JOURNAL OF VIROLOGY, Apr. 1987, p. 1116–1124
0022-538X/87/041116-09$02.00/0

Vol. 61, No. 4

Chemical and Immunological Characterizations of Equine Infectious Anemia Virus *gag*-Encoded Proteins

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Received 18 August 1986/Accepted 23 December 1986

The viral core proteins (p15, p26, p11, and p9) of equine infectious anemia virus (EIAV) (Wyoming strain) were purifed by reverse-phase high-pressure liquid chromatography. Each purified protein was analyzed for (i) amino acid content, (ii) N-terminal amino acid sequence, (iii) C-terminal amino acid sequence, and (iv) phosphoamino acid content. The results of N- and C-terminal amino acid sequence analysis of each *gag* protein, taken together with the nucleotide sequence of the EIAV *gag* gene (R. M. Stephens, J. W. Casey, and N. R. Rice, Science 231:589–594, 1986), show that the order of the proteins in the precursor is p15-p26-*-p11-p9, where a pentapeptide also found in the virus is represented by the asterisk. The data are in complete agreement with the predicted structure of the *gag* polyprotein and show the peptide bonds cleaved during proteolytic processing. The N terminus of p15 is blocked to Edman degradation. The p11 protein is identical to the nucleic acid-binding protein of EIAV previously isolated (C. W. Long, L. E. Henderson, and S. Oroszlan, Virology 104:491–496, 1980). High-titer rabbit antiserum was prepared against each purified protein. These antisera were used to detect the putative *gag* precursor ($Pr55^{gag}$) and intermediate cleavage products designated Pr49 (p15-p26-*-p11), Pr40 (p15-p26), and Pr35 (p26-*-p11) in the virus and in virus-infected cells. High-titer antisera to EIAV p15 and p26 showed cross-reactivity with the homologous protein of human T-cell lymphotropic virus type III/lymphadenopathy-associated virus.

Equine infectious anemia is a disease caused by chronic infection by a virus designated equine infectious anemia virus (EIAV) (24, 27, 28, 35). EIAV is a retrovirus (5) with a high-molecular-weight (sedimentation coefficient, 60S to 70S) genomic RNA (7, 12, 36), a viral reverse transcriptase with a preference for magnesium ions (1), and morphology similar to C-type retroviruses (12, 51). The virus has been subclassified as a lentivirus (12). Other members of the lentivirus subfamily of retroviruses are human immunosuppressive viruses (HIVs) including human T-cell lymphotropic virus type III (HTLV-III) (11) and type IV (26), lymphadenopathy-associated virus (2), and AIDS-associated retrovirus (31); simian immunosuppressive viruses including various simian T-lymphotropic viruses (10, 25, 26, 30) and Macaca nemestrina immunodeficiency virus (3); visna virus (48, 49); and caprine arthritis-encephalitis virus (37). Recently, the nucleotide sequence of a fragment of EIAV proviral DNA including all of the *gag* gene and part of the *pol* gene has been determined (50). The *gag* gene was identified as a long open reading frame that could code for a polypeptide of 486 residues designated $Pr55^{gag}$. The predicted amino acid sequence of EIAV $Pr55^{gag}$ shared some homology with the sequences of the predicted *gag* polypeptides of HIVs and visna virus.

EIAV has been shown to contain at least four major internal polypeptides (6, 23, 40) that are believed to be proteolytic cleavage products of $Pr55^{gag}$. The major internal proteins of EIAV have been designated p26, p15, p11, and p9 and have been isolated from disrupted virus by gel filtration chromatography in high concentrations of guanidine hydrochloride (34). The amino acid compositions and pIs of the isolated proteins were reported, but no amino acid sequence data were reported for the purified EIAV proteins.

In this report we describe the isolation of the major internal proteins of EIAV (p26, p15, p11, and p9) by reverse-phase high-pressure liquid chromatography (RP-HPLC). We report the amino acid composition and results of N- and C-terminal amino acid sequence analysis and phosphoamino acid analysis for each purified protein. These results are in agreement with the sequence predicted by translation of the nucleotide sequence of the EIAV *gag* gene and show the proteolytic cleavage sites and the order of structural proteins in $Pr55^{gag}$. Monospecific antiserum raised against each purified protein was used to investigate the state of *gag* polyproteins present in the virus and in virus-infected cells.

(Portions of this work were performed by R. C. Sowder in partial fulfillment of the requirements for the M.S. degree from Hood College, Frederick, Md., 1986.)

MATERIALS AND METHODS

Virus. EIAV (Wyoming strain) was grown in chronically infected equine fetal kidney (EFK) cells and purified by sucrose density gradient centrifugation as previously described (5); it was supplied by the Viral Resources Laboratory, Frederick Cancer Research Facility, National Cancer Institute, Frederick, Md.

Chemicals. All chemicals used in the liquid-phase spinning-up sequenator were purchased from Beckman Instruments, Inc., Palo Alto, Calif. Polybrene was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Guanidine hydrochloride (enzyme grade) was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Acetonitrile and 1-propanol were obtained from Burdick Jackson Laboratories, Inc., Muskegon, Mich. Trifluoroacetic acid (TFA) Sequanal grade was purchased from Pierce Chemical Co., Rockford, Ill.

