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Purification and N-Terminal Amino Acid Sequence Comparisons of Structural Proteins from Retrovirus-D/Washington and Mason-Pfizer Monkey Virus

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A new D-type retrovirus originally designated SAIDS-D/Washington and here referred to as retrovirus-D/Washington (R-D/W) was recently isolated at the University of Washington Primate Center, Seattle, Wash., from a rhesus monkey with an acquired immunodeficiency syndrome and retroperitoneal fibromatosis. To better establish the relationship of this new D-type virus to the prototype D-type virus, Mason-Pfizer monkey virus (MPMV), we have purified and compared six structural proteins from each virus. The proteins purified from each D-type retrovirus include p4, p10, p12, p14, p27, and a phosphoprotein designated pp18 for MPMV and pp20 for R-D/W. Amino acid analysis and N-terminal amino acid sequence analysis show that the p4, p12, p14, and p27 proteins of R-D/W are distinct from the homologous proteins of MPMV but that these proteins from the two different viruses share a high degree of amino acid sequence homology. The p10 proteins from the two viruses have similar amino acid compositions, and both are blocked to N-terminal Edman degradation. The phosphoproteins from the two viruses each contain phosphoserine but are different from each other in amino acid composition, molecular weight, and N-terminal amino acid sequence. The data thus show that each of the R-D/W proteins examined is distinguishable from its MPMV homolog and that a major difference between these two D-type retroviruses is found in the viral phosphoproteins. The N-terminal amino acid sequences of D-type retroviral proteins were used to search for sequence homologies between D-type and other retroviral amino acid sequences. An unexpected amino acid sequence homology was found between R-D/W pp20 (a *gag* protein) and a 28-residue segment of the *env* precursor polyprotein of Rous sarcoma virus. The N-terminal amino acid sequences of the D-type major *gag* protein (p27) and the nucleic acid-binding protein (p14) show only limited amino acid sequence homology to functionally homologous proteins of C-type retroviruses.

Mason-Pfizer monkey virus (MPMV) was first isolated from a rhesus monkey breast carcinoma (4). The virus buds from the cell membrane of infected cells by envelopment of preformed A particles, and the mature virions contain a cone-shaped, centrally located nucleoid. The virus-encoded reverse transcriptase requires magnesium rather than manganese as a divalent cation for full activity. These unique morphological and biochemical properties have led to the classification of MPMV as a D-type retrovirus. The virus is exogenous in macaques, since nucleic acid sequences homologous to the virus are present only in infected animals (1, 8).

Additional D-type retroviruses have also been isolated from the squirrel monkey, a New World primate (squirrel monkey retrovirus) (2, 13), and from the langur, an Old World monkey (45). Unlike MPMV, these viruses are endogenous, genetically transmitted retroviruses that are found in all cells of all animals of those species (1, 45). Nucleic acid hybridization studies have suggested that the langur virus is partially related to MPMV and that MPMV may have been derived from an endogenous virus of langurs or another closely related species (1).

The inoculation of MPMV into macaques has not led to tumor formation, but after inoculation of newborn rhesus monkeys, a disease spectrum characterized by lymphadenopathy, weight loss, diarrhea, and opportunistic infections has been observed (10). A similar syndrome has occurred spontaneously in various species of macaques housed at the New England, California, and Washington Regional Primate Research Centers (11, 20, 22). This disease, called simian acquired immunodeficiency syndrome, has been thought to be associated with a recently discovered group of D-type retroviruses that are related to MPMV (5, 25, 43). These newly isolated viruses have been referred to as retrovirus-D or simian acquired immunodeficiency syndrome virus. The simian acquired immunodeficiency syndrome disease at the Washington Regional Primate Research Center is also associated with a fibromatous tumor termed retroperitoneal fibromatosis which is characterized by an aggressive proliferation of highly vascularized fibrous tissue (11).

The immunological relatedness of retrovirus-D to the other D-type retroviruses (MPMV, langur virus, and squirrel monkey retrovirus) has been determined in specific radioimmunoassays for the major core protein (p27) and the envelope glycoprotein purified from MPMV. Retrovirus-D and langur virus share common antigenic determinants with MPMV p27 (25, 43). The retrovirus-D isolates from the three primate centers (Washington, New England, and California) have also been compared in radioimmunoassays to the MPMV gp70 and p10 proteins. The results show that antigens of retrovirus-D/Washington (R-D/W) are immunologically distinct from those present in retrovirus-D/New England and retrovirus-D/California (25, 43; L. Arthur, unpublished data). Of this family of related D-type retroviruses, R-D/W is the only isolate that is associated with retroperi-

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toneal fibromatosis. The tumor can be induced by inoculation of macaques with filtered cell culture media obtained from mammalian cell lines producing R-D/W (R. Benveniste, unpublished data).

Little is known about the molecular structure of the D-type retroviruses; the nucleic acid sequence of the viral genome is not known, and the N-terminal amino acid sequence of only one viral protein (MPMV p27) has been determined (29). As a first step toward elucidating the detailed molecular structures of the D-type retroviruses and to provide a better understanding of the relationships between various D-type isolates and the relationships between D-type retroviruses and other members of the retrovirus family, we have purified six structural proteins from MPMV and six structural proteins from R-D/W and determined the amino acid content and N-terminal amino acid sequences of these purified D-type viral proteins. Proteins isolated from each D-type virus were designated according to their molecular weights expressed in thousands, and wherever possible, the notation system used here is consistent with the protein designations used by others to denote the structural proteins of MPMV (36, 37, 46). The proteins isolated from each virus include p4, p10, p12, p14, and p27, as well as two phosphoproteins designated pp20 (R-D/W) and pp18 (MPMV). The data reveal that the p4, p10, p12, p14, and p27 proteins of R-D/W are related to but not identical to the comparable proteins of MPMV. The phosphoproteins of R-D/W and MPMV each contain phosphoserine but are unequal in size and have distinctly different N-terminal amino acid sequences and amino acid compositions. The data thus clearly indicate that R-D/W is related to MPMV but that these two similar D-type retroviruses may have different major phosphoproteins.

MATERIALS AND METHODS

Virus production. R-D/W virus was grown in dog thymus cells (FCf2Th) or a human lung adenocarcinoma cell line (A549) and harvested by centrifugation followed by sucrose density gradient centrifugation as previously described (1). MPMV was grown in A549 cells and purified in a similar fashion.

