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Overexpression and Substrate Specificity Studies of Phosphodeoxyribomutase and Thymidine Phosphorylase

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The gene encoding phosphodeoxyribomutase and thymidine phosphorylase [EC 2.4.2.4] from *Escherichia coli* were excised from plasmid pVH19 utilizing *Ava*I restriction enzyme and ligated to the expression vector pKK-226 containing the *E. coli tac* promoter. Introduction of the resulting plasmid pCB-11 into *E. coli* JM105 allowed for the overexpression of both enzymes upon induction with isopropylthiogalactoside. A 3-L fermentation of this *E. coli* strain produced 1500 and 75,000 units, respectively, of the mutase and nucleoside phosphorylase. Substrate specificity studies indicate that the mutase accepts D-ribose 5-phosphate and D-arabinose 5-phosphate as substrates in addition to its natural substrate 2-deoxyribose 5-phosphate. Thymidine phosphorylase showed, however, narrow substrate specificity on the pentose moiety. Only α-D-2-deoxyribose 1-phosphate was accepted as substrate. α-D-Ribose 1-phosphate and α-D-arabinose 1-phosphate are not acceptable based on a coupled enzymatic assay. D-2,3-Dideoxyribose 5-phosphate was not converted to nucleoside based on the coupled enzymatic reactions using phosphodeoxyribomutase and thymidine or purine nucleoside phosphorylase.

INTRODUCTION

Analogues of the naturally occurring nucleosides have been used extensively as antibiotics and as biological probes (*1–3*). A number of nucleosides including zidovudine (3′-azido-3′-deoxythymidine; AZT) and the 2′,3′-dideoxy derivatives of the naturally occurring nucleosides have been of considerable interest due to their selective inhibition of the retrovirus causative of acquired immunodeficiency syndrome (AIDS) (*4*).

Nucleosides are traditionally synthesized by chemical methods (*5*). Recently, strategies based on enzymes have been explored for the synthesis of natural, as well as unnatural nucleosides (*6–10*). Two strategies are generally employed, as illustrated in Fig. 1a. The first of these uses a pyrimidine nucleoside as the glycosyl donor and a purine or purine analogue as the glycosyl acceptor. In a one-pot reaction, pyrimidine nucleoside phosphorylase serves to generate α-D-ribose 1-phosphate (R-1-P) *in situ*, which then serves as a substrate for purine nucleoside phosphorylase, which accepts a free purine base as a second substrate to displace the phosphate and form the new nucleoside. A variation of this methodology is

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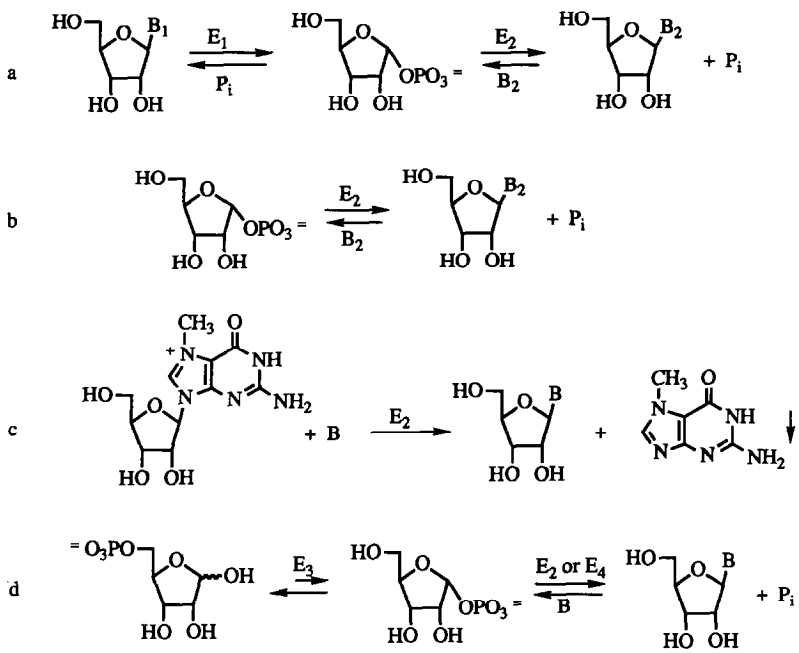


