Involvement of a Cellular Glycolytic Enzyme, Phosphoglycerate Kinase, in Sendai Virus Transcription*

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In vitro mRNA synthesis of Sendai virus is almost entirely dependent on the addition of cellular proteins (host factors). Previous studies indicated that the host factor activity from bovine brain was resolved into at least two complementary fractions, one of which may be tubulin. In this study, the host factor activity that stimulates the transcription in the presence of tubulin was further purified from bovine brain. This fraction was found to contain at least two complementary factors, and one of them was purified to a single polypeptide chain with an apparent Mr of 46,000 (p46). From the amino acid sequence, biochemical, and immunological analyses, p46 was identified as a glycolytic enzyme, phosphoglycerate kinase (PGK). Purified native PGK from rabbit and yeast, and a recombinant human PGK substituted for p46. Although, as previously suggested, tubulin was involved in the transcription initiation complex formation by being integrated into the complex, p46 and its complementary factor had little effect on the complex formation. On the other hand, when p46 and the complementary factor were added to the RNA chain elongation reaction from the isolated initiation complex formed with tubulin, mRNA synthesis was dramatically stimulated. The enzymatic activity per se of PGK did not seem to be required for its activity. West-‐Western blot analysis showed that PGK could directly interact with tubulin. These data suggest that PGK stimulates Sendai virus mRNA synthesis at the elongation step, probably through its interaction with tubulin in the initiation complex.

Sendai virus (SeV), a member of the Paramyxovirus family in the order Mononegavirales, contains a monopartite negative strand RNA genome, which consists of six genes encoding the viral proteins, nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), hemagglutinin-neuraminidase glycoprotein (HN), and large protein (L) (reviewed in Refs. 1–3). The viral envelope encloses a ribonucleoprotein complex (RNP), which mainly contains the RNA genome of 15.3 kilobases and three viral proteins: NP, P, and L. The RNA genome encapsidated with the NP proteins serves as the template to synthesize a positive strand leader RNA and mRNAs as well as a full-length positive strand anti-genomic RNA, which serves as the template for the negative strand RNA genome (1). The genetic information of the RNA genome is expressed through at least six monocistronic mRNA species. The P gene mRNA products which encode the C and V protein families, in addition to the P protein, use multiple translation initiation sites as well as an open reading frame newly generated by RNA editing (1). According to a current model (1), the viral RNA synthesis starts with a viral RNA-dependent RNA polymerase entering at the 3’ end of the genome RNA, where the viral RNA polymerase initiates the sequential synthesis of a leader RNA and mRNAs or synthesizes a full-length anti-genome RNA. However, little is known about the mechanism of mode switching of the RNA polymerase from transcription to replication, although it has been shown that three viral proteins, the NP, P, and L proteins are required for transcription and replication of the genome RNA (4–6) and that the L protein interacts with the P protein to form the polymerase complex for replication and transcription (4, 7). It has also been proposed that, during replication, P protein acts as a chaperon for the NP to encapsidate newly synthesized genome or antigenome RNAs (8).

Several studies using in vitro transcription systems of paramyxoviruses, e.g. human parainfluenza virus type 3 (HPIV3) (9, 10), mumps virus (11), measles virus (12), respiratory syncytial virus (13), rinderpest virus (14), and canine distemper virus (15), suggest the involvement of cellular proteins in the viral RNA synthesis in addition to viral proteins (reviewed in Refs. 16 and 17). We have established an accurate and efficient in vitro mRNA synthesizing system using purified SeV particles or viral RNP complexes, in which viral mRNA synthesis is almost completely dependent on the presence of host cell proteins (host factors) (18). We have demonstrated that cellular tubulin, which is essential for in vitro transcription of SeV, is integrated into the transcription initiation complex and activates viral mRNA synthesis (18, 19). The involvement of tubulin in SeV RNA synthesis was also supported by the inhibition of in vitro transcription of SeV by a monoclonal anti-tubulin antibody (20). On the other hand, De et al. (21, 22) showed that the polymeric form of actin binds viral RNP of human parainfluenza virus type 3 (HPIV3) and activates in vitro transcription. They also found that the HPIV3 RNPs are specifically localized on the actin microfilaments in the virus-infected cells (23). The participation of tubulin or actin in transcription has also been observed in measles virus (12) and respiratory syncytial virus (24, 25), respectively. Furthermore, cellular protein kinases such as casein kinase II and protein kinase A stimulate mRNA synthesis, potentially through phosphorylation of the viral mRNA polymerase (26).
kinase C isoform ζ have been shown to phosphorylate the P protein (26–30). However, the precise mode of action of these host factors in activating and regulating the RNA polymerase of paramyxoviruses remains to be studied. In addition, there seem to be other factors required for the transcription as well as replication of paramyxovirus genome that have not been identified yet (18, 19). Therefore, the purification and the functional analysis of each host factor may, of course, provide an important step toward understanding the molecular mechanism of transcription and replication of the paramyxovirus genome.

In this study, we purified the host factor activity complementary to tubulin, and showed it has at least two components, and we identified one of them as phosphoglycerate kinase (PGK), a glycolytic enzyme. This is the first example showing the involvement of a glycolytic enzyme in the transcription of paramyxoviruses. We have shown that PGK stimulates viral mRNA synthesis at the elongation step, probably through its interaction with tubulin integrated into the initiation complex. Tubulin stimulated both mRNA synthesis and leader RNA synthesis of SeV, while PGK failed to stimulate leader RNA synthesis, suggesting that mRNA and leader RNA synthesis may be regulated by different sets of host proteins.

