Conversion of Thymidylate Synthase into an HIV Protease Substrate*

Jean-Jacques Kupiec‡, Stephane Hazebrouck, Thirry Leste-Lasserre, and Pierre Sonigo‡

From the Génétique des Virus, ICGM-CNRS UPR 415, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, 75014 Paris, France

Thymidylate synthase (TS) is an essential enzyme of DNA metabolism. We have carried out an extensive insertional mutagenesis of the Escherichia coli TS gene (thyA) using three different methods. Insertion of exogenous sequences at unique restriction sites or at random positions produced defective mutants, whereas comparison of TS sequences from different species allowed us to identify six zones permissive for insertions of exogenous sequences. The insertion of Human immunodeficiency virus type 1 (HIV-1) protease substrate sequences into the permissive sites converted TS to an HIV-1 protease substrate, and the in vivo cleavage of these insertions by the cloned HIV-1 protease conferred a thymidylate synthase-deficient phenotype in some of our E. coli mutant strains. In agreement with crystallographic data, these results show that the permissive sites are located in regions of the TS protein not essential for enzyme activity and accessible to cleavage by HIV protease. These results also show that it is possible to control a growth phenotype in E. coli through the protease-mediated destruction of an essential metabolic enzyme. Because both wild type and thymidylate synthase-deficient phenotypes are selectable on the appropriate growth medium, these thyA mutants could be used for genetic selections of protease inhibitors and analysis of protease specificities.

Cleavage of a precursor polypeptide by a protease is a general mechanism for the regulation of physiological processes (1–4). Human immunodeficiency virus (HIV-1)1 provides an example. In retroviruses a viral protease is responsible for cleavage of the polypeptides encoded by the gag and pol genes, producing mature structural and enzymatic proteins. This process is essential for generating infectious virus particles (for review see Ref. 5). At the junctions of the protein domains, within the precursors, lie peptide target sequences that are recognized and cleaved by the virally encoded protease. Although some degree of similarity exists between these target sequences, no consensus sequence has been found to predict cleavability. Moreover, other sequences in cellular proteins have also been found to be cleaved by HIV-1 protease (Ref. 6 and references therein). In order to study the determinants of this substrate specificity, a system allowing the genetic selection of HIV protease activity is much needed. Therefore, we decided to insert HIV-1 protease target sites into thymidylate synthase (TS), which is a selectable marker in Escherichia coli. This could make possible the genetic selection of protease mutants with altered specificities or protease inhibitors and substrates from large libraries of random peptides (see Fig. 1 for a detailed explanation). In fact, it has already been shown that HIV-1 Gag and Gag-Pol precursor polypeptides are correctly processed by HIV-1 protease when they are co-expressed in E. coli (7, 8) and that an HIV-1 protease target sequence could be inserted into E. coli β-galactosidase (9). In this latter case, the enzyme retained its activity, and when the donor HIV-1 protease was coexpressed in the same strain, β-galactosidase was cleaved and inactivated. However, conditional growth has not been reported with this system. Similarly, HIV-1 protease, human rhinovirus protease 3C, or zucchini yellow mosaic virus protease target sequences have been inserted into E. coli proteins responsible for resistance to tetracycline or sensitivity to streptomycin (10–12). In these cases, coexpression of these mutants with their cognate protease impaired bacterial growth in the presence of the appropriate antibiotic. We thought that insertion of protease target sequences into TS could provide a valuable system because positive and negative selection media are available allowing the selection of both wild type and thymidylate synthase-deficient phenotypes (13, 14). These two types of selection should not only permit to search for inhibitors as in the case of previous systems based on antibiotic sensitivity phenotypes but also to study protease specificity (see Fig. 1 for an overview). TS is an essential enzyme of DNA metabolism catalyzing dTMP synthesis. Although TS has been characterized by various techniques, including x-ray crystallography (15, 16), insertional mutagenesis has not been done.

In order to obtain an E. coli strain with a thymidylate synthase-deficient phenotype conferred by HIV-1 protease activity, the first steps consist of mapping tolerant sites for insertion of exogenous sequences within the TS protein and to determine, among these sites, those that are accessible to the HIV-1 protease. We report here that we have proceeded to an extensive insertional mutagenesis of the E. coli TS gene (thyA) and found several regions permissive to insertions of HIV-1 protease target sequences. Further analysis showed that these insertions converted TS to an HIV-1 protease substrate and that the in vivo cleavage of these insertions conferred a thymidylate synthase-deficient phenotype in some of the E. coli mutated strains.