FIG. 1. Enzymatic methods for nucleoside synthesis E_1, uridine phosphorylase; E_2, purine nucleoside phosphorylase; E_3, phosphopentomutase; E_4, thymidine phosphorylase.

shown in Fig. 1b which involves the isolation of R-1-P. Isolation of this intermediate allows for greater ease in purification of the product nucleoside since the nucleoside which serves as pentosyl donor is not present in the final coupling step.

The disadvantages of the above enzymatic strategies are that they either require the presence of both pyrimidine and purine nucleoside phosphorylases or the isolation of R-1-P which is unstable. Moreover, both processes are reversible, that complicate product isolation and result in a low-yield production of the desired nucleoside. We have recently reported an improved procedure to overcome these drawbacks using 7-methylguanosine (or 7-methylinosine) as substrate in purine nucleoside phosphorylase-catalyzed synthesis of nucleosides (*10*) (Fig. 1c). In this transribosylation process, the leaving group 7-methylguanosine is extremely insoluble in the aqueous solution and precipitates out of solution, making the process essentially irreversible. 7-Methylguanosine is also a useful substrate for the synthesis of R-1-P catalyzed by purine nucleoside phosphorylase in the presence of inorganic phosphate.

Since R-1-P is relatively unstable and not readily available, it may be generated from the more stable and readily available precursor D-ribose 5-phosphate (R-5-P) with the use of (*11, 12*) phosphodeoxyribomutase as catalyst. The R-1-P generated could then be used *in situ* as a substrate for nucleoside phosphorylase (Fig. 1d). This combined enzymatic procedure may provide a new route to a number of nucleosides. It is known that purine nucleoside phosphorylase accepts a wide range of nucleoside substrates with modifications at the ribose and the purine

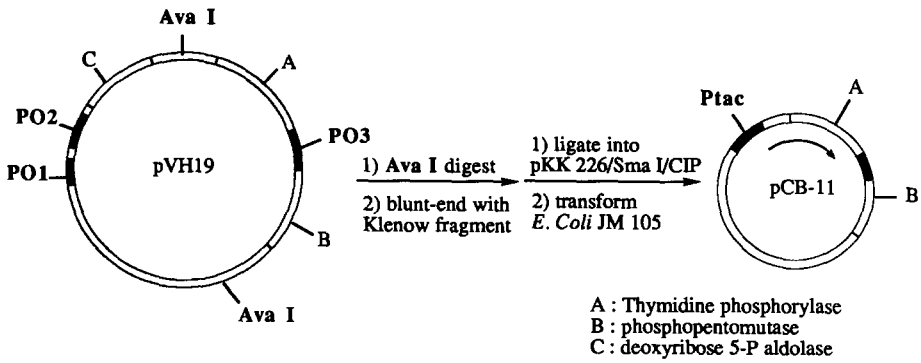


FIG. 2. Subcloning of the phosphopentomutase and thymidine phosphorylase genes.

base moieties (*6–10*). If phosphodeoxyribomutase also expresses relaxed substrate specificity, a number of unnatural nucleosides will become available based on this combined enzymatic process. Although purine nucleoside phosphorylase is commercially available and relatively inexpensive, both the mutase and pyrimidine nucleoside phosphorylase are not readily available. We here report the subcloning and overexpression of the genes encoding these two enzymes in *Escherichia coli,* and a preliminary study on their substrate specificity.

