Functional Characterization of RNA-Dependent DNA Polymerase and RNase H Activities of a Recombinant HIV Reverse Transcriptase†

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ABSTRACT: The DNA polymerase and RNase H activities of HIV reverse transcriptase are both essential for HIV replication. Although the two activities are both catalyzed by a single polypeptide, they are physically separate; i.e., the DNA polymerase resides in the N-terminal domain whereas the RNase H is localized in the C-terminal domain. The present study was undertaken to characterize the enzymatic properties of these two activities and to determine whether the two catalytic sites are also functionally distinct. We have observed that EGTA specifically stimulates, whereas $CaCl_2$ selectively inhibits, the RNA-dependent DNA polymerase activity but that neither compound has any effect on the RNase H activity of a recombinant HIV reverse transcriptase. The stimulation of the DNA polymerase activity by EGTA is dependent on the Mg^{2+} concentration; the greatest stimulation is observed at low Mg^{2+} concentrations. Similarly, the inhibition of DNA polymerase activity by Ca^{2+} is influenced by Mg^{2+} concentration. Ca^{2+} inhibition can be reversed by increasing Mg^{2+} concentrations, suggesting the possibility that $CaCl_2$ inhibits the reverse transcriptase activity by competing for a metal-binding site on the enzyme. The pyrophosphate analogue phosphonoformate selectively inhibits the polymerase activity but not the RNase H activity of HIV reverse transcriptase. In constrast, the RNase H activity can be selectively inhibited by deoxyadenosine 5′-monophosphate, whereas the DNA polymerase activity is not inhibited. These results suggest that the DNA polymerase and RNase activities are not only physically separate but that they are also functionally distinct.

Recently great interest has been focused on the reverse transcriptase of human immunodeficiency virus (HIV),[1] the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984). This enzyme is essential for viral replication and thus constitutes an ideal target for antiviral therapy (Mitsuya & Broder, 1987). In fact, inhibitors of this enzyme such as the triphosphate derivatives of azidothymidine (AZT) and dideoxynucleosides have been found to be effective in the treatment of AIDS.

HIV reverse transcriptase is comprised of two polypeptides of 66 and 51 kDa that have identical N-terminal amino acid sequences, suggesting that p51 is derived from p66 by proteolytic processing (diMarzo Varonese et al., 1986; Lightfoote et al., 1986). The removal of 15 kDa from the C-terminus of p66 results in the loss of RNase H activity as well as in a marked reduction in the polymerase activity (Hansen et al., 1988; Hizi et al., 1988). Recent studies have shown that p66 consists of two distinct domains, an N-terminal polymerase domain and a C-terminal RNase H domain (Johnson et al., 1986; Hansen et al., 1988; Tisdale et al., 1988; Schatz et al., 1989). Although recent in vitro studies have demonstrated that both the polymerase and the RNAase H activities proceed simultaneously (Schatz et al., 1990), it is not known how the DNA polymerase and RNase H activities are coordinated during viral replication. However, since they represent potential targets for antiviral agents, it is obvious that further characterization of the functional properties of both activities is essential, not only for structure–function studies but also for rational design of specific inhibitors of HIV reverse transcriptase.

The present studies have been undertaken to further characterize the enzymatic properties of the RNA-dependent DNA polymerase and RNase H activities of a recombinant HIV reverse transcriptase that has been expressed in *Escherichia coli* (Deibel et al., 1990).

Experimental Procedures

Ribo- and deoxyribonucleoside monophosphates and phosphonoformate (PFA) were obtained from Sigma Chemical Co. Poly(dT) and $(dT)_{12-18}$ were from Midland Certified Reagents. Poly(C)–agarose, dTTP, ddTTP, and poly(A) were purchased from Pharmacia LKB Biotechnologies. [^{3}H]Poly(A), 300 cpm/pmol AMP, was from Amersham. [^{3}H]dTTP was from NEN. AZTTP was a gift of Dr. Abdul Moshin Mian of the Department of Oncology, University of Miami. Monoclonal antibody M3364 to HIV reverse transcriptase was a gift of Dr. Lionel Resnick of Mount Sinai Medical Center, Miami.

