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Ribonucleoside Triphosphate-Dependent Pyrophosphate Exchange of Reovirus Cores

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Reovirus cores catalyze a ribonucleoside triphosphate (rNTP)-dependent pyrophosphate exchange reaction in the presence of all four rNTP species. When rNTP species are tested individually, only guanosine-5'-triphosphate supports pyrophosphate exchange.

Reovirus cores (subviral particles), formed by removing the outer viral capsomeres, possess at least two different enzymatic activities: a ribonucleic acid (RNA) transcriptase which synthesizes single-stranded RNA in the presence of a full complement of ribonucleoside triphosphates (rNTP species) (5, 7, 9, 12, 13), and a phosphatase activity that converts both rNTP molecules and deoxyribonucleoside triphosphates (dNTP molecules) to the corresponding diphosphates and inorganic phosphate (8). This report shows that reovirus cores catalyze a third activity, an rNTP-dependent pyrophosphate exchange reaction. These studies provided presumptive evidence for the presence of guanine (G) residues at the 5'-termini of the transcriptase products.

Type 3 reovirus (Dearing strain) was grown in mouse fibroblast L-929 cells as previously described (9). Virus particles were isolated, purified, and converted to viral cores by a procedure involving differential centrifugation and treatment with trypsin, Triton X-100, and sodium dodecyl sulfate (S. Silverstein and G. Acs, *unpublished data*). Conversion to viral cores was verified by both electron microscopy and the determination of buoyant density (9). The reaction mixtures for the assay of pyrophosphate exchange activity were virtually identical to those used for the RNA transcriptase assay (8, 9), and contained 2.0 mM ^{32}P-sodium-pyrophosphate (1 to 3 $\times$ 10^4 counts per min per nmole) and 2.5 to 12.5 μg of core protein. Incubation was at 37 C. Protein was determined by the method of Lowry et al. (10). All compounds used were commercial samples of analytical grade. ^{32}P-tetrasodium pyrophosphate was obtained from both New England Nuclear Corp., Boston, Mass., and from Amersham/Searle, Arlington Heights, Ill. The tetrasodium salts of ^{3}H-rNTP were products of New England Nuclear Corp.

In preliminary studies on pyrophosphate exchange in the presence of four rNTP species [adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytosine triphosphate (CTP), uridine triphosphate (UTP)], the reaction was found to be linear with time for at least 60 min, and linear with respect to core protein concentration in the range of 2.5 to 12.5 μg of protein per 0.10 ml of reaction mixture (30 min assay). The exchange reaction has an absolute requirement for Mg^{2+} (7.5 mM), and a *p*H optimum of 7.9. The Mg^{2+} and *p*H requirements are identical to those for RNA transcriptase (9). Although the presence of 0.25 M potassium-acetate stimulates transcriptase activity by about twofold, potassium-acetate is unnecessary for pyrophosphate exchange. To facilitate comparison, pyrophosphate exchange activity was assayed with 0.25 M potassium-acetate. In the presence of four rNTP species, optimal exchange occurred when each NTP was 2.0 mM. The same rate of exchange was observed when the pyrophosphate concentration was varied from 1.0 to 4.0 mM.

Data from two representative experiments on the rNTP requirements for pyrophosphate exchange are shown in Table 1. This exchange has an absolute requirement for exogenous rNTP molecules with the best activity observed in the presence of all four rNTP species. Individually, only GTP supports pyrophosphate exchange. This exchange is only stimulated by the addition of the other three rNTP molecules; single rNTP molecules or pairs are not stimulatory. There is no activity in a reaction mixture containing ATP + CTP + UTP. The activity with 8 mM GTP and 2 mM GTP is an average of 50 and 30%, respectively, of that observed in the presence of four rNTP species (2 mM each). In other experi-

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TABLE 1. *Ribonucleoside triphosphate requirements for pyrophosphate exchange*[a]

Ribonucleoside triphosphate added[b]	Concentration of triphosphate (mM)	Charcoal-adsorbable pyrophosphate (ribonucleoside triphosphates) (nmoles)	
		Expt 1	Expt 2
None		0.03	
ATP + CTP + UTP + GTP	2	0.90	1.09
ATP	8	0.03	
ATP	2	0.01	
CTP	8	0.00	
CTP	2	0.02	
UTP	8	0.00	
UTP	2	0.00	
GTP	8	0.40	0.60
GTP	2	0.22	0.39
GTP + ATP	2	0.25	0.38
GTP + CTP	2	0.20	0.35
GTP + UTP	2	0.21	0.34
ATP + CTP + UTP	2	0.00	
GTP + UTP + ATP	2		0.38
GTP + CTP + ATP	2		0.36
GTP + CTP + UTP	2		0.36

[a] Each reaction mixture contained 7.5 μg of reovirus core protein in a final volume of 0.10 ml. Experiment 1 was 30 min at 37 C with 2 mM ^{32}P-pyrophosphate (2.43×10^4 counts per min per nmole). Experiment 2 was 60 min at 37 C with 2 mM ^{32}P-pyrophosphate (1.60×10^4 counts per min per nmole). At time zero and at subsequent intervals, the reaction was stopped by cooling in ice, followed by the addition of 0.50 ml of Na-pyrophosphate (0.1 M), 0.10 ml of bovine serum albumin (1.0%), and 1.0 ml of trichloroacetic acid (10%). Samples were centrifuged in the cold for about 10 min at 12,000 × *g*. The entire supernatant fraction was mixed with 0.20 ml of activated charcoal (Mallinckrodt, N. F.; 15%, w/v) and 1.0 ml of 0.5 M sodium-acetate, *p*H 5.0. After 30 min in ice, the charcoal was removed by filtration through a moistened glass fiber filter (Whatman type GF/A). The charcoal was washed with four 15-ml portions of 0.1 N sodium acetate (*p*H 5.0) containing 0.01 M sodium pyrophosphate. Filters were dried, suspended in Liquiflor (New England Nuclear Corp., Boston, Mass.), and radioactivity was determined by scintillation counting. The same reovirus core preparation was used in both experiments; experiment 1 was performed two weeks prior to experiment 2.

