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La Crosse Virions Contain a Primer-Stimulated RNA Polymerase and a Methylated Cap-Dependent Endonuclease

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Purified La Crosse virions in vitro were found to transcribe their negative polarity (−) RNA genomes. This polymerase activity was stimulated by oligonucleotides such as $(A)_nG$, cap analogs such as $m^7GpppAm$, and natural mRNAs such as alfalfa mosaic virus RNA 4. For $(A)_nG$- and alfalfa mosaic virus RNA 4-stimulated reactions, evidence is presented that these RNAs stimulate activity by acting as primers for viral transcription. The cap analogs appear to stimulate activity via an alternative mechanism. Purified La Crosse virions were also found to contain an endonuclease which specifically cleaves alfalfa mosaic virus RNA 4 when this RNA contains a methylated cap group.

The genome of La Crosse virus (LAC), a member of the California encephalitis serogroup of the insect-transmitted *Bunyaviridae* (3), consists of three segments of single-stranded RNA of negative polarity [(−)], each contained within separate nucleocapsids which are labeled small (S), medium (M), and large (L). Genetic and molecular studies (10, 11) have led to the following gene assignments: the S segment codes for the N protein, the M segment codes for the two surface glycoproteins, and the L segment, by elimination, codes for the L protein which is located internally and thought to be part of the viral polymerase (19). In addition to the structural proteins, bunyavirus-infected cells also contain two nonstructural (NS) proteins, NS_s and NS_m, coded for by the S and M genome segments, respectively (8, 9).

The LAC S genome segment is 982 nucleotides long and codes for the N and NS_s proteins in overlapping reading frames (2, 8). The use of synthetic oligonucleotide primers and end-labeled DNA and genome RNA probes has revealed that transcription from the 3′ end of the LAC S (−) genome segment is more complicated than expected (21). Both primer extension and S1 nuclease mapping studies have detected the 5′ end of the major-message-sized transcript near the 3′ end of the (−) genome template and at least seven other minor transcripts, also putative mRNAs, which start internally within a 75-nucleotide-long region near this end. S1 nuclease mapping with 3′-end-labeled DNA strands which covered nucleotides 600 to 982 of the S genome segment detected a single 3′ end of the S genome transcripts in this region, at approximately position 886 (22). This finding has suggested that all mRNA-sized transcripts continued to position 886, which contains the sequence GUUUUU, similar to the mRNA termination signals found in the various influenza virus genome segments (28). Further, with 3′-end-labeled S genome RNA as a probe, three leader RNAs complementary to the 3′ end and extending for ca. 75, 95, and 115 nucleotides were also demonstrated (21). The presence of these leader RNAs suggested that the putative mRNAs which start internally are the result of the reinitiation of viral polymerase after termination of leader RNA synthesis.

Recently, the 5′ ends of the major S genome mRNAs of both snowshoe hare virus (SSH) and LAC have been determined more precisely by using defined oligodeoxynucleotides as specific primers for S genome positive polarity [(+)] RNA. For SSH, Bishop et al. (4) have extended the primer on total intracellular 12S RNA, cloned the primer extended transcripts as DNA, and then determined their sequences. Three of the clones reported by Bishop et al. (4) contain an additional 13 nucleotides beyond the precise 3′ end of the (−) genome template which varied in sequence from clone to clone. The sequences of the three clones then converged at the penultimate base of the (−) S genome template and continued as the sequence complementary to the S genome. In the case of LAC, the primer was extended on intracellular CsCl pellet RNA (RNA depleted of genome and antigenome nucleocapsids by CsCl density gradient centrifugation) in the presence of ddNTPs so that the sequence of the total S mRNA population could be determined directly (22). This experiment demonstrated that the sequence of the major LAC S mRNA was complementary to the 3′ end of its (−) genome template up to the penultimate base. The exact 5′ end of the mRNA, however, was found to extend 11 to 15 nucleotides beyond the 3′ end of the (−) genome template, and the sequence of this extension was also heterogeneous. Since these extensions could not have been specified by the SSH and LAC (−) genome templates, this finding has suggested that these bunyavirus S mRNAs start on a primer (4, 22), presumably of host origin, similar to the situation found during influenza virus transcription.

Although all (−) RNA viruses are thought to contain a virion polymerase which initiates the intracellular replicative cycle by primary transcription, this activity has been demonstrated in vitro only with the bunyaviruses Uukuniemi virus (27) and Lumbo virus (5). The possibility that intracellular bunyavirus transcription starts on a primer prompted us to reexamine bunyavirus polymerase activity in vitro, since this activity may be stimulated with primers and cap analogs as previously reported for influenza virus (6, 7, 24, 26). In this paper, we report that LAC virions in vitro do synthesize mRNA and that this activity can be stimulated by synthetic oligonucleotides such as $(A)_nG$, natural primers such as alfalfa mosaic virus (ALMV) RNA 4, and also cap analogs.

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We also provide evidence that the $(A)_nG$ and ALMV RNA 4 stimulation was due to these molecules acting as primers. For ALMV RNA 4-stimulated reactions, purified LAC virions were also shown to contain an endonuclease activity which cleaves ALMV RNA 4 at ca. 12 and 13 nucleotides from the cap group, consistent with the estimated size of the ALMV RNA 4-derived primer. The LAC endonuclease cleaves only ALMV RNA 4 species which contain a methylated cap group.

MATERIALS AND METHODS

Preparation of intracellular RNA. BHK cells were infected and harvested by the method described in reference 20, as modified by H. Lindsey-Regnary (Ph.D. thesis, Emory University, Atlanta, Ga., 1983). Cytoplasmic extracts of uninfected and LAC-infected BHK-21 cells were prepared and fractionated into CsCl pellet and banded material as previously described (21). The CsCl pellet RNA was adjusted to a concentration of 200 optical density units (ODU) per ml.