Reverse Transcriptase. Recombinant HIV reverse transcriptase was cloned and expressed as previously described (Deibel et al., 1990). The enzyme was purified essentially as described by Roth et al. (1985). The purified enzyme had a specific activity of approximately 100000 units/mg. One unit is defined as 1 nmol of dNMP incorporated per hour under standard assay conditions. Protein was determined according to Bradford (1976) with BSA as standard.

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[1] Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; PFA, phosphonoformic acid; AZT and AZTTP, 3′-azido-2′,3′-dideoxythymidine and its 5′-triphosphate; ddTTP, dideoxythymidine triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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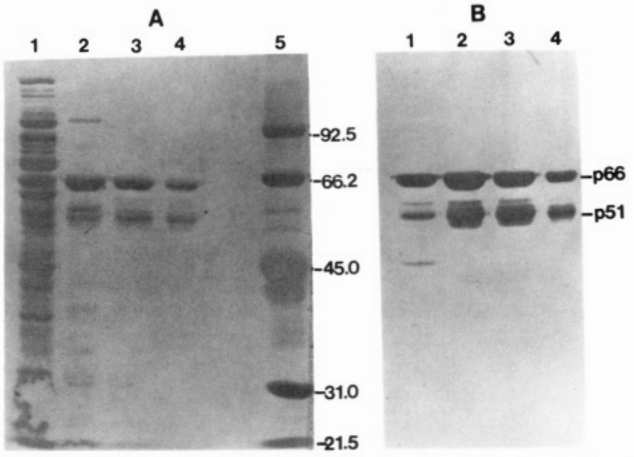

FIGURE 1: SDS–PAGE of fractions from a preparation of recombinant HIV reverse transcriptase. (A) Amido black stained blot. (B) Western blot. Lane 1, crude extract; lane 2; DEAE-cellulose; lane 3, phosphocellulose; lane 4, poly(C)–agarose; lane 5, low molecular weight markers.

SDA–PAGE and Immunoblotting. SDS–PAGE was performed according to Laemmli (1970) with a 4% stacking and a 10% resolving gel. After electrophoresis, resolved proteins were electrotransferred to a 0.45 μm nitrocellulose membrane (Towbin et al., 1979). The transferred proteins were detected by amido black staining, and HIV reverse transcriptase peptides were detected by immunoblotting with monoclonal antibody M3364 specific for HIV reverse transcriptase p66/51 (diMarzo Veronese et al., 1985, 1986). The immunoblot was developed by using a Vectorstain kit and diaminobenzidine as the chromophore as specified by the manufacturer (Vector Labs., Inc.).

Assay for RNA-Dependent DNA Polymerase Activity. The reaction mixture in a final volume of 0.1 mL contained 0.033 A_{260} unit of poly(A)/oligo(dT) (4:1); 40 mM Tris-HCl, pH 8.3; 2 mM $MgCl_2$; 150 mM KCl; 1 mM DTT; 0.025% NP-40; 0.1–0.2 unit of HIV reverse transcriptase; 2.5% glycerol; 0.2 mM EGTA; 8 μg of BSA; and 20 μM [^{3}H]TTP (2000 cpm/pmol). After incubation for 15 min at 37 °C, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976).

Assay for RNase H Activity. RNase H assays were carried out in a final volume of 0.05 mL and contained 40 mM Tris-HCl, pH 8.3; 16 mM $MgCl_2$; 58 000 cpm [^{3}H]poly(A)/poly(dT) (1:1); 4 μg of BSA; 3% glycerol; 0.02% NP-40; and 13 units of HIV reverse transcriptase. The reaction mixture was incubated at 37 °C, and 30-μL aliquots were applied to 2.4-cm circles of Whatman DE81 paper after 20 min of incubation. The circles were washed, dried, and counted as previously described (Que et al., 1978).

