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Isolation and characterization of a dideoxyguanosine triphosphate-resistant mutant of human immunodeficiency virus reverse transcriptase

(RNA-directed DNA polymerase/drug resistance/chain-terminator inhibitor/retroviral replication)

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ABSTRACT **The appearance of drug-resistant strains of viral pathogens is a major difficulty confounding current efforts to block viral infections. The identification and analysis of mutations responsible for drug resistance can provide important clues helpful in understanding the mechanisms of resistance and in the eventual development of better therapies. We have used a direct screening method to scan libraries of mutagenized genes encoding the reverse transcriptase of human immunodeficiency virus type 1, and have recovered a variant enzyme that is resistant to the chain-terminator inhibitor 2′,3′-dideoxyguanosine triphosphate. The single substitution mutation in this variant conferred broad crossresistance to a variety of other antiviral compounds currently in clinical trials. Virus carrying the mutation was fully infectious in cultured human lymphocytes. The replication of the mutant virus was highly resistant to phosphonoformic acid but did not show increased resistance to the prodrug dideoxyguanosine.**

The replication of the retroviral genome includes several reactions, each catalyzed by specific viral gene products, that are distinctive from steps in the normal replication of the host cell. Prominent among these unusual reactions is reverse transcription, the synthesis of a DNA copy of the RNA genome, mediated by the virion-associated enzyme reverse transcriptase (RT). Because this reaction is so central to retroviral replication, and because it is at least presumably dispensable for the host, it is a prime target of antiviral drugs aimed at blocking viral infection. Many of these compounds block the DNA polymerase activity of RT by chain termination: when incorporated into the growing 3′ end of the DNA product, these compounds block further elongation. The prodrugs 3′-azido-3′-deoxythymidine (zidovudine; AZT), dideoxycytidine (ddC), and dideoxyinosine (ddI) are all converted to the corresponding 5′-triphosphates by host kinases and then presumably incorporated into DNA by RT to block viral DNA synthesis.

Resistance to antiviral drugs arises rapidly in viruses of many types and can severely limit the use of these compounds in antiviral therapy (1, 2). Variants of human immunodeficiency virus type 1 (HIV-1) profoundly resistant to AZT have been isolated from AIDS patients undergoing prolonged treatment (3) and have been shown to contain specific mutations in the region of the viral *pol* gene encoding RT (4). These mutations are sufficient, when introduced into a cloned wild-type genome, to confer on the virus drug resistance for replication in culture. Curiously, the mutant RT isolated from the resistant virus, or expressed in bacterial systems, did not manifest any detectable changes in its sensitivity to inhibition by AZTTP as measured in standard DNA polymerase assays *in vitro*. This result suggests that some aspect of the activity of RT *in vivo* is significantly different from that measured *in vitro*.

A large number of mutant RTs have been generated by site-specific mutagenesis of cloned DNAs that express the enzyme in heterologous systems (5–7). Some mutants displaying altered sensitivity to the chain-terminator inhibitor AZTTP have been recovered in these efforts and characterized (8), and the effects of the mutations on the viral life cycle were determined after transfer of the mutations to viral genomes (9). While some of the mutant genomes could not replicate, others were viable and could be tested for resistance to the prodrug inhibitor for their replication in cell culture. The mutant viruses were not resistant in culture, and in fact some were hypersensitive to AZT (9). The findings further support the conclusion that there must exist profound differences between DNA polymerase assays carried out *in vitro* and the process of DNA synthesis in the infected cell.

The isolation of more such mutants resistant to the nucleoside analogues under evaluation for antiviral therapy is likely to be important in predicting the efficacy of these drugs, in determining the crossresistance of these mutant viruses to other drugs, in defining the spectrum of changes in the viral genome that can produce resistance, and in defining the site of action of the drugs on the RT enzyme. To accelerate the isolation and characterization of such drug-resistant mutants, we developed an *in situ* screening method for assaying the RT activity present in individual bacterial colonies expressing the cloned HIV enzyme (10). We have now used the method to isolate and characterize a mutant clone encoding a variant enzyme resistant to the chain-terminator inhibitor 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP).

