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PROPERTIES OF THE REVERSE TRANSCRIPTION OF SYNTHETIC AND HAMSTER RETROVIRAL RNA BY AVIAN AND HAMSTER VIRAL POLYMERASES

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SUMMARY: Reverse transcription of poly(rA)·oligo(dT) and of the viral RNA present in purified virions of a hamster retrovirus by hamster and avian reverse transcriptase has been examined under various reaction conditions. The results demonstrate that the hamster virus polymerase transcribes by a processive mechanism and that the size of the poly(dT) transcripts is not influenced by dTTP or enzyme concentrations. These and previous results show that initiation is the rate-limiting step. Both polymerases were markedly more active with Mn^{2+} than Mg^{2+} when tested on the hamster viral RNA template and produced the same "strong stop" cDNA with either metal ion. Evidence is presented which suggests that the metal ions affect the template activity of the polyribonucleic acids rather than the activity of the enzyme.

Studies of the properties of the avian and murine viral reverse transcriptases have shown that these polymerases are different structurally, biochemically and immunologically, although both perform similar functions *in vivo* (reviewed in 1,2). Previous work in our laboratory has demonstrated that the AMV retroviral polymerase transcribes by a processive mechanism on poly(rA) templates and that the processivity is influenced by factors which affect the stability of the enzyme·template·daughter strand complex such as temperature and ionic strength (3)

Considerable effort has been spent to develop techniques whereby complete cDNA transcripts of various RNA's can be produced in good yield. Reaction conditions have been studied to optimize Mn^{2+} or Mg^{2+}, deoxyribonucleoside triphosphate (dNTP) concentrations (4-9) and detergent concentrations (10). These efforts have produced infectious retroviral cDNA, although in very low yield (6,11). Despite these successes, many of the basic mechanisms of reverse transcription remain unclear, and an important obstacle to the production of full-length cDNA transcripts of retroviral RNA is that initiation is near the 5' terminus making the formation of secondary structures which allow transcription to switch over to the 3' terminus essential. There also have been reports of multiple initiation sites (8,12,13), transcription "helper" proteins (14), and effects dependent on various states of the enzyme (15-17).

dNTP, deoxyribonucleoside triphosphates; AMV, avian myeloblastosis virus; cDNA, complementary DNA; HaLV, hamster leukemia virus; HaSV, hamster sarcoma virus; HaRV, hamster retrovirus; DTT,dithiothreitol; vRNA, viral RNA.

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Prior publications (18,19) concerning hamster viral reverse transcriptases are based on the hamster-murine-rat pseudotypes (HaLV and HaSV) and the polymerases and templates of these pseudotypes may not be representative of the "hamster" enzyme RNA. The only other study done on a hamster type-C virus showed no detectable endogenous activity (20). In recent reports, we described the isolation of a hamster retrovirus (HaRV) demonstrated that it possesses endogenous reverse transcriptase activity and showed the HaRV polymerase to be biochemically similar to the murine viral polymerases and to have significant immunological cross-reactivity with MuLV polymerase (21,22). This paper examines the effects of reaction conditions on the transcription of endogenous and synthetic templates and shows that the metal ions have relatively little influence on enzyme activity but appear to affect the activity of the ribopolymers as templates. We also show that the HaRV polymerase transcribes in a processive manner and that dTTP and enzyme concentrations had almost no effects on transcript length. Particular attention was paid to the effect of metal ions since previous results from other laboratories are contradictory (4-8) and we had shown metal ions to have some effect on the AMV polymerase product using synthetic templates (3).

MATERIALS AND METHODS

Virus Preparation: The production and purification of HaRV polymerase have been previously described (21,22). Purified AMV reverse transcriptase was obtained through the NCI Office of Program Resources and Logistics, Viral Oncology Program.

Assay Procedures and Endogenous Reactions: Reverse transcriptase assays utilizing poly(rA)·oligo(dT) were done as described previously (3,22). Endogenous reactions were performed in TNDN [50mM Tris-HCl, pH 8.3; 60mM NaCl; 10mM DTT; 0.3% (v/v) NP-40] which contained $MnCl_2$, $MgCl_2$, deoxyribonucleoside triphosphates, oligodeoxyribonucleotide primers, and AMV polymerase as described in the text. Reactions were incubated for 120 min at 30° unless otherwise noted and harvested with trichloroacetic acid onto glass fiber filters or prepared for electrophoresis.

