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REVERSE TRANSCRIPTASE-ASSOCIATED RNase H. Part IV. Pyrophosphate does not inhibit RNase H activity of AMV DNA polymerase

Arun Srivastava and Mukund J. Modak

The Memorial Sloan Kettering Cancer Center,
1275 York Avenue,New York,N.Y.10021

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ABSTRACT:
Sodium pyrophosphate, a known inhibitor of DNA polymerases, strongly inhibits DNA synthesis directed by various synthetic and natural template-primers and catalysed by reverse transcriptases from AMV and RLV, but has no effect on the reverse transcriptase-associated ribonuclease H activity.The synthesis of both actinomycin D sensitive and insensitive DNA under the direction of 70 S AMV RNA or globin mRNA with high or low concentrations of substrate triphosphates is inhibited by the addition of pyrophosphate but under none of these conditions, could RNase H activity be suppressed by inclusion of pyrophosphate. These studies further establish that the site for RNase H function is distinct from the substrate binding site and that the target for pyrophosphate action is not the RNase H(template binding site).

INTRODUCTION:

RNA dependent DNA polymerases(RT) are unique DNA polymerases not only because they utilize RNA as template for DNA synthesis but also due to the fact that many of these enzymes express a nucleolytic activity,RNase H, specific for the RNA strand of an RNA:DNA hybrid[1]. We and others have presented some evidence to indicate the physical and mechanistic independence of synthetic and nucleolytic reactions catalysed by reverse transcriptases[2-6] and have found that modification of the substrate binding site with pyridoxal 5'phosphate,although severely inhibits substrate polymerization, has no effect on the associated nuclease function[7].Further investigations on the expression of RNase H revealed that the expression of nuclease activity may occur through the template binding site on RT[8,9]. Therefore, the inhibitor of RNase H function is also expected to inhibit polymerization reaction as well. Pyrophosphate, which has been known to be a DNA polymerase inhibitor[10], has recently been employed in a reverse transcription reaction to obtain full length copies of complementary DNA(cDNA). The mechanism of the production of long cDNA molecules was attributed to the inhibitory action of pyrophosphate on the RNase H function of reverse transcriptase[12-14].Pyrophosphate mediated inhibition of RNase H activity appeared highly unlikely,in view of the mechanism of the nucleolytic action and the known inhibitory action of pyrophosphate at the substrate level. We have, therefore,directly tested the effect of pyrophosphate addition on the polymerization and nuclease(RNase H) function of reverse transcriptase using AMV DNA polymerase as a model enzyme

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system, and have found that pyrophosphate has no effect on the RNase H activity expressed by AMV polymerase but it strongly inhibits both actinomycin D insensitive (primary strand) and actinomycin sensitive (secondary strand) synthesis of DNA on RNA templates.

MATERIALS AND METHODS

Materials: [^{3}H]-deoxynucleoside triphosphates were obtained from Amersham-Searle, Inc. Synthetic template-primers and unlabeled deoxynucleoside triphosphates were the products of P.L. Biochemicals, Inc. fd DNA was purchased from Enzobiochem. [^{32}p] 70S RNA from ASV was a kind gift from Dr. E. Stavenezer of this Institute. Rauscher murine leukemia virus (RLV) and Avian myeloblastosis virus (AMV) was supplied by the Division of Cancer, Cause and Prevention, N.C.I.

Reverse transcriptase from the above viruses were purified by poly rC-agarose chromatography, as described earlier[15-16]. E. coli RNA polymerase, holoenzyme, was prepared according to Burgess[17]. Homogenous DNA polymerase I from E. coli was a generous gift of Dr. L. Loeb. Calf thymus RNase H was purified as described earlier[18].

Synthesis of fd DNA: [^{3}H]-RNA hybrid: fd DNA: [^{3}H] RNA hybrid, the substrate for RNase H assays, was synthesized in a final volume of 2 ml containing 80 mM Tris-HCl (pH 7.8), 50 mM KCl, 10 mM DTT, 10 mM $MgCl_2$, 500 µg fd DNA, 1 mM each of ATP, CTP, UTP and 20 µM [^{3}H] GTP (3000 cpm/pmol) and 250 units of homogenous E. coli RNA polymerase. After 2 hrs of incubation at 37°C, the reaction mixture was placed in a 60°C water bath for 10 min, cooled slowly for 20 min and then placed in an ice bath. The DNA:RNA hybrid was purified by isopycnic banding on cesium sulfate gradient, desalted by Sephadex G-75 column chromatography and stored frozen at -20°C before use.

