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The Zinc Finger of Nucleocapsid Protein of Friend Murine Leukemia Virus Is Critical for Proviral DNA Synthesis In Vivo

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Nucleocapsid protein NCp10 of murine leukemia virus (MuLV) is encoded by the 3′ domain of *gag* and contains a zinc finger of the form Cys-X_2-Cys-X_4-His-X_4-Cys flanked by basic amino acids. In the course of virus assembly, NCp10 is necessary for core formation, and the zinc finger flanked by the basic residues is required for the packaging of the genomic RNA dimer. In vitro, NCp10 exhibits strong nucleic acid binding and annealing activities that appear to be critical for virus infectivity since NCp10 promotes dimerization of the viral RNA containing the E/DLS packaging-dimerization signal and annealing of replication primer $tRNA^{Pro}$ to the initiation site of reverse transcription (PBS). Recent in vitro studies have suggested that NCp10 may also play a role in proviral DNA synthesis. To investigate the function of NCp10 in proviral DNA synthesis in vivo, we developed a simple and convenient genetic packaging system consisting of two DNA constructs expressing the packaging components *gag-pol* and *env* of Friend MuLV and a Moloney MuLV-based *lacZ* vector with either the MuLV E+ or a rat VL30 E packaging signal. This system allowed us to examine the consequences of a set of mutations in NCp10 on a single round of recombinant virus replication. Most mutations in the N- or C-terminal domain of NCp10 do not significantly alter infectivity, while those in the zinc finger drastically impair infectivity. Analysis of the viral RNA content in virions showed that all mutations in the zinc finger decrease but do not abolish packaging of the recombinant genome. Interestingly enough, mutation of Y-28 to S (mutation Y28S) in the zinc finger results in RNA packaging at a level similar to that observed upon deletion of three prolines and three arginines in the C-terminal domain of NCp10 (mutant ΔPR3). However, mutant Y28S is noninfectious while mutant ΔPR3 is only threefold less infectious than the wild-type virus, which prompted us to examine the role of NCp10 protein in proviral DNA synthesis in vivo using these nucleocapsid mutants. PCR amplification was used to analyze viral DNA synthesized in newly infected cells, and results indicate that the Y28S zinc finger mutation impairs reverse transcription, thus suggesting that the nucleocapsid protein zinc finger plays a key role in proviral DNA synthesis in vivo.

The nucleocapsid (NC) protein (NCp10) of Friend murine leukemia virus (MuLV) is derived from the C terminus of the $Pr65^{gag}$ polyprotein precursor. It is a small basic protein of 56 amino acids containing a central Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC) motif capable of coordinating a zinc ion (10, 31, 39; for a review, see reference 11). Such zinc finger structures are conserved among all retroviral NC proteins known so far except those of spumaretroviruses (6, 9–11, 42), indicating that this domain probably plays a critical role in virus structure and replication. Mutational studies of NC proteins from MuLV, avian type C viruses, and human immunodeficiency virus (38) have indicated that most mutations altering the zinc finger motif cause defects in genomic RNA encapsidation (2, 7, 11, 15, 16, 18–20, 28–30); however, the mechanism underlying this defect is poorly understood. Another conserved characteristic of retroviral NC proteins is the presence of basic amino acids flanking the zinc finger. Earlier works have suggested that these basic residues are required for RNA recognition and binding leading to viral RNA dimerization and packaging (22). In a mutational study of MuLV, Rein et al. (38) confirmed that only the central domain of the NCp10 consisting of the zinc finger and flanking basic residues is required for viral RNA packaging.

In vitro, NCp10 of Moloney MuLV (MoMuLV), like NCp12 of avian sarcoma and leukosis virus and NCp7 of human immunodeficiency virus type 1 has nucleic acid annealing activities which promote viral RNA dimerization, primer tRNA annealing to the initiation site for viral DNA synthesis (PBS), and minus-strand DNA transfer (2, 4, 11, 35–37, 45). Genetic data support the notion that the function of NC protein is not restricted to genomic RNA dimerization and encapsidation (1, 15, 22, 28). Interestingly, a mutation of Y-28 to S (Y28S mutation) in the zinc finger of Mo-MuLV NCp10 was shown to moderately affect genomic RNA packaging but resulted in the production of noninfectious MuLV (28).

These observations prompted us to examine the role of NC protein in proviral DNA synthesis in vivo in a single round of virus replication. A simple genetic packaging system has been developed, consisting of two DNA constructs expressing the packaging components *gag-pol* and *env* of Friend MuLV (40) and of a retroviral vector with either the MuLV or the rat VL30 packaging-dimerization signal (E/DLS) (3, 43, 44). Mutations were created throughout NCp10, and their effects on virion production and infectivity were examined by using this genetic system.

Results show that none of the mutations altered virion production but that mutations in the zinc finger abolished infectivity. Biochemical analyses of the zinc finger mutant virions showed a 10- to 20-fold reduction in genomic RNA packaging but only a 2- to 3-fold decrease for mutant Y28S, in agreement with a previous report (28). Interestingly, a C-terminus dele-

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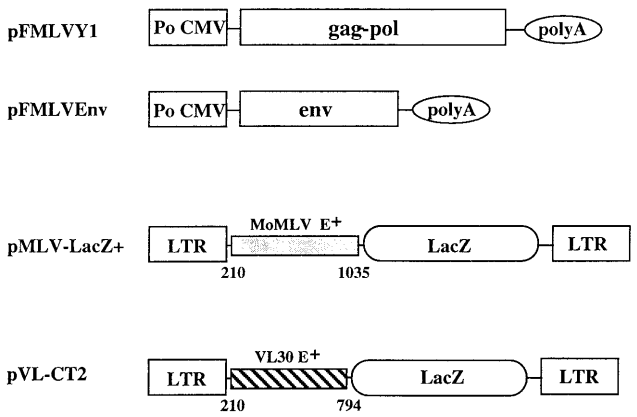


FIG. 1. Schematic representation of the plasmid DNA constructs. Plasmids pFMLVY1 and pFMLVEnv encode *gag-pol* (positions 627 to 5840) and *env* (positions 5783 to 7810), respectively, of the FB29 strain of Friend MuLV under the control of the CMV early promoter. The retroviral vectors contain the Mo-MuLV LTRs, *lacZ*, and either the homologous E/DLS packaging signal (positions 210 to 1035) for pMLV-LacZ+ or the rat VL30 E/DLS signal (positions 210 to 794) for pVL-CT2. polyA, polyadenylation signal of bovine growth hormone.

tion named ΔPR3 caused a two- to threefold reduction in genomic RNA packaging, and the mutant virus was only three- to fourfold less infectious than the wild type (WT) while zinc finger mutant Y28S was noninfectious. In an attempt to understand the defect in the zinc finger mutant, we analyzed viral DNA made in newly infected cells by means of quantitative PCR. Results indicate that proviral DNA synthesis started in zinc finger mutant viruses but accumulation was consistently lower than in the WT, and, moreover, it did not go to completion. These data suggest that an intact zinc finger in NCp10 protein is critical for proviral DNA synthesis in vivo.

