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Mutational Analysis of the Major Homology Region of Mason-Pfizer Monkey Virus by Use of Saturation Mutagenesis

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The major capsid (CA) protein of retroviruses possesses a stretch of 20 amino acids, called the major homology region (MHR), which is evolutionarily conserved and invariant in location within the primary sequence of the protein. The function of this region was investigated by examining the effect of random single-amino-acid substitutions within the central 13 positions of the MHR on the life cycle of Mason-Pfizer monkey virus (M-PMV), an immunosuppressive D-type retrovirus. When these mutants were subcloned into an M-PMV proviral vector and expressed in COS cells, one of two major phenotypes was observed. The first group, containing three mutants bearing drastic amino acid substitutions, was unable to assemble capsids in the cytoplasm of the host cell. The second and more common group of mutants was able to assemble and release virions, but these either displayed greatly reduced levels of infectivity or were completely noninfectious. Included within this second group were two mutants with unusual phenotypes; mutant D158Y exhibited a novel cleavage site for the viral protease that resulted in cleavage of the major capsid protein, p27 (CA), within the MHR, whereas mutant F156L appeared to have lost a major site for antibody recognition within the mature CA protein. The results of this mutagenic analysis suggest that changes in the MHR sequence can interfere with the assembly of viral capsids and block an early stage of the infection cycle of M-PMV.

Mason-Pfizer monkey virus (M-PMV) was originally isolated from a spontaneous mammary carcinoma in a rhesus monkey (12). While this exogenous virus has never been demonstrated to be oncogenic (18, 19), it has been associated with an acquired immunodeficiency syndrome in macaques (13, 33, 47). M-PMV is the prototype of the D-type retroviruses (19). The most prominent characteristic of this class of primate retroviruses is that an immature capsid is assembled within the cytoplasm of the infected cell and is subsequently transported to the plasma membrane, where it extrudes through the membrane and is released.

Three Gag-related protein precursors, Pr180, Pr95, and Pr78, can be identified in M-PMV-infected cells (6). $Pr180^{gag\text{-}pro\text{-}pol}$ has been shown to yield the RNA-dependent DNA polymerase (reverse transcriptase [RT]) (9, 46), while $Pr95^{gag\text{-}pro}$ is the precursor of the viral protease (46). $Pr78^{gag}$ is the major Gag polyprotein precursor; at the time of budding, it is cleaved to yield six structural proteins arranged in the following order: NH_2-p10-pp16/pp24-p12-p27-p14-p4-COOH (7, 26). The amino-terminal product, p10, is the membrane-associated matrix (MA) protein; pp16/pp24, the major phosphoprotein, and p12, a protein found only in D- and B-type retroviruses, have unknown functions; p27, the major capsid (CA) protein, is the major structural component of the viral core-shell; and p14, the nucleocapsid protein (NC), is a highly basic protein that is the major component of the ribonucleoprotein complex. The function of p4 is unknown.

The exact biological role of the major capsid protein remains uncertain, but an increasing number of reports indicate that this structural protein may function at different steps during the retroviral life cycle. The most widely accepted model of the molecular structure of a retrovirus, as originally formalized by Bolognesi et al. in 1978 (4), suggests that CA is the major constituent of the protein shell that surrounds the inner ribonucleoprotein core present in the mature viral particle. Numerous studies have provided data that strongly support this model, including direct biochemical analysis of intact viral particles, purified viral cores, and other viral substructure (3, 48, 49), cross-linking studies in which both intact virions and stripped viral cores were treated with various cross-linking reagents (37), and studies in which direct visualization techniques, such as immunoelectron microscopy, were used to determine the localization of the individual structural proteins in the virion (21, 22).

Because CA has such a prominent structural role and because it is the largest of the Gag cleavage products, it might be expected to play a major role in directing particle formation; however, the data relevant to this issue have been rather contradictory. Deletions and insertions into the CA domain of the Gag precursor of Moloney murine leukemia virus (M-MuLV) invariably resulted in loss of capsid assembly and particle release (23, 31, 44). In addition, recent work by Barklis and coworkers has demonstrated that the CA domain of the Gag polyprotein is essential for incorporation of Gag–β-galactosidase fusion proteins into wild-type MuLV particles (25, 29). On the other hand, studies on Rous sarcoma virus indicate that most of the CA coding domain can be deleted without blocking virion assembly (50). It is important to notice, however, that even in this system, an important assembly domain may lie near the carboxyl terminus of CA, since deletions that extend to this area of the protein destroy the ability of mutants to form stable virions.

Data obtained in different laboratories indicate that CA may also play an essential role in events that occur early in the replicative cycle of the virus. More specifically, it seems that CA remains associated with the viral genome during the

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initial stages of the infection. Since CA is not known to possess any enzymatic activity, it is reasonable to predict that its function is to form a "casing," which protects the DNA intermediates and the enzymatic machinery involved in reverse transcription and in the integration process, during these critical early stages of the life cycle. Hsu and coworkers have described a mutation that mapped to the CA coding region of the M-MuLV genome, which permits particle assembly but blocks the infection at a step prior to proviral DNA synthesis in the newly infected cell (27). Furthermore, the mouse locus Fv-1, which determines the N- and B-tropism of MuLV in mouse cells, is thought to encode a cellular factor that reduces the efficiency with which the proviral DNA is integrated in the genomic DNA of a nonpermissive host cell (11, 17, 28, 51). Naturally occurring amino acid changes, which are responsible for the N- or B-tropism of MuLV, have been mapped to the major capsid protein (p30)-coding region of this virus (15). Finally, the development of an assay that accurately reproduces the M-MuLV integration reaction in vitro has enabled Brown and coworkers to establish that unintegrated DNA, in extracts made from acutely infected cells, exists within an integration-competent, high-molecular-weight nucleoprotein complex (8); direct evidence indicates that this complex is derived from infecting virion cores and contains three viral proteins: RT, the integrase, and CA (5). It is interesting that similar results have been obtained recently with the Ty1 retrotransposon system in *Saccharomyces cerevisiae* (1, 16).

When the amino acid sequences of the Gag precursor proteins of distantly related retroviruses are compared, very little sequence homology can be found. One of the most-conserved sequences of Gag is the Cys-His box motif (C-X_2-C-X_4-H-X_4-C, where X_n indicates a stretch of n amino acid residues), which is present in one or two copies in the NC domain of all retroviruses and which appears to play a crucial role in the packaging and dimerization of the viral RNA genome (24, 34). The only other region of significant primary sequence homology between different Gag proteins is located in CA (36). This region, which has been designated the major homology region (MHR), is present in members of the *Lentiviridae* family (visna virus, human immunodeficiency virus types 1 and 2, and simian immunodeficiency virus), the complex *Oncoviridae* (human T-cell lymphotropic virus types I and II), and both type C (baboon endogenous virus [BaEV], MuLV, feline leukemia virus, and Rous sarcoma virus) and type B (mouse mammary tumor virus) retroviruses (50). It does not, however, appear to be conserved in the Gag-equivalent protein of Ty1. The percentage of primary structure identity along the entire MHR domain varies from virus to virus, but it is interesting that 6 of the 20 amino acid positions of the MHR are absolutely conserved or show a single conservative substitution in some viruses (Fig. 1). It is noteworthy that the MHR is found in a comparable position within the CA primary sequence of all retroviruses studied (50). The biological significance of the MHR is unknown, but it is reasonable to assume that its clear evolutionary conservation reflects an important functional role.

In an attempt to gain an understanding of the role of the MHR in the retroviral life cycle, we generated a series of mutations within the MHR of M-PMV. Expression of these mutant genomes in COS cells has allowed us to determine that, for the most part, they do not block the ability of the virus to assemble and release viral particles. Nevertheless, the majority of the mutations in this region are lethal. These results suggest that the MHR has a role in processes that occur early during infection.

	h					s			h		h		s	h	% Identity
M-PMV	V	K	Q	G	P	D	E	P	F	A	D	F	V	H	R	L	I	T	T	A	
SIV	V	K	Q	G	P	D	E	P	F	A	D	F	V	H	R	L	I	T	T	A	100%
HIV-1	I	R	Q	G	P	K	E	P	F	R	D	Y	V	D	R	F	Y	K	T	L	50%
RSV	I	M	Q	G	P	S	E	S	F	V	D	F	A	N	R	L	I	K	A	V	50%
HTLV-I	I	L	Q	G	L	E	E	P	Y	H	A	F	V	E	R	L	N	I	A	L	40%
FeLV	V	V	Q	G	K	E	E	T	P	A	A	F	L	E	R	L	K	E	A	Y	40%
BaEV	I	T	Q	G	K	D	E	S	P	A	A	F	M	E	R	L	L	E	G	F	40%
VISNA	V	K	Q	K	N	T	E	S	Y	E	D	F	I	A	R	L	L	E	A	I	40%
MoMuLV	I	T	Q	G	P	N	E	S	P	S	A	F	L	E	R	L	K	E	A	Y	35%
MMTV	L	K	Q	G	N	E	E	S	Y	E	T	F	I	S	R	L	E	E	A	V	35%

FIG. 1. Comparison of the MHRs of 10 different retroviruses. Positions of absolute identity in all 10 viruses are denoted by shaded boxes; positions of nearly absolute identity (one conservative substitution) are denoted by dashed boxes. The letters h and s denoted conserved hydrophobic and small amino acids, respectively. The percent identity is calculated with respect to the M-PMV sequence. SIV, simian immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; RSV, Rous sarcoma virus; HTLV-I, human T-cell lymphotropic virus type I; FeLV, feline leukemia virus; MMTV, mouse mammary tumor virus.

