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# REMOVAL OF ANTI-HUMAN IMMUNODEFICIENCY VIRUS 2′,3′-DIDEOXYNUCLEOSIDE MONOPHOSPHATES FROM DNA BY A NOVEL HUMAN CYTOSOLIC 3′ → 5′ EXONUCLEASE

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**Abstract**—A 3′ → 5′ exonuclease has been highly purified from the cytosol of human acute lymphoblastic leukemia H9 cells. The apparent molecular weight of this enzyme was approximately 50,000, as indicated by its sedimentation in glycerol gradients. The exonuclease did not copurify with DNA polymerase activity, required $MgCl_2$ for its exonucleolytic activity, and was inhibited by KCl above 60 mM. The enzyme was active on single-stranded DNA, DNA duplexes and DNA/RNA duplexes, and it was efficient at removing 3′-terminal mispairs from DNA. The products of the exonucleolytic reaction were deoxynucleoside 5′-monophosphates. The behavior of the exonuclease was examined on DNA terminated at the 3′ end with a variety of dideoxynucleosides that are potent against human immunodeficiency virus type 1. The exonuclease has a broad substrate specificity; however, the rate of the enzymatic reaction varied among the D dideoxynucleosides tested (ddAMP = ddCMP > d4TMP > AZTMP). Similarly, the enzyme was examined for its reactivity with DNA terminated by either the D or L enantiomers of ddC, SddC or FddC. The removal of analogs with the native D configuration was at least 6-fold more rapid than that of the L-compounds, and the type of structural modification had an impact on the rate at which the D enantiomers were removed (SddCMP > ddCMP > FddCMP). The monophosphate forms of AZT, D4T, L-FddC and L-ddC were potent inhibitors of the exonuclease at micromolar concentrations, while D-ddCMP partially inhibited the enzyme at millimolar concentrations. Based on its physical and enzymatic properties, this exonuclease represents a novel enzyme that may have an important role in determining the relative potencies of dideoxynucleosides against human immunodeficiency virus type 1.

*Key words:* 3′ → 5′ exonuclease; 2′,3′-dideoxynucleosides, anti-HIV-1

The efficacy of ddN† as therapeutic agents for HIV-1 has been demonstrated clearly [1–4]. The extent to which ddN can be introduced at the 3′-terminals of the viral DNA where they act as chain terminators is a critical factor in their ability to inhibit the replication of HIV-1. Since HIV-1 RT does not appear to have an associated 3′ → 5′ exonuclease activity, the event of chain termination would be dependent both upon the efficiency of incorporation by HIV-1 RT and by the intracellular level of the triphosphate metabolite of a given analog. However, in a recent study [5] with the triphosphate metabolites of the D and L enantiomers of SddC, we observed that although D-SddCTP has a lower $K_i$ for HIV-1 RT than L-SddCTP (3TCTP), more of its intracellular metabolite is required to achieve the same potency as L-SddCTP against HIV-1 in H9 cells. During this investigation, a 3′ → 5′ exonuclease that removed D-SddCMP from 3′-terminals of DNA six times faster than L-SddCMP was identified in the cytosol of H9 cells. In addition to its localization in the cellular cytoplasm where HIV-1 replication is initiated, the exonuclease was found to be reactive on DNA annealed to an RNA template, a substrate that is similar to the HIV-1 genome during its early stage of replication in the infected cell. On the basis of these findings, we postulated that the availability of a cellular exonuclease at the site of viral replication could be a key contributing factor to account for the increased antiviral action of L-SddC as compared with the D enantiomer [5]. These observations raised the possibility that the exonuclease may have a similar impact on other anti-HIV-1 ddN and thus may be involved in the reversibility of the antiviral action noted with this class of compounds. A broad substrate specificity would indicate that the exonuclease may decrease the chain termination potential and constitute a host-mediated determinant of the anti-HIV-1 potency of multiple ddN. It was also of interest to determine whether the exonuclease could be inhibited by the monophosphate form of ddN, which is a key metabolite derived from intracellular phosphorylation of a number of these anti-HIV-1 compounds [6, 7]. The relative susceptibility of the exonuclease to the monophosphate metabolites would also be expected to have an impact on the extent of chain termination and on chemotherapy using combinations of ddN.

