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Point Mutations in the Proximal Cys-His Box of Rous Sarcoma Virus Nucleocapsid Protein

PHILIPPE DUPRAZ,* SUZANNE OERTLE, CLAUDE MERIC,† PASCAL DAMAY, AND PIERRE-FRANÇOIS SPAHR

Department of Molecular Biology, University of Geneva, 30, Quai Ernest Ansermet, 1211 Genève 4, Switzerland

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To extend our previous studies of the function of the Cys-His box of Rous sarcoma virus NC protein, we have constructed a series of point mutations of the conserved or nonconserved amino acids of the proximal Cys-His box and a one-amino-acid deletion. All mutants were characterized for production of viral proteins and particles, for packaging and maturation of viral RNA, for reverse transcriptase activity, and for infectivity. Our results indicated the following. (i) Mutations affecting the strictly conserved amino acids cysteine 21, cysteine 24, and histidine 29 were lethal; only the mutant His-29→Pro was still able to package viral RNA, most of it in an immature form. (ii) Mutation of the highly conserved glycine 28 to valine reduced viral RNA packaging by 90% and infectivity 30-fold, whereas mutant Gly-28→Ala was fully infectious. This suggests a steric hindrance limit at this position. (iii) Shortening the distance between cysteine 24 and histidine 29 by deleting one amino acid abolished the maturation of viral RNA and yielded noninfectious particles. (iv) Substitution of tyrosine 22 by serine lowered viral RNA packaging efficiency and yielded particles that were 400-fold less infectious; double mutant Tyr-22Thr-23→SerSer had the same infectivity as Tyr-22→Ser, whereas mutant Thr-23→Ser was fully infectious. (v) Changing glutamine 33 to a charged glutamate residue did not affect virus infectivity. Similarities and differences between our avian mutants and those in murine retroviruses are discussed.

All retroviruses express a gene encoding their structural protein (*gag* gene). In the case of Rous sarcoma virus (RSV), the gene is expressed by translation of the genomic 35S mRNA in the cytoplasm of the infected cell. The product is a polyprotein precursor of 76-kilodalton $Pr76^{gag}$, which migrates to the plasma membrane of the host cell, where it is cleaved during virus assembly and budding to yield five mature proteins: MA, the matrix-associated protein; P-10, a protein of unknown function; CA, the major structural component of the capsid; NC, the nucleocapsid protein; and PR, the protease responsible for the stepwise proteolytic cleavage of the *gag* precursor (29; for a review, see references 5 and 50).

In the mature virion, only NC is found tightly associated with the genomic 70S RNA dimer (8, 9) in the nucleoid of the virion. Recent results have shown that MA does also bind to viral RNA but, in contrast to previous reports (32, 33), without apparent specificity (47). We have shown that NC of RSV can be cross-linked by UV light to the virion RNA, and we have sequenced the binding sites; only a small proportion of the NC molecules from RSV were found tightly bound to the genomic RNA dimer (7, 36). Purified NC from avian viruses binds RNA in vitro, but specificity for viral RNA has not been demonstrated to date (10, 30, 31, 46, 49).

All the various retroviral NCs have a conserved pattern of amino acids which was named the cysteine-histidine box (6). It can be represented by the formula Cys-X_2-Cys-X_4-His-X_4-Cys (Fig. 1), where X refers to the nonconserved amino acids between the conserved pattern. Many of these boxes contain one or two aromatic amino acids; the first is at position $n + 1$ or $n + 2$ (n = first cysteine of the box), and the second is after the histidine. A highly conserved glycine residue at $n + 7$ is also found in most Cys-His boxes.

Some retroviruses (such as murine leukemia virus) or retroid elements contain only one box, whereas other retroviruses (such as the avian or visna viruses, bovine leukemia virus, and the human retroviruses) have two boxes (6, 40). This motif is also found in human immunodeficiency virus NC (18) and in cauliflower mosaic virus (6), and a similar sequence is found in T4 gene 32 protein (52).

The Cys-His box shows strong similarity to the zinc finger motif of various nucleic acid binding proteins (3). However, whether retroviral NC protein binds zinc ions is controversial. In vitro, NC affinity for zinc has been demonstrated by zinc blotting (45), but in purified virions, significant amounts of metal are not detected (19). Spectroscopic studies of synthetic peptides corresponding to the Cys-His boxes of various retroviral NCs or of NC synthesized in vitro suggest that the Cys-His box binds zinc ions (18, 43, 48). Whether this is the specific-type binding seen in transcription factors and, if so, whether it plays a role in NC function or is a nonspecific binding to free SH groups that are in the protein remain to be elucidated.

Various mutations have been introduced in the Cys-His box of NC protein to study its function. We have shown that a linker insertion in the first box of RSV NC affects viral RNA maturation and packaging (39) and that deletion of the first box abolishes virus infectivity while deletion of the second reduces it by a factor of 200 (38). In another study (19), it was shown that mutations substituting all the strictly conserved cysteines with serine residues and the histidine with isoleucine abolish infectivity.

We undertook to determine, by using in vitro mutagenesis, whether amino acids other than those strictly conserved played a role in the function of the Cys-His box within NC protein. Since we have shown previously that the first box of RSV NC is more important in NC function (38), we intro-

* Corresponding author.
† Present address: Pasteur Mérieux Sérums et Vaccins, 69280 Marcy L'Etoile, France.

