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Articles

Epigenetic Pyrimidine Nucleotides in Competition with Natural dNTPs as Substrates for Diverse DNA Polymerases

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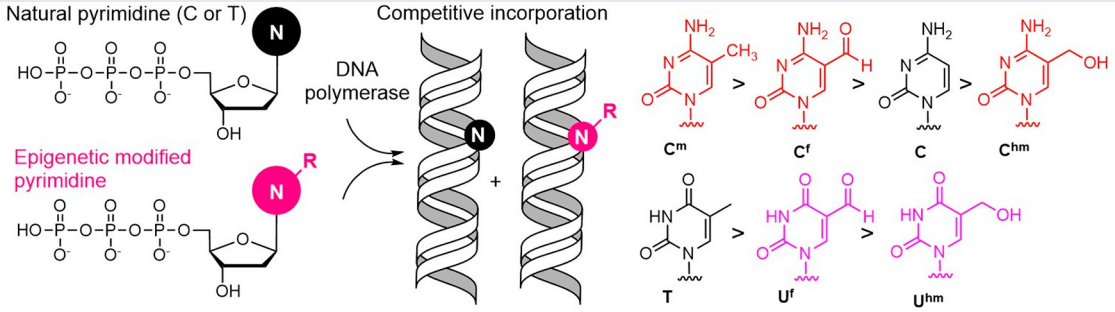

ABSTRACT: Five 2′-deoxyribonucleoside triphosphates (dNTPs) derived from epigenetic pyrimidines (5-methylcytosine, 5-hydroxymethylcytosine, 5-formylcytosine, 5-hydroxymethyluracil, and 5-formyluracil) were prepared and systematically studied as substrates for nine DNA polymerases in competition with natural dNTPs by primer extension experiments. The incorporation of these substrates was evaluated by a restriction endonucleases cleavage-based assay and by a kinetic study of single nucleotide extension. All of the modified pyrimidine dNTPs were good substrates for the studied DNA polymerases that incorporated a significant percentage of the modified nucleotides into DNA even in the presence of natural nucleotides. 5-Methylcytosine dNTP was an even better substrate for most polymerases than natural dCTP. On the other hand, 5-hydroxymethyl-2′-deoxyuridine triphosphate was not the best substrate for SPO1 DNA polymerase, which naturally synthesizes 5hmU-rich genomes of the SPO1 bacteriophage. The results shed light onto the possibility of gene silencing through recycling and random incorporation of epigenetic nucleotides and into the replication of modified bacteriophage genomes.

5-Methylcytosine (5mC) and its oxidized congeners, i.e., 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC), are epigenetic DNA modifications regulating gene expression.[1−3] The 5mC is synthesized in DNA through methylation catalyzed by DNA methyltransferases,[4] whereas the oxidative derivatives are formed through enzymatic oxidation by ten−eleven translocation (TET) enzymes.[5−7] The methylated 5mC is present in human genomic DNA in 3−6% of all cytosines, while the oxidized cytosine congeners are much less frequent in most tissues (although the 5hmC levels in the brain are quite high).[8,9] On the other hand, the biological role of 5-hydroxymethyluracil (5hmU) is not yet understood,[10] although it was found as a minor nucleobase in human stem cells[11] or in some types of cancer,[12] as well as in protozoan parasites.[13] Strikingly, in genomes of certain bacteriophages, 5hmU almost completely replaces thymine[14,15] due to inhibition of thymidylate synthase.[16] We found that the presence of 5hmU in the Pveg promoter significantly increases transcription with *Escherichia coli* RNA polymerase[17] and developed a transcription switch based on the photocaging and release of 5hmU in DNA.[18]

5-Formyluracil (5fU) is a product of oxidative damage of thymine that can cause mutations due to base-pairing with both A and G,[19,20] and the corresponding 5-formyl-2′-deoxyuridine triphosphate can be incorporated into DNA in the presence of dTTP.[21] DNA repair can release from DNA some modified pyrimidine nucleotides[22] that in principle can be phosphorylated to triphosphates and get randomly incorporated into genomic DNA by DNA polymerases. The oxidized epigenetic pyrimidines (5hmC, 5fC, 5caC, 5hmU, and 5fU) are probably too rare to significantly alter the genome