Chemicals. All reagents used in the liquid-phase spinning cup sequencer were purchased from Beckman Instruments, Palo Alto, Calif. Polybrene was purchased from Aldrich Chemical Co., Milwaukee, Wis. Guanidine-hydrochloride (enzyme grade) was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Acetonitrile, 1-propanol, ethylacetate, benzene, and butyl chloride were obtained from Burdick Jackson, Muskegon, Mo. Sequencer grade trifluoroacetic acid (TFA) was purchased from Pierce Chemical Co., Rockford, Ill.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10-to-20% gradient gels by the method of Laemmli (21). Proteins were visualized by staining with Coomassie brilliant blue R-250, Bio-Rad Laboratories, Richmond, Calif.

Amino acid analysis. Samples for amino acid analysis were hydrolyzed for 24 h in vacuo with 6 N HCl containing 0.1% liquid phenol and then dried by vacuum desiccation. Analysis was performed with a Durrum D-500 amino acid analyzer and ninhydrin detection of eluted amino acids.

***o*-Phosphoamino acid analysis.** Samples were hydrolyzed in vacuo in 4 N HCl at 110°C for 1.5 h. After the 4 N HCl was removed by evaporation under reduced pressure, the samples were dissolved in 0.006 N HCl and injected onto an anion exchange column (Synchropak AX300), and the *o*-phosphoamino acids were separated at 50°C by isocratic elution at a flow rate of 1.3 ml/min with 15 mM KH_2PO_4 adjusted to pH 3.0 with concentrated H_3PO_4 and made 12.5% (vol/vol) in methanol (48). Eluted phosphoamino acids were detected by fluorescence after reaction with orthophthalaldehyde reagent (32).

Liquid-phase sequencing. Semiautomated microsequencing was performed with an 890C Beckman sequencer updated and modified as previously described (15). Phenylthiohydantoin (PTH) derivatives of amino acid were quantitatively identified as previously described (14).

Separation of viral proteins by RP-HPLC. For reversed-phase high-pressure liquid chromatography (RP-HPLC), concentrated suspensions of purified virus (1 mg/ml) in 0.1 M sodium phosphate (pH 7.0) were saturated with guanidine hydrochloride, made 10% (vol/vol) in mercaptoethanol and kept at 4°C overnight. The resulting slightly turbid solution of disrupted virus was adjusted to pH 2.0 by addition of TFA and injected into a high-pressure liquid chromatograph (Waters Associates, Milford, Mass.), and viral proteins were separated on a μBondapak C_{18} column (Waters Associates). Eluted proteins were detected by measuring UV A_{206} with a model 450 variable-wavelength detector (Waters Associates) and collected manually to optimize separations and recoveries. Solvents were removed by lyophilization. Flow rate, elution solvent, and gradient conditions are given in the figure legends. After each separation of viral proteins, the RP-HPLC column was washed at 50°C with 120 ml of 1-propanol–water–TFA (90:10:0.1) at a flow rate of 0.2 ml/min. Columns were stored in 1-propanol or in acetonitrile containing 0.05% TFA. Columns were frequently monitored for their ability to separate a standard mixture of proteins and peptides (50 μg each of bovine serum albumin, ovalbumin, lysozyme, ribonuclease, methionine-enkephalin, and leucine-enkephalin). When necessary, columns were cleaned and repacked as previously described (18).

RESULTS

In previous publications, we have described the separation of structural proteins from C-type (17) and B-type (19) retroviruses by RP-HPLC. In this communication, we will describe the separation of the structural proteins of D-type retroviruses by RP-HPLC and show some of the structural differences between two similar D-type viruses (R-D/W and MPMV).

The proteins associated with purified R-D/W virus and MPMV were first compared by SDS-PAGE (Fig. 1). In the low-molecular-weight region of the gel (less than 30,000), the structural proteins associated with each virus appeared quite similar. Each lane contained prominent bands labeled p27, p14, p12, and p10. The mobilities of these bands in the R-D/W lane (Fig. 1, lane 1) appeared identical to the mobilities of the like bands in the MPMV lane (lane 2). As will be shown, these bands correspond to major structural proteins present in each virus preparation. In the region of the gel bracketed between 21,000 and 16,000 (Fig. 1, brackets) the two lanes appeared dissimilar. A band at 18,000 appeared more prominent in the MPMV lane (Fig. 1, lane 2) than in the R-D/W lane (lane 1), and a region of staining between 21,000 and 19,000 in the R-D/W lane (lane 1) was not apparent in the MPMV lane (lane 2). Each virus contained a phosphoprotein that migrated in this region of the gel and was present in the virus in amounts comparable to the amounts of p27, p14, p12 and p10 found in the virus. However, these phosphoproteins did not stain as intensely as did the other viral proteins with

Coomassie brilliant blue, and each phosphoprotein migrated as a wide band in SDS-PAGE. The R-D/W phosphoprotein migrated as a wide band extending over the region of the gel from 21,000 to 19,000, and the MPMV phosphoprotein migrated as a wide band extending over the region of the gel from 18,000 to 16,000. Here we will refer to the R-D/W phosphoprotein as pp20 and the MPMV phosphoprotein as pp18 (previously designated pp16-18 in reference 3).

The low-molecular-weight proteins associated with R-D/W were separated by elution from an RP-HPLC column with discontinuous gradients of acetonitrile (0.5% TFA) (Fig. 2). The eluate containing each UV peak was collected as indicated, and the proteins were identified by SDS-PAGE. The major viral protein (p14, pp20, p12, p4, p10, or p27) found in each UV peak is indicated in Fig. 2. The SDS-PAGE analysis of the eluted proteins is shown in Fig. 3. The purified viral proteins p14, pp20, p12, and p10 were used for chemical analysis without further purification. The viral protein p27 was further purified by rechromatography (RP-HPLC; data not shown). After rechromatography, the p27 was judged to be at least 95% homogenous by SDS-PAGE analysis. The p4 protein was not readily detected with Coomassie brilliant blue stain, but no other proteins were detected in the SDS-PAGE analysis of this column fraction (Fig. 3, lane 4). Moreover, the p4 protein gave a single symmetrical peak when rechromatographed (data not shown).

By visually comparing the staining intensities of the purified proteins (Fig. 3) with the staining intensities of the respective proteins seen in the whole-virus preparation (Fig. 1), we estimated that greater than 60% of the total amounts of the p14, p12, and p10 proteins present in the virus preparation were recovered from the chromatography step. However, it appears that only 30% or less of the total amount of p27 present in the virus preparation was recovered from the initial chromatography. The reason for the apparently low recovery of p27 is at present unknown.

