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# Analysis of the Polypeptides Synthesized in Rinderpest Virus-Infected Cells

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We have identified, by [$^{35}$S]methionine labeling, eight major induced proteins and a number of minor proteins in rinderpest virus-infected bovine kidney cells. The polypeptides ranged in molecular weight from 212 to 21.5 kDa. The majority of these polypeptides are virus specific, as demonstrated by immunoprecipitation with rabbit hyperimmune serum against rinderpest. Infected cells radiolabeled with glucosamine contained a 75-kDa polypeptide and a broad band migrating at 80 kDa, both identified as virus specific by immunoprecipitation. Phosphorylated virus-specific proteins of 65 kDa and a complex of polypeptides at 92.5 kDa were also identified. Monospecific and monoclonal antibodies against measles virus and canine distemper virus hemagglutinin, fusion protein, nucleocapsid protein, and phosphoproteins confirmed the identity of the corresponding rinderpest virus-specific polypeptides.

## INTRODUCTION

The *Morbilliviruses* form a genus within the Paramyxoviridae family and consist of measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV), and peste-des-petits ruminants virus (PPRV). It has been shown that MV and CDV have six viral structural proteins: L, phosphoprotein (P), hemagglutinin (H), nucleocapsid (N), fusion (F), and matrix (M), and post-translational modification of some of these polypeptides has been demonstrated (Rima, 1983; Scheid and Choppin, 1977). In MV and CDV the F ($F_0$) protein is cleaved to its biologically active form, $F_1$ and $F_2$. These two polypeptides may be held together in the virion by a disulfide bridge as described for other paramyxoviruses (Rima, 1983).

The genomic RNA of *Morbilliviruses* is single-stranded, of negative polarity, and transcribed into at least six mRNA species (Hall *et al.*, 1978; Udem and Cook, 1984; Yoshikawa *et al.*, 1986). Recently the mRNAs for most of the genes of MV and CDV have been cloned and sequenced (Alkhatib and Briedis, 1986; Barrett and Mahy, 1984; Barrett *et al.*, 1985; Bellini *et al.*, 1985, 1986; Billeter *et al.*, 1984; Gorecki and Rozenblatt, 1980; Gerald *et al.*, 1986; Richardson *et al.*, 1985, 1986; Rozenblatt *et al.*, 1982, 1985; Russell *et al.*, 1985; Wong and Hirano, 1986).

Monoclonal antibodies against many MV and CDV polypeptides have been used to antigenically compare MV, CDV, and RPV (Norrby *et al.*, 1985; Sheshberadaran *et al.*, 1986). These studies demonstrated that there was considerable epitopic relatedness among many of the viral proteins particularly the F protein (Norrby *et al.*, 1985; Sheshberadaran *et al.*, 1986).

There have, however, been few studies at the molecular level of RPV or PPRV. Several investigators have purified RPV and described the major structural proteins (Underwood and Brown, 1974; Prakash *et al.*, 1979; Sato *et al.*, 1981). The latter two groups of investigators and recently Diallo *et al.* (1987) also analyzed polypeptide synthesis in RPV- and PPRV-infected cells and identified five viral proteins, P, H, N, F, and M by polyacrylamide gel electrophoresis (PAGE) and immunoprecipitation. Sato *et al.* (1981) demonstrated that the H, but not the F, protein was glycosylated. To date the largest structural polypeptide found in purified MV and CDV, the L protein, has not been described in RPV-infected cells.

We have undertaken a study to identify and more completely characterize the viral proteins synthesized in RPV-infected cells. At least eight viral-specific polypeptides were identified in infected cells by PAGE analysis and immunoprecipitation. Post-translational modification of some of these proteins was shown when infected cells were labeled with [$^{3}$H]-glucosamine or [$^{32}$P]orthophosphoric acid. The identity of some of these proteins was further confirmed by immunoprecipitation with monoclonal and monospecific polyclonal antisera against MV and CDV polypeptides.

## MATERIALS AND METHODS

### Virus

Virulent RPV strain Kabete "O" was obtained from Dr. J. House of the Plum Island Animal Disease Center,

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and grown in monolayers of primary bovine kidney (BK) cells in Joklik's modified Eagle's media supplemented with 10% calf serum.

