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Human Immunodeficiency Virus Type 1 Reverse Transcriptase Expressing the K70E Mutation Exhibits a Decrease in Specific Activity and Processivity

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ABSTRACT

Adefovir dipivoxil [9-(2-(bispivaloyloxymethyl)phosphonylmethoxyethyl)adenine (bis-POM PMEA)], an oral prodrug of adefovir (PMEA), is currently in phase III clinical testing for the treatment of human immunodeficiency virus-1 (HIV-1) infection. Previous *in vitro* experiments have shown that HIV-1 recombinant viruses expressing either a K65R or a K70E mutation in reverse transcriptase (RT) have reduced sensitivity to PMEA and that the K70E mutant also has impaired replication capacity *in vitro*. Genotypic analyses of samples from patients enrolled in a phase I/II clinical trial of adefovir dipivoxil demonstrated that the K70E RT mutation developed in two of 29 patients during extended therapy. To further investigate the molecular mechanisms involved in the resistance to PMEA, we cloned, expressed, and purified HIV-1 RT enzymes carrying either the K65R or K70E and, for comparison, the M184V mutation. The K_m values of dNTPs for these mutant enzymes were not significantly altered from wild-type RT. The K_i values for the K65R mutant were increased from wild-type by 2–5-fold against a variety of inhibitors, whereas the K_i values for the M184V mutant were increased 12-fold specifically for 2′,3′-dideoxy-3′-thiacytidine (3TC) triphosphate. The K_i values for the K70E mutant were increased for PMEA diphosphate and 3TC triphosphate by 2–3-fold. These results are in agreement with antiviral drug susceptibility assay results. The three recombinant enzymes were also evaluated for their specific activities and processivities. All mutants were reduced in specific activity with respect to wild-type RT. In single-cycle processivity studies, the M184V mutant was, as expected, notably impaired. The K70E mutant was also slightly impaired, whereas the K65R mutant was slightly more processive than wild-type. These results with recombinant K70E RT are consistent with the reduced *in vitro* replication capacity of the K70E RT mutant of HIV-1 and further demonstrate that the K70E mutation confers minor PMEA and 3TC resistance to HIV-1.

The RT of HIV-1 is essential for HIV-1 replication because it generates the viral DNA that integrates into the cellular genome. Currently there are seven approved drugs for the treatment of AIDS that specifically target HIV-1 RT; five of these are nucleoside analogs: AZT, ddC, ddI, 3TC, and d4T. These RTIs block RT function both as competitive inhibitors with regard to dNTP substrate and as chain terminators of viral DNA synthesis. However, RTI-resistant HIV-1 has developed in patients receiving these treatments (Larder *et al.*, 1989; Rooke *et al.*, 1989; Gu *et al.*, 1992, 1994b; Kozal *et al.*, 1994; Lin *et al.*, 1994; Zhang *et al.*, 1994; Wainberg *et al.*, 1995) and this has been shown to result in increased viral loads (Schuurman *et al.*, 1995; Zazzi *et al.*, 1996). As viral load is strongly correlated with disease progression (Mellors *et al.*, 1996; O'Brien *et al.*, 1997), such increases are likely to reduce long-term clinical benefits. Therefore, new antiretroviral drugs with unique genetic resistance profiles need to be developed.

PMEA (adefovir) is an acyclic nucleoside phosphonate analog that functions as an RTI and is active against multiple retroviruses, including HIV-1 (Pauwels *et al.*, 1988; Cherrington *et al.*, 1996a), as well as other DNA viruses, including herpes viruses (De Clercq *et al.*, 1986; De Clercq *et al.*, 1987) and hepadnaviruses (Yokota *et al.*, 1994). PMEA has also shown potent antiviral activity as a prophylaxis in the simian immunodeficiency model of AIDS (Tsai *et al.*, 1994). An orally bioavailable prodrug, adefovir dipivoxil, has shown anti-HIV activity in phase I/II clinical trials (Deeks *et al.*, 1997) and is presently in phase III clinical trials for the treatment of AIDS and phase II trials for hepatitis B infections. PMEA is a

ABBREVIATIONS: RT, reverse transcriptase; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEApp, 9-(2-phosphonylmethoxyethyl)adenine diphosphate; HIV-1, human immunodeficiency virus type 1; HCMV, human cytomegalovirus; RTI, reverse transcriptase inhibitor; dNTP, deoxyribonucleotide triphosphate; 3TC, 2′,3′-dideoxy-3′-thiacytidine; 3TCTP, 2′,3′-dideoxy-3′-thiacytidine triphosphate; ddI, 2′,3′-dideoxyinosine; ddC, 2′,3′-dideoxycytidine; AZT, 3′-azido-3′-deoxythymidine; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; DTT, dithiothreitol; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline/0.5% Tween 20; BSA, bovine serum albumin; bp, base pair(s).