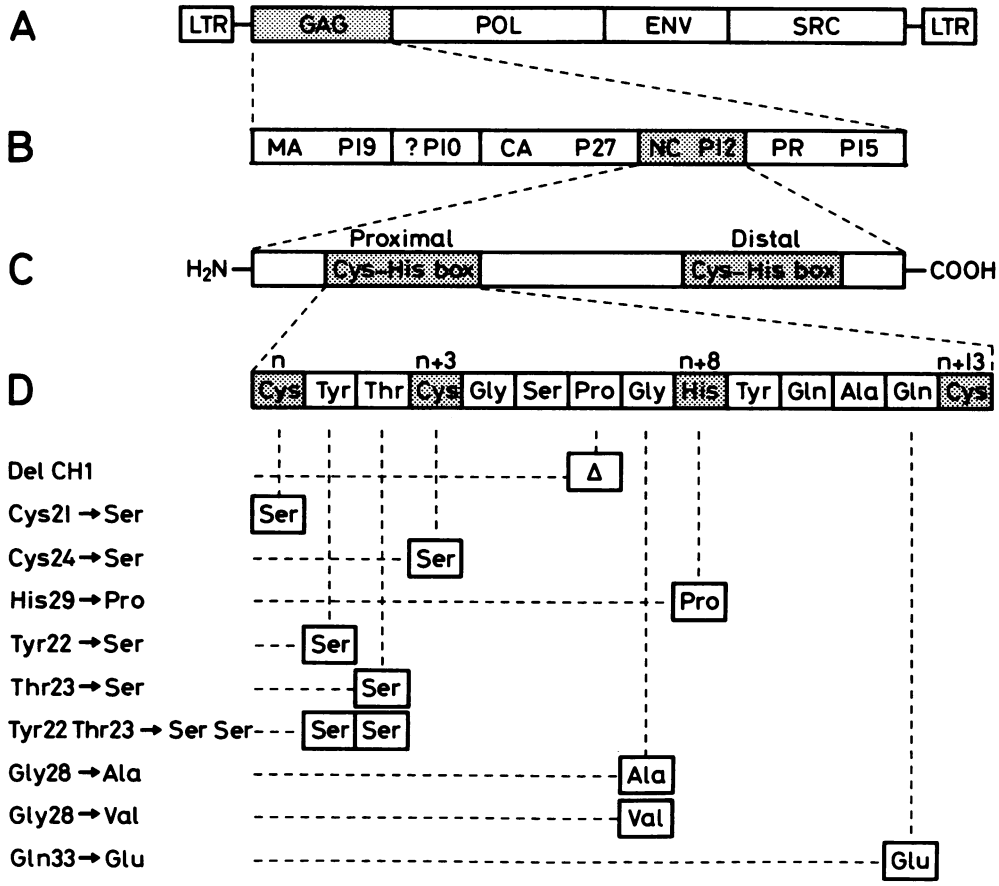


FIG. 1. Structure of *gag* mutants of the proximal Cys-His box of RSV NC. (A) Complete genome of RSV PrC as a linear provirus. The regions encoding the *gag*, *pol*, *env*, and *src* genes are shown in boxes. LTR, long terminal repeat. (B) Enlargement of *gag* gene. (C) Schematic representation of RSV NC (p12) showing the position of the Cys-His boxes. (D) Amino acid sequence of the wild-type proximal Cys-His box, with the conserved cysteines and histidine in shaded boxes, and the mutants constructed in this box. The mutants are numbered as follows. The position of the wild-type amino acid is numbered from the N-terminal end of RSV NC, and the arrow points to the amino acid change. Mutant delCH1 (Del CH1) is a precise deletion of Pro-27.

duced nine point mutations and a small deletion in the proximal Cys-His box of RSV NC protein; all the mutants were characterized in a transient transfection assay for production of viral proteins, RNA packaging and maturation, and reverse transcriptase activity and were tested for biologic infectivity. We report here the results of this study, some of which either differ from or extend the recently published observations made by a similar approach on mutations in murine retroviruses (17, 37).

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblasts prepared from CDI-EV-O eggs (Gs^- and Chf^-; Central Diergeneeskundig Institute, CDI Lelystad, The Netherlands) were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) at 37°C in an atmosphere supplemented with 5% CO_2.

Bacterial strains. *Escherichia coli* DH5α and CJ236 were grown according to the instruction of the mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). *E. coli* DH5α was rendered competent as previously described (44). Plasmid DNAs were purified from either small or large cultures by the alkaline lysis method and were further purified for transfection by equilibrium density gradient centrifugation in cesium chloride-ethidium bromide (44).

Cloned DNAs. Plasmid pAPrc has already been described (39). It contains a nonpermutated copy of the provirus RSV Prague C strain. Plasmid pAsPrc is a *SalI-Eco*RV subclone of pAPrc in pBR322 (39) containing the entire *gag* sequence. All the mutations were constructed in pBS*gag*, a 773-base-pair *PstI-Eco*RI fragment cloned in the phagemid vector bluescribe (+) (Stratagene, San Diego, Calif.).

Site directed mutagenesis. The following oligonucleotides were synthesized on a 381 A DNA synthesizer (Applied Biosystems) and purified as previously described (35): Cys-21→Ser, 3′-CCGAGAGGATGTGAACACCCAGGGGCCC-5′; Cys-24→Ser, 3′-CCGAGACGATGTGAAGACCCAGG GGCCC-5′; Tyr-22→Ser, 3′-CCGAGACGAGGTGAACAC CCAGGGGCCC-5′; Thr-23→Ser, 3′-CCGAGACGATGTC AACACCCAGGGGCCC-5′; Tyr-22Thr-23→SerSer, 3′-CC GAGACGAGGTCAACACCCAGGGGCCC-5′; His-29→Pro, 3′-GAACACCCAGGGGCCCTGGAATAGTCCGCGTCAC GGGC-5′; Gly-28→Ala, 3′-GTGAACACCCAGGGGCCG TGTAATAGTCCGC-5′; Gly-28→Val, 3′-GTGAACACCCA GGGGCCATGTAATAGTCCGCG-5′; Gln-33→Glu, 3′-GT GAACACCCAGGGGCCCTGTAATAGTCCGCCTCA CGGGC-5′. The mutants Cys-21→Ser, His-29→Pro, and Tyr-22Thr-23→SerSer were constructed as described by Zoller and Smith (54). For the others, the selection method described by Kunkel (27) was used with some modification.

Briefly, a single-stranded uracil-containing DNA template was obtained by introducing the phagemid pBS*gag* into *E. coli* CJ-236 (*dut ung*) and by infection with helper phage M13K07, as described by Vieira and Messing (51). The synthesis of the mutagenic strand was performed according to the mutagenesis kit instructions (Bio-Rad). The resulting double-stranded DNA was introduced into *E. coli* DH5α (dut^+ ung^+ $RecA^-$) by the $CaCl_2$ transformation protocol, and the bacteria were grown on Luria-Bertani + ampicillin (100 μg/ml) plates. Mutant delCH1 was constructed by digestion of the subclone pBS*gag* with *Sma*I and *Bam*HI. The *Bam*HI protruding end was filled in by using the Klenow fragment of DNA Pol I. After purification, the DNA was circularized by using T4 DNA ligase.

The introduction of the mutation was confirmed by DNA sequencing by using the dideoxy chain termination method (53). Elongation with avian myeloblastosis virus reverse transcriptase was primed by a synthetic oligonucleotide complementary to the 3′ end of the RSV NC (53). The mutated fragments were cloned back into pAsPrc by using the *Nco*I sites, and then the *Sal*I-*Eco*RV fragment of pAsPrc was introduced into pAPrc.

