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# Rous Sarcoma Virus Nucleic Acid-Binding Protein p12 Is Necessary for Viral 70S RNA Dimer Formation and Packaging

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**To study the function(s) of the Rous sarcoma virus nucleic acid-binding protein p12, we constructed mutants by using two restriction sites in the p12 proviral coding sequence of the Prague C strain to insert *Kpn*I synthetic linkers. The two restriction sites are in the same reading frame, which allowed us to construct a deletion mutant lacking the two conserved Cys-His regions and a duplication mutant containing three intact Cys-His boxes. These mutant DNAs were transfected into chicken embryo fibroblasts, and the viral particles produced in a transient assay were characterized biochemically and for infectivity. Our results indicate that the Rous sarcoma virus nucleic acid-binding protein p12 is necessary for genomic RNA packaging but not for particle assembly and is implicated in the formation of a stable 70S dimeric RNA. Moreover, the fact that one mutant was apparently able to package normal 70S RNA but was not infectious suggests a role for p12 during the infection process.**

All retroviruses carry a gene (*gag*) whose expression yields a polyprotein precursor of the mature viral *gag* structural proteins. For Rous sarcoma virus (RSV), the primary product of the *gag* gene is a 76-kilodalton precursor protein (Pr76) which is cleaved during virus assembly to yield five mature proteins, p19, p10, p27, p12, and p15. p19 is associated with the lipid envelope (28), p27 constitutes the core shell, and p15 is a protease responsible for the maturation of the precursor. p12 is a basic nucleic acid-binding protein which is found associated with the 70S RNA in the core of the virion (2, 11). We have shown that a small number of p12 molecules (and not p19 as previously reported [32]) can be cross-linked by UV light to the RNA in the virion, and we have identified and sequenced their binding sites (10, 24). However, no binding specificity of p12 for viral RNA has been demonstrated in vitro (12, 19, 20, 34).

In cells infected by wild-type RSV, viral RNA constitutes 10 to 20% of the poly(A)$^+$ mRNA (37). The transcribed 35S RNA is spliced into 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. Considerable work has been done to determine the packaging sequences at the RNA level (for a review, see reference 4), but the proteins which interact with these sequences have not been identified. Among the *gag* proteins, p12 is the best candidate for this interactive role, whether as an individual protein or as a constituent of Pr76 or one of its cleavage intermediates.

Amino acid sequence comparisons among the various retroviral nucleic acid-binding proteins has revealed the conservation of a pattern of cysteine and histidine residues (7, 25). These residues constitute a region that we refer to as the cysteine-histidine box, defined by the conserved positions of four residues, three cysteines and one histidine: if the first cysteine is designated as n, there is a second cysteine at the position n + 3, a histidine at n + 8, and a third cysteine at n + 13. Other residues are also highly conserved, particularly in the nonavian retroviruses (7). RSV p12 contains two Cys-His boxes in the same orientation, whereas murine leukemia virus p10 has only one. One Cys-His box is also found in the first open reading frame of a copia clone (25) but not in the yeast Ty element; both of these are transposable elements which can produce retrovirallike particles containing reverse transcriptase (13, 33). The same Cys-His box was recently found in the coat protein gene (ORF IV) upstream of the putative reverse transcriptase gene of cauliflower mosaic virus, a plant DNA virus which replicates via a reverse transcription step (8). A similar pattern of cysteines with one histidine was found in the T4 single-stranded DNA-binding protein coded by gene 32; however, the sequence is in the opposite orientation, and the histidine is at position n + 9 instead of n + 8 (41). The sequence is located between amino acids 72 and 116, a region containing most of the regularly spaced tyrosine residues which are thought to be involved in nucleic acid binding (40).

The presence of a conserved amino acid pattern in various nucleic acid-binding proteins prompted us to study its role in RSV replication by in vitro mutagenesis of the viral protein p12. We used two restriction sites present in the p12 coding sequence to insert *Kpn*I synthetic linkers; these two restriction sites are in the same reading frame, allowing the construction of a deletion mutant with the deletion spanning the two Cys-His boxes and a duplication mutant with three intact Cys-His boxes. These in vitro mutant DNAs were transfected into chicken embryo fibroblasts. The viral particles produced in a transient assay were characterized biochemically and for infectivity. Our results indicate that the RSV nucleic acid-binding protein p12 is necessary for genomic RNA packaging but not for particle assembly and is implicated in the formation of a stable 70S RNA dimer. Moreover, the fact that one mutant is apparently able to package normal 70S RNA but is noninfectious suggests a role for p12 during the infection process.

## 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

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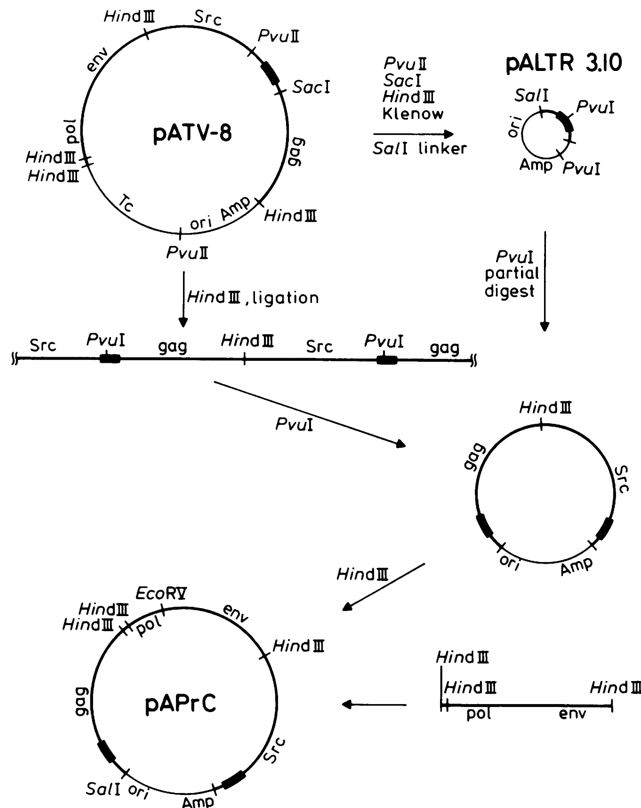


FIG. 1. Construction of infectious provirallike Pr-C clone from permuted pATV-8 clone. Details of the DNA manipulations are given in Materials and Methods. A *Sal*I linker was inserted 5′ to the first LTR to allow easy subcloning of the *gag* region in pBR322 by using the unique *Eco*RV site in the *pol* gene. The two LTRs are represented as black boxes.

medium (Imperial Laboratories) supplemented with 1 to 5% fetal calf serum (Sera-Lab) at 37°C in an atmosphere supplemented with 5% $CO_2$.