DNA polymerase assays: Assays were carried out in a final volume of 100 microliters and contained the following components: i) With synthetic template-primers: 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol (DTT), 50 µg bovine serum albumin, 0.5 µg of template-primer, 20 µM of appropriate [^{3}H]-deoxynucleoside triphosphate, adjusted to a final specific activity of 1000 cpm/pmol, 100 mM KCl, 8 mM $MgCl_2$ for AMV and E. coli DNA polymerase and 1 mM $MnCl_2$ for RLV polymerase. Incubation was done at 37°C for 30 min. ii) With 70S RNA template: 50 mM Tris-HCl (pH 7.8), 0.4 mM DTT, 25 µg/ml 70S RNA, 5 µg/ml oligo dT, 0.2 mM dATP, 0.2 mM dCTP either 0.2 mM [^{3}H]-dGTP (8Ci/mMol) or [^{3}H]-TTP (53 ci/mM), 8 mM $MgCl_2$ and 40 mM KCl. Incubation was done at 37°C for 90 min.

The reaction was terminated by addition of 5% (w/v) trichloroacetic acid containing 0.01 M sodium pyrophosphate. The acid insoluble precipitate was collected on Whatman GF/B filter papers, washed extensively with TCA containing pyrophosphate, water and finally with ethanol, dried and counted in a toluene based scintillation fluid[19].

RNase H assays: The reaction mixture contained, in a final volume of 100 microliters, 50mM Tris-HCl, pH 7.8, 1 mM DTT, either 10 pmol of fd DNA:(^{3}H) RNA hybrid (as total nucleotides) or 2.5 µg ^{32}P-70S RNA:cDNA hybrid(2,000 cpm/µg RNA), 8 mM $MgCl_2$ and 40 mM KCl. Reactions were initiated by the addition of enzyme fraction and incubated at 37 C for 60 minutes. Reactions were terminated by the addition of TCA and acid insoluble radioactivity was determined as described before.

RESULTS

Effect of pyrophosphate on the DNA polymerase activity: Both AMV and RLV reverse transcriptases were tested for their response to PPi addition with synthetic and natural template primers. The kinetics of DNA synthesis directed by AMV 70S RNA and poly(rC).$(dG)_{12-18}$ in the presence and absence of PPi is presented in figure 1 (data for RLV DNA polymerase are not shown). It is clear from the results that DNA synthesis directed by various template-primers is inhibited in the presence of PPi. Since a report in literature indicated that PPi inhibits only a small size class anticomplementary DNA synthesis while synthesis of cDNA is insensitive to PPi addition[12], we examined the effect of PPi on the DNA synthesis directed by AMV 70 S RNA in the presence and absence of actinomycin D. The results presented in figure 2 show that pyrophosphate inhibits synthesis of both complementary and anticomplementary DNA strands.

Since in the presence of high concentrations of substrate triphosphates, both rate and extent of DNA synthesis on RNA templates are known to be significantly increased[20-24]. We therefore examined the effect of PPi on DNA synthesis with low and high substrate concentration. Under both the substrate conditions, PPi was found to inhibit DNA synthesis(data not shown).

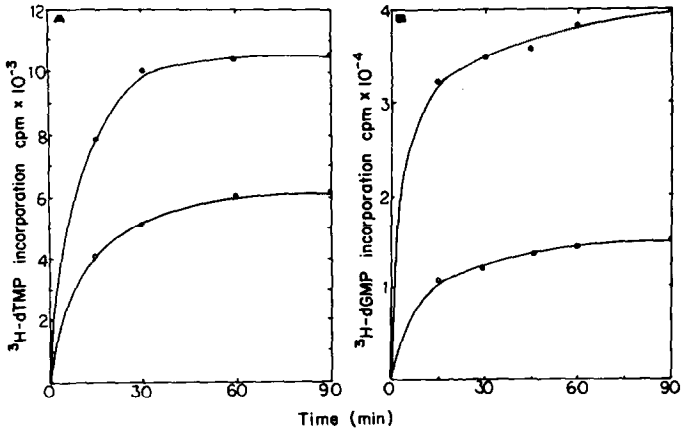

Fig. 1: Inhibition of DNA synthesis directed by various template primers and catalyzed by AMV polymerase in the presence of 4 mM pyrophosphate.
●—● control; o—o with 4 mM PPi
A. AMV 70S RNA·4S tRNA primer; [^{3}H]-dTTP substrate plus other dNTPs
B. poly (rC)·(dG)~15; [^{3}H]-dGTP substrate

