C-1 in vivo are similar to those observed in vitro. These relaxation properties can only be approximately fitted by the RRNN model calculation using a single effective correlation time τ_c. However, the strong field dependence of T_1 and the absolute values of T_2 are not adequately explained by this model. The MLSA calculation provides a much better fit to the relaxation times and accordingly gives more information about motions of the glycogen molecule. The results suggest that internal motions dominate the relaxation with an average correlation time of 3.9×10^{-9} s. The fit to the T_2 data shows that the overall molecular tumbling makes the strongest contribution to the T_2 relaxation but it is much less than expected from a rigid molecule, once again showing that the internal motions of glycogen are almost unrestricted throughout the molecule. This particular molecular nature of glycogen leads to the observation of narrow ^{13}C resonances which leads to the 100% ^{13}C NMR visibility.

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(Difluoromethylene)phosphates of Guanine Nucleosides as Probes of DNA Polymerases and G Proteins†

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ABSTRACT: 5′-Polyphosphates of N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine and -guanosine which contain a difluoromethylene group in place of a phosphoanhydride oxygen have been synthesized. 5′-[β,γ-(Difluoromethylene)triphosphates], including that of 2′-deoxyguanosine, were prepared by reaction of the corresponding 5′-phosphates, activated by 1,1′-carbonyldiimidazole, with difluoromethanediphosphonate. The 5′-[(difluoromethylene)diphosphate] of N^2-(*p*-*n*-butylphenyl)guanosine was prepared by treatment of a protected 5′-tosyl nucleoside with difluoromethanediphosphonate, followed by deprotection. Condensation of this nucleotide, activated with 1,1′-carbonyldiimidazole, with orthophosphate gave N^2-(*p*-*n*-butylphenyl)guanosine 5′-[(α,β-difluoromethylene)triphosphate]. Products were characterized by ^{31}P and ^{19}F NMR spectroscopy. The phosphonates were tested for their ability to displace [^{3}H]GDP from the GTP binding proteins cellular (EC) and oncogenic (Leu-61) Ha-*ras* p21, and for their ability to inhibit DNA polymerase α from Chinese hamster ovary cells. The p21s bound weakly to a triphosphonate when the CF_2 group was in the β,γ position, but not when it was in the α,β position, and they did not bind to the corresponding (difluoromethylene)diphosphate. In contrast, the CF_2 group had no effect on inhibition of DNA polymerase α by N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-[(β,γ-difluoromethylene)triphosphate]. 2′-Deoxyguanosine 5′-[(β,γ-difluoromethylene)triphosphate] was found to be a bona fide substrate for several DNA polymerases and had a lower apparent K_m than dGTP with *Bacillus subtilis* DNA polymerase III.

Direct study of nucleoside triphosphate bound forms of DNA polymerases and G proteins is limited by the ability of the proteins to alter the structure of the nucleotides. DNA polymerases in the presence of template-primers incorporate many dNTP derivatives with the release of pyrophosphate, and the GTPase activity of G proteins degrades GTP derivatives to the nucleoside 5′-diphosphate and orthophosphate. Numerous studies [see, for example, Burgers and Eckstein (1979), Blackburn et al. (1985), and Tucker et al. (1986)] have explored effects of modification of the triphosphate group of nucleotides to determine the electronic and stereochemical requirements for binding to relevant proteins, and to identify, among candidate analogues, those that might serve as stable

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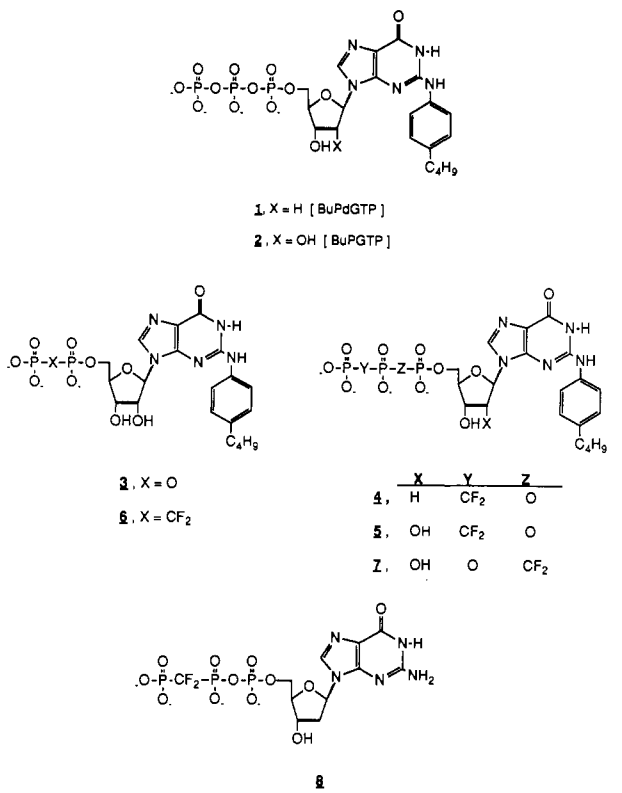
0006-2960/90/0429-6820$02.50/0

Chart I



crystallographic and spectroscopic probes of the proteins. In this context, Blackburn and co-workers have proposed that the difluoromethylene (CF_2) group may represent an "isopolar, isosteric" replacement for oxygen in the polyphosphate moiety of nucleotides (Blackburn et al., 1984). Among a series of β,γ-substituted ATP and GTP derivatives, for example, those with the CF_2 group replacing the β,γ-phosphoanhydride oxygen were most similar in chemical and physical properties to the natural nucleotides (Blackburn et al., 1984).

