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Coding Assignments of the Five Smaller mRNAs of Newcastle Disease Virus

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The polypeptide coding assignments for the five messengers of the 18S size class of Newcastle disease virus (NDV) RNA have been determined by cell-free translation of individual RNAs separated by gel electrophoresis. Listed in order of their decreasing electrophoretic mobilities in acid agarose-urea gels, the coding assignments of the RNAs were as follows: RNA 1, M protein; RNA 2, P protein; RNA 3, NP; RNA 4, F glycoprotein; and RNA 5, HN glycoprotein. RNA 2 also directed the synthesis of 33- and 36-kilodalton proteins, which were tentatively identified as being overlapping segments of the P protein. The 33- and 36-kilodalton polypeptides could be detected in infected cells, but not in purified virions of NDV. Since the other unique NDV RNA, a 35S species, has been shown previously to encode the viral L protein, these results complete the coding assignments of the six known NDV mRNAs.

The genome of the paramyxovirus Newcastle disease virus (NDV) is a single negative strand of RNA (3, 7, 22) with a molecular weight of 5.1 $\times$ 10^6 to 5.7 $\times$ 10^6 (23) and a sedimentation coefficient of 50S. The nucleocapsid contains a transcriptase (RNA-dependent ribonucleotidyl transferase, EC 2.7.7.6) (14, 20) that directs the synthesis of transcripts of subgenomic lengths. These transcripts anneal to the entire genome (26, 32), mostly contain 3′ polyadenylated (poly[A]) tails (39, 42, 43), and include mRNAs that code for viral structural proteins (9, 11, 12, 27).

The viral transcripts have been separated into three size classes with sedimentation coefficients of 35S, 22S, and 18S (4). The 35S size class contains unique sequences that anneal to 34 to 40% of the genome (26, 32, 37) and direct the synthesis of the large, 220,000-dalton L protein of the nucleocapsid (27). These observations show that this size class of RNA contains a single unique messenger.

The 18S size class anneals to the remainder of the genome (21, 26, 32) and on that basis presumably contains all of the unique mRNAs other than the L protein mRNA. This size class contains at least five or six electrophoretic species of RNA (10, 11, 21, 38, 43). In previous work (9, 11, 12, 27), the 18S mRNAs were shown to code for four structural proteins: the nonglycosylated membrane protein (M; apparent molecular weight, 41,000), a phosphorprotein of the nucleocapsid (P; 53,000) the major nucleocapsid protein (NP; 56,000), and a 67-kilodalton, nonglycosylated form (HN_{67}) of the hemagglutinin-neuraminidase glycoprotein (HN; molecular weight, ~74,000 for the glycosylated form). As will be shown in this paper, the 18S size class also encodes the polypeptide moiety of a sixth viral structural protein, the fusion glycoprotein (F). In vivo, the F glycoprotein consists of two disulfide-linked glycosylated subunits (F_1 and F_2; respective molecular weights, 53,000 and 10,000) that are generated by proteolytic cleavage of a glycoprotein precursor (F_0; molecular weight, 66,000) (33). The combined estimated polypeptide molecular weight (4.9 $\times$ 10^5) of the six viral proteins L, HN, F, NP, P, and M approaches the estimated maximum coding capacity of the NDV genome.

On the basis of RNA hybridization-competition experiments, the 22S size class does not contain additional unique sequences (40, 41). This size class has been shown to contain at least two distinct electrophoretic species (38, 43), and preliminary characterization suggested that these species are covalently linked 18S transcripts (40, 41), perhaps generated by transcriptional readthrough of adjacent genes (12). Polycistronic transcripts of this nature have

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been characterized for vesicular stomatitis virus (17).

The purpose of the experiments described here was to identify the polypeptides encoded by each electrophoretic band of viral 18S RNA. It was of particular interest to test whether any of the electrophoretic bands might encode more than one unique viral polypeptide and to test whether additional polypeptide products could be detected by the translation of separated mRNAs. A direct and apparently complete determination of RNA coding assignments has not been reported previously for a paramyxovirus.

Preliminary results of this study were presented at the Fourth International Symposium on Negative Strand Viruses, held in the U.S. Virgin Islands, 1980 (13).

