Human Parainfluenza Virus Type 3 Transcription In Vitro: Role of Cellular Actin in mRNA Synthesis

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Purified ribonucleoprotein complexes of human parainfluenza virus type 3 (HPIV-3) virions required, in addition to the viral proteins, soluble cytoplasmic proteins from uninfected cells for the synthesis of mRNAs in vitro. In contrast to Sendai virus transcription, in vitro RNA synthesis from HPIV-3 ribonucleoprotein complexes was not stimulated significantly by purified tubulin. Moreover, cytoplasmic extract depleted of tubulin by immunoprecipitation stimulated HPIV-3 transcription effectively, suggesting involvement of a host protein(s) other than tubulin in the HPIV-3 transcription process. The transcription stimulatory factor was purified from uninfected cell extract by conventional chromatography and was found to contain a major 43-kDa polypeptide. In Western blot (immunoblot) analysis, this protein reacted with antiactin antibody, suggesting that the 43-kDa polypeptide is actin. This possibility was further supported by its polymerization activity and properties of binding to blue-Sepharose and heparin-Sepharose columns. Furthermore, when the cell extract was depleted of actin by immunoprecipitation by antiactin antibody, the stimulatory activity was abolished, indicating an involvement of actin in the stimulation of HPIV-3 transcription. After purification from RNases, similar stimulatory activity associated with the 43-kDa protein was detected in other cell lines as well, including CV-1, HeLa, and BHK.

**Human parainfluenza virus type 3 (HPIV-3), a paramyxovirus, is an important pathogen that causes severe lower respiratory tract illness in children (3, 4, 6, 22). The genome of HPIV-3 is a single-stranded negative-sense RNA of 15,461 nucleotides contained within a helical nucleocapsid that serves as a template for the synthesis of six mRNAs (NP, P/C, M, F, HN, and L) catalyzed by the virion-associated RNA-dependent RNA polymerase (20). Analyses of proteins isolated from HPIV-3-infected cells, purified virions, and subviral complexes identified six structural proteins for HPIV-3 (28, 45, 46, 55, 62, 63). The viral envelope contains two glycoproteins, the fusion protein (F) and the hemagglutinin-neuraminidase protein (HN), and also an unglycosylated matrix protein (M). A rigid inner ribonucleoprotein complex (RNP) contains genomic RNA tightly bound with the nucleocapsid protein (NP), and associated with that are two proteins, the polymerase protein (L) and phosphoprotein (P). Based on nucleotide sequence analyses of the genes, the amino acid sequences for all these structural proteins are currently known (7, 10, 12, 14-18, 29, 33, 34, 43, 47, 49-52, 54). There are, in addition, viral nonstructural proteins; these include protein C, encoded within the P mRNA in an overlapping reading frame, and protein P-D, arising from an edited transcript of the P gene (15, 19, 28, 34, 46, 50, 55, 62). Although V protein is not detected in HPIV-3, other paramyxoviruses have been reported to produce this cysteine-rich nonstructural protein, which also arises from an edited or unedited transcript of the P gene (2, 13, 30, 38-40, 48, 56, 57, 59). The functions of these nonstructural proteins remain unknown, despite the fact that their conservation among different members of the paramyxovirus family suggests that they might play a biologically important role in the virus life cycle.**

From extensive in vitro studies on vesicular stomatitis virus (VSV), a prototype rhabdovirus, it has been established that the minimum structural unit required for transcription and replication is the viral RNP. Studies on in vitro transcription of paramyxoviruses have been scanty because of low efficiency of in vitro synthesis of mRNAs from the purified viral RNPs (25, 26, 42, 44). From in vivo and in vitro studies it is well established that transcription occurs sequentially from the 3′ to the 5′ end of the genome RNA, yielding a small leader RNA (55 nucleotides) and six capped and polyadenylated mRNAs in the order 3′ NP-P/C-M-F-HN-L 5′. The RNP-associated proteins are therefore likely to be involved in the synthesis and modification of viral transcripts. Recently, requirements for host cell proteins as positive transcription factors for in vitro RNA synthesis by negative-strand RNA viruses have been reported (9, 27, 36, 37). In separate studies, either tubulin or microtubule-associated proteins have been shown to be involved in those stimulatory processes (27, 36, 37).

