JOURNAL OF VIROLOGY, July 1993, p. 3826–3834
0022-538X/93/073826-09$02.00/0

Vol. 67, No. 7

Analysis of Deletions and Thermosensitive Mutations in Rous Sarcoma Virus *gag* Protein p10

PHILIPPE DUPRAZ* AND PIERRE-FRANÇOIS SPAHR

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

Received 16 September 1992/Accepted 25 March 1993

Rous sarcoma virus protein p10 is a *gag* component of the virion present in stoichiometric amount but of unknown function. To characterize this protein, a series of mutants of p10 with linker insertions or deletions was generated by site-directed mutagenesis of a cloned proviral DNA. The deletions and two of the linkers insertions, which disrupted proline pairs, reduced the yield of virus particles upon transfection. These two linker insertion mutants were moreover thermosensitive for this phenotype, producing fewer virus particles at 41°C than at 36°C. Examination of the intracellular viral proteins demonstrated that for all mutants, the amount of *gag* precursor was similar to the wild-type level. Moreover, the amount of mature *gag* CA that could be detected by this analysis was similar between each of the mutants and the wild type. This finding suggests that the transport of *gag* to the membrane and the initial stages of maturation were not affected by the mutations. The virus particles contained normal amounts of active reverse transcriptase, showing that the *gag-pol* polyprotein was incorporated and cleaved properly. Viral RNA was quantitatively and qualitatively similar in mutant and wild-type virions. However, the infectivity of the mutants virions differed; one of the thermosensitive linker insertions that had no effect on particle production at 36°C was nevertheless noninfectious at that temperature. Together, these data suggest that the p10 protein is involved in a late steps of virus maturation, possibly budding, and perhaps also in an early event of viral infection.

All retroviruses express a gene encoding their structural proteins (*gag* gene). In the case of Rous sarcoma virus (RSV), *gag* is expressed by translation of the genomic 35S mRNA in the cytoplasm of the infected cell. The product is a polyprotein precursor of 76 kDa ($Pr76^{gag}$) that migrates to the plasma membrane of the host cell, where it is incorporated into growing virus particle and probably cleaved during or just after budding into mature proteins. These proteins, beginning from the N terminus of the precursor, are MA, the matrix-associated protein; p2, a protein of unknown function; p10, also of unknown function; CA, the major structural component of the capsid; p1, of unknown function; NC, the nucleocapsid protein; and PR, the protease responsible for the stepwise proteolytic cleavage of the *gag* precursor (for a review, see reference 37).

Sequencing of the RSV Prague C (PrC) strain genome as well as amino acid sequence information revealed that in $Pr76^{gag}$ between p19 (MA) and p27 (CA) was the coding sequence for a protein of 62 amino acids with a predicted molecular weight of 6,800 (26). This protein (p10) was purified and sequenced from both avian myeloblastosis virus and RSV PrC virions, and its amino acid sequence was found to be consistent with that predicted from the genome of RSV PrC (9, 26). Later, it was found that an additional small peptide (p2) is present between MA and p10 (23, 35). The localization of protein p10 within the virion has not been determined but has been suggested to be between the envelope and the core (24). The protein is very unusual in its amino acid composition. It contains a large proportion of proline (12%) and glycine (20%) residues but neither lysine nor cysteine residues. In fact, metabolic labeling with [^{14}C] glycine was shown to preferentially label p10, and this technique was used to purify p10 (24). In sodium dodecyl sulfate (SDS)-gels, p10 migrates abnormally because of its composition (it migrates like a 12- to 15-kDa protein); the migration is even more bizarre in SDS-urea gels, with an apparent molecular size weight of 40 kDa (24). Apart from these biochemical properties, little is known about p10, including its function.

To investigate the function of this *gag* gene product in the RSV replication cycle, we have generated a series of deletion and linker insertion mutation of the p10 region of a cloned DNA copy of RSV PrC. These mutant DNAs were transfected into chicken embryo fibroblast cells (CEFs), and the effects of the mutations on viral proteins synthesis and virion assembly were determined biochemically and biologically. Characterization of the mutant virions produced after transient transfection suggested that p10 deletions and thermosensitive (*ts*) linker insertions reduced the yield of RSV virions and that these virions were defective in some other aspect, since they could not productively infect fresh CEFs. This analysis suggests a role for p10 in the budding processes and in an early step of infection.

MATERIALS AND METHODS

Cell culture. CEFs were prepared from virus-free embryonated eggs (Gs^- and Chf^-; SPAFAS, Inc., Norwich, Conn.) and grown in Dulbecco modified Eagle medium containing 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) at 35.5 or 41°C in an atmosphere supplemented with 5% carbon dioxide.

Bacterial strains. *Escherichia coli* DH5α (*dut*$^+$ *ung*$^+$ $RecA^-$) and CJ-236 (*dut ung*) were grown according to the instructions included with the mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). *E. coli* DH5α was rendered competent for transformation as previously described (25). Plasmid DNAs were purified from either small- or large-scale cultures by the alkaline lysis method and further purified for

* Corresponding author.

transfection either by density gradient centrifugation in cesium chloride-ethidium bromide or by polyethylene glycol precipitation (25).

Cloned DNAs. Plasmid pAPrc has already been described (19). It contains a nonpermutated copy of the provirus RSV PrC. Plasmid pAsPrc is a *Sal*I-*Eco*RV subclone of pAPrc in pBR322 containing the entire *gag* sequence (19). All mutations were constructed in pBS*gag*R1, a 2,395-bp *Eco*RI-*Eco*RI fragment cloned in the phagemid vector pBS(+) (Stratagene, San Diego, Calif.). For the RNase protection assay, plasmid pBSLSD was constructed as follows: an *Eco*RI-*Bam*HI fragment (nucleotides [nt] 9238 to 532 of pAPrc) was cloned into pBS(+) such that transcription under the control of the T3 promoter results in the synthesis of an antisense RNA (this construct is analogous to the previously described plasmid pAT-8K, in which the same RSV-specific sequence was cloned into pGEM-2 [1]).

