Tubulin: A factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs

(transcription factor)

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ABSTRACT Tubulin acts as a positive transcription factor for in vitro RNA synthesis by two different negative-strand viruses: Sendai virus, a paramyxovirus; vesicular stomatitis virus (VSV), a rhabdovirus. A monoclonal antibody directed against β-tubulin completely inhibited not only mRNA synthesis and RNA replication catalyzed in vitro by extracts of cells infected with either virus but also mRNA synthesis by detergent-disrupted purified virions. The synthesis of both a leader-like RNA and the NP mRNA directed by detergent-disrupted purified Sendai virions was shown to be totally dependent on the addition of purified tubulin. The addition of purified tubulin, although not required, also stimulated mRNA synthesis directed by detergent-disrupted VSV virions 2- to 7-fold. Finally, there appears to be an association between tubulin and the L protein of VSV, since both monoclonal and polyclonal antisera specifically immunoprecipitated not only tubulin but also the L protein of two different VSV serotypes from the soluble protein fraction of infected cells.

Two different groups of negative-strand RNA viruses, the rhabdoviruses (vesicular stomatitis virus (VSV)) and the paramyxoviruses (Sendai virus) share similar strategies for their reproduction (1, 2). Each virus carries within the virion an RNA-dependent RNA polymerase, composed of the L and NS subunits for VSV or the L and P subunits for Sendai virus (3, 4). When purified virus is treated with detergent, the disrupted virions transcribe first a leader RNA and then the mRNAs sequentially from the nucleocapsid RNA template (5–7). After viral mRNA and protein synthesis in the infected cell, the negative-strand genome RNA of either virus is replicated through a positive-strand complementary RNA intermediate (1, 2). Both the positive- and negative-genome length RNAs are encapsidated by the N (VSV) or NP (Sendai virus) proteins to form nucleocapsid structures concomitant with the synthesis of their respective genome RNA. We have developed cell-free systems for VSV (8) and for Sendai virus (9) that support both the faithful transcription and replication of the genome RNAs of the wild-type viruses and the replication of the corresponding defective-interfering (DI) viruses under conditions of mixed infection.

In this communication, we have exploited these in vitro systems to identify any host cell proteins that may be required in the process of viral RNA synthesis. It is known that the addition of extracts of uninfected cells will stimulate transcription directed by VSV or Sendai virus (5, 10, 11). We report here that tubulin appears to be one host protein that can act as a positive transcription factor for both of these viruses. We show that the addition of a monoclonal antibody directed against β-tubulin completely inhibited all viral RNA synthesis in extracts of cells infected with either VSV or Sendai virus. The addition of purified tubulin, moreover, was required for the in vitro synthesis of Sendai virus leader-like RNA and NP mRNA from detergent-treated virions. Similarly, the addition of tubulin to detergent-disrupted VSV, while not absolutely required for transcription, markedly stimulated VSV mRNA synthesis.

MATERIALS AND METHODS

Cells and Virus. The Indiana (I) serotype of VSV and an Indiana strain-specific DI particle, MS-T, were grown in baby hamster kidney (BHK) cells and purified as described (8). The Ogden New Jersey (NJ) serotype of VSV and an Ogden strain-specific DI particle, DI-OS, were obtained from M. Reichmann (12) and propagated as described above. Sendai virus (Harris strain) and a Sendai virus-derived DI particle, DI-H, were propagated in embryonated chicken eggs and purified as described (9).

Monoclonal Antibodies. A hybridoma cell line secreting monoclonal antibody to actin (C4) was selected as described by Ball et al. (13). The C4 anti-actin monoclonal antibody has been shown to be generally reactive toward all actins (J.L.L., unpublished observations). The hybridoma cell line secreting monoclonal antibody specifically to β-tubulin (3F3G2) was selected as described by Williams et al. (14). Ascites fluids were collected after interperitoneal injection of the hybridoma cell lines or the control myeloma SP-2 cell line into pristane-treated BALB/c mice. The inherent RNAse activity within the ascites fluids was completely inhibited by the addition of RNasin to the in vitro assays as described below.

