Effects of Base Analog Substitutions in the Noncoding dC of the 3′-d(CTG)-5′ Template Recognition Site of the Bacteriophage T7 Primase†

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ABSTRACT: The 63-kDa gene 4 protein (DNA primase) of bacteriophage T7 catalyzes the synthesis of the oligoribonucleotides pppACC(C/A) and pppACAC at single-stranded DNA recognition sites 3′-d[CTGG-(G/T)]-5′ and 3′-d(CTGTG)-5′, respectively. At these sites, the 3′-terminal deoxycytidine residue is conserved but noncoding; the 3′-dC residue is required to initiate catalytic synthesis of oligoribonucleotides, yet it is not used as a template residue for the synthesis of a complementary G residue in the RNA primer. We have examined the interactions between T7 primase and the functional groups of the 3′-dC residue by measuring the ability of the primase to catalyze the synthesis of oligoribonucleotides on synthetic single-stranded 20-mer templates [e.g., 3′-d(GCTATGGTGACTGGTAGTCG)-5′] that contain analogs of dC in the conserved pentanucleotide recognition site. Recognition sites containing 5-methyldeoxycytidine (m^5dC) or 1-(β-D-2′-deoxyribosyl)-2-pyrimidinone ($d^{H4}C$) substitutions for dC support oligoribonucleotide synthesis whereas those containing deoxythymidine (dT) and deoxyuridine (dU) substitutions do not. Oligoribonucleotide synthesis on the native template (containing dC) is inhibited competitively by the template containing a dT residue in the primase recognition site, 3′-[(N_{10})TTGGT(N_5)]-5′, with an apparent K_i of 1.30 ± 0.04 μM. Templates containing dU residues, 3′-[(N_{10})UTGGT(N_5)]-5′ and 3′-[(N_9)UTTGGT-(N_5)]-5′, affect both the apparent K_m and V_{max} parameters for oligoribonucleotide synthesis on the 3′-[(N_{10})CTGGT(N_5)]-5′ template.

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 (Ogawa & Okazaki, 1980), but they also often provide primers for initiation at origins of replication. DNA primases are physically and functionally associated with DNA helicases, in part, to make use of the translocation activity of the helicase in order to access recognition sites for primer synthesis on the single-stranded DNA (ssDNA)[1] template (see Kornberg & Baker, 1992).

The bacteriophage T7 primase and helicase are encoded as two colinear proteins, a full-length 63-kDa protein and a protein of 56 kDa that is translated from an internal start codon located in-frame, and 189 bases from the start codon of the 63-kDa gene 4 protein (Dunn & Studier, 1983). Both proteins bind to ssDNA in the presence of nucleoside triphosphates, preferentially dTTP, and translocate in the 5′ to 3′ direction along the DNA template using energy provided from NTP hydrolysis (Kolodner & Richardson, 1977; Tabor & Richardson, 1981; Matson & Richardson, 1983). Upon encountering duplex DNA, both proteins will function as helicases to unwind the DNA (Matson *et al.*, 1983). These activities are common to both gene products, and the site for NTP hydrolysis has been identified within the carboxyl-terminal half of the proteins (Notarnicola & Richardson, 1993; Patel *et al.*, 1994).

Only the 63-kDa gene 4 protein catalyzes template-directed synthesis of oligoribonucleotides (Bernstein & Richardson, 1988, 1989; Mendelman & Richardson, 1991). The 63-kDa protein binds 1 mol of zinc per mole of protein (Mendelman *et al.*, 1994). The unique 7-kDa amino-terminal domain of the 63-kDa gene 4 protein contains a single Cys_4 zinc-binding motif [Cys-Xaa_2-Cys-Xaa_{15}-Cys-Xaa_2-Cys] that enables the 63-kDa gene 4 protein to function as a primase (Bernstein & Richardson, 1988; Mendelman & Richardson, 1994). Similar metal-binding motifs may be conserved in all DNA primases, including those from bacteriophage, viral, prokaryotic, and eukaryotic sources (Mendelman *et al.*, 1994). The T7 primase synthesizes tetraribonucleotide primers pppACC-(C/A) and pppACAC at the primase recognition sequences 3′-d[CTGG(G/T)]-5′ and 3′-d(CTGTG)-5′, respectively (Scherzinger *et al.*, 1979; Romano & Richardson, 1979; Tabor & Richardson, 1981; Nakai & Richardson, 1986). T7 DNA polymerase then uses these primers to initiate DNA synthesis on ssDNA.