Site-directed mutagenesis. The following oligonucleotides (with the restriction sites in bold and the mutated nucleotides underlined) were synthesized on a 381A DNA synthesizer (Applied Biosystems) and purified as previously described (14) or by high-pressure liquid chromatography. The oligonucleotides used were as follows: ML1 (3′-CCTCGAACGC TCA**TGCGCA**GGCGGGC-5′), ML2 (3′-CCCCCCACTGTG TGGA**TGCGCA**GGCCCCCGCC-5′), ML3 (3′-GGGACCG C**TGCGCA**CCCCACCC-5′), ML4 (3′-CCGGGACTGACTG ACCCGT**TGCGCA**TCCCAGTCCC-5′), and ML5 (3′-CCC GCCTCGGT**TGCGCA**CCCGTACGCCC-5′).

A modification of the selection method of Kunkel (13) was used. Briefly, a single-stranded uracil-containing DNA template was obtained by introducing the phagemid pBS*gag*R1 into *E. coli* CJ-236 and by coinfection with helper phage M13K07 (Promega) as described by Vieira and Messing (34). The mutagenic strand was synthesized according to the mutagenesis kit instructions (Bio-Rad), the resulting double-stranded DNA was introduced into *E. coli* DH5α by the calcium chloride transformation protocol, and transformants were selected by growth on Luria-Bertani–ampicillin (100 μg/ml) plates (25). The introduction of all mutations was confirmed by dideoxy-chain termination DNA sequencing of plasmid DNA (38), using T7 DNA polymerase (Pharmacia).

Transfection and infectivity. CEFs, either freshly prepared or frozen in 15% glycerol, were used after two to seven passages. DNA transfection and virus infection were performed as described previously (18, 19). The medium was changed every 24 h and harvested between 2 and 3 days posttransfection or between 2 and 9 days postinfection.

Exogenous template reverse transcriptase assay. The exogenous template reverse transcriptase assay was performed on crude viral pellets (see below) by the method of Goff et al. (6b). The viral pellets resuspended in NTE (100 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA), corresponding to 1 plate-day of the production of one 100-mm-diameter petri dish in a transient assay, were lysed in 50 μl 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 mg of oligo(dT) per ml, 10 mg of poly(A) per ml, and 50 μCi of [α-^{32}P]dTTP (3,000 Ci/mmol) and incubated at 37°C for 1 h. Thereafter, 10 μl of the reaction mixture was spotted on dry DEAE-paper (DE81; 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) or exposed.

Protein analysis. Viral particles produced by the transfected or infected cells were purified by ultracentrifugation through a 20% sucrose cushion, and their protein content was analyzed by immunoblotting with polyclonal antibodies against RSV NC (p12), MA (p19), and CA (p27) as described previously (19). Intracellular viral proteins, produced after transfection, were analyzed by immunoprecipitation and immunoblotting with antibodies against RSV CA as previously described (19).

Purification of viral RNAs. The viral particles produced after transfection were purified by centrifugation through a sucrose cushion, and the RNA was extracted by lysis of the particles with SDS-proteinase K as described previously (5).

To quantitate the amount of RSV-specific RNA encapsidated by the virus after transfection, RNase protection assays were performed. Plasmid pBSLSD was digested with *Bst*EII and in vitro transcribed by using T3 polymerase and a commercial kit (Promega) according to the kit instructions. Virion-associated RNA was isolated as for Northern (RNA) analysis. The RNase protection was performed as previously described (25), the nuclease-resistant hybrid was analyzed on a denaturing polyacrylamide gel, and the product was detected by autoradiography. Transcription results in a 469-nt probe which after hybridization protects either a 429-nt (unspliced genomic RNA) or 293-nt (spliced RNA) fragment. The strength of the signal detected was determined by densitometric scanning of the autoradiograph.

RESULTS

Construction of mutation in RSV protein p10. We chose to insert linkers for the enzyme *Mlu*I in the coding region of p10 for the following reasons: first, *Mlu*I cuts only once the wild-type genome of RSV PrC (at nt 7901) and was thus practical for cloning purposes; second, the *Mlu*I recognition sequence introduces a basic Thr-Arg dipeptide, which would be predicted to affect significantly p10 since it is quite hydrophobic in nature (24). It should be noted that the insertion of linker ML2 disrupts a pair of proline residues and that mutation ML1 is also adjacent to two prolines (Fig. 1A). Proline residues are known to interrupt β sheets or α helices in protein structures (16); thus, we expected that these mutants would have an interesting phenotype. Mutant ML1 is a substitution of Gly-55 for Arg, but the mutation creates an *Mlu*I restriction site. By in vitro recombination between plasmids possessing the *Mlu*I sites, it was possible to make a series of deletions (Fig. 1B). Deletion D13 removes most of p10 (47 amino acids deleted) from nt 934 to 1075. The other deletions were designed to encompass either mostly hydrophobic regions (D23, 13 amino acids deleted; D45, 15 amino acid deleted) or more hydrophilic regions of p10 (D25, 10 amino acid deleted; D14, 9 amino acid deleted). After being cloned back into our full-length RSV PrC DNA clone (pAPrC) (19), all of the mutations were confirmed by DNA sequencing to ensure conservation of the reading frame and the correct mutagenesis, as described in Materials and Methods.

Deletions were not introduced nearer the N terminus of p10 because we wanted to avoid possible inhibition of Pr76^{gag} cleavage by PR at the p2-p10 junction. For similar reasons, we have left intact the last six amino acids at the C terminus, which might be important for PR recognition. Moreover, two regulatory elements have been described in the p10 region near its N terminus: an enhancer of long terminal repeat activity (2) and a negative regulator of splicing (1). The core sequence of the negative regulator of splicing ends approximately at nt 930 (17); the *gag* enhancer is less clearly defined, but strong activity is conferred by a fragment between nt 776 and 952 (Fig. 1B).