Accordingly, the present study was undertaken to characterize the exonuclease with emphasis on its substrate specificity. A highly purified form of this enzyme was examined for its general properties and for its interaction with DNA terminated by clinically available and experimental anti-HIV-1 ddN. The susceptibility of the exonuclease to monophosphate metabolites of AZT, D4T, and L-FddC, as well as to the D and L enantiomers of ddC, was also assessed.

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† Abbreviations: ddN, 2′,3′-dideoxynucleosides; HIV-1, human immunodeficiency virus type 1; ddCTP, 2′,3′-dideoxycytidine triphosphate; RT, reverse transcriptase; ssDNA, single-stranded DNA; SddC, 2′,3′-dideoxy-3′-thiacytidine; SddCTP, 2′,3′-dideoxy-3′-thiacytidine triphosphate; and FddCTP, 2′,3′-dideoxy-5-fluorocytidine triphosphate.

## MATERIALS AND METHODS

### *Materials and compounds*

The triphosphate forms of AZT and D4T were provided by the AIDS repository and Dr W. H. Prusoff, respectively; ddCTP and ddATP were obtained from Pharmacia LKB Biotechnology Inc. The D and L enantiomers of SddC were provided by Dr. C. K. Chu, Department of Medicinal Chemistry, University of Georgia, and the D and L enantiomers of FddC and L-ddC were synthesized by Dr T. S. Lin *et al.* as detailed elsewhere [8]; triphosphate forms of these compounds, as well as AZTMP, D4TMP, L-ddCMP, D-ddCMP and L-FddCMP, were prepared in our laboratory as described previously [9]. ssDNA cellulose was purchased from Sigma, DE52 anion exchange resin from Whatman, and *Escherichia coli* 16 S and 23 S ribosomal RNA from Boehringer Mannheim. Molecular weight standards used for ultracentrifugation in glycerol gradients were purchased from Pharmacia LKB Biotechnology Inc.

### *Cells*

The human H9 cells, an acute lymphoblastic leukemia T-cell line, were maintained in RPMI (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (JRH Biosciences) and 100 μg/mL kanamycin at 37° in 5% $CO_2$. The doubling time was approximately 16 hr, and the cells were routinely tsted for mycoplasma.

### *Exonuclease assay*

The exonuclease activity was determined by a sequencing gel assay as detailed elsewhere [5]. Briefly, the 3′-terminated substrates were prepared from a 20, 22, 23 or 24 base-long DNA primer with the following sequence:

3′-CAATTTTGAATTTCCTTAACTGCC-5′
24 1

The primers were synthesized, purified and labeled at the 5′-end with [γ-$^{32}$P]ATP (sp. act. ~500 Ci/mmol, Amersham) as previously described [5]. The radiolabeled primers were each annealed to a complementary rRNA template and terminated at their 3′ ends with the appropriate analog triphosphate in a standing start reaction catalyzed by HIV-1 RT as previously described [5]. Under these conditions, the 20mer was terminated with AZTMP or D4TMP at the 21st position, the 22mer was terminated with ddAMP at the 23rd position, and either the 23mer or the 24mer was terminated with the D or L enantiomer of ddCMP, SddCMP or FddCMP at the 24th or 25th position (the latter also encoding for a dCMP residue). Substrates with 3′-terminal mismatches were prepared by synthesizing the above 24mer where the 24th dCMP residue was replaced with either TMP, dGMP or dAMP and labeling the DNA at the 5′-end with [γ-$^{32}$P]ATP. For studying the reaction of products of the exonuclease, the 24mer was radiolabeled at the 3′-terminal with 10 μM [α-$^{32}$P]dCTP (sp. act. > 6000 Ci/mmol, Amersham) in a standing start reaction with HIV-1 RT. Following purification of all of the above primers through preparative 15% polyacrylamide/urea sequencing gels, portions of each primer were left in a single-stranded form or annealed to a complementary DNA (35mer) or rRNA sequence. Exonuclease assays were done in 10-μL reactions containing 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 0.18 μCi/mL 3′-terminated substrate, and 2 μL of various dilutions of fractions obtained from each purification step. The incubations varied from 5 to 120 min at 37°. Reactions were terminated by adding 4 μL of 98% formamide, 10 mM EDTA and 0.025% bromophenol blue. The samples were denatured at 100° for 5 min followed by rapid cooling on ice. The unreacted material as well as the reaction products were separated on 15% polyacrylamide/urea sequencing gels and visualized by autoradiography. The reaction products were quantitated on a Molecular Dynamics Densitometer. One unit of exonuclease activity is defined as the amount of enzyme required to remove 1 nmol of D-SddCMP from DNA at 37° in 1 hr.