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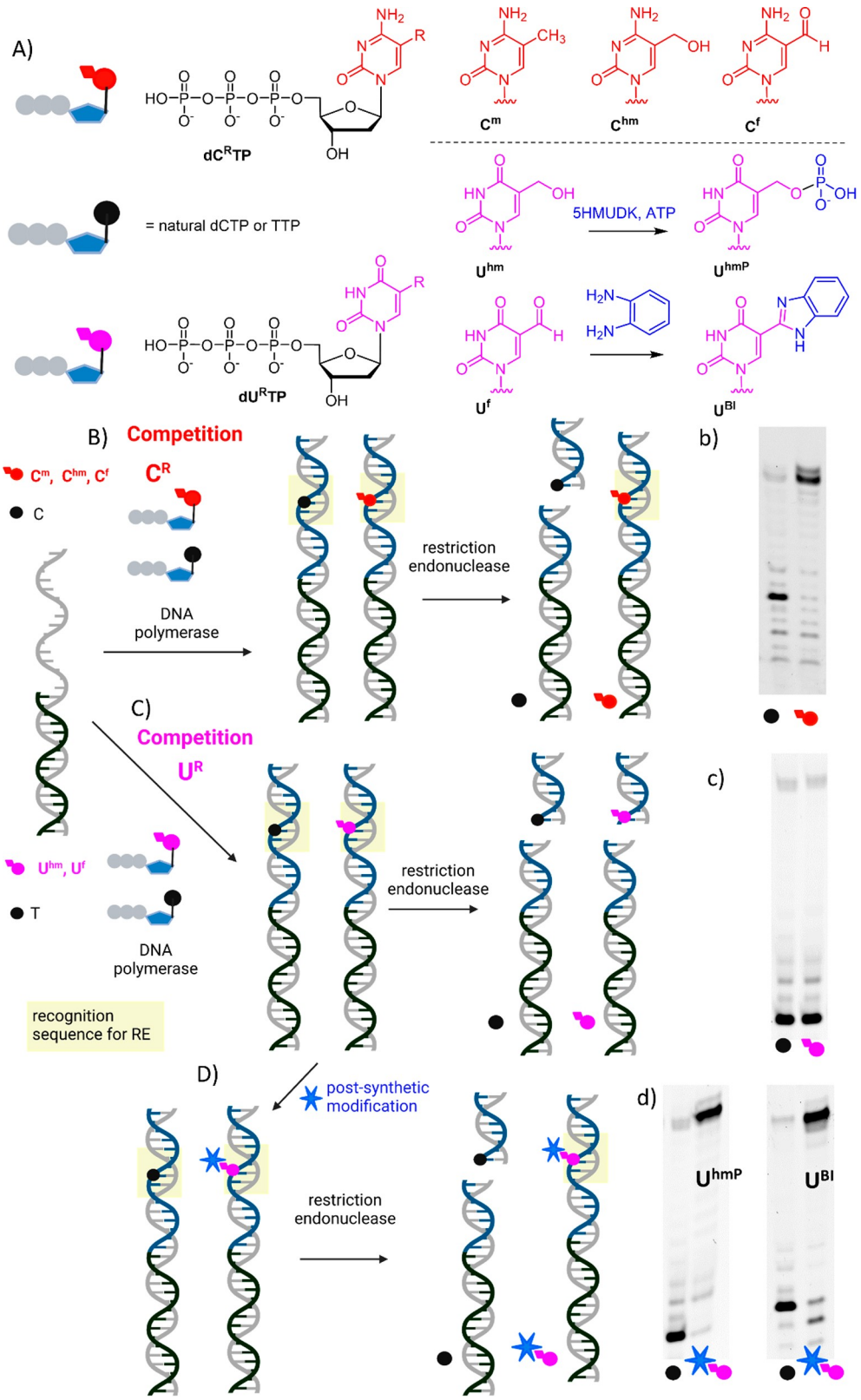

Figure 1. (A) Structures of dNTPs and postsynthetic labeling of U^{hm} and U^{f}; (B) competition PEX experiments with $dC^{R}TP$s followed by cleavage by RE; (C) competition PEX experiments with $dU^{R}TP$s followed by cleavage by RE; (D) competition PEX experiments with $dU^{R}TP$s followed by postsynthetic labeling and cleavage by RE; representative PAGE gels shown as b, c, and d.

through reincorporation, while the relatively frequent 5mC would cause significant gene silencing if randomly reincorporated. Indeed, experiments with the introduction of 5-methyl-dCTP to cells through microinjection or electroporation showed significant gene silencing.[23−25] To prevent the random reincorporation in normal healthy cells,[26] the 5mC 2′-deoxyribonucleoside monophosphate is deaminated to thymidine monophosphate by 5-methyl-dCMP deaminase,[27] and its

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phosphorylation by dCMP kinase is inhibited[28] to decrease the level of 5-methyl-dCTP.