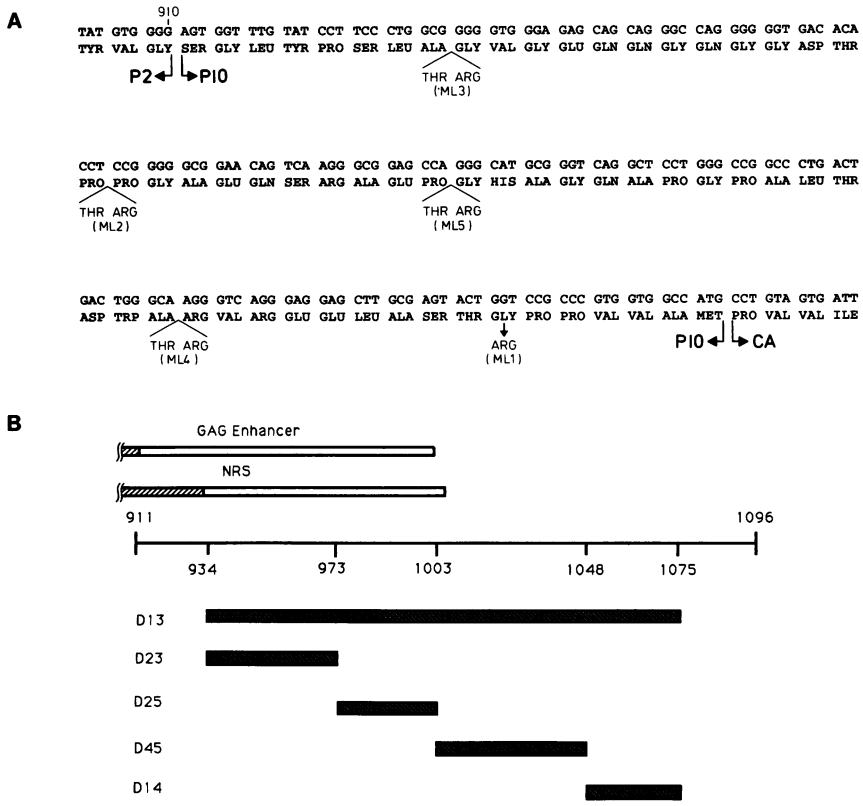

FIG. 1. (A) DNA and amino acid sequences of the p10 protein (26), with the 6-mer *Mlu*I linker insertions indicated. Protein p10 is located between p2 and CA in the RSV Pr76^{gag} polyprotein (23, 35). (B) Schematic representation of p10, with the positions of the linker insertions (at 934, 973, 1003, and 1048) and of the single amino acid substitution ML1 at 1075 indicated. The deletions are shown as shaded boxes and were obtained by in vitro recombination of the individual linker insertion mutants. The negative regulator of splicing (NRS) and *gag* enhancer element described are represented above; the hatched region represents the possible core sequences of these elements, but they extend further upstream into the MA-p2 region of the *gag* gene (1, 2).

Analysis of virion proteins. Viral mutant DNAs were transfected into CEFs by the DEAE-dextran protocol; after 24 h to 48 h, viral particles were purified and analyzed by immunoblotting with polyclonal anti-CA, anti-NC, or anti-MA sera as described in Materials and Methods. We first transfected our mutants at 36 to 37°C, which is the normal temperature for avian retroviruses. The results are shown in Fig. 2A and B and Table 1. The mutants with linker insertions (ML1 to ML5) did not show any significant differences in the amount of virus protein released in the medium compared with the wild type. The MA and CA proteins appeared to be fully processed. This finding suggests that cleavage between MA, p2, and p10 was not inhibited by the ML3 dipeptide insertion and that PR still recognized the p10-CA junction in mutant ML1. Thus, it is likely that there is no inhibition of Pr76^{gag} precursor processing in these five mutants.

The deletion mutants produced fewer virus particles than did the wild-type virus (Fig. 2A and B; Table 1); very weak signals for CA and MA could be detected for these mutant upon prolonged exposure of the blot (data not shown).

We repeated this test at a higher temperature to determine whether some of the linker insertions could lead to conditional mutants. Upon transfection at 41°C (nonpermissive temperature for avian retroviruses), the deletion mutations showed a phenotype similar to that at the permissive temperature (Fig. 2; Table 1). Processed CA was quantified by densitometric scanning of different autoradiographs obtained from the analysis of transfections at 41°C (some variation was observed between transfections). Deletion mutant D14 showed a 20- to 30-fold decrease in the amount of virus released in the medium in comparison with the wild type, while D13, D23, D25, and D45 produced 30- to 50-fold fewer virus particles. CA was fully processed in these mutants (Fig. 2C), suggesting that the proteolytic cleavage of the *gag* polyprotein precursor was not impaired by the deletions in p10.

In contrast, a dramatic decrease in the number of virions released into the medium was observed when the transfection was performed at the nonpermissive temperature for two of the linker insertion mutants. ML1 and ML2 showed a large reduction in the amount of virus produced at 41°C compared with the wild type (2.5 to 5% for ML1 and 1 to 2% for ML2). Thus, p10 mutants ML1 and ML2 are thermosensitive for particle release.

Linker insertions mutants ML3, ML4, and ML5 showed no difference in phenotype at 36 or 41°C; the differences in NC protein level detected is not specific for these mutants but is due to blotting problems, as NC does not adhere well to nitrocellulose membrane (these three mutants are fully infectious at both temperatures [see below]).

Synthesis of Pr76^{gag} precursor polyprotein in transfected cells. Two possible explanations for the phenotype of the deletion and *ts* linker insertion mutants are that they do not

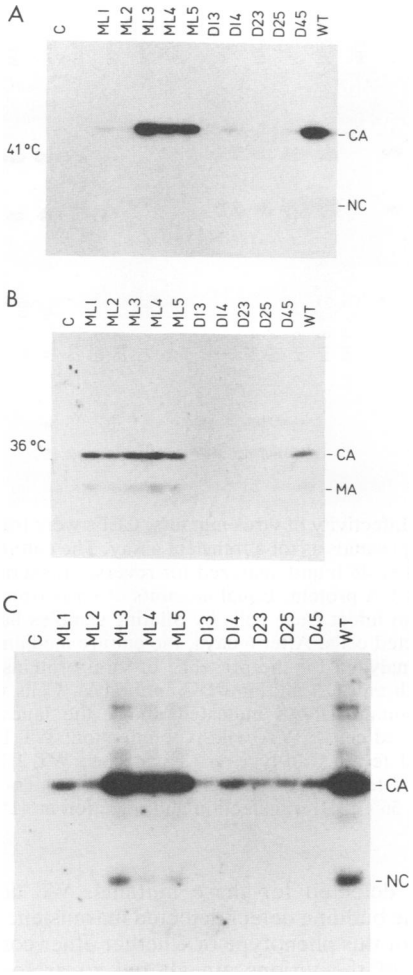

FIG. 2. Analysis of the virion *gag*-encoded proteins. Virions produced by the transfected cells at 36 or 41°C were purified as described in Materials and Methods. Viral proteins were resolved by SDS-polyacrylamide gel electrophoresis and then immunoblotted with polyclonal antibodies against RSV CA and NC for (A) or RSV CA and MA (B) and ^{125}I-labeled protein A. Cells were transfected with the mutant DNAs indicated above the lanes as described in Materials and Methods. C, control (cells transfected with no DNA); WT, wild-type DNA transfection. Panel C is a longer exposure of the blots shown in panel A.

