

# Purification of a terminal uridylyltransferase that acts as host factor in the *in vitro* poliovirus replicase reaction

(RNA synthesis/hairpin priming)

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**ABSTRACT** Poliovirus RNA polymerase requires a host factor to initiate RNA synthesis *in vitro*. The host factor was previously purified to near homogeneity from HeLa cells but was not assigned an enzymatic activity. This report describes the purification of a terminal uridylyltransferase that can act as host factor. By all criteria examined it is identical to the factor purified previously. It has the same molecular weight (68,000), chromatographic properties, and cellular localization. We present evidence that terminal uridylyltransferase can add uridine residues to the 3' poly(A) end of virion RNA and that these anneal back to the poly(A) and form a hairpin primer for polymerase.

Poliovirus has a 7500-nucleotide genome of positive polarity. Its 5'-terminal nucleotide is covalently linked to a protein (VPg), and the 3' end consists of a heterogeneous poly(A) tract averaging 75 nucleotides (1-6). Poliovirus encodes an RNA-dependent RNA polymerase, crude preparations of which can copy virion RNA *in vitro* (7, 8). Upon purification there is an absolute requirement for a primer (9-11). Added oligo(U) can anneal to the 3'-terminal poly(A) of virion RNA and prime RNA synthesis. A protein fraction from uninfected cells can replace the oligo(U) primer and provide the initiation function for the viral polymerase (9). This protein, "host factor," allows the viral polymerase to transcribe any poly(A)-containing RNA, with no clear specificity for virion RNA (11).

We have reported preliminary results that suggested that host factor is a terminal uridylyltransferase (TUTase) (12). We proposed that, *in vitro*, host factor uridylylates the 3'-terminus of virion RNA creating a hairpin primer recognized by poliovirus RNA polymerase. To test this hypothesis we have attempted to separate host factor and uridylyltransferase activities by further purification. Although our initial characterization of TUTase used enzyme preparations from rabbit reticulocytes (12), host factor has been purified from HeLa cells (10, 13). It is apparently a  $M_r$  68,000 cytoplasmic protein partitioned between ribosomal and soluble fractions. The approach taken here was to purify TUTase from the soluble phase of HeLa cells and test it for host factor activity. We find that host factor and TUT activities copurify through all procedures investigated.

## MATERIALS AND METHODS

Many of the procedures used in this report have been described (12).

**Purification of TUTase from HeLa Cells.** HeLa S3 cells were grown in suspension in Eagle's minimal essential medium (MEM) supplemented with 7% (vol/vol) horse serum, by the Massachusetts Institute of Technology Cell

Culture Center. Starting material for purification was  $6-9 \times 10^9$  cells. Cells were swollen in 100 ml of buffer I [10 mM Hepes-KOH, pH 8.0/15 mM NaCl/1.5 mM  $MgCl_2$ /5 mM 2-mercaptoethanol plus aprotinin (600 Kallikrein units/ml)] on ice, and disrupted with a Dounce homogenizer. The extract was centrifuged at  $27,000 \times g$  for 30 min at 2°C. The supernatant (cytoplasmic extract) was centrifuged at  $200,000 \times g$  for 2 hr at 4°C yielding a clear S200 supernatant fraction.

The S200 fraction was applied to a phosphocellulose column (Whatman P11), and the column was developed with a gradient of increasing salt. All TUTase activity bound to the column, and there was only one peak of activity in the eluent. TUTase activity eluted at about 360 mM KCl.

Peak fractions from the phosphocellulose column were pooled and diluted with buffer A [20 mM Hepes-KOH, pH 8.0/0.1 M EDTA/5 mM 2-mercaptoethanol/5% (vol/vol) glycerol] to bring the concentration of KCl down to  $\leq 50$  mM. This material was loaded onto a DEAE-Sephacel (Pharmacia) column. The column was washed thoroughly with buffer A/50 mM KCl, and protein was eluted with a gradient of increasing salt. All TUTase activity bound to the column, and there was only one peak of activity eluting between 80 and 120 mM KCl. Fractions with the highest activity were pooled and concentrated.

