Kinetics of Synthesis and Phosphorylation of Respiratory Syncytial Virus Polypeptides

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SUMMARY

The kinetics of synthesis of [35S]methionine-labelled respiratory syncytial virus-specific proteins were studied in CV-I cells infected at high multiplicity. Immuno-precipitated viral proteins resolved by SDS–PAGE were quantified by scanning fluorographs of protein bands. The nucleocapsid (N) protein was detectable by 2 h post-infection (p.i.), whereas the phospho- (P), matrix (M) and fusion (F₀) proteins and Vp24 (a matrix-like protein) were first detected between 4 and 6 h p.i. Synthesis of the glyco- (G) protein was first detected at 6 h p.i. and reached its peak synthesis rate at 10 h p.i. Virus-specific P, M and Vp24 proteins were phosphorylated in infected cells. The P protein was highly phosphorylated in purified virions whereas phosphorylated species of the M and Vp24 proteins were minor components. The phosphorylated form of the P protein was detected by monoclonal antibody precipitation, confirming the identity of this protein. The N protein was not phosphorylated in infected cells or in virions. Synthesis of [35S]methionine-labelled proteins preceded detectable 32P i labelling by several hours. The putative phosphorylated M protein was detected at 6 h p.i. before phosphorylated forms of P and Vp24 were seen. The timing of appearance of the phosphorylated species of P and Vp24 proteins in infected cells corresponded to the release of infectious virions from infected cell monolayers at 10 to 12 h p.i.

INTRODUCTION

Human respiratory syncytial (RS) virus is unique compared to other members of the paramyxovirus family because it contains 10 viral genes for which the coding assignments have been determined (Collins et al., 1984). RS virions and infected cells contain seven structural proteins (Wunner & Pringle, 1976; Levine, 1977; Pringle et al., 1981; Fernie & Gerin, 1982; Lambert & Pons, 1983) and, in addition, three small (M, 14K, 11K and 9-5K) non-structural gene products have been identified in infected cells (Huang et al., 1985). The non-structural proteins are sometimes found as minor components of purified virions (Collins et al., 1984; Huang et al., 1985). The genes for the 41K nucleocapsid (NP or N) protein, the 28K matrix (M) protein, the 34K phospho- (P) protein, Vp24 (a matrix-like protein), the 68K fusion (F₀) protein and the 84K to 90K glyco- (G) protein have been cloned (Elango et al., 1985; Collins et al., 1984; Wertz et al., 1985; Satake et al., 1985). In addition, transcriptional mapping by u.v. inactivation demonstrated that the gene order for the RS virus genome is 14K-11K-N-P-M-9-5K-36K(G)-F-24K-L (Dickens et al., 1984), where L is the large protein.

Although a great deal of work has been done to define the molecular biology of this virus, very little is known about the early events in RS virus replication. The first reported study of the kinetics of RS virus protein synthesis did not detect virus-specific polypeptides before 18 h post-infection (p.i.; Cash et al., 1979). More recent studies of RS virus protein synthesis have concentrated on the kinetics of glycoprotein synthesis and processing (Gruber & Levine, 1985; Fernie et al., 1985). High multiplicity experiments carried out in HeLa cells demonstrated that
the N protein was first detected at 11 h p.i. (Gruber & Levine, 1985). In the experiments described here, we were able to detect N protein synthesis as early as 2 h p.i.

Very little is known about the phosphorylation of RS virus proteins during virus replication. Therefore, it was of interest to identify the RS virus proteins phosphorylated in infected cells. Phosphorylation and dephosphorylation of negative strand virus proteins may play critical roles in regulating virus-specific functions in infected cells. Phosphorylation of vesicular stomatitis virus polypeptides during replication has been implicated as a possible regulating mechanism for transcription (Clinton et al., 1978, 1979; Sanchez et al., 1985). Influenza virus nucleocapsid protein phosphorylation has been shown to stimulate viral transcription (Kamata & Watanabe, 1977). It has been suggested that phosphorylation may also play a role in regulating assembly of nucleocapsids into virions (Lamb & Choppin, 1977).