### Preparation of radiolabeled lysates from virus-infected cells

Monolayers of primary BK cells, in 60-mm dishes, were infected with RPV at a multiplicity of infection of 0.001–0.05 $TCID_{50}$ per cell and then radiolabeled at varying times with different isotopes. Infected cell monolayers were washed twice with methionine-free medium, incubated in this medium for 30–60 min, and then labeled with 50 $\mu$Ci [$^{35}$S]methionine (New England Nuclear Corp., Boston, MA; >1000 Ci/mmol) in 0.5 ml methionine-free medium for 90–120 min. For labeling with [$^{3}$H]glucosamine (New England Nuclear Corp.; 32.5 Ci/mmol), infected cells were washed as described above but in media containing 1/10th the normal concentration of glucose. After incubation in this medium, cells were labeled for 1 hr in 0.5 ml media containing 100 $\mu$Ci [$^{3}$H]glucosamine. To radiolabel proteins with $^{32}$P, infected cells were washed and incubated in phosphate-free media and radiolabeled in these media with 150 $\mu$Ci [$^{32}$P]orthophosphoric acid (ICN Biomedicals, Inc., Costa Mesa, CA) for 2 hr. Infected cells were washed twice with phosphate-buffered saline, and lysed in 0.15 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.5, 0.0015 *M* $MgCl_2$, 1% Nonidet-P40, and 1% sodium deoxycholate. Nuclei were removed by centrifugation at 1500 rpm for 3 min and the cytoplasm was stored in aliquots at −70°.

The effect of tunicamycin on proteins synthesized in virus-infected cells was examined by incubating infected cells for 4 hr with 1 $\mu$g/ml of tunicamycin prior to radiolabeling. Cells were then washed and incubated in media minus methionine containing tunicamycin and radiolabeled for 1 hr with 120 $\mu$Ci [$^{35}$S]methionine in the presence of 1 $\mu$g/ml of tunicamycin. A 1 mg/ml stock solution of tunicamycin was prepared in dimethyl sulfoxide.

### Antisera

The RPV antisera used in this study were provided by Dr. J. Houce. Hyperimmune rabbit antiserum was prepared from rabbits infected with the Nakamura III lapinized strain of RPV. Convalescent serum was prepared by vaccination of cattle with the attenuated Kabete "O" Plowright strain of RPV. MV monospecific and monoclonal antibodies against the H, M, and N polypeptides and a CDV monoclonal antibody against the P protein were provided by Dr. E. Norrby (Sheshberadaran *et al.*, 1986; Varsanyi *et al.*, 1984). Dr. W. Bellini provided MV F monoclonal antibodies (Richardson *et al.*, 1986).

### Immunoprecipitation

Approximately equivalent amounts of radioactivity from cytoplasmic extracts of infected or uninfected cells were reacted with antiserum as previously described (Grubman *et al.*, 1984).

### Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed on 10% or 12.5% slab gels in the Tris–glycine buffer system (Grubman *et al.*, 1984; Laemmli, 1970). After electrophoresis the gels were fluorographed with sodium salicylate (Chamberlain, 1979), dried, and exposed to X-ray film at −70° (Bonner and Laskey, 1974) or directly dried and exposed to X-ray film with an intensifying screen for analysis of $^{32}$P-labeled polypeptides.

## RESULTS

### Identification of viral-specific proteins synthesized in infected cells

Viral protein synthesis was examined in BK cells infected at a low multiplicity with RPV by labeling with [$^{35}$S]methionine at 1, 2, 3, and 4 days after infection and examining lysates by PAGE. As seen in Fig. 1A (lanes 1 and 2) a number of new proteins were present as early as Day 1 after infection even though no cellular morphological changes were detected. The major additional protein has a MW of approximately 65 kDa and presumably is the viral N protein which is the major protein found in morbillivirus-infected cells (Rima, 1983). Proteins of 38.5 kDa (M protein) and minor amounts of a 212 kDa (L protein), 92.5 kDa (P protein), 77 kDa (H protein), and 62 kDa ($F_0$ protein) were also induced.

By the second day after infection, virus-induced syncytia are visible in 10–20% of the cell sheet. Approximately eight viral induced polypeptides are synthesized (lane 3) including a complex of three polypeptides of about 92.5 kDa and a band of 80 kDa (between the P and H proteins) appearing by 2 days after infection, but decreasing in intensity and becoming diffuse by 4 days. At 3 and 4 days after infection, when virus-induced morphological alterations are visible in greater than 50% of the cell sheet, viral-induced proteins are the major polypeptides synthesized (lanes 4 and 5). In this experiment there was a marked inhibition of host cell protein synthesis (compare Fig. 1, lanes 1 and 5). However, it should be mentioned that this level of inhibition did not always occur (data not shown). A polypeptide of approximately 21.5 kDa (C protein) was also found in infected cells when lysates were analyzed on a 12.5% gel (Fig. 1B).