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) and stored at −70°C. Secondary cultures of chicken embryo cells were prepared as described previously (11).

mRNA purification. Secondary cultures of chicken embryo cells were infected with 10 PFU per cell and incubated at 40.5°C. To label the viral RNA that was used for the preparative separation (Fig. 1), actinomycin D (2 μg/ml) was added to the infected cells at 3.25 h postinfection and [^{3}H]uridine (25 μCi/ml) was added at 4 h postinfection. At 9 h postinfection the cells were washed three times with cold phosphate-buffered saline and solubilized with two washes of cold solubilizing buffer (11) containing 1% (vol/vol) Triton N-101 and 0.5% (wt/vol) sodium deoxycholate instead of sodium dodecyl sulfate (SDS). The cells lysed within 3 to 4 min, leaving most nuclei intact and attached to the plate. The poly(A)-containing RNA fraction was isolated directly from the lysate by using oligodeoxythymidylic acid-cellulose as previously described (11). To prepare the mRNA used to generate cell-free system products shown in Fig. 5, the cells were exposed to 2 μg of actinomycin D per ml beginning 0.5 h before infection. At 7 h postinfection, the cells were solubilized, and the poly(A)-containing RNA was isolated as described above.

Agarose gel electrophoresis of RNA. Electrophoresis on preparative and analytical scales was performed, using 1.5% agarose gels in 0.025 M sodium citrate, pH 3.5, containing 6 M urea as described elsewhere (44). For the preparative separation, samples were collected automatically at 10-min intervals over a 36-h period of electrophoresis. The samples that contained detectable radioactivity were pooled into 15 fractions.

Cell-free translation of NDV mRNA. Viral mRNA was translated in micrococcal nuclease-treated rabbit reticulocyte lysates (28) in the presence of 100 to 500 μCi [^{35}S]methionine per ml as described previously (12), except that spermidine was included at a concentration of 0.4 mM. Incubation was at 30°C for 90 min. Reactions were terminated by the addition of six volumes of polyacrylamide gel sample buffer (24), and the samples were boiled immediately for 3 min.

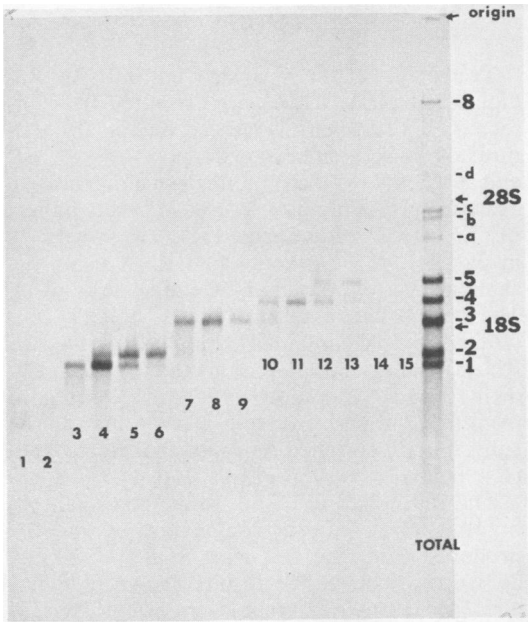

FIG. 1. Analytical agarose gel electrophoresis of fractions 1 to 15 of poly(A)-containing NDV mRNAs separated by preparative gel electrophoresis. For comparison, a sample of total poly(A)-containing NDV mRNA (total) was analyzed in the same gel. The NDV RNA bands are numbered 1 to 5 (18S RNAs), a to d (22S RNAs) and 8 (35S RNA), and the positions of 18S and 28S rRNAs from BHK cells are marked. The radioactive bands were visualized by fluorography.

Preparation of viral polypeptides in vivo. Secondary cultures of chicken embryo cells were infected with an input multiplicity of 5 PFU per cell, incubated for 9 h at 40.5°C, and exposed for 0.5 h to 50 μCi of [^{35}S]methionine per ml in component minimal essential medium (GIBCO Laboratories) containing 2.5% of the usual methionine content. The cells were washed three times with cold phosphate-buffered saline, lysed by the addition of polyacrylamide gel sample buffer, and the extracts were boiled immediately for 3 min.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 11.5% gels and the buffer system of Laemmli (24), with the exact details as described before (11, 12, 37).