Results reported here demonstrate that both the rates of synthesis and time of appearance of individual viral polypeptides differed. We postulate that some temporal control may be exerted over synthesis of either viral mRNAs or proteins. The order of appearance (synthesis) of immunoprecipitated [35S]methionine-labelled N, P and M viral proteins appeared to follow the order of genes in virion RNA (Dickens et al., 1984). Comparisons of the kinetics of protein synthesis and phosphorylation of viral proteins were also carried out.

**METHODS**

**Cells and virus.** CV-1 cells were grown in MEM supplemented with 10% heat-inactivated foetal bovine serum (Kansas City Biologicals). Virus infections and stocks were prepared as previously described (Lambert & Pons, 1983). The Long strain of RS virus was used in all experiments. RS virus infectivity was determined on HEp-2 monolayers using either agarose or methyl cellulose overlays as previously described (Lambert et al., 1980).

**Labelling and kinetics experiments.** CV-1 cell monolayers in 35 mm plates were infected with a plaque-purified stock of RS virus (Long strain) at a multiplicity of 5 p.f.u./cell. RS virus-infected CV-1 cell monolayers were pulse-labelled with either [35S]methionine (50 μCi/ml) in methionine-free MEM or with 32P, in citrate-buffered MEM (phosphate-free) containing Hanks' salts for 15 min at the indicated times p.i. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Lambert & Pons, 1983) and the viral polypeptides were immunoprecipitated as described below. Immunoprecipitated viral polypeptides were analysed by SDS-PAGE using the methods of Laemmli (1970).

**Immunoprecipitation and SDS-PAGE.** Immunoprecipitation of RS virus antigens in infected cell extracts made in RIPA buffer was carried out with anti-RS virus rabbit serum and IgG sorb (The Enzyme Center, Boston, Mass., U.S.A.) as previously described (Lambert & Pons, 1983). Viral proteins were released from IgG sorb into Laemmli SDS-PAGE buffer (Laemmli, 1970) by boiling for 2 min. IgG sorb was removed by centrifugation for 3 min in a microfuge (Eppendorf). For some experiments, RS virus proteins were precipitated with mouse serum against NP40-disrupted whole virus or with mouse monoclonal antibody (MAb) against the P protein. MAb in ascites fluid (designated L2) directed against RS virus P protein was kindly provided by Dr E. Walsh (Rochester University Medical School, Rochester, N.Y., U.S.A.). Immunoprecipitation of viral proteins with MAb against P protein was carried out by incubating 10 μl of a 1:100 dilution of MAb L2 for 4 h at 4 °C. Precipitation of RS virus protein-immune complexes was accomplished by adding Immunobeads containing rabbit anti-mouse IgG (Bio-Rad). Immunoprecipitated proteins were released from washed immunobeads by adding 50 μl SDS-PAGE sample buffer (Laemmli, 1970) then heating for 2 min in a boiling water-bath. Samples were analysed on 12.5% polyacrylamide gels.

**Quantification of RS polypeptide bands in fluorographs.** Optical scans of fluorographs of gels were made with a visible light densitometer (E-C Apparatus Corp.). Kodak X-Omat AR X-ray film was preflashed with light prior to exposure to gels to give a background OD of approximately 0-1 units, so that quantitative scans of fluorographs could be carried out (Laskey & Mills, 1975). Gel scan data were entered into an Apple computer via a VersaWriter digitizer drawing board (Versa Computing Inc.) and analysed by the computer. Integrated peak areas were determined to be proportional to protein band intensities in fluorographs by scanning twofold dilutions of labelled virion proteins separated by SDS-PAGE. The decrease in averages of band densities and peak areas was linear. Experimental peak area values were plotted against the time p.i. to give an estimate of the kinetics of [35S]methionine or 32P, labelling of viral proteins. Partial proteolytic digestions of [35S]methionine and 32P, labelled RS virus proteins were carried out as described previously (Wechsler et al., 1985b).