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis and vector system. To introduce random single-base-pair substitutions over the CA protein MHR, oligonucleotide-directed saturation mutagenesis (14) was done as follows. A mixed-sequence oligonucleotide was synthesized by contaminating each of the mononucleotide precursor reservoirs with 1% of each of the "mutant" monomers (i.e., the G reservoir was contaminated with 1% A, 1% C, and 1% T). The contamination percentage (p) used was determined on the basis of the length of the doped sequence (n) in order to optimize the frequency of single-substitution oligonucleotides in the heterogeneous population obtained at the end of the synthesis. The choice was made on the basis of the binomial distribution equation $N = n![N!(n - N)!]^{-1}(1 - p)^{n - N}p^N$, which permits the calculation of the expected frequency of the substitution class (N), when the length of the doped sequence (n) and the percentage of doping (p) at each nucleotide position are known (14, 32).

The heterogeneous population of oligonucleotides was used in a mutagenesis reaction by the method first developed by Kunkel (30). To perform the mutagenesis, a 1.5-kbp *Sst*I-*Bam*HI fragment from an infectious M-PMV proviral clone was subcloned into the replicative form of M13mp19 (35). This fragment contains the last two-thirds of *gag*, including the entire p27 (CA) coding sequence. After mutagenesis, the mutagenized region from 70 clones was sequenced (43), and 13 missense mutant clones were identified. An *Sst*I-*Bsp*HI fragment of 1.0 kbp was excised from the mutant phage replicative-form DNAs and substituted for the wild-type counterpart in the M-PMV expression vector pSHRM15 (39). This vector contains an infectious proviral clone of M-PMV, a simian virus 40 origin of replication, and the hygromycin resistance gene under the control of the simian virus 40 early promoter.

Nomenclature. Substituted amino acids and their positions were used to designate the mutants; for example, in mutant D153N, an aspartic acid at position 153 within the CA protein was substituted with an asparagine. The mutant designated DEL throughout this article carries an in-frame

deletion of three nonconsecutive nucleotides within the MHR coding region that results in the following amino acid sequence: Asp-153-Glu-Pro-Phe-Ala-Asp-Ser-Tyr-Glu-Ile-Ile-Thr-165 (the numbers indicate the positions of the first and last amino acids of the mutant sequence within CA; the underlined positions have been changed with respect to the wild-type sequence).

Cells, transfection protocols, metabolic labeling, immunoprecipitation, and gel electrophoresis. For the transient expression of viral proteins in COS-1 cells, the cells were transfected with viral genomic DNA (7 μg of DNA per 0.8 × 10^6 cells per 60-mm plate) by the modified calcium phosphate method (10). At 48 h posttransfection, the cells were pulse-labeled with 0.1 mCi of [^{3}H]leucine (0.25 mCi/ml, 156.0 Ci/mmol; Du Pont Co.) in leucine-free minimal essential medium and then chased in complete Dulbecco's modified Eagle's medium (D-MEM). Cells were lysed in lysis buffer A (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5]), and cell-associated viral proteins were immunoprecipitated with a rabbit anti-p27 antiserum, as described previously (6). Metabolically labeled viral particles were pelleted from the culture supernatants of pulse-chase-labeled cells by centrifugation at 80,000 rpm in a Beckman TLA 100 rotor for 15 min at 4°C, and the viral pellet was lysed in lysis buffer B (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5]). Virion-associated viral proteins were immunoprecipitated with a goat anti-M-PMV antiserum (Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md.) as described above.

Viral polypeptides immunoprecipitated with virus-specific antibodies were separated on either a 10 or a 12% polyacrylamide–SDS resolving gel, as described previously (6).

RT assay and cell-free infection of HeLa cells. To determine whether virions containing RT activity were released from cells expressing mutant viral genomes, COS-1 cells were transfected as described above with viral genomic DNA, and the growth medium was changed at 24 h posttransfection. After an additional 24 h, the culture fluids were harvested and clarified by centrifugation at 10,000 rpm in a Beckman JA20 rotor for 15 min at 4°C. RT-containing virions were pelleted by ultracentrifugation at 80,000 rpm in a Beckman TLA 100 rotor for 15 min at 4°C. Pellets obtained from 0.5 ml of COS-1 cell medium were disrupted in 15 μl of lysis buffer (0.05% Triton X-100, 0.1 M KCl, 0.002 M dithiothreitol, 0.05 M Tris-HCl [pH 8.0]). The RT assay was performed as described previously on a 7.5-μl portion of the disrupted virus pellet (9, 42).

The results obtained from each of two 60-mm plates per sample were averaged after subtraction of the background counts and after normalization for the relative levels of expression of the human growth hormone (hGH) antigen in the culture medium (see below). The results obtained for each of the mutants are expressed as a percentage of the wild-type counts.

The RT assay was also used to assess the infectivity of M-PMV mutant viral genomes. Culture fluids were harvested from COS-1 cells that had been transfected with either wild-type or mutant proviral DNA as described above. Equivalent amounts of RT-containing medium were used to infect HeLa cell monolayers. Virus infection was carried out in 100-mm plates (1.4 × 10^6 cells per plate) in 1.0 ml of complete growth medium containing 2.0 μg of Polybrene (hexadimethrine bromide; Sigma) per ml at 37°C for 1.5 h. Following infection, 9 ml of complete growth medium was added to each plate, and the cells were incubated for an additional 22 h in a 5% CO_2 incubator. The culture medium was changed every 24 h. Culture fluids from the infected HeLa cells were collected every 4 days, after which the cells were harvested by trypsinization, and 1.4 × 10^6 cells were reseeded onto fresh 100-mm plates. RT assays were carried out, as described above, on the pelletable material obtained from 3 ml of HeLa cell medium.

hGH assay for transfection efficiency. In order to normalize for the transfection efficiency and to be better able to compare the RT data obtained from the single MHR mutants, COS-1 cells were cotransfected, as described above, with either wild-type or mutant virus (7 μg of DNA per 0.8 × 10^6 cells per 60-mm plate) and with plasmid pXGH5, expressing the hGH gene under the control of the mouse metallothionein 1 promoter (1 μg of DNA per 0.8 × 10^6 cells per 60-mm plate) (45). The culture fluids were harvested as described above, and expression of the hGH antigen in 5-μl samples of culture supernatants was determined as suggested by the manufacturer (Allegro human growth hormone transient gene expression assay system; Nichols Institute Diagnostics).

Purification of viral particles and analysis of virus-associated proteins by SDS-PAGE. In order to perform a direct biochemical analysis of the viral particles released by COS-1 cells transiently expressing wild-type or mutant M-PMV proviral DNA, the extracellular virions were purified through a 24 to 48% (wt/wt) sucrose gradient as described previously with some modifications (6). The experiment was performed by transfecting three 100-mm plates of COS-1 cells (20 μg of DNA per 2.0 × 10^6 cells per plate) with either wild-type or mutant proviral DNA. At 48 h posttransfection, cells were pulse-labeled for 30 min with 0.26 mCi of a mixture of [^{35}S]methionine and [^{35}S]cysteine (1,232.7 Ci/mmol; protein labeling mix; Du Pont) in 1.0 ml of methionine- and cysteine-free D-MEM (GIBCO). At the end of the 30-min incubation, 2 ml of fresh methionine- and cysteine-free D-MEM supplemented with 2% fetal bovine serum (Biocell), 584 μg of glutamine per ml, 6.0 μg of methionine per ml, and 9.6 μg of cysteine per ml was added to each plate, and the incubation was continued for an additional 7.5 h. At the end of the labeling period, culture fluids were harvested from each of the plates, pooled, and clarified by centrifugation at 10,000 rpm in a Beckman JA20 rotor for 15 min at 4°C. Virus was concentrated 18-fold in an Amicon concentrator (Centriprep-30 concentrator; Amicon division, W. R. Grace & Co.), layered over a 24 to 48% preformed sucrose gradient (in TNE buffer), and centrifuged at 41,000 rpm in an SW41 rotor for 3 h at 4°C. Fractions (0.8 ml) were collected, and the refractive index of every fourth fraction was determined with a Bausch & Lomb ABBE 3L refractometer. The fractions were then diluted fourfold in TNE buffer (0.01 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.001 M EDTA), after which an RT assay was performed on one-third of each fraction as described above. Virus was pelleted from the remaining two-thirds of each fraction by centrifugation at 80,000 rpm in a TLA-100 rotor for 30 min at 4°C. The pellets were resuspended directly in protein loading buffer (0.05 M Tris-HCl [pH 6.8], 0.1 M dithiothreitol, 2% SDS, 0.2% bromophenol blue, 10% glycerol), and the viral proteins were resolved on either 12 or 15% polyacrylamide–SDS gels as described previously (6). After polyacrylamide gel electrophoresis (PAGE), the amounts of [^{35}S]methionine and [^{35}S]cysteine incorporated in the CA (p27) band throughout the gradient were measured with a radioanalytic imaging system (Ambis Systems Inc.).