Although a pentanucleotide sequence is required for the synthesis of full-length tetraribonucleotide primers, a minimal primase recognition site, 3′-d(CTG)-5′, is necessary and

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[1] Abbreviations: 2′-deoxy-5-methylcytidine (m^5dC); 1-(β-D-2′-deoxyribosyl)-2-pyrimidinone ($d^{H4}C$); 2′-deoxythymidine (dT); 2′-deoxyuridine (dU); 2′-deoxycytidine (dC); single-stranded DNA (ssDNA); high-performance liquid chromatography (HPLC).

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sufficient to support the synthesis of pppApC ribonucleotide dimers by the T7 primase (Mendelman & Richardson, 1991). The primases of bacteriophage T4 and *Escherichia coli* also initiate catalysis of oligoribonucleotides from conserved trinucleotide sequences. The T4 gp61 primase recognizes the sequence 3′-d(TTG)-5′ on ssDNA containing glucosylated (hydroxymethyl)cytosine residues, or the sequence 3′-d[T(T/C)G]-5′ on ssDNA lacking such cytosine modification (Cha & Alberts, 1986; Hinton & Nossal, 1987). Similarly, the *E. coli* DnaG protein recognizes the trinucleotide sequence 3′-d(GTC)-5′ and initiates the synthesis of primers beginning with pppApG (Hiasa *et al.*, 1989; Yoda & Okazaki, 1991; Swart & Griep, 1993). As in the case of the T7 primase, the initial dinucleotides synthesized by the T4 and *E. coli* enzymes are then extended by the primases to yield predominately pentanucleotides in the case of T4 (Nossal, 1980; Liu & Alberts, 1981) and a more heterogeneous population of oligoribonucleotides in the case of *E. coli* (Zechner *et al.*, 1992). In all three cases, the primases initiate synthesis at trinucleotide sequences in which the 3′-nucleotide of that sequence is essential for recognition but does not code for a residue in the product oligoribonucleotide.

The mechanism by which the common zinc motif of the prokaryotic DNA primases recognizes a trinucleotide sequence on ssDNA is presently unknown. One approach to investigate these interactions is the substitution of nucleotide analogs for those common residues normally found in the recognition sequence. This approach has proven valuable with a number of other proteins that bind to duplex DNA in a sequence-specific manner. Such examples include the *trp* repressor (Mazzarelli *et al.*, 1992; Smith *et al.*, 1994), restriction endonucleases such as *Rsr*I and *Eco*RI (Aiken & Gumport, 1991; Aiken *et al.*, 1991), and the T3 RNA polymerase (Schick & Martin, 1993). To determine which functional groups of the 3′-dC residue within a T7 primase recognition site are important for the initiation of RNA synthesis by the bacteriophage T7 primase, we have synthesized a series of 20-mer oligodeoxynucleotide templates containing single nucleotide analog substitutions and measured the ability of these incrementally modified templates to direct the synthesis of the corresponding oligoribonucleotide products.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Phosphoramidites. Common nucleoside phosphoramidites and synthesis supports were purchased from CPG, Inc. (Lincoln Park, NJ). Base labile fast deprotection phosphoramidites and supports were from BioGenex (San Ramon, CA). The phosphoramidite derivatives of 2′-deoxyuridine (dU) and 2′-deoxy-5-methylcytidine (m^5dC) were from Cruachem (Glasgow, Scotland). The phosphoramidite of 1-(β-D-2′-deoxyribosyl)-2-pyrimidinone ($d^{H4}C$) was prepared as previously described (Gildea & McLaughlin, 1989). Nonradioactive and radioactive nucleotides were purchased from Pharmacia LKB Biotechnology, Inc., and Du Pont-New England Nuclear Research Products, respectively.

DNA Templates. Oligodeoxynucleotides of the sequence 3′-d(GCTATGGTGAXTGGTAGTCG)-5′ (where the primase recognition site is underlined and dX refers to dC or dT) were synthesized by Oligos, ETC (Guilford, CT). Oligodeoxynucleotides containing m^5dC, or $d^{H4}C$, or dU for residue dX were synthesized as described under Methods. All oligodeoxynucleotides were eluted from a 20% polyacrylamide gel in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA, a portion of each purified oligodeoxynucleotide was labeled with [γ-^{32}P]ATP and polynucleotide kinase, and PAGE analysis was performed to verify homogeneity.

Enzymes. Bacteriophage T7 primase $G4A_{G64}$, described previously (Mendelman *et al.*, 1994), contains a Gly substitution for Met at amino acid position 64 of the T7 63-kDa gene 4 protein (Mendelman *et al.*, 1993). This mutation allows expression of the 63-kDa gene 4 protein and eliminates expression of the 56-kDa protein. The mutation has no detectable effect on the dTTPase, helicase, or primase activites of the 63-kDa gene 4 protein and functions *in vivo* to support T7 phage growth (Mendelman *et al.*, 1992, 1993). For convenience, we refer to the 63-kDa $G4A_{G64}$ protein as primase. Concentrations of gene 4 protein are based on a monomeric species of molecular weight 63 000 although the protein probably functions as a hexamer (Notarnicola & Richardson, 1993; Patel & Hingorani, 1993; Engelman *et al.*, 1995). Snake venom phosphodiesterase (*Crotalus durissus*) and calf-intestinal alkaline phosphatase were from Boehringer-Mannheim.

