JOURNAL OF VIROLOGY, Sept. 1988, p. 3328–3333
0022-538X/88/093328-06$02.00/0

Vol. 62, No. 9

Mutations in Rous Sarcoma Virus Nucleocapsid Protein p12 (NC): Deletions of Cys-His Boxes

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Received 25 February 1988/Accepted 25 May 1988

Rous sarcoma virus nucleocapsid protein p12 (NC) contains two conserved amino acid motifs, the Cys-His boxes, which constitute potential metal-binding domains. To try to understand the function of NC and of each of its Cys-His boxes during the viral life cycle, particularly in viral RNA packaging, we have used synthetic oligonucleotides to delete precisely either the proximal or the distal box, or both Cys-His boxes. The mutant DNAs were transfected into chicken embryo fibroblasts, and the virions produced in a transient assay were characterized biochemically for production of viral proteins and particles, RNA packaging, and infectivity. The results indicated the following. (i) The deletion of either the proximal or the distal box decreases the amount of viral RNA packaged in the particles and results in incomplete 70S dimer formation. (ii) The deletion of both boxes inhibits viral RNA packaging. (iii) The deletion of the proximal, but not the distal, box suppresses any detectable infectivity, while the deletion of the distal, but not the proximal, box lowers infectivity 100 to 200 times.

The structural proteins of retroviruses are encoded by the *gag* gene whose primary product is a polyprotein precursor. Pr76, the *gag* protein precursor of Rous sarcoma virus (RSV), is a 76,000-dalton protein that yields, upon proteolytic cleavage, the mature MA (p19), p10, CA (p27), NC (p12), and PR (p15) *gag*-encoded proteins (19). RSV NC is a basic protein which is associated with the 70S RNA in the core of the virion (2, 7). A small number of NC molecules can be cross-linked by UV light to the RNA in the virion, and we have identified and sequenced their binding sites (6). No binding specificity of RSV NC for viral RNA has been demonstrated in vitro (8, 20, 28, 29).

The primary transcription product of the integrated provirus is a 35S RNA which codes for the *gag* and the *gag-pol* polyprotein precursors. Splicing of the same RNA also yields two different subgenomic mRNAs coding for the envelope glycoproteins and for the transforming protein $pp60^{src}$. However, only the full-length 35S RNA is packaged in the viral particles as a 70S dimer. Thus, for the correct packaging of their genome, retroviruses need to discriminate against cellular and subgenomic viral RNA. Packaging sequences at the RNA level have been described previously (13, 14), but the proteins which interact with these sequences have not been identified. Among the *gag*-encoded proteins, RSV NC is the best candidate for this role, either as an individual protein or as a constituent of Pr76 or one of its cleavage intermediates. With a first set of mutations in RSV NC, we have shown that this protein is necessary for viral RNA packaging and formation of a stable 70S genomic dimer RNA (25).

Almost all the retroviral nucleic acid-binding proteins known so far possess a conserved pattern of cysteine and histidine residues (1, 4). These residues constitute what we have referred to as the cysteine-histidine box (25), which is defined by the conserved position of four residues, three cysteines and one histidine; if the proximal cysteine is designated as n, there is a distal cysteine at the position $n+3$, a histidine at $n+8$, and a third cysteine at $n+13$. Some other residues are also highly conserved, particularly a glycine located in front of the histidine. Another typical feature of these sequences is the presence of one or two aromatic residues, the proximal one at the position $n+1$ or $n+2$ and the distal one at $n+9$.

One Cys-His box is also found in a *Drosophila* copia clone (26) and in the coat protein gene of cauliflower mosaic virus (5). A similar pattern exists also in the T4 single-stranded DNA-binding protein coded by gene 32; however, the sequence is in opposite orientation, and the histidine is at position $n+9$, instead of $n+8$ (31).

By analogy to the "zinc fingers" present in many regulatory DNA-binding proteins (17), a possible structure for the Cys-His box as a metal-binding domain has been proposed (1) and it was shown (9) that the box of the T4 gene 32 product binds a Zn^{2+} ion with a structural function in single-stranded nucleic acid binding. Until now however, the association of a metal ion with the purified retroviral nucleic acid-binding proteins has not been shown.