In Vitro RNA Synthesis and Product Analysis. Cytoplasmic extracts of cells coinfected with Sendai virus and DI-H were prepared in a reaction mixture as described by Carlsten et al. (9). Cytoplasmic extracts of cells coinfected with either VSV (I serotype) and MS-T or VSV (NJ serotype) and DI-OS were prepared in a reaction mixture as described by Peluso and Moyer (8). The cell-free extracts were incubated at 30°C with RNasin (Promega Biotec) at 960 units/ml and [3H]UTP (250 μCi/ml; 1 Ci = 37 GBq; ICN) or [α-32P]UTP (300 μCi/ml; ICN) in the presence or absence of antibody for the times indicated in the text. The [3H]- or α-32P-labeled RNA products were isolated and analyzed by electrophoresis on acid urea/1.5% agarose gels and fluorography (8) or by 20% polyacrylamide/urea gel electrophoresis (15), respectively.

Immunoprecipitation. VSV-infected BHK cells were labeled from 2.5 to 4.5 hr after infection with [35S]methionine (25 μCi/ml; New England Nuclear). The soluble protein fraction was prepared from cytoplasmic extracts and immunoprecipitated with various antibodies, and the immunocomplexes were collected by binding to Staphylococ-

Abbreviations: VSV, vesicular stomatitis virus; I serotype, Indiana serotype of VSV; NJ serotype, New Jersey serotype of VSV; DI, defective interfering.

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**Acid-insoluble radioactivity (cpm)**

For presence of serotype)

| 30  | 15,150 | 12,260 |

**RESULTS**

**Effect of Selected Monoclonal Antibodies on Viral RNA Synthesis.** As a first approach to identify cellular factors that might be involved in negative-strand virus production, we asked whether monoclonal antibodies against selected cellular proteins might inhibit viral-specific RNA synthesis (Table 1). Extracts of cells infected with Sendai virus or VSV were prepared and incubated in the presence of individual monoclonal antibodies under reaction conditions that supported viral transcription and genome replication (8, 9). As a control, the addition of anti-mycelma cell ascites fluid to such extracts gave only a small inhibition of total Sendai virus or VSV RNA synthesis. The effect of an anti-actin monoclonal antibody was tested, because actin has been recently shown to be a transcription factor for cellular RNA polymerase II (16, 17) and actin is packaged within purified Sendai virions (18). The anti-actin monoclonal antibody had no effect on in vitro RNA synthesis by either virus (Table 1). A monoclonal antibody to β-tubulin, on the other hand, markedly inhibited total RNA synthesis in extracts of cells infected with either virus. The anti-β-tubulin monoclonal antibody, however, did not inhibit in vitro RNA synthesis by the virion-associated DNA-dependent RNA polymerase of rabbit poxvirus (19). These data suggest that tubulin may be involved in viral RNA synthesis directed by these two different groups of negative-strand RNA viruses.

**The Effect of Tubulin and the Anti-Tubulin Antibody on Sendai Virus RNA Synthesis.** We next wanted to determine whether the anti-tubulin antibody might show a selective inhibition toward different classes of Sendai virus RNA products. Cells were coinfected with Sendai virus and DI-H, and the RNA products synthesized in cytoplasmic extracts in either the presence or absence of antibody were analyzed by acid urea/agarose gel electrophoresis. Both the mRNA synthesis and genome RNA replication normally observed in extracts of virus-infected cells (Fig. 1, lane D) were completely inhibited by the addition of the anti-β-tubulin monoclonal antibody (lane E). Titration experiments showed that an increasing inhibition of all classes of viral RNA occurred with increasing amounts of antibody (data not shown).

We also tested the effect of the anti-tubulin antibody on transcription by purified Sendai virus. Under the same reaction conditions used for RNA synthesis in extracts of infected cells, we could detect no transcription by detergent-disrupted Sendai virus (Fig. 1, lane A). However, when the soluble protein fraction of uninfected cells was added to detergent-disrupted virus, the synthesis of the viral mRNAs was observed (lane B). The Sendai virus mRNA synthesis mediated by the addition of the soluble protein fraction was totally inhibited by the simultaneous addition of the anti-β-tubulin monoclonal antibody (lane C). These data suggest that cellular proteins are absolutely required for any transcription from purified Sendai virus and that tubulin may be one of the required components in the soluble protein fraction.

