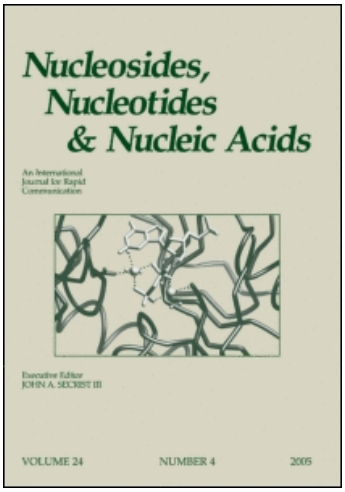

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Stephen Freese[a]; Jörg Hoheisel[b]; Hans Lehrach[b]; George Wright[a]
[a] Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA, U.S.A. [b] Imperial Cancer Research Fund, London, U.K.

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N^2-SUBSTITUTED-2'-DEOXYGUANOSINE 5'-TRIPHOSPHATES AS SUBSTRATES FOR *E. COLI* DNA POLYMERASE I

Stephen Freese,[1] Jörg Hoheisel,[2] Hans Lehrach,[2] and George Wright[1,*]

[1]Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655, U.S.A.
[2]Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K.

ABSTRACT

Several N^2-alkyl and N^2-phenyl 2'-deoxyguanosine 5'- triphosphates and 2-bromo-2'-deoxyinosine 5'-triphosphate were synthesized and tested as substrates for *E. coli* DNA polymerase I with a template:primer system requiring incorporation of 85 nucleotides. N^2-Methyl-dGTP and N^2-ethyl-dGTP were found to be efficiently incorporated in place of dGTP to give full length product. N^2-n-Hexyl-dGTP supported limited full length synthesis at high concentration, but N^2-phenyl- and N^2-(*p*-n-butylphenyl)-dGTP were poor substrates. 2-Bromo-2'-deoxyinosine 5'-triphosphate was a good substrate for pol I, and it was a replacement only for dGTP. Melting temperatures of oligodeoxyribonucleotides containing N^2-alkyl-dG residues, annealed to complementary single stranded DNA, were lower than that of the normal oligomer.

Many base-modified deoxyribonucleoside triphosphates serve as alternate substrates for DNA polymerases, and most of these possess substituents that emerge from the enzymes in the major groove of B DNA. DNA polymerase I (pol I) from *E. coli* has been extensively utilized for these studies, and, in principle, effects of modified substrates could be related to structural features of this enzyme observed in the solid state.[1] Modified substrates incorporated by pol I in which novel substituents project into the minor groove of DNA include 2'-deoxyinosine 5'-triphosphate (dITP),[2,3] 2-amino-2'-deoxyadenosine 5'-triphosphate,[3,4] and the related 2-aminopurine deoxyribonucleoside 5'-triphosphate.[3,5] These nucleotides, however, possess substituents at the 2-position that are normally present in the minor groove. We have been interested in the

chemistry of N^2-substituted guanine nucleosides and nucleotides because of their properties as selective inhibitors of DNA polymerases, thymidine kinases and G proteins.[6-9] Because substituents at the N^2 position of guanine should project into the minor groove of a DNA double helix containing such a modified residue, we wanted to test if N^2-substituted dGTP derivatives were substrates for DNA polymerases. We also considered that oligodeoxyribonucleotides containing N^2-alkyl-dG residues might be useful tools to study the physicochemical and biochemical effects of substituents in the minor groove of double stranded DNA.

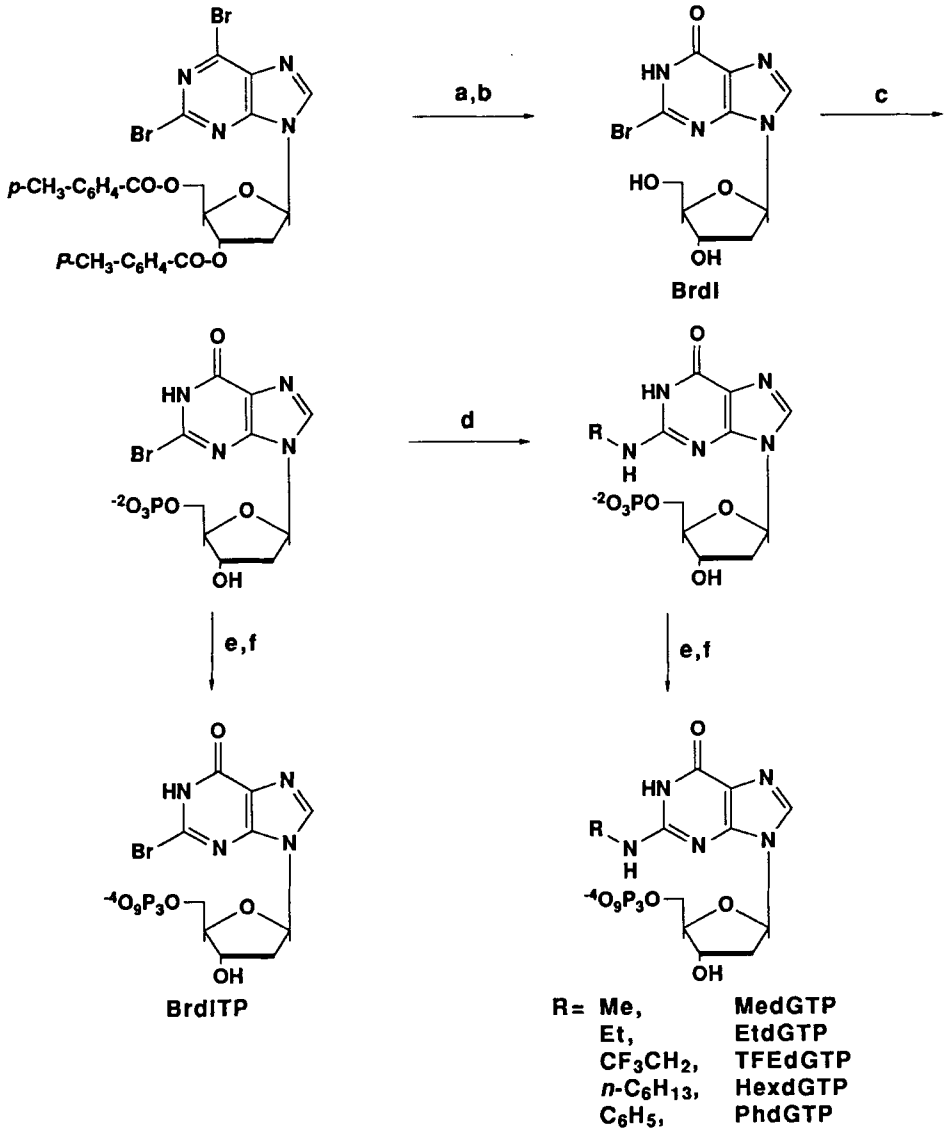
We report here the synthesis of several dGTP derivatives bearing substituents of different size on the exocyclic (N^2) amino group of the aglycone. 2-Bromo-2'-deoxyinosine 5'-phosphate (BrdIMP) was a convenient starting material to prepare N^2-substituted dGMP derivatives for subsequent conversion to the target nucleoside 5'-triphosphates. DNA polymerase I (Klenow fragment) from *E. coli* was chosen as a model enzyme for incorporation studies, and a template:primer of defined sequence was used to assess sequence dependence in the ability of the enzyme to incorporate the modified nucleotides. In this paper we demonstrate that N^2-alkyl-dGTPs are efficient substrates for pol I, but that N^2-phenyl-dGTPs are poor substrates. BrdITP itself was an efficient dGTP-specific substrate for pol I. GC rich oligonucleotides containing N^2-methyl-dG and N^2-ethyl-dG residues had lower T_m values than the natural oligomer, but that containing 2-BrdI residues hardly annealed to the complementary sequence above -5° C.

