A New Structural Protein for Newcastle Disease Virus

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(Accepted 28 April 1980)

SUMMARY

Proteins induced in Newcastle disease virus (NDV)-infected chick embryo fibroblasts (CEF) and proteins incorporated into virions grown in ovo were analysed by modified versions of a two-dimensional polyacrylamide gel electrophoresis system. The following previously described NDV-induced proteins were detected and resolved from host proteins: L (mol. wt. approx. 200K), HN (75K), F0 (66K), F1 (56K), NP (55K) and M (39K). Two additional polypeptides, NAP (nucleocapsid-associated protein, mol. wt. 56K) and a 36K mol. wt. protein were induced in NDV-infected cells. NAP but not 36K was found in purified virions. Radioactive labelling studies with 3H-glucosamine and 32P-orthophosphate demonstrated that neither NAP nor 36K is glycosylated, but that both are phosphorylated.

Variation in the isoelectric point and apparent mol. wt. of NAP among different strains was seen and exactly reproduced in both CEF and baby hamster kidney (BHK) cells. The synthesis of NDV-induced proteins including NAP and 36K was unaffected by actinomycin D, whereas the synthesis of host cell proteins was drastically reduced. These data are evidence that NAP and 36K are virus coded. Peptide analysis indicated that NAP, NP and F1 are unrelated polypeptides. The demonstration that NAP is virus coded, together with its phosphorylation and association with the nucleocapsid, suggest that NAP may be the NDV analogue of the P protein of Sendai virus.

INTRODUCTION

Conventional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separates polypeptides on the basis of their mol. wt., so that polypeptides with similar mol. wt. are not easily resolved. In order to detect any minor virus-induced polypeptides in NDV-infected CEF cells, and to resolve the F1 and NP polypeptides which have similar SDS-PAGE mobilities, two-dimensional gel electrophoresis (O'Farrell, 1975) was employed. This combines a first dimension separation of proteins on the basis of their isoelectric point with a second dimension separation by SDS-PAGE.

Nucleocapsids from the paramyxovirus Sendai virus, and the rhabdovirus vesicular stomatitis virus (VSV) contain three proteins: a structural nucleocapsid protein NP, a large protein L and a third protein called P in Sendai virus, NS in VSV. Virions of NDV, a paramyxovirus, contain analogues of NP and L, but evidence for an analogue of P (or NS) is less convincing. Hightower et al. (1975) have suggested that NDV has a protein of mol. wt. 47K, while Colonno & Stone (1976) have suggested a protein of mol. wt. 53K, which may be this analogue. Two-dimensional gel electrophoresis, with its greater resolving power than SDS-PAGE, presented the opportunity of detecting the NDV analogue of P and NS.
METHODS

Materials. RNase A from bovine pancreas, DNase I from bovine pancreas, α-chymotrypsin type I-S from bovine pancreas, actinomycin D and Tween 80 were obtained from Sigma, London. A 40% ampholine solution, pH 3.5 to 10, was from LKB, South Croydon, Surrey. All other chemicals were from BDH, Poole, Dorset. Medium 199, medium 199 lacking leucine, the baby hamster kidney (BHK) cell line BHK-21 and calf serum were from Flow Laboratories, Irvine, Scotland. X-ray films Kodirex KD59T and X-Omat XRPI were from Kodak, Manchester. L-(U-14C)-leucine (352 mCi/mmol), D-(1-3H)-glucosamine hydrochloride (2–6 Ci/mmol), L-(35S)-methionine (219 mCi/mmol) and 32P-orthophosphate (1 mCi/ml) were from the Radiochemical Centre, Amersham, Bucks.

