An RNA Polymerase Activity in Purified Rabies Virions

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SUMMARY

An RNA polymerase activity has been demonstrated in purified rabies virions. Efficiency of the reaction is low since the rate of incorporation was equal to 3 to 5 pmol of uridine per hour, per mg of protein. As with other mammalian rhabdoviruses the optimal temperature was 31 °C. Unlike vesicular stomatitis virus, manganese could be substituted for magnesium as a divalent cation, at an optimum concentration of 10 to 20 mM.

A transcriptase activity was first demonstrated by Baltimore et al. (1970) in purified Indiana serotype vesicular stomatitis virus (VSV). This observation was subsequently extended to the Cocal and New Jersey VSV serotypes and other rhabdoviruses such as Piry, Chandipura and Kern Canyon (Aalestad et al. 1971; Chang et al. 1974). A transcriptase activity has also been found in several fish rhabdoviruses: pike fry virus (Roy et al. 1975), infectious haematopoietic necrosis (IHN), the virus of haemorrhagic septicaemia (VHS; McAllister & Wagner, 1977) and spring viraemia of carp virus (P. Roy, personal communication).

Three facts suggested that rabies, like the other rhabdoviruses, is a negative strand virus: (i) its RNA is not infectious (Sokol et al. 1969); (ii) primary transcription could be detected in rabies infected cells in the presence of cycloheximide (Bishop & Flamand, 1975); (iii) single-stranded, short, polyadenylated molecules complementary to the genome and which are likely to be messengers for virus proteins were found in the cytoplasm of infected cells (Ermine & Flamand, 1977; Ermine, 1978). Nevertheless, previous attempts to demonstrate the presence of a transcriptase activity in rabies virions were unsuccessful, leading to the hypothesis that the molecular biology of the rabies virus was different from that of other rhabdoviruses. Suspecting that the lack of activity in the reaction in vitro could be due to alterations of the enzyme produced by experimental manipulation, we decided to re-investigate the problem using a purification procedure which preserves the infectivity of the virus to a great extent.

Virus production and purification were as follows: 20 roller bottles of BHK 21 cells (10^8 cells/bottle) were infected with the CVS strain of rabies virus at a m.o.i. of 0.05 p.f.u./cell. The infected cells were then incubated in Hanks’ medium supplemented with 0.5% lactalbumin hydrolysate, 5% calf serum and 0.2% NaHCO₃. After 24 h incubation at 36 °C supernatant fluid was discarded and replaced by the same medium without serum. Following incubation at 36 °C for 2 more days, the fluid portion was recovered, cooled in ice and centrifuged at 2800 g for 10 min. The pH was then adjusted to 7.2. The virus was purified by two successive precipitations with an ethylene oxide polymer in the presence of Ca^{2+} ions; the pellet was re-suspended in an EDTA-containing buffer (0.05 M-tris-HCl, pH 7.4, 0.05 M-EDTA) as described by Adamowicz et al. (1974). With this procedure 95% of the non-viral acid-insoluble material was removed, and the virus was concentrated 200 times. The concentrated virus suspension was then centrifuged through a linear gradient of 15 to 45% (w/v) sucrose in 0.02 M-tris-HCl, pH 7.4, 0.15 M-NaCl for 45 min at 120000 g at 4 °C.
An opalescent virus band was clearly seen 2/3 of the way down the gradient, corresponding to a density of 1.16 g/ml. The gradient was fractionated from the bottom. Fractions containing the virus were applied to a column of Sephadex G-75 which had been equilibrated in tris-NaCl buffer at 4 °C. The sample volume was equal to 1/10 column volume. Fractions containing 1.5 ml were collected and the two fractions containing most protein were used for the assay in vitro. The protein concentrations were between 2 and 4 mg/ml, as shown by absorbance at 280 nm and assays by the method of Lowry et al. (1951). Infectivity was determined as previously described (Flamand et al. 1977) and varied between 2 to 4 × 10⁹ p.f.u/ml, depending on the virus batch.