Considering these findings and the potential of the CF_2 group as a NMR reporter group for ligand–protein binding studies, we have synthesized several guanine nucleoside (difluoromethylene)phosphates, including several substituted on the exocyclic amino group with the *p-n*-butylphenyl group. The latter substituent confers potent and selective inhibitory activity against eukaryotic DNA polymerase α to the corresponding dGTP derivative, BuPdGTP[1] (**1**; see Chart I) (Khan et al., 1984). The analogous ribonucleoside triphosphate, BuPGTP (**2**), also bound and activated the G protein, bovine transducin (Kelleher et al., 1986). Although BuPdGTP (**1**) is probably not a substrate for DNA polymerase α (Khan et al., 1984), **2** was an efficient substrate for the GTPase activity of transducin (Kelleher et al., 1986). BuPGTP, among other N^2-substituted GTP derivatives, also binds to the G protein p21, the product of the *ras* oncogene family (this work and Noonan et al., unpublished results).

Given the ability of the parent nucleotides, **1** and **2**, to inhibit DNA synthesis catalyzed by pol α and to compete with GDP for binding to p21, respectively, we sought to compare the activity of the CF_2-substituted counterparts to assess residues critical to binding with the respective proteins. The results suggested that pol α tolerates the CF_2 group well in the β,γ position but that p21 tolerates CF_2 poorly at either the α,β or the β,γ position. The observation that a (difluoromethylene)phosphate could bind pol α with good affinity prompted us to synthesize the analogous dGTP derivative, $dGMPPCF_2P$ (**8**) and to study the response of several DNA polymerases to it. The results of this study indicate that this compound is a particularly efficient substrate for *Bacillus subtilis* DNA polymerase III.

Experimental Procedures

General. Melting points were determined on a Mel-temp apparatus and are uncorrected. Ultraviolet spectra were obtained with a Gilford Response spectrophotometer. ^{31}P NMR spectra were recorded at 102 MHz on Bruker WM250 and at 81 MHz on Bruker AC200 spectrometers; EDTA was added to improve resolution, and external phosphoric acid was used as reference. ^{19}F NMR spectra were obtained at 188.3 MHz with the Bruker AC200 instrument, and external trifluoroacetic acid was the reference. Routine ^1H NMR spectra were run at 60 MHz on a Perkin-Elmer R12B/Nicolet TT7 instrument with tetramethylsilane as internal reference. Analyses for C, H, and N were done by the Microanalysis Laboratory, University of Massachusetts, Amherst, and analyses for P were performed as described by Ames and Dubin (1960). Analytical and preparative HPLC of nucleotides were done with a Waters Model 600 gradient system and a Lambda-max Model 481 detector.

Materials. Anhydrous tetrahydrofuran and anhydrous diethyl ether were refluxed and distilled from sodium metal in the presence of benzophenone. Hexamethylphosphoramide (HMPA) was refluxed over calcium hydride and distilled at reduced pressure. All dried solvents were stored over molecular sieves (4 Å) in the dark. Dry pyridine was purchased from Aldrich, and ethanol-free chloroform was from EM Reagent Science. Glass-distilled water was used throughout. Thin-layer chromatography was done with Merck Kieselgel 60 F-254 analytical plates, and column chromatography employed silica gel (fine mesh, 230–400 ASTM). DEAE-Sephadex was purchased from Pharmacia, and all HPLC columns were from Rainin.

Proteins and Substrates. The Ha-*ras* proteins, p21 EC and the Leu-61 mutant, were gifts from Merck, Sharp and Dohme Research Laboratories, West Point, PA. DNA polymerase α from Chinese hamster ovary cells was purified as described (Khan & Brown, 1985), and pol α from calf thymus was immunopurified according to the method of Chang et al. (1984). *B. subtilis* pol III was the DEAE-cellulose fraction IV prepared as described (Barnes & Brown, 1979). *Escherichia coli* pol I was purchased from Boehringer. [^{3}H]GDP (11 Ci/mmol), [^{3}H]dGTP (37 Ci/mmol), [^{32}P]dGMP (1500 Ci/mmol), and [^{3}H]dTTP (21.5 Ci/mmol) were purchased from Amersham. $dGMPPCH_2P$ was purchased from Miles Laboratories, and other nonradioactive nucleotides were from Pharmacia P-L or from Sigma. Calf thymus DNA was from Worthington.

Tetraethyl difluoromethanediphosphonate was prepared by the method of Blackburn et al. (1981) and was further purified by HPLC with a Waters 6000A pump and differential re-

[1] Abbreviations: BuPdGTP, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; BuPGTP, N^2-(*p-n*-butylphenyl)guanosine 5′-triphosphate; $dGMPPCF_2P$, 2′-deoxyguanosine 5′-[β,γ-(difluoromethylene)triphosphate]; $dGMPPCH_2P$, 2′-deoxyguanosine 5′-(β,γ-methylenetriphosphate); HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; HMPA, hexamethylphosphoramide; TEAB, triethylammonium bicarbonate; TEA, triethylammonium; Ha-*ras*-p21, product of the Ha-*ras* gene; pol α, DNA polymerase α; pol I, DNA polymerase I; pol III, DNA polymerase III; CHO, Chinese hamster ovary; CF_2PP, difluoromethanediphosphonic acid; BuPGuo, N^2-(*p-n*-butylphenyl)guanosine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid. Other nucleotide inhibitor acronyms are explained, with reference to the structures, in Table I.

fractometer detector on a Microsorb 5-μm silica gel column (21.4 mm × 25 cm) using 80% ethyl acetate/20% cyclohexane as eluent. Difluoromethanediphosphonic acid (CF_2PP) was obtained by treatment of the ester with iodotrimethylsilane (Blackburn et al., 1981) and immediately converted to the ammonium salt. Aliquots were converted to the more soluble (tri-*n*-butylammonium) salt before each reaction. BuPdGTP (**1**), BuPGTP (**2**), and BuPGDP (**3**) were prepared as previously described (Wright & Dudycz, 1984; Kelleher et al., 1986).