Virus and cells. NDV strains HP, LK, Beaudette C and N were obtained from Professor C. F. Fox, University of California at Los Angeles, strain Ulster from Dr R. Avery, Warwick. Approx. 10^4 p.f.u. of NDV in 0.1 ml tris-saline buffer (0.025 M-tris-HCl 0.137 M-NaCl, 0.7 mM-Na2HPO4, pH 7.2) was injected into each 10 day-old embryonated egg. After 48 h at 37 °C, allantoic fluid was pooled and clarified by centrifugation at 10000 g for 10 min at 4 °C. Virus was pelleted by centrifugation at 77000 g for 2 h at 4 °C. The pellet was resuspended in tris-saline and further purified by centrifugation for 24 h at 284000 g at 4 °C on 10 to 30% (w/v) sucrose, 10 to 22.5% (w/v) potassium sodium tartrate gradients containing 50 mM-NaCl, 1 mM-EDTA, 10 mM-tris-HCl, pH 7.2, on an MSE superspeed 65 centrifuge using a 6 × 14 ml titanium swing-out rotor. The virus band was removed and virus pelleted at 77000 g for 2 h at 4 °C, resuspended in tris-saline and stored at −70 °C. Secondary monolayers of chick embryo fibroblasts (CEF) were grown in medium 199 supplemented with 5% calf serum. BHK cells were grown in medium 199 supplemented with 10% calf serum.

Radio-labelled virus and monolayers. 35S-methionine-labelled NDV was obtained by injecting seven eggs each with 100 μCi of 35S-methionine shortly after injection with NDV (strain Beaudette C). Radio-labelled virus was purified as for unlabelled virus. Fractionation of the virus into nucleocapsid and envelope was as described by Scheid & Choppin (1973). Fractions were then dialysed against 10 mM-tris-HCl, pH 7.0, containing 0.1 mM-MgCl2 and 0.5% (w/v) SDS (tris-SDS buffer).

Confluent monolayers of CEF or BHK cells in 30 mm plastic Petri dishes were mock infected with tris-saline or infected at 20 to 50 p.f.u./cell and allowed to adsorb for 30 min at 37 °C [except that the multiplicity with NDV N was undetermined, due to an inability to detect plaques consistently even when trypsin was included in the overlay medium as described by Nagai et al. (1976)]. The inoculum was replaced with 2 ml/dish of medium 199 supplemented with 5% dialysed calf serum for CEF cells or 10% dialysed calf serum for BHK cells. 10 × 199 medium lacking leucine was used in the preparation of this medium. If the monolayers were later to be labelled with 14C-leucine, the medium was supplemented with 1 μg/ml leucine; if with 3H-glucosamine, medium was supplemented with 10 μg/ml leucine; if with 32P-orthophosphate, medium was supplemented with 10 μg/ml leucine but phosphate was omitted. Medium was removed 6 h p.i. and replaced with 0.3 ml fresh medium containing the appropriate isotope. A 0.3 ml amount was found to be sufficient to prevent drying out of a 30 mm Petri dish for 3 h if the dish was tilted occasionally. For labelling with 14C-leucine, medium containing no leucine was supplemented with 10 μCi/ml of 14C-leucine. For labelling with 3H-glucosamine, medium containing 10 μg/ml leucine was supplemented with 100 μCi/ml of 3H-glucosamine. For labelling with 32P-orthophosphate, medium containing 10 μg/ml leucine but no phosphate was supplemented with 600 μCi/ml of 32P-orthophosphate. After 3 h, labelling medium was removed and the
monolayer washed three times with tris-saline. After removal of the tris-saline, the monolayer was scraped off into 0.75 ml of tris-SDS buffer, when two-dimensional electrophoresis was to be in mode (A) below. This sample was boiled for 2 min, incubated for 1 h at room temperature with 67 µg/ml RNase and 33 µg/ml DNase and then split into two portions before storage at −20 °C.

Alternatively, the sample was scraped off into 0.75 ml of buffer containing 8% (w/v) Tween 80, 5% (v/v) 2-mercaptoethanol, 2% ampholines, pH 3.5 to 10, and 9.5 M-urea, when two-dimensional electrophoresis was to be in mode (B) described below. This sample was incubated for 1 h at room temperature with 67 µg/ml RNase and 33 µg/ml DNase before storage at −20 °C.

Actinomycin D treatment of NDV-infected cells was as follows. Immediately after an inoculum of 5 p.f.u./cell of NDV strain HP was removed, it was replaced by 2 ml of medium containing 2 µg/ml actinomycin D. This medium was removed and replaced by 0.3 ml of 14C-leucine labelling medium containing 2 µg/ml actinomycin D 12 h p.i. Three h later this medium was removed and the sample was prepared for two-dimensional electrophoresis in mode (B). Another monolayer was infected, incubated and labelled under the same conditions except that all media lacked actinomycin D.