MATERIALS AND METHODS

Plasmid DNA construction. Plasmids pFMLVY1 and pFMLVEnv were constructed in order to provide the packaging structural viral components. Both plasmids derived from plasmid pRc/CMV′, a modified form of plasmid pRc/CMV (Invitrogen) in which the sequence from nucleotides 1284 to 3253, a *Bam*HI fragment, was eliminated (5). To construct plasmid pFMLVY1, the fragment from nucleotides 569 to 5867 containing *gag-pol* of Friend MuLV (40) was inserted in pRc/CMV′ between the cytomegalovirus (CMV) promoter and the bovine growth hormone polyadenylation signal. Similarly, the fragment from nucleotides 5642 to 8301 containing *env* of Friend MuLV has been inserted into pRc/CMV′ to generate plasmid pFMLVEnv.

Retroviral vectors pMLV-LacZ+ and pVL-CT2 have been described before (44) and contain the MuLV E/DLS (nucleotides 210 to 1035) and the VL30 E/DLS (nucleotides 205 to 794), respectively, inserted between the 5′ long terminal repeat (LTR) and the reporter *lacZ* gene (24). The dicistronic retroviral vector pVL-CBT2 has been previously described (5, 43) and contains the VL30 E/DLS (nucleotides 205 to 794) inserted between the human placental alkaline phosphatase (*plap*) and the neomycin phosphotransferase (*neo*) genes.

Mutagenesis. The 2.1-kb fragment spanning the *Pst*I and *Eco*RI sites of pFB29/B (40) (nucleotides 569 to 2857) was subcloned into M13mp18 (46), and the resulting plasmid was used as a target for site-directed mutagenesis as described by Kunkel (25). The following mutagenic oligonucleotides were used (superscript numerals indicate positions, underlined nucleotides represent changes responsible for the amino acid substitutions, and localization of deleted amino acids is indicated by hyphens): R8S, ^{2073}AGC GGG CAG AG**C** CAG GAT AG; R11S, ^{2081}GAG ACA GGA TAG **C**CA GGG AGG AGA; D22K, ^{2112}AGG CCC CAA CTC **AA**G CAC GAC CAG TG; Y28S, ^{2126}CAC GAC CAG TGT GCC **AG**C TGC AAA GAA AAG GG; Y28G, ^{2126}CAC GAC CAG TGT GCC **GG**C TGC AAA GAA AAG GG; H34C, ^{2147}C AAA GAA AAG GGA **TG**T TGG GCT AGA G; W35G, ^{2151}GAA AAG GGA CAT **G**GG GCT AGA GAT T; ΔPR1, ^{2178}CCC AAG AAG CCA AGA GGA-GGA CCA CGA CCC CAG; ΔPR2, ^{2169}AGA GAT TGC CCC AAG AAG-GGA-GGA CCA CGA CCC CAG G; and ΔPR3, ^{2169}AGA GAT TGC CCC AAG AAG-GGA-GGA-CCC CAG GCC TCC CTC C.

Mutations were confirmed by DNA sequence analysis. The fragment carrying the mutation was reinserted into plasmid pFMLVY1 containing full-length *gag-pol* of Friend MuLV.

Cell culture and DNA transfection. NIH 3T3 cells and an NIH 3T3 cell line containing pVL-CBT2 DNA (5, 43), named NIH 3T3-CBT2, were grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum at 37°C in the presence of 5% CO_2.

NIH 3T3 or NIH 3T3-CBT2 cells were transfected by the calcium phosphate method of Chen and Okayama (8). A total of 5×10^5 NIH 3T3 cells in a 90-mm-diameter plate were transfected with 5 μg each of pFMLVY1 (WT or mutant NCp10) and pFMLVEnv and 15 μg of either pMLV-LacZ+ or pVLCT2. The cells were incubated overnight at 35°C in a 3% CO_2 atmosphere. Then the medium was removed and the cells were washed twice with phosphate-buffered saline, refed, and incubated in a 5% CO_2 atmosphere at 37°C. To transfect NIH 3T3-CBT2 cells, 10 μg each of pFMLVY1 (WT or mutant NCp10) and pFMLVEnv were used.

RT assay and recombinant virus infectivity. Two days after transfection, reverse transcriptase (RT) activity in the supernatant was measured by using poly rA:dT as described previously by Goff et al. (17). Two methods were used to determine the infectivity of the recombinant virus. (i) Fresh NIH 3T3 cells were incubated for 24 h with virus-containing supernatant from transfected cells, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed as described previously (22). The viral titer was defined as the number of β-galactosidase-expressing cells generated by 1 ml of virus-containing supernatant (see Fig. 2B). (ii) An autoinfection assay exploited the ability of virions produced by the transfected cells to infect other cells in the same culture population. After DNA transfection, cells were divided every 2 to 3 days, and 1 day later half of the cells were stained with X-Gal. The percentage of cells expressing β-galactosidase (*lacZ*$^+$) at different times after DNA transfection was determined, and the results are shown below (see Fig. 2C).

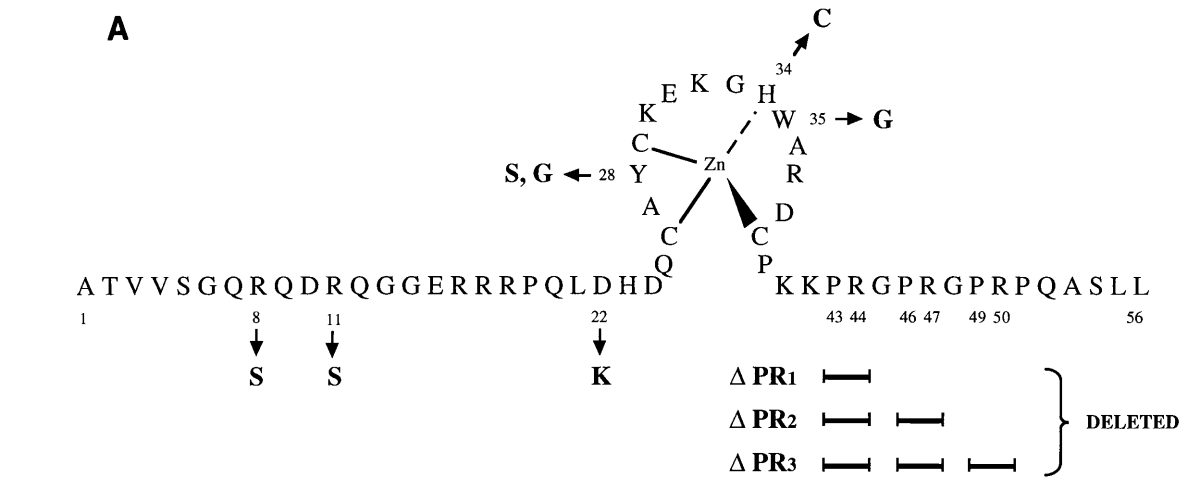
Western blot (immunoblot) analysis. Virus-containing medium was collected every 12 h between 2 and 4 days after DNA transfection. Virus was pelleted by ultracentrifugation at 25 krpm through a 20% sucrose cushion. The virus pellet was resuspended in STE buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) and kept at −20°C. An aliquot of the viral preparation was used for the RT assay (17). For Western analysis, the amount of viral particles present in the samples was normalized according to RT activity. The CAp30 and precursor proteins were detected by using anti-CAp30 goat antibodies (32) and an enhanced chemiluminescence (ECL) procedure (Amersham) according to the manufacturer's instructions. Quantitations were performed by laser scanning by reference to Mo-MuLV virions collected from 3T3-A9 cells as described previously (22), and 100% corresponded to CAp30 present in WT virions (see Fig. 3).