Transfection and infectivity. Chicken embryo fibroblasts, either freshly prepared or kept frozen in the presence of 15% glycerol, were used after two to seven passages. Transfection and infectivity were performed as described previously (38, 39). Exogenous template reverse transcriptase activity in virions purified from the supernatant medium of infected or transfected cells was measured as described previously (38).

Protein analysis. Viral proteins produced by the transfected or infected cells were analyzed by immunoprecipitation and immunoblotting with polyclonal antibodies against RSV NC (p12), MA (p19), and CA (p27) (36) as described previously (39).

Purification of viral RNA. The viral RNA of virions produced after transfection was purified as follows. Medium from transfected cells grown in two 100-mm culture dishes was harvested every 12 h and stored on ice. Cellular debris were removed by centrifugation at 15,000 × *g* for 10 min at 4°C and a sample was kept for immunoblotting analysis. Virus was pelleted through a cushion of 20% sucrose in NTE by centrifugation for 1.5 h at 35,000 rpm (160,000 × *g*) at 4°C in an SW40 rotor (Beckman Instruments, Inc., Fullerton, Calif.). It was critical not to freeze the harvested medium before RNA extraction.

Medium was carefully removed by aspiration to avoid contamination of the viral pellet with culture medium. The virions were lysed in a solution containing 100 mM NaCl, 50 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, 1% sodium dodecyl sulfate, 100 μg of proteinase K per ml, and 50 μg of yeast tRNA per ml for 20 min at 37°C. RNA was extracted twice with phenol-chloroform and once with chloroform and was then precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2). The pellet was rinsed once with 70% ethanol, dried under vacuum, and digested with 10 μg of RNase-free DNase I per ml for 20 min at 37°C to remove contaminating plasmid DNA. Viral RNA was analyzed by a nondenaturing Northern (RNA) blot procedure as previously described (24, 25).

RESULTS

Construction of 10 mutations in the proximal Cys-His box of RSV NC. The level of conservation of the different amino acids of the Cys-His boxes of retroviral NC proteins varies widely. The cysteine and histidine residues which define the motif are strictly conserved; some other residues also show a high degree of conservation, whereas other positions are more variable. The first box of retroviral NCs are more conserved than the second box, and, in the case of RSV, we have shown that it plays a more important role in NC function (38). To test the relative importance of each amino acid, we constructed a range of point mutations of the proximal Cys-His box of RSV NC.

The strictly conserved residues were mutated: Cys-21 to serine, Cys-24 to serine, and His-29 to a proline (see the legend to Fig. 1 for numbering and nomenclature). The mutations were designed to maintain the proposed secondary structure formed by the residues. The highly conserved Gly-28 was replaced by either an alanine or a valine residue to test the steric constraint of this residue in the Cys-His box.

Murine NC proteins have well-conserved aromatic residues in positions $n + 2$ and $n + 9$, whereas avian NCs have the aromatic residues at $n + 1$ and $n + 9$. We chose, therefore, to mutate either the Tyr-22 at $n + 1$ or the Thr-23 at $n + 2$ to serine residues to test the importance of both the aromatic residue and the amino acid at position $n + 2$ in avian Cys-His box function. A double mutant, Tyr-22Thr-23→SerSer, was also constructed to test whether the mutations have an additive effect.

The avian conserved Gln-33 residue at $n + 12$ was replaced by a glutamate residue to test whether a charged residue in this position modifies the box function, since most other retroviruses have an aspartate residue in this position.

Finally, since we have previously shown that a linker insertion between Pro-27 and Gly-28 affected viral RNA maturation (39), we deleted the Pro-27 to test whether distances between conserved amino acid residues were critical for NC function.

The mutations were constructed by oligonucleotide-mediated, site-directed mutagenesis, except for the deletion. The mutations were confirmed by DNA sequencing, and the mutagenic fragments were cloned back into the plasmid pAPrc, which contains a full-length copy of the wild-type RSV Prague C strain genome. The mutant DNA clones were transfected into cultured chicken embryo fibroblasts and characterized in a transient assay for the following parameters: (i) synthesis of the *gag* gene precursor protein, (ii) release of viral particles and their protein content, (iii) viral RNA content of the virions (quantitatively and qualitatively by a nondenaturing Northern blot procedure), and (iv) biological characterization for infectivity of virions produced in the transient assay.

Cells transfected with all mutants synthesized the *gag* precursor $Pr76^{gag}$ and released virion particles containing normal amounts of viral proteins. Viral mutant DNAs were transfected into chicken embryo fibroblasts by DEAE-dextran-mediated transfection, which was followed by a glycerol shock, as previously described (39). The transient expression of the viral sequence was analyzed 48 to 60 h later. As a control, we included the mutant Prc1, which has a dipeptide (Val-Pro) insertion in the proximal box at position $n + 7$ and was characterized previously (39). Cells from one 100-mm petri dish were lysed, and the *gag* precursor was immunoprecipitated from the cell lysate with a polyclonal antibody against CA (p27). The viral proteins were eluted and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was followed by an immunoblot with ^{125}I-labeled protein A and polyclonal anti-CA antibody. The results show that all of the mutants produced the viral *gag*