In an effort to understand the molecular mechanism of gene expression of HPIV-3, we recently developed an in vitro transcription system in which a requirement for host cell protein(s) was observed in the stimulatory process (9). We have now purified and characterized a 43-kDa host factor required for viral RNA synthesis which appears to be cellular actin.

**MATERIALS AND METHODS**

**Cells and viruses.** CV-1 and human lung carcinoma (HLC) cells (ATCC CCL 185, A549) were propagated in monolayers as described previously (9). HPIV-3 (HA-1; NIH 47885) was grown in CV-1 cells as described previously (9), except that the virus was collected at 40 h postinfection from spent medium, and freeze-thawing of cells was omitted to minimize host cell protein contamination. Sendai virus was obtained as a kind gift from A. Portner, St. Jude Children’s Hospital, Memphis, Tenn.
Purification of RNP s. RNPs were isolated from HPIV-3 and Sendai virus essentially as described elsewhere for VSV (8), with the following modifications. The purified virus (2 mg at 500 µg/ml) was disrupted in 10 mM Tris-hydrochloride (pH 8.0) containing 5% glycerol, 0.4 M NaCl, 1.85% Triton X-100, and 0.6 mM dithiothreitol (DTT) (final concentration) at room temperature for 10 min. RNP was sedimented by centrifugation in an SW 50.1 rotor at 40,000 rpm for 2 h through 30% (vol/vol) glycerol (1.5 ml) in 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.5) and 1 mM DTT onto a 100% glycerol (0.5 ml) cushion. RNP was collected from the top of the 100% glycerol cushion and stored in liquid nitrogen for subsequent use in transcription reactions.

Purification of transcription stimulatory factor. Soluble cytoplasmic extracts of uninfected HLC and CV-1 cells were prepared from 2 × 10^6 cells as described previously (9). Unless otherwise stated, all subsequent purification steps were carried out at 4°C. Solid ammonium sulfate was added to the soluble supernatant to 30% saturation, and the proteins were allowed to precipitate for 30 min on ice. After centrifugation at 10,000 × g for 30 min to remove the precipitate, the supernatant was brought to 50% saturation by adding solid ammonium sulfate. The precipitate was collected by centrifugation, dissolved in buffer A (50 mM HEPES-KOH [pH 7.5], 5 mM MgCl₂, 10 mM KCl, 1 mM DTT) and dialyzed overnight against the same buffer. Insoluble materials were removed by centrifugation at 10,000 × g for 10 min. The supernatant (30 to 50% ammonium sulfate fraction) was then loaded onto a phosphocellulose (P-11) column (3 by 2.5 cm) previously equilibrated with buffer A. The column was washed with 5 ml of buffer A, and the flowthrough and buffer wash were pooled. The phosphocellulose unbound fraction was loaded onto a DEAE-cellulose (DE-52) column (1.5 by 3 cm), equilibrated with buffer A. The column was washed with 20 ml of buffer A, and the bound proteins were eluted from the column by a linear 0 to 0.3 M NaCl (15 ml, total) gradient in buffer A. In each fraction, 0.15 ml of eluate was collected. The proteins eluted in fractions 75 to 90 were pooled as the purified transcription factor and stored at −20°C for subsequent use.

In vitro RNA synthesis and product analysis. In vitro transcription reaction as developed in our laboratory (9) was further modified as detailed below. A 50-µl reaction volume contained 100 mM HEPES-KOH, pH 8.0; 100 mM KCl; 5 mM MgCl₂; 1 mM DTT; 1 mM each ATP, GTP, and UTP; 10 µM CTP; 15 µCi of [α-³²P]CTP; 25 U of human placental RNAse inhibitor; 2 µg of RNP; and host proteins that varied for individual experiments. The reaction was carried out at 30°C for 3 h.