nucleotide analog that requires only two phosphorylation steps by cellular enzymes to become the active metabolite (PMEApp) in cells (Balzarini *et al.*, 1995; Robbins *et al.*, 1995). This novel phosphorylation requirement permits its activity in a wide variety of cell types, including resting T cells and cells of the monocyte/macrophage lineage (Shirasaka *et al.*, 1995; Perno *et al.*, 1996).

In vitro selection of HIV-1 in the presence of PMEA has resulted in the identification of two different mutations in RT with reduced sensitivity to PMEA, K65R, and K70E (Gu *et al.*, 1995; Cherrington *et al.*, 1996b). The K65R mutant of RT showed greater resistance to PMEA than the K70E mutant and was also cross-resistant to 3TC, ddC, and ddI *in vitro*. The K65R enzyme has been extensively characterized *in vitro* and showed decreased affinity for most RTIs, in agreement with antiviral susceptibility data (Gu *et al.*, 1994a). We now report that the K70E mutant enzyme shows only minor decreases in affinity for PMEApp, correlating with its 9-fold reduction in PMEA sensitivity in cell culture. Moreover, we observe that the K70E mutant RT is less active and less processive than wild-type RT, correlating with its reduced *in vitro* replication capacity (Cherrington *et al.*, 1996b). Of these two *in vitro* PMEA-selected mutations, only the K70E mutation has been observed to develop in two of 29 patients treated with adefovir dipivoxil for up to 1 year (Mulato *et al.*, 1998).

Materials and Methods

Cloning, expression, and purification of wild-type and mutant RT. The wild-type RT expression construct pRT66 was a gift from M. Wainberg and has been described by Gu *et al.* (1994a). Briefly, the *pol* sequences were polymerase chain reactions amplified from the HXB2D molecular clone of HIV-1 and then transferred into an expression vector pKK223–3 (Pharmacia Biotech, Piscataway, NJ). Appropriate initiation and stop codons were included in the polymerase chain reaction primers. Mutant expression vectors corresponding to K65R, K70E, and M184V RTs were subsequently generated by oligonucleotide-based site-directed mutagenesis of the pRT66 vector. All constructs were sequenced to verify correct nucleotide sequences. *Escherichia coli* JM109 were transformed with the wild-type or mutant constructs and then induced with 1 nM isopropyl β-D-thiogalactopyranoside. Purification was performed according to Hansen *et al.* (1987) using, sequentially, DEAE cellulose, phosphocellulose, and poly(rC)-agarose column chromatography.

K_i/K_m determination. The enzyme kinetic analyses were performed as described in Cherrington *et al.* (1995). The reaction mixtures for the DNA-dependent DNA polymerase function contained 50 mM Tris·HCl, pH 8.0, 5% glycerol, 1 mM DTT, 500 μg/ml BSA, 5 mM $MgCl_2$, 200 μg/ml activated calf thymus DNA (Pharmacia), 60 μM of each dNTP, and various concentrations of the appropriate [^{3}H]dNTP (30 Ci/mmol; Amersham, Arlington Heights, IL). For RNA-dependent DNA polymerase activity, a defined 86-bp RNA template was annealed to a 15-bp DNA oligonucleotide primer (Cherrington *et al.*, 1995). For all reactions, approximately 10^{-4} units of enzyme were used per 60-μl reaction (unit = incorporation of 1 nmol of [^{3}H]dNTP/hr at 37°). Kinetic constants were determined by fitting the initial rate data to Lineweaver-Burk plots using the KinetAsyst program (Think Technologies).