The quantitative recovery of the pp20 protein is difficult to judge by comparing the SDS-PAGE gels shown in Fig. 1 and 3 because the staining intensity of this protein band was found to greatly decrease with increasing time in the destaining solution. The gel shown in Fig. 3 was photographed after 20 h of destaining while the pp20 band was still clearly visible. The gel shown in Fig. 1 was more completely destained to permit convenient photography of the minor bands present in the preparation, but this resulted in the partial destaining of the phosphoprotein band in the R-D/W lane. However, it is apparent from the chromatogram shown in Fig. 2 that the area of the UV peak identified with pp20 is comparable to the areas of the other viral proteins, suggesting that purified pp20 is recovered from the virus preparations in amounts comparable to the amounts of the other viral proteins recovered. This suggestion is also supported by quantitative amino acid analysis of the purified proteins.

The low-molecular-weight proteins (p4, p10, p12, p14, p27, and pp18) associated with MPMV were separated by RP-HPLC (Fig. 4) by techniques and procedures similar to those described above for the separation of R-D/W proteins. The p14, p10, and p27 proteins of MPMV, purified as described in the legend to Fig. 4, were analyzed by SDS-PAGE without further purification. The pp18, p12, and p4 proteins were each taken for rechromatography as described in the legend to Fig. 4 (data not shown) before SDS-PAGE analysis. The results of SDS-PAGE analysis of the proteins (approximately 2 μg) collected in column fractions 1, 3, 4, 5, and 6 (Fig. 4) are shown in Fig. 5. Column fraction 2 (Fig. 4), containing the p4 protein, was omitted from the gel analysis shown in Fig. 5. The purified p14, pp18, p12, p10, and p27 proteins appeared to be at least 90% homogeneous when analyzed by SDS-PAGE (Fig. 5), and upon further analysis, each gave a single, unique N-terminal amino acid sequence (see below). The gel shown in Fig. 5 was soaked in destaining solvent for 48 h before photography, which resulted in a partial destaining of the pp18 protein (Fig. 5, lane 3). As with pp20 of R-D/W, the tendency for the phosphoprotein of MPMV (pp18) to partially destain during the destaining process made it difficult to estimate the abundance of pp18 in the gel shown in Fig. 1, lane 2.

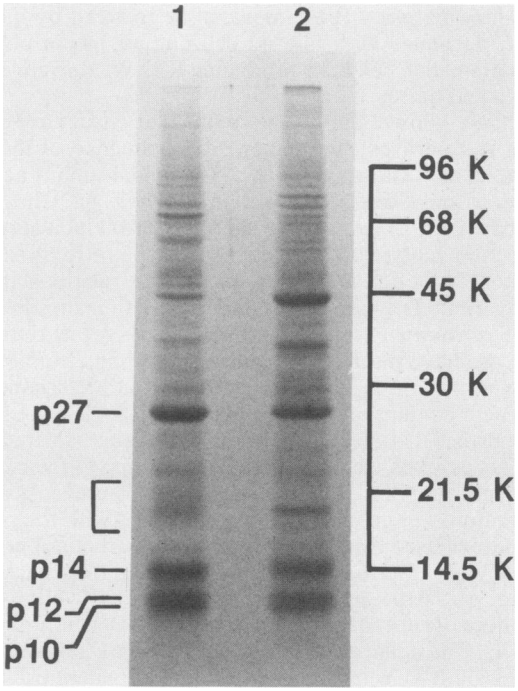

FIG. 1. SDS-PAGE analysis of proteins associated with purified R-D/W virus (lane 1) and MPMV (lane 2). Proteins were separated by electrophoresis through a 10-to-20% gradient slab gel prepared by the method of Laemmli (21). Each lane contains proteins from 5.0 μl of 1,000× concentrated and purified virus. The p27, p14, p12, and p10 proteins of R-D/W are indistinguishable from the homologous proteins of MPMV by mobility in SDS-PAGE. A Bracket ([) indicates the approximate migration of the viral phosphoproteins; however, the phosphoproteins of these D-type viruses do not migrate as discrete bands (see Fig. 6) and are not readily visualized by the staining procedure used here. The positions of standard molecular weight marker proteins (14.5K through 96K; low-molecular-weight standards supplied by Bio-Rad Laboratories) are indicated on the right. Proteins were visualized by staining with Coomassie brilliant blue.

A comparison of the apparent mobilities of R-D/W pp20 (Fig. 3, lane 2) and MPMV pp18 (Fig. 5, lane 2) suggests that the phosphoproteins of the two different viruses do not have the same apparent molecular weight. The mobilities of the R-D/W pp20 and MPMV pp18 proteins were compared directly on the same SDS-PAGE gel as shown in Fig. 6, where it is apparent that the R-D/W pp20 migrated more slowly than the MPMV pp18. In Fig. 3 and 6, the R-D/W pp20 appeared as a wide band extending from the 21,000 to the 19,000 region of the gel and, on close inspection, appeared to be composed of at least four evenly spaced narrow bands on an evenly stained background. The evenly

