Host cell proteins required for measles virus reproduction

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We have developed a cell-free system derived from measles virus-infected cells that supported the transcription and replication of measles virus RNA in vitro. The data suggest that tubulin may be required for these reactions, since an anti-β-tubulin monoclonal antibody inhibited viral RNA synthesis and the addition of purified tubulin stimulated measles virus RNA synthesis in vitro. Tubulin may be a subunit of the viral RNA polymerase, since two different anti-tubulin antibodies, one specific for the β- and another specific for the α-subunit of tubulin, coimmunoprecipitated the measles virus L protein as well as tubulin from extracts of measles virus-infected cells. Other experiments further implicated actin in the budding process during virus maturation, as there appeared to be a specific association of actin in vitro only with nucleocapsids that have terminated RNA synthesis, which is presumably a prerequisite to budding.

Introduction

The paramyxoviruses, measles and Sendai viruses, each contain a single-stranded RNA genome of the negative-strand sense. These viruses package an RNA-dependent RNA polymerase consisting of the L and P protein subunits, which catalyse transcription and replication of the nucleocapsid template containing the genome RNA encapsidated in the major nucleocapsid protein N and NP, respectively (Ray & Fujinami, 1987; Siefrid et al., 1978; Stallcup et al., 1979; Stone et al., 1971). The envelope proteins, F, H or HN and M, constitute the remaining structural proteins of the virion (Mountcastle & Choppin, 1977; Wechsler & Fields, 1978). There are, in addition, viral non-structural proteins whose functions are unknown: the C protein, which is synthesized in infected cells from an overlapping reading frame of the P mRNA (Bellini et al., 1985; Dethlefsen & Kolakofsky, 1983; Dowling et al., 1986; Shioda et al., 1983) and the V protein, which is translated from a measles virus RNA polymerase-edited transcript of the P gene (Cattaneo et al., 1989).

Transcription of the paramyxovirus genome RNA occurs from the 3' end to the 5' end of the genome, sequentially yielding the leader RNA, followed by the N and NP, P + C, M, F, H and HN and L mRNAs for measles and Sendai viruses, respectively (Dowling et al., 1986; Glazier et al., 1977; Leppert et al., 1979; Richardson et al., 1985; Rima et al., 1986; Shioda et al., 1983, 1986). We previously described in vitro RNA synthesis systems for both Sendai virus and vesicular stomatitis virus (VSV), a negative-strand RNA virus of the rhabdovirus family, which utilize cytoplasmic extracts of virus-infected cells permeabilized with lysolceithin (Carlson et al., 1985; Peluso & Moyer, 1983). These extracts accurately transcribe viral mRNAs and can also support genome RNA replication and encapsidation. An advantage of the cell-free transcription system is that it can be used in reconstitution experiments to identify and purify both viral and host proteins required for RNA synthesis (Baker & Moyer, 1988; Peluso & Moyer, 1988). We have adapted this methodology to prepare a cell-free system from measles virus-infected cells and show that it supports viral mRNA synthesis and genome RNA replication in vitro.

We have been interested in understanding the role of the host cell in negative-strand RNA virus infections. Our laboratory (Moyer et al., 1986), as well as that of Hill et al. (1986), has reported the requirement of components of the microtubule system for VSV and Sendai virus RNA synthesis. We have recently shown that tubulin is required for RNA synthesis by both Sendai virus and VSV (New Jersey), whereas the microtubule-associated proteins are required for VSV of both the New Jersey and Indiana serotypes, but not for Sendai virus (S. M. Horikami, R. Williams, Jr & S. A. Moyer, unpublished data). We report here that, like Sendai virus, measles virus also appears to require tubulin for RNA synthesis. In addition previous studies (Bohn et al., 1986; Stallcup et al., 1983) have suggested that actin, another cytoskele-
nal component, may be involved in measles virus maturation by budding from the plasma membrane. Actin is, in fact, packaged into completed virions (Tyrrell & Norrby, 1978; Wang et al., 1976). We report here that there was a tight association of actin with measles nucleocapsids in vitro, specifically upon the termination of RNA synthesis, which is presumably a prerequisite for budding.