ODPE analysis. For oligodeoxynucleotide primer extension (ODPE) analysis, oligodeoxynucleotide primers which correspond to nucleotides 38 to 50 and 136 to 147 from the 3′ end of the S (−) genome were gifts of J. Obijeski (Genetech, Inc.). The 5′ end of the primers were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The RNAs to be tested were mixed with 25 ng of labeled primer and ethanol precipitated. The reaction mixture was then resuspended in 7 μl of water and heat denatured at 90°C for 2 min. The reactions were then placed at 43°C, and the reaction conditions were changed to 50 mM Tris (pH 8.3), 8 mM MgCl, 0.08 M NaCl, and 16 U of avian myeloblastosis virus (AMV) reverse transcriptase in a total volume of 20 μl and incubated for 90 min. The reactions were then phenol extracted and ethanol precipitated and analyzed on an 8 or 12% polyacrylamide gel (29).

Preparation of purified LAC virions. BHK cells were infected with LAC virus at a multiplicity of 0.01 PFU per cell and the infection was carried out for 48 h at 37°C. The cellular supernatant was then harvested and cleared by low-speed centrifugation (10,000 × *g* for 20 min), and the virus was then precipitated by the addition of 2.3 g of NaCl and 7 g of polyethylene glycol 6000 per 100 ml, stirred for 4 h at 0°C, and collected by being centrifuged for 20 min at 10,000 × *g*. The pellet was then suspended in TNE, and the virus was banded in a glycerol-tartrate gradient (19) by centrifugation for 4 h at 35,000 rpm in an SW41 rotor. The visible virus band was then removed, diluted with TNE, pelleted through a 30% glycerol cushion in TNE (21), and suspended in TNE–10% glycerol at a concentration of 0.5 to 1 mg/ml. The virus was stored at −20°C and appeared to be stable to freezing and thawing.

LAC virion in vitro polymerase reaction. The in vitro reaction conditions were essentially those of Plotch and Krug (25), and the reaction mixture contained 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.1), 100 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.2% Nonidet P-40, 1 mM ATP, GTP, and CTP, 50 μM UTP containing 5 μCi of [α-^{32}P]UTP, 40 μg of rat liver ribonuclease inhibitor per ml, and purified LAC virions at 100 to 200 μg/ml. The reaction was incubated at 30°C, and incorporation was measured by trichloroacetic acid precipitation onto Whatman G/FC filters which were washed with 5% trichloroacetic acid containing 50 mM $Na_2P_2O_7$. For nonradioactive reactions, the UTP was raised to 1 mM and the reaction products of 100-μl reactions were recovered by centrifugation through a 20 to 40% CsCl gradient after disruption of the virions with 4 volumes of 0.5 M NaCl–1% Nonidet P-40–10 mM EDTA. The pelleted RNA was redissolved, recovered by ethanol precipitation, and dissolved in 50 μl of ET (1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.4).

Preparation of $(A)_nG$. A total of 2 mg of polyadenylate-guanylate (1.8 to 1; P-L Biochemicals, Inc.) in 400 μl of ET was digested with 100 U of ribonuclease T_1 and 6 U of calf intestinal alkaline phosphatase for 90 min at 37°C. The enzymes were digested with 100 μg of proteinase K in 0.2% sodium dodecyl sulfate for 30 min at 55°C and removed by two phenol extractions. The deproteinized RNA was then chromatographed on a 2-ml DE52 column by batch elution with 50, 150, and 333 mM and 1 M triethylammonium bicarbonate (pH 10). The eluted fractions were then dried in a vacuum centrifuge, dissolved in water, and dried two more times. The desalted fractions were then analyzed for chain lengths on cellulose thin-layer plates developed with 70% 1 M ammonium acetate–30% ethanol by using ApG and ApApG (P-L Biochemicals) as markers. Concentrations were determined by assuming that 1 mg was equal to 25 ODUs.

RESULTS

Effect of oligonucleotides and cap analogs on LAC virion polymerase activity. Because of the obvious similarities between the structures at the 5′ ends of bunyavirus and influenza virus mRNAs mentioned above and the large body of information already available for how influenza virus initiates its mRNA synthesis, we have chosen influenza virus as a model in planning our experiments. McGeough and Kitron (17) and Plotch and Krug (25) have demonstrated that guanosine nucleosides, nucleotides, and oliognucleotides stimulate the influenza virion polymerase reaction. The dinucleotide ApG can stimulate the influenza polymerase reaction as much as 100-fold and is thought to act as a primer by base pairing with the 3′-terminal OH-UpC of the influenza (−) genome segments (12, 17, 25). Since the LAC genome segments contain a similar 3′-terminal sequence (20), purified LAC virions were incubated as described above, plus and minus the oligonucleotides as shown in Table 1, and trichloroacetic acid-precipitable incorporation was determined. LAC virions alone did incorporate a low level of [^{32}P]UTP into acid-precipitable material. The dinucleotide ApG (but not GpA or ApA; data not shown) and the trinucleotide ApApG stimulated polymerase activity 1.4- to 4.1-fold and 1.2- to 13.2-fold, respectively (Table 1). The extent of stimulation varied from one virus preparation to another for unknown reasons, but the stimulation by these short oligonucleotides appeared greatest when the endogenous polymerase activity of the virions was lowest. Bouloy et al. (7) and Penn and Mahy (24) have demonstrated that cap analogs such as m^7GpppAm also stimulate the influenza virion polymerase reaction. However, unlike dinucleotides such as ApG, the cap analogs are not incorporated into the transcripts but presumably act through an alternative mechanism, as the cap analog and ApG stimulations are additive. Table 1 also demonstrates that these cap analogs similarly stimulate the LAC virion polymerase reaction from 2.6- to 4.5-fold, and when added together with ApG or ApApG, the stimulations are similarly additive.