Methods

High-Performance Liquid Chromatography (HPLC). HPLC was performed on a Beckman HPLC system using C-18 reversed-phase columns (ODS-Hypersil, 5 μm particle size, 120 Å pore, 4.6 or 9.4 × 250 mm). Solvent systems were as follows: buffer A = 50 mM triethylammonium acetate (pH 7.0), with an acetonitrile gradient; buffer B = 20 mM KH_2PO_4 (pH 5.5), with a methanol gradient. Flow rates were 1.5 and 3.0 mL/min for the 4.6 and 9.4 mm diameter columns, respectively. Detection was normally at 260 nm. All analyses were conducted at ambient temperature. Integration was performed with a Shimadzu C-R3A Chromatopac integrator.

Oligodeoxynucleotide Synthesis and Isolation. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. The oligonucleotides were synthesized at 1 μmol scale using β-cyanoethyl phosphoramidite methodology and long-chain alkylamine controlled-pore glass solid supports (Matteucci & Caruthers, 1981). The oligomer containing $d^{H4}C$ was prepared using "fast-deprotecting" phosphoramidites: phenoxyacetyl N-protection for dA and dG and isobutyryl N-protection for dC. The coupling time of the DNA synthesizer was increased from 30 s to 3 min for all modified phosphoramidites. The usual 3% trichloroacetic acid deblocking solution was replaced with 2% dichloroacetic acid for the entire assembly of the oligomer containing $d^{H4}C$. Each oligomer was synthesized with the 5′-terminal dimethoxytrityl group attached ("trityl ON") to facilitate purification.

After completion of the DNA synthesis, the solid supports were dried under vacuum and added to concentrated ammonium hydroxide (3.0 mL). After 4 h at 27 °C, the oligomer containing $d^{H4}C$ was decanted and concentrated to a small volume under reduced pressure in a rotary evaporator. The other oligomers were each heated at 50 °C for 16 h and then similarly concentrated. All oligomers were purified by

reversed-phase HPLC using a linear gradient of 14−45% acetonitrile in buffer A over 40 min. Appropriate fractions were collected and concentrated to low volume, and acetic acid (80%, 5 mL) was added to remove the 5′-terminal dimethoxytrityl group. After 20 min at 0 °C, EtOH/H_2O (1:1, 5 mL) was added, and the solutions were again reduced to a low volume. This process was repeated two additional times to remove most of the acetic acid. Each oligomer was then applied to a Sephadex G-10 column (2.7 × 27 cm) and eluted with H_2O (McLaughlin & Piel, 1984). The fractions containing the desalted oligomers were lyophilized and analyzed by reversed-phase HPLC using a linear gradient of 0−70% methanol in buffer B over 60 min. The oligomers containing dU and m^5dC eluted as a single peak. The oligomer containing $d^{H4}C$ required an additional purification, which was performed with a linear gradient of 0−35% acetonitrile in buffer A over 60 min. The appropriate fraction was collected and concentrated to dryness. Water (5 mL) was added, and the solution was lyophilized. The sample was lyophilized from H_2O (5 mL) two additional times to completely remove the volatile buffers.

Nucleoside Analyses. Approximately 0.4 A_{260} units (12 μg) of each purified oligomer was incubated overnight at 37 °C with snake venom phosphodiesterase (2.0 units) and alkaline phosphatase (2.0 units) in a total volume of 20 μL containing 100 mM Tris-HCl (pH 8.0) and 20 mM $MgCl_2$. A portion of this digestion mixture was analyzed directly by reversed-phase HPLC using a linear gradient of 0−70% methanol in buffer B over 60 min. Detection was at 260 nm, but the digestion mixture containing the 2-pyrimidinone ($d^{H4}C$) required an additional analysis at 310 nm to observe the modified base. Peaks were identified by coinjection with authentic standards. The following correction factors were applied to the integrated areas of the deoxynucleoside peaks to normalize the extinction coefficients at pH 5.5: dC = 7.8; $d^{H4}C$ = 5.8 (310 nm); dG = 12.5; dT = 8.4; dA = 13.1.