This material was applied to 10-30% glycerol gradients prepared in Buffer A/50 mM KCl, and these were centrifuged at 2°C in a Beckman SW41 rotor for about 40 hr at 40,000 rpm. Gradient fractions were collected from the bottom of the tube, and small portions were assayed for TUTase activity. Fractions with the highest activity were pooled.

TUTase purified through the glycerol gradient was further purified using a poly(A)-agarose column [AGPoly(A), Pharmacia]. Proteins were eluted with a gradient of increasing salt. TUTase eluted in a single peak between 100 and 200 mM KCl; the exact position of the peak varied slightly with different batches of poly(A)-agarose. Fractions with the highest activity were pooled. Protein concentration was estimated by electrophoresing the sample through denaturing polyacrylamide gels and comparing band intensity with standards of known concentration. The material was made 25% in glycerol and stored at -70°C. At this stage the enzyme could no longer tolerate more than several hours at 4°C.

To assay TUTase activity in the cytoplasmic extract and S200 it was necessary to remove factors that inhibited the assay (presumably RNA) by passing the protein over a small DEAE column in buffer A containing 150 mM KCl. TUTase does not bind to DEAE at 150 mM KCl.

**Replicase Reaction.** Replicase reactions were carried out as described (12), by using CTP as the labeled nucleotide. Purified HeLa TUTase at an estimated concentration of

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Abbreviations: TUTase, terminal uridylyltransferase; VPg, viral protein, genome-linked.

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about 1  $\mu\text{g}/\text{ml}$  replaced host factor and oligo(U). Reaction mixtures were incubated for 60 min.

**Isolation of Poly(A) from Virion RNA Incubated with TUTase.** Virion RNA was incubated with rabbit reticulocyte TUTase/host factor (12) under replicase reaction conditions in the absence of poliovirus polymerase. The reticulocyte enzyme was a gift of Dan Levin. The [ $\alpha$ - $^{32}\text{P}$ ]UTP labeled product was digested with RNase T1 (cleaves after guanosine residues to leave mono- and oligonucleotides with a 3' phosphate group) and isolated by binding to poly(U) immobilized on filters as described by Spector and Baltimore (5). This material was subjected to nearest neighbor analysis (12).

**Reverse Transcriptase Assay.** Reverse transcriptase reactions were carried out in 50  $\mu\text{l}$  of 22 mM Tris-HCl, pH 8.5/2 mM dithiothreitol/3 mM  $\text{Mg}(\text{OAc})_2$ /27 mM NaCl/0.7 mM dATP/0.7 mM dGTP/0.7 mM dCTP/0.7 mM UTP/40  $\mu\text{M}$  dTTP/1  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]dTTP (New England Nuclear)/2 units of RNasin RNase inhibitor (Promega Biotech, Madison, WI)/0.3  $\mu\text{g}$  of virion RNA/1.5 units of avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, St. Petersburg, FL), and, as indicated, either 20  $\mu\text{g}$  of oligo(U)/ml or about 0.5  $\mu\text{g}$  (total protein) of rabbit reticulocyte TUTase/host factor (12). The mixtures were incubated at

30°C for 60 min. Products were spotted onto DE-81 paper and analyzed as for the TUTase assay.

## RESULTS

TUTase has been identified as a contaminant in eukaryotic initiation factor 2 (eIF2) preparations from rabbit reticulocyte ribosomal salt wash (12). These fractions of eIF2 could replace host factor in the *in vitro* poliovirus replicase reaction. Preliminary experiments indicated that HeLa cells also contain a TUTase activity that is partially soluble and partially associated with ribosomes (12). To purify that activity further we first developed an assay that could quantitate the activity in crude fractions. This was accomplished by DEAE-Sephacel chromatography that removed apparent inhibitors (probably RNA). Using this treatment we found that 65% of the TUTase activity is soluble after high speed centrifugation (as found earlier for host factor; ref. 10) and chose to purify the soluble material.

