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Isolation of a Foot-and-Mouth Disease Polyuridylic Acid Polymerase and Its Inhibition by Antibody

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A template-dependent polyuridylic acid [poly(U)] polymerase has been isolated from BHK cells infected with foot-and-mouth disease virus (FMDV). Enzyme activity in a 20,000 × *g* supernatant of a cytoplasmic extract was concentrated by precipitation with 30 to 50% saturated ammonium sulfate. The poly(U) polymerase was freed of membranes by sodium dodecyl sulfate and 1,1,2-trichlorotrifluoroethane extraction, and RNA was removed by precipitation with 2 M LiCl. The solubilized poly(U) polymerase required polyadenylic acid as template complexed to an oligouridylic acid primer and Mg^{2+} for activity, but was inhibited by Mn^{2+}. Antisera from animals infected with FMDV had previously been shown to inhibit the activity of FMDV RNA replicase complexed to the endogenous RNA template. The same antisera also inhibited the activity of poly(U) polymerase. Antisera depleted of antibody by adsorption with the virus infection-associated antigen of FMDV no longer inhibited replicase and polymerase activities. The evidence suggests that FMDV RNA replicase, poly(U) polymerase, and the virus infection-associated antigen share a common protein.

The RNA of foot-and-mouth disease virus (FMDV), as well as other picornaviruses, replicates by way of an RNA-dependent RNA polymerase (replicase) found in the cytoplasm of infected cells (1, 3, 11). The FMDV replicase has been fractionated into two separate complexes of RNA and protein (2). One, a 20 to 70S component, synthesizes double-stranded RNA, and the other, a 100 to 300S component, synthesizes 37S single-stranded viral RNA. Attempts to prepare a template-dependent replicase were unsuccessful. The cell-free synthesis of virus-specific RNA by FMDV RNA polymerase was inhibited by antisera from infected guinea pigs but not by antisera from animals vaccinated with inactivated virus (12). This selective inhibition by antibody would suggest that one or more proteins of the polymerase complex are coded for by the virus genome.

The viral polypeptide isolated with the poliovirus replication complex has been reported to be an essential component of the poliovirus replicase (10). It has also been reported recently that poliovirus-infected cells have a polyadenylic acid [poly(A)]·oligouridylic acid [oligo(U)]-dependent polyuridylic acid [poly(U)] polymerase containing the same viral polypeptide as the poliovirus RNA replicase (7, 8). The poly(U) polymerase is assumed to function in the initial stage of viral replication in which a complete strand of negative (complementary) RNA is synthesized to become part of the replicative intermediate. The poly(U) polymerase would copy the poly(A) known to exist at the 3′ end of picornavirus RNA (4, 14) to form the poly(U) at the 5′ end of negative-strand RNA.

We report here the isolation of a soluble FMDV-specific RNA-dependent RNA polymerase that requires a poly(A) template combined with an oligo(U) primer. The activity of the poly(U) polymerase is inhibited by the same antisera that inhibit FMDV replicase.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney-21, clone 13 cells (American Type Culture Collection) were grown for 5 to 7 days in 2-liter Baxter bottles in a medium consisting of a modified Eagle salt solution, 0.02 M in Tris, containing 10% tryptose phosphate and 10% bovine serum (13). Cells were infected at a multiplicity of 50 PFU with FMDV, type A, subtype 12, in 10 ml of inoculum.

Preparation of cytoplasmic extract from infected cells. After 5 min of incubation at 37°C, 40 ml of growth medium was added and incubation was continued for 3.5 to 4.5 h (except when different times were required for kinetic studies). The bottles were cooled in an ice bath, and 50 ml of cold 0.14 M NaCl–0.01 M Tris-hydrochloride, pH 7.5, was added. Cells were scraped from the glass, collected by centrifugation, and washed once with the Tris-buffered saline. Cells (75×10^8) were suspended in 150 ml of cold 0.01 M NaCl–0.01 M Tris-hydrochloride, pH 8.0 (TN buffer), broken in a tight-fitting Dounce homogenizer, and centrifuged for 5 min at 5,000 × *g* to remove the nuclei. The nuclei were washed once with 10 to 15 ml of TN buffer, and the wash was added to the supernatant-cytoplasmic extract.