MATERIALS AND METHODS

Cloning, sequencing, site-directed mutagenesis, and immunoblotting were done according to standard procedures.

Plasmids

pTZthyA—The 1300-base pair thyA fragment was first subcloned from pBTAH2 (17) into the phagemid pTZ18R (Pharmacia Biotech Inc.) using HindII sites. The HindII sites were destroyed and replaced by Xhol sites by site-directed mutagenesis.

* This work was supported by grants from Agence Nationale pour la Recherche sur le SIDA and SIDACTION. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 33-1-40516438; Fax: 33-1-40517210.

1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; TS, thymidylate synthase.
Insertion of a protease target sequence within an essential protein and expression of the cognate protease confers a protease-dependent phenotype. Insertion within TS allows selection of both wild type and thymidylate synthase-deficient phenotypes on positive and negative selection media, respectively (see "Materials and Methods" for a description of these media). These two types of selection allow a wide range of applications. For example: 1) Protease inhibitors could be directly selected on the positive selection medium from an endogenously expressed library of random peptides. The expression of the protease confers a thymidylate synthase-deficient phenotype, but those cells expressing a protease inhibitor will revert to a wild type phenotype and grow as colonies. 2) Protease substrates could also be selected from random sequences but this time inserted into TS itself. Those cells carrying a substrate sequence inserted into TS will have a thymidylate synthase-deficient phenotype and grow as colonies on the negative selection medium. By means of this method, exhaustive collections of protease substrate sequences could be collected, allowing the analysis of proteases specificity and the design of inhibitors. 3) Protease mutants could also be easily analyzed. For example, substrate specificity could be analyzed for protease mutants that are resistant to a currently known inhibitor, allowing the design of a new and specific inhibitor.

**Fig. 1. Genetic selection of protease inhibitors and substrates.** Insertion of a protease target sequence within an essential protein and expression of the cognate protease confers a protease-dependent phenotype. Insertion within TS allows selection of both wild type and thymidylate synthase-deficient phenotypes on positive and negative selection media, respectively (see "Materials and Methods" for a description of these media). These two types of selection allow a wide range of applications. For example: 1) Protease inhibitors could be directly selected on the positive selection medium from an endogenously expressed library of random peptides. The expression of the protease confers a thymidylate synthase-deficient phenotype, but those cells expressing a protease inhibitor will revert to a wild type phenotype and grow as colonies. 2) Protease substrates could also be selected from random sequences but this time inserted into TS itself. Those cells carrying a substrate sequence inserted into TS will have a thymidylate synthase-deficient phenotype and grow as colonies on the negative selection medium. By means of this method, exhaustive collections of protease substrate sequences could be collected, allowing the analysis of proteases specificity and the design of inhibitors. 3) Protease mutants could also be easily analyzed. For example, substrate specificity could be analyzed for protease mutants that are resistant to a currently known inhibitor, allowing the design of a new and specific inhibitor.

pSUthyA—thyA was also cloned into pSU 18 (18) by subcloning the XhoI insert from pTZthyA into the SalI site of pSU 18.

pTZprt1 and pSUprt1—The 2865-base pair HIV-1 pol gene fragment, corresponding to the protease and reverse transcriptase domains, was subcloned from pBRT1prt1 (7) into pTZ19 (Pharmacia) and pSU19 (18), using the EcoRI and SalI sites.

pTZprt2 and pSUprt2—The 1470-base pair HIV-1 pol fragment, corresponding solely to the reverse transcriptase domain, was also subcloned from pBRT2prt2 (7) into pTZ19 and pSU19 using the EcoRI and SalI sites. pBRT1prt1 and pBRT2prt2 clones were kindly provided by the National Institutes of Health (AIDS Research and Reference Reagent Program).

**HIV-1 Protease Target Sequences**

Complementary oligonucleotides corresponding to HIV-1 protease target sequences were synthesized, hybridized, and cloned into thyA. The sequence for S1 is VSFNFPQITL (P6 protease junction of the HIV-1 Gag precursor); the DNA coding for this sequence has a HindIII site. The sequence for S2 is DROQTVSNFPQITLWQRPL (P6 protease junction of the HIV-1 Gag precursor). S3 is 50 amino acids from the P6 protease junction of the HIV-1 Gag precursor, encompassing S2. The sequence for S4 is IRRKVLFLDG (reverse transcriptase/integrase junction of the HIV-1 Gag precursor).