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Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (47) was performed on 10 to 20% gradient gels by the method of Laemmli (29). 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 D500 amino acid analyzer using ninhydrin detection of eluted amino acids. Cysteine was determined as cysteic acid after performic acid oxidation (22). Tryptophan was determined spectrophotometrically from the A_{294}.

O-Phosphoamino acid analysis. Samples were hydrolyzed in vacuo in 4 N HCl at 110°C for 1.5 h. After the 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; SynChrom, Inc., Linden, Ind.). The 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 (52). Eluted phosphoamino acids were detected by fluorescence after reaction with orthophthalaldehyde reagent (41).

Liquid-phase sequencing. Semiautomated microsequencing was performed with an updated 890B Beckman sequencer, as previously described (8, 16). Phenylthiohydantoin (PTH) derivatives of amino acids were quantitatively identified as previously described (15).

Separation of viral proteins by RP-HPLC. Concentrated suspensions of purified virus (1 mg/ml) in 10 mM Tris hydrochloride (pH 7.2)–100 mM NaCl–1 mM EDTA were adjusted to pH 2.0 by the addition of TFA and added to 5 volumes of saturated (at 23°C) guanidine hydrochloride. The resulting slightly turbid solution of disrupted virus was injected into a high-pressure liquid chromatograph (Waters Associates, Inc., Milford, Mass.) and separated by RP-HPLC on an Ultrapore RPSC (C_3) (Beckman) or μBondapak C_{18} column (Waters), as previously described for the separation of structural proteins from other retroviruses (9, 18, 20, 21, 39). Eluted proteins were detected by UV absorption at 206 nm (19) with a model 450 (Waters) variable-wavelength detector and collected manually to optimize separations and recoveries. Solvents were removed by lyophilization. The 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 acetonitrile containing 0.1% TFA. When necessary, proteins were rechromatographed on a Bakerbond wide-pore diphenyl column (J. T. Baker Research Products, Phillipsburg, N.J.). 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, RNase, Met-enkephalin, and Leu-enkephalin), as previously described (14). When necessary, columns were cleaned and repacked as previously described (19).

Immunization. EIAV p26 and p15 proteins purified by RP-HPLC were further purified by preparative SDS-PAGE. The protein bands were located in the gel by visualization in the presence of 0.1 M KCl (13), excised, minced, and injected directly into rabbits. EIAV p9 and p11 purified by RP-HPLC were mixed with Freund adjuvant for immunization. The immunizations were initiated with an injection of approximately 100 μg of purified protein, followed by seven booster injections of 50 μg of protein each, given at 2-week intervals. The immunized rabbits were bled out 1 week after the final booster injection, and the sera were tested for monospecific antibodies to purified EIAV proteins immobilized on diazopaper. Adsorbed antibodies were detected by complex formation with ^{125}I-labeled protein A followed by autoradiography.

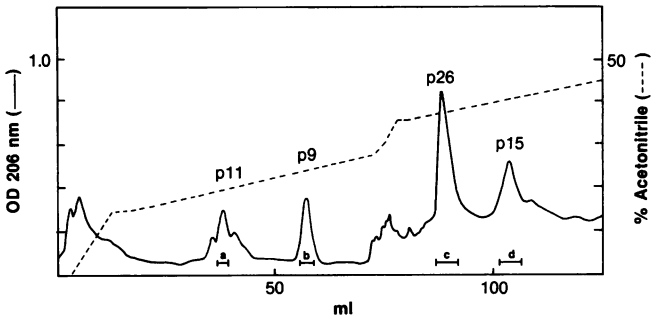

FIG. 1. Separation of EIAV proteins by RP-HPLC. Purified EIAV (Wyoming strain) (1.65 mg) was dissolved in 15 ml of 8 M guanidine hydrochloride containing 100 μl of β-mercaptoethanol and adjusted to pH 2.0 with the addition of TFA and then pumped at 1.0 ml/min through an Ultrapore RPSC column (4.6 by 75 mm). Proteins bound to the column were eluted at pH 2.0 with gradients of increasing concentrations of acetonitrile and detected by UV absorption at 206 nm. Column fractions a, b, c, and d were collected, lyophilized, rechromatographed on a Bakerbond diphenyl column as described above (see the text), and analyzed by SDS-PAGE (Fig. 2) to identify the major viral protein present in each UV peak. The major viral protein identified is indicated above each peak.

Immune-transfer analysis. Proteins separated by SDS-PAGE were transferred and fixed to diazo paper by transverse electrophoresis (44). The excess diazo groups were reacted with 10% powdered milk protein –0.1 M phosphate (pH 9), and antigens were detected by reaction with monospecific antisera against EIAV proteins. Antigen-antibody complexes were reacted with ^{125}I-labeled protein A and detected by autoradiography.

RESULTS

Proteins associated with purified EIAV were separated by RP-HPLC. A typical RP-HPLC chromatogram obtained from the separation of EIAV proteins is shown in Fig. 1. The proteins collected in effluent fractions a, b, c, and d (Fig. 1) were pooled with identical fractions from other separations of EIAV proteins performed in the same manner. Each pool of partly purified protein was lyophilized and rechromatographed by RP-HPLC on a Bakerbond diphenyl column (results not shown) by essentially the same procedure as that described for the chromatography shown in Fig. 1. The repurified proteins were then analyzed and identified by SDS-PAGE (Fig. 2A). The major viral protein identified with each UV peak is indicated in Fig. 1 (p11, p9, p26, and p15). The p11, p9, and p15 proteins (Fig. 2A) were taken for chemical analysis without further purification. The p26 protein (Fig. 2A) was rechromatographed as in the experiment shown in Fig. 1 except that a more shallow gradient of acetonitrile was used to elute the protein (results not shown). The repurified p26 protein was reanalyzed by SDS-PAGE (Fig. 2B) and taken for chemical analysis.