Recombinant RT quantification. The concentrations of the recombinant RT preparations were determined by quantitative immunoblots using a commercially available recombinant RT (Worthington Biochemical, Freehold, NJ) as a standard. The standard was diluted to a 1 ng/μl concentration and 2, 4, 8 and 16 μl were electrophoresed with 1–15 μl of the RT preparations of unknown concentration in a 10% sodium dodecyl sulfate polyacrylamide gel. A nitrocellulose immunoblot was prepared, blocked with 5% nonfat dry milk in PBS-T, incubated overnight with an anti-HIV-1 RT monoclonal antibody (Intracell, Cambridge, MA; 1:400 in PBS-T/1% BSA), and then incubated with Cy5-conjugated donkey antimouse IgG (Jackson ImmunoResearch, West Grove, PA; 1:300 in PBS-T/1% BSA). After washing, the blot was scanned at 600 V using red fluorescence in a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The density of the heterodimeric RT bands was within the linear range of the instrument and a plot of fluorescence intensity versus nanograms of the RT standard yielded a straight line. Values of the unknown wild-type and mutant RT concentrations were determined from the linear regression analysis by interpolation. This experiment was performed twice and the average values used for the specific activity studies.

Specific activity determination. The polymerase activity of 1–3 ng of each recombinant RT preparation was evaluated in duplicate using a synthetic poly(rA)/$p(dT)_{12–18}$ template/primer (Pharmacia Biotech). Each 50-μl reaction contained 15 μg/ml poly(rA)/$p(dT)_{12–18}$, 10 nM DTT, 50 mM Tris·HCl, pH 6.8, 60 mM KCl, 1 mM EDTA, and 10 mM $MgCl_2$. The reactions were begun by adding [α-^{32}P]dTTP (500 Ci/mmol, Amersham) to a final concentration of 180 nM. Aliquots of 15 μl were removed at 5, 10, and 20 min and applied directly onto Whatman 3-mm filter paper disks. The disks were washed three times for 10 min. each time in 5% trichloroacetic acid/1% sodium pyrophosphate, twice in 95% ethanol, then dried and counted in liquid scintillation fluid (Ready Safe; Beckman, Palo Alto, CA). The incorporated [α-^{32}P]dTTP was plotted as cpm versus time and the initial velocities determined from the slopes of the linear regression analyses. All values are presented as a percentage of the initial velocity of the wild-type recombinant RT with the percentage standard deviation of the duplicate samples also indicated.

Processivity assays. Heteropolymeric HIV RNA template was prepared from a *Xho*I linearized pHIV-PBS plasmid using the Ribo Max kit (Promega, Madison, WI) according to the manufacturer's instructions. pHIV-PBS contains the 970-bp *Bgl*II-*Sph*I fragment of the HXB2D molecular clone of HIV-1, corresponding to nucleotides 472-1442, and includes the R, U5 and 5′ *gag* portions of the genome. This plasmid is similar to that used by Arts *et al.* (1994). A second RNA template contained the truncated A/T rich 5′-untranslated RNA4 region of the alfalfa mosaic virus together with the 5′-end of the coding sequence from HCMV DNA polymerase. The 600-base RNA template was prepared by transcription of *Spe*I linearized pUL54–4 plasmid (Cihlar *et al.*, 1997). Double-stranded template/primer was prepared in batch by incubating the RNA templates (200 nM) with a DNA oligonucleotide primer (400 nM) for 10 min at 85°, then 10 min at 55° for annealing. The sequence for the HIV-1 oligonucleotide was 5′-GTC CCT GTT CGG GCG CCA-3′ and corresponded to the natural tRNA primer binding site. The HCMV *pol* oligonucleotide sequence was 5′-CCG CGA CCG CAC CGC CGG TCA-3′. The homopolymeric poly(rA)/$oligo(dT)_{18}$ template was prepared by annealing 25 nM poly(rA) (6000-bp average length; Boehringer/Mannheim, Indianapolis, IN) with 400 nM $oligo(dT)_{18}$, resulting in a dT primer for approximately every 375 bases of rA template. The processivity assays were carried out essentially as described by Arion *et al.* (1996). Briefly, 2 ng of the wild-type or mutant RT was preincubated at 37° for 15 min with 5 pmol of heteropolymeric or 2 pmol of homopolymeric template/primer (calculated in moles of primer) in a 10 nM DTT, 50 mM Tris·HCl, pH 6.8, 60 mM KCl, 1 mM EDTA, and 10 mM $MgCl_2$ reaction buffer. The 50-μl reactions were begun by adding the dNTPs with or without a quenching template/primer at a final concentration of 50 μM dATP, dCTP, and dGTP; 180 nM [α-^{32}P]dTTP (500 Ci/mmol) and 33 μg/ml poly(rC)/$p(dG)_{12–18}$ (Pharmacia Biotech). This concentration of the quenching poly(rC)/$p(dG)_{12–18}$ is in 18-fold molar excess to the heteropolymeric template/primer. For the poly(rA)/$oligo(dT)_{18}$ processivity reactions, only 16.7 nM [α-^{32}P]dTTP (500 Ci/mmol) and 33 μg/ml poly(rC)/$p(dG)_{12–18}$ was added. After 1 hr at 37°, the total incorporation was assessed in a