MATERIALS AND METHODS

Isolation of Mutant Clone. A plasmid expressing wild-type HIV RT, pHRTRX2 (11), was introduced into an *Escherichia coli* mutator strain carrying the *mutD5* mutation (strain LE30; gift of L. Enquist, DuPont) and was propagated in nutrient-poor medium (M9 plus Casamino acids). Cultures were grown for 14 hr in rich medium (Luria broth) to induce mutations, and plasmid DNA was prepared and used to transform nonmutator bacteria (HB101). Colonies were grown on plates containing M9 salts plus Casamino acids and tryptophan and were screened by the *in situ* colony screening procedure (10).

Inhibition of RT Activity *in Vitro* by ddGTP. Assays were performed with extracts from DNA polymerase I-deficient *E. coli* C2110 carrying a single chromosomally integrated copy of the appropriate expression plasmid, encoding either the

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; PFA, phosphonoformic acid; RT, reverse transcriptase.

wild-type or the mutant RT. The bacteria carrying the respective constructs were induced by tryptophan starvation for the production of the fusion protein, and extracts were prepared (6). The assays were carried out in duplicate in 50-μl of 50 mM Tris Cl, pH 8.0/10 mM $MgCl_2$/20 mM dithiothreitol/60 mM NaCl/0.05% Nonidet P-40 containing poly(rC) (10 μg/ml), oligo(dG) (5 μg/ml), 4.7 μM [α-^{32}P]dGTP [1 Ci (37 GBq)/mmol)], and various concentrations of ddGTP.

Inhibition by Other Compounds: Assays for Crossresistance. Except as noted, conditions were as for inhibition by ddGTP. For inhibition by ddTTP, template and primer were poly(rA) and oligo(dT), and substrate was [α-^{32}P]dTTP (5.6 μM); for ddCTP, template and primer were poly(rI) and oligo(dC), substrate was [α-^{32}P]dCTP (5 μM), and divalent cation was $MnCl_2$ (0.1 μM); for ddATP, template and primer were poly(rU) and oligo(dA), substrate was [α-^{32}P]dATP (5 μM), and divalent cation was $MnCl_2$ (2 mM). For both phosphonoformic acid (PFA) and AZTTP, primer and template were poly(rA) and oligo(dT), substrate was [α-^{32}P]dTTP (5.6 μM), and cation was $MgCl_2$ (10 mM). To test for dependence of pddGTPr-1 RT activity on divalent cation, template and primer were poly(rA) and oligo(dT), and substrate was [α-^{32}P]dTTP (5.6 μM).

Mapping of Mutation in ddGTPr-1 Clone. Two sets of plasmids were constructed by exchanging fragments between the wild-type and mutant clone. In the first set, the region encoding RT, flanked by unique *Bam*HI and *Xba* I sites, was exchanged between the two parents to produce plasmids EX 1 and EX 2. In the second set, a fragment containing a 478-base-pair *Bam*HI–*Eco*RV portion of the gene was exchanged to produce plasmids EX 3 and EX 4. Enzymes encoded by each plasmid were tested for sensitivity to ddGTP as described above. The nucleotide sequence of both strands of the *Bam*HI–*Eco*RV fragment was determined (12, 13).

Analysis of Kinetics of Viral Replication. Jurkat-tat cells (14) were infected with virus or transfected by the DEAE-dextran method and allowed to recover for 24 hr, and 10^6 cells (0.5 ml) were distributed in triplicate into medium (RPMI 1640/10% fetal bovine serum with antibiotics; 4.5 ml) containing various levels of antiviral drugs, ensuring identical input virus inocula for the different drug doses. Three milliliters of the medium was replaced after 24 hr and subsequently every 2–3 days (for medium with PFA) or daily (for medium with ddG). Cells were split 1:2 or 1:3 when their density exceeded 4–5 $\times$ 10^6 per ml and were handled identically for all samples within an experiment. Supernatants were analyzed for RT activity under standard conditions. Radioactivity incorporated was internally normalized to the peak activity for the given viral clone in the presence of no antiviral drug; the peaks were similar between mutant and wild type, $\approx 5 \times 10^4$ cpm/ml of unconcentrated supernatant. Mock infection or transfection showed no appreciable loss in cell viability at the highest drug concentrations used.

