0026-895X/00/051051-05$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:1051–1055, 2000 /13006/817851

Excision of β-D- and β-L-Nucleotide Analogs from DNA by the Human Cytosolic 3′-to-5′ Exonuclease

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Received September 30, 1999; accepted January 11, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The cytosolic 3′-to-5′ exonuclease from chronic lymphocytic leukemia cells was highly purified, and its ability to remove β-D- and β-L-nucleotide analogs from the 3′-end of DNA was determined. The relative rate of excision of β-D-ddCMP, β-L-ddCMP, β-L-FddCMP, β-L-SddCMP, β-L-Fd4CMP, and β-L-OddCMP from the 3′-end of a single-stranded oligonucleotide primer or a primer annealed with complementary DNA and/or RNA templates was assessed. The rate of excision of β-D-nucleotides from the 3′-end of DNA was higher than that of β-L-nucleotides, which could be partly attributable to the affinity of the enzyme to β-D-nucleotide-terminated DNA being 5-fold higher compared with that of β-L-nucleotide-terminated DNA. The rate of removal of β-L-Fd4CMP and β-L-OddCMP from the 3′-end of DNA was at least 8 to 10 times lower compared with that of β-L-SddCMP. HIV reverse transcriptase could elongate DNA primers after the removal of chain terminators by the cytosolic exonuclease. Concentrations of nucleoside 5′-monophosphate analogs that inhibit the cytosolic exonuclease by 50% were estimated. Among the nucleoside 5′-monophosphate analogs examined, β-L-Fd4CMP appeared to be the most effective inhibitor of the cytosolic exonuclease, with an ID_{50} value of 38 μM.

Dideoxynucleoside analogs in both β-D and β-L configuration have been shown to possess the activity against HIV and human hepatitis B virus (HBV) (Mitsuya et al., 1985; Belleau et al., 1989; Doong et al., 1991; Chang et al., 1992, 1993; Furman et al., 1992; Lin et al., 1994a,b, 1995; Zhu et al., 1998). The antiviral activity of nucleoside analogs is attributable to the preferential incorporation of their triphosphate forms into the 3′-end of viral DNA by viral reverse transcriptase (RT), which results in termination of viral DNA synthesis. The incorporation of nucleoside analogs into the 3′-end of DNA is critical for antiviral activity; therefore, the excision of these nucleoside analogs from DNA by 3′-to-5′ exonucleases may decrease their potential for chain termination and may reverse the action of these nucleoside analogs.

Several years ago, a 3′-to-5′ exonuclease from the cytosol of human acute lymphoblastic leukemia H9 cells was partly purified in this laboratory (Skalski et al., 1993, 1995). The removal of nucleotide analogs with the natural β-D configuration from the 3′-end of DNA was shown to occur much faster than that of the β-L-nucleotide analogs. These results could explain in part higher efficiency of action of β-L-SddC (3TC), β-L-ddC, and β-L-FddC against HIV reproduction compared with their β-D- counterpart.

We continue to investigate the human cytosolic 3′-to-5′ exonuclease and its role in the removal of β-L- and β-D-nucleotide analogs from the 3′-end of DNA. Special attention was focused on the excision of β-L-Fd4CMP and β-L-OddCMP. As was shown earlier, both β-L-OddC and β-L-Fd4C were even more potent inhibitors of HIV and HBV replication compared with β-L-SddC in cell culture and in vivo (Lee et al., 1995; Lin et al., 1996; Dutschman et al., 1998). The time to reappearance of HBV DNA after removal of β-L-Fd4C from cell culture is longer than that of β-L-SddC (Zhu et al., 1998). It was not clear how efficiently β-L-Fd4CMP or β-L-OddCMP can be removed from the 3′-end of DNA by the cytosolic exonuclease once they were incorporated into the 3′ termini.

In this study, we have highly purified the 3′-to-5′ exonuclease from acute lymphocytic leukemia cells of patients in leukemic blast crisis after leukophoresis. The relative rate of excision of β-L-OddCMP and β-L-Fd4CMP from the 3′-end of DNA was compared with that of β-D-ddCMP, β-L-ddCMP, β-L-FddCMP, and β-L-SddCMP. We also evaluated the affinity of the enzyme to DNA terminated with β-L- and β-D-nucleotide analogs. In addition, the susceptibility of the exonuclease to monophosphate metabolites of β-L- and β-D-nucleoside analogs was also assessed.