To demonstrate that the newly synthesized proteins were viral specific, lysates were immunoprecipitated

with either normal rabbit serum or hyperimmune rabbit serum against RPV. As shown in Figs. 2 and 3 (lane 2) proteins of 212 kDa (L), a complex of two minor and one major polypeptide of approximately 92.5 kDa (P), a broad band at 80 kDa (between P and H), 77 kDa (H), 65 kDa (N), 38.5 kDa (M), and 21.5 kDa (C), synthesized from 1 to 4 days after infection, were immunoprecipitated with polyclonal antiserum, but not with normal serum. Viral-induced polypeptides of 62 kDa ($F_0$) and 49.5 kDa ($F_1$) are inefficiently immunoprecipitated with either this antiserum (Fig. 2) or convalescent antiserum from cattle (data not shown).

Previous studies have shown that there is considerable homology between MV, CDV, and RPV polypeptides (Sheshbaradaran *et al.*, 1986). To help identify the viral-induced polypeptides, monospecific and monoclonal antibodies against MV and CDV proteins were used to immunoprecipitate RPV-infected cell lysates. Monospecific polyclonal antisera against MV H polypeptide precipitated both the diffuse 80-kDa (note arrowhead) and the 77-kDa polypeptides (Fig. 3, lane

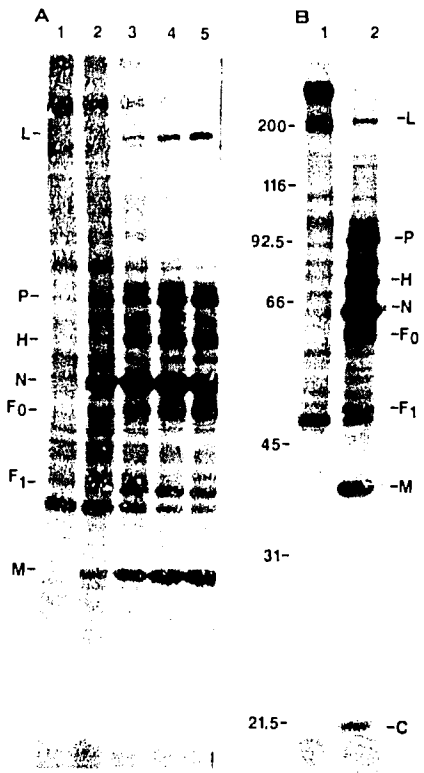


FIG. 1. Methionine labeling of RPV-infected BK cells. (A) Rinderpest-infected BK cells were labeled with [$^{35}$S]methionine for 1.5 hr in methionine-free media at 1, 2, 3, and 4 days after infection. Lysates were prepared and analyzed by PAGE on 10% slab gels. Mock-infected BK cells were similarly labeled and prepared for PAGE analysis. Lane 1, labeled BK cell lysate; lanes 2–5, RPV-infected cell lysates at 1, 2, 3, and 4 days after infection. (B) Analysis of RPV-infected cell lysates on a 12.5% slab gel. Lane 1, mock BK cell lysate; lane 2, RPV-infected cell lysate at 3 days after infection. The numbers on the left refer to molecular weight markers in kilodaltons.