filter-based assay as described above and the remainder of the reaction was stopped by adding a 4× formamide loading buffer and heating for 2 min at 95°. Samples were electrophoresed in a 7 M urea-6% polyacrylamide gel. The image was visualized and quantified by PhosphorImager analysis using the Storm 860 and Image Quant analysis software. Line profiles drawn down the lane were used to quantify all the product bands with the HIV RNA template and the poly(rA)/oligo(dT)$_{18}$ template, whereas box/volume analysis was utilized for the single bands generated from the HCMV RNA template. The efficiency of quenching, as determined by the addition of the quenching template/primer before the test template/primer, was >93%.

Results

Recombinant enzyme K_m/K_i analysis. The K_m values for three dNTPs and the K_i values for a panel of RT inhibitors were determined for wild-type *E. coli*-expressed recombinant HIV-1 RT using activated calf thymus DNA as template/primer (Table 1). The K_i/K_m ratios for each inhibitor, indicative of their relative inhibitory capacity, were similar to our previously published data using virion-associated wild-type RT (Cherrington *et al.*, 1996a). Therefore, *E. coli*-produced recombinant HIV-1 RT expressing the site-directed mutations K65R, K70E, and M184V were used for the subsequent enzymatic studies. In addition to the *in vitro* PMEA-selected K65R and K70E mutations, the M184V mutation was included in this analysis as it demonstrates notable 3TC resistance and has been shown to be deficient in processivity (Back *et al.*, 1996). As shown in Table 1, the K_m values for dATP, dCTP, and dTTP substrates were not significantly different among all four enzymes. The K_m results shown here with the K65R and M184V mutants are in agreement with results published previously that described the use of an RNA template (Gu *et al.*, 1994b; Ueno and Mitsuya, 1997). Seven different inhibitors were analyzed with the four enzymes and the corresponding K_i values were calculated (Table 1). In agreement with previous results, increased K_i values for most inhibitors with the K65R mutant were observed (Gu *et al.*, 1994b). In contrast, the K70E mutant showed only slight increases in K_i values for PMEApp and 3TCTP. The M184V mutant exhibited a strong and selective increase in K_i for 3TCTP, as expected from previous observations (Quan *et al.*, 1996). Kinetic analyses using a heteropolymeric RNA template with PMEApp and 3TCTP as inhibitors were also performed. In these studies, the increases in K_i values with PMEApp were quite similar to those observed with the DNA template. However, these increases were more pronounced with 3TCTP on the RNA template for the K65R RT (3.5-fold) and the M184V RT (35-fold) compared with wild-type. Thus, with regard to the K70E mutant, no significant changes in affinity for the natural dNTP substrates and minor decreases in affinity for the inhibitors PMEApp and 3TCTP were observed.

Specific activity analysis of recombinant enzymes. To determine the specific activity of the recombinant enzyme preparations, quantitative immunoblot analyses were performed as described in Materials and Methods. The RT concentrations ranged from 1–17 ng/μl in the wild-type and various mutant preparations. The DNA polymerase activity was measured using a synthetic poly(rA)/(dT)$_{12–18}$ template/primer over a 20-min initial rate reaction. The calculated initial velocities were then divided by the concentration of enzyme used in the assay to determine the specific activity of the recombinant RT preparations (Fig. 1). All three mutant enzymes were significantly impaired in their specific activity compared with wild-type enzyme, with M184V exhibiting only 35% of wild-type activity and the K70E exhibiting 70% of wild-type activity. In agreement with these results, a second preparation of the K70E RT (K70E #2) containing a 17-fold higher enzyme concentration was tested; it also exhibited only 74% of wild-type activity. Interestingly, the K65R enzyme also showed diminished specific activity, 57% of wild-type.