TUTase was then purified by conventional ion exchange chromatography and glycerol gradient centrifugation. Fig. 1 shows the chromatographic profiles; Table 1 shows the recovery and purification at various steps.

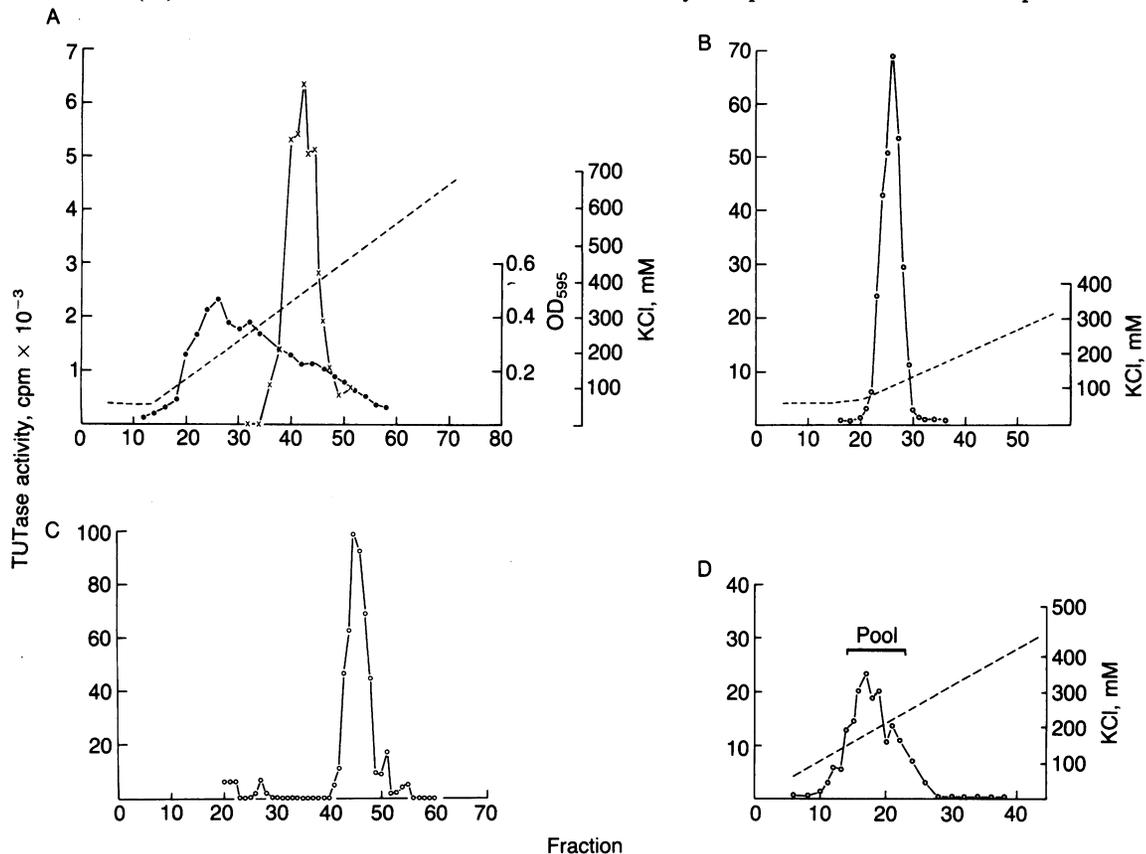


FIG. 1. Purification of TUTase. Incorporation of [ $\alpha$ - $^{32}\text{P}$ ]UTP by TUTase during various purification steps starting from postribosomal supernatant is shown. (A) Chromatography of step 2 TUTase on phosphocellulose. Postribosomal supernatant (S200, step 2) was applied to a column ( $2.6 \times 15$  cm) of phosphocellulose equilibrated with buffer A/50 mM KCl. Proteins were eluted with a 400-ml linear gradient of 50 mM to 1 M KCl (---) in buffer A, fractions (about 5.2 ml) were collected, and activity was measured ( $\times$ ). Protein concentrations were measured using the BioRad assay and shown here as  $\text{OD}_{595}$  ( $\bullet$ ). An  $\text{OD}_{595}$  of 0.4 was approximately equal to 1 mg of protein/ml. (B) Chromatography of step 3 TUTase on DEAE-Sephacel. Pooled phosphocellulose peak material (step 3) was diluted and applied to a column ( $1.6 \times 8$  cm) of DEAE-Sephacel equilibrated with buffer A/50 mM KCl. Proteins were eluted with an 80-ml linear gradient of 50–550 mM KCl in buffer A, 1.2-ml fractions were collected, and activity was measured. (C) Sedimentation of step 4 TUTase through a glycerol gradient. The peak fractions from DEAE-Sephacel chromatography (step 4) were pooled, and a portion was applied to a 10–30% glycerol gradient. Fractions of about 0.17 ml were collected and the activity was measured ( $\circ$ ). The activity sedimented at approximately 4S. Although the gradient used here was analytical rather than preparative it is representative of the results of a number of experiments. (D) Chromatography of step 5 TUTase on poly(A)-agarose. The pooled peak material from glycerol gradients (step 5) was diluted with buffer A/50 mM KCl and applied to a column ( $0.9 \times 15.6$  cm) of poly(A)-agarose equilibrated with buffer A/50 mM KCl. In other experiments smaller columns were used, with essentially the same results. Proteins were eluted with a 50-ml, 50–550 mM KCl gradient in buffer A (---), and 1.2-ml fractions were collected and assayed for activity.

Table 1. Purification of TUTase from HeLa cells