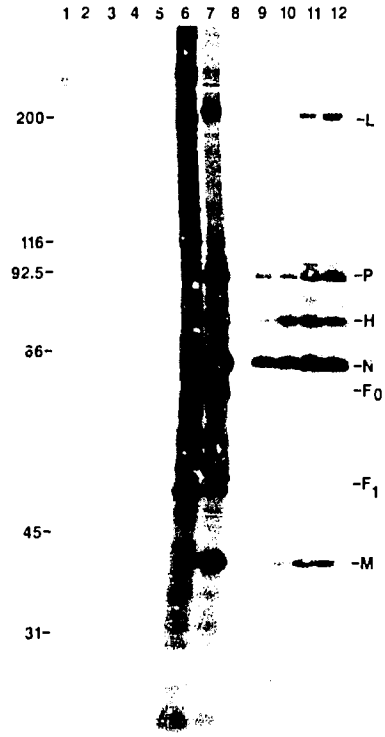


FIG. 2. Immunoprecipitation of methionine-labeled RPV-infected cell lysates. [$^{35}$S]methionine-labeled BK lysates prepared 1, 2, 3, and 4 days after RPV infection were immunoprecipitated with either a 1:40 dilution of normal rabbit serum or a 1:40 dilution of rabbit hyperimmune antisera against RPV and analyzed on 10% slab gels. Lanes 1–5 and 8–12, mock BK lysate and lysates 1, 2, 3, and 4 days after infection were immunoprecipitated with either normal or RPV specific serum, respectively. Lane 6, mock BK lysate prior to immunoprecipitation; lane 7, RPV lysate at 4 days after infection prior to immunoprecipitation. The numbers on the left refer to molecular weight markers in kilodaltons.

3), while a CDV monoclonal antibody against the phosphoprotein precipitated the 92.5-kDa polypeptide (Fig. 3, lane 7). The CDV monoclonal antibody also immunoprecipitated a number of other RPV-specific polypeptides (see Discussion). In a subsequent section we will present additional information supporting the immunological evidence that the 77- and 92.5-kDa polypeptides are the RPV hemagglutinin and phosphoprotein, respectively. Monoclonal antibodies against MV F protein very weakly immunoprecipitated both the $F_0$ and $F_1$ proteins (Fig. 3, lanes 8 and 9). Monoclonal antibodies against MV N immunoprecipitated the RPV N polypeptide (lanes 4 and 5), while an MV M monoclonal antibody did not immunoprecipitate any RPV proteins above background (lane 6), in contrast to previous reports (Sheshberadaran *et al.*, 1986).

## Glycosylated viral-induced polypeptides

Infected cells were labeled with [$^{3}$H]glucosamine at 1, 2, 3, and 4 days postinfection. The major glycosyl-

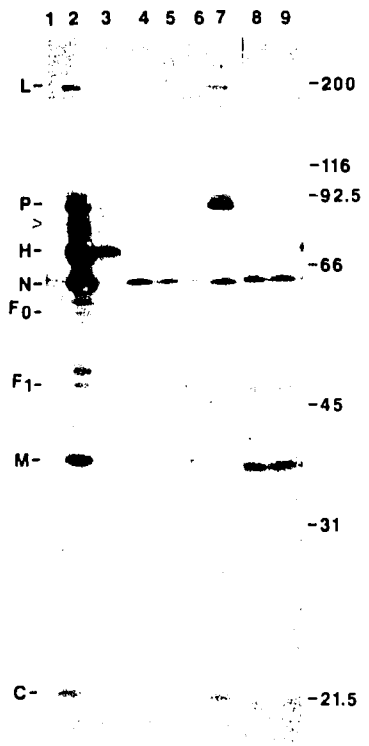


FIG. 3. Immunoprecipitation of [$^{35}$S]methionine-labeled RPV-infected cell lysates with MV and CDV antibodies. Lysates were immunoprecipitated with various antisera and analyzed on 12.5% slab gels. Lane 1, 1:40 dilution of normal rabbit serum; lane 2, 1:40 dilution of rabbit hyperimmune serum against RPV; lane 3, 1:4 dilution of monospecific antisera against MV H; lanes 4–5, 1:4 dilution of monoclonal antibodies against MV N (A1 B2 and R2 B4); lane 6, 1:4 dilution of a MV monoclonal antibody against M (19AG10); lane 7, 1:4 dilution of a CDV monoclonal antibody against P (3.803). Lanes 8–9, 1:4 dilution of monoclonal antibodies against MV F (84IM7 and 84IE3). Lanes 8–9 were exposed to X-ray film five times longer than lanes 1–7. The arrowhead in the left margin identifies the diffuse 80-kDa polypeptide. The numbers on the right refer to molecular weight markers in kilodaltons.

ated polypeptide, synthesized by 2 days, comigrated with a methionine-labeled polypeptide of 77 kDa (H) (Fig. 4, lanes 2 and 5). The second major glycosylated polypeptide, a broad band of approximately 80 kDa (note arrowhead), comigrates with a minor methionine-labeled polypeptide (lanes 2 and 6). Two minor glycoproteins of approximately 64 and 62 kDa are synthesized 3 days after infection. The 62-kDa glycoprotein appears to comigrate with the presumed fusion protein ($F_0$).

