dramatic effect on the regulation of translational initiation (Vaughn and Hansen, 1973) plus an effect on translational elongation as well. The availability of multiple forms of the aminoacyl-tRNA synthetases could facilitate regulation of such cellular events. By changing forms, the particulate synthetases could rapidly adapt to changing regulatory roles while continuing to carry out their classical function of aminoacylation of tRNA. Thus, the different forms of the enzyme may have different enzymatic or regulatory capabilities.

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Discrimination of DNA Polymerase and RNase H Activities in Reverse Transcriptase of Avian Myeloblastosis Virus

Marian Gorecki* and Amos Panet

ABSTRACT: The active sites in reverse transcriptase of avian myeloblastosis virus have been selectively modified by various chemical reagents. The DNA polymerase activity is very sensitive to hydrophobic sulfhydryl reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-hydroxymercuribenzoate but resistant to sulfhydryl reagents with hydrophilic properties. The RNase H activity, on the other hand, is resistant to both hydrophobic and hydrophilic sulfhydryl reagents, indicating the absence of cysteinyl residues essential for RNase H activity. *N*-Ethylmaleimide (NEM), an amino and sulfhydryl group specific reagent, inactivates both DNA polymerase and RNase H, the later activity being fourfold more stable. Polynucleotides, but not nucleotide triphosphates, protect the two enzymatic activities of reverse transcriptase against NEM. Since pretreatment of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) does not prevent *N*-ethylmaleimide from reacting with a residue necessary for DNA polymerase activity, two different reactive groups are probably involved with this enzymatic activity. The pH profile of reverse transcriptase inhibition by *N*-ethylmaleimide also suggests the involvement of two reactive groups essential for the DNA polymerase activity with apparent pK_as of 5.5 and 6.5. Only one reactive group with a pK_a of 7.5 is found associated with the RNase H activity.

Reverse transcriptase (RNA-dependent DNA polymerase) of RNA tumor viruses exhibits three distinct measurable activities in vitro, namely, those of a DNA polymerase (Baltimore, 1970; Temin and Mizutani, 1970), of RNase H (Molling, et al., 1971), and of specific binding of tRNA, the primer for DNA synthesis (Panet et al., 1975). Reverse transcriptase of avian myeloblastosis virus (AMV) is a complex of two polypeptides with molecular weights 65 000 (α) and 105 000 (β) (Grandgenett et al., 1973), the α subunit being derived from the β subunit by proteolytic cleavage (Gibson and Verma, 1974). Both DNA polymerase and RNase H activities were shown to reside in the isolated α subunit (Grandgenett et al., 1973). However, the binding of the primer $tRNA^{Trp}$ could not be demonstrated with the purified α subunit (Grandgenett et al., 1976; Haseltine et al., 1977).

Although AMV reverse transcriptase has been extensively studied, only scanty information is available regarding the properties of its active sites. In addition, it is not clear to what extent these sites, which arise from a single gene product, overlap each other. In this study, we demonstrate that the

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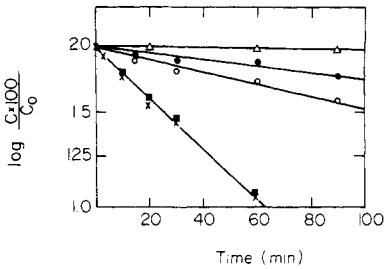

FIGURE 1: Effect of NEM on DNA polymerase activity and protection by polynucleotides. The reactions were conducted in two steps. (a) Reverse transcriptase (5 units) was incubated in an ice bath, in 150 μL of a mixture containing 50 mM Tris-HCl (pH 7.5) and 5 mM NEM. (b) At the indicated time intervals, 15-μL aliquots were withdrawn into tubes containing 5 μL of 160 mM DTT and left for 10 min in ice to destroy excess NEM. Residual DNA polymerase activity was then assayed, as described under Materials and Methods: (x-x) with NEM; (Δ-Δ) without NEM; (■-■) with NEM and dTTP (1 mM); (○-○) with NEM and poly(A)-oligo(dT) (120 μg/mL); (●-●) with NEM and chicken tRNA (1.3 mg/mL).