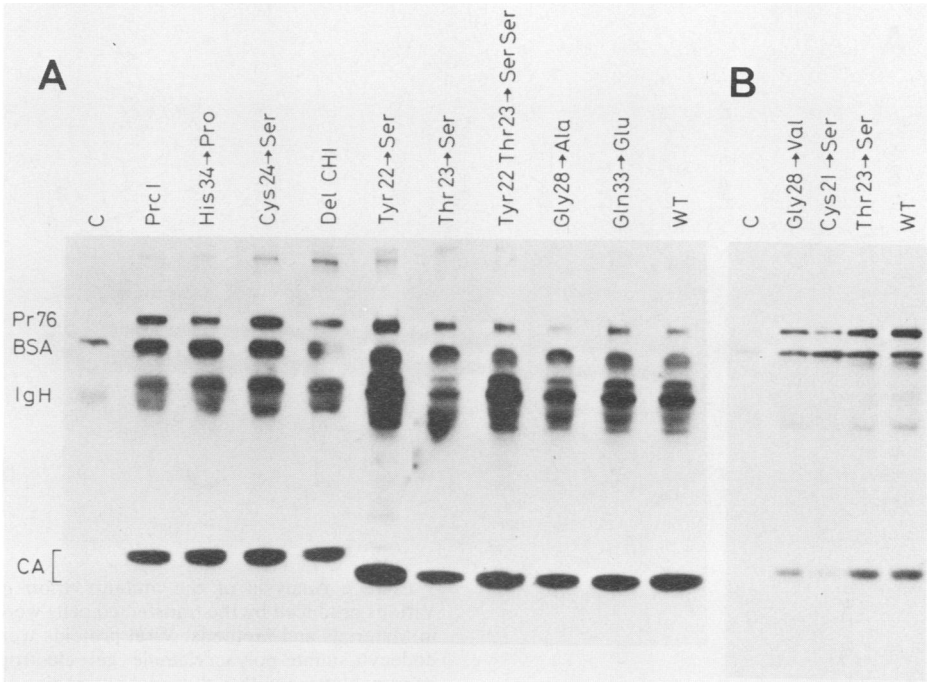


FIG. 2. Intracellular viral proteins from cells transfected with all the mutants. Cell lysates were immunoprecipitated with a polyclonal antibody against CA (p27), followed by a protein A-Sepharose adsorption. The eluted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28) and detected by immunoblotting with anti-CA (p27) serum and ^{125}I-labeled protein A. Autoradiography was for 24 h with an intensifying screen. (A) Lanes: C, control transfection with no DNA; Prc1, transfection with mutant Prc1 DNA (39). Other cells were transfected with the mutants indicated above the lanes. WT, Wild-type transfection. (B) The analysis was the same as described for panel A. Lane C, Control. Other cells were transfected with the mutants indicated above the lanes. WT, Wild type.

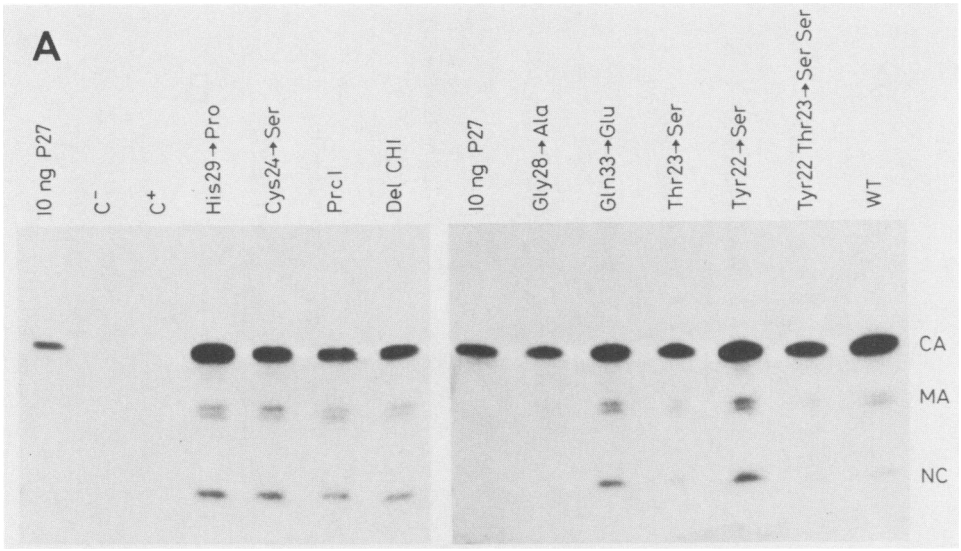
precursor Pr76^{gag} and its cleavage intermediates (Fig. 2). The presence of mature CA was probably because of abortive or budding virus particles bound to the cell membrane. The amount of expressed *gag* precursor varied slightly from one transfection to another but correlated well with the number of viral particles produced.

Virions produced in the transfection were analyzed to test whether maturation or packaging of viral *gag* proteins was affected by the mutation in NC. The viral particles were purified as described in Materials and Methods and analyzed by immunoblotting with polyclonal anti-NC, anti-MA, and anti-CA sera. All the mutants contained the same proportions of viral CA, MA, and NC *gag* proteins as the wild type (Fig. 3). No significant differences were detected in the mobility of or the amount of NC compared with the wild type. Furthermore, in all cases, reverse transcriptase activity correlated well with the amount of viral protein produced in the medium of the transfected cells, as determined by densitometric scanning of the autoradiograph of the immunoblot by using the CA protein as the standard (data not shown). The amount of CA protein was used to standardize the amount of virion in all subsequent experiments.

Mutation of the strictly conserved cysteines and histidine. In agreement with a report describing similar mutations of the conserved cysteines and histidine of avian myeloblastosis virus NC (19), the three similar mutants that we have constructed, Cys-21→Ser, Cys-24→Ser, and His-29→Pro, were all unable to replicate in our infectivity assays or to spread in the cultures (Fig. 4 and 5, lanes Cys21→Ser, Cys24→Ser, and His29→Pro), although Cys-24→Ser sometimes showed a thin band in the protein immunoblot with anti-CA. Analysis of the medium on day 6 after infection for reverse transcriptase activity indicated that mutant Cys-24→Ser was at least 1,000 times less infectious than the wild-type virus (Table 1).

To determine whether these mutations affected the packaging of viral RNA, nucleic acids were extracted from the virions released in the transient assay and analyzed by Northern blotting under conditions which preserve the secondary and tertiary structures of the genomic RNA (24, 25). Hybridization of the blot with a full-length RSV DNA probe showed that mutant Cys-21→Ser was devoid of viral RNA (Fig. 6, lane Cys21→Ser), whereas for mutant Cys-24→Ser a faint smear appeared consistently, showing that some degraded material was present. This suggests that in this mutant the secondary and tertiary structures of the RNA were almost lost. Surprisingly, mutant His-29→Pro was still able to package viral RNA (Fig. 6, lane His29→Pro). Normalized to the number of viral particles, this mutant packaged approximately twofold less viral RNA than the wild-type virus (Table 2); however, approximately 60% of the packaged viral RNA was present as an immature 35S RNA.