The effect of tunicamycin, a drug which inhibits asparagine-linked glycosylation, on viral protein synthesis was examined. After 3 days of infection cells were treated with tunicamycin and subsequently labeled with [$^{35}$S]methionine. As shown in Fig. 4 (lanes 2 and 3) the production of the 77-kDa glycosylated polypeptide (H) and a 49.5-kDa non-glucosamine-labeled polypeptide, comigrating with $F_1$, were inhibited by tunicamycin treatment. Two additional methionine-labeled polypeptides of about 80 and 55 kDa appear only after treatment (see arrowheads in left margin and lanes 2 and 3).

To determine if the glycosylated polypeptides and the two additional polypeptides found only in tunicamycin-treated cells are viral specific, lysates were immunoprecipitated with hyperimmune serum against RPV. The results shown in Fig. 5 demonstrate that in glucosamine-labeled cells only the broad band at 80 kDa and the major 77-kDa glycoprotein (H) are precipitated with this serum (lane 8), but not with normal serum (data not shown). In drug-treated cells the two additional methionine-labeled polypeptides as well as L, P, N, and M are precipitated specifically with hyperimmune serum (lane 3 and 7). However, M is inefficiently precipitated in drug-treated as compared to

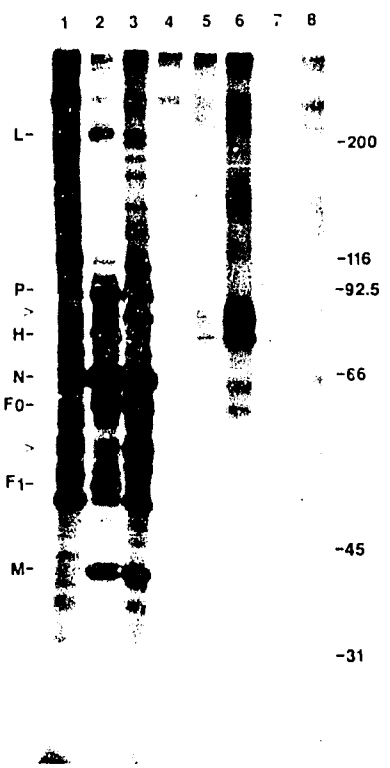


FIG. 4. Analysis of glucosamine-labeled RPV-infected BK cells. RPV-infected BK cells were radiolabeled with [$^3$H]glucosamine at 1, 2, 3, and 4 days after infection. Lysates were prepared and analyzed by PAGE on 10% slab gels. Infected BK cells were also pretreated with tunicamycin for 4 hr at 3 days after infection and radiolabeled with [$^{35}$S]methionine for 1 hr in the presence of tunicamycin and a lysate was prepared. Lane 1, [$^{35}$S]methionine labeled mock BK lysate; lane 2, [$^{35}$S]methionine-labeled RPV-infected lysate; lane 3, [$^{35}$S]methionine-labeled, tunicamycin-treated RPV-infected lysate; lanes 4–8, RPV-infected cell lysates labeled with [$^3$H]glucosamine at 1, 2, 3, and 4 days after infection and control BK cell lysate similarly labeled, respectively. The arrowheads in the left margin identify the new polypeptides found in drug-treated cells. The numbers on the right refer to molecular weight markers in kilodaltons.