### *Purification of the cytosolic exonuclease*

All steps were performed at 4° unless otherwise specified. Fractions from each chromatographic step were assayed for DNA polymerase activity [10] as well as for 3′ → 5′ exonuclease activity using 3′-D-SddCMP-terminated DNA/RNA substrates. Ten litres of exponentially growing H9 cells ($8 \times 10^5$–$1 \times 10^6$ cells/mL) was fractionated to obtain the cytosol, which was subjected to DE52 anion exchange, followed by ssDNA cellulose affinity columns as previously described [5]. The exonuclease activity eluted from the ssDNA cellulose column at approximately 0.4 M KCl, several fractions after the DNA polymerase peak. Following dialysis in Buffer B (50 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL of each leupeptin and pepstatin A), the exonuclease fractions were loaded onto a Mono Q HR 5/5 anion exchange column equilibrated with Buffer B. The column was washed with 5 mL of Buffer B, and the bound proteins were eluted in a 20-mL linear gradient of 0 to 0.8 M KCl in Buffer B. The exonuclease peak eluted in the void fractions, and the DNA polymerase peak was collected at 0.42 M KCl. The exonuclease fraction was reloaded on Mono Q, and the void fractions were chromatographed on a Mono S HR 5/5 cation exchange column using the same conditions as those for Mono Q. The exonuclease activity recovered from Mono S eluted at 0.32 M KCl. The enzyme was purified approximately 1200-fold from the H9 cytosol to a specific activity of $4.9 \times 10^4$ U/mg protein, as determined by analysis of exonuclease peaks from each purification step by densitometer. Aliquots of 100 μL of the Mono S preparation were diluted to 200 μL with Buffer B and 0.2 M KCl and layered on a 4.8-mL glycerol gradient (10–30%). Molecular weight standards were prepared at 25 μg/mL in 200 μL Buffer B and 0.2 M KCl. The gradients were centrifuged at 240,000 *g* in an SW 50.1 rotor at 4° for 36 hr. Fractions of 0.5 mL were collected for each gradient, and the exonuclease was identified by assaying fractions for activity on DNA/RNA substrates with DNA terminated by 3′-ddAMP or 3′-D-SddCMP. The positions of the molecular weight standards were determined by SDS/PAGE followed by staining in Coomassie Blue. Exonuclease recovered from glycerol

gradients was also analyzed by SDS/PAGE; however, the proteins were visualized by silver stain. The enzyme was aliquoted and stored at −80° in 0.5 mg/mL bovine serum albumin. Under these conditions, the exonuclease was stable for approximately 6 months. This preparation was used for all of the studies, and 0.034 U of the exonuclease was used in each assay.

## RESULTS

### *General properties of the cytosolic 3′ → 5′ exonuclease*

The exonuclease activity recovered from glycerol gradients sedimented at a position corresponding to approximately 50,000 Da as determined by testing the fractions on 3′-ddAMP-terminated DNA/RNA substrates (Fig. 1A). The active fraction contained one major band with a molecular weight of 50,000 (Fig. 1B). The enzyme was non-processive, as previously reported for the partially purified exonuclease [5]. The exonuclease required a divalent metal for activity with a marked preference for magnesium at 0.5 to 2.0 mM (Fig. 2A). Manganese, calcium and zinc could also be utilized; however, the exonucleolytic activity was reduced greatly (results not shown). The effect of KCl at concentrations above 20 mM was examined in Fig. 2B. The enzymatic activity was inhibited above 60 mM KCl.

The exonucleolytic removal of 3′-ddAMP was examined on ssDNA, DNA/duplexes and DNA/RNA duplexes in Fig. 3A. A comparison of the reaction products at 5 and 15 min indicated that the exonuclease was most reactive on ssDNA and least reactive on DNA/RNA substrates.