Step	Vol, ml	Total protein, mg	Total units	Yield, %	Fold
1. Cytoplasmic extract	43	680	—	—	—
2. Post-ribosomal supernatant	42	440	8780	(100)	(1)
3. Phosphocellulose	50	25	6155	70	12
4. DEAE-Sephacel	5.5	1.7	4315	49	130
5. Glycerol gradient	5.6	0.4	5915	67	740
6. Poly(A)-agarose	1.2	0.08	3845	44	2400

Quantitative data are shown for the six steps in a typical purification of TUTase from HeLa cells. Units are pmol of UTP incorporated during the standard reaction. Material from steps 1 and 2 was passed over DEAE-Sephacel to remove inhibitory substances before it was assayed.

Enzyme fractions during purification were analyzed by electrophoresis through a denaturing polyacrylamide gel and silver nitrate staining (Fig. 2). Only one major protein band was present in step 6 HeLa TUTase. When fractions containing enzyme activity from the glycerol gradient or poly(A)-agarose steps were analyzed by electrophoresis, this  $M_r$  68,000 protein was the only protein whose distribution correlated with TUTase activity (data not shown).

To assure that the activity we purified was really TUTase, the most highly purified fraction was incubated with several different RNA substrates, and [ $\alpha$ - $^{32}$ P]UTP labeled products were analyzed. Products were digested with ribonuclease T2, which leaves 3' mononucleotides, and fractionated on a thin layer chromatogram (nearest neighbor analysis; ref. 12). Appearance of a labeled nucleotide indicates transfer of the  $\alpha$ -phosphate of UTP to the 3' hydroxyl end of an RNA chain. Fig. 3 shows that the ends of polyribonucleotides can be uridylylated and that more than one UMP residue can be added, indicating that this TUTase is similar to that characterized (12).

Finally, step 6 HeLa TUTase was tested for activity in the *in vitro* poliovirus replicase reaction. The highly purified

HeLa TUTase, like the rabbit reticulocyte TUTase described (12), did act as host factor (Table 2). This result is not surprising because TUTase and host factor have similar, if not identical, properties in all procedures employed for purification (results presented here; refs. 10 and 13). Other chromatographic resins were tested, including ATP-agarose, UTP-agarose, and poly(C)-poly(I)-agarose, and host factor and TUTase copurified on all (data not shown).

