Uukuniemi Virus Contains an RNA Polymerase

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An RNA-dependent RNA polymerase activity has been found associated with Uukuniemi virions. The enzyme activity is expressed only after disrupting the virions with the nonionic detergent Triton X-100 and is absolutely dependent on Mn^{2+} , whereas Mg^{2+} is not required, a finding that distinguishes this polymerase from those of other enveloped minus-strand RNA viruses. Within the range pH 7.2 to 8.5 no distinct optimum was found. The optimum temperature was between 37 and 40 C. The reaction was not inhibited by actinomycin D, rifampin, or DNase, whereas RNase was completely inhibitory. The partially RNase-resistant product consisted of rather small-sized RNA, which contained sequences complementary to Uukuniemi virus RNA as shown by hybridization to the template L, M, and S RNA species of Uukuniemi virus.

Uukuniemi virus, a tick-borne arbovirus (8, 13), has recently been proposed as a possible member of the new large Bunyavirus group of arboviruses (12, 17). The structure of Uukuniemi virus (14-16, 18, 22), and some of the other possible members of this group (11, 12, 20; personal communications by Obijeski and Murphy [LaCrosse virus] and Lyons [Bunyamwera virus]; unpublished data of Saikku and Pettersson [Inkoo virus]), has been found to be of a novel type. The genome of Uukuniemi virus consists of three pieces of single-stranded RNA sedimenting at 29S (L), 22S (M), and 17S (S) (14). Whether the genome is segmented (like that of influenza or reoviruses), or whether the two smaller RNAs are derived from defective particles (as in vesicular stomatitis virus), is not known.

Another interesting finding is that the ribonucleoprotein (RNP) strands isolated from the virions seem in electron micrographs to be closed circles (16). They measure about 2.8 μm (L), 1.4 μm (M), and 0.7 μm (S) in length and are protected by the N protein against the action of pancreatic RNase (15). Circular RNPs have also been found in Lumbo virus, another member of the Bunyaviruses (20). Whether the RNAs in the RNPs of these viruses are covalently closed circles is not known.

The RNA species of Uukuniemi virus have not been found to be infectious (Pettersson and Saikku, unpublished data). This, and the detection of a virion-associated RNA polymerase in Lumbo virus (5), led us to search for a similar enzyme in Uukuniemi virus particles. Such polymerases have also been found in vesicular stomatitis (3), influenza (4), Newcastle disease (9), and Pichinde (7) virions, all of which are

enveloped minus-strand RNA viruses (2). In this paper we report evidence for the presence of such a polymerase in Uukuniemi virus and describe the optimal conditions for its activity *in vitro*.

MATERIALS AND METHODS

Cells and virus. The origin and cultivation of chicken embryo cells have been described previously (14). Stock virus was prepared from the prototype strain S23 of Uukuniemi virus after five clonings in primary cultures of chicken embryo cells (14).

Virus cultivation and purification. Monolayers of secondary chicken embryo cell cultures were infected at a multiplicity of about 5 PFU/cell as described previously (14). The virus was cultivated in Eagle minimum essential medium supplemented with 2% calf serum (10 ml/60-cm² petri dish). The virus was harvested at 24, 48, and 72 h postinfection. The culture fluid was replaced with fresh medium after each harvest. The virus-containing media (2 to 4 liters/batch) were pooled and cleared by centrifugation at 10,000 $\times g$ for 30 min. All centrifugations and procedures during the purification process were carried out at 4 C. The virus was concentrated by vacuum dialysis to about 200 to 300 ml. After centrifugation at 4,000 $\times g$ for 15 min, the virus was pelleted at 22,000 rpm for 60 min (Spinco SW27 rotor) through a 3-ml cushion of 15% (wt/wt) sucrose made in TN buffer (0.1 M NaCl, 0.05 M Tris, pH 7.4). The pelleted virus was eluted in TN buffer overnight, then resuspended gently with a Pasteur pipette and layered on a discontinuous sucrose gradient made in TN (15). Centrifugation was at 25,000 rpm for 3 h (SW27). The virus band was collected, and the virus was finally concentrated by pelleting at 22,000 rpm for 60 min and resuspended in 0.01 M Tris, pH 7.4. Virus protein content was estimated by the method of Lowry et al. (10).