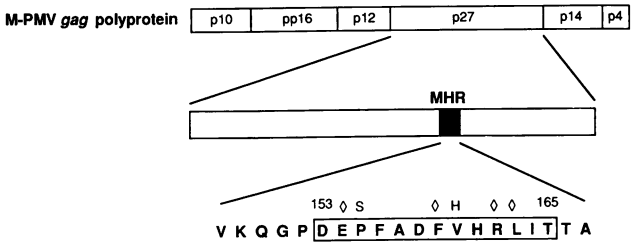

FIG. 2. Schematic diagram showing the position of the MHR within the M-PMV Gag polyprotein. The *gag*-encoded proteins are shown as they are linearly arranged within the Gag precursor. An enlargement of CA (p27) reveals the position of the MHR (solid box). The amino acid sequence of the M-PMV MHR is reported in the single-letter amino acid code. Numbers indicate amino acid positions within p27. Amino acid positions within the MHR are indicated as follows: ◇, highly conserved residues, S, conserved small residues; H, conserved hydrophobic residues. The amino acid sequence subjected to mutagenesis in the present study is boxed.

Electron microscopy. Electron microscopic analysis of COS-1 cells transfected with viral genomic DNA was carried out as described previously (41).

RESULTS

Saturation mutagenesis of the CA protein MHR. In order to understand the functional role of the MHR in the life cycle of M-PMV, we subjected the central 13 amino acid positions of this domain to oligonucleotide-directed saturation mutagenesis (Fig. 2). This technique, modified from the method first developed by Derbyshire et al. (14), allows the introduction of random single-base-pair substitutions over a sequence of interest. A 1.5-kb *Sac*I-*Bam*HI fragment of the M-PMV *gag* gene was subcloned into an M13-based vector (35) and subjected to mutagenesis (30) with a doped oligonucleotide spanning the coding region for amino acids 153 to 165 of the CA protein (see Materials and Methods). A total of 70 clones were sequenced, and 13 missense mutations plus an in-frame 1-amino-acid deletion/substitution mutant (DEL; see Materials and Methods) were identified (Fig. 3). These 14 mutant CA coding regions were cloned into an M-PMV expression vector, pSHRM15 (39), for phenotypic analysis.

Release of RT-containing virions from COS-1 cells transfected with mutant M-PMV genomes. To determine whether cells expressing M-PMV MHR mutants were able to release viral particles into the culture supernatant, mutant viral

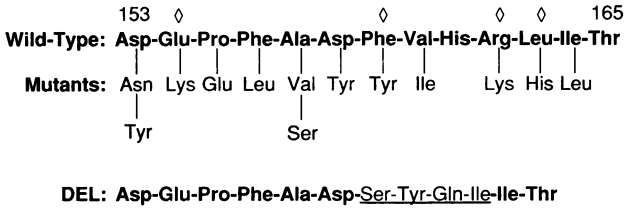

FIG. 3. MHR mutants. The sequence of the central portion of the wild-type M-PMV MHR is shown together with the mutations obtained by saturation mutagenesis (see Materials and Methods). The numbers above the wild-type sequence show the position of the region within p27 subjected to mutagenesis. Open diamonds indicate the positions of highly conserved amino acid residues within the MHR. DEL is the amino acid sequence resulting from an in-frame deletion of three non-consecutive nucleotides in the MHR coding region.

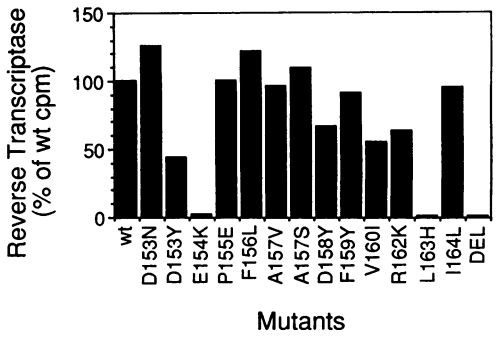

FIG. 4. Release of RT-containing viral particles by MHR mutant proviruses. COS-1 cell monolayers were transfected with either wild-type or mutant M-PMV genomic DNA. Culture medium was harvested at 48 h posttransfection and subjected to ultracentrifugation; the pelletable material was assayed for RT activity. RT results for each mutant are presented as a percentage of the wild-type (wt) activity, after subtraction of the background counts. Transfection efficiency was normalized by using the hGH assay (see Materials and Methods). Less than 2% of wild-type RT activity was observed in cells transfected with mutants E154K, L163H, and DEL. In contrast, cells transfected with all of the other mutant DNAs released virions containing between 45 and 128% of wild-type RT activity.

genomes were transfected into COS-1 cells. Culture medium was harvested at 48 h posttransfection and subjected to ultracentrifugation, and levels of RT activity associated with the pellets were determined (9, 42). In order to normalize for transfection efficiency, an hGH expression vector was cotransfected with the viral genomes (45). The normalized RT activity for each mutant is presented as a percentage of the wild-type activity. The results obtained in a representative experiment are presented in Fig. 4. A majority of the mutants showed pelletable RT activity (from 40 to 125% of wild-type activity). In contrast, mutants E154K, L163H, and DEL always showed less than 2% of wild-type RT activity. We interpret these results to mean that all but three of the amino acid substitutions in the MHR domain had no substantial effect on the ability of the mutants to release M-PMV viral particles into the culture supernatant of transfected cells.

Analysis of the infectivity of MHR mutants. Since virions were released from cells expressing the genomes of all but three of the MHR mutants, we determined whether the particles produced by these mutants were infectious. COS-1 cells were transfected with either wild-type or mutant DNAs, and at 48 h posttransfection, culture fluids were harvested. Samples of culture medium containing equivalent amounts of RT activity were used to infect HeLa cell monolayers. The spread of infectious virus through the HeLa cell cultures was monitored by the RT assay at various days postinfection (Fig. 5). With wild-type virus, the release of RT-containing virions could be detected by 3 days postinfection, and a rapid increase in enzymatic activity was observed until day 15. In contrast, in culture fluids of cells infected with mutant P155E, A157S, or I164L, the level of RT activity was significantly lower at each time point. Furthermore, no detectable RT activity was observed in the culture fluids of HeLa cells infected with any of the other MHR mutants that were capable of releasing virions. As an additional control that the observed differences in RT activity were not due to a dilution effect of the input virus, HeLa cells were infected with a fourfold dilution of the wild-type

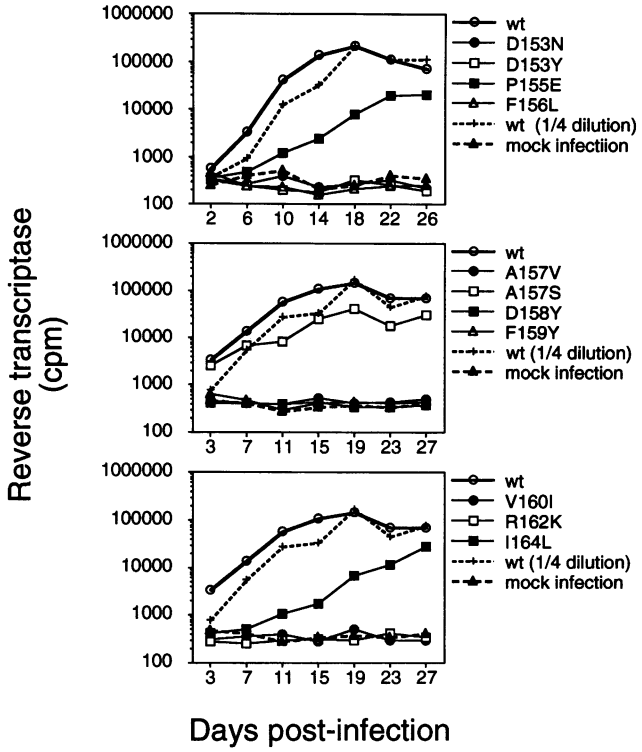

FIG. 5. Infectivity of MHR mutant virions. To determine the infectivity of viruses released from COS-1 cells transfected with wild-type or mutant genomes, virus-containing culture supernatants were harvested, and equivalent amounts of RT activity were used to infect HeLa cell monolayers. Culture medium from these infected HeLa cells was assayed at 4-day intervals, starting at either 2 (A) or 3 (B and C) days postinfection. Wild-type (wt)-virus-infected cells showed a rapid increase in RT activity 6 to 10 days after infection, reflecting the spread of infectious virus through the culture. Cell cultures infected with a fourfold dilution of wild-type virus showed a slight delay in the increase in RT activity, but the level at 18 days postinfection is identical to that of wild-type virions. In contrast, mutant virions P155E, A157S, and I164L, showed a marked reduction in RT activity at each time point. In all of the other mutant-virus-infected cells, no RT activity above that detected in uninfected cells was observed.

