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RNA-Dependent DNA Polymerase Activity in Five RNA Viruses: Divalent Cation Requirements

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Abstract. The RNA-dependent DNA polymerase(s) in murine leukemia, murine mammary tumor, and avian myeloblastosis viruses require manganese for optimal activity. The transcription of added synthetic polyribonucleotides is greatly enhanced when manganese is used in place of magnesium. A soluble RNA-dependent DNA polymerase activity has been released from murine leukemia particles in the presence of manganese and high detergent concentrations. Two RNA viruses, visna virus of sheep and a primate syncytial virus, not known to have tumor-producing ability, also contain the polymerase.

An RNA-dependent DNA polymerase has been demonstrated in RNA-containing tumor viruses[1,2] but not in several other RNA viruses.[3] Spiegelman *et al.*[4] have shown that use of synthetic templates, either double-stranded RNA or RNA·DNA hybrids, increases the sensitivity of detection of the enzyme(s). In attempting to use these templates with the Rauscher strain of murine leukemia virus, we encountered difficulty in stimulating enzymatic activity unless manganese was used as the divalent cation in place of magnesium. This study was undertaken to compare the effects of manganese and magnesium on DNA synthesis by a known RNA-dependent DNA polymerase in Rauscher leukemia virus. With the use of the appropriate divalent cation, enzymatic activity has been completely separated from intact Rauscher leukemia virus. In addition, using the more sensitive methods, a DNA polymerase has been demonstrated in two RNA viruses not yet shown to be oncogenic.

Materials and Methods. Viruses: Rauscher leukemia virus was prepared from leukemia-virus infected mice as previously described[3] and had a titer of $10^{8.3}$ plaque forming units (PFU)/ml. Avian myeloblastosis virus, produced in chickens, was supplied by Dr. J. W. Beard (Duke University) and had 10^{11} physical particles/ml. Murine mammary tumor virus was prepared as previously described and had 10^{11} physical particles/ml.[3] Visna virus[5] was grown in sheep testes cells and was supplied by Dr. L. Stone (NIH); its titer was $10^{8.0}$ PFU/ml. A syncytium-forming virus, isolated and maintained in primary African green monkey kidney cells, was supplied by Dr. W. Parks (NIH); the properties of the virus will be described in detail elsewhere (manuscript in preparation).

DNA polymerase assay: Poly r(A·U) and poly r(I·C) were purchased from Miles Research Co.; poly rA and poly rU were the gift of Dr. A. Beaudet (NIH). The polymer, $(dC \cdot rG)_n$, was generously provided by Dr. S. Spiegelman (Columbia University). RNase A was purchased from Calbiochem. Labeled deoxyribonucleotide triphosphates were from Schwarz BioResearch; unlabeled deoxyribonucleoside triphosphates were from P.L. Biochemicals. Triton X-100 was from Packard and Nonidet P-40 was a

gift from the Shell Chemical Co. RNA-dependent DNA polymerase was assayed as described[3]; details are provided in the appropriate legends.

Results. A comparison of the effects of magnesium acetate and manganese acetate on the rate of incorporation of [^{3}H]dTTP into DNA is shown in Fig. 1.