RESULTS AND DISCUSSION

Phosphodeoxyribomutase and thymidine phosphorylase are products of the inducible *deo* operon. The enzymes are produced constituatively in the *deo* regulatory mutant *E. coli* CGSC-5903 (*13*). Though this strain would be suitable for the production of the enzymes, purification would be tedious to obtain suitable specific activities for synthesis. The entire *deo* operon has, however, been cloned (*14*). Since the recombinant strain would produce the enzymes in much greater quantities than the constituative strain, it would be a better source for the enzyme. To this end, the plasmid pVH19 was obtained from Paul Valentin-Hansen of the Odense University of Denmark as a source for the genes of these two enzymes.

As shown in Fig. 2, the genes encoding phosphodeoxyribomutase and thymidine phosphorylase were excised utilizing *Ava*I restriction sites conveniently bracketing the genes. This step was to remove the two genes so they could be expressed without deoxyribose aldolase, which might serve to degrade pentose phosphates. Expression was achieved by ligating the 3-kb *Ava*I fragment into the expression vector pKK-226, which contains the strong *E. coli tac* promoter (*15*). Introduction of the resulting pCB-11 plasmid into *E. coli* JM105 allowed for control of gene expression where isopropyl thiogalactoside served as the inducer. A 3-liter preparation of this strain produced 27 g of wet cells. The cells were disrupted by means of a French press. Following centrifugation and precipitation of nucleic acids with streptomycin sulfate, a 40–65% ammonium sulfate cut was obtained. This

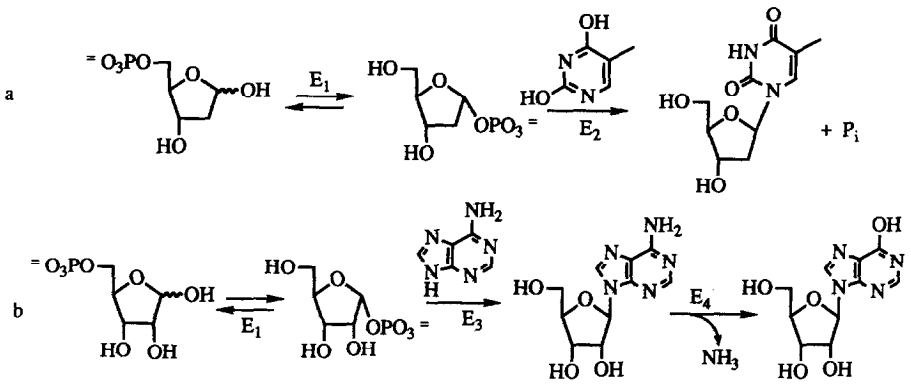
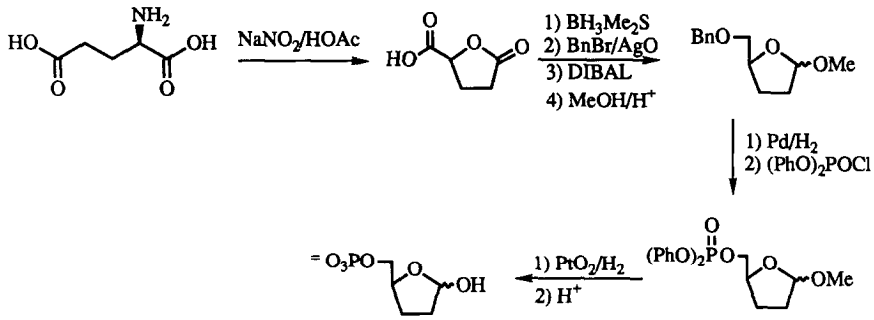


FIG. 3. Assays of phosphopentomutase and nucleoside phosphorylase activities. E_1, phosphopentomutase; E_2, thymidine phosphorylase; E_3, purine nucleoside phosphorylase; E_4, adenosine deaminase.

precipitant, after dialysis, contained 52 U/mg thymidine phosphorylase and 1 U/mg phosphopentomutase, for a total of 75,000 and 1,500 units, respectively.