The products of the exonucleolytic reaction were analyzed as previously described in this laboratory [11], using 25 base-long DNA substrates labeled with [α-$^{32}$P]dCTP at the 3′-end. Incubation of this substrate with the cytosolic exonuclease resulted in the production of a single product that comigrated with dCMP (Fig. 3B). Since no additional bands were detected, it is concluded that this enzyme has neither exonuclease activity nor 5′-directed activity and that the reaction products are deoxynucleoside 5′-monophosphates.

The proofreading activity of the exonuclease was tested on DNA duplexes with mispaired nucleotides at the 3′ terminals (Fig. 4). The removal of either 3′-dAMP or 3′-dGMP was comparable to that of the correctly paired 3′-dCMP (39–46% of the residues removed after 40 min). The exonuclease was most reactive with G-T mispairs; 67% of 3′-TMP was removed after 40 min.

### *Substrate specificity of the cytosolic 3′ → 5′ exonuclease*

The behavior of the exonuclease with clinically available anti-HIV-1 compounds was examined on DNA/RNA substrates as a function of time (Fig. 5). Analysis of the reaction products by densitometry revealed that 11% of 3′-AZTMP was removed after 60 min, whereas a similar amount of D4TMP residues (15%) was removed after 30 min. In contrast, 39% of 3′-ddAMP (a metabolite of ddI) and 38% of 3′-ddCMP moieties were removed from DNA after 30 min, indicating that the exonuclease is equally reactive on these two substrates.

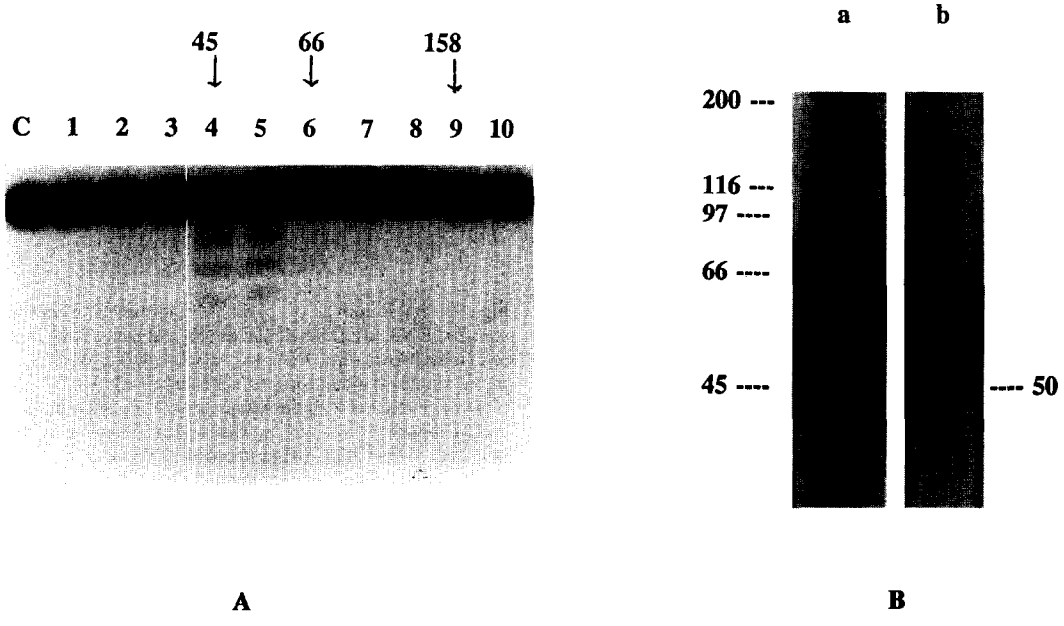


Fig. 1. Estimation of the molecular weight of the cytosolic exonuclease. (A) Exonuclease profile of fractions obtained from glycerol gradients. The exonuclease assays were done for 15 min on DNA/RNA substrates as outlined in Materials and Methods. The DNA component of the substrates was radiolabeled at the 5′-end and terminated with ddAMP at the 3′-end. Lane C represents the reaction in the absence of the exonuclease. The positions at which the molecular markers sedimented are indicted above the fraction numbers. (B) SDS/PAGE and silver stain of: high molecular weight standards (a); and fraction 4 from the glycerol gradient (b).