Methods

Cells and viruses. Measles virus (Edmonston strain) was grown in spinner HeLa cells (both generous gifts from Dr Peter Dowling, UMDNJ-New Jersey Medical School, Newark, N.J., U.S.A.), and a high titred virus stock was prepared as described by Udemb & Cook (1984). The measles virus experiments were carried out in the human cell line A549 (American Tissue Culture Collection).

Sendai virus (Harris strain) was grown in embryonated chicken eggs and purified as described previously (Carlsen et al., 1985). The preparation of Sendai virus-infected cell extracts and conditions for in vitro Sendai RNA synthesis were as described by Carlsen et al. (1985).

Measles virus in vivo RNA synthesis. Confluent 100 mm dishes of A549 cells were infected with measles virus at an m.o.i. of 5 at 37 °C. The cultures were labelled with [3H]uridine (50 μCi/ml; ICN) in the presence of actinomycin D (2 μg/ml) at various times after infection, as indicated in the text. At the end of the labelling period a cytoplasmic extract was prepared, after permeabilizing the infected cells with 2 ml lysolcithin (250 μg/ml) for 1 min at 4 °C as previously described by Carlsen et al. (1985), except the cells were scraped in HND buffer, containing 0.1 M-HEPES pH 8.1, 0.05 M-NH₄Cl, and 1 mM-DTT. The cytoplasmic extracts were digested with Proteinase K, the RNA was purified by phenol-chloroform extraction and then analysed by electrophoresis on 11 % polyacrylamide-SDS gels (Peluso & Moyer, 1985). The [3H]uridine incorporation was measured by liquid scintillation spectrometry.

Results

RNA synthesis in measles virus-infected A549 cells

The first steps in developing an in vitro RNA synthesis system for measles virus were to find the optimal cell line and determine the kinetics of viral RNA synthesis in vivo. A monolayer cell line is preferred for the methodology we employ for extract preparation. We tested several lines, including human cells (monolayer HeLa, A431 and A549) as well as monkey cells (Vero and LLMC-2) and the A549 cells were far superior (greater than fivefold) in terms of the total amount of measles virus RNAs synthesized in vivo. However, the yield of progeny virus from this cell line is very poor (data not shown). We determined that measles virus RNA synthesis in A549 cells peaked at 17 to 18 h after infection and all of the viral mRNAs, as well as genome RNA, were synthesized in the infected cells (data not shown). In addition, several readthrough RNA products, which are larger than the F mRNA, were also synthesized in vivo, as was previously observed (Dowling et al., 1986; Wong & Hirano, 1987; Yoshikawa et al., 1986) (See Fig. 1, lane 1).

In vitro measles virus RNA synthesis

To develop a cell-free transcription system, cytoplasmic extracts of measles virus-infected A549 cells were prepared in reaction mixture (see Methods) at 17 h after infection, the time of maximal RNA synthesis in vivo. By systematically varying the reaction components (data not
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Fig. 1. Measles virus in vitro RNA synthesis. Measles virus RNAs synthesized in vitro in two different experiments (lanes 2 and 3) are compared with either 3H-labelled RNAs from measles virus-infected A549 cells (lane 1) or 3H-labelled RNAs from Sendai virus-infected BHK cells (lane 4).

Fig. 2. Measles virus in vitro genome RNA synthesis. Micrococcal nuclease-resistant measles virus RNAs synthesized in vitro (lane 3) and in vivo (lane 2). Lane 1 shows marker 3H-labelled measles virus total RNA synthesized in vivo.