Several additional experiments were carried out to investigate properties of TUTase that might be related to priming of RNA synthesis. In the following experiments, step 6 rabbit reticulocyte enzyme (12) was used rather than HeLa TUTase, because it offered a much more concentrated form of the enzyme. To determine whether a short stretch of oligo(U) could be added to poliovirus RNA by the TUTase activity, RNA was incubated with the enzyme under replicase reaction conditions in the absence of viral polymerase. Digestion with RNase T1 liberated poly(A) from the viral RNA, which was then purified by binding to poly(U) immobilized on glass fiber filters. The poly(A)-enriched material was digested with RNase T2 for nearest neighbor analysis. As shown in Fig. 4, the products were uridine 3'-phosphate and adenosine 3'-phosphate in a ratio of about four to one,

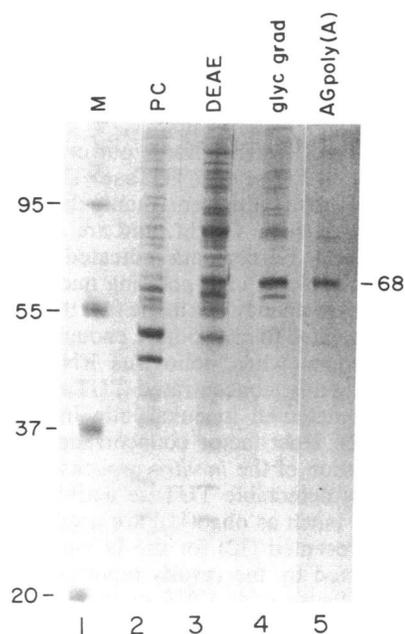


FIG. 2. NaDodSO<sub>4</sub>/PAGE of protein fractions during the purification of HeLa TUTase. Protein from each of the last four steps in the purification of HeLa TUTase was electrophoresed through a NaDodSO<sub>4</sub>/polyacrylamide gel and was stained with silver nitrate. Lanes 1–3 contained about 1  $\mu$ g of protein. Lanes: 1, molecular size standards in kDa; 2, step 3 (phosphocellulose peak); 3, step 4 (DEAE peak); 4, step 5 (glycerol gradient peak); 5, step 6 [poly(A) agarose peak].

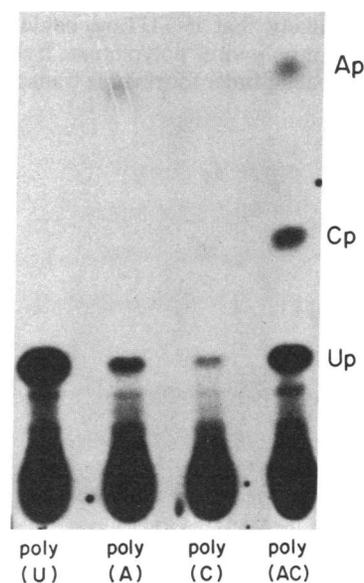


FIG. 3. Nearest neighbor analysis of products of HeLa TUTase. Step 6 HeLa TUTase was incubated with [ $\alpha$ - $^{32}$ P]UTP and different RNA homo- and heteropolymers. The products were isolated and digested with RNase T2 for nearest neighbor analysis. RNase T2 cleaves RNA nonspecifically to leave 3'-mononucleotides. The figure shows an autoradiogram of a chromatography plate. Ap, adenosine 3'-phosphate; Cp, cytidine 3'-phosphate; Up, uridine 3'-phosphate.

Table 2. HeLa TUTase can act as host factor in the *in vitro* poliovirus replicase reaction

Reaction	[ $\alpha$ - $^{32}$ P]CTP incorporated, pmol	
	Experiment 1	Experiment 2
Complete	1.03	0.86
minus TUTase	0.04	0.04
minus TUTase, minus polymerase	<0.01	<0.01
minus polymerase	<0.01	<0.01

Highly purified viral polymerase was incubated with step 4 HeLa TUTase in place of host factor under replicase reaction conditions.

indicating that on average those molecules to which UMP residues were added accepted five residues.