RNA polymerase assay. Purified Uukuniemi virus was treated with 1.0% Triton X-100 for 10 min at

room temperature before addition to the reaction mixture. The standard reaction mixture of 50 μ l contained: 12.5 μ g of virus protein, 50 mM Tris (pH 8), 5 mM MnCl_2 , 25 mM NaCl, 10 mM 2-mercaptoethanol, 0.5 mM ATP, CTP, and GTP (Sigma Chemical Co.), and 35 pmol of [^3H]UTP (17 Ci/mmol; New England Nuclear Corp.) (1 pmol = 5,872 counts/min). Incubation was at 37 C for 1 h. Increasing amounts of [^3H]UTP in the reaction mixture gave a linear increase in incorporation of radioactivity in the product RNA. Therefore, higher concentrations of [^3H]UTP and virus protein as well as longer incubation times were used in some cases. The reaction was stopped by addition of 0.5 ml of ice-cold 0.08 M sodium pyrophosphate and 1 ml of 10% trichloroacetic acid. Bovine serum albumin (50 μ g/tube) was added as a carrier. The precipitates were collected on membrane filters (Millipore Corp.) and washed with trichloroacetic acid, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation counter as described previously (15).

Reversed transcriptase assay. RNA-dependent DNA transcription activity was assayed as described by Baltimore (1) and Temin and Mizutani (21). Purified Uukuniemi virus was treated with 1% Triton X-100 at room temperature for 10 min and incubated in a 50- μ l standard assay mixture containing: 12.5 μ g of virus protein, 50 mM Tris (pH 8.3), magnesium acetate or alternatively manganese chloride at concentrations ranging from 1.5 to 12.0 mM, 60 mM NaCl, 20 mM dithiothreitol, 0.5 mM dATP, dCTP, and dGTP (Sigma Chemical Co.), and 17 pmol of [^3H]TTP (58 Ci/mmol) (The Radiochemical Centre, Amersham, England). After incubation at 37 C for 1 h the reaction was stopped and trichloroacetic acid-precipitable radioactivity was assayed as described above. Purified Triton X-100-disrupted Rous sarcoma virus (kindly supplied by A. Vaheri) was used as a positive control. Under the above conditions Rous sarcoma virus significantly stimulated the incorporation of [^3H]TTP into acid-insoluble form.

RNA extraction. RNA was extracted from purified virus and from the *in vitro* incubation mixtures with an equal volume of water-saturated phenol after addition of sodium dodecyl sulfate to a final concentration of 0.2%. The water phase was extracted with chloroform-octanol to remove the rest of the phenol, and the RNA was precipitated with ethanol at -20 C in the presence of 0.2 M NaCl.

RNA-RNA annealing. RNA samples were taken into 10 mM Tris, pH 7.5, and 1 mM EDTA, boiled for 90 s in a water bath, and quickly cooled on ice for 5 min, and NaCl was added to a final concentration of 0.4 M. In some cases sodium dodecyl sulfate (0.5%) was added to the mixtures. Annealing was carried out at 65 C for 3 to 5 h.

After annealing, the samples were treated with pancreatic RNase (2.7.7.16; Worthington Biochemicals Co.) (10 μ g/ml) for 30 min at 37 C, and trichloroacetic acid-precipitable radioactivity was determined as described previously (15).

Analysis of the product on sucrose gradient. The polymerase product was analyzed after phenol extraction on 15 to 30% (wt/wt) sucrose gradients

made in TSE buffer (0.1 M Tris, pH 7.4, 0.15 M NaCl, 0.001 M EDTA). Phenol-extracted ^{32}P -labeled Uukuniemi virus RNAs (14) were included in the samples as internal markers. Centrifugation was at 25,000 rpm for 17 h at 20 C in a Spinco SW27 rotor. Fractions (0.5 ml) were collected from below and half of them were treated with pancreatic RNase as described above. Trichloroacetic acid-precipitable radioactivity was determined as described previously (15).

