Nucleoside Analogue Substitutions in the Trinucleotide DNA Template Recognition Sequence 3′-(CTG)-5′ and Their Effects on the Activity of Bacteriophage T7 Primase†

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ABSTRACT: Bacteriophage T7 primase catalyzes the synthesis of the oligoribonucleotides pppACC(C/A) and pppACAC from the single-stranded DNA template sites 3′-d[CTGG(G/T)]-5′ and 3′-(CTGTG)-5′, respectively. The 3′-terminal deoxycytidine residue is conserved but noncoding. A series of nucleoside analogues have been prepared and incorporated into the conserved 3′-d(CTG)-5′ site, and the effects of these analogue templates on T7 primase activity have been examined. The nucleosides employed include a novel pyrimidine derivative, 2-amino-5-(β-2-deoxy-D-*erythro*-pentofuranosyl)pyridine (d2APy), whose synthesis is described. Template sites containing d2APy in place of the cryptic dC support oligoribonucleotide synthesis whereas those containing 3-deaza-2′-deoxycytidine (dc^3C) and 5-methyl-6-oxo-2′-deoxycytidine ($dm^{5ox}C$) substitutions do not, suggesting that the N3 nitrogen of cytidine is used for a critical interaction by the enzyme. Recognition sites containing 4-amino-1-(β-2-deoxy-D-*erythro*-pentofuranosyl)-5-methyl-2,6[1*H*,3*H*]-pyrimidione (dm^32P) or 2′-deoxyuridine (dU) substitutions for dT support oligoribonucleotide synthesis whereas those containing 5-methyl-4-pyrimidinone 2′-deoxyriboside ($d^{2H}T$) substitutions do not, suggesting the importance of Watson−Crick interactions at this template residue. Template sites containing 7-deaza-2′-deoxyguanosine (dc^7G) or 2′-deoxyinosine (dI) in place of dG support oligoribonucleotide synthesis. The reduced extent to which dc^7G is successful within the template suggests a primase−DNA interaction. Inhibition studies suggest that the primase enzyme binds "null" substrates but cannot initiate RNA synthesis.

DNA primases catalyze the template-directed *de novo* synthesis of oligoribonucleotides for use as primers by a DNA polymerase. A major role for DNA primases is in the synthesis of primers during the initiation of the synthesis of Okazaki fragments on the lagging strand (*1*), but DNA primases also often provide primers for initiation at origins of replication. The gene 4 protein of bacteriophage T7 encodes two collinear proteins from separate in-frame translational start sites, each of which binds single-stranded DNA (ssDNA)[1] during cell replication (*2*). The smaller 56-kDa protein possesses helicase activity, unwinding the parental DNA duplex. The larger 63-kDa protein (referred to hereafter simply as primase) possesses helicase activity but also a 63-aa N-terminal fragment that is responsible for the template-directed creation of short template-bound RNA strands (*3*−*5*). The primase enzyme is coupled to the helicase in this relatively small replisome so as to take advantage of the translational activity of the helicase, with associated dTTP hydrolysis (*6*−*8*).

Within a ssDNA sequence 3′-CTG-5′ is minimally sufficient for recognition and will support initiation and the synthesis of pppAC dimers by bacteriophage T7 primase (*5*). The template-bound tetramers pppACCA, pppACCC, and pppACAC are the minimum substrates recognized and extended by the T7 DNA polymerase (*9*−*11*). The 3′-cytidine of the 3′-CTG-5′ site is cryptic; it is essential for primase recognition but does not code for a residue in the product oligoribonucleotide.

The T7 primase is a hexamer that recognizes ssDNA through a Cys_4 zinc-binding motif in an equimolar ratio of zinc to protein (*3*, *12*). Structural analysis of these single Cys_4 "zinc ribbons" suggests that they have little in common with other zinc-binding motifs, with an almost entirely β-sheet secondary structure (*13*−*15*). The single Cys_4 zinc-binding ribbon observed in bacteriophage T7 is to date unique to enzymes involved in DNA replication/transcription, including other procaryotic and eucaryotic primases as well as transcription factors TFIIS, TFIIE, and TFIIB, among others. While the sequences outside the Cys_4 loop region can be highly substituted (*16*), chimeric T7/T4 and T7/T3 primases have indicated that the Cys_4 motif is not the sole determinant of sequence specificity (*17*). A truncated 271-aa N-terminal fragment of the gene 4 protein that lacks any helicase activity has been found to be the minimum requirement for native primase activity (*18*). Residues Asp_{31} and His_{33} within the Cys_4 loop have been found to play an essential role in sequence recognition, binding to the 3′-terminal cryptic cytosine (*19*). Asp_{31} binds to the C4 amino

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[1] Abbreviations: d2Apy, 2-amino-5-(β-2-deoxy-D-*erythro*-pentofuranosyl)pyridine; dc^3C, 3-deaza-2′-deoxycytidine; $dm^{5ox}C$, 4-amino-1-(β-2-deoxy-D-*erythro*-pentofuranosyl)-5-methyl-2,6[1*H*,3*H*]-pyrimidinone; dm^32P, 5-(β-D-2′-deoxyribosyl)-3-methyl-2[1*H*]-pyridinone; dU, 2′-deoxyuridine; $d^{2H}T$, 5-methyl-4-pyrimidinone 2′-deoxyriboside; dc^7G, 7-deaza-2′-deoxyguanosine; dI, 2′-deoxyinosine; ssDNA, single-stranded DNA.

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group of the cytosine (*19*), although previous studies have determined that the exocyclic amine of the cryptic 3′-cytidine was not a critical interaction for primase activity (*20*).

Primase binding sites for dTTP, required for helicase translocation, and binding sites for the template−triphosphate interactions have been proposed on the basis of modeling studies (*19*). It has been further determined that this unidirectional translocating gene 4 enzyme binds CTP prior to recognition site identification, suggesting that trinucleotide recognition by primase is at the third dG nucleotide, very likely based on traditional Watson−Crick hydrogen bonds (*19*). In the absence of a crystal structure, the mechanism by which these zinc motifs recognize a specific trimer on ssDNA remains largely speculative.

In the present work we have employed nucleoside analogues, substituted for individual residues within the trinucleotide recognition sequence, to probe the nature of the recognition of this conserved site. The use of such nucleoside analogues has proven valuable in the study of other ssDNA binding proteins (*21*−*25*) and has been already proven useful in the study of T7 primase−ssDNA interaction (*19*, *20*). A series of 20-mer oligodeoxynucleotides, each containing a single analogue nucleotide residue, were synthesized, and their abilities to direct the synthesis of the corresponding oligoribonucleotide primer products were evaluated.