By far the strongest stimulators of the influenza polymerase in vitro are natural mRNAs which contain the cap 1 structure at their 5′ end; these RNAs stimulate activity at molarities hundreds of times lower than oligonucleotides

TABLE 1. Effect of primers and cap analogs on LAC virion polymerase activity[a]

Expt	Additions	Sp act (10^3 cpm/5 μl)	Fold stimulation
1	None	3.0	
	A-G	4.2	1.4
	AAG	7.1	2.5
	$mGpppAm_2^2$	13.6	4.5
	A-G + $mGpppAm_2^2$	19.6	6.5 (5.9)
	AGG + $mGpppAm_2^2$	22.2	7.4 (6.9)
2	None	6.5	
	AAG	8.0	1.2
	$mGpppAm_2^2$	16.7	2.6
	AAG + $mGpppAm_2^2$	22.1	3.4 (3.8)
	ALMV4	46.6	7.2
	ALMV4 + $mGpppAm_2^2$	31.2	4.8 (9.8)
3	None	5.0	
	mGpppAm	18.8	3.8
	$mGpppAm_2^2$	21.5	4.3
	ALMV4	64.5	12.9
4	None	2.1	
	AAG	9.2	4.5
	None	1.3	
	AAG	5.2	4.0
5	None	0.97	
	A-G	3.98	4.1
	AAG	12.78	13.2

[a] Polymerase reactions were carried out as described in the text. Where indicated, A-G and AAG were added at 0.40 and 0.28 mM, respectively, $m^7GpppAm$ and $m^7GpppAm_2^2$ were added at 0.25 mM, and ALMV RNA 4 was added at 1.4 μM. Values for zero time controls or a reaction which contained 20 mM EDTA in addition (1,000 to 1,500 cpm) were subtracted from the values shown. The specific activity of the UTP was calculated at 6,400 cpm Cerenkov per pmol. The numbers in parentheses in the stimulation column show the expected stimulation if the effect of the primers and the cap analogs was additive. The reactions were incubated for 2.5 h at 30°C. Experiments 1, 2, 3, and 5 were each carried out with a different virus preparation; experiment 4 was carried out with two separate virus preparations.

such as ApG (6, 7). Krug and co-workers have shown that this stimulation is due to a virus-coded endonuclease contained within the influenza polymerase complex which generates an oligonucleotide primer containing the 5'-capped end of the mRNA and the first 12 to 14 nucleotides, which are then incorporated into the influenza transcripts (15, 26, 30, 31). LAC virions were therefore incubated with ALMV RNA 4 which contains the partially methylated cap structure m^7GpppG at its 5' end (14). In two experiments, 1.4 μM ALMV RNA 4 stimulated the LAC polymerase to 7.2 and 12.9 times the control level. Interestingly, when ALMV RNA 4 was added together with a cap analog, these stimulations were antagonistic rather than additive (Table 1).

Purified LAC virions thus contain an endogenous RNA polymerase which is stimulated by the same oligo- and polynucleotides as the influenza polymerase in vitro. To examine the products of the LAC virion polymerase, samples of the unstimulated (no oligonucleotides added) and ApG-stimulated (2.5-fold) reaction products were isolated after phenol extraction together with the virion (−) genome RNAs. After self-annealing, single-stranded RNAs were digested with RNase A in high salt, and the remaining double-stranded RNAs were examined by polyacrylamide gel electrophoresis (PAGE) (Fig. 1). Smears rather than discrete bands, 100 to 1,000 base pairs long, were seen in both the unstimulated and ApG-stimulated reactions. The proportion of transcript from each of the three LAC genome segments was not determined.

ApG-stimulated S genome polymerase products at the precise 3' end of the S (−) genome template. To determine where the ApG-stimulated reaction products had initiated RNA synthesis, the position of the 5' end of the in vitro transcripts relative to the 3' end of the S (−) genome template was measured. The LAC polymerase products were first isolated free of the virion (−) genomes by CsCl density gradient centrifugation under conditions where the (−) genome RNAs which were contained in helical nucleocapsids banded at a buoyant density of 1.3 (21; Lindsey-Regnary, Ph.D. thesis), but the unencapsidated reaction products pelleted through the gradient. To determine the position of the 5' end

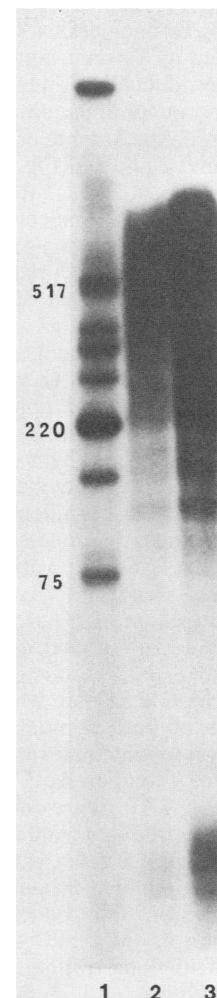

FIG. 1. Examination of chain length of LAC in vitro transcripts. The total RNAs from both an unstimulated (lane 2) and an ApG-stimulated (lane 3) 4-h polymerase reaction were isolated by phenol extraction after incubation of the reactions with 500 μg of proteinase K per ml in 0.2% sodium dodecyl sulfate–10 mM EDTA for 5 min at 60°C. The ethanol-precipitated RNAs were then digested with 10 μg of RNase A per ml in 2.5× buffer A (0.375 M NaCl, 25 mM Tris [pH 7.4], 2.5 mM EDTA) for 15 min at 25°C, followed by proteinase K digestion as above for a further 15 min at 25°C. The remaining double-stranded RNAs were recovered by ethanol precipitation and electrophoresed on an 8% 40:1 (acrylamide/bis-acrylamide ratio) nondenaturing gel along with end-labeled *Hin*fI digestion products ucts of pBR322 as markers (lane 1).