Northern (RNA) blot analysis. To examine the level of genomic RNA packaging, viral particles were collected from the medium of transfected NIH 3T3-CBT2 cells as described above. RNA was extracted from virion pellets, and RNA samples were normalized according to RT activity and analyzed by Northern blotting under denatured conditions as previously described (19, 33) using a ^{32}P-labelled DNA probe corresponding to the *neo* sequence (positions 1701 to 2098 in pVL-CBT2). Quantitations were performed by laser scanning, and 100% corresponded to recombinant genomic RNA present in WT virions (see Fig. 4).

PCR amplification of viral DNA synthesized in infected cells. To avoid plasmid DNA contaminations, NIH 3T3-CBT2 cells containing the integrated form of the retroviral vector pVL-CBT2 were transfected with pFMLVY1 (WT or mutant NCp10) and pFMLVEnv. A 10-μg amount of each plasmid was used for each transfection performed as described above. NIH 3T3 cells were infected for various periods with normalized quantities of virus-containing medium from transfected NIH 3T3-CBT2 cells. Controls included WT and WT1/10, in which cells were infected with normalized or 1/10 of the normalized WT virus-containing medium. Infected cells were lysed, and extrachromosomal DNA was extracted by the Hirt procedure (21). Samples corresponding to 10^5 cells infected with 0.5 ml of supernatant from transfected NIH 3T3-CBT2 cells were subjected to PCR amplification. Two pairs of PCR primers were used, N1701-N2098 and RU5-plap. Primer N1701 (5′-GTG GAG AGG CTA TTC GGC T-3′) and primer N2098 (5′-ATC CGA GTA CGT GCT CGC T-3′) are specific for *neo* (see Fig. 5). Primer RU5 (5′-GCA TCC GAA TCG TGG TCT C-3′) corresponds to the R-U5 junction of the MuLV LTR. Primer plap (5′-GGT TCC AGA AGT CCG GGT T-3′) is complementary to a sequence in the *plap* gene (see Fig. 5), inserted between the 5′ LTR and the VL30 sequence in the pVL-CBT2 vector. A total of 30 cycles were performed using the primer pair N1701-N2098, and 31 cycles were done using the primer pair RU5-plap. Each cycle consisted of 45 s of denaturation at 94°C, 45 s of annealing at 57°C, and 90 s of extension at 72°C. PCR-amplified DNA products were analyzed by 1.5% agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified (see Fig. 5).

RESULTS

A simple genetic packaging system derived from Friend MuLV. To analyze the structure-function relationships of NC protein NCp10 of MuLV in a single round of virus replication, we developed a simple and convenient packaging system (able



B

Mutant	pVL-CT2	pMLV-LacZ+
WT	2(±0.3)x10^4	1(±0.2)x10^4
R8S	2(±0.3)x10^4	1(±0.2)x10^4
R11S	1,7(±0.3)x10^4	0,9(±0.2)x10^4
D22K	0,4(±0.1)x10^4	0,3(±0.2)x10^4
Y28S	0 to10	0 to10
Y28G	0 to10	0 to10
H34C	0 to10	0 to10
W35G	0 to10	0 to10
Δ PR1	2(±0.2)x10^4	0,7(±0.1)x10^4
Δ PR2	1.3(±0.3)x10^4	0,5(±0.1)x10^4
Δ PR3	0,5(±0.2)x10^4	0,4(±0.1)x10^4

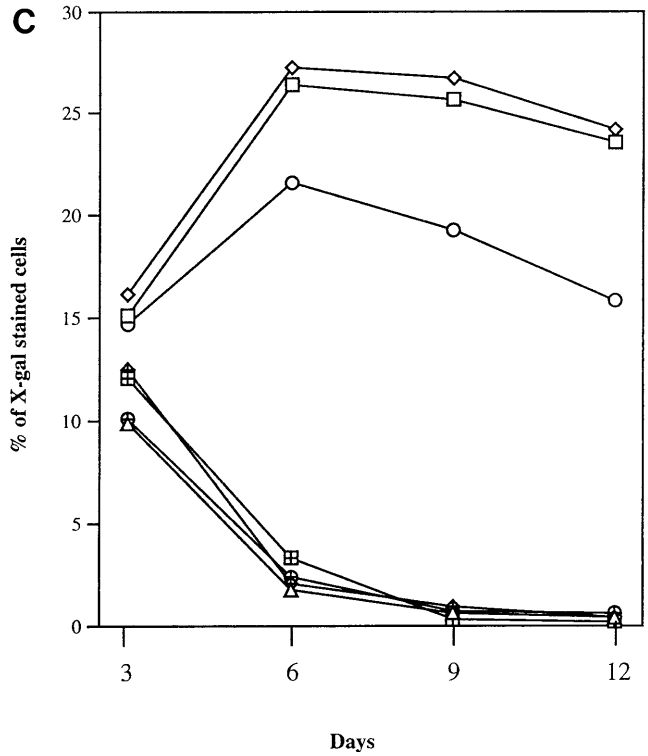


FIG. 2. Infectivity of recombinant viruses containing WT or mutant NC protein. (A) Amino acid sequence of NCp10 protein of Friend MuLV. Amino acid substitutions and deletions are indicated (arrows). NCp10 mutants have been named according to their position in the protein, i.e., R11S corresponds to the substitution of a serine for arginine at position 11. (B) Titers of recombinant viruses with WT or mutant NCp10 are expressed as infectious units per milliliter of virus-containing medium corresponding to the number of LacZ-positive cell foci. Substitutions are indicated as described above, and deletions of proline and arginine repeats corresponded to positions 43 and 44 (ΔPR1), positions 43, 44, 46, and 47 (ΔPR2), and positions 43, 44, 46, 47, 49, and 50 (ΔPR3). 0 to 10, the number of LacZ-positive cell foci varied from 0 to maximum 10. Standard errors are indicated and corresponded to at least three sets of experiments. (C) Autoinfection assay: plasmids pFMLVY1 with WT (□) or mutant NCp10 (R11S [◇], Y28S [⊞], Y28G [⊕], H34C [⊕], W35G [△], and ΔPR3 [○]), pFMLVEnv, and retroviral vector pVL-CT2 were simultaneously transfected into NIH 3T3 cells as described in Materials and Methods. Cell cultures were split every 3 days. One day later, one cell dish was stained with X-Gal and the percentage of stained cells was calculated. Data shown are from a typical experiment and agree with those reported in panel B.

to generate high-titer viral stocks, obviating the use of helper cell lines) based on the FB29 molecular clone of Friend MuLV (40). Two DNA constructs coding for *gag-pol* and *env* of Friend MuLV were used together with a retroviral vector containing either the MuLV or the rat VL30 E/DLS (3, 44) in cotransfection assays to transiently produce recombinant viruses. Similar systems derived from MoMuLV have been previously described (26, 34, 41).

