 8.0)–25 mM KCl–0.6 mM $MnCl_2$–0.25% (vol/vol) Nonidet P-40–10 mM dithiothreitol–5 μg of dT_{12-18} per ml–50 μg of poly(rA) per ml–156 μCi of [^{3}H]TTP (80 Ci/mmol; DuPont, NEN Research Products, Boston, Mass.) per ml. RT activities and infectivity results from a typical transfection of 293T cells are summarized in Table 1. Whereas the CCHH and wild-type particle yields from this transfection were essentially equivalent, the pDR56 clone (CCCC) produced ~2 to 20% of wild-type levels of virus

TABLE 1. Properties of mutant and wild-type Mo-MuLV

Virus	Virus from 293T cells[a]		Virus from K-NRK cells[b]		
	RT activity (cpm/ml)[c,d]	MuLV infectivity (FIU/ml)[e]	RT activity (cpm/ml)[f]	MuLV infectivity (FIU/ml)	KiSV infectivity (FFU/ml)[g]
Control[h]	0	$<10^0$	0	ND[i]	ND
CCCC mutant	9.29×10^5	$<10^0$	7.34×10^4	10^0	2×10^0
CCHH mutant	5.86×10^6	$<10^0$	1.12×10^5	$<10^0$	2×10^0
Wild type	6.33×10^6	4.5×10^5	5.47×10^5	6×10^4	4×10^4

[a] Transiently expressed virus.
[b] Virus from stably transfected K-NRK cells selected with G418.
[c] Counts per minute of [^{3}H]TTP incorporated per milliliter of culture fluid.
[d] A background of 1.16×10^3 cpm/ml has been subtracted from the RT values shown.
[e] Determined as described previously (13). FIU, focus-inducing units. Values are from the S^+L^- focus assay (4).
[f] A background of 3.05×10^3 cpm/ml has been subtracted from the RT values shown.
[g] FFU, focus-forming units. Values were determined as described in reference 24.
[h] 293T cells transfected with carrier DNA alone or K-NRK cells transfected with pGCcos3*neo* (13).
[i] ND, not determined.

particles, as determined by RT analysis. This decreased production of mutant particles was not observed in the Mo-MuLV mutants that were described previously (13). In an S^+L^- focus assay (4), neither of the mutants scored positive, whereas the wild-type virus had a titer of 4.5×10^5 focus-inducing units/ml (Table 1). This result is similar to those of our previous studies of Mo-MuLV NC mutants in which the ligand-binding or aromatic residues in the Zn^{2+} finger were altered (13).

Since formation of a focus in the S^+L^- assay requires several rounds of virus replication during the 5-day assay period, a mutant which replicated with low efficiency would not register in this test. Therefore, we also tested the infectivity of each of the two new mutants by transfecting them into cells containing a rescuable Kirsten sarcoma virus (KiSV) genome and analyzing particles produced by the transfected cells in the KiSV focus assay. Any KiSV particles which could infect an NIH 3T3 cell would be detected in this assay, since formation of a focus in this test requires only a single infection event (24). Stable transfectants of the mutants and wild type were obtained in K-NRK cells (1) as described previously (13). As shown in Table 1, the mutants were $\sim 10^4$-fold less infectious than the wild-type control in the KiSV focus assay as well as in the S^+L^- focus assay.

To determine whether proper protein expression and maturation (i.e., Gag precursor processing) were occurring, protein immunoblot analysis was performed on mutant and wild-type viruses as shown in Fig. 1B and C. The samples were adjusted for equivalent RT levels, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane, incubated with goat polyclonal antiserum to the $p30^{CA}$ protein, and visualized as described previously (12). The levels of $p30^{CA}$ observed by this analysis are similar for all of the clones, indicating that the ratios of $p30^{CA}$ to RT (or the ratios of *gag* to *pol* gene products) are similar among the mutant and wild-type samples. These studies also show that the viral protease is expressed and functions correctly in virus maturation, liberating $p30^{CA}$ from the $Pr65^{Gag}$ precursor. The Gag precursors from the mutant and wild-type clones are cleaved properly; however, there are some partially cleaved $Pr65^{Gag}$ products seen in the two mutants. We also tested the effects of the mutations on incorporation of Env proteins into virions. As shown in Fig. 1C, similar levels of *env* gene products in the mutant and wild-type viruses were observed when the blot was probed with goat polyclonal antiserum to $gp70^{SU}$.