Tryptic peptide mapping. [^{35}S]methionine-labeled polypeptides were separated by SDS-PAGE, excised from the dried gels, digested to completion with trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone (Worthington Diagnostics), and prepared for analysis as described before (11, 18, 37). The digests were spotted onto cellulose plates (20 by 20 by 0.1 cm; EM Laboratories), resolved in the horizontal dimension by electrophoresis at 400 V for 1 h, using pyridine-acetic acid-water (300:10:2,700 [vol/vol/vol]), pH 6.5, and in the vertical dimension by ascending chromatography for 4 h in amyl alcohol-isobutanol-propanol-pyridine-water (10:10:10:30:30 [vol/vol/vol/vol]). The plates were prepared for fluorography by impregnation with PPO (2,5-diphenyloxazole).

RESULTS

Translation of the separated 18S mRNAs. Radiolabeled NDV RNA was extracted from infected, actinomycin D-treated secondary cultures of chicken embryo cells, poly(A) selected, and subjected to electrophoresis in a preparative agarose gel containing 6 M urea in citrate buffer, pH 3.5 (44). The fractionated RNA was analyzed in parallel with unfractionated RNA using the same gel system on an analytical scale (Fig. 1). The gel profile of unfractionated poly(A)-containing mRNA contained bands 1 to 5 (the 18S RNAs), band 8 (35S RNA), and minor bands a to d (the 22S RNAs). Fractions 1 to 15, collected from the preparative gel, were found to be substantially enriched for individual electrophoretic bands of the 18S RNAs (Fig. 1).

The individual fractions were then analyzed by translation in reticulocyte lysates and the products were resolved using SDS-PAGE (Fig. 2). Comparison of the results shown in Fig. 1 and 2A demonstrated that fractions enriched for RNA band 1 (fractions 3 to 6) directed the synthesis of the M protein. Similarly, RNA bands 2, 3, and 4 correlated respectively with messenger activity for the P protein, NP protein, and an additional product, (apparent molecular weight, 52,000) which is identified in the following section as the cell-free system form of the F glycoprotein. More extensive analysis of the smaller cell-free system products (Fig. 2B) revealed that the RNA band that encoded the P protein also encoded two smaller polypeptides with apparent molecular weights of 36,000 (36K) and 33,000 (33K). These proteins had been estimated in a preliminary study to be 33K and 28K, respectively (13). Finally, the use of a longer film exposure (Fig. 2C) of the gel shown in Fig. 2A demonstrated that RNA 5 comigrated with messenger activity for HN_{67}, which has been previously identified as the unglycosylated form of the HN glycoprotein (9, 11).

The amount of cell-free HN and F synthesized in response to fractions 10 to 13 was low relative to the amount of radiolabel in RNAs 4 and 5, and although RNA 3 was barely detectable by fluorography in these fractions relative to RNAs 4 and 5, the NP protein was the principal translation product. A likely explanation is that the messengers encoding the two NDV glycoproteins, translated in vivo at the rough endoplasmic reticulum (35), are inefficiently translated in vitro on free polysomes relative to the mRNA coding for nonglycosylated proteins. Similar observations have been noted previously (15, 44).

In summary, the correlations between radiolabeled RNA and messenger activity provided the following coding assignments: RNA 1, M protein; RNA 2, P, 36K, and 33K proteins; RNA 3, NP; RNA 4, a 52K unglycosylated form of the F glycoprotein; and RNA 5, unglycosylated HN. The identities of the cell-free system products NP, HN_{67}, and M were previously established by peptide mapping (9, 11, 12). In this earlier work, a 53K product was initially thought to be the unprocessed polypeptide moiety of the F glycoprotein (9, 11) but was subsequently identified as the P protein (12, 36). The 33K, 36K, and 52K cell-free system products shown in Fig. 1 have not been reported previously and therefore were analyzed by peptide mapping as described in the following sections.

Peptide mapping of the product of RNA 4. Limited digest peptide mapping (8) of the 52K product of separated RNA 4 showed that it was not related to the NP, P, HN_{67}, M, or L proteins (not shown). Most of the limited digest fragments of the product of RNA 4 also did not comigrate with those of the glycoprotein F_0 (not shown). However, in our experience, limited digest mapping does not provide clear matches in the comparison of glycosylated polypeptides with their nonglycosylated counterparts, probably because the presence of carbohydrate side chains alters the susceptibility to proteolysis or peptide migration in polyacrylamide gels, or both.

