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Mutated K65R recombinant reverse transcriptase of human immunodeficiency virus type 1 shows diminished chain termination in the presence of 2′,3′-dideoxycytidine 5′-triphosphate and other drugs

(nucleoside analogs/viral drug resistance)

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ABSTRACT **A lysine-to-arginine substitution at amino acid 65 (K65R) in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is associated with resistance to 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI), and the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC). To further characterize the molecular basis of such resistance, we expressed the p66/p51 heterodimer of wild-type RT, K65R mutated RT, and a doubly mutated (K65R/M184V) RT in *Escherichia coli* and assessed the characteristics of nucleotide incorporation and chain termination in cell-free reverse transcription reactions in the presence and absence of various nucleoside triphosphate analogs. These reactions employed a HIV RNA template (HIV-PBS) that contained the primer binding sequence (PBS) and the U5 and R regions of HIV-1 genomic RNA and an oligodeoxynucleotide (dPR) complementary to the HIV-1 PBS as primer. The K65R and K65R/M184V RTs showed significantly decreased chain-termination effects during polymerization with the 5′-triphosphates of ddC, 3TC, 2′,3′-dideoxyadenosine, and AZT (3′-azido-3′-deoxythymidine) in comparison with wild-type RT. Detailed analysis with ddCTP and wild-type RT revealed that chain termination occurred at all guanines in the RNA template. However, the frequency of dideoxynucleoside triphosphate (ddNTP)-induced chain termination was decreased at certain guanines but not others in reactions catalyzed by K65R RT. Both the K65R mutant RT and wild-type RT had similar processive activity. These results indicate that decreased chain termination of K65R RT in the presence of ddNTPs is consistent with data obtained in viral replication assays.**

Genomic RNA of human immunodeficiency virus type 1 (HIV-1) is converted to double-stranded DNA by viral reverse transcriptase (RT). Nucleoside analogs, such as 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), and 2′,3′-dideoxycytidine (ddC), can competitively inhibit HIV-1 RT in their triphosphorylated state; termination occurs when they are incorporated into nascent elongating proviral DNA chains during reverse transcription. Both competitive inhibition and chain termination are thought to be responsible for the inhibitory activity of these drugs *in vivo* (1, 2). Specific mutations in RT are responsible for resistance to each of these nucleoside analogs (3–6). A leucine-to-valine change at codon 74 (L74V) is associated with altered substrate recognition and resistance to ddI (7). However, RT from AZT-resistant HIV-1 isolates and from recombinant RT molecules containing specific AZT resistance-conferring mutations did not show detectable resistance and/or decreased chain-termination effects in the presence of AZT 5′-triphosphate (AZTTP) in cell-free assays (8).

Site-directed mutagenesis has shown that a lysine-to-arginine substitution at codon 65 (K65R) confers resistance to ddC, the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine (3TC), and ddI, but not to AZT (6, 9). This mutation occurs within a generally conserved IKKK amino acid sequence motif (positions 63–66) located in the "fingers" subdomain of HIV-1 RT (10, 11). Studies with enzyme-neutralizing monoclonal antibody against a sequence at positions 65–73 in RT have shown that this region is involved in the binding of dNTP substrate during formation of the RT–template/primer–dNTP ternary complex (12).

Recombinant RT containing the K65R substitution had an increased K_m for dCTP and dATP and, more importantly, an increased K_i/K_m for ddCTP, 3TC 5′-triphosphate (3TCTP), and ddATP in comparison with wild-type RT, when either synthetic homopolymeric or natural heteropolymeric template/primer molecules were studied (13). This partially explains the observed combined resistance to ddC, 3TC, and ddI (a precursor of ddATP). A separate methionine-to-valine substitution at position 184 of RT (M184V) confers resistance to 3TC, ddI, and ddC (5, 14). However, this mutation did not display either synergistic or antagonistic effects with regard to drug resistance when combined in an infectious recombinant viral clone with K65R (6). Amino acid 184 is in the conserved YMDD motif (positions 183–186) that binds a divalent cation and forms part of the polymerase active site (11). We now report that recombinant K65R p66/p51 RT is significantly less prone than wild-type enzyme to incorporation of and chain termination by ddCTP, ddATP, 3TCTP, and AZTTP.