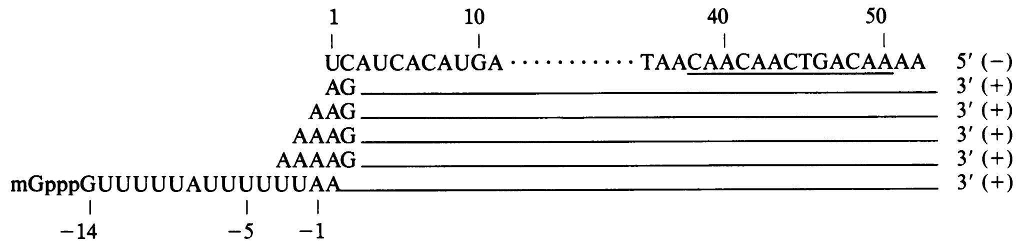

FIG. 2. Schematic representation of LAC in vitro transcripts. The top line shows the sequence at the 3′ end of the S (−) genome with the precise 3′ end at the left and numbered at position 1. The synthetic oligodeoxynucleotide used for ODPE analysis (position 38 to 50) is underlined. The middle four lines show the 5′ ends of the $(A)_nG$-stimulated reaction products (on the left) as deduced from this work. The bottom line shows the 5′ end of the ALMV RNA 4-stimulated transcript, assuming that the 5′ end of ALMV RNA 4 was transferred to the in vitro product. The minus numbers denote extension beyond the 3′ end of the S (−) genome.

of the transcript made in vitro, a synthetic oligodeoxynucleotide corresponding to nucleotides 38 to 50 of the (−) S genome RNA was used as a primer and extended on the in vitro (+) polymerase products by using reverse transcriptase (Fig. 2). To avoid subsequent ambiguities, this reaction will be referred to as ODPE. An example of such an experiment is shown in Fig. 3. Lanes 2 and 3 of Fig. 3A show the results of the ODPE analysis on CsCl pellet RNA from uninfected and LAC-infected BHK cells, respectively, as a control. Note that whereas no primer extension occurred on uninfected RNA, on LAC-infected CsCl pellet RNA the primer was extended both to a single band ca. 50 bases long, relative to the restriction fragment markers, as well as a group of four bands 12 to 15 bases longer. The band ca. 50 bases long was determined to represent extension to the precise 3′ end of (−) genome (position 1), since in a parallel experiment the primer was also extended on (+) antigenome RNA isolated from intracellular nucleocapsids. This extension yielded a single band which comigrated with the 50-base band (Fig. 3B, lanes 1 and 2). Since CsCl gradients appear to cleanly separate genome and antigenome nucleocapsids from other virus-specific RNAs, the band at position 1 in lane 3, Fig. 3A, probably represents leader RNAs or other (+) transcripts rather than antigenome RNA. The bands at −12 to −15 represent mRNAs with nontemplated 5′ extensions previously described.

The oligodeoxynucleotide primer was also extended on the reaction products of both a mock virion polymerase reaction, i.e., a reaction to which no ribonucleoside triphosphates were added (lane 1) and an ApG-stimulated polymerase reaction (lane 4, Fig. 3A). Interestingly, ODPE analysis of the mock polymerase products yielded a pattern identical to that of LAC-infected CsCl pellet RNA, whereas primer extension on the ApG-stimulated products yielded a greatly enhanced band at position 1 and bands around position 1 without any stimulation of the bands at −12 to −15. The unexpected finding of identical primer extensions on both mock-polymerase products and bona fide intracellular mRNAs (LAC-infected CsCl pellet RNA) could be due to either contaminating LAC transcripts or ribonucleoside triphosphates in the virus preparation or both. LAC virus does not produce high yields in tissue culture. The virus used in the mock-polymerase reaction represents the harvest from 12 plates of cells, whereas the LAC-infected CsCl pellet RNA used was the yield from 4/10 of a single plate, and other pleomorphic (−) RNA viruses such as Sendai virus are known to contain considerable amounts of cellular contaminants (13). Since the method used to determine the 5′ end of the transcripts is indirect, it cannot differentiate between de novo transcripts and preexisting ones. However, when excess EDTA was included in the polymerase reaction (with a separate virus preparation), bands representing the LAC transcripts were not detected (lane 1, Fig. 4A). By subtracting the pattern in lane 1 from that in lane 4, we conclude that

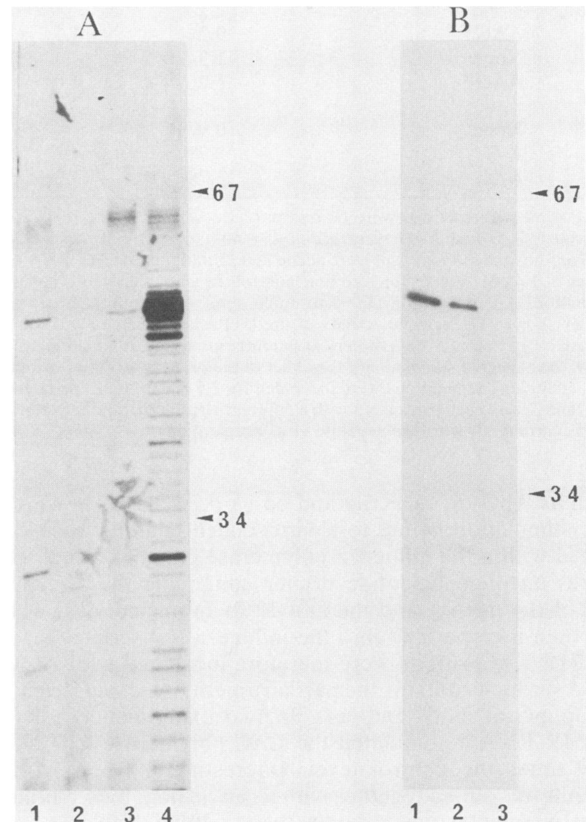

FIG. 3. ODPE analysis of mock- and ApG-stimulated polymerase products. (A) The products of nonradioactive 100-μl reactions were pelleted through CsCl density gradients and used for primer extension of end-labeled oligodeoxynucleotides as described in the text. Lanes 2 and 3, result of ODPE analysis of 1 ODU of uninfected and LAC-infected intracellular CsCl pellet RNA, respectively. Lanes 1 and 4, ODPE analysis of 40% of the mock- and ApG-stimulated reaction products, respectively. The numbers at the right refer to the marker positions of the *Msp*I digestion of pBR322. (B) ODPE analysis of RNA extracted from intracellular nucleocapsids banded in CsCl density gradients (lane 1), 20% of ApG-stimulated reaction products (lane 2), and 1 ODU of LAC-infected CsCl pellet RNA (lane 3). The tetramer band at position −12 to −15 is just visible below the 67-base marker in lane 3.