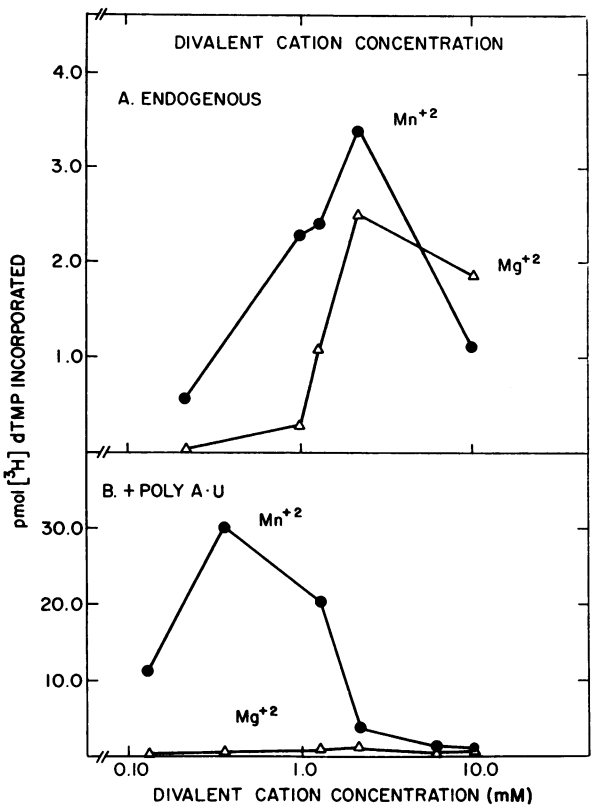

FIG. 1. (*A*) Each reaction mixture was incubated 90 min at 37°C and contained in 0.05 ml: 40 mM Tris·HCl, pH 7.8; 60 mM KCl; 2 mM dithiothreitol; 0.014% (v/v) Triton X-100; 1.5 μg viral protein manganese acetate or magnesium acetate as indicated; 20 μM [^{3}H-Methyl] dTTP (5000 cpm/pmol) and 500 μM dATP, dGTP, and dCTP. Incorporation, 0.08 pmol, in the absence of added divalent cation, is subtracted from each value. (*B*) Each reaction mixture, incubated for 25 min at 37°C, was as in *A* except: 0.04% Triton X-100; 0.5 μg viral protein; no unlabeled deoxyribonucleotide triphosphates; and 0.22 A_{260} Poly r(A·U).

At 4×10^{-3} M divalent cation, the synthesis of DNA from viral RNA present in intact virions occurred equally well with either magnesium or manganese (Fig. 1*A*) as previously reported.[6] However, at limiting concentrations of divalent cation (1×10^{-3} M), manganese was more effective. When the synthetic RNA polymer, poly r(A·U), was added as template (Fig. 1*B*) there was a 50- to 100-fold stimulation of activity with manganese; there was little polymer-dependent activity if magnesium was present. Similar results were obtained if the detergent was removed by dialysis after disruption of viral particles. The concentration of manganese (4×10^{-3} M) that was optimal for DNA synthesis from endogenous viral RNA was too high for optimal synthesis with the synthetic RNA template. For the polymer-stimulated reaction the manganese optimum was 6×10^{-4} M.

The requirements for synthesis of thymidylic acid or deoxyadenylic acid homopolymer from poly r(A·U) in the presence of manganese are shown in Table 1. The single-stranded polymers, poly rA or poly rU, could not substi-

TABLE 1. *Requirements for poly r(A · U) stimulated DNA synthesis.*

	pmol incorporated	
	[^{3}H]dTMP	[^{3}H]dAMP
Complete	2.40	0.10
− poly r(A · U)	<0.01	<0.01
− poly r(A · U) + poly rA	<0.01	<0.01
+ poly rU	<0.01	<0.01
− dATP	2.40	—
− TTP	—	0.20
+ dGTP, + dCTP	2.40	—
− Mn^{+2}	<0.01	<0.01

Each reaction mixture was incubated 30 min at 37 °C and contained in 0.05 ml: 40 mM Tris · HCl, pH 7.8; 60 mM KCl; 2 mM manganese acetate; 2 mM dithiothreitol; 0.028% (v/v) Triton X-100; 0.03 μg viral protein (Rauscher leukemia virus); 0.22 A_{260} of poly r(A · U), poly rA, or poly rU, as indicated; 20 μM [^{3}H-methyl]dTTP (5000 cpm/pmol) and 100 μM unlabeled dATP; or 20 μM [^{3}H]dATP (5000 cpm/pmol) and 100 μM unlabeled dTTP and, where indicated, 500 μM dGTP and dCTP.