Oligoribonucleotide Synthesis Assay. The assay for measuring oligoribonucleotide synthesis catalyzed by T7 primase was performed as described previously (Mendelman & Richardson, 1991) with the following modifications. The reaction mixture (10 μL) contained 50 mM potassium glutamate, pH 7.2, 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 [α-^{32}P]CTP (5 μCi), 300 μM ATP, 10 μM oligomer template, and T7 DNA primase (50 or 100 nM monomer, as indicated). 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 substrates and products, respectively (Mendelman & Richardson, 1991). The mixture was incubated for 1 h at 50 °C, and dephosphorylated products were separated on a 20% polyacrylamide gel containing 8 M urea. Oligoribonucleotide products were visualized by autoradiography of the dried gel. Competition experiments were performed by adding various concentrations of competitor DNA [0, 0.37, 0.75, 1.5, and 3 μM of 3′-d(GCTATGGTGXXTGGTAGTCG)-5′, where dXX is either dAT or dAU or dUT] to reactions containing buffer, 100 μM [α-^{32}P]CTP, 300 μM ATP, 2 mM dTTP, 50 nM primase, and 0.37, 0.75, 1.5, or 3 μM of the 3′-d(GCTATGGTGACTGGTAGTCG)-5′ oligomer template. Incorporation of [^{32}P]CMP into ApC dimers, ApCpC trimers, and ApCpCpA tetramers was measured directly from the dried gel with a PhosphorImager (Molecular Dynamics). Total incorporation of CMP into oligoribonucleotide product was calculated using a conversion factor that reflects one CMP per dimer and two CMP per trimer and tetramer.

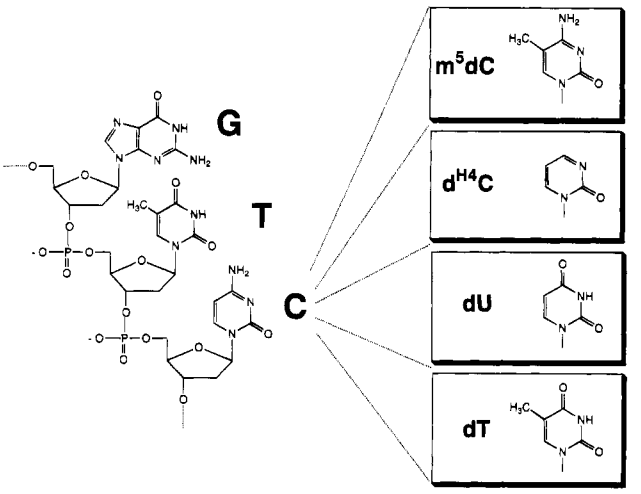

FIGURE 1: Summary of the structures of base analog substitutions in the 20-base oligomer templates.

RESULTS

Design, Synthesis, and Characterization of the Modified Oligodeoxynucleotides. The primase templates used in these studies require the use of synthetic oligodeoxynucleotides into which nucleotide analogs had been incorporated by chemical synthesis procedures. In previous studies (Mendelman & Richardson, 1991), we have described the template requirements for primer synthesis by the 63-kDa gene 4 protein. When a complete pentanucleotide recognition sequence is present on an oligodeoxynucleotide as short as 20 residues, T7 primase will catalyze the synthesis of tetraribonucleotides, provided that the oligodeoxynucleotide contains 10 residues to the 3′-side, and 5 residues to the 5′-side, of a primase recognition site. Consequently, all of the oligodeoxynucleotide templates used in this study contain the pentanucleotide primase recognition sequence 3′-d(CTGGT)-5′ flanked by 10 and 5 deoxynucleotide residues on the 3′- and 5′-sides, respectively. At this site, primase synthesizes the tetraribonucleotide pppACCA (Mendelman & Richardson, 1991).

Five 20-mer oligodeoxynucleotides of the general sequence 3′-d(GCTATGGTGACTGGTAGTCG)-5′ were synthesized, four of which contained single nucleotide substitutions by either 2′-deoxy-5-methylcytidine (m^5dC), 1-(β-D-2′-deoxyribosyl)-2-pyrimidinone ($d^{H4}C$), 2′-deoxyuridine (dU), or 2′-deoxythymidine (dT) for the 3′-dC residue of the primase recognition site 3′-d(CTGGT)-5′. The structures of the analogs are illustrated in Figure 1. The oligomers were assembled using conventional, automated solid phased based DNA synthesis methodology (Matteucci & Caruthers, 1981). The only difficulty in the synthetic procedures was the observation that the 2-pyrimidinone base was sensitive to extended periods of time in the concentrated aqueous ammonia deprotection solution, so "fast-deprotecting" phosphoramidite synthons were used to prepare this oligomer, greatly improving the final yield. Acid and base catalyzed