5-Substituted pyrimidine or 7-substituted 7-deazapurine dNTPs are generally good substrates for DNA polymerases and can be used for enzymatic synthesis of base-modified DNA.[29,30] We[31,32] and others[33,34] have shown that some alkenyl-, alkynyl-, or aryl-substituted dNTPs can even be better substrates for DNA polymerases than the canonical natural dNTPs in competitive experiments, and the enzyme kinetic experiments revealed that the K_M values of some modified dNTPs are lower than those of the natural dNTPs due to increased cation−π stacking interactions in the active site of the polymerase.[31,32] We have also developed[31,32] an assay to assess the ratio of incorporation of modified versus natural nucleotides based on the cleavage of certain DNA sequences with type II restriction endonucleases (REs) that can be selected not to cleave the modified sequence and to cleave the unmodified one.[35−37] To the best of our knowledge, there was no report of a systematic and quantitative study of competitive incorporation of the 2′-deoxyribonucleoside triphosphates (dNTPs) bearing the epigenetic pyrimidine modifications in the presence of the natural dNTP counterparts, and therefore, we performed this research and report it here.

RESULTS AND DISCUSSION

We selected five epigenetically relevant pyrimidine dNTPs for our study (Figure 1A). The **dC^{m}TP** is commercially available, whereas the **dC^{hm}TP**,[38,39] **dC^{f}TP**,[38,39] and **dU^{hm}TP**[40] are known compounds, but they were prepared by modified procedures. The hydroxymethylated dNTPs **dC^{hm}TP** and **dU^{hm}TP** were prepared by a modified protocol through triphosphorylation of 5-acetyloxymethyl-2′-deoxyuridine[41] or -2′-deoxycytidine (for details, see the Supporting Information). Known **dC^{f}TP** and new **dU^{f}TP** were prepared by triphosphorylation[40] of 5-formyl-2′-deoxycytidine[8] or -uridine[42] (see the Supporting Information).

The portfolio of nine tested enzymes included examples of four classes of DNA polymerases from different forms of life: Bst Large Fragment and Taq from the prokaryotic A family; KOD XL, Pwo, and Vent(exo-) from the prokaryotic B family; T4 and SPO1 as viral polymerases; as well as human α and β DNA polymerases as eukaryotic enzymes. The SPO1 polymerase was particularly interesting because it is responsible for the synthesis of phage DNA containing 5hmU in *Bacillus subtilis* infected by bacteriophage SPO1.[43,44] SPO1 polymerase was expressed and purified as described in the Supporting Information. All of the other DNA polymerases were purchased from commercial suppliers.

In order to use our previously developed method[31,32] for testing of the outcome of competition primer extension (PEX) experiments based on cleavage by REs, for each modified nucleobase we needed to select an RE that fully cleaves the natural DNA and should not cleave the sequence containing the modified nucleobase. We used 5′-FAM-labeled primers for the PEX, and the polyacrylamide gel electrophoresis (PAGE) analysis that separates the cleaved (shorter) and uncleaved (longer) oligonucleotides allowed an accurate quantification of the outcome of the competition experiments. For modified cytidine derivatives, we found BglII as a suitable RE since the presence of **dC^{m}**, **dC^{hm}**, or **dC^{f}** within the recognition sequence completely blocks the enzymatic cleavage (Figure 1B,b). However, the uridine derivatives, **dU^{hm}** and **dU^{f}**, do not inhibit restriction cleavage by the most commonly available REs[45,46] (e.g., EcoRI, EcoRV, or ScaI, Figure 1C,c), and therefore, we used a different approach based on postsynthetic reactions of **dU^{hm}** and **dU^{f}** in DNA to form a bulkier modification capable of blocking the RE cleavage (Figure 1D,d). We chose the formation of a benzimidazole ring through the reaction with *o*-phenylenediamine (*o*-PDA)[47] as a labeling method for **dU^{f}**. The PEX products prepared with **dU^{f}TP** were treated with *o*-PDA to quantitatively form the benzimidazole-labeled DNA (confirmed by MALDI-TOF analysis) that inhibited the restriction cleavage by ScaI. To quantify **dU^{hm}**, we first intended and tested its oxidation to **dU^{f}** with $KRuO_4$.[48] However, we observed significant damage and a loss of DNA. Therefore, we used phosphorylation of the hydroxymethyl group by 5-hydroxymethyl DNA kinase (5-HMUDK) as reported previously[18] for switching of transcription. The phosphorylated-**dU^{hm}** completely inhibited the restriction by ScaI, which allowed us to distinguish and separate the products (Figure 1D,d). In all cases, we used double normalization using a positive control of natural DNA (+, 0%) and fully modified control (M, 100%) to accurately calculate the outcome of the competitive experiments. This approach was particularly important in the case of postsynthetic modification of **dU^{f}**, where the formed bulkier product **dU^{BI}** inhibited the RE cleavage by ScaI only to 75−95%.