Mutations of the highly conserved glycine 28 at *n* + 7. The mutant Gly-28→Ala was analyzed as described above to determine whether the packaging of viral RNA was perturbed by the additional methyl group of the alanine residue. The results show that this mutant was still able to both package and mature the viral RNA correctly (Fig. 6, lane Gly28→Ala, and Table 2) and was fully infectious (Fig. 4 and 5, lane Gly28→Ala, and Table 1). We, therefore, constructed a further mutant with a sterically larger residue, valine, instead of glycine (Gly-28→Val). This mutant contained mature viral RNA but, normalized to the number of viral particles, packaged approximately 10-fold less viral RNA than the wild type (Fig. 6, lane Gly28→Val, and Table 2). It also showed reduced infectivity; analysis of the reverse



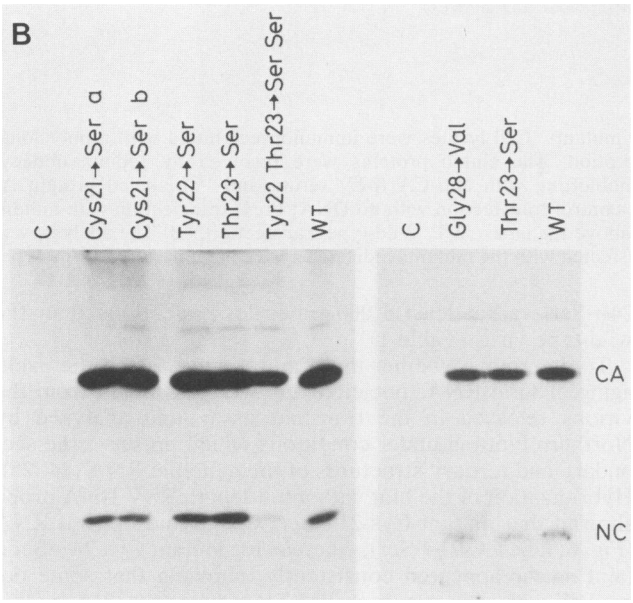


FIG. 3. Analysis of the mutant virion *gag*-encoded proteins. Virions produced by the transfected cells were purified as described in Materials and Methods. Viral proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with polyclonal antibodies against RSV NC (p12), MA (p19), and CA (p27) and ^{125}I-labeled protein A. (A) Lanes: 10 ng P27, 10 ng of high-pressure liquid chromatography-purified p27 protein; C^-, untransfected cells; C^+, control transfection with no DNA. Other cells were transfected with the mutants indicated above the lanes. WT, Wild-type transfection. Autoradiography was for 24 h with an intensifying screen. (B) The analysis was the same as described for panel A, except that only anti-CA and anti-NC antibodies were used. Lanes: C, control; Cys21→Ser a and Cys21→Ser b, transfection with two different clones of mutant Cys21→Ser. Other cells were transfected with the mutants indicated above the lanes. WT, Wild type. Autoradiography was for 24 h with an intensifying screen.

transcriptase activity and of the amount of CA in the virions indicated that the mutant Gly-28→Val was 20 to 30 times less infectious than the wild-type virus (Fig. 4 and 5, lane Gly28→Val, and Table 1).

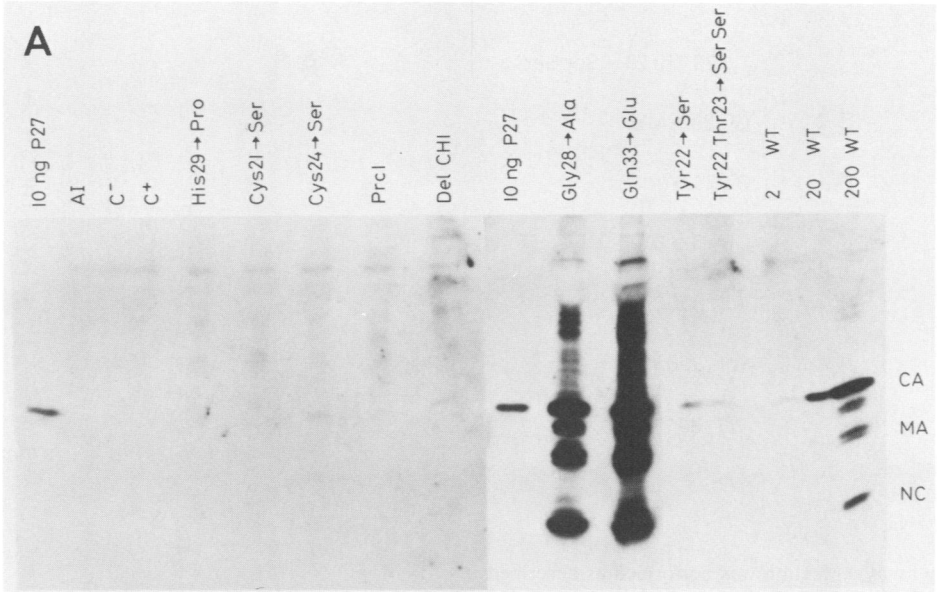
Distance between the cysteine at *n* + 3 and the histidine at *n* + 8 is critical for maturation of viral RNA. In view of the abnormal structure of the RNA packaged by mutant Prc1 (39), which has the dipeptide Val-Pro inserted between the proline at *n* + 6 and the glycine at *n* + 7, we have analyzed the phenotype of the viral RNA packaged by a new mutant, delCH1, which has a deletion of the proline at *n* + 6. The Northern blot shows that, although both of these mutants were still able to package viral RNA (approximately 50% less than the wild type), more than 30% of their RNA was in an immature 35S form (Fig. 6, lanes delCH1 and Prc1). The deletion mutant delCH1 was unable to replicate in our infectivity assay (Fig. 4 and 5, lane Del CH1). Therefore, insertion of two amino acid residues or deletion of one amino acid yielded similar phenotypic changes.

The aromatic amino acid tyrosine at *n* + 1 when mutated disturbed the packaging of viral RNA. Mutants Tyr-22→Ser, Thr-23→Ser and Tyr-22Thr-23→SerSer were all able to mature viral RNA, but less RNA was present in mutants Tyr-22→Ser and Tyr-22Thr-23→SerSer than in the wild type (Fig. 6, lanes Tyr22→Ser, Thr23→Ser, and Tyr22Thr23→SerSer, and Table 2). The mutant Thr-23→Ser contained normal amounts of viral RNA and was fully infectious, whereas mutants Tyr-22→Ser and Tyr-22Thr-23→SerSer showed a very low infectivity. The reverse transcriptase activity and Western blot (immunoblot) test for these mutants revealed that Tyr-22→Ser was approximately 400 times less infectious than the wild type and that mutant Tyr-22Thr-23→SerSer was 400 to 800 times less infectious (Fig. 4 and 5 and Table 1).