**RESULTS**

**Kinetics of synthesis of RS virus polypeptides in infected CV-1 cells**

Time-course of synthesis of RS virus proteins in CV-1 cells was determined by pulse labelling infected cell cultures for 30 min at 2 h intervals from 0 to 18 h p.i. Infected cell monolayers were
Synthesis kinetics of RS virus polypeptides

Fig. 1. Time-course of synthesis of [35S]methionine-labelled RS virus polypeptides in CV-1 cells. Monolayers of CV-1 cells were mock-infected or infected with the Long strain at a multiplicity of 5 p.f.u./cell for 1 h at 33 °C. Media were replaced and cells were incubated at 37 °C. At the indicated times (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 24 and 48 h p.i.) cells were pulse-labelled for 15 min with [35S]methionine (50 μCi/plate) in methionine-free MEM. After pulse labelling, monolayers were washed with ice-cold phosphate-buffered saline then disrupted with 0.5 ml RIPA buffer. Immunoprecipitated proteins from infected (I) and uninfected (U) cell lysates were analysed on 10% polyacrylamide gels under reducing conditions.

also pulse-labelled at 24 and 48 h p.i. Uninfected control cells were pulse-labelled at the same times and under identical experimental conditions. [35S]Methionine-labelled viral polypeptides were immunoprecipitated with hyperimmune rabbit anti-RS virus serum and separated on 10% polyacrylamide gels (Fig. 1). Viral proteins were identified as previously described (Lambert & Pons, 1983). Synthesis of the N protein was detected as early as 2 to 4 h p.i. The band running just ahead of the N protein was probably actin, since it was also present in the uninfected cell lanes. The P and M proteins were first detected at 4 to 6 h p.i., with the appearance of the P protein preceding that of the M protein. The fusion proteins (F₀ and F₁) were first detected at 6 and 8 h p.i., respectively. Because of its low methionine content, the large G protein was not well labelled but was detectable in the original fluorograph by 6 h p.i. Two minor proteins of approximately 14K and 11K (designated x and y respectively) were immunoprecipitated. The identity of these proteins is not yet known but since they were specifically precipitated from infected but not uninfected cell extracts, they most probably represent viral non-structural proteins. Because these protein bands were very faint, their synthesis rates were not analysed further.

In order to visualize more accurately the kinetics of synthesis of viral proteins, fluorographs of polyacrylamide gels were scanned using a visible light densitometer. Densitometric profiles were analysed by computer to determine the areas under the peak profiles of immunoprecipitated viral protein bands at the indicated times p.i. Peak areas, indicative of labelled protein band density, were used as an estimate of incorporation of radioactivity into protein bands. Band intensity of radiofluorographs has been shown to be directly related to visible light
kinetics of \([^{35}\text{S}]\)methionine-labelled viral polypeptide synthesis. Estimates of the amounts of labelled proteins detectable by immunoprecipitation in Fig. 1 were determined by scanning the X-ray film fluorographs of SDS–polyacrylamide gels with a densitometer as described in Methods. Band densities of specific proteins were compared by determining the area of peaks from densitometric scans by computer analysis and the values are plotted against time post-infection. (a) Kinetics of G (○), F₀ (■) and F₁ (▲) proteins. (b) Kinetics of N (▼), P (●) and M (■) proteins and Vp24 (○).

absorption of bands on the X-ray film and to the amount of radioactivity in the bands (Laskey & Mills, 1975).

Fig. 2(a) represents the rate of incorporation of \([^{35}\text{S}]\)methionine into immunoprecipitated viral glycoproteins. Synthesis rates of F₀ and F₁ were similar, although F₀ was detected first at 4 h p.i. The F₂ subunit of the fusion protein was not detected because it does not label well with \([^{35}\text{S}]\)methionine. The initial rate of synthesis of the G protein was similar to that of the uncleaved F₀ protein between 6 and 10 h p.i., after which the synthesis rate was reduced. A contributing factor to the apparent lag in detection of the G protein compared to F₀ protein was probably the fact that \([^{35}\text{S}]\)methionine labelling of the G protein is poor (Pringle et al., 1981; Dubovi, 1982). This makes an accurate estimate of the rate of synthesis of this protein difficult using this technique.