RESULTS

Chemistry. The Scheme summarizes the strategy for synthesis of the family of N^2-substituted dGTP derivatives. The approach was to develop a reactive intermediate for introduction of alkyl or aryl groups at the latest step possible in the sequence. The starting point was a protected 2,6-dibromopurine 9-β-deoxyribonucleoside which was available in high yield from the sodium salt glycosylation of 2,6-dibromopurine.[10] Deblocking of the nucleoside and hydrolysis of the 6-methoxy intermediate gave 2-bromo-2'-deoxyinosine (BrdI) in 56% overall yield. This nucleoside was converted to the 5'-mono- and triphosphates by standard methods (see Experimental Procedures).

Displacement by amines of the bromo group of BrdITP, the ideal ultimate intermediate, was attempted. BrdITP did undergo displacement

Scheme



Reagents: (a) NaOMe, MeOH (b) 0.1 M NaOH, reflux (c) $POCl_3$, $(MeO)_3PO$ (d) RNH_2, H_2O, Δ (e) 1,1'-carbonyldiimidazole, hexamethylphosphoramide (f) $\{P_2O_7^{-}HNBu_3\}$

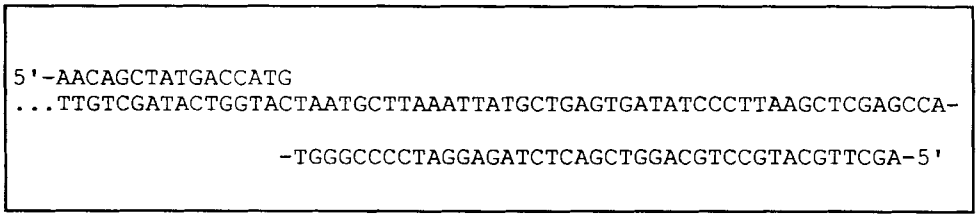


Figure 1. Structure of template:primer used in incorporation assays.

with methylamine and p-n-butylaniline, but significant decomposition to BrdIMP and the corresponding N^2-substituted dGMP derivatives occurred, as monitored by analytical HPLC (data not shown). BrdIMP, however, reacted with several alkylamines to give the N^2-alkyl-dGMPs in 60-80% yields, although the less reactive 2,2,2-trifluoroethylamine only gave 30% yield of the dGMP derivative, TFEdGMP. Reaction of BrdIMP with aniline gave N^2-phenyl-dGMP in 50% yield.[8] Each monophosphate, after activation by 1,1'-carbonyldiimidazole, was converted to the triphosphate by treatment with pyrophosphate. Yields and properties of N^2-substituted dGTP derivatives and of BrdITP are given in Experimental Procedures.

N^2-Substituted dGTP derivatives are substrates for pol I. The structure of the template region of the template:primer used for the experiments in this paper is shown in Figure 1. *E. coli* pol I under control assay conditions (see Figures 2 and 3) efficiently copied this template in the presence of the four natural dNTPs to give nearly exclusively full length product by the addition of 85 nucleotides to the primer (Figure 2, lane E). However, incubations in the absence of dGTP (see, for example, lane H of Figure 3) consistently showed primer extension beyond G sites, indicating significant misincorporation opposite C residues in the template. Pol I has been shown to misincorporate substrates in the absence of the complementary nucleotide,[11] and to extend resulting non-complementary primers in the presence of an excess of the following correct substrates.[12] Consequently, background misincorporation in the absence of dGTP allowed bypass of certain G sites and accumulation of products just before those sites, i.e. at C5, C16, A27 and A37.