The amino acid compositions of the EIAV p9, p11, p26, and p15 proteins purified as described above were determined (Table 1). Our results for the amino acid compositions

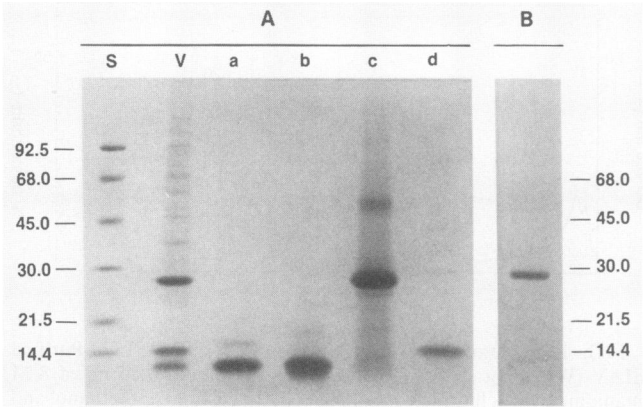

FIG. 2. (A) EIAV proteins purified from disrupted virus by RP-HPLC as described in the legend to Fig. 1 were rechromatographed on a Bakerbond diphenyl column (results not shown) before analysis by SDS-PAGE. Samples of proteins purified from pools a, b, c, and d (Fig. 1) were analyzed in the indicated lanes. Lane V, Unfractionated EIAV; lane S, molecular weight standards. (B) The partly purified preparation of EIAV p27 (panel A, lane c) was rechromatographed on a Bakerbond diphenyl column as in the experiment whose results are shown in Fig. 1 except that the protein was eluted on a more shallow gradient and then reanalyzed by SDS-PAGE as shown.

of p26 and p15 are in good agreement with the results of Montelaro et al (34). Our results for the amino acid composition of EIAV p11 are in good agreement with the amino acid composition of the DNA binding-protein purified from EIAV by Long et al (32).

The p26 and p15 proteins of EIAV had mobilities in SDS-PAGE that were consistent with their molecular weights based on amino acid analysis of the purified proteins (Table 1). The p9 and p11 proteins appeared to comigrate in the SDS-PAGE shown in Fig. 2; however, in other experiments we observed p11 migrating slightly more slowly than p9. The results of amino acid analysis (Table 1) suggest that p11 is composed of about 76 amino acid residues, with a calculated molecular weight of about 8,000, and that p9 is composed of about 51 residues, with a calculated molecular weight of about 6,000. The discrepancy between the molecular weights of p11 and p9 determined by mobility in SDS-PAGE and by amino acid analysis was probably due to the inaccuracy of the SDS-PAGE methods for the estimation of molecular weights of small proteins.

TABLE 1. Amino acid compositions of EIAV proteins

Amino acid residue(s)	Amt in protein[a]:			
	p26	p15	p11	p9
Asp + Asn	26 (26)	13 (13)	2 (2)	8 (8)
Thr	15 (14)	9 (9)	3 (3)	2 (2)
Ser	8 (7)	8 (8)	4 (4)	4 (4)
Glu + Gln	31 (32)	18 (18)	9 (9)	13 (13)
Pro	19 (18)	4 (3)	7 (7)	3 (3)
Gly	16 (16)	7 (6)	12 (12)	0 0
Ala	17 (16)	8 (8)	6 (6)	0 0
Val	8 (7)	8 (9)	2 (2)	4 (4)
Met	9 (11)	1 (2)	0 0	0 0
Ile	17 (19)	3 (3)	0 0	2 (2)
Leu	19 (16)	14 (14)	4 (4)	5 (5)
Tyr	4 (5)	2 (2)	1 (1)	2 (2)
Phe	8 (7)	4 (4)	4 (4)	0 0
His	3 (3)	1 (1)	2 (2)	1 (1)
Lys	13 (11)	13 (14)	11 (11)	6 (6)
Arg	17 (16)	4 (4)	3 (3)	0 0
Cys[b]	3 (3)	1 (1)	6 (6)	0 0
Trp[c]	3 (3)	4 (5)	0 0	1 (1)

[a] Moles of residue per mole of protein, rounded to the nearest integer. Values were determined by amino acid analysis of purified proteins after acid hydrolysis. The values in parentheses were taken from the translated proviral DNA sequence (50).

[b] Determined as cysteic acid.

[c] Determined by spectroscopic analysis.