Recombinant viruses were generated by NIH 3T3 cells co-transfected with DNA constructs pFMLVY1 and pFMLVEnv of Friend MuLV FB29 and a retroviral vector containing *lacZ* (see Materials and Methods) (Fig. 1). In each experiment, the efficiency of transfection was assessed 2 days after transfection by the percentage of cells expressing β-galactosidase. Virus-containing medium was used to infect fresh NIH 3T3 cells, and 1 day later infected cells expressing β-galactosidase were stained with X-Gal and counted to give the viral titer. A typical experiment gave an efficiency of transfection of about 15% and

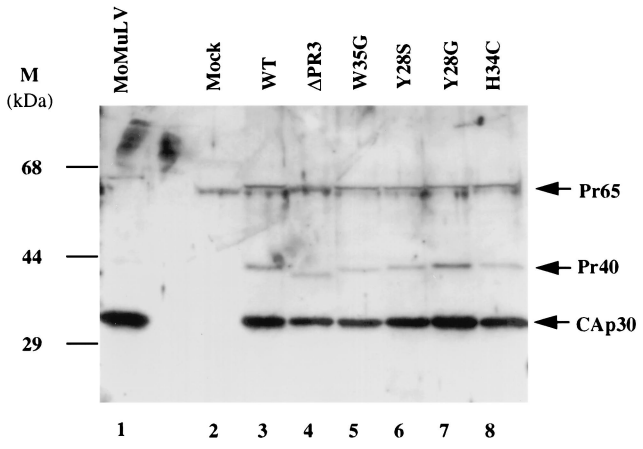


FIG. 3. Western blot analysis of mutant virions. Viral particles were pelleted from medium of transfected NIH 3T3 cells by ultracentrifugation through 5 ml of a 20% sucrose cushion. Viral proteins from equivalent quantities of virions, based on RT activities using poly rA:dT (see Materials and Methods), were separated on a 10% polyacrylamide gel in 0.1% sodium dodecyl sulfate and transferred onto nitrocellulose, and CAp30 and related proteins were detected by using anti-CAp30 antibodies and a chemiluminescence system (ECL; Amersham) according to the manufacturer's instructions. Lane 1, Mo-MuLV virions produced by 3T3 A9 cells; lane 2, mock transfection; lanes 3 to 8, recombinant virions with WT NCp10 or the indicated mutants. Relative values of virion CAp30 were obtained by laser scanning and found to be 100% (WT), 66% (ΔPR3), 60% (W35G), 105% (Y28S), 155% (Y28G), and 90% (H34C). It should be noted that mutants Y28S, Y28G, H34C, and W35G were very poorly infectious or noninfectious (Fig. 2B and C).

viral titers of about 1×10^4 infectious units (IU)/ml with the pMLV-LacZ+ vector bearing the MoMuLV E/DLS encapsidation signal and about 2×10^4 IU/ml with the pVL-CT2 vector containing the rat VL30 E/DLS encapsidation signal. These results are in agreement with previous observations of Torrent et al. (44) indicating that the rat VL30 packaging signal is more efficient than that of Mo-MuLV.

Mutations in NCp10 zinc finger drastically impair infectivity of MuLV and MuLV-VL30 recombinant viruses. To examine the functions of NCp10 in a single round of virus replication, substitutions and deletions were introduced into Friend MuLV NCp10 coding sequence in plasmid pFMLVY1 (Fig. 2A). The consequences of these mutations for recombinant virus infectivity were examined in NIH 3T3 cells (Fig. 2B). Substitution of serine for arginine at positions 8 and 11 in the N terminus of NCp10 (mutants R8S and R11S, respectively) did not alter infectivity (Fig. 2B). Substitution of lysine for asparagine at position 22 (mutant D22K), a residue close to the zinc finger, decreased infectivity by 5- to 10-fold. Furthermore, deletion of one or two proline-arginine (PR) residues located in the C-terminal region of NCp10 (mutants ΔPR1 and ΔPR2) only slightly altered infectivity (Fig. 2B). When all three PR repeats were deleted (mutant ΔPR3), infectivity was about 20 to 50% of that of the WT. However, mutations in the NCp10 zinc finger drastically impaired infectivity, since substitution of neutral residues for aromatic amino acids (mutants Y28S, Y28G, and W35G) or cysteine for histidine at position 34 (mutant H34C) resulted in the production of noninfectious or very poorly infectious viruses (from 0 to 10 IU/ml while WT virus was at 1×10^4 to 2×10^4 IU/ml; Fig. 2B). It is interesting that the effects of these NCp10 mutations on recombinant virus infectivity were found to be independent of the homologous or heterologous packaging signal used under the present conditions.

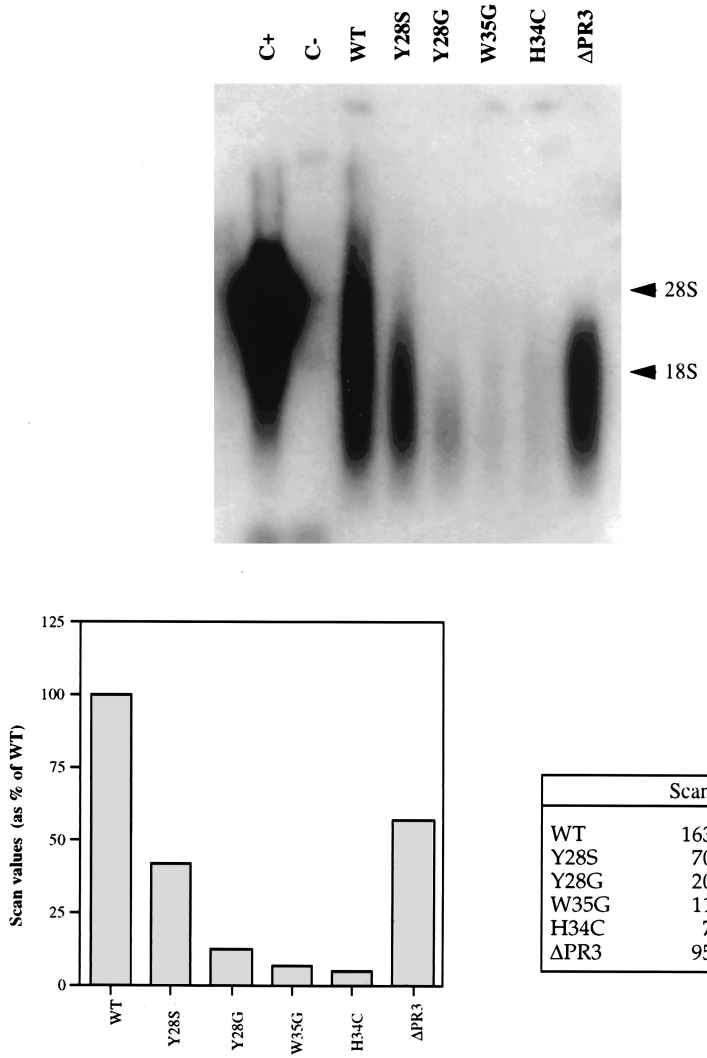
Infectivity of WT and NC mutant (R11S, Y28S, Y28G, H34C, W35G, and ΔPR3) viruses was also assessed by means of the autoinfection assay which examined the spread of infectious viruses within a cell population subjected to transfection with the three DNA constructs pFMLVY1, pFMLVEnv, and the LacZ retroviral vector (see Materials and Methods). Under these conditions, 10 to 15% of all NIH 3T3 cells transiently expressed β-galactosidase encoded by the retroviral vector at day 3 (Fig. 2C). Between days 3 and 6, infectious recombinant viruses were able to spread in this cell population, but not noninfectious recombinant viruses. As shown in Fig. 2C, 26 to 27% of the cells expressed β-galactosidase at days 6 and 9 when WT and mutant R11S recombinant viruses were produced and 19 to 21% of the cells expressed β-galactosidase with mutant ΔPR3. However, with mutants Y28S, Y28G, H34C, and W35G, the percentage of $lacZ^+$ cells decreased from 10 to 12% at day 3 to <1% at day 9, indicating that these NC zinc finger mutations were deleterious to the formation of infectious virions.