The results of incubation of the template:primer and pol I with dGTP, or selected N^2-substituted derivatives, and the other dNTPs are summarized

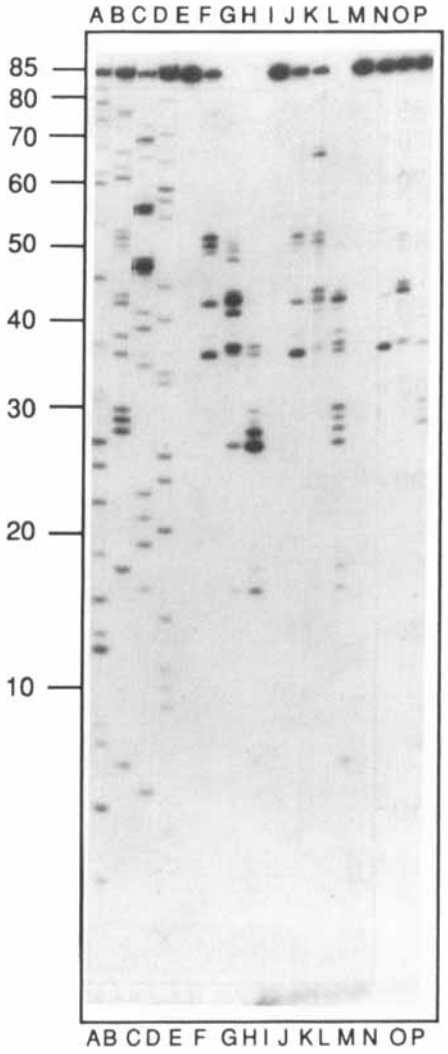


Figure 2. Autoradiogram of a gel from *E. coli* DNA polymerase I-catalyzed incorporation of N^2-substituted dGTP derivatives by template:primer. Lanes A-D: sequencing lanes for products ending with A, G, C and T, respectively. Lanes E-H: incubation for 40 min with 40 μM dATP, dCTP and dTTP, plus 40 μM dGTP (E), MedGTP (F), HexdGTP (G) or PhdGTP (H). Lanes I-L: incubation for 40 min with 40 μM dATP, dCTP and dTTP, plus 180 μM dGTP (I), MedGTP (J), HexdGTP (K) or PhdGTP (L). Lanes M-P: incubation for 20 min with 40 μM dATP, dCTP and dTTP, plus 40 μM dGTP (M), MedGTP (N), HexdGTP (O) or PhdGTP (P), followed by a 20 min chase with 40 μM dGTP.

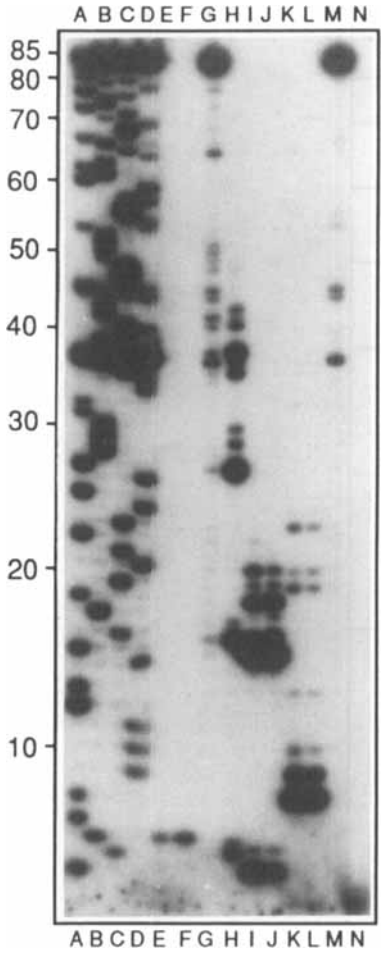


Figure 3. Autoradiogram of a gel from *E. coli* DNA polymerase I-catalyzed incorporation of BrdITP by template:primer. Lanes A-D: sequencing lanes for products ending with A, G, C and T, respectively. Incubations were done for 40 min with the additions indicated. Lanes E,F: 20 μM dGTP, dCTP and dTTP, plus 180 μM BrdITP (E) or no analog (F). Lanes G,H: 20 μM dATP, dCTP and dTTP, plus 180 μM BrdITP (G) or no analog (H). Lanes I,J: 20 μM dGTP, dATP and dTTP, plus 180 μM BrdITP (I) or no analog (J). Lanes K,L: 20 μM dGTP, dATP and dCTP, plus 180 μM BrdITP (K) or no analog (L). Lanes M,N: 20 μM dATP, dCTP,and dTTP, and 180 μM dGTP, with pol I (M) or without pol I (N).

in the autoradiograph of the gel in Figure 2. Lanes A-D represent standard dideoxy sequencing reactions, and show products terminating at A, G, C and T residues, respectively. The sequence of the fully copied product, numbered as shown for discussion purposes, can readily be read from Figure 2 as follows:

```
          5    10   15   20   25   30   35   40   45   50
 primerATTACGAATTTAATACGACTCACTATAGGGAATTCGAGCTCGGTACCCGGGG-
   55   60   65   70   75   80   85
-ATCCTCTAGAGTCGACCTGCAGGCATGCAAGCT-3'
```

As depicted in Figure 2, dGTP gave full length product with this template:primer (lanes E and I) without significant terminations. When MedGTP replaced dGTP in the assays, at both 40 and 180 μM (lanes F and J, respectively) substantial full length product was formed. Terminations occurred at G42 and G49-51, representing regions requiring two and four G incorporations, respectively. In contrast to these terminations, no products accumulated at the three G sequence G28-30 or the two G sequence G74-75, suggesting that secondary structure in addition to sequence effects may dominate terminations at sites requiring more than one MedGMP incorporation event. A single G site, G36, showed strong termination, even at high concentration of MedGTP (lane J). A chase step with dGTP (lane N) was able to extend all MedGMP terminated primers except that at G36. EtdGTP gave a pattern of incorporation and terminations essentially identical with those for MedGTP, but TFEdGTP, even at 180 μM, was not incorporated into the primer by pol I (results not shown).