Fig. 2(b) represents the rates of incorporation of \([^{35}\text{S}]\)methionine into immunoprecipitated N, P and M proteins and Vp24. By 12 h p.i. all the viral proteins detectable by immunoprecipitation were being synthesized at maximal rates. Rates of synthesis of N, P and M proteins were similar between 2 and 12 h p.i. During the early phase (2 to 6 h p.i.) of synthesis, the relative rate of synthesis of these proteins was in the order N > P > M until approximately 8 h p.i. when equivalent \([^{35}\text{S}]\)methionine labelling of these proteins was detected during a 15 min pulse labelling period. The rate of \([^{35}\text{S}]\)methionine incorporation into Vp24 was significantly less than that of the other three ‘internal’ (N, P and M) structural proteins. The peak synthesis rate of Vp24 was achieved by 12 h p.i. These results suggest that synthesis of the N protein may precede the synthesis of the other viral polypeptides by 1 to 2 h followed, in order, by the P and M proteins.

The multiphasic nature of the incorporation of \([^{35}\text{S}]\)methionine represented in Fig. 2(a, b) was observed in three separate experiments. It is not clear whether this is the result of a ‘cycling’ phenomenon of reduced viral protein synthesis followed by new bursts of protein synthesis, transient alterations in membrane transport of \([^{35}\text{S}]\)methionine, or variations in the efficiency of immunoprecipitation of viral proteins at various times p.i. The first phase of synthesis occurred from 2 to 12 h p.i., followed by a decrease in the amount of all proteins detected at 14 h p.i. This was followed by a slight increase in synthesis rates for all proteins with the rate for the M protein being highest at 18 h p.i. rather than at 12 h p.i. Synthesis of F₀ and F₁ demonstrated a similar profile, although at lower rates of \([^{35}\text{S}]\)methionine incorporation. A slight decline in the rate of synthesis for all viral proteins was observed between 24 and 48 h p.i. with the exception of the G protein which increased slightly during this period (Fig. 2a).

Kinetics of phosphorylation of RS virus polypeptides

As part of this study, we were interested in determining which viral polypeptides were phosphorylated in infected CV-I cells. It was also of interest to determine the kinetics of
phosphorylation of RS virus polypeptides for comparison with synthesis of [$^{35}$S]methionine-labelled proteins. These experiments were carried out as described for the [$^{35}$S]methionine experiments discussed above except that pulse labelling of infected cell monolayers with $^{32}$P, was carried out in phosphate-free medium. In control experiments, use of citrate-buffered media did not significantly alter the rate of [$^{35}$S]methionine incorporation into viral polypeptides or the release of labelled virions into the media (data not shown). For comparison, $^{32}$P, pulse-labelled mock-infected cells were immunoprecipitated using identical conditions (Fig. 3, lane 1).
Fig. 4. Kinetics of $^{32}$P incorporation into viral phosphoproteins. The relative amounts of phosphoproteins detectable by immunoprecipitation in Fig. 3 were determined by scanning the fluorograph of SDS–polyacrylamide gels. The band densities were quantified by computer analysis as described in Methods and plotted against time p.i. Labelled protein bands are P (■) and M (▲) proteins, Vp24 (●) and X, a 14K protein (○).

Infected cell extracts, from 12 h p.i. CV-1 cells pulse-labelled with $[^{35}$S]methionine, were also run as controls (Fig. 3, lane 11).