The nucleic acid-binding proteins of some retroviruses, like murine leukemia virus, contain one Cys-His box, whereas most retroviruses, such as RSV, bovine leukemia virus, visna virus, and the human retroviruses, have two boxes. This suggests that these boxes may constitute independent structures with independent functions. However, the variations observed in their amino acid composition suggest that they are not functionally equivalent. In the case of RSV, for example, there is little homology between the two boxes; only 6 amino acids out of 14 are conserved, including the 4 which define the pattern. Moreover, the proximal box contains two tyrosines, whereas the distal box has no aromatic residue (Fig. 1).

To further study NC in the replicative cycle of RSV, and in particular to define the respective role of the two Cys-His boxes in its function, we have used synthetic oligonucleotides to delete precisely the coding sequence of either one of the two boxes or both. The mutant DNAs were transfected into chicken embryo fibroblasts, and the viral particles produced in a transient assay were characterized biochemically and for infectivity.

The following results support the hypothesis deduced from the sequence comparison between the two boxes. (i)

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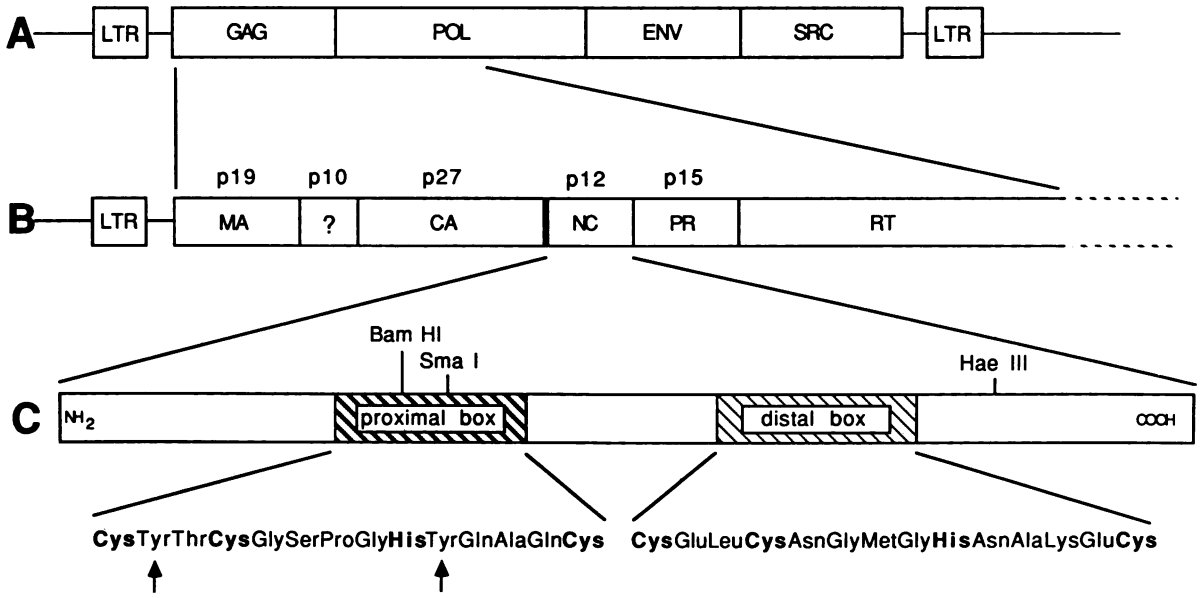

FIG. 1. (A) Complete genome of Pr-C as a linear provirus. The regions encoding the *gag*, *pol*, env, and *src* genes are shown in boxes. LTR, Long terminal repeat. (B) Enlargement of *gag* gene and N-terminal domain of *pol* gene. (C) Schematic representation of RSV NC (p12). The sequences of the two Cys-His boxes which were precisely deleted are represented under the two hatched boxes, with the conserved cysteine and histidine residues in boldtype face. The two aromatic residues in the proximal box are indicated by the arrows.

