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Identification of the P Proteins and Other Disulfide-Linked and Phosphorylated Proteins of Newcastle Disease Virus

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A unique abundant protein, designated P by analogy to the putative polymerase proteins of other paramyxoviruses, was identified in purified Newcastle disease virus. Under nonreducing conditions the P proteins could be separated from other viral proteins on sodium dodecyl sulfate-polyacrylamide gels. The P proteins were isolated from detergent-solubilized virions as 53,000- to 55,000-dalton monomers and disulfide-linked trimers. Distinct forms of P having four different isoelectric points and two different electrophoretic mobilities were resolved by two-dimensional electrophoresis. Two forms of P were phosphorylated, as were the nucleocapsid protein and non-glycosylated membrane protein. In addition to disulfide-linked forms of P, dimers of the hemagglutinin-neuraminidase glycoprotein and two disulfide-linked versions of the fusion glycoprotein were identified. Several electrophoretic variants of the nucleocapsid protein that were probably created by intrachain disulfide bonding were also isolated from virions under nonreducing conditions. The locations of the newly identified proteins were determined by detergent-salt fractionation of virions and by surface-selective radioiodination of the viral envelope. The P proteins were associated with nucleocapsids and were not detected at the surface of virions. Both forms of the fusion glycoproteins were on the exterior of the viral envelope. Herein the properties of the P proteins are compared with similar proteins of rhabdoviruses and other paramyxoviruses, and a role for multiple forms of proteins in the genetic economy of Newcastle disease virus is discussed.

The avian paramyxovirus Newcastle disease virus (NDV) contains four abundant and several minor species of protein (7). The major proteins include the hemagglutinin-neuraminidase (HN) and fusion ($F_{1,2}$) glycopolypeptides that form two populations of external projections on the lipoprotein envelope of virions (37, 44, 47), nucleocapsid proteins (NP), and non-glycosylated membrane (M) proteins. The M proteins are extrinsically associated with the inner surface of the envelope (28) and closely apposed to the flexible ribonucleoprotein helix that constitutes the core of virions (21, 31). Less abundant proteins include large (L) 220,000-dalton core-associated proteins, 47,000-dalton species of unknown location, and fragments of NP that range from 43,000 to 53,000 daltons (19, 35). Other well-characterized paramyxoviruses, Sendai virus and simian virus 5, contain four types of abundant proteins that appear to be functionally analogous to the major NDV proteins (7, 36, 46) and an additional core-associated protein, designated P because it is a putative component of the virion-associated RNA-dependent RNA polymerase (5, 45, 49).

Recently we identified a candidate for the P protein of NDV based upon its rapid assembly into virions (47a). Using cell-free protein-synthesizing systems programmed with mRNA transcribed by detergent-activated virions, Collins et al. (12) proved that P is encoded by the viral genome and showed that the gene specifying P was second in the transcriptional order (NP-P-F_0-M-HN-L). Herein, peptide mapping by high-voltage paper ionophoresis (9, 19) and by detergent-limited proteolysis (8) was used to prove that P is unique among known NDV proteins. The P proteins and additional disulfide-linked polypeptides were located in virions by fractionation and by radioiodination. Finally, each of the detectably phosphorylated viral proteins was identified by peptide mapping.

MATERIALS AND METHODS

Virus preparation and cell culture. Strain *AV* (Australia-Victoria, 1932) of NDV was grown in 10-day-old embryonated chicken eggs (Spafas, Inc.). Virus was purified from allantoic fluid as described previously (11, 18) and stored at −70°C in phosphate-buffered saline without divalent cations.

Secondary cultures of chicken embryo cells were prepared as described previously (6, 11). The cells were grown in NCI medium (GIBCO Laboratories) containing 6% calf serum under a humidified atmosphere of 5% CO_2 maintained at 37°C. Confluent cul-

tures in plastic 100-mm cell culture plates (7.5 × 10^6 cells per plate) were routinely used.

Preparation of radioactive virus. All radioisotopes were obtained from New England Nuclear Corp. except for L-[^{35}S]methionine, which was also from Amersham Corp. Cell cultures were infected with an input multiplicity of 10 PFU/cell. After a 45-min adsorption period, Eagle minimal essential medium containing 5% calf serum was added, and the cultures were incubated for 2 h at 37°C. Then the incubation medium was replaced by minimal essential medium containing 5% (0.75 mg/liter) of the normal concentration of methionine, 5% dialyzed calf serum, and 100 μCi of [^{35}S]methionine (500 to 1,300 Ci/mmol) per ml of medium. After a 15-h incubation at 37°C, the medium was collected from the radioactive cultures and clarified by low-speed centrifugation (15 min at 12,000 × *g*). Nonradioactive carrier virions were then added to the clarified medium. The virions were centrifuged for 1 h at 83,000 × *g* through a layer of 20% sucrose onto a 60% sucrose pad. The band at the interface of the sucrose layers was collected, diluted with phosphate-buffered saline, layered over a linear gradient of 20 to 65% sucrose in standard buffer, and centrifuged at 83,000 × *g* for 16 h in an SW27 rotor. The band of radioactive virions was removed from the gradient and diluted in phosphate-buffered saline, and virions were concentrated by differential ultracentrifugation. The resulting virus pellet was resuspended in a small volume of phosphate-buffered saline and stored at −70°C.

The same procedure was used to obtain purified virions labeled with [^{3}H]serine and [^{3}H]threonine except that infected chicken embryo cells were incubated in minimal essential medium lacking threonine and supplemented with 5% dialyzed calf serum, 50 μCi of L-[^{3}H]threonine (3.4 Ci/mmol), and 50 μCi of L-[^{3}H]serine (5 Ci/mmol) per ml of medium. To obtain ^{32}P-labeled virions, infected chicken embryo cells were incubated in phosphate-free minimal essential medium supplemented with 5% dialyzed calf serum and 200 μCi of $^{32}P_i$ per ml of medium.