³²P-labeled RNA products were analyzed after ethanol precipitation in a 5% polyacrylamide-urea gel. The gel was then exposed to X-ray film in the presence of intensifier at −80°C.

Protein analysis. The proteins in the RNP or in the cell extract were resolved by electrophoresis in a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (32), and the protein bands were visualized by staining with silver reagents (35).

For Western blot (immunoblot), the proteins were resolved in a 10% SDS-polyacrylamide gel and subsequently transferred to a nylon (GeneScreen) membrane (38). The blot was developed with specific antibody, and the complex was then detected by using horseradish peroxidase-conjugated second antibody and diaminobenzidine as the substrate.

Depletion of tubulin or actin from the soluble cytoplasmic fraction was done by incubating antibodies in the transcription buffer (100 µl, total volume) at 4°C overnight with different amounts of anti-β-tubulin (Boehringer Mannheim) or antiactin (Sigma) purified from RNAs by DEAE- Affiliate Blue column chromatography according to the manufacturer’s protocol (BioRad). Then, 20 µl of a 10% suspension of Formalin-fixed Staphylococcus aureus cells was added and incubated for another 1 h at 4°C. The cells were removed by centrifugation. The supernatant was concentrated by using a Centricron-10 microconcentrator (Amicon) adjusted to the transcription buffer and used in the transcription reaction. An antitubulin-Sepharose column was prepared by conjugating 50 µg of antibody with CNBr-activated Sepharose 4B (0.8 ml, packed volume) according to the manufacturer’s protocol (Pharmacia). The column was equilibrated with the transcription buffer, and proteins were allowed to bind for 2 h at 4°C. The bound proteins were eluted by using 0.1 M glycine-HCl buffer (pH 3.0) and neutralized immediately. The proteins were then concentrated and adjusted to the transcription buffer for subsequent use.

Actin was prepared from the soluble cytoplasmic extract of HLC cells by polymerization-depolymerization cycles done by the method of Gordon et al. (23). Purified bovine brain tubulin was obtained as a kind gift from Henry Sternlicht, Case Western Reserve University, Cleveland, Ohio.

RESULTS

Requirement of host proteins for mRNA synthesis by viral RNP. Our previous studies (9) demonstrated that RNP isolated from HPIV-3-infected cells after a certain stage of purification failed to synthesize mRNAs in vitro unless cytoplasmic extract from the uninfected cells was added during the transcription reaction. However, under similar transcription conditions with cellular proteins, no mRNA synthesis was observed when Triton-disrupted HPIV-3 virions were used. This was mainly because of the presence of contaminating RNase associated with the purified virions. To obtain viral RNP with relative ease and in large amounts for studying the role of host cell proteins on transcription, we developed a procedure which yielded highly purified RNP from virions with no appreciable RNase contamination. Although it contained nucleocapsid protein NP, polymerase protein L, and phosphoprotein P (Fig. 1A), the purified viral RNP did not synthesize mRNAs significantly when it was used in the transcription reaction (Fig. 1B). However, mRNA synthesis occurred only after the addition of soluble cytoplasmic proteins from uninfected HLC cells to the viral RNP and increased proportionately with increasing amounts of host cell extract (Fig. 1B). Thus, similar to the infected-cell RNP (9), the RNP purified from the virion also required host proteins for mRNA synthesis in vitro.

Transcription stimulatory activity of tubulin-depleted cell extract. Since previous studies (36, 37) indicated that cellular tubulin acts as a positive transcription factor for Sendai and measles viruses, we initially attempted to determine whether HPIV-3 had a similar requirement. Purified bovine brain tubulin was used in the transcription reaction to determine its effect on mRNA synthesis from viral RNP. Surprisingly, no significant stimulation of RNA synthesis was observed with increasing amounts of tubulin (up to 5 µg), whereas the same amount of tubulin significantly stimulated RNA synthesis from Sendai virus RNA up to 15-fold (Fig. 2), as reported earlier (37), suggesting that HPIV-3 transcription does not require tubulin.
To further confirm that tubulin is not the cellular component required for HPIV-3 transcription, the soluble cytoplasmic extract from HLC cells were depleted of tubulin in separate experiments. This was accomplished by slowly passing the cell extract through an immunoaffinity column containing antitubulin antibody. The bound proteins were eluted from the column, and the stimulatory activities of the bound and unbound proteins were tested in HPIV-3 transcription reactions. As shown in Fig. 3A, most of the stimulatory activity (>80%) was found to be present in the unbound fraction.

