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Human immunodeficiency virus reverse transcriptase substitutes for DNA polymerase I in *Escherichia coli*

(AIDS/3′-azido-3′-deoxythymidine)

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ABSTRACT **We present evidence that human immunodeficiency virus (HIV) reverse transcriptase (RT) can substitute for DNA polymerase I in bacteria. Expression of HIV RT enables an *Escherichia coli* mutant, *polA12 recA718*, containing a temperature-sensitive mutation in DNA polymerase I, to grow at a nonpermissive temperature. The plasmid pBR322 contains a DNA polymerase I-dependent origin of replication. Expression of HIV RT enables the same *E. coli* mutant to maintain this plasmid at a nonpermissive temperature. Furthermore, expression of HIV RT in this mutant renders it sensitive to 3′-azido-3′-deoxythymidine, a commonly used anti-AIDS drug that targets HIV RT. These combined findings on the genetic complementation of DNA polymerase I by HIV RT provide a bacterial assay to screen for drugs directed against HIV RT. Genetic complementation provides a method for positive selection of large numbers of functional HIV RT mutants for studies on structure–function relationships.**

The most useful drugs for the treatment of AIDS are nucleoside analogs that target the viral reverse transcriptase (RT) (1–3). The design of specific inhibitors for human immunodeficiency virus (HIV) RT should be facilitated by the recent establishment of a high-resolution crystal structure of HIV RT (4). However, the three-dimensional structure of the wild-type enzyme may not be sufficient to guide these efforts, since mutations in HIV RT arise frequently in infected individuals and render the virus resistant to nucleoside analogs and other antiviral therapies (5). Methods to determine the spectrum of HIV RT mutations present in viral isolates from individual patients and to study the effects of different mutations on drug resistance could be instrumental in guiding the design of therapies to prevent the emergence of drug-resistant viruses.

Escherichia coli DNA polymerase I (pol I) was the first DNA polymerase to be purified and has been the most extensively studied (6). pol I is a 109-kDa protein that possesses multiple catalytic activities. In addition to the polymerization of complementary deoxynucleoside triphosphates, pol I functions in proofreading errors in polymerization by catalyzing the exonucleolytic hydrolysis of DNA in the 3′ → 5′ direction and functions in degrading RNA primers on the lagging DNA strand through the action of its 5′ → 3′ exonucleolytic activity. We have previously reported that mammalian DNA polymerase β (pol β) can substitute for *E. coli* pol I in DNA replication by polymerizing nucleotides to fill in gaps between Okazaki fragments (7, 8). HIV RT catalyzes the synthesis of a cDNA using the viral RNA as a template and then copies the newly synthesized cDNA to produce a double-stranded DNA product (9). HIV RT contains an RNase H activity that hydrolyzes the RNA strand of a DNA·RNA hybrid, and this activity might serve to remove RNA primers during the course of DNA replication. This similarity in catalytic activities suggests that HIV RT might also substitute for *E. coli* pol I.

In this paper we establish that HIV RT can substitute for *E. coli* pol I in promoting cell growth of *polA12 recA718* cells and in plasmid replication. This genetic complementation provides a method for collecting a large number of HIV RT mutants for studies on the structure–function of HIV RT and for the design of drugs directed against HIV RT, as well as an approach using bacteria for the screening anti-AIDS drugs that target HIV RT.

MATERIALS AND METHODS

Strains and Media. pol I^{ts} refers to the SC18-12 *E. coli* B/r strain, which has the genotype *recA718 polA12 uvr155 trpE65 lon-11 sulA1*, and the pol I^+ strain has the genotype *uvr155 trpE65 lon-11 sulA1* (10). *E. coli* NM522 (Stratagene) was used for cloning and plasmid constructions. Difco nutrient agar (NA) containing NaCl (4 g/liter) was used for studies on genetic complementation. Nutrient broth (NB) was used for culturing pol I^+ and pol I^{ts} strains and was prepared by directions of Difco. Chloramphenicol (30 μg/ml) was used for the selection of pHSG576 derivative plasmids, carbenicillin (50 μg/ml) was used for maintenance of pBR322, and tetracycline (12.5 μg/ml) was used for growing pol I^+ and pol I^{ts} strains.

