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Reverse Transcriptase from Simian Foamy Virus Serotype 1: Purification and Characterization

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Chromatography on heparin-Sepharose, known for its affinity for nucleotide-binding polypeptides, was used to purify the viral RNA-dependent DNA polymerase (reverse transcriptase) from the core polypeptides of simian foamy virus type 1. This procedure allowed the recovery of highly purified enzyme with a high specific activity. The average molecular weight of this monomeric enzyme is 81,000 and is thus comparable to that found for other known primate retroviruses. Reverse transcriptase activity of simian foamy virus type 1 requires a ribonucleotide template as a primer or otherwise a DNA with 3'-OH ends. Other optimal conditions of activity are reviewed. Heat inactivation studies led to the concept of an enzyme with two loci, one specific for the substrate and the other for the template-primer.

Retroviridae, characterized by the presence of an RNA-dependent DNA polymerase (RDDP), also known under the name reverse transcriptase (RT), are grouped into three distinct subfamilies, the Oncornavirinae, the Lentivirinae, and the Spumavirinae (6). Whereas Oncornavirinae (e.g., type C, type D, and type B viruses), first isolated from various animal tumors, belong to the RNA tumor virus group, the Spumavirinae (foamy viruses) are characterized by their latency in vivo (8) and by their particular cytopathic effect in vitro (5). Parks et al. demonstrated by the study of one serotype of the simian foamy viruses (SFV) that this group of viruses contains a high-molecular-weight genomic RNA and an RDDP (16). To this date, only one report has been published concerning a brief study of the semipurified RT of a primate foamy virus, H4188, an isolate of human origin, which cross-reacts with SFV6 (11). To our knowledge, no complete purification of an SFV RDDP and no detailed biochemical characterization of such an enzyme has been reported. Therefore, we thought it of interest to investigate as to the best purification procedure for the RT of an SFV and to further characterize this enzyme.

SFV1 was kindly supplied by the National Institutes of Health (Bethesda, Md.). It was propagated in canine Cf2Th cells (14). Cultures were treated 24 h before infection with 5 μg of polybrene per ml (Sigma Chemical Co.) and inoculated at a multiplicity of infection of 1. Within an 8-day incubation period, when the cell monolayers presented a characteristic and generalized cytopathic effect, virus was harvested through three cycles of freezing at −80°C and subsequent thawing. Virions were purified by a method previously described (3). Briefly, the supernatant fluids of frozen and thawed cultures were clarified by centrifugation for 10 min at 10,000 × *g*, and the viral particles were pelletized by a 2-h centrifugation at 100,000 × *g*. The pellets were suspended in TNE buffer (100 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl–1 mM EDTA) and banded through a 20 to 70% (wt/vol) preformed sucrose density gradient at 100,000 × *g* for 2 h at 4°C. Purified virus with a buoyant density of 1.16 g/ml was collected, and the degree of purification was verified by electron microscopy. Uninfected dog thymus cells were treated in parallel and were used as controls in some of the experiments.

Extraction of envelope proteins was done by following the method of Strand and August (18) from 8.5 mg of purified virus, diluted at 2 mg/ml in TNE buffer. Core particles were disrupted by sonication and treated with a detergent mixture whose constituents were added successively in the following order: Nonidet P-40 (NP-40), 0.2%; sodium deoxycholate (DOC), 0.1%; potassium chloride, 0.5 M. The preparation was centrifuged for 20 min at 30,000 × *g* at 4°C. The supernatant containing the structural core polypeptides and an RT activity was subjected to ion-exchange column chromatography. The fractions from the DEAE-cellulose column showing a significant enzymatic activity (Fig. 1) were pooled and loaded onto a heparin-Sephar-