RESULTS

Recombinant HIV Reverse Transcriptase Is Functionally and Immunologically Identical with the Viral Enzyme. Purification of recombinant HIV reverse transcriptase expressed in *E. coli* resulted in an active enzyme that was predicted to differ from the virally encoded protein by only the addition of an N-terminal methionine (Deiber et al., 1990). Like the reverse transcriptase isolated from virions, the recombinant enzyme is a heterodimer of 66 and 51 kDa. Figure 1 shows the polypeptide structure of the purified enzyme and its reactivity with a monoclonal antibody to viral HIV reverse transcriptase.

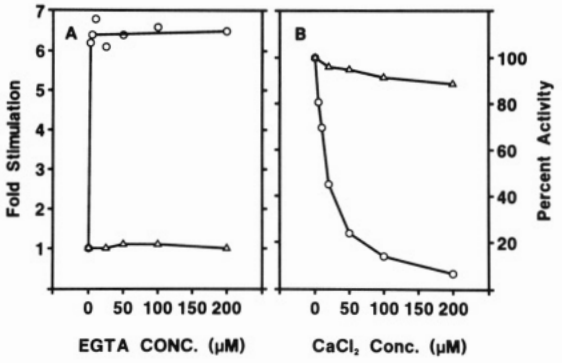

FIGURE 2: Effects of EGTA and $CaCl_2$ on DNA polymerase and RNase H activities of HIV reverse transcriptase. DNA polymerase (○) and RNase H (△) were assayed separately as described under Experimental Procedures. (A) The EGTA concentration was varied from 0 to 200 μM. 100% activity represents 8.2 pmol of [^{3}H]dTMP incorporated per 15 min of incubation for DNA polymerase activity and 48 pmol of [^{3}H]AMP hydrolyzed per 15 min by the RNase H activity. (B) The $CaCl_2$ concentration was varied from 0 to 200 μM. 100% activity represents 12.5 pmol of [^{3}H]TMP incorporated per 15 min for DNA polymerase activity and 52 pmol of [^{3}H]AMP hydrolyzed per 15 min by the RNase H activity.

The recombinant enzyme was found to have a pH optimum and divalent cation and monovalent cation optima identical with those reported for the viral enzyme and to have similar sensitivity to inhibitors such as phosphonoformate (PFA), dideoxy-TTP (ddTTP), and azido-TTP (AZTTP) (data not shown), suggesting that the recombinant enzyme is essentially identical with that isolated from virions (Hoffman et al., 1985).

Effects of EGTA and $CaCl_2$ on RNase H and RNA-Dependent DNA Polymerase Activities. Others have reported that EGTA stimulates HIV reverse transcriptase (Hoffman et al., 1985), and we have found that the recombinant enzyme is also markedly stimulated by EGTA. The effects of increasing concentrations of EGTA on both the RNase H and DNA polymerase activities of the recombinant enzyme are shown in Figure 2A. The rate of DNA synthesis is stimulated approximately 6-fold at 2.5 μM EGTA, and higher concentrations did not further increase the reverse transcriptase activity. However, EGTA has no effect on the RNase H activity up to a concentration of 200 μM.

Since EGTA is a Ca^{2+} chelator, we have investigated whether the stimulatory effect of EGTA on DNA polymerase activity is due to the removal of contaminating Ca^{2+} that is present in one or more assay reagents or in the purified enzyme and is inhibitory to the reverse transcriptase. The effects of $CaCl_2$ on both the reverse transcriptase and RNase H activities are shown in Figure 2B. The RNA-dependent DNA polymerase activity is very sensitive to inhibition by $CaCl_2$, being inhibited about 55% at 20 μM and 90% at 100 μM $CaCl_2$, whereas the RNase H activity is unaffected by the addition of $CaCl_2$.

Mechanism of Inhibition of RNA-Dependent DNA Polymerase Activity by $CaCl_2$. All DNA polymerases, including reverse transcriptase, absolutely require a divalent cation such as Mg^{2+} or Mn^{2+} for catalytic activity, and specific binding sites for these metal ions have been demonstrated in DNA polymerases (Derbyshire et al., 1988). Thus it is possible that the inhibition of reverse transcriptase activity by $CaCl_2$ is due to displacement of Mg^{2+} from a polymerase metal-binding site. The effects of $CaCl_2$ on the reverse transcriptase activity at different $MgCl_2$ concentrations are shown in Figure 3A. The enzyme is significantly more sensitive to inhibition by $CaCl_2$ at low (1 mM) than at high (10–20 mM) $MgCl_2$ concentrations. $CaCl_2$ (50 μM) inhibited reverse transcriptase activity