shown) the optimal conditions stated in Methods for in vitro measles virus RNA synthesis were determined, and include the key conditions of 0.1 M-HEPES pH 8.1 and 0.05 M-NH₄Cl, with an incubation period of 3 h at 30 °C. Utilizing this reaction mixture in two different experiments shown in Fig. 1, the cytoplasmic extract was shown to synthesize measles virus RNAs (lanes 2 and 3) identical to those synthesized in the infected cell (lane 1). By comparing the in vivo and in vitro synthesized measles RNAs with marker Sendai virus mRNAs (Fig. 1) and VSV mRNAs (data not shown), we calculated the sizes of the measles virus RNAs as 1900, 1900, 1500, 2200, 2000 and about 6000 nucleotides for the N, P, M, F, H and L mRNAs, respectively, and greater than 12000 nucleotides for the genome RNA. These values are in reasonable agreement with the sizes [nucleotides minus poly(A)] obtained by sequencing cDNA clones of several of the mRNAs; 1688, 1657, 1472 and 1949 for N, P + C, M and H, respectively (Alkhatib & Briedis, 1986; Bellini et al., 1985, 1986; Billeter et al., 1984; Gerald et al., 1986; Rozenblatt et al., 1985).

Since genome length RNA also appeared to be synthesized in vitro, we wanted to confirm that this product was encapsidated as it is in vivo. Cytoplasmic extracts from measles virus-infected A549 cells were prepared at 17 h post-infection, incubated in reaction mixture with [3H]UTP and the products were treated with micrococcal nuclease to digest any unencapsidated RNA. The micrococcal nuclease-resistant RNA species synthesized in vitro (Fig. 2, lane 3) and in vivo (lane 2) comigrated in the agarose-urea gel, with the largest product synthesized being greater than 12000 nucleotides. The encapsidation reaction did not require de novo protein synthesis, since no translation occurred in these reactions, but utilized the preformed proteins present in the cell extract. The micrococcal nuclease-resistant in vitro and in vivo product RNAs also banded in the same position as nucleocapsids at 1.31 g/ml on CsCl density
gradients (data not shown). It should be noted that, in addition to the full-length genome RNA, there were smaller nuclease-resistant RNAs that were also synthesized, particularly in vitro. Although the replication and encapsidation of the large measles virus genome RNA occurred in vitro, it appeared to be relatively inefficient with premature stop sites, a finding we also reported for wild-type Sendai virus genome RNA replication in vitro (Carlsen et al., 1985).

Inhibition of measles virus RNA synthesis by an anti-tubulin antibody

We previously reported that tubulin was necessary for Sendai virus, as well as VSV RNA synthesis in vitro (Moyer et al., 1986). It was therefore of interest to determine whether measles virus, a member of a different genus of the paramyxovirus family, also had a similar requirement. Extracts of measles virus-infected A549 cells were incubated with [3H]UTP in reaction mixture in the presence of individual monoclonal antibodies and the products were analysed by agarose gel electrophoresis. Compared with the control the anti-β-tubulin antibody completely inhibited all measles virus RNA synthesis (Fig. 3, lanes 1 and 2, respectively). In contrast, neither an anti-myeloma ascites fluid nor an anti-actin antibody had any effect on viral RNA synthesis (Fig. 3, lanes 3 and 4, respectively). In experiments using dilutions of the anti-β-tubulin antibody the amount of inhibition of measles virus RNA synthesis decreased with decreasing amounts of the antibody (data not shown). These data suggested that the host cell-derived tubulin might play a role in the synthesis of measles virus RNA.

Effect of tubulin on measles virus RNA synthesis in vitro

Following the separation of the measles virus-infected cytoplasmic extract into nucleocapsid and soluble protein fractions as described in Methods, one can assay the effect of purified components on transcription. The nucleocapsid fraction alone was capable of some viral RNA transcription in vitro, since it contained the RNA-N template, the viral RNA polymerase (Fig. 4, lane 1) and probably some tubulin (see Discussion). The addition of increasing amounts of the soluble protein fraction from infected cells stimulated synthesis of all the viral mRNAs (Fig. 4, lanes 2 to 5). The stimulation (2-9-fold increase) appeared to be saturated when the soluble protein fraction added to nucleocapsids was from an equivalent number of infected cells (Fig. 4, lane 3). These data suggested that one or more proteins from the infected cell were required for efficient measles virus RNA synthesis. It should be noted that measles genome RNA replication seemed to occur only inefficiently in the reconstituted reactions in comparison with the unfractionated extracts (Fig. 1 and 2).