**Bacterial cultures.** *Escherichia coli* HB101 (high-producer isolate 1035) was grown in L broth and was transformed to ampicillin resistance by the $CaCl_2$ method (22).

**Cloned DNA.** The original permuted clone of the RSV Prague C (Pr-C) strain, pATV8, was provided by R. Guntaka (16); its sequence has been published (30). The restriction sites were determined with the computer program Compseq, developed by A. Bairoch, Geneva, Switzerland.

**DNA constructions.** DNA constructions were done as described previously (22). The permuted clone of the Pr-C strain, pATV-8, was cut with *Pvu*II, diluted, and circularized. The subclone was then digested with *Sac*I and *Hin*dIII, repaired with the Klenow fragment of DNA polymerase, diluted, and circularized. A *Sal*I linker (Institut Pasteur, Paris, France) was then inserted at the unique *Pvu*II site. The subclone finally obtained, pALTR3.10, contains the pBR322 sequences between the *Pvu*II and *Hin*dIII sites and one RSV long terminal repeat (LTR) with a small part of the leader and end of the *src* gene. The large *Hin*dIII fragment from pATV8 containing a small portion of the *pol* gene, the *gag* gene, one LTR, the *src* gene, and part of the *env* gene was purified, ligated at a high plasmid concentration, and digested with *Pvu*I, which cuts in the LTR. These fragments were ligated with pALTR3.10 linearized with *Pvu*I in the presence of ethidium bromide (EtBr). The correct recombinant was then partially digested with *Hin*dIII and ligated with the *pol-env Hin*dIII fragment of pATV8. The resulting RSV Pr-C clone pAPr-C has the typical proviral structure, with two LTRs flanking the four viral genes, *gag, pol, env*, and *src* (Fig. 1). Tested by transfection, this clone was infectious. The pAPr-C plasmid was quite instable when maintained by bacterial colonies on agar at 4°C, although the host bacteria was a *recA*$^-$ strain. It was found that the best storage conditions are −70°C in 15% glycerol.

The last reconstruction step of pAPr-C also produced a polymerase-defective mutant (*pol* mutant) without the small *Hin*dIII fragment between positions 2738 and 2868. This 130-base-pair deletion results in a frameshift and a premature termination of the protein.

To construct the *env-src* deletion mutant, the sequences between the *Pvu*II sites at positions 5567 and 8671 of the wild-type pAPr-C plasmid were deleted. The *Eco*RI (position 6142)-*Mlu*I (position 7899) fragment was used as a specific probe for the deleted sequences.

The *Sal*I linker inserted 5′ to the left LTR could be used with the unique *Eco*RV site located in the *pol* gene to subclone the *gag* region and easily insert it back into the pAPr-C clone.

To linearize a plasmid containing more than one specific restriction site, the digestion was done with different quantities of enzyme and in the presence of 50 to 100 μg of EtBr per ml. The digested DNA was extracted three times with isoamyl alcohol to eliminate the EtBr and once with phenol-chloroform and subjected to electrophoresis overnight at low voltage on an agarose-Tris borate-EDTA (agarose-TBE) gel (22) without EtBr. The linear DNA was electroeluted into a dialysis bag and purified on DEAE-Sephacel (Pharmacia) before ligation.

**DNA sequence analysis.** Plasmids (5 μg) were digested with *Bam*HI and labeled by using the Klenow fragment of DNA polymerase and [$\alpha$-$^{32}$P]dATP. The labeled fragments were then cut with *Eco*RI and separated on a 1.5% agarose-TBE gel. The fragments of interest were electroeluted on NA 45 membranes (Schleicher & Schuell, Inc.) and subjected to chemical degradation by the method of Maxam and Gilbert (23).

**Transfection of chicken embryo fibroblasts.** Cells either freshly prepared from embryos or frozen in the presence of glycerol were used for transfection after two to seven passages. Cells frozen in a medium containing dimethyl sulfoxide could not be used because of the activation of endogenous retroviruses. DEAE-dextran-mediated transfection was done essentially as described (1), with some modifications. The day before transfection, cells in petri dishes with confluent growth were trypsinized and split into two or three portions per dish. At the time of transfection, the cells were approximately 70 to 90% confluent. The cells were washed with Tris-buffered saline (25 mM Tris hydrochloride, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $Na_2HPO_4$ [pH 7.4]) sterilized by filtration. DNA (up to 15 μg per 100-mm petri dish) was diluted in 0.8 ml of Tris-buffered saline, and 0.2 ml of Tris-buffered saline containing 1 mg of DEAE-dextran per ml was added. The DNA sample (1 ml per petri dish) was left on the cells for 30 min at 37°C, 10 ml of culture medium containing 5% fetal calf serum was added, and the cells were left for 4 to 5 h at 37°C. The cells were then shocked for 2 min in 15% glycerol, washed with culture medium, and incubated with 5 ml of culture medium containing 2.5% fetal calf serum. After 12 h, the medium was discarded; fresh medium (5 ml) was then added and was collected 24 h later. Fresh medium (5 ml) was again

added and was collected after 24 h (transient assay). For the long-term assay, cells were passaged once after 2 days and analyzed after 1 week, when the cells transfected with the wild-type plasmid appeared highly transformed.

**Antibodies.** Polyclonal antibodies against RSV p12, p19, and p27 were prepared as described previously (24) from viral proteins purified either by gel filtration in the presence of 6 M guanidinium hydrochloride (p27) or by reverse-phase high-pressure liquid chromatography (p12 and p19).

**Viral protein analysis.** Immediately after collection, the medium was centrifuged for 10 min at 16,000 × *g* in a Sorvall centrifuge (Ivan Sorvall, Inc.) to remove cellular debris and stored frozen. The virions were pelleted at high speed (Beckman SW60 rotor at 40,000 rpm for 40 min) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting (see below) with a mixture of polyclonal antibodies against p12, p19, and p27.

