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Reduction of the Potent DNA Polymerase III Holoenzyme 3′→5′ Exonuclease Activity by Template-Primer Analogues†

Mark A. Griep,‡ Jo Anna Reems, Mary A. Franden, and Charles S. McHenry*
Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262
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ABSTRACT: The DNA polymerase III holoenzyme of *Escherichia coli* contains a potent 3′→5′ exonuclease that removes the terminal nucleotide from a synthetic deoxyoligonucleotide primer with a half-life of approximately 2 s. Degradation of primers could not be effectively prevented by permitting the holoenzyme to "idle" at the primer terminus in the presence of limited deoxynucleoside triphosphates. To further characterize this exonuclease and to develop stable primers to facilitate experimental manipulations, we synthesized a series of twelve 25-mer oligonucleotides that differed only in the two 3′-terminal residues. The penultimate position contained either a CMP or a dCMP residue, while at the terminal position either AMP, dAMP, 2′,3′-dideoxyAMP, cordycepin (3′-dAMP), dAMPαS, or 2′,3′-dideoxyAMPαS was incorporated. No single change at either the 3′-penultimate or 3′-terminal positions resulted in a decrease in the exonuclease rate greater than 10-fold; however, combined changes at these two sites resulted in a strong synergistic effect. Placing a ribonucleotide at the penultimate position coupled by a phosphorothioate linkage to a terminal 2′,3′-dideoxynucleotide reduced the rate of exonucleolytic activity almost 30000-fold (half-life ~ 16 h). If only the ribonucleotide and phosphorothioate substitutions were made, a primer capable of being efficiently elongated was generated that exhibited a 500-fold increase in stability (half-life = 40 min). The elemental effect observed by substituting a nonbridging oxygen in the terminal phosphodiester bond for sulfur increased from 1.5 to 200 as other substitutions were made that decreased the exonuclease rate. This was consistent with a change in the rate-limiting step of the exonuclease reaction from a conformational change to the chemical step where the covalent bond is cleaved. At least part of this effect appears to be due to perturbations within the enzyme's active site and not solely due to changes in electrophilicity.

The DNA polymerase III holoenzyme is the major replicative polymerase of *Escherichia coli*. It contains a DNA polymerase III core plus auxiliary proteins that confer the special properties required of a true replicative complex. These properties include rapid polymerization rates, the ability to form a highly processive ATP-dependent clamp on the DNA template, the ability to interact with other replication proteins, and the apparent ability to function as an asymmetric dimer with distinguishable leading and lagging strand polymerases [for reviews, see Kornberg (1982) and McHenry (1988a,b)].

The DNA polymerase III core of holoenzyme[1] contains three subunits: α, ε, and θ of 130000, 27500, and 10000 daltons, respectively (McHenry & Crow, 1979). α, encoded

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* Author to whom correspondence should be addressed.
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[1] Abbreviations: SSB, *E. coli* single-stranded DNA-binding protein; ddATP, 2′,3′-dideoxyadenosine triphosphate; dATPαS, 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); ddATPαS, 2′,3′-dideoxyadenosine 5′-*O*-(1-thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; holoenzyme, *E. coli* DNA polymerase III holoenzyme; polIII′, *E. coli* DNA polymerase III′; PEI, poly(ethylenimine); 3′-[^{32}P]...NN, 25-mer primer radioactively labeled between nucleotides 24 and 25 at the 3′-end with either α-^{32}P or α-^{35}S in the phosphodiester or phosphorothioate linkage; "...", the 23 nucleotides preceding the two 3′-terminal nucleotides; NN, the 3′-penultimate and terminal nucleotide residues; ...dC, 24-mer 3′-terminated with 2′-deoxycytidine; ...rC, 24-mer 3′-terminated with cytidine; ...dCrA, 25-mer with a 3′-penultimate 2′-deoxycytidine and 3′-terminal adenosine; ...rCrA, 25-mer with a 3′-penultimate cytidine and 3′-terminal adenosine.

