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Influenza virus proteins: Identity, synthesis, and modification analyzed by two-dimensional gel electrophoresis

(temporal sequence/glycosylation/phosphorylation/cleavage)

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ABSTRACT **A modification of the two-dimensional protein electrophoresis system of O'Farrell was used to resolve influenza A virus proteins from each other and from host proteins in infected cells. Viral protein spots corresponding to the hemagglutinin proteins, neuraminidase, nucleocapsid protein, and nonstructural protein, were identified on the two-dimensional electrophoretogram. Use of the two-dimensional separation has allowed us to identify glycoprotein heterogeneity, to demonstrate directly the synthesis of neuraminidase, to analyze some viral proteins early after infection, and to demonstrate that the influenza virus NP and NS proteins are phosphorylated in infected MDCK cells.**

Influenza A-NWS virus infection of susceptible cells results in the synthesis of eight viral proteins: P1, P2, P3, HA 0, NP, NA, M, and NS (1–4). The HA 0 glycoprotein precursor is, in turn, cleaved into two final product polypeptides, HA 1 and HA 2 (5). The number of influenza virus protein species, and the similarities in molecular weights of several of them, make complete resolution of all 10 by use of a single electrophoretic gel system difficult. Thus, in our hands, the discontinuous sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide system of Laemmli (6) has failed to completely separate the NA protein from HA 1 and the M protein from HA 2 when applied to purified NWS virion preparations.

This problem of resolution is exacerbated in studies of viral protein synthesis in infected cells. Influenza virus demonstrates a relatively slow shut-off of host protein synthesis; with certain influenza virus strains host proteins may continue to be made as late as 6 hr after infection. Therefore, if influenza virus-infected cells are pulse-labeled with radioactive amino acids early after infection, solubilized, and run on a $NaDodSO_4$/polyacrylamide gel, a variable background of host synthesis is seen, often obscuring the minor viral proteins and making quantitation of even the major viral species difficult.

Studies *in vivo* of post-translational modifications of viral proteins have similarly been hampered by high backgrounds contributed by the host polypeptides. Such host protein modifications as phosphorylation may continue to occur long after cellular polypeptide synthesis has been severely inhibited by the virus, obscuring the viral specific processes.

The increased resolution of proteins attainable by the two-dimensional gel electrophoresis system developed by O'Farrell (7) seemed to have the potential of solving many of these problems. The system uses separation of proteins first by charge in an isoelectric focusing dimension, then by subunit molecular weight in a second, $NaDodSO_4$/polyacrylamide gel dimension. Using this system, we have successfully and quantitatively resolved the HA 0, HA 1, HA 2, NA, NS, and NP influenza virus proteins from each other and from the vast majority of cellular proteins. The resolution achieved has allowed us to analyze the synthesis of influenza glycoproteins early in the infectious cycle, to directly demonstrate the synthesis of neuraminidase protein, and to show that the nonstructural (NS) and nucleoproteins (NP) are phosphorylated in infected cells.

MATERIALS AND METHODS

Cells and Virus. Nonradioactive preparations of the NWS strain of influenza virus were grown in the allantoic cavity of embryonated chicken eggs and purified by the technique detailed (8). MDCK cells were infected with NWS as follows: MDCK cells were plated on 60-mm plastic tissue culture plates in 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered Dulbecco-modified Eagle's medium (DME-Hepes) supplemented with 10% fetal bovine serum (Irvine Scientific). Infection was begun when the cells reached half confluency. The medium was aspirated off, and the cells were washed twice with phosphate-buffered saline (P_i/NaCl). Three to five $\times 10^7$ plaque-forming units of NWS virus (representing a multiplicity of infection of 30–50) were then added to each plate in a small volume of DME-Hepes/2% bovine calf serum at 37°. At 1 hr after infection the volume was brought up to 5 ml per plate by addition of DME-Hepes/2% bovine calf serum.