To radioactively label viral glycoproteins, infected chicken embryo cells were incubated for 6 h in glucose-free NCI medium supplemented with 10 mM sodium pyruvate, 5% dialyzed calf serum, and 100 μCi of D-[2-^{3}H]mannose (18.4 Ci/mmol) per ml of medium. At the end of the incubation period, the radioactive medium was collected and clarified by low-speed centrifugation. The clarified medium was layered over 20% sucrose in standard buffer and centrifuged at 83,000 × *g* for 1 h in an SW27 rotor. The virion pellet was dissolved in polyacrylamide gel sample buffer lacking mercaptoethanol, heated for 5 min at 100°C, and stored at −20°C.

Radioiodination of virions. Surface-specific radioisotopic labeling of virions was carried out by lactoperoxidase-glucose oxidase-catalyzed iodination (radioiodination system, New England Nuclear Corp.). Virions were first purified by equilibrium density ultracentrifugation as described above and resuspended at a final concentration of 1 mg of protein per ml of phosphate-buffered saline. Approximately 10 μl of Na^{125}I (0.1 Ci/ml) was added to 100 μl of 0.05 M sodium phosphate buffer (pH 7.4) in a reaction vessel containing lactoperoxidase and glucose oxidase (New England Nuclear radioiodination reagent). The reaction was started with 25 μl of 1% β-D-glucose and immediately mixed with an equal volume of virus suspension. After incubation at room temperature for 30 min, the reaction was terminated by the addition of 10 μl of sodium *m*-bisulfite (10 mg/ml). Labeled virions were washed with phosphate-buffered saline, concentrated by differential ultracentrifugation, dissolved in gel sample buffer without mercaptoethanol, and stored at −20°C.

Isolation of nucleocapsids from virions. Purified virions labeled with [^{35}S]methionine were mixed with a disruption solution (1% Triton X-100, 0.4 M KCl, 0.0125 M Tris-hydrochloride at pH 6.8; aprotinin at 1,000 kallikrein inhibitor units [KIU] per ml) and incubated at room temperature for 20 min. Nucleocapsids were separated from solubilized polypeptides by equilibrium density gradient centrifugation (150,000 × *g*, 105 min), using a Beckman SW50.1 rotor. Linear D_2O-glycerol-sucrose gradients prepared as described by Colonno and Stone (14) were used. After centrifugation, gradient fractions containing the peak of radioactivity, which corresponded to a particulate band (1.27 g/cm^3), were pooled. Nucleocapsids were recovered by differential ultracentrifugation (110,000 × *g*, 1 h), dissolved in gel sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

SDS-PAGE. The radioactively labeled polypeptides of virions, isolated nucleocapsids, or infected cells were solubilized in gel sample buffer (0.0625 M Tris-hydrochloride [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 0.001% bromophenol blue) and heated at 100°C for 5 min. Samples to be analyzed under nonreducing conditions were dissolved in gel sample buffer lacking mercaptoethanol and heated as above. Proteins were separated on 11.5% polyacrylamide slab gels according to the procedure of Laemmli (24). Gels were run at a constant current of 20 mA for 16 h. The gels were then acid fixed and stained with Coomassie brilliant blue G-250 (Eastman Kodak Co.) or prepared for fluorography as described by Laskey and Mills (27). The gels were dried onto sheets of dialysis membrane (Bio-Rad Laboratories) and exposed to Kodak XR-5 X-ray film for 2 to 10 days at −70°C.

Molecular weights of the viral proteins were estimated on SDS-polyacrylamide gels, using the following markers: insulin (molecular weight, 5,733); phosphorylase *a* (92,000); the subunits of *Escherichia coli* RNA polymerase β' (165,000), β (155,000), and α (39,000), all purchased from Boehringer-Mannheim Co.; and molecular-weight markers (14,300- to 71,500-molecular-weight range) from BDH Chemicals, Ltd.

Isoelectric focusing-SDS-polyacrylamide gel electrophoresis. Two-dimensional analyses of viral proteins, using isoelectric focusing in the first dimension and SDS-PAGE in the second, were performed by the methods of O'Farrell (39) with minor modifications. A suspension of purified radioactive virions in phosphate-buffered saline was added to an equal volume of a twofold concentration of gel sample buffer and mixed with an equal volume of O'Farrell lysis buffer. Samples containing 10 to 25 μg of protein were layered onto isoelectric focusing gels which were cast

in either 100-μl micropipettes or glass tubes (1.7-mm inner diameter). Isoelectric focusing was carried out at 500 V for 8 h. The cylindrical gels were then applied to the tops of 11.5% polyacrylamide slab gels and subjected to electrophoresis for 18 h at a constant current of 20 mA.

Peptide mapping. Discrete bands of radioactively labeled viral proteins located by autoradiography were excised from dried gels and used for peptide mapping by the following procedures. (i) Mapping by detergent-limited proteolytic digestion was performed by the method of Cleveland et al. (8) except that gel slices were rehydrated in the presence of 1% dithiothreitol. Peptides generated by *Staphylococcus aureus* protease V8 (Miles Laboratories, Ltd.) at a concentration of 25 μg/ml were separated on a 15% polyacrylamide gel which was subsequently analyzed by fluorography. (ii) Tryptic peptides were produced as described previously (19) with minor modifications. Gel slices containing viral proteins were rehydrated in 1 ml of 1% ammonium bicarbonate containing 200 μg of trypsin-TPCK (trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone; Worthington Biochemicals Corp.). The rehydrated slices were crushed and incubated at 37°C with agitation for 24 h. After 8 and 16 h of incubation, each sample received an additional 0.1 ml of 1% ammonium bicarbonate solution containing 100 μg of trypsin-TPCK. Gel fragments were removed by centrifugation, and the samples were lyophilized. The resulting peptides were separated by high-voltage paper ionophoresis at pH 3.5 as described previously (16, 19). Autoradiograms of the peptide maps were scanned with a Joyce-Loebl Chromoscan densitometer.