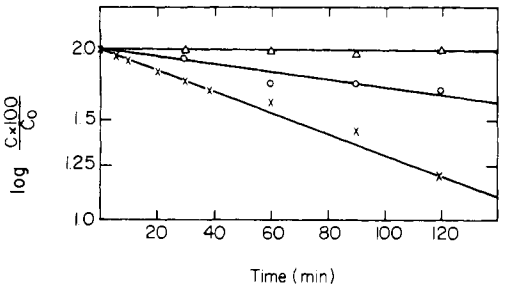

FIGURE 2: Effect of NEM on RNase H activity and protection by template primer. The reactions were conducted as described in Figure 1 with 10 units of enzyme (x-x) with NEM; (Δ-Δ) without NEM; (○-○) with NEM and poly(A)-oligo(dT) (120 μg/mL).

DNA polymerase and RNase H activities reside on independent active sites which may partially overlap but can be discriminated by suitable chemical modifications.

Materials and Methods

Poly(A) and oligo(dT) were gifts from Dr. Y. Lapidot of the Hebrew University, Jerusalem. Poly(dT) was purchased from Miles, dTTP was from P-L Biochemicals Inc., and [^{3}H]dTTP and [^{3}H]ADP were from The Radiochemical Centre, Amersham. Ethylenimine, 2-iodoacetic acid, and 2-iodoacetamide were from Fluka, DTNB and *p*-hydroxymercuribenzoate were from Sigma, and NEM was from Aldrich Chemical Co. NTCB (Degani and Patchornik, 1971) was a gift from Dr. Y. Degani of the Weizmann Institute, Rehovot. AMV was a gift from Dr. J. W. Beard.

[^{3}H]Poly(A) was synthesized in a reaction mixture (25 μL) containing 200 mM Tris-HCl[1] (pH 8.5), 20 mM $MgCl_2$, 1 μmol of [^{3}H]ADP (specific activity 100 cpm/pmol), and 1 unit of *E. coli* polynucleotide phosphorylase (a generous gift from Dr. H. Sorek of the Weizmann Institute, Rehovot). After 30 min at 30 °C, the mixture was adjusted to 0.3 M NaCl, and the product was extracted twice with phenol and precipitated with 2 volumes of ethanol. [^{3}H]Poly(A) was further purified by gel filtration on Sephadex G-100. The high-molecular-weight fractions from the void volume were collected and stored in liquid nitrogen.

Chicken liver tRNA was prepared according to Rogg et al. (1969). AMV reverse transcriptase was purified as described by Verma and Baltimore (1973); 20 mM potassium phosphate (pH 7.5) replaced Tris-HCl in all the buffers used during enzyme purification. The enzyme (α,β) from the phosphocellulose column was extensively dialyzed against 20 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.05 mM DTT, and 0.1% Triton X-100. Dialysis was terminated when the sulfhydryl content of the enzyme solution was less than 0.1 mM, as determined by the DTNB assay for SH groups (Ellmann, 1959). The enzyme was stored in liquid nitrogen.

DNA Polymerase Assay. Reaction mixtures (30 μL) contained 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 10 μg of bovine serum albumin, 10 μM [^{3}H]dTTP (specific activity 400 cpm/pmol), and 1 μg of poly(A)-oligo(dT). DTT and enzyme were added, as indicated in the figure legends. Under such conditions, DNA polymerase activity was linear for at least 40 min. After a 30-min incubation at 37 °C, 25-μL aliquots were spotted on filter papers (Whatman no. 1). The filters were washed three times with 5% trichloroacetic acid, followed by ethanol and ether, dried, and counted in toluene-based scintillation fluid. One unit of activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of dTMP per minute.

RNase H Assay. Reaction mixtures (50 μL) contained 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 10 μg of bovine serum albumin, 200 pmol of [^{3}H]poly(A) (specific activity 100 cpm/pmol), and 40 pmol of poly(dT). This mixture was heated at 80 °C for 4 min and cooled to room temperature, and DTT and enzyme were added as described in the figure legends. Incubation was carried out at 37 °C for 2 h. The reaction was terminated by the addition of 50 μL of 0.1 M sodium pyrophosphate containing 200 μg of bovine serum albumin and 7 μg of calf thymus DNA, followed by the addition of 5 μL of 100% trichloroacetic acid. After 10 min in ice, the sample was centrifuged for 3 min at 12 000*g*, and the supernatant was counted in dioxane-based scintillation fluid.