This work was supported by National Institutes of Health Grants CA-63477 and AI-42157.

ABBREVIATIONS: HBV, human hepatitis B virus; RT, reverse transcriptase; dNTPs, 2′-deoxynucleoside 5′-triphosphates; β-L-FddCMP, 5-fluoro-analog of β-L-ddCMP; β-L-Fd4CMP, 2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine 5′-monophosphate; β-L-SddCMP, β-L-2′,3′-dideoxy-3′-thiocytidine 5′-monophosphate; β-L-OddCMP, 2′,3′-dideoxy-3′dioxolane-cytidine 5′-monophosphate; β-D-d4TMP, 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-monophosphate; PAGE, polyacrylamide gel electrophoresis.

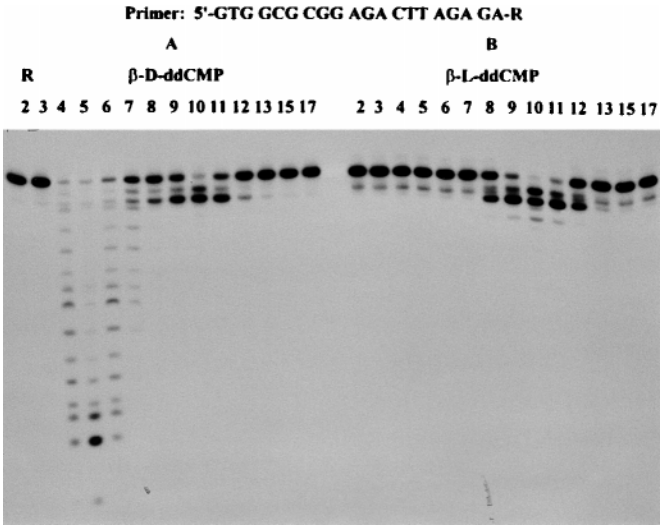

Fig. 1. Analysis of the exonuclease activity following Resource S purification step. A, β-D-ddCMP-terminated 20-mer primer shown above in a complex with 33-mer DNA template was used as a substrate in the exonuclease assay. B, β-L-ddCMP-terminated 20-mer primer as above in a complex with 33-mer DNA template was used as a substrate for exonuclease assay. Reaction conditions are described under *Experimental Procedures*. The reaction products were analyzed by a 20% denaturing PAGE and visualized by autoradiography.

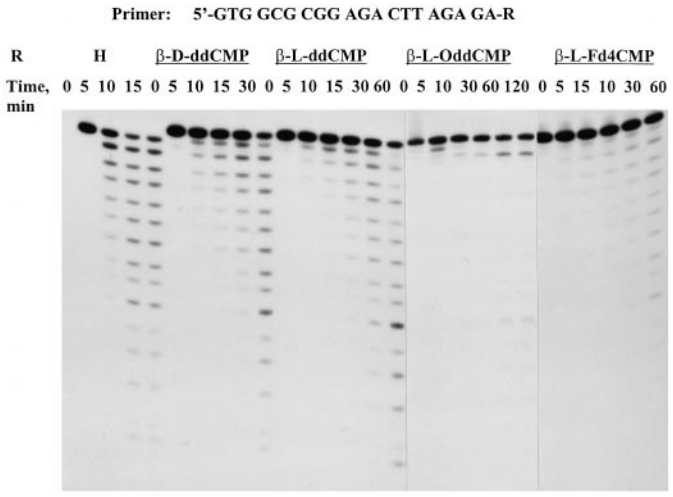

Fig. 2. Time dependence of degradation of DNA primers (shown above) with β-D- and β-L-nucleotide analogs at the 3′-end. Primers were annealed with DNA template as described under *Experimental Procedures*.