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                    5                   10                  15                  20
p11  Gln-Thr-Gly-Leu-Ala-Gly-Pro-Phe-Lys-Gly-Gly-Ala-Leu-Lys-Gly-Gly-Pro-Leu-Lys-Ala-
                   25
     Ala-Gln- X - X -Tyr-Asn-

                    5                   10                  15                  20
p9   Pro-Ile-Gln-Gln-Lys-Ser-Gln-His-Asn-Lys-Ser-Val-Val-Gln-Glu-Thr-Pro- X -Thr- X -
     Asn-Leu-Tyr-

                    5                   10                  15                  20
p27  Pro-Ile-Met-Ile-Asp-Gly-Ala-Gly-Asn-Arg-Asn-Phe-Arg-Pro-Leu-Thr-Pro-Arg-Gly-Tyr-
                   25
     Thr-Thr- X -Val-Asn-Thr-Ile-Gln-

p15  Blocked
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FIG. 3. N-terminal amino acid sequences of EIAV proteins. The N-terminal amino acid sequences were determined by automated Edman degradation of 3 to 4 nmol of each purified protein. The PTH-amino acid derivative produced by each Edman cycle was identified by HPLC, as previously described (15). Residues are shown only if the PTH-amino acid derivative was positively identified and recovered in an amount consistent with a 95% repetitive yield for the Edman degradation. Where the results of an Edman cycle failed to meet these criteria, an X is shown in the sequence. Edman degradation of EIAV p15 did not produce any detectable PTH-amino acid derivatives, indicating that the N-terminal amino group of this protein is unavailable or Edman degradation, and is therefore reported as blocked.

Each of the purified proteins of EIAV (p26, p15, p11, and p9) was also analyzed for phosphoamino acid content by a method using mild acid hydrolysis followed by separation and quantitation of phosphothreonine, phosphoserine, and phosphotyrosine by anion-exchange chromatography (52). Analysis of p26, p11, p15, and p9 gave no detectable peaks eluting in the region of the phosphoamino acids. The method of analysis would probably have detected 0.2 residues of phosphoamino acid per mol of protein. These results are consistent with reported negative results of in vivo labeling with [^{32}P]phosphate (34).

The purified proteins of EIAV (p26, p15, p11, and p9) were each taken for automated N-terminal Edman degradation, and the PTH-amino acid derivative obtained from each Edman cycle was identified by HPLC (15). The N-terminal amino acid sequences of p26, p11, and p9 deduced by Edman degradation are presented in Fig. 3. No PTH-amino acid derivatives were produced by Edman degradation of p15, indicating that this protein is blocked to Edman degradation and may have a derivatized N-terminal amino group.

Each of the purified EIAV proteins was digested with a carboxypeptidase for various times, and the liberated free amino acids were analyzed to determine residues at the C terminus of the protein. The results showing the rate of release of amino acid residues from EIAV p11, p9, p26, and p15 by enzymatic digestion with carboxypeptidase A are given in Table 2. The C-terminal amino acid sequence was suggested by the rate of release of residues and is shown in Fig. 4 for each EIAV protein.

The N-terminal amino acid sequences of the EIAV proteins given in Fig. 3 and the C-terminal amino acid sequences

TABLE 2. Amino acids released by carboxypeptidase digestion of EIAV proteins

Protein	Amino acid released	Amt of amino acid released (mol/mol of protein) with time (min) of digestion[a]:		
		1.0	5.0	20.0
p11	Phe	0.86	0.98	0.99
	Thr	0.10	0.47	0.99
	Gln[b]	0.10	0.25	0.58
	Lys		0.08	0.24
p9	Glu	0.41	0.88	1.00
	Trp	ND[c]	ND	ND
	Leu	0.15	0.63	1.26
	Asp		0.11	0.34
	Ser			0.22
p27	Leu	0.28	0.37	0.49
	Met[d]	0.18	0.44	0.77
p15	Tyr	0.12	0.32	0.62

[a] The data are for carboxypeptidase A digestion of p11, p27, and p15 and carboxypeptidase Y digestion of p9.

[b] The analytical system used could not distinguish between Gln and Asn, but Gln is compatible with the nucleotide sequence reported by Stephens et al. (50).

[c] Tryptophan was not determined by our analytical method but was predicted by the nucleotide sequence (50) as the penultimate residue of $Pr55^{gag}$ and p9.

[d] Determined as the sum of methionine and methionine sulfoxide.

given in Fig. 4, as well as the amino acid compositions (Table 1), were compared with the deduced amino acid sequence (50) of EIAV $Pr55^{gag}$ predicted by translation of the proviral DNA sequence (Fig. 5). The C-terminal residues of p15 and the N-terminal residues of p26 are identical to a contiguous sequence of residues from positions 124 to 152 in the predicted structure of $Pr55^{gag}$. Proteolysis between p15 and p26 occurred at a single peptide bond between residues 124 and 125. In a similar manner, it was shown that the C-terminal residues of p11 and the N-terminal sequence of p9 are identical to a contiguous amino acid sequence in $Pr55^{gag}$ (residues 432 to 458) and proteolysis between p11 and p9 occurred at a single peptide bond between residues 435 and 436. The C-terminal amino acid residues determined for the p9 protein align with the predicted C-terminal residues of $Pr55^{gag}$ except that our method of analysis could not determine tryptophan (the predicted penultimate tryptophan residue). The C-terminal residues of p26 and the N-terminal residues of p11 do not align with a contiguous predicted amino acid sequence in $Pr55^{gag}$. The C-terminal residue of p26 aligns with residue 354 of $Pr55^{gag}$, and the N-terminal residue of p11 aligns with residue 360. Thus, there are five residues predicted between the C terminus and p26 and the N terminus of p11. A peptide with an amino acid composition identical to the predicted composition of the five-residue segment between p26 and p11 was searched for and was found in early-eluting fractions of the chromatogram shown in Fig. 1.