MATERIALS AND METHODS

Materials. The p66/p51 heterodimers of wild-type RT plus RTs containing either the K65R mutation alone or both the K65R and M184V substitutions (K65R/M184V) were expressed in *Escherichia coli* JM109 and purified by HPLC to >95% (13). dNTPs, ddCTP, ddATP, ddITP, and AZTTP were purchased from Pharmacia. 3TCTP was kindly provided by Glaxo. [α-^{32}P]dATP, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from Amersham.

Construction and Transcription of the HIV-1 RNA Template. The HIV-1 RNA template transcription plasmid pHIV-PBS was constructed by cloning a 947-bp *Bgl* II–*Pst* I fragment

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTTP, AZT 5′-triphosphate; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; 3TC, (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine; 3TCTP, 3TC 5′-triphosphate; HIV, human immunodeficiency virus; RT, reverse transcriptase; PBS, primer binding sequence.

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containing both the primer binding sequence (PBS) and repeat (R and U5) regions of the 5′ long terminal repeat (LTR) (+473 to +1420 of pHXB2 cloned viral DNA) into the pSP72 transcription vector (Promega) (15). Fig. 1 illustrates the pHIV-PBS construct and the transcriptional products of priming, reverse transcription, and chain termination with either an 18-mer oligodeoxynucleotide (dPR) or human placental $tRNA_3^{Lys}$ (16) as primer. dPR contains the sequence 5′-GTCCCTGTTCGGGCGCCA-3′, which is complementary to the HIV-1 PBS. CsCl-purified pHIV-PBS DNA was linearized with *Acc* I and then transcribed by using the phage T7 promoter and the Megatranscript Kit (Ambion). The transcript generated is a 497-nt RNA that includes part of the R region, the complete U5 region, noncoding sequence, and part of the HIV *gag* gene.

ddNTP Incorporation/Chain Termination in Reverse Transcription Assays. The chain-terminating effects of various ddNTPs were assayed with HIV-PBS RNA as template and either dPR or $tRNA_3^{Lys}$ as primer. Reverse transcription reactions were performed in 20 μl containing 50 mM Tris (pH 7.8, 37°C), 75 mM KCl, 10 mM $MgCl_2$, and 250 μM dNTPs. HIV-PBS RNA template (50 nM) and [γ-^{32}P]ATP labeled dPR primer (125 nM) were included in the reaction. The reverse transcription mixture was first denatured at 85°C for 2 min, then cooled to 55°C for 8 min, to allow for specific annealing of primer to the RNA template, and then cooled again to 37°C, at which time recombinant HIV RT was added (42.5 nM). Reaction mixtures were incubated in the presence or absence of ddNTP (2.5–83 μM) for 1 hr at 37°C. Products were extracted with phenol/chloroform, passed through a Sephadex G-25 column, precipitated with ethanol, heated at 95°C for 5 min, and electrophoresed in a denaturing 5% polyacrylamide gel. Reverse transcription sequencing was performed with HIV-PBS RNA template, [γ-^{32}P]ATP-labeled dPR primer, and the K65R and wild-type RTs. Both wild-type and K65R RT possessed near-identical levels of enzymatic activity in the absence of inhibitors (13). The full-length (−) strong-stop DNA product of reverse transcription is 192 nt for reactions primed with dPR (15).

We also assessed the effects of 3TCTP, ddATP, ddITP, and AZTTP on chain termination at two concentrations, 25 and 83 μM, chosen for optimal activity in this regard, in the presence of 10 μCi (37 kBq) each of [α-^{32}P]dATP and [α-^{32}P]dCTP.