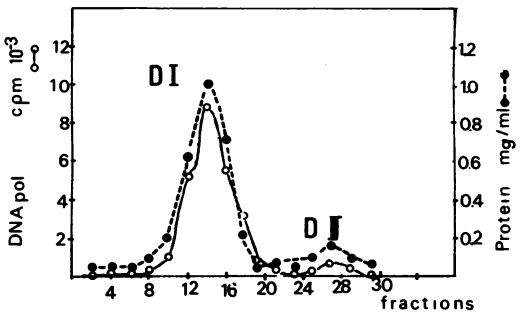

FIG. 1. DEAE-cellulose chromatography. Supernatant S (7 mg) obtained from disrupted core particles by a 20-min centrifugation at 30,000 × g was diluted in three times its volume of buffer 1 (10 mM $K_2PO^-_4$ [pH 7.2]–2 mM dithiothreitol [DTT]–0.2% NP-40–10% glycerol) before being pumped on a DEAE-cellulose column (5 by 1 cm) prepared by the method of Burgess (2) and equilibrated previously with buffer 1. The column was washed with three times its volume of buffer 1 before elution with 20 ml of buffer 2 (0.300 mM $K_2PO^-_4$ [pH 7.2]–2 mM DTT–0.2% NP-40); 0.5-ml fractions were collected at a rate of 5 ml/h. Protein (●) determination was done by the method of Bohlen et al. (1). RDDP (○) was assayed on 20-μl portions of each fraction, using the following reaction mixture: 50 mM Tris-hydrochloride (pH 7.5), 1 mM $MnCl_2$, 1 mM DTT, 40 mM KCl, 100 μg of BSA per ml, 0.5 mM [^{3}H]TTP (50 cpm/pmol), and 4 μg of rA_n-dT_{12-18} per ml. After 10 min at 37°C, the acid-insoluble product of the reaction was collected on 0.2 μM Sartorius filter disks (type SM-11-307) and processed for detection of radioactivity by liquid scintillation spectrometry (Intertechnique SL 30 apparatus). Two fractions of RDDP, DI and DII, were found.

ose column. The affinity chromatography led to a single pool of RDDP activity (Fig. 2). As shown by polyacrylamide gel electrophoresis analysis of materials from successive purification steps (Table 1; Fig. 3), a purified RT was

TABLE 1. Purification of the RDDP of SFV1[a]

Fraction or pool	Total protein (mg)	Sp act (U/μg of protein)[b]	% Yield	Purification rate
S	7	72.5	100	1
DI	0.52	249	7.4	3.4
H	0.22	5,677	3.1	78

[a] The RDDP of SFV1 was purified from supernatant S obtained after a 30,000 × g centrifugation of disrupted core particles as described in the text. Pool DI was obtained after chromatography on DEAE-cellulose. Pool H was obtained after chromatography on heparin-Sepharose and corresponds to a highly purified enzyme.

[b] One unit of activity is defined as the incorporation of 1 pmol of dTMP under the above conditions in 10 min. The specific activity of the enzyme is given in units per microgram of protein.

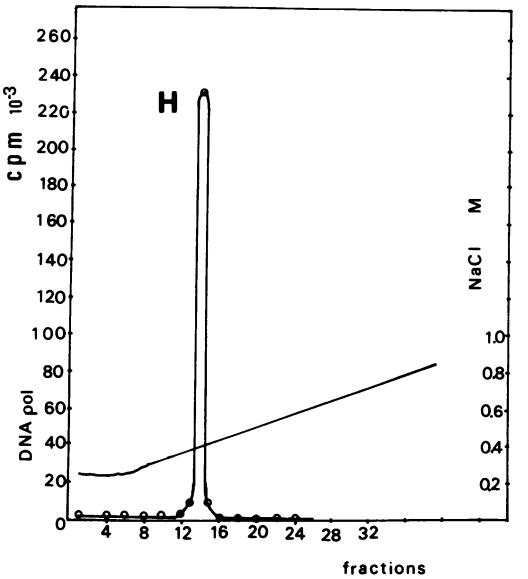

FIG. 2. Heparin-Sepharose affinity chromatography. Pool DI (0.5 mg of protein in 4 ml), obtained after DEAE-cellulose chromatography, was added on a heparin-Sepharose 4B column as described by Golomb et al. (7). The column, first equilibrated with B buffer (50 mM Tris-hydrochloride [pH 7.5]–2 mM DTT–0.1 mM EDTA–0.2% NP-40–20% glycerol) containing 180 mM NaCl, was then washed extensively with 240 mM NaCl in B buffer, before elution with a linear gradient of 240 to 1,000 mM NaCl in B buffer. Fractions of 0.5 ml were collected at 3 ml/h; 20-μl portions were assayed for RDDP activity as described in the text. Protein content was determined by the method of Bohlen et al. (1). Results are given in 10^{-3} cpm of incorporated [^{3}H]dTMP. A unique pool of enzymatic activity, H, was found (3 ml; 0.2 mg of protein).