Purified Triton-treated rabies virions were found to be capable of catalysing the incorporation of ³H-UTP into acid-insoluble material. That this activity is associated with rabies virus is shown by its co-sedimentation with complement fixation activity and infectivity in a sucrose gradient (Fig. 1). Furthermore, Fig. 1 reveals the presence of non-infectious but serologically active virus material exhibiting detectable RNA polymerase activity, which sediments in the region of truncated particles (fractions 6 to 9). The material which sedimented in fractions 12 to 15 of the gradient appeared clean by examination in the electron microscope and contained homogeneous bullet-shaped particles. Serological tests demonstrated this virus to be rabies. The protein profile of the virus material was comparable to that reported in the literature (Sokol et al. 1971).

The omission of one of the nucleoside triphosphates from the reaction in vitro resulted in considerable inhibition of incorporation of ³H-UTP. The reaction was absolutely dependent on the presence of Mg²⁺ or Mn²⁺, the optimum concentration for both ions being between 10 and 20 mM (Figs. 2a, b). Mg²⁺ and Mn²⁺ had apparently equivalent effects and their simultaneous presence did not lead to an increased effect. pH was the second critical factor of the reaction. The pH optimum of the reaction was 8.9 and total inhibition was obtained at acidic values (Fig. 2e). Incorporation of nucleoside triphosphates began to decrease at pH values greater than 9.15. The reaction was temperature dependent, being maximal at 31 °C (Fig. 2d). The efficiency of the reaction was enhanced by the presence of Triton X-100 and dithiothreitol, the optima being 10 mM and 3 mM respectively. Both compounds became inhibitory at higher concentrations. No incorporation occurred in the absence of Triton. Polymerase activity was depressed in the presence of monovalent cations such as NH₄⁺. Actinomycin D had no effect.

Under optimal conditions, the reaction was linear for 7 h before reaching a plateau. The efficiency of the reaction was low, based on the rate of incorporation of 3 to 5 pmol uridine/h/mg protein.

The reaction products were analysed at 2.5 h, i.e. during the linear phase of incorporation. SDS was added to a final concentration of 0.5% and the reaction mixture was passed through a column of Sephadex G-50 equilibrated in high salt buffer (HSB: 0.4 M-NaCl, 0.01 M-tris-HCl, pH 7.4, 2 mM-EDTA) containing 0.5% SDS and 50 μg/ml of dextran sulphate. Since the majority of TCA-insoluble radioactivity was eluted in the void volume of the column, this corresponded to molecules containing more than 100 nucleotides or to nascent chains still attached to their templates. This material was precipitated with alcohol and the resulting pellet recovered after centrifugation was dissolved in 0.2 ml of HSB. It was initially 60% resistant to RNase treatment and became 100% RNase resistant after self-hybridization for 2 days at 60 °C. Since the reaction products annealed to the template, this strongly suggests that the virion-associated polymerase is indeed a transcriptase. Product hybridization giving 100% protection of virus RNA to subsequent nuclease digestion would be the final evidence that the enzymatic activity is a rabies transcriptase. Unfortunately,
Fig. 1. Co-sedimentation of infectivity, complement fixation and polymerase activities of purified rabies virions in a sucrose density gradient. After two successive precipitations with an ethylene oxide polymer, partially purified virions were centrifuged through a linear sucrose gradient of 15 to 45% (w/v) sucrose for 45 min at 120,000 g. The gradient was fractionated from the bottom into 17 fractions of 1.5 ml. Fifty µl of each fraction were used to prime an in vitro reaction (125 µl total) containing 0.1 µmol each of ATP, CTP and GTP and 0.01 µmol UTP+10 µCi of 3H-UTP (Saclay-specific radioactivity 25 Ci/mmol), 8 µmol of Bicin, pH 8.9, 1 µmol of MnCl₂, 4 µmol of NaCl, 0.6 µmol of dithiothreitol and 0.02 µl of Triton N-101. Incubation temperature was 31 °C. The reaction was stopped by adding 20 µl of a saturated solution of sodium pyrophosphate, 0.5 ml of high salt buffer (0.01 M-tris-HCl, pH 7.4, 0.4 M-NaCl, 2 mM-EDTA) containing 1% SDS and 4 ml of cold 5% trichloracetic acid. Precipitates were collected on membrane filters (Selectron, 45 µm), washed ten times with cold trichloracetic acid and the radioactivity determined in an Intertechnique liquid scintillation spectrometer. CF antigens were titrated with a Kolmer microtechnique using a hyperimmune anti-rabies guinea pig serum diluted to contain 4 units. The reciprocal of the highest initial dilution of antigen yielding no haemolysis was taken as the number of complement fixing units (c.f.u.) per 0.025 ml. Infectivity, complement fixation and polymerase activities were determined on three separate gradients in the same experiment. ● ● ●, Infectivity; ▼——▼, CF activity; ▼—▼, radioactivity.
Fig. 2. Influence of the concentrations of Mg$^{2+}$ and Mn$^{2+}$, of temperature and of pH on polymerase activity associated with purified rabies virions. Virions were purified by two successive precipitations with an ethylene oxide polymer followed by centrifugation through a 15-45% sucrose gradient as described in Fig. 1 and in the text. Fractions containing the virus were applied to a column of Sephadex G75. The two fractions most rich in protein were used for the assay. Except for the variable studied, the conditions of the reaction in vitro were as described in Fig. 1. (a) Influence of the Mg$^{2+}$ concentration (mM); (b) influence of the Mn$^{2+}$ concentration (mM); (c) influence of pH; (d) influence of temperature (°C).

