JOURNAL OF VIROLOGY, March 1978, p. 912-916
0022-538X/78/0025-0912$02.00/0

Vol. 25, No. 3
Printed in U.S.A.

Spring Viremia of Carp Virus RNA and Virion-Associated Transcriptase Activity

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Received for publication 1 November 1977

An RNA-dependent RNA polymerase activity has been demonstrated for spring viremia of carp virus (SVCV). The optimal temperature for in vitro synthesis of RNA was 20 to 25°C. The SVCV enzyme activity was stimulated when the methyl donor *S*-adenosyl-L-methionine was included in the reaction mixture. *S*-adenosyl-L-methionine was not particularly effective in stimulating the virion RNA polymerase activity of vesicular stomatitis virus or pike fry rhabdovirus. The 5′ nucleotide of the SVCV viral RNA is pppAp.

Several fish diseases have been shown to be caused by viruses (8, 25), at least four of which are rhabdoviruses: spring viremia of carp virus (SVCV), infectious hematopoietic necrosis virus of salmonid fish (IHNV), viral hemorrhagic septicemia virus of rainbow trout (VHS virus), and pike fry rhabdovirus (PFR). The salmonid viruses, IHNV and VHS virus, resemble rabies virus in their protein structure (15, 17, 18) and have been shown to have virion-associated RNA-dependent RNA polymerase activities with both lower temperature optima and different divalent cation requirements than vesicular stomatitis virus (VSV) (19). Pike fry rhabdovirus has a protein structure like VSV (22) and has a virion-associated RNA-dependent RNA polymerase which can be assayed in vitro (22). Evidence has been obtained indicating that this enzyme functions as a transcriptase for synthesis of viral complementary RNA in infected cells (22). SVCV has three major structural proteins, a glycoprotein, G, a putative membrane protein, M, a nucleocapsid protein, N, as well as two minor proteins, L and NS (16, 23). Two phosphoproteins are found in SVCV preparations (23); one is an NS-type protein, whereas the other, although having a similar electrophoretic mobility to the viral N protein in tube gels, can be distinguished from the N protein by slab gel electrophoresis (P. Roy and J. P. Clewley, *Negative Strand Viruses and the Host Cell*, in press). In this communication we report an SVC virion-associated RNA-dependent RNA polymerase that is stimulated in in vitro assays by *S*-adenosyl-L-methionine (SAM). Also we report that the 5′ nucleotide of SVCV RNA is pppAp.

Monolayers of fathead minnow (FHM) cells were grown at 25°C in Eagle minimum essential medium supplemented with 10% fetal calf serum (12, 22). Baby hamster kidney cells (BHK, clone 21) were grown in the same medium at 38°C. SVCV was cloned by standard techniques in FHM cells (5). SVCV or PFR were grown in monolayers of FHM or BHK cells at 25°C, whereas VSV was grown in BHK cells at 33°C. [^{3}H]uridine (100 μCi/ml) or [^{32}P]sodium orthophosphate (50 μCi/ml) was added to the growth medium for preparing labeled viral RNA. Purification of the viruses from clarified supernatant fluids by polyethylene glycol precipitation and subsequent equilibrium and velocity gradient centrifugation has been described previously (20).

The presence of a virion RNA-dependent RNA polymerase is characteristic of negative-strand viruses (2, 3) and, indeed, is necessary for the synthesis of complementary messenger RNA in infected cells (3). The presence of this enzyme in SVCV preparations has been demonstrated by both in vitro and in vivo analyses. The procedure used to assay an SVC virion RNA polymerase followed the methods described previously for VSV (1) and PFR (22), using the incorporation of [α-^{32}P]ATP into an acid-insoluble product to monitor the de novo RNA synthesis. For SVCV, optimal conditions for the in vitro assay were similar to those reported for VSV and PFR (1, 22), except that the temperature optimum was between 20 and 25°C (Fig. 1). Since the normal host for SVCV is a freshwater carp, the low temperature optimum probably reflects an adaptation of the virus to growth in a poikilothermic animal. The RNA polymerase activity was linear for at least 4 h of incubation (data not shown). The inclusion of 0.5 to 1 mM SAM in the SVCV reaction mixture stimulated enzyme activity (Fig. 2), but did not appreciably stimulate the virion polymerase of VSV or PFR. This result for SVCV is similar to that originally reported for cytoplasmic polyhedrosis virus (11),

although cytoplasmic polyhedrosis virus transcriptase is stimulated to a much greater extent. It is not known whether the stimulation by SAM relates to a block in transcription reinitiation due to the accumulation of unmethylated products. Analysis of the 5'-terminal structure of SVCV mRNA's synthesized in the presence and absence of SAM should answer this question.