The mutase activity in this enzyme mixture was assayed by coupling the phosphotransfer reaction with the synthesis when thymidine phosphorylase and excess of thymine are present in the reaction mixture. This is illustrated in Fig. 3a. 2-Deoxyribose 1-phosphate is the natural substrate for thymidine phosphorylase and the production of the nucleoside thymidine can be monitored at 290 nm (*16*). As expected, 2-deoxyribose 5-phosphate was demonstrated to be a substrate in the coupled system. Thymidine phosphorylase was similarly assayed based on the reactions shown in Fig. 3a with an excess of 3-deoxyribose 5-phosphate. Ribose 5-phosphate and arabinose 5-phosphate produced no nucleosides when monitored in the same fashion. Furthermore, no product was indicated by TLC even after extended incubation. This indicated that this enzymatic route would not be feasible for the production of ribosyl or arabosyl pyrimidine nucleosides. This result confirms that of Krenitsky (*7*) which indicated that arabosyl pyrimidine nucleosides are very poor substrates for thymidine phosphorylase with activities 1/200 that of thymidine. These results, however, did not confirm that α-D-arabinose 1-phosphate and α-D-ribose 1-phosphate were not produced in the mutase reactions.

To further investigate if the mutase accepts D-arabinose 5-phosphate and D-ribose 5-phosphate as substrates, purine nucleoside phosphorylase was used in the coupled systems as shown in Fig. 4. It was observed that both D-ribose 5-phosphate and D-arabinose 5-phosphate were substrates for the mutase under saturation kinetics conditions. Previous report (*11*) also indicated that R-1-P is a substrate for the mutase. 2,3-Dideoxyribose 5-phosphate (see Scheme 1 for preparation) was, however, not a substrate in either purine nucleoside phosphorylase or thymidine phosphorylase coupled reactions. Whether or not it is a substrate for the mutase is not clear. It is clear that the coupled enzymatic process is not a suitable route to dideoxynucleosides from dideoxyribose 5-phosphate. Table 1 summarizes the relative activities of various pentose 5-phosphates tested as substrates for phosphodeoxyribomutase.



SCHEME 1. Synthesis of D-2,3-dideoxyribose 5-phosphate.

CONCLUSION

Phosphodeoxyribomutase and thymidine phosphorylase from *E. coli* have been overexpressed in *E. coli* JM105. Substrate specificity studies indicate that the mutase also accepts D-ribose 5-phosphate and D-arabinose 5-phosphate as substrates, in addition to its natural substrate D-2-deoxyribose 5-phosphate. Thymidine phosphorylase, however, showed a narrow substrate specificity on the pentose moiety; α-2-deoxyribose 1-phosphate was the only substrate observed in the coupled assay system. Coupling with purine nucleoside phosphorylase, phosphodeoxyribomutase may be useful for the synthesis of purine nucleoside analogues containing 2-deoxyribose, arabinose,or ribose moiety in the sugar component. D-2,3-Deoxyribose 5-phosphate was not a substrate in the coupled enzymatic systems for the synthesis of either purine or pyrimidine nucleosides.

EXPERIMENTAL SECTION

Materials and Biochemicals

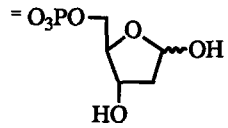
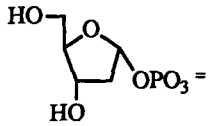
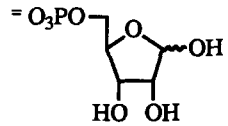
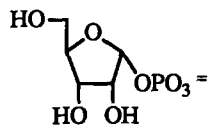
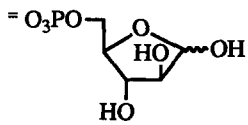
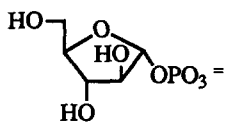
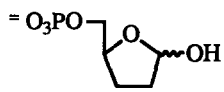
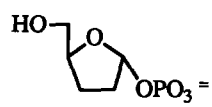
LB and 2XTY were used as rich growth media (*17*), and MA containing 0.2% carbon source was used as minimal growth media for *E. coli* (*18*). Restriction endonucleases, T_4 DNA ligase and T_4 polynucleotide kinase were from New England Biolabs and Bethesda Research Laboratories. Klenow fragment for DNA polymerase I was obtained from Amersham. Plasmid pVH19 was obtained from P. Valentin-Hansen (*14*). Plasmid DNA was purified from *E. coli* as described (*19*). All other DNA manipulations were performed using standard procedures (*18*). D-2-Deoxyribose 5-phosphate was prepared from a deoxyribose 5-phosphate aldolase catalyzed reaction (*20*). D-Arabinose 5-phosphate was prepared from D-arabinose catalyzed by hexokinase (*21*).