TABLE 2. Transcription of various template-primers by purified SFV1 DNA polymerase[a]

Template-primer	pmol of incorporated dTMP per min
rA_n-dT_{12-18}	50
rC_n-dG_{12-18}	18
rU_n-dA_{12-18}	7.2
Activated DNA	10.2
dA_n-dT_{12-18}	5.5
dC_n-dG_{12-18}	3.7

[a] The conditions employed were the same as those described for the RDDP, except for the substitution of each chosen template-primer, with each assay using the same amount of enzyme. In the assay with activated DNA, the substrate mixture included, other than [^{3}H]TTP, 0.5 mM concentrations of dGTP, dATP, and dCTP. Activities are expressed as the initial rate of reaction (picomoles of deoxynucleotide monophosphate incorporated per minute).

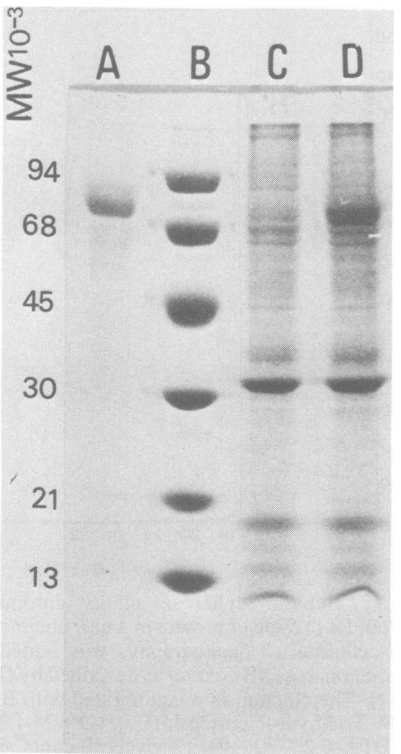

FIG. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins was carried out according to Laemmli (10), using continuous 8 to 16% polyacrylamide vertical slab gels. The gels were then treated as described by Weber and Osborn (21). Samples analyzed were: lane C, purified virus; lane D, pool DI obtained after DEAE-cellulose chromatography; lane A, pool H obtained after heparin-Sepharose chromatography; lane B, marker proteins. The molecular weight (MW) markers used were: phosphorylase b, 94,000; BSA, 68,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; trypsin inhibitor, 21,000; cytochrome *c*, 13,000.

obtained with a high yield after heparin-Sepharose chromatography. As demonstrated elsewhere (4, 9, 17), this affinity of the enzyme for heparin indicates its high nucleic acid-binding properties.

The molecular weight of the purified SFV1 RT was estimated by sedimentation analysis as 81,000 (Fig. 4). This size is comparable to that of the RT of human foamy virus isolate H4188 (11).

The purified enzyme was tested with a battery of six different polynucleotides to establish which of them is the most efficient template-primer for the enzymatic reaction. By evaluation of the rate of initial incorporation, it appeared that the enzyme kinetics vary in a wide range depending on the polynucleotide employed. The highest activity was obtained with polyriboadenylate-oligodeoxythymidylate (rA_n-dT_{12-18}) (Table 2). Divalent cation requirements were investigated by using Mn^{2+} and Mg^{2+}. Catalytic activity was greater in the presence of Mn^{2+}.

The optimal pH for enzymatic activity under the reaction conditions employed (rA_n-dT_{12-18} and Mn^{2+}) is 7.50 ± 0.30.

The purified enzyme retains its full activity during 6 months when stored at −20°C in 50% (vol/vol) glycerol in the presence of bovine serum albumin (BSA). In the absence of BSA, it loses 55% of its activity within 10 days.

Purified RT from SFV1 was exposed to thermal inactivation in the presence or absence of