Isolation of poly(U) polymerase. In the procedure developed in this laboratory, the cytoplasmic extract was spun for 30 min at 20,000 × *g* and the sedimented membrane fraction, which contained less than 1% of the poly(U) polymerase activity (as will be seen later in Table 2), was discarded. The appropriate volume of a saturated solution of ammonium sulfate in TN buffer (AS) was added to the supernatant fluid to 30% saturation, and the mixture was kept at 0°C for a minimum of 1 h. The pellet obtained from centrifuging for 10 min at 10,000 × *g* was discarded, and AS was added to the supernatant to 50% saturation. After treatment as before, the pellet obtained by centrifugation contained most of the enzyme activity. The pellet was dissolved in 5 ml of 0.2% sodium dodecyl sulfate, and the solution was emulsified with a Vortex mixer for 1 min with twice the volume of 1,1,2-trichlorotrifluoroethane (Genesolv-D, Allied Chemical). The emulsion was centrifuged for 5 min at 20,000 × *g*, and the aqueous phase was collected and extracted with Genesolv-D two additional times. An equal volume of 4 M LiCl in TN buffer was added to the final aqueous phase, and the mixture was allowed to stand at −11°C for 18 to 20 h. The precipitated single-stranded and replicative intermediate RNA was removed by centrifuging for 30 min at 20,000 × *g*. The supernatant was dialyzed against repeated changes of TN buffer and could be stored as such at 4°C with little loss in activity for a period of 6 to 8 weeks. Other poly(U) polymerase preparations were stored at −60°C as such or in the presence of 10% glycerol for 10 to 12 months without any significant loss in activity.

Poly(U) polymerase and replicase assays. A modification of the Flanegan-Baltimore procedure (7) was used to determine poly(U) polymerase activity. A 50-μl portion of enzyme was assayed in a final volume of 125 μl of solution containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.0), 15 mM magnesium acetate, 0.08 mM UTP, 20 μg of poly(A) per ml (Miles Laboratories), 10 μg of oligo(U)$_{10-20}$ per ml (Collaborative Research), 4 mM phosphoenolpyruvic acid, 3.5 IU of pyruvate kinase per ml, and 10 μg of actinomycin D per ml. For the assay of replicase activity, the poly(A) and oligo(U) were replaced by 0.2 mM ATP, GTP, and CTP. Five microcuries of [^{3}H]UTP (specific activity, 27 Ci/mmol) in 50% ethanol was evaporated to dryness with a stream of nitrogen for use in each assay. After incubation for 60 min at 37°C, the reaction was stopped by the addition of 0.5 ml of saturated sodium pyrophosphate, 1.0 ml of 10% trichloroacetic acid, and 100 μg of carrier RNA. The acid-insoluble labeled enzymatic product was collected on Whatman GF/A filters and counted in Formula 950-A scintillation fluid (New England Nuclear) with a Beckman LS-335 counter.

Antisera. Antisera, antigen-absorbed sera, and purified immunoglobulin G (IgG) fractions were prepared from live virus-vaccinated animals as previously described (12). Guinea pig antisera to FMDV type A, subtype 12, and type O, subtype 1, were used as such. Type A antisera were also treated with an excess of the virus infection-associated antigen (6) shown to be immunologically related to the RNA-dependent RNA polymerase (12). Purified IgG fractions free of RNase were prepared from whole and 140S (intact virion) antigen-adsorbed antisera to FMDV types A and O by passage through DEAE-cellulose and Sephadex G-100 columns.

RESULTS

Isolation of poly(U) polymerase activity and its separation from replicase activity. Poly(U) polymerase activity was detected in the crude cytoplasmic extract from infected cells in significant amounts at 2.5 h postinfection, reached a peak of activity at 3.5 h which remained relatively stable for the next hour, and then decreased from 5 h onward. Replicase activity measured at the same time behaved similarly, except for a more rapid decrease in activity following the peak value at 3.5 h, in agreement with previously reported results (12). As shown in Table 1, the ratio of poly(U) polymerase to replicase activity increased with time to a maximum value at 4.5 to 5 h postinfection, essentially because of the more rapidly decreasing activity of the replicase.