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by the *dnaE* gene, is the catalytic polymerase subunit (Gefter et al., 1971; Welch & McHenry, 1982). The *dnaQ* (*mutD*) gene product, ϵ, is responsible for the 3′→5′ proofreading exonuclease activity of DNA polymerase III (Horiuchi et al., 1978; Cox & Horner, 1983; Echols et al., 1983; DiFrancesco et al., 1984; Scheuermann & Echols, 1984). The α and ϵ subunits act synergistically, stimulating the activity of one another in an $\alpha\epsilon$ complex or within the $\alpha\epsilon\theta$ DNA polymerase III core complex (Maki & Kornberg, 1987; Studwell & O'-Donnell, 1990). Both in vitro and in vivo studies indicate that the proper functioning of the 3′→5′ exonuclease of holoenzyme is required to maintain the fidelity of DNA replication (Cox, 1976; Horiuchi et al., 1978; Kunkel et al., 1979; Fersht et al., 1982; Fersht & Knill-Jones, 1983; Fowler et al., 1986).

In this paper, we establish that DNA polymerase III holoenzyme possesses an extremely rapid 3′→5′ exonuclease. This potent exonuclease frustrated several investigations that depended on our ability to form stable polymerase–template-primer complexes. To obtain primers that are sufficiently stable for quantitative experimental manipulations, we have synthesized a series of primer analogues and have characterized their influence on the exonucleolytic reaction. These analogues not only provided practical solutions to experimental difficulties but provided significant insight into the mechanism of the 3′→5′ exonuclease within the *E. coli* replicative complex.

Materials and Methods

Proteins and Enzymes. *E. coli* DNA polymerase III holoenzyme was prepared by the method of Oberfelder and McHenry (1987). Two holoenzyme preparations (387 000 and 458 000 units/mg) were utilized in this study, and no significant differences were noted. SSB was isolated from an *E. coli* overproducer, RLM727 (constructed by and gift of Roger McMacken), by procedures described in Griep and McHenry (1989). The following enzymes were purchased: Sequenase version 2.0 DNA polymerase (U.S. Biochemical Corp.), terminal deoxynucleotidyl transferase (Amersham), and T4 polynucleotide kinase (New England Biolabs).

Nucleotides. M13mp18 single-stranded DNA was isolated by the procedure of Johanson and McHenry (1984). An extinction coefficient of 7370 (M nucleotide)$^{-1}$ cm^{-1} at 260 nm was used to determine the concentration of template and template-primer (Berkowitz & Day, 1974). The unlabeled nucleotides, ATP, dATP, ddATP, dATPαS, and ddATPαS, were obtained from Pharmacia. Cordycepin (3′-dATP) and dAMP were purchased from Sigma. [α-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol), and [γ-^{32}P]ATP (4500 Ci/mmol) were purchased from ICN. [α-^{32}P]Cordycepin (5000 Ci/mmol), [α-^{35}S]dATPαS (500 Ci/mmol), and [α-^{35}S]ddATPαS (1320 Ci/mmol) were supplied by New England Nuclear. [α-^{32}P]ddATP (>5000 Ci/mmol) was obtained from Amersham. Stock solutions used for 3′-terminal labeling of oligonucleotides of 10 μM dATP, 10 μM ddATP, 20 μM dATPαS, 10 μM ddATPαS, and 13 μM cordycepin were diluted with either [α-^{32}P]dATP, [α-^{32}P]ddATP, [α-^{35}S]dATPαS, [α-^{35}S]ddATPαS, or [α-^{32}P]cordycepin to obtain specific activities of 11 000, 8600, 400, 1900, and 360 cpm/fmol, respectively. Cytidine and adenosine controlled-pore glass supports and cytidine 5′-(β-cyanoethyl diisopropylphosphoramidite) were obtained from either Peninsula Labs or MilliGen/Biosearch.

Synthesis and Purification of Oligonucleotides. Four oligonucleotides were synthesized on a Biosearch DNA synthesizer Model 8600. Deoxycytidine or cytidine controlled-pore glass supports were used to synthesize two 24-mers that 3′-terminated with dC and rC, respectively. Adenosine controlled-pore glass support was used to synthesize two 25-mers that 3′-terminated with either dCrA or rCrA. The 3′-penultimate rC was incorporated by use of standard phosphoramidite chemistry with the 2′-hydroxyl protected with a *tert*-butyldimethylsilyl protecting group (Chou et al., 1989). The sequence for each of the four oligomers was identical for the first 23 5′-deoxynucleotides. All four oligomers were complementary to M13mp18 at positions 991–1015 (Yanisch-Perron et al., 1985). Stock primer concentration was determined with an extinction coefficient of 220 000 (M primer)$^{-1}$ cm^{-1}, which was calculated according to Borer (1975).