Cells were lysed in RIPA buffer (50 mM Tris hydrochloride, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 20 mM EDTA [pH 8.5]), and the lysate was immunoprecipitated with a polyclonal antibody against p27 followed by protein A-Sepharose (Pharmacia) adsorption. The eluted proteins were then resolved by SDS-PAGE and labeled by immunoblotting (see below) with the same antibody and $^{125}$I-labeled protein A.

For the immunoblotting reactions (modified from the method of Burnette [3]), the resolved proteins were electrotransferred onto nitrocellulose in 0.19 M glycine–25 mM Trizma base–20% methanol [pH 8.3] at 250 mA overnight in a Bio-Rad Transblot apparatus. The filters were stained with 0.2% Ponceau S dye in 3% trichloroacetic acid, treated for 1 h in A1 buffer (10 mM Tris hydrochloride [pH 7.5], 100 mM $MgCl_2$, 0.1% Triton X-100, 0.5% bovine serum albumin) to block the binding capacity of the paper, and incubated for 2 h with the antibodies diluted 500 times in A1. After three 5-min washings in A2 buffer (same as A1 but with 0.1% bovine serum albumin), the filters were incubated for 1 h in 5 ml of A2 buffer containing 1.5 $\mu$Ci of $^{125}$I-labeled protein A (specific activity, 43 mCi/mg). The filters were then washed three times for 10 min in A2 buffer containing 0.5 M LiCl and autoradiographed for several days at 4°C or overnight at −70°C with an intensifying screen. Cyanogen bromide-activated paper was prepared as described previously (27) and used with a discontinuous electrophoresis gel system in the presence of SDS (system J4179, modified from the method of Neville and Glossmann [26]). The filters could be reused with other antisera after being washed in 0.2 M glycine hydrochloride (pH 2.2; 10 min) and in 9 M guanidinium hydrochloride–2% 2-mercaptoethanol (10 min) at room temperature (I. Bolivar, University of Geneva, personal communication).

**Northern blotting.** At 48 to 60 h after transfection, the cells were trypsinized, washed with culture medium containing 5% fetal calf serum, lysed in 20 mM Tris hydrochloride–1% SDS–0.5 M LiCl–5 mM EDTA, and incubated with proteinase K for 1 h at 37°C. The nucleic acids were extracted three times with phenol-chloroform, and the RNA was precipitated overnight at −20°C in 3 M LiCl. The RNA pellet was dissolved in water containing 1% SDS and precipitated once with ethanol before analysis on a nondenaturing agarose-TBE gel containing 0.5 $\mu$g of EtBr per ml. The RNA was electrotransferred onto a GeneScreen nylon membrane (New England Nuclear Corp.) in 25 mM sodium phosphate buffer (pH 6.5) (Transblot apparatus, Bio-Rad). The wet nylon membrane was then UV irradiated for 2 min at a distance of 5 cm from two germicide lamps (15 W each), prehybridized, and hybridized as described previously (18). The freshly prepared prehybridization solution contained 50% twice recrystalized and deionized formamide (Fluka), 1 M NaCl, 10× Denhardt solution, 50 mM Tris hydrochloride (pH 7.5), 0.1% sodium $PP_i$, 1% SDS, and 10% dextran sulfate (molecular weight, 500,000; Pharmacia). The solution was heated at 60°C and filtered while hot through a 0.45-$\mu$m HA membrane filter (Millipore Corp.) in a Swinnex-25 filter holder (Millipore) with a syringe. Salmon sperm DNA was heat denatured at 75°C for 15 min in 85% formamide and added to the filtrate at a final concentration of 100 $\mu$g/ml. The GeneScreen membrane was prehybridized for 16 h at 42°C in a sealed bag with 0.15 ml of solution per $cm^2$ of membrane with slow constant rocking. The nick-translated $^{32}$P-labeled probes were heat denatured at 75°C for 15 min in a volume of prehybridization solution (without NaCl, dextran sulfate, and carrier DNA) equal to one-fifth of that used for prehybridization, and the solution was added to the bag containing the prehybridization solution and the GeneScreen membrane. After hybridization was performed for 24 h at 42°C, the membrane was washed for 5 min at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS and for 60 min at 42°C with 0.2× SSC.

The RNA contained in the virions was extracted from the crude viral pellets (see viral protein analysis) in the presence of 5 $\mu$g of carrier *E. coli* tRNA, as described above for cellular RNA but without the 3 M LiCl precipitation step. To eliminate contaminating plasmids, the RNA was digested for 20 min at 37°C with 10 $\mu$g of RNase-free DNase I per ml (commercial DNase I treated with Macaloid, as described previously [22]).

**Exogenous template reverse transcriptase assay.** The exogenous template reverse transcriptase assay was performed on crude viral pellets (see viral protein analysis) by the method of Goff et al. (14). The pellets corresponding to one-third of the production of one 100-mm petri dish in a transient assay were lysed in 50 $\mu$l of a reaction cocktail containing 50 mM Tris hydrochloride, 20 mM dithiothreitol, 5 mM $MgCl_2$, 50 mM NaCl, 0.05% Nonidet P-40, 5 $\mu$g of oligo(dT) per ml, 10 $\mu$g of poly(A) per ml, and 50 $\mu$Ci of [$\alpha$-$^{32}$P]dTTP (3,000 Ci/mmol) and incubated at 37°C for 0 to 20 min. Every 5 min thereafter, 10 $\mu$l of the reaction mixture was spotted on dry DEAE paper (DE-81; Whatman, Inc.). The paper was then immediately washed three times in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) for 15 min and once in 95% ethanol, dried, and counted (Cerenkov).

## RESULTS

**Construction of RSV *gag* protein p12 mutants.** An infectious Pr-C provirallike clone with two LTRs was constructed from a permuted clone as described in Materials and Methods (Fig. 1). The RSV sequence predicts the existence of two *Sma*I restriction sites in the *gag-pol* subclone (*Sal*I-*Eco*RV) constructed in pBR322, one in the p19 coding sequence at nucleotide position 523 and the other in p12 at position 1924, in the first Cys-His box. Among the numerous *Hae*III restriction sites, the three sites present in the p12 coding sequence are clustered 3′ to the second Cys-His box at positions 2047, 2081, and 2086 (Fig. 2). The *gag* subclone was linearized by partial digestion in the presence of EtBr with *Sma*I or *Hae*III and ligated to a hexamer *Kpn*I linker. The plasmids were circularized and screened for the correct linker insertion positions.