N^2-(p-n-Butylphenyl)-2′-deoxyguanosine 5′-[β,γ-(Difluoromethylene)triphosphate] (**4**). A solution of the bis(triethylammonium) salt of N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-phosphate (Wright & Dudycz, 1984) (190 mg, 0.28 mmol) in HMPA (3 mL) was stirred with 1,1′-carbonyldiimidazole (228 mg, 1.4 mmol) for 4.5 h at room temperature. A solution of bis(tri-*n*-butylammonium) difluoromethanediphosphonate (259 mg, 0.27 mmol) in HMPA (4 mL) was added to the solution of activated monophosphate, and the solution was stirred at room temperature for 20 h. After slow dilution with cold water, the solution was applied to a DEAE-Sephadex column (4.5 cm × 28 cm) which was developed with a linear gradient of 0.2–1.0 M triethylammonium bicarbonate (TEAB) during 16 h at a flow rate of 2.66 mL/min. Fractions 100–137 (15 mL each) containing the product were combined, concentrated, and purified again on a DEAE-Sephadex column (4.5 cm × 28 cm) with a linear gradient of 1.0–2.0 M TEAB. Fractions centered at ca. 1.1 M TEAB containing pure product were combined and lyophilized to give 146 mg (50%) of **4** as the triethylammonium (TEA) salt. Anal. Calcd for $C_{45}H_{84}F_2N_9O_{12}P_3$: P, 8.62. Found: P, 8.81.

N^2-(p-n-Butylphenyl)guanosine 5′-[(β,γ-Difluoromethylene)triphosphate] (**5**). In a manner analogous to the above reaction, condensation of difluoromethanediphosphonate with BuPGMP (Kelleher et al., 1986) (75 mg, 0.11 mmol) gave, after DEAE-Sephadex chromatography as described for **4**, 54 mg (45%) of **5** as the TEA salt. Anal. Calcd for $C_{45}H_{84}F_2N_9O_{13}P_3$: P, 8.49. Found: P, 8.53.

2′-Deoxyguanosine 5′-[(β,γ-difluoromethylene)triphosphate] (**8**). dGMP, sodium salt, was converted to the pyridinium salt with Dowex 50W-X8 (pyridinium form). The latter salt was coevaporated with tri-*n*-butylamine and lyophilized. The resulting tri-*n*-butylammonium salt (88 mg, 0.12 mmol) was dissolved in HMPA (5 mL) by heating at 45 °C for 16 h. 1,1′-Carbonyldiimidazole (205 mg, 1.27 mmol) was introduced, and the solution was stirred at rt for 24 h. Methanol (50 μL) was added to decompose excess 1,1′-carbonyldiimidazole. The tri-*n*-butylammonium salt of CF_2PP (188 mg, 0.197 mmol) dissolved in HMPA (2 mL) was added to the solution of activated dGMP, and the combined solution was stirred at rt. Progress of the reaction was followed by TLC (silica gel plates, developed twice in *i*-PrOH/NH_4OH/H_2O, 6:3:1). After 30 h the reaction mixture was cooled in an ice bath and ice/water (6 mL) was added. The diluted solution was applied to a DEAE-Sephadex column (4.5 cm × 28 cm), and the column was developed with a linear gradient of 0.2–2.0 M TEAB, as described in the synthesis of **4**. Fractions containing the major product (ca. 1.2 M TEAB) were combined and evaporated. The product was lyophilized to give 120 mg (93%) of **8** as the TEA salt. Anal. Calcd for $C_{35}H_{72}F_2N_9O_{12}P_3$: P, 8.89. Found: P, 8.60.

N^2-(p-n-Butylphenyl)-2′,3′-O-(methoxymethylidene)-guanosine. Trimethyl orthoformate (3 mL) was added to a mixture of N^2-(*p*-*n*-butylphenyl)guanosine (Wright & Dudycz, 1984) (800 mg, 1.93 mmol) and *p*-toluenesulfonic acid monohydrate (740 mg, 3.89 mmol). Dimethyl sulfoxide (2 mL) was added to effect solution, and the mixture was stirred at rt for 3 h. A solution of sodium methoxide in methanol (1 M) was added gradually to neutralize the reaction mixture. The precipitate was applied to a silica gel column (2 cm × 25 cm) prepared in chloroform, and the product was eluted with 15% methanol in chloroform (150 mL). Removal of solvents left 659 mg (75%) of pure product as a mixture of diastereomers: mp 236–241 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 6.19, 6.09 (2 H, s, orthoformate protons); 7.98, 8.03 (2 H, s, H-8s). Anal. Calcd for $C_{22}H_{27}N_5O_6$: C, 57.76; H, 5.95; N, 15.31. Found: C, 57.08; H, 5.99; N, 15.12.

N^2-(p-n-Butylphenyl)-2′,3′-O-(methoxymethylidene)-5′-tosylguanosine. A mixture of the above product (250 mg, 0.55 mmol) and tosyl chloride (320 mg, 1.68 mmol) in dry pyridine was stirred at room temperature to effect solution. After the mixture stood in a refrigerator for 24 h, the solution was placed directly on a silica gel column (2 cm × 25 cm) prepared in chloroform, and the product was eluted with 10% methanol in chloroform (100 mL) to give 240 mg (72%) of colorless solid: mp 195–200 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 7.10 (d, 4 H) and 7.50 (d, 4 H) (*p*-tosyl protons overlapped with *p*-*n*-butylphenyl aromatic protons), 2.41 (s, 3 H, tosyl CH_3). Anal. Calcd for $C_{29}H_{33}N_5O_8S$: C, 56.94; H, 5.44; N, 11.45. Found: C, 56.80; H, 5.44; N, 11.47.