EXPERIMENTAL PROCEDURES

Materials

^{1}H NMR spectra were obtained on Varian spectrometers (300, 400, and 500 MHz). NMR samples contained tetramethylsilane as the internal standard unless otherwise specified. Rotary evaporations under reduced pressure were accomplished using Buchi systems. Thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} precoated on aluminum sheets (EM Separations Technology). Flash column chromatography was run on silica gel 60, particle size 0.040−0.063 (EM Separations Technology). Silica-coated preparative chromatography plates were Uniplate 20 × 20 cm and 1000 μm precoated on glass (Analtech, Inc). Dry solvents were purchased from Aldrich Chemical Co. and used without further purification. UV scans were performed on a Perkin-Elmer 8452A diode array spectrophotometer scanning from 180 to 820 nm.

The four common 2′-deoxynucleotide phosphoramidites and 3′-terminal nucleoside-bound controlled pore glass (CPG) supports were purchased from Glen Research (Sterling, VA). The phosphoramidite derivatives of 2′-deoxyuridine (dU) and 2′-deoxyinosine (dI) were acquired from Cruachem (Glasgow, Scotland). Nonradioactive and radioactive nucleotide triphosphates were purchased from Pharmacia LKB Biotechnology, Inc., and ICN, respectively. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC) was performed on a Beckman HPLC system using C-18 reversed-phase columns (ODS−Hypersil, 5 μm particle size, 120 Å pore) detecting at 260 nm. UV analyses of oligodeoxynucleotides were performed on a Perkin-Elmer Lambda 3B UV−vis spectrophotometer. Snake venom phosphodiesterase (*Crotalus dursissus*) and calf intestine alkaline phosphodiesterase were from Boehringer-Mannheim. Integration of HPLC chromatograms was performed with a Shimadzu C-R3A Chromatopac integrator.

Bacteriophage T7 primase $G4A_{G64}$ was obtained from the laboratory of Dr. Charles C. Richardson at Harvard Medical School's Department of Biological Chemistry and Molecular Pharmacology. The protein provided contains a Gly substituted for Met at amino acid position 64 of the gene 4 protein which eliminates expression of the collinear 56-kDa helicase protein.

Denaturing polyacrylamide gels (20% monomer; 8 M urea) were run at 50 × 38 × 0.04 cm using an electrolytic buffer of 1 mM EDTA and 45 mM Tris−borate, pH 8.4. Radioactivity was visualized using a Molecular Dynamics 425 phosphorimager.

Methods

*2-Amino-5-iodopyridine (**2**).* The iodo compound **2** was prepared by Ogura's method (*26*). A mixture of 2-aminopyridine (**1**) (2.4 g, 25 mmol), periodic acid dihydrate (0.86 g, 3.75 mmol), and iodine (2.7 g, 10.7 mmol) was heated in a solution of acetic acid (60 mL), water (3 mL), and sulfuric acid (0.5 mL) at 80 °C for 4 h. The mixture was poured into 10% aqueous $Na_2S_2O_3$ solution to remove unreacted iodine and extracted with ether. The extract was washed with 10% aqueous NaOH, dried over potassium carbonate, and concentrated *in vacuo*. The residue was purified by flash chromatography (ethyl acetate/hexanes, 5:2; R_f = 0.64). Recrystallization from ethanol gave colorless crystals of compound **2** (4.4 g, 80%). UV−vis: λ_{max} (CH_3OH) 247 [ϵ = 46 330 L/(M cm)], 314 [ϵ = 7970 L/(M cm)] nm. IR (KBr): 3377 (s), 3301 (s), 3144 (s, br), 3012 (m), 1640 (s), 1577 (s), 1545 (s), 1483 (s), 1381 (s), 1312 (s), 1256 (s), 1142 (s), 1086 (s), 998 (s), 828 (s), 526 (s), 457 (s) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ = 8.21 (s, 1H), 7.62 (d, J = 8 Hz, 1H), 6.35 (d, J = 8 Hz, 1H), 4.51 (s, 2H) ppm. ^{13}C NMR ($CDCl_3$): δ = 157.30, 153.73, 145.31, 110.96, 78.00 ppm. mp 128−129 °C. HRMS: calculated for $C_5H_4IN_2$ (M + 1), 220.9576; found, 220.9576.

*5-(β-D-Glyceropentofuran-3′-ulos-1′-yl)-2-aminopyridine (**5**).* A mixture of bis(dibenzylideneacetone)palladium (0.115 g, 0.2 mmol) and tris(pentafluorophenyl)phosphine (0.213 g, 0.4 mmol) in acetonitrile (60 mL) was stirred under nitrogen at room temperature for 30 min. *N*,*N*-Diisopropylethylamine (1.4 mL, 8 mmol), 1,4-anhydro-2-deoxy-3-*O*-[(1,1-dimethylethyl)diphenylsilyl]-D-erythro-1-enitol (**3**) (*27*) (1.42 g, 4 mmol), and **2** (0.880 g, 4 mmol) were added, and the resulting reaction solution was refluxed under nitrogen at 95 °C for 30 h. Volatiles were removed *in vacuo*, and the residue was purified by flash chromatography (dichloromethane/methanol, 9:1; R_f = 0.37) to yield intermediate **4** (1.6 g, 90%) as a colorless foam slightly contaminated by a trace amount of *N*,*N*-diisopropylethylamine. To a solution of compound **4** (1.6 g, 3.6 mmol) in THF (20 mL) at 0 °C was added acetic acid (0.88 mL, 16 mmol) followed by 8 mL of a l M solution of tetra-*n*-butylammonium fluoride in THF (8 mmol). The desilylation reaction was completed in 40 min, as revealed by TLC analysis. The volatiles were removed, and the residue was separated by flash chromatography (dichloromethane/methanol, 9:1; R_f = 0.23) to afford compound **5** (0.67 g). The yield for the conversion of **3** to **5** (two steps) was 80%.

Characterization of crude **4**: ^{1}H NMR ($CDCl_3$) δ = 7.86−7.83 (m, 2H), 7.77−7.72 (m, 3H), 7.47−7.41 (m, 6H), 7.07 (dd, 1H, J = 2.4 Hz), 6.27 (d, 1H, J = 8.4 Hz), 5.43 (d, 1H,

J = 2.8 Hz), 4.67−4.65 (m, 1H), 4.22−4.20 (m, 1H), 3.85−3.80 (m, 2H), 1.09 (s, 9H) ppm.