Processivity of recombinant enzymes on heteropolymeric RNA templates. Reverse transcription by HIV-1 RT proceeds with multiple pauses and repeated cycles of association/dissociation of the enzyme from the template/primer (Klarmann *et al.*, 1993). To assess the DNA polymerase activity of RT in a single cycle of processivity, an enzyme activity assay was performed in the presence of an excess of a quenching template/primer that cannot incorporate labeled dNTP (Arion *et al.*, 1996). The single-cycle processivity of wild-type, M184V, K65R, and K70E RT was measured using two different heteropolymeric RNA templates, one derived from HIV-1, which generates the (–) strand strong-stop DNA, and the other derived from the HCMV *pol* gene. In both cases, a DNA oligonucleotide was used as a primer and the products of the reactions were separated by electrophoresis. As shown in Fig. 2A, RT processivity on the HIV RNA template was highly abortive, with a ladder of bands punctuated by preferential bands. However, full-length product of 191 nucleotides was achieved in a minority of reverse transcripts. To normalize for total enzyme activity, each band in each

TABLE 1

K_m and K_i values for wild-type and the K70E, M184V, and K65R mutants of HIV-1 RT

K_m values are averages from 3–10 separate experiments. The average standard error for these K_m values is 13.2%. K_i values are averages from three to six separate experiments. The average standard error for these values is 16.0%. Numbers in brackets are the fold increases in K_i values from wild-type. Fold increases of < 2-fold are not statistically significant.

Recombinant HIV-1 RT	K_m			K_i					
	dATP	dCTP	dTTP	PMEApp	3TCTP	AZTTP	ddCTP	d4TTP	ddATP
					μM				
Wild-type	0.49	0.61	0.80	0.07	1.16	0.08	0.14	0.03	0.29
K65R	0.34	0.57	0.60	0.36	1.33	0.25	0.26	0.1	0.75
				[5.1]	[<2]	[3.1]	[<2]	[3.2]	[2.6]
K70E	0.54	0.74	1.03	0.19	2.66	0.09	0.22	0.05	0.33
				[2.7]	[2.3]	[<2]	[<2]	[<2]	[<2]
M184V	0.61	0.70	0.81	0.08	14.35	0.06	0.24	0.03	0.42
				[<2]	[12.4]	[<2]	[<2]	[<2]	[<2]

lane of the gel was quantified using a PhosphorImager. In this way, the proportion of product achieving full-length size relative to the sum total of all the product bands was determined and this value ranged from 1.3 to 3.1% for the various enzymes. Results from such analyses were averaged from three experiments and are presented in Fig. 3A as the percentage of wild-type. These results demonstrated that the M184V mutant was notably impaired in processivity, the K70E mutant slightly impaired, and the K65R mutant slightly enhanced in single-cycle processivity.

Fig. 2B shows the single-cycle processivity results using the HCMV RNA template. Using this RNA template, processivity proceeded relatively unimpaired with only a single full-length product band observed at 98 nucleotides. To best normalize for total enzyme activity with this template/primer, the intensities of the bands shown in Fig. 2B were divided by the intensities of bands generated in the absence of the quenching reagent for each enzyme (data not shown). In the absence of quenching, all enzymes exhibited similar activities. Using this analysis, the amount of full-length single-cycle product ranged from 2.7 to 6.6% of the total unquenched product and is shown in Fig. 3B as the percentage of wild-type. Thus, using two different heteropolymeric RNA templates, one that was characterized by numerous RT dissociations and one that allowed efficient procession to a full-length, 98-nucleotide product, the relative results of the processivity experiments were the same: the M184V mutant processed substantially less than wild-type, the K70E mutant processed slightly less than wild-type, and the K65R mutant processed slightly more than wild-type.

Processivity of the K70E mutant on a poly(rA)/oligo(dT)$_{18}$ template. Analyses of single-cycle processivity were performed with a homopolymeric poly(rA)/oligo(dT)$_{18}$ template/primer under conditions of limited dNTP to quantify extension length differences in processivity. The products of this primer extension assay were separated on a DNA sequencing gel and quantified by PhosphorImager analysis.

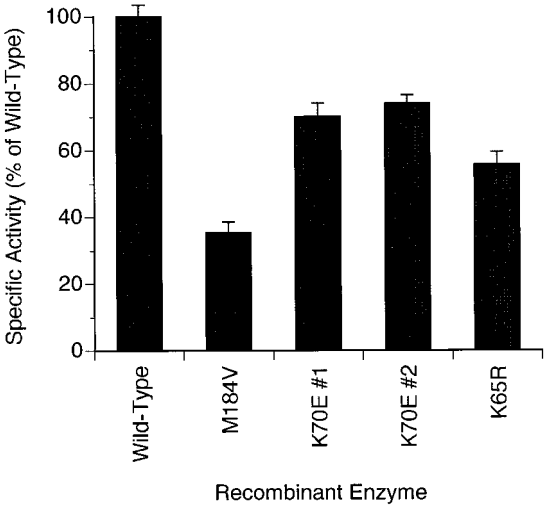

Fig. 1. Specific activity of recombinant enzymes. RNA-dependent DNA polymerase activity was assessed using a synthetic poly(rA)/p(dT)$_{12–18}$ template/primer for wild-type RT and the M184V, K70E and K65R mutants of RT. Two independent *E. coli* preparations of the K70E mutant (#1 and #2) having a 17-fold difference in enzyme concentration were analyzed. All specific activities are expressed as a percentage of wild-type specific activity, as measured in duplicate, with the corresponding percent standard deviations indicated.