Taken together, these data clearly show that only mutations in the zinc finger of Friend MuLV NC protein were able to strongly impair infectivity (Fig. 2).

Biochemical analysis of the virons produced. The ability of NC mutants to release functional *gag-pol*-related proteins in the supernatant was assayed by means of RT activity (17). RT activity measured in the supernatant containing virions produced by cells transfected with mutant DNAs was at a level similar to that of the WT (data not shown), indicating that the mutations did not significantly impair virion production. In order to confirm these results, equivalent amounts of viral particles were collected by ultracentrifugation and virion proteins were analyzed by Western blotting using anti-CAp30 antibodies (28). The amounts of CAp30 were similar in WT and mutant recombinant MuLV virions (differing by a factor of 2 at the most; Fig. 3). Moreover, the ratios of fully processed CAp30 to *gag* polyprotein precursors Pr65 and Pr40 were similar for WT and mutant recombinant virions (Fig. 3). These results indicate that the NCp10 mutations examined did not significantly impair virion production and maturation.

To examine the influence of NC mutations on genomic RNA packaging, viral particles were collected from 48 to 96 h after transfection of NIH 3T3-CBT2 cells, an NIH 3T3 cell line containing the pVL-CBT2 retroviral vector (5, 44), with pFMLVY1 (WT or mutant NCp10) and pFMLVEnv. The amounts of virions collected and pelleted were normalized according to RT activity, and viral RNAs were purified and analyzed by Northern blotting under denaturing conditions using a ^{32}P-labelled *neo* sequence as the probe (see Materials and Methods). Mutations in the N-terminal domain of NCp10 did not significantly alter the level of genomic RNA packaging compared with that of the WT virus (results not shown). Mutations in the NCp10 zinc finger caused either an extensive reduction (10- to 20-fold decreases for mutants Y28G, H34C, and W35G) or a slight reduction (a 2.5-fold decrease for mutant Y28S) in genomic RNA packaging (Fig. 4). Deletions in the C-terminal domain of NCp10 did not modify the level of genomic RNA packaging for mutants ΔPR1 and ΔPR2 (data not shown) and resulted in a 40% decrease only for mutant ΔPR3 (Fig. 4). It is of particular interest that mutant viruses ΔPR3 and Y28S contain similar levels of genomic RNA (Fig. 4), but ΔPR3 was infectious (about 0.5×10^4 IU/ml) while Y28S was very poorly infectious (0 to 10 IU/ml) (Fig. 2B and C). These observations prompted us to examine the effects of NCp10 zinc finger mutations on viral DNA synthesis in vivo.

Analysis by PCR amplification of viral DNA synthesis in infected cells. NIH 3T3-CBT2 cells were transfected with WT or NCp10 mutant pFMLVY1 and pFMLVEnv. Subsequently,



	Scan values	% of WT
WT	16387.73	100.00
Y28S	7031.85	41.76
Y28G	2014.65	11.96
W35G	1104.15	6.56
H34C	738.48	4.39
ΔPR3	9573.31	56.85

FIG. 4. Northern blot analysis of genomic RNA in recombinant NC mutant virions. Viral particles from transfected NIH 3T3-CBT2 cells were pelleted by ultracentrifugation, amounts were normalized according to the RT activities, and RNAs were purified and then analyzed as described in Materials and Methods. Lanes: 1, cellular RNA (20 ng) of NIH 3T3-CBT2 cell line (positive control [C+]); 2, cellular RNA (20 ng) of untransfected NIH 3T3 cells (negative control [C−]); 3 to 8, WT or the indicated mutants. The positions of 28 and 18S rRNA markers are indicated on the right.

NIH 3T3 cells were incubated with equivalent quantities of virus produced by the transfected NIH 3T3-CBT2 cells for 30 min, 6 h, and 24 h (see Materials and Methods). Extrachromosomal DNA corresponding to 10^5 cells was used to amplify by PCR the viral DNA synthesized in infected cells. In order to analyze early and late viral DNA synthesis, two PCR primer pairs were used (Fig. 5A). PCR with the N1701-N2098 primer pair, specific for *neo*, can detect early minus-strand cDNA transcripts following the first-strand transfer, and PCR with the RU5-plap primer pair detects only late viral DNA synthesis since it needs completion of the second-strand transfer and further extension of the viral DNA strands (23). To facilitate quantitation, two levels of WT virus, a normalized level and 1/10 of that level, were used in these assays (Fig. 5).

Minus-strand DNA started to be detectable 30 min after infection with the WT and NC mutants, indicating that initiation and first-strand transfer did take place (Fig. 5B). As shown in Fig. 5B, newly made viral DNAs detected with the Neo primer pair were found to accumulate 6 and 24 h after infection with WT and ΔPR3 viruses. However, accumulation of newly made DNA was about 10 times lower with Y28S and H34C mutant viruses (Fig. 5B). Six hours after infection, the late steps of viral DNA synthesis as analyzed by PCR using the RU5-plap primer pair were detected for the WT and ΔPR3 viruses but not for the zinc finger mutants Y28S and H34C. At 24 h, DNA corresponding to the 5′ viral domain was observed with WT and ΔPR3 viruses. In contrast, no DNA corresponding to this domain was detected with Y28S and H34C mutant viruses (Fig. 5B).