Results

Inhibition of DNA Polymerase and RNase H Activities by NEM. To examine the functional amino acid residues in the active sites of AMV reverse transcriptase, we used the sulfhydryl and amino group specific reagent NEM. Purified reverse transcriptase was preincubated with NEM, and the reaction was stopped at various times by the addition of DTT, destroying reagent excesses. Residual activity of DNA polymerase and RNase H was then assayed. Figures 1 and 2 demonstrate that both activities are inhibited at a linear rate when plotted as log (C/C_0) vs. time of preincubation (where C_0 is the initial activity and C is the residual activity). From the half-life determination, DNA polymerase activity appears 3.6-fold more sensitive to NEM than that of RNase H activity. Incubation of the enzyme in the presence of polynucleotides, such as poly(A)-oligo(dT) template primer or chicken liver tRNA, resulted in partial protection of both activities against NEM. Protection was dependent on the amount of tRNA added; thus, 1.3 mg/mL of total tRNA slowed down the polymerase inactivation by a factor of 10 (Figure 1), while tRNA at a concentration of 0.13 mg/mL only halved the inactivation rate (data not shown). On the other hand, nucleotide triphosphate, another substrate of the DNA polymerase reaction, did not affect the inactivation rate (Figure 1). It should be mentioned that the interaction of NEM with the enzyme exhibits very strong temperature dependence. Thus, the half-lives of polymerase inactivation were 31 and 5 min, at 0 and 25 °C, respectively. We have carried out the preincubations

[1] Abbreviations used are: AMV, avian myeloblastosis virus; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoate; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

TABLE I: Effect of Different Reagents on DNA Polymerase and RNAse H Activities.[a]

Reagent	Concn (mM)	Incubation time (min)	DNA polymerase residual act. (%)	RNase H residual act. (%)
None			100	100
NEM[b]	8	30	28	52
DTNB	1	2	9	96
p-Hydroxymercuribenzoate	1	2	3	87
NTCB	1	30	53	111
Iodoacetic acid[b]	10	60	95	97
Iodoacetamide[b]	10	60	102	94
Ethylenimine[b]	10	60	104	100

[a] Reverse transcriptase in 100 μL of 0.1 M Tris-HCl, pH 7.4, was incubated at 4 °C with the reagents depicted in the table. Residual DNA polymerase and RNase H activities assays were carried out as described under Materials and Methods (100% activity equals 5 enzyme units for DNA polymerase and 10 units for RNase H). [b] Excess of reagents was destroyed by the addition of DTT to a final concentration of 20 mM.

TABLE II: Sequential Inactivation of DNA Polymerase by DTNB and NEM and Reactivation by DTT.[a]

Addition			DNA polymerase residual act. (%)
DTNB	NEM	DTT	
−	−	−	100
+	−	−	7.4
−	−	+	100
+	−	+	87.8
+	+	−	2.7
+	+	+	40
−	+	+	40

[a] Reverse transcriptase in 0.1 M Tris-HCl, pH 7.4, was incubated with 1.4 mM DTNB for 10 min at 4 °C and NEM was added to a final concentration of 5 mM; after additional incubation for 30 min, DTT was added to a final concentration of 20 mM. Residual activity was then determined by the addition of DNA polymerase reaction mixture components, as described under Materials and Methods (100% activity equals 5 enzyme units).

at 0 °C, since at room temperature the dialyzed enzyme, not protected by thiols, rapidly loses activity in solution.