Characterization of **5**: UV−vis λ_{max} (CH_3OH) 239 [ϵ = 15 570 L/(M cm)], 300 [ϵ = 3910 L/(M cm)] nm; IR (KBr) 3440 (s), 3325 (s, br), 3204 (s, br), 3053 (w), 2950 (w), 2921 (w), 2881 (w), 2857 (w), 2823 (w), 1761 (s), 1634 (s), 1611 (s), 1513 (s), 1421 (s), 1323 (s), 1161 (s), 1103 (s), 844 (s), 775 (m) cm^{-1}; ^1H NMR (CD_3OD) δ = 7.97 (d, 1H, J = 2.4 Hz), 7.68 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.8 Hz), 6.61 (d, 1H, J = 8.8 Hz), 5.08 (dd, 1H, J_1 = 6.0 Hz, J_2 = 11.2 Hz), 3.98 (t, 1H, J = 3.2 Hz), 3.82 (d, 2H, 3.2 Hz), 2.75 (dd, 1H, J_1 = 6.0 Hz, J_2 = 17.2 Hz), 2.45 (dd, 1H, J_1 = 11.2 Hz, J_2 = 17.2 Hz) ppm; ^{13}C NMR (CD_3OD) δ = 215.56, 160.99, 146.67, 138.19, 126.09, 110.44, 84.38, 76.89, 62.04, 46.19 ppm; mp 140 °C dec; HRMS, calculated for $C_{10}H_{13}N_2O_3$ (M + 1), 209.0926; found, 209.0926.

*2-Amino-5-(β-2-deoxy-D-erythro-pentofuranosyl)pyridine (**6**).* To a solution of compound **5** (624 mg, 3 mmol) in acetonitrile (20 mL) and acetic acid (20 mL) at 0 °C was added sodium triacetoxyborohydride (3.18 g, 15 mmol). The reaction was complete within 20 min, as revealed by TLC analysis. The reaction was quenched with a 50% ethanol/water solution (10 mL). Volatiles were then removed *in vacuo*, and the residue was separated by column chromatography (methanol/dichloromethane, 1:4; R_f = 0.29) to afford compound **6** (541 mg, 86%). UV−vis: λ_{max} (CH_3OH) 239 [ϵ = 35 560 L/(M cm)], 300 [ϵ = 9560 L/(M cm)] nm. IR (KBr): 3339 (s, br), 3220 (s, br), 3024 (s), 2987 (w), 2930 (m), 2873 (m), 1634 (s), 1564 (s), 1507 (s), 1413 (s), 1357 (s), 1268 (m), 1218 (m), 1092 (s), 1054 (s), 1030 (s), 948 (m), 828 (m), 651 (m). ^1H NMR (D_2O): δ = 7.64 (s, 1H), 7.31 (d, 1H, J = 8.4 Hz), 6.41 (d, 1H, 8.4 Hz), 4.77 (dd, 1H, J_1 = 4.8 Hz, J_2 = 9.6 Hz), 4.16−4.10 (m, 1H), 3.75−3.70 (m, 1H), 3.46−3.41 (m, 2H), 1.90−1.77 (m, 2H) ppm. ^{13}C NMR (D_2O): δ = 159.13, 145.40, 138.61, 126.52, 111.32, 87.86, 79.13, 73.96, 63.19, 42.45 ppm. HRMS: calculated for $C_{10}H_{14}N_2O_3$ (M + 1), 210.1004; found, 210.1004.

*2-{N-[1-(Dimethylamino)methylidine]amino}-5-(β-2-deoxy-D-erythro-pentofuranosyl)pyridine (**7**).* To a solution of compound **6** (210 mg, 1 mmol) in methanol (10 mL) was added *N*,*N*-dimethylacetamide dimethyl acetal (405 mL, 3 mmol), and the reaction mixture was stirred at 78 °C for 7 h. Volatiles were removed in vacuo, and the resulting residue was separated by column chromatography (dichloromethane/methanol, 4:1; R_f = 0.45) to afford compound **7** (255 mg, 91%), a highly hydroscopic sticky solid. UV−vis: λ_{max}(CH_3OH) 240, 256, 321 nm. IR (KBr): 3358 (s, br), 3010 (w), 2923 (w), 2873 (w), 1610 (m), 1590 (s), 1550 (s), 1476 (m), 1394 (m), 859 (m). ^1H NMR ($CDCl_3$): δ = 8.27 (d, 1H, J = 2.4 Hz), 7.53 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.0 Hz), 6.71 (d, 1H, J = 8.0 Hz), 5.09 (dd, 1H, J_1 = 5.6 Hz, J_2 = 10.0 Hz), 4.40 (dt, 1H, J_1 = 5.6 Hz, J_2 = 2.8 Hz), 3.98−3.95 (m, 1H), 3.78−3.69 (m, 2H), 3.05 (s, 6H), 2.57 (s, br, 2H), 2.20 (ddd, 1H, J_1 = 2.4 Hz, J_2 = 5.6 Hz, J_3 = 13.2 Hz), 2.02−1.95 (m, 4H) ppm. ^{13}C NMR ($CDCl_3$): δ = 162.98, 159.55, 146.36, 136.16, 130.19, 117.83, 87.79, 78.03, 73.33, 63.30, 43.50, 38.47 ppm. HRMS: calculated for $C_{14}H_{22}N_3O_3$ (M + 1), 280.1661; found, 280.1662.