synthesize Pr76^{gag} in sufficient amounts and that Pr76^{gag} is trapped in cell cytoplasm. To test these hypotheses, we transfected our mutants DNAs as described above at the nonpermissive temperature; after 48 h, the cells from two 100-mm-diameter petri dishes were lysed, and the *gag* precursor was immunoprecipitated from the cell lysate with a polyclonal antibody against CA. Eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western immunoblotting with the anti-CA antibody. We have detected an antigenically related protein in untransfected cells with a size slightly less than that of Pr76^{gag}. This band may be that of an endogenous *gag* protein that is incompetent for budding or a nonspecific serum protein, as it never interfered negatively or positively with our mutants during weeks of wild-type or mutant infections. All mutants synthesized *gag*-related proteins in amounts comparable to those of the wild-type protein (Fig. 3A).

TABLE 1. Structures and biological activities of mutants of RSV p10 protein[a]

Protein[b]	Deleted bases or position of linker insertion[c]	Virion produced at[d]:		RT released from infected CEFs at[e]:	
		36°C	41°C	36°C	41°C
Prc6 (wt)		+	+	+++	+++
*ts*ML1[f]	1075	+	−	+++	−
*ts*ML2	973	+	−	+/−	−
ML3	934	+	+	+++	+++
ML4	1048	+	+	+++	+++
ML5	1003	+	+	+++	+++
D13	934–1075	−	−	−	−
D14	1048–1075	−	−	−	−
D23	934–973	−	−	+	−
D25	973–1003	−	−	−	−
D45	1003–1048	−	−	++	−

[a] For all mutants transiently transfected in CEFs, the relative reverse transcriptase activity, determined as the amount of reverse transcriptase activity released (in counts per minute) versus CA protein level (in densitometric units) detected by scanning densitometry of the protein immunoblot at 41°C, was 100% of the wild-type level (48 h after transfection).

[b] ML1 to ML5, linker insertions; D13 to D45, deletions.

[c] Numbered from the sequence of RSV PrcC (26).

[d] +, wild-type level; −, less than 5% of wild-type level.

[e] Reverse transcriptase (RT) activity as a proportion of the wild-type level. +++, 100%; ++, 20 to 50%; +, 1 to 5%; +/−, low and sometime not above background; −, not detected.

[f] A single-amino-acid substitution (Gly-55→Arg) that introduced an *Mlu*I restriction site.

Deletion mutant D13 synthesized a Pr76^{gag} protein whose mobility was significantly faster than that of the wild type but whose size is consistent with the deletion of 47 amino acids, migrating at about 70 to 72 kDa. In the case of mutants D14 and D23, mobility was also increased but appeared to be greater than would be predicted from the 9- and 13-amino-acid deletions, respectively. The novel *gag* precursors of mutants D25 and D45 migrated approximately at the same position as did the wild-type Pr76^{gag}. Mutants ML1 and ML2 synthesized a precursor with a mobility similar to that of the wild type (mutants ML3, ML4, and ML5 were not studied because they displayed no alteration in particle formation). With a longer exposure of this blot, processed CA protein was detected for all mutants, with some variations in the amount compared with the wild type (Fig. 3B). However, these variations were not reproducible between transfections. From an average of 10 different transfections, the amount of processed CA protein detected in these protocols is not affected in the mutants. Thus, the observed differences in virus particle release are not due to precursor accumulation in the cell cytoplasm or precursor instability. Furthermore, it appears but needs to be confirmed that the precursor is transported to the plasma membrane correctly, since the interactions between *gag* precursors leading to particle maturation and precursor cleavage (which occurs only during or after budding) is not different from the wild-type interactions in this analysis.

Infectivity of mutant viruses. To determine the infectivity of the mutants, virus produced 48 h after transfection of CEFs with the mutant plasmids was collected and used to infect fresh CEFs. Equal numbers of virions were used for the infection with each mutant, as determined by the level of CA and reverse transcriptase activity in the medium. After 6 days, the culture medium above the infected cells was analyzed for viral particle production by immunoblotting or

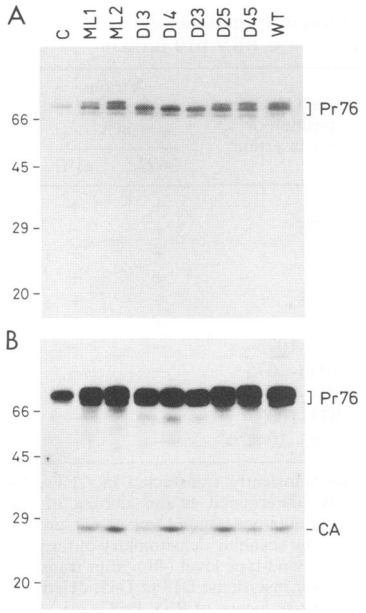

FIG. 3. Intracellular viral proteins of cells transfected with viral mutants. Cell lysates were immunoprecipitated with a polyclonal antibody against CA (p27) and then subjected to protein A-Sepharose adsorption. The eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting with anti-CA (p27) serum and ^{125}I-labeled protein A. Cells were transfected with the mutant DNAs indicated above the lanes. C, control (cells transfected with no DNA); WT, wild-type DNA transfection. Panels B represent a longer exposure of the blot shown in panel A. Sizes are indicated in kilodaltons.