Processivity of Reverse Transcription. Processivity assays were performed in a time course using both wild-type and K65R mutant RTs. HIV-PBS RNA (50 nM) and [γ-^{32}P]ATP-labeled dPR primer (125 nM) were preincubated with 42.5 nM RT in 50 mM Tris, pH 7.8/75 mM KCl/10 mM $MgCl_2$ for 3 min at 37°C. Then, 100 μg of poly(U·G) RNA (as a trap for RT) and 250 μM dNTPs (final concentration) in the same buffer were added to initiate polymerization. Samples (10 μl) were removed from 80-μl reaction solutions and transferred to 1 μl of 0.5 M EDTA at each time point. One-third of the volume of reaction products was loaded onto a denaturing 5% polyacrylamide gel and analyzed by exposure to x-ray film.

RESULTS

Processivity Analysis of Reverse Transcription with Wild-Type and Mutated RTs. HIV RT frequently pauses during polymerization at sites prior to completion of reverse transcription. These sites are commonly found in homopolymeric stretches of template or in areas of RNA template secondary structure (17). In Figs. 2 and 3*a*, full-length (−) strong-stop DNA product (i.e., 192 nt; open arrows) is found at the top of the gels. In Fig. 2, the other bands represent intermediate products generated due to pausing along the HIV RNA genomic template. Similarly, these bands are found in lanes representing no-drug situations in Fig. 3*a*.

Processivity studies using HIV-PBS RNA template and dPR as primer in a time course showed that the K65R and wild-type RTs possessed similar processive efficiency (Fig. 2). However, the K65R mutated RT seemed to synthesize full-length (−) strong-stop DNA slightly faster than wild-type RT (Fig. 2), possibly due to a slightly higher V_{max} for dCTP assessed with heteropolymeric template/primer (13). No significant differences between the two enzymes were observed with regard to pause sites (Figs. 2 and 3*a*). Due to their similar processivities, both the wild-type and K65R mutated RTs yielded similar levels of (−) strong-stop DNA product in the absence of drug. Interestingly, both RTs shared the same major "hot" pause site at a CCCGUCU located between two bulges containing similar repeated sequences, GUUGUGUG and GUGUGUG (Figs. 2 and 3 *a* and *c*; open triangles). RNA secondary structure for the HIV-PBS template was determined by the Zucher formula (18).

Characterization of K65R RT and Altered Chain Termination Kinetics in Cell-Free Reverse Transcription Assays. To analyze chain-termination effects in polymerization reactions catalyzed by both wild-type and K65R RTs, we used [γ-^{32}P]ATP-end-labeled primer dPR and the HIV-PBS RNA template. Transcripts were electrophoresed in a sequencing gel, alongside the products of a reverse transcription sequencing reaction, to determine specific sites of chain termination by ddCTP during cell-free reverse transcription. Nucleotides were counted from the beginning of the R region (Fig. 3 *a* and *c*).

Using HIV-PBS RNA template, we found that chain termination occurred at all G sites, when ddCTP was used as a chain terminator (see sequencing ladder at right in Fig. 3*a*). With increasing concentrations of ddCTP in the presence of wild-type RT, we observed a progressive decrease in levels of