A charged amino acid at *n* + 12 in the box does not perturb NC function. When the glutamine residue at *n* + 12 was changed to a charged residue, glutamate, viral RNA was packaged and matured correctly (Fig. 6, lane Gln33→Glu, and Table 2). Infectivity of this mutant was not reduced compared with the wild type (Fig. 4 and 5, lanes Gln33→Glu, and Table 1).

DISCUSSION

Several aspects of the specificity of retroviral RNA packaging remain to be elucidated. One of these is the importance of the various conserved and nonconserved amino acids in



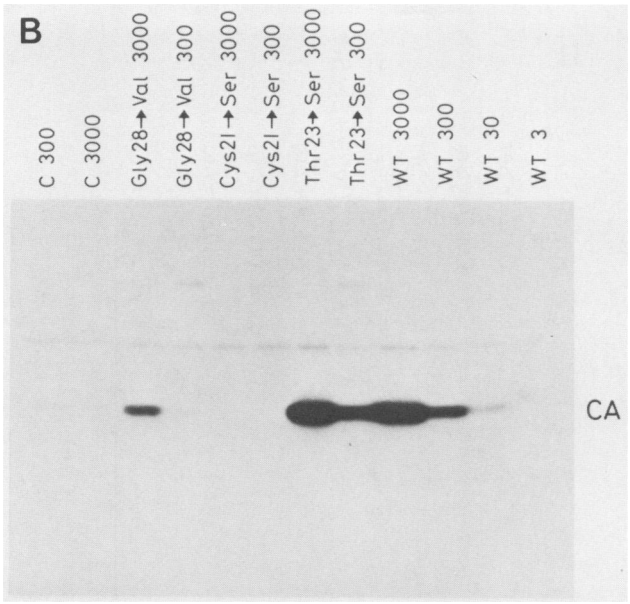


FIG. 4. Infectivity of the virus mutants. Chicken embryo fibroblasts were transfected with the mutant plasmids as for a transient assay. The culture media were collected after 60 h and were used to infect fresh cells. After 6 days, the medium was analyzed for the presence of viral proteins by immunoblotting with anti-CA, anti-MA, and anti-NC antibodies. (A) Lanes: 10 ng P27, 10 ng of high-pressure liquid chromatography-purified p27 protein; C^-, mock infection; C^+, infection with 2 ml of medium harvested from the mock-transfected cells; 2 WT, infection with 2 μl of the medium harvested from the cells transfected with the wild-type plasmid; 20 WT, infection with 20 μl of the same medium; 200 WT, infection with 200 μl of the same medium. Other cells were transfected with 2 ml of medium from cells transfected with the mutants indicated above the lanes. Autoradiography was for 48 h with an intensifying screen. (B) The analysis was the same as described for panel A, except that only anti-CA antibodies were used. Lanes: C 300 and C 3000, infection with, respectively, 300 and 3,000 μl of medium harvested from the mock-transfected cells; Gly28→Val 3000 and Gly28→Val 300, infection with 3,000 and 300 μl, respectively, of medium from Gly28→Val-transfected cells; Cys21→Ser 3000 and Cys21→Ser 300, infection with 3,000 and 300 μl, respectively, of medium from Cys21→Ser-transfected cells; Thr23→Ser 3000 and Thr23→Ser 300, infection with 3,000 and 300 μl, respectively, of medium from Thr23→Ser-transfected cells; WT 3000, WT 300, WT 30, and WT 3, infection with, respectively, 3,000, 300, 30, and 3 μl of medium harvested from wild-type-transfected cells.

the Cys-His box of the NC protein. Examination of the published amino acid sequences of the Cys-His boxes shows that among the various retroviruses, preferential amino acids are used in certain positions of the box (6, 18). This preference is more apparent when the viruses are classified according to their hosts. We have therefore used site-directed mutagenesis to investigate the role of the amino acids in the box function.

RSV NC has two Cys-His boxes, and the mutations described here affect only the proximal box. In a previous study, we obtained results that suggest an additive function of the two motifs (38). As a consequence, the observed phenotypes are expected to be less stringent than in an NC protein with only one box, such as the Moloney murine leukemia virus (MoMuLV) NC.

As already observed for other mutants of the Cys-His box of RSV NC (19, 38, 39), no interference with the production of viral *gag* proteins, their maturation, or the assembly of a virion particle was detected. Furthermore, all the mutants contained wild-type levels of reverse transcriptase activity as determined by an exogenous assay, suggesting that the expression of the *pol* gene products was unaffected. Most of our mutants block virus replication, as already described for similar mutants in MoMuLV, human immunodeficiency virus type 1, and RSV NC proteins (1, 17, 19, 37).

The strong phenotypic change observed when Cys-21, Cys-24, or His-29 was mutated was not unexpected in view of the well-demonstrated biological importance of these residues in NC function from RSV NC (19) and MoMuLV NC (17). However, analysis of the viral RNA packaged by our mutant His-29→Pro revealed an interesting difference compared with the cysteine mutants, as it still packaged a large amount of viral RNA, most of which was in an immature 35S monomeric form.

It has already been noted (3, 43, 45, 48) that the Cys-His motif is reminiscent of the zinc finger motif found in many

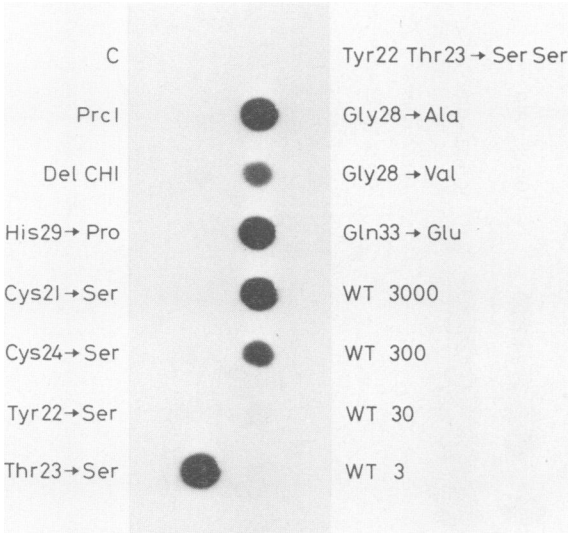


FIG. 5. Infectivity assay. Infection was performed as described in the legend to Fig. 4 with 3,000 μl of filtered medium harvested from each mutant transfection, and four days after infection the reverse transcriptase activity was measured in the supernatant medium by the dot blot procedure (16). Nomenclature was as described in the legend to Fig. 4B.