Therefore, the glycoprotein F_0 and the product of RNA 4 were compared by tryptic peptide fingerprinting. For this analysis, the cell-free translation products had been prepared by using total, rather than separated, viral RNA. The total translation products were separated by SDS-PAGE, and the product of RNA 4 was identified, by the criteria of electrophoretic mobility and limited digest pattern, as a diffuse band migrating ahead of the P protein.

The F_0 glycoprotein generated a pattern of six methionine-containing peptides (numbered 1 to 6; Fig. 3). The pattern of [^{35}S]methionine-containing tryptic peptides for the product of RNA 4 (labeled F_{vitro} in Fig. 3) was a composite of F_0-specific peptides (numbered 1 to 6) and peptides derived from NP. This result established the identity of the RNA 4 product as a cell-free system form of the F glycoprotein, and showed that under the conditions of SDS-PAGE in this experiment cell-free F comigrated with an NP fragment.

Peptide mapping of the 33K and 36K proteins. The identities of the 36K and 33K proteins were also investigated by peptide mapping. These proteins, like the P protein, were the translation products of RNA 2. Band 2 might therefore contain one, two, or three species of mRNA. It was unlikely that 36K or 33K were the translation products of a specific fragment of a larger mRNA contaminating band 2, because the messenger preparation had been purified by oligo-

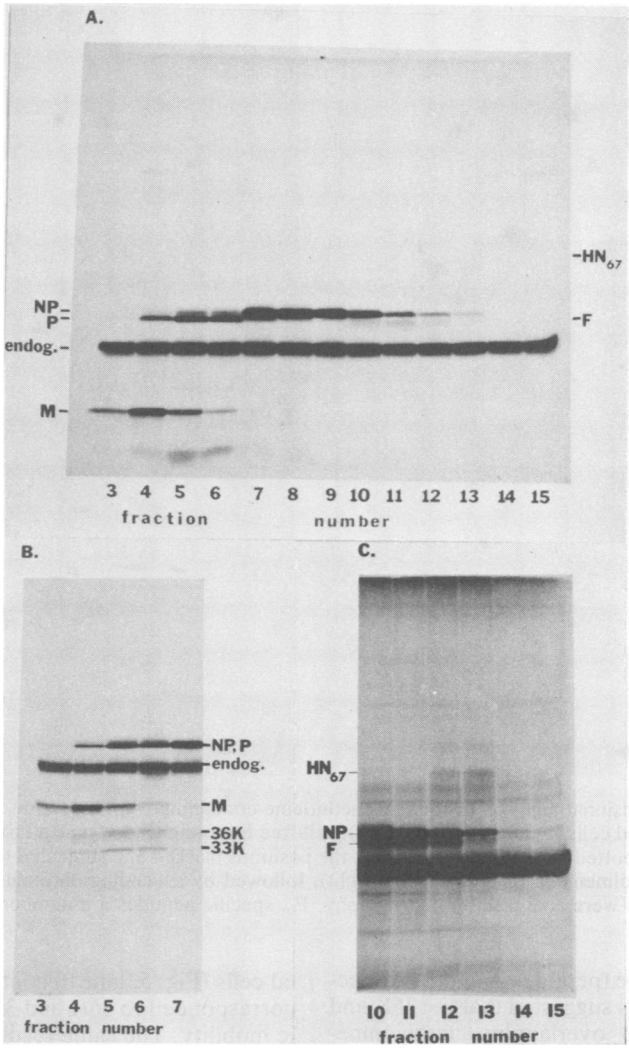

FIG. 2. SDS-PAGE of [^{35}S]methionine-containing polypeptides synthesized in response to NDV RNA fractions 3 to 15. Fractions were individually translated in reticulocyte lysates, and the protein products were analyzed with 11.5% polyacrylamide gels and fluorography. For the gel represented in (A) electrophoresis was for 16 h, and a longer film exposure of part of the same gel is shown in (C). (B) is a fluorogram of part of a second gel that was subjected to electrophoresis for 11 h. The viral products and the major endogenous reticulocyte product (endog) are marked. The endogenous reticulocyte product was a major product because low concentrations of viral mRNAs and relatively long film exposures were used. The translation products of fractions 1 and 2 contained only endogenous reticulocyte products and are not shown.

deoxythymidylic acid-cellulose chromatography (which selects for intact poly[A] 3′ termini) and analyzed by cell-free translation (which usually selects for intact 5′ termini). Thus, it was likely that 36K and 33K were products of intact mRNAs and were either unique or related to the P protein.