These data indicate that the second DNA strand transfer did not occur or was very inefficient in mutant viruses Y28S and H34C (compare lanes wild type 1/10, Y28S, and H34C in Fig. 5B, right panel).

DISCUSSION

Nucleocapsid protein of MuLV is a structural component of the virion NC in which several hundred NCp10 molecules are in tight association with the genomic RNA dimer (11). In addition, NCp10, like the NC protein of avian and human

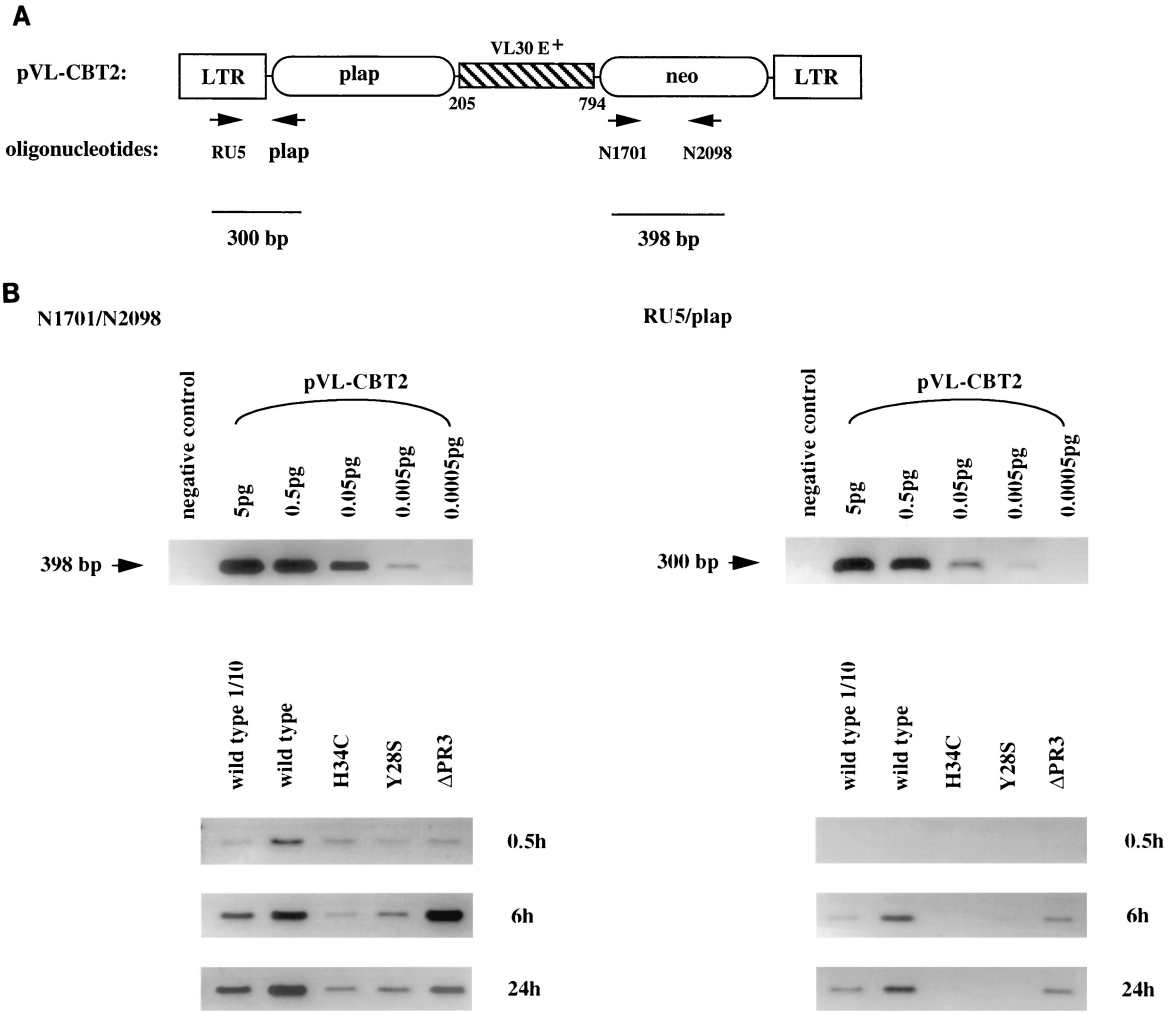


FIG. 5. Viral DNA synthesized in NIH 3T3 cells examined by PCR amplification. NIH 3T3 cells were infected by recombinant viruses as described in Materials and Methods. Extrachromosomal DNA was prepared by the method of Hirt (21), and a fraction of it was subjected to PCR amplification using two primer pairs. (A) Schematic representation of the retroviral vector pVL-CBT2 and location of the two primer pairs. N1701 and N2098 are specific for the Neo sequence and allow amplification of minus-strand cDNA following the first strand transfer (23). RU5 and plap are specific for the 5′ LTR and phosphatase sequences and therefore can direct amplification of viral DNA only after the second transfer (23). The sizes of PCR-amplified fragments are also indicated. (B) Analysis of the DNA fragments generated upon PCR amplification. NIH 3T3 cells were infected for 0.5, 6, and 24 h with recombinant viruses produced by NIH 3T3-CBT2 cells. DNA from uninfected NIH 3T3 cells was used as a negative control. Serial dilutions of plasmid DNA pVL-CBT2, 0.0005 to 5 pg (equivalent to 10^2 to 10^6 molecules), were used as positive controls. Mutants and WT are indicated above the lanes. A linear response of PCR amplification was obtained from 0.0005 to 0.05 pg of plasmid pVL-CBT2 using both N1701-N2098 and RU5-plap primer pairs. Relative fluorescence values for the PCR-amplified DNAs were obtained by scanning and are given below. Note that relative values are approximate and each number (taken as 100%) is ±15%. (i) Early cDNA transcripts (N1701-N2098 primer pair). At 0.5 h: WT, arbitrarily set as 100%; WT 1/10, 15%; H34C, 7%; Y28S, 3%; ΔPR3, 8%. At 6 h: WT, 200%; WT 1/10, 100%; H34C, 12%; Y28S, 22%; ΔPR3, 250%. At 24 h: WT, 500%; WT 1/10, 180%; H34C, 25%; Y28S, 80%; ΔPR3, 160%. (ii) Late cDNA transcripts (RU5-plap primer pair). At 6 h: WT, arbitrarily set as 100%; WT 1/10, 10%; H34C, 0%; Y28S, 0%; ΔPR3, 30%. At 24 h: WT, 160%; WT 1/10, 50%; H34C, 0%; Y28S, 2%; ΔPR3, 55%.

retroviruses, has nucleic acid binding and annealing activities that are directly implicated in several steps of the viral life cycle. For example, it has been found that NCp10 promotes dimerization of MuLV RNA containing the E/DLS and annealing of primer $tRNA^{Pro}$ to the initiation site for viral DNA synthesis (PBS) in vitro (11, 22, 35–37). NCp10 mutations that cause a strong defect in viral RNA binding and dimerization and in primer $tRNA^{Pro}$ annealing to the PBS in vitro result in a large decrease in genomic RNA packaging and initiation of viral DNA synthesis in vivo (11, 14, 22).