The observation that SVCV possesses a virion RNA polymerase suggests that, as for VSV and PFR (10, 22), the viral enzyme should be capable of directing the intracellular synthesis of viral complementary RNA. To test this, a preparation of [^{3}H]uridine-labeled SVCV was used to infect confluent FHM (or BHK) cell monolayers, and, as shown in Fig. 3A, the presence and amount of viral complementary RNA were determined as described previously (22). It was found that the growth of SVCV in terms of released virus from either cell line was essentially equivalent (Fig. 3B). The kinetics of viral complementary RNA obtained from the two host cell lines was also similar, although it appeared that the overall accumulation of viral complementary RNA was

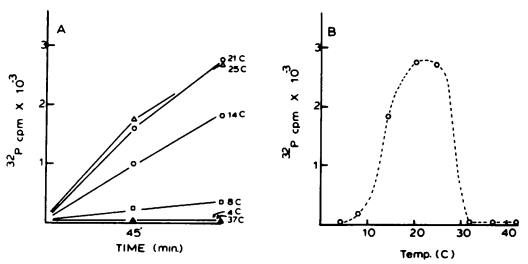

FIG. 1. *RNA polymerase activity of SVCV. The endogenously templated RNA polymerase activity associated with SVCV was measured at different temperatures of incubation (A) as described previously (1, 22). The temperature optimum of the enzyme activity (B) was determined for the 90-min reaction product.*

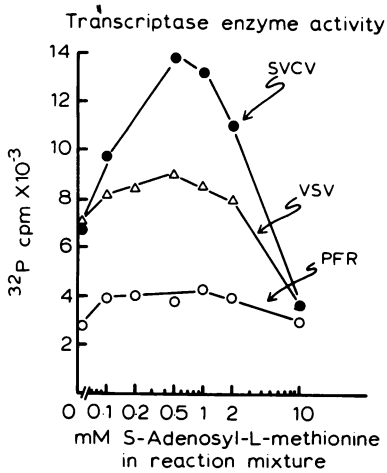

FIG. 2. *Effect of SAM concentration on VSV, PFR, and SVCV virion RNA polymerases. The incorporations of [^{32}P]AMP into product RNA by standard reaction mixtures containing [α-^{32}P]ATP (specific activity, 5 mCi/μmol), various concentrations of SAM, and templated by VSV, PFR, or SVCV were determined as described previously (22). The PFR and SVCV reaction mixtures were incubated at 20°C, whereas the VSV reaction mixture was incubated at 28°C. The [^{32}P]AMP incorporations, which were linear for 4 h of incubation, are plotted.*

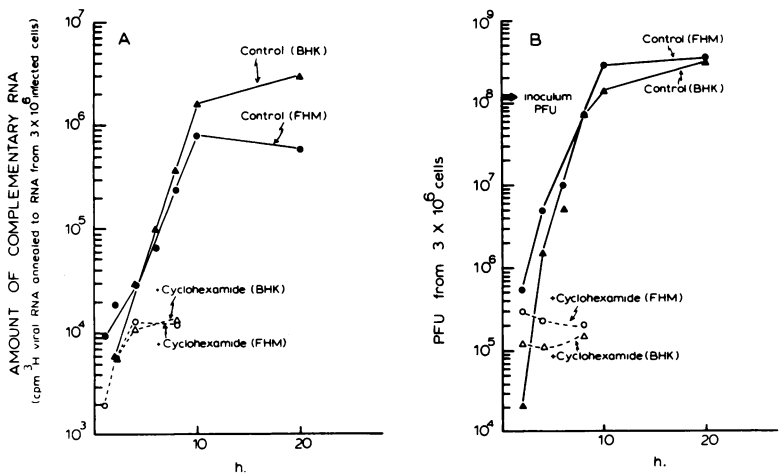

FIG. 3. *The in vivo synthesis of viral complementary RNA by SVCV in BHK and FHM cells. The intracellular viral complementary RNA produced by SVCV in BHK- or FHM-infected cells was determined at various times postinfection (A) as described previously (9, 12). The cell monolayers were incubated at 20°C in the presence or absence of 100 μg of cycloheximide per ml, and the release of infectious virus into the supernatant fluids (B) was monitored by plaque assays as described previously (5, 22).*

greater in the BHK-infected cells than in the FHM-infected cells (Fig. 3A). Under conditions in which addition of cycloheximide reduced de novo protein synthesis by at least 95% (data not shown), substantial accumulations of viral complementary RNA were observed in cycloheximide-treated cells, suggesting that the virion polymerase is capable of synthesizing viral complementary RNA in the absence of viral-directed protein synthesis.