RESULTS

Isolation and Characterization of Mutant HIV-1 RT. To isolate a drug-resistant variant of the HIV-1 RT, we generated a library of random mutants and screened for the desired phenotype. The parent for the mutagenesis was the bacterial expression plasmid pHRTRX2, expressing the HIV RT as an enzymatically active fusion protein under control of the *trp* operon (11). The plasmid was mutagenized by transfer into a mutator strain of bacteria (LE30) carrying the *mutD5* allele (15) and growth in high levels of thymidine for 14 hr. Under these conditions, a *lacZ* indicator gene on a control plasmid was mutagenized sufficiently in parallel experiments to yield 0.2–1.0% Lac− mutants. Nonmutator bacteria (HB101) were then transformed with pools of the mutagenized DNA, plated for individual colonies, and lifted to nitrocellulose filters.

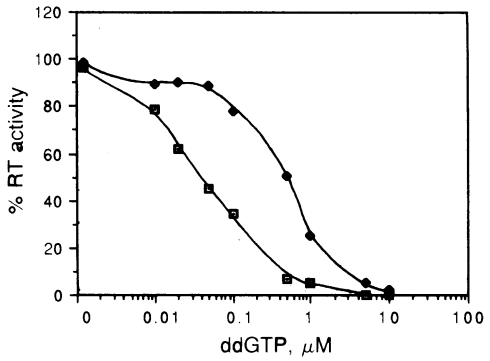

FIG. 1. Inhibition of wild-type (pHRTRX2, ⊡) and the pddGTPr-1 variant (◆) RT by ddGTP.

After induction of the *trp* operon, the bacteria on the filter were screened for RT activity in the presence of 1 μM ddGTP, a level that inhibits the wild-type enzyme $\approx$95%. Of 50,000 colonies screened, we recovered one clone, pddGTPr-1, that consistently tested as drug-resistant.

Cultures containing the mutant and wild-type clones were induced for expression, lysates were prepared, and conventional RT assays were carried out in various concentrations of ddGTP (Fig. 1). The IC_{50} value was at least 10 times higher for the mutant enzyme. The overall yield of enzyme present in the extracts was comparable to the wild type, based on Coomassie blue staining of the proteins after PAGE. The specific activity of the mutant enzyme in the absence of inhibitor was similar to (50–100%) that of the wild type.

To identify the mutation responsible for the resistance, chimeric RT plasmids were first generated by exchanging DNA fragments between pddGTPr-1 and the wild-type parent (Fig. 2). The enzymes encoded by these hybrid genes were then tested for resistance in the standard assay. In the first pair of hybrids, created by exchanging the entire RT sequence, the drug-resistance phenotype was found to segregate with the mutant RT fragment, demonstrating that the mutation lies in the RT coding region and not in the *trpE* fusion partner or the regulatory regions. A second pair of hybrids showed that the mutation was located in the 5′

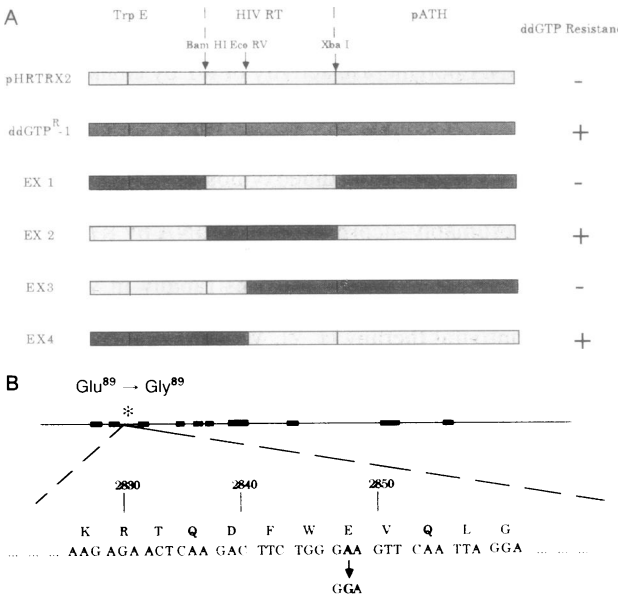

FIG. 2. (*A*) Localization of the mutation in the pddGTPr-1 RT by construction of hybrid RTs. Portions of the wild-type and the variant plasmid were exchanged by using selected restriction sites. (*B*) Identity of the point mutation.

portion of the gene, upstream of the *Eco*RV site at position 427. Sequence analysis showed a single nucleotide change in this portion of the gene. The mutation was an A-to-G transition, changing the GAA triplet (Glu^{89}) to GGA (Gly). Thus, substitution of a single residue in the protein was responsible for the altered phenotype.