MuLV NC protein is highly basic, with a zinc finger which exhibits a strong affinity for Zn^{2+} (9, 11, 13). The solution structure of NCp10 reveals that the central domain of the protein, consisting of the zinc finger motif and flanking basic residues, has a well-defined globular structure, while the N- and C-terminal domains are flexible (13). Interestingly, substitutions of serine for cysteine that affect the zinc finger structure (Fig. 2A) or of neutral residues for the aromatic amino acids, Y-28 and W-35 (Fig. 2A), that change the side chains of the zinc finger (13) resulted in a strong decrease in genomic RNA packaging and the production of poorly infectious or noninfectious viruses (Fig. 2B) (6, 11, 18–20, 28). Moreover, substitutions of neutral residues for basic amino acids flanking the zinc finger impaired genomic RNA packaging as well as $tRNA^{Pro}$ annealing to the PBS, and mutant viruses were poorly infectious (22). However, NCp10 mutations such as the Y28S mutation (Fig. 2A) (28) did not cause a strong decrease in genomic RNA packaging, but the mutant viruses were not

infectious (28). In addition, NCp10 was found to cooperate with RT for viral DNA synthesis in vitro (2, 11, 12). These observations prompted us to perform mutations thought to change the structure of the zinc finger or the basic nature of the N- or C-terminal domain of NCp10 and examine their effects on virion production and infectivity and on viral DNA synthesis in vivo.

For this purpose, a simple genetic packaging system was derived from the highly infectious FB29 clone of Friend MuLV (40). Two plasmids were constructed to allow expression of the packaging components *gag-pol* and *env*. In addition, two MoMuLV-based vectors with either the MuLV E+ or a rat VL30 E packaging signal were available (43, 44). Simultaneous transfection of cells with the three DNAs, the two packaging constructs and one retroviral vector, resulted in the production of recombinant viruses (Fig. 2) (26, 34, 41). This convenient genetic system should permit an extensive analysis of the genetic elements involved in viral core formation, genomic RNA dimerization, and encapsidation and in the early steps of virus replication leading to the synthesis of the double-stranded proviral DNA and its integration into the host genome.

Mutations in the N- or C-terminal domain of NCp10 did not extensively alter infectivity, while those in the zinc finger resulted in the production of very poorly infectious or noninfectious viruses (Fig. 2). The level of genomic RNA packaging was decreased by 10- to 20-fold with almost all deleterious mutations in the zinc finger (Fig. 2). One exception is the Y28S mutant, in which packaging was 40% of the WT level, in agreement with a previous report (28), and similar to that in mutant ΔPR3, in which three prolines and three arginines have been deleted in the C-terminal domain of NCp10. However, mutant Y28S was noninfectious or very poorly infectious, whereas mutant ΔPR3 was only threefold less infectious than the WT virus (Fig. 2). Viral DNA synthesis in newly infected cells was analyzed by PCR amplification, and the results show that the Y28S zinc finger mutant, but not mutant ΔPR3, appears to be impaired in its ability to complete viral DNA synthesis (Fig. 5). Minus-strand DNA synthesis occurred, but plus-strand DNA was not detected under these conditions. Plus-strand DNA synthesis also appeared to be inhibited in mutant H34C (Fig. 5).

These results show that NC protein NCp10 of MuLV most probably plays a key role in viral DNA synthesis, as suggested earlier by in vitro data for MoMuLV and human immunodeficiency virus type 1 (2, 11, 12). These results also favor the notion that the central globular domain of NCp10 (13), consisting of the zinc finger and flanking basic residues, is a crucial structure necessary not only for the packaging of the genomic RNA (22, 38) but also for its conversion into double-stranded DNA by RT. Work is in progress to define residues in the central globular domain of NCp10, such as tyrosine, that are crucial for the synthesis of a complete double-stranded viral DNA.

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REFERENCES

1. **Aldovini, A., and R. A. Young.** 1990. Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. J. Virol. **64:**1920–1926.
2. **Allain, B., M. Lapadat-Tapolsky, C. Berlioz, and J. L. Darlix.** 1994. Transactivation of the minus strand DNA transfer by nucleocapsid protein during reverse transcription of the retroviral genome. EMBO J. **13:**973–981.
3. **Bender, M. A., T. D. Palmer, R. E. Gelinas, and A. D. Miller.** 1987. Evidence that the packaging signal of Moloney murine leukemia virus extends into the *gag* region. J. Virol. **61:**1639–1646.
4. **Berkowitz, R. D., J. Luban, and S. P. Goff.** 1993. Specific binding of human immunodeficiency virus type 1 Gag polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. J. Virol. **67:**7190–7200.
5. **Berlioz, C., and J. L. Darlix.** 1995. An internal ribosomal entry mechanism promotes translation of murine leukemia virus Gag polyprotein precursors. J. Virol. **69:**2214–2222.
6. **Bess, J. W., P. J. Powell, H. J. Issaq, L. J. Schumack, M. K. Grimes, L. E. Henderson, and L. O. Arthur.** 1992. Tightly bound zinc in human immunodeficiency virus type 1, human T-cell leukemia virus type 1, and other retroviruses. J. Virol. **66:**840–847.
7. **Bowles, N. E., P. Damay, and P.-F. Spahr.** 1993. Effect of rearrangements and duplications of the Cis-His motifs of Rous sarcoma virus nucleocapsid protein. J. Virol. **67:**623–631.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
9. **Cornille, F., Y. Mely, D. Ficheux, I. Salvignol, D. Gérard, J. L. Darlix, M.-C. Fournié-Zaluski, and B. Roques.** 1990. Solid phase synthesis of the retroviral nucleocapsid protein NCp10 of Moloney murine leukemia virus and related zinc-fingers in free SH forms: influence of zinc chelation on structural and biochemical properties. Int. J. Pep. Protein Res. **36:**551–558.
10. **Covey, S. N.** 1986. Amino acid sequence homology in *GAG* region of reverse transcribing elements and the coat protein gene of cauliflower mosaic virus. Nucleic Acids Res. **14:**623–633.
11. **Darlix, J.-L., M. Lapadat-Tapolsky, H. de Rocquigny, and B. Roques.** 1995. First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J. Mol. Biol. **254:**532–537.
12. **Darlix, J. L., A. Vincent, C. Gabus, H. de Rocquigny, and B. Roques.** 1993. Transactivation of the 5′ to 3′ viral DNA strand transfer by nucleocapsid protein during reverse transcription of HIV-1 RNA. C. R. Acad. Sci. Ser. III Sci. Vie **316:**763–771.
13. **Déméné, H., N. Jullian, N. Morellet, H. de Rocquigny, F. Cornille, B. Maigret, and B. P. Roques.** 1994. Three dimensional 1H NMR structure of the nucleocapsid protein NCp10 of Moloney murine leukemia virus. J. Biol. NMR **4:**153–170.
14. **De Rocquigny, H., D. Ficheux, C. Gabus, B. Allain, M. C. Fournie-Zaluski, J. L. Darlix, and B. P. Roques.** 1993. Two short basic sequences surrounding the zinc finger of nucleocapsid protein NCp10 of Moloney murine leukemia virus are critical for RNA annealing activity. Nucleic Acids Res. **21:**823–829.
15. **Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Göttlinger.** 1993. Mapping of functionally important residues of a cysteine-histeine box in the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **67:**6159–6169.
16. **Dupraz, P., S. Oertle, C. Méric, P. Damay, and P.-F. Spahr.** 1990. Point mutations in the proximal Cys-His box of Rous sarcoma virus nucleocapsid protein. J. Virol. **64:**4978–4987.
17. **Goff, S., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of rapid assay for release of virion reverse transcriptase. J. Virol. **38:**239–248.
18. **Gorelick, R. J., D. J. Chabot, A. Rein, L. E. Henderson, and L. O. Arthur.** 1993. The two zinc fingers in the human immunodeficiency virus type 1 nucleocapsid protein are not functionally equivalent. J. Virol. **67:**4027–4036.
19. **Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein.** 1988. Point mutations of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific recognition by a zinc finger-like protein sequence. Proc. Natl. Acad. Sci. USA **85:**8420–8424.
20. **Gorelick, R. J., S. M. Nigida, Jr., J. W. Bess, Jr., L. O. Arthur, L. E. Henderson, and A. Rein.** 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. J. Virol. **46:**3207–3211.
21. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
22. **Housset, V., H. de Rocquigny, B. P. Roques, and J. L. Darlix.** 1993. Basic amino acids flanking the zinc finger of Moloney murine leukemia virus nucleocapsid protein NCp10 are critical for virus infectivity. J. Virol. **67:**2537–2545.
23. **Hu, W., and H. Temin.** 1990. Retroviral recombination and reverse transcription. Science **250:**1227–1233.
24. **Kalnins, A., K. Otto, U. Rüter, and B. Müller-Hill.** 1983. Sequence of the lacZ gene of Escherichia coli. EMBO J. **2:**593–597.
25. **Kunkel, T. A.** 1985. Rapid and efficient site-directed mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
26. **Landau, N., and D. R. Littman.** 1992. Packaging system for rapid production of murine leukemia virus vectors with variable tropism. J. Virol. **66:**5110–5113.
27. **Lapadat-Tapolsky, M., H. de Rocquigny, D. van Gent, B. P. Roques, R. Plasterk, and J. L. Darlix.** 1993. Interactions between HIV-1 nucleocapsid

