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A Screen in *Escherichia coli* for Nucleoside Analogs That Target Human Immunodeficiency Virus (HIV) Reverse Transcriptase: Coexpression of HIV Reverse Transcriptase and Herpes Simplex Virus Thymidine Kinase

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Human immunodeficiency virus (HIV) reverse transcriptase substitutes for temperature-sensitive DNA polymerase I (Pol I^{ts}) in *Escherichia coli*, providing a screen for anti-HIV reverse transcriptase nucleoside analogs in bacteria. Since phosphorylation of nucleosides in *E. coli* is limited to thymidine and its derivatives, we coexpressed herpes simplex virus thymidine kinase, an enzyme that phosphorylates a wide variety of nucleoside analogs, together with HIV reverse transcriptase. Coexpression of herpes simplex virus thymidine kinase and HIV reverse transcriptase rendered Pol I^{ts} cells sensitive to dideoxycytidine. Studies with different nucleoside analogs indicate that this bacterial screening system is able to select and identify nucleoside analogs that specifically target HIV reverse transcriptase.

Human immunodeficiency virus (HIV) reverse transcriptase (RT) is a major target for the chemotherapy of HIV infection. Most clinically approved anti-HIV RT drugs are nucleoside analogs that are incorporated by HIV RT and terminate DNA synthesis, thereby preventing viral replication (14). However, emergence of resistant viral mutants early in the course of therapy with nucleoside analogs such as zidovidine (AZT) frequently limits their effectiveness (5, 13). That misincorporation by HIV RT is a major source of the high mutation rate of the virus is in accord with in vitro studies indicating that HIV RT is the most error-prone DNA polymerase thus far reported (1, 2, 20). We have demonstrated that HIV RT can functionally substitute for DNA polymerase I in *Escherichia coli* (11). Expression of HIV RT allows an *E. coli* mutant harboring a temperature-sensitive DNA polymerase I (Pol I^{ts}) to grow at the nonpermissive temperature. We have proposed that this genetic complementation system could serve as a safe and rapid screen for nucleoside analogs directed against HIV RT. However, the system would be limited by the narrow substrate specificity of nucleoside kinases present in the bacterial host. In the case of AZT, a thymidine analog, phosphorylation to the monophosphate is catalyzed by *E. coli* thymidine kinase (TK) (3, 15); further phosphorylation to the triphosphate is catalyzed by nonspecific nucleoside monophosphate kinases (4), and as a result, *E. coli* strains harboring HIV RT are sensitive to AZT. In contrast, dC and its analogs are not incorporated into DNA in *E. coli* because the bacteria lack a dC kinase (16), and these compounds are not substrates for *E. coli* TK. Lack of a dC kinase and other nucleoside kinases in *E. coli* and the restricted substrate specificity of *E. coli* TK limits the types of nucleoside analogs that can be screened in *E. coli*.

To extend the utility of the *E. coli* complementation system to a broader spectrum of nucleoside derivatives, such as dC analogs, we coexpressed HIV RT and herpes simplex virus (HSV) TK. HSV TK phosphorylates a wide variety of nucleoside analogs, including ddC (6a), cytosine arabinoside (araC) (6), acyclovir (3, 7), and gancyclovir (3), as well as thymidine analogs such as AZT (15). We tested whether coexpression of HSV TK and HIV RT confers sensitivity to several analogs that are not phosphorylated by *E. coli* enzymes.

Coexpression of HIV RT and HSV TK in Pol I^{ts} cells. pRT-TK, a dual plasmid that expresses both HIV RT and HSV TK, was assembled by inserting the *tk* gene into a low-copy-number plasmid, pHIVRT (Fig. 1A), that contains the gene for HIV RT under the control of *lac* promoter/operator (11). HIV RT and HSV TK encoded by these plasmids are transcribed as a single mRNA from the *lac* promoter/operator. To measure the expression of HIV RT and HSV TK, extracts were prepared from Pol I^{ts} cells harboring pHIVRT, pTK, or pRT-TK. After electrophoresis, Western blotting (immunoblotting) was carried out by using antibodies against HIV RT and HSV TK. As seen in Fig. 1B, immunopositive bands from cells harboring pHIVRT (lane 2) and pTK (lane 4) comigrated with purified HIV RT (lane 1) and purified HSV TK (lane 3), respectively. A nonspecific band migrating nearly coincident with HSV TK was observed in the extract of Pol I^{ts} cells carrying pHIVRT (lane 2); its intensity was less than that of the HSV TK in lane 3. Most importantly, extracts of cells harboring pRT-TK (lane 5) exhibited two immunopositive bands that migrate coincident with the individual purified viral proteins.