We also depleted the cell extract of tubulin by increasing the amounts of antitubulin antibody and then removing the tubulin-antibody complex by precipitation with formalin-fixed S. aureus cells. Again, transcription stimulatory activity in the tubulin-depleted cell extract was decreased only 15% when 2 μg of antitubulin antibody was used for immunoprecipitation. There was no further decrease in the activity after precipitation of tubulin with 4 μg of antibody (Fig. 3B). It is important to note that the antibody precipitated more than 95% of the tubulin present in the cell extract, as evaluated by Western blot analysis (data not shown). Thus, the results given above clearly indicate that the stimulatory activity resides in a host protein(s) not related to tubulin.

A 43-kDa protein stimulates transcription in vitro. In order to characterize the transcription stimulatory factor, the cytoplasmic extract of HLC cells was fractionated as described in Materials and Methods (see Table 1 for a summary). In an attempt to quantitate transcription stimulatory activity, we have defined 1 U as the amount of stimulatory activity which is required for maximal transcription under the standard assay conditions described in Materials and Methods. Consequently, the specific activity is expressed as the inverse of the amount of protein necessary for maximal transcription under these conditions. It is noteworthy that an apparent loss of stimulatory activity was observed in each of the purification steps. In the purification procedure used, transcription stimulatory activity was first enriched by precipitation with ammonium sulfate at two different saturations (0 to 30% and 30 to 50%). Although the activity was present in both fractions, more than 70% of the activity was present in the 30 to 50% fraction. Therefore, the 30 to 50% fraction was used for further purification of the stimulatory factor. After dialysis, the proteins were loaded onto a phosphocellulose column, where the stimulatory activity was found to be present in the unbound fraction (compare Fig. 4, lanes PC-11). Next, the protein (PC-11) was loaded onto a DE-52 column previously equilibrated with buffer A. The bound proteins were eluted with a linear salt gradient (Fig. 4A), and
the stimulatory activities of different fractions were tested in the standard transcription reaction (Fig. 4B). The proteins eluting at 0.18 to 0.28 M NaCl contained stimulatory activity, and protein analysis in an SDS-polyacrylamide gel showed a single major polypeptide of 43 kDa present in those fractions (Fig. 4). These results suggest that transcription stimulatory activity may be associated with the 43-kDa protein.

Characterization of 43-kDa protein as actin. Since paramyxoviruses have been reported to interact with the cytoskeletal protein actin and since the molecular weight of the putative host factor was very similar to that of actin, we first attempted to characterize the 43-kDa protein by its reactivity with antiactin antibody. The proteins present in the pooled DE-52 fractions containing stimulatory activity were allowed to react with antiactin antibody in a Western blot analysis. The 43-kDa protein and also a few high-molecular-weight proteins reacted with the antibody (Fig. 5), indicating their actinlike nature, which was further confirmed by virtue of the similarity of the 43-kDa protein to actin in the following properties: the polypeptide was able to polymerize in the presence of buffer containing 0.1 M KCl, 5 mM MgCl₂, and 1 mM ATP (23); it was present predominantly in the unbound fraction of a heparin-Sepharose column (11); and it bound strongly in a blue-Sepharose column (11; data not shown). Note that the polypeptide migrating slightly faster than actin (Fig. 5A) was identified as creatine phosphokinase from the lysis buffer used during concentration of the DE-52 fractions.