The determined amino acid compositions of p15, p11, and p9 are in excellent agreement with the predicted compositions of these proteins based on the determined proteolytic cleavage sites and predicted amino acid sequence of $Pr55^{gag}$ (values in parentheses in Table 1). The predicted amino acid composition of p15 includes two methionine residues (residues 1 and 87), but amino acid analysis showed only one methionine residue. This suggests, but does not prove, that the initiator methionine may be removed from the mature protein. The experimentally determined values for p26 are in fair agreement with the predicted values, but the agreement is not as good as that obtained for the other proteins. Much of the observed deviation may have been due to inherent inaccuracies of amino acid analysis of larger proteins, but the possibility of minor discrepancies between the predicted composition based on the DNA sequence of a noninfectious clone and the observed composition of a protein from a replication-competent virus cannot be entirely excluded. Thus, it appears that p15, p26, p11, p9, and the pentapeptide could account for all residues of $Pr55^{gag}$ predicted by the nucleotide sequence of the EIAV *gag* gene, except the initiator methionine. The results show that the structure of $Pr55^{gag}$ is p15-p26-*-p11-p9, where the pentapeptide is represented by an asterisk.

Rabbits were immunized with the purified EIAV proteins to prepare monospecific antisera directed against each of the EIAV proteins (anti-p26, anti-p15, anti-p11, and anti-p9). The monospecific antisera were used to examine the virus and virus-infected cells for related proteins.

Purified whole EIAV was disrupted, separated by SDS-PAGE, and transferred to diazo paper (44), and viral antigens on individual strips of paper were detected by reaction with each of the monospecific antisera (anti-p15, anti-p26, anti-p9, or anti-p11). Antigen-antibody complexes were visualized by radioautography after reaction with ^{125}I-labeled protein A. The results of a 4-h exposure of the realigned strips are shown in Fig. 6A, and the results of an 18-h exposure of the upper parts of the same strips are shown in Fig. 6B. In Fig. 6A it can be seen that each of the monospecific antisera reacted with a unique set of viral proteins. The anti-p15 serum (lane 1) reacted with p15 and a lower-molecular-weight band (approximately 4,000) and also detected bands labeled Pr40, Pr49, and Pr55. The anti-p26 serum (lane 2) detected bands labeled Pr40, Pr49, and Pr55 but did not detect bands at 15,000 or 4,000. In addition, the anti-p26 serum detected bands labeled Pr35 and p26 and lower-molecular-weight bands of about 23,000, 22,000, and 18,000. The anti-p9 serum (lane 3) detected bands labeled Pr55 and p9 but did not detect any other bands. The anti-p11 serum (lane 4) detected a band labeled p11 but did not detect any other viral protein bands. However, in other experiments (Fig. 6C) using lower dilutions of anti-p11, it was possible to detect a weak reaction with Pr49 and Pr35. The monospecific nature of the anti-p15, anti-p26, anti-p9, and anti-p11 sera is indicated by the data shown in Fig. 6 and was confirmed by testing their reactivity against purified viral proteins (data not shown).

Protein	C-terminal amino acid sequence
p11	Lys-Gln-Thr-Phe-OH
p9	(Leu,Ser)Asp-Leu-Trp-Glu-OH
p27	Met-Met-Leu-OH
p15	-Tyr-OH

FIG. 4. C-terminal amino acid sequences of EIAV proteins. The C-terminal amino acid sequences were deduced from the results of partial enzymatic digestions with carboxypeptidase (Table 2). Where the sequence of residues was not determined, the residues are reported in parentheses. Under the conditions of amino acid analysis, Asn and Gln were not separated from each other. The residue marked by an asterisk is discussed in Table 2, footnote *c*.

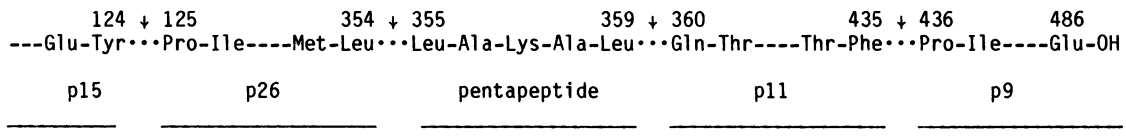

FIG. 5. Proteolytic cleavage sites in EIAV Pr55^{gag}. The peptide bonds in Pr55^{gag} cleaved by proteolysis were determined by aligning the N- and C-terminal amino acid sequences of p26, p11, and p9 and the C-terminal residue of p15 with the deduced amino acid sequence of EIAV Pr55^{gag} (50). The peptide bonds cleaved are indicated by triple dots and an arrow. The numbers above residues indicate the positions of the residues in the deduced amino acid sequence of Pr55^{gag}. The cleavage products p15, p26, pentapeptide, p11, and p9 are indicated by solid lines and were all isolated from mature viruses.

The proteins precipitated from EIAV-infected EFK cells with anti-p26 serum and detected by immune transfer with anti-p26 serum are shown in Fig. 7, lane 2. Proteins present in whole EIAV were also included in the same gel as positive controls (Fig. 7, lane 1). The proteins visualized in Fig. 7, lane 1 are equivalent to those visualized in Fig. 6, lane 2. Very little p26 was detected in the infected-cell cultures (Fig. 7, lane 2), suggesting that mature viral particles were effectively separated from the cells before the immune precipitation. Precipitations performed with anti-p15 serum and detected with anti-p26 serum gave results identical to those shown in Fig. 7, except that p26 was not detected. Precipitations and detections with preimmune rabbit sera gave no detectable bands at this level of exposure. The data presented in Fig. 7 show that proteins equivalent to Pr55, Pr49, and Pr40 were present in the EIAV-infected cells. However, the infected cells appear to have contained a higher proportion of Pr40 (relative to Pr55 and Pr49) than was found in the virus.