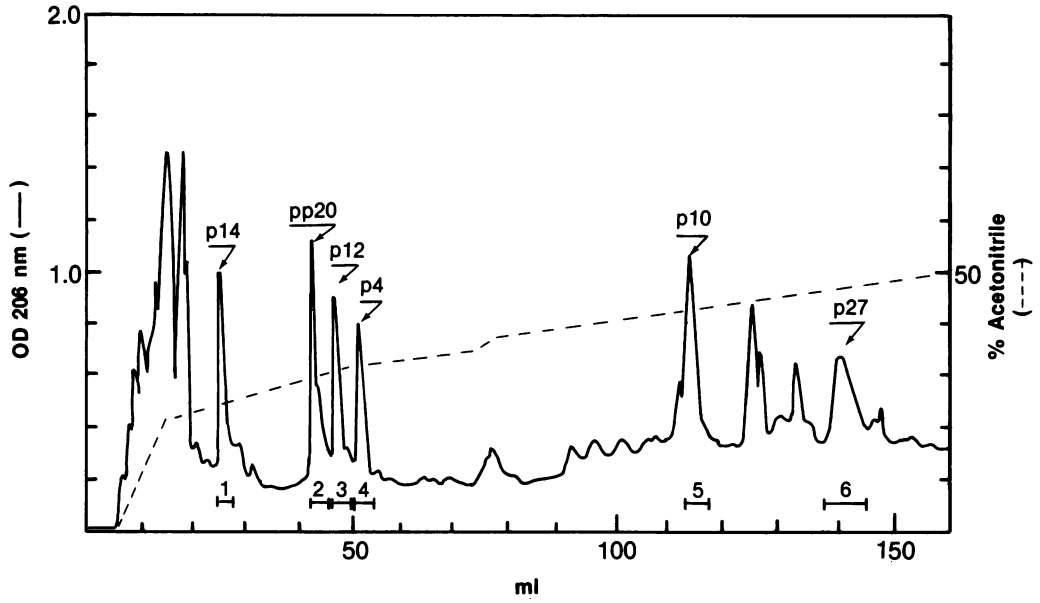

FIG. 2. Chromatographic separation of R-D/W proteins by RP-HPLC. R-D/W virus (2.0 ml; 1,000× concentrate containing 2.0 mg of protein) was applied to the column and eluted at a flow rate of 1.0 ml/min at pH 2.0 (0.5% TFA) with gradients of increasing concentration of acetonitrile as indicated. Peaks of A_{206} (⊢—⊣) were collected. The viral proteins present in the labeled UV peaks were identified by SDS-PAGE (see Fig. 3). OD_{206}, Optical density at 206 nm.

spaced bands were most easily seen during the destaining process. The MPMV pp18 also appeared as a wide band but did not appear to be composed of evenly spaced narrow bands.

The results of amino acid analysis of the various purified R-D/W and MPMV proteins are presented in Table 1, together with the estimated minimum molecular weights of the proteins based on their amino acid analysis data. The minimum molecular weights of the virus-associated p10, p12, and p27 proteins are in close agreement with the molecular weights of these proteins as determined by their SDS-PAGE mobility (Fig. 1). The minimum molecular weight of the p14 proteins based on amino acid analysis is about 11,000, compared with a molecular weight of approximately 14,000 based on their SDS-PAGE mobility. This apparent discrepancy is probably due to an overestimate of the molecular weight of p14 as determined by the SDS-PAGE mobility. The p14 of MPMV has been shown to be a nucleic acid-binding protein with a basic isoelectric point (24). The amino acid composition of MPMV p14 listed in Table 1 is in good agreement with the previously reported amino acid composition of the nucleic acid-binding protein of MPMV (24). Other basic retroviral proteins with nucleic acid-binding properties have also shown anomalously slow mobilities in SDS-PAGE. For example the p10 of Rauscher leukemia virus has a molecular weight of 6,347 as determined by amino acid sequence (15) but has been designated p10 on the basis of mobility in SDS-PAGE.

The more hydrophilic R-D/W and MPMV proteins were also analyzed for phosphoamino acids by a semiquantitative method. The pp20 protein of R-D/W and the pp18 protein of MPMV were found to contain phosphoserine but no detectable phosphothreonine or phosphotyrosine. The amount of phosphoserine contained in the phosphoproteins could not be accurately determined because of losses incurred during hydrolysis. However, it was our impression that the

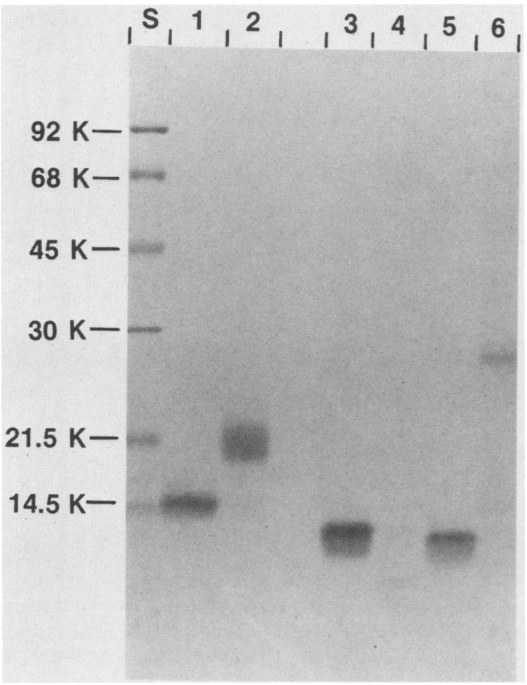

FIG. 3. SDS-PAGE analysis of R-D/W structural proteins purified by RP-HPLC (Fig. 2). Samples of fractions 1 through 4 (4.8% each), fraction 5 (4.2%), and fraction 6 (2.4%) (Fig. 2) were applied to gel lanes 1 through 6 (indicated according to pool number). Lane S contains molecular weight marker proteins (14.5K through 92K). The SDS-PAGE analysis was performed as described in the legend to Fig. 1.

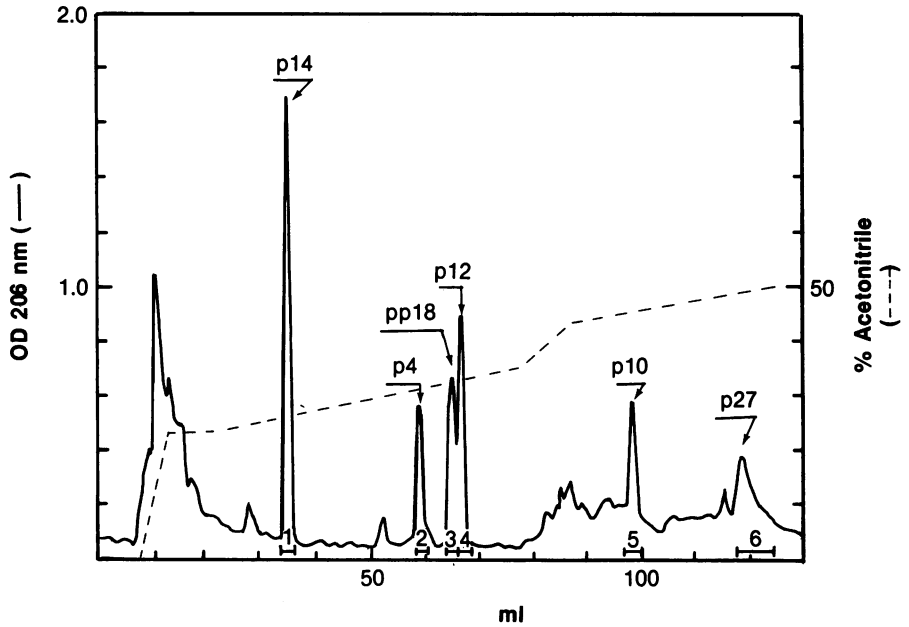

FIG. 4. Chromatographic separation of MPMV proteins by RP-HPLC. MPMV (2.0 ml; 1,000× concentrate containing 2.0 mg of protein) was applied to the column and eluted as described in the legend to Fig. 2. Column fractions (⊢⊣) were pooled. The purified proteins in each labeled peak were identified by SDS-PAGE as shown in Fig. 5. OD_{206}, Optical density at 206 nm.

phosphoproteins could contain at least 1 mol of phosphoserine per mol of protein. No phosphoamino acids were detected in the other R-D/W and MPMV proteins analyzed (p14, p12, and p4).