RESULTS

Separation of the polypeptides of virions by SDS-PAGE under reducing and nonreducing conditions. Polypeptides labeled with either [^{35}S]methionine or [^{3}H]mannose were extracted from purified virions and resolved by SDS-PAGE. Viral proteins solubilized in the presence of 2-mercaptoethanol (reducing conditions) separated into five major size classes of [^{35}S]methionine-labeled polypeptides (Fig. 1a) and two [^{3}H]mannose-labeled components (Fig. 1b), the smaller being a doublet. Based on earlier studies in which similar patterns were obtained (11, 31, 36, 38), the individual bands corresponded to the following polypeptides listed in order of increasing electrophoretic mobility: the high-molecular-weight L protein, the HN glycoprotein that contained both radioactive methionine and mannose, the major nucleocapsid protein NP, the F_1 glycoprotein region that was marked by both radiochemicals, and the nonglycosylated M protein. Occasionally a minor 55,000-dalton band that migrated between NP and the F_1 region was also resolved (Fig. 1a). The 10,000-dalton F_2 subunit of the fusion glycoprotein was not retained on these gels, and a minor 47,000-dalton polypeptide observed in previous studies was not detected (9, 19).

Different electrophoretic patterns were obtained if polypeptides labeled with either [^{35}S]methionine (Fig. 1c) or [^{3}H]mannose (Fig. 1d) were extracted from virions in the absence of 2-mercaptoethanol (nonreducing conditions). The 74,000-dalton HN band was not detected (Fig. 1c); instead, a multimer containing both radioactive methionine and mannose appeared in the high-molecular-weight region of the gel. This new band was identified as HN by comparing the limited digest peptide map of the 74,000-dalton HN (Fig. 2a) obtained from reducing gels with that of the multimer (Fig. 2b).

In addition to HN, the mobility of the other major glycopolypeptide F_1 decreased under nonreducing conditions. All of the [^{3}H]mannose-labeled F_1 shifted out of the 53,000-dalton region of gels and migrated in two closely spaced bands centered at about 64,000 daltons (labeled $F_{1,2}$ in Fig. 1c). Limited-digest peptide mapping confirmed that both the larger (Fig. 2c) and the smaller (Fig. 2d) components of the doublet were

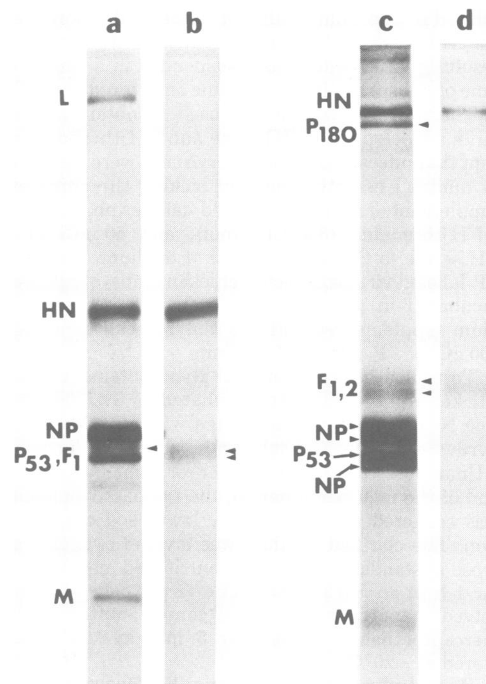


FIG. 1. *Fluorograms of [^{35}S]methionine-labeled (a, c) and [^{3}H]mannose-labeled (b, d) polypeptides extracted from purified virions and analyzed by SDS-PAGE under reducing (a, b) and nonreducing (c, d) conditions. Details of radioisotopic labeling, virus purification, and polyacrylamide gel electrophoresis are described in the text. Arrowheads on the right side of the slots mark the positions of the following partially resolved polypeptides: (a) P_{55}, (b) F_1 doublet, (c) $F_{1,2}$ doublet and the P_{180} doublet.*

related to the F_0 precursor glycoprotein isolated from infected cells (Fig. 2e).

In contrast to the behavior of the [^{3}H]mannose-labeled polypeptides, only part of the [^{35}S]methionine-labeled species shifted out of the 53,000-dalton region of nonreducing gels (Fig. 1c). The peptide map (Fig. 2f) of the polypeptides that remained in this region was different than the maps of F (Fig. 2c–e) and NP (Fig. 2g). The new map appeared to be a composite of contaminating peptides derived from NP and those from an additional protein. When the peptide map (Fig. 2h) of a pair of closely spaced disulfide-linked proteins (P_{180}) that contained radioactive methionine (Fig. 1c) but not mannose (Fig. 1d) were compared, the peptides of this map matched the unique peptides in the map of the remaining 53,000-dalton polypeptides. These data suggested that virions contained an additional class of polypeptides which could be isolated as 180,000-dalton multimers and 53,000-dalton monomers under nonreducing conditions. Peptides derived from these proteins dominated limited-digest peptide maps (Fig. 2i and j) of 53,000-dalton polypeptides extracted from virions of infected cells and analyzed by SDS-PAGE under reducing conditions.

The 220,000-dalton L protein was not detected by SDS-PAGE under nonreducing conditions (Fig. 1c). It probably remained in complexes which were too large to enter the gel. It has been reported that disulfide bonds can form in polypeptides by autooxidation during solubilization (17). Treatment of virions with 0.01 M iodoacetamide before disruption had no effect on NDV polypeptide patterns in nonreducing gels. Therefore, the disulfide-linked polypeptides which we have identified were probably not generated by oxidation of polypeptides during sample preparation.