Cells were labeled with ^{14}C-labeled amino acids at the times indicated by aspirating the infecting medium from the plate, washing the cells with P_i/NaCl, and then adding 2 ml of DME-Hepes (containing no unlabeled carrier amino acids) plus 20 μCi of a uniformly ^{14}C-labeled protein hydrolysate (Amersham Searle Corporation; 56 Ci/atom carbon) per plate. At the end of a 20- to 30-min pulse, the infected cells were washed twice with P_i/NaCl and harvested with a rubber policeman. Pulse chases were performed as above, except that at the end of the 30-min labeling period, the cells were washed twice with P_i/NaCl and overlaid with unlabeled complete DME-Hepes/2% bovine calf serum. The cells were then incubated for the indicated chase period and harvested as above.

Infected cells were labeled with ^{32}P as follows: At 5.5 hr after infection, the medium was removed and the cells were washed twice with DME-Hepes containing 2% dialyzed bovine calf serum and lacking inorganic phosphate. The cell monolayer was then overlaid with 2 ml of the above medium plus 500 μCi of carrier-free $H_3{}^{32}PO_4$ (ICN Chemical and Radioisotope Division). After 1 hr, the cells were washed twice with P_i/NaCl and harvested as before.

Two-Dimensional Electrophoresis. Samples were prepared for electrophoresis by a modification of the method described

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DME-Hepes, Dulbecco-modified Eagle's medium containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; P_i/NaCl, phosphate-buffered saline.

(9). Sonically disrupted cellular extracts were pretreated with 10 μg each of pancreatic RNase A and DNase I for 1 hr at 4° C prior to $NaDodSO_4$ solubilization to decrease the viscosity of the sample and to reduce streaking in the isoelectric focusing dimension.

Nine microliters of sample (either purified influenza virions or influenza virus-infected MDCK cells), containing up to 200 μg of protein, were added to 3 μl of 10% $NaDodSO_4$ and 1.5 μl of 0.5 M Tris·HCl, pH 6.8. The samples were then incubated at 70 °C for 20 min to fully solubilize the proteins, and cooled. Solid urea (Schwarz/Mann ultrapure grade) was then added to a final concentration of 9 M, and 2 vol of lysis buffer [9.5 M urea/2% Bio-Rad (pH 3–10) Biolyte ampholytes/5% 2-mercaptoethanol] containing 8% wt/vol Nonidet P-40 were added.

Isoelectric Focusing. The isoelectric focusing gels were prepared as detailed by O'Farrell (7). Gels were 9 M in urea (Schwarz/Mann ultrapure grade), 4% acrylamide, 0.216% *N,N*-methylene bisacrylamide, and 2% Bio-Rad (pH 3–10) ampholytes. After addition of ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine to initiate polymerization, the gel solution was poured into 0.2 cm inner diameter × 13 cm glass tubes, allowing 1 cm at the top for the sample volume. The gels were both pre-electrophoresed and run with the acid buffer (10 mM H_3PO_4) in the upper electrode chamber and the alkaline buffer (20 mM NaOH) in the lower electrode chamber. Pre-electrophoresis was conducted for 15 min at 200 V, 30 min at 300 V, and 30 min at 400 V. The samples were then loaded onto the top of the polymerized gels (acid end) and overlaid with 10 μl per gel of sample overlay buffer [9 M urea/1% Bio-Rad (pH 3–10) ampholytes] and upper electrode buffer in turn. Loading the samples on the acid end of the gels rather than the basic end as described by O'Farrell resulted in better resolution of basic proteins. Focusing was performed for 12–16 hr at 400 V, plus one additional hour at 800 V. The gels were then removed from their tubes under air pressure and prepared for the second dimension separation.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The isoelectric focusing gels were pre-equilibrated with $NaDodSO_4$ sample buffer (10% wt/vol glycerol/5% 2-mercaptoethanol/2.3% wt/vol $NaDodSO_4$/63 mM Tris·HCl, pH 6.8) by shaking them in 10 ml of the buffer for 2 hr prior to loading atop the second dimension. The second dimension $NaDodSO_4$/polyacrylamide slab gel was poured as described previously for analytical single dimension electrophoresis (8). A 5% stacking, 10% running polyacrylamide gel system was used. The running gel measured approximately 16 × 12 × 0.1 cm. Two centimeters of stacking gel was poured on top of the running gel, yielding a flat surface 1 mm below the top of the notched gel plate. The first dimension tube gel was sealed across the top of the slab with 1% agarose in $NaDodSO_4$ sample buffer. Molecular weight marker proteins, which had been polymerized into short segments of polyacrylamide gel identical in composition and diameter to the focusing gels and stored frozen until use, were then sealed adjacent to the focusing gel with the same agarose/$NaDodSO_4$ sample buffer solution. After the agarose had hardened, the slab was placed in a gel apparatus, bromophenol blue tracking dye was added to the upper electrode buffer, and the gel was run at 20 mA until the dye front reached the end of the slab. The gel was then fixed, stained, and dried as described (8). Autoradiography was performed with Kodak No-Screen x-ray film.