The insertion of a linker at position 1924 (Pr-C 1, Fig. 2C and D) resulted in the addition of the dipeptide Val-Pro in the

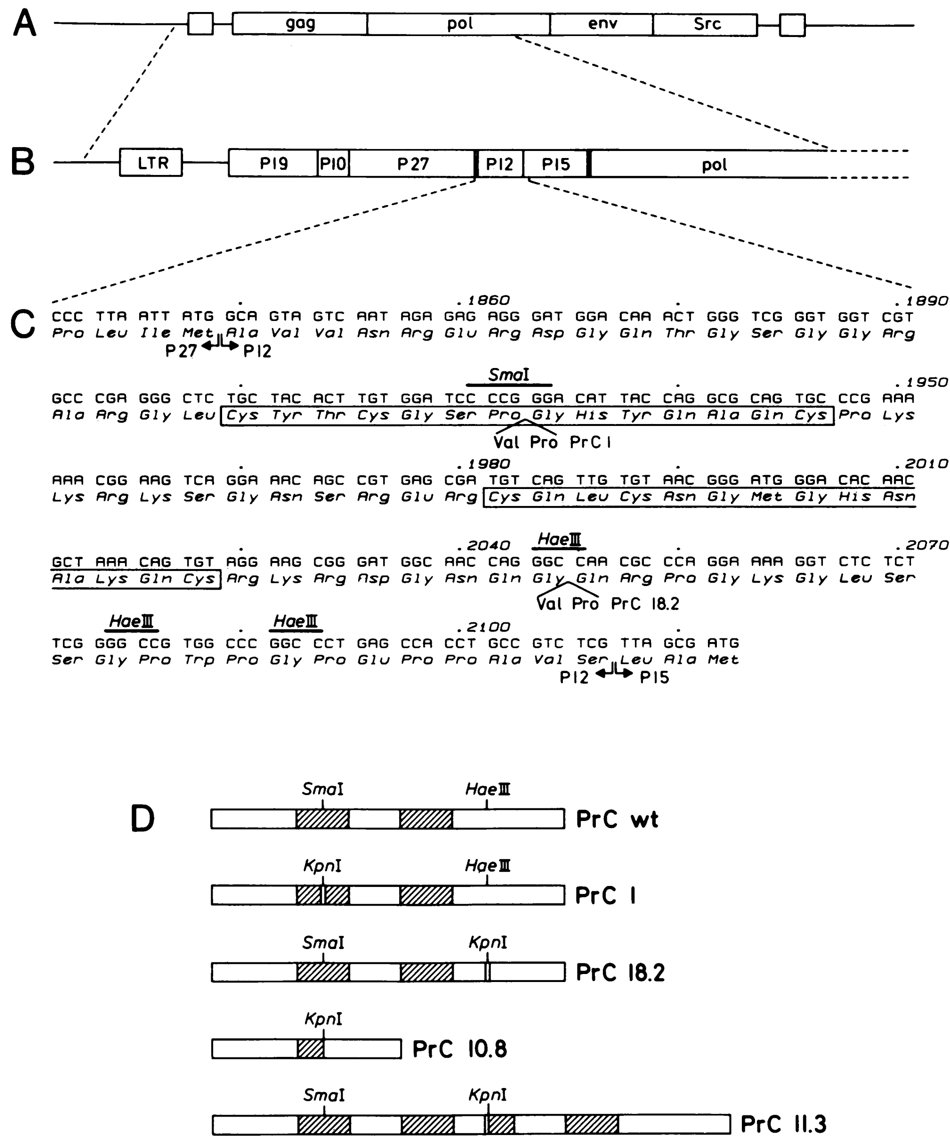


FIG. 2. Structure of *gag* mutants of Pr-C. (A) Complete genome of Pr-C as linear provirus. The regions encoding the *gag, pol, env,* and *src* genes are shown in boxes. (B) Enlargement of *gag* gene and N-terminal domain of *pol* gene. (C) DNA and amino acid sequences (30) of coding strand of p12 protein, with the recognition sites for the two enzymes which were used to insert the linkers. The two amino acids coded by the 6-mer *Kpn*I linkers inserted in the sequence are also indicated. The two other mutants, Pr-C 10.8 and Pr-C 11.3 were obtained by in vitro recombination of the linker insertion mutants. The two Cys-His regions are shown in boxes. (D) Schematic representation of the four mutant p12 proteins coded by Pr-C 1, Pr-C 10.8, Pr-C 11.3, and Pr-C 18.2 compared with the wild-type (wt) protein. The restriction sites of the corresponding DNA sequence are also indicated. The Cys-His conserved regions, or the remaining parts of them, are indicated by hatched boxes.

first Cys-His box, between the amino acids Pro and Gly just in front of the conserved His. As a consequence, one of the essential features of the Cys-His box, namely the constant distance between the conserved amino acids, was modified.

With *Hae*III, linker insertion was observed only at position 2047 (Pr-C 18.2, Fig. 2C), eight amino acids after the end of the second Cys-His box. In the mutant Pr-C 18.2, the insertion again resulted in the addition of the dipeptide Val-Pro. The two mutants were sequenced in the region of the insertions. In each case, only one linker was found without any modification of the reading frame. The cleavage positions of the two restriction sites used to insert the linkers were in the same reading frame, and we were therefore able to construct two other mutants by in vitro recombination.

One mutant had lost the sequence between positions 1923 and 2047 (Pr-C 10.8, Fig. 2C and D), representing the second half of the first Cys-His box, the second box, and three basic amino acid clusters. The encoded protein consists of only 50 (instead of 89) amino acids.

In the second mutant (Pr-C 11.3, Fig. 2C and D), the same sequence, from positions 1924 to 2046, was duplicated starting at position 2047, after the *Kpn*I linker. This new p12 protein is made up of 132 (instead of 89) amino acids, three intact Cys-His boxes, and three more basic amino acids clusters.

**Transient assay of p12 mutants upon transfection of chicken embryo fibroblasts.** To test the mutants, the altered proviral sequences were introduced into chicken embryo fibroblasts

by DEAE-dextran-mediated transfection. The medium and the cells were collected between 48 and 60 h after transfection (transient assay) and analyzed by immunoblotting with a mixture of polyclonal antibodies against p12, p19, and p27. Because of the small amount of produced virions, the particles were simply pelleted at high speed. However, a control analysis performed on sucrose gradient-purified mutant virions showed that they had approximately the same density as that of the wild-type virions (data not shown). Intracellular viral proteins were analyzed by immunoprecipitation followed by immunoblotting.