Plasmids. pHSG576 is a low-copy-number plasmid containing a chloramphenicol-resistance marker (11). pHIVRT is the corresponding plasmid expressing HIV RT. It was generated from pJS-RT (Joann B. Sweasy, Yale University, New Haven, CT) by using a 5′ primer containing a *Hin*dIII site, a ribosomal binding site and 15 nucleotides at the 5′ N terminus of HIV RT sequence, and a 3′ primer containing 18 nucleotides at the 3′ C terminus and an *Eco*RI site. After amplification by the polymerase chain reaction, the product was fused to the *lac* promoter/operator in pHSG576 by digestion with *Hin*dIII and *Eco*RI. A 61-bp noncoding DNA sequence was inserted at the *Hin*dIII site to reduce the expression levels. D186N (pHIVRT-DN) and T215Y HIV RT (pHIVRT-TY) mutants were constructed by site-directed mutagenesis (B.K. and T. R. Hathaway, unpublished data), and expression of these two mutant proteins in the pol I^{ts} strain was quantitated by Western blot analysis (data not shown). pβL is pHSG576 derivative plasmid expressing rat pol β (8).

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Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; pol I, DNA polymerase I; pol β, DNA polymerase β; AZT, 3′-azido-3′-deoxythymidine; IPTG, isopropyl β-D-thiogalactoside.

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Complementation. The pol I^{ts} strain was transformed with plasmid pHSG576, pHIVRT, or pHIVRT-DN and was grown in NB medium to logarithmic phase at 30°C. Thereafter, ≈10 μl (10^6 cells) was introduced at the center of an agar plate, and an inoculation loop was gradually moved from the center to the edge of the plate as the plate was rotated. This procedure yielded a higher density of colonies in the center than at the edge of the plates. Duplicate plates were incubated at 30 or 37°C for 48 hr. To determine complementation efficiency of HIV RT, cultures of the pol I^{ts} strain harboring either pHSG576 or pHIVRT were diluted with NB medium and plated. Duplicate plates were incubated at 30 or 37°C, and after a 48-hr incubation visible colonies were counted. In experiments on the maintenance of pBR322, the pol I^{ts} strain harboring plasmid pHSG576 or pHIVRT and the pol I$^+$ strain with pHSG576 were transformed with pBR322. The cultures were grown at 30°C to logarithmic phase in NB medium containing tetracycline, chloramphenicol, carbenicillin, and isopropyl β-D-thiogalactoside (IPTG; 1 mM) as indicated above. The transformed cells were plated on NA plates containing carbenicillin, and the plates were incubated at 30, 37, 39, or 42°C for 48 hr.

3′-Azido-3′-deoxythymidine (AZT) Sensitivity. *E. coli* pol I^{ts} cells harboring pβL, pHIVRT, or pHIVRT-TY were grown, and about 300 cells were plated onto NA plates containing 0, 50, 100, 150, 200, or 250 nM AZT (Sigma). Duplicate plates were incubated at 30 or 37°C for 48 hr. The percentage of cell survival at each AZT concentration is the ratio of the number of colonies obtained at 37°C to the number of colonies obtained at 30°C times 100%.

Positive Genetic Selection. A mixture of pHIVRT and pHSG576 (1:200) was used to transform the *polA12 recA718* strain. Transformation efficiency was determined by incubating the transformed cells at 30°C. For genetic selection, 4000 transformed cells at a density of about 100 cells per plate were incubated at 37°C for 48 hr. Colonies grown at 37°C were isolated, and plasmids were characterized by *Eco*RI restriction digestion, which discriminates between plasmid DNA from pHSG576 and pHIVRT.

Western Blot Analysis. Cells expressing HIV RT or pol β were grown to logarithmic phase and then incubated with or without IPTG (1 mM) for 3 hr. The harvested cells were mixed with SDS stop buffer and were applied to SDS/12% PAGE gels. Proteins in the gel were transferred to nitrocellulose filters, and HIV RT and rat pol β were visualized (8). The approximate number of molecules per cell was estimated by coelectrophoresis with graded amounts of purified HIV RT (12, 13) and rat pol β (Joann B. Sweasy). Secondary antibody against p66 HIV RT was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health); monoclonal antibody to HIV-1 RT protein (p66) from Epitope (Beaverton, OR). Secondary antibody against rat pol β was provided by Joann B. Sweasy.