Phosphorylation of nucleosides. We measured the ability of extracts of Pol I^{ts} cells harboring plasmids that express HIV RT, HSV TK, or both to phosphorylate thymidine, AZT, or dC. As seen in Table 1, extracts of *E. coli* carrying the parent plasmid, pHSG576, or each of the modified plasmids phosphorylated thymidine at approximately equal rates. Similarly, AZT was phosphorylated at similar rates by each of the extracts, suggesting that the endogenous TK is the major activity that phosphorylates AZT. Phosphorylation of AZT by *E. coli* TK is in accord with previous results (15). In contrast, significant phosphorylation of dC was observed only with extracts of cells carrying plasmids that encode HSV TK, suggesting that HSV TK expression confers the ability to phosphorylate dC on

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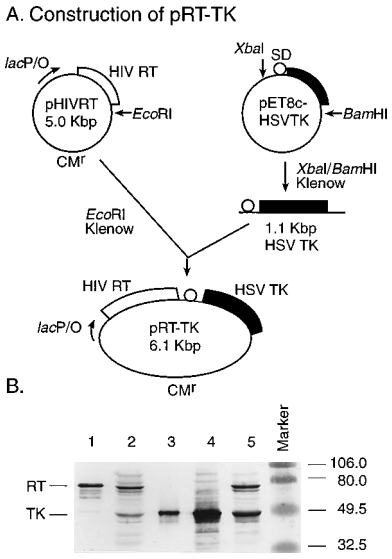

FIG. 1. Expression of HIV RT and HSV TK in *E. coli* Pol I^{ts} cells. (A) Construction of pRT-TK expressing HIV RT and HSV TK. The HSV *tk* gene containing the ribosomal binding site (SD) was obtained from pET8c-HSVTK (M. Black, Darwin Molecular Corp.). The 1.1-kb *Xba*I-*Bam*HI fragment of pET8c-HSVTK containing the HSV *tk* gene was blunt ended by Klenow fragment, and this fragment was inserted into the blunt-ended *Eco*RI site of pHIVRT, generating the dual construct pRT-TK. pTK is pHSG576 containing the 1.1-kb HSV *tk* gene at the *Sma*I site. pHIVRT, pRT-TK, and pTK each contain the DNA polymerase I-independent pSC101 replication origin. Pol I^{+} refers to an *E. coli* strain with the genotype *trpE65 uvr-155 lon-11 sulA1. E. coli* NM522 (Stratagene) was used as a host for plasmid construction. Pol I^{ts} refers to the *E. coli* B/r strain SC18-12, with the genotype *polA12 recA718 trpE65 uvr-155 lon-11 sulA1*. Nutrient broth was used for culturing Pol I^{+} and Pol I^{ts} strains. Chloramphenicol (30 μg/ml) was used for the selection of pHSG576 derivatives, and tetracycline (12.5 μg/ml) was used for the culture of Pol I^{+} and Pol I^{ts} strains. Isopropythiogalactopyranoside (IPTG; 1 mM) was used for the induction of viral protein expression from plasmids. *lac*P/O, *lac* promoter/operator. (B) Western analysis. Pol I^{ts} cells containing pHIVRT and pRT-TK were grown to log phase at 37°C, and Pol I^{ts} cells containing pTK were grown to log phase in nutrient broth containing antibiotics at 30°C. Protein expression was induced at an optical density at 600 nm of 0.1 by the addition of IPTG (1 mM) 3 h prior to harvest. Approximately 2×10^6 cells for pRT-TK and pTK analysis and 10^6 cells for pHIVRT analysis were loaded on a sodium dodecyl sulfate–12% polyacrylamide gel (21). HIV RT and HSV TK were visualized by using primary antibodies against HIV RT p66 (monoclonal; from P. Yoshihara, NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases) and HSV TK (polyclonal; from W. Summers, Yale University), secondary antibodies conjugated to alkaline phosphatase (goat anti-mouse immunoglobulin G for anti-HIV RT and goat anti-rabbit immunoglobulin G for anti-HSV TK, Bio-Rad), and Western detection reagents (Bio-Rad). Lanes: 1, purified HIV RT (this study); 2, HIV RT from Pol I^{ts} cells with pHIVRT; 3, HSV TK in vitro translated (M. Black, Darwin Molecular Corp.); 4, HSV TK from Pol I^{ts} cells with pTK; 5, HIV RT and HSV TK from Pol I^{ts} cells with pRT-TK; 6, molecular weight markers (positions indicated in kilodaltons).