Size Analysis of Poly(dT) Transcripts: Poly(rA) templates 1100 nucleotides in length were prepared on a calibrated high pressure porous glass bead column as previously described (3). $Poly(rA)_{1100}$, $oligo(dT)_{12-18}$, dTTP, and AMV polymerase were used at the indicated concentrations in 50mM Tris-HCl, 0.2mM $MnCl_2$, 60mM NaCl, 5mM DTT and 0.2% NP-40 at pH 8.3. Incubations were done at 25° for 120 min and terminated in a boiling waterbath for 2 min. The sizes of the poly(dT) transcripts were determined by high pressure liquid chromatography(3).

Sample Preparation for Gel Electrophoresis: Endogenous reactions were terminated by the addition of an equal volume of buffer-saturated phenol. After 2-3 phenol extractions, the aqueous phases were precipitated with 3 volumes of cold 95% ethanol and stored overnight at -15° prior to centrifugation. The pellets were resuspended in 15µl of 0.4N sodium hydroxide and kept at 37° for 4 h to degrade the RNA. After neutralization with EDTA, sodium acetate and magnesium acetate were added to final concentrations of 0.3M and 10mM, respectively, in a final volume of 150µl. The samples were reprecipitated with 3 volumes of 95% ethanol, stored overnight at -15°, pelleted, rinsed with cold 70% ethanol and vacuum dried. The dry pellets were redissolved in 10mM phosphate buffer, pH 7.0, and an equal volume of dimethylsulfoxide was added, followed by glyoxal to 1M with heating (23)

Polyacrylamide Gel Electrophoresis: Electrophoresis of cDNA and markers was done on 15% uniform polyacrylamide slab gels in 10mM phosphate buffer, pH 7.0. Gels were cast at an acrylamide:bisacrylamide ratio of 29:1. After degassing, polymerization was initiated with 50µl TEMED and 333µl of 10% ammonium persulfate per 100ml solution. The gels were stored overnight and prerun for 60 min prior to sample application. Power was applied until the bromophenol blue had run to the bottom of the gels. The gels were removed from their holders, fixed in 10% trichloroacetic acid and prepared for fluorography (24). Yeast tRNA and Hind III nuclease fragments of λ-DNA were labelled with ^{32}P as described by Maniatis *et al.* (25).

RESULTS AND DISCUSSION

Processive reverse transcription by HaRV polymerase: We have previously reported that reverse transcription by AMV polymerase is processive (3). Using the same techniques, similar experiments were done with the HaRV polymerase to demonstrate that a mammalian reverse transcriptase also has a processive mode of action (fig. 1). The details and rationale of the processivity assay have been published elsewhere (3). After 10 and 60 min incubations, poly(dT) transcripts with average lengths of 122 and 135 nucleotides, respectively, were made. If the polymerase was distributive, an average length of 17 nucleotides (2 + oligo$(dT)_{12-18}$ ≃ 17) would be predicted based on the incorporation of 3% of the total dTTP after 60 min. Even if all the dTTP had been incorporated in a distributive manner, the transcripts would be only 50 nucleotides long. These results demonstrate the processivity of HaRV polymerase. As previously shown for the AMV polymerase, maximum size transcripts were made in a short time which is consistent with initiation being the rate determining step. The only other experiments done with mammalian DNA polymerases, the α and β DNA polymerases from calf thymus, demon-

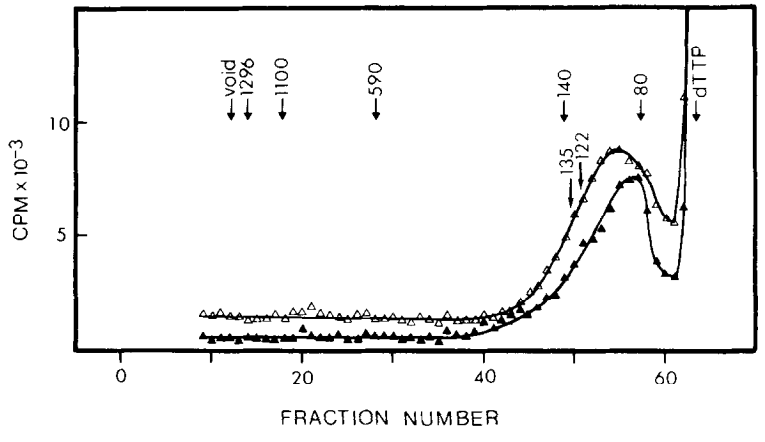

Fig. 1. Elution profile of poly(dT) strands after 10 min (▲——▲) and 60 min (△——△) incubation at 30° using 40nM poly$(rA)_{1100}$, 20nM oligo$(dT)_{12-18}$, 1.0µM dTTP, a hamster type-C viral polymerase and 100µM Mn^{2+}. The incubation buffer also contained 5mM DTT, 0.25% NP-40, 60mM NaCl and 50mM Tris-HCl at pH 8.3. Heavy arrows at the top of the figure mark the elution positions of markers of the indicated lengths. Small arrows indicate the average lengths of the poly(dT) transcripts if they are assumed to be homogeneous.