The R-D/W and MPMV proteins were further compared by N-terminal amino acid sequence analysis. Each purified protein was subjected to N-terminal Edman degradation, and the newly formed PTH-amino acid from each Edman cycle was identified by RP-HPLC. The experimentally determined N-terminal amino acid sequences of the purified R-D/W and MPMV proteins are presented in Fig. 7.

The p14 proteins of R-D/W and MPMV have identical N-terminal amino acid sequences through residue 15. No

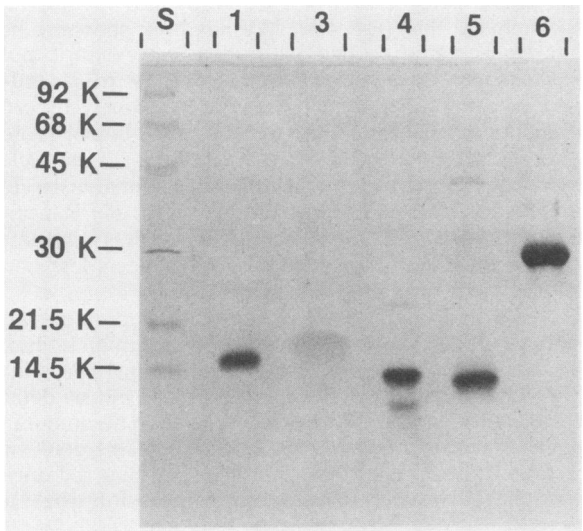

FIG. 5. SDS-PAGE analysis of MPMV structural proteins purified by RP-HPLC. Samples (approximately 2 μg) of protein obtained from column pools 1, 3, 4, 5, and 6 (Fig. 4) were taken for analysis. Gel lanes are numbered according to pool numbers shown in Fig. 4. The protein from pools 3 and 4 shown in lanes 3 and 4 was rechromatographed before SDS-PAGE analysis. Pool 2 contained p4 (see the text) and was not included in the SDS-PAGE analysis shown. Lane S contains molecular weight marker proteins (14.5K through 92K).

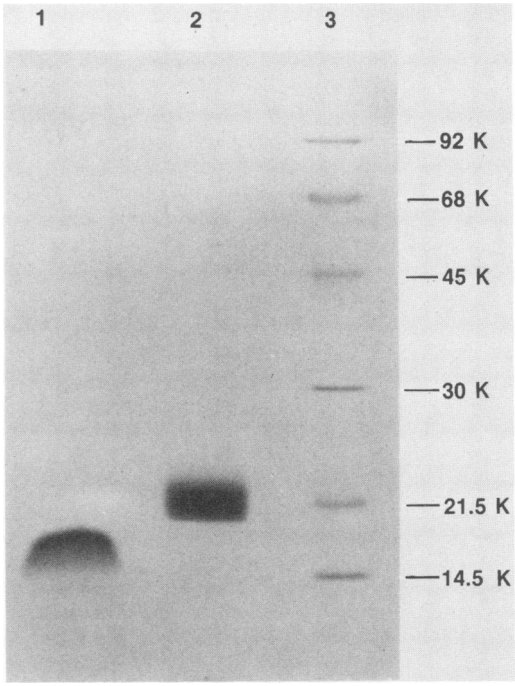

FIG. 6. Comparisons of MPMV pp18 (lane 1) and R-D/W pp20 (lane 2) mobilities on SDS-PAGE. Conditions were as described in the legend to Fig. 1. Molecular weight marker proteins (14.5K to 92K) are shown in lane 3.

TABLE 1. Amino acid analysis of proteins purified from R-D/W and MPMV

Residue	Mol of residue per mol of protein[a]:											
	p14		pp20	pp18	p12		p4		p10		p27	
	R-D/W	MPMV	R-D/W	MPMV	R-D/W	MPMV	R-D/W	MPMV	R-D/W	MPMV	R-D/W	MPMV
Aspartic acid	13.0 (13)	12.2 (12)	28.0 (28)	24.7 (25)	7.3 (7)	6.4 (6)	4.1 (4)	4.0 (4)	11.1 (11)	9.3 (9)	23.9 (24)	24.7 (25)
Threonine	2.7 (3)	2.7 (3)	9.9 (10)	13.2 (13)	5.7 (6)	7.3 (7)	2.0 (2)	2.0 (2)	4.6 (5)	4.7 (5)	19.3 (19)	17.7 (18)
Serine	5.0 (5)	3.1 (3)	16.0 (16)	8.0 (8)	10.1 (10)	5.2 (5)	3.9 (4)	2.8 (3)	2.6 (3)	3.6 (4)	11.3 (11)	15.1 (15)
Glutamic acid	8.9 (9)	10.9 (11)	20.7 (21)	15.4 (15)	21.4 (21)	22.7 (23)	6.0 (6)	6.0 (6)	11.8 (12)	12.1 (12)	26.3 (26)	26.9 (27)
Proline	8.0 (8)	8.2 (8)	18.0 (18)	30.6 (31)	6.7 (7)	8.5 (9)	6.5 (7)	7.0 (7)	5.0 (5)	4.8 (5)	13.4 (13)	12.3 (12)
Glycine	10.8 (11)	11.3 (11)	4.8 (5)	4.2 (4)	5.6 (6)	6.9 (7)	1.1 (1)	1.0 (1)	6.0 (6)	6.0 (6)	22.7 (23)	21.2 (21)
Alanine	6.6 (7)	8.8 (9)	14.6 (15)	14.1 (14)	1.7 (2)	7.3 (7)	2.0 (2)	2.6 (3)	3.8 (4)	5.0 (5)	28.4 (28)	26.0 (26)
Valine	1.6 (2)	2.0 (2)	6.2 (6)	4.2 (4)	3.7 (4)	3.3 (3)	3.8 (4)	3.7 (4)	6.4 (6)	7.3 (7)	12.4 (12)	13.1 (3)
Methionine	1.2 (1)	0	6.2 (6)	2.3 (2)	1.2 (1)	0	0	0	1.2 (1)	1.2 (1)	4.5 (5)	3.2 (3)
Isoleucine	0	1.3 (1)	4.2 (4)	0	8.0 (8)	7.4 (7)	1.0 (1)	0	4.6 (5)	3.3 (3)	10.7 (11)	7.3 (7)
Leucine	4.3 (4)	2.9 (3)	10.6 (11)	6.5 (7)	11.6 (12)	11.0 (11)	1.1 (1)	1.2 (1)	8.2 (8)	7.8 (8)	17.2 (17)	19.9 (20)
Tyrosine	1.1 (1)	1.0 (1)	4.3 (4)	5.3 (5)	2.5 (3)	0	1.0 (1)	1.0 (1)	3.6 (4)	3.8 (4)	9.8 (10)	8.7 (9)
Phenylalanine	3.8 (4)	4.0 (4)	2.0 (2)	3.0 (3)	1.3 (1)	1.4 (1)	1.9 (2)	1.9 (2)	6.1 (6)	5.5 (6)	10.0 (10)	10.4 (10)
Histidine	4.0 (4)	5.7 (6)	1.9 (2)	3.0 (3)	4.0 (4)	5.1 (5)	0	0	1.4 (1)	1.7 (2)	4.8 (5)	5.0 (5)
Lysine	12.8 (13)	13.6 (14)	14.7 (15)	8.8 (9)	12.9 (13)	14.1 (14)	0	1.1 (1)	10.4 (10)	8.7 (9)	12.9 (13)	13.2 (13)
Arginine	5.7 (6)	3.0 (3)	4.8 (5)	3.6 (4)	5.3 (5)	2.8 (3)	0	0	4.0 (4)	4.4 (4)	11.7 (12)	12.0 (12)
Cystine-O_3[b]	6.2 (6)	7.7 (8)	0	0	0	2.4 (2)	0	0	2.0 (2)	1.8 (2)	3.2 (3)	3.7 (4)
Tryptophan	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mol wt	10,600	10,720	18,540	15,900	12,580	12,210	3,745	3,745	10,680	10,490	26,210	26,010