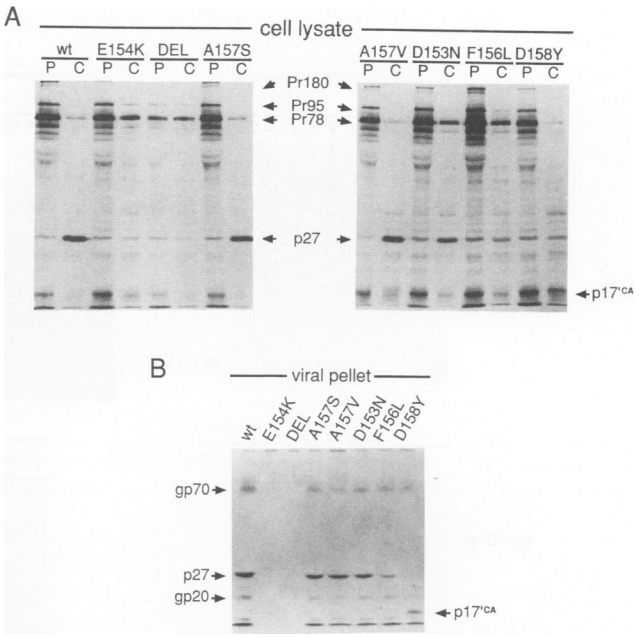

FIG. 6. Immunoprecipitation of cell- and virion-associated viral proteins. (A) To investigate the biosynthesis and processing of the Gag precursors in cells transiently expressing MHR mutants, COS-1 cells transfected with either wild-type (wt) or mutant viral genomes were pulse-labeled with [^{3}H]leucine for 20 min (lanes P) and chased for 4 h (lanes C) at 48 h posttransfection. Virus-specific proteins were immunoprecipitated from the cell lysates with anti-p27 antiserum and analyzed on a 12% protein gel. The positions of Pr78^{gag} (the major Gag precursor), Pr95$^{gag\text{-}pro}$, Pr180$^{gag\text{-}pro\text{-}pol}$, p27, the major capsid protein, and p17$'^{CA}$, a presumptive cleavage product of p27, are indicated. (B) Extracellular virions were pelleted from the culture fluids after a 4-h chase, and virion-associated proteins were immunoprecipitated with anti-M-PMV antiserum and analyzed on a 12% protein gel. The positions of p27, the envelope glycoproteins gp70 and gp20, and p17$'^{CA}$ are shown.

virus stock. We observed a delay of 3 to 4 days in the increase in RT activity, but the enzymatic activity reached a peak identical to that obtained with the undiluted wild-type-virus-containing medium. We conclude that all of the MHR mutant virions are either noninfectious or greatly reduced in infectivity compared with wild-type M-PMV.

Synthesis, processing, and release of viral proteins from cells expressing MHR mutants. To determine whether the amino acid changes introduced in the MHR have any effect on the stability of Gag polyproteins or on their processing into mature structural proteins, a pulse-chase experiment was performed on COS-1 cells transfected with either wild-type or mutant viral genomes. Cells were pulse-labeled for 20 min with [^{3}H]leucine or pulse-labeled and then chased in medium containing cold leucine for 4 h. Virus-specific proteins present both in the cell lysate and in the extracellular virions were then immunoprecipitated and resolved by SDS-PAGE (Fig. 6). In wild-type-M-PMV-infected, pulse-labeled cells, bands migrating at the expected positions of viral polyproteins (Pr180$^{gag\text{-}pro\text{-}pol}$, Pr95$^{gag\text{-}pro}$, and Pr78^{gag}) were detected (Fig. 6A). After a 4-h chase, a band corresponding to p27, the major capsid protein, appeared as a processing product of the Gag polyprotein (Fig. 6A). In virions that were released during the 4-h chase, a characteristic pattern of mature viral proteins was observed (Fig. 6B).

In cells transfected with the A157S, A157V, or D153N mutant viral genome, Gag polyproteins were synthesized and processed to mature protein products in a wild-type manner (Fig. 6A). Furthermore, virions were detected in the culture medium of cells expressing these mutants in a manner similar to that seen with wild-type virus (Fig. 6B). An identical phenotype was observed with mutants D153Y, P155E, F159Y, V160I, R162K, and I164L (data not shown). These results demonstrate that the amino acid changes introduced in the MHR of this group of mutants have no discernible effect on the synthesis of M-PMV proteins, their assembly into intracytoplasmic A-type particles (ICAPs), or the release and maturation of viral particles from the host cell.

In contrast, while normal levels of the three Gag polyproteins were synthesized in cells transfected with mutants E154K (Fig. 6A) and L163H (data not shown), none of them were processed to the mature products (Fig. 6A). During the chase period, a significant amount of Pr78^{gag} and Pr95$^{gag\text{-}pro}$ remained stable in the cells, and no virions were detected in the culture medium (Fig. 6, E154K). A similar phenotype

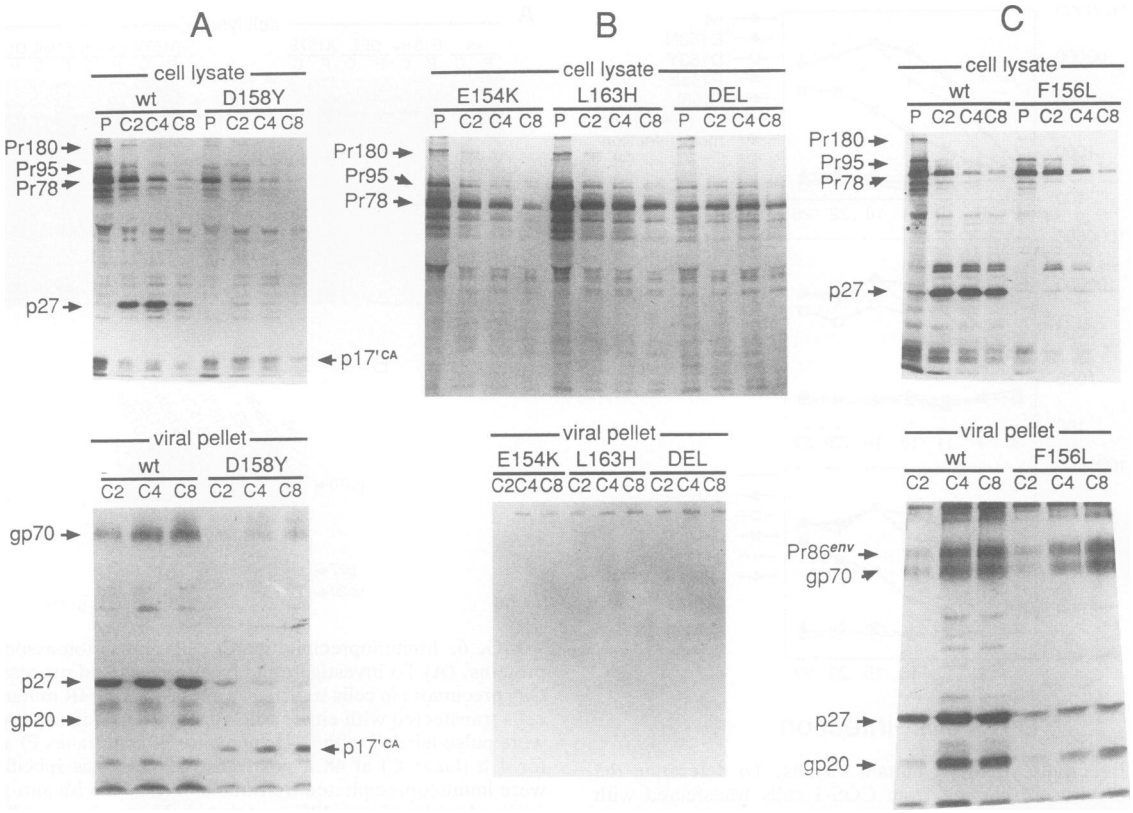

FIG. 7. Kinetics of processing and turnover of wild-type and mutant Gag polyproteins. To determine the efficiency of Gag polyprotein processing or the rate of their turnover, COS-1 cells were transfected with wild-type (wt) or mutant genomic DNAs. At 48 h posttransfection, the cells were pulse-labeled for 20 min with [^{3}H]leucine (lanes P). At the end of the pulse-label or after 2 h (lanes C2), 4 h (lanes C4), or 8 h (lanes C8) of chase, Gag precursors and p27 were immunoprecipitated from the cell lysates with anti-p27 antiserum and analyzed on a 12% protein gel (cell lysate). To determine the efficiency and the rate at which virus and incorporated proteins were released, extracellular virions were pelleted from the culture supernatants after 2, 4, or 8 h of chase, and virus-specific proteins were then immunoprecipitated with anti-M-PMV antiserum (viral pellet). The positions of Pr180$^{gag\text{-}pro\text{-}pol}$, Pr95$^{gag\text{-}pro}$, Pr78^{gag}, p27, p17$'^{CA}$, and the envelope glycoproteins Pr86^{env}, gp70, and gp20 are indicated. Data are from two separate experiments.

was observed with mutant DEL (Fig. 6). The results of the pulse-chase experiments described to this point are consistent with the RT assay data (Fig. 4).

In pulse-labeled cells transfected with mutants D158Y and F156L, bands corresponding to the three Gag polyproteins (Pr78, Pr95, and Pr180) were detected at levels comparable to those in the wild type (Fig. 6A). After a 4-h chase period, the amounts of the Gag precursors decreased at a rate similar to that in the wild type, but a much reduced level of both cell- and virion-associated CA (p27) was observed (Fig. 6A, D158Y and F156L, lane C; Fig. 6B, D158Y and F156L). This result was discordant with the results of the RT assay, which had indicated that wild-type levels of RT-containing viral particles were released into the medium of transfected cells (Fig. 4).

In the case of mutant D158Y, a virus-specific protein of approximately 17 kDa (here indicated as p17$'^{CA}$) was detected both in the cell lysate and in the extracellular virions (Fig. 6). This band was not observed as a mature viral product in wild-type-transfected cells, nor was it present in wild-type virions released into the culture medium. Its origin is investigated in more detail below.