In the case of HexdGTP no full length primer product was formed at 40 μM (Figure 2, lane G), but a substantial amount was formed at 180 μM (lane K). Strong terminations occurred at the double G sequence G42-43 and at C41 preceding this sequence. The several terminations occurring with HexdGTP at sites just preceding G sites, i.e. at C16, A27, A37 and C41, although similar to those observed in the absence of dGTP (see above), may result from inhibition of pol I by the compound (see below). As found for MedGTP and EtdGTP, no terminations were observed at the three G sequence G28-30. An abundant primer remained only at G36 after the dGTP chase (lane O).

PhdGTP gave no full length product at either 40 or 180 μM (lanes H and L). Single residues could be incorporated, however, because

terminations occurred at each G incorporation site up to G42. As found for HexdGTP, terminations occurring at sites immediately before a PhdGMP residue was to be incorporated, for example at C16 and A27, could result simply from prior bypass of G sites. However, the appearance of weak bands at G29 and G30, as well as at G36, G38 and, at 180 μM, G42 (lane L), indicates no absolute block to PhdGTP incorporation by pol I in this system, even at sites requiring sequential G incorporations. Indeed, most primers could be extended efficiently to full length product following the dGTP chase (lane P). The related N^2-(*p*-n-butylphenyl) derivative, BuPdGTP, at 40 μM, gave results essentially identical to those with PhdGTP.

BuPdGTP is a potent and selective inhibitor of eukaryotic DNA polymerase α,[6] and the termination results cited above for the related nucleotides HexdGTP and PhdGTP suggested that these nucleotides might inhibit pol I in addition to acting as substrates. Assays of pol I involving incorporation of [^{3}H]TTP into activated DNA in the absence of dGTP, as described previously,[9] showed that HexdGTP, PhdGTP and BuPdGTP at 500 μM each inhibited enzyme activity by 19%, 34% and 52%, respectively. In the same assay, MedGTP stimulated [^{3}H]TTP incorporation by 73% at 100 μM, but had no effect at 500 μM. We, thus, conclude that substrate efficiency and inhibitory potency of N^2-substituted dGTPs are inversely related. Studies of these properties are in progress with a simpler template:primer system which will allow detailed kinetics to be determined at a single incorporation/inhibition site.

2-Bromo-dITP specifically replaces dGTP with pol I. The substrate potential of BrdITP and its fidelity as a dGTP analog were tested by incubating the template:primer with *E. coli* pol I, 20 μM of three natural dNTPs and no or 180 μM BrdITP, but in the absence of each dNTP in turn. The results are summarized in Figure 3, in which lane M is a positive control (20 μM dATP, dCTP, dTTP and 180 μM dGTP). Lanes E and F illustrate the reaction run in the absence of dATP but with BrdITP (lane E) or no analog (lane F); clearly, BrdITP did not replace dATP in this reaction. When BrdITP replaced dGTP, however, predominately full length product DNA was observed (lane G), compared to background incorporation in the absence of dGTP (lane H). BrdITP could not replace dCTP (lane I) or dTTP (lane K) in primer extension reactions. The ability of BrdITP to be incorporated into DNA specifically in place of dGTP suggests not only that the third hydrogen bond in the G:C base pair is not essential for

incorporation [for example, dITP can be incorporated enzymatically into DNA[2,3]], but that repulsion between the 2-bromo group of the purine and the 2-oxo group of the pyrimidine is insufficient to alter base recognition in the 2-BrdI:dC base pair.

T_m of RdGMP-containing oligodeoxyribonucleotides. Normal and MedG-, EtdG- and BrdI-containing 5'-GCGGCCGC oligomers (the *Not*I restriction endonuclease recognition sequence) were prepared by a fill in reaction with *E. coli* pol I of a template:primer system in the presence of [α-^{32}P]dCTP, and the oligomers were released by treatment of product with uracil-DNA glycosylase[13] (see Experimental Procedures). Since the first G (5' to 3') of the oligomer originally was part of the primer molecule, analogs were incorporated at positions 3, 4 and 7 only. Oligomers were annealed to partially exonuclease III-digested single stranded pJDH118 DNA bearing a *Not*I site.[14] Aliquots of the mixture were spotted across a DEAE-cellulose thin layer chromatography plate, and a temperature gradient was applied across the plate (see Experimental Procedures). Development of the plate with buffer caused single strand oligomer to travel with the solvent front, but bound oligomer remained at the origin.

Plots of counts remaining at the origin as a function of the temperature at each spot gave the typical T_m curves shown in Figure 4. It is apparent that the T_m of MedG-containing oligomer, 20° C, was 10° lower than that of the normal oligomer. (Results for the EtdG-containing oligomer were indistinguishable from those for MedG.) The decrease in duplex stability for the alkylated oligomers may reflect a combination of reduced hydrogen bonding strength in each RdG:dC base pair in the duplex and disturbance of the hydration spine in the minor groove of the (presumed) B DNA structure.[15] The oligomer containing BrdI residues had a strongly reduced T_m. At the lowest temperature achievable (-5° C), only about 10% of BrdIno-containing oligomer was bound to single stranded DNA (Figure 4).