[a] Values are given as moles of residues per mole of protein and the value rounded to the nearest integer is given in parentheses. Values were calculated from the mole percentage of amino acid residues by assuming a molecular weight consistent with SDS-PAGE mobility of the protein and adjusted to give the least departure from the nearest integer values.

[b] Determined after performic acid oxidation of cystine residues.

[c] ND, Not determined.

PTH-amino acid derivative could be identified for positions 16 and 18 of either R-D/W or MPMV p14. The only observed difference in the amino acid sequences of the p14s occurred at position 19, where MPMV had a glutamic acid residue in place of an asparagine in R-D/W. This substitution is compatible with the differences observed by amino acid analysis of the two p14s (Table 1).

The N-terminal amino acid sequences of the phosphoproteins of R-D/W (pp20) and MPMV (pp18) were distinct from each other (Fig. 7). This result is compatible with the observation that these two viral proteins differed in apparent molecular weight (Fig. 6) and amino acid composition (Table 1).

The N-terminal amino acid sequences of the p12 protein from R-D/W and the p12 protein from MPMV were nearly identical to each other except for substitutions in MPMV of an alanine for serine at position 14, a glutamic acid for glycine at position 24, and an alanine for glutamine at position 28 (Fig. 7). The data in Table 1 reveal that MPMV p12 contains more alanine by amino acid composition than does R-D/W p12.

The p4 peptides from R-D/W and MPMV each contain about 35 residues (Table 1). The N-terminal amino acid sequence of each peptide was determined for 26 of the 35 residues (Fig. 7). The amino acid sequence of R-D/W p4 differed from the amino acid sequence of MPMV p4 by substitutions at the following five positions in MPMV p4; position 9 (asparagine for serine), position 10 (lysine for serine), position 16 (serine for asparagine), position 18 (proline for isoleucine), and position 26 (asparagine for proline). These substitutions account for all of the differences in amino acid compositions between R-D/W p4 and MPMV p4 except for one alanine in MPMV p4. This observation suggests that there is at least one additional position in the unsequenced C-terminal nine residues of the p4 peptides in which R-D/W p4 differs from MPMV p4. Thus, the p4 peptides of R-D/W and MPMV differ by at least six amino acid substitutions out of 35 residues.

The N-terminal amino acid sequences of the p27 proteins from R-D/W and MPMV were determined and compared for the first 24 residues of each protein (Fig. 7). Comparison of the N-terminal amino acid sequences of R-D/W and MPMV p27 proteins revealed only a single amino acid substitution at position 6, a valine in MPMV for a methionine in R-D/W.

The p10 proteins of R-D/W and MPMV were each subjected to 20 cycles of N-terminal Edman degradation, but no PTH-amino acid derivatives were obtained from either protein. This strongly suggests that these proteins are covalently modified at their N-terminal amino group resulting in an amino terminus that is blocked to Edman degradation.

To investigate possible relationships between the amino acid sequence of D-type retroviral protein and amino acid sequences of other proteins, the N-terminal amino acid sequences of the R-D/W and MPMV proteins listed in Fig. 7 were compared with the known amino acid sequences of *gag* precursor polyproteins of other retroviruses, including Moloney murine leukemia virus (42), Moloney murine sarcoma virus (47), feline leukemia virus (12), baboon endogenous virus (M-7) (44), bovine leukemia virus (34), Rous sarcoma virus (RSV) (40), human T-cell leukemia virus-I (41), and murine mammary tumor virus (9) and also to the *env* precursor polyprotein of human T-cell leukemia virus-I

Viral Protein	N-Terminal Amino Acid Sequence
p14	
R-D/W	Ala-Ala-Ala-Phe-Ser-Gly-Gln-Thr-Val-Lys-Asp-Phe-Leu-Asn-Asn- X -Asn- X -Asp-Arg-Gly-
MPMV	" " " " " " " " " " " " " " " " " " Glu X "
phosphoprotein	
R-D/W	Ala-Ala-Val-Thr-Gln-Thr-Gln-Lys-Ile-Leu-Lys-Val-Ser-Ser-Gln-Thr-Asp-Leu-Arg-Asp-Lys-Ser-Gln-Asn-Ser-
MPMV	Leu-Thr-Ala-Gln-Thr-Ser-Lys-Asp-Pro-Glu-Asp-Pro-Asn-Pro-Ser-Glu-Val-Asp-Trp-Asp-Gly-Leu-Glu-
R-D/W	Glu-Met-Asp- X -Ile-Ser-Leu-
p12	
R-D/W	Ala-Val-Val-Asn-Pro-Lys-Glu-Glu-Leu-Lys-Glu-Lys-Ile-Ser-Gln-Leu-Glu-Glu-Gln-Ile-Lys-Leu-Glu-Gly-Leu-
MPMV	" " " " " " " " " " " " " Ala " " " " " " " " " Glu "
R-D/W	His-Gln-Gln-Leu-Ile-Ile-Arg-Leu-Gln
MPMV	" " Ala " " " X " "
p4	
R-D/W	Gly-Ala-Val-Ser-Phe-Val-Pro-Ala-Ser-Ser-Asn-Asn-Pro-Phe-Gln-Asn-Leu-Ile-Glu-Pro-Pro-Gln-Glu-Val-Gln-Pro
MPMV	" " " " " " " " Asn-Lys " " " " " Ser " Pro " " " " " " " Asp
p27	
R-D/W	Pro-Val-Thr-Glu-Thr-Met-Asp-Gly-Gln-Gly-Gln-Ala-Trp-Arg-His-His-Asn-Gly-Phe-Asp-Phe-Thr-Val-Ile
MPMV	" " " " " Val " " " " " " " " " " " " " " " " " X " "