The 24-mers used in subsequent 3′-end labeling reactions were cleaved from the controlled-pore glass support, deprotected by standard ammonium hydroxide treatment (Hagerman, 1985), and purified by chromatography on a C18 HPLC column (Waters). A 38-min (1 mL/min) gradient was run from 10% acetonitrile–90 mM triethylammonium acetate, pH 7.0, to 30% acetonitrile–70 mM triethylammonium acetate, pH 7.0, following a 2-min isocratic step of the initial solvent. The absorbance of each fraction was monitored at 260 nm. The collected oligomer was dried in a Savant Speed Vac, suspended in 95% ethanol, and evaporated a second time. The oligomers were dissolved in 10 mM HEPES, pH 7.5, containing 1 mM EDTA and stored at −20 °C.

The 25-mers used in subsequent 5′-end labeling reactions were purified on 20% (w/v) acrylamide–8 M urea gels (Hagerman, 1985). Oligomer ...dCrA was treated as described by Hagerman (1985). Oligomer ...rCrA was deprotected with 30% ammonium hydroxide/ethanol for a period of 48 h at room temperature and dried down. Removal of the *tert*-butyldimethylsilyl 2′-protecting group on the penultimate ribonucleotide was accomplished by further treatment in 1.0 M tetrabutylammonium fluoride in tetrahydrofuran (16 h, ambient temperature) followed by chromatography on Dowex 50W-X8 (0.8 × 3.8 cm) (Sigma) and Sephadex G-25 (0.8 × 3.8 cm) (Sigma) (Chou et al., 1989).

Sequenase 3′-End Labeling of Template-Primer Analogues. Eight template-primer analogues were prepared by hybridizing 800 nM of primer ...dC or ...rC to 400 nM M13mp18 in 50 mM HEPES, pH 7.5, and 200 mM NaCl at 75 °C for 5 min. Mixtures were cooled slowly to room temperature (1.5 °C/min). By use of a modification of the procedure of Tabor and Richardson (1987), the 3′-termini were labeled by incubation of 200 nM primer-template with either 1.0 M [α-^{32}P]dATP, [α-^{32}P]ddATP, [α-^{35}S]dATPαS, or [α-^{35}S]ddATPαS and 3 units of Sequenase/pmol of primer-template in 10 mM magnesium acetate and 50 mM HEPES, pH 7.5, for 30 min at 37 °C. Enzyme was thermally inactivated (10 min, 65 °C). The resulting labeled template-primer analogues were separated from unincorporated nucleotides on a Bio Gel A-5m column (1.0 × 3.0 cm) (Bio-Rad) equilibrated in 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, and 1 mM EDTA. The DNA concentration was determined spectrophotometrically by measuring the absorbance of the pooled radioactive peak fractions at 260 nm. The resulting template-primer analogues are summarized in Table I.

Terminal Deoxynucleotidyl Transferase 3′-End Labeling of Template-Primer Analogues. Terminal transferase was used to add a single 3′-terminal [α-^{32}P]cordycepin residue to oligomers ...dC and ...rC. Reactions (20 μL) containing 10 pmol of either oligo ...dC or ...rC, 500 nM [α-^{32}P]cordycepin, 100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 0.5 mM DTT, and 68 units of terminal deoxynucleotidyl transferase (Tu & Cohen, 1980; Yousaf et al., 1984) were

incubated (2 h, 30 °C) with subsequent thermal inactivation of the enzyme (5 min, 90 °C). The resulting labeled primers were annealed to the M13mp18 template in 300 mM NaCl, purified, and quantitated as described above. The template-primer analogues were designated 3′-[^{32}P]...dCcordycepin and 3′-[^{32}P]...rCcordycepin (Table I).