**EXPERIMENTAL PROCEDURES**

### Materials

ATP, GTP, UTP, and CTP were purchased from Yamaso Shoyu, Japan. Haptyate C was obtained from Clarkson Chemical Co. [α-32P]UTP (400 Ci/mmol), [α-32P]GTP (400 Ci/mmol), Blue-Sepharose CL-6B, Heparin-Sepharose CL-6B, mouse monoclonal anti-chicken brain β-tubulin antibody (IgG1, clone DM1B), horseradish peroxidase (HRP)-linked protein A, and the ECL Western blotting system were from Amersham Pharmacia Bieiot. N-[N-Tris-(hydroxymethyl)methyl ketone (TPCK)-treated bovine pancreatic trypsin was from Worthington. Yeast (Saccharomyces cerevisiae) phosphoglycerate kinases (PGK) and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Roche Molecular Biochemicals. Rabbit muscle PGK, 3-phosphoglycerate, and t-glucose-3-phosphate were from Sigma. Nickel-nitritotriacetic acid-agarose and 4 CN membrane peroxidase substrate system were obtained from Qiagen and Kirkegaard & Perry Laboratories, respectively. HRP-conjugated goat anti-mouse IgG polyclonal antibody was from Promega Immobilon. Polyvinylidene difluoride (PVDF) membrane was from Millipore. Freund's complete adjuvant and Freund's incomplete adjuvant came from Difco. 2-Fluoro-1-methylpyridinium toluene-4-sulfonate-activated Freund's complete adjuvant and Freund's incomplete adjuvant came from Difco. HRP-conjugated goat anti-mouse IgG polyclonal antibody was from Promega Immobilon. Polyvinylidene difluoride (PVDF) membrane was from Millipore. Freund's complete adjuvant and Freund's incomplete adjuvant came from Difco. Cellulofine was the product of Seikagaku Corp. from Difco. 2-Fluoro-1-methylpyridinium toluene-4-sulfonate-activated Freund's complete adjuvant and Freund's incomplete adjuvant came from Difco. HRP-conjugated goat anti-mouse IgG polyclonal antibody was from Promega Immobilon. Polyvinylidene difluoride (PVDF) membrane was from Millipore. Freund's complete adjuvant and Freund's incomplete adjuvant came from Difco. Cellulofine was the product of Seikagaku Corp.

### Viruses

Sendai virus strain Z, propagated in the allantoic cavity of 10-day-old chick embryos, was purified as described by Shibata et al. (31). Vesicular stomatitis virus strain New Jersey was kindly supplied by Dr. H. Shibuta (Institute of Medical Science, University of Tokyo, Tokyo, Japan).

**Preparation of Bovine Brain Extract (S100 Fraction)**

The 100,000 × g supernatant fraction (S100) from bovine brain was prepared as described by Takagi et al. (19).

**Purification of Cellular Tubulin**

Microtubular proteins were prepared form bovine brain through three assembly-disassembly cycles as described by Shelanski et al. (32). Purified tubulin free from microtubule-associated protein was obtained as a heterodimer by phosphocellulose column chromatography of microtubular proteins as reported by Weigarten et al. (33).

**In Vitro Transcription of SeV and Vesicular Stomatitis Virus (VSV)**

**In vitro** mRNA synthesis of Sendai virus or vesicular stomatitis virus was carried out under standard conditions as described by Mizumoto et al. (18), with 50 μM [α-32P]UTP (3,000–5,000 cpm/pmol) and 6 μg of purified Sendai virus particles, or with 50 μM [α-32P]UTP (500–1,000 cpm/pmol) and 1 μg of purified vesicular stomatitis virus particles. After incubation for 120 min at 30 °C, the reaction mixtures were treated with proteinase K. The transcripts were extracted with phenol-chloroform and precipitated with ethanol, and then electrophoresed in an 1.2% agarose gel after denaturation with glyoxal. In vitro plus leader RNA synthesis of Sendai virus was carried out with 10 μg [α-32P]GTP (40,000–50,000 cpm/pmol) and 6 μg of virus particles under the same conditions for mRNA synthesis except that UTP was omitted, as described elsewhere (2). After incubation for 120 min at 30 °C, the RNA products were denatured with formamide, and analyzed by electrophoresis in a 20% polyacrylamide gel containing 8% urea.

**Preparation of Sendai Virus Ribonucleoprotein (RNP)**

Purified Sendai virus particles (300 μg) were solubilized by incubation in 500 μl of a solution containing 40 mM HEPES-KOH (pH 7.9 at 20 °C), 30 mM NaCl, 30 mM KCl, 6 mM MgCl2, 2 mM DTT, and 0.1% Nonidet P-40 for 30 min at 30 °C. The reaction mixture was centrifuged in a 2-ml tube in a Beckman TLS55 rotor containing 750 μl cushion A (30% [v/v] glycerol, 20 mM Tris-HCl (pH 7.9), 70 mM KCl, 10 mM MgCl2, 2 mM DTT, and 0.05% Nonidet P-40) layered on a 750-μl cushion B (50% [v/v] glycerol, 20 mM Tris-HCl (pH 7.9), 70 mM KCl, 10 mM MgCl2, and 2 mM DTT), at 100,000 × g for 120 min at 4 °C. The RNP pellets were resuspended in 60% sucrose/TNE (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA), and then subjected to in vitro transcription. The amounts of the pelleted viral RNP used for transcription were determined by adjusting NP protein contents in each RNP to that of 6 μg (total protein) of virus particles using SDS-polyacrylamide gel electrophoresis.

**Isolation of Transcription Initiation Complexes**

The transcription initiation complex was isolated as described by Takagi et al. (19). Purified Sendai virus particles (30 μg) were incubated for 30 min at 30 °C with the host factor in a 125-μl reaction mixture containing 40 mM HEPES-KOH (pH 7.9 at 20 °C), 30 mM NaCl, 50 mM KCl, 6 mM MgCl2, 2 mM DTT, and 0.1% Nonidet P-40, and the reaction was centrifuged in a 0.8-ml ultraclear tube (Beckman), which contained two cushions, A and B (200 μl each), at 100,000 × g for 120 min at 4 °C in a Hitachi P55ST-2 rotor with adapters. The viral RNP pellets were resuspended in cushion B and were subjected to the elongation reaction without additional host factors.

**Purification of Host Factors**

All operations were performed at 4 °C.