Since the anti-β-tubulin antibody appeared to inhibit measles virus RNA synthesis, a confirmation of this result would be to show that the addition of tubulin had a stimulatory effect. In fact, purified tubulin alone did stimulate (2.3-fold) measles virus transcription, although the effect was primarily on the comigrating N and P mRNAs, with a smaller stimulation of the other mRNAs (Fig. 4, lanes 6 to 9). These data suggested that tubulin may be one, but perhaps not the only, factor in the soluble protein fraction utilized for measles virus RNA synthesis in vitro.
Tubulin appeared to stimulate measles virus transcription so we wanted to determine whether there might be an association of tubulin with either of the viral polymerase subunits, as we previously reported for VSV (Moyer et al., 1986). The $^3$H and $^{35}$S double-labelled soluble protein fraction from measles virus-infected cells was incubated with various antibodies and the immunoprecipitated proteins were analysed by SDS-PAGE. Compared with the control anti-myeloma ascites fluid (Fig. 5, lane 4), the anti-$\beta$-tubulin monoclonal antibody and an anti-$\alpha$-tubulin peptide antibody (Gundesen et al., 1984) both specifically coimmunoprecipitated tubulin and the measles virus L protein (Fig. 5, lanes 2 and 3, respectively). The L protein was identified on the basis of its size and its comigration with the presumptive viral L protein immunoprecipitated with serum from an SSPE patient (Fig. 5, lane 1). The position of tubulin was identified by its immunoprecipitation from an uninfected cell extract with the anti-$\beta$-tubulin antibody (data not shown). The anti-tubulin antibodies also immunoprecipitated another, apparently host protein, with a much lower $M_r$ of approximately 30K. These data suggested that tubulin was associated with the measles virus L protein in the soluble protein fraction of the
Fig. 6. The association of actin with Sendai and measles virus nucleocapsids in the absence of RNA synthesis. Sendai (a) or measles (b) virus nucleocapsids isolated from extracts of infected cells were incubated in the appropriate reaction mixtures containing the [3H]leucine and [35S]methionine double-labelled soluble protein fraction from Sendai virus-infected cells (a, lanes 2 and 3) or measles virus-infected cells (b, lanes 1 and 2) or uninfected cells (a, lanes 4 and 5 and b, lanes 3 and 4) in the presence (a, lanes 3 and 5 and b, lanes 2 and 4) or absence (a, lanes 2 and 4 and b, lanes 1 and 3) of RNA synthesis (see Methods and text). Lanes 1 (a) and 5 (b) are 3H-labelled purified Sendai virus and measles virus, respectively, with the viral proteins identified. Ac indicates the 43K actin protein.

infected cell and therefore may be a subunit of the measles virus RNA polymerase.

Association of proteins with RNA during replication

We demonstrated above that measles virus genome RNA replication and encapsidation occurred in the cell-free system (Fig. 2). To identify the viral protein(s) associated with the nucleocapsid product, unlabelled measles virus nucleocapsids isolated from infected cells were incubated in reconstitution experiments with the [3H]leucine and [35S]methionine double-labelled soluble protein fraction from Sendai virus-infected cells (a, lanes 2 and 3) or measles virus-infected cells (b, lanes 1 and 2) or uninfected cells (a, lanes 4 and 5 and b, lanes 3 and 4) in the presence (a, lanes 3 and 5 and b, lanes 2 and 4) or absence (a, lanes 2 and 4 and b, lanes 1 and 3) of RNA synthesis (see Methods and text). Lanes 1 (a) and 5 (b) are 3H-labelled purified Sendai virus and measles virus, respectively, with the viral proteins identified. Ac indicates the 43K actin protein.