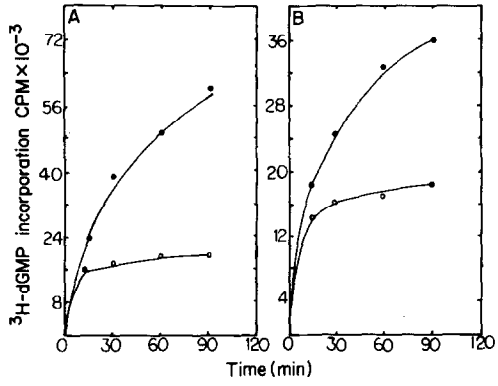

Fig. 2: Pyrophosphate (4mM) inhibition of AMV DNA polymerase activity with AMV 70S RNA·$(dT)_{10}$
●—● control; o—o with 4 mM PPi
A. DNA synthesis was measured by [^{3}H]-dGMP incorporation in the presence of all 4 dNTPs.
B. Only complementary DNA synthesis was measured under this condition by ^{3}H-dGMP incorporation since 100 µg/ml actinomycin D was present in both control and test reactions.

Effect of pyrophosphate on RNase H activity: A typical dose response of PPi on the polymerase and RNase H activity expressed by AMV polymerase is shown in figure 3. For comparison, E.coli DNA polymerase I (fig 3B) is also included since it also expresses RNAse H type activity[25-26]. The results clearly show that the RNase H activity associated with both AMV and E.coli polymerases is insensitive to PPi addition, while polymerization reaction is strongly inhibited. Similar results were obtained when polymerization of dNTPs(synthesis) and subsequent degradation of template RNA was monitored using ^{32}P-labeled ASV RNA as a template(Table 1). Addition of either actinomycin D or PPi or both

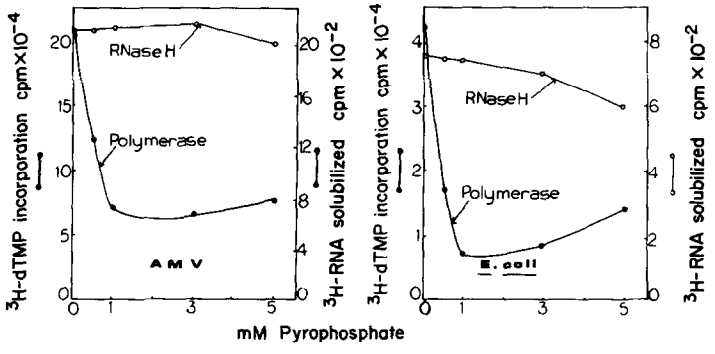

Fig. 3: Dose response of pyrophosphate influence on AMV and E. coli DNA polymerases.
A. AMV polymerase assayed with $(rA)_n$-$(dT)_{10}$ template-primer and RNase H assayed with fd DNA: [^{3}H] RNA hybrid.
B. E. coli DNA polymerase I assayed in the similar manner.

Table 1 : Effect of pyrophosphate on AMV DNA polymerase catalyzed synthetic and nucleolytic reactions.

Reaction Mix	Polymerase[a]		RNase H[b]	
	^{3}H-dTMP incorporation (CPM)	% Activity	^{32}p-RNA solubilization (CPM)	% Activity
Control	12162	100	755	100
+PPi *	5100	41.9	953	126.2
+Act.D	7473	61.4	900	119.2
+PPi+Act.D	3490	28.7	1144	151.5

[a]Assay was done as described under Materials and Methods. The reaction was stopped by chilling in an ice-bath followed by the addition of 10 mM EDTA. The template RNA was alkali digested (0.3M NaOH at 37°C for 4 hrs), and [^{3}H]-labeled DNA product was precipitated with 10% TCA after neutralization.

[b]Assay was done as described under Materials and Methods. The reaction mixture contained unlabeled dNTPs.

* Abbreviations used: PPi- pyrophosphate, AMV- Avian myeloblastosis virus,RLV-Rauscher murine leukemia virus,RNase H- Ribonuclease H, DTT-Dithiothreitol, dNTPs-deoxyribonucleoside triphosphates,TCA-Trichloroacetic acid, Act.D-Actinomycin D.

had no effect on the RNase H activity as judged by the degradation of ^{32}P-labeled 70 S RNA template during the synthesis.