As shown in Fig. 3, $^{32}$P-labelled polypeptides, immunoprecipitated with RS virus antiserum, were resolved by 10% SDS–PAGE. A phosphorylated band, which we presumed to represent the phosphorylated form of the M protein, was first detected at 6 h p.i. This band migrated significantly slower than the $[^{35}$S]methionine-labelled M protein in these gels although a minor $[^{35}$S]methionine band was observed in the same region (Fig. 3, lane 11, arrow) suggesting that only a small portion of the M protein was phosphorylated. This result is consistent with that reported for the phosphorylated species of Sendai virus M protein which migrates significantly slower than its unphosphorylated form (Graves, 1981). Phosphorylated Vp24 was first detectable at 10 h p.i.

The earliest phosphorylated protein seen in these gels was a minor immunoprecipitated phosphorylated protein band (designated X) which was observed throughout the time-course experiment. The identity of the X phosphoprotein is uncertain at present but it might represent one of the non-structural viral proteins since this band was not detected in immunoprecipitates from uninfected cells (Fig. 3, lane 1).

Scans of fluorographs of these gels were carried out as described above for Fig. 2, and band density values for the three viral phosphoproteins (P, M and Vp24) were plotted against time p.i. (Fig. 4). Phosphorylated P protein was only slightly visible by 6 h p.i. whereas, at the same time point, approximately twice as much of the putative phosphorylated M protein was immunoprecipitated. The rate of phosphorylation of the M protein exceeded that of the P protein until 12 to 14 h p.i. when the amount of immunoprecipitated phosphorylated P protein increased approximately threefold (Fig. 4). The rate of $^{32}$P labelling of the P protein increased steadily between 12 and 18 h p.i. The phosphorylated species of Vp24 was first detected at 10 h p.i. and peaked at 16 h p.i. By 18 h p.i. $^{32}$P labelling of Vp24 had decreased by about 30%. Rates of $^{32}$P labelling of both M protein and Vp24 were similar in that both had decreased by 18 h p.i. whereas the phosphorylation rate of the P protein had increased.

Comparison of kinetics of protein synthesis and phosphorylation of the P and M proteins and Vp24

Rates of protein synthesis (determined by $[^{35}$S]methionine labelling) and phosphorylation ($^{32}$P labelling) of the P and M proteins and Vp24 from 2 to 18 h p.i. were directly compared
Synthesis kinetics of RS virus polypeptides

Fig. 5. Comparison of the kinetics of protein synthesis represented by $[^{35}S]$methionine incorporation (■) and of phosphorylation of the same proteins, represented by $^{32}P$ incorporation (○). Data were obtained from Fig. 2 and 4. Protein band densities were determined by scanning fluorographs of polyacrylamide gels as described in Methods. The graphs show a comparison of the kinetics of synthesis and phosphorylation for the P protein (a), M protein (b) and Vp24 (c).

Using data from the protein gels shown in Fig. 2 and 4 (Fig. 5a to c). Synthesis of $[^{35}S]$methionine-labelled proteins preceded detectable $^{32}P$ labelling by about 7 h if one compares labelling at 50% of maximal incorporation rates. This phenomenon was most apparent for phosphorylation of the P protein (a) and Vp24 (c). The putative phosphorylated M protein (b) was very poorly labelled with $^{32}P$, relative to $[^{35}S]$methionine labelling, as compared to either P protein or Vp24. The appearance of phosphorylated species of the P protein (8 to 12 h p.i.) closely parallels the timing of release of infectious virions from infected cell monolayers under one-step growth conditions (Lambert et al., 1980).