**Nomenclature of thyA Mutants**

The first three letters designate the site of insertion (i.e., ISE means insertion at site E) and the two following characters designate the sequence inserted (i.e., ISES1 means S1 inserted at site E).

Oligonucleotide-directed Mutagenesis

Mutagenesis was made according to Kunkel et al. (19). Oligonucleotides were synthesized to introduce an HpaI site into thyA at sites A–G (Fig. 2). Annealing and extension reactions were performed on the single stranded form of the pTZthyA phagemid DNA. Mutants were termed ISA-1SG, respectively.

Δ-thyA E. coli Strains, Selection Media, and Phenotypes

β-1083 is the HB101 Δ-thyA::Em auxotroph for leucine, proline, and thymidine. It grows at 30 °C. β-1308 is the Δ-thyA::Em derivative of the wild type MG 1655 E. coli K12 (F− λ−). Minimal medium contains citric acid (1.6 g/liter), MgSO4 (0.4 g/liter), α-Mannitol (10 g/liter), NH4Cl (2 g/liter), and K2HPO4 (17.4 g/liter). Müller Hinton medium (Difco) is a rich medium devoid of thymidine. Positive selection medium is minimal or Müller-Hinton medium. Negative selection medium contains minimal or Müller-Hinton medium complemented with thymidine (100 μg/ml) and trimethoprim (20 μg/ml in minimal medium and 200 μg/ml in Müller-Hinton medium). Nonselective medium is minimal or MüllerHinton medium complemented with thymidine (100 μg/ml). Wild type and thymidylate synthase-deficient phenotypes grow on positive and negative selection media, respectively. Mixed phenotypes grow on both media.

Antibodies and Protein Extracts for Immunoblotting Experiments

Antibody A is a polyclonal serum from a rabbit injected with the whole TS protein (kindly provided by Dr. F. Maley, Albany, NY). We also prepared antibody B, a polyclonal serum from a rabbit injected with a synthetic peptide corresponding to amino acids 241–264 of the E. coli TS protein. Protein extract preparation, pellets corresponding to 30-ml cultures of β-1083 bacteria transfected with one of the pTZthyA mutants (OD = 1) were resuspended in 2 ml of buffer (0.04 mM phenylmethylsulfonyl fluoride, 4.8 mM 2-mercaptoethanol, 2 mM Tris-HCl, pH 7.5) and sonicated until all the cells were disrupted (4 × 30 s). Cellular debris was removed by centrifugation (5 min at 13,000 × g), and 50 μl of glycerol were added.

Measurement of Thymidylate Synthase Activity

We measured tritium released from [3H]dUMP after the enzyme reaction was terminated, as described by Roberts (20). For protein extract preparation, pellets corresponding to 30-ml cultures of β-1083 bacteria transfected with one of the pTZthyA mutants (OD = 1) were resuspended in 2 ml of buffer (0.04 mM phenylmethylsulfonyl fluoride, 4.8 mM 2-mercaptoethanol, 2 mM Tris-HCl, pH 7.5) and sonicated until all the cells were disrupted (4 × 30 s). Cellular debris was removed by centrifugation (5 min at 13,000 × g), and 50 μl of glycerol were added.
Protein concentration was determined in the extracts by the Biuret method (BCA kit, Pierce).

RESULTS

Mapping of Sites Permissive for Insertion of Exogenous Sequences within Thymidylate Synthase—Insertions of a S1 peptide coding sequence (corresponding to a 10-amino acid-long target sequence in the Gag-Pol precursor; see “Materials and Methods”) were made at unique restriction sites within the thyA gene, but all the mutants thus generated by this method had a thymidylate synthase-deficient phenotype (data not shown). Wild type mutants were also screened in libraries made by insertion of a S1 peptide coding sequence into double stranded breaks created at random position in thyA DNA by DNaseI digestion. Onewild typemutant was obtained by using this procedure (see below). Finally, we analyzed alignments of TS sequences from different species. Although this protein is highly conserved, seven sites can be identified where insertions have spontaneously occurred during the evolution of some species (see Fig. 2 and Ref. 15). We thought that these sites might also accommodate artificial insertions. Thus, seven E. coli thyA mutants containing one HpaI restriction site at each of these seven sites were constructed by site-directed mutagenesis.