*5-[β-2-Deoxy-5'-O-(4,4-dimethoxytrityl)-D-erythro-pentofuranosyl]-2-{N-[1-(dimethylamino)methylidine]amino}-pyridine (**8**).* To a solution of compound **7** (168 mg, 0.60 mmol) in anhydrous pyridine (30 mL) at room temperature was added 4,4-dimethoxytrityl chloride (265 mg, 0.78 mmol). The reaction mixture was stirred at room temperature. The reaction was completed in 12 h, as revealed by TLC analysis. The mixture was concentrated under reduced pressure, dissolved in dichloromethane (30 mL), and washed with saturated sodium bicarbonate solution (3 × 20 mL) and water (3 × 20 mL). The organic portion was dried over sodium sulfate overnight. The drying reagent was removed by filtration, and the solution was concentrated *in vacuo*. The residue was purified by flash chromatography (dicloromethane/methanol, 9:1; R_f = 0.40) to give compound **8** as a colorless foam (315 mg, yield 89%). UV−vis: λ_{max}(CH_3OH) 235, 267 nm. IR (KBr): 3390 (br), 3050 (w), 3030 (w), 2999 (w), 2930 (m), 2920 (m), 2867 (w), 2836 (w), 1610 (s), 1590 (s), 1470 (s), 1394 (s), 1300 (m), 1243 (s), 1174 (s), 1035 (s), 8349 (m), 752 (m), 588 (m) cm^{-1}. ^1H NMR ($CDCl_3$): δ = 8.26 (d, 1H, J = 2.1 Hz), 7.57 (dd, 1H, J_1 = 2.1 Hz, J_2 = 8.4 Hz), 7.45 (d, 2H, J = 7.2 Hz), 7.34 (d, 4H, J = 8.7 Hz), 7.29−7.19 (m, 3H), 6.81 (d, 4H, J = 8.7 Hz), 6.68 (d, 1H, J = 8.4 Hz), 5.11 (dd, 1H, J_1 = 5.7 Hz, J_2 = 10.2 Hz), 4.44−4.42 (m, 1H), 4.07−4.02 (m, 1H), 3.78 (s, 6H), 3.34 (dd, 1H, J_1 = 4.8 Hz, J_2 = 9.9 Hz), 3.24 (dd, 1H, J_1 = 5.4 Hz, J_2 = 9.9 Hz), 3.05 (s, 6H), 2.19 (ddd, 1H, J_1 = 1.5 Hz, J_2 = 5.7 Hz, J_3 = 13.2 Hz), 2.04 (ddd, 1H, J_1 = 6.0 Hz, J_2 = 10.2 Hz, J_3 = 13.2 Hz), 1.59 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): δ = 163.28, 159.17, 158.35, 146.60, 144.81, 136.06, 135.69, 130.11, 129.69, 128.21, 127.84, 126.78, 117.58, 113.16, 86.46, 86.26, 78.16, 74.57, 64.78, 55.43, 43.94, 38.28, 16.21 ppm. mp 77−79 °C. HRMS: calculated for $C_{35}H_{40}N_3O_5$ (M + 1), 582.2968; found, 582.2966.

*5-[β-2-Deoxy-3-O-(2-cyanoethyl-N,N-diisopropylphosphino)-5-O-(4,4-dimethoxytrityl)-D-erythro-pentofuranosyl]-2-{N-[1-(dimethylamino)methylidine]amino}pyridine (**9**).* To a solution of compound **8** (210 mg, 0.36 mmol) and *N*,*N*-diisopropylethylamine (0.25 mL, 1.5 mmol) in anhydrous dichloromethane (10 mL) was added dropwise 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.12 mL, 0.54 mmol) at room temperature. The reaction mixture was stirred for 2 h, methanol (1 mL) was added, and stirring was continued for 20 min. Volatiles were removed by rotary evaporation *in vacuo*, and the residue was isolated by flash chromatography (dichloromethane/methanol, 9:1; R_f = 0.48) to give compound **9** slightly contaminated by compounds from the quenching reaction. Compound **9** was precipitated by hexanes to yield the product (196 mg, 70%) as a colorless foam consisting of a 1:1 mixture of diastereoisomers. UV−vis: λ_{max} (CH_3OH) 205, 234, 268 nm. IR(KBr): 3056 (w), 3031 (w), 2968 (s), 2930 (s), 2873 (m), 2836 (m), 2256 (m), 1627 (s), 1596 (s), 1508 (s), 1250 (s), 1180 (s), 972 (m), 834 (m), 733 (m), 798 (m) cm^{-1}. ^{31}P NMR ($CDCl_3$): δ 144.82, 144.65 ppm. mp 80 °C dec. HRMS: calculated for $C_{44}H_{57}N_5O_6P$ (M + 1), 782.4046; found, 782.4050.

Nucleoside Standards and Phosphoramidites. Syntheses of nucleoside standards were carried out as previously described for 5-(β-D-2′-deoxyribosyl)-2-aminopyridine (d2APy) (*30*), 3-deaza-2′-deoxycytidine (dc^3C) (*31*), 5-methyl-6-carboxy-2′-deoxycytidine ($dm^{5ox}C$) (*32*), 5-(β-D-2′-deoxyribosyl)-3-methyl-2-pyridinone (dm^32P) (*33*), 2-deoxy-2′-deoxythymidine ($d^{2H}T$) (*34*), and 7-deaza-2′-deoxyguanosine (dc^7G) (*33*).

DNA Synthesis. Oligonucleotides were synthesized from 1 μmol of bound nucleoside on 500 Å silica supports using conventional automated phosphoramidite chemistry (*34*) and according to methods described previously (*20*). Oligodeoxynucleotides were created of the sequence 3′-(GCTATGGTGAC^{x}TGGTAGTCG)-5′ (where C^{x} is alternatively dC, d2APy, dm^{5ox}C, or dc^{3}C), 3′-(GCTATGGTGACT^{x}GGTAGTCG)-5′ (where T^{x} is alternatively d^{2H}T or dm^{3}2P), or 3′-(GCTATGGTGACTG^{x}GTAGTCG)-5′ (where G^{x} is alternatively dI or dc^{7}G). Controlled pore glass and protecting groups were removed from the dry oligomers containing the nucleoside bases d2APy, d^{2H}T, dm^{3}2P, dI, and dc^{7}G using concentrated ammonium hydroxide at 50 °C for 12 h. Deprotection of the nucleosides dm^{5ox}C and dc^{3}C required a 16 h incubation in concentrated aqueous ammonia at 60 °C. Purification of these oligonucleotides was accomplished by HPLC (trityl on).

Approximately 0.4 A_{260} unit (12 μg) of each purified oligomer was incubated overnight at 37 °C with snake venom phosphodiesterase (2.0 units) and calf intestinal alkaline phosphatase (2.0 units) in a total volume of 20 μL containing 100 mM Tris-HCl (pH 8.0) and 20 mM $MgCl_2$. Strand purity and snake venom digestion were analyzed by HPLC (trityl off). Peaks were identified by co-injection with authentic standards. Oligomers containing dm^{5ox}C could not be fully digested with snake venom phosphodiesterase but were effectively digested with S1 nuclease.

Enzymes. Bacteriophage T7 primase G4A$_{G64}$ contains a Gly substituted for Met at amino acid position 64 of the gene 4 protein *(35, 36)*. This mutation allows expression of the 63-kDa gene 4 protein but eliminates expression of the collinear 56-kDa gene 4 protein. The mutation has no detectable effect on the dTTPase, helicase, or primase activities of the 63-kDa gene 4 protein and functions *in vitro* to support T7 phage growth (*37*). For convenience, we refer to the 63-kDa G4A$_{G63}$ protein as primase. Concentration calculations of the gene 4 protein were based on the monomeric protein of molecular mass 63 kDa although the protein functions *in vivo* as a hexamer (*37*−*39*).