Monoclonal antibody precipitation of P protein and comparison of $[^{35}S]$methionine- and $^{32}P$-labelled virion proteins

RS virus-infected CV-1 cells were labelled with either $[^{35}S]$methionine or $^{32}P$, from 0 to 24 h p.i. in order to compare viral proteins present in virions and immunoprecipitates of infected cell
Fig. 6. Comparison of MAb immunoprecipitation of the [35S]methionine- and 32P-labelled P protein of RS virus-infected cells and the proteins of purified virions. Virus-infected CV-1 cells were labelled with 50 μCi/ml [35S]methionine in methionine-free medium or with 50 μCi/ml 32P, in phosphate-free medium from 0 to 24 h p.i. then lysed in RIPA buffer. Virions in the media were harvested and partially purified by pelleting through a 10 ml 30% sucrose cushion in a SW28 rotor for 1.5 h at 25000 r.p.m. Viral proteins were resolved in 12.5% polyacrylamide gels. Fluorographs of [35S]methionine- (lanes 1 and 3) and 32P-labelled (lanes 2 and 4) proteins immunoprecipitated with either hyperimmune mouse serum made against NP40-lysed whole virus (a, lanes 1 and 2), or with anti-P protein MAb L2 (a, lanes 3 and 4). (b) For comparison, virion proteins were run in the same gels. Lane 1 is [35S]methionine-labelled purified virions and lane 2 is 32P-labelled purified virions grown in CV-1 cells. Dashed lines connect M and Vp24 bands with their phosphorylated forms.

extracts. In order to resolve better the proteins in the region of the P and M proteins, these samples were analysed on 12.5% polyacrylamide gels (Fig. 6a, b). Cell extracts from [35S]methionine- and 32P-labelled infected cells were immunoprecipitated with either hyperimmune mouse anti-RS virus serum (Fig. 6a, lanes 1 and 2) or with a MAb directed against the P protein (Fig. 6a, lanes 3 and 4). Antigen–antibody complexes were precipitated with
rabbit anti-mouse IgG Immunobeads. Monoclonal antibody against the P protein precipitated both $[^{35}S]$methionine and $^{32}$P-labelled P protein, demonstrating that this phosphorylated band was indeed the P protein (Fig. 6a, lanes 3 and 4). Virions released into the media of these cultures were harvested by pelleting virus through a 30% sucrose cushion. Virus pellets were resuspended in SDS–PAGE sample buffer (Laemmli, 1970) and the proteins were separated in the same gel as described above (Fig. 6b). The major $^{32}$P-labelled protein found in virions as well as in infected cells was the P protein (Fig. 6b, lane 2). The phosphorylated band above the P protein, but below the N protein, probably represents the phosphorylated form of actin. One-dimensional peptide mapping of $[^{35}S]$methionine- and $^{32}$P-labelled proteins using the method of Cleveland et al. (1977) suggested that Vp24 and the M and X phosphoproteins do not represent breakdown products of the P protein since all these peptides had different patterns compared to digests of P protein (data not shown). Proof of the identity of the putative M phosphoprotein must await direct identification by immunoprecipitation with anti-M protein MAb.

**DISCUSSION**

We have investigated the time-course of synthesis and phosphorylation of RS virus proteins immunoprecipitated from CV-1 cells infected at high multiplicity. The initial investigation of the kinetics of RS virus protein synthesis was carried out by Cash et al. (1979). Viral proteins were not observed until 18 to 24 h p.i., probably because the m.o.i. was low. Later, Gruber & Levine (1985) demonstrated that the N protein was the first viral protein to appear at 11 h p.i. in RS virus-infected HeLa cells followed by appearance of other proteins at 16 h p.i. Although a high m.o.i. was used, Gruber & Levine (1985) did not use immunoprecipitation for detection of virus-specific proteins. Consequently, high background levels of cell proteins made it difficult to observe low levels of viral protein synthesis early in the infectious cycle.

The study reported here is the first detailed investigation of the time-course of synthesis of RS virus proteins in cells infected at high multiplicity using immunoprecipitation for detection of virus-specific polypeptides. The N protein, which is one of the three most abundant RS virus proteins found both in infected cells and in virions, was the first protein detected (at 2 h p.i.) The early synthesis of these three proteins (2 to 12 h p.i.) took place at similar rates except that N protein was detected earlier and was followed in order of appearance by the P and M proteins. The timing of detection of RS virus N, P and M proteins appeared to coincide with the gene order of these proteins (Dickens et al., 1984) and suggests that temporal control of virus protein synthesis may be exerted at either the transcriptional or translational level.