With the methodology in hand, we screened all modified **dN^{R}TP**s in the competition with the natural dNTP counterparts (always at ratios 1:1 or 10:1) in the presence of each of the selected DNA polymerases. Typical outcomes of the experiments are shown in Figure 2, which shows the PAGE

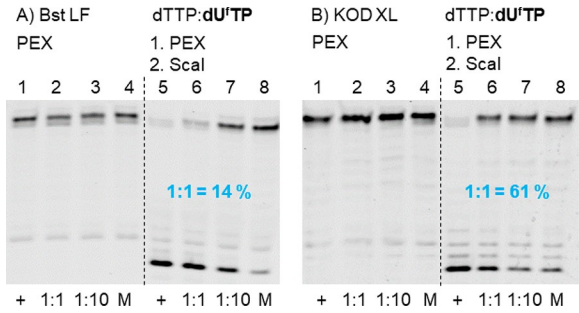

Figure 2. PAGE analyses of PEX experiments with Bst Large Fragment and KOD XL DNA polymerases and with **dU^{f}TP**. Lane 1 (+): product of PEX using natural dNTPs after *o*-PDA labeling. Lanes 2 (1:1) and 3 (1:10): products of PEX using three natural dNTPs and corresponding ratio of dTTP/**dU^{f}TP** after *o*-PDA labeling. Lane 4 (M): product of PEX using **dU^{f}TP** after *o*-PDA labeling. Lane 5 (+): product of PEX using natural dNTPs after *o*-PDA labeling and cleavage by ScaI. Lanes 6 (1:1) and 7 (1:10): products of PEX using three natural dNTPs and corresponding ratio of dTTP/**dU^{f}TP** after *o*-PDA labeling and cleavage by ScaI. Lane 8 (M): product of PEX using **dU^{f}TP** after *o*-PDA labeling and cleavage by ScaI.

analysis of the PEX reactions (at different ratios) and then cleavage by the RE (ScaI). The percentage of modified pyrimidine in the DNA sequence was calculated from the ratio of the intensity of the uncleaved (slower) and cleaved (faster) products. All other PAGE analyses for all **dN^{R}TP**s and all other polymerases are shown in the Supporting Information (Figures S4−S10), and all results are summarized in Tables 1 and 2. From the modified **dC^{R}TP**s, its 5-methyl derivative (**dC^{m}TP**) was found to be a superior substrate (better than natural dCTP) for all tested polymerases except for SPO1. The highest

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Table 1. Competitive PEX Experiments (Percentage of Incorporation of Modified dN^R)[a]

enzyme	Bst LF		Taq		KOD XL		Pwo		Vent(exo-)	
dNTP/**dN^{R}TP**	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
dC^{m}TP	76 (7)	97 (1)	71 (1)	97 (1)	66 (6)	96 (2)	78 (3)	97 (3)	73 (5)	95 (3)
dC^{hm}TP	11 (3)	56 (2)	17 (3)	72 (2)	47 (5)	86 (1)	53 (1)	90 (2)	50 (2)	89 (2)
dC^{f}TP	42 (3)	88 (2)	62 (3)	90 (4)	77 (3)	94 (3)	76 (1)	97 (1)	-[b]	-[b]
dU^{hm}TP	11 (2)	59 (1)	11 (3)	54 (3)	42 (3)	91 (2)	35 (4)	83 (2)	30 (1)	83 (0)
dU^{f}TP	14 (2)	63 (1)	19 (1)	67 (3)	61 (3)	86 (5)	59 (5)	88 (3)	57 (5)	88 (3)

[a]Standard deviations are in the parenteses. All experiments were performed in triplicate. [b]No PEX product was observed.