Two-dimensional gel electrophoresis (A). A modified form of the O'Farrell (1975) procedure was employed. Tween 80 was used in the isoelectric focusing (IEF) dimension instead of NP40, and 2% ampholines, pH 3.5 to 10, were used rather than a mixture of different ampholines. Sample preparation was based on that of Ames & Nikaido (1976). A labelled sample was boiled for 30 s and allowed to cool. A 30 µl amount was mixed with 30 mg urea and 30 µl of buffer containing 8% (w/v) Tween 80, 9.5 M-urea, 5% (v/v) 2-mercaptoethanol and 2% (w/v) ampholines, pH 3.5 to 10. This gave a final sample vol. of 80 µl. Isoelectric focusing was for 4000 V h at room temperature. Measurement of pH gradients, equilibration of the isoelectric focusing gels and running of the second dimension gels were as described by O'Farrell (1975). Second dimension gels were run in a Bio-Rad model 220 dual vertical slab gel apparatus with 10 cm of 10% acrylamide separating gel and 2 cm of 4% acrylamide stacking gel. Gels were run for approx. 3.5 h at 40 mA/slab, until the dye front almost reached the bottom of the separating gel and were then either prepared for fluorography as described by Bonner & Laskey (1974) or fixed in 10% (v/v) acetic acid and dried down for autoradiography. Kodirex KD59T was used for autoradiography and X-Omat XRPI for fluorography.

Two-dimensional gel electrophoresis (B). This was based on the method of O'Farrell (1975) and is similar to the modification reported by O'Farrell et al. (1977) as non-equilibrium pH gradient electrophoresis. Isoelectric focusing gels contained 2% Tween 80 instead of NP40, and 2% ampholines, pH 3.5 to 10. 30 µl amounts of radioactive samples were loaded at the anode (phosphoric acid) terminal of isoelectric focusing gels and electrofocused for 7 h at 350 V at room temperature. Equilibration of these gels and running of the second dimension were as described by O'Farrell (1975). After completion of the second dimension electrophoresis, gels were either fixed in 10% (v/v) acetic acid and dried down for autoradiography, or prepared for fluorography as described by Bonner & Laskey (1974).

Peptide fingerprinting of NP, F1 and NAP. A modification of the procedure of Cleveland et al. (1977) was used. A two-dimensional gel of 14C-leucine-labelled NDV-infected CEF cells was set up as described in (A) above, except that a well was made at one end of the agarose used to cement the first dimension rod to the second dimension slab. This well was filled with purified NDV in final sample buffer (Laemmli, 1970) and was used to form a guide strip. The gel was then run as described above. At the end of the run, the guide strip of polyacrylamide was cut off and stained with Coomassie brilliant blue. The remainder
of the gel was stored at 4 °C. The guide strip was destained and the position of the NP/F\textsubscript{1} band was used to calculate the distance that NP, NAP and F\textsubscript{1} had migrated into the remainder of the gel. This calculation was necessary because the guide strip swelled slightly during the staining and destaining procedure. A strip of polyacrylamide was cut out across the remainder of the gel at the calculated position of migration of NP, NAP and F\textsubscript{1}. This strip of polyacrylamide also contained chick proteins of similar mol. wt. and is referred to as the 56K strip. This strip was equilibrated for 30 min in 0.0625 M-tris-HCl, pH 6.8, containing 0.5% (w/v) SDS and 0.001% (w/v) bromophenol blue (SE buffer). For proteolysis, special glass plates were made for the Bio-Rad slab gel apparatus. These plates were hollowed out at the top to allow room for the 56K strip and the chymotrypsin in agarose described below. 2 cm of stacking gel and 10 cm of 15% polyacrylamide separating gel were used to resolve the peptides generated. The equilibrated 56K strip was placed into the hollow of the glass plate, above the stacking gel. The remaining space in the hollow was filled with 4 ml of 1.5 mg/ml chymotrypsin in 0.7% (w/v) agarose in SE buffer kept molten at 45 °C. After the agarose had set, the uncooled gel was run until the marker dye reached the bottom of the stacking gel. The current was then switched off, the cooling chamber of the apparatus was filled with water pre-warmed to 37 °C and the apparatus placed into an incubator at 37 °C. This allowed proteolytic cleavage of the proteins which were loaded in the 56K strip, but which were now stacked tightly with chymotrypsin. After 45 min in the incubator the apparatus was removed, the cooling chamber emptied and the current through the apparatus restarted. The gel was run until the marker dye reached the bottom of the separating gel, with an ample supply of cooling water, and was then prepared for fluorography.