A potential problem with using $[^{35}S]$methionine labelling for time-course kinetics experiments is that viral proteins contain different amounts of this amino acid. Accurate comparisons of synthesis rates for individual viral proteins must take this into account. We did not correct our data for this difference in methionine content since this was not known for the Long strain of RS virus. However, ratios of the N, P and M proteins in RS virions (Long strain) or in infected cells are similar when proteins are labelled with either $[^{3}H]$amino acids or $[^{35}S]$methionine. The estimates for the rate of synthesis of these three proteins should, therefore, be accurate. Differences in amounts of immunoprecipitated individual viral proteins could also reflect differences in the relative serum concentration of immunoglobulins specific for each viral protein. This was not considered to be a problem in the experiments reported here because immunoprecipitations were carried out with antibody in vast excess.

An important part of this study was concerned with identifying virus-specific phosphoproteins as well as correlating the time-course of phosphorylation with viral protein synthesis. Cash et al. (1979) identified a 35K RS virus protein as the only phosphorylated species in infected cells. We previously reported that RS virions contain one highly phosphorylated protein of similar size, designated the P protein (Lambert & Pons, 1983). However, results of the present study show that at least three (and possibly four) virus-specific proteins (P, M, Vp24 and X) were phosphorylated in infected cells with the P protein being the most highly phosphorylated species in infected cells and virions. The earliest phosphorylated protein detected was the putative M protein, based on limited proteolytic digest analysis. In addition, partial chymotryptic digests of
The phosphorylated forms of P, M and X proteins and Vp24 demonstrated that they are not related proteins (data not shown).

Rates of phosphorylation differed for each of the proteins and phosphorylation occurred much later than their initial period of maximal synthesis. The timing of increased phosphorylation of P, M and Vp24 polypeptides may correspond with the timing of virus budding at 8 to 10 h p.i. (Lambert et al., 1980). The discrepancy in the synthesis and the timing of phosphorylation of the P and M proteins and Vp24 suggests that either virus-specific protein kinase activity was not high in the early phase of infection or that these proteins were not accessible to these enzymes until later in the infectious cycle. Another possibility is that P protein, for example, may be phosphorylated during assembly (budding) of RS virions. Because RS virus is highly cell-associated, labelled virions (i.e. budded but still attached to cells) would not have been distinguished from intracellular P protein immunoprecipitated from RIPA cell extracts in the experiments described here. Future experiments designed to determine the possible role of phosphorylation of RS viral proteins in virus assembly will need to address this issue.

An interesting difference in the pattern of phosphorylation of RS virus proteins compared to the human parainfluenza virus type 3 (HPIV3) proteins was noted during the course of these studies. The HPIV3 P, N and M virion proteins are phosphorylated (Wechsler et al., 1985b) whereas the intracellular HPIV3 M protein is not (Wechsler et al., 1985a). In experiments reported here for RS virus (Fig. 3 and 6), the intracellular putative M protein of RS virus was more highly phosphorylated than the M protein present in virions. We did not detect phosphorylation of the N or L proteins in RS virions or in infected cells. The L, N and P proteins are the most likely proteins to be involved in transcription, since these proteins are closely associated with purified RS virus nucleocapsids (Wunner & Pringle, 1976; Peeples & Levine, 1979; Huang et al., 1985; D. M. Lambert, unpublished observation). The phosphorylation pattern of RS virus polypeptides differed from that of most other paramyxoviruses in that the N protein was not phosphorylated either in infected cells or in virions.

Further studies are needed before the role of viral protein phosphorylation in either RS virus transcription or virus assembly can be assessed. Also, it is not known at present whether phosphorylation of RS viral proteins is mediated by cellular protein kinases or a virus-specific kinase. It will be important to compare the results of in vitro phosphorylation reactions using purified virions and nucleocapsids to determine whether a virus-associated protein kinase is responsible for this phosphorylating activity. These experiments are currently underway.

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