Table 2. Competitive PEX Experiments (Percentage of Incorporation of Modified dN^R)[a]

enzyme	T4		SPO1		human pol α		human pol β	
dNTP/**dN^{R}TP**	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
dC^{m}TP	87 (4)	98 (1)	42 (3)	77 (0)	58 (7)	91 (5)	71 (3)	90 (5)
dC^{hm}TP	78 (3)	96 (2)	14 (2)	58 (2)	46 (2)	90 (0)	17 (3)	67 (2)
dC^{f}TP	81 (8)	96 (5)	38 (6)	72 (5)	74 (3)	94 (3)	26 (5)	69 (7)
dU^{hm}TP	20 (1)	73 (3)	27 (3)	68 (7)	17 (3)	71 (8)	11 (1)	66 (4)
dU^{f}TP	30 (2)	82 (10)	51 (2)	86 (3)	47 (2)	90 (7)	10 (1)	45 (5)

[a]Standard deviations are in the parentheses. All experiments were performed in triplicate.

incorporation percentage was achieved with T4 polymerase, which incorporated **dC^m** almost exclusively (87%). The 5-formylcytidine triphosphate (**dC^{f}TP**) was also found to be a superior substrate for T4, Taq, KOD XL, Pwo, and human pol α enzymes. On the other hand, we did not observe any traces of the PEX product with **dC^{f}TP** using Vent(exo-) polymerase, which could be caused by a possible Schiff-base cross-link formation[49] from the aldehyde group with a lysine of the enzyme. The hydroxymethylated **dC^{hm}TP** was generally a somewhat worse or comparable substrate (compared to dCTP) except for the viral T4 polymerase that preferred even this nucleotide over its natural counterpart. Modified **dU^{R}TP**s were generally worse substrates than the modified **dC^{R}TP**s. **dU^{f}TP** was a slightly better substrate than dTTP only for prokaryotic KOD XL, Pwo, and Vent(exo-) with ca. 60% incorporation of **dU^f**, whereas for other polymerases it was a worse substrate than dTTP. The **dU^{hm}TP** was the worst substrate for all tested polymerases with maximum incorporation of only 42% (for KOD XL).

To verify the results of competitive PEX experiments, we performed the kinetics of single nucleotide incorporations with all modified **dN^{R}TP**s as well as their natural counterparts (Figure 3). The steady state kinetics model was employed using a 15-nt FAM-labeled primer and a 16-nt template designed for incorporation of only one single dNTP; hence the mixture contained only one studied dNTP or **dN^{R}TP**, and no additional dNTPs were present. The PAGE separation of the primer and product allowed an accurate quantification of the intensity of the FAM-labeled ONs by densitometric analysis of the fluorescence of the two bands.[31,32] We chose five different polymerases across the selected families for a confirmation kinetics study: Bst Large Fragment and Taq (prokaryotic A family), Vent(exo-) (prokaryotic B family), human polymerase β (eukaryotic), and SPO1 polymerase (viral). The modified dNTPs underwent 3 min PEX reactions followed by densitometric analysis of the formed product resolved on PAGE. In the case of SPO1 polymerase with **dC^{m}TP** and **dC^{f}TP**, the single-nucleotide-extended product was accompanied by an $n + 1$ product through a nontemplated addition of another nucleotide, and in those cases we included both

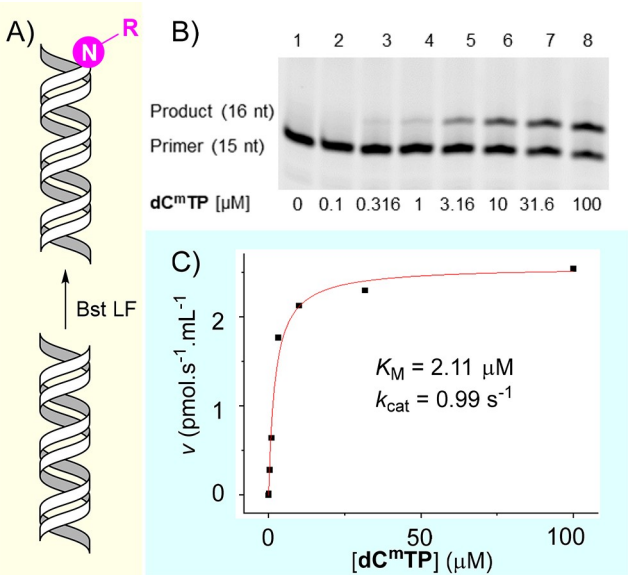

Figure 3. Principle of steady state kinetics of single nucleotide incorporation. (A) PEX experiment employing DNA polymerase and dNTP of various concentration in constant time (3 min). (B) PAGE analysis of the outcome. (C) Michaelis–Menten function and corresponding K_M and k_{cat} values obtained by fitting the values from PAGE analysis.

products in the calculations. The data were fitted into the Michaelis–Menten equation which provides K_M, corresponding to affinity of the substrate and enzyme, and k_{cat}, corresponding to rate of the reaction. The ratio k_{cat}/K_M indicates the substrate activity of the dNTP with the polymerase. Finally, we determined the discrimination rate calculated as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$, which shows the comparison with the natural counterpart and, therefore, the preference of the enzyme for the natural or modified dNTP.