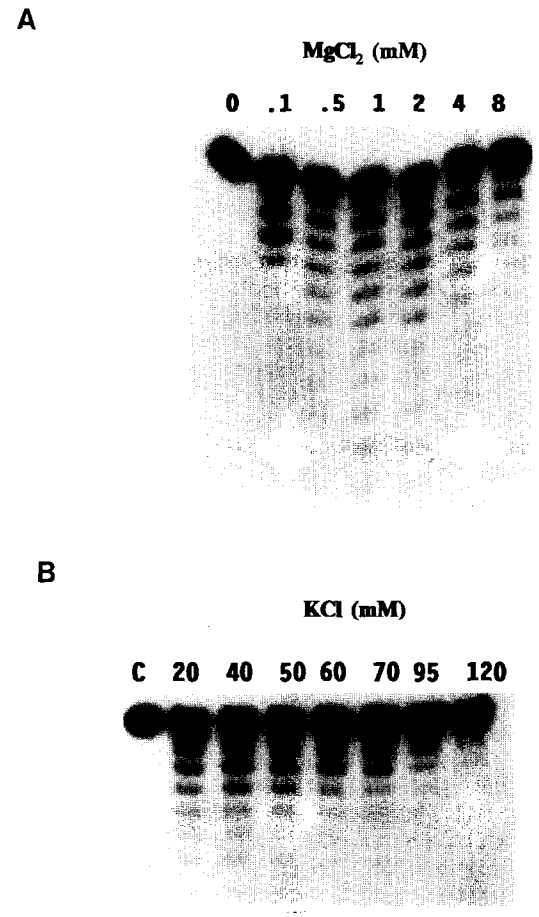


Fig. 2. Divalent metal requirement and salt profile of the cytosolic exonuclease. (A) The exonucleolytic removal of 3′-D-ddCMP from 5′-radiolabeled DNA was examined on DNA/RNA duplexes at the indicated concentrations of $MgCl_2$. (B) Effect of KCl on the exonuclease. The excision of 3′-D-SddCMP from 5′-radiolabeled DNA was assayed on DNA/RNA substrates in the presence of increasing concentrations of KCl. The reaction in lane C was performed in the absence of exonuclease. The exonuclease reactions were carried out for 1 hr as described in Materials and Methods.

The lower activity with DNA terminated by the two TMP analogs was not due to sequence-related factors since ddTMP and dTMP were each removed more efficiently than D4TMP (results not shown). Thus, the cytosolic exonuclease is capable of removing structurally diverse ddNMP from DNA/RNA substrates although the efficiency of removal varies among the analogs.

The reactivity of the exonuclease was tested subsequently on DNA/RNA substrates with DNA terminated by either D or L enantiomers of ddCMP, SddCMP or FddCMP as a function of time (Fig. 6). In all cases, the enzyme was more than six times slower at removing the L than the D enantiomers, as indicated by comparing the reaction products at 120 and 20 min, respectively. Additionally, among the D enantiomers, the addition of a sulfur group at the 3′ position resulted in removal of approximately 1.5-fold more of the residue as compared with 3′-D-ddCMP. The modification to FddCMP decreased the removal by approximately 2-fold from that observed for D-ddCMP.