Pyrophosphate has been used as an additive to reverse transcriptase reactions as a means of obtaining the increased size and yield of the cDNA product [11-13]. The observed size increase of the product has recently been postulated to be due to the inhibition of RNase H activity associated with reverse transcriptase[14]. Since our results could not be explained on the basis of the above mentioned postulate, we undertook more rigorous examination of pyrophosphate influence on the expression of both polymerase and polymerase associated RNase H activity. In addition to using identical conditions to those described earlier,[12,13,14] we examined the effect of pyrophosphate with changing divalent metal ion, salt and substrate triphosphate concentrations as well as using buffer systems at varying pH values. The size of the cDNA product obtained with 70S RNA or globin mRNA catalyzed by AMV polymerase, both in the presence and absence of 4 mM PPi, was determined by alkaline sucrose density gradient centrifugation. Although a significant increase in the cDNA size synthesized on both the templates was observed in the presence of PPi, (data not shown), under none of the above conditions PPi had any inhibitory effect on the expression of the associated RNase H activity. Furthermore, the data presented in

Table 2: Pyrophosphate influence on polymerase-coupled RNase H activity.

Hybrid		Polymerase [^{3}H]-dGMP or dTMP incorporated (CPM)	Polymerase % Activity	RNase H [^{32}P] or [^{3}H] RNA solubilized (CPM)	RNase H % Activity
[^{32}P]RNA:cDNA[a]	-PPi	29118	100	976	100
	+PPi	7057	24.2	2228	228.3
[^{3}H]RNA:fd DNA[b]	-PPi	3710	100	2280	100
	+PPi	1700	45.8	2390	104.8

[a]For this experiment, [^{32}P]-labeled ASV 70S RNA was used as a template in a regular reaction mixture and after 60 min incubation at 37°C, [^{3}H]-dGTP was added together with or without 4 mM PPi. Incubation was continued for additional 30 min and incorporation of [^{3}H]dGMP and degradation of [^{32}]-RNA was determined as described under Materials and Methods.

Labeled RNA degradation in the absence of synthesis (minus dNTPs) amounts to < 5%.

[b]In this experiment, DNA synthesis was determined by measuring alkali resistant, TCA precipitable incorporation of [^{3}H]-dTMP. RNase H activity was measured in the presence of non-radioactive dNTPs.

Table 2 clearly indicate that inclusion of PPi into the reaction mixture containing either AMV 70S RNA: cDNA hybrid or fd DNA:RNA hybrid as the template-primers impairs the synthetic reaction but has no effect on the associated RNase H activity. In fact, PPi causes a stimulation of the RNase H activity assayed with 70S RNA:cDNA hybrid.

The data in Table 3 further demonstrate the inability of PPi to inhibit the RNase H activity associated with AMV polymerase assayed under two differ-

Table 3: Pyrophosphate influence on AMV and calf thymus RNase H activity.

RNase H from	RNase H activity with fd DNA:[^{3}H]-RNA hybrid: pH (7.4-7.8) ^{3}H-RNA solubilized -PPi	pH (7.4-7.8) ^{3}H-RNA solubilized +PPi	pH 8.3 ^{3}H-RNA solubilized -PPi	pH 8.3 ^{3}H-RNA solubilized +PPi
AMV	2499(100)	2281(91.3)	2672(100)	2758(103.2)
Calf thymus	3323(100)	3076(92.6)	3318(100)	3358(101.2)

Assays were done as described under Materials and Methods except that buffer of desired pH was employed in the reaction mixture. Figures in parentheses represent percent enzyme activity.

ent pH conditions. We also included an RNase H, isolated from calf thymus, which has no polymerase activity associated with it, and found that under identical conditions, calf thymus RNase H is also refractory to PPi action.

DISCUSSION

A detailed examination of pyrophosphate influence on the expression of polymerase associated RNase H activity seemed warranted since pyrophosphate mediated inhibition of the nucleolytic activity was implicated into a probable mechanism of the production of long cDNA products. While we have been able to confirm the phenomenon of the production of long cDNA copies of RNA templates synthesized in the presence of PPi, we have not been able to obtain any evidence to substantiate PPi inhibition of RNase H activity implicated to explain the phenomenon. Inhibition of nucleases, other than RNase H, either associated or contaminating with reverse transcriptase, are most likely the target of PPi action. Indeed, a recent report describing the association of endonucleolytic activities with AMV polymerase and their inhibition by PPi, strongly supports this notion[27].