RESULTS

Complementation by HIV RT for *E. coli* pol I. The *E. coli* double mutant *polA12 recA718* contains a temperature-sensitive pol I (pol I^{ts}) (10). This mutant is unable to grow at 37°C in rich media at low cell density due to a failure of pol I to join Okazaki fragments during lagging-strand DNA synthesis (7, 8, 10). This cell density-dependent temperature-sensitive phenotype is demonstrated (Fig. 1) by plating concentric dilutions of *E. coli polA12 recA718* in nutrient agar (7, 10). In this experiment, 2×10^6 *E. coli* cells were introduced at the center of a plate by using a 10-μl inoculation loop. The plate was rotated, and the loop was gradually moved to the periphery to display the bacteria in a diverging spiral of increasing dilution. Wild-type *E. coli, polA*$^+$ *recA*$^+$ (pol I$^+$), containing the parent plasmid (pHSG576), which lacks the HIV RT gene (11), is able to grow at both at 30°C and 37°C (Fig. 1*A*), whereas the pol I^{ts} mutant containing the parent plasmid (pHSG576), which lacks the HIV RT gene, grows at 30°C at all dilutions tested but can only grow at 37°C at the high density in the center of the plate (Fig. 1*B*). This growth deficit can be complemented in the pol I^{ts} mutant by a plasmid expressing HIV RT (pHIVRT); the infected *E. coli* are able to grow at 37°C at low density near the periphery of the plate (Fig. 1*C*). HIV RT D186N contains a mutation at the substrate binding site and expresses a nonfunctional RT (14). As shown in Fig. 1*D*, the *E. coli* double mutant harboring the plasmid that expresses D186N HIV RT (pHIVRT-DN) is unable to grow at 37°C, further indicating that genetic complementation of pol I by HIV RT requires HIV RT to be active.

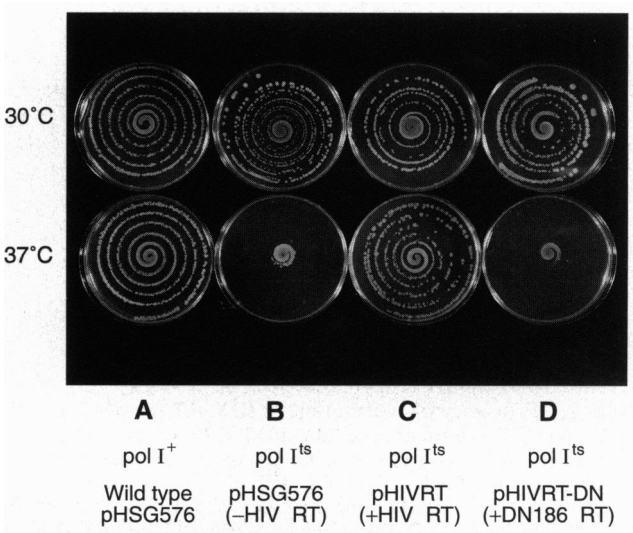

FIG. 1. Functional complementation of *E. coli* pol I by HIV RT. *E. coli polA*$^+$ *recA*$^+$ (JS295; pol I$^+$) and *polA12 recA718* (JS200; pol I^{ts}) were transformed with pHSG576, a low-copy-number plasmid containing a pol I-independent pSC101 replication origin and a chloramphenicol-resistance gene (11). The *polA12 recA718* strain (tetracycline resistant) was transformed with pHIVRT and pHIVRT-DN. Transformed cells were grown to logarithmic phase in NB containing tetracycline (12.5 μg/ml), chloramphenicol (30 μg/ml), and IPTG (1 mM); then 2×10^6 cells were deposited and diluted by rotation with a 10-μl inoculation loop on a nutrient agar plate containing the same concentrations of tetracycline, chloramphenicol, and IPTG. Duplicate plates were incubated at 30°C or 37°C for 48 hr. (*A*) Growth of the wild-type *E. coli polA*$^+$ *recA*$^+$ strain with the parent plasmid, pHSG576, at 30°C and 37°C. (*B*) Growth of the *polA12 recA718* strain with pHSG576 at 30°C and 37°C. (*C*) Growth of the *polA12 recA718* strain with pHIVRT at 30°C and 37°C. (*D*) Growth of the *polA12 recA718* strain with pHIVRT-DN at 30°C and 37°C.

