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Published on Web 11/14/2002

Enzymatic Phosphorylation of Unnatural Nucleosides

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Received August 7, 2002

Abstract: In an effort to expand the genetic alphabet, a number of unnatural, predominantly hydrophobic, nucleoside analogues have been developed which pair selectively in duplex DNA and during enzymatic synthesis. Significant progress has been made toward the efficient in vitro replication of DNA containing these base pairs. However, the in vivo expansion of the genetic alphabet will require that the unnatural nucleoside triphosphates be available within the cell at sufficient concentrations for DNA replication. We report our initial efforts toward the development of an unnatural in vivo nucleoside phosphorylation pathway that is based on nucleoside salvage enzymes. The first step of this pathway is catalyzed by the *D. melanogaster* nucleoside kinase, which catalyzes the phosphorylation of nucleosides to the corresponding monophosphates. We demonstrate that each unnatural nucleoside is phosphorylated with a rate that should be sufficient for the in vivo replication of DNA.

Introduction

In an effort to expand the genetic alphabet by supplementing the natural base pairs dA:dT and dG:dC with an unnatural base pair, we have synthesized and characterized a variety of predominantly hydrophobic nucleobases analogues.[1−6] Several of the unnatural base pairs formed between these hydrophobic nucleobases are stable in duplex DNA and are also inserted, proofread, and extended efficiently and selectively by DNA polymerases in vitro.[7] On the basis of these results, as well as pioneering studies from the Kool lab,[8,9] it is apparent that the requirements for duplex stability and replication do not limit the genetic code to hydrogen bonded (H-bonded) base pairs, and hydrophobic interactions may be sufficient to control information storage and retrieval. The in vivo replication of DNA containing these unnatural base pairs requires that the unnatural nucleoside triphosphates be available intracellularly at sufficient concentrations for DNA synthesis. Thus, we have begun to examine different pathways for intracellular activation of unnatural nucleosides. These studies of unnatural nucleoside activation may also aid in the design of pharmacologically active nucleoside analogue drugs that also rely on cellular activation.

The simplest strategy to supply *E. coli* with unnatural nucleoside triphosphates is to supplement the growth media with the unnatural nucleosides. It is possible that these small, hydrophobic molecules will either passively diffuse or be actively transferred across the lipophilic cell membrane and become trapped inside the cell, provided that they are phosphorylated by cellular kinases of the nucleoside salvage pathway.[10] In this pathway nucleosides are converted to the corresponding triphosphates by the successive action of nucleoside, monophosphate, and diphosphate kinases. This pathway produces nucleoside triphosphates in sufficient concentrations for a variety of cellular functions. For example, in mammalian cells, DNA repair, mitochondrial DNA synthesis in G1 phase cells, and the activation of antiviral and cytostatic nucleoside analogues,[11,12] all rely on nucleoside triphosphates from the salvage pathway.

The first step in the synthesis of nucleoside triphosphates is the nucleoside kinase catalyzed transfer of the γ-phosphate from a donor molecule (ATP) to the nucleoside C5′−OH acceptor to yield the nucleoside monophosphate. In some cases, this is the rate-limiting step in the pharmacological activation of nucleoside drug analogues.[13] Thus, a variety of antiviral therapies are based on the ability of a virus-encoded kinase to phosphorylate a broader range of substrates than the host cellular kinases. For example, acyclovir is selectively activated by herpes simplex virus type-1 thymidine kinase (HSV-1 TK),[14] which

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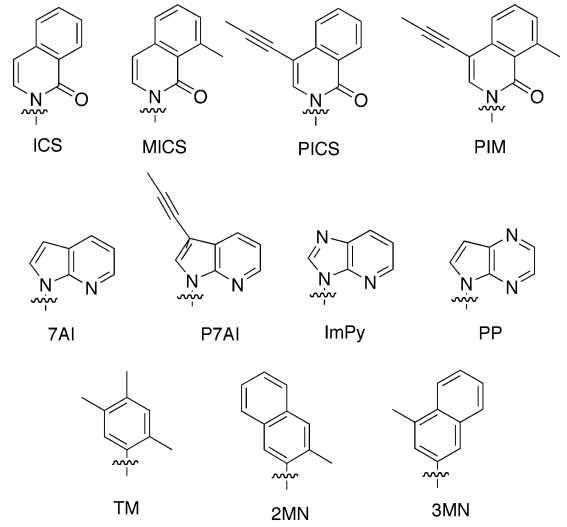
10.1021/ja028050m CCC: $22.00