![Fig. 2. Position of permissive insertion sites along the E. coli TS protein. A, multiple alignment of TS sequences from 17 species. Sequences were extracted from the SwissProt Data Bank, and the alignment was performed using the clustal software. Asterisks indicate conserved amino acids. E. coli, E. coli; B. subtilis, Bacillus subtilis; TN4003, transposon 4003 (Staphylococcus aureus); L. casei, Lactobacillus casei; B. phi 3T, bacteriophage phi-3T; BT4, bacteriophage T4; Rnorv, Rattus norvegicus; Athal, Arabidopsis thaliana; Scere, Saccharomyces cerevisiae; VZvir, varicella-zoster virus; P. carinii, Pneumocystis carinii; HT4, herpesvirus atelis; L. major, Leishmania major; Calb, Candida albicans; Pchab, Plasmodium chabaudi. The seven sites (A–G) where insertions have spontaneously occurred in some species were chosen for insertion of HIV protease target sequences in E. coli TS. B, insertions map. IS means insertion. Numbers indicate the amino acid immediately preceding the insertions. E. coli TS is 264 amino acids long.](image-url)
quently, these Hpa restriction sites were used to introduce various inserts.

Two double stranded adaptors corresponding to two distinct 10-amino acid HIV-1 protease target sequences, termed S1 and S4, were separately introduced into each of these mutants (see "Materials and Methods"). Fourteen mutants were therefore constructed. The phenotypes of these mutants, carried by the high copy number pTZ 18 plasmid, were determined in the constructed. The phenotypes of these mutants, carried by the "Materials and Methods"). Fourteen mutants were therefore constructed. The phenotypes of these mutants, carried by the high copy number pTZ 18 plasmid, were determined in the

| Sequence | S1 | S2 | S3 | S4 | S1 |
|----------|----|----|----|----|----|
| ISA, B, C, E, F | mixed | mixed | thya− | mixed | thya− |
| ISD | wt | mixed | wt | mixed | thya− |
| ISG | thya− | thya− | thya− | thya− |

Thymidylate Synthase Conversion into HIV Protease Substrate

Phenotypes of thyA mutants carrying one insertion

These phenotypes were analysed in the β-1083 E. coli strain grown on minimal medium. ISA–G, insertion sites (Fig. 2). S1 and S4, 10-amino acid-long inserts. S2, 20-amino acid-long insert. S3, 50-amino acid-long insert (see "Materials and Methods" for a description). wt, wild type phenotype. thya−, thymidylate synthase-deficient phenotype.

Theory to at least a 25-fold reduction in the production of TS protein. As shown in Table 1, all of the mutants that had a mixed phenotype when carried by the pTZ vector had a thymidylate synthase-deficient phenotype when carried by the pBR vector. Those bearing an insert at site D, which had a wild type phenotype when carried by pTZ 18, had a mixed phenotype when carried by pBR. These results show that the TS mutants have a reduced thymidylate synthase activity. They also demonstrate that it is possible to change the phenotype of E. coli by adjusting, within reasonable limits, the level of expression of the mutants. In fact, subcloning the mutant sequences from pTZ 18 into pBR. 322 reduced the level of thymidylate synthase activity below the threshold necessary for growth on a positive selection medium, with the exception of mutants with an insert at D site.

Taken together, the results of the insertional mutagenesis of thyA show that site D is more permissive for the insertion of exogenous sequences than any other site we have identified.

Finally, to test the plasticity of TS further, we made two insertions in the same molecule, each of them at a different permissive site. This could also improve TS cleavage by the HIV-1 protease and/or modulate the activity of TS. For this purpose, we constructed seven "double mutants" that carry two HIV-1 target sequences. All these double mutants have a thymidylate synthase-deficient phenotype (data not shown). The structure of TS proteins with two insertions is probably too profoundly perturbed to maintain the integrity of the catalytic site.

Effect of Insertions on the Activity of Thymidylate Synthase—In order to get a more quantitative estimate, thymidylate synthase activity was measured in crude extracts of proteins prepared from bacteria expressing the different thyA mutants. (see "Materials and Methods"). Twelve thyA mutants bearing S1 or S4 inserts were tested. They all had a very low activity estimated to be <1% compared with the wild type. This loss of activity must be attributed to a reduction in enzymatic activity of the mutated enzymes rather than to a reduction in their half-life. Indeed, immunoblotting experiments (see below) did not show a reduction in the quantity of TS expressed by the thyA mutants. These thyA mutants were overexpressed because of the plac promoter and because pTZ 18 vector is a high copy number plasmid (500 copies/cell). Thus, it is not surprising that in spite of their highly reduced activity, they ensured growth on the positive selection medium. Moreover, other mutants with <1% activity relative to wild type have already been shown to grow on the positive selection medium (13, 22).