**Step 1: Hydroxylapatite Column Chromatography—Bovine brain extract (S100) (36 mg, 3.6 ml) was loaded onto a hydroxylapatite (Haptyate C) column (inner diameter, 1.6 × 16 cm) pre-equilibrated with 1 mM potassium phosphate buffer (1 mM KH2PO4-K2HPO4 (pH 6.9 at 20 °C), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 20% glycerol) as described by Mizumoto et al. (18), and proteins were step-eluted with 75, 150, 300, and 600 mM potassium phosphate buffer at a flow rate of 24 mM/l. Proteins in each fraction were concentrated with ammonium sulfate (80% saturation) and then used for in vitro transcription assays. The 75, 150, 300, and 600 mM potassium phosphate eluates were referred to as HA75, HA150, HA300, and HA600, respectively. For further purification of host factors in HA75, hydroxylapatite column chromatography was done in four batches without ammonium sulfate precipitation.

The HA75 fractions from four batches were combined and then dialyzed against TEMG (20 mM Tris-KCl (pH 7.9 at 20 °C), 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 20% glycerol) containing 20 mM KCl. Approximately 172 ml (0.5 mg/ml) of the HA75 was obtained from 14.4 ml of S100 fraction.

**Step 2: Blue-Sepharose Column Chromatography**—A portion (43 mg, 86 ml) of the HA75 fraction was loaded onto a Blue-Sepharose column (inner diameter, 1.6 × 18 cm) pre-equilibrated with TEMG/20 mM KCl, and proteins were step-eluted with TEMG containing 20, 150, and 300 mM KCl at a flow rate of 18 ml/h. The 20, 150, and 300 mM KCl eluates were referred to as BS20, BS150, and BS300, respectively. This column gave two active, complementary fractions, BS20 and BS150. The BS150 fractions from two batches were combined and dialyzed against TEMG/150 mM KCl. The dialysate of BS150 (50 ml, 0.26 mg/ml) was subjected to Heparin-Sepharose column chromatography. The BS20 fraction was kept at –80 °C.

**Step 3: Heparin-Sepharose Column Chromatography**—A batch (6.5 mg, 25 ml) of the BS150 fraction was loaded onto a Heparin-Sepharose column (inner diameter, 0.8 × 16 cm) pre-equilibrated with TEMG/150 mM KCl. After washing the column with 10 ml of the same buffer, proteins were eluted with a 50-ml linear gradient of 150–350 mM KCl in 2 ml.

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TEMG at a flow rate of 6 ml/h and fractions of 0.54 ml were collected. The active fractions (220 mM KCl eluate, referred to HS220) containing a single polypeptide with $M_r$ of 46,000 (p46) (0.035 mg/ml) were pooled and stored at -80 °C.

**Amino Acid Sequence Analysis**

Highly purified p46 (90 μg, ~2 nmol) was digested with TPCK-treated trypsin (2 μg at 1:45 ratio (enzyme/substrate; w/w) in 180 μl of a trypsin-digestion buffer (50 mM Tris-HCl (pH 8.0), 10 mM CaCl$_2$) at 30 °C overnight. The tryptic peptides were fractionated by reverse-phase high performance liquid chromatography. The individual peptides were sequenced by automated Edman degradation using an Applied Biosystems model 477A sequencer. The obtained amino acid sequences were compared with sequences in the protein data base (PIR) by using the BLAST program.

**Assay for Phosphoglycerate Kinase Activity**

PGK activity was assayed in a coupled reaction with GAPDH reaction as described by Lee (34). The assay was performed at room temperature (~25 °C) in a total volume of 0.5 ml containing 0.1 x Tris-HCl (pH 7.9), 10 mM MgCl$_2$, 0.15 mM Na$_2$EDTA, 2 mM ATP, 6 mM 3-phosphoglycerate, 0.1 mg/ml BSA, 50 μg of GAPDH, and 5–30 ng of PGK.

**Preparation of Antibody against Rabbit Muscle PGK**

Rabbit muscle PGK (300 μg, obtained from Sigma) was mixed with an equal volume of Freund’s complete adjuvant and injected subcutaneously into a New Zealand White rabbit (2 kg) four times at about 2-week intervals. After 11 weeks from the first immunization, polyclonal rabbit muscle PGK antibodies were affinity-purified using a rabbit muscle PGK-immobilized affinity column that had been prepared by coupling of PGK to 2-fluoro-1-methylpyridinium toluene-4-sulfonate-activated Cellulofine according to the manufacturer’s protocol.

**Bacterial Expression of Human PGK-1**

The coding sequence of human PGK-1 (GenBank accession nos. L00159 and L00160) was amplified from human leukocyte RNA (35) by RT-PCR using a sense primer, 5’-CCA GGA TCC ATG TCG CTT TCT AAC AAG C-3’ (BamHI site underlined) and an antisense primer, 5’-GCT ACA GCT ATT GAT GAG AGC ATC CAC-3’ (BglII site underlined). The amplified PCR fragments of 1269 base pairs were digested with BamHI and BglII, inserted into BamHI and BglII sites of the pQE-16 plasmid (Qiagen), and sequenced to confirm their sequences and orientations. The resultant plasmid, pQE-hPGK, was transformed into Escherichia coli strain XL-1-Blue. E. coli XL-1-Blue harboring the plasmid pQE-hPGK were grown in LB medium containing ampicillin (100 μg/ml) at 37 °C until the A$_{600}$ reached 0.6. Expression of the recombinant human PGK with the hexahistidine tag at the carboxyl terminus was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM and cells grown for another 2 h before harvesting. The His-tagged human PGK was purified using a nickel-nitriilotriacetic acid-agarose column according to the manufacturer’s protocol (Qiagen).