Nucleocapsid and Envelope Protein Isolation. One hundred microliters (360 μg of protein) of egg-grown, purified NWS virions were mixed with 200 μl of 3% Triton X-100 in flu buffer (100 mM NaCl/10 mM Tris·HCl, pH 7.6/1 mM EDTA) and agitated at room temperature for 2 min. After being cooled to 4°, the detergent-disrupted virions were loaded on top of a 4-ml sucrose cushion (10% sucrose/2% Triton X-100, in flu buffer), and the nucleocapsids were pelleted through the cushion by centrifugation in a Spinco SW 56 rotor at 40,000 rpm for 2 hr. The material above the sucrose cushion contained the viral envelope proteins. The crude nucleocapsids present in the pellet were re-extracted with 2% Triton X-100 in flu buffer, repelleted, and solubilized with sample buffer.

RESULTS

Influenza Virion Proteins Are Resolved by Two-Dimensional Gel Electrophoresis. Coomassie blue staining of a two-dimensional gel electrophoretogram of solubilized influenza virions reveals the presence of several protein spots (Fig. 1*A*). These spots have been correlated with the known viral proteins by several independent means. The primary method involved a comparison of the apparent molecular weights of the spots resolved in the two-dimensional electrophoretogram with the positions of influenza virion proteins coelectrophoresed as size markers in the $NaDodSO_4$ dimension. Ambiguities remaining in the assignment of specific spots to known viral