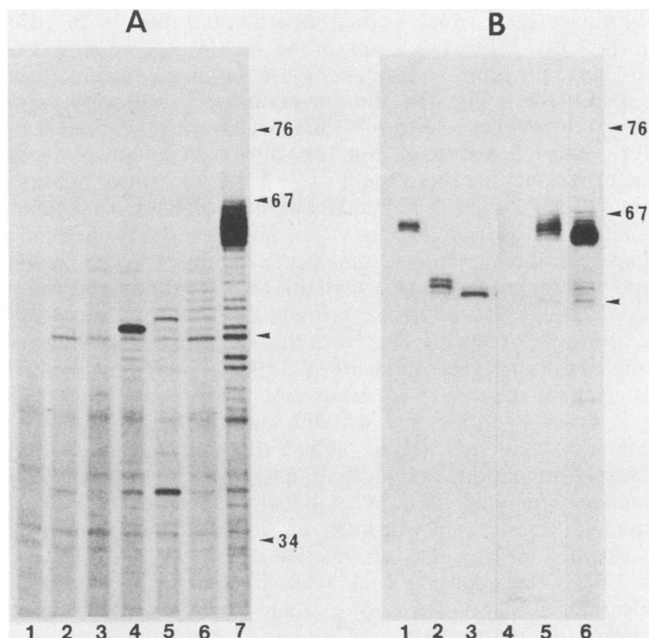

FIG. 4. ODPE analysis of $(A)_nG$- and ALMV RNA 4-stimulated reactions. (A) The products of 100-μl reactions containing an additional 20 mM EDTA (lane 1), no additions (lane 2), and 0.4 mM $(A)_1G$, $(A)_2G$, $(A)_{3-5}G$, or $(A)_{3-9}G$ (lane 3 to 6, respectively) were recovered through CsCl gradients, and 40% of each reaction was analyzed by ODPE. Lane 7, ODPE analysis of 1 ODU of intracellular LAC CsCl pellet RNA. The arrow shows position 1 as determined in Fig. 3B (see text). (B) ODPE analysis of 40% of the ALMV RNA 4-stimulated reaction products (lane 1), 40% of the $(A)_{3-5}G$- and $(A)_2G$-plus-mGpppAm-stimulated reaction products (lanes 2 and 3, respectively), and 1 ODU of intracellular uninfected and LAC-infected CsCl pellet RNA (lanes 4 and 5, respectively). Lane 6, Longer exposure of lane 1.

the major de novo transcripts of the ApG-stimulated reaction began at position 1, or the precise 3′ end of the S genome, with minor transcripts on either side of position 1. This can be seen more clearly in Fig. 3B, lane 2, which shows a lesser exposure of the ODPE analysis of the ApG-stimulated in vitro reaction.

$(A)_nG$ and ALMV RNA 4: primers for LAC transcription in vitro. The question of whether in the $(A)_nG$-stimulated reactions the oligonucleotides were acting as primers was also examined by ODPE analysis. If indeed the ApG-stimulated reactions began transcription at the precise 3′ end of the S genome template with the 5′ ApG base pairing with the 3′-terminal HO-UpC and acting as a primer, then analysis of the AAG-stimulated reaction products as described above should lead to transcripts whose 5′ ends started at position −1, the AAAG-stimulated reactions at position −2, etc. (Fig. 2). LAC polymerase reactions were therefore carried out as described in Fig. 4A with an additional 20 mM EDTA to inhibit de novo RNA synthesis (lane 1), no additions (lane 2), ApG (lane 3), $(A)_2G$ (lane 4), $(A)_{3-5}G$ (lane 5), or $(A)_{3-9}G$ (lane 6), and the reactions products were isolated as above and analyzed by ODPE. The pattern of primer extensions on intracellular CsCl pellet RNA in a parallel reaction is shown in lane 7. Note that in lane 7 the same pattern of bands at −12 to −15 was seen as before, but a group of doublet bands around position +1 was detected rather than one prominent band as in lane 3 of Fig. 3A. The reasons for this variation are not clear (see below), but the band representing the precise 3′ end of the S genome was again determined by ODPE analysis on antigenome RNA and found to be the lower band of the middle doublet (arrow, Fig. 4). When the primer was extended on the reaction products of the unstimulated and $(A)_nG$-stimulated reactions, the unstimulated and the ApG-stimulated reactions yielded mostly a single band at position +1 (lanes 2 and 3), the AAG-stimulated reaction yielded an additional strong band at position −1 (lane 4), and the $(A)_{3-5}G$- and $(A)_{3-9}G$-stimulated reactions yielded additional bands at −2 to −3 and −2 to −6, respectively (lanes 5 and 6). Also note that at this exposure, no bands in this region were observed when excess EDTA was added to the reaction before the virus (lane 1). It therefore appears that the unstimulated LAC virion polymerase reaction initiates RNA synthesis at the precise 3′ end of the (−) genome template, presumably with ATP, and that the $(A)_nG$-stimulated reactions initiate RNA synthesis by the ApG portion of each oligonucleotide base pairing with the 3′ end of the (−) genome and acting as a primer.