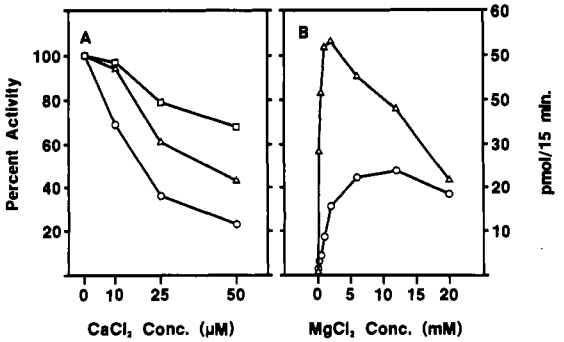

FIGURE 3: Effect of $MgCl_2$ concentration on the inhibition of RNA-dependent DNA synthesis by $CaCl_2$ and on its stimulation by EGTA. Assays for reverse transcriptase were carried out as described under Experimental Procedures. (A) The concentration of $CaCl_2$ was varied as indicated in the absence of EGTA and the presence of either 1 mM (○), 10 mM (△) or 20 mM (□) $MgCl_2$. (B) The $MgCl_2$ concentration was varied as indicated in the absence (○) or presence (△) of 0.2 mM EGTA.

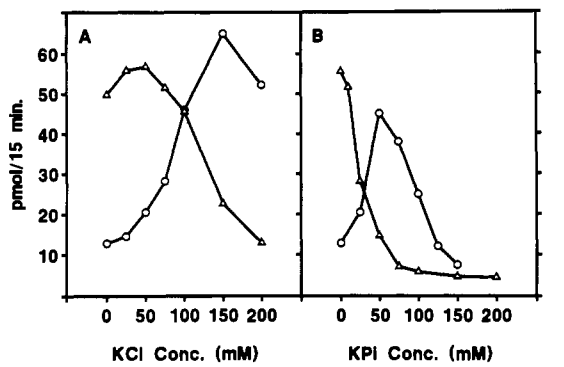

FIGURE 4: Effects of KCl and KP_i on RNA-dependent DNA polymerase and RNase H activities. The assay conditions were as described under Experimental Procedures. (A) The KCl concentration was varied as indicated. (B) The KP_i concentration was varied as indicated. DNA polymerase (○), RNase H (△).

77% at 1 mM, 57% at 10 mM, and 32% at 20 mM $MgCl_2$. This suggests that Ca^{2+} inhibits reverse transcriptase by competing with Mg^{2+} for a metal-binding site on the enzyme.

Also consistent with this mechanism of inhibition is the observation that the stimulation of reverse transcriptase activity by EGTA is dependent on the Mg^{2+} concentration. As shown in Figure 3B, EGTA stimulates reverse transcriptase activity approximately 7-fold at 0.5 mM $MgCl_2$ but only 1.6-fold at 8 mM $MgCl_2$. Furthermore, the optimal Mg^{2+} concentration is greatly influenced by the addition of EGTA. In the absence of EGTA, the concentration of Mg^{2+} required for maximal activity is broad and ranges from 8 to 12 mM, whereas in the presence of 0.5 mM EGTA the optimal magnesium concentration is 1–2 mM.

Effects of KP_i and KCl on Reverse Transcriptase and RNase H Activities. The effects of increasing concentrations of KCl on both the reverse transcriptase and RNase H activities are shown in Figure 4A. The rate of DNA synthesis is markedly stimulated by KCl, and at the optimal concentration of 150 mM it is stimulated about 5-fold. In contrast, RNase H activity is only slightly stimulated at low KCl concentrations, and concentrations greater than 100 mM are inhibitory. The effect of KCl on the reverse transcriptase activity is identical with that reported for authentic virion enzyme (Hoffman et al., 1985).