Effect of pH on NEM Reaction. The inactivation of the NEM reactive group was studied by reacting the enzyme with NEM at various pHs, followed by a DNA polymerase and RNase H activity assay. Figure 3a shows that NEM inactivates polymerase activity over a wide pH range. The bell-shaped curve of pH dependence of inactivation implicated two different reactive groups, one with a pK_a between 5 and 6 and another with a pK_a of about 6.5. Since the reactivity of NEM toward amino acid side chains in the low pH range has not been established, it is difficult to infer about the identity of the first reactive residue. The second one with a pK_a of 6.5 may be an amino or sulfhydryl group with an unusually low pK_a. In contradistinction, NEM inhibition of RNase H activity followed a different pattern (Figure 3b). At pH 5.5–6.0 the enzyme was unaffected, but at higher pH inactivation was rapid with a midpoint at pH 7.5. These data further visualize the different properties of the NEM reactive groups in the active sites of reverse transcriptase. It should be mentioned here that the preincubation of the enzyme at low pH in acetate buffer or at neutral pH in phosphate buffer somewhat destroyed enzyme activities. Therefore, residual activities were calculated

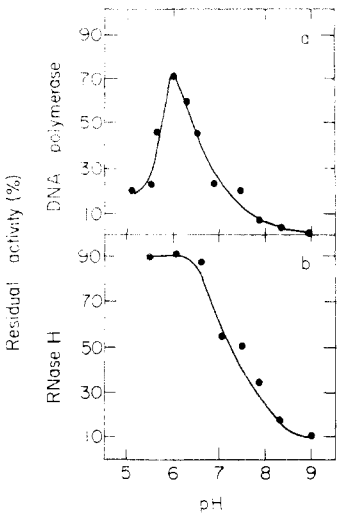

FIGURE 3: pH dependence of reverse transcriptase inactivation by NEM. Enzyme (5 units for DNA polymerase and 10 units for RNase H) was preincubated at 4 °C with NEM (1 mM for inhibition of DNA polymerase and 8 mM for RNase H) at various pHs, 20 mM sodium acetate for pHs 5 to 6, 20 mM potassium phosphate for pHs 6.0 to 6.5, and 20 mM Tris-HCl for pHs 7.0–9.0. Excess NEM was destroyed by DTT (40 mM) and residual activity was assayed as described under Materials and Methods. To prevent a possible effect of pH on the assay, Tris buffer concentration in the assay mixture was increased to 0.1 M and the pH in the assay was thus kept between 8.1 and 8.3.

on the basis of the activities measured after identical treatment but in the absence of NEM.

Differential Inhibition of DNA Polymerase and RNase H Activities by Various Sulfhydryl Reagents. Reverse transcriptase was preincubated with various reagents, followed by assays for both DNA polymerase and RNase H activities (Table I). DTNB, which reacts exclusively with -SH groups in proteins yielding the mixed disulfide product (Elmann, 1959), was found to be a potent inhibitor of DNA polymerase activity without affecting RNase H. An almost complete and instantaneous loss of DNA polymerase activity was observed even at low DTNB concentrations (0.2 mM). *p*-Hydroxymercuribenzoic acid, which binds to cysteine residues through the mercury (Boyer, 1954), is another example of a selective potent inhibitor of DNA polymerase activity. These two reagents introduce a bulky substituent into the polymerase active site, and steric hindrance might prevent them from similarly reacting within the RNase H site. To eliminate this possibility, we tested NTCB, a sulfhydryl reagent which introduced a small cyano group onto the cysteinyl residues (Degani and Patchornik, 1971). This latter reagent also inhibited polymerase activity without affecting that of RNase H. It should be mentioned, however, that the latter reagent affects the polymerase activity only after prolonged incubation, due to its low reactivity compared to DTNB. The common feature of these three reagents is their hydrophobic moiety which probably directs them to the active site of polymerization. This notion was further supported by the total lack of reactivity of hydrophilic sulfhydryl reagents such as iodoacetic acid, iodoacetamide, and ethylenimine toward AMV reverse transcriptase. They exert no effect on enzymatic activity even at high concentrations and after prolonged incubation (Table I).