The results are summarized in Tables 3–7. Generally, the kinetics results fit well to the data obtained by competition assays. Low K_M values and high discrimination rates of **dC^{m}TP** for Bst LF, Taq, and Vent(exo-) and Human β polymerase confirmed the superior substrate activity of this nucleotide

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Table 3. Bst Large Fragment Kinetics

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})	rate[a]
dCTP	2.91 (0.86)	1.07 (0.05)	0.366	1.00
dC^{m}TP	1.89 (0.20)	1.00 (0.01)	0.531	1.45
dC^{hm}TP	18.8 (4.6)	0.96 (0.07)	0.051	0.14
dC^{f}TP	15.9 (3.9)	1.17 (0.10)	0.073	0.20
dTTP	6.41 (1.19)	0.90 (0.06)	0.140	1.00
dU^{hm}TP	41.5 (14.4)	0.41 (0.08)	0.0098	0.069
dU^{f}TP	63.9 (4.4)	1.31 (0.16)	0.021	0.15

[a]The rate is defined as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$. All experiments were performed in triplicate; only **dU^{hm}TP** experiments were performed in duplicate.

Table 4. Taq Kinetics

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})	rate[a]
dCTP	5.65 (0.35)	0.54 (0.05)	0.096	1.00
dC^{m}TP	3.18 (0.19)	0.49 (0.08)	0.15	1.62
dC^{hm}TP	170 (38)	0.81 (0.18)	0.0047	0.05
dC^{f}TP	3.73 (1.30)	0.43 (0.10)	0.11	1.19
dTTP	9.37 (0.65)	0.45 (0.01)	0.049	1.00
dU^{hm}TP	27.3 (4.9)	0.17 (0.04)	0.0061	0.13
dU^{f}TP	23.1 (3.1)	0.45 (0.05)	0.019	0.40

[a]The rate is defined as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$. All experiments were performed in triplicate, only **dC^{hm}TP** experiments were performed as duplicate.

Table 5. Vent(exo-) Kinetics

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})	rate[a]
dCTP	5.42 (1.55)	0.48 (0.22)	0.089	1.00
dC^{m}TP	2.98 (0.90)	0.61 (0.19)	0.206	2.33
dC^{hm}TP	7.67 (2.55)	0.65 (0.20)	0.084	0.95
dC^{f}TP[b]	-	-	-	-
dTTP	3.95 (1.20)	0.59 (0.21)	0.149	1.00
dU^{hm}TP	9.60 (2.74)	0.56 (0.08)	0.058	0.39
dU^{f}TP	5.94 (1.01)	0.89 (0.12)	0.150	1.01

[a]The rate is defined as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$. [b]Not performed since this polymerase die not give PEX products with **dC^{f}TP**. All experiments were performed in triplicate.

Table 6. Human Polymerase β Kinetics

	K_M (μM)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_M ($\times 10^{-3}$ s^{-1} μM^{-1})	rate[a]
dCTP	1.88 (0.84)	5.62 (0.36)	2.99	1.00
dC^{m}TP	1.65 (0.45)	6.34 (0.60)	3.84	1.29
dC^{hm}TP	9.76 (2.50)	5.51 (0.19)	0.564	0.19
dC^{f}TP	7.72 (3.25)	5.53 (0.24)	0.716	0.24
dTTP	10.9 (3.4)	5.47 (0.05)	0.501	1.00
dU^{hm}TP	28.8 (4.9)	4.16 (0.31)	0.144	0.29
dU^{f}TP	13.6 (1.6)	3.22 (0.11)	0.238	0.47

[a]The rate is defined as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$. All experiments were performed in triplicate.

compared to natural dCTP. Also **dC^{f}TP** was confirmed to be a very good substrate for most of the enzymes (though less efficient than **dC^{m}TP**), and with Taq polymerase, it also showed a lower K_M value than natural dCTP and a higher discrimination rate of 1.19. Conversely, **dC^{hm}TP** was incorporated less effectively with the selected enzymes achieving its highest discrimination rate of ca. 0.95 with Vent(exo-) polymerase. The **dU^{f}TP** showed a comparable rate