Experimental Procedures

Materials and Compounds. The triphosphate form of β-L-Fd4C was synthesized at Vion Pharmaceuticals, Inc. (New Haven, CT). β-L-SddCTP was a gift from Prof. R. F. Schinazi (Veterans Affairs Medical Center, Decatur, GA). β-D-ddCTP was obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). All other nucleoside 5′-triphosphate analogs as well as 5′-monophosphate analogs were synthesized in our laboratory as described previously (Chang et al., 1992). The 20-base DNA primer (5′-GTG GCG CGG AGA CTT AGA GA-3′) and 33-base DNA primer (5′-CCC GCC CCA AAT GTC TCT AAG TCT CCG CGC CAC-3′) were purchased from Integrated DNA Technologies, Inc. (Coravelle, IA). The 33-base RNA template with the same nucleotide sequence as for DNA template was synthesized on an Applied Biosynthesis 380A DNA synthesizer at the Yale University oligonucleotide synthesis facility (New Haven, CT). The large fragment of DNA polymerase I (Klenow fragment-exo$^-$) was obtained from United States Biochemical Corp. (Cleveland, OH). The HIV RT was purchased from Worthington Biochemical Corp. (Freehold, NJ); T4 polynucleotide kinase was from Biolabs (Beverly, MA); [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) and [α-^{32}P]dCTP (specific activity 6000 Ci/mmol) were from Amersham (Arlington Heights, IL).

Purification of Cytosolic 3′-to-5′ Exonuclease and Exonuclease Assay. Human cytosolic 3′-to-5′ exonuclease was purified from 3 g of cytosol fraction of acute lymphocytic leukemia cells of patients in blast crisis after leukophoresis. Purification procedure included DE52 anion exchange, phosphocellulose P11, single-stranded DNA cellulose, Mono Q HR 5/5, and Resource S chromatography columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ) as described earlier (Skalski et al., 1995). Specific activity of the enzyme was defined as the amount of enzyme required to release 5 pmol of [^{32}P]dCMP from poly(dC)·oligo[^{32}P]dCMP per milligram of protein in 15 min at 37°C. The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.1, 1 mM $MgCl_2$, 1 mM dithiothreitol, 35 pmol of poly(dC)·oligo[^{32}P]dCMP, 0.1 mg/ml BSA, and 2 μl of enzyme (0.12 U). Poly(dC) was labeled at 3′-end with [α-^{32}P]dCTP by terminal nucleotidyltransferase to get poly(dC)·oligo[^{32}P]dCMP (Sambrook et al., 1989).

Preparation of 3′-End-Terminated DNA Oligonucleotides. The DNA oligonucleotide primer was labeled at the 5′-position with T4 polynucleotide kinase using [γ-^{32}P]ATP, as described elsewhere (Sambrook et al., 1989). The radiolabeled oligonucleotide was annealed to 33-mer template and terminated at its 3′-end with β-D-ddCMP, β-L-FddCMP, β-L-Fd4CMP, β-L-OddCMP, or β-L-SddCMP using Klenow fragment-exo$^-$ or HIV RT. The reaction mixture contained 5 to 10 pmol of primer template, 10 μM dNTP analog, 0.5 U of Klenow fragment, or 2 U of HIV RT, and buffer according to manufacturer's instructions. The yield of the reaction was estimated by denaturing SDS-polyacrylamide electrophoresis (PAGE). In some experiments, terminated oligonucleotide was excised from the gel, recovered from the gel slice, and purified through a Sephadex G-25 spin column (Boehringer Mannheim, Indianapolis, IN). In the latter case, the purified 3′-terminated primer was annealed to a 1.5-fold molar excess of 33-base complementary DNA and/or RNA template to generate the DNA/DNA or DNA/RNA hybrids or was used as a single-stranded DNA primer terminated with nucleotide analogs at the 3′-end.

DNA Primer Extension Assay. The reaction mixture contained 1 nM 33-mer oligonucleotide template annealed to the primer terminated with nucleotide analog, 0.12 U of the 3′-to-5′ exonuclease, and buffer as described above. The reactions were performed at 37°C up to 30 min after the addition of 10 μM dNTPs, 9 mM $MgCl_2$, and 2 U of HIV RT. The reactions were then incubated for an additional 15 min. Reaction products were separated on 20% denaturing PAGE and visualized using autoradiography.

Interaction of 3′-to-5′ Exonuclease with the Primers Terminated with Nucleotide Analogs. To determine the relative affinity of the exonuclease to the terminated primer, 1 nM 5′-[^{32}P] primer annealed with 33-mer DNA template was incubated with 0.12 U of exonuclease in the presence of different concentrations of 33-mer template in a complex with nonradioactive primers terminated with β-D-ddCMP or β-L-ddCMP for 15 min at 37°C. Nonradioactive natural DNA primer was used as a control. The reaction products were analyzed by 20% denaturing PAGE and visualized by autoradiography, and the rate of degradation was quantified with a computer densitometer as described below.