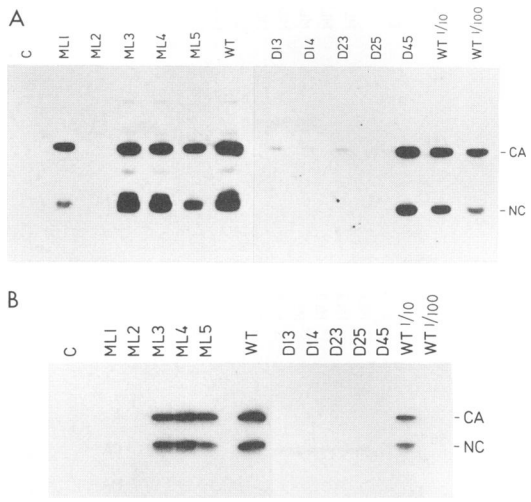

FIG. 4. Infectivity of virus mutants. CEFs were transfected with the mutant plasmids as for a transient assay. The culture media were collected after 48 h and analyzed for reverse transcriptase activity and level of CA protein. Equal amounts of virus from each mutant were used to infect fresh cells by diluting samples harvested from the transfected cells. After 6 days, the culture medium above these cells was analyzed for the presence of viral proteins by immunoblotting with anti-CA and anti-NC antibodies. Cells were infected with the mutant DNAs indicated above the lanes. C, control (mock-infected cells); WT, wild-type infection; WT 1/10, infection with 10-fold fewer wild-type virus particles; WT 1/100, infection with 100-fold fewer wild-type virus particles. (A) Transfection and infection at 36°C; (B) transfection and infection at 41°C.

by measuring reverse transcriptase activity in the medium. In the first set of infections, particles produced at 36°C were used to infect CEFs at 36°C. Four linker insertion mutants (ML1, ML3, ML4, and ML5) and deletion mutant D45 were similar in infectivity to the wild type (Fig. 4A and 5; Table 1). The *ts* mutant ML2 released very few virus particles (Fig. 4A), and the reverse transcriptase activity was more than 100-fold lower (Fig. 5; Table 1). Cells infected with deletion mutant D23 produced few virions (Fig. 4A and 5); cells infected with mutants D13, D14, and D25 appeared to release some particles (Fig. 4A), but no virion-associated reverse transcriptase activity could be detected (Fig. 5; Table 1).

In a second set of infections, virus particles produced by transfection at 41°C were used to infect fresh CEFs maintained at 41°C during the whole infection time (6 days). As above, the infection was standardized for the number of particles before infection. Analysis of the medium for virion-associated mature CA and NC proteins revealed that the *ts* mutants ML1 and ML2 were noninfectious whereas mutants ML3, ML4, and ML5 were similar in infectivity to the wild type (Fig. 4B). Cells infected with the five deletion mutants did not release any virus into the medium (Fig. 4B). For all mutants, the results were confirmed by measuring the reverse transcriptase activity in the medium above these cells (Fig. 5; Table 1).

From these results, it appears that the virions produced by ML3, ML4, and ML5 are wild type in phenotype. The virion produced by mutants D13, D14, D23, and D25 were defective in some way so as to either reduce strongly or abolish their infectivity in CEFs. We have no clues to the block of infectivity detected for these mutants. We do not know whether the budding defect detected in transient transfection accounts for this phenotype or whether other components or structures of the virions are altered so as to lower their infectivity (see below). Mutants *ts*ML1 and *ts*ML2 were variously affected by temperature; *ts*ML1 was fully infectious at 36°C and noninfectious at 41°C, whereas *ts* ML2 showed a strong reduction in infectivity at both temperatures. D45 was more infectious at 36°C than at 41°C and might also be somehow thermosensitive.

Reverse transcriptase activity of the virions produced by transient transfection. The lack of infectivity described for most of our mutants could be explained if during virus assembly the *gag-pol* precursor was excluded from virions

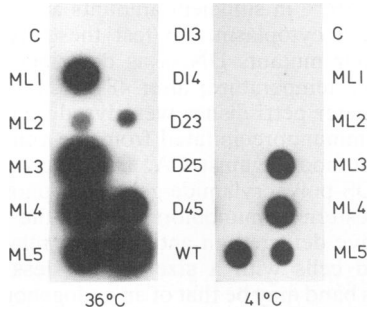

FIG. 5. Infectivity assay. Infections were performed as described in the legend to Fig. 4; 6 days after infection, reverse transcriptase activity was measured in the supernatant medium by the dot blot procedure as described in Materials and Methods. Nomenclature is as described in the legend to Fig. 4.

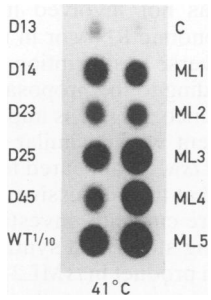

FIG. 6. Exogenous reverse transcriptase assays on particle released by cells transfected at 41°C. Virus particles were purified by centrifugation through a sucrose cushion, and the crude viral pellet was resuspended in NTE. An aliquot was analyzed by Western blotting to determine the quantity of CA present; the rest was used in the assay as described in Materials and Methods. Cells were transfected with the mutant DNAs indicated above the lanes. C, control (cells transfected with no DNA); WT1/10, 10-fold dilution of a wild-type DNA transfection. Relative amounts of CA protein (in densitometric units) loaded: C, 0; ML1, 2.5; ML2, 2.4; ML3, 49.6; ML4, 34.3; ML5, 35.3; D13, 1.2; D14, 3.4; D23, 1.2; D25, 2.8, D45, 2.7; WT1/10, 5.2.