Further Characterization of the ddGTPr-1 Mutation. The mutant enzyme was tested for crossresistance to a spectrum of other inhibitors of DNA polymerases using the appropriate ribohomopolymer templates. To reduce the background activity of DNA polymerase I detected on some of the templates, the pddGTPr-1 construct was transferred to a PolA-independent plasmid and introduced into a $PolA^-$ bacterial host (strain C2110; ref. 16). Extracts were prepared and assayed in the standard reaction in the presence of various levels of inhibitors. In each case, the enzymes were assayed on the appropriate substrates with optimal concentrations of divalent cation (Fig. 3; summarized in Table 1). The mutant enzyme was approximately as resistant to the chain terminators ddTTP and ddCTP as it was to ddGTP, with IC_{50} values 8 and 20 times that of the wild type. The mutant was marginally resistant to ddATP and showed significant resistance to the clinically important (17) compound AZTTP (Fig. 3*B*). Studies with other AZTTP-resistant RTs have shown that some such mutants are also resistant to PFA, a pyrophosphate analog (8). The pddGTPr-1 enzyme was highly resistant to PFA, with an IC_{50} value 25-fold higher than the wild-type enzyme assayed on poly(rA) template. The mutant enzyme was extraordinarily resistant to PFA when assayed on other templates, with an IC_{50} value as much as 500-fold higher on the poly(rC) template (data not shown).

Table 1. Resistance of RT to various inhibitors

Inhibitor	IC_{50}, μM		Fold resistance
	Wild type	Variant	
ddGTP	0.04	0.5	12.5
ddATP	0.05	0.15	3
ddTTP	0.10	2.0	20
ddCTP	0.05	0.4	8
PFA	0.20	5.0	25
AZTTP	0.05	0.25	5

The RT of HIV-1, like most other RTs, displays a characteristic preference for Mg^{2+} as divalent cation for maximal activity; the RTs of the murine leukemia viruses are unusual in preferring Mn^{2+} on homopolymer templates. To test the pddGTPr-1 mutant for altered cation requirements, extracts were assayed under standard conditions but with various concentrations of Mn^{2+} and Mg^{2+}. The mutant showed