Quantitation of the Excision Reactions. The bands on X-ray, representing the excision of nucleotide analogs, were quantified with a computer densitometer (Molecular Dynamics, Sunnyvale, CA) as described previously (Mendelman et al., 1990). The bands were scanned, and the efficiency of the removal of nucleotide analogs from the 3′-end of DNA was expressed as a function of time or substrate concentration for different experiments. The relative rate of excision

TABLE 1

Relative rate of excision of β-L- and β-D-nucleotide analogs from the 3′-end of DNA primers by the human cytosolic 3′-to-5′ exonuclease

The rate of excision was defined as a time (minute) causing the removal of 20% of nucleotide analogs from the 3′-end of DNA primer. Values and S.D. are from one experiment repeated at least three times with close results.

Primer Template	R						
	H	β-D-ddCMP	β-L-ddCMP	β-L-FddCMP	β-L-SddCMP	β-L-Fd4CMP	β-L-O-ddCMP
ssDNA[a]	1.5 ± 0.1	3.7 ± 1.4	21 ± 16	27 ± 10	7.8 ± 2.0	>60	>60
dsDNA	2.2 ± 0.4	3.5 ± 0.5	6 ± 9	20 ± 9	6.3 ± 1.4	>60	>60
DNA/RNA	9.2 ± 1.1	13.3 ± 3.4	30 ± 6	31 ± 10	24.4 ± 2.6	>60	>60

[a] ssDNA, sequence of 20-mer DNA primer shown in Fig. 1; dsDNA and DNA/RNA, 20-mer annealed to 33-mer DNA or RNA templates with the sequence as shown under *Experimental Procedures*.

of nucleotide analogs was defined as a time (minute) causing the removal of 20% nucleotide analog from the 3′-end of primer under conditions described above. The incubation time was chosen so to have a linear dependence between the degradation of DNA and time.

Results

Purification and General Characteristic of the 3′-to-5′ Cytosolic Exonuclease. The 3′-to-5′ exonuclease was highly purified from cytosolic fraction of cells obtained by leukophoresis from patients with chronic lymphocytic leukemia. The procedure included DE-52, phosphocellulose, single-stranded DNA cellulose, Mono Q HR 5/5, and Resource S column chromatography steps as described previously (Skalski et al., 1995). The cytosolic 3′-to-5′ exonuclease was shown to have a broad substrate specificity, but the rate of the removal of nucleotide analogs from the 3′-end of DNA was higher for the analogs with the native β-D configuration than that for the β-L configuration (Skalski et al., 1995). In this study, after every chromatography step the fractions were analyzed for the presence of the 3′-to-5′ exonuclease activity, using oligonucleotides terminated with β-D- and β-L-nucleotide analogs. The chromatography on Resource S column clearly demonstrated the presence of two nuclease peaks (Fig. 1). The first peak (fractions 4–7) was eluted from the column at 0.3 M KCl and excised β-D-ddCMP residue from the 3′-end of DNA primer was much faster compared with that of β-L-ddCMP. The second nuclease (fractions 10–11) was eluted at 0.52 M KCl and removed both β-L- and β-D-nucleotide analogs with approximately the same efficiency. As can be seen in Fig. 1A, the pattern of oligonucleotide degradation for two exonucleases is different. The differences are represented by the intensity of bands formed after the removal of nucleotide analogs. The exonuclease found in peak 1 (fractions 4–7) excised nucleotides consecutively. However, the exonuclease found in peak 2 (fractions10–11) results in the accumulation of product degradation after analog excision. The same pattern of degradation was observed when β-D-ddCMP- (A) or β-L-ddCMP-terminated (B) oligonucleotides were used as substrates. Here, we have focused our attention on the first peak, which had the same general characteristic as was demonstrated before (Skalski et al., 1995). The characterization of the exonuclease from the second peak will require additional investigation. After the last step of purification, targeted enzyme (fractions 4–7) was free of DNA polymerases, DNA endonucleases, and 5′-to-3′ exonuclease. In the latter case, 20-mer oligodeoxynucleotide primer annealed to 33-mer DNA template was labeled at the 3′-end with [α-^{32}P]dCMP by Klenow fragment-exo$^-$) and used as a substrate for 5′-to-3′ exonuclease. No fragments shorter than 20-mer oligonucleotide were discovered (data not shown). The specific activity of the 3′-to-5′ exonuclease after the last step of purification was 2400 U/mg protein.