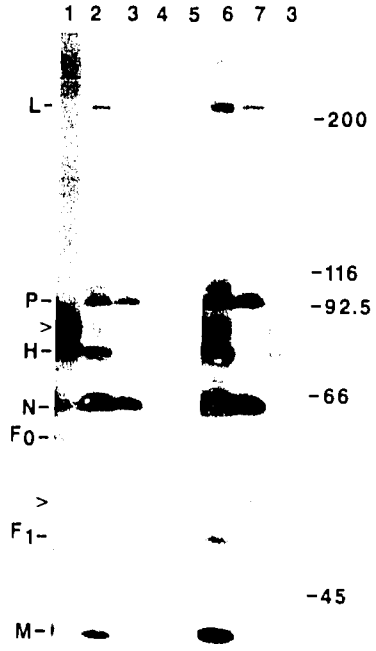


FIG. 5. Immunoprecipitation of glucosamine-labeled cell lysates. Mock BK or RPV-infected BK cell lysates were immunoprecipitated with hyperimmune rabbit antisera against RPV and analyzed by PAGE on 10% slab gels. Lane 1, RPV-infected cell lysate labeled with [$^3$H]glucosamine at 3 days after infection; lane 2, RPV-infected cell lysate labeled with [$^{35}$S]methionine at 3 days after infection and immunoprecipitated with a 1:40 dilution of rabbit hyperimmune antisera against RPV; lane 3, RPV-infected, tunicamycin treated cell lysate labeled with [$^{35}$S]methionine at 3 days after infection and immunoprecipitated as described for lane 2, lane 4, RPV-infected cell lysate labeled with [$^3$H]glucosamine at 3 days after infection and immunoprecipitated as described for lane 2; lane 5, mock BK cell lysate labeled with [$^3$H]glucosamine and immunoprecipitated as described for lane 2; lanes 6–8, A longer exposure of lanes 2–4. The arrowheads in the left margin refer to the new polypeptides found in drug-treated cells. The numbers on the right refer to molecular weight markers in kilodaltons.

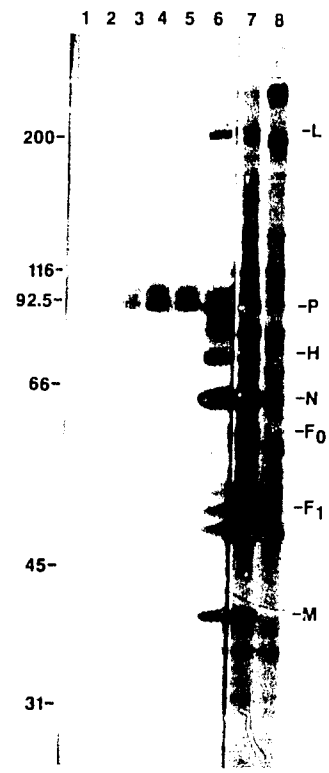


FIG. 6. Immunoprecipitation of $^{32}$P-labeled RPV-infected cell lysates. Mock BK or RPV-infected cell lysates were immunoprecipitated with a 1:40 dilution of rabbit hyperimmune antisera against RPV and analyzed on 10% slab gels. Lane 1, immunoprecipitation of $^{32}$P-labeled mock BK cell lysate; lanes 2–5, immunoprecipitation of RPV-infected lysates labeled with $^{32}$P at 1, 2, 3, and 4 days after infection, respectively; lane 6, immunoprecipitation of RPV-infected cell lysate labeled with [$^{35}$S]methionine; lane 7, RPV-infected cell lysate labeled with [$^{35}$S]methionine; lane 8, mock BK cell lysate labeled with [$^{35}$S]methionine. The numbers on the left refer to molecular weight markers in kilodaltons.

non-drug-treated lysates (Fig. 5, lanes 2 and 3, also 6 and 7).

### Phosphorylated viral-induced polypeptides

Infected BK cells were radiolabeled with $^{32}$P for 2 hr at 1, 2, 3, and 4 days postinfection. By 2 days postinfection a number of newly labeled proteins are apparent and the pattern of phosphorylated polypeptides remained the same throughout the period of labeling (data not shown). Immunoprecipitation of the lysates with hyperimmune serum precipitated only the complex of bands at 92.5 kDa (P) and the minor band at 65 kDa (N) (Fig. 6). No phosphorylated polypeptides reacted with normal serum (data not shown).

## DISCUSSION

In this report we have examined the proteins synthesized in RPV-infected BK cells by a variety of procedures and have identified at least eight viral-specific polypeptides (Table 1). Previous studies with RPV

TABLE 1

MOLECULAR WEIGHTS OF VIRAL-INDUCED PROTEINS IN RINDERPEST-INFECTED CELLS

| Polypeptide | Molecular weight | Post-translational modification |
|---|---|---|
| L | 212,000 | — |
| P | 92,500 | Phosphorylated |
| H | 77,000 | Glycosylated |
| N | 65,000 | Phosphorylated |
| $F_0$ | 62,000 | Glycosylated (?) |
| $F_1$ | 49,500 | — |
| M | 38,500 | — |
| C | 21,500 | — |

identified a number of proteins found in purified virus by PAGE analysis (Prakash *et al.*, 1979; Underwood and Brown, 1974). Sato *et al.* (1981) and Diallo *et al.* (1987) utilized immunoprecipitation to identify four proteins in purified virus and five in RPV infected cells, i.e., P, H, N, F, and M, and also demonstrated that the H, but not the $F_1$, protein is glycosylated. Our results confirmed the above data and in addition we have identified the RPV L and C proteins and examined post-translational modification of the P, H, and $F_0$ polypeptides.