To determine if the CCCC and CCHH mutants packaged viral RNA, transiently expressed virus samples were examined by Northern (RNA) analysis (Fig. 2). RNA was isolated, fractionated, transferred to a nitrocellulose filter, and hybridized as described previously (12). For these experiments, viral pellets were disrupted as described by Fu and coworkers (10). A ^{32}P-labeled nick-translated pRR88 probe was used for hybridizations. The probe was prepared using a nick translation system from Life Technologies (Gaithersburg, Md.). Blots were washed at high stringency with four 250-ml washes with 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate at 65°C. Radiolabeled filters were visualized by autoradiography. Samples were adjusted for equal RT levels. Both of the mutants contain wild-type levels of full-length genomic RNA. These results are remarkable since the infectivity of the mutants remains undetectable. This is the strongest evidence to date that the Zn^{2+} fingers perform roles in the infection process other than genomic RNA recognition and packaging.

Inspection of the CCCC lane in Fig. 2 reveals a smear of low-molecular-weight material which hybridizes with the probe. While this appears to be degraded viral RNA, an alternative possibility is that it is plasmid DNA carried over from the transfection. This seem plausible since ~7-fold more supernatant was required to give the same RT activity in the CCCC pellet as in the wild-type or CCHH pellets. To test this possibility directly, we repeated the experiment except that viral RNA was purified by pelleting virus, disrupted in guanidine isothiocyanate, through a 96% (wt/vol) CsCl cushion as described previously (7). This procedure rigorously separates and

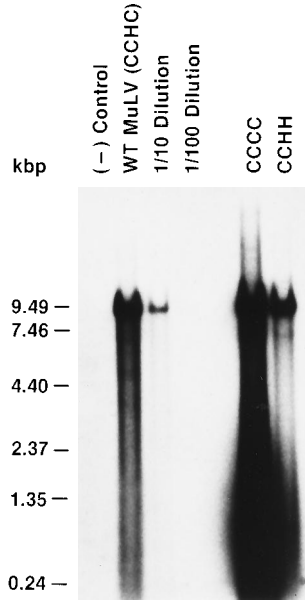

FIG. 2. RNA blot analysis of transiently expressed mutant and wild-type virus particles from 293T cells. Particles were analyzed for Mo-MuLV genomic RNA by using a full-length pRR88 probe that was ^{32}P labeled by nick translation. All samples were adjusted for equal RT levels (1.3×10^7 cpm), fractionated, and treated as described in the text. Dilutions (10- and 100-fold) of the CCHC (wild-type [WT]) samples were also tested. (−), pelleted supernatants from 293T cells transfected with sheared salmon sperm DNA. The RNA markers are indicated on the left. The large smear at the bottom of the blot in the CCCC lane is degraded DNA that was carried over from the transfection of the 293T cells.

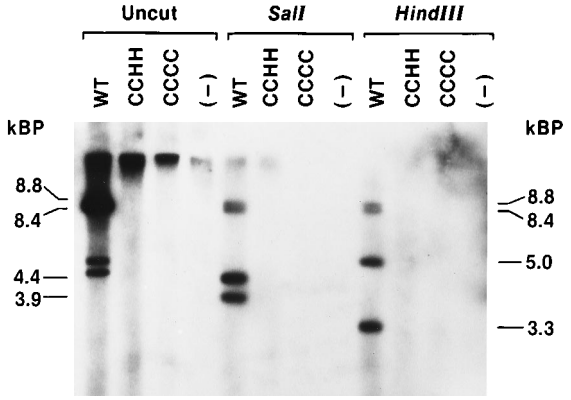

FIG. 3. Southern blot analysis of Hirt supernatant-fractionated DNA from mutant and wild-type virus-infected NIH 3T3 cells. Undigested, *Sal*I-digested, and *Hin*dIII-digested Hirt supernatant-fractionated DNA samples from NIH 3T3 cells infected with wild type (WT; CCHC), CCHH, and CCCC viruses were analyzed. Blots were probed a full-length pRR88 probe that was ^{32}P labeled by nick translation. Samples and the respective restriction endonuclease treatments are identified above the lanes. (−), Hirt supernatant-fractionated DNA from mock-infected NIH 3T3 cells. The calculated sizes of linearized bands are indicated at the sides.

purifies RNA from protein and DNA. Mutant and wild-type samples with the same levels of full-length genomic RNA (in the absence of any contaminating DNA) were compared. There was no longer a large smear of degraded material at the bottom of the CCCC lane when the blot was hybridized with the ^{32}P-labeled pRR88 probe (data not shown). The full-length proviral clones pDR56 (CCCC), pDR57 (CCHH), and pRR88 (wild type; CCHC) were also transfected into K-NRK cells to determine if the mutants could package heterologous viral genomes as was observed previously (13). There appeared to be no defect in genome packaging when blots were hybridized with either the Ki-*ras* probe, pSW11.1, or the Mo-MuLV probe, pRR88, as described previously (reference 13 and data not shown).