**RESULTS**

Fig. 1 to 5 were obtained by two-dimensional electrophoresis in mode (A) and refer exclusively to NDV strain Beaudette C. Fig. 6 and 7 were obtained by two-dimensional electrophoresis in mode (B). The base to acid orientation is left to right in all figures.

Two-dimensional gel separations of the proteins of 35S-methionine-labelled NDV grown in ovo are shown in Fig. 1. L, HN, F\textsubscript{1}, NP, NAP and a trace of actin (Ac) can be seen in undisrupted virions. NP and NAP are components of the nucleocapsid, although L protein and some NAP have been lost. HN is a component of the envelope, but F\textsubscript{1} has been lost and high mol. wt. material, HMW (which may be an aggregate), is produced. When analysed by conventional SDS–PAGE, the envelope fraction was shown to contain HN, F\textsubscript{1} and M (not shown). M protein enters the isoelectric focusing gel very poorly, as found in Sendai virus by Raghow et al. (1978). The charge heterogeneity of glycoproteins HN and F\textsubscript{1} reported for Sendai virus by Raghow et al. (1978) and the glycoproteins of influenza virus by Privalsky & Penhoet (1978) is not as clear in these separations of NDV proteins. A comparison of protein synthesis in NDV-infected with that in mock-infected CEF cells is shown in Fig. 2. L protein enters the gel poorly, which may be due to aggregation or insolubility of high mol. wt. material in the isoelectric focusing gel. HN is a rather broad spot, and this may hide the charge heterogeneity of glycoproteins of other viruses. F\textsubscript{0} can be seen in infected cells although it was absent from virions. F\textsubscript{1}, NP and NAP can clearly be distinguished and both NP and NAP exhibit charge heterogeneity. M protein is seen only as a spot at the extreme basic end of the gel. This may merely represent insolubility in the isoelectric focusing gel. A polypeptide 36K which appears as a doublet is also induced in infected cells. Increased synthesis of certain CEF polypeptides has been reported following infection with SV5 and Sendai virus by Peluso et al. (1977). When CEF cells are infected with NDV, there is a decrease in the level of host protein synthesis (Hightower & Bratt,
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Fig. 1. Fluorograph of two-dimensional polyacrylamide gel separations of egg-grown NDV virion polypeptides labelled with $^{35}$S-methionine. (a) Whole virus; L protein and M protein are faint streaks into the gel. (b) Nucleocapsid fraction containing NP and NAP. (c) Envelope fraction containing HN, F$_1$ and high mol. wt. material, HMW. Mol. wt. values $\times 10^3$ are indicated.

1974) but, as with other paramyxoviruses, enhanced synthesis of certain host polypeptides is seen. These are presumably the same polypeptides whose synthesis is enhanced by SV5 or Sendai virus infection, and mol. wts. of 96K, 86K and 78K agree well with the 99K, 97K, 86K and 78K estimated by Peluso et al. (1977).

To determine which NDV-induced protein of mol. wt. 56K is F$_1$ we labelled NDV-infected and mock-infected cells with $^3$H-glucosamine. Two-dimensional separations are shown in Fig. 3. HN and F$_1$ can be clearly identified, but F$_0$ is rather diffuse. In addition to these expected glycoproteins, a polypeptide that labels only slightly with $^{14}$C-leucine labels
Fig. 2. Autoradiograph of two-dimensional polyacrylamide gel separations of (a) NDV-infected and (b) mock-infected CEF cells labelled with $^{14}$C-leucine. The pH values are given along the top, mol. wt. in the centre. Virion proteins L, HN, F₁, NAP, NP and M are indicated, as well as the F₀ precursor protein and a non-structural polypeptide 36K. Chick cell proteins, the synthesis of which is enhanced by NDV infection, are indicated with their mol. wt. ($\times 10^{-3}$) in (b): 96, 86 and 78. Actin is also shown in (b).