In conclusion, we have clearly demonstrated that RNase H site is not the target of PPi action. Since RNase H activity appears to be expressed through the template binding site[8,9], PPi mediated polymerase inhibition may not be due to interference with the template binding function of reverse transcriptases. In fact, PPi may be used as a selective reagent to 'turn on' the RNase H function of reverse transcriptases by inhibiting the synthetic reaction.

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REFERENCES:

1. Mölling,K.,Bolognesi,D.P.,Bauer,W.,Busen,W.,Plassman,H.W. and Hausen,P.(1971) Nature (New Biol.),234, 240-243.
2. Leis,J.P.,Berkower,I. and Hurwitz,J.(1973) In DNA Synthesis in vitro (Wells,R.D. and Inman,R.B.,eds.), 287-308, Baltimore.
3. Brewer,L. and Wells,R.D.(1974) J.Virol.,14, 1494-1498.
4. Verma,I.M.,Mason,W.S.,Drost,S.D. and Baltimore,D(1974) Nature, 251, 27-31.
5. Wang,L.H. and Levinson,W.E.(1978) Bioinorganic Chem., 8, 535-540.
6. Modak,M.J. and Srivastava,A.(1979) J.Biol.Chem., 254, 4756-4759.
7. Modak,M.J.(1976) Biochem.Biophys.Res.Commun., 71, 180-187.
8. Modak,M.J. and Marcus,S.L. (1977) J.Virol., 22, 243-246.
9. Marcus,S.L.,Smith,S.W. and Modak,M.J. (1978) J.Virol., 27, 576-581.
0. Kornberg,A. (1974) In DNA Biosynthesis, W.H.Freeman and Co., San Francisco.

11. Kacian,D.L. and Myers,J.C. (1976) Proc.Natl.Acad.Sci.,U.S.A.,73, 2191-2195.
12. Kacian,D.L. and Myers,J.C. (1976) Proc.Natl.Acad.Sci.,U.S.A.,73, 3408-3412.
13. Myers,J.C.,Spiegelman,S. and Kacian,D.L. (1977) Proc.Natl.Acad.Sci.,U.S.A., 74, 2840-2843.
14. Myers,J.C. and Spiegelman,S. (1978) Proc.Natl.Acad.Sci.,U.S.A., 75, 5329-5333.
15. Marcus,S.L.,Modak,M.J. and Cavalieri,L.F. (1974) J.Virol., 14 , 853-859.
16. Modak,M.J. and Marcus,S.L. (1977) J.Biol.Chem., 252, 11-19.
17. Burgess,R.R.(1969) J.Biol.Chem., 244, 6160-6167.
18. Stavrianopolous,J.G.,Gambino-Giuffrida,A. and Chargaff,E. (1976) Proc.Natl. Acad.Sci.,U.S.A., 73, 1087-1091.
19. Modak,M.J. (1976) Anal.Biochem., 75, 340-344.
20. Imazumi,T.,Diggelman,H. and Schrrer,K. (1973) Proc.Natl.Acad.Sci.,U.S.A., 70, 1122-1126.
21. Efstratidadis,A.,Maniatis,T.,Kafatos,F.C.,Jeffrey,A. and Vournakis, J.N. (1975) Cell, 4, 367-378.
22. Monahan,J.J.,Harris,S.E.,Woo,S.L.,Robberson,D.L. and O'Malley,B,W. (1976) Biochem., 15, 223-233.
23. Rothenberg,E. and Baltimore,D. (1978) J.Virol., 17, 168-174.
24. Marcus,S.L. and Modak,M.J. (1976) Nucl.Acid Res., 3, 1473-1486.
25. Shekman,R.,Wickner,W.,Westergaard,O.,Brutlag,D.,Geider,K.,Bertsch,L.L. and Kornberg,A. (1972) Proc.Natl.Acad.Sci.,U.S.A., 69, 2691-2695.
26. Leis,J.P.,Berkower,I. and Hurwitz,J. (1973) Proc.Natl.Acad.Sci.,U.S.A., 70, 466-470.
27. Samuel,K.P.,Papas,T.S. and Chirikjian,J.G. (1979) Proc.Natl.Acad.Sci., U.S.A., 76, 2659-2663.