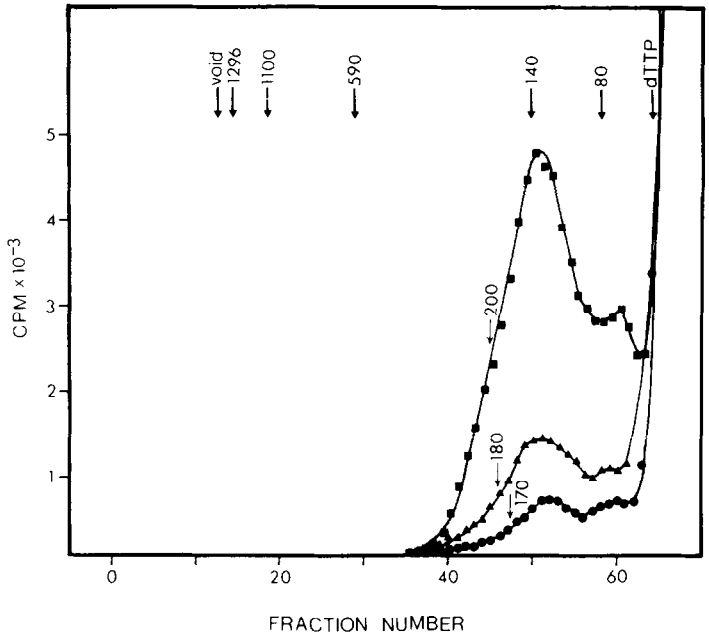

Fig. 2. Size distribution of the poly(dT) products made by AMV polymerase from $\overline{poly(rA)}_{1100}$·oligo(dT)$_{12-18}$. All reactions contained 20nM poly(rA)$_{1100}$, 0.2mM Mn^{2+} and were incubated for 120 min at 25°. Other reactant concentrations were:

	■—■	▲—▲	●—●
AMV polymerase	4.0nM	2.0nM	1.0nM
oligo(dT)$_{12-18}$	40nM	20nM	10nM
^{3}H-dTTP (41 Ci/mmol)	2.0μM	1.0μM	0.5μM

Heavy arrows at the top of the figure mark the elution positions of markers of the indicated lengths. Small arrows indicate the average lengths of the poly(dT) transcripts if they are assumed to be homogeneous.

strated that they were 'quasi-processive' since they incorporated only 8-11 nucleotides before enzyme dissociation (26).

Influence of dTTP at sub-K_m levels on transcript length: Using AMV polymerase in the poly(rA)$_{1100}$·oligo(dT)$_{12-18}$ system, it was found that varying the dTTP concentration from 0.5 to 2.0μM, while maintaining constant ratios of polymerase, oligo(dT) and dTTP, had almost no effect on the lengths of the poly(dT) transcripts, although the increase in quantity of product was related to the increase in the concentrations of reactants and polymerase (fig. 2). Concentrations of dTTP below the 5μM K_m (27) were chosen to maximize their effect on the reaction. Enzyme concentration also had little effect on the size of the products, although the quantity of transcript was proportional to enzyme concentration. The small difference in the size distribution of the poly(dT) demonstrates that the enzyme and dTTP concentrations effect quantitative rather than qualitative changes in transcription. All of these facts are consistent with initiation and not polymerization being the rate determining step. We have also shown elsewhere (3)

that product size is dependent on the metal ions, ionic strength and temperature using poly(rA)·oligo(dT).

Influence of detergent: Although maximum endogenous activity was observed at an NP-40 detergent concentration of 0.038% (data not shown), a concentration of 0.3% was used to insure that the vRNA template would be accessible to the exogenous AMV polymerase. Using purified AMV and HaRV polymerases, we have found that enzyme activity on poly(rA) templates is independent of detergent concentration over a range of 0-1% NP-40. Thus, the detergent 'optima' reported for endogenous reactions are probably a reflection of the detergent concentrations which make the viral envelope permeable to reactants, but do not remove the viral coat which would make the genome susceptible to nuclease digestion and alter the secondary structure required for transcription to switch from the 5' end to the 3' end of the genome.