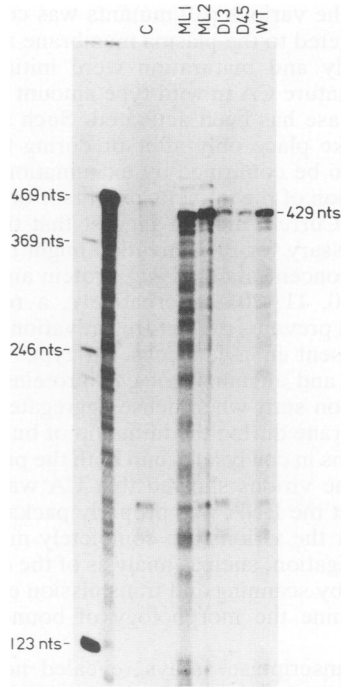

FIG. 7. Viral RNA content of virions produced after transient transfection at 36°C. The virions were purified, and the viral RNA was extracted as described in Materials and Methods. The RNA was analyzed by RNase protection assay as described previously (25) with a 469-nt probe complementary to the leader and the beginning of the *gag* gene, encompassing the splice donor site. After hybridization, the 35S genomic RNA protects a fragment of 429 nt. Cells were transfected with the mutant DNAs indicated above the lanes. C, control (cell transfected with no DNA); WT, wild-type transfection. 469nts indicates the probe position; a 123-nt ladder was used as a size marker. Relative amounts of CA protein (in densitometric units) loaded: C, 0; ML1, 25; ML2; 23.5; D13, 3.34; D45, 2.8; WT, 22.9.

or was not processed correctly so as to either reduce or abolish reverse transcriptase activity. Therefore, the reverse transcriptase activity was determined for particles produced by transfection at 41°C for each of the p10 mutants and wild-type virus, using the rapid dot assay described in Materials and Methods, and quantitated by scintillation counting (Fig. 6). Each of the deletion mutants released reverse transcriptase activity into the medium. The *ts* mutants also released some activity. The relative reverse transcriptase activity of the mutant particles was similar to that of the wild type (Table 1). The activity was expressed relative to the number of virions in the medium, measured by densitometric scanning of the autoradiographs of the immunoblots (Fig. 2C), using CA protein as the standard. Although some variations were detected in the analysis of the deletion mutants, it does not appear that the defect in the infectivity of the deletion mutants and *ts* mutants could be attributed to a defect in the synthesis, packaging, and processing of the *gag-pol* polyprotein.

Analysis of viral RNA in mutant virions. To determine whether the noninfectious mutant virions produced after transfection contained viral genomic RNA, we purified virus particles produced by a transfection at 36°C and extracted their viral RNAs as described in Materials and Methods. Cold viral RNA was then analyzed by RNase protection assay using an in vitro-labeled RNA probe complementary to part of the leader and *gag* gene, encompassing the splice donor sequence. Viral genomic RNA was quantitated by scanning densitometry of the autoradiograph and expressed relative to the amount of CA determined by Western blotting as described above. The results (Fig. 7) showed that *ts* mutants ML1 and ML2 contained wild-type levels of RNA. The relative packaging of mutants D13 and D45 was similar to that of the wild type. Thus, the particles produced in transient transfection were not defective for viral RNA packaging. Furthermore, the viral RNA isolated from these mutants migrated as the 70S dimer in a nondenaturing Northern blot (preliminary results).

DISCUSSION

The mutations that we have generated in protein p10 showed that although in some cases virus release was greatly reduced, those viruses which were released appeared to be correctly formed. Our data are consistent with previous reports showing that the major assembly domains of the RSV *gag* gene reside only in the MA, MA-p2, and CA-NC domains (6, 36, 37), while the PR domain may enhance the efficiency of virion assembly (21, 22, 32). One large deletion of *gag* (mutant R-3C [37]), in which two amino acids of p2, all of p10, and 26 amino acids of CA were removed, was reported to have no effect on the rate of particle formation and that the density of these core particles formed by expressing the *gag* gene in a simian virus 40-based expression vector was the same as that for the wild type (37). Thus, our results are different from these findings since in our case, fewer virus particles were released into the medium than for wild-type virus. Nevertheless, the amounts of processed capsid and *gag* precursor in the cell lysate were the same as for the wild type. We have not tested whether the mutant particles sedimented at the same density as did the wild type on a sucrose gradient, but since viral particles from the deletion mutants could be pelleted through a 20% sucrose cushion, there was not a gross reduction in their density.

We interpret these data as an indication that the *gag*

precursor of the various p10 mutants was correctly synthesized and targeted to the plasma membrane and that at least virus assembly and maturation were initiated, since the presence of mature CA in wild-type amount is an indication that the protease has been activated. Such an activation is thought to take place only after or during budding. These results need to be confirmed by examination of the subcellular localization of the mutants precursor or by pulse-chase experiments. Current models suggest that the dimerization which is necessary for PR activation might be induced both by a critical concentration of *gag* protein and by an activation signal (10, 11, 20). Alternatively, a repressor of PR activity which prevents premature activation of the protease might be present in avian cells. In type C retroviruses, accumulation and alignment of *gag* proteins necessary for PR dimerization start when dense aggregates appear at the plasma membrane during the initiation of budding. Analysis of viral proteins in cell lysates and both the protein and RNA contents of the virions showed that CA was matured correctly and that the RNA was properly packaged and dimerized. Whether the virions are completely matured requires further investigation, such as analysis of the cell surface and virion pellets by scanning and transmission electron microscopy to examine the morphology of bound and released virions.

Reverse transcriptase assays revealed normal levels of active enzyme in the virions. Thus, the *gag-pol* precursor is also expressed and incorporated into the virions and is probably proteolytically processed normally. This finding again supports our hypothesis that qualitatively, virion maturation is not impaired in our p10 mutants. Similar phenotypes for linker insertion and deletion mutants of p12 of Moloney murine leukemia virus (MoMuLV) (also rich in proline and glycine) have been described. Particle formation was unaffected by a deletion at the MA-p12 boundary (nt 944 to 1088), and the reverse transcriptase was fully active (4). Similarly, with linker insertion in p12, MoMuLV Gag-β-galactosidase fusion constructs could be assembled into mature virions (8). However, no defects in the budding process were demonstrated for these mutants. It could be that p12 of MoMuLV is a more flexible protein than p10 or that regions other than those mutated would show similar phenotypes (p12 is considerably larger than p10 and contains the well-conserved retroviral sequence Pro-Pro-Pro-Tyr that is present in p2 of RSV and therefore may have properties of both proteins).

Three of the linker insertions in RSV p10 (ML3, ML4, and ML5) had no apparent effect on virus phenotype. Some linker insertions in MoMuLV p12 have also been shown to have no effect on the infectivity of MoMuLV (15).