To examine whether or not TUTase could attach uracil nucleotides to the 5'-terminal peptide VPg, step 6 rabbit reticulocyte enzyme was incubated with synthetic VPg (14) and [ $\alpha$ - $^{32}$ P]UTP under TUTase reaction conditions. Several concentrations of VPg were used, ranging from 30 to 150  $\mu$ M. At the end of the reaction, the material was analyzed by electrophoresis through a thin layer cellulose plate at pH 3.5. In this system, UTP migrates toward the anode and VPg (both uridylylated and nonuridylylated forms) migrates toward the cathode (15). No uridylylated VPg could be detected by autoradiography of the thin layer plate (data not shown).

To assess the likelihood that a short stretch of uridylic acid residues added to the 3' terminal poly(A) of poliovirion RNA could fold back and prime polymerization, we exploited a different nucleic acid polymerase activity. Retroviral reverse transcriptase synthesizes DNA copies of RNA molecules. This enzyme has been characterized extensively, and its primer requirements are well understood (16–18). The primer *in vivo* is a tRNA molecule that specifically hybridizes to an initiation site within the genome of the retrovirus (19, 20). *In vitro* either oligo(U) or oligo(dT) can be used to prime synthesis from 3' poly(A) in template RNA; oligo(dT) sequences as small as four nucleotides long are efficient primers (17). It seemed likely that if TUTase could synthesize a foldback primer for poliovirus polymerase, it should likewise synthesize a foldback primer for reverse transcriptase. Table

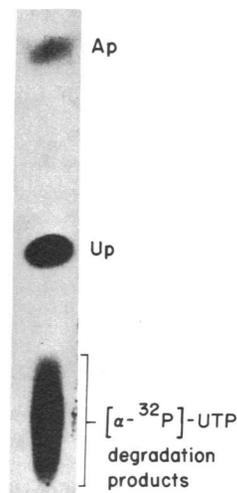


FIG. 4. Addition of UMP residues to the 3' end of virion RNA by TUTase. Virion RNA was incubated with TUTase as described in the text. Label associated with the 3' terminal poly(A) was analyzed by nearest neighbor analysis. The ratio of radioactivity in uridine 3'-phosphate (Up) to adenosine 3'-phosphate (Ap) spots was about 4:1.

Table 3. Stimulation of reverse transcriptase by TUTase

Reaction	[ $\alpha$ - $^{32}$ P]dTTP incorporated, pmol
Complete	79.0
minus oligo(U)	1.2
minus oligo(U), plus TUTase	26.5
minus oligo(U), minus reverse transcriptase, plus TUTase	0.8

Complete reaction consists of poliovirion RNA template, oligo(U) primer, reverse transcriptase, UTP, and all four dNTPs.

3 shows that this is the case. In the absence of a primer, reverse transcriptase incorporates very little dTTP. Oligo(U) can function as a primer for efficient synthesis. The step 6 rabbit reticulocyte enzyme containing TUTase activity can replace oligo(U) as an initiator for reverse transcriptase, just as it can replace oligo(U) in the poliovirus polymerase assay. The step 6 rabbit reticulocyte enzyme alone cannot incorporate significant amounts of dTTP. These results support the hypothesis that it is the TUTase activity in step 6 rabbit reticulocyte enzyme that acts as host factor—it seems unlikely that any other activity in this highly purified preparation could serve to initiate synthesis by two quite different polymerases.

Another group reported that host factor was a double-stranded RNA-dependent protein kinase (21). We could not detect kinase activity in step 6 TUTase from HeLa cells or step 6 enzyme from rabbit reticulocytes, although both of these preparations had very high host factor activity. Reactions were carried out in the presence and absence of low levels of double-stranded RNA, with added highly purified eukaryotic initiation factor 2 (data not shown). We also tested authentic double-stranded RNA-dependent protein kinase from rabbit reticulocytes for host factor activity. In two independent experiments we could not see any stimulation of the replicase reaction (data not shown). These data do not support the hypothesis that double-stranded RNA-dependent protein kinase and host factor are the same enzymes.