because of the low efficiency of the reaction, there is not enough product to hybridize all the templates present in the in vitro assay.

The RNA polymerase reaction primed by rabies virus requires the presence of Mg$^{2+}$ or Mn$^{2+}$. For VSV Indiana, it has been demonstrated that the reaction has an absolute requirement for Mg$^{2+}$ which cannot be replaced by Mn$^{2+}$ (Aalestad et al. 1971). In this respect Kern Canyon virus is similar to VSV. The transcriptases of New Jersey, Cocal, Chandipura, Piry and pike fry viruses also require Mg$^{2+}$ (Chang et al. 1974; Roy et al. 1975) but it is not known if Mn$^{2+}$ can substitute for Mg$^{2+}$. In the case of the two salmonid rhabdoviruses
IHN and VHS, Mn\(^{2+}\) is a suitable divalent cation substitute for Mg\(^{2+}\) (McAllister & Wagner, 1977). This fact, coupled with the observation that their protein patterns are quite similar to that of rabies and are quite different from those of the other rhabdoviruses, suggests that these three viruses could be closely related. The optimal temperature of 31 °C for the polymerase reaction is similar to that observed for the other rhabdoviruses except the fish viruses (Roy et al. 1975; McAllister & Wagner, 1977). The RNA polymerase activity demonstrated in purified rabies virions \textit{in vitro} is low compared to that of other rhabdoviruses. Several hypotheses may be advanced to explain this observation. Since enzyme activity is rapidly inhibited by detergents, a relatively low concentration of Triton X-100 was used (0.01%). Under these conditions, it is probable that the membranes were not completely solubilized, yielding either a few particles having transcriptase activity or particles which are all transcribed with a low efficiency. The observation of a relatively high pH optimum for the reaction strengthens this argument, since it is known that alkaline pH disrupts virus particles (for review, see Fiszman, 1976). In addition, nucleoside triphosphatase activities have been demonstrated in all rhabdovirus preparations thus far investigated (Roy & Bishop, 1971). These enzymes are inhibited by Triton N-101, which is generally used at a final concentration of 1%. Although such enzymes have not yet been demonstrated in rabies virions, it is not excluded that they could be present and still active at a detergent concentration of 0.01%; this would lead to a reduction of the size of the nucleoside triphosphate pool. Furthermore, nucleoside mono- and diphosphates could inhibit the reaction \textit{in vitro}. An alternative explanation is that the reaction mixture was contaminated by RNase. Since these enzymes are inhibited at alkaline pH, the reaction would thus be maximal at pH 8.9. A fourth possibility is that the virus polymerase requires a cellular factor for its efficient functioning. Experiments are currently in progress to increase the efficiency of the reaction \textit{in vitro}.

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REFERENCES


Short communications


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