In Vivo Cleavage of Insertions by HIV-1 Protease—Protein extracts were prepared from E. coli cells expressing the various thyA mutants with or without HIV-1 protease (prt+ or prt− respectively; see "Materials and Methods") and analyzed by immunoblotting with TS-specific antibodies (Fig. 3). In all the tested mutants, the 30.5-kDa TS band showed a high reduction in intensity in cells expressing HIV-1 protease, whereas this is not the case with wild type TS used as a control (Fig. 3C). In some experiments the cleavage products were not detected (Fig. 3A), probably due to their rapid degradation, as is the case in the E. coli (11, 23). However, bands of 21 and 20 kDa, corresponding to one of the two expected cleavage products, could be seen in several other experiments with ISB and ISC mutants, respectively (Fig. 3, B and C). The other cleavage product was not detected; either the product was not recognized by the antibodies or it was degraded. These data indicate that HIV-1 protease is able to recognize and cleave, in this heterologous context, the two target sequences at various positions within the TS protein.

Thymidylate Synthase Cleavage by HIV-1 Protease Confers a
Thymidylate Synthase Conversion into HIV Protease Substrate

Fig. 3. In vivo cleavage of insertions within thymidylate synthase. β-1083 cells were transformed by two constructs, a pTZ thyA mutant and a pSU prt plasmid expressing either an active (+) or a defective (−) HIV protease protein. For each thyA mutant, as well as for thyA wild type, the quantity of cells was normalized in positive and negative selection medium. On the plus medium the plating efficiency was very low and prevented us from estimating growth differences. This could be due to the combination of two factors: the reduced viability of cells with a thyA mutant and the toxicity of HIV protease in E. coli (11, 24). As illustrated in Table II, there was a 230- and 140-fold induction of prt1 ISFS1 and prt2 ISES1 cell growth on the negative selection medium. The other mutants did not exhibit the thymidylate synthase-deficient phenotype, probably because the levels of thymidylate synthase activity and/or cleavage by the protease were insufficient.

DISCUSSION

Comparison of TS sequences from different species enabled us to identify six sites permissive for insertions within the TS protein. We were able to insert 20 or even 50 amino acids in the case of site D, showing that insertions at these sites allow a residual enzymatic activity that is still sufficient for bacterial growth. These results are in agreement with crystallographic data that show that the natural insertions that occurred spontaneously during the evolution of some species correspond to surface loops and do not contribute to the active site (15, 16). All the insertions made in other regions of TS protein failed to produce an active TS. Because TS is a highly structured and conserved protein, it is likely that the insertions markedly disturbed the core structure of the protein and therefore abolished enzymatic activity. We also observed the in vivo cleavage of TS by HIV-1 protease for two different target sequences inserted at the six different permissive sites, whereas wild type TS was unaffected. This result demonstrates that these insertions are accessible to HIV protease and that cleavage of the natural substrate sequences can occur in this heterologous context. In vivo cleavage in a heterologous context has already been reported for the S1 sequence (9) but not S4. It is remarkable that cleavage occurred in six different contexts, suggesting that accessibility is the only parameter important for cleavage besides the target sequence itself. This cleavage conferred a protease-dependent phenotype for two of these mutants. In the other mutants the extent of TS cleavage was probably too low for phenotypic switch. A nonspecific toxic effect of HIV-1 protease on E. coli growth has been reported (11, 24). This nonspecific toxic effect of HIV-1 protease cannot account for growth induction on the negative selection medium. ISFS1 and ISES1 prt+ cells exhibited background growth between 4 × 10⁻³ and 7 × 10⁻³, respectively, on the negative selection medium. These values are in the same range as the background growth reported in another study using HIV-1 protease and the tetracy-

Table II

HIV-1 protease-dependent phenotypes of thyA mutants in the β-1308 E. coli strain

|                  | plus medium | minus medium |
|------------------|-------------|--------------|
| prt+, ISES1      | ND          | 42           |
| prt−, ISES1      | ND          | 0.3          |
| prt+, ISFS1      | ND          | 70           |
| prt−, ISFS1      | ND          | 0.3          |
| prt+, thyA−      | <0.001      | 100          |
| prt−, thyA−      | <0.001      | 100          |
| prt+, thyA+      | 100         | <0.001       |
| prt−, thyA+      | 100         | <0.001       |
Thymidylate Synthase Conversion into HIV Protease Substrate

cline resistance protein as a target (10) but higher than the $10^{-6}$ background observed with the zucchini yellow mosaic virus protease (12). The background in our TS system could probably be considerably reduced by optimizing the ratio of expression of TS relative to HIV-1 protease and therefore improving the rate of TS cleavage.