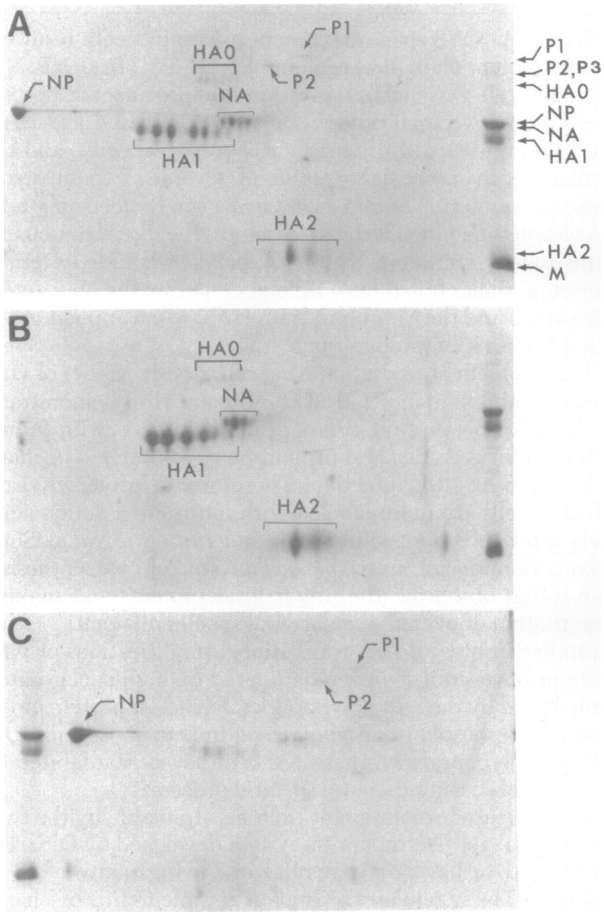

FIG. 1. Two-dimensional gel electrophoretograms of influenza virus proteins. Preparations of influenza viral proteins were subjected to two-dimensional gel electrophoresis. Solubilized influenza virions were applied adjacent to the isoelectric focusing gels to provide parallel viral protein molecular weight markers for the $NaDodSO_4$ dimension. Subsequent to electrophoresis, the gels were stained with Coomassie blue. In this, and all subsequent figures, the basic end of the isoelectric focusing gel is oriented to the left, and the $NaDodSO_4$ dimension is run from top to bottom. (*A*) Whole virion proteins; (*B*) virion envelope proteins; (*C*) virion nucleocapsid proteins.

polypeptides were resolved by correlating the subviral distributions of the two-dimensional gel spots with those of the known influenza proteins.

For example, one of the most abundant virion polypeptides is the nucleoprotein (NP) NP is the predominant protein constituent of purified nucleocapsid preparations and has an apparent molecular weight on a one-dimensional $NaDodSO_4$/polyacrylamide gel of 60,000 (2, 10). A predominant protein spot on our two-dimensional electrophoretograms of influenza virions migrates as a polypeptide of molecular weight 60,000 (Fig. 1*A*) and is localized exclusively in preparations of isolated nucleocapsids (Fig. 1*C*). This spot is therefore identified as the NP protein. NP protein is known to be rich in arginine residues (11); this is consistent with the alkaline isoelectric point of the spot in question.

By a similar process, we have identified eight of the ten influenza proteins as protein spots on a two-dimensional electrophoretogram. The recoveries of HA 0, NP, HA 1, NA, and HA 2 proteins in the two-dimensional gel system are virtually quantitative when compared to their recoveries in a single-dimension $NaDodSO_4$ gel. The M protein does not appear to focus within the pH range used here. [In more recent experiments using the O'Farrell nonequilibrium isoelectric focusing technique (12), we have been able to resolve the influenza M protein as an extremely basic polypeptide of 25,000 molecular weight.] We have been unable to identify P3 protein as a discrete spot, and the recoveries of P1 and P2 proteins are low in this system.

Viral Glycoproteins Are Heterogeneous. Multiple protein spots were detected in areas of the two-dimensional gels that correspond to the expected positions of the viral glycoproteins based on their molecular weights (Fig. 1*A*). Solubilization of the viral envelope proteins showed that these multiple spots were envelope components (Fig. 1*B*). The known influenza virus envelope proteins consist of the neuraminidase NA (molecular weight = 58,000), the precursor hemagglutinin HA 0 (molecular weight $\cong$ 80,000), and its cleavage products HA 1 (molecular weight $\cong$ 56,000) and HA 2 (molecular weight $\cong$ 26,000) (5, 10). The tentative assignments of these spots to specific proteins, made on the basis of molecular weight, were confirmed by analyzing the synthesis and processing of these components in infected cells. Fig. 2*A* presents an autoradiogram of a two-dimensional gel electrophoretogram of influenza virus-infected cells that had been pulse-labeled for 30 min with radioactive amino acids immediately after infection. Uninfected control cells yield an identical pattern (not shown). Fig. 2*C* shows an autoradiogram of an extract of influenza virus-infected cells labeled in the same manner, but beginning at 6 hr after infection. Spots with migrations characteristic of two of the four virion envelope proteins (HA 0 and NA) and not seen in Fig. 2*A* are visible in this autoradiogram (the other virus-specific polypeptides visible, as well as Fig. 2*B*, will be discussed later). The apparent molecular weights of these spots, and their labeling after a short pulse, confirm the identity of these proteins as HA 0 and NA, as indicated. Fig. 2*D* presents a similar autoradiogram of infected cells labeled with radioactive amino acids for 30 min at 4 hr after infection, followed by a 2-hr chase with nonradioactive amino acids. This chase period is sufficient to allow the processing of the HA 0 precursor into its product polypeptides. Examination of Fig. 2*D* reveals the presence of two additional groups of spots that are absent in the simple pulse experiment. These spots are coincident with the positions of the two remaining virion envelope proteins and have the same apparent molecular weights as HA 1 and HA 2. These HA 1 and HA 2 spots are not present in extracts of uninfected cells pulse-labeled and chased in the same manner, nor in infected cells pulsed at 4 hr and not subjected to a chase (autoradiograms not shown). The molecular weights of these spots, their association with the viral envelope, and their delayed appearance in infected cells confirm their assignment to the HA 1 and HA 2 proteins, respectively.