RNA Synthesis Assay. The assay for measuring oligoribonucleotide synthesis was carried out as described previously (*20*). The reaction mixture (10 μL) contained 50 mM potassium glutamate, pH 7.5, 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg/mL bovine serum albumin, 2 mM dTTP, 100 μM CTP ([α-^{32}P]CTP = 33 nM; 5.0 μCi), 300 μM ATP, 10 μM oligomer template, and 10 nM monomer bacteriophage T7 DNA primase. The reaction mixture was incubated for 1 h at 37 °C and then terminated by the addition of *N*-ethylmaleimide (10 mM final concentration) and alkaline phosphatase (10 units) to inhibit T7 primase and to dephosphorylate the ribonucleotide products, respectively. The mixture was incubated for 1 h at 50 °C, and dephosphorylated products were separated on a 20% polyacrylamide gel containing 8 M urea. The gels were dried under high vacuum, and the oligoribonucleotide products were visualized using a phosphorimager.

Inhibition of Synthesis Assay. The assay for exploring the nature of oligoribonucleotide synthesis was carried out similarly to the method described previously (*20*). The reaction mixture (50 μL) contained 50 mM potassium glutamate, pH 7.5, 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg/mL bovine serum albumin, 70 μM CTP ([α-^{32}P]CTP = 50 nM; 7.6 μCi), 300 μM ATP, 32 μM oligomer template, 6.0, 3.0, 1.5, and 0.75 μM strand containing dm^{5ox}C at the site of cryptic dC, and 200 nM monomer bacteriophage T7 DNA primase. An aliquot of 10 μL of the reaction mixture incubating at 37 °C was removed at 4, 8 12, 16, and 20 min to a "stop" solution consisting of *N*-ethylmaleimide (10 mM final concentration) and alkaline phosphatase (10 units). The stop mixture was incubated for 1 h at 50 °C, and dephosphorylated products were separated on a 20% polyacrylamide gel containing 8 M urea. The gels were dried under high vacuum, and the oligoribonucleotide products were visualized using a phosphorimager.

RESULTS

Previous studies have described the template requirements for primer synthesis by the 63-kDa gene 4 protein (*20*). T7 primase will catalyze the synthesis of di-, tri-, and tetraribonucleotides from a template as short as 20 nucleotides in length, provided that the sequence includes the appropriate pentanucleotide recognition sequence position such that there are 10 residues to the 3′-side and 5 residues to the 5′-side. Nine 20-mer oligodeoxynucleotides of the general sequence 3′-d(GCTATGGTGACTGGTAGTCG)-5′ were synthesized, eight of which contained a single modified nucleotide substitution at the dC, dT, or dG sites underlined.

Design, Synthesis, and Characterization of the Modified Oligonucleotides. One critical component of the recognition process between a protein and a substrate involves hydrogen-bonding interactions. The nucleoside analogues incorporated into the 3′-CTG-5′ recognition site were all designed to incrementally alter the nature of the potential hydrogen-bonding interactions available to the T7 primase. We were particularly interested in the functional groups present on the cryptic dC residue, so critical for the initiation reaction but not itself transcribed. We designed and prepared three analogue dC residues, each one featuring a single removed functional group that did not otherwise alter the remaining two functional groups. These analogues included the d^{H4}C (*20*), dc^{3}C (*29*), and d2APy (*28*) derivatives illustrated in Figure 1. We additionally used a fourth analogue, dm^{5ox}C (*30*) (Figure 1), in which all three functional groups of dC are present, but the tautomeric form of the N3 nitrogen is altered due to the presence of the O6 carbonyl. In addition to the cryptic dC residue, we incorporated three analogues into the adjacent dT residue, including dU, dm^{3}2P (*31*), and d^{H2}T (*32*) (see Figure 1). The first analogue simply eliminates the methyl group at position 5. The second is a 2-pyridone nucleoside (dm^{3}2P), which lacks the O2 carbonyl but otherwise maintains normal hydrogen-bonding characteristics. The third derivative (d^{H2}T) is similar to dm^{3}2P but, as a pyrimidine heterocycle, alters the tautomeric nature of the N3 nitrogen. Only two analogues were positioned in the third sequence position in place of dG: dI, in which the exocyclic amino group is absent, and dc^{7}G, from which the N7 nitrogen was removed (Figure 1).

The synthesis of most of the nucleoside analogues has been reported, the exception being the d2APy analogue. To delete the O2 carbonyl and maintain the N4 amino group and N3 nitrogen in their common tautomeric forms, it was necessary to prepare this analogue as the C-nucleoside. While we could obtain small quantities of the desired material from a synthesis that would protect the exocyclic amino group of