Pr55 shares antigenic determinants with p15, p26, and p9 and has an apparent molecular weight of 55,000, in good agreement with the sum of the molecular weights of p15 (ca. 14,000), p26 plus the pentapeptide (ca. 27,500), p9 (ca. 6,000), and p11 (ca. 8,000), which is equal to 55,500. Pr49 contains antigenic determinants common to p15, p26, and p11 but not to p9 and has an apparent molecular weight of 49,000, in agreement with the sum of the molecular weights for p15, p26, and p11 (ca. 49,500). Pr40 shares antigenic determinants with p15 and p26, and its apparent molecular weight of 39,000 is within experimental error for the sum of the molecular weights of p15 and p26 (ca. 41,500). Pr35 shares antigenic determinants with p26 and p11 but not with p15 or p9, and its apparent molecular weight is 35,000, in good agreement with the sum of the molecular weights of p26 (plus the pentapeptide) and p11 (ca. 35,500).

We interpret these data by assuming that Pr55 is the *gag* precursor polyprotein of EIAV and that Pr49, Pr40, Pr35, p15, p26, p9, and p11 are all derived by proteolytic cleavage of Pr55. The data are consistent with this assumption and the proven gene order for EIAV Pr55^{gag} (p15-p26-*-p11-p9), as shown in Fig. 8. The fact that anti-p11 serum did not react with Pr55 remains unexplained but suggests that major antigenic determinants for p11 may involve the terminal amino or carboxyl group or both.

In other experiments, EFK cells infected with EIAV were incubated with [^{3}H]myristic acid and examined for the presence of metabolically labeled Pr55, as previously described for the detection of other retroviral *gag* precursor polyproteins (45, 46). These experiments failed to show detectable amounts of myristylated Pr55.

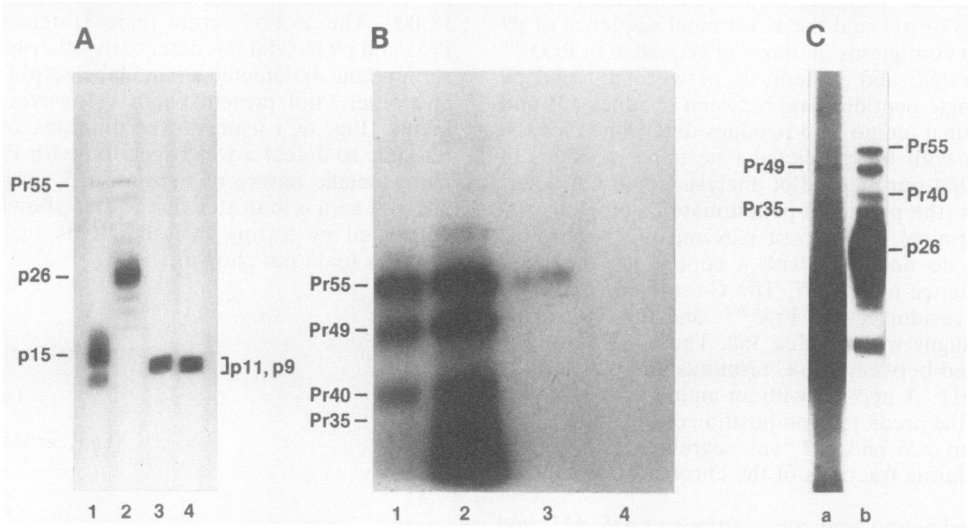

FIG. 6. Antigens present in EIAV detected by immune transfer with monospecific antisera to p15, p26, p9, and p11. EIAV proteins were separated by SDS-PAGE in a slab gel apparatus with a 12-cm-wide application well. After separation, the proteins were transferred and fixed to diazo paper. Vertical strips cut from the diazo paper were stained individually with 1:1,000 dilutions of monospecific antisera to p15 (lanes 1), p26 (lanes 2), p9 (lanes 3), and p11 (lanes 4) and reacted with ^{125}I-labeled protein A. The strips were then realigned, and the antigen-antibody complexes were detected by radioautography. (A) Results of a 4-h exposure of the realigned strips to film; (B) upper parts of the realigned strips exposed to the film for 18 h; (C) results obtained with anti-p11 serum at a 1:100 dilution (lane a) aligned with a strip reacted with anti-p26 serum (lane b). In other experiments performed as described above, radiolabeled molecular weight marker proteins were run in an adjacent lane to calibrte the gel for estimation of the apparent molecular weights of the viral antigens (results not shown).