Taken together, the results of these experiments unambiguously define the viral glycoproteins and indicate extensive charge heterogeneity for all of them. This heterogeneity is unlikely to be an artifact of the $NaDodSO_4$ solubilization technique used since virtually identical results have been obtained using the unmodified O'Farrell procedure, which does not use $NaDodSO_4$ solubilization prior to the isoelectric focusing step.

Viral Glycoproteins Are Synthesized Early after Infection. Analysis of viral protein synthesis in infected cells, particularly early after infection, has been hampered by the continuing synthesis of cellular proteins, which tends to obscure less abundant viral species when a one-dimensional separation is used. Resolution of viral glycoproteins has proved particularly difficult because they tend to form rather diffuse bands in $NaDodSO_4$ gel electrophoretograms. Several groups of investigators have reported a temporal control of viral protein synthesis in influenza-infected cells, reporting that the synthesis of the nucleocapsid (NP) and nonstructural (NS) proteins occurs early in infection, considerably before any of the viral glycoproteins (refs. 13 and 14; discussed in ref. 15). Other investigators have reported the early synthesis of almost all the viral proteins (16, 17), although these investigators were unable to resolve the NA protein. The superior resolution of the two-dimensional gel system has allowed us to re-examine this problem. As discussed previously, Fig. 2 presents a series of autoradiograms of cells pulse-labeled with ^{14}C-labeled amino acids at various times after viral infection. Fig. 2*A* shows the pattern obtained from influenza-infected cells labeled immediately after infection. Fig. 2*C* shows an analogous labeling of cells 6 hr after infection. The viral NP, HA 0, and NA proteins are clearly evident in this electrophoretogram, as are a series of spots at approximately 23,000 daltons present in the lower left-hand quadrant which we attribute to the viral nonstructural (NS) protein. This identification is made on the basis of the following criteria: (*i*) the absence of this species in purified virus particles and in uninfected cells (1); (*ii*) its occurrence as a major influenza virus-induced protein in infected cells (16); (*iii*) its molecular weight (1); and (*iv*) its localization in infected cell nuclei (18, 19) (autoradiograms not shown).

Fig. 2*B* presents an analysis of proteins synthesized in cells 1.5 hr after influenza virus infection. Three of the viral proteins resolved by this technique (NP, NS, and HA 0) are being synthesized in detectable amounts at this time. Since the synthesis of HA 0 is of particular interest, we have enlarged areas containing this glycoprotein from a series of autoradiograms of gels derived from cells at various times after infection (Fig. 3). Starting at 1.5 hr after infection (Fig. 3*B*), a new spot appears, which is absent in cells labeled immediately after infection (Fig. 3*A*). This spot (HA 0) becomes increasingly predominant with time as its synthesis becomes enhanced and the synthesis of the host polypeptides diminishes (Fig. 3 *C* and *D*). We have quantitated the amounts of HA 0 and NP made at various times after infection by excising the appropriate spots from the gels and measuring the associated radioactivity. Our results show that the relative rates of synthesis of these two proteins remain essentially constant throughout the infection cycle. Neuraminidase, because it is synthesized in smaller amounts than either of the above proteins, could only be clearly demonstrated subsequent to 2.5 hr after infection. Once detected, its synthesis also roughly parallels that of NP and HA 0 (unpublished data).