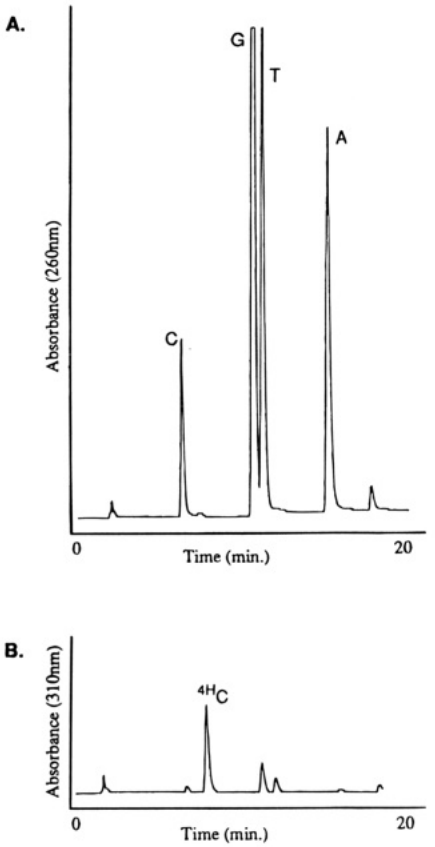

FIGURE 2: Nucleoside compositional analysis of oligomer containing $d^{H4}C$. HPLC chromatograms of the products from the enzyme digestion of the oligomer containing $d^{H4}C$: (A) detection at 260 nm showing dC, dG, dT, and dA; (B) detection at 310 nm showing $d^{H4}C$.

reactions of related pyrimidinones have been observed previously (Wightman & Holy, 1973; Altermatt & Tamm, 1985; Gildea & McLaughlin, 1989; Iocono *et al.*, 1990; Kuimelis & Nambiar, 1994). After deprotection, the 5′-terminal dimethoxytrityl-containing oligomers were purified by reversed-phase HPLC, detritylated, and desalted by gel filtration. The oligomer containing $d^{H4}C$ required a second HPLC purification after the final detritylation step. A nucleoside compositional analysis of each oligomer (Eadie *et al.*, 1987) produced the expected results and verified that the modified nucleosides were present in the oligomer in the proper ratios. Due to the different optical properties of the 2-pyrimidinone base ($\lambda_{max} = 303$ nm), the HPLC analysis of the digestion mixture was performed at both 260 and 310 nm (Gildea & McLaughlin, 1989). The HPLC chromatograms of the digested oligomer containing the $d^{H4}C$ residue are illustrated in Figure 2.

Primase Activity Using Native and Analog Templates. Each of the oligomers described above were tested in the oligoribonucleotide synthesis assay to determine the effects on primase activity resulting from the presence of the cytidine analogs in the primase recognition sequence (Figure 3). In this assay, primase (100 nM) was incubated with an excess of oligonucleotide template (10 μM) and ATP, and [α-^{32}P]-labeled CTP. Additionally, the optimal nucleotide for stimulation of binding to the ssDNA templates, and for translocation along the template, is dTTP, and this nucleotide was also a component of the reaction mixtures although it was not incorporated into any of the product RNAs. Following incubation, the oligoribonucleotide products were

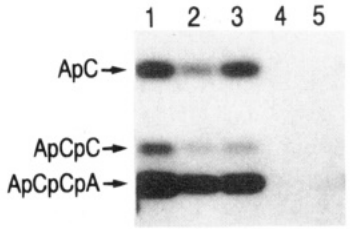

FIGURE 3: Oligoribonucleotide synthesis by T7 primase on templates with various primase recognition sites. The template 3′-d(GCTATGGTGAXTGGTAGTCG)-5′ contains the following nucleotides at position X: in lanes 1, dC; 2, $d^{H4}C$; 3, m^5dC; 4, dU; 5, dT. The oligonucleotide synthesis assay was carried out as described under Experimental Procedures. Each reaction mixture contained 10 μM template as determined by spectroscopy of the stock solution prior to addition or by labeling the 5′-termini of the oligonucleotides with ^{32}P and measuring their recovery in the gel. The relative migration of ApC, ApCpC, and ApCpCpA di-, tri-, and tetraribonucleotide products is indicated to the left. The identity of the different species is known from previous studies (Mendelman & Richardson, 1991; Hine & Richardson, 1994). The autoradiograph was exposed for 1 h.

dephosphorylated, separated in a 20% polyacrylamide gel, and visualized by autoradiography of the dried gel. As shown in Figure 3, T7 primase catalyzes the synthesis of pppAC, pppACC, and pppACCA with control templates containing the 3′-dC residue, as expected (Mendelman & Richardson, 1991; Hine & Richardson, 1994). Although dimers, trimers, and tetramers are synthesized in this reaction, only tetraribonucleotides can be used as primers by the T7 DNA polymerase (Romano & Richardson, 1979). Since several parameters can influence the ratio of the three species (Mendelman & Richardson, 1991), we have used the sum of di-, tri-, and tetraribonucleotides to measure the amount of synthesis.