The distribution of the cDNA product lengths for the wild-type and K70E RT mutant are shown in Fig. 4. The median cDNA length for these distributions was 25.5 nucleotides for the wild-type RT and 22 nucleotides for the K70E RT mutant. This median decrease of 3.5 nucleotides for the K70E mutant is similar to the reported decrease of 2–10 nucleotides for the M184V mutant under similar experimental conditions (Back *et al.*, 1996). These analyses using a synthetic homopolymeric poly(rA)/oligo(dT)$_{18}$ template/primer agree with the observations using the heteropolymeric RNA template/primers and demonstrate a reduced processivity for the K70E RT mutant.

Discussion

The K70E mutant of HIV-1 RT has been selected *in vitro* by PMEA and has been observed to develop in two of 29 patients undergoing extended therapy in a phase I/II clinical trial of adefovir dipivoxil (Mulato *et al.*, 1998). HIV-1 recombinant

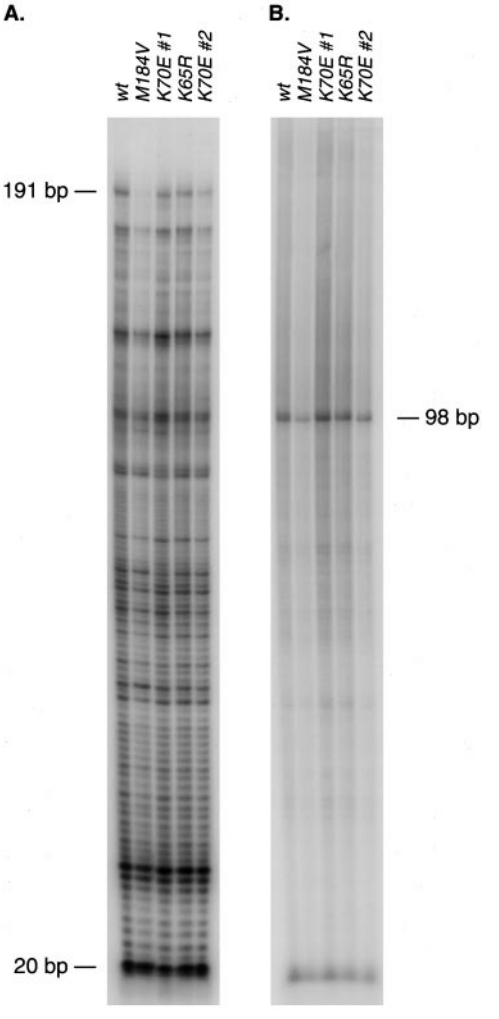

Fig. 2. Single-cycle processivity of recombinant enzymes using a heteropolymeric HIV RNA (A) or heteropolymeric HCMV RNA (B) template. The resulting single-cycle polymerase products from reactions containing the wild-type RT, M184V, K65R, and two independent preparations of K70E mutant RT (#1 & #2) are shown. Processivity reactions utilized an excess of unlabeled quenching template/primer to assure single-cycle processivity in the labeled products. Full-length product for the HIV RNA template is 191 nucleotides (A) and for the HCMV RNA template is 98 nucleotides (B). Extended primer lengths were determined by simultaneously electrophoresing dideoxy chain terminating DNA sequencing reactions.

viruses containing this mutation have also been shown to result in diminished sensitivity to PMEA and 3TC *in vitro* (Cherrington *et al.*, 1996b). The enzymatic analyses using recombinant K70E RT presented here showed minor decreases in affinity for PMEApp and 3TCTP, with K_i values increasing by approximately 2.5-fold using a DNA or RNA template. The magnitudes of these increased K_i values are in agreement with the moderate decreases in drug sensitivities observed with the K70E recombinant virus in cell culture. The specific activity of K70E RT was also reduced compared with wild-type. Finally, in single-cycle processivity assays, the K70E RT demonstrated moderately reduced processivity compared with wild-type RT. These results support the published observation of the reduced *in vitro* replication capacity of HIV-1 expressing the K70E RT mutation (Cherrington *et al.*, 1996b). Interestingly, an AZT-associated mutation at the same amino acid, K70R, is reported to not alter the replication capacity of HIV expressing K70R (Sharma and Crumpacker, 1997), suggesting an amino acid specificity for this phenotype.