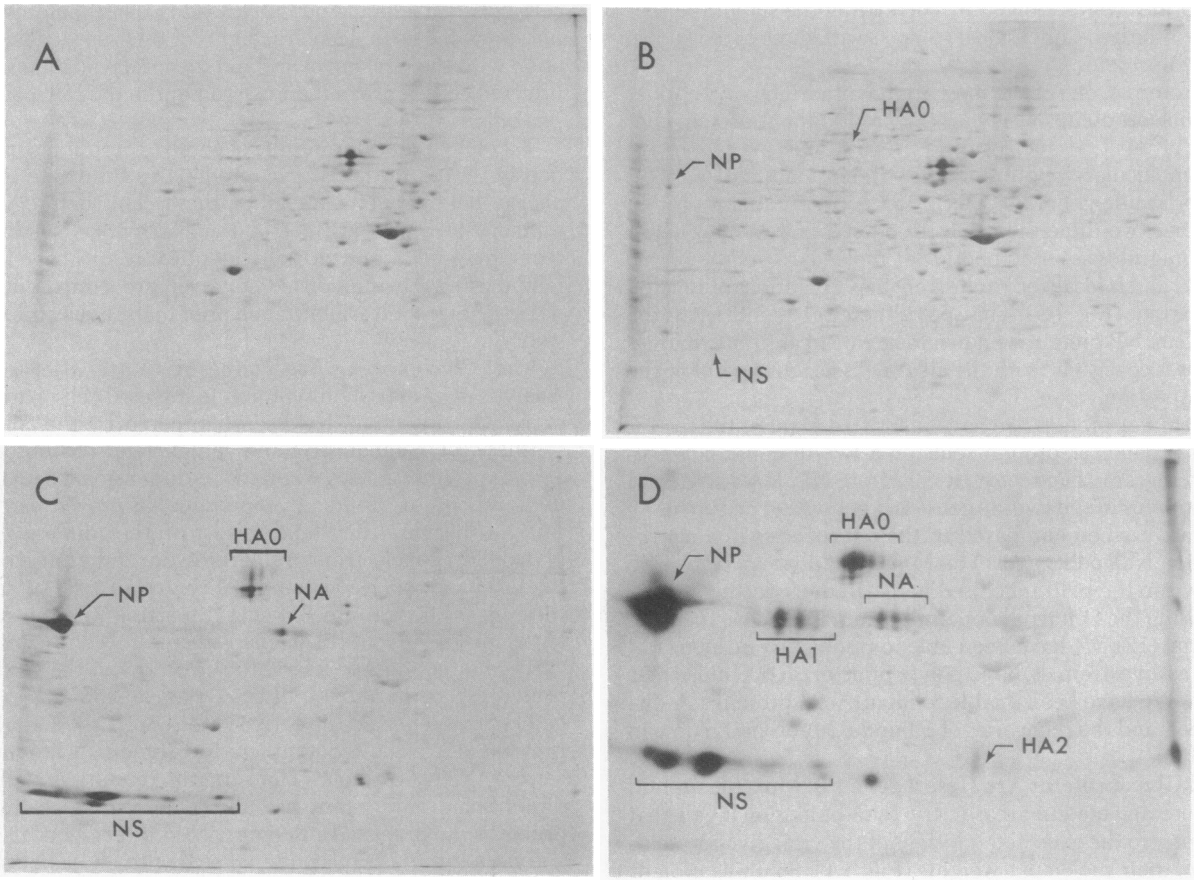

FIG. 2. Extracts of MDCK cells, infected with influenza virus and labeled with ^{14}C-labeled amino acids, solubilized, and subjected to two-dimensional gel electrophoresis followed by autoradiography. (*A*) Cells labeled for 30 min, beginning immediately after infection by NWS influenza virus. (*B*) Influenza virus-infected cells labeled for 30 min, beginning 1.5 hr after infection. (*C*) Influenza virus-infected cells labeled for 30 min, beginning 6 hr after infection. (*D*) Influenza virus-infected cells labeled for 30 min at 4 hr after infection, followed by a 2-hr chase with nonradioactive amino acids, as described in the text.