The relationships among these three proteins were investigated by tryptic peptide mapping (Fig. 4). The P protein was found to contain at least 10 [^{35}S]methionine-containing tryptic peptides (numbered 1 to 10), and in some separations, peptides 1, 2 and 10 appeared to separate partially as doublets. The 36K protein contained three labeled peptides (1, 10, and A), and mixing experiments with the digest of the P protein (Fig. 4) showed that peptides 1 and 10 were common to both 36K and P. Similarly, the 33K protein contained three peptides (1, 10, and B). Mixing experiments (Fig. 4) showed that peptides 1 and 10 were common to P, 36K, and 33K and that the 36K and 33K proteins each contained a

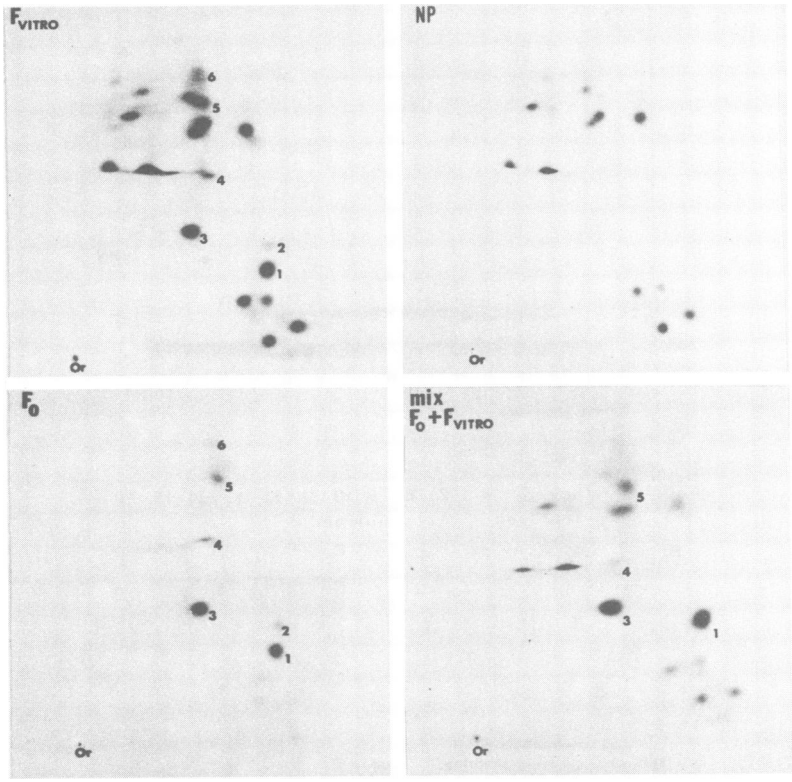

FIG. 3. Two-dimensional analysis of the [^{35}S]methionine-containing tryptic peptides of the F_0 glycoprotein extracted from infected cells (F_0), cell-free NP (NP), cell-free F protein (F_{vitro}), and a mixture of F_0 and cell-free F. The digests were spotted onto cellulose plates at the positions marked and subjected to electrophoresis at pH 6.5 in the horizontal dimension (cathode to the right), followed by ascending chromatography in the vertical dimension. The plates were analyzed by fluorography. F_0, specific peptides are numbered 1 to 6.

single unique peptide (peptides A and B, respectively). These results suggested that the 36K and 33K proteins were overlapping, methionine-poor fragments of the P protein. Since the 36K and 33K proteins could be labeled by the incorporation of formyl [^{35}S]methionyl-tRNA$_F$ in vitro (not shown), they probably represented *N*-terminal fragments of the P protein. However, the conclusion that the 33K, 36K, and P proteins are related is based on the comigration of two out of three methionine-containing tryptic peptides (Fig. 4). The single nonmatching peptides for 33K and 36K presumably are unique because of posttranslational modifications or because they contain the C termini. Alternatively, it is possible that nonrelated polypeptides could generate two matching peptides. Thus, since the matches were not extensive and contained differences, the identifications of 36K and 33K should be considered tentative.

