JOURNAL OF VIROLOGY, June 1977, p. 839-843
Vol. 22, No. 3

Printed in U.S.A.

Virion RNA Polymerases of Two Salmonid Rhabdoviruses

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Received for publication 26 January 1977

RNA-dependent RNA polymerases were found to be associated with two salmonid rhabdoviruses: infectious hematopoietic necrosis (IHN) virus and the virus of hemorrhagic septicemia (VHS). The protein composition of these rhabdoviruses closely resembles that of rabies virus rather than that of vesicular stomatitis virus (McAllister and Wagner, 1975). The optimal temperature for in vitro transcription was found to be ~18°C for IHN virus and ~15°C for VHS, closely approximating optimal temperatures for growth of these viruses in salmonid cells. Unlike vesicular stomatitis virus, manganese ion (1 mM) could be used as a divalent cation substitute for magnesium ion (5 mM). The in vitro transcription products of IHN and VHS viruses hybridized completely to the homologous genome but not at all to the heterologous genome.

Although all rhabdoviruses studied to date appear to have virion-associated RNA-dependent RNA polymerases (11, 20), the degree of transcription activity varies considerably among different rhabdovirus serotypes (1, 4, 11, 19, 20). In vitro transcription of relatively high specific activity has been demonstrated in the vesicular stomatitis (VS) virus subgroup, including VS virus-like fish rhabdoviruses (4, 19). In contrast, only minimal in vitro transcriptase activity, under non-physiological conditions, has been detected in rabies virus (A. Flamand, personal communication). The protein structure of two salmonid rhabdoviruses, infectious hematopoietic necrosis (IHN) virus and the virus of hemorrhagic septicemia (VHS), has been found to resemble closely that of rabies virus (8, 12, 13) rather than that of VS virus. These observations prompted us to investigate the functional in vitro parameters of the transcriptases of these two rabies-like fish rhabdoviruses.

Continuous cell lines (7) derived from the embryonic tissues of chinook salmon (*Oncorhynchus tshawytscha*; CHSE-214) and steelhead trout (*Salmo gairdneri*; STE-137) were used to propagate IHN virus and VHS virus (Danish F_1 strain) (14, 15), respectively. Both cell lines were grown at 18°C in plastic flasks (75-cm^2 surface, Falcon) using Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum. The appropriate cell line was infected as previously described (13) and incubated at 18°C for IHN virus production or at 14°C for VHS virus production. Infected cells were incubated with MEM containing 5% fetal calf serum. If virions were to be radioactively labeled, the overlay medium contained either [^{3}H]uridine (5 μCi/ml) or [^{14}C]uridine (1 μCi/ml). Media from infected cells were harvested 90 h postinfection, and cell debris was removed by centrifugation at 1,500 × *g* for 20 min. Virions were concentrated by centrifugation at 80,000 × *g* for 90 min in an SW27 rotor. The pellets were resuspended in reticulocyte standard buffer (RSB; 10 mM KCl, 1.5 mM $MgSO_4$, and 10 mM Tris-hydrochloride [pH 7.4]) and layered over linear, preformed 0 to 40% sucrose gradients containing 1 M NaCl, 1 mM EDTA, and 20 mM Tris-hydrochloride (pH 7.4). After centrifugation at 44,000 × *g* for 60 min in an SW27 rotor, the visible virion band was removed by side puncture. Virions were pelleted by centrifugation at 80,000 × *g* for 90 min in an SW27 rotor, resuspended in RSB (pH 8.0) containing 10% glycerol, and stored on ice.

[^{14}C]uridine (447 mCi/mmol) was obtained from New England Nuclear, Boston, Mass., and [^{3}H]uridine (20 Ci/mmol) and [^{3}H]UTP (16 Ci/mmol) were obtained from Schwarz/Mann, Orangeburg, N.Y. Nucleoside triphosphates, dithiothreitol (DTT), and actinomycin D were obtained from Calbiochem, La Jolla, Calif. RNase T_1 (310,000 U/mg), Sephadex G-50 (medium), and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo. RNase A (500 U/mg) was obtained from Worthington Biochemical Corp., Freehold, N.J. Media and serum were obtained from Grand Island Biological Co., Grand Island, N.Y.