Viral Nucleocapsid and Nonstructural Proteins Are Phosphorylated in Infected Cells. We have reported that the NP protein of influenza virus is phosphorylated at a serine residue (20). To examine the phosphorylation of this component in infected cells, we turned to the two-dimensional electrophoretic system described above when a one-dimensional $NaDodSO_4$ electrophoresis system proved to be inadequate due to a high background of host cellular protein phosphorylation. We pulse-labeled cells with [^{32}P]orthophosphate and examined the resulting phosphorylated proteins by two-dimensional electrophoresis followed by autoradiography of the stained gel. Fig. 4*A* shows that a large number of ^{32}P-labeled proteins are detected in extracts of uninfected cells. Fig. 4*B* shows that many of the same spots are labeled in cells 5.5 hr after influenza virus infection. In addition, phosphorylation of the viral NP protein is clearly evident and, rather unexpectedly, two new phosphorylated species appear at positions coincident with the more acidic ^{14}C-labeled amino acid-labeled NS protein spots (shown in Fig. 2*C*). We have previously shown that the phosphate present on the NP protein of virions is labile to bacterial alkaline phosphatase (20). Fig. 4*C* demonstrates that this is also true of the cell-associated NP protein and most of the cellular phosphoproteins, whereas the NS phosphate label is refractile to the same treatment. We have mapped the tryptic peptides of each of the NS species and shown that they are identical to each other with the exception of the phosphorylated peptides, that the tryptic peptides differ from those of M protein, and that the most acidic NS spot has twice as much phosphate per mole of protein as does the other phosphorylated component (unpublished data).

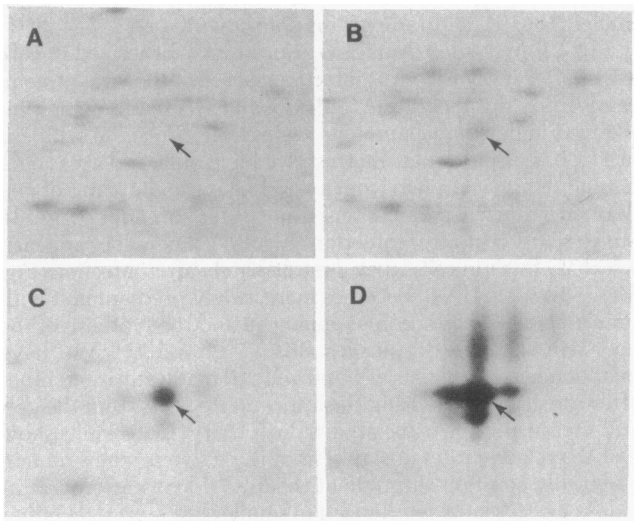

FIG. 3. Detail of the HA 0 regions of Fig. 2 demonstrating synthesis of the HA 0 protein early after influenza virus infection. Arrow indicates location HA 0 occupies in a two-dimensional gel. (*A*) Zero time; (*B*) 1.5 hr after infection; (*C*) 3 hr after infection (electroretogram not shown in Fig. 2); (*D*) 6 hr after infection.