The poly(U) activity is associated with smooth cytoplasmic membranes found in the 20,000 × *g* supernatant (Table 2). Unlike the procedure for poliovirus in which the major portion of the membrane-bound poly(U) polymerase was sedimented at 20,000 × *g* (8), less than 5% of the starting activity is apparently sedimented under these conditions in the FMDV system. The sedimented pellet may contain inhibitors of poly(U) polymerase activity, since recoveries in the 20,000 × *g* supernatant sometimes exceed 100%. Poly(U) polymerase activity is apparently quantitatively precipitated be-

TABLE 1. *Comparison of kinetics of poly(U) polymerase and endogenous replicase production in FMDV-infected BHK cells*[a]

h postinfection	Relative activity, % of peak value		Poly(U) polymerase/replicase activity
	Poly(U) polymerase	Replicase	
2.5	14.5	16.1	2.30
3.5	100[b]	100[c]	2.55
4	91.5	84.3	2.77
4.5	89.0	57.3	3.97
5	69.1	43.5	4.05
6	51.5	33.8	3.88

[a] Cytoplasmic extracts were prepared from infected cells at each designated time as described in the text, and aliquots were used for the two assays. Protein concentrations (9) ranged from 8.1 to 10.4 mg/ml.

[b] Poly(U) polymerase activity at 3.5 h was 153 pmol of [^{3}H]UMP incorporated per 50 μl of enzyme after incubation for 60 min at 37°C

[c] Endogenous replicase activity at 3.5 h was 60.0 pmol of [^{3}H]UMP incorporated per 50 μl of enzyme after incubation for 60 min at 37°C.

TABLE 2. *Recovery of poly(U) polymerase and its separation from endogenous replicase activity*[a]

Cell fraction	Vol (ml)	Activity (pmol of [^{3}H]UMP incorporated)		Recovery of poly(U) polymerase (%)
		Replicase	Poly(U) polymerase	
A. Used in recovery procedure				
Cytoplasmic extract	150	34.4	152	100
20,000 × *g* supernatant	165	18.9	171	124[b]
30% AS supernatant	236		142	147
30–50% AS precipitate[c]	9		35.6	1.4[c]
2 M LiCl supernatant[d]	26	2.1	261	29.8
B. Discarded				
20,000 × *g* pellet	20	77.4	44.5	3.9
30% AS precipitate	20		22.5	2.0
50% AS supernatant	330		0.4	<1.0
2 M LiCl precipitate	6	50.5	121	3.2

[a] Recovery procedure and assay methods are described in the text. Precipitates were resuspended in TN buffer unless otherwise stated. Samples containing ammonium sulfate (AS) and LiCl were dialyzed before assay.
[b] Recoveries at this step ranged from 88 to 138%.
[c] Dissolved in 0.2% sodium dodecyl sulfate in TN buffer. Sodium dodecyl sulfate as well as 0.5% sodium deoxycholate in 1.0% Nonidet P-40 inhibited the assay at this and other steps.
[d] Protein concentration (9) was 10.2 mg/ml; 510 μg was used per assay.

tween 30 and 50% saturation with ammonium sulfate. Not shown in Table 2 is the fact that 65% of the activity in the 20,000 × *g* supernatant can be sedimented at 78,000 × *g* after 1.5 h, and about 90% can be sedimented after 10 h at 100,000 × *g*.

It has been reported that the presence of even trace amounts of phospholipids interfered with the dissociation of encephalomyocarditis virus replicase complex into enzyme and template RNA (15). In the FMDV system reported on here, it was also essential to first remove phospholipids by Genesolv-D extraction to enable the single-stranded and replicative intermediate RNA to be precipitated by 2 M LiCl. The detergent-solubilized 30 to 50% ammonium sulfate precipitate containing poly(U) polymerase activity was so extracted. If phospholipids were not removed by Genesolv-D extraction, the poly(U) polymerase activity was distributed erratically between the 2 M LiCl precipitate and supernatant fractions. Recovery of poly(U) polymerase activity cannot be accurately related to the starting activity. The active replicase complex may consist of the poly(U) polymerase bound to an endogenous RNA template (8), and stripping off of this RNA template during the isolation procedure may convert replicase activity into poly(U) activity.