The results showed that all of the mutants synthesized particles containing *gag*-related proteins (Fig. 3). However, it was reproducibly observed that Pr-C 11.3, the duplication mutant, produced two- to fourfold lower levels of viral proteins in the medium than did the other clones after transfection with equal amounts of DNA (Fig. 3, lane j). On the other hand, Pr-C 10.8, the deletion mutant, released at least as many and generally more virions than did the wild-type virus (Fig. 3, lane i).

Analysis of the viral proteins in transfected cells with the anti-p27 serum (Fig. 4) showed that the *gag* precursor proteins and their cleavage intermediates were produced by all of the mutants; the presence of mature p27 was probably due to particles bound to the cell membrane since, in this experiment, adsorbed virus was not removed by trypsinization. The duplication mutant Pr-C 11.3 produced a larger precursor but in lower quantity, possibly because the mutant protein is less stable than the normal one (lane e). The shorter precursor produced by Pr-C 10.8 appeared to be more abundant than the wild-type Pr76 (lane d). This was probably not due to increased stability of the precursor since the amount of cleaved p27 was also higher.

The deleted p12 protein produced by Pr-C 10.8 was not detected on a normal immunoblot with nitrocellulose paper

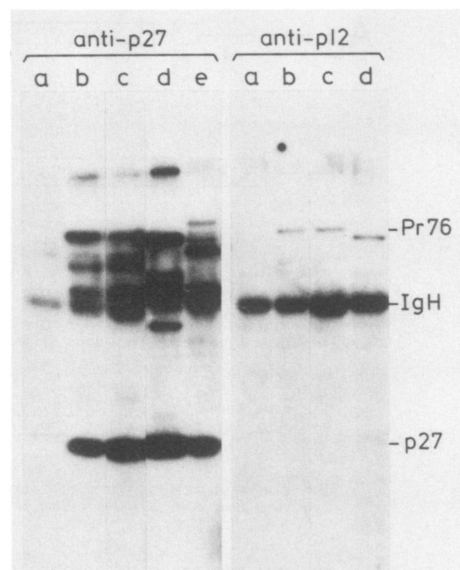


FIG. 4. Intracellular viral proteins after 60-h transient transfection assay. The cell lysates were immunoprecipitated with a polyclonal antibody against p27 followed by protein A-Sepharose adsorption. The eluted proteins were resolved by SDS-PAGE and labeled on an immunoblot with anti-p27 serum or with anti-p12 serum and $^{125}$I-labeled protein A. Lanes: a, controls; b, wild type; c, Pr-C 1; d, Pr-C 10.8; e, Pr-C 11.3. IgH, immunoglobulin heavy chain.

(Fig. 3, lane i). However, it is possible that, because of its small size, the mutant protein p12 was not retained on the nitrocellulose. Therefore, we tried to detect the mutant protein after covalent linkage to cyanogen bromide-activated paper. The serum raised against p12 did not react with any

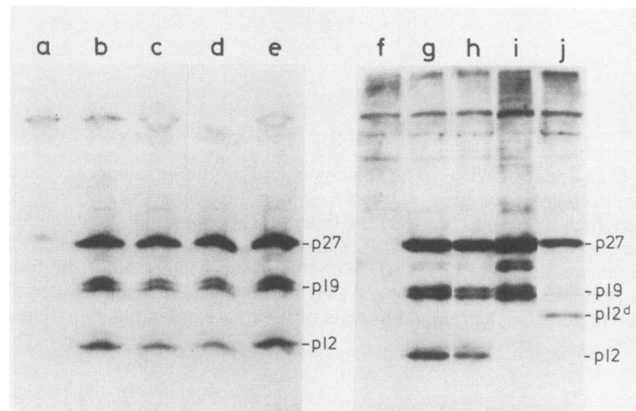


FIG. 3. Virion *gag* proteins produced after transient transfection assay. The cells were transfected in the presence of 200 μg of DEAE-dextran per ml and glycerol shocked. The medium was collected as described in Materials and Methods, centrifuged to eliminate cellular debris, and frozen. The virions were pelleted at high speed and analyzed by SDS-PAGE followed by an immunoblot with $^{125}$I-labeled protein A and polyclonal antibodies against p12, p19, and p27. One-third of the viruses produced by one 100-mm petri dish were sufficient for detecting the viral proteins by overnight exposure. Lanes: a and f, control transfections with no DNA; b and g, transfection with the wild-type plasmid; c, transfection with a reverse transcriptase-defective mutant; d and h, transfection with Pr-C 1; e, transfection with Pr-C 18.2; i, transfection with Pr-C 10.8; j, transfection with Pr-C 11.3. The novel 15-kilodalton p12 protein is indicated as p12$^{d}$.

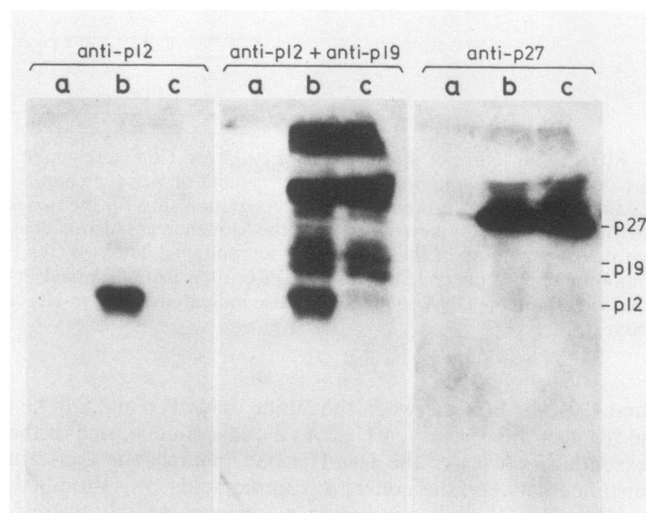


FIG. 5. Immunoblot analysis of wild-type and Pr-C 10.8 *gag* proteins on cyanogen-activated paper. The virion proteins were separated by electrophoresis on a 15% polyacrylamide gel, electrotransferred to cyanogen-activated paper, and detected with polyclonal antibodies and $^{125}$I-labeled protein A. The filter was probed with anti-p12 serum, autoradiographed, and then probed with anti-p19 serum. The same filter was washed as described in Materials and Methods and probed with anti-p27 serum. Lanes: a, control transfection without DNA; b, wild-type plasmid; c, Pr-C 10.8.