Fig. 3. Fluorograph of two-dimensional polyacrylamide gel separations of (a) NDV-infected and (b) mock-infected CEF cells labelled with $^{3}$H-glucosamine. In (a), HN, F₀ and F₁ are indicated, as well as additional glycosylated material with apparent mol. wt. 62K (gp62) and 40K (gp40).
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Fig. 4. Autoradiography of two-dimensional polyacrylamide gel separations of (a) NDV-infected and (b) mock-infected CEF cells labelled with 32P-orthophosphate. In (a), NAP, NP and 36K are indicated.

heavily with 3H-glucosamine. This has an apparent mol. wt. of 62K and is indicated as gp62 in Fig. 3. A further observation was that some 3H-glucosamine label accumulated in a position similar to that of M protein in Fig. 2 and this is indicated as gp40.

To determine whether the charge heterogeneity of NP, NAP and 36K was due to phosphorylation, we labelled NDV-infected and mock-infected cells with 32P-orthophosphate. Two-dimensional separations are shown in Fig. 4. It is clear that this charge heterogeneity is associated with phosphorylation. Phosphorylation of L or M proteins as described for Sendai virus by Lamb & Choppin (1977) cannot be seen. Neither of these proteins fully enters the isoelectric focusing dimension and it is not justified to conclude that L and M proteins of NDV are not phosphorylated. NAP shows resolution in the isoelectric focusing dimension which may be due to various levels of phosphorylation. There is also resolution in the SDS dimension of phosphorylated species which could be due to phosphorylation at different sites on the polypeptide altering the conformation and affecting mobility in the SDS dimension.

It has been suggested that the nucleocapsid protein of NDV may be synthesized as a precursor molecule which is normally rapidly cleaved to the final mol. wt. (Hightower & Smith, 1978). There is no pulse-chase relationship between NAP and NP (not shown). To determine whether NAP is related to NP, peptide fingerprints were obtained as described in Methods and are shown in Fig. 5. There is little correspondence between the peptides generated from NAP and those from NP and we conclude that NAP and NP are not related. 56K refers to the peptides generated from a chick cell protein of mol. wt. close to that of NP, NAP and F₁.

Fig. 6. compares NAP induced by five strains of NDV in CEF (b to f) and BHK (h to l) cells. The relevant areas of mock-infected CEF and BHK cells are (a) and (g), respectively. The NAP proteins from strains HP (b and h), LK (c and i) and Beaudette C (d and j) appear identical in isoelectric point and apparent mol. wt. The NAP of strain N (e and k) is slightly
more basic and has a slightly lower apparent mol. wt. and the NAP of strain Ulster (f and l) is appreciably more basic and of still lower apparent mol. wt. than those of strains HP, LK and Beaudette C. It should be noted that this variation in NAP is dependent on the strain of virus, and is independent of the host cell. For example, the difference in mol. wt. and isoelectric point of NAP between strains HP and Ulster is identical in CEF and BHK cells (Fig. 6, b : f = h : l).

Actinomycin D inhibits DNA-dependent RNA synthesis so that if NAP were host coded but synthesized in response to NDV infection, actinomycin D should prevent its production. Two CEF monolayers infected with NDV strain HP were labelled with 14C-leucine 12 to 15 h p.i. Actinomycin D (2 μg/ml) was present in the medium above one of these monolayers throughout the post-infection incubation and labelling periods. Two-dimensional separations are shown in Fig. 7. Host cell protein synthesis was drastically reduced in actinomycin-treated infected cells (b), but synthesis of NDV-coded polypeptides was unaffected. Synthesis of NAP was unaffected by actinomycin treatment, although more NAP was found in the phosphorylated state.

