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Difluorotoluene, a Nonpolar Isostere for Thymine, Codes Specifically and Efficiently for Adenine in DNA Replication

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The high specificity of incorporation of nucleotides into DNA by polymerase enzymes is crucial for maintaining fidelity of information transfer in cellular replication. The initial insertion event is the first point at which mutations of the genome are avoided.[1,2] Mismatched pairing at this step occurs on the level of only ~1 in 10^3–10^5 insertions, indicating a selectivity of at least 4 kcal/mol.[1] It is clear that polymerases enhance the selectivity of nucleotide choice at the active site relative to the much lower pairing differences observed at the duplex terminus in the absence of enzyme.[2]

While many of the kinetic details of replication have been studied in recent years,[3] the precise physical origins of this selectivity enhancement are poorly understood. Mechanisms involving both kinetic and binding selectivity between correct and incorrect nucleotides have been proposed.[3] Base–base hydrogen bonding, base stacking, base pair geometry, and interactions between the enzyme, DNA, and nucleotides have all been invoked as potentially important interactions; however, the relative importance of these different effects remains unclear. Many DNA nucleotide analogs with altered or reduced H-bonding potential have been examined as substrates for polymerases;[4] most of those analogs are quite poor substrates, and result in less discriminate incorporation fidelity than do the natural nucleotides. This general finding has been used as evidence that the number and strength of hydrogen bonds in a given pair determine efficiency and fidelity of DNA synthesis. Indeed, most if not all current models for replication fidelity hold that the specificity of hydrogen bonds formed in the new base pair is a central contributor to the observed selectivity.

Here we present evidence, however, that a DNA polymerase can exert high fidelity even when a base pair completely lacks conventional hydrogen bonds. The difluorotoluene nucleoside **1** has recently been constructed as a nonpolar shape mimic for natural thymidine (**2**).[5,6] Its "base" moiety cannot measurably form paired complexes with adenine derivatives even in chloroform, a solvent in which H-bonded complexes are much more stable than they are in water.[7] When placed within a DNA strand paired opposite adenine, moreover, it actually destabilizes the helix by ~4–5 kcal relative to thymine at the same position. In addition, **1** shows no inherent pairing selectivity among the four natural bases, also consistent with its nonpolar, non-hydrogen-bonding nature (ref [7] and work in progress). We felt therefore that **1** would serve as a good test for the importance of thymidine's hydrogen bonding groups on fidelity, because **1** lacks the strongly localized charges but retains nearly the exact steric shape of the natural molecule. If, as current models suggest, such polar interactions are important for achieving high fidelity, then **1** would be expected to be very inefficient and highly nonselective as a template for replication.

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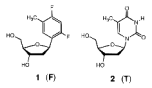
We were surprised to find, however, that nucleoside **1** (also called F) serves as a very good template for DNA synthesis. Initial single-nucleotide insertion experiments with the Klenow fragment of DNA Polymerase I (*exo*-) and a 23nt primer (Figure 1) indicated that at 20 μM dNTP concentrations, adenine, and only adenine, is inserted well opposite F (not shown). We then carried out more extensive studies using multiple nucleotide synthesis with a template sequence containing F, T, or an abasic nucleoside (**ϕ**) at the sixth position downstream of an 18nt primer (Figure 1). An equimolar mixture of dATP, dCTP, dTTP, and dGTP at 20 μM each was used, and short and long time points were examined. The results show that replacement of thymine in the template by **1** has little or no measurable effect on DNA synthesis; in both cases the primer is efficiently extended to the end of the template, with no pause either before or after the site in question even at the shortest (2 min) time point. By comparison, substitution in the template by an abasic nucleoside causes a strong, long-lived stop immediately after a nucleotide (deoxyadenosine) is inserted at that position.



1 (F) 2 (T)

To examine quantitatively the ability of nonpolar analog **1** to serve as a template for DNA synthesis, we carried out kinetic measurements of single-nucleotide insertions with the same enzyme. The 23nt template sequence is shown in Figure 1A. Steady-state methods[8] were used to determine the efficiency (V_{max}/K_m) of insertion of natural nucleotides opposite T or opposite **1** in the same sequence (Table 1). Examination of relative efficiencies for each of the four bases allows a quantitative measure of fidelity (selectivity of nucleotide insertion) at the position in question.[8,9]