We next examined whether antiactin antibody could be used to deplete actin from the cell extract and thereby
remove its stimulatory activity. As expected, after depletion by immunoprecipitation with 10 and 20 μl of antiactin antibody, the stimulatory activities in the resulting cell extracts were decreased by 50 and 90%, respectively (Fig. 6). This again suggested that an actinlike protein(s) is involved in this transcription stimulation process. Finally, we purified actin from soluble cytoplasmic extract as a polymerized complex and then depolymerized it in an appropriate buffer. The depolymerized actin thus isolated was more than 90% pure (Fig. 7A) and was found to stimulate transcription efficiently (Fig. 7B). Thus, the results given above indicate that cytoplasmic actin is involved in the stimulation of HPIV-3 transcription.

Transcription stimulatory activity in other cell lines. In our previous study we were unable to detect HPIV-3 transcription stimulatory activity in crude soluble cytoplasmic extract of CV-1 cells, possibly because of contaminating RNase activity. Since actin is a ubiquitous protein, we decided to purify the transcription stimulatory activity from CV-1 cells. Similar to that observed in the HLC cells, the stimulatory activity was detected in DE-52 fractions, and the elution pattern was exactly the same as in HLC cells (Fig. 8B). Protein analysis showed that those fractions contained the 43-kDa protein and, in addition, several high-molecular-weight proteins, a few of them possibly representing multi-meric forms of actin. Similar results were obtained when the 43-kDa polypeptide was purified and assayed in an HPIV-3 transcription reaction from HeLa and BHK cells (data not shown).

FIG. 5. Western blot analysis of purified transcription stimulatory factor using antiactin antibody. Aliquots (20 μl) of ammonium sulfate and DE-52 (pooled fractions 75 to 90) were electrophoresed in a 10% SDS-polyacrylamide gel and electroblotted to nylon membrane. (A) Coomassie blue-stained protein pattern. Actin, purified bovine muscle actin (1.5 μg). (B) Immunoblot after reaction with antiactin antibody followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin as the second antibody and staining with diaminobenzidine-primidazole.

FIG. 6. Effect of actin-depleted cell extract on transcription. Soluble cytoplasmic extract (Mock Sup; 40 μg) of uninfected HLC cells was depleted of actin by immunoprecipitation with antiactin antibody and Formalin-fixed S. aureus cells as indicated. The resulting cell extract was used in transcription reactions, and the 32P-labeled RNA products were analyzed in a 5% polyacrylamide–urea gel.

DISCUSSION

In a previous report we demonstrated a requirement of host cell extract for the synthesis of mRNAs from HPIV-3 RNP purified from the infected cells in an in vitro transcription system (9). In this study we have used soluble cytoplasmic extract from uninfected HLC cells for purification and characterization of the host transcription stimulatory factor and have demonstrated that a 43-kDa protein is required for the synthesis of mRNAs in vitro. We have also shown that even though RNP purified from the virions contained all the polypeptides required for transcription (NP, L, and P), it was virtually inactive for RNA synthesis unless the 43-kDa host factor was added to it. The 43-kDa polypeptide was confirmed to be cellular actin by a series of experiments: (i) highly purified 43-kDa protein reacted with antiactin antibody, as shown by Western blot analysis (Fig. 5); (ii) in vitro mRNA synthesis was virtually abolished when the cell extract was depleted of actin with antiactin antibody (Fig. 6); and (iii) protein purified from the extract by the standard purification procedure developed for actin, i.e., monomerpolymer formation (23), stimulated in vitro transcription (Fig. 7). In a separate series of experiments, we exploited...
some unique properties of actin to characterize the 43-kDa host factor. These include (i) its inability to bind to a heparin-Sepharose column but its strong binding to a blue-Sepharose column, (ii) its polymerization-depolymerization behavior, and (iii) its strong binding affinity to DNase I (data not shown). Thus, the 43-kDa host factor for HPIV-3 transcription is confirmed to be cellular actin.