Subunit composition of the disulfide-linked multimers. To determine whether the complexes isolated by SDS-PAGE under nonreducing conditions were composed of identical or electrophoretically distinct subunits, a two-dimensional polyacrylamide gel analysis was used. Viral proteins were first separated under nonreducing conditions and then electrophoresed in a second dimension under reducing conditions. Polypeptides in which disulfide bonding had no major influence on mobility were distributed along a diagonal line passing through M and the major NP spot (Fig. 3). Both glycopolypeptides were off the diagonal. After reduction the HN multimer, which had an apparent molecular weight of 192,000, yielded a single 74,000-dalton species. Therefore, it probably was a homogeneous dimer. Two-dimensional analysis revealed that both $F_{1,2}$ bands were composed of 53,000- to 55,000-dalton F_1

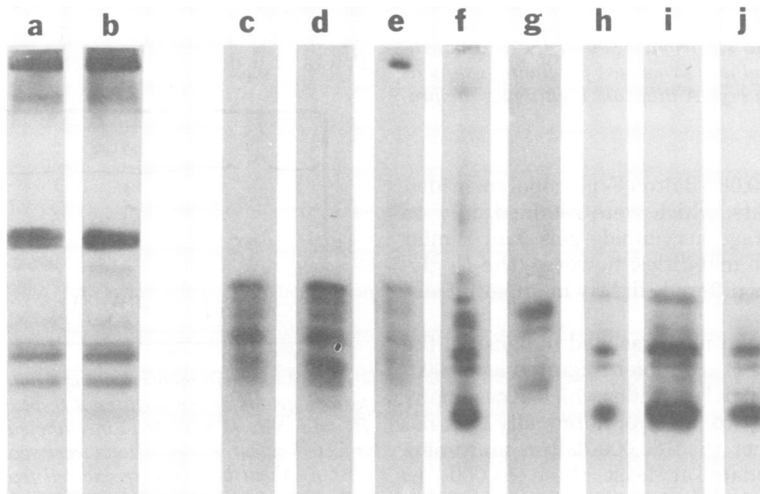


FIG. 2. *Fluorograms of the limited-digest peptide maps of [^{35}S]methionine-labeled polypeptides. Radioactively labeled infected cell extracts were prepared as described previously (11), and radioactive virions were prepared as described in the text. Individual bands were excised from gels similar to those shown in Fig. 1 and treated with S. aureus protease V8. The resulting peptides were separated by SDS-PAGE on 15% polyacrylamide gels. Except for the samples designated otherwise, proteins were taken from virions. Maps of the following proteins were compared: (a) HN from infected cells; (b) HN multimer; (c) upper band of $F_{1,2}$ doublet; (d) lower band of $F_{1,2}$ doublet; (e) F_0 from infected cells; (f) 53,000-dalton polypeptides; (g) NP; (h) P_{180} doublet; (i) 53,000-dalton polypeptides; (j) 53,000-dalton polypeptides from infected cells. Samples in (a), (e), (g), (i), and (j) were taken from reducing gels; samples in (b), (c), (d), (f), and (h) were from nonreducing gels.*

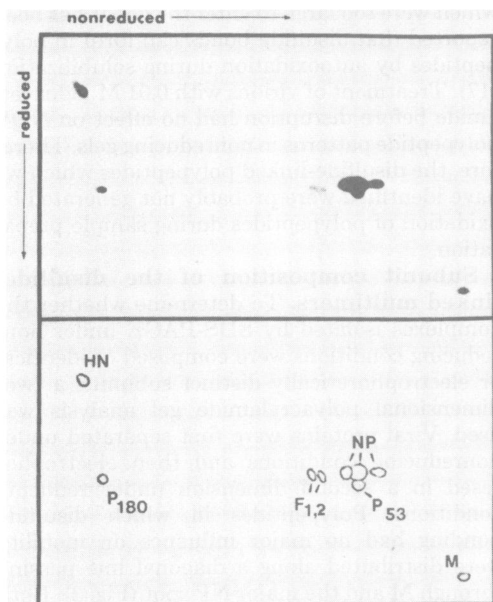


FIG. 3. *Fluorogram of [^{35}S]methionine-labeled polypeptides separated by two-dimensional SDS-PAGE. Radioactive polypeptides extracted from virions were resolved first by SDS-PAGE under nonreducing conditions in a cylindrical 9% polyacrylamide gel (horizontal dimension). Separation of the polypeptides was similar to that shown in Fig. 1c. The gel was then equilibrated in a buffer of 0.5 M Tris-hydrochloride (pH 8.8)–0.4% SDS–2% mercaptoethanol. The gel was mounted on a 10% polyacrylamide slab gel and subjected to electrophoresis in the vertical dimension. The gel was processed for fluorography as described in the text. A diagram identifying the proteins is shown.*

subunits and 10,000-dalton F_2 glycopolypeptides. The F_2 subunits, which were retained only on higher-percentage acrylamide gels, had similar electrophoretic mobilities; however, the F_1 glycopolypeptides differed slightly in apparent size (see Fig. 1b).

After reduction in the second dimension, the subunits of the P_{180} doublet migrated as 53,000- to 55,000-dalton polypeptides which occasionally resolved into two electrophoretically distinct species. Each of the 180,000-dalton multimers could accomodate at least three 53,000- to 55,000-dalton subunits.

Three electrophoretically distinct forms of NP, all having peptide maps similar to the one shown in Fig. 2g, were often resolved on nonreducing gels (Fig. 1c and 3). Varying degrees of intrachain disulfide bonding in NP probably created these variants, which included one form with a higher mobility than reduced NP (50). After electrophoresis in the reducing dimension of two-dimensional SDS-PAGE, each form of NP acquired the same electrophoretic mobility (Fig. 3). Therefore, the NP species which had a smaller apparent size under nonreducing conditions was a conformational variant and not a degradation product.

Tryptic peptide analysis of the P proteins. The preceding polyacrylamide gel analyses and limited-digest peptide mapping studies suggested that the additional polypeptides, which have been designated P with a subscript indicating the apparent molecular weight of different forms (P_{53}, P_{55}, P_{180}), might be unique viral proteins that had not been identified before. The tryptic peptides of all known NDV proteins were compared previously by high-voltage paper ionophoresis (9, 19). Therefore, this method was chosen for evaluation of the P proteins as well.