To quantitate the efficiency with which HIV RT substitutes for pol I, the plating efficiencies at 30 and 37°C using the pol I^{ts} mutant harboring either the parent plasmid, pHSG576, or a plasmid that expresses active HIV RT, pHIVRT, were measured. With increasing dilutions, *E. coli* with pHSG576 (HIV RT$^-$) are progressively defective in growth at 37°C (compare the number of colonies at 30°C and 37°C in Fig. 2). In contrast, *E. coli* expressing HIV RT form an equal number of colonies at 30°C and 37°C at all dilutions tested (Fig. 2). These results suggest that expression of HIV

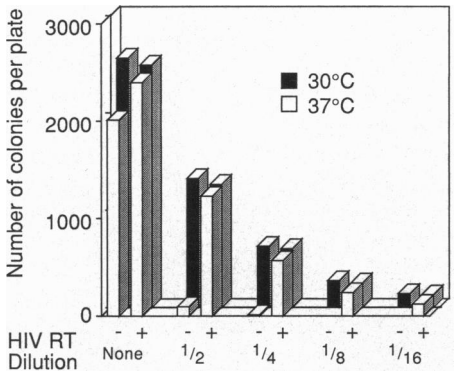

FIG. 2. Efficiency of substitution of HIV RT for pol I. *E. coli polA12 recA718* containing either pHSG576 or pHIVRT was grown to 2×10^8 cells per ml at 30°C. The indicated number of cells per plate in progressive dilutions was plated on nutrient agar containing tetracycline, chloramphenicol, and IPTG as given in *Materials and Methods*. Duplicate plates were incubated for 48 hr at 30°C (solid bars) or 37°C (open bars), and colonies were scored. The results given are averages from three different experiments. In the same type of experiment with *polA12 recA718* cells expressing rat pol β, the plating efficiency at a dilution of 1/16 (about 150 cells per plate) at 37°C was about 65% of that obtained at 30°C (data not shown). In NA plates without IPTG, the efficiency of the pol I^{ts} cells expressing rat pol β drastically decreased at low cell density, as was observed for pol I^{ts} cells containing pHSG576 (data not shown). In contrast, pol I^{ts} cells expressing HIV RT were able to grow at 37°C even in the absence of induction with a plating efficiency close to 80%. The pol I^{ts} strain containing the DN186 HIV RT mutant shows a decrease in plating efficiency at low cell density when grown at 37°C, similar to that observed with pol I^{ts} containing pHSG576 (data not shown).

RT fully complements the growth defect at elevated temperature and low density exhibited by the *polA12 recA718* strain.

We tested whether this genetic complementation by HIV RT can be used as a positive selection for active HIV RT mutants by introducing a transforming mixture (1:200) of plasmids expressing (pHIVRT) and not expressing (pHSG576) HIV RT into the *polA12 recA718* strain and selecting colonies that can grow at 37°C. Approximately 4000 transformed cells were incubated at 37°C for 48 hr at a cell density of about 100 cells per plate. Fifteen of 17 colonies tested that grew at 37°C contained pHIVRT. This experiment demonstrates that colonies expressing HIV RT can be selected from mixed populations by growing at 37°C at low cell density.

Table 1. Replication of pBR322 by HIV RT

	Plating efficiency, %		
	polA$^+$ *recA*$^+$	*polA12 recA718*	
Temp., °C	pHSG576	pHSG576	pHIVRT
37	94	0.5	27
39	91	<0.1	11
42	88	<0.01	<0.1

The *polA12 recA718* mutant strain harboring pHSG576 or pHIVRT and the *polA*$^+$ *recA*$^+$ wild-type strain containing pHSG576 were transformed with pBR322. Transformed cells were grown to 2×10^8 cells per ml in NB containing tetracycline (12.5 μg/ml), chloramphenicol (30 μg/ml), IPTG (1 mM), and carbenicillin (50 μg/ml). Cells were diluted and plated on nutrient agar plates containing tetracycline, chloramphenicol, carbenicillin, and IPTG as described above. Duplicate plates were incubated at 37, 39, and 42°C for 48 hr. Plating efficiencies (%) of each strain are shown as the ratio of the colony number at 37, 39, or 42°C to the colony number at 30°C times 100%.

Therefore, functional complementation of *E. coli* pol I by HIV RT can be used to identify active HIV RT mutants by positive genetic selection.