Scheme 1. Unnatural Nucleotides.



also phosphorylates a broad range of nucleoside analogues with modifications in both the furanose ring and the nucleobase. Another deoxynucleoside kinase with a broad substrate tolerance is the kinase from *D. melanogaster* (*Dm*-dNK).[13,15] *Dm*-dNK is 14% identical to HSV-1 TK and is the only known kinase in *Drosophila*.[13,15] *Dm*-dNK was the first kinase shown to phosphorylate all four natural deoxyribonucleosides with reasonable efficiency, although it is most active with pyrimidine nucleosides.

The replication of DNA containing an unnatural base pair in *E. coli* might be based on phosphorylation of the unnatural nucleoside precursors by endogenous salvage enzymes. However, preliminary examination of the endogenous nucleoside kinase activities in *E. coli* whole cell lysates was not promising. Furthermore, unlike HSV-1 TK and *Dm*-dNK, there is little evidence that *E. coli* nucleoside kinases are capable of phosphorylating a broad range of substrates.[16−19] Therefore, instead of focusing on bacterial kinases, we examined the activity of HSV-1 TK and *Dm*-dNK toward the unnatural nucleosides shown in Scheme 1. Preliminary kinetic characterization of both HSV-1 TK and *Dm*-dNK demonstrated that the unnatural nucleosides were more efficiently phosphorylated by *Dm*-dNK. Thus, we initiated a more complete characterization of the specificity and catalytic efficiency of *Dm*-dNK toward the unnatural nucleosides. A comparison of steady-state kinetic rate data obtained for a variety of unnatural nucleosides allowed the effects of base structure and hydrophobicity to be examined. The results suggest that *E. coli* transformed with a plasmid encoding *Dm*-dNK should be capable of providing unnatural monophosphates at physiologically relevant concentrations.

Experimental Section

Abbreviations. HSV-1 TK, herpes simplex virus type-1 thymidine kinase; *Dm*-dNK, kinase from *Drosophila melanogaster*; AZT, 3′-azido-3′-deoxythymidine; dA, deoxyadenosine; dT, deoxythymidine; dG, deoxyguanosine; dC, deoxycytidine; KF, Klenow fragment of DNA polymerase I; pol β, DNA polymerase β.

Kinase Expression. A plasmid containing the *Dm*-dNK gene was kindly provided by Prof. Magnus Johansson (Karolinska Institute, Sweden). The gene was subcloned into the pET-15b expression vector, and confirmed by sequencing. This expression plasmid results in fusion of the carboxy terminus of the protein to a $(His)_6$ tag. Following transformation of BL21(DE3) pLysS *E. coli* (Stratagene), protein was overexpressed by IPTG induction and purified by affinity chromatography with a Ni−NTA affinity column (Novagen). The protein was dialyzed extensively against 50 mM Tris (pH 8.0), 1 mM DTT, 50 mM NaCl buffer. The size and purity of the recombinant protein was determined by SDS−PAGE. The protein concentration was determined using the Bio-Rad protein assay with BSA as a standard. Approximately 2 mg of pure protein was obtained from a 1 L culture. All nucleoside analogues were synthesized as reported previously,[1−5] with the exceptions of dT, dA, AZT, and acyclovir which were obtained from Sigma.

Kinetic Assay. Nucleoside monophosphorylation was assayed with standard literature protocols based on the phophorylation of radiolabeled nucleosides.[18,20] However, to rapidly screen many different unnatural nucleosides an alternative assay was developed that is based on supplementing the ATP phosphate donor pool with a small amount of γ-^{32}P labeled ATP. Product formation was monitored by chromatographic separation and subsequent quantitation of the ^{32}P labeled monphosphorylation product. This assay worked well for *Dm*-dNK, which has a high affinity for ATP ($K_{M(ATP)} = 2\ \mu M$),[15] allowing assays to be run under saturating conditions with only 50 μM of total ATP. The assay was less reproducible for the HSV-1 TK enzyme, which has a higher $K_{M(ATP)}$ (100 μM)[21,22] (necessitating higher concentrations of ATP and complicating the quantification of the product relative to ATP).