The Cys-His boxes are modular elements which play additive roles in viral RNA packaging and 70S dimer structure formation. (ii) The proximal box, but not the distal one, is absolutely required for infectivity.

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblasts prepared from Valo eggs (Lohmann Tierzucht, Cuxhaven, Federal Republic of Germany) were grown in Dulbecco modified Eagle medium containing 1 to 5% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) at 37°C in an atmosphere supplemented with 5% CO_2.

Bacterial strains. *Escherichia coli* HB101 was grown in L broth and was transformed to ampicillin resistance by the $CaCl_2$ method (22). *E. coli* JM101 was kept on minimal medium supplemented with 1 mM thiamine-HCl (22).

Oligonucleotide synthesis and purification. Oligonucleotides were synthesized either on a Beckman System I DNA synthesizer, or on a Pharmacia Gene Assembler, or on an Applied Biosystems 381 A DNA synthesizer and were deprotected according to the instructions given by the manufacturer. They were further purified according to the procedure described previously (21). The following oligonucleotides were synthesized: CM0, 20-mer 5'GGGCGATCTT TATGTTCCAT3' (primer for sequencing); CM1, 40-mer 5'CCTGACTTCCGTTTTTTCGGGAGCCCTCGGGCACG ACCAC3' (proximal box, deletion); CM2, 20-mer 5'ATC TCCCGGGGATCCACAAG3' (proximal box, selection); CM3, 39-mer 5'GGTTGCCATCCCGCTTTCTTCGCTCA CGGCTGTTTCCTG3' (distal box, deletion); CM4, 20-mer 5'CTGTTAGCGTTGTGTCCCA3' (distal box, selection).

Site-directed mutagenesis. The mutations were constructed in the clone ΔgagBS, a *Pst*I-*Eco*RI subclone of the clone pAPr-C (12, 25) in the phagemid Bluescribe(+) (Stratagene, formerly Vector Cloning Systems, San Diego, Calif.). The ΔgagBS plasmid was introduced into the *E. coli* JM101 strain, and single-stranded DNA was obtained upon infection with M13 VCS strain provided in the kit.

Single-stranded DNA purification. The single-stranded DNA was purified from the polyethylene glycol-precipitated bacteriophages by two phenol extractions, one ether extraction, and ethanol precipitation in the presence of 0.3 M sodium acetate.

Mutagenesis. The mutations were constructed by using the method of "priming all the way round" as described previously (33). The template (1 μg) and the labeled primer (10 pmol) were annealed together in 10 μl of buffer containing 10 mM Tris hydrochloride (pH 8.0) and 10 mM $MgCl_2$. The tube containing the sample was placed in a small beaker of hot water (80°C) and let cool to room temperature for about 30 min. Dried [α-^{32}P]dATP (5 μCi) was taken up with the annealing mix, and the following substances were added: 1 μl of 10× TM buffer (100 mM Tris [pH 8.0], 100 mM $MgCl_2$), 1 μl of 5 mM rATP, 1 μl of 5 mM deoxynucleoside triphosphates, 1 μl of 100 mM dithiothreitol, 4 μl of water, 10 U of T4 DNA ligase (Anglian Biotechnology, Colchester, Great Britain), and 2 U of the Klenow fragment of DNA polymerase (Anglian Biotechnology). After 12 to 14 h of incubation at 12°C, 80 μl of 10 mM Tris hydrochloride (pH 8.0)–10 mM EDTA was added to the extension-ligation reaction, and then 100 μl of 13% polyethylene glycol 6000–1.6 M NaCl. The mixture was left on ice for 15 min and spun in a microfuge for 5 min. The supernatant was removed completely, and the pellet was dissolved in 180 μl of water. A 20-μl quantity of 2 M NaOH was added to the sample, which was left at room temperature for 5 min, and then placed on ice for 2 min. The sample was then loaded on a 5 to 20% sucrose gradient and centrifuged for 2.5 h at 38,000 rpm, 4°C, in a SW60 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). About 20 200-μl fractions were collected from the bottom of the tube, neutralized with 1/10th volume of 3 M sodium acetate, and counted. The closed circular DNA ran at about fractions 6 through 10, in front of the single-stranded template and the unincorporated ATP. The closed circular DNA fractions were pooled, 10 μg of carrier tRNA was added, and the nucleic acids were precipitated with

ethanol. The DNA was suspended in water, and half of it was used, either immediately, or after digestion with a restriction endonuclease to transform *E. coli* HB101 bacteria made competent by the $CaCl_2$ method (22).