Transcription of HaRV vRNA by AMV and HaRV Polymerases: A prevalent attitude concerning the endogenous activity of mammalian type-C viral reverse transcriptases suggests that Mg^{2+}-containing reactions yield a small amount of long cDNA, while the Mn^{2+}-containing reactions produce larger amounts of short cDNA product. Since the reverse transcriptase from AMV is known to be highly active in the presence of either Mg^{2+} or Mn^{2+} on poly(rCm) and poly(rA) templates while the mammalian type-C polymerases are not active with Mg^{2+}, we compared the activity and product size of the AMV polymerase with that of the HaRV enzyme using HaRV vRNA in the presence of Mg^{2+} or Mn^{2+}. Our previous results using poly(rA) have shown that the AMV enzyme is processive with either metal, but that the size and quantity of the product is affected by the metal (3).

As seen in Table 1, the HaRV enzyme, like other mammalian type-C polymerases, has very low activity in the presence of Mg^{2+} relative to Mn^{2+}-containing reactions Preparations of purified HaRV were either untreated or heat-treated, which inactivated the HaRV polymerase, prior to use in the reactions. In every instance where AMV polymerase was used, an amount of enzyme was added so that its poly(rA)·oligo(dT) activity was equal to that of the HaRV polymerase present. The addition of purified AMV reverse transcriptase to the reactions containing unheated detergent-lysed HaRV significantly increases the quantity of DNA product, both with and without oligo(dT). More dramatic increases are found when the AMV polymerase is used with heat-treated HaRV virions, in which the HaRV polymerase has been inactivated. Possible explanations are that the tRNA primers of the endogenous reaction are not all effectively annealed, interference due to unknown secondary structure is relieved, or degradative enzymes are inactivated.

When tested in the presence of Mg^{2+} (Table 1), HaRV reverse transcriptase and the combination of HaRV and AMV reverse transcriptases are much less active than in Mn^{2+} tests. Various concentrations and ratios of Mg^{2+} to dNTP's have been

Table 1

Endogenous DNA Synthesis by HaRV and AMV Polymerases
With and Without Mn^{2+}, Mg^{2+}, oligo(dT)$_{12-18}$, and dNTP's

		pmol deoxyribonucleotide incorporated					
		1.88mM Mg^{2+}			0.2mM Mn^{2+}		
oligo(dT)	dNTP[1]	HaRV	HaRV+AMV	AMV[2]	HaRV	HaRV+AMV	AMV[2]
-	-	0.04	-	-	0.07	-	-
+	-	0.05	-	-	0.42	-	-
-	+	0.14	0.22	1.28	0.86	1.96	2.31
+	+	0.16[3]	0.28	1.96	3.74	5.59	7.36

(1)Final individual concentrations of dATP, dCTP, and dGTP in Mn^{2+} experiments were 0.031mM; in Mg^{2+} experiments the dNTP's were 0.63mM each. 10μCi (2.0μM) of [^{3}H] dTTP was used per reaction. (2)HaRV polymerase inactivated by heating to 65° for 1 h followed by slow cooling to 30° prior to addition of AMV polymerase. (3)Contribution due to poly(dT) synthesis has been subtracted.

tested (Table 2). None of the combinations was particularly active although an optimal activity was observed at a Mg^{2+} to dNTP ratio of 4 to 1. The high activity of the AMV reverse transcriptase on synthetic templates in the presence of Mg^{2+} is clearly lost when vRNA is supplied as the template. Since AMV is quite active on the vRNA in the presence of Mn^{2+}, the loss of activity with Mg^{2+} suggests that Mg^{2+} reduces the activity of the vRNA as a template. This is in agreement with data showing that metal ions alter the secondary structure of polyribonucleotides (9), which possibly changes their template activity or affinity for polymerase (28)

Electrophoresis of vRNA transcripts: Transcripts were also electrophoresed on a 15% uniform polyacrylamide gel to facilitate the separation of low molecular

Table 2

Endogenous DNA Synthesis by HaRV in the Presence of Mg^{2+}

0.1μg oligo(dT)	mM Mg^{2+}	mM dNTP[1]	Mg^{2+}/dNTP	pmol dNMP
-	1.88	1.89	1:1	0.14
-	1.88	0.94	2:1	0.31
-	3.76	0.94	4:1	0.41
-	5.64	0.94	6:1	0.20
-	3.76	0.31	12:1	0.11
+	1.88[2]	1.89	1:1	0.16
+	3.76	0.94	4:1	0.59
+	5.64	0.94	6:1	0.27

(1)Total concentration of dATP, dCTP, dGTP with 2.0μM ^{3}H-dTTP (10μCi). (2) Contribution due to poly(dT) synthesis has been subtracted.