Plasmid Replication. The *E. coli polA12 recA718* double mutant is unable to maintain the plasmid pBR322 at the nonpermissive temperature since pol I is required in the initiation of DNA replication at the ColE1 origin (6, 10). We asked whether HIV RT could also substitute for pol I in ColE1 plasmid replication. *E. coli polA12 recA718* harboring the parent plasmid or the plasmid expressing HIV RT was transformed with pBR322 and plated at 37°C in nutrient agar containing carbenicillin. The pol I^+ wild-type strain harboring both the parent plasmid and pBR322 grows equally well at 30°C and 37°C (Table 1). In contrast, growth of the pol I^{ts} strain containing the parent plasmid and pBR322 at 37°C is limited (plating efficiency = 0.5%). The pol I^{ts} mutant containing the parent expressing HIV RT and pBR322 exhibits a 54-fold higher plating efficiency (plating efficiency = 27%) at 37°C than does the pol I^{ts} mutant without HIV RT. The plating efficiency of both decreases as the incubation temperature increases (Table 1). The deficiency of the pol I^{ts} mutant in maintaining ColE1 plasmids is partially complemented by HIV RT. In a similar type of experiment, *E. coli polA12 recA718* expressing the RT from murine leukemia virus exhibited a 15-fold higher plating efficiency at 37°C than at 32°C (D. G. Ennis and R. J. Crouch, personal communication).

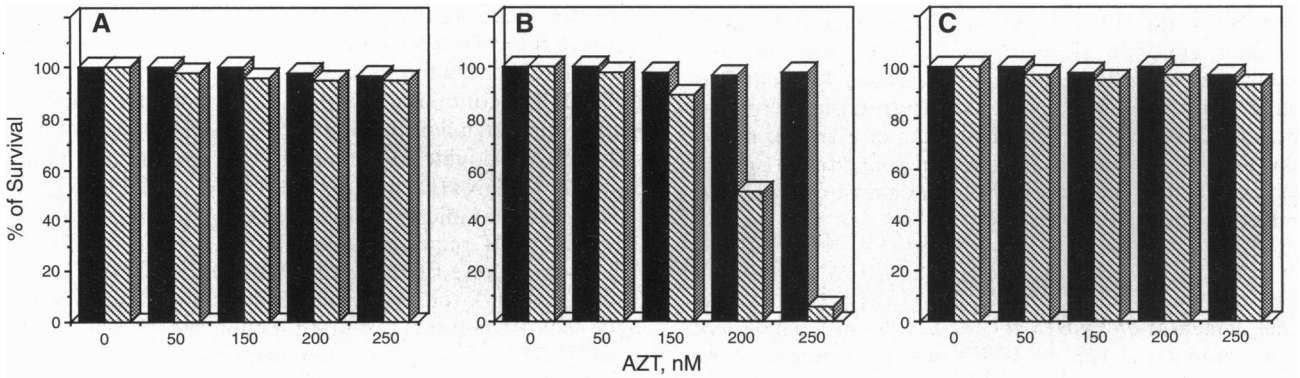

FIG. 3. Inhibition of HIV RT complementation by AZT. Approximately 300 *E. coli* cells expressing the designated proteins (*A*, rat pol β; *B*, wild-type HIV RT; *C*, TY215 HIV RT) were plated in duplicate on NA containing tetracycline, chloramphenicol, and IPTG (as described in Fig. 1) and AZT (0, 50, 150, 200, and 250 nM) and were incubated at 30°C (solid bars) or 37°C (hatched bars) for 48 hr. The percent survival is the ratio of the number of colonies formed in the presence to the number formed in the absence of AZT times 100%. In studies similar to those in Fig. 1, T215Y HIV RT mutant from pHIVRT-TY is able to complement the growth defect of pol I^{ts} mutant at nonpermissive temperature to the same extent as the wild-type HIV RT.