Figure 4B shows the effects of KP_i on the reverse transcriptase and RNase H activities. The effects of KP_i on reverse transcriptase activity are similar to those of KCl, and the optimal KP_i concentration is 50 mM. At the pH of the reaction mixture (8.3), the ionic strength of KP_i is 3 times that of KCl; thus maximal reverse transcriptase activity occurs at the same ionic strength whether chloride or phosphate is the anion. In contrast to the reverse transcriptase activity, the ribonuclease H activity is extremely sensitive to inhibition by KP_i, with 50% inhibition occurring at 25 mM and 90% inhibition at 80 mM KP_i.

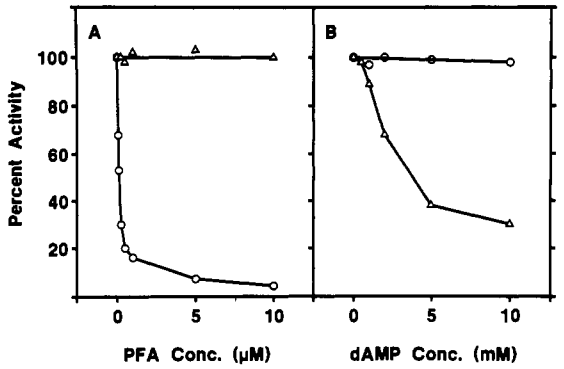

FIGURE 5: Effects of PFA and dAMP on RNase H and RNA-dependent DNA polymerase activities of HIV reverse transcriptase. DNA polymerase activity (○) and RNase H activity (△) were assayed separately as described under Experimental Procedures except for the addition of (A) PFA or (B) dAMP, as indicated, and 4 mM $MnCl_2$ was substituted for 16 mM $MgCl_2$ in the RNase H assay in (B).

Both RNA-Dependent DNA Polymerase and RNase H Activities Can Be Selectively Inhibited. It has been shown that PFA, a pyrophosphate analogue, is a potent inhibitor of HIV reverse transcriptase. Kinetically it acts as a competitive inhibitor of pyrophosphorolyis with PP_i as variable substrate, a noncompetitive inhibitor of polymerization with dNTP as variable substrate, and an uncompetitive inhibitor of polymerization with template/primer as variable substrate (Vrang & Oberg, 1986). Thus, it was of interest to determine whether PFA has any effect on the RNase H activity of HIV reverse transcriptase as an approach to determining the functional relationship between the DNA polymerase and RNase H activities. The effects of increasing concentrations of PFA on both the DNA polymerase and RNAse H activities of cloned HIV reverse transcriptase are shown in Figure 5A. Increasing concentrations of PFA resulted in an increasing inhibition of poly(dT) synthesis. The RNA-dependent DNA polymerase activity is inhibited approximately 50% by 0.2 μM and 95% by 5 μM PFA. However, PFA has no effect on the RNase H activity up to a concentration of 10 μM. These results suggest that the active sites for the RNA-dependent DNA polymerase and RNase H activities are functionally distinct.

We have previously demonstrated that nucleoside 5′-monophosphates, which are products of the proofreading 3′ to 5′ exonuclease activity of DNA polymerase I of *E. coli*, are selective inhibitors of the exonuclease activity but have no effect on the DNA polymerase activity (Que et al., 1978). Recent studies by Starnes and Cheng (1989) have shown that the products of the RNase H activity of virion HIV reverse transcriptase are also nucleoside 5′-monophosphates. Thus, we looked for product inhibition of RNase H activity by nucleoside 5′-monophosphates. The effects of several nucleotides were determined. We found that, whereas AMP did not inhibit RNase H activity at concentrations up to 10 mM (data not shown), dAMP did inhibit the RNase H activity of reverse transcriptase, although the IC_{50} was found to be relatively high (3 mM). The effects of dAMP on both the polymerase and RNase H activities of HIV reverse transcriptase are shown in Figure 5B. It can be seen that dAMP is a selective inhibitor

of the RNase H activity but has no effect on the RNA-dependent DNA polymerase activity of the reverse transcriptase. These findings further suggest that the active sites for the polymerase and RNase H activities are functionally distinct.