The method used to assay VS virus transcrip-

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tase activity (9) was modified slightly for IHN and VHS. The virus preparations in RSB containing 10% glycerol (0.4 ml) were mixed with 0.1 ml of 2× Triton-high salt solubilizer (18.7% glycerol, 3.74% Triton X-100, 1.2 mM DTT, 4.4 mM KCl, 0.66 mM $MgSO_4$, 1.44 M NaCl, and 4.4 mM Tris-hydrochloride [pH 8.0]). After 2 min at 0°C, each preparation was combined with an equal volume of reaction mixture consisting of 1.3 mM DTT, 8 mM magnesium acetate, 1.4 mM each of ATP, CTP, and GTP, 0.133 mM [^{3}H]UTP (230 μCi/μmol), and 50 mM Tris-hydrochloride, pH 8.0. Samples (0.1 ml) were pipetted into test tubes, capped, and incubated at the appropriate temperature and for the appropriate time before the reaction was terminated by the addition of 0.6 ml of 67 mM sodium pyrophosphate and 0.1 ml (200 μg) of yeast RNA. Duplicate samples were immediately mixed with 0.5 ml of ice-cold 25% trichloroacetic acid and placed on ice for 30 min. Precipitable materials were collected on 0.45-μm-pore-size membrane filters and processed as previously described (5, 9). The radioactivity incorporated into newly synthesized RNA was determined with a Beckman LS-230 liquid scintillation system and corrected with the assistance of a double-label computer program.

RNA species were extracted from intact, purified virions and from in vitro polymerase reaction mixtures freed by centrifugation of nucleocapsid templates (5, 9). In brief, RNA made 1% with respect to sodium dodecyl sulfate was extracted with water-saturated phenol-chloroform-isoamyl alcohol (1:1:1%) for 60 min at 25°C. The aqueous phase was removed, and the RNA was precipitated at −20°C by the addition of 2.5 volumes of 100% ethanol. No carrier was added to virion RNA, the concentration of which was determined by absorbance at 260 nm. Carrier RNA was added to in vitro transcription products, which were freed of residual [^{3}H]UTP by passage through a Sephadex G-50 column equilibrated with 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 20 mM Tris-hydrochloride (pH 7.4).

Table 1 demonstrates the various conditions under which IHN and VHS transcriptase activity was optimal. In vitro RNA synthesis was measured by [^{3}H]UMP incorporation into a trichloroacetic acid-precipitable product over a 1-h period. The RNA polymerase of each virus was found to require nonionic detergent, divalent cations, all four nucleoside triphosphatases, and a reducing agent. The DTT requirement appeared to be more stringent for VHS than for IHN virus. However, the activity of VHS transcriptase was usually greater than twice that of the IHN enzyme under optimal conditions. A magnesium ion concentration of 5 mM proved optimal for both viruses. In addition, manganese ions at a concentration of 1 mM could substitute for magnesium with near maximum activity for the IHN enzyme but with 40% reduced activity for the VHS enzyme. The addition of both divalent cations in varying concentrations did not further stimulate RNA synthesis (data not shown).

Temperature dependence was also examined as another parameter for determining optimal in vitro transcriptase activity. Both IHN and VHS fish rhabdoviruses infect poikilotherms, which live at ambient temperatures of 20°C or below. Transcriptase activity in complete reaction mixtures was measured for both IHN and VHS at temperatures ranging from 0 to 31°C. Figure 1 shows that the temperature optima for transcriptase activity were ~18°C for IHN virus and ~15°C for VHS virus. It is also of interest that residual polymerase activity can be detected at 0°C, particularly for VHS, whereas virtually no enzymatic activity was present at 24°C. By comparison, the temperature optimum of the VS viral transcriptase is 28 to 31°C (2).