Analysis of the kinetics of Gag polyprotein turnover and processing in MHR mutants. The metabolic labeling experiments described above showed that the processing of Gag polyproteins was altered in the MHR mutants E154K, L163H, DEL, F156L, and D158Y. Furthermore, in the case of F156L and D158Y, these experiments demonstrated an apparent inconsistency with the findings of the RT assay (see above and Fig. 4 and 6). Since the experiments presented in Fig. 6 examined only a single time point, they did not answer questions about the efficiency of the maturation event or the rate of turnover of unprocessed precursor molecules. To clarify these issues, we performed a time course pulse-chase experiment (Fig. 7). COS-1 cells were transfected with wild-type or mutant proviral DNAs or mock transfected, as indicated. At 48 h posttransfection, the cells were pulse-labeled for 20 min or pulse-labeled and chased in complete medium for 2, 4, or 8 h. At each time point, virus-specific proteins present in both cell lysates and extracellular virions were analyzed by immunoprecipitation and SDS-PAGE.

The processing of wild-type Gag polyproteins was detected following a 2-h chase as indicated by the appearance of CA (p27) in both cell lysates and extracellular virions (Fig. 7A and C, lanes wt/C2). The amount of cell-associated p27 increased following a 4-h chase (Fig. 7A and C, lanes wt/C4); half of the initially synthesized Gag precursor (Pr78^{gag}) was processed by 2 h, consistent with the kinetics of Gag processing described previously (41). Following an 8-h chase, the bulk of the Gag polyprotein had been processed,

and by this time most of the mature CA protein (p27) was found in the medium (Fig. 7A and C, compare cell lysate, lanes wt/C8, and viral pellet, lanes wt/C8). The amounts of the virion-associated envelope glycoproteins gp70 and gp20 in the culture supernatant increased during the 8-h chase with kinetics similar to those displayed by the accumulation of CA (Fig. 7A and C, viral pellet, lanes C2 to C8).

The Gag polyproteins of mutants E154K and L163H were stably expressed in the cells without being released into the culture supernatants and without being processed to mature products even after an extended chase (Fig. 7B, cell lysate, lanes P to C8). The Gag polyproteins of these mutants were turned over with a half-life of approximately 4 h, and even after 8 h, 10 to 25% of the initially synthesized molecules were still cell associated. Although the initial levels of Gag polyprotein synthesized in cells expressing mutant DEL were significantly lower than wild-type levels (Fig. 7, compare panel A, wt, and panel B, DEL, lanes P), the major Gag precursor displayed greater stability and a very reduced rate of turnover (Fig. 7B, DEL, compare lanes P, C2, C4, and C8). The latter observation is in accordance with the findings of the pulse-chase experiment presented in Fig. 6.

In cells transfected with mutant D158Y, the processing of the major Gag polyprotein ($Pr78^{gag}$) followed the wild-type pattern, as judged by the rate at which the precursor disappeared from the cell lysates. However, in this case, much smaller amounts of both cell-associated and released CA were detected at all time points (Fig. 7A, D158Y, lanes C2 to C8). Starting at 2 h, a virus-specific protein of 17 kDa that cross-reacted with the anti-p27 antiserum was detected both in the cell lysates and in the pelletable material from the culture supernatants (Fig. 7A, D158Y, cell lysate and viral pellet, lane C2). This protein thus appeared to be a chase product of CA and was observed to accumulate in the extracellular viral particles throughout an 8-h chase (Fig. 7A, D158Y, viral pellet, lane C8).

Cells expressing the F156L mutant also displayed decreased CA levels in both the cell lysates and the viral pellets after an extended chase period. However, the amount of viral glycoproteins ($Pr86^{env}$, gp70, and gp20) that accumulated in the pellet during the 8-h chase was similar to that found in wild-type particles (Fig. 7C, viral pellet, compare wt and F156L). The latter result was in accordance with the RT assay data (Fig. 4) and indicated that normal amounts of viral particles were being released into the culture supernatants.

Biochemical analysis of released M-PMV mutant virions. To further investigate the phenotypes of mutants D158Y and F156L, we performed a direct biochemical analysis of radiolabeled virions released from COS-1 cells expressing these mutants.

COS-1 cells were transfected with either wild-type, F156L, or D158Y mutant genomic DNA, and at 48 posttransfection, the cells were metabolically labeled for 8 h with a mixture of [^{35}S]methionine and [^{35}S]cysteine. Released virus in the culture fluids was centrifuged on a continuous 24 to 48% (wt/wt) sucrose gradient as described in Materials and Methods, and fractions were collected. An RT assay was performed on an aliquot of each fraction. The viral particles from the remaining portion of each fraction were pelleted by ultracentrifugation, and their protein profile was analyzed by SDS-PAGE.

A peak of RT activity was obtained from wild-type M-PMV DNA-transfected cells at a density of 1.16 g/ml (Fig. 8A). A similar peak was obtained from F156L mutant DNA-transfected cells (Fig. 8C). The presence of viral particles of the expected density (40) was confirmed by direct analysis of the protein content of the high-speed pellets obtained from each sucrose fraction. With both the wild type (Fig. 8A and B) and mutant F156L (Fig. 8C and D), p27 was detected at the expected density in the gradient (1.16 g/ml). In contrast to the immunoprecipitation experiments, it was clear from this direct analysis of virion proteins that the F156L mutant virions contained wild-type levels of p27. These results indicated that the F156L mutation had no effect on virus assembly, but after precursor cleavage, the amino acid substitution caused the loss of a major epitope within the CA protein.

A similar analysis of the polypeptide composition of sucrose gradient-purified D158Y mutant virions from transfected COS-1 cells is presented in Fig. 9. Wild-type M-PMV virions of the expected density were detected in the supernatant of transfected COS-1 cells, as demonstrated by the presence of a peak of p27 between fractions 4 and 7 (density, 1.15 to 1.16 g/ml) as described above. In contrast, in the D158Y mutant virions which sedimented to the same region of the sucrose gradient, significantly less p27 was found; in its place, two new proteins were observed, the $p17^{'CA}$ protein, seen previously in pulse-chase experiments (Fig. 6 and 7), and a 10-kDa protein ($p10^{'CA}$), not present in wild-type virions. The sizes of these novel products indicate that the D158Y mutation generated a new cleavage site for the viral aspartyl protease within the MHR region, so that during maturational cleavage, p27 is further cleaved to $p10^{'CA}$ and $p17^{'CA}$.

Electron microscopic analysis of CA mutants. The experiments described above suggested that different mutants might be blocked at distinct stages in the virus replication cycle. To obtain additional information on the defects in the MHR mutants, we carried out electron microscopic studies of COS-1 cells transfected with wild-type and mutant viral genomes (Fig. 10 and Table 1). In wild-type-M-PMV-genome-transfected cells, preassembled capsids were observed scattered throughout the cytoplasm and could be seen at the plasma membrane in the process of budding; extracellular immature particles were found adjacent to the cell. In addition, occasional capsids could be observed assembling at the plasma membrane as they budded from the cell (data not shown).

In cells transfected with mutants E154K and L163H, no evidence of preassembled capsids could be observed in the cytoplasm, and no particles were found in the process of budding from the plasma membrane (Table 1). Some of the cells expressing the E154K mutant proteins were characterized by the presence of electron-dense patches underlying the plasma membrane (Fig. 10A; Table 1) that were never observed either in wild-type-M-PMV-expressing cells or in nontransfected cells. These results are consistent with the data presented above and suggest that the block imposed by the E154K and L163H mutations on viral replication occurs before the formation of preassembled capsids has taken place.

When the other MHR mutant DNAs were transfected into COS-1 cells, a similar phenotype was observed in every instance (Table 1). The majority of the cells displayed wild-type characteristics (Fig. 10B and C); however, in some of the cells, aberrant structures, such as particles with type C morphology (Fig. 10D), filamentous structures (Fig. 10E and F), and double- and multiple-cored particles (Table 1), could be observed in the same cell as particles that were indistinguishable from their wild-type counterparts. The significance of this result will be discussed below.