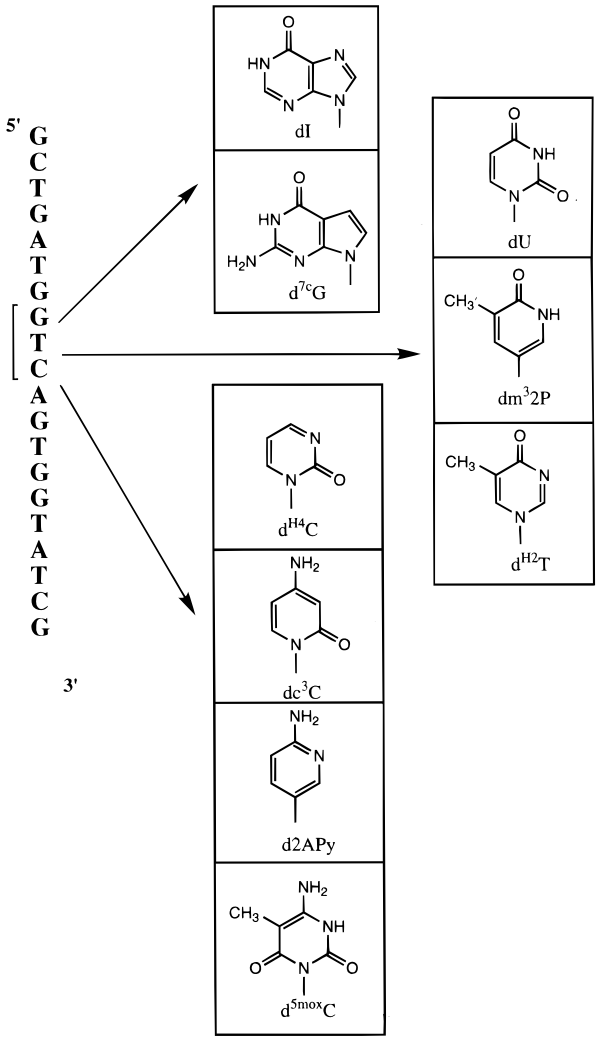
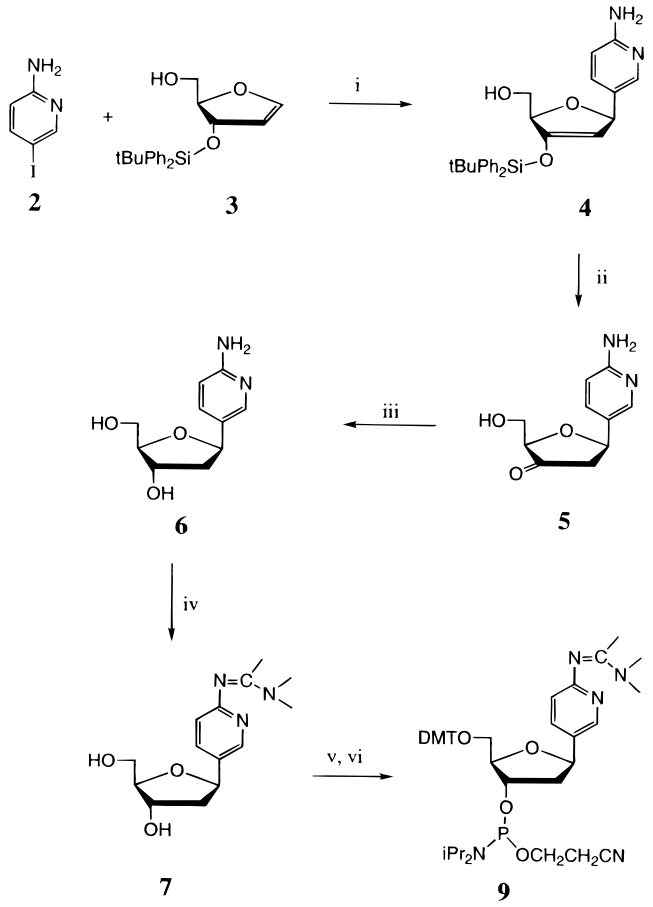


FIGURE 1: Structures of the nucleoside analogues and the position of substitution in the 20-base oligonucleotide template.

2-amino-5-iodopyridine with the common dC protecting group benzamide, deprotection in hot (60 °C) ammonia proved to be difficult (see Supporting Information). We chose therefore a more base-labile amidine protection of the exocyclic amine, described in Scheme 1, which resulted in oligonucleotides that could be fully deprotected within 18 h. Nucleoside analysis was performed with detection at 260 and 300 nm (Figure 2). The related C-nucleoside m^32P was prepared in a similar manner, as described elsewhere (*31*). The remaining syntheses were completed using known procedures.

Primase Activity Using Native and Analogue Templates. Each of the 20-mers possessing a single analogue residue was evaluated for RNA synthesis capabilities relative to the native template to determine the importance of specific functional groups (Table 1). In the presence of excess oligonucleotide template (10 mM), ATP, [α-^{32}P]CTP, and dTTP, the primase enzyme catalyzes the synthesis of pppAC, pppACC, and pppACCA as expected. PAGE analysis of the products was preceded by treatment of the mixture with alkaline phosphatase to remove the terminal triphosphate residues. The observed anomalous mobility effects (Figure 3) for the product oligonucleotides (gel mobilities: ApCpCpA > ApCpC > ApC) have been previously noted (*9*, *20*), and the nature of this anomaly has been subsequently defined

Scheme 1[a]



[a] Reagents: i, $(dba)_2Pd^0/Ph_3P/iPr_2EtN/CH_3CN$; ii, $nBu_4N^+F^-/CH_3COOH/THF$; iii, $NaB(O_2CCH_3)_3H/CH_3COOH/CH_3CN$; iv, $(CH_3)_2NC(OCH_3)_2CH_3/CH_3OH$; v, DMTCl/$C_5H_5N$; vi, $[(CH_3)_3CH]_2NP(Cl)OCH_2CH_2CN/iPr_2EtN/CH_2Cl_2$.

(40). The ratios of di-, tri-, and tetraribonucleotide products are altered by a number of parameters (*9*), so calculations of priming effectiveness were taken to be the total sum of labeled nucleotide incorporated as a percentage of that occurring for the native template as previously described (*20*). The effects resulting from a given analogue can be categorized as having a slight or no effect (activity at least 40% that of native) or having dramatic effects (activity ≤5% that of native).

Functional Group Requirements at the Cryptic 3′-dC. As we have reported previously (*20*), replacement of the cryptic 3′-dC with the analogue that eliminates its exocyclic amino group ($d^{H4}C$; see Figure 1) results in a 50% reduction in template activity, but this sequence is still a functioning substrate. Similarly, primase recognition sites lacking the O2 carbonyl at the 3′-dC site, those containing d2APy in place of dC, also support RNA synthesis, albeit at a level reduced some 60% relative to the native template. On the other hand, essentially no RNA products from primase activity were observed with templates containing dc^3C or $dm^{5ox}C$ (less than 1% and less than 5%, respectively). In the sequence containing dc^3C the N3 nitrogen at the 3′-dC site has been eliminated and replaced with C−H. With the $dm^{5ox}C$ substitution, the tautomeric form of this N3 nitrogen has been converted from an imino to an amido functionality. These results suggest that all three of the functional groups normally involved in Watson−Crick base pairing are moderately or

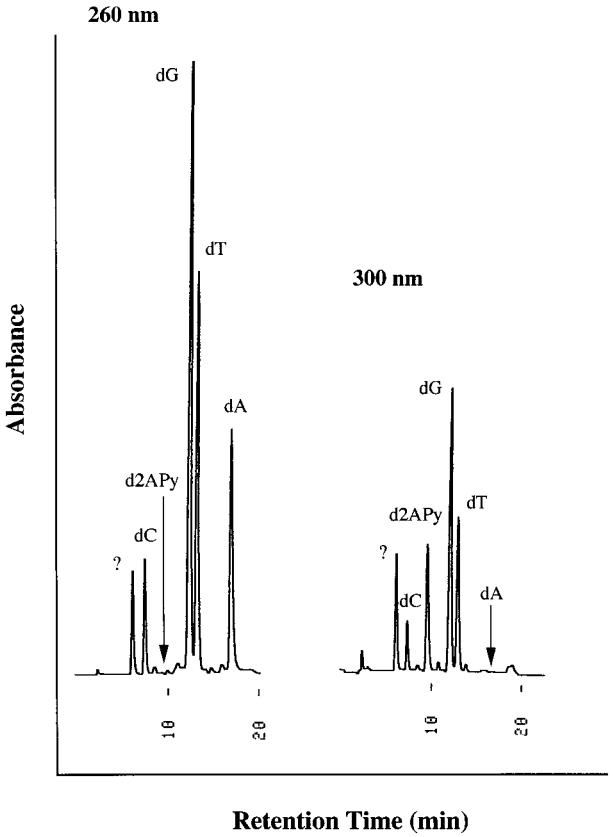


FIGURE 2: HPLC analysis of the snake venom phosphodiesterase/alkaline phosphatase digestion (see Methods) of the 20-mer 3′-[GCTATGGTG(2APy)TGGGTAGTCG)-5′. Left panel: detection at 260 nm. Right panel: detection at 300 nm.