DISCUSSION

At least two interacting factors may determine if a modified nucleotide can be incorporated into DNA. The enzyme active site may be incapable of binding and incorporating the analog, or the double helical DNA containing the newly incorporated nucleotide may not be able to anneal sufficiently to support further incorporation. The results of this work

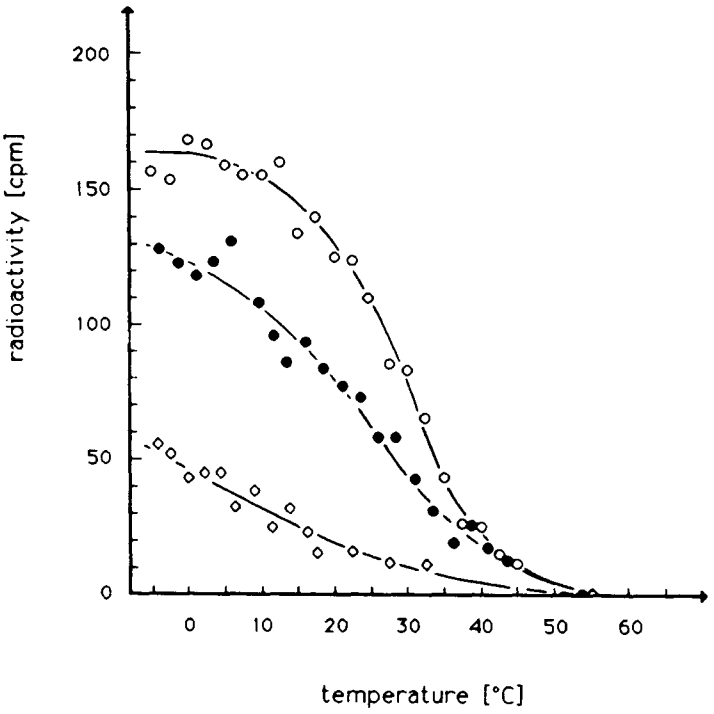


Figure 4. T_m determination for *Not*I oligomer 5'-GCGGCCGC and MedG- and BrdI-containing *Not*I oligomers annealed to pJDH118 plasmid DNA. Thin layer chromatography of ^{32}P-labelled oligomer:plasmid DNA mixtures was done as described in Experimental Procedures, with the temperature gradient as indicated. Counts of labelled oligomer retained at the origin are plotted against the temperature at each position. The results are the average of four experiments. ○, *Not*I oligomer; ●, MedG-containing *Not*I oligomer; ◇, BrdI-containing *Not*I oligomer.

suggest that groups as large as n-hexyl, phenyl, and even p-(n-butyl)-phenyl, on the exocyclic amino group of dGTP do not prevent the analogs from serving as substrates for *E. coli* pol I. However, the larger substituents confer inhibitory properties on the nucleotides leading to accumulation of primers terminated before a dGTP incorporation site. The possible role of the 3' to 5' exonuclease activity of pol I in modulating the net incorporation at individual sites is under study.

Support of full length primer synthesis by HexdGTP at high concentration shows that an n-hexyl group in the minor groove does not appear to be too large to allow proper annealing of the DNA product. Clearly, pol I accommodates poorly a phenyl group in the minor groove of

product DNA, and the enzyme is reluctant to accept two in sequence (Figure 2). These observations indicate a stricter size limitation for incorporation of nucleotides with minor groove substituents than for those with major groove substituents, at least for incorporation catalyzed by pol I.[16] However, substituent-dependent effects on double stranded DNA structure and stability may be more important than enzyme structure for incorporation of N^2-substituted dGTPs. For example, AMV reverse transcriptase incorporated the analogs shown in Figure 2 with similar efficiencies as reported here for pol I (results not shown).

The ability of 2-bromo-dITP to serve as an efficient, dGTP-specific substrate for pol I (Figure 3) indicates that the DNA containing the BrdI residues must also anneal sufficiently to support continued polymerization. Perturbation of the double stranded structure, such as tilting of 2-BrI:C base pairs due to repulsion between the 2-bromo and 2-oxo groups in the bases, respectively, apparently had little effect on recognition of the template:primer by pol I.

B form DNA contains a hydration spine in the minor groove which stabilizes the structure,[15] and alkyl groups in the minor groove might be expected to disrupt this network of water molecules. Self-complementary oligomers containing a single MedG residue, or the other minor groove modified nucleosides 2'-deoxyinosine and 3-deaza-2'-deoxyadenosine, have been synthesized chemically;[17] CD spectra of these and related[18] duplexes indicated that they indeed exist as B form DNA. In the present study hybridization strength of hemi-alkylated duplex DNA derived from RdGTPs was found to be lower than that of the normal duplex (Figure 4). In contrast, a self-complementary decamer containing a single MedG residue in each strand was reported to have a T_m 4° higher than that of the dG-containing oligomer.[17] Study of the variation of T_m of oligomers derived from other substituted dGTPs, in which electronic and steric effects of substituents may be varied, may help reveal the factors involved in stabilization of the duplex form. In addition, questions about the role of minor groove determinants for binding of duplex DNA with proteins and small molecules may be answered with such reagents, as has been reported for the interaction of restriction endonucleases with substituents in the major groove of DNA (see, for example, refs. 3, 18). Indeed, a chemically prepared oligomer containing a single MedG residue was resistant to cutting by restriction endonuclease *Sau*3AI but almost normally sensitive to *Mbo*I, even though the enzymes have the same recognition sequence.[17]

The ability of N^2-substituted dGTP derivatives to serve as substrates for DNA polymerases provides a convenient source of defined oligodeoxyribonucleotides containing specific substituents in the minor groove of DNA. The ability of BrdITP to be incorporated into DNA specifically in place of dGTP suggests a strategy to introduce N^2 substituents directly into oligomers by the reaction of BrdI-containing oligonucleotides with amines. Studies with such a model oligonucleotide are in progress.