Colony screening. A total of 150 to 200 colonies were screened with oligonucleotides complementary to the deletions. The colonies were transferred to nitrocellulose filters, which were then treated with alkali, neutralized, and baked at 80°C in vacuo (22). The screening oligonucleotide (150 pmol) was labeled with 10 μCi of [γ-^{32}P]ATP and T4 polynucleotide kinase for 30 min at 37°C in 50 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$–3mM dithiothreitol. The mixture was then diluted with 3 to 10 ml of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and filtered through a 0.45-μm-pore-size nitrocellulose filter and stored frozen. The filters were prewet in 6× SSC for 5 min and prehybridized for 5 min at 65°C in a solution containing 10× Denhardt solution, 6× SSC buffer, and 0.2% sodium dodecyl sulfate, without shaking. The filters were then rinsed in 6× SSC and hybridized in disposable plastic petri dishes, colonies face down, for 1 h at room temperature. The filters were washed with 100 ml of 6× SSC three times for 5 min at room temperature and then once at 50°C for 5 min. The filters were covered with Saran Wrap and autoradiographed. The plasmid DNA of three to six colonies showing no hybridization was analyzed by restriction enzyme digestion, and one candidate was sequenced.

DNA sequencing. The mutated plasmids were sequenced either by procedure of Maxam and Gilbert (23) or by Sanger dideoxy-chain termination using a synthetic oligonucleotide complementary to the end of the RSV NC coding sequence (CM0) or one of the screening oligonucleotides (CM4) and avian reverse transcriptase (32).

Transfection. Chicken embryo fibroblasts, either freshly prepared or frozen in the presence of 15% glycerol, were used for transfection after two to seven passages. Transfection was performed as described previously (25).

Protein analysis. Viral proteins produced by the transfected cells were analyzed as described previously (25) by immunoprecipitation and immunoblotting (3) with polyclonal antibodies against RSV NC (p12), MA (p19), and CA (p27) (24).

Northern (RNA blot) analysis. The viral RNA content of the produced particles was analyzed by nondenaturing Northern blot analysis as described previously (15, 16).

Exogenous template reverse transcriptase assay. The test was performed as described previously (25) on virus pelleted from 5 ml of culture medium (10).

Infectivity. Chicken embryo fibroblasts were plated at a cell density of about 750,000 cells per 100-mm-diameter Petri dish the day before use. The virus stocks were filtered before use through a 0.45-μm-pore-size disposable polysulfone Acrodisc filter unit (Gelman Sciences, Inc., Ann Arbor, Mich.) to avoid the transfer of cells. Just before infection, the cells were treated for 1 h at 37°C in culture medium without serum containing 25 μg of DEAE-dextran per ml. The cells were infected with various dilutions of the virus stocks in culture medium without serum for 2 h at 37°C. The culture medium was then changed for fresh culture medium containing 5% fetal bovine serum. After 2 days (I+2), the serum concentration was lowered to 2%, and 2 days later (I+4), the serum concentration was lowered to 0%. Six days after infection, the culture medium was harvested and analyzed for reverse transcriptase activity and viral proteins.

RESULTS

Deletion of the Cys-His boxes. To remove precisely the proximal or the distal box, or both Cys-His boxes in RSV NC, we used the technique of site-directed mutagenesis with synthetic oligonucleotides. To obtain single-stranded DNA containing the NC coding sequence, the *Sal*I-*Eco*RV *gag-pol* subclone of the plasmid pAPr-C (12, 25) in pBR322 was further subcloned into the phagemid Bluescribe(+) by using the restriction sites *Pst*I-*Eco*RI. The Bluescribe vector is a pBR322-derived plasmid containing the M13 replication and packaging sequences that allow the packaging of single-stranded plasmid DNA into phage capsids upon infection of the *E. coli* host by the phage M13.