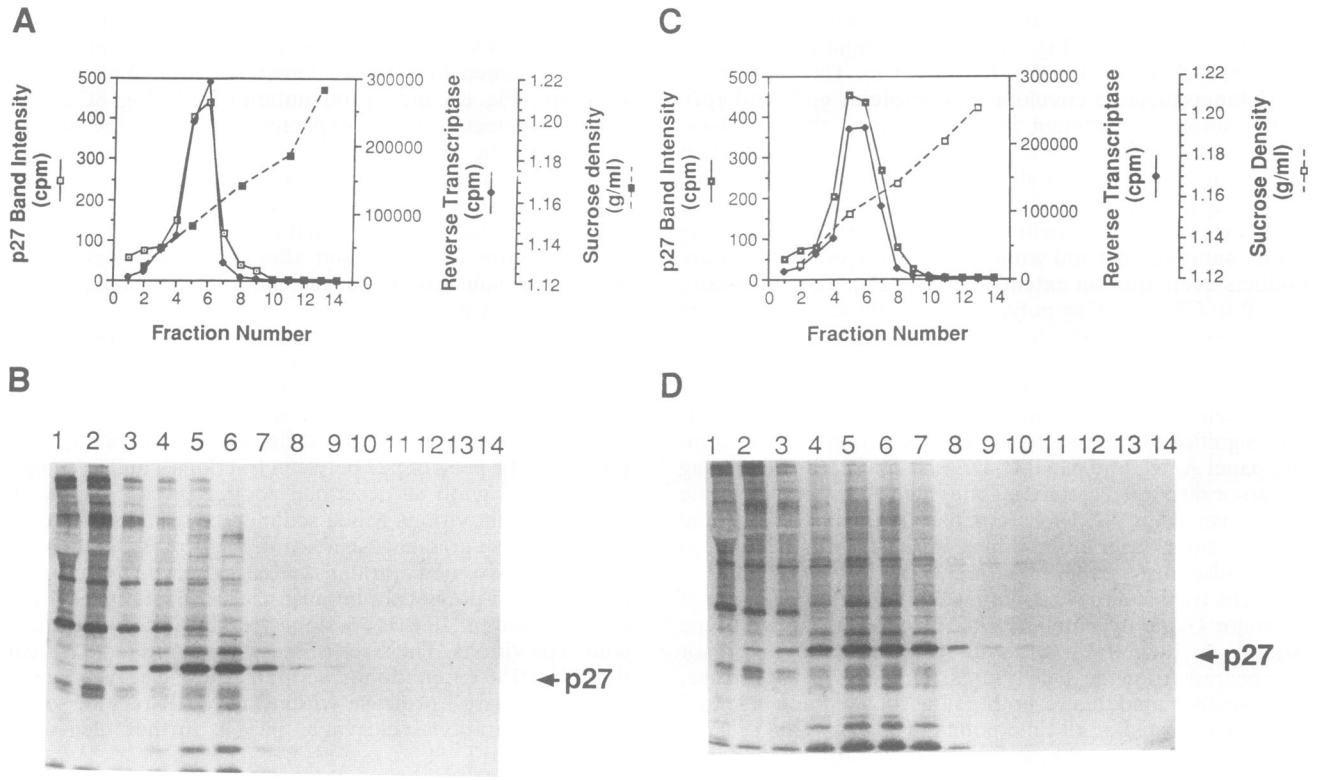

FIG. 8. Analysis of the protein content of viral particles released from F156L mutant-transfected cells. COS-1 cells transfected with either wild-type (wt) (A and B) or F156L mutant (C and D) genomic DNA were metabolically labeled for 8 h with a mixture of [^{35}S]methionine and [^{35}S]cysteine at 48 h posttransfection, and concentrated (see Materials and Methods) culture supernatants were loaded on a linear 24 to 48% (wt/wt) sucrose gradient. (A and C) Fractions (0.8 ml) were collected from the top of the gradient, and an RT assay was performed on an aliquot of each fraction. Quantitation of p27 band intensity by radioanalytic imaging is presented for both the wild type and mutant F156L. (B and D) Viral particles were collected by ultracentrifugation from each of the fractions, and their protein content was analyzed by SDS-PAGE (12% protein gel) and autoradiography of the dried gel. The position of p27, the major capsid protein, is indicated.

DISCUSSION

One of the few conserved regions of the Gag polyprotein of retroviruses has been designated the MHR (50). It spans 20 amino acid residues of CA, and within its length, 6 positions are either absolutely conserved or have single conserved substitutions. The biological significance of this homology is not clear, but it is reasonable to suppose that this evolutionarily conserved domain has a role in one of the events of the retrovirus life cycle in which CA is thought to be involved.

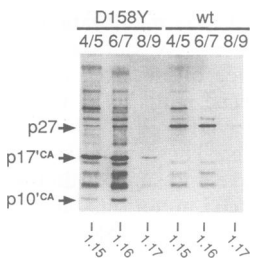

FIG. 9. Analysis of the protein content of viral particles released from D158Y mutant-transfected cells. COS-1 cells transfected with either wild-type (wt) or D158Y mutant genomic DNA were labeled, and virions were fractionated from the supernatant as described in the legend to Fig. 7. Fractions expected to contain M-PMV virions (4 to 9) were pooled in groups of two (4 plus 5, 6 plus 7, and 8 plus 9); each of the pooled fractions was subjected to ultracentrifugation, and the protein profile of the viral pellet was analyzed on a 15% polyacrylamide–SDS gel. The average sucrose density of each of the pooled fractions is indicated at the bottom of the gel (in grams per milliliter). The positions of p27, p17$^{'CA}$, and p10$^{'CA}$ are indicated.

Saturation mutagenesis of M-PMV MHR. This study represents a first attempt to understand the possible biological function of the MHR in the life cycle of retroviruses by means of mutational analysis. Since information about this conserved domain was very limited, we chose to use a mutagenesis method that gave equal weight to each position rather than engineer specific amino acid changes at single positions. For this reason, we used a saturation mutagenesis technique to introduce a series of random missense changes along the central 13 amino acids of M-PMV MHR. With this method, in a single mutagenesis reaction, we were able to obtain 13 different single-amino-acid substitutions along the MHR; all but two (positions 161 and 165) of the positions were changed, and two different independent substitutions were obtained at positions 153 and 157.

Phenotypes of the MHR mutants. The results presented in this article show that the MHR mutants fall into two major phenotypic classes. The first group contains three very drastic mutations that make the mutants unable to release viral particles from the host cell. The first of these mutations (E154K) changes a negative charge to a positive one in an absolutely conserved position of the MHR. The second

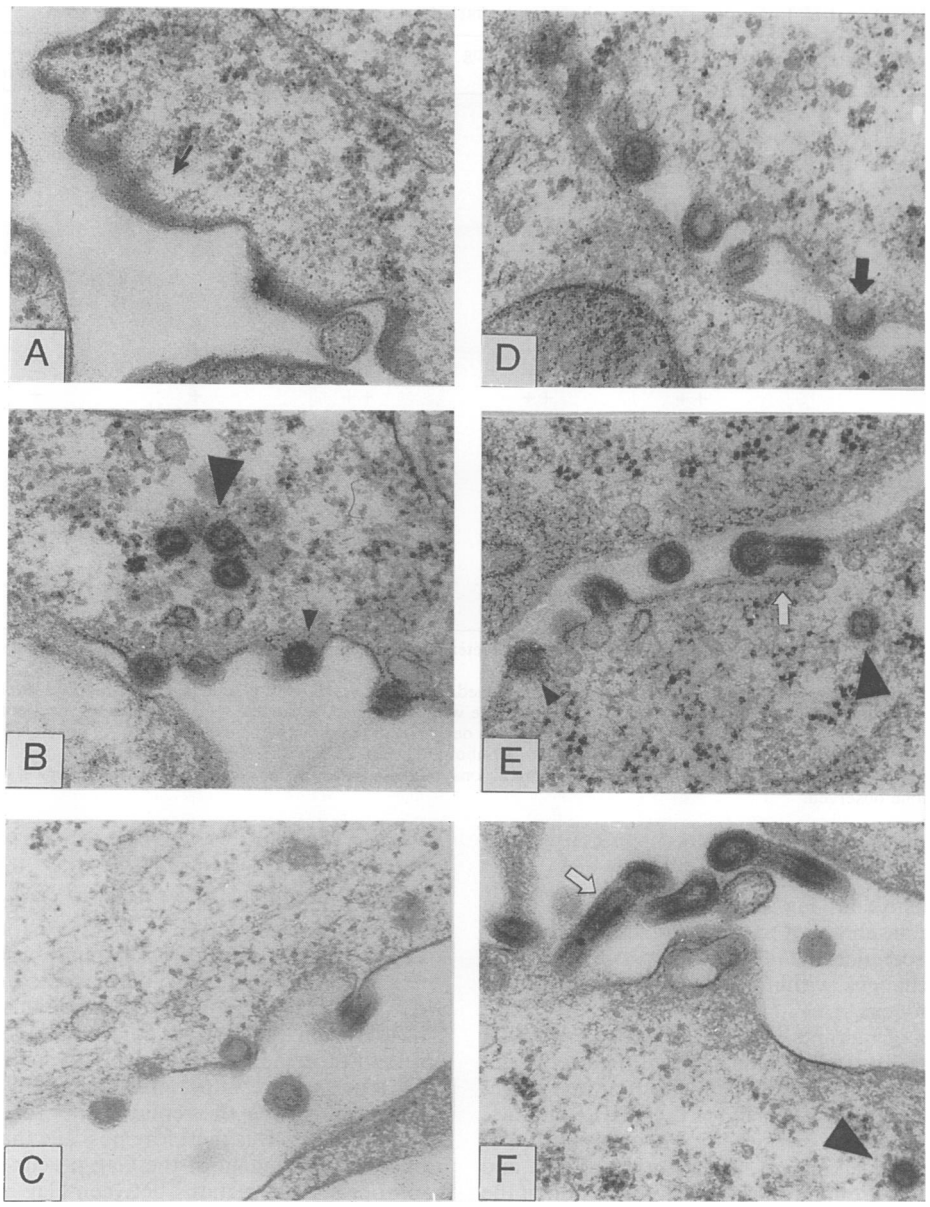

FIG. 10. Electron micrographs of COS-1 cells expressing mutant viral genomes. Thin sections of COS-1 cells which had been transfected with MHR mutant genomes were examined under the electron microscope to determine the morphology of the mutant viruses. (A) In mutant E154K-transfected cells, occasional electron-dense "patches" (arrow) could be observed. (B, C, D, E, and F) The majority of cells expressing MHR mutants capable of releasing virions displayed only wild-type-like structures, such as ICAPs (large arrowheads), budding type D particles at the plasma membrane (small arrowheads), and immature released particles in the immediate vicinity of the cells. A minority of the cells transfected with assembly-competent MHR mutant DNAs displayed various anomalous structures alongside the normal ones. Such structures included filamentous budding structures (open arrows) and budding particles with type C morphology (solid arrow). Representative sections from mutants I164L (B), V160I (C), A157V (D), D158Y (E), and F156L (F) are shown. Magnification, ×75,000.