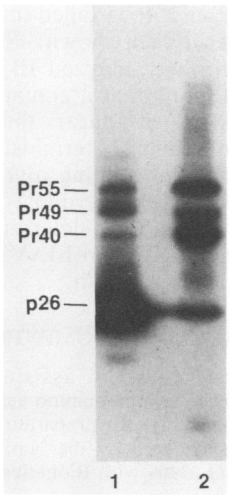

FIG. 7. Antigens in EIAV-infected EFK cells precipitated with anti-p26 serum and detected with anti-p26 serum. EFK cells infected with EIAV were grown to confluency in a T-150 flask, rinsed 3 times with serum-free medium, lysed with lysing buffer (5.0 ml), and centrifuged at 5,000 × *g* and the supernatant was reacted with 10 μl of anti-p26 serum plus 80 μl of a 50% (wt/vol) suspension of protein A-Sepharose beads for 18 h at 37°C. The Sepharose beads were collected by centrifugation, washed 7 times with lysing buffer, and suspended in SDS-PAGE starting buffer, and the eluted proteins were separated by SDS-PAGE. Lane 1, 10 μl of EIAV; lane 2, proteins collected from infected EFK cells by precipitation with anti-p26 serum. After separation by SDS-PAGE, the proteins were transferred to diazo paper and detected by immune transfer with anti-p26 serum as in Fig. 5, lane 2.

The monospecific antisera (anti-p15, anti-p26, anti-p11, and anti-p9) were all tested in immune transfer against separated proteins present in various purified retroviruses including HTLV-I, HTLV-III, avian reticuloendotheliosis virus, Rous sarcoma virus, Moloney murine leukemia virus, mouse mammary tumor virus, Mason-Pfizer monkey virus, and retrovirus-D/Washington. Positive reactions were detected between anti-EIAV p26 serum and HTLV-III p24 (Fig. 9, lane 1) and between anti-EIAV p15 serum and HTLV-III p17 (Fig. 9, lane 4). The amount of virus applied to each lane was adjusted to give approximately equal Coomassie brilliant blue staining bands for HTLV-III p24 and EIAV p26 (determined in separate experiments not shown). Thus, the obvious differences seen in the intensities

gag Protein	Polyprotein Structure				Antigenic Activity			
	p15	p26	p11	p9	p15	p26	p11	p9
Pr55	———	———	———	———	+	+		+
Pr49	———	———	———		+	+	(+)	
Pr40	———	———			+	+		
Pr35		———	———			+	(+)	

FIG. 8. Proposed proteolytic processing scheme for generation of Pr49^{gag}, Pr40^{gag}, and Pr35^{gag} from Pr55^{gag}. The first horizontal line represents Pr55^{gag} and shows the proposed order of the viral *gag* proteins (p15, p26, p11, and p9) in the precursor. The other horizontal lines represent suggested structures of intermediate cleavage fragments of Pr55^{gag}, proposed on the basis of antigenic reactivity indicated by plus signs in the appropriate right-hand column and apparent molecular weights based on SDS-PAGE mobility. Plus signs in parentheses indicate weak reactions detected with antisera diluted 1:100 (Fig. 6C).

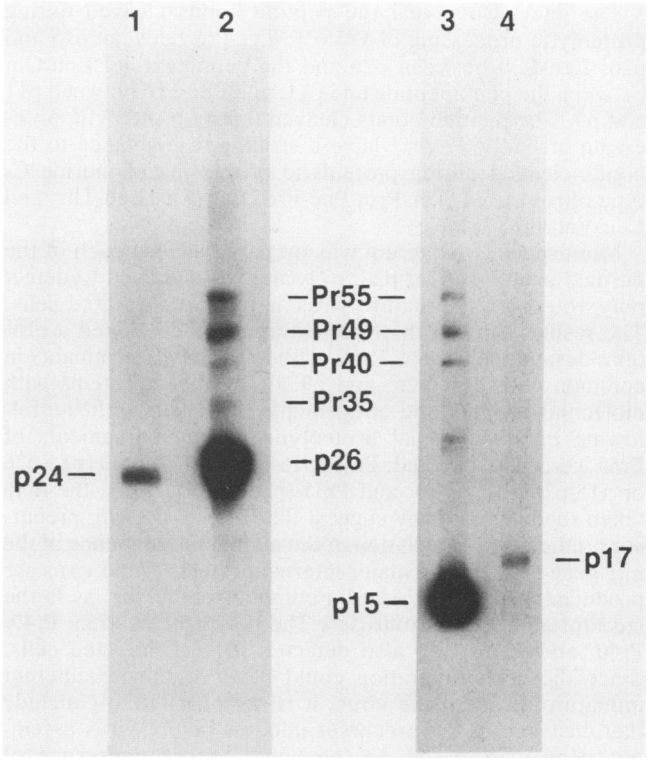

FIG. 9. Antigens present in HTLV-III detected with antisera prepared against purified EIAV p15 and p26. Viral proteins were separated, transferred, fixed to diazo paper, and detected as described in the legend to Fig. 6. Lanes 1 and 4, HTLV-III; lanes 2 and 3, EIAV. In lanes 1 and 2 antigens were detected with anti-p26 serum and in lanes 3 and 4 antigens were detected with anti-p15 serum.

of immunoblot bands for EIAV p24 and HTLV-III p26 (Fig. 9, cf. lanes 2 and 1) and for EIAV p15 and HTLV-III p17 (Fig. 9, cf. lanes 3 and 4) probably reflect weak cross-reactivity between the specific EIAV antiserum and the homologous HTLV-III protein. In other experiments (data not shown), purified HTLV-III p24 and p17 were used to confirm the identities of the cross-reactive proteins.