Primase recognition sites containing m^5dC or $d^{H4}C$ in place of dC support oligoribonucleotide synthesis by the primase, albeit to a lesser extent, whereas essentially no products of primase activity were observed with templates containing dT or dU (Figure 3). Measurement of the amount of total [^{32}P]CMP incorporation into the RNA products indicated that templates containing m^5dC, $d^{H4}C$, dT, or dU in place of the noncoded dC residue supported oligoribonucleotide synthesis at relative levels of 80%, 50%, <0.1%, and <0.1%, respectively. In addition to the data obtained in the 1 h synthesis assay (Figure 3), the products synthesized off each of the templates at various times during the 1 h incubation were analyzed in an identical manner. At shorter times of incubation there is proportionally less synthesis of oligoribonucleotides, but the relative amounts remain constant (data not shown).

Inhibition of Primer Synthesis by Inactive Templates. Two modified recognition sites, 3′-d(TTGGT)-5′ and 3′-d(UTGGT)-5′, did not support oligoribonucleotide synthesis catalyzed by the primase. To determine whether the gene 4 protein bound these modified sites or, after binding, was simply unable to intiate RNA synthesis, we examined the inhibitory effects of these two templates on oligoribonucleotide synthesis reactions which employed the native 3′-d(CTGGT)-5′ recognition site. Oligoribonucleotide synthesis on the native template increases linearly from DNA template concentrations of 0.5 to 10 μM in reactions containing 50 nM primase. Conditions for which the nucleotide substrates became saturating for the enzyme were determined in reactions containing 50 nM primase and 1 μM oligodeoxy-

Table 1: Apparent K_m and Relative V_{max} for Oligoribonucleotide Synthesis on Template Containing a 3′-d(CTGGT)-5′ Recognition Site in the Presence of Inactive Primase Recognition Sites[a]

	competitor template			
	3′-d(TTGGT)-5′		3′-d(UTGGT)-5′	
competitor (μM)	K_m, app (μM)	V_{max} (pmol/min)	K_m, app (μM)	V_{max} (pmol/min)
none	1.3	41	1.3	41
0.375	1.4	42	1.1	36
0.75	2.5	45	1.4	30
1.5	3.2	45	1.4	24
3.0	4.4	45	2.1	20

[a]Each of the two oligonucleotides containing an inactive modified recognition site, 3′-d(TTGGT)-5′ and 3′-d(UTGGT)-5′, were added at the indicated concentrations to oligoribonucleotide synthesis assays containing the 3′-d(CTGGT)-5′ template, and the total amount of oligoribonucleotides synthesized were determined (see Experimental Procedures). The apparent K_m and V_{max} were determined from double-reciprocal kinetic plots.

nucleotide in the standard oligoribonucleotide synthesis assay. The apparent K_m for ATP was determined to be 180 ± 5 μM, and the apparent K_m for CTP was found to be 26 ± 1 μM under these reaction conditions (data not shown).

Inhibition of oligoribonucleotide synthesis on the template containing the 3′-d(CTGGT)-5′ site by the template containing the 3′-d(TTGGT)-5′ sequence was determined by assaying reaction mixtures containing 100 μM CTP, 300 μM ATP, 2 mM dTTP, and 50 nM primase. Increasing concentrations of the 3′-d(TTGGT)-5′ template progressively inhibited oligoribonucleotide synthesis on the native 3′-d(CTGGT)-5′ template by affecting almost solely the apparent K_m parameter characterizing the reaction (Table 1). The direct (Figure 4A) and double-reciprocal (Figure 4B) kinetic plots of the velocity and substrate data indicate that the 3′-d(TTGGT)-5′ template competitively inhibits oligoribonucleotide synthesis on the 3′-d(CTGGT)-5′ template. An apparent K_i of 1.30 ± 0.04 μM was calculated for the 3′-d(TTGGT)-5′ template from two independent determinations. These results suggest that inhibition occurs through random binding of the primase to the oligonucleotide (see Discussion).

The 3′-d(UTGGT)-5′ template also inhibits oligonucleotide synthesis on the 3′-d(CTGGT)-5′ template (Figure 5), but the character of its inhibitory effects differs from that of the 3′-d(TTGGT)-5′ template. The values calculated for apparent K_m and relative V_{max} are given in Table 1. Unlike the competition experiments using the 3′-d(TTGGT)-5′ template, both the apparent K_m and the relative V_{max} values for total primer synthesis are affected by the presence of the 3′-d(UTGGT)-5′ template. Changes in the apparent K_m and relative V_{max} were also observed in a related competition experiment in which a dU residue immediately precedes the 3′-d(TTGGT)-5′ site [e.g., 3′-d(UTTGGT)-5′] (data not shown). The results from these experiments reflect a more complex mode of binding for templates that contain dU; even under substrate saturating conditions, some nonspecific binding between the primase and the analog sequences appears to be present.