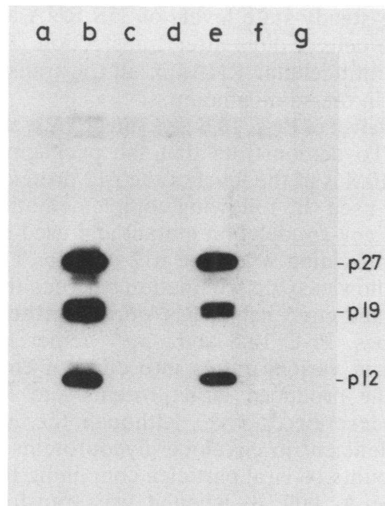


FIG. 6. Infectivity of mutants. The cells were transfected as described in Materials and Methods and split 2 days later. The culture medium was collected 1 week later and processed as described in the legend to Fig. 4. Lanes: a, control transfection; b, wild-type plasmid; c, mutant with a defective reverse transcriptase; d, Pr-C 1; e, Pr-C 18.2; f, Pr-C 10.8; g, Pr-C 11.3.

protein in Pr-C 10.8 particles (Fig. 5, lane c), although p19 and p27 were present in the same amount as that in the wild-type virus. The very dark bands at the top of Fig. 5, specific for the anti-p19 serum, were probably due to cross-reaction of the anti-p19 serum with the viral glycoproteins gp37 and gp85. To verify that the anti-p12 serum could still recognize the mutant p12, total proteins in cells transfected by the wild-type Pr-C strain and the mutants Pr-C 1 and Pr-C 10.8 were analyzed by immunoprecipitation with anti-p27 serum followed by an immunoblot either with anti-p12 or anti-p27 serum. Both sera recognized the *gag* precursor protein (Fig. 4). The anti-p12 serum gave a weaker reaction, but it was identical for the wild-type and mutant precursor proteins. This suggests that the antigenicity of the p12 protein was not affected by the deletion. These observations support the conclusion that the cleaved mutant protein p12 is missing or is reduced to an undetectable level in the viral particles.

As expected, the mutant Pr-C 11.3 produced a p12 protein with a slower mobility than that of the wild-type protein. The novel protein migrated at a position of 15 kilodaltons, consistent with the presence of a 41-amino-acid duplication (Fig. 3, lane j). The p12 proteins produced by the mutants Pr-C 1 and Pr-C 18.2 had the same mobility as that of the wild-type protein (Fig. 3, lanes b, d, g, and h). No aberrant cleavage product was observed in the particles produced. In the transfected cells, as well as in the viral pellets, there were some nonviral proteins that cross-reacted with the antisera (Fig. 3; compare the controls [lanes a and f] with the wild-type Pr-C [lanes b and g]) because of the single purification step used for the preparation of the viruses. The rabbit immunoglobulin heavy chains also reacted on the immunoblot after the immunoprecipitation (Fig. 4), but because of their size, they did not interfere with the assay.

**A fully infectious p12 mutant.** To test for infectivity of the mutants, the cells were passaged 2 days after transfection to allow secondary infections. The release of viruses was analyzed 4 days later when the cells infected by the wild-type virus appeared highly transformed. The viral proteins were detected as described above. Transformed cells and viral proteins were produced in the same amounts by the wild-type virus and by Pr-C 18.2, which has a linker inserted after the second Cys-His box (Fig. 6, lanes b and e). Transfection by the other mutants did not produce any transformed cells or viral particles, indicating that the mutants are replication defective. As a control, a mutant defective for reverse transcription (see Materials and Methods) was used in the same experiment (Fig. 6, lane c). Under the experimental conditions (few passages to avoid possible recombinations with endogenous proviruses or an excision of the inserted linker or duplicated sequences), we did not detect any residual infectivity.

**All mutants contain normal amounts of active reverse transcriptase.** Reverse transcriptase activity was tested with the crude viral pellets of the p12 mutants, the wild-type virus, and the *pol* mutant by using the rapid dot assay as described in Materials and Methods. To quantify the results, the initial polymerization rate (0 to 5 min) was measured and taken as the reverse transcriptase activity of the produced particles. These values were compared to the amount of viral proteins produced in the medium, measured by scanning the autoradiographs of the immunoblots, with the p27 protein as a marker. The results showed reverse transcriptase activity consistent with the amount of particles released in the medium (data not shown). Thus, the defect in the infectivity of the mutants Pr-C 1, Pr-C 10.8, and Pr-C 11.3 could not be attributed to a defect in the synthesis or maturation of the reverse transcriptase.

**Analysis of viral RNA in mutant virions and transfected cells.** Since the mutant protein p12 is an RNA-binding protein and since three of the p12 mutants were noninfectious but produced particles containing normal amounts of reverse transcriptase, it was important to test for the presence of viral RNA in these virions.

RSV full-length RNA exists in the form of a monomer

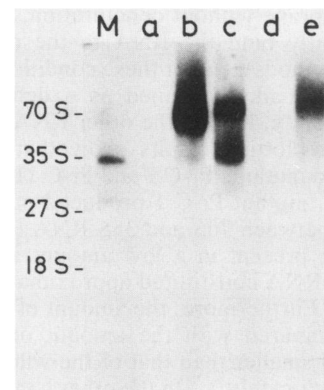


FIG. 7. Viral RNA content of mutant virions. The cells were transfected and the medium was collected as described in Materials and Methods; the medium was then centrifuged for 10 min at 16,000 $\times$ *g* to eliminate cellular debris and stored frozen. After being thawed, the virions were pelleted at high speed, and the RNA was phenol extracted, digested with RNase-free DNase I, size fractionated on a nondenaturing 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with the nick-translated pATV-8 plasmid containing RSV coding sequences. Lanes: M, total cellular RNA from cells transfected with the wild-type plasmid (the positions of the 18S and 27S rRNA were determined by methylene blue staining); a, control transfection without DNA; b, wild-type plasmid; c, Pr-C 1; d, Pr-C 10.8; e, Pr-C 11.3.