The results show, remarkably, that analog **1** promotes efficiency and fidelity of nucleotide insertion nearly as high as that observed for natural thymine (Table 1). The apparent efficiency of insertion of deoxyadenosine opposite **1** is only ~4-fold lower than that opposite T. In addition, the selectivity for insertion of A rather than C, T, or G is surprisingly similar (Figure 2): With T in the template, A is preferred by 3.0–4.5 log units over the other three bases, and with **1** in the template, A is preferred by 2.9–4.2 log units. These data indicate that the energetic barrier leading to the newly synthesized A–F base pair is within 0.9 kcal of that for the A–T pair. Moreover, A is inserted with ~3.6–6.0 kcal of selectivity (relative to C, T, G) opposite F in the template. By comparison, the results for the abasic nucleoside in the template show that A is incorporated opposite an abasic site nearly two orders of magnitude less efficiently than across from **1** (Table 1), and the abasic site causes a strong stall in further strand elongation, while **1** does not (Figure 1). In addition, our measured fidelity of A insertion opposite F is much higher than reported values opposite an abasic site.[10] Thus, our findings with analog F are quite distinct from the known “A rule”, in which adenine is preferentially incorporated opposite abasic sites.[10,11]

These observations suggest that conventional hydrogen bonds may not be necessary for high efficiency and fidelity in DNA synthesis, and that shape complementarity may play a more important role in fidelity than previously believed.11 We cannot rule out a very weak contribution from an unconventional C–F···H–N hydrogen bond in the F–A base pair being synthesized;[12] however, these effects must be quite small since our previous studies have shown clearly that difluorotoluene is a highly hydrophobic species which (i) does not pair with adenine even in chloroform, (ii) is strongly destabilizing when paired with adenine in DNA, and (iii) shows no inherent pairing selectivity in the absence of the enzyme.[7]

Previous polymerase experiments with hydrogen bonding-impaired analogs of natural DNA bases have nearly always resulted in poor nucleotide insertion or elongation.[4] Those earlier analogs have considerable steric alterations (relative to the natural bases) resulting from

alkylation or removal of H-bonding groups. We hypothesize that the reason that analog **1** is so successful in DNA replication is that it retains the steric shape and conformation of the natural nucleoside. It will be useful in continuing studies to compare analog **1** with closely related structures to evaluate the importance of the shape mimicry. Future studies will also address the kinetic behavior using pre-steady-state methods, and will explore the generality of F–A base pair recognition by different polymerases and in different sequence contexts. It will also be of interest to investigate whether the nucleotide triphosphate derivative of **1** is viable in DNA synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. (a) Kornberg, A.; Baker, TA.; Freeman, WH., editors. DNA Replication. 2nd ed.. New York: 1992. p. 113 (b) Loeb LA, Kunkel TA. Annu. Rev. Biochem 1982;52:429. [PubMed: 6214209]
2. Petruska J, Goodman MF, Boosalis MS, Sowers LC, Cheong C, Tinoco I Jr. Proc. Natl. Acad. Sci. U.S.A 1988;85:6252. [PubMed: 3413095]
3. (a) Kuchta RD, Benkovic P, Benkovic SJ. Biochemistry 1988;27:6716. [PubMed: 3058205] (b) Kuchta RD, Mizrahi V, Benkovic P, Johnson KA, Benkovic SJ. Biochemistry 1987;26:8410. [PubMed: 3327522] (c) Petruska J, Sowers LC, Goodman MF. Proc. Natl. Acad. Sci. U.S.A 1986;83:1559. [PubMed: 3456600] (d) Petruska J, Goodman MF, Boosalis MS, Sowers LC, Cheong C, Tinoco I Jr. Proc. Natl. Acad. Sci. U.S.A 1988;85:6252. [PubMed: 3413095] (e) Mendelman LV, Boosalis MS, Petruska J, Goodman MF. J. Biol. Chem 1989;264:14415. [PubMed: 2474545] (f) McClure WR, Jovin TM. J. Biol. Chem 1975;250:4073. [PubMed: 1092683] (g) Travaglini EC, Mildvan AS, Loeb LA. J. Biol. Chem 1975;250:8647. [PubMed: 1102540] (h) Fersht AR. Proc. Natl. Acad. Sci. U.S.A 1979;76:4946. [PubMed: 159450] (i) El-Deiry WS, So AG, Downey KM. Biochemistry 1988;27:546. [PubMed: 3280024] (j) Donlin MJ, Johnson KA. Biochemistry 1994;33:14908. [PubMed: 7993917]
4. (a) Freese E. J. Mol. Biol 1959;1:87. (b) Sowers LC, Fazakerley GV, Eritja R, Kaplan BE, Goodman MF. Proc. Natl. Acad. Sci. U.S.A 1986;83:5434. [PubMed: 3461441] (c) Eritja R, Horowitz DM, Walker PA, Ziehler-Martin JP, Boosalis MS, Goodman MF, Itakura K, Kaplan BE. Nucleic Acids Res 1986;14:8135. [PubMed: 3095793] (d) Singer B, Spengler S. Biochemistry 1981;20:1127. [PubMed: 7013784] (e) Strazewski P, Tamm C. Angew. Chem., Int. Ed. Engl 1990;29:36. (f) Shibutani S, Takeshita M, Grollman AP. Nature 1991;349:431. [PubMed: 1992344] (g) Ward DC, Reich E. J. Biol. Chem 1972;247:705. [PubMed: 4550757]
5. Schweitzer BA, Kool ET. J. Org. Chem 1994;59:7238–7242. 1995, 60, 8326. [PubMed: 20882116]
6. (a) Ren X-F, Schweitzer BA, Sheils CJ, Kool ET. Angew. Chem 1996;108:834–837.Angew. Chem., Int. Ed. Engl 1996;35:743–746. [PubMed: 20882114] (b) Ren RX-F, Chaudhuri NC, Kool ET. J. Am. Chem. Soc 1996;118:7671–7678. [PubMed: 20865136]
7. Schweitzer BA, Kool ET. J. Am. Chem. Soc 1995;117:1863–1872. 1996, 118, 931. [PubMed: 20882111]
8. Goodman MF, Creighton S, Bloom LB, Petruska J. Crit. Rev. Biochem. Mol. Biol 1993;28:83–126. [PubMed: 8485987]
9. The steady-state method does not address what is the actual rate-limiting step in DNA synthesis; for example, it is known that the rate-limiting step for incorporation of some natural pairs is dissociation from the template.[3a] Nonetheless, the method does allow for accurate relative comparisons of overall insertion efficiency regardless of which step is rate limiting.[8]
10. (a) Sagher D, Strauss B. Biochemistry 1983;22:4518–4526. [PubMed: 6354260] (b) Randall SK, Eritja R, Kaplan BE, Petruska J, Goodman MF. J. Biol. Chem 1987;262:6864–6870. [PubMed:

3571289] (c) Takeshita M, Chang C-N, Johnson F, Will S, Grollman AP. J. Biol. Chem 1987;262:10171–10179. [PubMed: 2440861]

11. From the present data alone we cannot rule out some “A rule”-like influences on selectivity of dATP insertion; however, very recent studies with the triphosphate analog of **1** also support our proposed model Moran S, Ren RX, Kool E. Manuscript in preparation.
12. Howard JAK, Hoy VJ, O’Hagan D, Smith GT. Tetrahedron 1996;52:12613–12622.

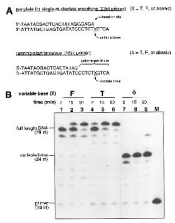

Figure 1.

(A) Sequences of DNA template/primer duplexes in this study. Compound **1** is designated **F** (from difluorotoluene). (B) Products of "running-start" DNA synthesis on synthetic duplex templates containing analog **1** or thymidine at the sixth position downstream of the 18nt primer 3′ end. Autoradiogram of 15% denaturing polyacrylamide gel electrophoretic analysis of products of DNA synthesis, using a ^{32}P-5′-end-labeled 18nt primer as shown in part A. Conditions are given in the text and in Supporting Information.

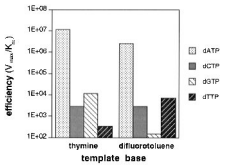

Figure 2.
Histogram of fidelity of nucleotide insertion opposite **T** and isostere **F** (compound **1**) with the 23nt primer template. Data are taken from Table 1.

Table 1

Steady-State Kinetic Parameters for Insertion of Single Nucleotides into a Template–Primer Duplex by the KF (*exo*-) Polymerase[a]

template base (X)	nucleotide triphosphate	K_m (μM)	V_{max} (% min^{-1})	efficiency (V_{max}/K_m)	fidelity[b]
T	dATP	3.4 (1.4)	36 (4)	1.1×10^7	1
	dCTP	2100 (1400)	5.8 (2.2)	2.8×10^3	0.00026
	dGTP	210 (60)	2.4 (0.5)	1.1×10^4	0.0011
	dTTP	190 (30)	0.062 (0.002)	3.3×10^2	0.00003
F	dATP	20 (4)	49 (3)	2.5×10^6	1
	dCTP	450 (140)	1.3 (0.1)	2.9×10^6	0.0012
	dGTP	180 (50)	0.027 (0.002)	1.5×10^2	0.00006
	dTTP	170 (30)	1.2 (0.04)	7.1×10^3	0.0028
abasic (ϕ)	dATP	69 (12)	3.0 (0.1)	4.3×10^4	0.0041[c]

[a] Conditions: 5 μM template–primer duplex (23nt template), 13–200 nM enzyme, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/mL bovine serum albumin, incubated for 1–20 min at 37 °C in a reaction volume of 10 μL (see Supporting Information for details). Standard deviations are given in parentheses.

[b] Efficiency relative to dATP insertion opposite T (entries 1–4) or dATP insertion opposite F (entries 5–8).

[c] Value relative to dATP insertion opposite T.