N^2-(p-n-Butylphenyl)guanosine 5′-[(Difluoromethylene)-diphosphate] (**6**). A mixture of the above 5′-tosyl compound (160 mg, 0.26 mmol) and the TBA salt of difluoromethanediphosphonic acid (425 mg, 0.446 mmol) in HMPA (3 mL) was stirred at 65 °C. Progress of the reaction was monitored by HPLC on a SynChropak AX-100 column (4.6 mm × 25 cm). After 24 h water was added, and the mixture was filtered through a 0.45-μm filter. The product was isolated by HPLC on a SynChropak AX-100 column (10 mm × 25 cm) in a gradient of 30% acetonitrile in water to 30% acetonitrile in 0.5 M ammonium bicarbonate during 35 min at a flow rate of 2 mL/min. The resulting blocked diphosphonate (112 mg, 61%) was dissolved in 3 N hydrochloric acid (pH ca. 1) and stirred at room temperature for 3 h. The formate esters so obtained (Griffin et al., 1967) were hydrolyzed by addition of concentrated ammonium hydroxide to pH 8 followed by stirring for 0.5 h. The solution was passed through a DEAE-Sephadex column (2 cm × 25 cm), and the column was eluted with a gradient of 0.2–1.0 M TEAB. The product emerged at ca. 1.0 M TEAB, yielding 51 mg (47%) of **6** as the TEA salt. Anal. Calcd for $C_{39}H_{69}F_2N_8O_{10}P_2$: P, 9.37. Found: P, 9.29.

N^2-(p-n-Butylphenyl)guanosine 5′-[α,β-(Difluoromethylene)triphosphate] (**7**). The TEA salt of **6** (48 mg, 41 μmol) was first changed to the pyridinium salt by passage through Dowex 50W-X8 (pyridinium form) and then was converted to the tri-*n*-butylammonium salt. The latter was dissolved in HMPA (2 mL) and stirred at room temperature with a solution of 1,1′-carbonyldiimidazole (64 mg, 390 μmol) in HMPA (2 mL). After 2.5 h a solution of mono(tri-*n*-butylammonium) phosphate (109 mg, 0.38 mmol) in HMPA (2 mL) was added, and the solution was stirred under nitrogen at room temperature for 16 h. The mixture was chilled and ice/water (10 mL) was added slowly. After filtration through a 0.45-μm filter, the solution was chromatographed on a SynChropak AX-100 column (10 mm × 25 cm) with a gradient of 30% aqueous acetonitrile to 30% acetonitrile in 0.5 M ammonium bicarbonate during 35 min at a flow rate of 8 mL/min. The product was eluted at ca. 0.4 M ammonium

Table I: ^{31}P and ^{19}F NMR Data[a]

compound	chemical shifts (ppm)				coupling constants (Hz)				
	$\delta_{P\alpha}$	$\delta_{P\beta}$	$\delta_{P\gamma}$	δ_F	$J_{P\alpha,P\beta}$	$J_{P\beta,P\gamma}$	$J_{P\alpha,F}$	$J_{P\beta,F}$	$J_{P\gamma,F}$
CF_2PP	5.47						79		
BuPGDP (3)[b]	−11.1	−9.9			20.8				
BuPGMPCF$_2$P (6)	3.1	4.8		−44	54		89	81	
BuPGTP (2)[b]	−11.3	−22.7	−8.3		20.3	20.3			
BuPGMPCF$_2$PP (7)	3.8	−6.6	−10.5	−45	61	28	81	89	
BuPGMPPCF$_2$P (5)	−11.2	−5.7	2.9	−45	32	60		91	81
GMPPCF$_2$P[c]	−10.2	−1.8	4.5	−43	31.7	57.3		89	73
BuPdGMPPCF$_2$P (4)	−11.2	−5.1	3.0	−44	30	60		91	81
dGMPPCF$_2$P (8)	−11	−5.7	3.0	−44	30.8	59		91	81

[a] Spectra were obtained in unbuffered aqueous solutions containing 10% D_2O as lock signal by use of the instruments described under Experimental Procedures. Chemical shifts were measured from external phosphoric acid and trifluoroacetic acid for ^{31}P and ^{19}F, respectively. [b] From Kelleher et al. (1986). [c] From Blackburn et al. (1984).

bicarbonate, and lyophilization yielded 15 mg (48%) of **7** as the ammonium salt. Anal. Calcd for $C_{21}H_{40}F_2N_9O_{13}P_3$: P, 12.25. Found: P, 12.25.

Synthesis of [α-^{32}P]dGMPPCF$_2$P. [^{32}P]dGMP (specific activity 1500 Ci/mmol) was purified by HPLC on a Synchropak AX-100 column (4.6 mm × 25 cm) in a gradient of 35% aqueous acetonitrile to 0.5 M ammonium bicarbonate in 35% acetonitrile during 30 min at a flow rate of 2 mL/min. Fractions eluting at ca. 0.2 M buffer were pooled and adjusted with unlabeled dGMP to a specific activity of 4 Ci/mmol. The labeled dGMP was converted to **8** as described above, and the resulting product was purified by HPLC on a SynChropak AX-100 column (4.6 mm × 25 cm). Using the gradient described above, pure [α-^{32}P]-**8** as the ammonium salt eluted at ca. 0.25 M ammonium bicarbonate. Analytical HPLC and TLC of the product indicated a radiopurity of 99.9%.

Binding of Nucleotides to p21. A filter binding assay adapted from those of Gibbs et al. (1984) and Tucker et al. (1986) was used. One microgram of p21 was incubated at 37 °C for 45 min in 50 μL of a mixture containing 50 mM Tris·HCl (pH 6.8), 10 mM dithiothreitol, 0.5 mM $MgCl_2$, 100 mM NH_4Cl, 5 mM EDTA, and 2 μM [^{3}H]GDP (specific activity 10 Ci/mmol). Appropriate amounts of test compounds dissolved in water or an equal volume of water were included during the incubation period. Exchange reactions were quenched by the addition of 1.5 mL of a cold solution of 1 mM $MgCl_2$ in 50 mM Tris·HCl (pH 6.8). The solutions were filtered through nitrocellulose disks (BA85, Schleicher and Schuell), and the disks were washed with quenching solution (2 × 1.5 mL). The disks were dried, and filter-bound radioactivity was determined by scintillation counting of each disk in 5 mL of Omnifluor (New England Nuclear).