Assay requirements of the poly(U) polymerase and its viral specificity. The poly(U) polymerase required poly(A) as the template and oligo(U) as the primer to be able to synthesize poly(U) from labeled UTP. Poly(U) was not synthesized in the absence of either template or primer (Table 3). Replacing the labeled UTP by labeled CTP (data not shown) resulted in a complete loss of activity. This lack of incorporation of noncomplementary nucleotide eliminates the possibility of nonspecific terminal addition of nucleotides by the LiCl supernatant. Mg^{2+} was essential for enzyme activity and could not be replaced to any significant extent by Mn^{2+}; the presence of 2.0 mM and 15 mM Mn^{2+} yielded only 11 and 9.3%, respectively, of the original activity. Mn^{2+} inhibited poly(U) activity in the presence of Mg^{2+}, and only 6.6% of the original activity remained when an equimolar (15 mM) amount of Mn^{2+} was added. Mn^{2+} has also been previously reported to inhibit the endogenous replicase activities of FMDV (11) and other picornaviruses (3). When the energy generating system of pyruvate kinase and phosphoenolpyruvic acid was absent, less than 15% of the original polymerase activity remained.

As was seen in Table 2, the LiCl supernatant had no significant activity when poly(A) and oligo(U) were replaced (as in the replicase assay) by the ribonucleoside triphosphates ATP, GTP, and CTP. When the ribonucleoside triphosphates were added to poly(A) and oligo(U) in the assay (data not shown), there was no change in the original activity. Thus, the LiCl supernatant does not contain any endogenous RNA template.

LiCl supernatants from uninfected cells incorporated only 3.0 pmol of [^{3}H]UMP under assay conditions in which supernatants from infected cells incorporated 297 pmol, and this uninfected

cell activity was not sensitive to changes in the assay substrate (Table 3). Additional evidence that uninfected cell polymerase activity is not significant was seen in assays with bovine serum albumin or sperm whale myoglobin in place of cellular extracts. There was a nonspecific incorporation of [^{3}H]UMP dependent on protein concentration in the assay mixture. Bovine serum albumin and sperm whale myoglobin incorporated 3.1 to 3.5 pmol of [^{3}H]UMP when 500 μg of the proteins was used in the assay and incorporated 9.8 to 10.8 pmol when 5,000 μg of protein was used. The LiCl supernatants from infected and uninfected cells contain from 9 to 12 mg of protein per ml, which corresponds to 460 to 600 μg of protein in the assay. Thus, LiCl supernatants from uninfected cells have no identifiable polymerase activity.

Kinetics of assay and proportionality of enzyme activity to protein concentration. The rate of incorporation of [^{3}H]UMP at 37°C by a LiCl supernatant enzyme preparation was linear for the first 30 min and increased thereafter at a diminishing rate for the next 30 min (Fig. 1). At the lower temperature of 29°C, linearity of incorporation continued for a longer time, as has been reported for poliovirus (7). The enzyme preparation decreased in activity proportionally to protein concentration (data not shown) when assayed for 60 min at 37°C at protein concentrations per assay of 540, 360, 180, and 90 μg, respectively. Thus, incorporation of [^{3}H]UMP is linear with enzyme concentration over this range.