**Immunoblotting**

SDS-polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (36). For Western blotting, proteins were resolved by electrophoresis in a 10% polyacrylamide gel containing SDS, and electroblotted onto a PVDF membrane. The membrane was probed with monoclonal β-tubulin antibody (1:500 dilution) and HRP-conjugated goat anti-mouse IgG polyclonal antibody for the detection of tubulin, or with affinity-purified polyclonal PGK antibody (1:100 dilution) and HRP-linked protein A for the detection of PGK. The immunocomplex was detected by ECL detection system. To detect the protein band(s) that interacts with tubulin by West-Western blotting, the blotted membrane was first blocked with 3% BSA in a binding buffer (20 mM HEPES-KOH (pH 7.9)), 100 mM KCl, 5 mM MgCl$_2$, 5 mM 2-mercaptoethanol) at 4 °C overnight, and then incubated with or without highly purified tubulin (50 μg/ml) in the binding buffer containing 0.1% BSA at room temperature for 3 h. After extensive washing of the membrane, tubulin on the membrane was detected by using anti-β-tubulin monoclonal antibody and 4-chloro-1-naphthol detection system.

**Protein Concentration**

Protein concentrations were determined by the methods of Bradford (37) using BSA as the standard.

**RESULTS**

**Purification of the Host Factors—Cellular proteins (host factors) have been considered to have essential roles in the in vitro mRNA synthesis of SeV. We have previously shown that the host factor activity in the bovine brain extract is separated into two complementary fractions by hydroxylapatite column chromatography (18). These two fractions synergistically stimulated the in vitro mRNA synthesis of SeV, and one could be replaced by highly purified tubulin (18). In order to identify and characterize the host factor(s) that acts complementally to tubulin, we attempted to purify the active components from the bovine brain extract. In this study, hydroxylapatite column chromatography was performed as the first step essentially as described by Mizumoto et al. (18) with some modifications. Bovine brain extract (S-100 fraction) was loaded onto a hydroxylapatite (Hypatite C) column, and proteins werestep-eluted with 75, 150, 300, and 600 mM potassium phosphate buffer, as illustrated in Fig. 1A. In these conditions, the host
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FIG. 2. Isolation of a host factor, p46, by Heparin-Sepharose column chromatography. A, the 150 mM KCl eluate (BS150, 6.5 mg) from Blue-Sepharose column was loaded onto a Heparin-Sepharose column (inner diameter, 0.8 × 9.6 cm) pre-equilibrated with TEMG/150 mM KCl. After washing the column with 10 ml of the same buffer, proteins were eluted with 50 ml of linear gradient of 150–350 mM KCl in TEMG. The aliquot (0.3 µl) of each column fraction was subjected to in vitro mRNA synthesis with Sendai virus particles (6 µg) in the presence of HA300 (10 µg) and BS20 (8.0 µg). The transcription-stimulatory activity is expressed as the amounts of [32P]UMP incorporated into the 18 S mRNA band (○). The activity was eluted as a single peak at 220 mM KCl. B, proteins in each column fraction were analyzed by electrophoresis in a 10% SDS-polyacrylamide gel followed by Coomassie Brilliant Blue staining. Lanes 1–3 indicate 30 µg of bovine brain extracts S-100 (BE), 20 µg of Hypatite C column 75 mM potassium phosphate eluate (HA75), and 3.0 µg of Blue-Sepharose column 150 mM KCl eluate (BS150), respectively. Lanes 4–10 indicate 6-µl aliquots of column fractions. The positions of marker proteins are shown on the left.

Identification of p46 as PGK—To identify p46, we performed amino acid sequence analysis of tryptic peptides of p46 and then searched for similar sequences in the protein data base (PIR) using the BLAST program. Surprisingly, amino acid sequences of three peptides were highly homologous to those of a eukaryotic glycolytic enzyme, PGK (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (Fig. 4A). These sequences almost completely matched the sequences of mammalian PGK, such as mouse PGK-1 (38) and human PGK-1 (39). The molecular weights of various PGKs were reported to be 46,000 ± factor activity was separated as observed before (18) into two complementary fractions, the 75 and 300 mM potassium phosphate eluates (referred to HA75 and HA300, respectively). Western blot analysis with monoclonal anti-tubulin antibody revealed that tubulin was mainly detected in HA300, but almost not in HA75 (data not shown). Although the addition of HA75 alone (Fig. 1B, lane 2) or HA300 alone (lane 8) to the in vitro transcription mixture containing SeV particles (lane 1) gave weak transcription-stimulatory activities, the combinatorial addition of HA75 and HA300 synergistically stimulated the reaction to give 18 S size transcript (lane 9). To purify the host factor(s) contained in HA75, the fraction was loaded onto a Blue-Sepharose column, and proteins were step-eluted with 20, 150, and 300 mM KCl (Fig. 1A). The 20, 150, and 300 mM KCl eluates are referred to as BS20, BS150, and BS300, respectively. When BS fractions were added to the transcription reaction mixture without HA300, each BS fraction alone (Fig. 1B, lanes 3–5) or in combination (lanes 6 and 7) supported little, if any, stimulatory activity. However, the simultaneous addition of BS20 and BS150 (lane 13) or all Blue-Sepharose column fractions (lane 14) in the presence of a saturating amount of HA300 restored the transcription-stimulatory activity to levels comparable to that obtained with the combination of HA75 and HA300 (lane 9), while each Blue-Sepharose column fraction alone failed to stimulate transcription (lanes 10–12), even in the presence of HA300. These results indicate that the host factor activity in HA75 can be further separated into the BS20 and BS150 fractions.