Although the 36K and 33K proteins do not appear to be unique, they were not simply cell-free system artifacts. Gel profiles of [^{35}S]methionine-containing polypeptides from NDV-infected cells (Fig. 5, lane b) contained two bands that corresponded to 36K and 33K by electrophoretic mobility. The same result was obtained when NDV-infected cells were solubilized in gel sample buffer containing the protease inhibitors aprotinin (50 μg/ml) and phenylmethylsulfonyl flouride (1 mM) (not shown). Limited digest peptide mapping confirmed the relationships between the in vivo and cell-free system polypeptides (not shown). 36K and 33K were not detectable in gradient-purified virions (not shown) and were therefore considered to be nonstructural proteins. Chambers and Samson (6a) recently reported two nonstructural proteins for NDV that probably are the same as the 36K and 33K proteins described here.

Previously, we showed that 36K and 33K were encoded by transcripts synthesized in vitro by detergent-activated NDV virions, demonstrating that these polypeptides are virus specific. In this respect, they differed from several other polypeptides from NDV-infected cells that lacked counterparts of equal intensity among the polypeptides of uninfected cells (Fig. 5, lanes a

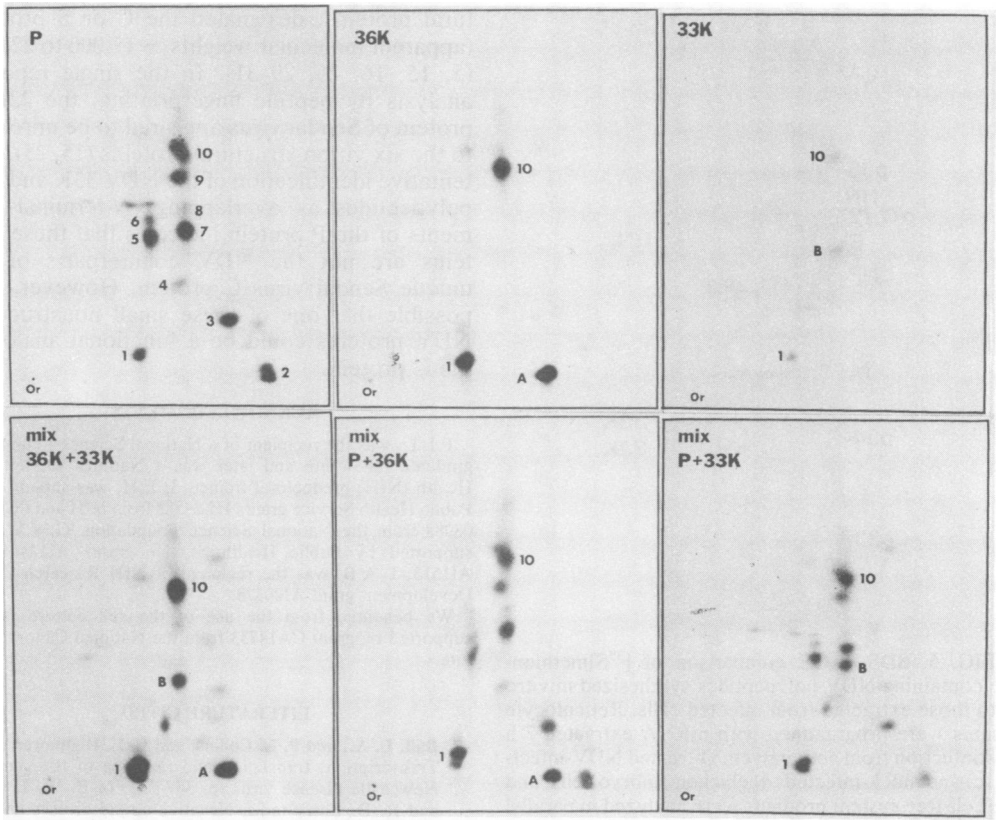

FIG. 4. Two-dimensional analysis of the [^{35}S]methionine-containing tryptic peptides of the P, 36K, and 33K proteins synthesized in reticulocyte lysates. The digests were spotted individually or in mixtures onto cellulose plates and analyzed by electrophoresis and chromatography as described in the legend to Fig. 3. Tryptic peptides specific to the P protein are numbered 1 to 10, the peptide unique to 36K is lettered A, and the peptide unique to 33K is lettered B.

and b; bands p88, p72, p71, p34, and p23). These were identified by peptide mapping as cell-specific polypeptides whose synthesis was stimulated by NDV infection (12) and a variety of other treatments (19; data not shown).