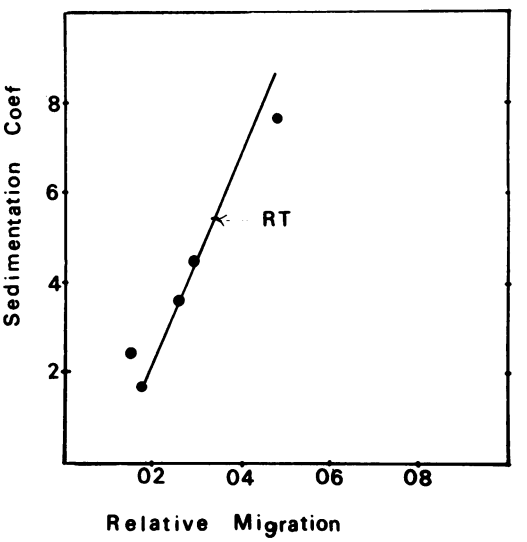

FIG. 4. Determination of the molecular size (sedimentation constant S_{20}) of the RDDP of SFV1 by centrifugation in a sucrose density gradient as described by Martin and Ames (12). Linear density gradients of 5 to 20% (vol/vol) sucrose in 50 mM Tris-hydrochloride–1 mM DTT–0.1 mM EDTA (final pH 7.5) were prepared in Beckman ultracentrifuge SW40.1 rotor tubes. Samples of 100 μg of purified enzyme (H) diluted in the same buffer were loaded onto a density gradient with 100 μg of BSA (S_{20}, 4.43; molecular weight, 68,000). A parallel density gradient was loaded with the same amount of BSA as well as with 100 μg of each of the following markers (from Worthington Diagnostics): cytochrome *c* (horse heart) (S_{20}, 1.70; molecular weight, 13,400), soya bean trypsin inhibitor (S_{20}, 2.38; molecular weight, 21,000), ovalbumin (chicken egg) (S_{20}, 3.55; molecular weight, 45,000), and yeast alcohol dehydrogenase (S_{20} 7.5; molecular weight, 141,000). The gradients were centrifuged for 16 h at 105,000 × *g* at 4°C and then cytochrome *c* was assayed by measuring the optical absorption at 400 nm. BSA, ovalbumin and trypsin inhibitor were assayed by optical absorption at 280 nm. Yeast alcohol dehydrogenase was assayed by the method of Vallee and Hoch (19), with evaluation of the reduction kinetics followed by measure of the optical absorption based on the following reaction: $RCH_2OH + NAD \rightarrow RCHO + NADH_2$.

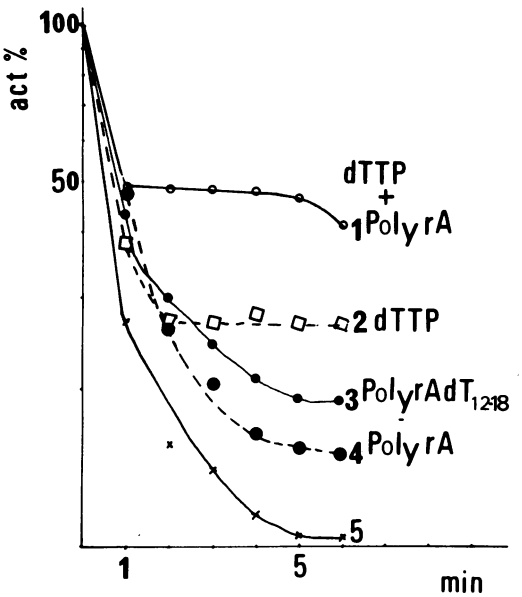

FIG. 5. Protection of the RDDP against heat inactivation. A 1-μg sample of enzyme was inactivated at 42°C at various time intervals, in the following medium: 50 mM Tris-hydrochloride (pH 7.2)–1 mM $MnCl_2$–40 mM KCl with 100 μg of BSA per ml. The following comparative assays were done, with either the enzyme alone or in the presence of substrate or template, or both. (1) Presence of substrate (dTTP, 1 mM) and of template polyriboadenylate (PolyrA) (8 μg/ml). (2) Presence of substrate (dTTP, 1 mM). (3) Presence of template-substrate, rA_n-dT_{12-18} (8 μg/ml. (4) Presence of template polyriboadenylate (8 μg/ml). (5) Absence of any of the former substrates or templates. After the given incubation time at 42°C, the enzyme was cooled at 0°C during 5 min. Then, RDDP activity was determined, using standard assays.

various compounds required for maximal activity (Fig. 5). A synergistic protective effect was noted when all of the components were present. A 2.5-fold protection of the enzyme was observed in the presence of both matrix and substrate as compared with the enzyme alone. The protection offered by the substrate alone was inferior to that found with the template-primer. Thus, it appears that the enzyme has two different loci, one specific for the template-primer and the other specific for the substrate. Protection in the presence of the template-primer rA_n-dT_{12-18} as compared with that seen with the template polyriboadenylate alone reveals an attachment of the enzyme to the 3'-OH of the primer oligodeoxythymidylate. This fact suggests that a function of the primer is to assure a more active attachment of the RT to the template-primer, forming a stable complex. All these results taken together led us to the concept of an RT with separate loci for the substrate and template-primer and presenting a pocket-like conformation in the presence of both template-primer and substrate (15).

At present, further investigations are under way to determine whether the RT polypeptide of SFV1 has not only an RDDP activity with the characteristics here defined but also an RNAse H activity, such as that demonstrated as characteristic for the major known Retroviridae (13, 20).

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