Substrate Specificity of the Cytosolic Exonuclease. The ability of the 3′-to-5′ exonuclease to remove β-D- and β-L-nucleotides from the 3′-end of DNA was examined on a single-stranded oligonucleotide primer, or primers annealed to complementary DNA or RNA templates. The degradation of regular DNA with the same sequence was used as a control. Figure 2 shows the time dependence of the removal of β-D-ddCMP, β-L-ddCMP, β-L-Fd4CMP, and β-L-OddCMP from the 3′-end of DNA primer annealed with 33-mer DNA template. As can be seen, all nucleotide analogs could be removed from the 3′-end of primers, but the rate of excision varied among the substrates. Degradation of β-D-ddCMP-terminated primer was only 2- to 4-fold less compared with that of natural primer and faster than that of β-L-ddCMP. The result correlated with that published previously from this laboratory (Skalski et al., 1995). β-L-Fd4CMP and β-L-OddCMP were much poorer substrates for exonuclease even in comparison with β-L-ddCMP. The relative rate of excision of β-D-ddCMP, β-L-ddCMP, β-L-FddCMP, β-L-SddCMP, β-L-Fd4CMP, and β-L-OddCMP from the 3′-end of single-stranded DNA and that annealed with DNA or RNA templates was estimated using the method described earlier (Mendelman et al., 1990). The results are presented in Table 1. The exonuclease was the least active with the DNA primer annealed to the RNA template for all nucleotide analogs examined. Table 1 also shows that the rate of excision of nucleotide analogs was similar, when either a single-stranded DNA primer or a duplex with DNA template was used. Among β-L-nucleotides at DNA termini, β-L-SddCMP was the most susceptible substrate to excision. The introduction of fluoro atom at the 5-position of cytidine did not change its susceptibility to removal by the exonuclease. The excision rates of nucleotide analogs from the 3′-termini of the DNA primers by the cytosolic exonuclease decreased in the following order: β-D-ddCMP > β-L-SddCMP > β-L-ddCMP ≥ β-L-FddCMP ≫ β-L-OddCMP ≥ β-L-Fd4CMP.

Inhibition of the 3′-to-5′ Exonuclease Activity by DNA Terminated with β-D- and β-L-Nucleotide Analogs. The different rates of excision of β-L- and β-D-nucleotide analogs from the 3′-end of DNA by the exonuclease might be attributable to the different affinities for the exonuclease to DNA terminated with β-L-nucleotides compared with those terminated with β-D-nucleotides. To address this question, the degradation of 5′-^{32}P-20-mer primer in the presence of increasing amounts of nonlabeled 20-mer primer with β-D- or β-L-ddCMP at the 3′-end was assessed (Fig. 3). The 50% inhibition of 5′-^{32}P-20-mer DNA primer degradation by the β-D-ddCMP-terminated primer was observed at the molar

ratio of 1:3 and that by β-L-ddCMP-terminated primer at the molar ratio higher than 1:10. As expected, the 50% inhibition of 5′-^{32}P primer degradation by natural nonterminated primer was found to be at the ratio of about 1:1. These results indicate a somewhat greater affinity of the enzyme to the primer terminated with β-D-nucleotide analogs compared with those terminated with β-L-nucleotide analogs.

The Primer Extension by HIV RT after Removal of Nucleotide Analogs from the 3′-End of Primer. To ensure that after the removal of nucleotide analog from the 3′-end of DNA by the exonuclease HIV RT would be able to extend the repaired DNA primer, a DNA chain elongation reaction was performed. The reaction mixture included cytosolic exonuclease, HIV RT, terminated primer-template complex, and four dNTPs. Figure 4 illustrates that HIV RT could elongate primers after the removal of β-D-ddCMP, β-L-ddCMP, or β-L-Fd4CMP from the 3′-end by the cytosolic exonuclease. Incubation of a terminated primer with the exonuclease in the absence of HIV RT resulted in the appearance of oligonucleotide bands with shorter lengths (lanes 2, 7, 12), which correlates with the results presented in Fig. 1. Because of the lack of exonuclease activity in HIV RT, incubation of HIV RT with terminated primers in the presence of four dNTPs causes neither the elongation of the primer nor the excision of nucleotide from the 3′-end (lanes 4, 9, 14). When both 3′-to-5′ exonuclease and HIV RT were included in the reaction mixture containing template in a complex with terminated primer and four dNTPs, the products of polymerization as well as excision were revealed (lanes 3, 8, 13). These results indicate that the removal of chain terminators by the cytosolic exonuclease generates a correctly base-paired 3′-termini that can be used effectively by HIV RT.