Discussion

Recently there have been several reports of successful expression of cloned HIV reverse transcriptase in *E. coli* in large quantities and in soluble form (Larder et al., 1987; Hizi et al., 1988). More recently Deibel et al. (1990) have also succeeded in the cloning and expression of HIV reverse transcriptase in *E. coli* and demonstrated that the sensitivities of the cloned enzyme to various inhibitors are identical with those reported for authentic virion HIV reverse transcriptase. We have shown that the functional properties of the reverse transcriptase are also similar to those of the viral enzyme; e.g., it is markedly stimulated by EGTA, exhibits a magnesium optimum of 1–2 mM in the presence of EGTA, is stimulated by KCl with an optimum of 150 mM, thus has a pH optimum of 8.3. In addition, the enzyme prefers Mg^{2+} over Mn^{2+} with poly(A)/oligo(dT) as template/primer; i.e., the rate of DNA synthesis with Mn^{2+} is only one-tenth of that with Mg^{2+} (unpublished observation). We have used this recombinant enzyme to further characterize the RNA-dependent DNA polymerase and RNase H activities, with the ultimate goal of understanding how these two activities are coordinated during viral DNA replication. The present studies on the distinctive enzymatic properties of the DNA polymerase and ribonuclease H activities should lead to a better understanding of the mechanism of HIV retroviral replication and may provide necessary information for the rational design of specific inhibitors of both the reverse transcriptase and RNase H catalytic activities.

The present studies demonstrate that $CaCl_2$ specifically inhibits the reverse transcriptase activity but not the RNase H activity of HIV reverse transcriptase and that the inhibition can be reversed by increasing $MgCl_2$ concentrations, suggesting the possibility that Ca^{2+} inhibits reverse transcriptase by competing with Mg^{2+} for a metal-binding site on the enzyme. These studies further demonstrate that EGTA stimulates reverse transcriptase activity, particularly at low Mg^{2+} concentrations, but has no effect on RNase H activity. Furthermore, the Mg^{2+} optimum for reverse transcriptase is influenced by whether or not EGTA is present in the reaction mixture. The stimulation by EGTA presumably is due to the removal of contaminating Ca^{2+} present in either the reagents or the enzyme preparation.

We have also considered that possibility that the inhibition of reverse transcriptase activity by $CaCl_2$ could be due to the activation of a calcium-dependent protease resulting in the degradation of the protein and the loss of DNA polymerase activity. The following observations argue against this possibility: (1) inhibition of reverse transcriptase by $CaCl_2$ is specific for the polymerase activity whereas one would expect both the RNase H and DNA polymerase activities to be affected if the effects of Ca^{2+} were mediated by proteolysis; (2) preincubation of the enzyme in the absence of EGTA did not affect the stimulation of reverse transcriptase activity by the subsequent addition of EGTA; and (3) protease inhibitors such as leupeptin had only minimal effects on the polymerase activity; i.e., only a 50%–100% stimulation was observed as compared to a 7-fold stimulation by EGTA.

The present studies also demonstrate that the RNA-dependent DNA polymerase activity of the recombinant HIV reverse transcriptase is very sensitive to inhibition by PFA; 50% inhibition was observed at 0.2 μM. The IC_{50} is very similar to the values of 0.5 and 0.1 μM reported by Vrang and Oberg (1986) and Sandstrom et al. (1985), respectively, though somewhat lower than the value of 2.0 μM reported by Sarin et al. (1985).

Previous studies demonstrated that the N-terminal half of HIV reverse transcriptase has significant homology with bacterial and viral DNA polymerases, whereas the C-terminus of the enzyme is homologous to bacterial RNase H (Johnson et al., 1986), suggesting that the active sites for the reverse transcriptase activity and RNase H activity are physically distinct. The present findings that the reverse transcriptase activity can be selectively inhibited by PFA and Ca^{2+} and the RNase H activity by dAMP suggest that these two catalytic sites are also functionally distinct.

The mechanism by which RNase H is inhibited by dAMP is not known. However, the observation that the RNase H activity can be inhibited by deoxynucleoside 5′-monophosphates provides a structural basis for designing specific inhibitors of this enzymatic activity. RNase H has been shown to be essential for viral replication and thus is potentially an important target for anti-HIV therapy. Furthermore, the demonstration that the reverse transcriptase activity and the RNase H activity are functionally distinct and that each can be selectively inhibited by specific inhibitors suggests the possibility of developing combination antiviral therapy.