In the absence of actinomycin, F₀ cannot be detected in these NDV-infected cells (7a),
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Fig. 6. Segments of two-dimensional fluorographs containing NAP induced by five strains of NDV. (a to f) CEF cells, (g to l) BHK cells. (a, g) mock-infected; (b to f) and (h to l) infected with NDV strains HP (b and h), LK (c and i), Beaudette C (d and j), N (e and k) or Ulster (f and l). The arrows indicate a host cell protein as a reference point.

but F_0 can be seen in the presence of actinomycin (7b). A possible explanation for this and the higher levels of phosphorylated NAP in actinomycin-treated cells is given in the Discussion.

DISCUSSION

NDV has a very similar polypeptide composition to another paramyxovirus, Sendai virus, except that Sendai virus has a polypeptide P with no known analogue in NDV. We suggest that NAP is the NDV analogue of P. The properties NAP shares with P are firstly that it is associated with the nucleocapsid and secondly that it is phosphorylated. These properties are also shared with the NS protein of vesicular stomatitis virus. NAP has remained undiscovered presumably because its mol. wt. is so similar to that of F_1 and NP that it is impossible to resolve by one-dimensional SDS–PAGE.

Hightower et al. (1975) presented evidence that NDV induced a small amount of a protein 47K and Ball et al. (1978) suggested that 47K was the NDV analogue of the P protein.
of Sendai virus, but NAP is induced in quantities similar to HN (Fig. 2, 6 and 8) so it is unlikely that we have merely described a shift in the apparent mol. wt. of the same protein. We have not detected synthesis of 47K induced by any of the five strains of NDV described in this study, nor by NDV strains Texas, Herts, AV, B, F or Queensland (not shown). The presence of a protein in the NDV viron with identical isoelectric point and mol. wt. to chick actin (apparent mol. wt. 45K) may explain the presence of a protein of similar mol. wt. (47K) in the virion. Evidence has recently been presented for the presence of actin in other paramyxovirus virions: Sendai virus (Lamb & Choppin, 1978) measles virus (Tyrrell & Norrby, 1978) and mumps virus (Orvell, 1978). 47K may be a trace of actin in the NDV virion, but it must be added that Hightower et al. (1975) found that 47K has a different tryptic peptide fingerprint to actin. Colonno & Stone (1976) described a protein of mol. wt. 53K in an NDV transcriptive complex and suggest that this may be the NDV analogue of the NS protein of VSV. Although this is closer to the mol. wt. of NAP, Hightower et al (1975) have described fragments of NP, in particular a polypeptide of mol. wt. 53K, in the NDV virion and in infected cells. We also detect these fragments of NP in our preparations of NDV, although the faint streaks they form into the two-dimensional gels of Fig. 1 are not visible in the photographs.

The resolution of phosphoderivatives of NAP in the SDS dimension which are presumably phosphorylated to the same extent, leads to speculation about whether phosphorylation at different sites on the protein has functional significance. It would, for instance, be interesting to determine whether the pattern of phosphorylation of NAP differs at early and late times p.i. when there is a change from transcription to replication of the NDV genome. Clinton et al. (1978) found that the more highly phosphorylated forms of the NS protein of VSV do not bind to virus cores either from infected cells or virions and speculated that a phosphorylated form of NS protein may regulate RNA synthesis or regulate the switch between transcription and replication.

Actinomycin D increased the amount of F₀ in NDV (strain HP)-infected cells and increased the proportion of phosphorylated forms of NAP. The length of the treatment with actinomycin was such that it is possible that there was a decline in the level of protease
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responsible for the $F_0 \rightarrow F_1$ cleavage and in the level of phosphatase responsible for converting phosphorylated forms of NAP to the unphosphorylated form.

The amounts of gp62 and gp40 vary among strains of NDV. Preliminary pulse-chase and peptide fingerprint data suggest that they are breakdown products of HN, rather than aberrant cleavage products of $F_0$ (Madansky & Bratt, 1978) and it is interesting to note that Örvell (1978) found that HN of mumps virus could be cleaved *in vitro* by trypsin to yield a fragment of mol. wt. 40K. These glycosylated polypeptides and the non-glycosylated polypeptide 36K are under further investigation.

This research was supported by a research grant from the Wellcome Trust. P. Chambers is the recipient of a Science Research Council Postgraduate Award.

REFERENCES


*(Received 12 February 1980)*