Inhibition of the 3′-to-5′ Exonuclease by 5′-Monophosphates of Nucleotide Analogs. As was shown (Skalski et al., 1995), 5′-monophosphates of some nucleotide analogs inhibited the cytosolic exonuclease. Using a computer densitometer, we estimated the efficiency of β-D-FddCMP, β-L-FddCMP, β-L-Fd4CMP, β-D-d4TMP, and β-L-OddCMP to inhibit the cytosolic exonuclease. Table 2 shows 50% inhibition of the degradation of natural oligonucleotide primer in a complex with template by dNMP analogs. β-L-Fd4CMP was the most potent inhibitor of the exonuclease; 50% inhibition of primer degradation was achieved at 38 μM. The inhibitory activity for β-L-OddCMP and β-L-FddCMP was 10-fold less compared with that of β-L-Fd4CMP.

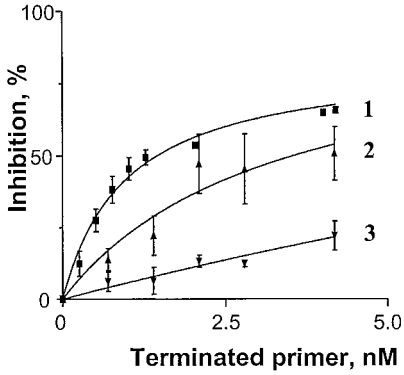

Fig. 3. Inhibition of degradation of 5′-^{32}P-20-mer primer in a complex with DNA template by natural (1), β-D-ddCMP- (2), or β-L-ddCMP-terminated primers (3) in a complex with DNA template. Reaction mixture contained 0.12 U of enzyme, 0.9 nM 5′-^{32}P-20-mer primer-template complex, and different amounts of nonradioactive natural or terminated primer annealed with template. The reaction products were analyzed by a 20% denaturing PAGE and visualized by autoradiography. Bands on the film were scanned with a densitometer, and the results were presented as a percentage of inhibition of primer degradation versus concentration of terminated primer. Time of reaction, 10 min.

Discussion

The amount of dideoxynucleotide analogs presented at the DNA termini depends not only on the efficiency of the incorporation of the compounds by DNA polymerases but also on the rate of excision by 3′-to-5′ exonucleases. There have been several reports describing the isolation of exonucleases (Kwan, 1977; Skarnes et al., 1986; Belyakova et al., 1993; Perrino et al., 1994; Skalski et al., 1995; Mazur and Perrino, 1999) that were free of DNA polymerase activity. Although the physiologic role of 3′-to-5′ exonucleases should be eluci-