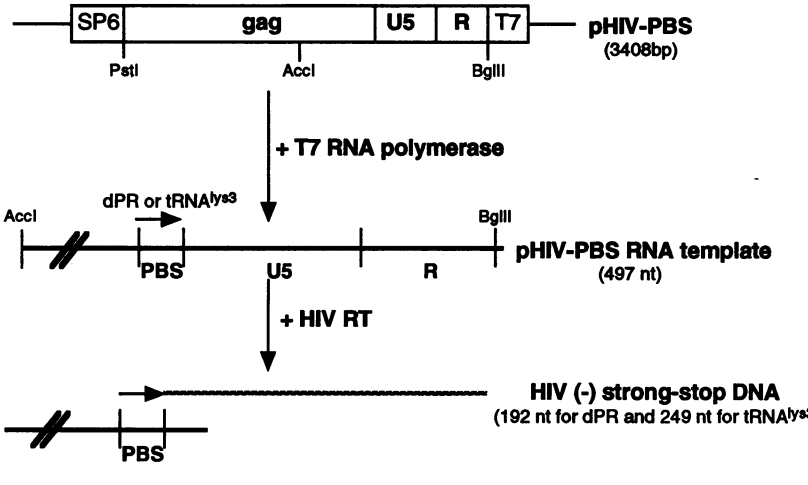

FIG. 1. Schematic representation of the recombinant plasmid pHIV-PBS, RNA template, and products of *in vitro* reverse transcription. pHIV-PBS was constructed by cloning a *Pst* I–*Bgl* II fragment of HIV-1 which contains part of the *gag* gene, the complete U5 region, and part of the R region—i.e., nt 473–1420 of pHXB2 DNA, into pSP72 vector (15). pHIV-PBS was linearized with *Acc* I to yield a transcript of 483 nt (HIV-PBS RNA template) under control of a T7 promoter. The full-length (−) strong-stop DNA products of reverse transcription are 192 and 249 nt for reactions primed with dPR or $tRNA_3^{Lys}$, respectively.

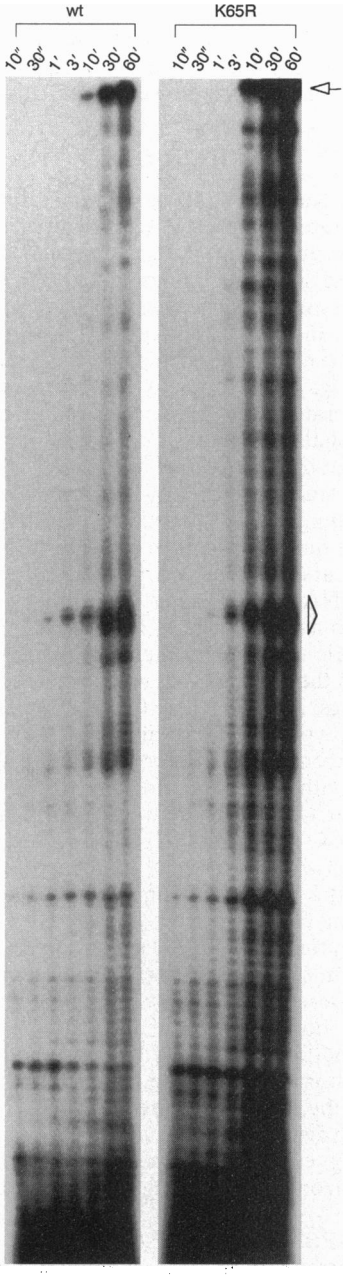

FIG. 2. DNA chain elongation processivity of the wild-type (wt) and mutated K65R RTs in reverse transcription using HIV-PBS RNA template, [γ-^{32}P]ATP-labeled primer dPR, and poly(U·G) RNA as a RT trap in a time-course reaction. Ten microliters of reaction solution was removed and added to 1 μl of 0.5 M EDTA to stop reactions at each time shown (10 sec to 60 min). Products were analyzed in a denaturing 5% polyacrylamide gel. Open arrow, full-length (−) strong-stop DNA; open triangle, major pause site.

(−) strong-stop DNA. Only 2% of the control level of synthesis was seen at 80 μM ddCTP (i.e., one-third the concentration of dCTP that was present). In contrast, significantly more synthesis of (−) strong-stop DNA (35% of control) was generated by the K65R RT at the same concentration of ddCTP (Fig. 3 *a* and *b*). Chain termination effected by ddCTP occurred in a concentration-dependent fashion for wild-type and K65R RTs (Fig. 3*b*). Similar concentration-dependent effects were obtained with ddATP, ddITP, AZTTP, and 3TCTP (data not shown).