Subcloning of Genes Coding for Phosphopentomutase and Thymidine Phosphorylase

Plasmid pVH19 was digested with the restriction endonuclease *Ava*I. A 3-kb *Ava*I fragment was isolated and blunt-ended with the Klenow fragment of DNA polymerase. This fragment was cloned into the *Sma*I site of the expression vector

TABLE 1

Relative Rates of Phosphopentomutase-Catalyzed Conversion of D-Pentose 5-Phosphate to α-D-Pentose 1-Phosphate Based on Coupled Enzymatic Assays

Substrate	Products	Rel. rate
		1.0[a]
		0.84
		0.92
		--[b]

[a] 39 U/mg based on the coupled assay (1 U = 1 μmol of substrate was converted at the maximum velocity).

[b] No purine or pyrimidine nucleoside was observed in the coupled assay.

pKK-266, yielding pCB-11, which was transformed into *E. coli* JM105. The orientation with respect to the *tac*promoter was verified by *Hind*III digestion. All procedures followed the protocols described previously (*17, 18, 20*).

Enzymatic Assays

Purine nucleoside phosphorylase and thymidine phosphorylase were assayed as previously described (*16*). Phosphodeoxyribomutase was assayed using a coupled enzymatic systems as described in Fig. 3 (*11, 12*). A solution (495 μl) containing 0.1 M Tris, 0.1 mM $MnCl_2$, 3.8×10^{-6} M glucose 1,6-diphosphate, 0.14 mM adenine, 0.7 unit phosphopentomutase, 0.1 unit purine nucleoside phosphorylase, and 2.7 units adenosine deaminase was prepared and incubated at room temperature for 3 min. Sugar phosphate (50 μl of an 80 mM solution, ribose 5-phosphate, deoxyribose 5-phosphate, arabinose 5-phosphate, or dideoxyribose 5-phosphate) was added. The absorbance change at 265 nm was monitored where $\varepsilon = 8.1\ mM^{-1}\ cm^{-1}$.

Production of Phosphodeoxyribomutase and Thymidine Phosphorylase

A 3-liter preparation of LB media containing 100 μg/ml ampicillin was inoculated with 3 ml overnight culture containing pCB11. After 6-h growth, isopropyl thiogalactoside was added to a final concentration of 2 mM. The culture was then incubated for an additional 12 h. The cells, 27 g, were collected by centrifugation and processed as described previously (*20*) to obtain cell lysates. The 40–65% ammonium sulfate precipitate contained 52 U/mg thymidine phosphorylase and 1 U/mg deoxyribomutase, for a total of 75,000 and 1500 units, respectively. Further purification of phosphodeoxyribomutase was carried out according to the literature procedure (*11*) to give 705 units with 39 U/mg of specific activity.