Table 1: RNA Primer Synthesis on Analogue Templates of 5′-GCTGATGGTCAGTGGTATCG-3′

		activity (%)
dC analogues	$d^{H4}C$	50
	$dc^{3}C$	<1
	d2APy	40
	$dm^{5ox}C$	<5
dT analogues	dU	80
	$dm^{3}2P$	80
	$d^{H2}T$	<1
dG analogues	dI	70
	$dc^{7}G$	40

critically necessary at the nontranscribed cryptic 3′-dC residue for effective primase action.

Functional Group Requirements at dT in the Recognition Sequence. Primase recognition sites containing dU in place of dT (loss of the C5 methyl group) function essentially as native templates. Similarly, the introduction of the $dm^{3}2P$ analogue in place of dT (loss of the O2 carbonyl) results in a template that is able to support RNA synthesis by the primase enzyme at a level of 80% of that observed for the native template. On the other hand, in the presence of the related derivative $d^{2H}T$, essentially no RNA products resulting from primase activity were observed (less than 1%). These results suggest that the hydrogen-donating N3 nitrogen of thymidine is an essential contact site—either to the enzyme or to the complementary dA residue. Conversely, the enzyme has little regard for the presence of the O2 carbonyl at this site.

Functional Group Requirements at dG in the Recognition Sequence. The third position of the conserved 3′-CTG-5′

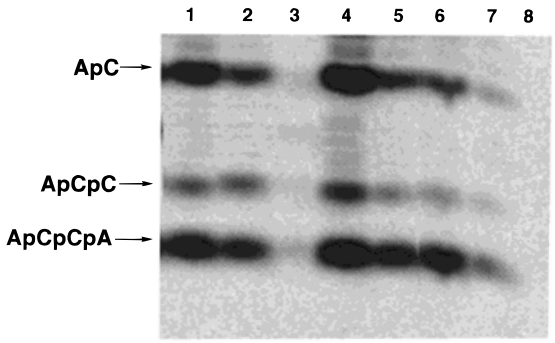


FIGURE 3: Oligoribonucleotide synthesis by T7 primase and analysis by denaturing PAGE. The template 3′-(GCTATGGTGCTGGGTAGTCG)-5′ contains the following modifications at the underlined trinucleotide recognition site: lane 1, T = $dm^{3}2P$; lane 2, G = $dc^{7}G$; lane 3, T = $d^{2H}T$; lane 4, dC; lane 5, G = dI; lane 6, T = dU; lane 7, C = $dm^{5ox}C$; lane 8, C = $dc^{3}C$. The oligonucleotide synthesis assay was carried out as described under Methods. Results of C = d2APy are not shown.

recognition site codes for the incorporation of CTP by the primase enzyme. It has previously been determined that CTP is bound to the primase enzyme prior to recognition of the trimer binding site and that recognition is facilitated by dG−CTP Watson−Crick interaction (*19*). Primase recognition sites containing $dc^{7}G$ or dI in place of dG both retain at least two of the three Watson−Crick functional groups, and both support RNA synthesis. The template containing $dc^{7}G$ exhibits activity that is reduced by some 60%, while that containing dI is only reduced by 30%, both relative to the fully native template. The reduced efficiency of the $dc^{7}G$ analogue is likely unrelated to Watson−Crick base pairing since all three hydrogen-bonding groups are available. Similarly, the dI analogue possesses two of the G-C hydrogen-bonding triad and still efficiently promotes RNA synthesis.

Effects of Inactive Templates. Templates that are unable to promote RNA synthesis could do so by two different pathways: one, the enzyme may be unable to bind the altered substrate, and two, the enzyme is bound but unable to process the analogue template. To discriminate between these two possibilities, we have examined the ability of primase to synthesize RNA dimers, trimers, and tetramers from the native template in the presence of varying concentrations of the inactive templates. Because multiple polymerization steps were involved in the synthesis of the three different targets, it was difficult to obtain precise Michaelis−Menten parameters. Additionally, the assay was complicated by discriminating between the incorporated dCTP from the large quantity of unincorporated triphosphate. Nevertheless, an examination of initial velocity data (e.g., see Figure 4) for the three inactive templates suggests that they all functioned as moderate inhibitors of the native template with concentrations necessary for a 50% reduction in K_i of approximately 0.15 μM.

DISCUSSION

The primase enzyme is nearly homologous in sequence regardless of species, from the simplest bacteriophage through higher eucaryotes. The unusual nature of the zinc motif, the trinucleotide recognition sequence, and the cryptic 3′ residue are hallmarks of this protein throughout the species. These enzymes are unusual in that they contact ssDNA in a