[^{35}S]methionine-labeled polypeptides from virions and infected cells were separated on SDS-polyacrylamide gels like those in Fig. 1. Individual bands were cut from the gel, exhaustively digested with trypsin, and analyzed by paper ionophoresis at pH 3.5. Densitometer scans of the resulting autoradiograms are shown in Fig. 4. From previous studies it was known that the 53,000-dalton region of reducing gels contained F_1 and that fragments of NP were occasionally present. The tryptic maps in Fig. 4 showed that P was distinct from either of these polypeptides. Here, the map of F_0 was used to represent F-gene products. The tryptic map of P was also different from the maps of L, HN,

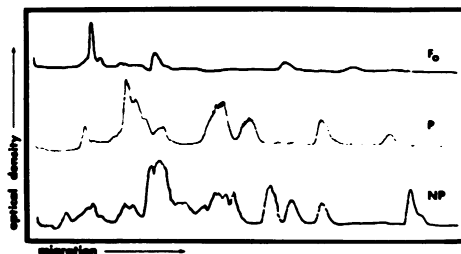


FIG. 4. *Densitometer tracings obtained from autoradiograms of the methionyl tryptic peptides of F_0, P, and NP. [^{35}S]methionine-labeled proteins from infected cultures and virions were separated by SDS-PAGE. Viral bands were excised from the gels and exhaustively digested with trypsin as described in the text. The resulting peptides were separated by high-voltage paper ionophoresis at pH 3.5. The maps of $F_{1,2}$ from virions and F_0 from infected cells were indistinguishable, so only the latter was shown. Likewise, P_{180} and P_{53} (except for contaminating NP peptides) taken from nonreducing gels of virions and P_{53} from reducing gels of infected cell samples had similar maps, and only the latter was shown. The source of radioactive NP was an extract of infected cells.*

and M (data not shown; see 19 for comparable maps). Thus, with the possible exception of the 47,000-dalton protein, which was not detected in this study, the P proteins were not related to any of the known NDV proteins.

Location of the P and $F_{1,2}$ proteins in virions. Colonno and Stone (14) found 53,000-dalton polypeptides associated with NDV nucleocapsids that had been isolated from virions of strain Beaudette *C* under conditions which preserved transcriptase activity. Based on this observation, we considered the P protein of strain *AV* to be a likely candidate for a core-associated protein. As a first step in the isolation of cores, the sensitivities of the proteins of purified virions of strain *AV* to extraction by 1% Triton X-100 alone or in combination with increasing amounts of KCl were determined. Four classes of proteins were obtained. Detergent alone solubilized HN and both forms of $F_{1,2}$. In the presence of increasing concentrations of salt, M was solubilized first (0.1 M KCl), followed by a group of polypeptides (L, P_{180}, P_{53}) which were more tightly bound to cores, leaving only NP, which remained bound to RNA even in 1.0 M KCl. Optimum conditions for the selective removal of most envelope-associated proteins were 1% Triton X-100 and 0.4 M KCl. These conditions were milder than those used by Colonno and Stone (1% Triton X-100, 0.75 M KCl) for the isolation of transcriptase-active cores. The different optima for the preservation of core-associated proteins may reflect strain-specific differences in the affinity of proteins for the nucleocapsid.

To analyze the polypeptides of the nucleocapsid, purified virions containing radioactive proteins were solubilized in 1% Triton X-100–0.4 M KCl, and the nucleocapsids were separated from free polypeptides by equilibrium density ultracentrifugation. A single peak of radioactivity was obtained in the glycerol-sucrose-D_2O gradient at a density of 1.27 g/cm^3, the characteristic density of NDV nucleocapsids (14, 33). This material was collected and compared with solubilized virions by SDS-PAGE (Fig. 5a and b). The following polypeptides were associated with isolated cores in lesser amounts: high-molecular-weight polypeptides which comigrated with L, 53,000- and 50,000-dalton components, and a trace of M-size protein. To show that the 53,000-dalton core-associated polypeptides were not NP fragments, the limited-digest peptide maps of these polypeptides (Fig. 5c) were compared with those of authentic P (Fig. 5d) and NP (Fig. 5e). The core-associated 53,000-dalton polypeptides were found to be P proteins; however, the 50,000-dalton species was identified as an NP fragment (data not shown). P_{180} was also identified in

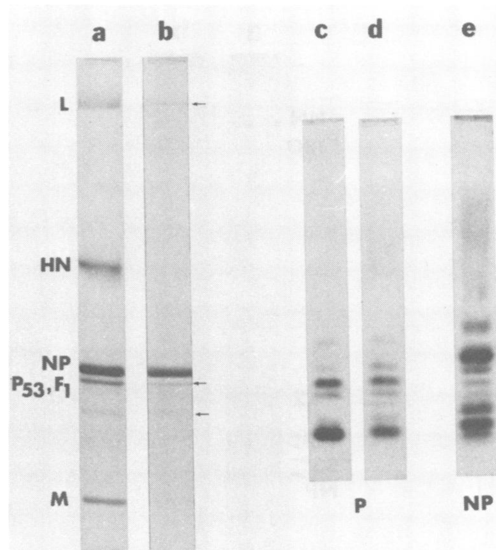


FIG. 5. *Analyses of the proteins of nucleocapsids. Radioactive nucleocapsids were released from virions by 1% Triton X-100 in 0.4 M KCl and purified by equilibrium density ultracentrifugation. The isolated nucleocapsids were solubilized in gel sample buffer containing mercaptoethanol, and the [^{35}S]methionine-labeled polypeptides were separated by SDS-PAGE. An autoradiogram is shown of a slab gel in which [^{35}S]methionine-labeled polypeptides from virions (a) prepared as in the legend to Fig. 1 were compared with those of nucleocapsids (b). Nucleocapsid proteins were analyzed further by limited-digest peptide mapping as described in the legend to Fig. 2. A fluorogram of the peptide map of P_{53} (c) obtained from purified nucleocapsids was compared with the maps of P_{53} (d) and NP (e) taken from virions.*

nucleocapsids analyzed by SDS-PAGE under nonreducing conditions (not shown). The identity of the L-size polypeptide in cores could not be verified by limited-digest mapping due to its scarcity and large size.