[b] Abbreviations: ATP, adenosine triphosphate; CTP, cytosine triphosphate; UTP, uridine triphosphate; and GTP, guanosine triphosphate.

ments, GTP (8 mM) exchange with pyrophosphate was linear for at least 60 min at 37 C, and indifferent to the presence of 0.25 M potassium-acetate; increasing the GTP concentration to 16 mM had no effect on activity.

The findings that the optimal conditions for pyrophosphate exchange resemble those for RNA transcriptase and that individually only GTP supports pyrophosphate exchange suggest that G may be present at the 5′-termini of the transcriptase products. This was verified in subsequent experiments (D. H. Levin, G. Acs, and S. Silverstein, *unpublished data*). Attempts to show pyrophosphorolysis of reovirus double-stranded RNA by using ^{3}H-RNA labeled cores have so far been unsuccessful (J. T. Wachsman, *unpublished data*). The 5′-terminus of the RNA bacteriophage Qβ is known to contain at least a tetra-G (1, 3, 4). Although the Qβ RNA polymerase also catalyzes an rNTP-dependent pyrophosphate exchange reaction, there is no exchange in the presence of any single rNTP; minimally, a mixture of both ATP and GTP is required for exchange (1). It is tempting to speculate on the possibility that at least a portion of the reovirus-associated GTP exchange is the result of some function unrelated to the presence of double-stranded RNA and that GTP has a unique role in the binding of transcriptase to viral RNA.

Although dNTP molecules are substrates for reovirus core phosphatase (8), a mixture of four dNTP species (2 mM each) does not support pyrophosphate exchange in the standard assay mixture, nor are they substrates for reovirus transcriptase (9). dGTP alone (8 mM) does not support exchange either under standard assay conditions or when 7.5 mM Mg^{2+} is replaced by 2 mM Mn^{2+}. A study of RNA transcriptase activity showed that it is strongly inhibited by pyrophosphate, with approximately 90% inhibition

observed in the presence of 2 mM pyrophosphate. Comparative assays showed that the rate of pyrophosphate exchange is approximately one-fourth the rate of RNA synthesis in the absence of pyrophosphate.

An experiment to determine the extent of pyrophosphate exchange with individual rNTP molecules in the presence of all four rNTP species is shown in Table 2. Exchange with GTP accounts for almost 40% of the total, which is in agreement with the data of experiment two, Table 1, also involving a 60-min incubation at 37 C. This suggests that the rate of GTP (2 mM) exchange is the same either singly or in the presence of all four rNTP species. Exchange with ATP and UTP accounts for 21% and 12%, respectively, of the total. In a similar experiment with Qβ RNA polymerase (1), pyrophosphate exchange with GTP and ATP accounted for 33% and 51%, respectively, of the total exchange. We are presently unaware of the factors that determine these differences. It is clear, however, that the degree of participation of individual rNTP species in the exchange reaction is not related to the overall base composition of reovirus double-stranded RNA (2, 11) or the single-stranded RNA transcriptase products (7). In both single- and double-stranded reovirus RNA, A and U are each present in higher concentration than G.

TABLE 2. *Extent of pyrophosphate exchange with individual ribonucleoside triphosphates (rNTP), after incubation in the presence of all four triphosphates*[a]

Ribonucleoside triphosphate	Pyrophosphate (nmoles) exchanged per nmole of nucleoside triphosphate recovered from charcoal	Total exchange
		%
GTP	0.013	39.5
ATP	0.0068	20.7
CTP	0.0091	27.7
UTP	0.0040	12.1

[a] The reaction mixture contained 15 μg of reovirus core protein, a mixture of 4 rNTP species (2.0 mM each), and 2.0 mM ^{32}P-pyrophosphate (1.6×10^4 counts per min per nmole), in a final volume of 0.10 ml. The following tritiated triphosphates were used: ATP-*G*-^3H = 1.4×10^3 counts per min per nmole; UTP-*5*-^3H = 4.2×10^2 counts per min per nmole; CTP-*5*-^3H = 2.5×10^2 counts per min per nmole; GTP-*8*-^3H = 3.8×10^2 counts per min per nmole. Incubation was for 60 min at 37 C. The reaction was stopped and the rNTP molecules were adsorbed to charcoal as described in the legend of Table 1. The rNTP molecules were eluted from charcoal with cold 40% acetone containing 0.1% NH_4OH. The eluate was concentrated by flash evaporation at 37 C, and the individual rNTP molecules were separated by chromatography on water-washed polyethylenimine cellulose thin-layer sheets (Brinkmann Instruments, Inc., Westbury, N.Y.), by using 0.85 M potassium phosphate (*p*H 3.4) as the developing solvent (6). The position of each rNTP was determined by ultraviolet absorption, and the sheets were then cut into 0.5-cm strips in the direction of solvent flow. Each strip was analyzed for ^3H and ^{32}P by scintillation counting. The recovery of ^3H was used to correct for the unequal adsorption and elution of rNTP molecules from charcoal and for the calculation of the extent of pyrophosphate exchange per nmole of rNTP.

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