Some of our mutants do not productively infect fresh CEFs. It was reported several years ago that MoMuLV p12 specifically interacted with the homologous viral RNA and that it was tightly bound to it (27–31). Sen and Todaro (30, 31) have proposed that p12 plays a role in encapsidation of the genomic RNA. Thus, one possibility to explain the phenotype of our mutants was that RNA packaging or maturation was affected. However, we found that deletion mutants D13 and D45, *ts*ML1, and *ts*ML2 contained normal amount of viral RNA and that this RNA was correctly matured (preliminary results). This finding strongly suggested that p10 is not involved in the selection, packaging, and maturation of the RNA. Crawford and Goff (4) have described p12 deletion mutants that were demonstrated to have normal endogenous minus-strand strong-stop DNA and plus-strand DNA synthesis. They concluded that the C terminus of p12 was not involved in the packaging and maturation of the genomic RNA or in reverse transcription. Nevertheless, no reverse transcription product was detected in infected cells, leading to the proposal that an early step of infection, probably uncoating, was impaired. The phenotype of *ts*ML2 is consistent with a similar block in early entry. Virions produced by *ts*ML2 appeared to be wild type-like for all parameters tested at the permissive temperature but were noninfectious. We are currently investigating *ts*ML2 virions for endogenous strong-stop DNA synthesis and intracellular reverse transcription product in *ts*ML2-infected CEFs to test whether *ts*ML2 can integrate in cells. The deletion mutants and *ts*ML1 do not productively infect CEFs at 41°C, probably as a result of the same block in virus budding observed after transfection, but the data do not exclude the possibility that there is also impairment of an early step of infection. Even if mutant D45 does not produce more particles at 36°C than at 41°C, it is more infectious at that temperature. We have no hypothesis to explain this somewhat puzzling phenotype, but it would mean that the few particles that are produced at 36°C are fully infectious while the rest are trapped in the cell and that D45 is also a kind of *ts* mutant. In fact, if ML1 and D45 particles are produced at 36°C and then shifted to 41°C, they can infect the cells at 41°C. Thus, it appears that the *ts* phenotype is not inducible after budding (6a), supporting the view that p10 may be involved in some core protein folding and cell membrane-virus interactions.

It is interesting to note that the two *ts* mutations are those in the vicinity of pairs of proline residues. Mutant *ts*ML1 changes a small glycine to a large basic arginine residue next to the proline pair, while *ts*ML2 introduced two amino acids between the prolines. Proline is an amino acid which strongly affects secondary structure. Proline-rich regions in proteins may be particularly important since they usually interrupt classic secondary structures like β sheets or α helices and are associated with surface loops and turns, proline pairs usually being present in β turns (16). The large number of prolines in p10 suggests that this protein plays a structurally important role in determining $Pr76^{gag}$ conformation and is likely to play a regulatory role in the conformational switches that may control the budding steps. In human immunodeficiency virus type 1, a lentivirus that buds similarly to RSV, two polypeptide have been shown to be implicated in budding: the auxiliary protein Vpu and the C-terminal polypeptide p6 of the $Pr55^{gag}$ polyprotein. Deletion of Vpu was shown to inhibit virion release at the membrane, but the particles that did not detach appeared to be mostly mature (12, 33). The Vpu deletion mutants were as infectious as the wild-type virus, confirming that the maturation steps necessary for infectivity were not affected (12). Vpu is not related to *gag* proteins and is not proline rich but rather is an integral membrane protein and is not incorporated into progeny virions. In contrast, human immunodeficiency virus type 1 p6 is a proline-rich polypeptide and is incorporated into virion. Complete deletion of p6 or substitution of two adjacent proline residues with isoleucine was shown to reduce the rate of budding (7). Virions that did not detach from the cells were observed by electron microscopy to be anchored to the cell membrane by a thin tether. Additionally, most of these mutant virions appeared immature. Infectivity of the deletion mutant was greatly reduced, while that of the proline mutant was reduced only slightly (7). These phenotypes are strikingly similar to those of some of the p10 mutants described here.

Two regulatory elements have been characterized in the RSV p10 region of the genome. At the DNA level, an

enhancer has been mapped between nt 776 and 998 and shown to increase chloramphenicol acetyltransferase activity by a factor of 20 in pSVCAT or only 3 in pRSVCAT constructs (2). Footprinting analysis demonstrated protection of the sequence between nt 813 and 872 from the start site of transcription, while chloramphenicol acetyltransferase assays indicated a significant drop in transcription resulting from deletion of the region between nt 776 and 952 (less than 50%) (2, 3). At the RNA level, a negative regulator of splicing has been mapped between nt 707 and 930; deletion led to a decrease in the level of unspliced genomic RNA to less than 10% of the normal level (1, 17). We do not believe that these elements have had any significant effect on the phenotype of our mutants; ML3, the mutant in which the linker is inserted closest to these elements, is fully infectious, while D13, a near-full-length deletion of p10, shows neither a decrease in synthesis of Pr76^{gag} nor an imbalance between the amounts of CA and RNA compared with the wild type.

In summary, the mutational analysis of the p10 coding sequences reported here demonstrates that p10 is required for a late step in virus maturation, probably budding from the cell surface, and that critical amino acid sequences and motifs are required in the p10 region of the Pr76^{gag} polyprotein to control this final step of assembly. p10 could be needed to fold properly CA or the virion core; indeed, the phenotype of *ts*ML2 suggests a role for p10 in an early step of infection that might be due to a defect in some aspect of core protein folding. Another explanation would be that the mutants have some defects in glycoprotein packaging. Further experiments are under way to fully answer these questions.

ACKNOWLEDGMENTS

The expert technical assistance of P. Damay is gratefully acknowledged. We thank N. Bowles for critical reading of the manuscript and helpful discussions, D. Rifat for oligonucleotide synthesis, and O. Jenni for photographs and figures.

This work was supported by grant 31-29983.90 from the Swiss National Science Foundation.

REFERENCES

1. **Arrigo, S., and K. Beemon.** 1988. Regulation of Rous sarcoma virus RNA splicing and stability. Mol. Cell. Biol. **8**:4858–4867.
2. **Arrigo, S., M. Yun, and K. Beemon.** 1987. *cis*-acting regulatory elements within the *gag* genes of avian retroviruses. Mol. Cell. Biol. **7**:338–397.
3. **Carlberg, K., T. A. Ryden, and K. Beemon.** 1988. Localization and footprinting of an enhancer within the avian sarcoma virus *gag* gene. J. Virol. **62**:1617–1624.
4. **Crawford, S., and S. P. Goff.** 1984. Mutations in the *gag* proteins p12 and p15 of Moloney murine leukemia virus block early stages of infection. J. Virol. **49**:909–917.
5. **Dupraz, P., S. Oertle, C. Méric, P. Damay, and P.-F. Spahr.** 1990. Point mutations in the proximal Cys-His box of Rous sarcoma virus nucleocapsid protein. J. Virol. **64**:4978–4987.
6. **Dupraz, P., and P.-F. Spahr.** 1992. Specificity of Rous sarcoma virus nucleocapsid protein in genomic RNA packaging. J. Virol. **66**:4662–4670.