Table 7. SPO1 Kinetics

	K_M (μM)	k_{cat} ($\times 10^{-4}$ s^{-1})	k_{cat}/K_M ($\times 10^{-4}$ s^{-1} μM^{-1})	rate[a]
dCTP	0.56 (0.15)	8.18 (0.39)	14.67	1.00
dC^{m}TP	0.41 (0.02)	4.08 (0.18)	9.98	0.68
dC^{hm}TP	0.49 (0.03)	4.37 (0.25)	8.87	0.60
dC^{f}TP	0.41 (0.11)	4.86 (0.40)	11.91	0.81
dTTP	0.54 (0.19)	3.13 (0.20)	5.75	1.00
dU^{hm}TP	0.83 (0.25)	2.84 (0.06)	3.44	0.60
dU^{f}TP	0.54 (0.07)	3.73 (0.34)	6.97	1.21

[a]The rate is defined as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$. All experiments were performed in triplicate.

of incorporation to dTTP with Vent(exo-) and SPO1 polymerases. On the other hand, **dU^{hm}TP** with low discrimination rate values was only rarely incorporated in competition with dTTP by all tested polymerases, including the SPO1 polymerase. Even at a 10:1 ratio, **dU^{hm}TP** gave only 68% incorporation, and it needed as much as a 100:1 ratio to reach 91% incorporation. This result is consistent with the model where the almost exclusive replacement of T with 5hmU in genomic DNA of the SPO1 bacteriophage is due to efficient inhibition of the TTP biosynthesis and the high abundance of **dU^{hm}TP**[14] rather than to the preference of the SPO1 DNA polymerase for **dU^{hm}TP**.

In conclusion, we have prepared five **dN^{R}TP**s containing epigenetic pyrimidines, **dC^{m}TP**, **dC^{hm}TP**, **dC^{f}TP**, **dU^{hm}TP**, and **dU^{f}TP**, and systematically studied their substrate activities in competitive PEX experiments in the presence of natural dCTP or dTTP, using an assay based on cleavage of the PEX product mixtures with REs. For quantification of **dU^{hm}** and **dU^{f}**, we developed a modified assay based on postsynthetic labeling followed by RE cleavage. The results of competitive PEX assays were verified by detailed kinetic studies with four classes of DNA polymerases. The study has revealed that **dC^{m}TP** is a superior substrate compared to dCTP with almost all tested DNA polymerases due to the higher affinity of this nucleotide to the active site of the enzymes (manifesting in lower K_M values compared to dCTP). Also, **dC^{f}TP** was a superior substrate for several polymerases, whereas **dC^{hm}TP**, **dU^{hm}TP**, and **dU^{f}TP** were worse substrates than dCTP or dTTP, but still significant incorporations were observed at the 1:1 ratio with their natural counterparts. These results indicate that **dN^{R}TP**s containing the epigenetic pyrimidines, which can be formed through DNA repair and a salvage pathway, could get randomly incorporated into genomic DNA by polymerases and thus modify the epigenetic profile of the genome. This could be most relevant for the most abundant **dC^{m}**, whose triphosphate **dC^{m}TP** is a superior substrate for DNA polymerases. Apparently, the efficient deamination[27] and inhibition of phosphorylation[28] of **dC^{m}MP** are absolutely crucial for preventing the random gene silencing due to endogenous DNA methylation. Suitable delivery of **dC^{m}TP** into cells through transport systems[50] or triphosphate prodrugs[51] might be used to induce gene silencing. On the other hand, the **dU^{hm}TP** is a rather poor substrate (worse than dTTP), even for SPO1 DNA polymerase that synthesizes the 5hmU-rich genome of the SPO1 bacteriophage, and the virus crucially needs inhibition of dTTP synthesis to achieve the efficient incorporation of 5hmU into its DNA.

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METHODS

A full experimental section with methods and characterization of compounds is given in the Supporting Information. Only selected typical procedures are given below.

Competitive Assay: Incorporation of Modified dC^{R}TP Employing Human DNA Polymerase α and β. Primer annealing: The primer Prim248short-FAM was mixed with the template TempBglII−C (1.5-fold excess) in aqueous Tris·HCl buffer (pH 7.5, 50 mM), DTT (5 mM), and $MgCl_2$ (5 mM) to obtain a 1.0 μM final concentration of the primer. The annealing was performed in a thermal cycler. The sample was incubated at 95 °C for 5 min and then allowed to slowly cool down to 25 °C over 60 min. Prepared primed-TempBglII−C was stored at −20 °C.