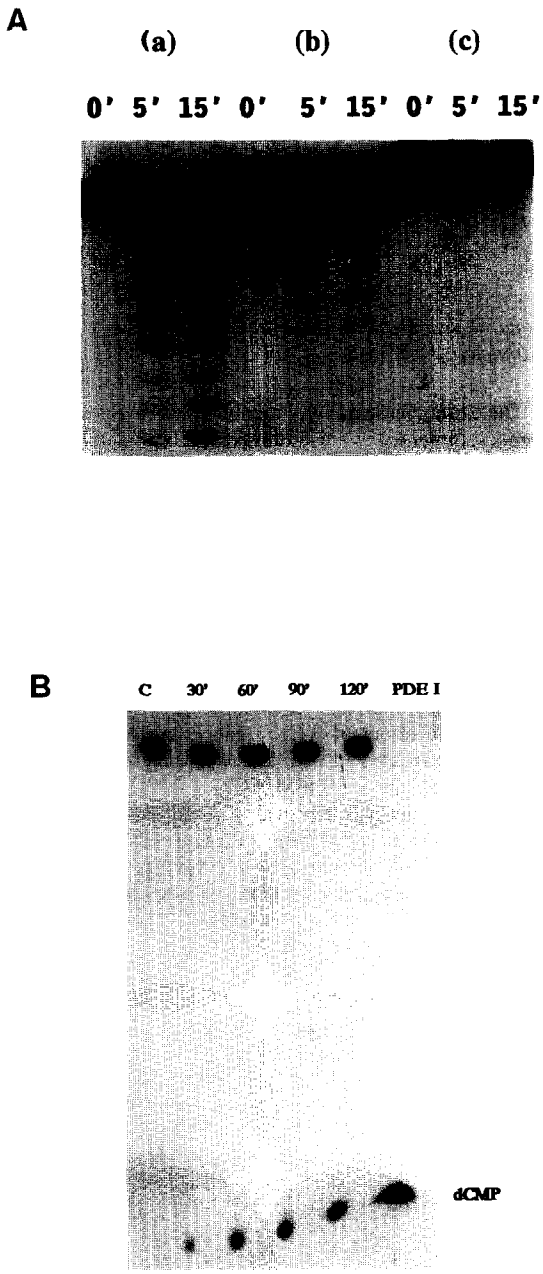


Fig. 3. (A) Removal of 3′-ddAMP terminals from: single-stranded DNA (a); DNA duplexes (b); and DNA/RNA duplexes (c). In each case, the DNA was radiolabeled at the 5′-end, left in the single-stranded form or annealed to RNA or DNA templates, and reacted with the exonuclease as detailed in Materials and Methods (B). Reaction products of the exonuclease. Single-stranded 3′-[$\alpha$-$^{32}$P]dCMP-terminated DNA was reacted with the cytosolic exonuclease for the indicated times as described in Materials and Methods. The substrate was also reacted with $1 \times 10^{-3}$ U of phosphodiesterase I (PDE I) for 5 min at 25° in 50 mM Tris-HCl, pH 8.0, and 15 mM $MgCl_2$. Under these conditions, dCMP was the only reaction product of PDE I.

*Effect of dideoxynucleoside analog monophosphates on the activity of exonuclease*

The removal of 3′-D-ddCMP from DNA/RNA duplexes was examined in the presence of several free ddNMP in Fig. 7. L-FddCMP was the most potent in-

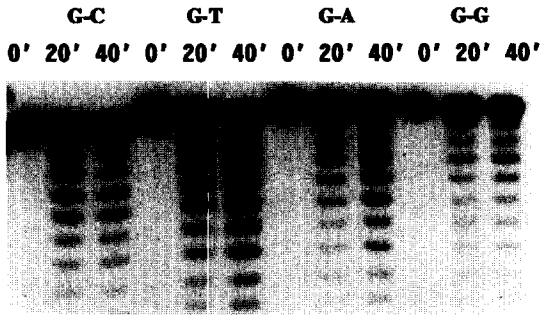


Fig. 4. Excision of 3′-terminal mismatches from DNA duplexes. DNA (24mer) radiolabeled at the 5′-end contained dCMP, dAMP, dGMP or TMP at the 3′ terminal. Each sequence was annealed to a 35mer DNA template that contained a dGMP residue at the 24th position from its 3′-end. The assays were performed with the cytosolic exonuclease for the indicated times as outlined in Materials and Methods.

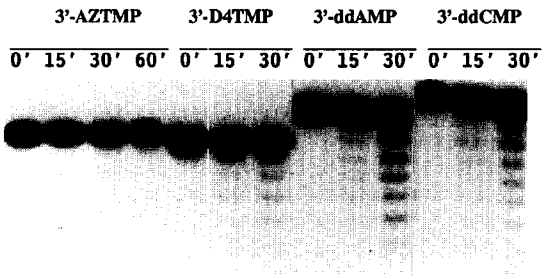


Fig. 5. Substrate specificity of the cytosolic exonuclease. Removal of clinically available anti-HIV-1 dideoxynucleoside monophosphates from DNA was determined as a function of time. All assays were perfomred with DNA/RNA substrates where the DNA was radiolabeled at the 5′-end and terminated with the indicated analog at its 3′ terminal as described in Materials and Methods.

hibitor of the exonuclease and, at 20 μM, completely prevented the removal of 3′-D-ddCMP residues from DNA. A comparable decrease in the 3′ → 5′ exonucleolytic activity was observed at 5 μM L-FddCMP, 25 μM D4TMP, 50 μM AZTMP, and 100 μM L-ddCMP. D-ddCMP was a relatively weak inhibitor of the exonuclease at concentrations up to 5 mM.