The involvement of the cytoskeletal protein actin in HPIV-3 transcription is similar to the findings from other studies on paramyxoviruses (Sendai and measles viruses), in which another cytoskeletal protein, tubulin, has been shown to stimulate transcription in vitro (36, 37). However, the involvement of actin in the HPIV-3 system makes this system unique and specific. It is quite apparent that tubulin cannot replace actin in HPIV-3 transcription in vitro, whereas it is an excellent transcription factor for Sendai virus transcription (Fig. 2) (37). The precise molecular basis for this specificity is unclear. It is important to mention that a highly acidic protein, polyglutamic acid, has been shown to activate Sendai virus transcription in vitro (60; unpublished observation). HPIV-3 transcription is similarly activated by polyglutamic acid (data not shown). Thus, it seems that the negative charge of actin or tubulin somehow plays a role in paramyxovirus transcription. Similar involvement of the acidic domain of the phosphoprotein (P) of VSV has been implicated in transcription activation in vitro (5). The intriguing question is why actin and tubulin, both being acidic in nature, differentially activate HPIV-3 and Sendai virus, respectively. It is possible that actin preferentially binds to HPIV-3 RNP whereas tubulin is specific for Sendai virus RNP. In fact, we have observed a strong association of actin with HPIV-3 RNP in in vitro studies in which actin-bound RNP was easily pelleted by centrifugation through a glycerol gradient (data not shown). Specific intracellular interaction of VSV L protein with tubulin has recently been demonstrated (37). It would be interesting to determine the exact mechanism by which cytoskeletal proteins interact with paramyxoviruses and play an important role in RNA synthesis. It is tempting to speculate that the interaction of actin

![Image](https://example.com/image.png)
with HPIV-3 template in its monomeric form is followed by polymerization that creates an acidic sheet which interacts with the NP protein-bound genome template to facilitate transcription by RNA polymerase. Thus, it seems likely that the host protein(s) modifies the template rather than functioning as a polymerase subunit, as postulated for tubulin in Sendai virus transcription (37).

It is important to note that other minor proteins were also present in the purified actin fraction, although some of these proteins represent multimeric forms of actin (Fig. 5) mediated by actin-binding proteins. It is worth mentioning here that at least 20 different actin-binding proteins have been reported (31, 41). In our continued attempt to purify the 43-kDa monomeric form of actin to evaluate its transcription stimulatory activity, we routinely encountered an irreversible inactivation of this function. It could be because (i) actin requires other actin-binding proteins for the stimulatory activity, as it does for regulated polymerization-depolymerization and other modification activities (31, 41); (ii) the monomeric form of actin is more active than the polymeric form, and in the absence of an actin-binding protein(s) the monomeric form is highly unstable (11); or (iii) the actin becomes inactivated because of a lack of ATP in the buffers used during purification. At this point it is not possible to ascertain precisely which molecular form of actin is involved in this process or to determine whether there is any role for an actin-binding protein(s). It is also noteworthy that commercially available actin does not stimulate HPIV-3 transcription in vitro (data not shown). We have observed that this actin preparation was partly soluble and failed to polymerize in vitro under conditions in which actin purified from cytoplasmic extract polymerized efficiently. Thus, it is possible that the transcription activation property of actin is directly linked to its capability for monomer-polymer formation. Experiments are under way to clarify these possibilities.

Since the transcription stimulatory factor has been characterized as actin, the question of whether actin remains associated with the viral nucleocapsid during its life cycle arises. Previous studies indicated that paramyxoviruses remain attached to the cytoskeletal structure during synthetic and maturation processes, and a role for cytoskeletal proteins in primary transcription has been suggested (1, 21, 24, 53). In addition, actin has been found in virions of paramyxoviruses (61). In fact, the purified HPIV-3 RNP used in our studies contained a trace amount of actin, as seen by Western blot analysis (data not shown), which may account for the basal level of transcription activity of the RNP without the cytoplasmic extract (Fig. 4B). Although the current belief is that actin is involved in the maturation and budding processes of these viruses, the role of these cytoskeletal proteins in viral RNA synthesis has not been investigated critically (1, 53). Furthermore, the involvement of cytoskeletal proteins in HPIV-3 reproduction has not yet been studied. Thus, a detailed study of the role of actin in HPIV-3 transcription would certainly shed light in the interesting area of host-virus interaction and virus reproduction.

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