Reactive Residues in the DNA Polymerase and RNase H Active Sites. Results of two experiments suggested that NEM and DTNB inhibited DNA polymerase activity by reacting with different residues in the active site. Polynucleotides protected the polymerase activity against NEM, but not against

DTNB, and the rates of inactivation caused by these two reagents differ markedly. This notion was further supported by carrying out the inhibition of reverse transcriptase using DTNB and NEM sequentially (Table II). We took advantage of the observation that DTT restored polymerase activity after enzyme interaction with DTNB, but not with NEM, and asked whether NEM could still interact with an enzyme which had already been treated with DTNB. The experiment was conducted in the following way: Enzyme treated with DTNB under conditions ensuring a total polymerase inactivation was subsequently reacted with NEM and the reaction pursued to a point where 40% of the original polymerase activity was inhibited (as determined in a control reaction where NEM was allowed to react with the enzyme in the absence of DTNB). The reactions were terminated by adding DTT, and residual DNA polymerase activity was assayed with poly(A)-oligo(dT) as template primer. When DTNB alone was used as inhibitor, DTT restored about 90% of the enzymatic activity. On the other hand, when the enzyme was first completely inactivated by DTNB, followed by the addition of NEM, DTT restored the level of the enzymatic activity to only 40% of the control. This result suggests that when the enzyme is inactivated with DTNB NEM can still interact irreversibly with an essential group, probably different from that which has been saturated with DTNB. An alternative possibility that NEM merely replaces the 2-nitro-5-thiobenzoyl moiety from the enzyme can be ruled out, since NEM is an electrophilic reagent, unable to react with a nucleophilic-mixed disulfide. For instance, incubation of NEM and DTNB at pH 7–8 does not lead to the release of 2-nitro-5-thiobenzoic acid (unpublished observation). The essential residue in the polymerase active site reactive toward NEM may be either another cysteinyl residue or, more likely, the reactive group with a pK_a = 7.5 mentioned previously (Figure 3).

Stability of DNA Polymerase and RNase H to High Hydrogen Concentration. The data presented so far dealt mainly with selective inactivation of the DNA polymerase. None of the inhibitors described preferentially affected the activity of RNase H. The latter, however, could be selectively inhibited by exposing the enzyme to low pH. In the experiment described in Figure 4, we have examined the effect of low pH (4.0–5.5) on the two enzyme activities. RNase H was completely inactivated by short preincubation of the enzyme at pH below 4.5. This activity is sensitive over a narrow pH range and at pH 5.0 only 50% of the activity was lost. On the other hand, DNA polymerase was not influenced by preincubations over a wider pH range (4.3–8.3) and sharp inactivation was observed only below pH 4.3. This sort of inactivation caused by preincubation at low pH is irreversible and probably depends upon conformational changes in the active sites of the enzyme molecule. The site responsible for DNA polymerase activity is more stable than that of RNase H. The stability to a high concentration of hydrogen is therefore different from the stability of these activities to thermal inactivation where RNase H activity is about fourfold more stable than DNA polymerase (Verma et al., 1974; Papas et al., 1974).

The inactivation caused by pH is not time dependent, and similar profiles were obtained whether enzyme was brought to the given pH and immediately adjusted to pH 8.3 for the assay or preincubated for either 1 or 2 h at low pHs before the assay.

Discussion

Several temperature-sensitive replication mutants of avian sarcoma virus have been shown to be defective in their reverse transcriptase (Verma et al., 1974). Both DNA polymerase and RNase H activities were affected by the mutations, implying that they were both constituents of the same gene product. In the present study, we have demonstrated by selective inactivation of DNA polymerase and RNase H that these activities of reverse transcriptase are manifested by separate sites, although there is some overlapping between them. The polymerase site includes an essential cysteinyl residue which reacts with hydrophobic sulfhydryl reagents such as DTNB and not with hydrophilic sulfhydryl reagents like iodoacetamide. Such differential reactivity suggests a hydrophobic milieu for this indispensable cysteine residue. Similar restricted reactivity of -SH groups in a hydrophobic environment which hinders the access of hydrophilic reagents has been observed in several proteins (Friedmann, 1973; Schechter et al., 1975).

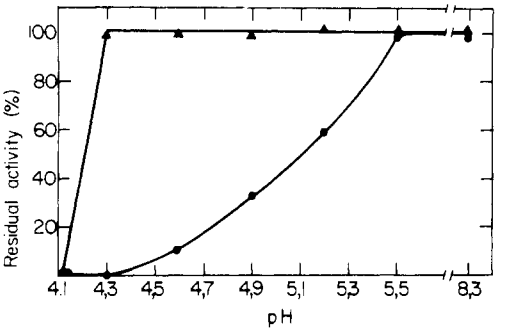

FIGURE 4: Effect of pH on reverse transcriptase activities: (■-■) RNase H; (▲-▲) DNA polymerase. Enzyme (5 units for DNA polymerase and 10 units for RNase H) was mixed at 4 °C with sodium acetate buffer to give a final concentration of 20 mM at a given pH and, subsequently, the mixture was assayed as described under Materials and Methods. Tris buffer concentration in the assay mixture was increased to 0.1 M to maintain the pH between 8.1 and 8.3.

