Kinetics of Synthesis of Influenza Virus Ribonucleoprotein Structures

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SUMMARY

The synthesis of influenza virus ribonucleoprotein structures (RNPs) in infected chick embryo cells was analysed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium deoxycholate which resolves the RNPs into five size classes. A relatively small proportion of total RNPs accumulated in the nucleus but free NP protein was found there in large amounts over the period 1.5 to 4 h post-infection. In contrast, by 4 h post-infection, all cytoplasmic NP was complexed into RNP structures. At early times, during a 15 min pulse of \[^{35}\text{S}\]methionine, nearly all the newly synthesized NP was incorporated into RNPs but by 4 h the majority of pulse-labelled NP was present as free protein. However, the proportion of free NP:NP in RNPs remained constant over the 1.5 to 4 h post-infection period, indicating that there was a delay before the NP synthesized later in infection was assembled into RNP structures. Individual RNP size classes were predominantly cytoplasmic and accumulated at similar rates but were not produced in equimolar amounts. The rates of synthesis of individual RNPs were in general agreement with their rates of accumulation with the remarkable exception of RNP d (containing RNA 7, the matrix protein gene). This was synthesized nearly 10-fold faster but accumulated at the same rate as the other RNPs. Possibly RNP d is more rapidly degraded than the other RNPs.

The influenza virus genome consists of eight separate segments of single-stranded negative-sense RNA (RNA\(^-\), McGeoch et al., 1976; Palese & Schulman, 1976). In the infected cell these RNA segments are transcribed into two forms of complementary plus-sense RNA (RNA\(^+\)): a truncated copy which serves as messenger RNA (mRNA) to direct the synthesis of virus proteins and a complete transcript (cRNA) which serves as template for the synthesis of negative-sense virus RNA (Hay et al., 1977a, b). Where the various forms of virus-coded RNA are synthesized is unclear, but there is evidence that the cell nucleus is the probable site of transcription of virus mRNA (Armstrong & Barry, 1974; Taylor et al., 1977; Mark et al., 1978, 1979; Barrett et al., 1979).

Within the virion the RNA segments are complexed with nucleoprotein (NP; Pons et al., 1969) to form separate ribonucleoprotein (RNP) complexes (Duesberg, 1969; Compans et al., 1972). In extracts from infected cells, RNPs containing both RNA\(^+\) and RNA\(^-\) have been found (Pons, 1971, 1975) and it seems reasonable to suppose that all intracellular virus-induced RNA may be present in RNP complexes. Apart from trace amounts of other virus proteins the principal component of intracellular RNPs is the NP protein (Caliguiri & Gerstein, 1978; Rees & Dimmock, 1981 b).

A number of studies has been made of the kinetics of synthesis and accumulation of virus-specific RNA (Scholtissek & Rott, 1970; Hay et al., 1977a; Barrett et al., 1978, 1979; Mark et al., 1978) and proteins (Skehel, 1972, 1973; Meier-Ewert & Compans, 1974; Lamb & Choppin, 1976; Inglis et al., 1976, 1978; Inglis & Mahy, 1979) in influenza virus-infected cells, but these studies have, with the exception of Krug (1972) and Krug & Etkind (1973, 1975), largely tended to ignore the fact that the RNA and protein may combine to form RNP complexes. We have previously described an electrophoretic procedure for the analysis of influenza virion RNPs which allows the separation of the eight RNP structures into five size
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Fig. 1. Accumulation of label into total NP protein (●) or NP associated with all RNPs combined (○) in infected cells labelled with [35S]methionine either continuously from 1 h post-infection (a, b) or pulse-labelled for 15 min at the times shown (c, d). Nuclear (a, c) and cytoplasmic (b, d) fractions were obtained using the nuclear monolayer technique and analysed for proteins and RNPs by PAGE as described in the text. Following autoradiography, bands were cut from the gels and radioactivity determined by liquid scintillation counting. The sum total of radioactivity in the five RNP classes is presented here. The left-hand scale refers to continuous labelling and the right-hand scale to pulse labelling.

classes (Rees & Dimmock, 1981a, b). We have used this procedure here to investigate the synthesis of virus RNPs in infected cells.