Under optimal conditions for in vitro transcription of IHN and VHS virions, incorpora-

TABLE 1. *RNA polymerase activity in IHN and VHS virions in vitro*

Reaction condition[a]	Enzyme activity (pmol UMP incorporated/mg viral protein/h)	
	IHN (18°C)[b]	VHS (14°C)[c]
Complete mixture	1,040	2,145
−CTP	12	111
−Triton X-100	50	0
−DTT	580	157
−Magnesium	0	0
−Magnesium, + manganese (1 mM)	952	1,292

[a] The RNA polymerase activity of IHN and VHS viruses was monitored under various reaction mixture conditions. The complete reaction mixture contained 7.4×10^{-4} M DTT, 5×10^{-3} M magnesium acetate, 1.44×10^{-1} M NaCl, 0.37% Triton X-100, 5.87% glycerol, 7×10^{-4} M each of ATP, CTP, and GTP, and 6.7×10^{-5} M [^{3}H]UTP (230 μCi/μmol) in 3×10^{-2} M Tris-hydrochloride, pH 8.0. Samples were incubated at the desired temperature, and incorporation of label into trichloroacetic acid-precipitable material was determined by liquid scintillation spectroscopy. Reaction mixtures lacking CTP, Triton X-100, DDT, or magnesium and reaction mixtures substituting manganese (1×10^{-3} M) for magnesium were assayed in a similar manner.

[b] IHN virus RNA polymerase reaction at 18°C.

[c] VHS virus RNA polymerase reaction at 14°C.

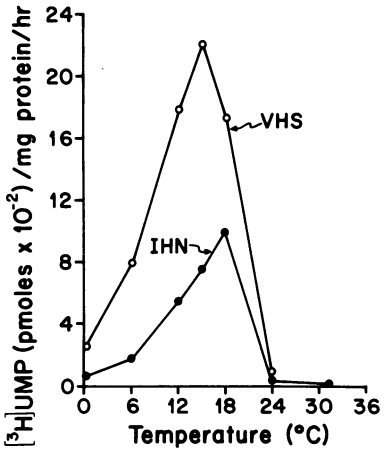

FIG. 1. *In vitro RNA polymerase activity of IHN and VHS viruses at different temperatures. The in vitro RNA polymerase activity of IHN virus and VHS virus was determined under optimal conditions (see Table 1) at seven incubation temperatures from 0 to 31°C. Activity was monitored over a 90-min incubation period. ^3H transcription products were recovered by trichloroacetic acid precipitation and membrane filtration.*

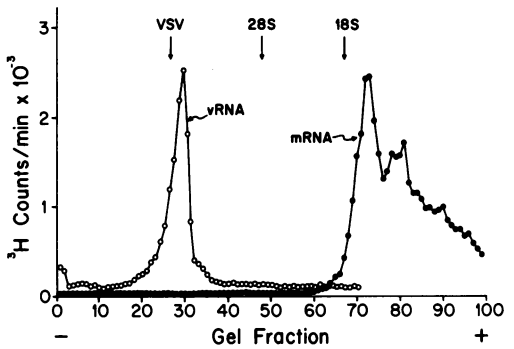

FIG. 2. *Analysis of IHN virion RNA (vRNA) and in vitro IHN transcripts (mRNA) by polyacrylamide gel electrophoresis run on separate gels. Phenol-extracted [^3H]RNA of purified IHN virions was subjected to electrophoresis on 2% polyacrylamide-agarose gels at 5 mA per gel for 2.5 h along with ^{14}C-labeled marker VS virion RNA and 28S + 18S ribosomal RNA (arrows). IHN ^3H transcription products were phenol-extracted from an in vitro RNA synthesis reaction at 18°C for 2 h after removal of nucleocapsids by centrifugation at 135,000 × g for 90 min in an SW50.1 rotor. In vitro transcribed mRNA freed of residual [^3H]UTP was subjected to electrophoresis on a separate 2% polyacrylamide-agarose gel for 3 h at 5 mA per gel along with ^{14}C-labeled 28S and 18S ribosomal markers (arrows). Longer electrophoresis accounts for farther migration of mRNA.*

tion of [^3H]UMP was linear for 2 h. At various intervals, samples were withdrawn from the incubation mixture, and the viral nucleocapsids were removed by centrifugation at 135,000 × *g* for 90 min in an SW50.1 rotor. Supernatant samples were then layered over preformed 10 to 30% sucrose gradients and centrifuged at 65,000 × *g* for 17 h in an SW41 rotor. Fractions were collected, and trichloroacetic acid-precipitable radioactivity was assayed. The RNA species synthesized in vitro by IHN virus accumulated in two peaks of approximately 14 to 17*S* and 9 to 13*S* over a period of 2 h. No larger RNA species could be detected (data not shown).