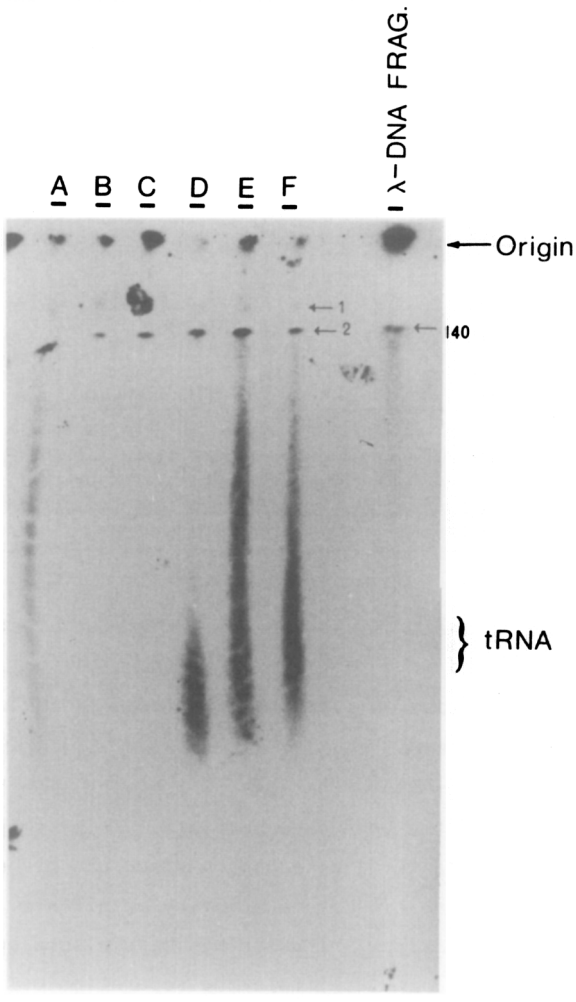

Fig. 3. Electrophoresis of alkaline hydrolyzed cDNA transcripts on a 15% uniform polyacrylamide gel. All reactions contained 2μM[^{3}H]-dTTP and HaRV virions either untreated (UNT) or heated (ΔT) at 65° for 60 min with slow cooling. Incubation was at 30° for 120 min. The reaction conditions are summarized below:

Lane	A	B	C	D	E	F
HaRV	UNT	ΔT	ΔT	ΔT	ΔT	UNT
AMV	-	+	+	+	+	+
Mn	0.2mM	-	0.2mM	-	0.2mM	0.2mM
Mg	-	1.88mM	-	1.88mM	-	-
dNTP's	31μM	0.63mM	31μM	0.63mM	31μM	31μM
oligo	0.1μg	-	-	0.1μg	0.1μg	0.1μg

weight cDNA (fig. 3). In addition to larger products which did not enter the gel, two distinct products, a minor and a major band, marked by arrows 1 and 2, respectively, were always found regardless of the polymerase or reaction conditions. These represent the 'strong stop' cDNA products found by other investigators in endogenous reactions of type-C viruses (29). The size of the major spot (#2)

relative to the nuclease digested λ marker is estimated to be 140 nucleotides which is similar to the murine strong-stop cDNA (29). The addition of oligo(dT) to the reactions resulted in the appearance of small heterogeneous products on the profiles. Electrophoresis of transcripts on a 4-15% gradient polyacrylamide gel demonstrates that most transcripts synthesized by AMV or HaRV polymerase in the absence of oligo(dT) are between 100 and 400 nucleotides in length (data not shown).

The results presented here and in a previous report (3) suggest that all retrovirus DNA polymerases transcribe in a processive manner. We propose that the complexity of retroviral endogenous reactions is due in part to the opposing actions of many of the reaction conditions. For example, low incubation temperature and ionic strength favor long transcripts, but inhibit initiation which is the rate-limiting step. The addition of exogenous polymerase would compensate for slower initiation, but requires high detergent levels which would make the genome accessible to nucleases as well as the exogenous polymerase. Still to be resolved are the conflicting results concerning the high activity of AMV polymerase in the presence of Mg^{2+} on synthetic templates vs. very low activity with Mg^{2+} on viral RNA templates. The use of Mg^{2+} which is reported to enhance transcript length, possibly by favoring the circularization of the genome, also reduces the total quantity of cDNA product.

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