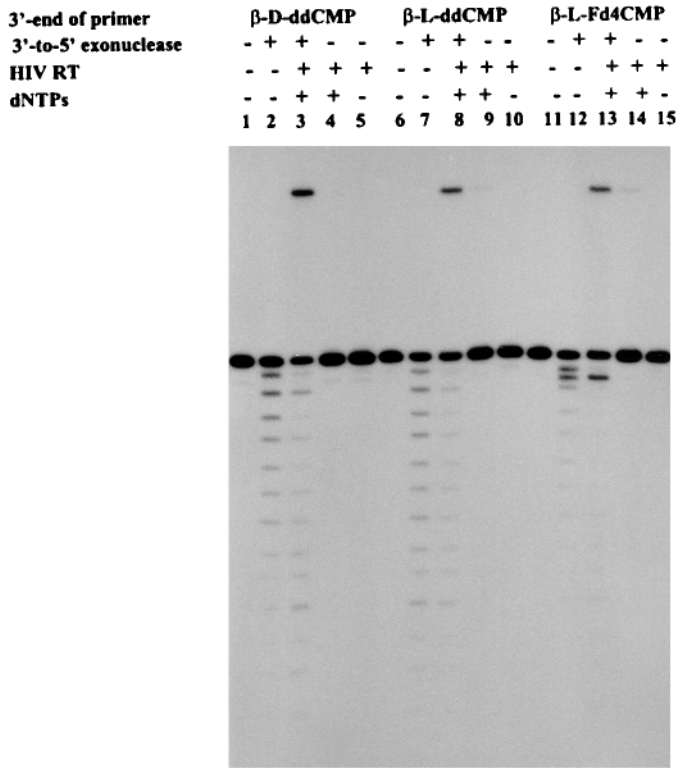

Fig. 4. Primer extension by HIV RT after removal of terminators from the 3′-end of primer by cytosolic 3′-to-5′ exonuclease. The indicated DNA substrates were incubated at 37°C for 15 min for β-D-ddCMP-terminated primer or 30 min for β-L-ddCMP and β-L-Fd4CMP with the 3′-to-5′ exonuclease (0.12 U) followed the addition of 2.7 U of HIV RT and 10 μM dNTPs. Reaction mixtures were then incubated for an additional 15 min. The reaction products were analyzed by a 20% denaturing PAGE and visualized by autoradiography.

TABLE 2

Concentration of 5′-monophosphates of nucleoside analogs that inhibits cytosolic 3′-to-5′ exonuclease by 50%

Values and S.D. are from one experiment repeated at least three times with close results.

ID_{50}				
β-D-d4TMP	β-D-FddCMP	β-L-Fd4CMP	β-L-OddCMP	β-L-FddCMP
		μM		
86 ± 15	84 ± 10	38 ± 9	400 ± 59	320 ± 50

dated, it was suggested that these exonucleases might function in multiple pathways to generate correct 3′ termini that supports additional steps as polymerization or ligation. Excision of incorrectly polymerized nucleotides by proofreading 3′-to-5′ exonucleases is an important mechanism to minimize errors during DNA synthesis. Additionally, exonucleases could play an important role in removing therapeutically important nucleotide analogs from the 3′-end of viral DNA, which causes the reversibility of their action. The main characteristics and substrate specificity of the cytosolic exonuclease have been reported (Skalski et al., 1995). Here, we have estimated the rate of excision of β-D-ddCMP, β-L-ddCMP, β-L-FddCMP, β-L-Fd4CMP, β-L-SddCMP, and β-L-OddCMP from the 3′-end of DNA primer or primer annealed to DNA or RNA templates. The exonuclease was least reactive on DNA/RNA substrates for all analogs examined, which supports the results published earlier (Skalski et al., 1995). Novel nucleotide analogs, β-L-OddCMP and β-L-Fd4CMP, proved to be poor substrates for the cytosolic exonuclease. The rate of their excision was about 8- to 10-fold less than that of β-D-ddCMP or β-L-SddCMP (Table 1). A comparison of the rate of removal of different nucleotide analogs from DNA by the cytosolic exonuclease has shown that a modification in the sugar conformation plays an important role in retarding the excision process. The inversion of orientation of the furanosyl residue of nucleotides from β-D into β-L configuration has a major impact on exonuclease activity. The introduction of sulfur in the 3′-position of sugar residue improved the excision rate while both nucleoside analogs remained in β-L configuration. β-L-SddCMP at 3′-end of DNA is removed faster than β-L-ddCMP. The result indicates also that an alteration in the carbohydrate moiety of nucleoside analogs makes them more resistant to removal by the cytosolic exonuclease. The substitution of hydrogen for fluorine at the 5-position of cytidine did not dramatically modify the exonuclease activity: β-L-ddCMP and β-L-FddCMP at the 3′ termini of DNA were equally good substrates for the exonuclease. Lower affinity of the exonuclease to the primer terminated with β-L-ddCMP compared with the primer terminated with β-D-ddCMP could partly explain the differences in the excision of β-L- and β-D-nucleotide analogs from the 3′-end of DNA primer (Fig. 4). The different efficiencies of removal of β-L-Fd4CMP and β-L-SddCMP from the 3′-end of DNA by the cytosolic exonuclease could partly explain the longer time for resynthesis of HBV genome after the removal of β-L-Fd4C from media than that after the removal of β-L-SddC (Zhu, 1998). We could not exclude that other factors or other exonucleases might play an important role in removal of nucleotide analogs from the 3′-end of DNA. The excision of the 3′-terminal nucleotide analog by HIV RT was observed under conditions that restricted DNA chain extension. In the latter case, the pyrophosphorolysis catalyzed by HIV RT was involved in this process (Meyer et al., 1998).

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