Two oligonucleotides were synthesized for the deletion of each Cys-His box: one deletion oligonucleotide complementary to 20 nucleotides on each side of the box, and one screening oligonucleotide complementary to the sequence to be deleted. The mutations were introduced into the Bluescribe subclones by using the "all the way round" priming technique, and the closed circular DNA molecules were purified on an alkaline sucrose gradient (33). The closed circular DNA was then introduced into *E. coli* by transformation, and the mutant plasmid was detected by hybridization of the bacterial colonies with the screening oligonucleotides. In the case of the proximal box, the presence of a unique *Bam*HI restriction site in the sequence to be deleted allowed us to also select directly for Pr-C del(1) mutants by *Bam*HI digestion of the closed circular DNA before transformation. The mutated DNA region was sequenced, and the corresponding full-length pAPr-C plasmids were reconstructed in two cloning steps.

Production of viral particles unaffected by deletion of Cys-His boxes. To test the mutants, the altered proviral sequences were introduced into chicken embryo fibroblasts by DEAE-dextran-mediated transfection, followed by a glycerol shock as previously described (25). The transient expression of the viral sequences was analyzed 48 to 60 h later; the produced particles were pelleted by centrifugation and analyzed biochemically and for infectivity. The mutant Pr-C 1, which has a Val-Pro insertion in the proximal box at position n+7 and was characterized previously (25), was included as a control. Viral proteins were detected in the transfected cells and in the virions either by immunoprecipitation, followed by immunoblotting (intracellular viral proteins), or by immunoblotting only (virion proteins) with polyclonal antibodies against the *gag*-encoded proteins NC (p12), MA (p19), and CA (p27).

The results showed that the wild type and all mutants produced particles containing the same amount of *gag*-related proteins; the amount of mutated p12 protein packaged into the virions appears to be identical to the wild type, although the signal corresponding to the protein with the two boxes deleted was weaker. The deletions in the NC result in an increased mobility corresponding to the size of the deletion (Fig. 2). The analysis of the intracellular viral proteins did not show any significant difference between the mutants and the wild type (Fig. 3). The lower amount of intracellular *gag* precursor and cleaved CA (p27) observed for mutant Pr-C 1 is probably due to an artifact of the immunoprecipitation, since the extracellular amount of viral proteins appears normal (Fig. 2 and 3, lanes c). Equivalent amounts of active reverse transcriptase were detected in pelleted virions (data not shown) by using the exogenous template assay (10).

Viral RNA content of the mutant virions. In view of the

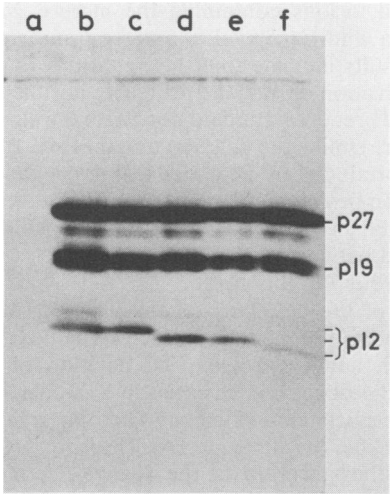

FIG. 2. Virion *gag*-encoded proteins produced in a transient transfection assay. The cells were transfected in the presence of DEAE-dextran and glycerol shocked. The medium was collected as described in Materials and Methods. The virions were pelleted by centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18), followed by immunoblotting with polyclonal antibodies against RSV NC (p12), MA (p19), and CA (p27) and ^{125}I-labeled protein A. Lanes: a, control transfection with no DNA; b, wild type; c, Pr-C 1; d, Pr-C del(1); e, Pr-C del(2); f, Pr-C del(1,2).