DISCUSSION

DNA primases from at least three sources, bacteriophage T7, bacteriophage T4, and *E. coli*, all initiate oligonucleotide

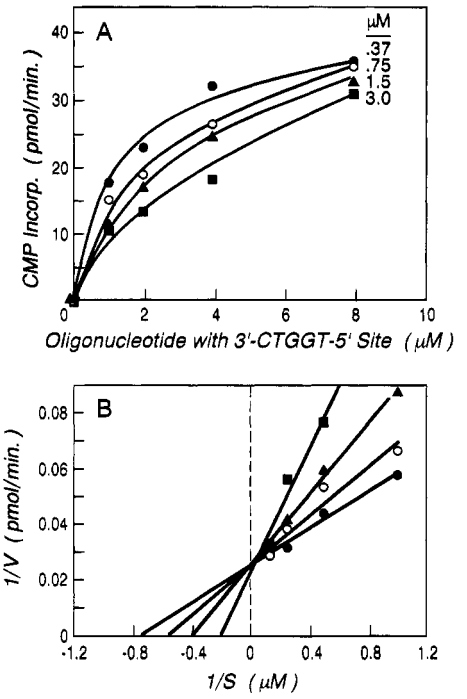

FIGURE 4: Inhibition of primer synthesis on the 3′-d(CTGGT)-5′ template by the competitor 3′-d(TTGGT)-5′ template. Various concentrations of the 3′-d(CTGGT)-5′ template were incubated with (●) 0.37 μM, (○) 0.75 μM, (▲) 1.5 μM, or (■) 3 μM of the competitor 3′-d(TTGGT)-5′ template. Reactions were performed, and total CMP incorporation was calculated as described under Experimental Procedures. (A) direct plot; (B) double-reciprocal plot with linear least-squares fit to the data.

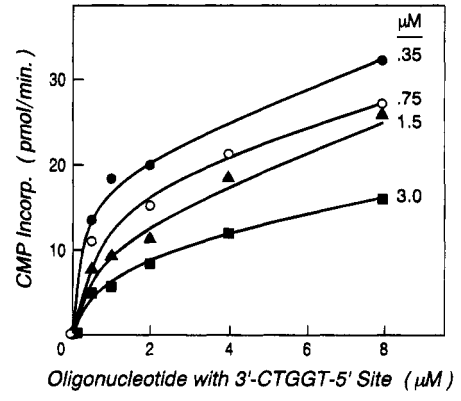

FIGURE 5: Direct plot of inhibition of primer synthesis on the 3′-d(CTGGT)-5′ template by the competitor 3′-d(UTGGT)-5′ template. Increasing concentrations of 3′-d(CTGGT)-5′ template were incubated with (●) 0.35 μM, (○) 0.75 μM, (▲) 1.5 μM, or (■) 3 μM of the competitor 3′-d(UTGGT)-5′ template. Reactions were performed, and total CMP incorporation was calculated as described under Experimental Procedures.

synthesis from trinucleotide recognition sequences, 3′-d(CTG)-5′, 3′-d(TTG)-5′, and 3′-d(GTC)-5′, respectively. In all three cases, the terminal 3′-nucleotide residue is essential for primer synthesis but does not serve as a template residue. The recognition of specific sequences in DNA by proteins containing a zinc motif is known to occur in several other biological processes. The Cys_2His_2 zinc fingers of the Zif268 (Pavletich & Pabo, 1991) and the human oncogene product GLI (Pavletich & Pabo, 1993) make major contacts with three of the four bases on a single side of the double helix. An alternative metal-binding motif, the Cys_4 zinc motif, is found in many proteins such as the human transcription elongation factor TFIIS. The Cys_4 zinc motif of this protein

forms a zinc ribbon (Qian *et al.*, 1993) that interacts with ssDNA, dsDNA, ssRNA, and RNA−DNA heteroduplexes (Agarwal *et al.*, 1991). Preliminary studies show that a peptide containing the first 63 amino acids of the T7 primase forms a zinc-dependent secondary structure with a CD spectrum that is almost identical to that of the Cys_4 zinc motif of TFIIS (X. Qian, M. Weiss, L. V. Mendelman, and C. C. Richardson, unpublished results). The fact that the zinc motifs of both proteins recognize sequences in single-stranded DNA reinforces the similarities between the two motifs.