DISCUSSION

Translation of NDV RNAs separated by gel electrophoresis provided the following coding assignments: RNA 1, M protein; RNA 2, P, 36K, and 33K proteins; RNA 3, NP; RNA 4, unglycosylated F; and RNA 5, unglycosylated HN.

These identifications, together with our earlier work (1, 12) and the previous detection of a putative leader RNA (2), provide the following picture of NDV transcription products. By analogy with vesicular stomatitis virus, the nonmessenger leader RNA is probably encoded at the 3′ terminus of the genome, and this is followed, in order, by genome sequences encoding RNA 3, RNA 2, (RNA 1, RNA 4), RNA 5, and RNA 8. It is possible that transcription products representing additional genomic sequences remain to be identified. For example, by analogy with vesicular stomatitis virus (34), a nonmessenger "trailer" RNA might be encoded at the 5′ terminus of the genome. However, since the 18S RNAs (RNAs 1 to 5) and 35S RNA (RNA 8) together anneal to essentially the entire genome (26, 32), it seems likely that RNAs 1 to 5 and 8 contain all of the viral mRNAs. Furthermore, oligonucleotide complexity analysis indicated that each electrophoretic band of 18S and 35S NDV RNA contained a single unique major transcript (38), and each encoded a single unique polypeptide. Together, these results suggest that RNAs 1 to 5 and 8 each consists of a single mRNA, and that these six species represent all of the viral messengers.

The coding assignments outlined above account for the six known unique NDV proteins L, HN, F, NP, P and M (6, 18, 37). In initial studies (9, 18) an additional, minor polypeptide (the 47K protein) appeared to be unique on the basis of peptide mapping. But such a protein was not

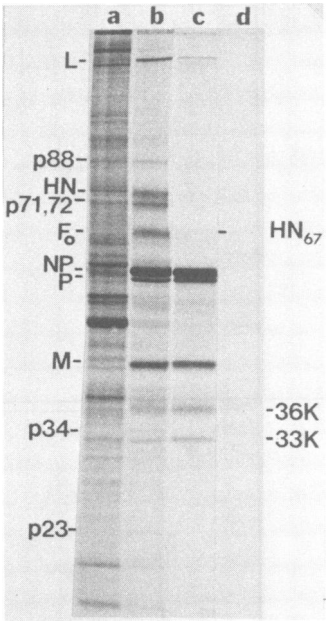

FIG. 5. SDS-PAGE comparison of [^{35}S]methionine-containing NDV polypeptides synthesized in vitro with those extracted from infected cells. Reticulocyte lysates were programmed with mRNA extracted 7 h postinfection from actinomycin D-treated NDV-infected (c) and mock-infected (d) chicken embryo cells, and the cell-free system products were analyzed in parallel with [^{35}S]methionine-containing polypeptides extracted at 10 h postinfection from mock-infected (a) and NDV-infected (b) chicken embryo cells. An 11.5% polyacrylamide gel was used and was analyzed by fluorography. The positions of virus-specific and virus-stimulated cell-specific polypeptides are shown.

detected in subsequent studies (1, 6, 11–13, 37) or in the work presented in this paper. Apart from the 33K and 36K proteins, no candidates for additional virus-specific polypeptides have been detected with the use of conditions of SDS-PAGE that would resolve products as small as 2,500 to 3,000 daltons (not shown), the use of total rather than poly(A)-selected mRNA from NDV-infected cells to program cell-free translation (not shown), incorporation of a mixture of 15 [^{3}H]-labeled amino acids rather than [^{35}S]methionine in vitro and in vivo (not shown), and two-dimensional gel analysis of NDV virions (37) and extracts of infected cells (6). These observations indicate that if additional NDV proteins remain to be identified, they must be of low abundance.

The identities of the 33K and 36K proteins are of particular interest because several paramyxoviruses (Sendai, mumps, measles, and canine distemper viruses and simian virus 5) have been reported to encode one or two small nonstructural proteins, designated the C or S proteins (apparent molecular weights, ~17,000 to 22,000) (5, 15, 16, 25, 29–31). In the single reported analysis by peptide fingerprinting, the 22K C protein of Sendai virus appeared to be unrelated to the six virion structural proteins (15, 25). Our tentative identification of the NDV 33K and 36K polypeptides as overlapping, *N*-terminal segments of the P protein indicates that these proteins are not the NDV counterparts of the unique Sendai virus C protein. However, it is possible that one of these small nonstructural NDV proteins could be a functional analog of the C protein.

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