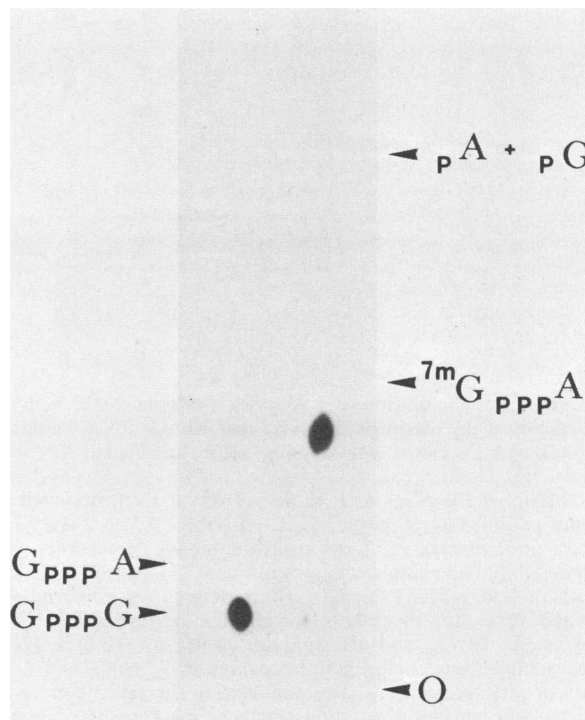

FIG. 5. Preparation and characterization of cap-labeled ALMV RNA 4. ALMV RNA 4 (50 μg) in 110 μl of 150 mM sodium acetate (pH 5.3) was reacted with 0.9 mM sodium periodate for 30 min at 0°C in the dark. The oxidized RNA was recovered by ethanol precipitation, and the cap group was β-eliminated by incubation with 100 μl of 0.3 M aniline–10 mM sodium acetate (pH 5) for 3 h at 25°C. The decapped RNA was recovered by ethanol precipitation, and 5-μg samples of the RNA were then recapped with 10 μM [α-^{32}P]GTP (400 Ci/mM) in the presence and absence of 100 μM *S*-adenosylmethionine, using the vaccinia virus guanylyl- and methyltransferases as previously described (18). The reactions were then phenol extracted, chromatographed on Sephadex G-50, and recovered by ethanol precipitation. Samples (5,000 cpm) of the capped (left lane) and capped and methylated (right lane) RNAs were then digested with 10 μg of nuclease P1 in 1 mM sodium acetate (pH 5.3) for 2 h at 37°C and electrophoresed on DE81 paper at pH 3.5 along with the markers indicated. O, Origin of the electrophoretogram; the anode is at the bottom.

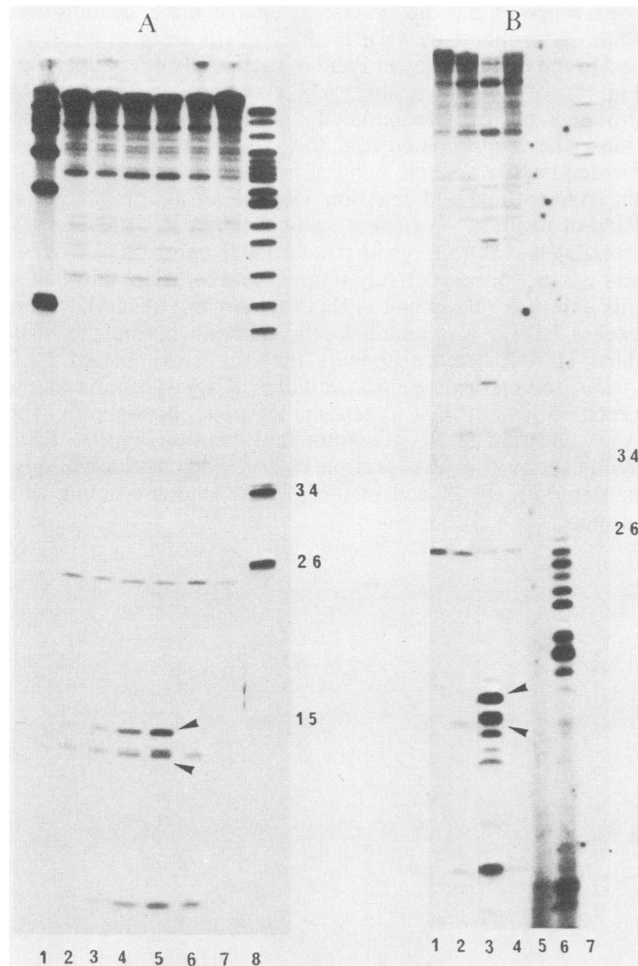

FIG. 6. Characterization of LAC virion endonuclease activity. (A) Samples of the methylated cap-labeled ALMV RNA 4 containing 20,000 cpm Cerenkov (ca. 0.5 pmol) were incubated at 30°C in 20 μl of 50 mM HEPES (pH 8.1)–100 mM KCl–6 mM $MgCl_2$–1 mM dithiothreitol–0.2% Nonidet P-40–40 μg of rat liver ribonuclease inhibitor per ml–3 μg of purified LAC virions for 0.5, 1, 2 and 5 min (lanes 2 to 5, respectively). As controls, identical reactions were incubated for 20 min either with an additional 40 mM EDTA (lane 6) or without LAC virions (lane 7). The reactions were then phenol extracted, recovered by ethanol precipitation in the presence of 20 μg of carrier tRNA, and electrophoresed on a 7 M urea–12.5% polyacrylamide sequencing gel. Lanes 1 and 8, *Hin*fI and *Msp*I digests of pBR322 markers. The numbers on the right refer to the lengths of the smaller restriction fragment markers. (B) Samples containing 20,000 cpm Cerenkov of either unmethylated (lanes 1 and 2) or methylated (lanes 3 and 4) cap-labeled ALMV RNA 4 were incubated for 20 min as described above either in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of purified LAC virions. In a separate experiment, LAC virions were incubated in 25-μl reactions as above with the addition of 10 μM [α-^{32}P]GTP (400 Ci/mM) either in the presence (lane 6) or absence (lane 5) of 10 μg of unmodified ALMV RNA 4. The reactions were then treated as described above. Lane 7; *Msp*I digestion of pBR322 as markers.