The purposes of the following experiment were to verify the internal location of the P proteins in virions by a method which did not involve fractionation and to determine whether both forms of $F_{1,2}$ were on the surface of virions. Purified virions were labeled with ^{125}I by using a combination of lactoperoxidase and glucose oxidase to selectively incorporate the radioisotope into proteins on the virion surface. The iodinated virions were solubilized and analyzed by SDS-PAGE under nonreducing conditions (Fig. 6b) along with marker polypeptides from [^{35}S]methionine-labeled virions (Fig. 6a). Although other viral proteins contained trace amounts of label, only the HN dimer and both forms of $F_{1,2}$ were readily accessible to radioio-

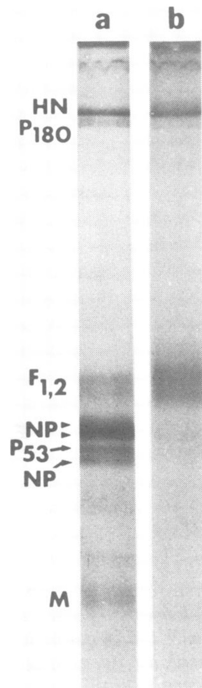


FIG. 6. *Identification of proteins on the surface of virions. Purified virions were labeled with ^{125}I in lactoperoxidase-glucose oxidase-catalyzed reactions. The radioiodinated virions were solubilized in gel sample buffer without mercaptoethanol, and the polypeptides were separated by SDS-PAGE. A fluorogram is shown of a slab gel in which [^{35}S]methionine-labeled polypeptides from virions (a) prepared as in the legend to Fig. 1 were compared with the ^{125}I-labeled polypeptides (b).*

dination, findings which support existing data on the arrangement of proteins in NDV virions based on radioiodination (30).

Identification of the phosphorylated proteins of virions. With the finding that the NDV P proteins were core associated, these proteins began to resemble the P phosphoproteins of other paramyxoviruses. No studies of phosphoproteins have been reported for NDV. Therefore, all of the detectably phosphorylated proteins of virions were identified. First, virions were collected from infected chicken embryo cell cultures exposed to phosphate-deficient medium supplemented with $^{32}P_i$. Polypeptides were extracted from the ^{32}P-labeled virions and analyzed by SDS-PAGE under nonreducing conditions. Three size classes of polypeptides contained radioactive phosphorus (Fig. 7b). These species comigrated with [^{35}S]methionine-labeled P_{180}, NP, and M extracted from virions (Fig. 7a). Due to the broad distribution of ^{32}P-containing material in the 50,000- to 56,000-dalton range, no distinct forms of either NP or P_{53} could be identified. No radioactive phosphorus was detected in L polypeptides analyzed on reducing gels (data not shown).

To establish that the radioactive phosphorus was associated with NDV polypeptides rather than with contaminating RNA, phospholipids, or cellular proteins, individual bands were excised from the gel (Fig. 7b) and subjected to limited-digest peptide mapping. Since radioactive phosphate is covalently linked to serine and threonine in other paramyxoviral proteins (26), the maps of the ^{32}P-labeled NDV polypeptides (Fig. 7d, f, and h) were compared with those of authentic viral proteins that were labeled with [^{3}H]serine and [^{3}H]threonine (Fig. 7c, e, and g). The peptides in the map of the 56,000-dalton ^{32}P-labeled species (Fig. 7d) were a subset of those in authentic NP (Fig. 7c), and the map of authentic M (Fig. 7e) matched that of the 41,000-dalton ^{32}P-labeled material (Fig. 7f). However, the peptides of the 180,000-dalton ^{32}P-labeled material (Fig. 7h) did not match the maps of either [^{3}H]serine-[^{3}H]threonine-labeled (Fig. 7g) or [^{35}S]methionine-labeled (Fig. 7i) P_{180} proteins.

Multiple phosphorylated and non-phosphorylated forms of the P proteins. Because the identification of putative P phosphoproteins by limited-digest peptide mapping (Fig. 7g–i) yielded equivocal results, the 53,000-dalton size class was analyzed further by using isoelectric focusing–SDS-PAGE. The polypeptides of [^{35}S]methionine-labeled and ^{32}P-labeled virions were separated in parallel first on cylindrical gels by isoelectric focusing and then on polyacrylamide slab gels. The regions of the gels containing the P proteins are shown in Fig. 8. Four major spots containing [^{35}S]methionine were detected. Each of the spots was excised from the gel and further analyzed by limited-digest peptide mapping (Fig. 8). The maps of spots 1 through 3 were indistinguishable from each other and from the map of authentic P. The map of spot 4 matched that of authentic P except for a single peptide (marked by arrow) which had a larger apparent size than its nearest neighbor in the P map. This peptide was probably responsible for the lower electrophoretic mobility of spot 4 relative to the other spots. Spot 4 probably corresponded to the minor 55,000-dalton polypeptides (marked by arrowhead in Fig. 1a) that migrated between NP and P_{53} on one-dimensional gels. The 55,000-dalton polypeptides were shown to be P related by limited-digest peptide mapping (data not shown). The pattern and relative intensities of the spots marked in Fig. 8 were reproducible among several different preparations of radioactive virions, and a similar pattern was obtained