The virion RNA of SVCV, labeled with ^{32}P, was digested by RNase T1, and, as shown in Fig. 4, the resulting oligonucleotides were resolved by two-dimensional gel electrophoresis (7, 8). The final positions of the two reference dye markers included in the digest are indicated by X (Fig. 4; bromophenol blue, top center, and xylene cyanol FF, lower left). These dye markers allow us to compare the SVCV fingerprint with the fingerprints of other rhabdovirus RNAs that have been published (6; VSV Indiana, VSV New Jersey, Cocal and Chandipura). The oligonucleotide fingerprint of SVCV is distinguishable from these serologically unrelated rhabdoviruses even though the number of the large oligonucleotides (i.e., those below the bromophenol blue marker) is approximately the same as in the fingerprints of the other rhabdoviruses (6). This

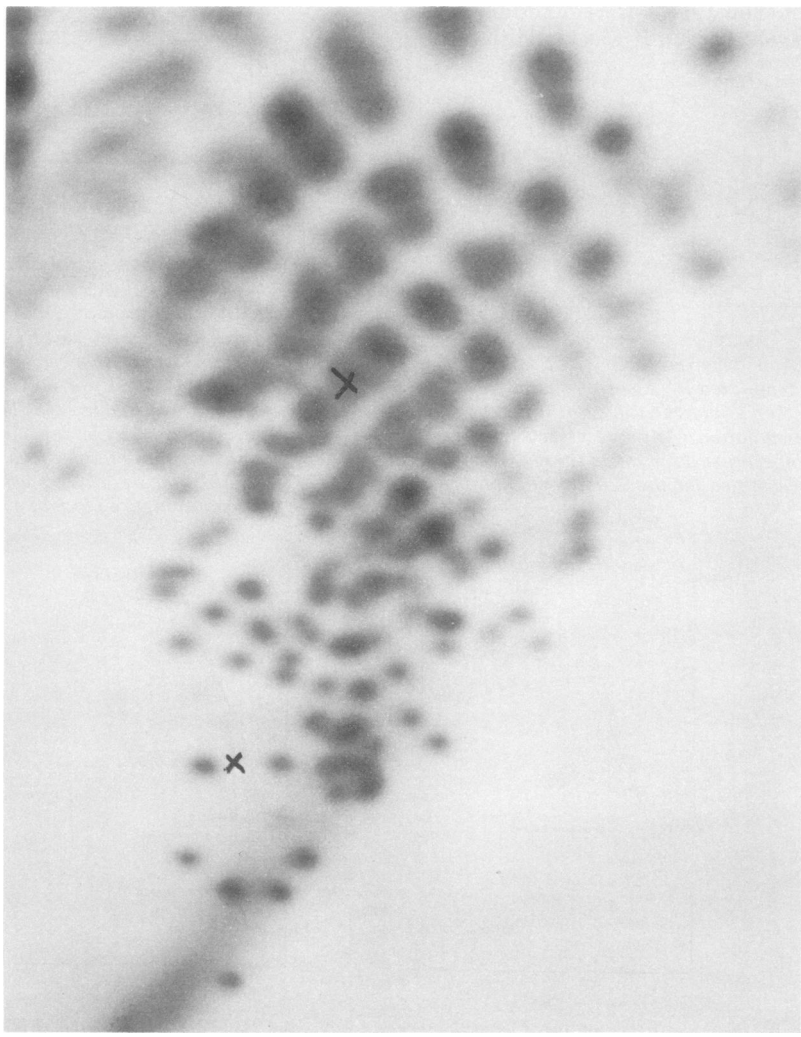

FIG. 4. *Oligonucleotide fingerprint of SVCV B particle RNA. A total of 5×10^6 cpm of RNA extracted from purified virions was digested with RNase T1 and resolved by two-dimensional gel electrophoresis as described previously (6, 9). The first dimension was from left to right and the second from bottom to top. The positions of the two dye markers are indicated (see text).*

suggests that SVCV RNA may have a similar nucleotide complexity to the viruses of the vesicular stomatitis group, although confirmation of this must await compositional and recovery analyses of the large oligonucleotides.