Assay of DNA Polymerases. All assays measured the incorporation of the labeled dNMP moiety of a radioactive dNTP into a cold trichloroacetic acid (TCA) insoluble form, using activated calf thymus DNA as template-primer. DNA polymerase α from Chinese hamster ovary (CHO) cells was assayed as described by Khan and Brown (1985), and calf thymus pol α was assayed as described by Higer et al. (1987). Assay mixtures (25 μL each) contained 0.1 mM EDTA, 40 mM dithiothreitol, 0.25 mg/mL BSA, 10 mM $MgCl_2$, 0.4 mg/mL activated DNA, and either 50 mM Tris·HCl, pH 8.0 (CHO pol α), or 20 mM potassium phosphate, pH 7.2 (calf thymus pol α). Assays contained 50 μM each of dATP, dCTP, and dGTP and 20 μM [^{3}H]dTTP (250 cpm/pmol). Reactions were initiated by the addition of 0.1 unit of enzyme and incubated at 37 °C for 0.5 h.

Assays for *E. coli* pol I and *B. subtilis* pol III were done as described by Barnes and Brown (1979). Assay mixtures (25 μL each) contained 30 mM Tris·HCl, pH 7.6, 10 mM magnesium acetate, 4 mM dithiothreitol, 20% glycerol, 0.4 mg/mL activated DNA, 25 μM each of dATP, dCTP, and dGTP, and 10 μM [^{3}H]dTTP (625 cpm/pmol). Reactions were initiated by the addition of 0.05 unit of enzyme and incubated at 30 °C for 10 min. One unit of polymerase activity is the amount of enzyme that incorporates, in the above assay conditions, 1 nmol of labeled dNTP into a cold TCA-insoluble form.

The truncated, dGTP-deficient assay (Wright & Brown, 1976), when employed, measured the incorporation of [^{3}H]-dTMP into a cold TCA-insoluble form in the absence of dGTP, using otherwise standard conditions described above. Inhibitory or substrate properties of nucleotide derivatives were assessed in the truncated, dGTP-deficient assay. K_i values of **1** and **4**, compounds that are competitive with dGTP, were obtained directly from log concentration–inhibition curves in the absence of dGTP (Wright & Brown, 1976). In experiments in which nucleotide derivatives were substrates, SC_{50} values representing the concentration of added nucleotide that caused half-maximal stimulation of label incorporation were obtained from log concentration–stimulation curves.

Results

Synthesis and Properties of Nucleoside (Difluoromethylene)phosphates. Previous syntheses of nucleoside β,γ-methylenetriphosphates (Blackburn et al., 1984) involved reaction of the nucleoside 5′-phosphoromorpholidates with the appropriate diphosphonate in pyridine solution. By analogy with our syntheses of **1** and **2** (Wright & Dudycz, 1984; Kelleher et al., 1986), we activated the corresponding monophosphates in situ in hexamethylphosphoramide (HMPA) with 1,1′-carbonyldiimidazole and treated these with the soluble (tri-*n*-butylammonium) salt of difluoromethanediphosphonic acid (CF_2PP). After 20 h at room temperature the products were purified by chromatography on DEAE-Sephadex, providing BuPdGMPPCF$_2$P (**4**) and BuPGMPPCF$_2$P (**5**) in 50% and 45% yields, respectively. An identical method using dGMP produced, after 30 h reaction time, a 93% yield of dGMPPCF$_2$P (**8**). The structures of these and other nucleoside phosphonates were confirmed by ^{31}P and ^{19}F NMR spectra (see Table I).

Preparation of the α,β-CF_2 derivative of BuPGTP, compound **7**, required first the synthesis of the diphosphonate BuPGMPCF$_2$P, **6**. However, direct condensation in pyridine between N^2-(*p*-*n*-butylphenyl)guanosine (BuPGuo) and CF_2PP activated with 1,1′-carbonyldiimidazole gave a poor yield (<5%) of **6**. Activation of the 5′-OH group of the nucleoside by introduction of the *p*-toluenesulfonyl (tosyl) group, a strategy that was successful in the synthesis of polyphosphates and polyphosphonates of adenosine (Dixit & Poulter, 1984), was then attempted. Although 5′-tosyl derivatives of guanosines are prone to cyclize to the corresponding 3,5′-anhydro-

guanosines (Schattka & Jastorff, 1972), we felt that the presence of the bulky *p-n*-butylphenyl group at N^2 might hinder cyclization. Direct reaction of BuPGuo with *p*-toluenesulfonyl chloride in pyridine, however, gave a mixture of mono- and ditosylates. Consequently, the 2′- and 3′-hydroxyls of BuPGuo were first protected by the 2′,3′-*O*-(methoxymethylidene) group (Griffin et al., 1967), chosen because of the mild conditions required for its ultimate removal. The protected nucleoside, isolated as a diastereomeric mixture in 75% yield, reacted smoothly with *p*-toluenesulfonyl chloride to give the 5′-tosyl derivative in 72% yield. Reaction of N^2-(*p-n*-butylphenyl)-2′,3′-*O*-(methoxymethylidene)-5′-tosylguanosine with the TBA salt of CF_2PP in HMPA at 65 °C gave the blocked 5′-diphosphonate in 61% yield. It was deblocked by standard methods (Griffin et al., 1967) to give BuPGMPCF$_2$P (**6**) in 47% yield. The final target compound, BuPGMPCF$_2$PP (**7**), was obtained in 48% yield by reaction of **6**, activated with 1,1′-carbonyldiimidazole in HMPA, with the TBA salt of orthophosphoric acid.