One of the two complementary fractions from Blue-Sepharose column, BS150, was further purified through a Heparin-Sepharose column. As shown in Fig. 2A, proteins were eluted with a linear gradient of 150–350 mM KCl, and the transcription-stimulatory activity of each fraction was assayed in the presence of HA300 and BS20. The activity was eluted as a single peak at 220 mM KCl. As seen in Fig. 2B, the active fractions (fractions 110–126) contained a single polypeptide with an apparent Mr of 46,000 (p46), which was coeluted with the transcription-stimulatory activity (lanes 5–9). To investigate the complementarity of the host factors for the transcription stimulation in a more purified system, p46 and BS20 were subjected to an in vitro transcription system reconstituted with highly purified tubulin instead of HA300 using viral particles or viral RNP (Fig. 3). In the case of transcription with virions (Fig. 3B), the addition of BS20 alone (lane 2), p46 alone (lane 3), or their combination (lane 4) caused a weak stimulation activity. In the presence of a saturating amount of highly purified tubulin, which by itself had some stimulatory activity (lane 5), neither BS20 (lane 6) nor p46 (lane 7) altered the reaction. However, the combination of three factors, BS20, p46, and tubulin, resulted in significant stimulation of mRNA synthesis (lane 8). In order to characterize the host factors using a more purified viral transcription machinery, the viral RNP complex was isolated from detergent-disrupted viral particles as described under “Experimental Procedures” (Fig. 3A, lane 2) in which most of the envelope glycoproteins were removed, and used for transcription in place of viral particles (Fig. 3C). Transcription was similarly and effectively stimulated depending on the addition of three factors: tubulin, p46, and BS20 (lane 8). These data suggest that the simultaneous presence of p46, tubulin, and unknown factor(s) in BS20 is required for the maximal transcription of the SeV genome, and that these factors act directly on the viral RNP to stimulate transcription.

Identification of p46 as PGK—To identify p46, we performed amino acid sequence analysis of tryptic peptides of p46 and then searched for similar sequences in the protein data base (PIR) using the BLAST program. Surprisingly, amino acid sequences of three peptides were highly homologous to those of a eukaryotic glycolytic enzyme, PGK (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (Fig. 4A). These sequences almost completely matched the sequences of mammalian PGK, such as mouse PGK-1 (38) and human PGK-1 (39). The molecular weights of various PGKs were reported to be 46,000 ±
Fig. 4. Identification of p46 as phosphoglycerate kinase. A, the tryptic peptides of p46 were fractionated by reverse-phase high performance liquid chromatography. Three (peptides 1–3) of the obtained peptides were subjected to the amino acid sequence analysis, followed by the homology search from the protein data base using the BLAST program. Partial amino acid sequences of the three peptides were shown with vertical lines. B, partial amino acid sequences of the three peptides were shown with vertical lines. C, Western blot analysis for PGK. BS150 (lane 1, 3.0 μg) and Heparin-Sepharose column fractions (lanes 2–8, each 6.0 μg) were immunoblotted with an affinity-purified anti-rabbit muscle PGK polyclonal antibody. In lane 9, a mixture of PGKs (each 0.1 μg) from rabbit muscle and yeast was used as the positive control.

Fig. 5. SeV mRNA synthesis-stimulatory activity of p46 and PGK from various sources. A, p46 (lane 1, 0.5 μg), rabbit muscle PGK (lane 2, 0.5 μg), yeast PGK (lane 3, 0.5 μg), and recombinant human PGK (lane 4, 0.4 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and stained by Coomassie Brilliant Blue. The positions of marker proteins are shown on the left. B, various amounts (10, 30, and 100 ng) of p46 (●), rabbit muscle PGK (○), yeast PGK (□), or recombinant human PGK (▲) were added to SeV transcription reactions supplemented with tubulin (3.0 μg) and BS20 (8.0 μg). Glyceraldehyde-3-phosphate dehydrogenase (▲) from rabbit muscle was used as the negative control. The transcription-stimulatory activity is expressed as the amounts of [32P]UMP incorporated into the 18 S mRNA band.

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PGK catalyzes the reversible interconversion of ADP + 1,3-bisphosphoglycerate to ATP + 3-phosphoglycerate. To assess p46 as bovine PGK, we examined the enzymatic activity of PGK at various steps during purification of p46. As shown in Table I, a majority of PGK activity was copurified with transcription-stimulatory activity of p46. The specific activity as the enzyme of the final column fraction, HS220 (481 units/mg protein) was comparable to that of rabbit muscle PGK (431 units/mg protein) or of yeast (S. cerevisiae) PGK (646 units/mg protein). An effective purification of PGK (p46) was achieved by three steps of column chromatography with an increase of 125-fold in the specific activity and with 59% recovery. Furthermore, PGK activity was coeluted with the transcription-stimulatory activity from a Heparin-Sepharose column (Fig. 4B). Since p46 was not separated from the other complementary factor(s) until Blue-Sepharose column chromatography, the specific activities of transcription in the presence of BS20 and tubulin were measured for the last two steps of purification. The final step of purification led to a 15-fold increase in the specific activity of transcription-stimulatory activity, which was nearly comparable to a 13-fold increase in PGK activity. As shown in Fig. 4C, affinity-purified polyclonal anti-rabbit muscle PGK antibodies, which reacted with rabbit muscle PGK and yeast one (lane 9), recognized p46 in BS150 (lane 1) as well as in HS fractions (lanes 3–7).

To investigate whether the transcription-stimulatory activity of p46 can be replaced by PGK from other sources, highly purified PGKs from rabbit muscle or Saccharomyces cerevisiae instead of p46 were added to the transcription reaction in the presence of complementary factors, tubulin and BS20 (Fig. 5). Each preparation from rabbit muscle (lane 2) or yeast (lane 3) contained a single polypeptide with approximate Mr values of 43,000 or 48,000, respectively (Fig. 5A). As shown in Fig. 5B, PGK either from rabbit muscle or from yeast was also capable of stimulating SeV transcription in the presence of tubulin and BS20 in a dose-dependent manner as was p46, with a similar specific activity. By contrast, the addition of the rabbit muscle type GAPDH with tubulin and BS20 had no effect on the SeV transcription. To further provide evidence in support of a direct involvement of PGK in the SeV transcription, we tested whether a recombinant PGK can substitute for p46. We constructed human PGK-1 expression vector to produce His-tagged recombinant protein and purified the protein to almost homogeneity, which had an apparent Mr of 46,000 (Fig. 5A, lane 4) corresponding to the calculated Mr (46,112) of the tagged protein. The purified recombinant PGK exhibited the PGK activity (363 units/mg protein). As seen in Fig. 5B, the purified recombinant human PGK also stimulated the SeV transcription reaction in the presence of tubulin and BS20 with a specific activity similar to those obtained with native PGKs. From these biochemical and immunological data, we conclude that p46 is the bovine PGK.