EXPERIMENTAL PROCEDURES

General. Nuclear magnetic resonance spectra were recorded at ambient temperature on Bruker WM-250 and AC-200 spectrometers. Tetramethylsilane served as internal reference for ^1H spectra, and capillaries of 8.5% phosphoric acid and of trifluoroacetic acid were used as external references for ^{31}P and ^{19}F spectra, respectively. Characteristic ^1H resonances only are presented for all new compounds; all other resonances were as expected. Ultraviolet spectra were obtained with a Gilford Response spectrophotometer. Ion exchange chromatography was performed with 2x20 cm columns of A-25 DEAE-Sephadex (Pharmacia), and 12 mL fractions were collected at a flow rate of 3 mL min^{-1}. Nucleotides were analyzed for phosphorus content, after ashing, by the method of Ames and Dubin.[19] All other elemental analyses were performed by the Microanalysis Laboratory, University of Massachusetts, Amherst. The syntheses of N^2-phenyl-2'-deoxyguanosine 5'-phosphate (PhdGMP) has been reported;[8] N^2-(*p*-n-butylphenyl)-dGTP (BuPdGTP) was prepared as described.[6]

2-Bromo-6-methoxy-9-(2-deoxy-β-D-ribofuranosyl)purine. 2,6-Dibromo-9-[2-deoxy-3,5-di-(*p*-toluyl)-β-D-ribofuranosyl]purine[10] (17.8 g, 28 mmol) was dissolved in a solution of sodium methoxide (56 mM) in methanol (1 L) at room temperature. After 2.5 h the solution was neutralized with glacial acetic acid and evaporated under reduced pressure. The residue was mixed with silica gel (70-230 mesh, 100 g) and applied to a column of silica gel (4.5x40 cm) in chloroform. Elution was performed with 3 L of chloroform followed by a gradient of chloroform to 10% methanol in chloroform. Product eluted at a methanol concentration of 7%. After evaporation of solvents, a gummy solid was obtained which was crystallized from methanol to give 6.6 g (68%) of colorless product, mp 125-127°C. UV (methanol) λ_{max} 258.5 nm (ϵ 12300). ^1H NMR (Me_2SO-d_6) δ 8.58 (s, 1H, C8-H), 6.34 (pseudo

t, 1H, C1'-H; J_{avg} = 6.6 Hz), 4.08 (s, 3H, OCH_3). Anal. Calcd for $C_{11}H_{13}N_4O_4Br$: C, 38.28; H, 3.80; N, 16.23; Br, 23.15. Found: C, 38.44; H,3.80; N, 16.18; Br, 23.45.

2-Bromo-9-(2-deoxy-β-D-ribofuranosyl)hypoxanthine (2-bromo-2'-deoxyinosine, BrdI). A solution of the above 6-methoxy compound (140 mg, 0.41 mmol) in 0.1 M NaOH (50 mL) was heated at reflux. After 30 min the solution was brought to room temperature, adjusted to pH 8 with glacial acetic acid, and applied to a DEAE-Sephadex column which had been equilibrated with 0.1 M ammonium bicarbonate. After washing with 0.5 L of the same buffer, elution was done with a linear gradient of 0.1 to 0.6 M ammonium bicarbonate during 4 h. The main peak, which eluted at a buffer concentration of 0.3 M, was evaporated to obtain 116 mg (82%) of colorless product as the ammonium salt. Free BrdI was prepared by reverse phase HPLC (C-18 column; 7.5% acetonitrile/0.05% trifluoroacetic acid in water): mp 120-125° (dec.). UV (pH 6.8 and 13) λ_{max} 257.3 nm (ϵ 12200), (pH 3) 253.5 nm (ϵ 11400). ^1H NMR (Me_2SO-d_6) δ 13.2 (br s, 1H, N1-H), 8.30 (s, 1H, C8-H), 6.23 (pseudo t, 1H, C1'-H; J_{avg} = 6.7 Hz). Anal. Calcd. for $C_{10}H_{11}N_4O_4Br \cdot 0.5H_2O$): C, 35.31; H, 3.56; N, 16.47; Br, 23.49. Found: C, 35.22; H, 3.64; N, 16.28; Br, 24.3.

2-Bromo-9-(2-deoxy-β-D-ribofuranosyl)hypoxanthine 5'-phosphate (BrdIMP). Phosphoryl chloride (35 μL, 0.37 mmol) was added to a cold (0°C) solution of ammonium BrdI (110 mg, 0.32 mmol) in trimethyl phosphate (2.0 mL). After 3 h at 0°C the solution was neutralized with 1 N sodium hydroxide, and this solution was applied to a DEAE-Sephadex column equilibrated with 0.2 M triethylammonium bicarbonate (TEAB). Elution was done with a linear gradient of 0.2 to 1 M TEAB during 8 h. Fractions eluting at 0.5 M TEAB were pooled and evaporated under reduced pressure. The residue was dissolved in water and lyophilized to give 96 mg (49%) of BrdIMP as the bis(triethylammonium) (TEA) salt. UV (H_2O) λ_{max} 254.4 nm (ϵ 12170). ^{31}P NMR (D_2O) δ 3.89 (t, J = 5.0 Hz); ^1H NMR (D_2O) δ 8.29 (s, 1H, C8-H), 6.38 (pseudo t, 1H, C1'-H; J_{avg} = 6.8 Hz). Anal. Calcd. for $C_{22}H_{42}N_6O_7BrP$: P, 5.05. Found: P, 4.84.