The cysteine residue in the polymerase site also reacts with NTCB, a reagent capable of introducing a cyano substituent onto -SH groups (Degani and Patchornik, 1971). This compound is probably reactive by virture of its benzoate moiety which directs the small cyano group into a hydrophobic pocket in the enzyme. The finding that NTCB affects selectively polymerase activity suggests that inactivation by the sulfhydryl reagents tested was due to the substitution of an essential cysteinyl residue rather than to steric hindrance by bulky sulfhydryl reagents.

The polymerase site contains additional amino acid residues, reactive toward NEM but not DTNB. The second essential group might be a reactive amino moiety, since NEM is known to react with both $-NH_2$ and -SH groups in proteins. For example, the lysine in the active site of NADP-dependent glutamate dehydrogenase from *Neurospora crassa* is reactive toward NEM (Blumenthal and Smith, 1973). Another amino group specific reagent, pyridoxal 5′-phosphate, has been recently shown to inhibit the polymerase activity of AMV reverse transcriptase by forming a Schiff base with a free amino group in the enzyme (Modak, 1976a,b; Papas et al., 1977). However, NEM and pyridoxal 5′-phosphate seem to react with different groups, since dTTP which protects against pyridoxal 5′-phosphate has no effect on the NEM inhibition. Moreover, NEM inactivates both polymerase and RNase H activities, whereas pyridoxal 5′-phosphate has no effect upon the latter activity (Modak, 1976a).

We have further attempted to identify the residue reacting with NEM by studying the pH dependence of DNA polymerase and RNase H inactivation. The pK_a value of the reactive group (about 6.5 for DNA polymerase and 7.5 for RNase H) computed from these experiments could be attributed either to amino or sulfhydryl residues in the polymer-

ization site. Although these values are lower than the pK_a (8–10.5) usually found for similar groups in proteins (Friedmann, 1973), it is not unique, as the reaction of NEM with the essential lysine in NADP-dependent glutamate dehydrogenase shows also a low pK_a value of 7.5 (Blumenthal and Smith, 1973). The other vulnerable amino acid residue required for DNA polymerase activity, which reacted with NEM with pK_a of 5.5, could be histidine. However, the identification of the chemical nature of all NEM reactive groups requires further studies. The pH profile found for RNase H inactivation by NEM is probably not due to interaction with a cysteine residue, as none of the tested specific sulfhydryl reagents affected this activity. However, the amount of enzyme available today does not permit direct chemical analysis of these reactive groups. Since the essential -SH group in the polymerase site has no apparent function in the RNase H activity, it may be concluded that the two activities have independent sites. The NEM reactive group, possibly a lysine residue, might be common to both active sites, since the pK_a is similar for both activities and polynucleotides protect both of them against NEM. The deoxynucleotide triphosphate binding site, deduced from pyridoxal 5′-phosphate inactivation (Papas et al., 1977; Modak, 1976b), probably comprises only part of the polymerization site and does not participate in the RNase H site. The concept of independent active sites for DNA polymerase and RNase H was further supported by our experiments in which differential inhibition of the RNase H activity was demonstrated. Thus, incubation of reverse transcriptase at low pH selectivity inhibits the RNase H activity. Brewer and Wells (1974) have shown that salts such as NaF or KCl, in the reaction mixture, also preferentially inhibit the RNase H activity, but it is not clear from these studies whether the effect is reversible or not. We have found that short incubation of the enzyme at pH 4.3–4.5 inactivates in a nonreversible fashion RNase H activity, without affecting the DNA polymerase. This behavior may turn out to be useful for two purposes: the synthesis of cDNA without concomitant degradation of the RNA template and in vitro studies concerning the role of RNase H in reverse transcription of the RNA tumor virus genome.

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