TABLE 3. *Assay requirements of LiCl supernatant preparations from infected and uninfected cells*

Assay changes	% of control assay[a]	
	Infected cells	Uninfected cells
−poly(A)	1.2	83
−oligo(U)	1.2	86
−pyruvate kinase and phosphoenolpyruvic acid	14	
−Mg^{2+}	1.9	86
−Mg^{2+}, +0.2 mM Mn^{2+}	4.2	
−Mg^{2+}, +0.5 mM Mn^{2+}	5.8	
−Mg^{2+}, +2.0 mM Mn^{2+}	11	
−Mg^{2+}, +15 mM Mn^{2+}	9.3	
+Mg^{2+}, +0.2 mM Mn^{2+}	95	
+Mg^{2+}, +0.5 mM Mn^{2+}	87	
+Mg^{2+}, +2.0 mM Mn^{2+}	39	
+Mg^{2+}, +15 mM Mn^{2+}	6.6	

[a] Assay was run as described in the text. The infected cell preparation incorporated 297 pmol of [^{3}H]UTP per 50 μl (452 μg of protein) of enzyme after incubation for 60 min at 37°C. The uninfected cell preparation incorporated 3.0 pmol of [^{3}H]UTP under the same assay conditions.

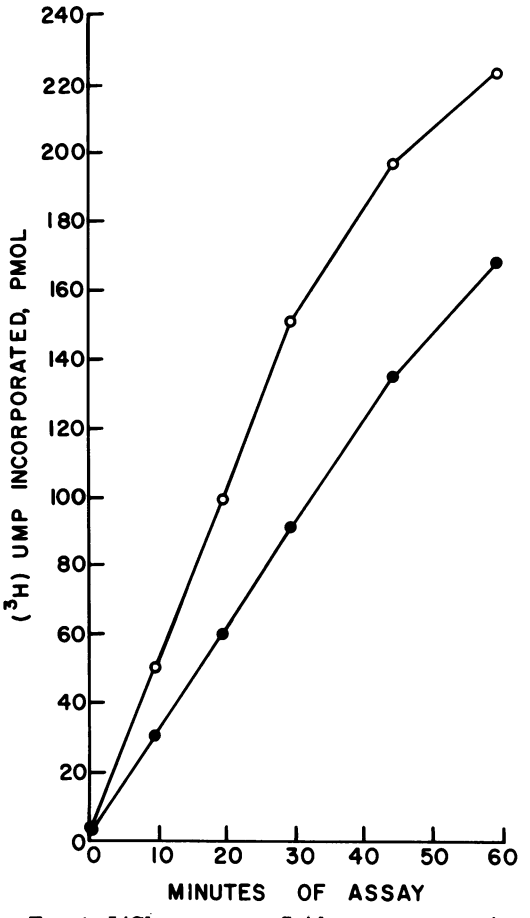

FIG. 1. *LiCl supernatant fluid enzyme preparation was assayed as described in the text, at 37 and 29°C. Samples were taken after incubation for 10, 20, 30, 45, and 60 min, and the acid-insoluble enzyme products counted for activity. Rate of incorporation of [^{3}H]UMP at 37°C* (○) *and at 29°C* (●).

Inhibition of poly(U) polymerase activity by antibody. Previous work in this laboratory had shown that the endogenous replicase activity induced by infection with type A FMDV was inhibited in vitro by antisera and IgG from animals infected with FMDV types A and O. Since poly(U) polymerase activity is also induced in FMDV-infected cells, as has been shown in this report, sera from infected animals should also contain antibodies against poly(U) polymerase. To test this hypothesis, antisera, IgG samples, and antigen-adsorbed preparations as listed in Table 4 were used against LiCl supernatants. Poly(U) polymerase activity was inhibited from 14 to 92% by antibody preparations from type A- and type O-infected animals as well as by IgG from antisera depleted of antibody to 140S virion antigen. However, antisera depleted of antibody

TABLE 4. *Inhibition of poly(U) polymerase activity by antibody*

Antibody preparation added to enzyme assay	% Reduction of control assay[a]	
	Expt 1	Expt 2
Guinea pig antisera, FMDV, O_1[b]	32.8	92.2
Guinea pig antisera, FMDV, A_{12}[b]	14.5	68.0
Antisera to A_{12}, depleted of antibody by VIA antigen adsorption[b]	0	0
IgG from A_{12} antisera		82.5
IgG from A_{12} antisera, depleted of antibody by 140S antigen adsorption	22.9	64.1
IgG from O_1 antisera, depleted of antibody by 140S antigen-adsorption		83.2

[a] Equal volumes of LiCl supernatant and antibody preparation were incubated for 2 h at 0°C (experiment 1) and for 30 min at 37°C followed by 24 h at 4°C (experiment 2) before use in assay.