A correlation between processivity and replication capacity has also been observed for the 3TC-associated M184V RT mutation (Boyer and Hughes, 1995; Back *et al.*, 1996). In our experiments, the M184V mutant again demonstrated significant impairment in processivity. However, despite the demonstrated *in vitro* replication deficiency (Back *et al.*, 1996), the M184V RT mutant HIV-1 is readily selected for in patients after 3TC treatment begins and is associated with a gradual return toward baseline viral loads (Schuurman *et al.*, 1995; Wainberg *et al.*, 1995). Thus, under the *in vivo* selective pressure of 3TC treatment, the replication deficient M184V virus is more fit than wild-type HIV-1. The replication impairment, however, may contribute to the gradual character of the increase in viral load. This slow return toward baseline viral load contrasts with resistance to the non-nucleoside RTI nevirapine, which is marked by a sharp return toward baseline viral loads (de Jong *et al.*, 1997). In the case of the K70E mutation, both the replication and processivity impairment, as well as the reduction in susceptibility to PMEA, are less notable than with the M184V mutation and 3TC. These results suggest that the K70E mutant of HIV-1 might estab-

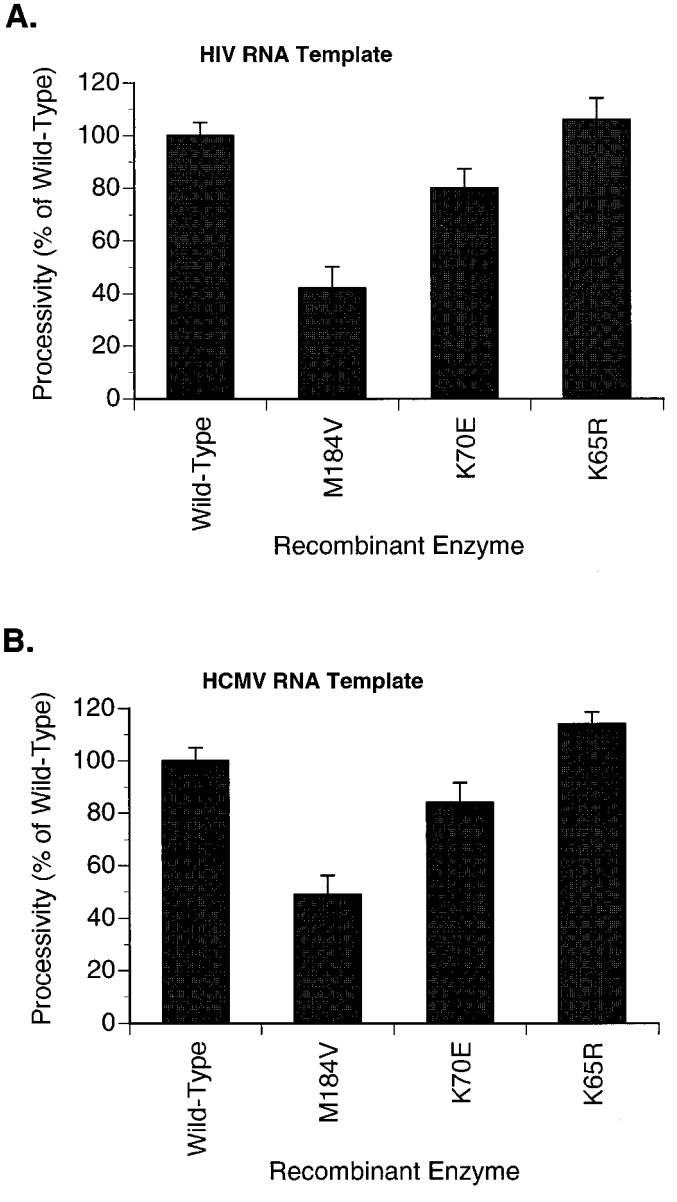

Fig. 3. Activity normalized single-cycle processivity results. A, As described in the results section, the quantity of full-length, 191-nucleotide product in Fig. 2A was divided by the sum of all products in that lane to determine the percent full-length product, thereby internally normalizing for enzyme activity. For the HIV RNA template, the M184V, K70E, and K65R mutants processed to full-length product at 42%, 80%, and 106% of wild-type, respectively. B, For the HCMV RNA template, to normalize for enzyme activity, the quantity of full-length, 98-nucleotide product in Fig. 2B was divided by the total quantity of unquenched product, as determined in parallel reactions performed in the absence of quenching template/primer (not shown). For this template, the M184V, K70E, and K65R mutants of RT processed to full-length product at 49%, 84%, and 114% of wild-type RT, respectively. All values are expressed as a percentage of wild-type RT processivity, with the two K70E preparations averaged together, and the percent standard deviation from three separate experiments indicated. The K70E mutant RT #1 processed slightly better than K70E #2 preparation. However, their combined values averaged over the three independent experiments as shown here demonstrated a significant decrease in processivity compared with wild-type, as indicated by the standard error bars.

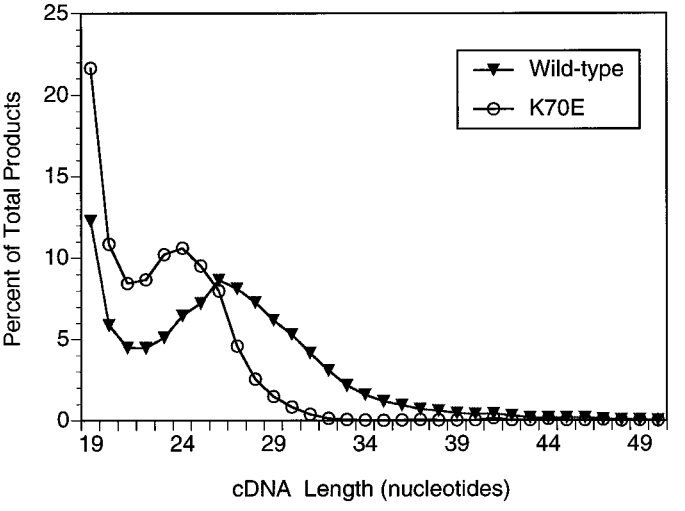