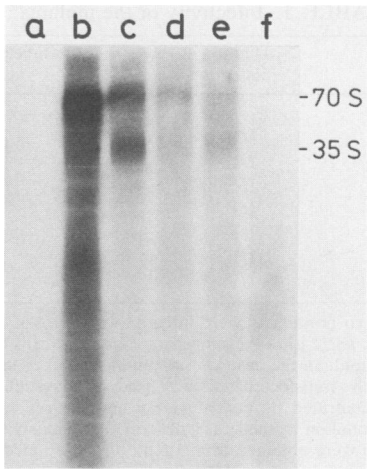

FIG. 4. Viral RNA content of the virions produced in a transient transfection assay. The virions were pelleted and digested with proteinase K in the presence of sodium dodecyl sulfate, the RNA was phenol extracted, digested with RNase-free DNase 1, size fractionated on a nondenaturing 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with the nick-translated pATV-8 plasmid containing the RSV coding sequence. Lanes: a, control transfection with no DNA; b, wild type; c, Pr-C 1; d, Pr-C del(1); e, Pr-C del(2); f, Pr-C del(1,2). The position of the 35S viral RNA was determined from the cellular RNA, and the position of 70S RNA was determined from the wild-type virus in lane b.

abnormal structure of the RNA packaged by Pr-C 1 (25), we have also analyzed the RNA packaging function of the deletion mutant virions by nondenaturing Northern blotting (16, 25). The deletion of either the proximal or the distal box resulted in the same phenotype; the total amount of viral RNA present in the particle was identical in both mutants but appeared lower than in the wild type or the mutant Pr-C 1 (Fig. 4, lanes d and e). As already observed in the case of this mutant, the 35S RNA, instead of being present in low levels compared with the 70S dimer (lane b) constituted approximately 50% of the viral RNA (lane c). The signals produced by the mutants with two boxes deleted, Pr-C del(1) and del(2) (lanes d and e), however, appeared more diffuse than that of Pr-C 1 (lane c), suggesting that the RNA has a less well-defined secondary and tertiary structure. The simultaneous deletion of the two boxes in Pr-C del(1,2) almost abolished viral RNA packaging (lane f), a phenotype similar to that of the deletion p12 mutant Pr-C 10.8 (25). The lane was, however, not as blank as the control and seemed to contain some degraded material.

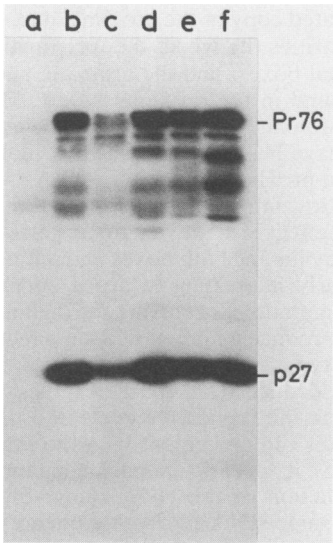

FIG. 3. Intracellular viral proteins. The cell lysates were immunoprecipitated with a polyclonal antibody against CA (p27), followed by protein A-Sepharose adsorption. The eluted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by immunoblotting with anti-CA (p27) serum and ^{125}I-labeled protein A. Lanes: a, control transfection with no DNA; b, wild type; c, Pr-C 1; d, Pr-C del(1); e, Pr-C del(2); f, Pr-C del(1,2).

The mutant without the distal Cys-His box still infectious. To monitor the infectivity of the mutants, chicken embryo fibroblasts were transfected with the mutant plasmids as for a transient assay. The culture medium was collected after 60 h, and various dilutions were used to infect fresh chicken embryo fibroblasts. After 6 days, the culture medium was analyzed for reverse transcriptase activity and viral proteins (Table 1 and Fig. 5). The results indicated that no replication of the mutants lacking either the proximal or both Cys-His boxes could be detected (Fig. 5, lanes g and i). The mutant having the proximal, but not the distal, box was weakly infectious (Fig. 5, lane h). The reverse transcriptase activities (Table 1) and the amount of proteins indicate that Pr-C del(2) was 100 to 200 times less infectious than the wild type. The electrophoretic mobility of the p12 protein produced 6 days after infection by this mutant was identical to that observed in the transient assay, indicating that no obvious reversion to the wild type occurred.