The frequency of termination at the first two G residues on the HIV-PBS RNA template after the primer terminus was about 50% for wild-type RT but was <10% for K65R RT when high concentrations of ddCTP were employed (see sequencing ladder at right in Fig. 3*a*). Termination frequency with wild-type RT became gradually reduced as chain elongation continued, because less polymerization in this region had occurred. In contrast, with K65R RT less of a chain termination effect was seen at certain sites than at others (Fig. 3 *a* and *c*). For both wild-type and K65R RTs, chain termination occurred more frequently immediately before the putative major pause site than after it (Fig. 3 *a* and *c*, open triangles). In addition, pausing may have been decreased at each of two sites with K65R at increasing concentrations of ddCTP (see arrows). These two pause sites (A and U) occurred in A+U-rich regions of the HIV-PBS template (Fig. 3*c*).

Effect of the K65R Substitution on Incorporation of ddNTPs and Chain Termination. We next assessed the effects of ddCTP, 3TCTP, ddATP, ddITP, and AZTTP on chain termination in polymerization reactions catalyzed by wild-type, K65R, and K65R/M184V RTs. In the absence of drug, the expected 192-nt full-length (−) strong-stop DNA fragment was transcribed when dPR was used as primer (open arrow; Fig. 4*a*, lanes 1, 12, and 23). In wild-type RT reactions primed by dPR, we found that ddCTP at 83 μM caused chain termination by >98%; i.e. the amount of (−) strong-stop DNA generated was only about 2% of that produced in reactions with the same enzyme in the absence of drug (Fig. 4*a*, lane 6; Fig. 4*b*). With ddCTP at 25 μM, the amount of (−) strong-stop DNA generated was <10% of that made by untreated controls (Fig. 4*a*, lane 7; Fig. 4*b*). In contrast, in the case of K65R RT (Fig. 4*a*, lanes 17 and 18; Fig. 4*b*), about 7 times more (−) strong-stop DNA product was generated at 83 μM and at 25 μM ddCTP than was the case for wild-type RT. When we studied the doubly mutated K65R/M184V RT, a further slight increase in levels of synthesis of (−) strong-stop DNA was observed (Fig. 4*a*, lanes 28 and 29; Fig. 4*b*). Although 3TC and ddC have similar IC_{50} profiles with regard to HIV replication (6), we found that 3TCTP was a far less efficient chain terminator than ddCTP in our assay system (Fig. 4*a*, lanes 8 and 9; Fig. 4*b*). 3TCTP also behaved as an inefficient chain terminator in the presence of the mutated RTs *in vitro* (Fig. 4*a*, lanes 19, 20, 30, and 31; Fig. 4*b*).

Although ddA is not a good inhibitor of HIV replication in tissue culture, we found that ddATP (the intracellular active form of ddI) caused chain termination as efficiently as ddCTP in these reactions (Fig. 4*a*, lanes 10 and 11; Fig. 4*b*). In addition, ddITP, not found as a phosphorylated form of ddI in cells, caused chain termination in reactions with wild-type RT, albeit at lower efficiency than ddATP (Fig. 4*a*, lanes 4 and 5; Fig. 4*b*). Both of the mutated RTs displayed resistance to chain termination by ddATP or ddITP to an extent similar to that seen with ddCTP (Fig. 4*a*, lanes 15, 16, 21, 22, 26, 27, 32, and 33; Fig. 4*b*).

High concentrations of AZTTP inhibited the generation of full-length (−) strong-stop DNA by >97% in the case of wild-type RT (Fig. 4*a*, lane 2; Fig. 4*b*) and by 90% when the two mutated RTs were studied (Fig. 4*a*, lanes 13 and 24; Fig. 4*b*). At lower concentrations, chain elongation efficiency was only 10% for wild-type RT (Fig. 4*a*, lane 3; Fig. 4*b*) but was >35% for K65R and K65R/M184V mutant RTs (Fig. 4*a*, lanes 14 and 25; Fig. 4*b*). Similar results were obtained with $tRNA_3^{Lys}$ as primer (data not shown).