Functional Properties of the Host Factors—It is interesting to see whether the enzymatic activity of PGK is directly involved in the transcription-stimulatory activity or not. We tested the effects of 3-phosphoglycerate, a substrate, and di-glycerol-3-phosphate, a competitive inhibitor against 3-phosphoglycerate,
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Table I

| Fraction | Protein (mg) | PGK activity | SeV transcription |
|----------|--------------|--------------|------------------|
|          | Specific activity (units/mg) | Total activity (units) | Yield % | Specific activity (nmol/mg) | Total activity (nmol) | Yield % |
| BS25     | 143          | 3.87         | 553              | 100    | 1.29                       | 16.8             | 100    |
| HA75     | 40           | 6.34         | 543              | 98     | 1.93                       | 13.0             | 21     |
| BS150    | 13           | 38.6         | 502              | 91     | 19.4                       | 79.4             | 100    |
| HS220    | 0.68         | 481          | 327              | 59     | 14.0                       | 59.4             | 100    |

a From 20 g of bovine brain.
b The total activity of the BS150 was assumed to be 100%, because p46 was not separated from other complementary factor(s) until the step of Blue-Sepharose column chromatography.

on the transcription-stimulatory activity of p46 in the range of 1–3 mM (Table II), since the $K_m$ value for 3-phosphoglycerate (34, 40) and the $K_m$ value for Dl-glycerol-3-phosphate (41) were reported to be 1 and 0.7 mM, respectively. Increasing the concentrations of both reagents had little effect on the transcription-stimulatory activity of p46. Thus, the enzymatic activity per se of PGK does not seem to be required for its activity.

To elucidate the functions of the host factors in SeV mRNA synthesis, their effects on transcription initiation and elongation reactions were examined. First, viral particles were incubated with the host factors in various combinations without four NTPs, followed by ultracentrifugation to obtain viral RNPs (initiation complexes) as the pellets. Four NTPs were then added to the isolated viral RNPs to determine their mRNA synthesis (19). When virus particles were preincubated with highly purified tubulin instead of bovine brain extract, the isolated viral RNP could also synthesize viral mRNAs (lane 5). These data further confirmed the notion that tubulin plays an important role in the initiation complex formation (19). In contrast, when virus particles were preincubated with BS20 (lane 2), p46 (lane 3), or their combination (lane 4), neither RNP pellets obtained by centrifugation supported mRNA synthesis. BS20 and p46 had little effect on the initiation complex formation with tubulin (lanes 6–10). We analyzed these RNP pellets by electrophoresis in a SDS-polyacrylamide gel followed by silver staining (Fig. 6B) and Western blotting using anti-tubulin antibodies as the probe (Fig. 6C). All the active initiation complexes formed in the presence of tubulin contained nearly identical amounts of tubulin (Fig. 6B, lanes 7–10), which was confirmed by Western blotting (Fig. 6C, lanes 7–10), indicating that neither BS20 and p46 affected integration of tubulin into the initiation complexes. Thus, it seems that p46 and the unknown factor(s) in the BS20 fraction participate in transcription after the initiation complex formation.

We analyzed the effect of host factors on the mRNA chain elongation reaction from the isolated initiation complexes formed in the presence of tubulin (Fig. 7). The initiation complexes were prepared by incubating virus particles with or without highly purified tubulin, followed by ultracentrifugation, and then subjected to transcription reactions in the presence or absence of the host factors. As mentioned above, the initiation complex formed with tubulin showed a transcription activity, although at a low level, without addition of the other host factors (Fig. 7, lane 6), and further addition of tubulin to the complex had no effect on transcription activity (lane 10), indicating that a saturating amount of tubulin was integrated into the initiation complex. Either BS20 alone (lane 7) or p46 alone (lane 8) also failed to stimulate the transcription from the isolated initiation complex. However, when p46 (PGK) and BS20 were simultaneously added to the RNA chain elongation reaction from the isolated initiation complex, mRNA synthesis was dramatically stimulated (lane 9). On the other hand, addition of p46 (PGK) and BS20 to RNA chain elongation reaction from the RNP, which had not been preincubated with tubulin, showed only a little mRNA synthesizing activity (lane 4). From these data, it seems that p46 and BS20 act cooperatively on the tubulin-containing active initiation complex at the level of RNA chain elongation.

To investigate whether these factors can interact with tubulin, West-Western blot analysis was performed using highly purified tubulin as the probe (Fig. 8). Fig. 8A shows the patterns of protein transfer of BS20, p46, tubulin, and virus particles from the gel to a PVDF membrane monitored by Amido Black staining. For West-Western blotting, proteins were sim-
TABLE II

| Additions | Transcription-stimulatory activity (pmol) |
|-----------|-----------------------------------------|
| None      | 0.31                                    |
| 3-phosphoglycerate | 0.35                                    |
| 1 mM      | 0.33                                    |
| 2 mM      | 0.34                                    |
| 3 mM      | 0.32                                    |
| 10 mM 3-phosphoglycerate | 0.32                                    |
| 2 mM 3-phosphoglycerate | 0.32                                    |
| 3 mM 3-phosphoglycerate | 0.29                                    |

**FIG. 7.** Effect of p46 and BS20 on the chain elongation step. The initiation complex were prepared by incubating SeV particles (300 μg) without (lanes 1–5) or with (lanes 6–10) tubulin (150 μg), followed by ultracentrifugation as described under “Experimental Procedures.” Aliquots of the RNP pellets (corresponding to 6 μg of staining with SeV particles) were subjected to the chain elongation reaction in the presence of indicated factors. Factors used were tubulin (3.0 mM), p46 (0.1 mM), and BS20 (8.0 μg). After elongation reaction, RNAs were analyzed by agarose gel electrophoresis and autoradiographed.