tute for the duplex, poly r(A · U). Incorporation of dTTP was not affected by the presence or absence of other triphosphates. Very little incorporation of [^{3}H]dATP occurred with poly r(A · U), indicating preferential transcription of the adenylic acid strand of the duplex. In other studies (not shown) no incorporation of [^{3}H]UTP was detected when it was substituted for dTTP. These results are in agreement with experiments characterizing the enzymatic reactions in avian myeloblastosis virus in the presence of magnesium.[4]

Our earlier studies of DNA synthesis with Rauscher leukemia virus in the presence of magnesium demonstrated a critical Triton X-100 concentration (0.014%) for maximal activity; at 0.20% Triton virtually no activity was detectable. The effect of varying the Triton concentration, with either manganese or magnesium present, is shown in Fig. 2; the critical range of detergent concentrations is summarized in Table 2. With magnesium, the endogenous reaction (in the absence of added copolymer) showed a sharp dependence upon detergent concentration (Fig. 2*A*). In contrast, activity was preserved even at a much higher Triton concentration (0.2%) with manganese as the divalent cation; activity was not detectable at this concentration of Triton with magnesium. Homopolymer synthesis with added poly r(A · U) and manganese (Fig. 2*B*) was not maximal until 0.028% Triton. Activity was low at 0.014%, the concentration at which the endogenous reaction was optimal. The higher detergent concentration needed for the polymer-stimulated reaction probably reflects the need to disrupt the virus particle sufficiently to allow interaction between added template and enzyme(s). At no concentration of magnesium or Triton tested was magnesium as effective as manganese for the poly r(A · U)-stimulated reaction.

Prior studies on the requirements for DNA synthesis with endogenous viral RNA have been performed with magnesium in the assay mixture; the requirements were reinvestigated with manganese and 0.10% Triton X-100 (Table 3). Maximal incorporation required all four deoxyribonucleoside triphosphates. The complete inhibition of product formation by RNase A contrasts with earlier studies in which difficulty was encountered in fully inhibiting DNA synthesis with added RNase.[1,2] Presumably, complete RNase susceptibility is

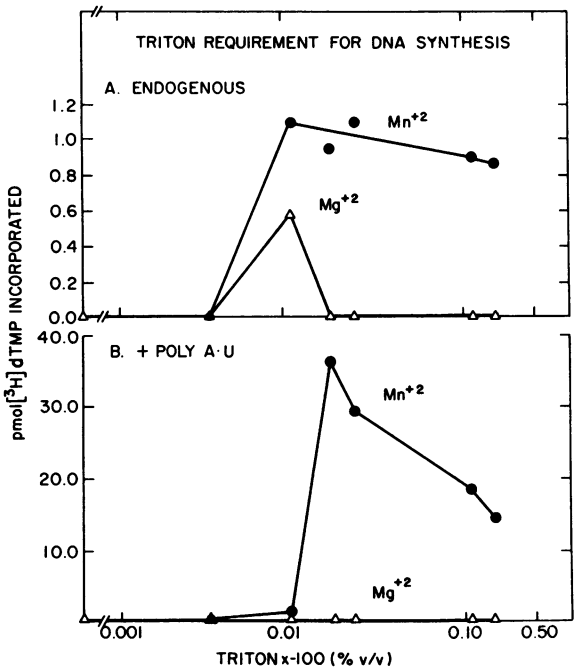

FIG. 2. (*A*) Each reaction as in Fig. 1*A*, except: 60 min at 37°C; either 2 mM manganese acetate or 10 mM magnesium acetate as indicated; 0.5 μg of viral protein; and Triton X-100 as indicated. (*B*) Each reaction was incubated for 25 min at 37°C and contained components as in *A*, except also 0.22 A_{260} poly r(A·U) and no dATP, dCTP, or dGTP.

possible in the more fully disrupted virus because of the greater accessibility of the viral RNA to added RNase.