DISCUSSION

The inhibitory effects of dideoxynucleoside inhibitors are believed to follow from the incorporation of ddNTPs into nascent DNA and the resulting chain termination of reverse transcription (2). A number of mutation sites in HIV-1 RT are responsible for resistance to AZT, ddI, ddC, and 3TC (3–6, 9, 14).

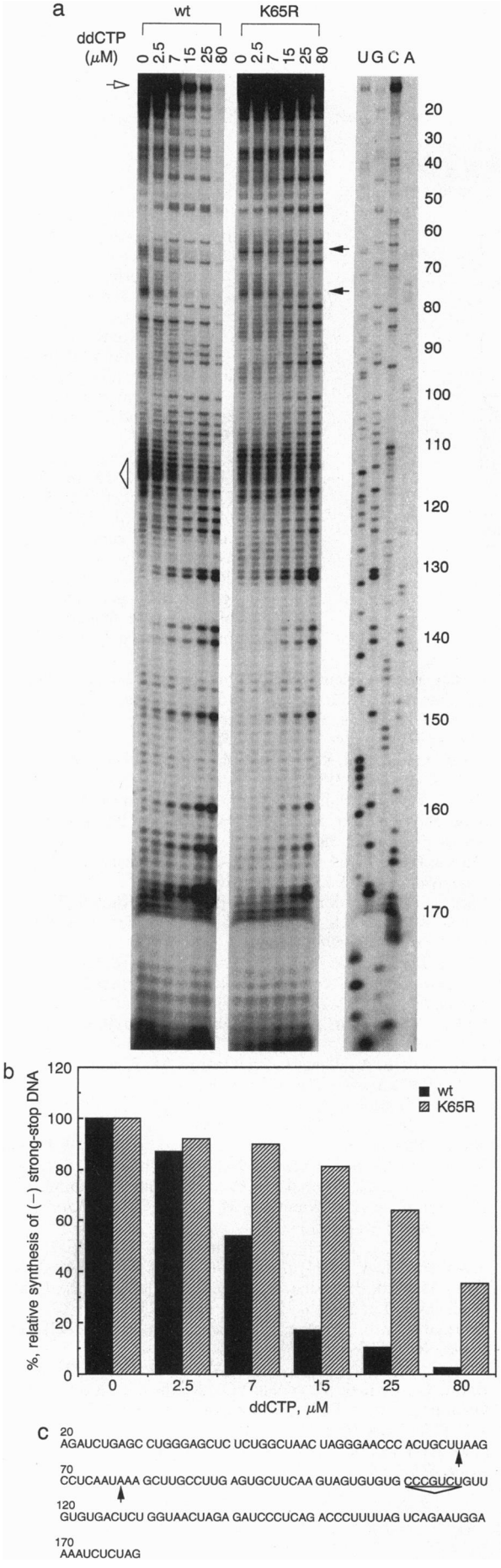

FIG. 3. Chain-termination characteristics of ddCTP with wild-type and K65R RTs. (*a*) Chain-termination sequencing was assayed with HIV-PBS RNA template and [γ-^{32}P]ATP-labeled dPR primers with either wild-type (wt) or K65R RT in the presence of various concentrations of ddCTP. Open arrow, full-length (−) strong-stop DNA; open triangle, major pause site; solid arrows, decreased pausing with increased concentrations of ddCTP for K65R RT. Numbers indicate RNA sequences in the HIV-PBS template, counted from the beginning of the R region. (*b*) Results calculated from the intensities of bands of the full-length (−) strong-stop DNA products shown in *a*. Band intensities obtained in the presence of different concentrations of ddCTP were divided by those obtained in the absence of drug. (*c*) Sequence of the HIV-PBS RNA template (same markings as in *a*).

