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Phosphorylation of the Nucleoprotein of an Avian Influenza Virus

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SUMMARY

High resolution polyacrylamide gel electrophoresis (PAGE) of chick embryo fibroblast cells infected with the avian influenza virus FPV-Rostock revealed two distinct polypeptides migrating in the region of the nucleoprotein (NP). One-dimensional fingerprinting of these polypeptides showed that they were both nucleoprotein, and [32P]orthophosphate labelling revealed that they differed with respect to their state of phosphorylation. Pulse-chase studies using [35S]methionine indicated that phosphorylation of a certain proportion of NP occurs rapidly after synthesis and is associated with transport to the nucleus. Nucleoprotein which remained in the cytoplasm was predominantly non-phosphorylated. Both the phosphorylated and the non-phosphorylated types of NP were found in ribonucleoprotein complexes (RNPs) of different densities isolated on renografin gradients, but RNPs isolated from the nucleus contained much more phosphorylated NP than those from the cytoplasm. The kinase responsible for nucleoprotein phosphorylation appears to be influenced by temperature of incubation of the infected cells.

INTRODUCTION

Phosphoproteins are ubiquitous in biological systems where they are believed to play an important role as mediators of metabolic regulation (Greengard, 1978; Nilsen-Hamilton & Hamilton, 1979). For example, the phosphorylation of histone proteins during the cell cycle probably reflects the action of regulators of DNA synthesis (Gurley et al., 1977), and phosphorylation of ribosome initiation factors can influence mRNA translation in eukaryotic cells (Levin et al., 1976; Farrell et al., 1977). Phosphoproteins have also been found in many virus systems, either as structural components of the virion (Tan & Sokol, 1972; Sokol & Clark, 1973; Rosemond & Moss, 1973; Privalsky & Penhoet, 1977) or as infected cell polypeptides (Russell et al., 1972; Lamb & Choppin, 1977; Schaffhausen & Benjamin, 1979; Wilcox et al., 1980). Most frequently, virus-specified phosphoproteins seem to be associated with nucleic acid (Levinson et al., 1977; Wilcox et al., 1980), an observation which has led to suggestions that phosphorylation may be involved in the regulation of virus gene expression and genome replication (Kamata & Watanabe, 1977; Clinton et al., 1978).

For influenza viruses, it has been reported that the largest non-structural protein (NS) and the major nucleocapsid protein (NP) are phosphoproteins (Privalsky & Penhoet, 1977, 1978). Although relatively little is known about the function of NS, the observation regarding NP phosphorylation is in keeping with the idea that an active regulation of its function is effected through this process (Kamata & Watanabe, 1977). The influenza nucleoprotein is known to be a component of both replicative and structural ribonucleoprotein complexes (Caliguiri & Gerstein, 1978), it is transported in and out of the nucleus (Breitenfeld & Schafer, 1957; Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Krug & Etkind, 1973; Hay & Skehel, 0022-1317/82/0000-5005 $02.00 © 1982 SGM
1975; Flawith & Dimmock, 1979), and is associated with virus-specific RNAs of both polarities (Pons, 1971, 1975). The precise role of phosphorylation in the regulation of these events is unclear, although some experiments suggest that, at least in vitro, NP phosphorylation may be a necessary preliminary to primary transcription (Kamata & Watanabe, 1977).

We report here an investigation into the synthesis and phosphorylation of influenza virus nucleoprotein using polyacrylamide gel electrophoresis (PAGE) conditions under which the phosphorylated form can be separated from its non-phosphorylated precursor. We found that phosphorylation is greatly influenced by the temperature at which replication takes place and occurs rapidly after, but not during, NP synthesis.

We were unable to associate phosphorylated NP uniquely with subclasses of RNP with different polymerase contents, but we did observe an enrichment of phosphorylated NP within the nucleus. The possibility that the kinase involved is virus-induced is discussed.

METHODS

Cells and viruses. Chick embryo fibroblasts (CEF) were prepared from 11-day-old embryos as described by Borland & Mahy (1968), except that Pronase (25 µg/ml) was used for tissue digestion, and Lavitt medium containing 10% foetal calf serum was used for growth of cells. The avian influenza virus strain A/fowl plague virus/Rostock/34 (H7N1) (FPV-Rostock) was propagated in the allantoic cavity of 11-days incubated fertile hens’ eggs.