protein and viral DNA may have important functions in the viral life cycle. Nucleic Acids Res. **21:**831–839.
28. **Méric, C., and S. Goff.** 1989. Characterization of Moloney murine leukemia virus mutants with single amino acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. **63:**1558–1568.
29. **Méric, C., E. Gouilloud, and P. F. Spahr.** 1988. Mutations in Rous sarcoma virus nucleocapsid protein p12 (NC): deletions of Cys-His boxes. J. Virol. **62:**3328–3333.
30. **Méric, C., and P. F. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60:**450–459.
31. **Morellet, N., N. Jullian, H. de Rocquigny, B. Maigret, J. L. Darlix, and B. P. Roques.** 1992. Determination of the structure of the nucleocapsid protein NCp7 from the human immunodeficiency virus type 1 by H-1 NMR. EMBO J. **11:**3059–3065.
32. **Oroszlan, S., C. L. Fisher, T. B. Stanley, and R. V. Gilden.** 1970. Protein of the murine C-type RNA tumour viruses: isolation of a group-specific antigen by isoelectric focusing. J. Gen. Virol. **8:**1–10.
33. **Ottmann, M., C. Gabus, and J. L. Darlix.** 1995. The central globular domain of the nucleocapsid protein of human immunodeficiency virus type 1 is critical for virion structure and infectivity. J. Virol. **69:**1778–1784.
34. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
35. **Prats, A. C., V. Housset, G. de Billy, F. Cornille, B. Roques, and J. L. Darlix.** 1991. Viral annealing activities of the nucleocapsid protein of Moloney murine leukemia virus are zinc independent. Nucleic Acids Res. **19:**3533–3541.
36. **Prats, A. C., C. Roy, P. Wang, M. Erard, V. Housset, C. Gabus, C. Paoletti, and J. L. Darlix.** 1990. *cis* elements and *trans*-acting factors involved in dimer formation of murine leukemia virus RNA. J. Virol. **64:**774–783.
37. **Prats, A. C., L. Sarih, C. Gabus, S. Litvak, G. Keith, and J. L. Darlix.** 1988. Small finger protein of avian and murine retroviruses has nucleic acid annealing activity and positions the replication primer tRNA onto genomic RNA. EMBO J. **7:**1777–1783.
38. **Rein, A., D. P. Harvin, J. Mirro, S. M. Ernst, and R. J. Gorelick.** 1994. Evidence that a central domain of nucleocapsid protein is required for RNA packaging in murine leukemia virus. J. Virol. **68:**6124–6129.
39. **Schiff, L. A., M. L. Nibert, and B. N. Fields.** 1988. Characterization of a zinc blotting technique: evidence that a retroviral gag protein binds zinc. Proc. Natl. Acad. Sci. USA **85:**4195–4199.
40. **Sitbon, M., B. Sola, L. Evans, J. Nishio, S. F. Hayes, K. Nathanson, C. F. Garon, and B. Chesebro.** 1986. Hemolytic anemia and erythroleukemia, two distinct pathogenic effects of Friend MuLV: mapping of the effects to different regions of the viral genome. Cell **47:**851–859.
41. **Soneoka, Y., P. M. Cannon, E. E. Ramsdale, J. C. Griffiths, G. Romano, S. M. Kingsman, and A. J. Kingsman.** 1995. A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res. **23:**628–633.
42. **Summers, M. F., L. E. Henderson, M. R. Chance, J. W. Bess, Jr., T. L. South, P. R. Blake, I. Sagi, G. Perez-Alvarado, R. C. Sowder III, D. R. Hare, and L. O. Arthur.** 1992. Nucleocapsid zinc fingers detected in retroviruses: EXAFS studies of intact viruses and the solution-state structure of the nucleocapsid protein from HIV-1. Protein Sci. **1:**563–574.
43. **Torrent, C., C. Berlioz, and J. L. Darlix.** Stable MLV-VL30 dicistronic retroviral vectors with a VL30 or MoMLV sequence promoting both packaging of genomic RNA and expression of the 3′ cistron. Submitted for publication.
44. **Torrent, C., C. Gabus, and J. L. Darlix.** 1994. A small and efficient dimerization/packaging signal of rat VL30 RNA and its use in murine leukemia virus-VL30-derived vectors for gene transfer. J. Virol. **68:**661–667.
45. **Tsuchihashi, Z., and P. O. Brown.** 1994. DNA strand exchange and selective DNA annealing promoted by the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **68:**5863–5870.
46. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.