In vitro ^3H-labeled RNA transcripts of IHN and VHS and virion ^3H-labeled RNAs were also analyzed by electrophoresis on 2% polyacrylamide gels along with ^{14}C-labeled VS virion RNA (42*S*) and 28*S* and 18*S* ribosomal RNA markers. Figure 2 shows characteristic electropherograms of IHN virion RNA, which migrates at about 40*S*, and IHN in vitro transcripts, which showed two major peaks of 15–17*S* and 12–14*S*. No larger mRNA (±28*S*) species could be detected under these conditions. The heterogeneous low-molecular-weight species probably represent incomplete transcripts or degraded larger RNA species or both. Similar patterns were exhibited by VHS virion RNA and in vitro transcripts (data not shown).

The results of the in vitro product analysis led us to characterize the RNA species synthesized in IHN virus-infected CHSE-214 cells. Monolayer cultures were treated for 2 h with actinomycin D (3 μg/ml) before infection with IHN virus at a multiplicity of ~1. The monolayer cultures were then incubated at 18°C for 22 h in MEM containing [^3H]uridine (50 μCi/ml) and actinomycin D (3 μg/ml). The cells were disrupted with 1% Nonidet P-40, and the RNA in the cytoplasmic fraction was recovered by phenol extraction. The RNA was then fractionated by affinity chromatography on oligodeoxythymidylic acid [oligo(dT)]-cellulose. The adenylated RNA was then centrifuged for 17 h at 65,000 × *g* in a linear 10 to 30% sucrose gradient; fractions were ethanol-precipitated and analyzed by electrophoresis on 2% acrylamide gels. By these techniques, 28*S* mRNA and heterogeneous 12–17*S* RNA could be detected (data not shown).

The mRNA$^-$ species synthesized in vitro by IHN and VHS virions, as well as polyadenylated mRNA synthesized in IHN-infected CHSE-214 cells, were analyzed by hybridization to negative-strand IHN and/or VHS virion RNA. RNA-RNA hybridization was assayed by mixing 25 μl of unlabeled virion RNA (2 μg) with 25 μl of [^3H]RNA ethanol-precipitated from infected cells or from the polymerase reaction mixture freed of nucleocapsids by centrifugation. Reaction mixtures were boiled for 30 s, chilled on ice, and made 0.4 M with respect to

NaCl. After incubation for 150 min at 60°C, aliquot samples were treated with 18 μg of RNase A and 17 U of RNase T_1 at 37°C for 30 min. Carrier yeast RNA and pyrophosphate were added to each sample, which was then precipitated with trichloroacetic acid and collected on membrane filters.

Data shown in Table 2 reveal complete hybridization of IHN and VHS in vitro transcription products to homologous virion RNA. However, no complementarity was observed by cross-hybridization of IHN and VHS mRNA with either heterologous virion RNA, indicating no detectable homology between IHN and VHS RNA templates. Low levels of in vitro mRNA self-annealing point to the efficacy of the heat denaturation procedure and removal of virion template RNA from the transcription mixture. In contrast, [^{3}H]RNA synthesized in IHN-infected cells gave far less clear results. Even after purification of polyadenylated mRNA on an oligo(dT) column, the 28*S* mRNA was almost equally resistant to RNase in the absence or presence of virion RNA. However, the smaller (12–17*S*) IHN mRNA synthesized in vivo did exhibit considerable annealing to homologous virion RNA and relatively little self-annealing.

TABLE 2. *Hybridization of IHN and VHS in vitro transcription products to homologous and heterologous virion RNA, and of in vivo IHN polyadenylated mRNA species to homologous virion RNA*

[^{3}H]mRNA transcript	% RNase resistance of annealing mixtures[c]		
	mRNA alone	mRNA + IHN virion RNA	mRNA + VHS virion RNA
In vitro			
IHN product[a]	1.0	100.0	0.4
VHS product[a]	0.3	0.4	103.0
In vivo			
Poly(A) 28*S* IHN product[b]	18.3	22.5	
Poly(A) 12–17*S* IHN product[b]	3.7	72.3	

[a] [^{3}H]labeled RNA was synthesized under optimal conditions for transcription of IHN and VHS during a 2-h incubation period. Nucleocapsid templates were removed by centrifugation; residual [^{3}H]UTP was removed by gel filtration.