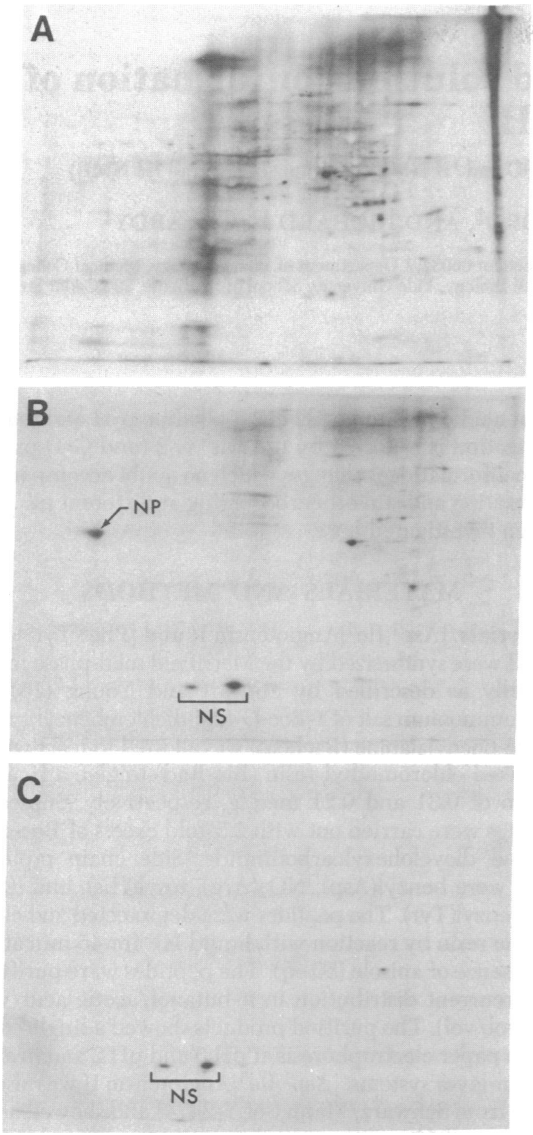

FIG. 4. Influenza virus-infected MDCK cells labeled for 1 hr with $^{32}P_i$ as detailed in the text, treated with RNase A and DNase I (10 μg of each for 1 hr at 4°), and subjected to two-dimensional electrophoresis. The ^{32}P-labeled components were visualized by autoradiography of the fixed and stained gels. (*A*) Uninfected MDCK cells. (*B*) Cells labeled 5.5 hr after influenza virus infection. (*C*) Equal aliquot of the extract in *B* but treated with 1.2 units of *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp.) in 0.1 M Tris-HCl (pH 8.0) at 37° for 1 hr.

DISCUSSION

The increased resolution afforded by the two-dimensional gel electrophoretic system has enabled us to perform a more critical analysis of the nature, synthesis, and modification of many of the proteins of influenza virus. This technique has allowed us to directly demonstrate the synthesis of viral neuraminidase, to identify heterogeneity of viral glycoproteins with respect to apparent net charge, and to show that viral glycoproteins are synthesized early in the infection cycle.

The molecular basis for the different isoelectric points displayed by the viral glycoproteins is not understood. However, similar kinds of heterogeneity have been noted in other biological systems (7). Since influenza virus glycoproteins do not contain sialic acid, the observed heterogeneity cannot be due to variations in the amount of this charged carbohydrate (21). It is possible that the multiple forms are due to different degrees of sulfation, since Compans and Pinter (22) have reported nonstoichiometric amounts of sulfate associated with preparations of influenza virus glycoproteins. Our analyses have unambiguously demonstrated the synthesis of viral hemagglutinin protein very early after infection, in contrast to some models of temporal control of viral protein synthesis that have been suggested. The results obtained in this study of viral protein synthesis are supported by measurements of the levels of individual influenza viral mRNAs in infected cells. These studies demonstrate a virtually parallel accumulation of all of the viral messages on polysomes after infection except for the matrix protein message, which increases late in infection relative to the others (P. A. Tekamp and E. E. Penhoet, unpublished data).

The demonstration of the phosphorylation of the nucleocapsid and nonstructural proteins in infected cells and the ability to separate the phosphorylated from the nonphosphorylated forms of the same protein (NS) should provide a critical tool for further examination of the functions of these proteins and their modifications in the viral life cycle. Two-dimensional gel analysis of temperature-sensitive mutants of influenza virus should be particularly useful in this regard.

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