We have proposed that the T7 primase makes at least three contacts between an N-terminal Cys_4 zinc ribbon and the trinucleotide recognition site, 3′-d(CTG)-5′, on single-stranded DNA. In the present study we describe the results of our approach to investigate these interactions by substitution of nucleotide analogs for those residues normally found in the recognition sequence. We have initially investigated the functional groups on the base of the 3′-dC residue within the sequence 3′-d(CTG)-5′ for their importance in the initiation of primer synthesis by the T7 primase. Oligoribonucleotide synthesis was measured on a series of 20-mer oligodeoxynucleotide templates containing various nucleotide analog substitutions for the noncoding dC residue of the pentanucleotide T7 primase recognition sequence 3′-d(CTG-GT)-5′. Future studies will examine the role of the functional groups of the other two nucleotides in primase recognition.

Templates containing an m^5dC substitution for dC are able to support oligoribonucleotide synthesis at an efficiency 80% of that observed with dC. Thus, the addition of a methyl group at position C-5 on the pyrimidine ring only marginally affects the interactions required for binding by primase and the initiation of RNA synthesis. This result supports earlier observations that primer synthesis occurs on denatured DNA isolated from bacteriophage T4 (Matson & Richardson, 1983), which contains glucosylated 2′-deoxy-5-(hydroxymethyl)cytidine residues in place of 2′-deoxycytidine (Revel, 1983). Replacement of the amino group at position C-4 with a single hydrogen atom ($d^{H4}C$) results in a 50% reduction in primase activity. This observation implies that interactions between the protein and template that are responsible for initiating synthesis have been altered. Nonetheless, primer synthesis is still significant on the $d^{H4}C$-containing template, suggesting that the amino group of cytosine is not an essential contact for primase activity.

The most striking effects are observed when dU or dT replaces dC in the recognition site: oligoribonucleotide synthesis was not detected (<0.1% of native) on these modified templates. A comparison of the structures of dC, dU, and dT (Figure 1) illustrates that, in contrast to dC, both dT and dU contain a carbonyl oxygen (a hydrogen bond acceptor) that replaces the amino group (a hydrogen bond donor) at the C-4 position. Additionally, the hydrogen bonding character at the N-3 nitrogen of the base is changed from that of a hydrogen bond acceptor to that of a donor as a result of the corresponding tautomeric change at this site. These results suggest that primase interactions with the conserved 3′-dC residue in the recognition site may also take place through contact with a hydrogen bond acceptor at the N-3 position of dC. Interactions taking place as a result of hydrogen bond donors or acceptors at the C-4 exocyclic functional group and N-3 nitrogen of the dC residue residue are also those involved in classic Watson−Crick base pairing interactions in native duplex DNA. It remains to be determined whether the interactions between primase and the conserved dC residue occur through direct protein−DNA contacts that mimic Watson−Crick hydrogen bonding interactions.

T7 primase activity on a template with a 3′-d(CTGGT)-5′ site is inhibited by templates containing the sequences 3′-d(TTGGT)-5′, 3′-d(UTGGT)-5′, and 3′-d(UTTGGT)-5′, but the kinetic characterization of this inhibition differs. The template containing dT (in place of dC) competitively inhibits primer synthesis, effectively acting like a null substrate in the active site; the apparent K_m relative to the dC-containing template increases approximately 4-fold, with little change in the relative V_{max}. Templates containing dU, however, affect both the apparent K_m and relative V_{max} during oligoribonucleotide formation. These changes are characteristic of a mixed type of inhibition, but could also be achieved by a suicide substrate if more than one active site is present on the enzyme.

T7 primase is an enzyme of multiple subunits (Mendelman *et al.*, 1993; Notarnicola & Richardson, 1993; Patel & Hingorani, 1993) that forms hexamers on ssDNA (Notarnicola & Richardson, 1993; Hingorani & Patel, 1993). Studies by electron microscopy show that ssDNA passes though the center of the gene 4 protein hexamer (Engelman *et al.*, 1995), and nuclease protection studies suggest that gene 4 covers approximately 30 bases on ssDNA (Hingorani & Patel, 1993). Since the relative V_{max} for the catalysis of oligoribonucleotide synthesis on the wild-type 20-base template is affected by the presence of either of the 20-base templates containing dU, we conclude that the T7 primase is capable of interacting with more than one of the 20-base templates at a time. The results also illustrate that catalysis by the T7 primase is exquisitely sensitive to specific functional group contacts with the template strand, and activity can be dramatically altered by a ssDNA oligomer containing a single altered functional group within the recognition site. An understanding of the interaction of the primase zinc motif with groups on the bases composing the trinucleotide recognition sequence provides an approach toward developing specific inhibitors of primases and consequently specific inhibitors of DNA replication. Such inhibitors could provide for the development of a novel class of chemotherapeutic drugs.

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