[b] CHSE-214 cells were infected with IHN virus (multiplicity of infection ≈1) for 22 h in the presence of [^{3}H]uridine (50 μCi/ml) and actinomycin D (3 μg/ml). Polyadenylated mRNA in cell cytoplasm was recovered from an oligo(dT) column and separated into 28*S* and 12–17*S* fractions by centrifugation for 17 h at 65,000 × *g*. Poly(A), Polyadenylic acid.

[c] Annealing mixtures of 25 μl of IHN or VHS virion RNA (2 μg) and 25 μl of [^{3}H]mRNA were incubated at 60°C for 150 min. Alternate samples were treated with 18 μg of RNase A and 17 U of RNase T_1 for 30 min at 37°C. Percentage of RNase-resistant to untreated [^{3}H]mRNA was calculated from ratio of RNA trichloroacetic acid-precipitated onto membrane filters.

These studies demonstrate quite active endogenous transcriptase activity associated with virions of IHN and VHS rhabdoviruses, both of which infect salmonid species of fish. These transcriptases exhibit certain properties similar to those of the VS virus group of rhabdoviruses that infect homoiotherms. The salmonid rhabdoviruses use a nucleocapsid template, and their requirements for nonionic detergent, all four nucleoside triphosphates, and divalent cations appear to resemble all rhabdoviruses and other negative-strand viruses (1–4, 19, 20). As in the case with other rhabdoviruses, only complementary RNA was found to be synthesized by in vitro transcription of IHN and VHS viruses. The molecular species of virion RNAs of IHN and VHS as well as their in vitro and in vivo mRNA transcripts resemble those of VS virus, as shown by polyacrylamide gel electrophoresis (16, 17). Failure to detect any cross-complementarity between the transcripts and genome RNAs of IHN and VHS is not too surprising. Previous studies revealed no antigenic relationships between IHN and VHS by cross-neutralization tests (14). In fact, only minor degrees of RNA complementarity have been detected by hybridization of antigenically related Indiana, New Jersey, and Cocal serotypes of VS virus, and no RNA homology could be demonstrated among more distantly related rhabdoviruses (18).

In other respects, the virion transcriptases of IHN and VHS exhibit properties quite different from those of other rhabdoviruses. Although the salmonid rhabdoviruses contain an L protein, they do not contain an NS protein (13), both of which are essential for in vitro transcription of VS virus (6). In fact, the protein composition of salmonid rhabdoviruses more closely resembles that of rabies virus (8, 12, 13), which exhibits barely detectable transcriptase activity in vitro (A. Flamand, personal communication). A major characteristic of the transcriptase of VS virus is complete dependence on the presence of Mg^{2+} and inhibition of in vitro RNA synthesis when Mn^{2+} is included in the reaction mixture (1–3). Our data clearly demonstrate that Mn^{2+} (1 mM) can effectively substitute for Mg^{2+} (5 mM) in the in vitro transcription of IHN (92% of Mg^{2+} optimal RNA synthesis) and of VHS transcription (60% of Mg^{2+} optimal RNA synthesis). The presence of both divalent cations in varying combinations did

not further stimulate salmonid rhabdovirus transcription. The most striking feature of the properties exhibited by salmonid rhabdovirus transcription is their temperature optimum, which is usually 28° to 31°C for most rhabdoviruses. In vitro RNA synthesis was found to reach maximal efficiency at 18°C for IHN virus and at 15°C for VHS virus. These optimal temperatures for in vitro polymerase activity closely parallel the optimal temperature for replication of IHN and VHS viruses in host salmonid cell lines (10, 21).

This research was supported by Public Health Service grant AI-11112 from the National Institute of Allergy and Infectious Diseases, by grant VC-88 from the American Cancer Society, and by grant BMS 72-02223 from the National Science Foundation. P. E. McAllister was a Postdoctoral Fellow of the National Institute of Allergy and Infectious Diseases (F32 AI-00139).

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