As previously demonstrated for MV and CDV (Rima, 1983), we have identified a high-molecular-weight polypeptide of approximately 212 kDa (L) and a phosphoprotein (P) in RPV-infected cells. The P protein in RPV-infected cell lysates is approximately 92.5 kDa and is clearly of higher apparent molecular weight than its counterpart in MV- and CDV-infected cells which was initially identified as a 70- to 73-kDa phosphoprotein. The MV and CDV gene for this protein has recently been cloned and sequenced and its primary amino acid sequence specifies a protein of molecular weight 53.9 kDa (Bellini *et al.*, 1985) or 54.9 kDa (Barrett *et al.*, 1985). The identity of the P polypeptide in RPV infected cells was confirmed by $^{32}$P-labeling and immunoprecipitation with a CDV monoclonal antibody. It is of interest to note that the P protein consists of a complex of three to four bands as shown by labeling with either [$^{35}$S]methionine or $^{32}$P. This observation may simply reflect various levels of phosphorylation of the P protein. Similar results have been reported in MV-infected cells (Graves *et al.*, 1978).

We have also identified a virus-specific polypeptide of 21.5 kDa which is immunoprecipitated with the same CDV monoclonal antibody to the P protein, as well as with RPV hyperimmune serum. Bellini *et al.* (1985) and Barrett *et al.* (1985) have demonstrated that the MV and CDV P gene has a second overlapping open reading frame which can code for a 186 amino acid protein termed the C protein. This protein was identified in MV-infected cells with antisynthetic peptide antibodies (Bellini *et al.*, 1985). The P monoclonal antibody used in this study was obtained from Dr. E. Norrby and it was reported that this antibody precipitated only the P and not the C protein in CDV-, MV-, and RPV-infected cell lysates (Sheshberadaran *et al.*, 1986). The observation that this antibody precipitates the L, N, and 21.5-kDa proteins in addition to the P protein in RPV-infected cells may simply reflect the presence of complexes in our lysates. These complexes may have been removed by Sheshberadaran *et al.* (1986) who used high-speed centrifugation to prepare lysates. In contrast, our protocol removed only nuclei. We suggest that the 21.5-kDa polypeptide is the RPV C protein. This protein is not glycosylated (data not shown) and therefore presumably is not $F_2$ which is reported to be glycosylated but poorly labeled with methionine in MV and CDV cells (Rima, 1983).

As reported for MV and CDV (Rima, 1983), the N protein is the major methionine-labeled polypeptide in RPV-infected cells and appears to be phosphorylated, but at a low level. The M protein is also a major RPV-specific protein.

In RPV-infected cells, we have identified four major glycosylated polypeptides. However, only a diffuse band at 80 kDa and the 77-kDa polypeptide (H) are immunoprecipitated with RPV hyperimmune serum. The 77- as well as the 80-kDa polypeptide were confirmed to be the H protein by immunoprecipitation with monospecific polyclonal antisera against MV H. The 80-kDa polypeptide may be a more highly glycosylated form of the 77-kDa protein, but this possibility has to be confirmed by further analysis. The appearance of the 77-kDa H polypeptide is completely inhibited by tunicamycin treatment and instead a sharp band of higher molecular weight (80 kDa) appears. This band is inefficiently immunoprecipitated with RPV serum. At present the relationship of the 80- and 77-kDa H proteins is unclear. Similar results have been reported for the measles H protein after tunicamycin treatment (Stallcup and Fields, 1981).

We have identified the uncleaved fusion protein ($F_0$) and the larger subunit of this proteolytically cleaved polypeptide ($F_1$). $F_0$, but not $F_1$, is labeled with glucosamine. Preliminary observation of a glycosylated polypeptide of less than 20 kDa may indicate that we have also identified the smaller subunit, $F_2$, of the fusion protein (data not shown). Neither $F_0$ nor $F_1$ is efficiently immunoprecipitated with hyperimmune or convalescent RPV serum, but monoclonal antibodies against MV fusion protein weakly immunoprecipitated both polypeptides. Synthesis of $F_1$ is completely inhibited in tunicamycin-treated cells and a higher-molecular-weight protein (55 kDa) appears. It is conceivable that this polypeptide is nonglycosylated $F_0$ which may not be susceptible to cleavage into $F_1$ and $F_2$.

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