FIG. 7. N-terminal amino acid sequences of MPMV and R-D/W proteins purified as described in the legends to Fig. 2 and 4 (see the text). At positions where the MPMV protein has the same residue as the R-D/W protein, the residue is listed in the R-D/W row and indicated by ditto marks (") in the MPMV row. An X in a sequence indicates a position where we could not make a positive identification of a PTH-amino acid derivative. The N-terminal amino acid sequence of each viral protein was determined by Edman degradation of 3 to 6 nmol of purified protein. The PTH-amino acid derivative obtained at each Edman cycle was quantitatively and qualititatively identified by high-pressure liquid chromatography (14). The quantitative recovery of PTH-amino acids was compatible with an overall repetitive yield of 96% for each Edman cycle from the analysis of each of the proteins listed above except for the p14 proteins. In the analysis of both MPMV and R-D/W p14, the repetitive yield of PTH-amino acid was 96% for cycles 1 through 15, but the quantity of PTH-amino acids recovered from cycles 17 and on was only about 10% of the expected value. The reason for the sharp decrease in the yield of PTH-amino acid after step 16 is unknown at present. The N-terminal amino acid sequence of MPMV p27 reported here is in complete agreement with our previously reported N-terminal sequence for this protein (29) except that in our previous publication, the residues at positions 13 and 16 were not positively identified, and the report contained typographical errors in positions 14 and 20.

(41), Moloney murine leukemia virus (42), RSV (40), bovine leukemia virus (35), and murine mammary tumor virus (33). The D-type virus sequences were also compared with those of all the proteins in the Georgetown data base by the Search Program on the National Institutes of Health computer (23).

These comparisons revealed statistically significant amino acid sequence homology between the N-terminal amino acid sequence of R-D/W pp20 and a peptide segment found in the *env* precursor polyprotein of RSV (Fig. 8). The N-terminal amino acid sequence of R-D/W pp20 did not show statistically significant amino acid sequence homology (alignment scores greater than 3.0) with any other known retrovirus amino acid sequences.

The N-terminal amino acid sequences of all other D-type *gag* proteins given in this report failed to show statistically significant amino acid sequence homologies with any other known amino acid sequences, including sequences of retroviral origin. However, the N-terminal residues of these D-type p27 proteins (Pro-Val-) are common with the N-terminal residues of HTLV-I p24 (31) and avian C-type p27 (26) and are similar to the N-terminal residues of all known mammalian C-type retrovirus p30 proteins (Pro-Leu) (29). The N-terminal alanine residue of these D-type nucleic acid-binding proteins (p14 proteins) is common to all other known retroviral nucleic acid-binding proteins from the murine C-type retroviruses (15) and RSV (K. S. Misono, F. S. Sharief, and J. Leis, Fed. Proc. **39**:1611, 1980). These observations suggest that the enzyme catalyzing the proteolytic cleavages of D-type *gag* precursor polyproteins may show a substrate specificity similar to that of other mammalian and avian C-type retrovirus proteases (7, 27, 28, 30, 49).

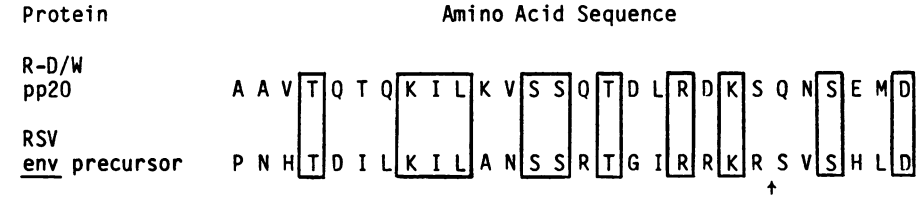

FIG. 8. Amino acid sequence homology between R-D/W pp20 and RSV *env* precursor polyprotein. The N-terminal amino acid sequence of 28 residues of R-D/W pp20 is shown aligned with a sequence of 28 residues found in the predicted amino acid sequence for the *env* precursor of RSV (residues predicted by nucleotides 6202 through 6286; see reference 40). Positions in the alignment containing identical residues in each sequence are indicated by boxes. The RSV sequence shown here spans the proteolytic cleavage site (arrow) between the viral gp85 (residues to the left of the arrow) and gp36 (residues to the right of the arrow).

DISCUSSION

Six proteins (p27, p14, p12, p10, p4, and a phosphoprotein) were purified from R-D/W and also from MPMV by high-pressure liquid chromatography. The results of SDS-PAGE and N-terminal amino acid sequence, amino acid, and phosphoamino acid analyses of these purified proteins provide a set of molecular data with which to characterize and compare these D-type retroviral isolates with each other and with other related viruses.

In previous studies, metabolic radiolabeling techniques have been used to identify the following MPMV proteins: p27, p14, p12, and p10 (29, 36, 37); two glycoproteins designated gp20 and gp70 (37); a phosphoprotein designated pp16-18 (here referred to as pp18); and a small protein designated p6 (3) that may be equivalent to the p4 protein described here. Antiserum raised against MPMV-p27 precipitated a high-molecular-weight protein from virus-infected cells that is believed to be the *gag* precursor designated $Pr78^{gag}$ (3, 38, 39). Recently, Bradac and Hunter (3) have used tryptic peptide mapping of radiolabeled MPMV proteins to show that p27, p14, p12, p10, and pp18 are all *gag* proteins derived by proteolytic cleavage of $Pr78^{gag}$. MPMV p4 may also be a *gag* protein since, as shown here, it is found in the virus in molar amounts comparable to those of the other known proteolytic cleavage products of $Pr78^{gag}$. The sum of the estimated molecular weights given in Table 1 for the MPMV proteins p14, pp18, p12, p4, p10, and p27 is 79,075, which is in good agreement with the estimated molecular weight of $Pr78^{gag}$.