eucaryotic transcription factors and in the bacteriophage T4 gene 32 protein, in which biochemical and biophysical studies have shown that zinc(II) has a structural function (13–15). If one assumes that the Cys-His motif in retroviral NC binds zinc ions, then the mutants Cys-21→Ser and Cys-24→Ser may have a different structure because of their inability to bind zinc, which does not allow the specific contact with viral RNA necessary for packaging. Mutant His-29→Pro may still bind zinc with weak affinity and maintain the ability to package but not to mature the viral RNA. However, the fact that a mutant of a strictly con-

TABLE 1. Infectivity of the mutants

Virus and dilution at infection[a]	Reverse transcriptase activity (cpm, 10^1)
Control	259
Wild type	
1/1	30,305
1/10	8,389
1/100	831
1/1,000	310
Prc1	301
DelCH1	321
Cys-21→Ser	291
Cys-24→Ser	321
His-29→Pro	328
Tyr-22→Ser	286
Thr-23→Ser	41,824
Tyr-22Thr-23→SerSer	284
Gly-28→Ala	27,032
Gly-28→Val	5,661
Gln-33→Glu	29,635

[a] Chicken embryo fibroblasts were infected with 2 ml of culture medium containing 2,000, 200, 20, or 2 μl of the filtered supernatant from the cells transfected with the wild-type virus or, in the case of the mutants, 2 ml of undiluted, filtered supernatant from the transfected cells. Four days after infection, the virions were concentrated by centrifugation of 1 ml of medium and the reverse transcriptase activity of the pellet was measured as described previously (38).

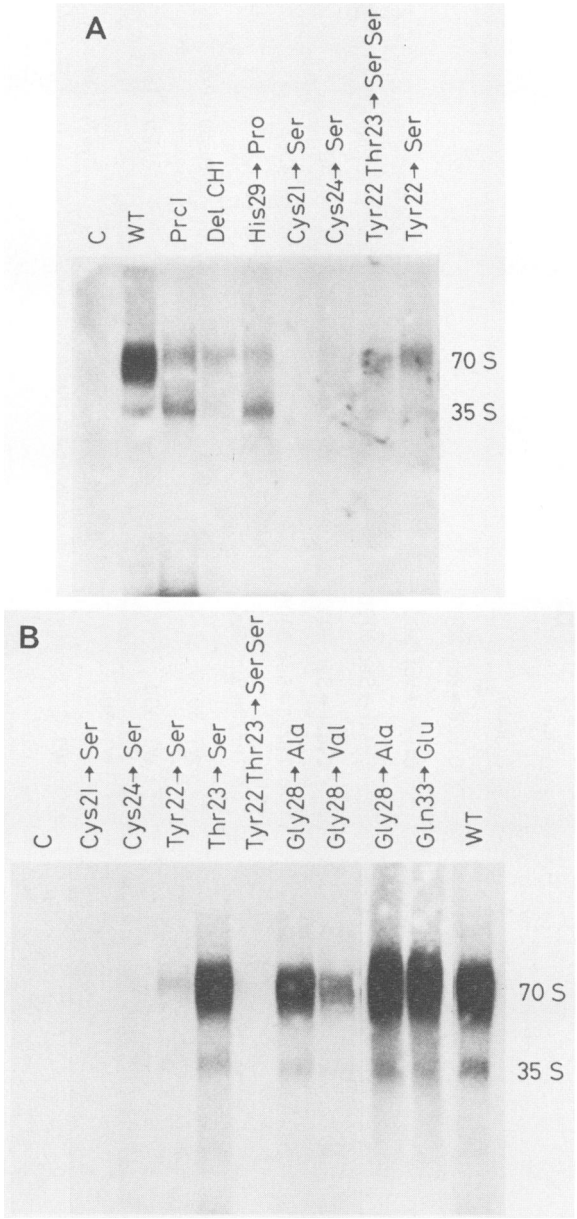


FIG. 6. Viral RNA content of the virions produced in a transient transfection assay. The virions were purified and viral RNA was extracted as described in Materials and Methods. After size fractionation on a nondenaturing 0.8% agarose gel, the RNA was electrotransferred to a nylon membrane and hybridized with a probe specific for RSV RNA (plasmid pAPrc) ^{32}P-labeled by random primer extension. (A) Lanes (and relative amounts of CA protein [in densitometric units] loaded): C, control transfection with no DNA; WT, transfection with wild-type DNA, 9.6; Prc1, 5.6; Del CHI, 18.7; His29→Pro, 13.2; Cys21→Ser, 11.2; Cys24→Ser, 16.8; Tyr22 Thr23→SerSer, 6.7; Tyr22→Ser, 14.3. The cells were transfected with the mutants indicated above the lanes. Exposure time was 18 h with an intensifying screen. (B) The analysis was the same as described for panel A. Lanes (and relative amounts of CA protein [in densitometric units] loaded): C, control; Cys21→Ser, 1.8; Cys24→Ser, 7.9; Tyr22→Ser, 5.2; Thr23→Ser, 4.7; Tyr22Thr23→SerSer, 3; Gly28→Ala, 5; Gly28→Val, 3; Gln33→Glu, 3.1; WT, 8. Exposure time was 24 h with an intensifying screen.

TABLE 2. Viral RNA content of the virions

Virus	% Total viral RNA[a]	% of RNA	
		70S	35S
Control			
Wild type	100	95	5
Prc1	50	52	48
delCH1	25	70	30
Cys-21→Ser			
Cys-24→Ser	1		
His-29→Pro	50	34	66
Tyr-22→Ser	25	87	13
Thr-23→Ser	100	98	2
Tyr-22Thr-23→SerSer	10	85	15
Gly-28→Ala	100	98	2
Gly-28→Val	10	98	2
Gln-33→Glu	100	98	2

[a] Viral RNA was purified and analyzed by a nondenaturing Northern blot procedure. The viral RNA was quantitated by scanning densitometry of the autoradiographs of the Northern blot. All results are normalized to the amount of CA protein obtained by densitometric scanning of the protein immunoblot autoradiographs and are expressed as a proportion of the wild type.

served amino acid was still able to package viral RNA raises the possibility that this motif does not have a restricted structural role in zinc binding, as previously suggested (3, 18) or that zinc does not play a role in NC RNA packaging function. In fact, spectroscopic studies on purified avian myeloblastosis virus NC (19) or from in vitro-synthesized MoMuLV NC (43) have shown that no major structural changes occur on binding of zinc by NC proteins. On the other hand, the contribution of the unaffected, distal Cys-His box to the phenotype is not known. It is conceivable that substitutions of the Cys residues of the proximal box destabilize the whole protein so much that it becomes unable to bind RNA, whereas a substitution of the His residue does not interfere so much with the distal box. A similar phenomenon could explain the differences between the phenotypes of the Cys mutants and Prc-del1 (38). The whole proximal box is missing from this mutant, but RNA is still packaged, albeit in reduced amounts.