During HIV replication, reverse transcription of (−)-strand DNA is initiated from a $tRNA_3^{Lys}$ primer annealed to viral genomic RNA at the PBS (16, 19). To mimic natural conditions, we used a cell-free system that included an RNA template containing the PBS, U5, and R regions of the HIV-1 genome and either dPR or human $tRNA_3^{Lys}$ as primer. The incorporation/chain-termination effects of a number of ddNTPs were assessed with wild-type RT and mutated K65R and K65R/M184V RTs. The latter two enzymes displayed reduced chain-termination effects in the presence of ddCTP, 3TCTP, ddATP, AZTTP, and ddITP in these assays, consistent with results that showed that the K65R RT had significantly increased K_i/K_m ratios for these ddNTPs in comparison with wild-type RT (13). No major differences in chain termination were observed between the K65R and K65R/M184V RTs. Similarly, the K65R/M184V mutated HIV-1 did not have a higher IC_{50} for ddC, ddI, and AZT than did K65R alone in virus replication assays (6).

We have shown that the effect of dideoxynucleosides on HIV-1 RT is limited to ddNTPs that are complementary to relevant template bases, suggesting that competitive inhibition by ddNTPs in the absence of chain termination is unlikely to play a significant role in the action of these compounds. Since the K65R mutation is located within the conserved IKKK motif, thought to play a role in the binding of dNTPs (12) and enzyme–template/primer interaction (11), it is not surprising that a mutation in this region would confer cross resistance among several different ddNTPs.

As a function of chain elongation, the wild-type RT showed a gradually decreased frequency of chain termination caused by ddCTP at all G sites in the RNA template. In contrast, for K65R RT we observed a decreased frequency of ddCTP-mediated chain termination at some G sites but not others (Fig. 3*a*). Similar results were obtained with other dideoxynucleoside inhibitors (data not shown). Differences in chain termination may be related to RNA secondary structure, since chain termination occurred less commonly before noncomplementary base-bulge structures than after them. Further analysis will be necessary to resolve whether a lower efficiency of chain termination at certain sites might be related to the nucleoside resistance encoded by the K65R mutation through altered interactions with template/primer, binding of dNTP substrates, or both.

Both the K65R and K65R/M184V substitutions also showed decreased chain termination with AZTTP, consistent with enzymatic studies that the mutated RT had a higher K_i/K_m for AZTTP than wild-type RT (13). However, recombinant viruses containing K65R and/or K65R and M184V mutations did not display diminished sensitivity to AZT when tested in tissue culture assays (5, 6, 9). However, RTs containing mutations associated with AZT resistance did not show reduced chain termination for AZTTP compared with wild-type RT (8). The IC_{50} of AZT in culture is about 1/10th that for ddC and 3TC (5, 6), although both AZTTP and ddCTP performed equally well as chain terminators for wild-type and mutated RTs in our cell-free system. This may reflect different