Other methods. The purity of the virus preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (15). Hemagglutination activity was measured at pH 5.3 as described by Saikku (19).

RESULTS

General properties of Uukuniemi virus RNA polymerase. Purified, partially disrupted Uukuniemi virus was found to catalyze the incorporation of [^3H]UTP into acid-precipitable form. This activity was associated with Uukuniemi virions as shown by its cosedimentation with the virus hemagglutinating activity in a purification gradient (Fig. 1). Furthermore, the incorporation of radioactivity was linearly dependent on increasing virus protein concentration in the reaction mixture (Fig. 2). The polymerase activity was only expressed if the virus was first disrupted with a nonionic detergent like Triton X-100, suggesting that the enzyme is located inside the virus particle. Because essentially no activity was detected separate from the virus peak in the purification gradient (Fig. 1) and the solubilization of the viral envelope was absolutely required, it is unlikely that a contaminating cellular protein adhering to the viral surface and cosedimenting with the virions could be responsible for the activity.

The acid-precipitable material was considered to be RNA since no product was made unless all four nucleoside triphosphates were present in the reaction, which excluded the possibility of random polynucleotides [including poly(U)] being synthesized and suggests that the transcription is dependent on Uukuniemi virus RNA template (Table 1). In addition, no incorporation of radioactivity was seen when pancreatic RNase was added to the reaction, whereas DNase, actinomycin D, and rifampin had no effect on the reaction (Table 1), suggesting that the virion enzyme is an RNA polymerase catalyzing RNA-dependent RNA transcription. This was further confirmed by assaying disrupted Uukuniemi virions for their ability to incorporate [^3H]TTP into acid-insoluble form in the presence of dATP, dCTP, and dGTP in a reversed transcriptase reaction (1, 21). No incor-

FIG. 1. Association of RNA polymerase activity with Uukuniemi virus. Uukuniemi virus was located in a discontinuous sucrose gradient (ref. 16 and text) by measuring the hemagglutinating activity in 1-ml fractions. Aliquots (10 μ l) of the same fractions containing at most 20 μ g of virus protein were assayed for viral polymerase in standard 50- μ l reactions. [3 H]UMP incorporated into acid-precipitable form was measured. The background radioactivity (10 to 15%), obtained in identical incubations lacking virus protein, has been subtracted from the values presented in this and all the subsequent figures. Bottom to the left. Symbols: [3 H], $\bullet$; hemagglutinating activity, $\circ$.

poration was observed (for details see Materials and Methods).

Optimal conditions. A striking feature of the polymerase is its absolute dependence on Mn^{2+} , the optimum concentration being about 4 to 5 mM (Fig. 3). Mg^{2+} did not replace Mn^{2+} , nor did it increase the enzyme activity; high concentrations (above 10 mM) were in fact inhibitory (Table 2). The activity was not significantly affected by the presence or absence of monovalent cations (Na^+ and K^+) or reducing agents like 2-mercaptoethanol (Table 1). No distinct pH optimum was found for the enzyme, although pH 8.0 repeatedly resulted in slightly better activity (Table 3).

The incubation temperature had a considerable effect on the polymerase activity. At all temperatures tested (22, 30, 37, and 40 C) the reaction continued for about 3 h, but with differ-

FIG. 2. Relationship between Uukuniemi virus protein concentration and polymerase activity levels. Standard reaction mixtures of 50 μ l contained from 12.5 to 37.5 μ g of virus protein. Incubation was for 1 h at 37 C, and [3 H]UMP incorporated into acid-precipitable form was determined.