The kinetic assays were performed with 1−5 nM kinase in 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL BSA. This reaction buffer was supplemented with 50 μM of ATP that was spiked with 0.1% $\gamma-^{32}$P labeled ATP. Reactions were performed with varying concentrations of nucleoside substrates. Twenty microliter reactions were incubated (37 °C for 1−10 min) and terminated by heating (95 °C for 5 min). One microliter of the reaction was then spotted on polyethyleneimine cellulose F thin-layer chromatography sheets (J. T. Baker), and the nucleosides and product monophosphates were separated using 66% isobutyric acid, 33% water, and 1% NH_4OH. Radioactivity was quantitated using a PhosphorImager (Molecular Dynamics), with overnight exposures and ImageQuant software (Molecular Dynamics). The steady-state kinetic data were evaluated by plotting the initial rates against nucleoside concentration and fitting the data to the Michaelis−Menten equation. Data presented are the average of three independent experiments.

Results and Discussion

Phosphorylation of Unnatural Nucleosides. Steady-state kinetic data for the *Dm*-dNK phosphorylation of deoxythymidine (dT), deoxyadenosine (dA), and thirteen unnatural nucleosides are reported in Table 1. The unnatural nucleosides tested included AZT and acyclovir, both of which have modified furanose rings, and eleven unnatural nucleosides with hydrophobic "bases" (Scheme 1). Eight of the unnatural hydrophobic nucleosides are based on the isocarbostyryl (**ICS**) or 7-azaindole (**7AI**) rings, or derivatives thereof (d**ICS**, d**PICS**, d**MICS**, d**7AI**, d**PP** and d**3MN**). The incorporation of each nucleoside into DNA has been characterized and a number of them form base pairs that are promising candidates for the replication of

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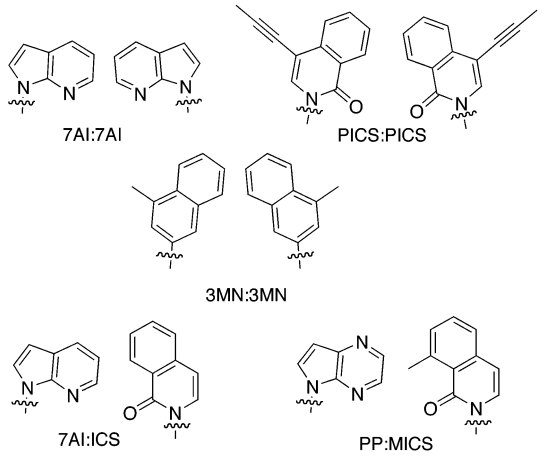
Table 1. Kinetic Parameters for *Dm*-Dnk toward Different Substrates[a]

nucleoside	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
dT	4.8 ± 1.0	1.6 ± 0.5	3.0×10^6
dA	5.2 ±.0	247 ± 50	2.1×10^4
d**ICS**	3.0 ± 0.5	152 ± 30	2.0×10^4
d**PICS**	0.8 ± 0.2	94 ± 10	8.5×10^3
d**MICS**	3.7 ± 0.5	92 ± 20	4.0×10^4
d**PIM**	1.5 ± 0.5	85 ± 10	1.8×10^4
d**7AI**	0.8 ± 0.2	686 ± 100	1.2×10^3
d**PP**	1.4 ± 0.4	306 ± 50	4.6×10^3
d**ImPy**	0.7 ± 0.2	510 ± 100	1.4×10^3
d**P7AI**	2.3 ± 0.2	75 ± 30	3.1×10^4
d**TM**	0.16 ± 0.02	682 ± 70	2.3×10^2
d**3MN**	0.20 ± 0.03	438 ± 123	4.6×10^2
d**2MN**	1.3 ± 0.2	105 ± 40	1.2×10^4
AZT	0.028 ± 0.005	15 ± 2	1.8×10^3
Acyclovir	n.d.[b]	n.d.[b]	$\leq 1.0 \times 10^2$

[a] See text for experimental details. [b] Not determined independently due to the slow rate of reaction.

Scheme 2. Unnatural Base Pairs.



unnatural DNA, including the **7AI**:**7AI**,[2,4,7] **PICS**:**PICS**,[1,2] and **3MN**:**3MN**[3] self-pairs and the **ICS**:**7AI**[2] and **PP**:**MICS**[4] heteropairs (Scheme 2). The unnatural nucleosides d**TM**, d**2MN**, d**P7AI**, d**ImPy**, and d**PIM** were also studied as kinase substrates in order to evaluate the effects of alternative base structures and hydrophobicity.