DISCUSSION

Previous studies have shown that EIAV is composed of at least four proteins (6, 23, 34, 40) believed to be associated with the internal structure of the virus. In this study we purified four major proteins (p11, p9, p26, and p15) and a pentapeptide from EIAV by RP-HPLC by using essentially the same techniques and procedures previously used to purify major internal structural proteins from B-type (21), C-type (18), and D-type (20) retroviruses and other retroviruses such as HTLV-I(9, 39).

Each purified protein was analyzed for its N- and C-terminal amino acid sequences and amino acid and phosphoamino acid content. The proteins were located in the deduced structure of the EIAV Pr55^{gag} polyprotein by aligning the deduced amino acid sequence with the experimentally determined amino acid sequences. This gave the order of the proteins in the structure of Pr55^{gag} as p15-p26-*-p11-p9, where a pentapeptide between p26 and p11 is represented by the asterisk, and also showed that all portions of Pr55^{gag} except the initiator methionine were accounted for among the purified proteins and peptides.

The data also reveal the peptide bonds cleaved during proteolytic processing of $Pr55^{gag}$: Tyr-Pro between p15 and p26; Leu-Leu between p26 and the pentapeptide; Leu-Gln between the pentapeptide and p11; and Phe-Pro between p11 and p9. The peptide bonds cleaved during proteolytic processing of EIAV $Pr55^{gag}$ have a striking resemblance to the bonds cleaved during proteolytic processing of murine C-type retroviruses (Tyr-Pro, Phe-Pro, Leu-Ala, Leu-Thr, and Leu-Val) (18, 38).

Monospecific antiserum was prepared against each of the purified proteins (p15, p26, p11, and p9) and used to detect polyproteins in the mature virus and in virus-infected cells. The results showed that the mature virus contained a protein, designated here as Pr55, with antigenic determinants in common with p15, p26, and p9. In addition, proteins with molecular weights and antigenicity consistent with the following putative partial proteolytic cleavage fragments of Pr55 were also detected: Pr49 (p15-p26-*-p11), Pr40 (p15-p26 or p15-p26-* or both), and Pr35 (p26-*-p11). Thus, the data taken together strongly suggest that Pr55 is the *gag* precursor predicted by translation of the nucleotide sequence of the *gag* gene and that the major internal proteins of the virus are produced by proteolysis of a common precursor, as is the case for all other retroviruses. The polyproteins Pr55, Pr49, Pr40, and Pr35 were also detected in virus-infected cells. Since the cell preparation could have contained adherent immature forms of the virus, it is not possible to conclude that cleavage of the precursor initiates before virus assembly. However, based on the apparent preponderance of $Pr40^{gag}$ found in the cell preparation, it appears that proteolytic cleavage between p15 and p26 may be a slow step in proteolytic processing. A kinetic analysis would be needed to firmly establish the relative rates of cleavage of various peptide bonds and to confirm precursor-product relationships implied here.

The mature virus also contained traces of polypeptides with apparent molecular weights of 23,000, 22,000, and 18,000 detected with anti-p26 serum and a 4,000-molecular-weight polypeptide detected with anti-p15 serum. These polypeptides were not further characterized but probably represent minor proteolytic cleavage fragments of p26 and p15.

A relationship between EIAV and HIV was first suggested on the basis of immunological cross-reactivity observed with anti-EIAV sera and HIV p24 (4, 33). The distant but distinct relationship was shown in detail by a comparison of the nucleotide sequences of HIV (42) and EIAV (50). In this study, we confirmed the original observations of antigenic cross-reactivity with more defined antisera by showing that anti-EIAV p26 serum reacted with HTLV-III p24 and in addition showed that anti-EIAV p15 serum reacted with HTLV-III p17.

Many retroviruses including mammalian C-type, D-type, B-type, HTLV-I, bovine leukemia virus, and HTLV-III have *gag* polyproteins that are modified by a two-step mechanism involving proteolytic removal of the initiator methionine followed by covalent attachment of a myristyl moiety [$CH_3(CH_2)_{12}CO$-] to the newly formed N-terminal glycine residue (17, 45, 46). It has recently been shown that myristylation requires an N-terminal glycine residue and is essential for assembly or budding or both of the murine C-type virus (43). The predicted N-terminal amino acid sequence of $Pr55^{gag}$ is Met-Gly-Asp-Pro-Leu-Thr (50). EIAV p15 appears to be lacking the initiator methionine (Table 1) and has a blocked or modified N terminus. However, when EIAV-infected cells were incubated in the presence of [^{3}H]myristic acid, Pr55 failed to incorporate significant radioactivity (results not shown). Recently, in collaboration with A. Schultz we analyzed EIAV p15 directly by chemical means and found that it contains no myristic acid (unpublished data). Thus, it appears that EIAV, like avian retroviruses, does not require N-terminal myristylation of its *gag* precursor. Factors preventing myristylation of EIAV Pr55 and the nature of the N-terminal blocking group are unknown. It will be of interest to determine the nature of the amino-terminal blocking group in EIAV Pr55 and its role in viral assembly or budding or both.

ACKNOWLEDGMENTS

We thank Cathy Hixson for assistance in performing phosphoamino acid and C-terminal amino acid analyses and Cheri Rhoderick and Carolyn Phillips for preparation of the manuscript.

This research was sponsored by the National Cancer Institute under contract NO1-CO-23909 with Bionetics Research, Inc.

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