Chemicals and isotopes. L-[35S]methionine and [α2P]orthophosphate were obtained from Amersham International. Acrylamide (2 × crystallized), bisacrylamide and cycloheximide were purchased from Serva. p-Fluorophenylalanine, phenylmethylsulphonyl fluoride (PMSF) and aprotinin were obtained from Sigma.

Labelling of infected cell polypeptides. At various times after infection with 50 to 100 p.f.u./cell, infected cell polypeptides on a single 1 cm diam. well of a Costar 24-well plate were pulse-labelled with L-[35S]methionine at 40 µCi/ml in methionine-free minimal essential medium (MEM), as described by Inglis et al. (1976). At the end of the labelling period cells were either harvested, or incubated in pulse-chase medium containing cold L-methionine and then harvested directly into Laemmli sample buffer containing 1 mM-PMSF and 20 units aprotinin/ml. Samples were immediately heated to 96 °C for 2 min, and applied to a Laemmli polyacrylamide gel.

Cell fractionation. Infected cells were fractionated after lysis by Dounce homogenization in a hypotonic RNA extraction buffer B4 (10 mM-tris–HCl pH 7.4, 10 mM-NaCl, 1 mM-MgCl₂, 1 mM-CaCl₂, 1 mM-triethanolamine (Inglis et al., 1977), containing 1% Nonidet P40 (NP40). The lysed cells were centrifuged at 2500 g for 5 min and the supernatant removed and designated ‘cytoplasm’. The pellet was resuspended in B4 containing 1% NP40 and 0.5% sodium deoxycholate and left 5 min on ice with occasional vortex mixing. The mixture was centrifuged at 2500 g for 5 min and the supernatant removed and discarded. This washing procedure was repeated and the final pellet was designated ‘nuclei’. Where necessary, nuclei were disrupted by sonication.

Isolation of ribonucleoprotein complexes. Cytoplasmic or disrupted nuclear fractions were layered on to a discontinuous renografin gradient exactly as described by Caliguiri & Gerstein (1978), and centrifuged at 35 000 rev/min for 16 h at 4 °C in a Beckman 40 Ti rotor.

Electrophoresis conditions. Slab gels (20 × 20 cm) were cast between glass plates separated by 1 mm-thick perspex spacers. Either 15% acrylamide–0.087% bisacrylamide, or 7% acrylamide–0.19% bisacrylamide, in 0.38 M-tris–HCl pH 8.7, 0.1% SDS, was polymerized using 0.003% TEMED and 0.003% ammonium persulphate. The 2 cm-high stacking gel was 3% acrylamide–0.26% bisacrylamide in 250 mM-tris–HCl pH 6.8. Gels were electrophoresed in a Raven Scientific slab gel apparatus at a constant current of 40 mA, until the
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bromophenol blue dye marker had reached the bottom. Electrophoresis buffer contained 6.06 g tris base, 28.8 g glycine and 1 g SDS per litre.

RESULTS

Two forms of nucleoprotein in infected cells incubated at 40.5 °C

Analysis of the polypeptides induced in CEF monolayers which had been infected with FPV-Rostock and incubated at 40.5 °C for 4.5 h and then pulse-labelled for 15 min with [35S]methionine, under conditions where minimal protein was applied to the gel, revealed a double-band migrating in the region of NP (Fig. 1). Previous PAGE analyses of this same FPV-Rostock strain had revealed only a single nucleoprotein species (Inglis et al., 1976; Almond et al., 1977); it was necessary, therefore, first to determine the identity of both these bands and to re-establish the homogeneity of this particular cloned virus stock.

A number of subclones of FPV-Rostock derived from the master stock used in Fig. 1, as well as many multiply plaque-purified recombinants derived from this and subsequent clones (Almond & Barry, 1979), were examined. A similar FPV-Rostock NP doublet was observed in all cases (data not shown). To verify that both these [35S]methionine species represented nucleoprotein, one-dimensional peptide maps were prepared by the method of Cleveland et al. (1977) after the bands had been excised from a dried 15% polyacrylamide gel. The autoradiograph (Fig. 2) reveals that although there are one or two peptides showing small differences in their migrations (arrowed) the map patterns are virtually identical.