## DISCUSSION

In addition to the exonuclease described both in the present work and previously [5], there have been several other reports describing exonucleases that can also be isolated free of DNA polymerase activity [12–17]. However, of these, only one has been identified in the cytoplasm of human cells [12]. A comparison of the physical and enzymatic properties of these DNases with the H9 exonuclease indicates that the protein we have described may represent a novel activity. Although we have observed other nucleases in partially purified cytosol preparations, the enzyme described in this work represents the most abundant exonuclease species with a preference for DNA terminated with D-SddCMP.

The significance of cytosolic exonucleases to cellular function is unclear. It is possible that such proteins are required at specific stages of DNA metabolism and are transported to the relevant cellular compartment at those times. Another possibility is that they are involved in the regulation of RNA levels. The definition of the biological role of the exonuclease is beyond the scope of the present work and will be addressed in the future.

The highly purified preparation of the exonuclease sediments at a rate corresponding to 50,000 Da in glycerol gradients, and it contains only one major band, which has a molecular weight of approximately 50,000. This indicates that the cytosolic enzyme is likely to be a monomer provided that the protein identified on SDS/PAGE is indeed the exonuclease. This will require further investigation. Although the exonuclease is least reactive with terminated DNA in the presence of RNA templates, its ability to interact with this type of substrate may be of particular relevance since it most closely mimics the predominant form of the HIV-1 genome during its initial replication in the cytoplasm of the infected cell. The exonuclease may be able to act as a proofreading enzyme as suggested by its preference for at least one type of 3′-terminal mispair (G-T) over correctly paired residues. This property may have implications in the fidelity of HIV-1 RT-catalyzed DNA elongation. In fact, an earlier study demonstrated that the most frequent error during DNA replication catalyzed by HIV-1 RT is a dGMP-TMP mispair that results in a transition of G to A [18]. Since errors during HIV-1 replication are thought to give rise to viral heterogeneity, the possible impact of the exonuclease on the fidelity of HIV-1 RT is presently being examined in more detail.

Studies of its substrate specificity indicate that the cytosolic exonuclease has a broad range of reactivity on ddNMP-terminated DNA/RNA sequences. This property is consistent with the idea that this cellular factor may functionally interact with replicating viral DNA to reverse chain termination not only by D-SddC but by other ddN as well. The exonuclease is susceptible to the monophosphate forms of AZTMP, D4TMP, L-FddCMP and L-ddCMP. More importantly, the inhibition by these metabolites is observed at micromolar concentrations that may be physiologically achievable. For instance, the intracellular concentration of AZTMP following exposure of HIV-1-infected H9 cells to therapeutic doses of AZT is approximately 1 mM [6], which is twenty times greater than the concentration that inhibits the exonuclease. It is conceivable that the anti-HIV-1 action of AZT could be due to the mechanism of "self-potentiation," which is common for this group of compounds [19–22]. This term refers to the frequently noted inhibition of cellular enzymes by nucleoside analogs, which results in their enhanced pharmacological action. In this case, self-potentiation may occur from an increase in the extent of the incorporation of AZT into viral DNA subsequent to inhibition of the exonuclease by the AZTMP metabolite. Moreover, the inhibition of ddCMP removal from DNA terminals by AZTMP could partially explain earlier reports about the synergistic interaction between ddC and AZT against HIV-1 observed in H9 cells and in peripheral blood mononuclear cells derived from individuals [23, 24].

Results obtained in this work indicates that the cytosolic exonuclease has several properties that may be relevant to the anti-HIV-1 action of ddN analogs and, possibly, to the fidelity of the viral replication in the infected cell. A clearer definition of the extent to which this