All results were obtained using the avian influenza virus A/FPV/Rostock/34 (H7N1; FP/R) and chick embryo fibroblast (CEF) cells in 5-cm Petri dishes seeded with 9 × 10⁶ cells (Morser et al., 1973). Cells were inoculated with virus in allantoic fluid to give a multiplicity of infection of 25 and incubated at 37 °C for 1 h. Times post-infection were measured from the time of addition of the virus. For continuous radiolabelling the inoculum was removed and replaced with 2 ml/plate of prewarmed Glasgow-modified minimal essential medium (GMEM) containing 10% normal methionine concentration and 50 μCi [35S]methionine (800 to 1200 Ci/mmol; Amersham International). Cells for pulse labelling received 3 ml prewarmed 199 medium containing 5% calf serum and, at 10 min prior to labelling, the medium was replaced with 2 ml prewarmed buffered Earle’s saline (BES). Cells were then pulsed with 25 μCi [35S]methionine in 0.5 ml BES for 15 min. At each time post-infection one dish of cells was taken and nuclear and cytoplasmic fractions prepared using the nuclear monolayer technique (Hudson & Dimmock, 1977; Possee et al., 1982). Proteins were analysed by PAGE as described by Cook et al. (1979) using the buffer system of Laemmli (1970) and RNPs were separated on 3 to 4% acrylamide gradient gels (Rees & Dimmock, 1981a) with a modified electrophoresis buffer containing 0-02 m-NaCl, 0-001 m-EDTA, 0-05 m-tris–HCl pH 7-4 and electrophoresis at 40 mA. All RNPs identified by this procedure co-migrated with those from virions and no others were found in infected cells (Rees & Dimmock, 1981a, b).

Since NP is the major protein component of intracellular virus RNPs separated by electrophoresis (Rees & Dimmock, 1981b) we determined what proportion of total NP was associated with RNP complexes at various times up to 4 h post-infection in cells labelled
continuously from 1 h (Fig. 1 a, b) or pulse-labelled for 15 min at various times (Fig. 1 c, d) with $[^{35}\text{S}]$methionine. Nuclear and cytoplasmic fractions were analysed by PAGE and, following autoradiography, RNP and NP bands were cut from the gels and radioactivity determined. It was assumed that the total $^{35}$S counts in RNP structures represented NP protein associated with the RNA since analysis of the polypeptides of intracellular virus RNPs separated by PAGE has shown NP to be the major component (Rees & Dimmock, 1981 b). For the purpose of this report we have defined as free NP protein any NP which is not associated with RNPs, i.e. radioactivity in NP protein determined on a protein gel less radioactivity in RNPs determined on a RNP gel. In cells labelled continuously, NP protein accumulated in both the nuclear and cytoplasmic fractions. In the nuclei (Fig. 1 a) the levels of radioactivity associated with RNPs remained low and the curves for RNP and total NP diverged, indicating a net accumulation of free NP protein. At early times, approx. 80% of the total nuclear NP was associated with RNP structures, but by 4 h post-infection this value had decreased to 10%. The cytoplasmic fractions (Fig. 1 b) showed an opposite trend with the accumulation of label in RNPs closely following that of NP protein. At 1-5 and 2 h post-infection about 55% of the total cytoplasmic NP protein was associated with RNPs but by 4 h all the cytoplasmic NP was in RNP complexes. Combining the data from nuclear and cytoplasmic fractions showed that the overall percentage of total NP protein associated with RNPs did not alter between 1.5 and 4 h post-infection, with a value of 50 to 60%.

The incorporation of $[^{35}\text{S}]$methionine into newly synthesized NP during a 15 min pulse increased with time post-infection in both nuclear (Fig. 1 c) and cytoplasmic (Fig. 1 d) fractions. In the nucleus the association of pulse-labelled NP with RNP structures remained fairly constant so that at 1-5 h all of the newly synthesized NP was in RNPs, but by 4 h only 9% of the NP was complexed with RNA. In the cytoplasmic fractions a similar, although less pronounced, divergence between total pulse-labelled NP and NP associated with RNPs could be seen. At 1.5 h post-infection about 84% of the newly synthesized NP had been incorporated into RNPs but by 4 h this proportion had decreased to about 30%.

The accumulation of $[^{35}\text{S}]$methionine into each of the five size classes of virus RNPs extracted from cells labelled continuously from 1 h post-infection is shown in Fig. 2 (a). All the RNPs were predominantly cytoplasmic, showing low levels of accumulation in the nucleus. The rates of accumulation of individual RNPs were similar, but they did not appear to be synthesized in equimolar amounts since by 4 h post-infection the total accumulation could be seen to vary by two- to threefold. The partitioning of RNPs between the nucleus and cytoplasm also varied, as shown by the ratios of cytoplasmic to nuclear radioactivity at 4 h. RNPs a, b, c and d had similar ratios, between 6-4:1 and 9-6:1, while the ratio for RNP e was 23:1.