The possibility of a precursor/product relationship between the two species was investigated using short pulse and pulse-chase labelling conditions. Fig. 3 shows infected cell polypeptides labelled at 4.5 h post-infection for 2, 5 and 10 min, and similar samples chased by incubation in a medium containing unlabelled methionine for a further 10 min. The faster migrating NP was the predominant species after a 2 min pulse, suggesting that this is the primary gene product. Appearance of the band labelled NPp is subsequent, indicating that its formation is due to post-translational modification of NP. Fig. 4 shows that this modification, giving rise to decreased electrophoretic mobility, is due, at least in part, to phosphorylation of the newly synthesized polypeptide. The comparison of [35S]methionine and [32P]orthophosphate-labelled products reveals that only the slowest migrating band is phosphorylated and, therefore, the two nucleoproteins differ in this respect.

It has been reported that the NP of the strain of influenza virus is phosphorylated only once at a serine residue (Privalsky & Penhoet, 1981). The single phosphorylated NP band detected in these studies is in accord with this result.

Interpretation of results illustrated in Fig. 2, 3 and 4 leads to the conclusion that phosphorylation occurs rapidly, soon after nucleoprotein synthesis. Not all the newly synthesized NP becomes phosphorylated, however, since even after chase periods of up to 2 h the relative abundance of the two species did not change significantly from that shown in Fig. 3, track 10 chase, i.e. after a 10 min chase period (data not shown).

Influence of temperature on nucleoprotein phosphorylation

In influenza virus-infected cells incubated at 40.5 °C, the two forms of NP were present in approximately equimolar proportions. Similar analyses of cells incubated at 34 °C and 37 °C, however, revealed that in both cases the non-phosphorylated NP was the major species (compare Fig. 5 b track 6 with Fig. 5 a, track 6; 37 °C not shown). In some cases prolonged exposure of autoradiographs was necessary to detect NPp at 34 °C. Pulse-chase experiments at these two temperatures confirmed this observation and indicated that, as at 40.5 °C, the relative proportions of the two NP forms did not change significantly even after the chase periods of up to 2 h (data not shown).
Fig. 1. PAGE analysis of polypeptides from FPV-Rostock-infected chick embryo fibroblasts at 4-5 h post-infection after incubation at 40-5 °C. Cells on a single well of a Costar 24 multi-well plate were pulse-labelled for 15 min with methionine-free MEM containing 40 μCi/ml L-[35S]methionine. After the labelling period cells were washed twice with ice-cold saline and dissolved in 200 μl Laemmli sample buffer. Approximately 1/10th of this material was loaded on to the 15% acrylamide gel and electrophoresed for approx. 4 h at 40 mA constant current.

Fig. 2. One-dimensional polypeptide fingerprints prepared by the method of Cleveland et al. (1977) of the two species of nucleoprotein illustrated in Fig. 1. The two NP bands were excised from a dried 15% polyacrylamide gel, rehydrated and placed directly into the slot of a new 15% polyacrylamide Laemmli gel. Chymotrypsin was added to the sample just prior to electrophoresis overnight at 50 V constant voltage as described previously (Almond & Barry, 1979). Lane 1, NP₁ (slowest migrating nucleoprotein band); lane 2, NP₂ (fastest migrating nucleoprotein band).
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Fig. 3. Investigation of the synthesis of the two NP species by pulse–chase experiments. FPV-Rostock-infected cells 4.5 h post-infection at 40.5 °C were pulse-labelled with L-[35S]methionine (100 μCi/ml) in methionine-free MEM for 2, 5 and 10 min, and dissolved in Laemmli sample buffer. Parallel samples, after washing with warm Hanks' balanced salt solution, were chased for 10 min with complete medium. UC, Uninfected control cells.

Fig. 4. Comparison of [35S]methionine and [32P]orthophosphate-labelled infected cell polypeptides. FPV-Rostock-infected cells were pulse-labelled 4 h post-infection at 40.5 °C with [35S]methionine (40 μCi/ml) or [32P]orthophosphate (500 μCi/ml). Cells were harvested and prepared for electrophoresis after washing twice with ice-cold saline. Electrophoresis was on a 15% acrylamide gel for 4 h at 40 mA constant current. Track 1, [35S]methionine-labelled infected cell polypeptides; track 2, [32P]orthophosphate-labelled infected cell polypeptides; track 3, [32P]orthophosphate-labelled uninfected cell polypeptides.
Temperature shift experiments (not shown) indicated that the temperature of synthesis greatly influenced the extent of phosphorylation, whereas the temperature of incubation after synthesis was complete had little effect. These differences are not related to differences in time of the replication cycle at these temperatures since the proportions of NP: NP<sub>p</sub> remain stable up to 14 h post-infection (data not shown).