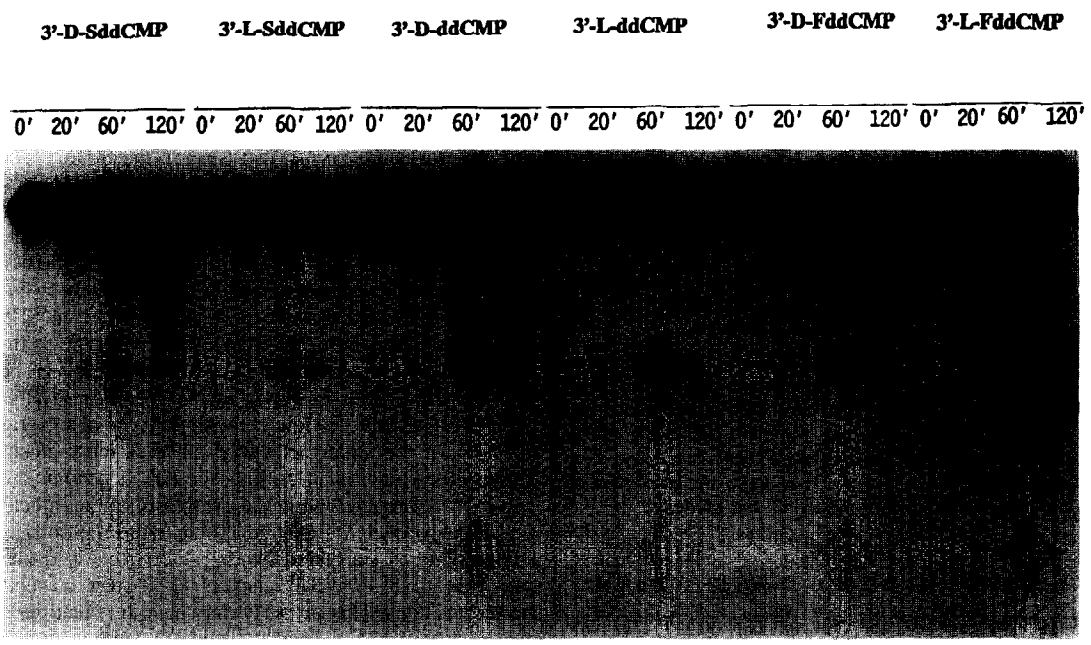


Fig. 6. Comparison of exonucleolytic activity on D- and L-enantiomer-terminated DNA. The exonucleolytic removal of anti-HIV-1 dideoxynucleoside monophosphates from DNA was examined as a function of time using DNA/RNA substrates. The DNA was radiolabeled at the 5′-end and terminated by the indicated D or L enantiomer at the 3′-end. The assays were performed as described in Materials and Methods.

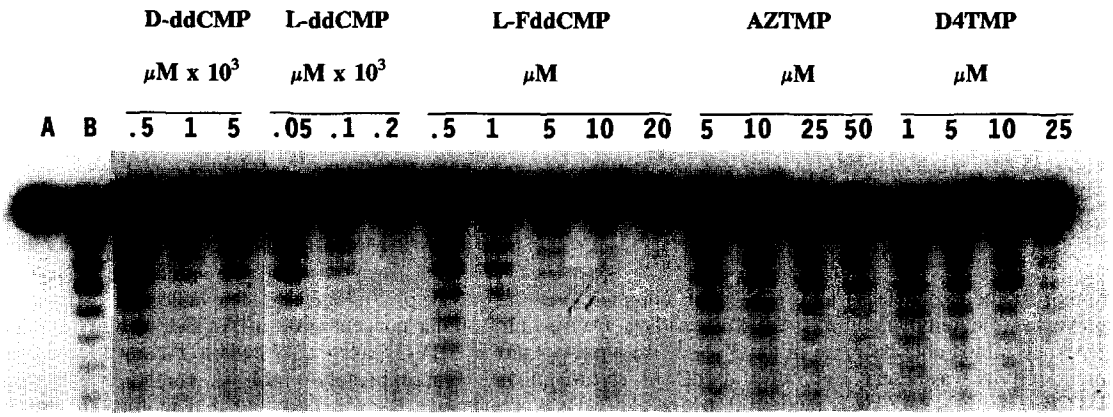


Fig. 7. Excision of 3′-D-ddCMP in the presence of dideoxynucleoside monophosphates. The enzyme assays were performed for 60 min with 0.01 μM (3′-terminals) 3′-D-ddCMP-terminated DNA (radiolabeled at the 5′-end) annealed to complementary RNA as described in Materials and Methods. Lanes A and B represent reactions in the absence of the exonuclease or dideoxynucleoside monophosphates, respectively.

enzyme is involved in these processes requires further molecular characterization of the exonuclease, which is presently underway.

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