5′-End Labeling of Template-Primer Analogues Using T4 Polynucleotide Kinase. Five 5′-end labeled template-primer analogues were generated with T4 polynucleotide kinase according to a procedure described by Maniatis et al. (1982). The radioactively labeled 25-mers, ...dCrA or ...rCrA, were annealed to template, purified, and quantitated as described above. These two substrates (Table II) were termed 5′-[^{32}P]...dCrA and 5′-[^{32}P]...rCrA. The other three 5′-end labeled substrates were constructed by annealing the 5′-end labeled 24-mers (...dC or ...rC) to M13mp18. The annealed primer was then extended by one nucleotide with Sequenase to add either of the nonradioactive nucleotides, dATP or dATPαS. After primer extension the substrates were purified and quantitated. These three template-primer analogues (Table II) were defined as 5′-[^{32}P]...dCdA, 5′-[^{32}P]...rCdA, and 5′-[^{32}P]...rCdAαS.

Examination of 20% polyacrylamide gels indicated that no detectable incorporation of extra nucleotides occurred during 3′-end labeling with either Sequenase or terminal deoxynucleotidyl transferase.

Elongation Assay. Holoenzyme elongation was determined by a modification of previously described procedures (Johanson & McHenry, 1984; Griep & McHenry, 1989). The final reaction premix contained 2.0 nM template-primer, 3′-[^{35}S]...rCdAαS, in 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 10 mM DTT, 10 mM magnesium acetate, 48 μM dATP, dCTP, and dGTP, and 18 μM [^{3}H]dTTP (sp act. of 400 cpm/pmol of dTTP). After the premix was equilibrated to 30 °C for at least 2 min, holoenzyme was added, and at various time points 12.5-μL aliquots were removed. The reaction was stopped, and the DNA was collected and quantitated as previously described (Johanson & McHenry, 1980). Background contributions from ^{35}S were subtracted from the ^{3}H counts. One unit of holoenzyme was defined as the amount needed to incorporate 1 pmol of (total) nucleotide per minute into acid-precipitable DNA under conditions where all other components were saturating (Johanson & McHenry, 1980; Bouché et al., 1975).

Exonuclease Assays. The holoenzyme 3′→5′ exonuclease rate for each template-primer analogue was measured in assays initiated by addition of saturating levels of the holoenzyme. A premix that contained the following components was preincubated for at least 2 min at 30 °C: 50 mM HEPES, pH 7.5, 100 mM potassium glutamate, 10 mM DTT, 500 μM ATP, 10 mM magnesium acetate, 2.0 nM primed template, and 2.0 μg of SSB/nmol of nucleotide. Three aliquots (12.5 μL) were removed prior to the addition of holoenzyme. After the addition of the polymerase, 12.5-μL aliquots were withdrawn at various time points and quenched with a final concentration of 140 mM EDTA. Quenched reaction mixtures were transferred onto Whatman DE-81 filters and batch washed (three times, 5 min in 0.3 M ammonium formate, pH 7.8, and 10 mM sodium pyrophosphate; once in deionized water; and once in 95% ethanol). The filters were dried, and the amount of radiolabel remaining was measured by scintillation counting. For kinetic measurements, infinity points were determined by adding 3–4 units of holoenzyme/fmol of primed template and allowing the reaction to proceed for 10 half-lives. An additional amount of holoenzyme (3–4 units/fmol of primed template) was added, and the reaction was allowed to proceed for another 10 half-lives. Three aliquots (12.5 μL) were removed, and the radiolabel was quantitated as described above.

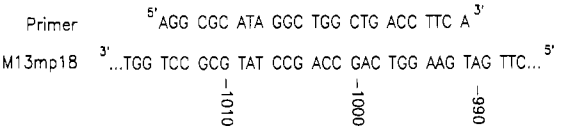

FIGURE 1: Nucleotide sequence of the 25-nucleotide primer and its M13mp18 complementary strand. The template strand M13mp18 is numbered according to Yanisch-Perron et al. (1985).

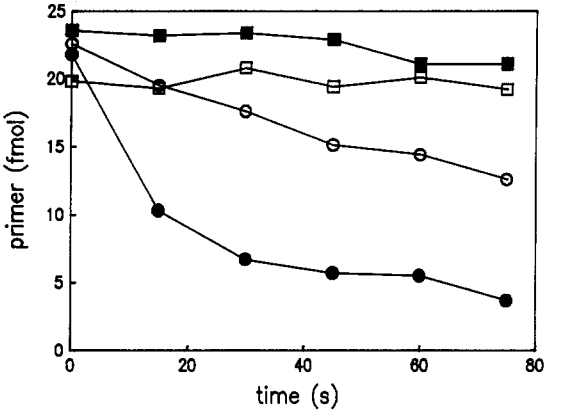

FIGURE 2: Time course of the DNA polymerase III holoenzyme exonuclease reaction. The exonuclease reaction was monitored as described under Materials and Methods with 5′-[^{32}P]...dCdA. The nucleotides added to the reaction were as follows: no dNTPs (●); dATP and dTTP (○); dATP, dTTP, and dGMP (■); four dNTPs (□).