mutation (L163H) is in a position of nearly absolute identity and substitutes a polar residue for a hydrophobic amino acid. The third mutation (DEL; see Materials and Methods) is an in-frame deletion of three nonconsecutive nucleotides, which results in the deletion of one amino acid residue and the substitution of four more in the central portion of the MHR. When these three mutants were subjected to a pulse-chase analysis, we observed that synthesis of the viral proteins appeared to be normal but the Gag precursors were not processed, consistent with a complete block in virion release. Furthermore, we noticed that in all three cases, the Gag precursor appeared to be relatively stable. When COS-1 cells transfected with the E154K and L163H mutant genomes were analyzed by electron microscopy, no evidence of capsid assembly was observed. These results suggest that these mutations block the formation of viral particles in COS-1 cells without affecting the stability of the precursor. Interestingly, in some of the cells expressing mutant E154K, we observed the presence of electron-dense patches underlying the plasma membrane. This is consistent with the

TABLE 1. Summary of morphological characteristics of MHR mutants in COS-1 cells

Virus	ICAPs in cytoplasm[a]	Type D budding particles[b]	Electron-dense patches[c]	Type C budding particles[d]	Filamentous structures[e]	Other anomalous formations[f]
Wild type	+++	+++	−	+	−	−
Partially infectious, assembly-positive mutants						
P155E	+++	++	++	+	−	−
A157S	+++	+++	−	+	+	+
I164L	+++	+++	−	+	+	+
Noninfectious, assembly-positive mutants						
D153N	++	++	+	−	−	−
D153Y	+	++	++	+	−	+
A157V	++	++	+	+	−	−
F159Y	++	++	+	−	−	−
V160I	++	++	++	+	+	+
R162K	n.d.[g]	n.d.	n.d.	n.d.	n.d.	n.d.
Noninfectious, assembly-negative mutants						
E154K	−	−	+	−	−	−
L163H	−	−	−	−	−	−
DEL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Other mutants						
F156L	++	++	−	−	+	−
D158Y	++	++	++	+	+	+

[a] The presence of ICAPs and their distribution within the cytoplasm were determined. Symbols: +++, wild-type levels, clusters present; ++, reduced levels, clusters present; +, scattered ICAPs; −, none observed.
[b] The presence of budding particles with type D morphology was determined. Symbols: +++, wild-type levels; ++, reduced levels; −, none observed.
[c] The presence of electron-dense patches underneath the plasma membrane was determined. Symbols: ++, numerous; +, occasional; −, none observed.
[d] The presence of budding particles with apparent type C morphology was determined. Symbols: +, some observed; −, none observed.
[e] The presence of tubular structures (Fig. 9C and D) was determined. Symbols: +, some observed; −, none observed.
[f] The presence of other anomalous structures, such as double immature virions, multiple immature virions, and oversized particles, was determined. Symbols: +, some observed; −, none observed.
[g] n.d., not determined.

accumulation of misfolded Gag precursor proteins that are unable to direct the assembly of capsids and are transported incorrectly to the cellular membrane. Nevertheless, it is clear that drastic changes within this region can block the capsid assembly process.

The second class of mutants contains MHR variants that exhibit normal synthesis and processing of viral proteins and virion release but are either greatly impaired in their infectivity or noninfectious. The majority of the MHR mutants, including two with relatively conservative changes at two of the most conserved positions of this region (F159Y and R162K), belong to this group. The protein composition of the mutant virions appeared to be normal, and an RNase protection experiment performed on viral particles released from COS-1 cells expressing wild-type and mutant viral genomes revealed that these mutant virions contained wild-type levels of M-PMV genomic RNA (data not shown). Thus, it appeared that these mutants were defective at some stage beyond virus assembly, release, and maturation.

When COS-1 cells expressing this class of mutants were analyzed by electron microscopy, the majority of the cells were found to display viral structures typical of type D retroviruses. However, we did observe a mixture of aberrant and normal viral structures in a minority of them. The former included filamentous budding structures, double or multiple particles, and oversized particles, all of which were associated with the plasma membrane. One possible explanation for these results is that the amino acid changes introduced in the MHR affect the manner in which the Gag precursors interact with each other and that this defect is emphasized by the overexpression of the viral proteins in COS-1 cells. Preliminary studies in HeLa cell lines that stably expressed an integrated wild-type M-PMV or mutant genome indicated that at the lower levels of protein expression observed in these cells, the bulk of assembling particles had wild-type morphology. Thus, as we will discuss in more detail below, it is likely that the more conservative substitutions in the MHR region can lead to conformational changes that alter protein-protein interactions at both the precursor and mature CA levels. More importantly, changes in the MHR region dramatically alter the replication potential of an apparently normal virion. It is therefore possible that the MHR region is located in a domain of the Gag precursor that both defines appropriate interactions between Gag molecules and is important for events that occur following virus entry during the next round of replication.

The MHR region is located near a region of the CA protein that in MuLV defines the N- or B-tropism of the virus, a phenotype that is characterized by a defect in the integration of nascent proviral DNAs in resistant cells (15, 28). Furthermore, a CA mutation that was found by Hsu et al. to block retrotranscription in newly infected cells maps in the same region of the protein (27). Thus, it is possible that most of the MHR mutations described here affect analogous CA protein functions for M-PMV. Future experiments are aimed at defining the specific defect in these MHR mutants in establishing infection.

MHR mutations can result in alterations in the processing and folding of the CA protein. A subset of the second class of MHR variants contains two mutants, F156L and D158Y, that showed unusual phenotypes. Both of these mutants released viral particles that contained normal amounts of viral RNA (data not shown) but were noninfectious. In addition, cells transfected with either F156L or D158Y

genomic DNA synthesized a wild-type complement of viral proteins. However, the processing of the major Gag precursor and the protein content of the virions appeared to be altered, as judged by the results of pulse-chase experiments. In fact, while the levels of mutant $Pr78^{gag}$ still present in the cells after the chase period were much lower than those synthesized initially, the amounts of p27 produced and released in the form of viral particles were much lower than in the wild type. Interestingly, this result was inconsistent with the results of RT analyses, which demonstrated the release of wild-type amounts of viral particles from transfected COS cells. A direct analysis of the protein composition of virions purified from the culture supernatants of F156L DNA-transfected cells showed that this mutant was able to release wild-type amounts of virions and that these virions contain normal amounts of nonimmunoreactive CA (p27). This result demonstrates that the substitution of a leucine for a phenylalanine at position 156 of CA disrupts what seems to be a single major epitope of this protein without impairing the assembly of ICAPs or the release and maturation of mutant virions. Surprisingly, the same mutation does not detectably alter the ability of the anti-p27 antiserum to recognize the Gag precursor. This indicates that the precursor conformation is tightly maintained in the immature virion and that major conformational changes occur within the CA domain following cleavage of the Gag polyprotein during maturation.

In the case of mutant D158Y, the pulse-chase experiments and the biochemical analysis of released virions presented in this article demonstrated that the mutant Gag precursor is processed to yield two anomalous products. The first one is a protein of approximately 17 kDa ($p17^{'CA}$) and is cross-reactive with antibodies that recognize CA. This protein appeared to be a chase product of p27 and is identifiable in both cell-associated fractions and released virions. The smaller one is a protein of about 10 kDa ($p10^{'CA}$) and is not recognized by either the rabbit serum raised against gel-purified p27 or the goat serum raised against whole M-PMV. For this reason, $p10^{'CA}$ was observed only when the protein content of purified mutant viral particles was determined.

These findings strongly suggest that the D158Y mutation introduces an extra viral protease cleavage site in the CA domain of the Gag precursor and that this site is recognized during the maturation of the released particles to yield $p17^{'CA}$ and $p10^{'CA}$. The sizes of the cleavage products suggest that the new cleavage site is located within the MHR. In addition, this hypothesis is consistent with a survey of retroviral protease cleavage sites by Pettit et al. (38). This predicts that the substitution of a tyrosine for an aspartic acid at position 158 of CA would increase the probability that this region of the precursor would be recognized as a viral protease cleavage site.

The observation that $p10^{'CA}$ is not recognized by either of the antisera used in this study is consistent with the notion that CA possesses a single major epitope and suggests that this epitope is located upstream of the MHR. Interestingly, a prediction of human immunodeficiency virus type 1 capsid protein (p24) conformation based on the structure of picornaviral VP2 coat protein (2, 20) suggests that the MHR, at least in human immunodeficiency virus type 1, is found at the boundary between the F β-sheet, which is thought to be buried inside the protein, and an N-terminally located loop that is thought to be located on the outside of the molecule. Thus, changes in the MHR could affect the way in which this loop might be exposed to antibody binding. Our observation that the D158Y mutant can be cleaved by the viral protease indicates that the MHR region is at the surface of the CA domain (at least within Pr78) and within a sufficiently open configuration for the protease to access this new cleavage site.