Fig. 4. Single-cycle processivity using a homopolymeric poly(rA)/$(dT)_{18}$ template. The products of a primer extension processivity assay for the wild-type and K70E mutant RT (#1) were quantified with a PhosphorImager and normalized to 100%. The percentages of cDNA product at the various extension lengths, ranging from 19 to 50 nucleotides, are shown. The median extension length for the wild-type and the K70E mutant were 25.5 and 22 nucleotides, respectively. Similar results were observed in three independent experiments under the same conditions.

lish itself quite slowly in response to adefovir dipivoxil treatment, consistent with our limited clinical observations.

The observation presented here of slightly increased processivity for the K65R mutant of HIV-1 RT confirms an earlier observation by Arion *et al.* (1996). These authors suggested that the increased processivity of the K65R mutant may be caused by reduced template/primer dissociation, hence better elongation. Our observation of the reduced specific activity of K65R in conjunction with its increased processivity also suggests altered association/dissociation characteristics. Thus, although the K65R mutant seems less catalytically active, enhanced template/primer binding characteristics may serve to promote single-cycle processivity. Interestingly, although the K65R mutant can develop *in vivo* in response to ddC or ddI therapy in a minority of the patients (Gu *et al.*, 1994b; Zhang *et al.*, 1994; Winters *et al.*, 1997), the K65R mutation has not developed in any patient treated with adefovir dipivoxil to date. This is curious because the *in vitro* susceptibility of the K65R mutant to PMEA is more notably reduced than that of the K70E mutant to PMEA.

Of the two patients who developed the K70E mutation during extended adefovir dipivoxil therapy, one of the patients was undergoing monotherapy and both showed continued viral load suppression during treatment (Mulato *et al.*, 1998). Although anecdotal, this is noteworthy, because viral load often rebounds once resistance mutations can be defined in plasma-derived virus (Schuurman *et al.*, 1995; Zazzi *et al.*, 1996). Possibly, the reduced RT processivity and specific activity, as well as the reduced *in vitro* replication capacity of the K70E mutant may, *in vivo*, balance the minor changes in K_i and IC_{50} values for PMEA, resulting in continued drug effectiveness as measured by HIV-1 RNA levels in plasma. The larger ongoing clinical trials should more clearly establish the role of the K70E mutation, if any, in clinical resistance to adefovir dipivoxil therapy.

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