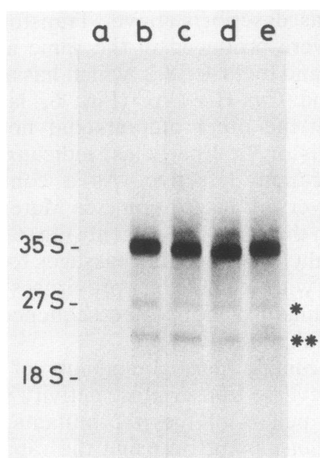


FIG. 8. Steady-state levels of viral RNA transcripts in transfected cells. The cells were transfected and used 60 h later. Total RNA was extracted and analyzed, after size separation in a nondenaturing agarose gel, on a Northern blot with nick-translated pATV-8 as a probe. Methylene blue staining of the filter was used to control the amount of loaded RNA and determine the positions of the 18S and 27S rRNA. The position of the 35S RNA is indicated. The two other RNAs (* and **) are probably *env* and *src* subgenomic mRNAs. Lanes: a, control transfection; b, wild-type plasmid; c, Pr-C 1; d, Pr-C 10.8; e, Pr-C 11.3.

(35S) or a dimer (70S). It is known that the RNA from virus harvested at intervals of 12 h or more carries hidden breaks (4) that are revealed by denaturation before analysis. Thus, the classical Northern technique performed under denaturing conditions cannot be used to detect the conformation of the genomic RNA contained in viral particles. For that purpose we used a recently developed modification of the Northern technique (E. W. Khandjian and C. Méric, Anal. Biochem., in press) based on the separation of the RNA on a nondenaturing agarose-TBE gel, electrophoretic transfer on a nylon membrane without denaturation, and UV irradiation to covalently bind the RNA to the membrane (see Materials and Methods). Under these conditions, a 70S RNA carrying hidden breaks remained as a defined band and hybridized efficiently, like all the other RNAs tested.

The Northern blotting results show that the wild-type virus and the two mutants Pr-C 1 and Pr-C 11.3 contain viral RNA. However, mutant Pr-C 1 produced particles with an abnormal ratio between 70S and 35S RNA (Fig. 7, lane c); instead of being present in a low amount as in wild-type virions, the 35S RNA constituted approximately 50% of the packaged RNA. Furthermore, the amount of packaged viral RNA, when compared with the amount of proteins produced, appeared smaller than that of the wild-type virus by a factor of approximately 2. On the other hand, mutant Pr-C 11.3 viral particles contained apparently normal 70S viral RNA (Fig. 7, lane e) and had an approximately normal RNA-to-protein ratio. Small differences in the electrophoretic mobilities of the packaged mutant RNAs, which could not be easily explained by their sizes, were also observed (Fig. 7, 70S RNA in lanes c and e). Mutant Pr-C 10.8, which encodes an internally deleted p12 protein, produced particles without viral RNA (Fig. 7, lane d).

To analyze the intracellular viral RNA, total cellular RNA was extracted from trypsinized cells (most of the viruses bound outside to the cell membrane are eliminated by trypsinization at 37°C) and analyzed as described above to determine the steady-state levels of 35S RNA and 25S *env* mRNA in the cells 2 days after transfection (Fig. 8). The viral-specific intracellular RNAs in all the transfected cells were present in the same amounts.

**Packaging defect of Pr-C 10.8 genomic RNA is at the level of p12 protein.** To demonstrate that the packaging defect of mutant Pr-C 10.8 is at the level of the p12 protein and not of the RNA, we used the following complementation test. We constructed a *env-src* deletion mutant and used it as a $gag^+$ helper virus providing wild-type p12 protein. This deletion was done to allow easy discrimination between the Pr-C 10.8 mutant RNA and $gag^+$ helper RNA on a Northern blot with different probes. Pr-C 10.8 and $gag^+$ helper DNAs were cotransfected in various ratios into chicken embryo fibroblasts, and the produced viral proteins and RNAs were analyzed as described above. Although the $gag^+$ helper mutant was deficient in envelope glycoproteins, it did produce low amounts of viral particles containing RNA dimers (Fig. 9, probe A, lane d) when it was transfected alone. However, the presence of a functional *env* product appeared important for the production of viral particles since the amount of the helper-specific p12 protein released in the medium increased when the *env* mutant was complemented with the Pr-C 10.8 p12-defective mutant (data not shown). An *env-src* specific probe allowed us to detect Pr-C 10.8 RNA packaged in hybrid particles (Fig. 9, probe B, lanes e and f), although it appeared either poorly structured or partially degraded (Fig. 9, probe B, lane e). With this probe, no hybridization signal could be detected with the deleted $gag^+$ helper alone (Fig. 9, probe B, lane d).

## DISCUSSION

The aim of this work was to create various RSV mutants with altered protein p12 and to compare their properties with those of the wild-type virus to determine the role of this nucleic acid-binding protein. We constructed mutants in this region, prompted by the observation that a pattern of

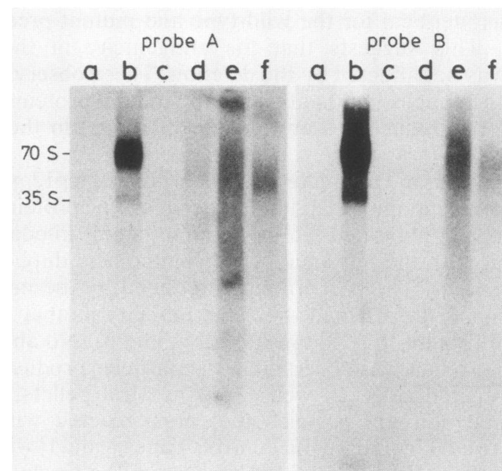


FIG. 9. Complementation of packaging defect of Pr-C 10.8 by wild-type p12 protein. The cells were transfected with the various plasmids, and the culture medium was collected 60 h later. The RNA contained in the virions was analyzed as described in the legend to Fig. 7. Lanes: a, control transfection; b, wild-type plasmid; c, Pr-C 10.8; d, p12 helper plasmid deleted in the *env* and *src* genes; e, Pr-C 10.8 and helper plasmid in a 1:1 ratio; f, Pr-C 10.8 and helper plasmid in a 2:1 ratio. Probe A, nick-translated pATV-8 plasmid; probe B, nick-translated *Eco*RI-*Mlu*I fragment specific for Pr-C 10.8 and wild-type virus RNA.

cysteines and histidines is conserved in the nucleic acid-binding proteins of all known retroviruses (7). To assay these mutants, we extended the use of the transient transfection assay to the study of retroviral mutant proteins. This assay was used because it has not been possible to obtain chicken cells permanently transfected with replication-defective mutants (6). This limitation does not exist in the murine system, in which stably transfected cell lines have been used to study the functions of various murine leukemia virus proteins (9, 15, 31).