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REFERENCES

1. **Adams, S. E., J. Mellor, K. Gull, R. B. Sim, M. F. Tuite, S. M. Kingsman, and A. J. Kingsman.** 1987. The function and relationship of Ty-VLP proteins in yeast reflect those of mammalian retroviral proteins. Cell **49**:111–119.
2. **Argos, P.** 1989. A possible homology between immunodeficiency virus p24 core protein and picornaviral VP2 coat protein: prediction of HIV p24 antigenic sites. EMBO J. **8**:779–785.
3. **Bolognesi, D. P., H. Gelderblom, H. Bauer, K. Molling, and G. Huper.** 1972. Polypeptides of avian RNA tumor viruses. V. Analysis of the virus core. Virology **47**:567–578.
4. **Bolognesi, D. P., R. C. Montelaro, H. Frank, and W. Schafer.** 1978. Assembly of type C oncornaviruses: a model. Science **199**:183–186.
5. **Bowerman, B., P. O. Brown, J. M. Bishop, and H. E. Varmus.** 1989. A nucleoprotein complex mediates the integration of retroviral DNA. Genes Dev. **3**:469–478.
6. **Bradac, J., and E. Hunter.** 1984. Polypeptides of Mason-Pfizer monkey virus. I. Synthesis and processing of the *gag* gene products. Virology **138**:260–275.
7. **Bradac, J. A., and E. Hunter.** 1986. Polypeptides of Mason-Pfizer monkey virus. III. Translational order of proteins on the *gag* and *env* gene specified precursor polypeptides. Virology **150**:503–508.
8. **Brown, P. O., B. Bowerman, H. E. Varmus, and J. M. Bishop.** 1987. Correct integration of retroviral DNA in vitro. Cell **49**:347–356.
9. **Chatterjee, S., J. Bradac, and E. Hunter.** 1985. A rapid screening procedure for the isolation of non-conditional replication mutants of Mason-Pfizer monkey virus: identification of a mutant defective in *pol*. Virology **141**:65–76.
10. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
11. **Chinsky, J., and R. Soeiro.** 1981. Fv-1 restriction of Friend leukemia virus: analysis of unintegrated proviral DNA. J. Virol. **40**:45–55.
12. **Chopra, H. C., and M. M. Mason.** 1970. A new virus in a spontaneous mammary tumor of a rhesus monkey. Cancer Res. **30**:2081–2086.
13. **Daniel, M. D., N. W. King, N. L. Letvin, R. D. Hunt, P. K. Sehgal, and R. C. Desrosiers.** 1984. A new type D retrovirus isolated from macaques with an immunodeficiency syndrome. Science **223**:602–605.
14. **Derbyshire, K. M., J. J. Salvo, and N. D. F. Grindley.** 1986. A simple and efficient procedure for saturation mutagenesis using mixed oligodeoxynucleotides. Gene **46**:145–152.
15. **DesGroseillers, L., and P. Jolicoeur.** 1983. Physical mapping of the Fv-1 tropism host range determinant of BALB/c murine leukemia viruses. J. Virol. **48**:685–696.
16. **Eichinger, D. J., and J. D. Boeke.** 1988. The DNA intermediate in yeast Ty1 element transposition copurifies with virus-like

particles: cell-free Ty1 transposition. Cell **54**:955–966.
17. **Faller, D. V., and N. Hopkins.** 1978. T1 oligonucleotide maps of the N-, B-, and B to NB-tropic murine leukemia viruses derived from BALB/c. J. Virol. **26**:143–152.
18. **Fine, D. L., J. C. Landon, R. J. Pienta, M. T. Kubicek, W. F. Loeb, and H. C. Chopra.** 1975. Responses of infant rhesus monkey to inoculation with Mason-Pfizer monkey virus materials. J. Natl. Cancer Inst. **54**:651–658.
19. **Fine, D. L., and G. Schochetman.** 1978. Type D primate retroviruses: a review. Cancer Res. **38**:3123–3139.
20. **Garry, R. D.** 1990. Extensive antigenic mimicry by retrovirus capsid proteins. AIDS Res. Human Retroviruses **6**:1361–1362.
21. **Gelderblom, H. R.** 1991. Assembly and morphology of HIV: potential effect of structure on viral function. AIDS **5**:617–638.
22. **Gelderblom, H. R., E. H. S. Hausmann, M. Ozel, G. Pauli, and M. A. Koch.** 1987. Fine structure of HIV and immunolocalization of structural proteins. Virology **156**:171–176.
23. **Goff, S., and L. I. Lobel.** 1987. Mutants of murine leukemia viruses and retroviral replication. Biochim. Biophys. Acta **907**: 93–123.
24. **Gorelick, R. J., S. M. Nigida, J. S. Bess, L. O. Arthur, L. E. Henderson, and A. Rein.** 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. J. Virol. **64**:3207–3211.
25. **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.
26. **Henderson, L. E., R. Sowder, G. Smythers, R. E. Benveniste, and S. Oroszlan.** 1985. Purification and N-terminal amino acid sequence comparisons of structural proteins from retrovirus-D/Washington and Mason-Pfizer monkey virus. J. Virol. **55**:778–787.
27. **Hsu, H., P. Schwartzberg, and S. P. Goff.** 1985. Point mutation in the p30 domain of the *gag* gene of Moloney murine leukemia virus. Virology **142**:211–214.
28. **Jolicoeur, P., and E. Rassart.** 1980. Effect of Fv-1 gene product on the synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. J. Virol. **33**:183–195.
29. **Jones, T. A., G. Blaug, M. Hansen, and E. Barklis.** 1990. Assembly of *gag*-β-galactosidase proteins into retrovirus particles. J. Virol. **64**:2265–2279.
30. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
31. **Lobel, L. I., and S. P. Goff.** 1984. Construction of mutants of Moloney murine leukemia virus by suppressor-linker insertional mutagenesis: position of viable insertion mutation. Proc. Natl. Acad. Sci. USA **8**:4149–4153.
32. **Makris, J. C., P. L. Nordmann, and W. S. Reznikoff.** 1988. Saturation mutagenesis using double-stranded oligonucleotides containing complementary mixed-sequence single strands. DNA Protein Eng. Tech. **1**:36–38.
33. **Marx, P. A., D. H. Maul, K. G. Osborn, N. W. Lerche, P. Moody, L. J. Lowenstine, R. V. Henrickson, L. O. Arthur, R. V. Gilden, M. Gravell, W. T. London, J. L. Sever, J. A. Levy, R. J. Munn, and M. B. Gardner.** 1984. Simian AIDS: isolation of a type D retrovirus and transmission of the disease. Science **223**:1083–1086.
34. **Meric, C., E. Gouilloud, and P. Spahr.** 1988. Mutations in Rous sarcoma virus nucleocapsid protein p12 (NC): deletions of Cys-His boxes. J. Virol. **62**:3328–3333.
35. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
36. **Patarca, R., and W. A. Haseltine.** 1985. A major retroviral core protein related to EPA and TIMP. Nature (London) **318**:390.
37. **Pepinsky, R. B., D. Cappiello, C. Wilkowsky, and V. M. Vogt.** 1980. Chemical crosslinking of proteins in avian sarcoma and leukemia viruses. Virology **102**:205–210.
38. **Pettit, S. C., J. Simsic, D. D. Loeb, L. Everitt, C. A. Hutchison III, and R. Swanstrom.** 1991. Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the P1 amino acid. J. Biol. Chem. **266**:14539–14547.
39. **Rhee, S. S., H. Hui, and E. Hunter.** 1990. Preassembled capsids of type D retroviruses contain a signal sufficient for targeting specifically to the plasma membrane. J. Virol. **64**:3844–3852.
40. **Rhee, S. S., and E. Hunter.** 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61**:1045–1053.
41. **Rhee, S. S., and E. Hunter.** 1991. Amino acid substitutions within the matrix protein of type D retroviruses affect assembly, transport and membrane association of a capsid. EMBO J. **10**:535–546.
42. **Sacks, T. L., S. G. Devare, G. T. Blennerhassett, and J. R. Stephenson.** 1978. Nonconditional replication mutants of type C and type D retroviruses defective in *gag* gene-coded polyprotein post-translational processing. Virology **91**:352–363.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
44. **Schwartzberg, P., J. Colicelli, M. L. Gordon, and S. P. Goff.** 1984. Mutations in the *gag* gene of Moloney murine leukemia virus: effects on production of virions and reverse transcriptase. J. Virol. **49**:918–924.
45. **Selden, R. F., K. Burke-Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore.** 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol. Cell. Biol. **6**:3173–3179.
46. **Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson.** 1986. Nucleotide sequence of Mason-Pfizer monkey virus: an immunosuppressive D-type retrovirus. Cell **45**:375–385.
47. **Stromberg, K., R. E. Benveniste, L. O. Arthur, H. Rabin, W. E. Giddens, H. D. Ochs, W. R. Morton, and C. Tsai.** 1984. Characterization of exogenous type D retrovirus from a fibroma of a macaque with simian AIDS and fibromatosis. Science **224**:289–292.
48. **Stromberg, K., N. E. Hurley, N. L. Davis, R. R. Rueckert, and E. Fleissner.** 1974. Structural studies of avian myeloblastosis virus: comparison of polypeptides in virion and core component by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Virol. **13**:513–528.
49. **Teramoto, Y. A., R. D. Cardiff, and J. K. Lund.** 1977. The structure of mouse mammary tumor virus: isolation and characterization of the core. Virology **77**:135–148.
50. **Wills, J. W., and R. C. Craven.** 1991. Form, function and use of retroviral Gag proteins. AIDS **5**:639–654.
51. **Yang, W. K., J. O. Kiggans, D. Yang, C. Ou, R. W. Tennant, A. Brown, and R. H. Bassin.** 1980. Synthesis and recircularization of N- and B-tropic retroviral DNA in Fv-1 permissive and restrictive mouse cells. Proc. Natl. Acad. Sci. USA **77**:2994–2998.