**Phosphorylated nucleoprotein in infected cells**

The experiments described above indicate that two distinct populations of nucleocapsid protein molecules exist in influenza virus-infected cells, and that their relative abundance is greatly influenced by the temperature of incubation. Previous work on ribonucleoprotein (RNP) complexes in infected cells had similarly revealed two distinct populations, in this case differing in their polymerase (P) proteins: nucleocapsid protein ratios (Caliguiri & Gerstein, 1978). The possibility that phosphorylation may play a role in the demarcation of these two RNP classes was therefore investigated. Structural and transcribing ribonucleoprotein complexes were prepared by centrifugation in renografin gradients from whole cells, and cytoplasmic and nucleoplasmic fractions.
Fig. 6. Location of the two species of nucleoprotein in FPV-Rostock-infected chick embryo fibroblasts. (a) Subclasses of ribonucleoprotein complexes separated on renografin gradients (Caliguiri & Gerstein, 1978), from nucleus and cytoplasm of cells pulse-labelled for 30 min with [35S]methionine 4.5 h post-infection at 40.5 °C. Track 1, 1.21 g/ml RNPs isolated from cytoplasm; track 2, 1.26 g/ml RNPs isolated from cytoplasm; track 3, 1.21 g/ml RNPs isolated from nucleus; track 4, 1.26 g/ml RNPs isolated from nucleus. (b) Total polypeptides from nuclei (track 1) and cytoplasm (track 2) of cells pulse-labelled for 30 min 4.5 h post-infection at 40.5 °C. Track 3, uninfected control cells.

The polypeptide components of these complexes were analysed by PAGE under conditions that separate the phosphorylated and non-phosphorylated forms of NP (Fig. 6a). No obvious differences in the relative proportions of NP:NP$_p$ were evident between the two types of RNP.
complexes. However, the relative proportions of the NPs did differ between RNP complexes isolated from cytoplasm and those isolated from nuclei. This was confirmed by a straightforward nuclear/cytoplasmic cell fractionation and PAGE analyses of each fraction (Fig. 6b).

Thus, these experiments indicate that there is a locational division of the two NP types but no apparent functional division in relation to implicated (Caliguiri & Gerstein, 1978) transcribing versus structural RNP complexes. This finding was somewhat surprising in the light of previous reports that NP phosphorylation is an essential preliminary to transcription \textit{in vitro} of the WSN strain of influenza virus (Sugiyama \textit{et al.}, 1976). With FPV-Rostock, however, we have been unable to detect efficient phosphorylation of NP using \([\gamma^{32}\text{P}]\text{ATP}\) in actively transcribing detergent-disrupted virions \textit{in vitro} at 34, 37 or 40.5°C.
Thus, we have been unable to obtain evidence of a role for phosphorylation in RNP-associated RNA synthesis. It does seem, however, that NP\(_p\) is just as capable as NP of fulfilling a structural role in RNP complexes since its proportion in these structures reflects approximately its proportion in the nucleus or cytoplasm as a whole.

**The nucleoprotein kinase**

Available data do not allow us to distinguish between the possibilities that the protein kinase involved in NP phosphorylation is virus or cellular in origin. The following observations, however, would be consistent with the notion that the kinase is virus-induced. First, a time course of protein synthesis in FPV-Rostock-infected cells shows that, at early times post-infection, almost no NP\(_p\) is made during the 15 min pulse period, whereas at later times (e.g. 4 h post-infection) phosphorylation proceeds rapidly (Fig. 5). Secondly, directly after removal of a cycloheximide block of protein synthesis, under which only primary transcription of the virus genome occurs (Lamb & Choppin, 1976), nucleoprotein kinase activity is not observed during a 15 min pulse (Fig. 7). Finally, under conditions where the amino acid analogue fluorophenylalanine is included in the tissue culture medium throughout infection, no NP\(_p\) is made during a 15 min pulse-labelling period at 4 h post-infection (data not shown).