A rapid kinase assay was developed based on the transfer of $\gamma-^{32}$P from ATP to the nucleoside substrate. The kinetic constants obtained for substrates dT and dA using the assay were compared to previously reported literature values.[13] Native substrate dT is phosphorylated most efficiently ($k_{cat}/K_M = 3.0 \times 10^6$ $M^{-1}s^{-1}$), whereas phosphorylation of dA is approximately 100-fold less efficient ($k_{cat}/K_M = 2.1 \times 10^4$ $M^{-1}s^{-1}$), predominantly due to an elevated K_M (1.6 μM and 247 μM for dT and dA, respectively). These data are in good agreement with literature values.[13]

The enzymatic phosphorylation of the unnatural nucleosides was then investigated. *Dm*-dNK phosphorylates AZT with moderate efficiency ($k_{cat}/K_M = 1.8 \times 10^3$ $M^{-1}s^{-1}$). Although the enzyme binds this nucleoside analogue with a reduced affinity compared to dT ($K_M = 15$ μM and 1.6 μM for AZT and dT, respectively), the decrease in efficiency is largely due to a reduced rate of catalytic turnover ($k_{cat} = 2.8 \times 10^{-2}$ s^{-1} vs 4.8 s^{-1} for AZT and dT, respectively). *Dm*-dNK shows no detectable activity toward the acyclovir nucleoside.

The enzymatic phosphorylation of **ICS** analogues shown in Scheme 1 was then evaluated. Overall, the **ICS** analogue nucleosides were found to be good substrates for *Dm*-dNK (Table 1). d**ICS** is phosphorylated much more efficiently than either AZT or acyclovir; the rate is actually equivalent to that of dA ($k_{cat}/K_M = 2.0 \times 10^4$ $M^{-1}s^{-1}$). Like dA, the decreased rate of phosphorylation of d**ICS**, relative to dT ($k_{cat}/K_M = 3.0 \times 10^6$ $M^{-1}s^{-1}$) results from weaker binding of the nucleoside ($K_M = 152$ μM and 1.6 μM for d**ICS** and dT, respectively). Substitution of the **ICS** ring was found to have small effects on the phosphorylation efficiency. C3-methyl substitution (d**MICS**), C7-propynyl substitution (d**PICS**), and both C3 and C7 substitutions (d**PIM**) of the **ICS** ring were examined. The increased bulk of C3-methyl substitution of d**MICS** resulted in a 2-fold increase in k_{cat}/K_M. However, while the additional steric demands of the C7-propynyl group of d**PICS** resulted in a slight decrease in K_M, the decrease was more than offset by an almost 4-fold decrease in k_{cat}. Taken together, these effects result in an efficiency that is 2-fold decreased relative to d**ICS**. d**PIM**, containing both C2-methyl and C7-propynyl substitutions, was converted to the monophosphate with the same efficiency as d**ICS**, presumably due to the effects of the two substitutions on k_{cat}. In all cases, the increased bulk at the **ICS** ring decreased the K_M of the enzyme for the substrate, but the effect on $k_{cat}$was variable. Consistent with this notion, d**PIM** is the most tightly bound of the hydrophobic base analogues examined ($K_M = 85$ μM).

The phosphorylation of the purine-like unnatural nucleosides d**7AI**, d**PP**, d**ImPy**, and d**P7AI** were also characterized (Table 1). d**7AI** is not as efficiently phosphorylated by the nucleoside kinase as is d**ICS**. The d**7AI** phosphorylation efficiency (k_{cat}/K_M) was 1.2×10^3 $M^{-1}s^{-1}$. This order of magnitude reduction in rate, relative to phosphorylation of dA, resulted from a 6.5-fold reduction and a 2.8-fold increase in k_{cat} and K_M, respectively. Apparently, H-bond acceptors (N1, N3, N7, or the exocyclic amine) or associated polarizability effects make dA a better phosphorylation substrate than the azaindole ring of d**7AI**. Structural studies (see below) with purine analogue substrates have implicated that important nucleobase-protein H-bonding interactions exist involving the N1 and exocyclic C2 substituents, as well as the N7 atom. To assess the importance of these interactions, we examined the effect of aza-substitution of the indole ring at positions 4 (d**PP**) and 3 (d**ImPy**), corresponding to the purine 6 and 7 positions. Aza-substitution at C3 of the azaindole ring resulted in little change in k_{cat}/K_M (1.2×10^3 $M^{-1}s^{-1}$ and 1.4×10^3 $M^{-1}s^{-1}$ for d**7AI** and d**ImPy**, respectively). Aza-substitution at C4 (d**PP**) resulted in an almost 4-fold increase in k_{cat}/K_M (4.6×10^3 $M^{-1}s^{-1}$) due to an increase in both k_{cat} and a decrease in K_M. It therefore appears likely that H-bonding or electronic interactions between the protein and the N1/exocyclic C2 substituent are moderately important for activity.