host cells. Lack of phosphorylation of dC by extracts of cells containing either the parent plasmid or pHIVRT (i.e., less than 0.5% of that observed for HSV TK-expressing cells) confirms that *E. coli* does not have an enzyme that phosphorylates dC (16) and confirms that the substrate specificity of *E. coli* TK is restricted. HSV TK can also phosphorylate ddC and araC, although the activities for ddC (6a) and araC (6) are much lower than that for dC (6).

Sensitivity to AZT. The effect of HSV TK on sensitivity to AZT was determined by comparing survival of Pol I^{ts} cells expressing HIV RT either alone or together with HSV TK. Complementation of Pol I^{ts} cells by pHIVRT or by the dual plasmid pRT-TK was measured as the ratio of plating efficiencies at 30 and 37°C. In this experiment, the plating efficiencies at 37°C of Pol I^{ts} cells harboring pHIVRT and pRT-TK were

TABLE 1. Phosphorylation of thymidine, AZT, and dC by *E. coli* extracts[a]

Substrate	Kinase activity (fmol/min)			
	pHSG576	pHIVRT	pRT-TK	pTK
Thymidine	8,150	7,917	8,200	9,367
AZT	1,333	1,472	1,442	1,548
dC	<3	<3	742	795

[a] Assay mixtures contained radiolabeled nucleoside (1 μM [^{3}H]thymidine 50 μM [^{3}H]dC, or 10 μM [^{3}H]AZT; all from Moravek, Brea, Calif.), 12.5 mM ATP, 100 mM sodium phosphate (pH 6), 30 mM magnesium acetate, and 5 μl of a lysed cell extract equivalent to 10^6 cells in a total volume of 10 μl. After incubation at 37°C for 30 min, the reaction was stopped by adding 40 μl of cold water and spotted onto a Whatman DE81 filter (3). Filters were washed three times with 4 mM ammonium formate (pH 7.5) containing 10 μM thymidine, once with water, and then twice with 95% ethanol and dried (9). Radioactivity was determined by scintillation counting; counting efficiency was approximately 2%.

96 and 94%, respectively, of that at 30°C. These results indicate that coexpression of HSV TK does not interfere with the ability of HIV RT to substitute for DNA polymerase I. Sensitivity of transformed cells to AZT was determined by plating cells on nutrient agar (NA) containing increasing concentrations of AZT. As seen in Fig. 2A, Pol I^{ts} cells expressing HIV RT are sensitive to AZT only at the restrictive temperature of 37°C, at which survival is dependent on the expression of HIV RT. Coexpression of HSV TK did not alter sensitivity to AZT (Fig. 2B). This observation is not surprising for several reasons. First, *E. coli* TK has been shown to phosphorylate AZT, and its activity in vivo may exceed that resulting from expression of HSV TK. Second, AZT phosphorylation may not be the limiting determinant of lethality. Rather, phosphorylation of AZT monophosphate by host kinase activity, incorporation into DNA by HIV RT, or nucleolytic degradation at replication forks could, either separately or in complex interaction with yet other factors, limit induction of lethality. Third, feedback inhibition of both host and HSV TKs by dTTP may guarantee a constant rate of production of AZT monophosphate, regardless of total cellular TK activity. Both *E. coli* TK (17) and HSV TK (6) are regulated by feedback mechanisms involving TTP.