**DISCUSSION**

Polyacrylamide gel electrophoresis of \(^{35}\text{S}\)methionine-labelled influenza virus-infected chick embryo fibroblasts revealed two distinct species of nucleoprotein. These were shown to be related to each other by one-dimensional peptide mapping. Labelling with \(^{32}\text{P}\)orthophosphate revealed that the slowest migrating nucleoprotein, designated NP\(_p\), was phosphorylated whereas the faster migrating band, NP, was not. Phosphorylation was the only difference detected between the two NP species and probably accounts for their different migrations in the gel systems used. The possibility that other differences exist, such as the state of acetylation, sulphation or hydroxylation, that would account for this migrational difference cannot be ruled out. However, evidence of nucleoprotein modifications of these types have not been reported and phosphorylation of polypeptides resulting in migrational changes in SDS gels have been seen by others (Wilcox et al., 1980; Lamb & Choppin, 1977).

In contrast to reports using other virus strains (Privalsky & Penhoet, 1978), no phosphorylation of the NS polypeptide was detected. The only virus-specified polypeptide which became labelled after incubation with \(^{32}\text{P}\)orthophosphate was nucleoprotein.

Separation of the two forms of NP after \(^{35}\text{S}\)methionine labelling allowed phosphorylation to be monitored by pulse–chase experiments. Results indicated that addition of phosphate to the unmodified NP occurred rapidly within 10 min of synthesis and the proportion of NP remaining unphosphorylated did not alter significantly in the following 2 h. The extent of conversion of NP to NP\(_p\) was dependent on the temperature at which NP was synthesized and this proportion was substantially greater at 40-5 °C than at 34 °C. No NP species of mobilities intermediate between those of NP and NP\(_p\) were observed. The relative proportion of NP and NP\(_p\) synthesized also varied at different times post-infection, the non-phosphorylated NP being the predominant species at earlier times, and an increasing proportion of NP\(_p\) being synthesized later in infection (i.e. after 2 h).

Attempts to correlate the phosphorylation process with a role in the demarcation of ribonucleoprotein complexes involved in RNA synthesis versus those playing a structural role proved unsuccessful. Neither RNPs previously implicated as actively transcribing, nor those believed to be structural (Caliguiri & Gerstein, 1978), were exclusively associated with either form of NP. This observation was somewhat surprising since earlier studies had suggested that NP phosphorylation may be important in virus transcription *in vitro* (Kamata &...
Watanabe, 1977). We have been unable to repeat the observations of NP phosphorylation in vitro using [γ-32P]ATP as a component of a transcription mixture with our strain of virus.

Isolation of RNPs from nucleus and cytoplasm and straightforward total protein analysis of nuclear and cytoplasmic fractions did, however, reveal the NP_p was primarily associated with the nucleus. One possibility to explain this observation is that the phosphorylation event may facilitate transport of either free NP or RNP complexes across the nuclear membrane, or alternatively, it could be that the kinase involved is active only in the nucleus. Within either of these interpretations the kinetics of phosphorylation are consistent with the recent observations of Briedis et al. (1981), who have shown conclusively that a large proportion of NP is transported to the nucleus soon after its synthesis. It might be speculated, therefore, that the relatively low abundance of NP_p early in infection means that RNPs or free NP molecules are mostly cytoplasmic at these times. This may relate to the temporal synthesis of various classes of RNA during the replicative cycle. Unfortunately, in common with Caliguiri & Gerstein (1978), we have also been unable to recover polymerase activity from RNAs after centrifugation on renografin gradients. It would be of value to examine the nature of the RNA product produced by nuclear and cytoplasmic RNPs, since it is possible that phosphorylation may influence polymerase specificity to accommodate the alternative functions of template, messenger and virion RNA synthesis (Skehel & Hay, 1978).

Another interpretation of the observation of low NP_p levels early in infection is that this may reflect a lower amount of nucleoprotein kinase in the cell at these times. This interpretation, together with the results of experiments using cycloheximide and amino acid analogues, are consistent with the idea, but do not prove, that the kinase is virus-induced. The identity of the kinase and the precise functional role of nucleoprotein phosphorylation in influenza virus replication, however, require further investigations.

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