The addition of a C3 propynyl group to the azaindole ring (d**P7AI**) had a significant positive effect on phosphorylation efficiency. The added propynyl group resulted in a 3-fold increase in k_{cat} and a 9-fold decrease in K_M, relative to d**7AI**, leading to a k_{cat}/K_M for phosphorylation that is greater than that for either dA or d**ICS**. In fact, d**P7AI** is phosphorylated only 100-fold less efficiently than dT (3.1×10^4 $M^{-1}s^{-1}$ and 3.0×10^6 $M^{-1}s^{-1}$ for d**P7AI** and dT, respectively), 20-fold faster than

AZT, and at least 300-fold faster than acyclovir. Apparently, the *Dm*-dNK active site is able to accommodate the increased steric demands of the propynyl group of d**P7AI** (see below).

To further address the role of hydrophobicity, we examined d**TM**, d**2MN**, and d**3MN** as substrates for *Dm*-dNK. The d**TM** nucleoside is an approximate shape mimic of the enzymes natural substrate, dT, but is only inefficiently phosphorylated (2.3×10^2 $M^{-1}s^{-1}$) due to both a reduced k_{cat} (nearly 20-fold), as well as an increased K_M (4.5-fold) relative to d**ICS**. As discussed below, *Dm*-dNK is expected to make numerous H-bonding and hydrophobic contacts with the base analogue. The absence of H-bond donor and acceptors, along with the minimal aromatic surface area, is apparently sufficient to render d**TM** a poor kinase substrate. The increased aromatic surface area of d**3MN** does not result in increased phosphorylation relative to d**TM**. However, the d**2MN** nucleoside, with the same surface area as d**3MN**, is phosphorylated 50-fold faster than is d**TM**, due to an increase in k_{cat} (8-fold) as well as a decrease in K_M (6-fold). These data demonstrates that both nucleobase H-bonding groups and shape play important roles in substrate recognition.

Protein-Nucleobase Interactions. The crystal structure of *Dm*-dNK bound to deoxycytidine was recently solved.[23] The enzyme has an α/β architecture with a central five-strand parallel sheet. The substrate dC is located in a deep pocket at the C-terminus of the β-sheet. The cytosine base is packed between F111 on one face and W57 and F80 on the other face. Other residues involved in hydrophobic packing are M69, Y70, V84, M88, A110, and M118. The base also makes two hydrogen bonds to Q81 via N3 and N4, whereas the 2-carbonyl oxygen of dC is hydrogen bonded to two water molecules.

In addition to the *Dm*-dNK structure, several structures have been reported for HSV-1 TK.[21,22,24−26] With approximately 30% of amino acid sequence similarity, the two enzymes show similar structure with several critical residues conserved in the substrate binding site.[13] A structural basis for the HSV-1 TK polyspecificity has been well documented and involves the reorganization of several amino acid side chains in the nucleobase binding site that allows for efficient packing of different substrates. When bound to dT, the thymine base makes three H-bonding contacts with the protein, two between the nucleobase O4 and N3 atoms and the donor and acceptor functionalities of Q125, respectively (this residue is conserved in *Dm*-dNK as Q81; hereafter, the corresponding *Dm*-dNK residues are given in parentheses for comparison).[13] The 2-carbonyl oxygen of dT also forms a water-mediated hydrogen bond with R176 (S123). The thymine base is hydrophobically packed by M128 (V84), I100 (deleted in *Dm*-dNK), W88 (W57), Y132 (M88), R163 (R111) and Y172 (R119). HSV-1 TK is able to phosphorylate uracil derivatives with bulky C5 substituents by readjustments of the hydrophobic pocket, including W88 (W57), R163 (R111), A167 (V115), and particularly Y132 (M88).[27] Structural rearrangements of the *Dm*-dNK residues may accommodate the propynyl group of d**P7AI** and facilitate phosphorylation. To accommodate guanosine analogues, HSV-1 TK is again able to conformationally reorganize, especially at M128 (V84), I100 (deleted), Y172 (R119), and M231 (Y179) and efficiently pack the modified nucleobases.[24,26] The H-bonding of Q125 (Q81) to the substrate is preserved in these complexes involving contacts with the guanine N1 and O6 atoms. The 6-carbonyl group of the purine also hydrogen bonds to the guanidinium group of R176 (S123). It is apparent that H-bonding groups located in the N1/O6 region of purine scaffolds mediate important interactions with HSV-1 TK residues Q125 (Q81) and R176 (S123). Similar interactions between *Dm*-dNK residues S123 and Q81 may facilitate the phosphorylation of d**PP**, relative to d**7AI**. Moreover, as discussed above, structural rearrangements involving W57, R111, V115, M88, V84, R119, and Y179 may allow for favorable interactions between the protein and the d**P7AI** propynyl group that result in the efficient activation of this nucleoside.