Discussion

We have described here the development of a cell-free transcription/replication system to study the viral and host components required for measles virus RNA synthesis. The basic methodology involving lyssolecithin
permeabilization of infected cells, which had previously been successful for the establishment of *in vitro* replication systems for both VSV (Peluso & Moyer, 1983) and Sendai virus (Carlsen et al., 1985), also worked very well for measles virus. Perhaps the key feature yielding this transcription system with our methodology was the selection of a monolayer cell line, A549, which gave a high level of viral RNA synthesis *in vitro*. This appeared to be achieved, in part, by the fact that progeny measles virus formation was very limited, resulting in the accumulation of viral nucleocapsid templates within the infected cell. The cell-free system appeared to synthesize all the measles virus mRNAs with the correct sizes and supported at least some replication of the genome RNA and its encapsidation with N protein (Fig. 1, 2 and 6). Although specific activities cannot be determined in cell extracts, on a per cell basis the relative levels of RNA synthesis in the various systems is VSV > measles virus > Sendai virus.

Measles virus RNA replication *in vitro* did not require *de novo* protein synthesis, but utilized the preformed proteins present in the infected cell at the time of extract preparation. It should be noted that the ability to replicate genome RNA appeared to be substantially reduced in the reconstitution reactions, although transcription could be fully restored (Fig. 2, 4 and 6). Perhaps a labile component required for RNA replication was lost in the period required for the separation of the subfractions. By analogy with the VSV and Sendai virus replication systems (Carlsen et al., 1985; Peluso & Moyer, 1983) *in vitro* measles virus RNA synthesis (Fig. 1) probably did not represent initiation, but rather the elongation of mRNAs and genome RNA already initiated *in vivo*. However, this point needs to be further investigated.

While this work was in progress, Ray & Fujinami (1987) reported the *in vitro* synthesis of measles virus mRNAs employing intracellular nucleocapsids isolated from infected HeLa cells. Although the reaction conditions in the two systems are generally similar there are some differences with regard to various components and incubation conditions. These differences are perhaps attributable to the different methodologies employed in each case. As in our reconstitution experiments, Ray & Fujinami (1987) did not observe *in vitro* RNA replication from the isolated nucleocapsids by RNA labelling experiments.

We have been particularly interested in understanding the role of the host cell, particularly the components of the cytoskeleton, in the reproduction of negative-strand RNA viruses. As we previously reported for VSV and Sendai virus (Moyer et al., 1986) tubulin also appeared to be an essential component for measles virus RNA synthesis, since an anti-β-tubulin antibody inhibited both viral transcription and replication *in vitro* (Fig. 3) and the addition of purified tubulin stimulated viral transcription (Fig. 4), although the level was not impressive (about threefold). The inhibition did not appear to be non-specific, since this anti-β-tubulin antibody did not inhibit transcription by the poxvirus RNA polymerase (data not shown). The reason the stimulation with tubulin was not greater, as expected from the antibody result, appeared to be that endogenous tubulin was present in the nucleocapsid fraction. Some polymerized tubulin co-pelleted with the intracellular nucleocapsids during the fractionation procedure (unpublished data) and some tubulin was probably attached to the nucleocapsid via its association with L protein (Fig. 5). Thus it is likely that these sources of endogenous tubulin in the reaction were responsible for both the level of RNA synthesis seen in the absence of any additions (Fig. 4, lane 1), as well as the low level of stimulation observed with the addition of soluble protein or purified tubulin (Fig. 4). Future experiments employing purified measles virus should be of interest in this regard.