We next asked whether purified bovine brain tubulin (20) alone could replace the soluble protein fraction of uninfected cells and stimulate RNA synthesis directed by purified Sendai virus. As described (Fig. 1), in the absence of any additions, detergent-disrupted Sendai virus did not synthesize any mRNAs (Fig. 2, lane A), while the addition of the soluble protein fraction from uninfected BHK cells allowed transcription (lane B). The addition of purified tubulin alone partially replaces the mixture of cellular proteins and leads to the synthesis of at least some NP mRNA, the first of the 3' sequentially transcribed Sendai mRNAs (lane C). There was also the synthesis of numerous sub-mRNA sized products, which may result from premature termination during NP mRNA synthesis.

Since these data suggested that tubulin stimulated the natural course of transcription of Sendai virus (i.e., from the 3' end of the genome), we measured the effect of tubulin on the synthesis of viral leader RNA, the first product of transcription, which precedes the synthesis of the first (NP)

**Table 1. Inhibition of viral RNA synthesis by monoclonal antibodies**

<table>
<thead>
<tr>
<th>Monoclonal antibody (ascites)</th>
<th>Extracts of cells infected with Sendai virus</th>
<th>VSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>15,860</td>
<td>13,061</td>
</tr>
<tr>
<td>Anti-mycelma (SP-2)</td>
<td>12,037</td>
<td>10,924</td>
</tr>
<tr>
<td>Anti-actin</td>
<td>15,150</td>
<td>12,260</td>
</tr>
<tr>
<td>Anti-β-tubulin</td>
<td>615</td>
<td>2,478</td>
</tr>
</tbody>
</table>

BHK cells were infected with either Sendai virus or VSV (I serotype) and at 12 or 4 hr postinfection, respectively, cytoplasmic cell extracts were prepared in reaction mixture as described (8, 9). An extract of $1 \times 10^6$ infected cells in 200 μl was incubated in the presence of [3H]UTP with 4 μl of the monoclonal antibody first at 4°C for 30 min to allow antibody–antigen complex formation and then at 30°C for 2 hr (Sendai virus) or 1.5 hr (VSV), and the trichloroacetic acid-insoluble radioactivity (cpm) was determined.
mRNA (7). Purified detergent-disrupted Sendai virus was incubated under various conditions and the small RNA products were analyzed by electrophoresis on 20% polyacrylamide/urea gels. In the absence of any additional components, Sendai virus synthesized little or no small RNAs (Fig. 2, lane D) or mRNA (Figs. 1 and 2). When the soluble protein fraction from uninfected cells was added to Sendai virions, however, a rather heterogeneous population of small (35–45 nucleotides) leader-sized RNAs (Fig. 2, lane E) in addition to the mRNAs (Figs. 1 and 2) were synthesized. The addition of purified tubulin could replace the cell protein and resulted in the synthesis of an approximately equal amount of a more discrete leader-like RNA ~40 nucleotides long as well as smaller amounts of products of ~35 and ~33 nucleotides (Fig. 2, lane F). These results suggest that, under the assay conditions used here, tubulin is necessary and sufficient for the initiation and synthesis of both the Sendai virus leader-like RNA and some of the adjacent NP mRNA.

The Effect of Anti-Tubulin Antibody and Tubulin on VSV RNA Synthesis. We then performed experiments similar to those described above for Sendai virus using two different serotypes of VSV. Detergent-treated purified VSV (I serotype) incubated in the absence of additional components synthesized viral mRNAs (Fig. 3, lane A), and this reaction was totally inhibited by the addition of the anti-tubulin monoclonal antibody (lane B). In addition, both the transcription and RNA replication that occurred in extracts prepared from cells coinfected with either VSV (I serotype) and MS-T or VSV (NJ serotype) and DI-OS (lanes C and E, respectively) were also totally inhibited by the anti-tubulin monoclonal antibody (lanes D and F).