[b] Normal sera occasionally inhibited the assay because of the presence of RNase, but the amount of inhibition was always less than the values for the antisera. VIA, Virus infection-associated antigen.

to the virus infection-associated antigen (6) (which has been shown not to be FMDV type specific [6] and to share a common antigenicity with FMDV replicase [12]) were no longer able to inhibit poly(U) polymerase activity. This latter result is strongly suggestive of a common antigenicity between FMDV RNA replicase and poly(U) polymerase.

DISCUSSION

Antibody preparations which previously had been shown to inhibit in vitro activity of FMDV RNA replicase are now shown to also inhibit the in vitro activity of poly(U) polymerase. When such antibody preparations were depleted of antibody by adsorption with the third antigenic component of FMDV (the virus infection-associated antigen), they could no longer inhibit replicase and poly(U) polymerase activity. These results are evidence for a common antigen shared by the RNA replicase, poly(U) polymerase, and virus infection-associated antigen.

In the only other data reported for picornaviruses, Flanegan and Baltimore (8) have suggested that the poliovirus polypeptide designated p63 is active as a poly(U) polymerase either as a monomer protein or complexed to NCVP2, its apparent precursor. The same protein, p63, when tightly bound to its negative-strand endogenous RNA template, is responsible for replicase activity. The shared antigenicity of the FMDV replicase and polymerase can be attributed to their possession of the same protein. In addition, the products of in vitro translation of FMDV RNA have been tested with antisera containing antibody against virus infection-associated antigen, and the immunoprecipitates contained a major peptide of approximately 56,000 daltons (5).

Other comparisons can be made of FMDV RNA replicase and poly(U) polymerase preparations. Their respective activities reach a maximum after 3.5 h in infected cells, but replicase activity decreases more rapidly thereafter, perhaps because of degradation of the endogenous RNA template (7). Both activities require Mg^{2+} and are inhibited by Mn^{2+}. Replicase activity is completely sedimented at 78,000 × *g* for 1.5 h and 30% so at 20,000 × *g* for 30 min, whereas poly(U) polymerase activity is only 65% and less than 5%, respectively, sedimented under the same conditions. Replicase activity is not stimulated by the addition of template, whereas poly(U) polymerase activity is dependent upon a poly(A) template and an oligo(U) primer. It was necessary to extract the 30 to 50% saturated AS precipitate with Genesolv-D to remove phospholipid membranes followed by precipitation of single-stranded and replicative intermediate RNA with 2 M LiCl to eliminate all the replicase activity from the poly(U) polymerase preparation.

The above findings are all in agreement with suggestions made previously in this paper that the FMDV genome induces the formation of a poly(A)-dependent poly(U) polymerase in infected cells able to synthesize the poly(U) sequence of negative-strand RNA. Apparently, the firmly bound complex of poly(U) polymerase protein and endogenous negative-strand viral RNA as template then functions to replicate the viral genome.

It is of interest that the major portion of poliovirus poly(U) polymerase from HeLa cells has been reported (7, 8) to sediment at 20,000 × *g* and to be separable from bound membranes and template by detergent extraction and 2 M LiCl precipitation, whereas almost all FMDV poly(U) polymerase activity from BHK cells remained in the 20,000 × *g* supernatant fluid and required Genesolv-D extraction in addition to detergent and 2 M LiCl treatment to separate bound membranes and template. However, both poly(U) polymerases required oligo(U) as a primer for activity.

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ADDENDUM IN PROOF

Since this paper was accepted for publication, it has been reported that a virus-coded protein of molecular weight 56,000 has been isolated from the RNA-dependent RNA polymerase replication complex in FMDV-infected cells, which appears to be the same protein as the virus infection-associated antigen (J. F. E. Newman, B. Cartwright, T. R. Doel, and F. Brown, J. Gen. Virol. **45**:497-507, 1979).

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