We next examined the position of the 5′ end of the products of the ALMV RNA 4-stimulated reaction. Krug and co-workers (26) have shown that natural mRNAs such as ALMV RNA 4 stimulate influenza virion transcription by first being cut by a virion endonuclease to generate a primer containing the capped 5′ end of the RNA and the first 12 to 14 nucleotides, which is then incorporated into the in vitro transcripts. When the 5′ end of the ALMV RNA 4-stimulated LAC polymerase products were similarly examined by ODPE (lane 1, Fig. 4B), the vast majority of transcripts were found to begin at −12 to −14, similar to intracellular mRNAs (cf., lanes 5 and 6 of Fig. 4B) but with somewhat less heterogeneity in this region. The LAC virion polymerase thus appears to be stimulated by natural mRNAs in a manner analogous to the influenza virion polymerase. In the same experiment we examined the effect of adding the cap analog mGpppAm to the $(A)_2G$- and the $(A)_{3-5}G$-stimulated reactions. The results of this experiment are shown in lanes 3 and 2 of Fig. 4B, respectively. Note that the presence of the cap analog does not alter the pattern of the 5′ ends of the in vitro transcripts relative to the transcripts made in their absence (cf., lanes 4 and 5 of Fig. 4A with lanes 3 and 2 of Fig. 4B). Since we have shown in Table 1 that the cap analog and $(A)_nG$ stimulations are additive, it seems likely that the cap analogs stimulate the LAC polymerase reaction differently than $(A)_nG$, possibly through an allosteric mechanism as suggested for influenza virions (24).

LAC virions contain a methylated cap-dependent endonuclease. As mentioned above, Krug and co-workers have shown that influenza virions contain an endonuclease which cleaves natural mRNAs such as coat protein mRNAs of brome mosaic virus and ALMV to generate an oligonucleotide containing the 5′-capped end of the mRNA and the first 13 or so nucleotides, which can then be used as a primer to initiate influenza mRNA synthesis (26). The influenza endonuclease has also been shown to require a methylated 5′-capping group on its RNA substrate for activity (26). To investigate whether LAC virions contain a similar endonuclease, which is responsible for the generation of a primer from ALMV RNA 4 estimated to be 12 to 14 nucleotides in length, ALMV RNA 4 uniquely radiolabeled in the cap group both as the unmethylated and methylated derivatives was prepared and analyzed by nuclease P1 digestion and electrophoresis on DEAE paper. As shown in Fig. 5, the unmethylated cap-labeled ALMV RNA 4 yielded a single spot which comigrated with marker GpppG (left lane), whereas the methylated RNA yielded mostly a single spot (right lane) which migrated just behind the markers mGpppA or mGpppAm, which do not separate in this system, consistent with the cap group mGpppG or mGpppGm. It seems likely that the methylated cap-labeled ALMV RNA 4 contains the cap group mGpppGm, since the vaccinia virus extract used to prepare the cap-labeled RNAs contains both the 7-methyl- and 2-*O*-methyltransferase activities (16, 18).

The experiment demonstrating the LAC virion endonuclease and describing some of its properties is shown in Fig. 6, in which the cap-labeled ALMV RNA 4 was incubated under the LAC virion polymerase reaction conditions but without added triphosphates. Lanes 2 to 5 of panel A show the kinetics of the LAC endonuclease activity on the methylated cap-labeled ALMV RNA 4, which is cleaved to mostly a doublet band (marked with arrows) ca. 13 and 14 nucleotides long relative to the 15-base *Msp*I restriction fragment marker, and a much smaller band which is ca. six to seven nucleotides long. The kinetics appear linear for the first 5 min, and the reaction is complete by 10 min (not shown). A control reaction which was incubated for 20 min without LAC virions (lane 7) shows no bands in this size range. Another control reaction which was incubated for 20 min in the presence of LAC virions but which contained 40 mM EDTA in addition showed some cleavage to the bottom band of the doublet and to the 6- to 7-mer but no detectable top

doublet band (lane 6). The cleavage of the methylated cap-labeled ALMV RNA 4 is thus dependent on the presence of purified LAC virions and most of the cleavage observed appears to be dependent on divalent cations. In addition, the LAC virion endonuclease activity is a relatively fast reaction compared with the virion RNA polymerase activity, which is linear for up to 2.5 h under these incubation conditions (not shown), and like the influenza endonuclease activity (26), it is apparently not coupled to RNA synthesis.

The possible requirement for a methylated cap structure in the endonuclease reaction was investigated by incubating both unmethylated (lanes 1 and 2, Fig. 6B) and methylated cap-labeled ALMV RNA 4 (lanes 3 and 4) in the presence (lanes 1 and 3) and absence (lanes 2 and 4) of LAC virions for 20 min. Lane 3 of Fig. 6B shows the same endonuclease pattern as seen for shorter incubations of the methylated ALMV RNA 4 (Fig. 6A), but the longer reaction here demonstrated that cleavages on either side of the major doublet also occur, but at a lesser frequency. Incubation of the unmethylated ALMV RNA 4 with LAC virions (lane 1) on the other hand did not lead to any detectable cleavage of this RNA. These results demonstrate that the endonuclease activity of purified LAC virions, similar to that of influenza virus, will not cleave an RNA which does not contain a methylated capping group.