Unlike purified Sendai virus, detergent-disrupted purified VSV (NJ serotype) carried out transcription in the absence of any added components (Fig. 4, lane A). In the case of this serotype, the product was primarily the N mRNA. By direct quantitation of the radioactivity in the N mRNA bands, the effects of either purified tubulin or soluble cell protein could be tested. The addition of bovine serum albumin or actin (control proteins) to the reactions catalyzed by VSV (NJ serotype) caused either a small increase (14%; Fig. 4, lane F) or a decrease (lane G), respectively, in N mRNA synthesis. The addition of the soluble protein fraction of uninfected cells stimulated the synthesis of four of the five VSV (NJ serotype) mRNAs >4-fold (lane B), similar to published results for VSV (I serotype) (5, 10). The addition of increasing amounts of purified tubulin alone stimulated increasing VSV mRNA synthesis up to 2-fold (lanes C–E). The stimulatory effect of tubulin was most pronounced on N mRNA synthesis, but M and NS mRNA synthesis also increased. In various experiments, the tubulin-directed stimulation of transcription varied from 2- to 7-fold with VSV of either the I or NJ serotypes (data not shown).
Biochemistry: Moyer et al.

FIG. 4. Tubulin stimulation of RNA synthesis from purified VSV (NJ serotype). Detergent-disrupted VSV (NJ serotype) (20 µg) was incubated at 30°C for 90 min in reaction mixture (8) with [3H]UTP in a final volume of 200 µl either alone (lane A) or in the presence of the soluble protein fraction from 3 x 10⁶ uninfected BHK cells (lane B), 4 µg (lane C), 10 µg (lane D), and 20 µg (lane E) of purified bovine brain tubulin, 10 µg of bovine serum albumin (lane F), or 10 µg of depolymerized actin (lane G). The RNA was purified and analyzed as described in Fig. 1. N, M, and NS show the positions of the respective viral mRNAs for these viral proteins.

Immunoprecipitation of Cell Extracts with Anti-Tubulin Antibodies. For purified VSV, the anti-β-tubulin antibody inhibited the transcription that occurs in the absence of any exogenous components, suggesting either that tubulin is packaged within the virus and is associated with a viral protein involved in transcription or that there is a tubulin-like epitope within one of the VSV proteins. We have tested whether β-tubulin is present within purified VSV preparations by immunoblot analysis. We were able to detect, relative to known standards, 0.15 µg of β-tubulin in 147.5 µg of VSV (NJ serotype) virion protein. However, within the sensitivity of the assay, no β-tubulin was detectable in VSV (I serotype) (data not shown). Utilizing the molecular composition of VSV determined by scanning transmission electron microscopy (22), this imposes a limit of ~4 molecules of tubulin for the 50 molecules of the L protein in the VSV (NJ serotype) particle. We could not measure the level of β-tubulin in VSV (I serotype) because β-tubulin and the VSV (I serotype) NS protein comigrate on the gel (see Fig. 5), which may inhibit effective transfer of the tubulin to the nitrocellulose. The fact that the anti-tubulin antibody did inhibit transcription of purified VSV (I serotype) (Fig. 3, lane B) does suggest, however, that some β-tubulin is also present in this virus.

We next tested whether either the anti-β-tubulin monoclonal antibody or a polyclonal rabbit anti-tubulin IgG might recognize any VSV-specific proteins. Uninfected cells or cells infected with either VSV (I serotype) or VSV (NJ serotype) were labeled with [35S]methionine and the soluble protein fractions of cytoplasmic cell extracts were then prepared and immunoprecipitated with the various antibodies. From uninfected cells, as expected, both the monoclonal and polyclonal anti-tubulin antibodies immunoprecipitated tubulin as well as small amounts of two smaller unknown cellular proteins (Fig. 5, lanes A and B). The rabbit anti-tubulin IgG was of lower titer and immunoprecipitated considerably less tubulin (~10%) than did the monoclonal antibody.

The immunoprecipitation of the soluble protein fractions from VSV-infected cells with rabbit anti-VSV antiserum showed that for each serotype all five viral proteins (L, G, NS, N, and M) were present in the sample (Fig. 5, lanes C and G). Interestingly, the anti-β-tubulin monoclonal antibody specifically immunoprecipitated the VSV L protein (a subunit of the viral RNA polymerase) in addition to tubulin from the soluble protein fractions of cells infected with either VSV serotype (lanes D and H). Similarly, the weaker polyclonal rabbit anti-tubulin IgG also immunoprecipitated the VSV L proteins (lanes E and I), but the reaction was not as strong and, therefore, required longer autoradiographic exposure times for visualization (7 days as opposed to 1 day; lanes F and J). In this and other similar experiments, we have consistently observed that the anti-β-tubulin monoclonal antibody immunoprecipitated the L protein of the NJ serotype better than that of the I serotype of VSV. In addition, for VSV (I serotype), but not the NJ serotype, there appeared to be some coprecipitation of other viral proteins (N and M) in addition to the L protein and tubulin.