Methyl 5-O-Benzyl-2,3-dideoxypentofuranoside

To 46 ml of dry toluene was added 4.7 g (22.8 mmol) (*S*)-γ-benzyloxymethyl-γ-butyrolactone (*22*). The solution was cooled to −78°C and 25 ml of a 1 M solution of DIBAL in hexane was added dropwise with stirring over 1 h. After being stirred for an additional 5 min, the reaction was quenched with the addition of 4.5 ml of methanol and allowed to warm to room temperature. Ethyl acetate (35 ml) and saturated $NaHCO_3$ (4.6 ml) were added and the mixture was stirred for 1 h. Na_2SO_4 (20 g) was added and the mixture was stirred overnight, filtered, and concentrated to yield the lactol. This compound was dissolved in 50 ml dry methanol, 1 g of oven-dried Dowex-50 (H^+) was added, and the mixture was shaken at 40°C overnight. The Dowex was removed by filtration and the mixture was concentrated under vacuum. Flash chromatography with EtOAc/$CHCl_3$ (8/2) as mobile phase gave 2 g of the product, 40% yield. $[\alpha]_D$ +14.0° (*c* 3.2, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 1.8 (m, 4H, CH_2CH_2) 3.27, 3.31 (2s, 3H, O–CH_3) 3.45 (m, 2H, –O–CH_2–) 4.36 (m, 5H, C_6H_5–). ^{13}C NMR/APT (50 MHz, $CDCl_3$) δ 25.9, 26.3, 31.9, 32.7 (CH_2–CH_2), 54.3, 54.6 (–CH), 76.7, 76.9 (OCH_3), 72.4, 73.3 ($2CH_2$) 105.2, 105.5 (CH) 127.5, 127.6, 127.7, 128.3, 138.3 (–C_6H_5).

Methyl 5-O-Diphenylphospho-2,3-dideoxy-D-pentofuranoside

Two grams of the aforementioned compound was dissolved in 20 ml methanol and 400 mg 10% palladium-on-carbon was added. The mixture was hydrogenated at 50 psi overnight. The catalyst was removed by filtration. TLC indicated removal of the benzyl group, R_f = 0.4 EtOAc/$CHCl_3$ (6/4). The solution was reduced to dryness and the residue was dissolved in 10 ml dry pyridine. Diphenyl phosphochloridate, 18 mmol, 2 eq. was added and the solution was stirred for 18 h. The reaction was quenched by the addition of 1 ml H_2O. Methylene chlodire, 50 ml, was added and the solution was extracted with 50 ml each of water, 1 M HCl, saturated bicarbonate, and brine. The methylene chloride layer was dried over $MgSO_4$ and reduced under vacuum to obtain an oil. Flash chromatography with ethyl acetate yielded 2 g of the title compound in 68% yield: $[\alpha]_D$ +14.8 (c 2.9, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$) δ 1.8 (m, 4H, CH_2–CH_2) 3.2, 3.22 (s, 3H, OCH_3) 4.2 (m, 3H, –CH, –CH_2) 4.9 (d, 1H, CH) 7.2 (m, 10H, C_6H_5–).

Dideoxyribose 5-Phosphate

One gram of the aforementioned compound was dissolved in 25 ml methanol. Platinum oxide, 400 mg, was added and the mixture was hydrogenated at 50 psi with vigorous stirring for 3 days. Disappearance of the starting material based on TLC (silica gel) at R_f 0.63 EtOAc/$CHCH_3$ (6/4) and appearance of a new spot, non-uv active, near the origin, as visualized with *p*-anisaldehyde reagent, indicating hydrogenolysis. The catalyst was removed by filtration and the solution concentrated under vacuum. Water, 50 ml, was added to the residue, along with 2 g Dowex-50 (H^+). The mixture was shaken at 40°C for 8 h and the catalyst was removed by filtration. The pH of the solution was adjusted to 7, extracted with 10 ml ethyl acetate, and the aqueous solution was lyophilized to yield the title compound as a mixture of anomers, as a hygroscopic white solid (≈300 mg): ^{1}H NMR (200 MHz, D_2O) δ 1.8 (m, 4H, CH_2–CH_2) 3.35 (m, 2H, CH_2) 4.3 (m, 1H, CH) 5.35 (m, 1H, CH). ^{13}C NMR (50 MHz, D_2O) δ 24.7, 24.8, 32.4, 32.9 (CH_2–CH_2) 67.98 (d, $^2J_{cp}$ = 6.2 Hz, CH_2) 69.21 (d, $^2J_{cp}$ = 6 Hz, CH_2) 77.44 (d, $^3J_{cp}$ = 8.1 Hz, CH) 79.08 (d, $^3J_{cp}$ = 8.3 Hz, CH) 98.4, 98.6 (–CH).