**FIG. 8.** Detection of tubulin-binding proteins by West-Western analysis. A, BS20 (lanes 1, 20 μg), p46 (lanes 2, 0.5 μg), SeV particles (lanes 3, 6 μg), and tubulin (lanes 4, 0.5 μg) were resolved by electrophoresis in a 10% SDS-polyacrylamide gel. After electrophoresis, proteins on the gel were transferred to an Immobilon P membrane, followed by staining with Amido Black. Positions of viral proteins (NP, P, F1, and HN) and marker proteins are shown on the right. B, after the pretreatment of the membrane without (lanes 1–4) or with (lanes 5–8) tubulin (50 μg/ml), tubulin remaining on the membrane was detected by using an anti-chicken brain β-tubulin monoclonal antibody. Closed and open arrowheads indicate the positions of tubulin-binding proteins (p52 and p27) in BS20 (lane 5) and p46 (lane 6), respectively.

**FIG. 9.** Effects of tubulin, p46, and BS20 on leader RNA synthesis of SeV. In vitro plus leader RNA synthesis of SeV was carried out with 6 μg of virus particles under the same conditions for mRNA synthesis except that one of the nucleotide substrates, UTP, was omitted and in the presence of indicated factors. Factors used were tubulin (3.0 mM), p46 (0.1 μg), and BS20 (8.0 μg). After transcription reaction, 32P-labeled short transcripts were resolved by electrophoresis in a 20% polyacrylamide gel containing 8 M urea and detected by autoradiography. Lane 1 indicates transcription products with virus particles alone. Closed arrowhead indicates the band of the major prematurely terminated leader RNA species (about 30 nucleotides). The positions of the xylene cyanol (XC) and bromphenol blue (BPB) dyes are shown on the left.
Phosphoglycerate Kinase Is Required for SeV Transcription

In our in vitro mRNA synthesizing system using purified SeV particles, viral mRNA synthesis was almost completely dependent on the presence of host cell proteins (host factors), one of which was suggested to be tubulin, a cytoskeletal protein (18). In this study, we attempted to purify the host factor(s) which acts complementarily to tubulin to stimulate viral mRNA synthesis and found the activity can be separated into two complementary fractions (BS20 and BS150) by Blue-Sepharose column chromatography (Fig. 1). These results suggest that p46 (PGK) and factor(s) present in BS20 may be specifically involved in the mRNA synthesis of SeV.

DISCUSSION

In our in vitro mRNA synthesizing system using purified SeV particles, viral mRNA synthesis was carried out under the same conditions for SeV using 1 μg of VSV particles instead of SeV particles in the presence of various factors as indicated. The following amounts of factors were added to the reaction: tubulin (3.0 μg), p46 (0.1 μg), and BS20 (8.0 μg). After reaction, 32P-labeled transcripts were resolved by electrophoresis in an 1.2% agarose gel and detected by autoradiography. Lane 1 indicates transcription products with virus particles alone (VSV alone).

In contrast, BS20 potently repressed tubulin-mediated leader RNA synthesis (lane 6), while p46 (PGK) failed to stimulate tubulin-mediated leader RNA synthesis with (lane 8) or without (lane 7) BS20. In addition, no significant effect of these factors on the mRNA synthesis of VSV was observed (Fig. 10). These results suggest that p46 (PGK) and factor(s) present in BS20 may be specifically involved in the mRNA synthesis of SeV.

The addition of 3-phosphoglycerate, a substrate, or DL-glycerol-3-phosphate, a substrate analogue inhibitor, did not affect transcription (Table II). Transcription of SeV was stimulated by both mammalian PGKs and yeast PGK, although the identity of overall primary amino acid sequences between mammalian PGK and yeast PGK is about 64% (38, 42).

Moyer et al. (20) reported that SeV transcription is inhibited by a monoclonal antibody against β-tubulin in an in vitro transcription system with SeV-infected cell lysate. They also mentioned that when the cell extract from uninfected BHK cells was added to detergent-disrupted SeV particles, several classes of viral mRNA were synthesized. By contrast, although addition of purified tubulin alone to SeV particles led to the synthesis of leader-like RNA and mRNA for the 5'-proximal NP gene, downstream mRNAs were not transcribed. Similar results were reported for measles virus (12, 16). Thus it seems that an additional factor(s) may be required for complete transcription of these viral genomes. These observations are in agreement with our results presented before and in this work. At present, however, it is not clear whether the complementary factors PGK (p46) and BS20 preferentially stimulate transcription of downstream mRNAs rather than that of mRNA for the 5'-proximal NP gene.

We have previously shown that a transcription initiation complex formed with bovine brain extract contains tubulin molecules (about 250 copies/genome), and that the amounts of tubulin integrated into the complexes correlate with their transcriptional activity (19). In the present work, we showed that highly purified tubulin is also integrated into the viral RNP complex at the transcription initiation step and activates transcription from the complex (Fig. 6), confirming that tubulin is directly involved in transcription initiation complex formation. By contrast, p46 (PGK) and BS20, when added together, dramatically stimulated chain elongation from the initiation complex formed with purified tubulin, without affecting the initiation complex formation (Fig. 7). Moreover, direct interactions between p46 (PGK) and tubulin was shown by West-Western blot analysis. These observations suggest that p46 (PGK) stimulates SeV mRNA synthesis at the elongation step, probably through the interaction of p46 (PGK) with tubulin that has been integrated into the initiation complex. Alternative possibility is that p46 (PGK) and BS20 may function in the reutilization of the viral RNA polymerase.