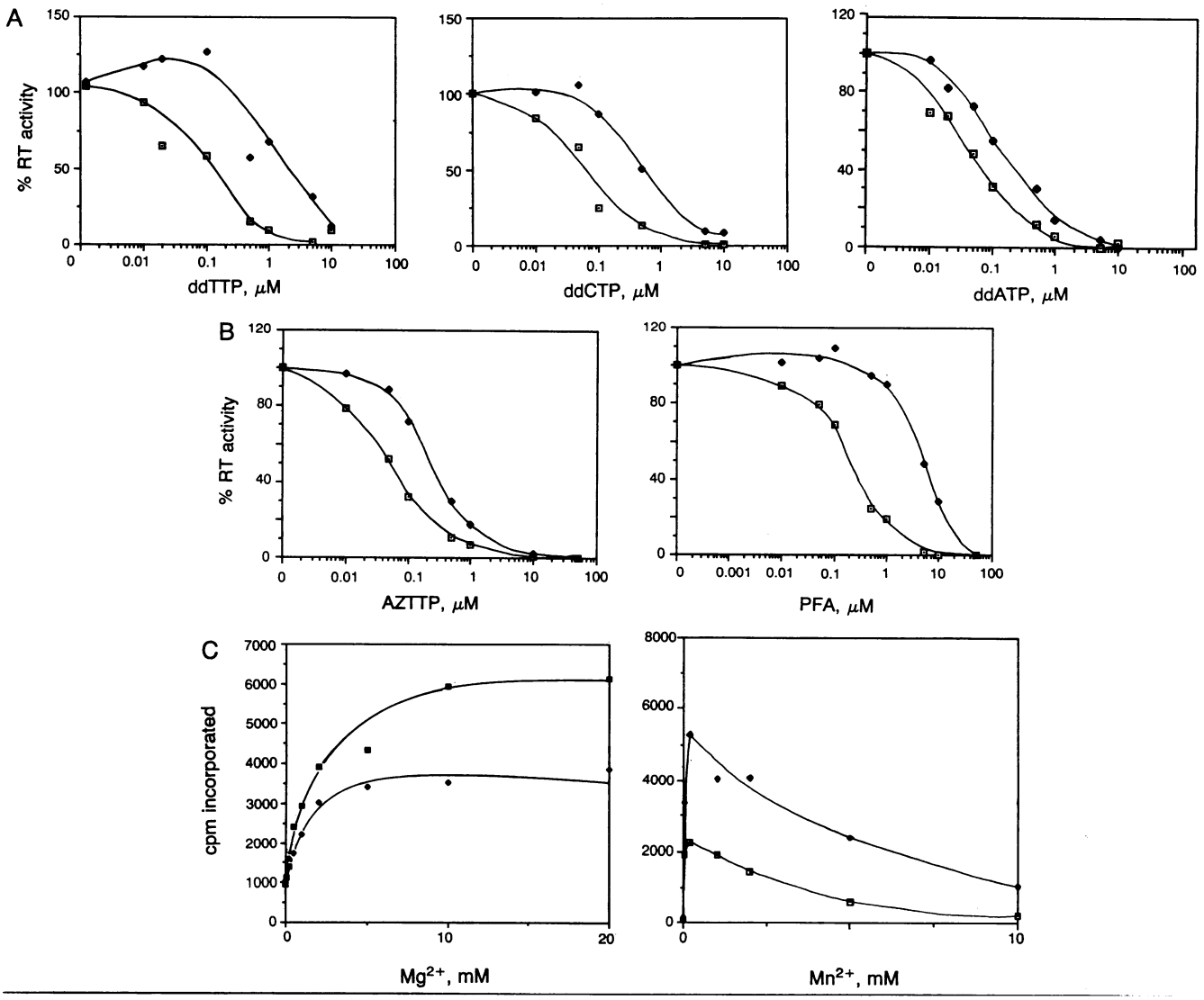

FIG. 3. Properties of wild-type (□) and pddGTPr-1 (◆) RT. (*A*) Crossresistance to ddNTPs. (*B*) Crossresistance to other inhibitors. (*C*) Dependence on divalent cation.

slightly low or normal activity compared with the wild-type at all concentrations of Mg^{2+} but was significantly more active than wild type with Mn^{2+} (Fig. 3*C*). The overall shapes of the curves, and the optimal concentrations, were similar for the two enzymes.

Replication of Viruses Bearing the ddGTPr-1 Mutation. To determine whether the mutation in pddGTPr-1 was compatible with virus replication, we excised a DNA fragment containing the mutation from pddGTPr-1 and used it to replace the corresponding fragment of two complete proviral DNAs, pHXB2 and pR7/3/BH10 (18, 19). The mutant DNAs were introduced into Jurkat-tat cells (14), and the appearance of virus was monitored by examination of the cultures for cytopathic effect and by assay for the appearance of RT activity in the culture medium. The mutant DNAs induced the formation of virus with kinetics and yield indistinguishable from those of the respective wild-type parental DNAs. There was no discernible difference in the cytopathic effect induced by the corresponding mutant and wild-type viruses. To determine whether the virion-derived enzyme would display the same altered drug sensitivity as the bacterially derived enzyme, we harvested virus from the culture supernatants and carried out RT assays in various levels of ddGTP. The enzyme extracted from the mutant virions displayed the same profile of increased drug resistance as the mutant bacterial enzyme (data not shown). Thus, the mutation was fully compatible with normal virus replication, was stably retained by the mutant virus during replication in culture, and conferred the same resistance on the enzyme, as measured by assays *in vitro*.