ACKNOWLEDGMENT

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REFERENCES

1. SUHADOLNIK, R. J. (1970) Nucleoside Antibiotics, Wiley, New York.
2. SUHADOLNIK, R. J. (1979) Nucleoside as Biological Probes, Wiley, New York.
3. DOLIN, R. (1985) *Science* **227,** 1296.
4. DAHLBERG, J. E., MITSUYA, H., BLAM, S. B., BRODER, S., AND AARONSON, S. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2469.
5. WALKER, R. T., DECLERRY, E., AND ECKSTEIN, F. (1979) Nucleoside Analogs: Chemistry, Biology, and Medicinal Applications, Plenum, New York.
6. KRENITSKY, T. A., RIDEOUT, J. L., CHAO, E. Y., KOSZALKA, G. W., GURNEY, F., CROUCH, R. C., COHN, N. K., WOLBERG, G., AND VINEGAR, R. (1986) *J. Med. Chem.* **29,** 138.
7. KRENITSKY, T. A., KOSZALKA, G. W., TUTTLE, J. V., RIDEOUT, J. L., AND ELION, G. B. (1981) *Carbohydr. Res.* **97,** 139.
8. UTAGAWA, T., MORISAWA, H., YAMANAKA, S., YAMAZAKI, A., YOSHINAGA, F., AND HIROSE, Y. (1985) *Agric. Biol. Chem.***49,** 3239.
9. KRENITSKY, T. A., KOSZALKA, G. W., AND TUTTLE, J. V.(1981) *Biochemistry* **20,** 3615.
10. HENNEN, W. J., AND WONG, C.-H. (1989) *J. Org. Chem.* **54,** 4692.
11. HAMMER-JESPERSON, K., AND MUNCH-PETERSON, A. (1970) *Eur. J. Biochem.* **17,** 397.
12. LEER, J. C., AND HAMMER-JESPERSON, K. (1975) *Biochemistry* **14,** 599.
13. ROY, S., HIYAMA, Y., TORCHIA, D. A., AND COHEN, J. S. (1986) *J. Am. Chem. Soc.* **108,** 1675.
14. VALENTIN-HANSEN, P., AIBA, H., AND SCHUMPERLI, D. (1982) *EMBO J.* **1,** 317.
15. AMMAN, E., BROSIUS, J., AND PTASNE, M. (1983) *Gene* **25,** 165.
16. KRENITSKY, J., TUTTLE, J., AND KOSZALKA, G. (1976) *J. Biol. Chem.* **251,** 4055.
17. DAVIS, L. G., DIBNER, M. D., AND BALTEY, J. F. (1986) Basic Methods in Molecular Biology, Elsevier, New York.

18. MANIATIS, T., FRITSCH, E. F., AND SAMBROOK, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. BIRNHOIM, H. C., AND DOLY, J. (1979) *J. Nucleic Acids Res.* **7,** 1573.
20. BARBAS, C. F., III, WANG, Y.-F., AND WONG, C.-H. (1990) *J. Am. Chem. Soc.* **112,** 2013.
21. BEDNARSKI, M. D., CRANS, D. C., DICOSIMO, R., SIMON, E. S., STEIN, P. D., AND WHITESIDES, G. M. (1988) *Tetrahedron Lett.* **24,** 427.
22. TANIGUCHI, M., KOGA, K., AND YAMADA, S. (1974) *Tetrahedron* **30,** 3547.