TABLE 1. General properties of Uukuniemi virus RNA polymerase

Reaction mixture	[3 H]UMP (pmol incorporated/mg of protein)
Complete ^a	30 ^b
-Triton X-100	0
-2-Mercaptoethanol	27
-ATP	0
-GTP	2
-CTP	2
-ATP, GTP, CTP	3
-MnCl ₂	2
-MnCl ₂ , +MgCl ₂ (8 mM)	3
-NaCl	22
-NaCl, +KCl (25 mM)	28
+RNase (10 μ g/ml)	5
+DNase (20 μ g/ml)	22
+Actinomycin D (5 μ g/ml)	25
+Rifampin (20 μ g/ml)	26

^a Complete reaction mixture contained 12.5 μ g of virus protein in a total assay volume of 50 μ l. Incubation was at 37 C for 1 h.

^b [3 H]UMP incorporated into acid-precipitable form was measured, and the background radioactivity was subtracted from the values presented as described under Fig. 1.

ent kinetics (Fig. 4). The reaction was most rapid and efficient at 37 and 40 C. During the

FIG. 3. Dependence on Mn^{2+} of the Uukuniemi virus polymerase activity. Standard 50- μ l reactions contained increasing concentrations of $MnCl_2$ as indicated in the figure. After incubation for 1 h at 37 C the acid-insoluble radioactivity was determined.

TABLE 2. Effect of Mg^{2+} on Uukuniemi virus polymerase

$MgCl_2$ (mM)	$[^3H]UMP$ (pmol incorporated/mg of protein) ^a	
	5 mM $MnCl_2$	No $MnCl_2$
0	24	0.6
3	21	0.9
6	19	0.8
9	25	0.6
12	22	1.8
15	17	2.2
20	9	Not done

^a 50- μ l reaction mixtures with the $MgCl_2$ concentrations indicated were incubated in the presence or absence of 5 mM $MnCl_2$. $[^3H]UMP$ incorporated into acid-insoluble form is shown.

TABLE 3. Effect of pH on Uukuniemi virus RNA polymerase

pH	$[^3H]UMP$ (pmol incorporated/mg of protein) ^a
7.2	21
7.5	17
8.0	26
8.5	17

^a 50- μ l reaction mixtures containing 50 mM Tris at the indicated pH values were incubated at 37 C for 1 h and the incorporation of $[^3H]UMP$ into acid-precipitable form was determined.

first 3 h of incubation the amount of product at 22 C was 20 to 30%, and at 30 C about 50% of that at 37 or 40 C.

Preliminary characterization of the prod-

FIG. 4. Effect of temperature and incubation period on the Uukuniemi virus polymerase activity. Duplicate 100- μ l reactions containing 50 μ g of virus protein were incubated at 22, 30, 37, and 40 C for 5 h. Duplicate samples were taken at 0.5- to 2-h intervals, and the $[^3H]UMP$ incorporated into acid-precipitable form was determined. Symbols: 22 C, Δ ; 30 C, $\blacktriangle$; 37 C, $\bullet$; 40 C, $\circ$.

uct. After phenol extraction, the product RNA made in vitro in a 2-h incubation reaction at 37 C was found to be about 40% RNase resistant. Because there was still a 100- to 1,000-fold excess of template after the in vitro incubation, it is very likely that part of the product RNA annealed to the template during the phenol extraction and ethanol precipitation and thus gave rise to the RNase resistance observed. To verify this, the product RNA was analyzed in a sucrose gradient (Fig. 5). About 30% of the product cosedimented with the Uukuniemi virus L, M, and S RNAs, 66 to 82% of this material being resistant to RNase. After melting the product at 100 C for 90 s and quenching on ice, only 10% remained RNase resistant. Similar sucrose gradient analysis of this heated product showed the strands to have been separated from their template. They were recovered as small-sized RNA sedimenting slower than 7S (data not shown). About 70 to 80% of the radioactivity in these strands was converted to RNase-resistant form if they were annealed back to their template after melting. The above results