To determine whether the mutant viruses would manifest drug resistance in their replication in culture, we measured the kinetics of release of virus in infected cultures maintained in various levels of drugs. Virus replication was initiated in human lymphocytic cell lines either by transfection with mutant DNA or by infection with virus supernatants, and after 24 hr the cultures were divided into replicate dishes. The cultures were maintained in medium with various levels of two inhibitors: the cognate prodrug, ddG, and the drug showing the highest crossresistance, PFA. Culture supernatants were harvested at various times postinfection and assayed under standard conditions for virion-associated RT. The mutant virus was profoundly resistant to PFA as compared with the wild type (Fig. 4*A*). In cultures containing 10 and 100 μM PFA, replication of the wild-type virus was significantly slowed, while that of the mutant was unaffected; at 1000 μM PFA, replication of the wild-type virus was almost completely suppressed, while that of the mutant was only delayed. We conclude that the mutation, isolated on the basis of its effect on RT activity *in vitro*, could confer a biologically significant phenotype on virus replication *in vivo*. In contrast to these results with PFA, the mutant virus was not detectably more resistant to ddG than the wild type (Fig. 4*B*). As ddG was increased, both wild-type and mutant viruses were similarly inhibited: at 5 μM drug, the replication of both viruses was delayed, and at 50 μM drug, replication of both was strongly suppressed. Thus, significant resistance to some drugs, but not all, was conferred on the virus by the mutation. This finding is similar to results with several AZTTP-resistant mutants (8).

DISCUSSION

Previous efforts to isolate drug-resistant variants of the HIV-1 RT have depended either on the recovery of drug-resistant virus from infected patients (3) or on *in vitro* mutagenesis of HIV RT expression constructs and subsequent testing of individual mutants for resistance (8). The direct screening of large numbers of randomly mutagenized clones as described here can rapidly identify variants with