Table 4 shows a comparison of the stimulation of the DNA polymerase of Rauscher leukemia virus by poly (dC·rG), poly r(I·C), and poly r(A·U) with magnesium and manganese. Incorporation with each polymer was tested at the optimal concentration of each cation. For each polymer, manganese was the preferred divalent cation. However, a direct comparison between the stimulation achieved with the three polymers may be unreliable, since different properties of the polymers, such as degree of helix formation or polymer size, may effect the reaction.

Two possible applications of the use of synthetic RNA polymers to detect RNA-dependent DNA polymerase activity are: (1) to aid in purification of the enzyme(s) and (2) to test RNA viruses other than RNA viruses known to induce tumors for the presence of such enzymatic activity. Our earlier studies,[3]

TABLE 2. *Triton concentrations for DNA synthesis.*

Triton (% v/v)	pmol [3H]dTMP incorporated: Endogenous reaction Mg+2	Endogenous reaction Mn+2	Poly r(A·U) Mg+2	Poly r(A·U) Mn+2
0.014	0.65	1.10	<0.01	0.35
0.028	<0.01	0.80	0.02	36.0
0.040	<0.01	1.10	<0.01	29.0

Components in the reaction as in Fig. 2. Both the endogenous reaction and the template-stimulated reaction are measurements of rate. The endogenous reaction contained all four deoxyribonucleoside triphosphates and was incubated for 60 min; the template-added reaction contained only [3H-methyl]dTTP (no other deoxyribonucleotide triphosphates) and was incubated for 25 min. The zero time value of 0.10 pmol of [3H]dTMP is subtracted from each value; it is the same with either magnesium or manganese.

TABLE 3. *Requirements for DNA synthesis from endogenous template with high triton.*

	pmol [^{3}H]dTMP incorporated
Complete	1.18
− dATP	0.04
− dATP, dCTP, dGTP	0.05
− Mn^{+2} (2×10^{-3} M)	<0.01
− Mn^{+2} + Mg^{+2} (2×10^{-3} M)	<0.01
− Mn^{+2} + Mg^{+2} (1×10^{-2} M)	<0.01
− Triton X-100	0.06
− Virus	<0.01
+ RNase A (1 mg/ml)	<0.01

Each reaction mixture, incubated 60 min at 37°C, was as given in Table 1, except: 0.1% (v/v) Triton X-100; 500 μM unlabeled deoxyribonucleotide triphosphates and 20 μM [^{3}H-Methyl]dTTP (5000 cpm/pmol) and 1.0 μg of viral protein. The [^{3}H]dTMP incorporated at zero time (0.10 pmol) is subtracted from each value.

TABLE 4. *Cation preferences for DNA synthesis with different polymers.*

Polymer	pmol incorporated [^{3}H]dGMP Mn^{+2}	pmol incorporated [^{3}H]dGMP Mg^{+2}
Poly dC·rG (0.003 A_{260})	18.0	<0.02
Poly r(I·C) (0.005 A_{260})	5.7	<0.01
	[^{3}H]dTMP	
Poly r(A·U) (0.006 A_{260})	27.8	<0.01

Reactions as in Table 3 except: 0.04% Triton X-100; 20 μM [^{3}H-methyl]-dTTP (5000 cpm/pmol) or 20 μM [^{3}H]dGTP (3000 cpm/pmol), and the manganese acetate for poly r(A·U) was 6×10^{-4} M, its optimum; the manganese acetate included for poly r(I·C) and poly(dC·rG) was 2×10^{-4} M, their optima. The concentration of magnesium used for all polymers was 1×10^{-2} M; higher or lower concentrations of magnesium were no more effective. The zero time value of 0.17 pmol of [^{3}H]dTMP is subtracted from all TMP incorporation; the zero time value of 0.20 pmol of [^{3}H]-dGMP is subtracted from all dGMP incorporation.