Susceptibility to AZT. We investigated whether the presence of HIV RT would render the *E. coli* mutant susceptible to AZT, a nucleoside analog that preferentially inhibits the viral RT and is extensively used for the treatment of AIDS. In these studies we compared AZT susceptibility of *E. coli* harboring plasmids that express HIV RT and pol β. As shown in Fig. 3*A*, pol I^{ts} expressing rat pol β is able to grow even in high concentrations of AZT, whereas the pol I^{ts} strain expressing HIV RT does not grow at high AZT concentrations (Fig. 3*B*). These results are in accord with the known preferential incorporation of AZT by HIV RT compared to that exhibited by pol β (15). The range of AZT concentrations in NA that differentiates between *E. coli* expressing HIV RT and pol β is 200–300 nM, while larger differences in sensitivity to AZT between the two enzymes in *in vitro* assays have been reported (15–17). T215Y, an HIV mutant obtained from a patient treated with AZT, contains a mutation in the RT gene that renders HIV RT resistant to AZT *in vivo* (18). As shown in Fig. 3*C*, cells expressing the T215Y AZT-resistant mutant are able to grow at high concentrations of AZT.

DISCUSSION

The results presented here indicate that HIV RT can substitute for temperature-sensitive pol I in *E. coli* DNA replication. *E. coli* pol I fills single-strand gaps between Okazaki fragments that are discontinuously synthesized during lagging-strand replication and also hydrolyzes RNA primers at the 5′ ends of Okazaki fragments (6). The most direct explanation for our findings is that DNA-dependent catalysis by HIV RT fills gaps between these DNA fragments. The RNase H activity of HIV RT could remove the RNA primers and facilitate completion of gap synthesis. The ability of HIV RT to substitute for pol I in plasmid replication indicates that HIV RT may initiate plasmid replication since pol I is believed to synthesize a continuous stretch of 400 nucleotides at the replication origin of pBR322 (6).

Our previous studies indicated that rat pol β is able to complement the temperature-sensitive phenotype for growth at 37°C exhibited by *polA12 recA718* (7, 8). Complementation of *E. coli* pol I requires induction of rat pol β by IPTG (7, 8). In contrast, a high level of complementation by HIV RT in the same strain can be achieved without induction by IPTG (plating efficiency = 60%). The basal level of expression of HIV RT, but not pol β, is sufficient to complement the *E. coli* DNA pol I temperature-sensitive phenotype. The enhanced ability of HIV RT to substitute for pol I^{ts} is not simply due to the amount but reflects some intrinsic property of the enzyme. The number of HIV RT molecules per cell in the uninduced and induced states as estimated from Western blots is about 20 and 100, respectively, while the number of rat pol β molecules per cell is slightly greater, about 50 and 300 (data not shown). However, we do not know the number of active molecules of either enzyme present in each cell. A number of mechanisms can be invoked for the greater ability of HIV RT than pol β to substitute for *E. coli* pol I. First, HIV RT is moderately processive (19, 20), whereas pol β is entirely distributive (21); it dissociates from the template after each nucleotide addition step. The processivity displayed by HIV RT may be sufficient to fill the gaps between Okazaki fragments on the lagging strand without the dissociation of the enzyme from the template. Second, HIV RT, but not pol β, is capable of strand displacement, and this could release the RNA primer of the Okazaki fragments from the RNA·DNA hybrid, rendering it susceptible to cellular ribonucleases. Third, the higher efficiency of complementation could also result from the RNase H activity of HIV RT, which serves to remove RNA primers at the 5′ terminus of Okazaki fragments. The 5′ → 3′ exonuclease activity of *polA12* believed to hydrolyze RNA primers is not active even at nonpermissive temperatures (22).

The establishment of a positive genetic selection assay for HIV RT in bacteria has three important implications. First, it should facilitate the collection of a large number of active mutants of HIV RT. Characterization of mutant HIV RTs will facilitate studies on structure–function relationships, understanding the evolution of HIV RT, and designing nucleoside analogs that are effective in the treatment of AIDS and are less susceptible to the development of resistant mutants. Second, it can be used to monitor patients for the emergence of mutations in HIV RT that render the virus resistant to therapy. The gene for HIV RT can be amplified from the blood of infected individuals, inserted into *E. coli*, and tested for drug resistance. Third, the induction of sensitivity of *E. coli* to AZT by means of HIV RT-dependent growth brings into focus the possibility of using *E. coli* as a vehicle for evaluating the potency of nucleoside analogs. This system will be facilitated by transfecting *E. coli* with human nucleoside kinases that phosphorylate analogs directed against the viral RT.

Note Added in Proof. Expression of murine leukemia virus RT has also been shown to restore survival of *polA12 recA718* mutant strains at the nonpermissive temperature (D. G. Ennis and R. J. Crouch, personal communication).

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