All nucleotides were characterized for structure and purity by ^{31}P and ^{19}F NMR spectra, the data for which are summarized in Table I, and by ^1H NMR spectra (data not shown), analytical HPLC, and phosphorus analyses. Relevant NMR data for the parent nucleotides BuPGDP and BuPGTP, and for the β,γ-CF_2 derivative of GTP (Blackburn et al., 1984), are included in Table I for comparison. The CF_2 group imparts considerable downfield shifts to directly bonded phosphorus nuclei; cf. δ 5.47 in CF_2PP vs δ −10.6 in pyrophosphate (Blackburn et al., 1981). Downfield shifts are seen in both P resonances of **6** relative to the diphosphate **3**. ^{31}P chemical shifts in the β,γ-CF_2 triphosphonates, **4**, **5**, and **8**, are consistent with those reported by Blackburn et al. (1984) for $GMPPCF_2P$ (Table I) and show that CF_2 has little effect on the chemical shift of the more remote α phosphorus. In the α,β-CF_2 derivative **7** the directly bonded P nuclei were again shifted downfield relative to **2**, but the γ P was unchanged. ^{19}F NMR chemical shifts in all analogues were essentially identical with those of CF_2PP (Table I).

Characteristic changes in ^{31}P–^{31}P ^2J coupling constants were observed in the phosphonates. A 3-fold increase of ^2J between carbon-linked relative to oxygen-linked P nuclei was seen for **6** and for the corresponding resonances in the α,β (**7**) and β,γ (**4**, **5**, **8**) bridged nucleotides. In the β,γ-CF_2 compounds, the ^2J values for the oxygen-bridged P nuclei increased about 50%, from 20.3 Hz in **2** to 30–32 Hz in the phosphonates. A somewhat lower increase in P_β–P_γ coupling constant was observed in **7**. ^{19}F–^{31}P ^2J values were different for the two P nuclei directly bonded to the CF_2 group. J_{F-P} for coupling with a β phosphorus was greater than those for coupling with either α or γ P in all cases except for the diphosphonate (**6**).

The β,γ-(difluoromethylene) nucleotides **4**, **5**, and **8** and BuPGMPCF$_2$P (**6**) showed stabilities comparable to those of typical nucleoside tri- and diphosphates, both in the solid state in a desiccator and in frozen (−20 °C) aqueous solutions. The α,β derivative BuPGMPCF$_2$PP (**7**), however, was more labile. For example, after incubation of freshly purified compounds at 10 mM in the p21 assay buffer (see Experimental Procedures) at 37 °C for 45 min, **7** gave 12% of a product coeluting (HPLC) with the diphosphonate, **6**. In contrast, **5** produced <3% of a product coeluting with BuPGMP, and **6** was unchanged under these conditions.

Binding of Nucleoside Phosphonates with p21. The competition assay for p21 permits convenient determination of relative binding affinities, K_{rel}, defined as the ratio of concentration of [^{3}H]GDP to that of analogue at which 50% inhibition of [^{3}H]GDP binding is observed (Tucker et al., 1986). Tested in this way, GTP displayed affinities to p21s similar to those of GDP (Table II). The data of Table II show that BuPGTP (**2**) and BuPGDP (**3**) bound to cellular (EC) Ha-*ras*-p21 with affinities 3-fold higher than those of GDP or GTP. Both **2** and **3**, furthermore, had increased affinity for the oncogenic Leu-61 mutant of Ha-*ras*-p21, with 6-fold higher affinities than the corresponding guanine nucleotides.

Table II: Binding of Nucleotides to p21[a]

compound	K_{rel}[b] EC p21	K_{rel}[b] Leu-61 p21
GTP	1.1	1.2
BuPGDP (**3**)	3.0	6.0
BuPGMPF$_2$P (**6**)[c]		
BuPGTP (**2**)	3.3	6.1
BuPGMPCF$_2$PP (**7**)[c]		
BuPGMPPCF$_2$P (**5**)	0.023	0.028

[a] Compounds over a range of concentrations were allowed to compete with 3 μM [^{3}H]GDP in the presence of 1 μM p21. Conditions and workup of the assays are described under Experimental Procedures. Control binding to p21 corresponded to 0.8–0.9 pmol of GDP/pmol of p21. [b] K_{rel} is the ratio of concentrations of [^{3}H]GDP (3 μM) to that of analogue which caused 50% inhibition of [^{3}H]GDP binding. [c] Less than 10% inhibition at 300 μM.

The results of Table II illustrate the dramatic decrease in affinity of the (difluoromethylene)phosphates, **6**, **7**, and **5**, for both proteins relative to the corresponding di- and triphosphates. Neither the diphosphonate **6** nor the α,β-triphosphonate **7** bound to either p21, even at concentrations 100-fold higher than that of GDP. The β,γ-triphosphonate **5** bound to both EC p21 and Leu-61 p21 but with 50-fold and 40-fold lower affinities, respectively, than those of GTP. The drastic decrease of affinities to p21 of **6**, **7**, and **5** relative to the di- and triphosphates (**3** and **2**, respectively) emphasizes the importance of the phosphoanhydride oxygens in binding with p21 and, likely, with other G proteins.

Inhibition of DNA Polymerase α by a (Difluoromethylene)phosphate. We investigated the influence of CF_2 groups in dNTPs on binding to DNA polymerases by taking advantage of the potent and selective inhibition of animal cell pol α by N^2-(*p-n*-butylphenyl)guanine nucleotides. BuPdGTP (**1**) inhibits pol α from different sources competitively with dGTP with K_i values of 1–10 nM (Khan et al., 1984; Fry & Loeb, 1986) but is apparently not an effective substrate for the enzyme. Assays of pol α from CHO cells in the absence of dGTP (see Experimental Procedures) showed that the β,γ-difluoromethylene analogue **4** inhibited the enzyme with K_i = 0.007 μM, a potency nearly identical with that of the triphosphate **1** (0.005 μM). This result is in stark contrast to the results for p21 binding (see above), where the β,γ-CF_2 group of **5** decreased its ability to bind the G protein relative to the triphosphate.