Implications for Unnatural Nucleobase Design. Several trends emerge from an analysis of *Dm*-dNK phosphorylation rates that may facilitate the future design of nucleoside analogues that are efficiently phosphorylated. Electronic effects resulting from aza-substitution are apparent but generally small. Structural effects are found to be more significant. The larger nucleosides d**2MN** and d**3MN** have the same hydrophobic surface area relative to d**TM**, but the additional aryl ring is oriented differently with respect to the glycosidic bond. Although the larger hydrophobic surface area of d**3MN**, relative to d**TM**, affords no increase in the reaction rate, the same increase in surface area of d**2MN** results in a 50-fold increase. The addition of a propynyl group had a similarly variable effect. Addition of a C7-propynyl group to the **ICS** ring had little effect, whereas the addition of the same group to the C3 position of the **7AI** ring resulted in a 26-fold increased rate of phosphorylation. The disparate effects that result from the same increase in hydrophobic surface area reflect the importance of substituent orientation. It seems likely that a carefully designed increase in hydrophobic surface area may generally facilitate unnatural nucleoside monophosphorylation.

d**7AI** has emerged as a particularly interesting unnatural nucleobase due to the recent demonstration that DNA containing the unnatural **7AI**:**7AI** self-pair may be efficiently replicated in vitro.[7] We have demonstrated that the d**7AI** nucleoside is phosphorylated by *Dm*-dNK with a rate that should supply the cell with sufficient concentrations of the monophosphate for further processing. However, derivatives that maintain replication efficiency and fidelity, and also improve activation of the nucleoside may be desirable. In this regard, d**PP** and d**P7AI** are particularly interesting, and their behavior implies that further derivatization may result in an unnatural nucleoside that is a good substrate for both kinase and polymerase enzymes. For example, the combination of C3 propynyl- and C4 aza-substitution, which each individually increase phosphorylation efficiency by *Dm*-dNK, might cooperatively increase the rate of phosphorylation. Moreover, aza- and alkyl-substitution at other indole positions might also result in more efficient activation of the nucleoside. Further experiments are currently in progress to evaluate these possibilities. Additional modifications of the **ICS** scaffold, by aza- and alkyl-substitution are also being investigated.

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Nature has evolved a system of information storage and retrieval based on complementary nucleobase H-bonds. However, there is no reason H-bonding should be uniquely capable of supporting the intermolecular interactions required for information storage and replication. We have demonstrated that hydrophobicity, known to be a dominant force in proteins,[28] is also capable of providing the intermolecular interactions required for nucleic acid structure and function, including base pair stability in duplex DNA, enzymatic triphosphate insertion, proofreading, and extension. We have recently developed a binary polymerase system, comprised of KF and pol β, that is capable of the in vitro replication of DNA containing three base pairs.[7] It is now apparent that the unnatural nucleoside analogues may also be efficiently phosphorylated to their corresponding monophosphates by *Dm*-dNK. Therefore, the first component of an unnatural phosphorylation pathway in *E. coli* could be based on transforming the bacteria with a plasmid coding for the Dm-dNK, and supplementing the growth media with the desired unnatural nucleosides. Although several important enzymes remain to be identified, including mono- and diphosphate kinases, *Dm*-dNK joins KF and pol β as part of an evolving system for the in vivo replication of DNA containing three base pairs.

Acknowledgment. The research described in this report was supported by the National Institutes of Health (GM 60005 to F.E.R.) and the Skaggs Institute for Chemical Biology (F.E.R. and P.G.S.).

JA028050M

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