N^2-Methyl-2'-deoxyguanosine 5'-phosphate (MedGMP). A solution of BrdIMP, TEA salt, (60 mg, 0.1 mmol) in water (10 mL) and a solution of methylamine in diethyl ether (5 mL, 11% w/v, 18 mmol) were sealed in a teflon-lined steel bomb and heated at 100°C for 16 h. The reaction mixture was brought to room temperature and applied to a DEAE-Sephadex column equilibrated

with 0.1 M TEAB. Elution was done with a linear gradient of 0.1 to 0.5 M TEAB during 8 h. Fractions eluting at 0.3 M buffer were pooled and evaporated under reduced pressure. The residue was dissolved in water and lyophilized to give 33 mg (71%) of MedGMP as the bis(triethylammonium) salt. [Elemental analysis was performed using the bis[tri(n-butyl)ammonium] (TBA) salt prepared as described below for the synthesis of triphosphates.] UV (H_2O) λ_{max} 253.5 nm (ϵ 13600). ^{31}P NMR (D_2O) δ 1.18 (t, J = 5.4 Hz). ^1H NMR (D_2O) δ 8.06 (s, 1H, C8-H), 6.42 (pseudo t, 1H, C1'-H; J_{avg} = 6.8 Hz), 2.96 (s, 3H, N-CH_3). Anal. Calcd. for $C_{23}H_{43}N_6O_7P$: P, 5.67. Found: P, 5.43.

N^2-Ethyl-2'-deoxyguanosine 5'-phosphate (EtdGMP). A mixture of BrdIMP, TEA salt (150 mg, 0.24 mmol), ethylamine (1 mL, 15 mmol) and water (8 mL) was heated in a bomb at 100° C for 15 h. The reaction mixture was adjusted to pH 7 with acetic acid and applied to a DEAE-Sephadex column. Elution was done with a gradient of 0.1 to 0.6 M ammonium bicarbonate during 8 h. Evaporation of fractions eluting at 0.35 M buffer afforded 99 mg (85%) of EtdGMP, ammonium salt, as a colorless solid. UV (H_2O) λ_{max} 255.0 nm (ϵ 15030). ^{31}P NMR (D_2O) δ 3.70 (t, J = 5.4 Hz). ^1H NMR (D_2O) δ 8.04 (s, 1H, C8-H), 6.39 (pseudo t, 1H, H-1'; J_{avg} = 4.9 Hz), 3.42 (q, 2H, N-CH_2; J = 7.2 Hz), 1.23 (t, 3H, CH_3; J = 7.2 Hz). Anal. Calcd. for $C_{12}H_{24}N_7O_7P$: P, 7.57. Found: P, 7.63.

N^2-(2,2,2-Trifluoroethyl)-2'-deoxyguanosine 5'-phosphate (TFEdGMP). A mixture of BrdIMP, TEA salt (200 mg, 0.33 mmol), 2,2,2-trifluoroethylamine (657 mg, 6.6 mmol) and water (5 mL) was heated in a bomb at 100° C for 13 h. The reaction mixture was filtered and subjected to preparative HPLC (C-18 column, 2 x 35 cm) with a gradient of 0.05% trifluoroacetic acid (TFA)/water to 0.05% TFA/25% acetonitrile/water during 36 min. Fractions containing the major product were lyophilized to give 33 mg (30%) of TFEdGMP, free acid, as a colorless solid. UV (H_2O) λ_{max} 252.5 nm (ϵ 13930). ^{31}P NMR (Me_2SO-d_6) δ 0.97 (t, J = 5.5 Hz). ^1H NMR (Me_2SO-d_6) δ 11.0 (bd s, 1H, N1-H), 8.10 (s, 1H, C8-H), 7.05 (t, 1H, N^2-H; J = 6.3 Hz), 6.24 (pseudo t, 1H, C1'-H; J_{avg} = 6.7 Hz), 4.21 (m, 2H, N-CH_2). ^{19}F NMR (Me_2SO-d_6) δ 33.06 (t, J = 8.9 Hz). The compound was converted to the ammonium salt by chromatography on DEAE-Sephadex with a gradient of 0.1 M to 0.6 M ammonium bicarbonate during 8 h; product eluted at 0.35 M buffer. Anal. Calcd for $C_{12}H_{21}N_7O_7FP$: P, 6.69. Found: P, 6.69.

N^2-(n-Hexyl)-2'-deoxyguanosine 5'-phosphate (HexdGMP). A solution of BrdIMP (131 mg, 0.21 mmol) and n-hexylamine (0.2 mL, 2.0 mmol) in water (10 mL) and 2-methoxyethanol (4 mL) was heated at reflux for 20 h. The solution was cooled and applied to a DEAE-Sephadex column equilibrated with 0.2 M TEAB. Elution was done with a gradient of 0.2 to 1 M TEAB during 8 h. Fractions eluting at 0.3 M buffer were evaporated, and the residue was dissolved in water and lyophilized to give 70 mg (62%) of product as the TEA salt. UV (H_2O) λ_{max} 254.4 nm (ϵ 10300). ^{31}P NMR (D_2O) δ 3.90 (t, J = 5.2 Hz). ^1H NMR (D_2O) δ 8.02 (s, 1H, C8-H), 6.36 (pseudo t, 1H, C1'-H; J_{avg} 6.9 Hz), 3.34 (t, 2H, N-CH_2), 1.32 (m, 8H, $(CH_2)_4$), 0.86 (t, 3H, CH_3). Anal. Calcd. for $C_{22}H_{41}N_6O_7P$: P, 5.82. Found: P, 5.57.