Although the mutants packaged wild-type levels of full-length genomic RNA, they were nevertheless noninfectious. This may be due to a defect in the ability of the virus to complete reverse transcription processes. To test this hypothesis, Hirt supernatant-fractionated nucleic acid was analyzed to determine whether proviral cDNAs could be synthesized from mutant and wild-type viruses. One hundred sixty milliliters of culture fluid from transiently expressing 293T cells was clarified and applied to 90% confluent 150-cm^2 flasks containing NIH 3T3 cells which had been pretreated with 20 μg of DEAE-dextran per ml in complete growth medium for 20 min at 37°C. Hirt supernatant DNA (16) was isolated 24 to 48 h later. Ten micrograms (calculated by A_{260}) of Hirt supernatant nucleic acid was untreated or digested with *Sal*I or *Hin*dIII and separated by electrophoresis on a 1% agarose gel. The DNA was transferred onto nylon-supported nitrocellulose (BA-S NC; Schleicher & Schuell, Keene, N.H.) as described by Southern (31). Prehybridization and hybridization (with a ^{32}P-labeled nick-translated pRR88 probe) using formamide and dextran sulfate were carried out as described previously (22). As expected (Fig. 3), the lanes with the wild-type-infected samples contained linear viral cDNA (8.4 kbp in the uncut lane, 4.4 and 3.9 kbp in the *Sal*I lane, and 5.0 and 3.3 kb in the *Hin*dIII lane) along with one- and two-long terminal repeat circles (8.4- and 8.8-kbp bands, respectively, in both the *Sal*I and *Hin*dIII lanes). These same cDNA species could be detected in an infection of NIH 3T3 cells when the wild-type viral inoculum was diluted 10-fold (data not shown). In contrast, viral cDNA was not detected in either of the lanes corresponding to the CCHH or CCCC mutant; lanes from these two samples were indistinguishable from the mock-infected control lane.

As indicated above, prior studies have replaced Zn^{2+}-coordinating residues with residues incapable of Zn^{2+} coordination (residues other than Cys or His). These studies have shown that retroviral Zn^{2+} fingers are involved in RNA packaging and strongly suggested that they have one or more additional functions (13, 20). In essence, the present results show that fingers in which the Cys at residue 39 is replaced by His or in which the His at residue 34 is replaced by Cys retain the ability to function in RNA packaging but are presumably defective in the other function(s) performed by the Zn^{2+} finger.

Although previous results indicated that mutants which had lost the ability to coordinate Zn^{2+} exhibited a second defect in addition to their defect in RNA packaging, the absence of viral RNA in the great majority of mutant particles made it extremely difficult to analyze the nature of this defect. The CCCC and CCHH mutants should now allow identification of the precise location in the virus life cycle that is blocked by these mutations. In turn, identifying the defect would add to our understanding of the functions of the Zn^{2+} finger in NC proteins. Our results show that the mutant particles are blocked before or during reverse transcription.

Present knowledge of the properties of NC suggests a number of possibilities for the second defect in the mutants. The genomic RNA in mutant particles might lack primer tRNA at the primer-binding site (23); the genomic RNA might be in the immature rather than mature conformation (11); reverse transcription might be blocked at a strand transfer step (3, 37) or be impeded at a region of secondary structure in genomic RNA (36). Evidence also exists that the NC proteins of HIV, equine anemia infectious virus, and murine leukemia virus are cleaved within the Zn^{2+} finger sequence by PR during early stages of infection (21, 27, 35); it is possible that the mutant NC proteins are not cleaved and that this defect underlies functional defects in reverse transcription. Experiments to test all of these possibilities are in progress.

Perhaps the most surprising and significant aspect of the current results is the extraordinary sensitivity of the retroviral Zn^{2+} finger to alteration by mutation: even a change of a Cys to a His residue (or vice versa), presumably retaining the ability of the array to coordinate a Zn^{2+} ion and preserving its function in RNA packaging, is essentially a lethal mutation for the virus. The results raise the possibility that, for reasons not yet understood, the CCHC configuration is the only one capable of performing its functions in virus replication. Thus, if antiviral agents reacting with the array could be devised, the virus might be unable to counter these agents by generating mutants resistant to them. Indeed, Rice et al. have described agents which inactivate HIV-1, apparently by reacting with Zn^{2+} fingers in the NC protein (25, 26).

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