DISCUSSION

In this work, we have tried to understand the respective contribution of each of the two Cys-His boxes to the

TABLE 1. Infectivity of the mutants[a]

Virus	Dilution at infection	Reverse transcriptase activity (cpm)
Control		58
Wild type	1/10	7,160
	1/100	636
	1/1,000	118
Pr-C 1	1/1	64
Pr-C del(1)	1/1	63
Pr-C del(2)	1/1	401
Pr-C del(1,2)	1/1	58

[a] Chicken embryo fibroblasts were infected with 2 ml of culture medium containing 200, 20, and 2 μl of the supernatant from the cells transfected with the wild-type plasmid. In the case of the mutants, 2 ml of undiluted filtered supernatant from the transfected cells was used. Six days after infection, the virions were concentrated by centrifugation and the reverse transcriptase activity was measured on the pellets as described previously (10). The values shown in the table were obtained from 1.6 ml of culture medium.

function(s) of RSV NC (p12). We have used a molecular genetic approach and have constructed straightforward mutations of these conserved motifs, the precise deletion of either the proximal or the distal box, or both Cys-His boxes.

As observed with other mutations in RSV NC already characterized (25), the deletions of the Cys-His boxes had no effect on the release of the viral particles. All of the mutants tested yielded virions containing the mature *gag*-encoded proteins and a wild-type level of reverse transcriptase activity. These results indicate that the mutations in p12 neither affect the activation of p15, the protease, nor the maturation of the *gag-pol* precursor, and do not affect the stability of the *gag* precursor. Unlike the deletion mutant Pr-C 10.8 (25), the deleted NC produced by Pr-C del(1,2) is packaged into the virions and can be detected on an immunoblot. According to the results published previously, it can be concluded that the region located between the two boxes is necessary to retain the mature protein in the virion.

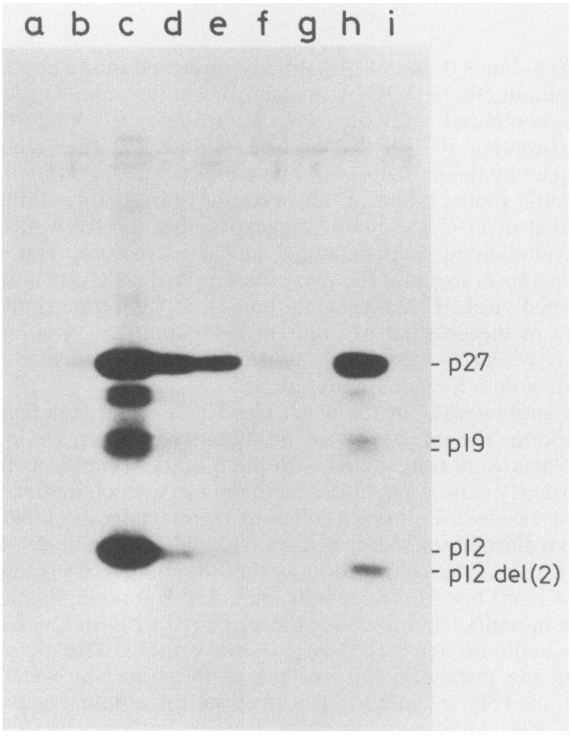

FIG. 5. Infectivity of the mutants. Chicken embryo fibroblasts were transfected with the mutant plasmids as for a transient assay. The culture media was collected after 60 h and was used to infect fresh cells. After 6 days, the medium was analyzed for the presence of viral proteins by immunoblotting. Lanes: a, mock infection; b, infection with 2 ml of medium from the mock-transfected cells; c, infection with 20 μl of the medium harvested from the cells transfected with the wild-type plasmid; d, infection with 2 μl of the same medium; e, infection with 1 μl of the same medium; f, g, h, and i, infection with 2 ml of medium harvested from cells transfected with Pr-C 1, Pr-C del(1), Pr-C del(2), and Pr-C del(1,2), respectively.