## DISCUSSION

The results presented here reinforce our original suggestion (12) that HeLa host factor is a TUTase. The two activities copurify through many different manipulations, have the same apparent molecular weight, and are distributed similarly within the cell. Earlier data indicated that host factor alone was not capable of incorporating nucleotides (9). We believe that the discrepancy lies in the fact that TUTase must be highly concentrated to incorporate enough labeled UTP to give a strong signal when poliovirus RNA is used as a substrate. Sufficiently concentrated TUTase to produce a signal was first detected in eukaryotic initiation factor 2 preparations (12). Host factor concentrations adequate for maximal stimulation of the *in vitro* replicase reaction, however, only show detectable TUTase activity when certain RNA molecules [such as oligo(U)] are used as acceptors.

The model presented (12) for the *in vitro* action of host factor is supported by the results reported here. It seems likely that host factor adds UMP residues to the 3' end of virion RNA, which can anneal to poly(A) and form a hairpin primer for RNA synthesis by viral polymerase. Five residues are added, on average, to the 3' end of virion RNA. A five-base-pair adenosine-uridine stem may not be stable at 30°C under the conditions of the *in vitro* replicase reaction. Nonetheless, TUTase preparations can also initiate for a different primer-dependent polymerase, reverse transcriptase. It seems likely that protein in the TUTase preparation (presumably the transferase itself) stabilizes the initiation

complex. Host factor (21) and TUTase (unpublished results) both bind to double-stranded RNA agarose resins better than to single-stranded RNA agarose resins.

TUTases and poly(U) polymerases have been described in a variety of tissues and organisms, ranging from plants to humans (12, 22–32). Association with ribosomes has been a consistent theme. There is no known cellular process that would require this type of enzyme. Small RNAs transcribed by eukaryotic RNA polymerase III are known to carry short (2–5 nucleotides long) stretches of uridylic acid at their 3' termini during some phase in their life cycle (33, 34). Although these uridylic acid residues may be genetically encoded, in at least one case they are added posttranscriptionally (35). A  $M_r$  50,000 protein specifically binds small RNA molecules that have been uridylylated at the 3' end but does not bind the same molecules lacking poly(U) tails (33, 36–38). The protein was initially identified as the antigen for a certain class of autoantibodies ("anti-protein La") made by patients with the disease systemic lupus erythematosus (39). Lupus antibodies have been described that are directed against a variety of ribonucleoprotein particles (40). The anti-protein La subset is unique in that the RNA components of the particles are extremely heterogeneous, and may include all RNA polymerase III transcripts. The function of the La antigen is unknown, but it is possible that it is related to the function of TUTase.

The results presented in this paper provide further evidence for our earlier suggestion that host factor is a TUTase that initiates poliovirus RNA synthesis *in vitro* by creating a hairpin primer for viral polymerase. Although other models have been proposed for initiation *in vitro*, we believe this is the most probable. The genome-linked protein, VPg, does not prime polymerization in this reaction (unpublished results). In contrast to the results of Morrow *et al.* (21), we find no protein kinase activity associated with host factor activity. These facts suggest that host factor-dependent RNA synthesis by poliovirus RNA polymerase is initiated by a TUTase activity and is highly analogous to oligo(U)-dependent RNA synthesis.

We thank Daniel Levin for his gift of step 6 eIF2 and double-stranded RNA-dependent protein kinase. Margaret Baron synthesized the VPg peptide used in one experiment. The Massachusetts Institute of Technology Cell Culture Center cultured HeLa cells for purification. We thank Bernard Mathey-Prevot for his advice on protein purification. N.C.A. was partially supported by a Medical Scientist Training Program grant from the National Institute of General Medical Sciences (2T 32 GM07753-06); this work was also funded by a grant from the National Institute of Allergy and Infectious Diseases (AI22346).

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