The lack of effect of the β,γ-CF_2 group on pol α inhibition by **4** suggests that the γ-phosphate group does not react appreciably with the enzyme in the enzyme/template-primer/inhibitor complex. This result is consistent with our observations that the related 5′-diphosphate, BuPdGDP (Khan et al., 1984), was equipotent with the corresponding 5′-triphosphate as a pol α inhibitor. The catalytic mechanism of dNTP polymerization involves attack of the 3′-OH group of the primer terminal nucleotide on the α phosphorus of the dNTP bound in the enzyme/template-primer complex as the Mg^{2+} chelate (Burgers & Eckstein, 1979). Assuming that the N^2-(*p-n*-butylphenyl) nucleotides bind the active site of pol α in a conformation similar to that of dNTP substrates, the lack of effect of the β,γ-CF_2 group implies considerable

Table III: Response of DNA Polymerases to dGMPPCF$_2$P (**8**)[a]

condition	[^{3}H]dTMP incorporation (pmol)		
	calf thymus pol α	*E. coli* pol I	*B. subtilis* pol III
–dGTP	27	3.4	6.1
+10 μM dGTP	40	14	12.4
–dGTP, +10 μM **8**	27	3.6	15.3
+100 μM dGTP	80	31	20
–dGTP, +100 μM **8**	30	5	25

[a] Enzymes were assayed with activated calf thymus DNA in the presence of [^{3}H]dTTP, dATP, and dCTP as described under Experimental Procedures, with the additions as indicated.

Table IV: dGMPPCF$_2$P (**8**) vs dGTP as Substrates for DNA Polymerases[a]

products	dGMP incorporation (pmol)					
	pol α		pol I		pol III	
	dGTP[b]	**8**[c]	dGTP[b]	**8**[c]	dGTP[b]	**8**[c]
cold TCA insoluble	40	6.6	60	5.5	38	43
base stable, cold TCA insoluble[d]	40	4.8	60	5.5	38	44
hot TCA soluble[e]	0.05	0.05	0.05	0.05	0.3	0.3

[a] Activated calf thymus DNA was used in appropriate truncated, dGTP-deficient assays containing dATP, dCTP, and dTTP (see Experimental Procedures). [b] [^{3}H]dGTP, 20 μM (specific activity 580 cpm/pmol). [c] [α-^{32}P]dGMPPCF$_2$P, 20 μM (specific activity 340 cpm/pmol). [d] The portion of the cold TCA insoluble material that remained insoluble in cold TCA following treatment with 0.5 M NaOH at 37 °C for 1 h. [e] The portion of the cold TCA insoluble material that remained insoluble in cold TCA after treatment with TCA at 100 °C for 0.5 h.

electronic and/or steric tolerance in the triphosphate binding site of pol α in contrast to the strict requirements of the analogous site in p21 (see above).

dGMPPCF$_2$P Is an Efficient Substrate for B. subtilis DNA Polymerase III. The lack of influence of the β,γ-CF$_2$ group on pol α inhibition by **4** prompted us to ask what the effect of such a replacement would be on the properties of a conventional dNTP substrate. We wondered if the dGTP analogue **8** would bind to DNA polymerases generally and what the consequences of binding might be. Therefore, we compared the response to **8** of three distinct DNA polymerases, calf thymus pol α, *E. coli* pol I, and *B. subtilis* pol III. The enzymes were assayed in truncated reactions lacking dGTP, and the effects of addition of dGTP or an equal concentration of dGMPPCF$_2$P (**8**) on the incorporation of [^{3}H]dTTP into activated DNA were measured. The results are summarized in Table III. In each case [^{3}H]dTMP incorporation was stimulated by the addition of dGTP to a level characteristic for each enzyme in these conditions. However, each enzyme responded differently to **8**. Pol α failed to respond to the analogue even at 100 μM. The pol I catalyzed reaction was stimulated weakly by the compound at 100 μM but failed to respond to it at 10 μM, a concentration at which dGTP supported half-maximal [^{3}H]dTMP incorporation. The pol III catalyzed reaction, in contrast to those of the other two enzymes, was stimulated by dGMPPCF$_2$P at both 10 and 100 μM, in each case apparently more than by dGTP itself.

The results of these experiments suggested that the responses of the three polymerases reflected their ability to use **8** as a source of dGMP for polymerization. To confirm this suggestion, we synthesized [α-^{32}P]-**8** and analyzed the capacity of each enzyme to incorporate the [^{32}P]dGMP portion of the analogue into activated DNA. Table IV summarizes the results of these experiments. In the conditions used each enzyme incorporated label from [α-^{32}P]-**8** in rough proportion to its efficacy as a stimulator of [^{3}H]dTMP incorporation in truncated assays (cf. Table III). Pol III used the analogue as effectively as it did [^{3}H]dGTP, whereas pol I and pol α used it with efficiencies of one-sixth and one-eleventh, respectively, of those of dGTP. Analysis of the radioactive product resulting from incorporation of ^{32}P in all cases revealed it to be base-stable and hot TCA labile (Table IV), as expected for a radioactive dNMP in phosphodiester linkage in DNA.

Table V: Kinetic Analysis of dGMPPCF$_2$P (**8**) and dGMPPCH$_2$P as Substrates for *B. subtilis* DNA Polymerase III[a]

substrate	assay mode	K_m (μM)[b]	SC_{50} (μM)[c]	V_{max} (pmol)
dGTP	direct[b]	10		30
dGTP	indirect[c]		9	34
dGMPPCF$_2$P (**8**)	direct	3		35
dGMPPCF$_2$P (**8**)	indirect		4	33
dGMPPCH$_2$P	indirect		18	16

[a] Assays employed activated calf thymus DNA and truncated, dGTP-deficient incubation conditions as described under Experimental Procedures. [b] The direct assay utilized several concentrations of [^{3}H]-dGTP or [α-^{32}P]-**8** as labeled precursor; apparent K_m represents the concentration of dGMP donor required to achieve half-maximal incorporation of label. [c] The indirect assay utilized [^{3}H]dTTP as labeled precursor, and the SC_{50} is the concentration of dGMP donor required to achieve half-maximal stimulation of [^{3}H]dTMP incorporation.