At a more general level our data show that it should be possible to induce a selectable phenotype in E. coli through the protease-mediated destruction of an essential metabolic enzyme and therefore that the genetic selection of bioactive molecules from large libraries of peptides is feasible. E. coli strains with a protease-dependent growth phenotype have previously been described (10–12). In these systems the proteases induce antibiotic-sensitive phenotypes that provide a means of selecting protease inhibitors, as is the case with TS on the positive selection medium (Fig. 1). In this type of selection the destruction of a target by a protease inhibits bacterial growth. As we have shown in this study, TS destruction induces bacterial growth on the negative selection medium. Because of this property, our engineered E. coli strain could be used not only for the selection of protease inhibitors but also for the study of protease specificity.

Acknowledgments—We are very grateful to Dr. Frank Maley (Albany, NY) for providing us with a TS antiserum used in most of the immunoblotting experiments of this study; Drs. A. Bailone and R. Devoret (Institut Curie, France) and Drs. P. Marière and W. Saurin (Institut Pasteur, Paris) for helpful discussions and exchange of ideas; and Drs. B. Shacklett, G. Pancino, P. Benaroch, C. Vaquero, and J. Richardson for helpful reviewing of this article. We also acknowledge Prof. D. Strosberg (University of Paris, France) for constant support. Our colleague, B. Lemeignan, gave us the Δ-thyA E. coli strains. She died suddenly not long ago, and we think of her fondly and often.

REFERENCES
1. Beynon, R. J., and Bond, J. S. (eds) (1994) Proteolytic Enzymes, IRL Press at Oxford University Press, Oxford
2. Scharpe, S., De Meester, I., Hendriks, D., Vanhoof, G., van Sande, M., and Vriend, G. (1991) Biodrime (Paris) 73, 121–126
3. Helen, C. U. T., and Winner, E. (1992) Curr. Opin. Biotechnol. 3, 643–649
4. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
5. Fitzgerald, P. M. D., and Springer, J. P. (1991) Annu. Rev. Biophys. Biophys. Chem. 20, 299–320
6. Tomasselli, A. G., and Heinekisson, R. L. (1994) Methods Enzymol. 241, 279–301
7. Farmerie, W. G., Loeb, D. D., Casavant, C. A., Hutchison, C. A., Edgell, M. H., and Swanstrom, R. (1987) Science 236, 305–308
8. Debourck, C., Gorniak, J. G., Strickler, J. E., Meek, T. D., Metcalf, B. W., and Rosenberg, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8903–8906
9. Baum, E. Z., Bebenitiz, G. A., and Gluzman, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 10023–10027
10. Block, T. M., and Grafstrom, R. H. (1990) Antimicrob. Agents Chemother. 34, 2337–2341
11. McCall, J. O., Kadam, S., and Katz, L. (1994) Bio/Technology 12, 1012–1015
12. Balint, R. F., and Plooy, I. (1995) Bio/Technology 13, 507–510
13. Belfort, M., and Pedersen-Lane, J. (1984) J. Bacteriol. 160, 371–378
14. Stacey, K. A., and Simson, E. (1965) J. Bacteriol. 90, 554–555
15. Hardy, L. W., Finer-Moore, J. S., Montfort, W. R., Jones, M. O. Santi, D. V., and Stroud, R. M. (1987) Science 235, 448–454
16. Matthews, D. A., Villafani, J. E., Johnson, C. A., Smith, W. W., Welsh, K., and Freer, S. (1990) J. Mol. Biol. 214, 937–948
17. Belfort, M., Maley, G., Pedersen-Lane, J., and Maley, F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4914–4918
18. Martinez, E., Bartoldine, B., and de la Cruz, F. (1988) Gene(Amst.) 68, 159–162
19. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
20. Roberts, D. W. (1966) Biochemistry 5, 3546–3548
21. Carreras, C. W., Costi, P. M., and Santi, D. V. (1994) J. Biol. Chem. 269, 12444–12446
22. Michaels, M. L., Kim, C. W. Matthews, D. A., and Miller, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3957–3961
23. Garber, J. L. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 422–426
24. Baum, E. Z., Bebenitz, G. A., and Gluzman, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5573–5577