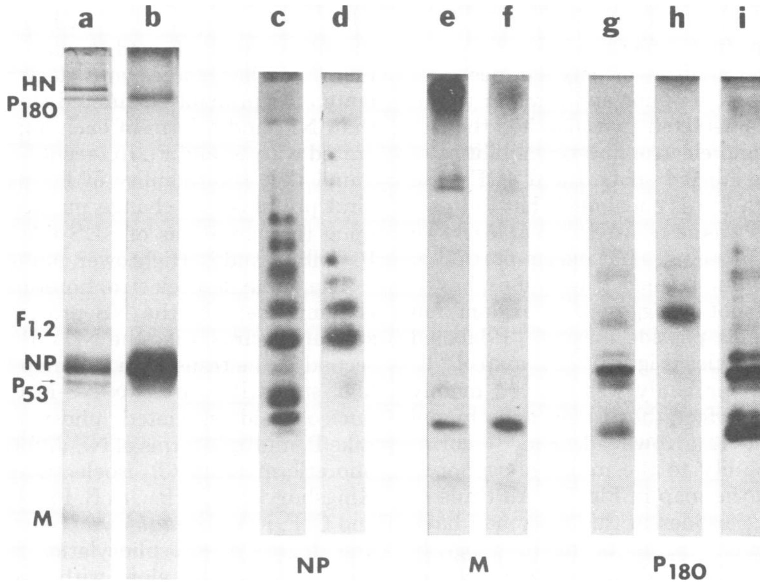


FIG. 7. *Analysis of phosphorylated proteins. [35S]methionine-labeled polypeptides (a) and 32P-labeled polypeptides (b) were extracted from purified virions and separated by SDS-PAGE under nonreducing conditions. A fluorogram of the resulting slab gel is shown. Limited-digest peptide maps were prepared as described in the legend to Fig. 2 from NP (c), M (e), and P_{180} (g) labeled with [3H]serine-[3H]threonine, and from NP (d), M (f), and P_{180} (h) labeled with $^{32}P_i$. A map of P_{53} (i) labeled with [35S]methionine was prepared for comparison. A fluorogram of the mapping gel is shown.*

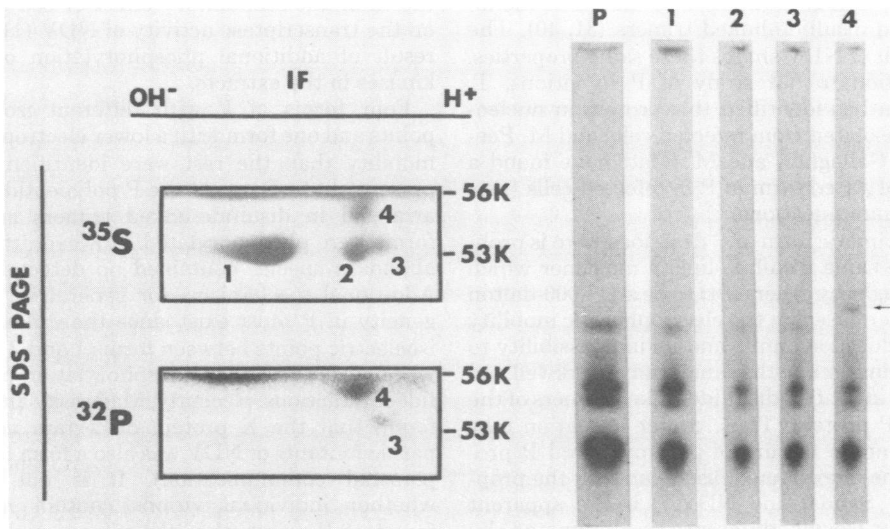


FIG. 8. *Isoelectric focusing-SDS-PAGE and limited digest peptide maps of the P proteins. [35S]methionine-labeled and 32P-labeled polypeptides were extracted from virions and analyzed by isoelectric focusing (horizontal) followed by SDS-PAGE (vertical) under reducing conditions. Fluorograms of the regions of the two slab gels between pH 5.7 and 6.4 and from 51,000 to 56,000 daltons are shown. Limited-digest peptide maps were prepared as described in the legend to Fig. 2 except that individual spots (designated 1-4) were excised from the dried slab gel containing [35S]methionine-labeled proteins. Fluorograms of the maps of spots 1-4 along with a map of [35S]methionine-labeled P_{53} from infected cells were compared. The arrow beside slot 4 marks the position of the largest peptide.*

with [^{35}S]methionine-labeled polypeptides from infected cultures (not shown).

In the parallel analysis of polypeptides from ^{32}P-labeled virions, a minor and a major radioactive spot were detected that matched the isoelectric points and electrophoretic mobilities of [^{32}S]methionine-labeled spots 3 and 4 (Fig. 8). From these data it was concluded that radioactive phosphorus was associated with at least two forms of the P proteins. That only a portion of the [^{35}S]methionine-labeled P was phosphorylated, primarily spot 4, suggested an explanation for the ambiguous peptide map of ^{32}P-labeled 180,000-dalton species (Fig. 7h). This map of ^{32}P-labeled peptides probably was derived mainly from spot 4, and in fact, the displaced methionyl peptide of spot 4 (arrowhead, Fig. 8) corresponded in mobility to the major phosphorylated peptide in the map in Fig. 7h. All three of the ^{32}P-labeled peptides in this map may have been overlapping fragments having slightly lower mobilities than the corresponding [^{35}S]methionyl peptides derived from spots 1 through 3.

DISCUSSION

Initial characterization of the NDV P proteins was guided by the known properties of the P proteins of Sendai virus. The latter proteins are phosphorylated (25, 26), are associated with nucleocapsids obtained from virions (32) and infected cells (22), and can be isolated as monomers and disulfide-linked trimers (31, 40). The P protein of NDV shares these same properties. In addition to our study of P in virions, T. Morrison has identified this protein in nucleocapsids isolated from infected cells and M. Peeples, P. Gallagher, and M. Bratt have found a phosphorylated form of P in infected cells (personal communications).

The trimeric form of P described here is probably the same disulfide-linked multimer which was thought by others (31) to be a 147,000-dalton NP trimer based on the electrophoretic mobility of its reduced subunits and its inaccessibility to radioiodination. In the same study Markwell and Fox (31) identified disulfide-linked trimers of the Sendai P protein. Thus, trimer formation may be a common feature of paramyxoviral P proteins. One unexplained discrepancy in the properties of Sendai and NDV P is the apparent molecular-weight difference as estimated by SDS-PAGE: the molecular weight of the Sendai P polypeptide is 78,000 (31), whereas that of the NDV P is 53,000.

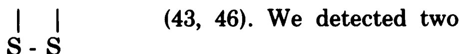
Comparisons of the peptide map of P with published maps revealed that the 53,000-dalton NDV polypeptides synthesized in cell-free systems were P-gene products and not an unglycosylated form of F as previously suggested (9, 11). Also, earlier maps of F_0 and F_1 appeared to be composites of the F_0 and P maps presented here (19). In earlier studies both electrophoretic variants of P probably exhibited slower mobilities than NP, and a form of each apparently comigrated with F_0 and F_1. In recent studies we have found that the mobility of P relative to other viral proteins can change markedly depending upon the conditions of SDS-PAGE (G. Smith, P. Collins, and L. Hightower, unpublished data).