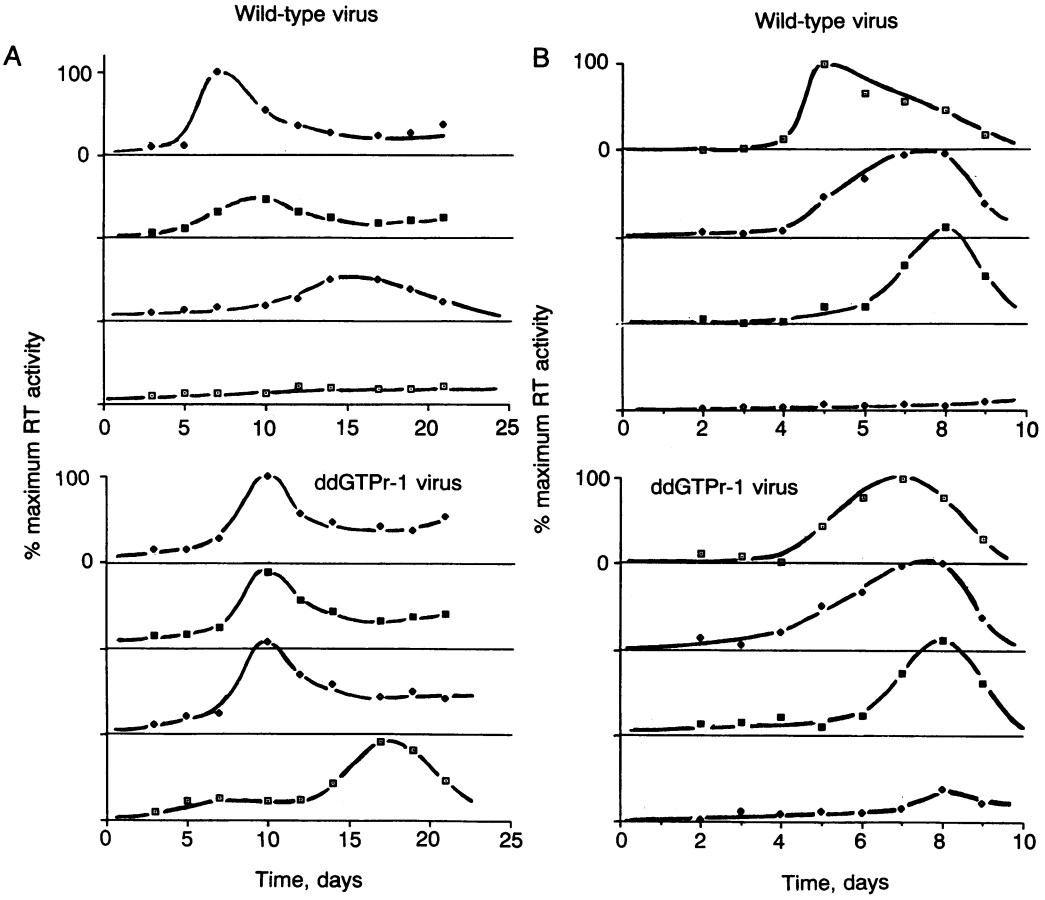

FIG. 4. Kinetics of replication of HIV-1 containing the ddGTPr-1 mutation, determined in the presence of various levels of inhibitors. (*A*) RT released after infection of lymphocytes with either wild-type or ddGTP-resistant HIV-1 (R3BH10) as indicated, in the presence of 0 μM (◆), 10 μM (□), 100 μM (◇), or 1000 μM (⊡) PFA. (*B*) RT released after transfection of lymphocytes with cloned proviral DNAs and growth in the presence of 0 μM (⊡), 0.1 μM (◆), 5 μM (□), or 50 μM (◇) ddG. Aliquots of the supernatant culture medium were assayed for RT activity as a measure of progeny virus at the indicated times postinfection.

desired properties. The ease and relatively high frequency (1 in 50,000) with which a ddGTP-resistant mutant was recovered in this study suggests that similar mutants resistant to many compounds can also be generated.

RT is an important target for antiviral therapy directed against AIDS (20), and thus it is noteworthy that a single point mutation was able to confer on the HIV-1 RT a significant level of drug resistance. The mutation frequency of retroviral replication (21, 22), attributable to the high error rate of RT (23, 24), is such that variants arise extremely rapidly and come to predominate in patients undergoing antiviral treatment. It would be alarming if such simple mutations can confer a broad crossresistance to other inhibitors *in vivo*. The result suggests that mutations like that in pddGTPr-1 might appear as combination drug treatments are applied, and that drugs of distinct classes should be used to avoid the appearance of viruses exhibiting simultaneous resistance (25, 26).

We speculate that the mutation affects the binding of all these phosphorylated inhibitors without severely altering the binding of the natural dNTPs. Thus, the natural Glu^{89} residue may be located at or near the triphosphate binding site or may indirectly perturb the structure of that site. That the mutation affects the requirement for divalent cation, presumably bound to the triphosphate, supports this notion. This residue is conserved among the known isolates of HIV-1, HIV-2, and various simian immunodeficiency viruses, though not among more distantly related retroviruses (27). The mutation is near, though not identical, to some of the mutations found in AZT-resistant variants of HIV-1 isolated from patients, causing changes at amino acids 67, 70, 215, and 219 (ref. 4). Ultimately, when the three-dimensional structure of a member of the RT family is known, mutations like that in pddGTPr-1 will be important in locating binding sites and understanding the detailed mechanism of action of the enzyme.

It is curious that the ddGTP-resistance mutation confers on the virus resistance to PFA, but not to ddG, for its replication in culture. Similarly, mutants of HIV RT that are resistant to AZTTP have been found not to confer resistance to AZT on the virus (9). It is possible that in the case of chain-terminator inhibitors in general, resistance measured *in vitro* may not correlate with resistance measured *in vivo*; the assay conditions used in the *in vitro* assays may simply be inappropriate, but alternatively it may be that there is a fundamental difference in the mechanism of action of the drugs between the two settings. It may be, for example, that the primary mechanism of inhibition *in vivo* is not through chain termination alone but rather through effects on enzyme elongation rate or processivity, or on specific steps in retroviral reverse transcription such as strong stop DNA translocation. It might also be that the presence of other viral proteins at the time of reverse transcription *in vivo* could significantly alter the response of the enzyme to inhibitors. The approach described here will allow the rapid characterization of mutants of RT resistant to various clinically relevant drugs and may assist in the understanding of the precise mechanisms by which chain-terminator inhibitors interfere with HIV replication.

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