Competitive incorporation of dCTP vs **dC^{R}TP** (R = **f**, **hm**, **m**) by PEX: The reaction mixture (30 μL) contained primed-TempBglII−C (0.1 μM primer, 0.15 μM template), human polymerase (α or β, 1.5 U), and natural dNTPs (dGTP, dTTP, and dATP, 100 μM); for a ratio of 1:1, dCTP (50 μM) and **dC^{R}TP** (50 μM); for a ratio of 1:10, dCTP (10 μM) and **dC^{R}TP** (100 μM); for positive control sample, dCTP (100 μM); for modification control sample, **dC^{R}TP** (100 μM); BSA (0.1 mg mL^{-1}); glycerol (10%); DTT (5 mM); and $MgCl_2$ (5 mM) in aqueous Tris·HCl buffer (pH 7.5, 50 mM). The mixture was incubated at 37 °C for either 2 h (human polymerase α) or 1 h (human polymerase β) and then divided into 2 × 15 μL. The stop solution (15 μL) was added to the first portion, and the mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S5, lanes 1, 2, 3, and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Cleavage by BglII: The second portion of the PEX product (15 μL) was mixed with NEBuffer 3.1 (3 μL) and BglII (20 U). The mixture was incubated at 37 °C for 60 min and then stopped by the addition of stop solution (to reach a 30 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S5 in Supporting Information).

Competitive Assay: Incorporation of dU^{f}TP Employing Taq DNA Polymerase. Competitive incorporation of dTTP vs **dU^{f}TP** by PEX: The reaction mixture (40 μL) contained primer Prim248Short-FAM (0.15 μM), template TempSp-T (0.225 μM), Taq polymerase (1 U), natural dNTPs (dGTP, dCTP, and dATP, 100 μM); for ratio 1:1, dTTP (50 μM) and **dU^{f}TP** (50 μM); for ratio 1:10, dTTP (10 μM) and **dU^{f}TP** (100 μM); for positive control sample, dTTP (100 μM); and for modification control sample, **dU^{f}TP** (100 μM) in a reaction buffer provided by a supplier. The mixture was incubated at 60 °C for 30 min.

Labeling of **dU^f** by o-PDA: To the mixture after PEX reaction (40 μL), a freshly prepared aqueous solution of *o*-PDA (100 mM, 2 μL, at 37 °C) was added. The mixture was incubated at 37 °C for 5 h and then divided into 2 × 21 μL. The stop solution (19 μL) was added to the first portion; the mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S6, lanes 1, 2, 3, and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Cleavage by SphI: The second portion of the PEX product (21 μL) was mixed with CutSmart Buffer (2 μL) and SphI-HF (20 U). The mixture was incubated at 37 °C for 30 min and then stopped by the addition of stop solution (to reach 40 μL total volume). Products of the cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S6, lanes 5, 6, 7, and 8).

Steady State Kinetics Assay. Reaction mixtures (10 μL) contained 5′-6-FAM-labeled primer Prim248Short-FAM (1 μM) and template Oligo1-termC (1 μM) for **dC^{R}TP** incorporation or Oligo1-termT (1 μM) for **dU^{R}TP** incorporation (1 μM) and DNA polymerase (Table S4) in a reaction buffer provided by a supplier. Reactions were initiated by the addition of various concentrations of natural or modified **dN^{R}TPs**, and the mixtures were incubated for 3 min at temperatures corresponding to the DNA polymerase of interest (Table S4). The final **dN^{R}TPs** concentrations in the samples were 0, 0.1, 0.316, 1, 3.16, 10, 31.6, and 100 μM. Reactions were stopped by the addition of 10 μL of stop solution. Products were denatured at 95 °C for 5 min and separated using 20% denaturing PAGE (Figures S11−S17 in the Supporting Information). Kinetic parameters (k_{cat} and K_M) were determined by fitting data to the Michaelis−Menten equation using Microsoft Excel and OriginPro 2021. The ratio of catalytic efficiency of modified **dN^{R}TP** with respect to natural dNTP was calculated as $(k_{cat}/K_M)_{modified}/(k_{cat}/K_M)_{natural}$. In the cases when a double band of the product was observed, the slower band (product N+1 of nontemplated addition of another nucleotide) was also included in the calculation. All experiments were performed in triplicate.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00342.

Full experimental section with synthetic procedures and characterization of all compounds, biochemical methods and procedures, figures of all PAGE analyses, and copies of NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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