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Characterization of DNA Metabolizing Enzymes in Situ following Polyacrylamide Gel Electrophoresis†

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ABSTRACT: We have detected the in situ activities of DNA glycosylase, endonuclease, exonuclease, DNA polymerase, and DNA ligase using a novel polyacrylamide activity gel electrophoresis procedure. DNA metabolizing enzymes were resolved through either native or SDS–polyacrylamide gels containing defined ^{32}P-labeled oligonucleotides annealed to M13 DNA. After electrophoresis, these enzymes catalyzed in situ reactions and their [^{32}P]DNA products were resolved from the gel by a second dimension of electrophoresis through a denaturing DNA sequencing gel. Detection of modified (degraded or elongated) oligonucleotide chains was used to locate various enzyme activities. The catalytic and physical properties of Novikoff hepatoma DNA polymerase β were found to be similar under both in vitro and in situ conditions. With 3′-terminally matched and mismatched [^{32}P]DNA substrates in the same activity gel, DNA polymerase and/or 3′ to 5′ exonuclease activities of *Escherichia coli* DNA polymerase I (large fragment), DNA polymerase III (holoenzyme), and exonuclease III were detected and characterized. In addition, use of matched and mismatched DNA primers permitted the uncoupling of mismatch excision and chain extension steps. Activities first detected in nondenaturing activity gels as either multifunctional or multimeric enzymes were also identified in denaturing activity gels, and assignment of activities to specific polypeptides suggested subunit composition. Furthermore, DNA substrates cast within polyacrylamide gels were successfully modified by the exogenous enzymes polynucleotide kinase and alkaline phosphatase before and after in situ detection of *E. coli* DNA ligase activity, respectively. Several restriction endonucleases and the tripeptide (Lys-Trp-Lys), which acts as an apurinic/apyrimidinic endonuclease, were able to diffuse into gels and modify DNA. This ability to create intermediate substrates within activity gels could prove extremely useful in delineating the steps of DNA replication and repair pathways.

Many DNA replication, repair, and restriction processes involve enzyme complexes composed of multifunctional and multimeric subunits. For exmaple, *Escherichia coli* DNA polymerase I is a monomeric protein (M_r = 103 000) that contains three distinct enzyme activities: polymerase, 3′ to 5′ exonuclease, and 5′ to 3′ exonuclease (Jovin et al., 1969). In contrast, *E. coli* DNA polymerase III holoenzyme contains polymerase and 3′ to 5′ exonuclease activities as separate subunits, designated α and ϵ with molecular weights of 140 000 and 27 000, respectively (Maki & Kornberg, 1985; Scheuermann & Echols, 1984). Recently, all five classes of eukaryotic DNA polymerases, α (Cotterill et al., 1987), β (Mosbaugh & Meyer, 1980; Mosbaugh & Linn, 1983), γ (Kunkel & Soni, 1988; Kunkel & Mosbaugh, 1989; Kaguni & Olson, 1989; Insdorf & Bogenhagen, 1989), δ (Byrnes et al., 1976; Crute et al., 1986), and ϵ (Burgers et al., 1989, 1990), have been reported to contain polymerase and 3′ to 5′ exonuclease activities; however, their catalytic subunit compositions have not been determined in all cases. Many DNA metabolizing enzymes function in multistep biochemical pathways that are coordinated by interactive catalytic activities. In some cases multiple enzyme activities are exhibited by a single polypeptide whereas in others a multimeric enzyme functions as a complex (Weiss, 1981; Sancar & Sancar, 1988; Modrich, 1989).

Assignment of catalytic activities to specific polypeptides or complexes remains an important objective for elucidating the mechanisms of DNA replication and repair. Resolution and detection of enzyme activities in situ following polyacrylamide gel electrophoresis are powerful tools in this regard (Spanos et al., 1981; Mezzina et al., 1984, 1987). Methods described within this paper and elsewhere (Blank et al., 1982,

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