In parallel experiments, we observed that AZT concentrations of up to 4 μM did not inhibit growth at 30°C, a temperature at which DNA polymerase I^{ts} is active. In addition, growth of wild-type Pol I^{+} cells carrying pRT-TK (Fig. 2B) or pHIVRT (data not shown) was not inhibited by AZT at 37°C. These observations indicate that AZT triphosphate is not stably incorporated into DNA by *E. coli* DNA polymerases under the experimental conditions.

Pol I^{ts} cells expressing HIV RT or HIV RT and HSV TK are insensitive to araC. AraC is a terminator of DNA synthesis. In eukaryotic cells, it is phosphorylated to the triphosphate and incorporated by DNA polymerases but extended with very low efficiency (18). The araC sensitivity of Pol I^{ts} cells expressing HIV RT alone or both HIV RT and HSV TK was determined by plating the transfected cells on NA plates containing graded amounts of the analog. HSV TK has been reported to efficiently phosphorylate araC (6). However, even with the dual vector, growth was not diminished at the highest araC concentrations tested (Fig. 2C). The most direct explanation is that even though HSV TK phosphorylates araC, HIV RT does not utilize the triphosphate as a substrate. In accord with this explanation, we did not observe elongation of a DNA template in an in vitro primer extension assay using purified HIV RT and araC triphosphate in place of dCTP (our unpublished data). This inability of HIV RT to incorporate araC into DNA

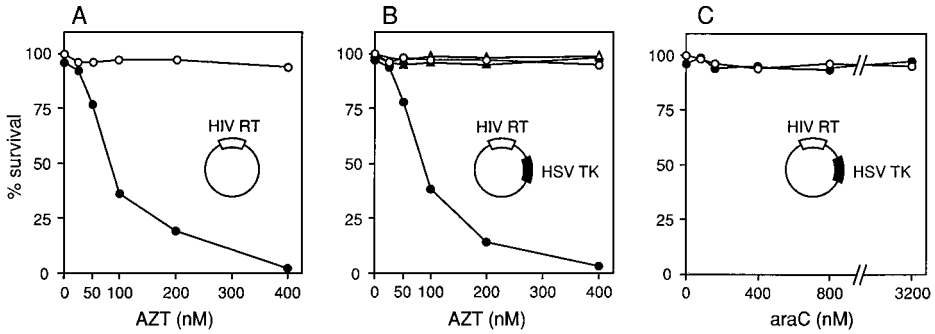

FIG. 2. AZT and araC sensitivity of Pol I^{ts} cells expressing HIV RT and HSV TK: genetic complementation of Pol I^{ts} by HIV RT. Pol I^{ts} cells were transformed by pHIVRT or pRT-TK. After determination of transformation efficiency at 30°C, about 250 transformed cells were spread on NA plates containing antibiotics and IPTG. Duplicate plates containing NA (23 g/liter; Difco) with NaCl (4 g/liter) were incubated at 30 and 37°C for 48 h in the presence of the indicated concentrations of AZT (Sigma) or araC (Sigma) in addition to antibiotics and IPTG. The percent survival at different concentrations of each nucleoside analog was determined as the ratio of colonies with and without analog at 30 and 37°C. (A) Sensitivity of Pol I^{ts} cells with pHIVRT to AZT at 30°C (○) and 37°C (●); (B) sensitivity of Pol I^{ts} cells with pRT-TK to AZT at 30°C (○) and 37°C (●) and sensitivity of Pol I^{+} cells with pHIVRT to AZT at 30°C (△) and 37°C (▲); (C) sensitivity of Pol I^{ts} cells with pRT-TK to araC at 30°C (○) and 37°C (●).

is unexpected because HIV RT is promiscuous in deoxynucleoside triphosphate selectivity.