DISCUSSION

The data presented here suggest that tubulin is a cofactor necessary for RNA synthesis by two different classes of negative strand viruses, VSV and Sendai virus. A monoclonal antibody against β-tubulin totally inhibited all in vitro
viral RNA synthesis directed either by extracts of infected cells or from the detergent-disrupted purified viruses (Table 1, Figs. 1 and 3). In the case of Sendai virus, there was no transcription of either leader-like RNAs or the mRNAs from purified virus unless either tubulin or the soluble protein fraction from uninfected cells was added. Tubulin, therefore, appears to be a required cellular protein, which is consistent with the observation that the anti-β-tubulin antibody totally inhibited the Sendai virus RNA synthesis observed in the presence of the cell protein (Fig. 1). Moreover, the addition of purified tubulin alone could stimulate purified Sendai virus to synthesize at least leader-like RNA to the same extent as cell protein. In addition, some adjacent NP mRNA could also be synthesized, although not with the same efficiency observed with cell protein. The differences in the abilities of tubulin and the soluble cell protein to stimulate the synthesis of NP mRNA and the other viral mRNAs suggest that an additional cell protein(s) may also be required for the complete transcription of the Sendai virus genome RNA.

We have not yet conclusively determined that the small RNAs synthesized by Sendai virus in the presence of host cell protein or tubulin are leader RNAs, but their sizes and kinetics of synthesis are consistent with this interpretation. Using a different type of analysis, Lepper et al. (7) reported that purified Sendai virus synthesized a leader RNA of 55 nucleotides in vitro, while in vivo they identified two leader RNA products of ≈31 and ≈55 nucleotides. All of these observations suggest that tubulin has a primary role in the initiation of transcription directly at the 3′ end of the Sendai virus genome.

Other investigators (7, 23, 24) have reported, in contrast to what we report here, that purified Sendai virus alone can synthesize the viral mRNAs and leader RNA in the absence of any added cellular components. Preliminary experiments suggest that the source of the virus may be the explanation for this apparent discrepancy. The Sendai virus used here was grown in embryonated chicken eggs and did not contain detectable tubulin by immunoblot analysis. The virus used by the other investigators, however, was propagated in tissue culture cells, and we have shown that significant levels of tubulin are packaged in Sendai virions grown in at least some cell lines. We suggest, therefore, that the transcription observed with tissue culture-grown virus occurs because the tubulin is packaged and present in such preparations, whereas the use of egg-grown virus would necessitate that tubulin be added as a cofactor for transcription.

Unlike Sendai virus, purified VSV required no addition of host cell protein for transcription (although both tubulin and soluble cell protein stimulated transcription). However, the anti-tubulin monoclonal antibody completely inhibited the reaction catalyzed by purified virions (Fig. 3). The addition of purified tubulin to detergent-treated VSV stimulated transcription only 2- to 7-fold, unlike the absolute requirement for tubulin (or soluble cell protein) observed with Sendai virus (Figs. 2 and 4). In immunoblot analyses, we have found that purified VSV (NJ) does contain small amounts of tubulin, which could account for the observations described above. We have also shown that two different anti-tubulin antibody preparations communoprecipitate the L protein of both serotypes of VSV as well as tubulin from the soluble protein fraction of infected cells (Fig. 5). The immunoprecipitation of the L protein by the anti-tubulin antibody could result from an epitope shared by tubulin and the L proteins of both VSV serotypes, or it could reflect a direct association between the two proteins. In preliminary protein immunoblot experiments, we have detected no crossreactivity of the anti-β-tubulin monoclonal antibody with any of the VSV proteins. In addition, a computer search of the GenBank database failed to reveal any homology between either α- or β-tubulin and the VSV (1 serotype) L protein sequences reported by Ponstingl et al. (25), Kraus et al. (26), and Schubert et al. (27), respectively, suggesting that there is no common epitope and that coprecipitation results instead from an association between these proteins.

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