The mutations that we produced in the RSV *gag* protein p12 had surprisingly little effect on the release of viral particles. All of the mutants tested yielded viral particles containing the mature *gag* proteins and a wild-type level of reverse transcriptase activity. The duplication mutant Pr-C 11.3 produced two to four times less viral protein in the medium than did the wild-type virus. Analysis of the intracellular viral protein indicated that the larger *gag* precursor protein produced by this mutant is less abundant than the wild type precursor. Since the amount of 35S mRNA in the cells transfected with this mutant was not affected, the cause of the low viral protein synthesis was probably the instability of the mutant precursor. The absence of any detectable polypeptide derived from p12 in mature Pr-C 10.8 mutant viruses suggests that this protein does not play a role either in precursor cleavage or in the budding or assembly of the virion proteins.

The reverse transcriptase activities measured in the particles correlated well with the amount of *gag* proteins, indicating that the *gag-pol* precursor is synthesized and cleaved correctly in the mutants, whether or not viral RNA is present. The same result has been obtained with other RNA packaging-defective mutants (17, 21).

An intriguing aspect of retrovirus physiology is the need for diploid RNA (5). Although a role for the dimer during reverse transcription has been postulated, there is no genetic evidence to support this hypothesis. The RNA sequences involved in the formation of the dimer have also been implicated in RNA packaging (29). However, the biological test used in these experiments could not discriminate between a role for the dimer in packaging and a later role during reverse transcription. However, it is likely that the formation of the dimer requires a protein as a cofactor since (i) a dimer was not obtained in vitro from 35S RNA, (ii) the formation of the dimer is tightly coupled to budding and is stabilized during the first minutes of the existence of the free particle (35), and (iii) an effect of the mutations on dimer formation at the RNA level is unlikely because Pr-C 18.2, which has the same linker inserted as does Pr-C 1, shows a wild-type dimer structure on nondenaturing agarose gels. The results obtained with one of the mutants, Pr-C 1, clearly show that p12 plays a role as a cofactor in dimer formation. The fact that the ratio between 35S and 70S RNA was not absolutely constant from one experiment to another suggests that the particles may contain a labile dimer structure which was partially destroyed during the extraction procedure. In wild-type particles such a labile structure is present during the first minutes after budding and is then matured into stable 70S RNA (35). This suggests that the p12 mutant Pr-C 1 is no longer able to mature its genomic RNA.

One of the most interesting aspects of p12 mutagenesis is the investigation of the function of the Cys-His box. This box is found in all retroviral nucleic acid-binding proteins and also in coding sequences of *Drosophila* copia transposable elements and, in a slightly different form, in the T4 gene 32 product. These proteins have various properties in common: (i) they bind single-stranded nucleic acids (19, 20, 34, 36, 40), (ii) they have nucleic acid-unwinding features (36, 40), and (iii) they are involved in DNA synthesis, either from DNA (40) or RNA. However, for the retroviruses, the last function has been observed only in vitro (36). The importance of this sequence was experimentally demonstrated in this study by the fact that three of the four mutants with modifications in this conserved region were not infectious. The fourth mutant, modified in another part of the protein, was infectious. In mutant Pr-C 1, the dipeptide Val-Pro is inserted near the middle of the first box, between the second cysteine and the histidine; this results in the modification of the relative positions of the conserved residues. The second box in the protein is unaffected. A tempered effect of the mutation was expected since the murine and feline nucleic acid-binding proteins have only one Cys-His box. Indeed, this mutation has no dramatic effect on the amount of packaged viral RNA, but the function responsible for the formation of the 70S dimer is partially defective. However, the boxes do not appear to have a simple additive effect since another mutant (Pr-C 11.3) with a 43-amino-acid duplication resulting in three intact boxes produced particles containing apparently normal 70S RNA but was still not infectious.

It is of course difficult to deduce the role of the Cys-His boxes from the phenotypes of only two mutants. Nevertheless, the boxes seem to be implicated in the formation of the 70S dimer, probably through the RNA-binding and -unwinding activities of the protein, properties well characterized in vitro (19, 20, 34, 36). Protein p12 and its Cys-His boxes must also play another role essential during infection, probably during reverse transcription, since the duplication mutant Pr-C 11.3, which contains apparently normal 70S RNA and reverse transcriptase is nevertheless replication defective. Mutations most probably affect single-stranded nucleic acid-binding and -unwinding properties by inducing conformational changes in the protein.

In mutant Pr-C 10.8, p12 has lost 39 of 89 amino acids making up the two boxes and many basic amino acid residues. As expected, the particles produced do not contain detectable viral RNA. Such a phenotype could be due to a packaging defect either at the protein or RNA level. However, no packaging signal could be found at the RNA level near the end of the *gag* gene (29), and when the defective p12 protein of Pr-C 10.8 was complemented by normal p12, the mutant RNA was packaged into particles. This shows that it is the alteration in the protein and not the deletion of the RNA sequence which is responsible for the packaging-defective phenotype. The fact that the viral RNA is absent does not prove that p12 is responsible for its selection but only for its packaging into the particles.

The remaining 50 amino acids of the Pr-C 10.8 deleted p12 protein could not be detected either as a small polypeptide or as a collection of partially processed molecules. It is unlikely that the deleted p12 protein has lost its antigenicity, since the anti-p12 serum reacted with the uncleaved Pr-C 10.8 *gag* precursor as strongly as with the wild type. This suggests that if the deleted p12 protein is present in the viral particles, it is in reduced amounts not detectable with our technique or as small cleaved peptides. If the deleted protein is not found in the viral core, p12 is probably cleaved before organization of the core as a closed structure. However, it has been shown that the cleavage of the other *gag* proteins is not a prerequisite to particle formation and budding (39). As a consequence, we still do not know whether the RNA is primarily bound by Pr60 or Pr66 (a cleavage intermediate lacking p15) (38) or by the matured p12. A mutant without

p15 but with a normal p12 sequence might provide a partial answer to this question. The construction of such a mutant is under way.

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