De et al. (21) purified a host factor for in vitro transcription of human parainfluenza virus type 3 (HPIV3), a paramyxovirus closely related to SeV, from the cytoplasmic extract of uninfected cells, and identified it as actin, a cytoskeletal protein. Furthermore, they found that both polymeric and monomeric forms of actin are integrated into the viral RNP complex, but only the polymeric form can activate HPIV3 transcription, and HPIV3 transcription is not stimulated by tubulin (22). In our SeV mRNA synthesizing system, highly purified actin also stimulated mRNA synthesis, but specific activity of tubulin dimer was 7-fold higher than that of the actin monomer (data not shown). On the other hand, they reported that GAPDH, a glycolytic enzyme, interacts with the 3'-terminal leader sequence of the genome as well as the plus leader RNA (43). It is interesting to note that GAPDH catalyzes formation of 1,3-bisphosphoglycerate, which is in turn the substrate for its downstream enzyme, PGK, in the sequential glycolytic reaction. It was reported that GAPDH is able to interact with not only PGK (44) but also tubulin (45). However, significant effect on the transcription with SeV particles was not observed by the addition of rabbit muscle type GAPDH in the various combinations with the host factors (tubulin, PGK, and BS20) for SeV transcription (Fig. 5, data not shown). We could not detect
GAPDH in SeV particles either by Western blotting with anti-GAPDH antibody or by the assay for its enzymatic activity (data not shown).

Most of the glycolytic enzymes have been shown to interact with filamentous actin (46–49) as well as microtubules (50–52), suggesting that it is a common feature of these enzymes to bind to cytoskeletal proteins. We also demonstrated that PGK interacts with tubulin (Fig. 8). However, physiological significance of binding of the glycolytic enzymes to cytoskeletal proteins is still unknown. Many glycolytic enzymes were suggested to interact with the carboxyl-terminal tails of the tubulin subunits (53), which is important sites for regulation of the assembly of microtubules through the binding of microtubule-associated proteins (reviewed in Ref. 54). Previous studies suggested that the carboxyl-terminal tails of the tubulin subunits, which are highly negatively charged and exposed on the surface of the tubules, may be involved in SeV transcription (16, 18). It is important to investigate the significance of the acidic tails of tubulin in the transcription-stimulatory activity and the recruitment of other host factors such as PGK. Tubulin may have multiple roles in SeV mRNA synthesis, e.g., tubulin 1) dissociates M protein, which acts as the negative regulator for RNA synthesis from the viral RNP, 2) stabilizes the RNP structure to maintain the template as a transcriptionally active state; and 3) recruits other host factors to the viral RNP. It remains to be seen at which precise steps of viral transcription PGK and BS20 act, and to which viral or cellular proteins they interact.

Our studies indicate that tubulin functions as a common host factor for both mRNA synthesis and plus leader RNA synthesis of SeV, while PGK acts specifically for mRNA synthesis (Fig. 9). Moyer et al. (20) also reported that purified tubulin stimulates the synthesis of leader-like RNA from the detergent-disrupted SeV particles. Interestingly, crude BS20 fraction, which exhibits the mRNA synthesis-stimulatory activity, was found to strongly repress leader RNA synthesis. Preliminary purification of BS20 revealed that both activities are separated into different fractions (data not shown). It is interesting to note that the negative factor which inhibits leader RNA synthesis, was found to be distinct from an RNase or a proteinase, and that it may act specifically to inhibit plus leader RNA synthesis, because this factor did not inhibit in vitro mRNA synthesis of SeV or of VSV. In addition, we previously reported an inhibitory factor that potently inhibits SeV mRNA synthesis from rat liver extract (55). Thus, mRNA synthesis as well as plus leader RNA synthesis may be regulated by both positive and negative factors.

The specific action of the host factors, tubulin, PGK, and BS20, in SeV mRNA synthesis also comes from the following observations. These factors did not show any effect on the in vitro mRNA synthesis with VSV particles (Fig. 10). Although either purified VSV particles or the viral RNP from virions are capable of transcribing mRNA in vitro, soluble cell extracts increase the level of mRNA synthesis (56). We also observed that in vitro transcription with VSV particles was stimulated 2–3-fold either by the addition of bovine brain extract or HeLa cell extract (data not shown), suggesting that at least tubulin, PGK, and BS20 are not responsible for the stimulation VSV transcription by crude cell extracts in our system. Hill et al. (57) reported that in vitro transcription and replication of VSV was stimulated by microtubule-associated proteins derived from HeLa cells or bovine cell, but not by tubulin. By contrast, Moyer et al. (20) reported that in vitro transcription of VSV was stimulated by purified tubulin and was inhibited by a monoclonal antibody against β-tubulin. At present, we do not know the reason for this discrepancy in the requirement of the host factors for in vitro mRNA synthesis of VSV. Recently, a reconstituted VSV transcription system was created using genome RNA encapsidated with nucleocapsid protein (N) and recombinant L and P proteins (58). In those studies, it was revealed that phosphorylation of P protein with cellular casein kinase II (59) and the association of L protein with protein synthesis elongation factor EF-1 (60) are essential for mRNA synthesis of VSV, and these host factors are packaged within the viral particles. Therefore, to elucidate precise functions of the host factors for transcription of SeV, it is important to develop a transcription system reconstituted with highly purified viral proteins and host factors.

Identification of a host factor as PGK will be helpful for understanding not only the regulatory mechanisms of SeV RNA synthesis but also the precise replication site of SeV in the cell. Since PGK is abundant and ubiquitous in cytoplasm of eukaryotic cells, its usage as a transcription factor seems to be feasible for the cytoplasmic replication of SeV. It is important to note that PGK was also identified as a subunit of primer recognition proteins, which are cofactors of DNA polymerase α and may have a role in lagging strand DNA replication in nuclei (61, 62). These observations including the involvement of PGK in cellular DNA replication and in viral transcription have suggested that PGK may contribute to regulation of multiple cellular as well as viral processes in addition to glycolysis. In this regard, a detailed study along the precise function of PGK and other host factors for SeV multiplication should lead to better understanding of host-virus interaction processes. Bacterially expressed recombinant PGK will make it possible to map the domain(s) of PGK required for the SeV transcription through site-directed mutational analysis. Experiments are in progress to address the significance of these interactions by delineating the role of PGK and BS20 in SeV transcription using a reconstituted RNA synthesizing system.

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