Coexpression of HSV TK renders HIV RT-expressing Pol I^{ts} cells sensitive to ddC. ddC is a terminator of DNA synthesis currently used in the treatment of AIDS. Incubation of Pol I^{ts} cells expressing HIV RT alone did not result in decreased survival at the restrictive temperature at which growth is dependent on HIV RT (Fig. 3A). In contrast, coexpression of HSV TK renders cells sensitive to ddC, but only at the restrictive temperature (Fig. 3B). Incubation with ddC did not inhibit the growth of Pol I^{ts} cells at 30°C (Fig. 3B), and Pol I^{+} cells expressing both viral proteins were not inhibited by ddC (data not shown). These observations imply that inhibition of growth is dependent on both the phosphorylation of ddC by HSV TK and incorporation of the ddCTP by HIV RT. Several observations suggest that ddCTP synthesized in HSV TK-expressing Pol I^{ts} cells is incorporated into DNA by HIV RT but not by DNA polymerase I under our experimental conditions. First, growth of Pol I^{+} cells expressing HSV TK or both HIV RT and HSV TK is not inhibited by ddC (data not shown). Second, at the permissive temperature at which complementation by HIV RT is not required for growth, Pol I^{ts} cells expressing HSV TK and HIV RT are insensitive to ddC (Fig. 3B). Third, at 37°C, a nonpermissive temperature at which HIV RT is required for DNA replication, Pol I^{ts} cells expressing pHIVRT are resistant to ddC; however, coexpression of HSV TK confers sensitivity to ddC at this temperature (Fig. 3B). Resistance to ddC of cells employing Pol I for growth could be due to inefficient incorporation or to excision by the 3′-to-5′ exonuclease activity of the enzyme. The latter is unlikely, however, because absence of a 3′-OH group at ddCMP termini blocks removal by DNA polymerase I and most exonucleases that have been purified extensively.

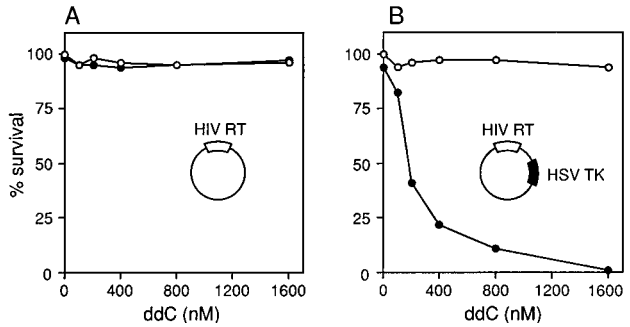

FIG. 3. Sensitivity of Pol I^{ts} cells expressing HIV RT and HSV TK to ddC. Percent survival was determined as indicated in the legend to Fig. 2. (A) Sensitivity of Pol I^{ts} cells with pHIVRT to ddC (Sigma) at 30°C (○) and 37°C (●); (B) sensitivity of Pol I^{ts} cells with pRT-TK to ddC at 30°C (○) and 37°C (●).

In summary, coexpression of HIV RT and HSV TK in Pol I^{ts} cells allows use of *E. coli* to screen dC analogs as well as dT analogs that target HIV RT. These studies provide an essential step in increasing the utility of this bacterial complementation system. Current methods for screening anti-HIV RT drugs heavily rely on human cell culture systems infected with live HIV (10, 19). These methods require extensive safety precautions and are expensive and inefficient. An alternative method utilizing an in vitro primer extension assay to measure incorporation of nucleoside analogs into DNA by purified HIV RT has been used (10, 19). Whether this assay accurately reflects the conditions under which HIV RT catalyzes DNA synthesis in cells needs to be determined. The bacterial screen for HIV RT inhibitors discussed here may be appropriate as a primary screen because it is safe, inexpensive, and efficient enough to process large numbers of candidate drugs. Moreover, the effects of various concentrations of inhibitors can be rapidly assessed on agar plates that contain inhibitor gradients (8). Other nucleoside kinases can now be expressed together with HIV RT, and ultimately, a series of tester strains can be established to analyze different sets of potential inhibitors of HIV RT.

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