In contrast to our results (Fig. 4) Ray & Fujinami (1987) reported no stimulation of measles RNA synthesis by an extract of uninfected cells, as monitored by the incorporation of [32P]UMP into TCA-precipitable material. The reason for this discrepancy is unclear, although their assay may not be as sensitive as monitoring individual mRNAs. The suggested involvement of tubulin we observed in measles virus RNA synthesis was particularly intriguing, since both VSV and Sendai virus also required microtubule components for RNA synthesis (Moyer et al., 1986; Hill et al., 1986) and in addition both rhabdoviruses (Chatterjee et al., 1984) and paramyxoviruses (Hamaguchi et al., 1985) seemed to require positioning of the subviral components on the cytoskeleton during the productive infection *in vivo*. To understand how tubulin could be exerting its effect we tested for its possible interaction with viral proteins. In fact two different anti-tubulin antibody preparations coimmunoprecipitated both tubulin and the measles virus L protein (Fig. 5). These same antibodies also coprecipitated tubulin and the VSV L protein (Moyer et al., 1986 and data not shown). In contrast we detected no such interaction with the Sendai virus L protein and tubulin in a similar experiment (data not shown). Similar data with two different antibodies with non-overlapping, unique epitopes strengthens our conclusion that tubulin and the measles virus L protein must interact in a relatively stable fashion. It seems unlikely that two different tubulin epitopes would be contained in both the measles virus and VSV L proteins. The anti-α-tubulin peptide antibody immunoprecipitated less tubulin and measles virus L protein, since it apparently had a much...
lower titre than the monoclonal anti-β-tubulin antibody. It should also be noted that the anti-SSPE sera immunoprecipitated only small amounts of the measles virus L protein and no detectable tubulin (Fig. 5, lane 1). Tubulin was probably not detectable in the latter case, since the anti-L activity in this serum was weak and tubulin (and all host cell proteins) was poorly labelled late in measles virus infection.

Previous experiments have suggested that another cytoskeleton component, actin, was essential for the reproduction of paramyxoviruses, specifically for budding. First, actin was found to be packaged within the virion of both measles and Sendai viruses (Tyrrell and Norrbjörn, 1978; Wang et al., 1976) and biochemical data showed that actin appeared to bind specifically to the M protein of Sendai and Newcastle disease viruses (Giuffre et al., 1982). Stallcup et al. (1979) showed that treatment of infected cells with cytochalasin B, which disrupted actin filaments, prevented virion formation and resulted in the accumulation of viral nucleocapsids within the infected cell. From the data it was postulated that actin filaments might be essential for the transport of the nucleocapsid from the cytoplasm to the cell surface where budding occurred. Further support for this idea came from electron microscopic studies by Bohn et al. (1986), who showed that in cytoskeleton preparations of measles virus-infected cells the growing end of the actin filament protruded into budding virus particles and was in close association with viral nucleocapsids. These authors suggested that the vectorial growth of the actin filaments was involved both in the transport of the nucleocapsid to the surface of the cell and for budding itself.

The results presented here suggested that actin was tightly bound to both Sendai and measles nucleocapsids in vitro specifically when RNA synthesis had been terminated (Fig. 6). The actin binding was extremely stable, since the CsCl centrifugation step used for purification of the nucleocapsids normally removed all the viral proteins except N from the nucleocapsid. It should be noted that we were able to inhibit RNA synthesis only by the addition of EDTA to the reactions because of endogenous nucleotides in the cell extracts. EDTA may potentially cause some aberrant protein–RNA interactions, but similar experiments with VSV did not result in actin bound to VSV nucleocapsids, suggesting that our observation is specific for paramyxoviruses.

We propose that it is the transcriptionally silent paramyxovirus nucleocapsids that bind to the actin filaments for transport and budding, and the actin is incorporated into progeny virions. The actin would then be released when new RNA synthesis begins upon subsequent infection with the virus. How is viral RNA synthesis terminated in the cell prior to budding? In the case of VSV the M protein appeared to function, in part, to inhibit transcription both in vivo and in vitro (for a review see Banerjee, 1987). However, VSV did not appear to utilize actin filaments for maturation, since actin was not packaged in the virion, cytochalasin B had no effect upon virus yield (Gentry & Busserau, 1980) and finally we found no actin binding to VSV nucleocapsids under any conditions in vitro (data not shown). Indirect evidence suggested that the paramyxovirus M protein, which was known to interact with actin in vitro (Giuffre et al., 1982) may have also inhibited RNA synthesis in vitro (Marx et al., 1974). However, our data suggested that the M protein need not necessarily be present, since when RNA synthesis was artificially inhibited in vitro actin bound tightly to silent nucleocapsids in the presence of uninfected cell protein (Fig. 6). Further experiments are in progress to understand the mechanisms by which these various cytoskeletal components participate in the reproduction of paramyxoviruses.

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