without synthetic templates and with magnesium, had shown that after treatment of a virus with 0.01% Triton, enzymatic activity cosedimented in sucrose gradients with intact virions. At 0.02% Triton, enzyme activity could be found also in the denser viral core and a lighter "soluble" fraction.[7] Virions treated with 0.2% Triton had no detectable activity. With 0.2% Triton treatment of virions in the presence of manganese, as shown in the sucrose gradient in Fig. 3,

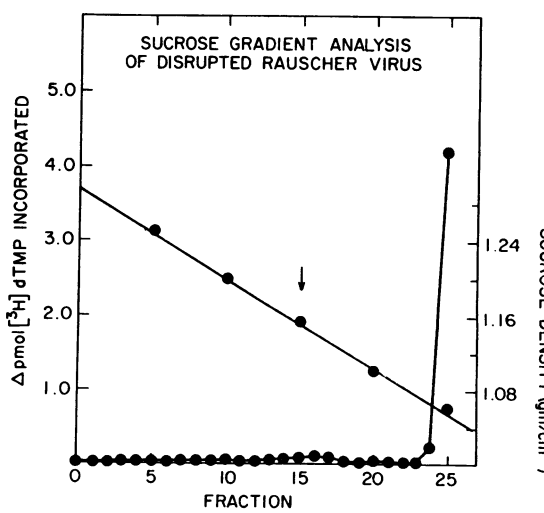

FIG. 3. Rauscher leukemia virus (9 μg) was incubated at 0°C for 15 min in 0.30 ml containing: 0.2% (v/v) Triton X-100; 2 mM manganese acetate and 2 mM dithiothreitol. The entire reaction was then layered on a 15–60% sucrose gradient containing 20 mM Tris·HCl, pH 7.8, 50 mM KCl; 2 mM manganese acetate; and 2 mM dithiothreitol. The gradient was centrifuged in an SW-41 rotor at 40,000 rpm for 90 min at 4°C. Fractions of 0.50 ml were collected and 25 μl of each fraction was assayed at 37°C for 60 min as in Fig. 2*B*, except manganese, not magnesium, was present.

enzyme activity could now be detected; all activity was present at the top of the gradient. When manganese was used, recovery of total activity upon treatment with either 0.02% or 0.2% Triton was comparable.

The generality of these conditions for observing polymer-stimulated DNA polymerase activity in several different viruses was examined (Table 5). Rauscher leukemia virus, murine mammary tumor virus, and avian myeloblastosis virus were found to have similar characteristics; manganese was the preferred cation in each case. However, another virus recently demonstrated to have an RNA-dependent DNA polymerase, visna virus,[8] showed a different divalent cation preference for the poly r(A·U) stimulated reaction; magnesium was the preferred cation when tested over a wide range of concentrations. Nevertheless, even with the less-preferred Mn^{++}, the polymer-stimulated activity could still be detected. A primate syncytium-forming virus, recently isolated by Parks *et al.* (manuscript in preparation) showed cation requirements similar to the three known tumor viruses. Whether the differences in divalent cation requirements for the polymer-stimulated reaction are related to the biological properties of these viruses is not yet clear. Vesicular stomatitis virus and an influenza virus preparation grown in SV-40-transformed mouse cells had no detectable polymer-stimulated DNA synthesis with either cation.

TABLE 5. *Comparison of divalent cation requirements for DNA synthesis by different RNA viruses.*

Virus (μg protein)	Polymer-dependent incorporation (pmol [^{3}H]-dTMP)	
	Mg^{+2}	Mn^{+2}
Murine leukemia (0.5 μg)	0.05	50.00
Avian myeloblastosis (1.0 μg)	0.08	8.15
Murine mammary tumor (1.8 μg)	0.16	0.66
Visna (6.2 μg)	4.10	0.15
Primate syncytial (5.0 μg)	0.01	7.80