6a. **Dupraz, P., and P.-F. Spahr.** Unpublished data.

6b. **Goff, S., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J. Virol. **38**:239–248.

7. **Göttlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutation affecting the p6 *gag* protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88**:3195–3199.
8. **Hansen, M., L. Jelinek, S. Whiting, and E. Barklis.** 1990. Transport and assembly of *gag* proteins into Moloney murine leukemia virus. J. Virol. **64**:5306–5316.
9. **Hunter, E., J. C. Bennet, A. Bhown, R. B. Pepinsky, and V. M. Vogt.** 1983. Amino-terminal amino acid sequence of p10, the fifth major Gag polypeptide of avian sarcoma and leukemia viruses. J. Virol. **45**:885–888.
10. **Jaskolski, M., M. Miller, J. K. M. Rao, J. Leis, and A. Wlodamer.** 1990. Structure of the aspartic protease from Rous sarcoma virus retrovirus refined at 2-angström resolution. Biochemistry **29**:5889–5898.
11. **Katoh, I., Y. Ikawa, and Y. Yoshinaka.** 1989. Retrovirus protease characterized as a dimeric aspartic proteinase. J. Virol. **63**:2226–2232.
12. **Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein.** 1990. The human immunodeficiency virus type 1-specific protein *vpu* is required for efficient virus maturation and release. J. Virol. **64**:621–629.
13. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
14. **Lo, K. M., S. S. Jones, N. Hackett, and H. G. Khorana.** 1984. Specific amino acid substitutions in bacterioopsin: replacement of a restriction fragment in the structural gene by synthetic DNA fragments containing altered codons. Proc. Natl. Acad. Sci. USA **81**:2285–2289.
15. **Lobel, L. I., and S. P. Goff.** 1984. Construction of mutants of Moloney murine leukemia virus by suppressor-linker insertional mutagenesis: positions of viable insertion mutations. Proc. Natl. Acad. Sci. USA **81**:4149–4153.
16. **MacArthur, M. W., and J. M. Thornton.** 1991. Influence of proline residues on protein conformation. J. Mol. Biol. **218**:397–412.
17. **McNally, M., R. Gontarek, and K. Beemon.** 1991. Characterization of Rous sarcoma virus intronic sequences that negatively regulate splicing. Virology **185**:99–108.
18. **Méric, C., E. Gouilloud, and P.-F. Spahr.** 1988. Mutation in Rous sarcoma virus nucleocapsid protein p12 (NC): deletion of Cys-His boxes. J. Virol. **62**:3328–3333.
19. **Méric, C., and P.-F. Spahr.** 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. J. Virol. **60**:450–459.
20. **Miller, M., M. Jaskolski, J. K. M. Rao, J. Leis, and A. Wlodamer.** 1989. Crystal structure of a retroviral protease proves relationship to aspartic protease family. Nature (London) **337**:576–579.
21. **Oertle, S., N. Bowles, and P.-F. Spahr.** 1992. Complementation studies with Rous sarcoma virus *gag* and *gag-pol* polyprotein mutants. J. Virol. **66**:3873–3878.
22. **Oertle, S., and P.-F. Spahr.** 1990. Role of the *gag* polyprotein precursor in packaging and maturation of Rous sarcoma virus genomic RNA. J. Virol. **64**:5757–5763.
23. **Pepinsky, R. B., R. J. Mattaliano, and V. M. Vogt.** 1986. Structure and processing of the p2 region of avian sarcoma and leukemia virus *gag* precursor polyproteins. J. Virol. **58**:50–58.
24. **Pepinsky, R. B., and V. M. Vogt.** 1983. Purification and properties of a fifth major viral *gag* protein from avian sarcoma and leukemia viruses. J. Virol. **45**:648–658.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–859.
27. **Sen, A., C. J. Sherr, and G. J. Todaro.** 1976. Specific binding of the type C viral core protein p12 with purified viral RNA. Cell **7**:21–32.
28. **Sen, A., C. J. Sherr, and G. J. Todaro.** 1977. Phosphorylation of murine type C viral p12 proteins regulates their extent of binding to the homologous viral RNA. Cell **10**:489–496.
29. **Sen, A., C. J. Sherr, and G. J. Todaro.** 1978. Endogenous feline (RD114) and baboon type C viruses have related specific RNA-binding proteins and genome binding sites. Virology **84**:99–107.
30. **Sen, A., and G. J. Todaro.** 1976. Specificity of in vitro binding of primate type C viral RNA and the homologous viral p12 core

protein. Science **193:**326–328.
31. **Sen, A., and G. J. Todaro.** 1977. The genome-associated, specific RNA binding protein of avian and mammalian type C viruses. Cell **10:**91–99.
32. **Stewart, L., and V. M. Vogt.** 1991. *trans*-acting viral protease is necessary and sufficient for activation of avian leukosis virus reverse transcriptase. J. Virol. **65:**6218–6231.
33. **Strebel, K., T. Klimkait, and M. A. Martin.** 1988. A novel gene of HIV-1, vpu, and its 16 kilodalton product. Science **241:**1221–1223.
34. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**1–11.
35. **Vogt, V. M., R. B. Pepinsky, and L. E. Southard.** 1985. Primary structure of p19 species of avian sarcoma and leukemia viruses. J. Virol. **56:**31–39.
36. **Weldon, R. A. J., C. R. Erdie, M. G. Oliver, and J. J. Wills.** 1990. Incorporation of chimeric Gag protein into retroviral particles. J. Virol. **64:**4169–4179.
37. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral gag proteins. AIDS **5:**639–654.
38. **Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman.** 1985. Rapid and easy sequencing of large linear double stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. **2:**89–94.