ALMV RNA 4 extracted from purified virions contains at its 5′ end the partially methylated cap group mGpppG (14). As shown above, this RNA is a potent stimulator of the LAC virion polymerase reaction, yielding transcripts whose 5′ ends began 12 to 14 nucleotides beyond the 3′ end of their (−) genome template. This suggests that this RNA was cleaved to produce fragments of this size which were then used as primers for RNA synthesis. It should therefore be possible to also demonstrate the LAC endonuclease activity by using this nonradioactive ALMV RNA 4 in a polymerase reaction which contained only a single radioactive ribonucleoside triphosphate. Under these conditions the cleaved ALMV RNA 4 primer should be extended by the virion polymerase only with this single triphosphate. GTP was chosen as the single radiolabeled triphosphate because of the analogies both in the 3′-end sequences of the genomes and the apparently similar endonuclease activities of influenza and LAC viruses (20, 26). The results of such an incubation are shown in lane 6 of Fig. 6B, and those of a parallel reaction in which the ALMV RNA 4 was omitted are shown in lane 5. The presence of ALMV RNA 4 in such a polymerase reaction which contained only [^{32}P]GTP as added triphosphates led to the appearance of a group of bands, ca. 16 to 24 nucleotides long, which were entirely absent when the mRNA was omitted (lane 5). The reasons for this heterogeneity of extension are not clear, but possible explanations include the presence of small amounts of contaminating ribotriphosphates in the purified LAC virions and that the RNA containing the partially methylated cap group (mGpppG) is cleaved more heterogenously than ALMV RNA 4 containing a mGpppGm cap group. Nevertheless, this experiment demonstrates that ALMV RNA 4, which stimulates the LAC polymerase reaction as described above, is also cleaved by purified LAC virions and that the cleavage products can be extended for a few nucleotides in the presence of limiting amounts of ribotriphosphates. In addition, at least some of the endonuclease cleavage products therefore contained 3′ OH ends. The above experiments thus demonstrate that LAC virions contain an endonuclease activity which is remarkably similar to that previously described for influenza virus (26) and suggest that the oligonucleotide products of this reaction can be used as primers for the initiation of RNA synthesis by the LAC virion polymerase.

DISCUSSION

The work described in this paper has demonstrated that purified LAC virions in vitro synthesize (+) transcripts and that this polymerase activity can be stimulated by (i) oligonucleotides such as $(A)_nG$ which act as primers and are incorporated into the in vitro transcripts, (ii) cap analogs such as mGpppAm, by a different mechanism than the $(A)_nG$ stimulation since their effects are additive, and (iii) natural mRNAs such as ALMV RNA 4. The stimulation of LAC polymerase by ALMV RNA 4 is presumably due to the action of an endonuclease which generates a primer 12 to 14 nucleotides long, since ODPE analysis of these in vitro transcripts demonstrated that their 5′ ends began 12 to 14 nucleotides beyond the 3′ end of the S genome. The presence of such an endonuclease activity in purified LAC virions, which cleaves ALMV RNA 4 ca. 12 and 13 nucleotides from the cap group, has also been demonstrated. Again, analogous to the influenza virion endonuclease, the LAC endonuclease cleaves only ALMV RNA 4 which contains a methylated cap group. The in vitro polymerase activity of LAC virions is thus remarkably similar to that of influenza virus. In addition, the previous demonstration that bunyavirus mRNAs synthesized in vivo contain 11 to 15 nontemplated nucleotides at their 5′ end which are heterogeneous in sequence (4, 22), similar to intracellular influenza mRNAs, again suggests that bunyaviruses and influenza viruses initiate the synthesis of their mRNAs by a similar mechanism, i.e., the capture of the 5′ end of a host mRNA in vivo and its use as a primer. The primer derived from host mRNA presumably represents the 5′ end of the mRNA containing the methylated cap structure required for the efficient translation of mRNAs in eucaryotic cells.

ODPE analysis of intracellular S genome-coded RNAs which had pelleted through CsCl density gradients, i.e., those RNAs which were not assembled into nucleocapsid structures, has demonstrated a rather varied array of transcripts with differing 5′ ends. By using primer extension as a guide to relative amounts, by far the most abundant transcripts are those whose 5′ ends are at position −10 to −15, i.e., the major S mRNA. However, transcripts whose 5′ ends are at position 1 and around position 1 are also seen. The relative amounts of these latter transcripts have varied from experiment to experiment, but the results shown in Fig. 4 in which a pattern of doublet bands is detected at position 4 and 3, 1 and −1 and −3 and −4 are more representative of our findings (they are also visible on an overexposure of Fig. 3). Whether these latter transcripts are also initiated by primers intracellularly is as yet unknown. Recently, Bishop et al. (4) have analyzed the 5′ ends of SSH total intracellular 12S RNA also by using a synthetic deoxyoligonucleotide primer, but the extended primers were then cloned and sequenced as DNA. These authors reported the sequence of 5 clones. As mentioned in the introduction, three clones contained a 13-nucleotide-long sequence at the 5′ end beyond position 1 which varied from clone to clone and probably represents the major S mRNA of SSH. The remaining two clones, however, had 5′ ends which began not at position 1 but at position 3 or 4 (the ambiguity is due to the tailing procedure used in the cloning). These latter clones presumably reflect transcripts which are analogous to the lower doublet band described above for intracellular LAC S

genome transcripts. Thus, transcripts whose 5′ ends are at positions 3 and 4 appear to be present in both LAC- and SSH-infected cells. The significance of these minor transcripts in bunyavirus-infected cells remains cloudy.

A curious feature of bunyavirus replication is the apparent requirement for on-going protein synthesis not only for genome replication (1, 22, 23) but also for transcription (1, 23). One possible interpretation of this unusual finding regarding transcription is that intracellular bunyavirus transcription both was primer dependent and requires an unstable host factor to generate the primer (22). The work described in this paper, however, casts doubt on this interpretation, since purified LAC virions can both cleave ALMV RNA 4 and use the products of this cleavage as primers in vitro. In addition, the ALMV RNA 4-stimulated virion polymerase reaction reported here clearly does not require on-going protein synthesis. The transcriptional requirement for on-going protein synthesis in bunyavirus-infected cells thus remains without explanation.

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