The phenotype of the mutants Gly-28→Ala and Gly-28→Val suggests that the conservation of the glycine at $n + 7$ in NC is not essential, as was predicted from its high level of conservation in Cys-His boxes. A similar mutant (Gly-7→Val) has been described in MoMuLV NC (37). In that case, however, the mutant viruses produced do not package viral RNA and are not infectious. This difference could be explained by a structural difference between the RSV and MoMuLV Cys-His boxes; in the RSV NC proximal box, the glycine is adjacent to two noncharged amino acids (Pro at $n + 5$ and Ser at $n + 6$), whereas in MoMuLV the glycine is close to two large, charged residues (Glu at $n + 5$ and Lys at $n + 6$). The structure of the polypeptide chain in this region may, therefore, be less sterically restricted in RSV than in MoMuLV with the hydrophobic Val residue not in the proximity of charged amino acids. Nevertheless, the glycine residue at $n + 7$ has been highly conserved in RSV even if it can be replaced, without a detectable effect, by alanine or, with a moderate effect, by valine, suggesting a selective advantage for this glycine. Since the three-dimensional structure of the NC is not known, the results described above highlight the difficulty in interpreting some of the mutagenesis data in which amino acids that do not appear related in the primary sequence may in fact be related in the tertiary structure.

Aromatic residues are found in many of the Cys-His boxes, usually at $n + 1$ or $n + 2$ and at $n + 9$. The phenotypes of our mutants show that the tyrosine at $n + 1$ in RSV NC is important for viral RNA packaging. Mutation of the tyrosine residue at $n + 2$ in MoMuLV NC shows the same phenotype. Indeed, spectroscopic studies predict that a large side chain from either position $n + 1$ or position $n + 2$ could occupy nearly the same orientation in space in the proposed three-dimensional Cys-His box model (18). It has also been suggested that this tyrosine plays a role in the selection of the RNA to be packaged (17, 37).

Changing the glutamine residue at position $n + 12$ to a charged residue (Gln-33→Glu) does not affect RSV NC function. The reverse mutation in MoMuLV (Asp-12→Asn) (37) confirms that the presence of either a charged or an uncharged residue in this position of the box does not alter the infectivity of the virus.

We have already described a dipeptide Val-Pro insertion mutant of the RSV proximal Cys-His box that packages viral RNA, but 50% was monomeric, compared with approximately 5 to 10% in wild-type virions (Prc1) (39). The phenotype displayed by the mutant delCH1 suggests again that the geometry of the motif, specifically the distances within the conserved Cys-His pattern, is critical. However, we cannot rule out the possibility that the deleted proline residue itself has an important structural function. This confirms, therefore, the role of RSV NC in the maturation of the genomic RNA inside the virion. No such phenotype has been described so far for mutants of MoMuLV NC.

The mechanism that ensures the specific packaging of retroviral RNA has not yet been elucidated. Although it seems clear that, at the protein level, NC plays a role in the specific recognition of the RNA, there is no evidence that the discrimination between cellular RNA and viral RNA is entirely due to the interaction of NC with viral RNA. In vivo, one mutant has been described that shows an RNA packaging defect. This quail cell line, SE21Q1B, produces virions containing large amounts of cellular RNA instead of viral RNA (12, 34). It has been suggested that some part of the $Pr76^{gag}$, in addition to the mutation affecting the packaging sequence in the leader, was also mutated (M. Linial and A. D. Miller, Curr. Top. Microbiol. Immunol., in press).

Studies have shown that *cis*-acting RNA packaging signals are located near the leader, close to the dimer linkage site and primer binding site sequences, which are involved in the dimerization of 35S RNA and binding of the $tRNA^{trp}$ primer of reverse transcription, respectively (21–23, 26). There is also evidence, in the case of MoMuLV, that a sequence in the *gag* gene may be required in *cis* for packaging (2). Recent in vitro studies suggest that NC interacts with packaging signals located near the dimer linkage site and the primer binding site sequences in the leader of RSV and MoMuLV (4, 7, 41). Direct evidence that these sequences bind NC in vivo has not been obtained to date, although experiments with UV cross-linking have shown that there is a binding site for RSV NC near the dimer linkage site in the leader (7).

We have already described mutants of RSV NC which are able to package a relatively large amount of 70S genomic RNA but are not infectious (Prc11.3 and Prc1 [39]). The fact that mutants delCH1, His-29→Pro, and Tyr-22→Ser showed the same phenotype again suggests that NC played a role in the early events of reverse transcription. Work is in progress to test whether the 70S dimer in these mutants lacks the $tRNA^{trp}$ primer of reverse transcription, as already reported for mutant Prc1 (42).

NC proteins are involved in another aspect of viral matu-

ration. In the nucleoid, the genomic RNA is coated by NC protein, suggesting that NCs also have a nonspecific binding function, probably involving sequences outside the Cys-His box. This hypothesis is supported by in vitro studies showing that the nonspecific binding function of purified MoMuLV NC is not lost when the cysteine residues are oxidized (20).

In conclusion, the site-directed mutagenesis of the Cys-His box of retroviral NCs emphasizes the importance of this motif in the viral replication cycle and points to a role in the recognition, packaging, and maturation of progeny viral RNA. It is, however, likely that this region of the NC protein is not solely responsible for the specificity of viral RNA recognition, since mutations between the two boxes of RSV NC also yield noninfectious particles that are devoid of viral RNA (10, 11). Finally, even though this remains to be demonstrated experimentally, it is likely that it is the polyprotein precursor $Pr76^{gag}$ that recognizes the viral RNA before particle formation. Thus, it is highly probable that the structure of both the Cys-His box and other regions of the NC domain depends on the overall structure of the polyprotein and, therefore, that other determinants outside the NC domain are involved in specific RNA recognition as well. An in vitro system with native NC or $Pr76^{gag}$, as well as mutants affecting the processing or the structure of the precursor $Pr76^{gag}$ in vivo, would help in answering this question.

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