The virion RNA has been further investigated by determining its 5'-terminal nucleotide. RNA extracted from purified ^{32}P-labeled virus preparations was chromatographed through a 4% agarose column to remove low-molecular-weight contaminants (13), precipitated with ethanol, and stored at −20°C until required. The RNA was digested with RNase T2 (14) and mixed with 5 mg of a pancreatic RNase digest of chicken embryo RNA, and, as shown in Fig. 5A, the digest was resolved by DEAE-cellulose column chromatography at pH 8.0 (4, 21, 24). The tetranucleotide isopleth, indicated by arrows in Fig. 5A, which was expected to contain the ^{32}P-labeled 5' terminus (13), was recovered after conversion to its barium salt by precipitation with ethanol. These nucleotides were subsequently converted to their hydrogen form by use of ion-exchange resin, Dowex-50 (H^+) (13, 21). The recovered oligonucleotides were digested with alkaline phosphatase to determine if the labeled phosphate residues could be removed by this enzyme (14). Subsequent chromatography on DEAE-cellulose (Fig. 5B) showed that all the label was converted to inorganic phosphate, suggesting that the label was not resident in internal phosphates or terminal-capped or capped-methylated nucleotide sequences. A second fraction of the tetranucleotide isopleth from the DEAE-cellulose column was also analysed by thin-layer chromatography on PEI cellulose, using optical quantities of the markers ppppG, ppppA, and pppG (21). All the label was recovered comigrating with the adenosine tetraphosphate marker (Fig. 5B inset), indicating that the 5'-terminal nucleotide of SVCV RNA is pppAp.

These studies demonstrate that SVCV possesses a virion RNA polymerase activity similar to those of other rhabdoviruses (1, 4, 15, 22). Its temperature optimum is lower than VSV, and in that respect resembles the virion polymerases of IHNV, PFR and VHS virus (15, 22). The stimulation of the SVCV transcriptase activity by SAM may indicate that SAM is involved in a regulatory function for messenger RNA synthesis, although that will have to await further analyses (11). The synthesis of viral complementary RNA in vivo in the presence of cycloheximide suggests that the virion polymerase is in-

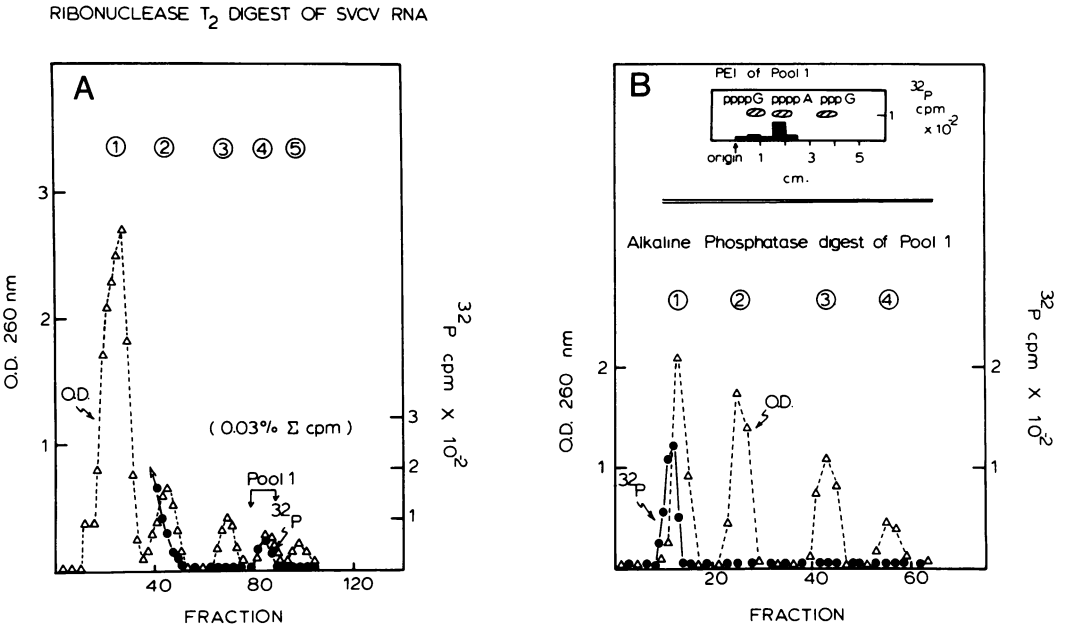

FIG. 5. *Identification of the SVCV RNA 5'-terminal nucleotide by DEAE-cellulose column chromatography. ^{32}P-labeled SVCV viral RNA was digested with RNase T2, and the resulting nucleotides were resolved on DEAE-cellulose, at pH 8, with a pancreatic RNase digest of chicken embryo fibroblast RNA (A) (13, 14). The 5'-terminal nucleotide fragment was recovered, and a portion was treated with alkaline phosphatase and rechromatographed on DEAE-cellulose column (B) as described previously (13, 14, 21). A second sample of termini was analyzed by thin-layer chromatography on PEI cellulose (B inset) as described previously (21).*

volved in viral complementary RNA synthesis in infected cells (3, 10). The oligonucleotide fingerprint and the 5′ nucleotide of pppAp agree with the concept of SVCV as a negative-strand virus (2, 3).

We thank Reginald Anderson for excellent technical assistance.

This study was supported by Public Health Service grants AI 13686 and AI 13042 from the National Institute of Allergy and Infectious Diseases.

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