The data for the R-D/W proteins presented in this report strongly suggest that these proteins are homologs of the MPMV *gag* gene products. R-D/W contains proteins similar in molecular weight, amino acid composition, and chromatographic mobility to MPMV p14, p10, p12, p4, and p27 (Fig. 1, 2, and 4; Table 1). In addition, R-D/W contains a protein (pp20) with phosphorylated serine residue(s) analogous to MPMV pp18. The N-terminal amino acid sequences of four of the R-D/W proteins (p4, p12, p14, and p27) are unique when compared with each other but are highly homologous to the N-terminal amino acid sequences of the corresponding proteins from MPMV (p4, p12, p14, and p27) (Fig. 7). The p10 proteins from both viruses are blocked to N-terminal Edman degradation. The phosphoprotein of R-D/W (pp20) and the phosphoprotein of MPMV (pp18) (Fig. 6) differ in size and show no significant amino acid sequence homology in their N-terminal segments (Fig. 7). However, in their physical and chemical properties, R-D/W pp20 and MPMV pp18 appear similar to each other in that both migrate as a wide band in SDS-PAGE, do not retain Coomassie brilliant blue stain as well as do other proteins, and are eluted from the RP-HPLC column with approximately the same concentration of acetonitrile (30 to 35%). In view of the common properties shared by the phosphoproteins of R-D/W and MPMV and the high degree of amino acid sequence homologies between the other compared viral *gag* proteins, we believe that it is probable that conserved regions will be found in the complete amino acid sequences of pp20 and p18. Since the p27, p14, p12, p4, and p10 proteins of R-D/W are all shown to be related to the respective *gag* proteins of MPMV, and the phosphoproteins of R-D/W and MPMV appear functionally homologous, we suggest that all the R-D/W proteins reported here are cleavage products of a *gag* precursor polyprotein analogous to MPMV $Pr78^{gag}$. This suggestion has now been confirmed by comparing all the amino acid sequences reported here (except the p4 proteins) to a partial nucleotide sequence of the *gag* gene of retrovirus-D/California, a D-type retrovirus closely related to R-D/W and MPMV (G. Heidecker, personal communication). However, it is uncertain whether the observed differences between the *gag* phosphoproteins of R-D/W and MPMV reflect different proteolytic cleavage sites in the *gag* precursors or differences between the amino acid sequences of the *gag* precursors of R-D/W and MPMV.

Except for the phosphoproteins, the compared N-terminal amino acid sequences of the homologous MPMV and R-D/W *gag* proteins differ from each other by about 10%. Assuming a similar degree of homology is maintained throughout each pair of proteins, the *gag* precursors of R-D/W and MPMV may be approximately 80 to 90% identical, except for the regions occupied by the phosphoproteins. This conclusion is consistent with the divergency of 12 to 18% at the nucleotide sequence level suggested by studies of the thermal stability of the hybrid between MPMV and R-D/W DNA (43).

Computer-assisted comparisons of the N-terminal amino acid sequences of D-type *gag* proteins to known *gag* and *env* sequences of other retroviruses revealed statistically significant sequence homologies between R-D/W pp20 and a segment of the *env* precursor of RSV. The homology with R-D/W pp20 is present in a segment of the RSV *env* precursor that spans a proteolytic cleavage site between the envelope glycoproteins (gp85 and gp36) of RSV (Fig. 8, arrow). Amino acid residues in the pp20 sequence were identical to residues in the RSV sequence at 11 of 28 compared positions. The probability of observing this degree of amino acid sequence homology by random selection was calculated to be less than 1 in 10^7 from an alignment score of 5.22 given by the ALIGN program (6). At the present, the biological and phylogenetic implications of the observed homology are subjects for speculation.

The N-terminal amino sequences of the D-type retrovirus *gag* proteins reported here are insufficient to establish a phylogenetic relationship between the *gag* proteins of primate D-type retroviruses and other known retroviral *gag* proteins. Nevertheless, a relationship between the primate D-type *gag* proteins and other retroviral *gag* proteins is

suggested. MPMV Pr78^{gag} has been shown to incorporate myristic acid, as do the *gag* precursors of many other mammalian retroviruses (39). In the murine retroviruses, the site of myristylation has been identified as the N-terminal amino group of the *gag* precursor and its cleavage product, p15 (16). The D-type p10 proteins are as hydrophobic as the p15 proteins of murine virus and are blocked to N-terminal Edman degradation. It is probable that the D-type p10 proteins represent the N-terminal portion of the *gag* precursor and are N-terminally myristylated. The D-type phosphoproteins may be functionally homologous to the phosphoproteins of other retroviruses. The D-type p14 proteins isolated here appear similar if not identical to the protein previously described by Long et al. (24) as a basic protein with nucleic acid-binding properties present in MPMV. Typically, retroviral nucleic acid-binding proteins contain at least three cysteine residues in a highly conserved sequence, and in some cases, the sequence is repeated (15, 27). The D-type p14 proteins contain six cysteine residues (Table 1), suggesting that these proteins may contain the repeated sequence. The N-terminal proline residue of the D-type p27 proteins is common to the major *gag* protein of all B- and C-type retroviruses, which shows that this common feature of the proteolytic cleavage mechanism extends to D-type viruses.

The methods and data presented in this report provide a molecular basis for the comparison of the *gag* proteins from different isolates of D-type virus. Similar studies with the structural proteins of other D-type retroviruses, including squirrel monkey retrovirus, langur virus, and the isolates of retrovirus-D from the California and New England Primate Centers, will reveal any structural similarities between these viruses. Since the various D-type isolates are associated with a variety of diseases in primates, detailed structural studies may help to elucidate the molecular nature of this pathogenic diversity.

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ADDENDUM IN PROOF

The nucleotide sequences of R-D/California (P. Marx and P. Luciw, personal communication) and of MPMV (E. Hunter, J. Engler, L. Perez, and C. Barker, personal communication) have confirmed that the p4 protein is encoded in the *gag* gene. The nucleotide sequence of the MPMV *gag* gene also shows a translated sequence homologous with the N-terminal amino acid sequence of R-D/W pp20 upstream from the sequence coding for the N-terminal sequence of MPMV pp18.

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