The anomalous electrophoretic behavior of P is reminiscent of the NS protein of vesicular stomatitis virus. Like the NDV P protein, NS is second in the transcriptional map (1, 2) of vesicular stomatitis virus (N-NS-M-G-L) and is a nucleocapsid-associated phosphoprotein (48). Like P, multiple forms of NS differing in electrophoretic mobility (3), isoelectric points (D. W. Kingsbury and C. Hsu, *in* B. Fields, R. Jaenisch, and C. F. Fox, *Animal Virus Genetics*, in press), and degree of phosphorylation have been reported (10, 23). NS along with L is necessary for the transcriptase activity of vesicular stomatitis virus, and the more highly phosphorylated form of NS is the more active in a reconstituted transcriptase assay (23). It is tempting to speculate that the approximately 30-fold-lower levels of transcriptase activity in NDV compared to vesicular stomatitis virus (20) is a reflection of underphosphorylation of P. It is possible that the dramatic stimulatory effect of cell extracts on the transcriptase activity of NDV (11) is the result of additional phosphorylation of P by kinases in the extracts.

Four forms of P with different isoelectric points and one form with a lower electrophoretic mobility than the rest were identified in the present study. Some of the P polypeptides were arranged in disulfide-linked trimers and two forms were phosphorylated. However, the most abundant species contained no detectable ^{32}P. Additional mechanisms for generating heterogeneity in P must exist since the difference in isoelectric points between forms 1 and 2 (Fig. 8) was not due to either phosphorylation or disulfide interactions. Recently, Madansky and Bratt found that the X protein of certain noncytopathic mutants of NDV was also a form of P (29; personal communication). It is not known whether individual virions contain multiple forms of P or whether the virus population is heterogeneous in this respect. The significance of the multiple forms is also unknown, but their existence suggests possible explanations for several dilemmas. Nucleocapsids of NDV contain a variety of enzymatic activities, including a nucleotidyl transferase (20), polyadenylic acid-adding activity (51), and methylating (13) and capping activities (15). In addition, nucleocapsid

proteins may be needed as nucleases or terminators for mRNA synthesis and as nuclease inhibitors or antiterminators to effect the transition from mRNA to genome-length positive-strand synthesis. Other structural roles in virion assembly and template conformation can be anticipated. Yet, only three types of viral protein (NP, L, and P) have been identified in nucleocapsids. Post-translational modification of proteins such as P may provide NDV with mechanisms for fitting a small number of gene products to a variety of different enzymatic and structural roles. A second dilemma is that the P proteins appear to be too abundant in nucleocapsids to be involved solely in transcriptase activity. For Sendai virus, an estimated 200 copies of P compared to 20 copies of L are in cores (41). Perhaps only one of the forms of P is a component of the transcriptase, whereas most of P may have other roles. Recently, V. Chinchar and A. Portner (personal communication) found that Sendai nucleocapsids containing a 40-kilodalton proteolytic fragment of P retained most of their enzymatic activity, suggesting either a greater enzymatic role for the L protein (19, 52) or that only part of the P molecule is required.

Beyond previous observations that reducing agents destroy infectivity, hemagglutinating activity, and cell-fusing activity (40, 46), little is known about the roles of disulfide bonding in determining the fine structure and biological activities of the nucleocapsids and glycoproteins of NDV. It is known that HN glycopolypeptides form homodimers in some strains of NDV, but not in others (34). For strain *AV* we showed that HN was predominantly in the form of disulfide-linked dimers. It is also known that the active form of the fusion glycoprotein ($F_{1,2}$) contains F_2 linked to F_1 in the following order: H_2N-F_2--F_1-COOH



(43, 46). We detected two electrophoretically distinct forms of $F_{1,2}$ in strain *AV*. The molecular basis for this difference in mobility is unknown; however, the alteration affected F_1 and not F_2. Therefore, several possibilities can be eliminated, including the presence of an uncleaved N-terminal signal sequence on one form of F_2 and the possibility of different cleavage sites between F_2 and F_1 which would have altered the mobility of each fragment.

We established that both forms of $F_{1,2}$ were on the surface of virions by using radiochemical methods. The presence of both forms of $F_{1,2}$ on virions could explain an unusual property of strain *AV*. Unlike many NDV strains, *AV* does not mediate fusion from without, the virion-associated cell-fusing activity of paramyxoviruses (4). However, *AV*-infected cells undergo fusion, demonstrating that this strain can express fusion activity. And since $F_{1,2}$ is necessary for viral penetration (37, 45), at least some of the fusion proteins on *AV* virions must be active. If one form of $F_{1,2}$ were inactive in membrane fusion but still able to compete with the active form for cellular receptors, fusion from without might not occur. Consistent with this hypothesis, only one form of $F_{1,2}$ was found in strains *HP* (31; our unpublished data) and Hickman (46), both of which cause fusion from without.

Whereas the molecular architecture of P, $F_{1,2}$, and HN featured interchain disulfide bonds, intrachain linkages occurred in the NP population. Evidence from several recent studies indicates that substantial chemical and conformational heterogeneity exists among the major polypeptides of the nucleocapsid of Sendai virus. Two phosphorylated species of NP coexist with the non-phosphorylated form (26), and NP displays considerable heterogeneity in isoelectric focusing gels (42). The heterogeneity in chemical cross-linking reactivity that NP exhibits has been ascribed to the conformational freedom inherent in flexible ribonucleoprotein complexes such as the paramyxoviral core (41). Our studies indicate that nucleocapsid proteins of NDV also exist in several forms. Three electrophoretic variants of NP were detected by SDS-PAGE under nonreducing conditions, suggesting that heterogeneity occurs at the level of intrachain disulfide bond formation. Furthermore, at least part of the NPs were phosphorylated. The variability in disulfide bonding within the NP population may reflect requirements for conformationally distinct subclasses of NP in the assembly of the core or folding of the flexible helix into a stable conformation in virions.

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