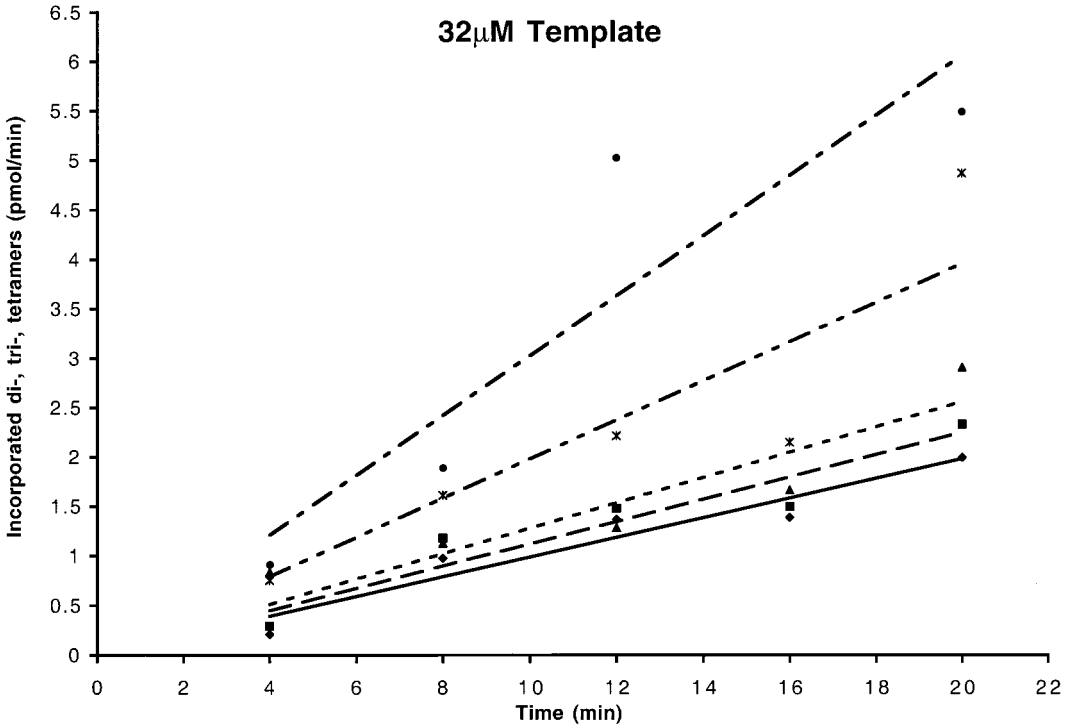


FIGURE 4: Effects of varying concentrations of 3′-d(GCTATGGTGAm^{5ox}CTGGTAGTCG)-5′ on the ability of the primase enzyme to synthesize RNA oligonucleotide from the native template 3′-d(GCTATGGTGACTGGTAGTCG)-5′ at a constant concentration of 32 μM. Inhibitor oligonucleotide concentrations are labeled as follows: 6 μM (—), 3 μM (— —), 1.5 μM (- - -), 0.375 μM (— - - —), and 0 μM (— - —).

site-specific manner, unlike the vast majority of sequence-specific binding proteins that recognize dsDNA. A major difference in the two types of recognition processes is that in the former the functional groups on the Watson−Crick face of the bases are available as a site for contact by the enzyme. In targeting a dsDNA recognition site, these same functional groups are only available as accessed by binding in one of the DNA grooves, and the central functional groups are not available at all.

Previous studies have deduced that the exocyclic amine of the cryptic cytidine is not a critical interaction for primase activity (*20*). It has been further proposed that the quiescence observed when dT or dU was in the 3′-termini of the recognition sequence is due to the conversion from amino to carboxyl at the 4 position and/or that it is due to the tautomeric conversion from proton acceptor to donor at the N3 nitrogen. Modeling studies have identified what appear to be critical amino acid residues in the Cys_4 loop region of the primase enzyme and suggested a mechanism for ssDNA recognition by the protein (*19*).

We have employed a series of single nucleotide analogues that alter or delete specific functional groups in the trinucleotide binding site to further analyze the nature of ssDNA−protein contacts. One analogue, the 2APy derivative, represents a new nucleoside, and its synthesis has been described in detail. Although this derivative contains a carbon−carbon linkage between the heterocycle and the sugar, this linkage permits the 2-pyridine heterocycle to exist in the amino rather than imino tautomer and function as an effective mimic of dC, but one that lacks the O2 carbonyl. The synthesis of templates containing the d2APy nucleoside analogue depended on selection of a base-labile protecting group for the exocyclic amine of the pyrimidine base. Initial efforts to synthesize the phosphoramidite using palladium chemistry and a benzoylamino protection scheme (see Scheme 1 in Supporting Information) proved unsatisfactory when ammonia deprotection efforts at 60 °C required more than 3 weeks to realize only 50% of the desired template. Use of the amidine protecting group (see Scheme 1) resulted in a simple high-yield synthesis of the nucleoside phosphoramidite that was readily deprotected when present in an oligonucleotide.

The analogue work reported here suggests that the N3 of the cryptic cytosine is of critical importance to the function of the primase enzyme at 3′-CTG-5′ sites. With either the deletion of this functional group (replacement by C−H, dc^{3}C) or its conversion to the N−H tautomer (dm^{5ox}C), dramatic losses in polymerization ability are observed. These results suggest a critical interaction to the N3 nitrogen functioning as a hydrogen bond acceptor. This type of interaction is one that could easily be employed for single-stranded but not double-stranded DNA since in the latter case the functional group would be buried in the center of the dG-dC base pair and essentially unavailable. There appear to be minor effects present with the loss of other functional groups, most notably the O2 carbonyl at the cryptic dC. The effects on polymerase activity resulting from the loss of this carbonyl bear some similarity to the proposed noncritical interaction between the N4 amino group of the cryptic cytosine (*20*) and Asp_{31} (*19*). However, the slight 2-fold loss in activity could also be accounted for by subtle changes in the nature of the substrate as the result of the altered functional group character. Other factors that cannot be adequately measured in the present study include effects of pK_a changes, dipole moment, and sugar and/or base conformations.

Our work also illustrates the importance of the N3 H of the thymidine in the center of the 3′-CTG-5′ sequence.

Whether this requirement implicates a protein contact or simply the need to form a Watson−Crick base pair with the incoming ATP monomer cannot be determined from this study. Although the need to form such Watson−Crick interactions has been the assumed mechanism for template-directed DNA and RNA syntheses, recent work (*29*, *41*, *42*) with "shape" analogues suggests that interstrand hydrogen bonding may be less critical than otherwise expected. The loss of one Watson−Crick functional group from the dG in the third sequence position has little effect upon polymerization, but in this case two of the three Watson−Crick interactions are maintained. However, the slight loss in activity with the incorporation of dc^7G for dG suggests a small loss in the nature of the protein−nucleic acid interaction. The data may implicate a weak contact at this site, but other effects as noted above might also be responsible for such slight changes in polymerase activity. Certainly the use of hydrogen-bonding contacts to the N7 nitrogen of dG is a common motif observed in the structures of a number of sequence-specific protein−dsDNA complexes (*43*, *44*).

Finally, the qualitative inhibition studies suggest that the inactive substrates are bound by the enzyme, not surprising given the number of nonspecific contacts likely present between the 20-mers and the protein. However, the mild inhibitory effects observed for these sequences with the native template suggest that the protein binds the analogue sequences but, presumably as a result of missing protein−DNA contacts, is unable to effectively initiate RNA polymerization.

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SUPPORTING INFORMATION AVAILABLE

Syntheses and experimental data, including a scheme, for compounds **1** and **6**−**9** using alternate protecting groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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