FIG. 5. Gradient analysis of the product transcribed by Uukuniemi virus RNA polymerase *in vitro*. The RNA transcript made *in vitro* in a 2-h incubation at 37 C and labeled with [3 H]UMP was extracted with phenol and after precipitation with ethanol analyzed on a 15 to 30% (wt/wt) sucrose gradient made in TSE. Centrifugation in a SW27 rotor, narrow tubes, was at 25,000 rpm for 17 h at 22 C. Radioactivity was determined from 0.5-ml fractions before and after treatment with pancreatic RNase (10 μ g/ml) for 30 min at 37 C and 0.2 M NaCl. 32 P-labeled Uukuniemi virus RNA was used as an internal marker to show the migration of L, M, and S RNAs (arrows). Bottom of the tube to the left. 3 H before (●) and after (○) RNase treatment.

suggest that the radioactivity cosedimenting with the L, M, and S RNAs represents small RNA fragments that are partially annealed to their templates and thus contain sequences complementary to all RNA species. The amount of double strandness in the hybrids is apparently too small to cause any significant change in the sedimentation property of the template RNAs.

DISCUSSION

In this paper we report the presence of an RNA-dependent RNA polymerase associated with Uukuniemi virions. This polymerase, like all the others described for enveloped minus-strand RNA viruses, is probably located inside the virus particle, since solubilization of the

viral envelope is a prerequisite for the expression of enzyme activity. Polyacrylamide gel electrophoresis of purified virus resolves only one nucleocapsid polypeptide (N) and two envelope polypeptides (G_1 and G_2) (15, 22). It is unlikely that the polymerase activity resides in either of the two glycoproteins. Whether the N protein, or some other as yet undetected viral polypeptide, is the polymerase has still to be investigated.

Some features of the optimal conditions for enzyme activity distinguish the Uukuniemi virus polymerase from that of other viruses. The most striking one is the total dependence on Mn^{2+} and the rather high optimum concentration required (4 to 5 mM). Some other polymerases also need Mn^{2+} ions, e.g., influenza (4) and Lumbo (5) virus polymerases. Their activity is, however, considerably reduced if Mg^{2+} is omitted, whereas Uukuniemi virus polymerase is not affected by the presence or absence of Mg^{2+} . Alternatively it is possible that Mn^{2+} is at least partly necessary to block the action of an unknown inhibitor of the Uukuniemi virus polymerase reaction. The results may thus be an *in vitro* artifact, not reflecting the true situation in the cell.

Uukuniemi virus polymerase functions at rather high temperatures, the optimum being around 37 to 40 C, in contrast to most of the other virion polymerases, which are more active at around 30 C (4, 7, 9). Whether the high temperature optimum is characteristic for the polymerases of the Bunyaviruses as a whole remains to be studied. Preliminary reports indicate that Lumbo virus polymerase also requires a high temperature for optimal activity (5).

The efficiency of the transcription reaction *in vitro* is rather low compared with that of vesicular stomatitis virus (3) but is of the same order as that of Newcastle disease (9), Pichinde (7), and Lumbo (5) virus polymerases. Uukuniemi virus polymerase functions linearly for about 2 h and at a reduced rate for another hour. The product molecules are not full-sized copies of the templates, but consist mainly of small RNA fragments. These are true transcripts of the template RNA, since they anneal to it. Even if a part of the product RNA is always found associated with all three RNA species, the L, M, and S RNAs (14), it is not clear from the present experiments whether all of the RNAs are transcribed by the virion polymerase or whether only one of them is a functional template.

A virion RNA polymerase has now been reported to be present in two representatives, Uukuniemi virus and Lumbo virus (5), of the

proposed large Bunyavirus group of arboviruses. Uukuniemi virus is the prototype of the Uukuniemi group (8) and Lumbo virus is a member of the California encephalitis group (17). These two viruses are not serologically related but have some structural features in common. The finding of a polymerase in viruses of both these groups is additional evidence that they are closely related and also suggests that all members of the Bunyavirus group may contain an RNA genome of the minus-strand polarity.

The fact that the genomes of the togaviruses and Bunyaviruses seem to be of different polarity suggests that the members of these two large groups of arboviruses replicate by a different mechanism and that they probably are phylogenically distinct from each other (2).

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