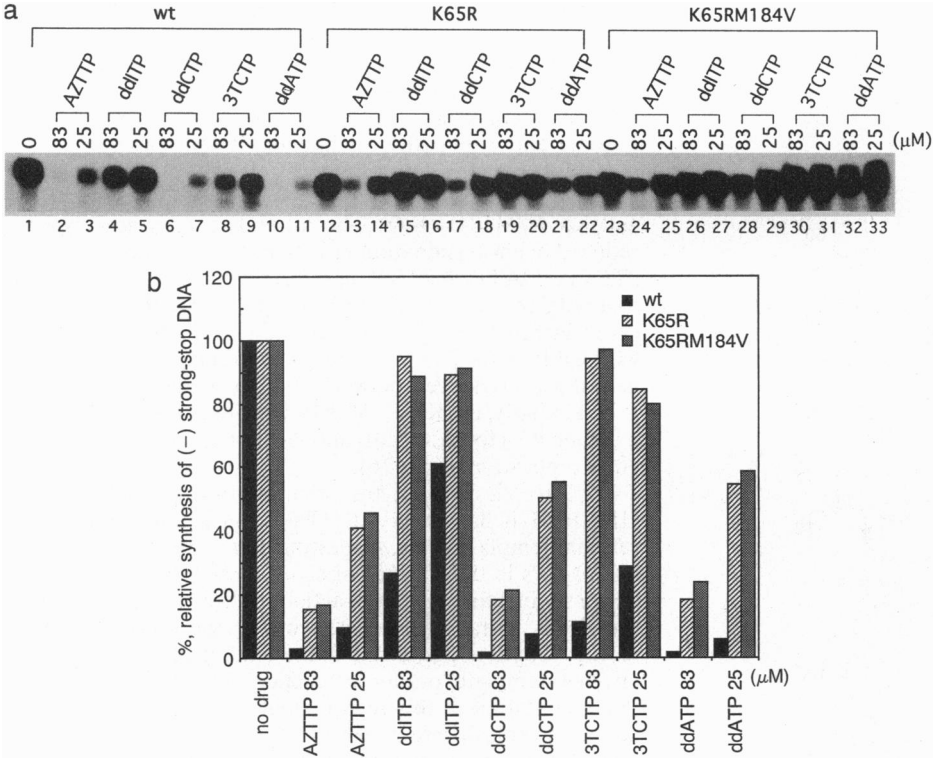

FIG. 4. Effect of various ddNTPs on incorporation/chain termination. (*a*) Full-length (−) strong-stop DNA product of 192 nt of reverse transcription reactions that were primed with dPR using the HIV-PBS RNA template. Wild-type (wt) RT (lanes 1–11), K65R RT (lanes 12–22), or K65R/M184V RT (lanes 23–33) were employed in their natural heterodimeric p66/p51 forms. Reactions were performed in either the absence (lanes 1, 12, and 23) or presence of AZTTP (lanes 2, 3, 13, 14, 24, and 25), ddITP (lanes 4, 5, 15, 16, 26, and 27), ddCTP (lanes 6, 7, 17, 18, 28, and 29), 3TCTP (lanes 8, 9, 19, 20, 30, and 31), or ddATP (lanes 10, 11, 21, 22, 32, and 33). (*b*) Results calculated from band intensities as analyzed by phosphor imaging (Bio-Rad). Relative intensities of full-length products generated in the presence of drugs were divided by those obtained in the absence of drugs.

kinetics of phosphorylation of distinct nucleoside analogs (20); alternatively, factors other than competitive inhibition and chain termination may be involved.

3TC has about the same IC_{50} as ddC in culture (6, 14), but its triphosphate form showed much less efficient chain termination than did ddCTP *in vitro*, consistent with data that 3TCTP has a higher K_i than ddCTP (13). In contrast, the IC_{50} of ddI is about 10 times that of ddC in culture (5, 6, 9, 14), yet ddATP, the intracellular active form of ddI, had the same efficiency of chain termination as ddCTP in our assay.

The processivity of HIV-1 RT ranges from about 1 to 300 nt on both RNA and DNA templates and is significantly less than that of RTs of other retroviruses (17). HIV-1 RT was found to preferentially pause in runs of guanine and cytosine residues during (−)-strand synthesis on RNA templates (17). Although our data are consistent with such findings, we also found a major pause site at a CCCGUCU sequence located between two similar GUUGUGUG and GUGUGUG sequences in the RNA template, with both wild-type and K65R mutated RTs. However, in the case of K65R but not wild-type RT, the frequency of pausing during synthesis of (−)-strand DNA was diminished at two A+U-rich major pause sites but not at others with increasing concentrations of ddCTP. Other dideoxynucleoside inhibitors also caused a lower frequency of pausing at these sites (data not shown). Thus, the K65R mutation did not significantly alter processivity but caused alterations in incorporation of chain-terminating substrates during RNA-dependent DNA synthesis.

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