The effect of the deletions on the viral RNA packaging function supports the model of the Cys-His boxes as modular units with additive roles. The deletion of either the proximal or the distal box resulted in a similar phenotype, reduced RNA packaging efficiency and abnormal structural maturation of the 70S dimer RNA. The effect of these two deletions on the structure of the viral RNA is more pronounced than the one observed in the case of Pr-C 1, which has a Val-Pro insertion in the proximal box. This suggests that the insertion of these two amino acids had only a limited disturbing effect on the structure of the proximal box. It is also possible that the deletions have general long-range negative effects on the protein which interfere with the binding of RNA and which explain the lower packaging efficiency when one of the two Cys-His boxes is deleted. In both cases, however, these results show that both boxes are necessary for the formation of a stable genomic dimer. The absence of packaging observed when both boxes are deleted suggests that at least one box is necessary for viral RNA packaging and that the basic amino acids located outside the boxes are not sufficient for that function.

The fact that one mutant is still infectious, although weakly, indicates that a nucleocapsid protein containing only the proximal Cys-His box is able to provide the minimal functions necessary for replication. This result is not surprising in view of the similarities between the various retroviral Cys-His boxes (1), which suggest that the distal box is a degenerated copy of the proximal one. Indeed, there are more similarities between the proximal boxes than between the distal boxes, and the aromatic amino acids are preferentially found in the proximal boxes. Moreover, retroviruses like Moloney murine leukemia virus and feline leukemia virus have NC with only one box motif, which can be classified as a proximal box.

The relatively low infectivity of Pr-C del(2) is possibly due to the disturbing effect of some remaining parts of the protein that functioned by linking both boxes and are now useless. It will be particularly interesting to see if spontaneous deletions and point mutations occurring during multiple rounds of infection can produce a mutant with an infectivity close to that of the wild type.

What can we deduce from these results concerning the role of NC during the replicative cycle and the function of the Cys-His boxes? In agreement with the mutants already characterized (25), it appears that packaging the viral RNA is not the only function of the NC protein. Pr-C del(1) and del(2) have the same RNA packaging phenotype, but only one of these mutants is able to replicate. The occurrence of a major problem during reverse transcription due to the abnormal structure of the RNA is unlikely, since both mutants show the same defect on a nondenaturing Northern blot. As we have already proposed (25), the retroviral NC probably functions in the infection process as a cofactor during reverse transcription. More mutants inside and out-

side the boxes will, however, be necessary to define precisely the contributions of the various regions of the protein.

Concerning the Cys-His boxes, these results suggest a double role: (i) a structural function allowing the basic residues to bind to the nucleic acid and (ii) a binding function of the box itself, probably mediated by the intercalation of the aromatic residues between the bases, as in the T4 gene 32 product (27, 30). In the case of murine leukemia virus NC (p10), the tryptophan residue at $n+9$ in the Cys-His box has also been implicated in RNA binding by fluorescence quenching (11). This binding function could be critical during reverse transcription, perhaps for the unwinding function of the protein. This is, however, only a working hypothesis, and more mutations will be necessary to prove it.

The results obtained so far have given no indication that, in vivo, NC is specifically binding to RSV RNA. RSV NC is clearly necessary for packaging, but another viral protein could also be involved. The characterization of various point mutations in the Cys-His boxes will perhaps give an answer to this question.

ACKNOWLEDGMENTS

We are grateful to A. Sussman and Beckman Instruments International in Geneva, Switzerland for the synthesis of some of the oligonucleotides used in this work. We thank Pascal Damay for remarkable technical assistance, Otto Jenni for photographs and plates, Pamela Schwartzberg Vinayaka Prasad, and Steve Goff for critical reading of the manuscript.

One of us (E.G.) gratefully acknowledges the financial support of the Sandoz Stiftung, Basel, Switzerland. This research was supported by grant 3.079.084 from the Swiss National Science Foundation.

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