Each reaction mixture was incubated at 37°C and contained in 0.05 ml: 40 mM Tris·HCl, pH 7.8; 60 mM KCl; either 2 mM manganese acetate or 10 mM magnesium acetate as indicated; 2 mM dithiothreitol; 0.22 A_{260} poly r(A·U); and 20 μM [^{3}H-Methyl]-dTTP (5000 cpm/pmol); reaction mixtures containing the different viruses were incubated with detergent as follows: Murine leukemia, 0.04% Triton X-100 for 30 min; primate syncytial virus, murine mammary tumor virus, and visna virus, 0.2% Triton X-100 for 90 min. Avian myeloblastosis virus was first incubated in 0.2% Nonidet P-40 and 200 mM dithiothreitol at 0°C for 10 min[4]; the reaction was then run for 30 min. A parallel incubation, minus polymer, was run for each virus, and the values obtained (0.05 pmol, except for the primate virus, 0.40 pmol) were subtracted in each case.

Discussion. Earlier studies have shown the marked stimulatory effect of synthetic RNA templates for RNA-dependent DNA polymerase activity of RNA tumor viruses.[4] The present studies show that with such templates both the particular cation and its concentration are critical for maximal activity. At either 4×10^{-3} M magnesium or 4×10^{-3} M manganese, DNA synthesis from endogenous viral RNA is optimal. If the divalent cation concentration is lowered, or if higher detergent concentrations are used, manganese is the preferred cation. When synthetic polymers are added, manganese again is preferred and the optimum concentration is lower than for the endogenous reaction; the optima are: 6×10^{-4} M for poly r(A·U); 2×10^{-4} M for poly r(I·C); 2×10^{-4} M for $(dC \cdot rG)_n$.

The first step in the reaction within the tumor virus particle is the synthesis of DNA from single-stranded viral RNA to form an RNA·DNA hybrid; a second step leads to the formation of double-stranded DNA.[9]

$$\text{RNA} \xrightarrow{1} \text{RNA·DNA} \xrightarrow{2} \text{DNA·DNA}$$

The synthetic RNA·DNA hybrid, $(dC \cdot rG)_n$, directs the synthesis of a homopolymer containing dGMP[4] and reflects the second step of the reaction. Which step of the reaction is reflected in transcription of double-stranded RNA is unresolved at this time.

For both the endogenous reaction and for the synthetic polynucleotide-stimulated reaction, manganese is the preferred cation. The preference for manganese, particularly with synthetic RNA·RNA polymers, and the differences in divalent cation optima with different polymers, have been observed also with the *DNA-dependent RNA polymerase* from *Micrococcus luteus*, formerly *M. lysodeikticus*.[10,11] Similarly, *DNA-dependent RNA polymerases* from adult rat liver and sea-urchin embryos require manganese for optimal activity.[12]

RNA-dependent RNA polymerases have been found in reovirus[13] and vesicular stomatitis virus.[14] A DNA-dependent RNA polymerase has been found in vaccinia virus.[15] In these cases, polymerase activity has been shown in viral cores, but a "soluble" activity has not yet been reported. In the present work, with the use of manganese and synthetic polymers, we have been able to disrupt Rauscher leukemia virus with a high concentration of detergent and to release a "soluble," polymer-dependent, RNA-dependent DNA polymerase activity.

The conditions that are optimal for detecting polymer-stimulated activity in the murine leukemia virion are effective for the murine mammary tumor virus and the avian myeloblastosis virion. Each of these viruses readily produces tumors when inoculated into its natural host. DNA polymerase activity has now been found in two RNA viruses that are not yet known to have tumor-producing potential. One of these, visna virus, is responsible for a chronic, progressive, neurologic disease in sheep; its enzymatic activity differs from that of the known tumor viruses in that its polymer-dependent reaction shows a marked preference for magnesium. The other virus, a primate syncytium-forming virus, has enzymatic properties that so far are not distinguishable from the known oncogenic RNA viruses. Whether the syncytium-forming virus produces tumors in its natural host has yet to be determined. The conditions described for both the endogenous and the polymer-stimulated reaction may be applicable for the characterization of other viruses isolated from cancers and other chronic diseases of animals and man.

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