To define the efficacy of **8** as a pol III substrate, we determined its apparent K_m and V_{max}, both directly through the use of the α-^{32}P compound and indirectly through the effect of **8** on [^{3}H]dTTP incorporation. The results, summarized in the upper two entries of Table V, compare these values with those obtained for dGTP. In the direct assay the incorporation of label from [α-^{32}P]-**8**, like that from dGTP, displayed typical Michaelis–Menten kinetics (curves not shown). dGMPPCF$_2$P was apparently a better substrate than dGTP, giving a significantly lower K_m value but approximately the same V_{max}. The SC_{50} (stimulatory) and V_{max} values estimated indirectly for both nucleotides were in close agreement with, respectively, the K_m and V_{max} values found by direct assessment of incorporation of labeled **8** and dGTP.

The ability of pol III to accept the "isopolar, isosteric" CF$_2$ group in place of the β,γ-phosphoanhydride oxygen of dGTP prompted us to inquire if it would be equally tolerant of the more hydrophobic CH$_2$ group in dGMPPCH$_2$P. We compared the latter compound to **8** with respect to its ability to stimulate [^{3}H]dTMP into activated DNA in the dGTP-deficient truncated assay. The results, included in the last entry of Table V, indicated that dGMPPCH$_2$P was active as an enzyme substrate, but considerably less so than **8** or dGTP. In these conditions dGMPPCH$_2$P supported a level of polymer formation only half of that achieved by **8** and had an SC_{50} nearly 5-fold greater. dGMPPCH$_2$P at 100 μM had no effect on pol α or pol I in the truncated, dGTP-deficient assay (data not shown).

Discussion

The different responses of the G protein, Ha-*ras* p21, and DNA polymerases to CF$_2$ groups in the 5′-polyphosphate group of guanine nucleotides suggest very different modes of ligand binding with the two classes of proteins. CF$_2$ was not a conservative replacement for either bridging oxygen with respect to binding to p21 (Table II), but it had little effect on the ability of an N^2-(*p*-*n*-butylphenyl)guanine deoxyribonucleotide to inhibit CHO cell DNA polymerase α.

G proteins bind both GTP and its hydrolysis product, GDP, with high affinity ($K_D \sim 10^{-8}$ M for p21s) and do not appreciably bind GMP. This observation underscores the strict

requirement of p21 for a 5′-di- or 5′-triphosphate group and is consistent with the loss of binding affinity when substitutions in them are made (Table II; Scherer et al., 1989). EPR studies and experiments with phosphorothioates of GDP and GTP concluded that a β-phosphate oxygen, but not α-phosphate oxygens, was involved in metal-coordinated binding with p21 (Feuerstein et al., 1987). Indeed, related "β,γ" isosteres, guanosine 5′-(β,γ-imidotriphosphate) and guanosine 5′-(β,γ-methylenetriphosphate), were reported to have affinities of one-tenth and one-hundredth, respectively, that of GDP for a c-Ha-*ras* p21, $p21_c$ (Scherer et al., 1989). In this context, the ability of these and compound **5** to bind, although weakly, to both forms of p21 is consistent with the minimal contribution of the γ-phosphate of GTP to the binding affinity, as reflected in the similar affinity of GTP and GDP for p21s. We have proposed (Noonan et al., unpublished work) that the *p-n*-butylphenyl group of **2** and other N^2 substituents on GTP enhance binding with p21, especially with the Leu-61 mutant form of p21, through hydrophobic interactions with the proteins. The similar decrease in affinity of **5** for both EC and Leu-61 p21 suggests that this interaction may only partially compensate for the loss of binding caused by the β,γ-CF_2 group. Although the α,β-(difluoromethylene) isosteres of BuPGDP and BuPGTP did not bind at all to p21s, the only 50- and 40-fold reductions in affinity of **5** for the EC and Leu-61 mutant p21s encourage us to explore **5** as a potential nonhydrolyzable probe for NMR studies of triphosphate-bound forms of p21s.

DNA polymerases bind significantly with substrate dNTPs only when directed by a template-primer (Bryant et al., 1983), and generally with lower affinities ($K_m \sim 10^{-6}$ M) compared with G protein–ligand binding. In the compounds tested for inhibition of DNA polymerase α, a major source of affinity to the enzyme is the N^2-(*p-n*-butylphenyl) group; the lack of effect of the β,γ-CF_2 group on the potency of **4** is consistent with greater flexibility in the triphosphate binding region of the DNA polymerase compared with that of the G protein. Indeed, the substrate analogue **8** was a bona fide substrate for three different DNA polymerases.

Compound **8**, $dGMPPCF_2P$, although incorporated into activated DNA by three DNA polymerases (Table III), was an efficient replacement for dGTP only with *B. subtilis* pol III (Table IV). In the latter case the β,γ-CF_2 group actually facilitated incorporation of the analogue, perhaps by increasing the susceptibility of the α P to nucleophilic attack by the primer 3′-OH group, or by lowering the activation energy for release of the leaving group, difluoromethanediphosphonate. We also observed that the methylene derivative, $dGMPPCH_2P$, stimulated DNA synthesis in the absence of dGTP only with pol III (Table V), indicating a wide tolerance of this enzyme for electronic or other structural alterations in the triphosphate group of substrates.

No significant homologies have been observed between *E. coli* pol I and a family of DNA polymerases with conserved structure including animal cell pol α [see, for example, Wang et al. (1989)]. Preliminary evidence suggests that the putative polymerase domain of *B. subtilis* DNA polymerase III, in addition, shares no strong homology with the polymerase domain of either group (Barnes et al., 1989). The unique response of this enzyme to **8** indicates that a novel active site structure and mechanism for dNTP incorporation may be employed by *B. subtilis* pol III. Compounds related to **8** can be synthesized and used to explore such possible differences.

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