The rates of synthesis of individual RNPs were investigated in cells pulsed for 15 min with $[^{35}\text{S}]$methionine at various times post-infection (Fig. 2 b). All the newly synthesized RNPs were predominantly cytoplasmic and the proportion of each RNP in the nucleus remained almost constant throughout infection. These results are in general agreement with those obtained by Krug (1972) in a study of the kinetics of synthesis of total RNP in MDCK cells infected with the WSN strain of influenza virus. RNPs a, b and c showed similar trends with an initial increase in their rates of synthesis, but by 2·5 to 3 h post-infection, the rates reached a plateau. The rate of synthesis of RNP e reached a peak at 2 h post-infection and then declined, while the rate of synthesis of RNP d (containing RNA 7, encoding the matrix protein) continued to increase.

Our results show that the majority of NP in the cytoplasm is in RNPs, especially at 4 h post-infection, while the majority of NP in the nucleus is present as free protein. This contrasts with the results obtained using the WSN strain of influenza virus and MDCK cells by Krug & Etkind (1973) who found that 80 to 85% of the NP in the nucleoplasm of cells
Fig. 2. Top panel: electrophoretic separation of RNPs (a to e) extracted from uninfected (U) and infected (I) CEF cells labelled continuously with \([^{35}S]m\)ethionine from 1 to 3.5 h post-infection as described in the text. \((a, b)\) Accumulation of label in RNPs in the nuclear (○) or cytoplasmic (●) fractions of infected cells continuously labelled from 1 h post-infection \((a)\) or pulse-labelled for 15 min at the times shown \((b)\) and processed as in Fig. 1. Letters a to e refer to RNPs a to e. All counts have been corrected for RNP size, based on the length of RNA contained in each RNP complex (Sleigh et al., 1979; Rees & Dimmock, 1981a); RNPs a, b, c, d and e contain RNAs 1 to 3, RNA 4, RNAs 5 and 6, RNA 7, and RNA 8 respectively.

labelled for 10 min at 4 h post-infection and chased for 60 min was in RNP structures. Combining the data from nuclear and cytoplasmic fractions of cells labelled continuously shows that the overall percentage of NP in RNPs remains constant, so that the differences between the nucleus and cytoplasm can be explained by progressive partitioning of free NP in the nucleus and NP complexed in RNPs in the cytoplasm. The evidence from pulse labelling suggests that, as the infection progresses, a lower proportion of newly synthesized NP is incorporated into RNPs. However, this may merely reflect a lower rate of association between newly synthesized NP and RNA relative to NP synthesis since the proportion of NP in RNPs does not alter in cells labelled continuously. This apparent delay between the synthesis of NP and its incorporation into RNPs may be related to the delay between the synthesis of this protein and its incorporation into virus particles in cells pulse-labelled and
chased from 4 h post-infection as described by Hay & Skehel (1975). The reasons for the accumulation of free NP in the nucleus and its function in the virus multiplication cycle, if any, are unknown, although it is apparently able to migrate from the nucleus again (Flawith & Dimmock, 1979), probably as a free protein and not necessarily complexed with RNA (P. J. Rees & N. J. Dimmock, unpublished results). In this system, fluorescent antibody staining shows that NP antigen is present in nuclei throughout infection (Flawith & Dimmock, 1979) and we have never observed nuclei free of antigen as described by Fraser (1967) and Maeno & Kilbourne (1970).

The synthesis of individual RNP structures appears to be under separate control since clear differences in their total accumulation, their nuclear–cytoplasmic ratios and their rates of synthesis at different times post-infection can be detected. Comparison between the relative rates of synthesis of the RNPs determined by pulse labelling and the relative amounts of each RNP accumulated in the host cell show similar trends, with the exception of RNP d. The rate of synthesis of this RNP shows a continuous increase up to 4 h post-infection which is not reflected in the accumulation in the cell. The reason for this discrepancy is not clear, but RNP d might be more susceptible to degradation than other RNPs. It did not appear to be transported from the cell (data not shown). RNP d contains RNA 7, the M protein gene, and possibly its polyadenylated cRNA. Since M protein becomes the most abundant virus polypeptide late in infection (Skehel, 1972, 1973; Inglis et al., 1976; Inglis & Mahy, 1979) the results obtained for RNP d may reflect increased rates of synthesis and turnover of M protein messenger RNA.

Our results do not allow us to distinguish between the nucleus and cytoplasm as possible sites of RNP synthesis, and we do not yet know if the RNA contained in the RNP complexes separated by electrophoresis is vRNA, cRNA or mRNA, although preliminary evidence suggests that between 10 and 20% of the RNPs contain polyadenylated RNA (P. J. Rees & N. J. Dimmock, unpublished results).

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