Synthesis of triphosphates. The monophosphates prepared above and PhdGMP (55 μmol each) were converted to the TBA salts by passage through ion exchange resin (Dowex 50W-8X, TBA form, 1 mL). After lyophilization each monophosphate and 1,1'-carbonyldiimidazole (45 mg, 275 μmol) were dissolved in hexamethylphosphoramide (HMPA) (1 mL), and the solution was stirred at rt for 7 h. Unreacted 1,1'- carbonyldiimidazole was destroyed by addition of methanol (35 μL), and a solution of tri(n-butyl)ammonium pyrophosphate (Sigma) (124 mg, 275 μmol) in HMPA (2 mL) was added dropwise. After stirring at rt for 24 h, the reaction mixture was diluted with 0.2 M TEAB (10 mL) and applied to a DEAE-Sephadex column which had been equilibrated with 0.2 M TEAB. Elution with a linear gradient of 0.2 - 1.5 M TEAB during 8 h, followed by concentration and lyophilization of the appropriate fractions, gave the nucleoside triphosphates as the bis(TEA) salts as summarized below:

Product	Yield	^{31}P NMR, δ			UV(H_2O)	
	%	α	β	γ	λ_{max}	ϵ
BrdITP	68	-10.90	-21.85	-5.96	257.0	13360
MedGTP	38	-11.07	-22.41	-8.13	253.0	12060
EtdGTP	62	-11.22	-22.97	-10.68	255.0	15150
TFEdGTP	54	-11.20	-22.74	-10.07	252.8	12650
HexdGTP	53	-10.93	-22.0	-6.07	254.5	15100
PhdGTP	30	-12.54	-24.37	-11.91	275.2	19100

Template:primer construction. Plasmid pTZ18R[20] was cut with *Hin*dIII, and about 300 nucleotides were removed from each 3' terminus with exonuclease III to create single strand template (Figure 1). The "reverse sequencing

primer" (Figure 1; New England Biolabs) was labelled at the 5' end with [γ-^{32}P]ATP using T4 polynucleotide kinase.[21] 3.4 μg of template were mixed with 0.4 μg of primer in 156 μL of buffer; the final buffer concentrations in the reactions were 33 mM Tris-acetate, pH 7.9, 66 mM potassium acetate and 10 mM magnesium acetate.[22] The mixture was heated to 100°C for 3 min and slowly cooled to rt in a waterbath.

DNA polymerase reactions. To 156 μL of template:primer solution were added 13 μL of 0.1 M dithiothreitol. To each 14 μL of this solution was added 1 μL of *E. coli* DNA polymerase I, Klenow fragment (1 U/μL, Boehringer, reagent grade). Aliquots of 3.5 μL of each mixture were added to 2 μL of nucleoside triphosphates to initiate DNA synthesis or sequencing reactions. Sequencing was carried out by the standard dideoxy-method[23] but with double the concentration of 2',3'-dideoxynucleotides for improved band intensities at low molecular weight. For DNA synthesis the final nucleotide concentrations were 40 μM each of dATP, dCTP and dTTP, and either 40 μM or 180 μM of dGTP or modified dGTP derivative. Incubations were for 40 min at 28°C; when a chase was done, 1 μL of a dGTP solution was added after 20 min to give 40 μM dGTP. Reactions were terminated by addition of 5 μL of load buffer (80% formamide, 20 mM Tris-borate, pH 8.3, 1 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanole) to each reaction. The samples were boiled for 3 min and immediately chilled on ice. Two μL samples were loaded in the slots of a 12% polyacrylamide gel in 8M urea, and electrophoresis was carried out at 25 V/cm in TBE buffer (90 mM Tris-borate, pH 8.8, 2.5 mM EDTA).

Assays for inhibition of pol I activity employed, in a volume of 25 μL, 0.01 unit of enzyme, 25 μM dATP and dCTP, 10 μM [^{3}H]TTP (5000 cpm/pmol), and 400 μg/mL activated calf thymus DNA. After incubation for 15 min at 30 °C in the presence or absence of RdGTPs, reaction mixtures were processed as described previously.[9]

*Not*I oligomer. Probes consisting of the *Not*I recognition sequence were generated and labelled to high specific activity with [α-^{32}P]dCTP as described by Craig et al.[24] Using the template 5'-GCGGCCGCACCGGCCGGCC, the primer 5'-GGCCGGCCGGUG was extended by *E. coli* pol I with dGTP or dGTP derivative and labelled dCTP. The probe was released by digestion with uracil-DNA glycosylase, which was a kind gift of Tomas Lindahl. (The actual cleavage, after the removal of the uracil base, is due to the presence of DNA endonuclease IV, which produces nicks on the 5' side of

the sugar-phosphate bond.[13]) Subsequently, the labelled probes were excised from a 20% polyacrylamide/8 M urea gel.

Melting temperature determination. The *Not*I oligomers (8.7 fmol each) were mixed with 0.94 pmol plasmid pJDH118 DNA[14] which had been cut with *Hin*dIII and digested with exonuclease III to create single stranded DNA bearing the *Not*I site of its extended polylinker. The mixtures in 116 μL of 180 mM NaCl were heated to 90° C and slowly cooled to 0° C and stored at 2° C overnight. One half μL aliquots were spotted on DEAE-cellulose thin layer chromatography plates (Polygram). The plates were dried at rt and sandwiched between two aluminum plates which were cooled at the left and heated at the right ends by circulating the liquid of two thermostatted waterbaths through them. The temperatures were measured in both waterbaths and on the outer surface of the aluminum plates. After elution with 8xSSC (1xSSC: 150 mM NaCl, 15 mM Na acetate, pH 7.5), counts of ^{32}P remaining at each origin were determined. Maximum normal oligomer bound to plasmid, at the lowest temperature, was 30% of the total applied.

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