Data Analysis. The amount of remaining radioactive primer (fmol) was plotted on semilogarithmic graph paper as a function of time. The amount of label remaining at the infinity time point was subtracted from each experimental data point prior to plotting. After it was demonstrated that the reactions accurately followed first-order kinetics for two or three half-lives, the rate constant (k_{exo}) and half-life ($t_{1/2}$) were calculated from $k = 0.693/t_{1/2}$ (Jencks, 1969).

Thin-Layer Chromatography Assay. Primer-templates were incubated as described above for the exonuclease assay. Aliquots were removed prior to addition of holoenzyme and again at 2.5 min after the addition of holoenzyme (75 half-lives). The reactions were terminated as described. Nonradioactive dATP and dAMP were added. Samples were concentrated, spotted onto PEI–cellulose plates (Baker), and developed in 0.3 M potassium phosphate, pH 7.0 (Mizrahi et al., 1986). After the plates were dried, positions for dATP and dAMP were visualized by fluorescence quenching. Radiolabeled products were detected by autoradiography and quantitated by scintillation counting.

RESULTS

The Potent Holoenzyme 3′→5′ Exonuclease Activity. Several of the current investigations in our laboratory are predicted on the ability to form stable holoenzyme–primer-template complexes. From previous work we knew that RNA primers synthesized on single-stranded DNA by DnaG primase formed stable complexes with holoenzyme (Johanson & McHenry, 1980). However, we found that DNA primers annealed to single-stranded templates formed considerably less stable complexes. For our initial studies, the DNA-primed template was constructed by synthesizing an oligodeoxynucleotide that was complementary to nucleotides 991–1015

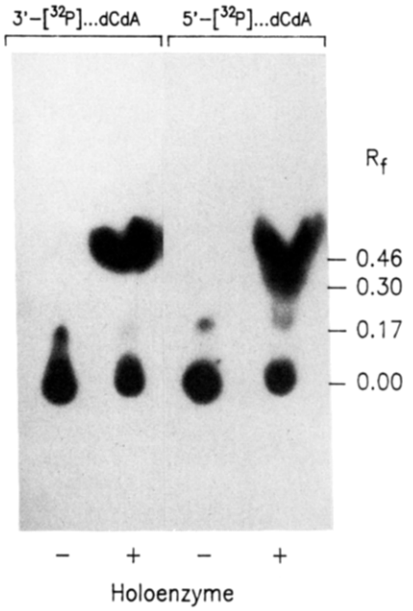

FIGURE 3: Products of DNA polymerase III holoenzyme exonulcease reaction. PEI–cellulose thin-layer chromatograms were run as described under Materials and Methods. R_f values are indicated to the right of the chromatogram. Digestion of 3′-[^{32}P]...dCdA by holoenzyme generated only dAMP (R_f = 0.46) (85% of the radioactivity). Following holoenzyme digestion of 5′-[^{32}P]...dCdA, two products were generated with R_f values of 0.46 and 0.30 (75% of the radioactivity). There was a trace of unreacted [γ-^{32}P]ATP (R_f = 0.17) substrate remaining from the end labeling reaction.

of the single-stranded circular plasmid M13mp18 (Figure 1). The 5′-end labeled DNA primer, 5′-[^{32}P]...dCdA, was rapidly degraded in less than 1 min (Figure 2). When all four dNTPs were added to the mixture, no degradation of the primer was observed (Figure 2), indicating that elongation was faster than the rate of hydrolysis. To preclude degradation of the DNA primers without fully elongating them, we attempted to "idle" (nucleotide insertion/excision) the holoenzyme at the 3′-terminus by adding only two of the dNTPs. The two dNTPs, dATP and dTTP, that were used corresponded to the 3′-terminal nucleotide of the oligomer and the next nucleotide to be inserted (Figure 1). The addition of these two dNTPs reduced the rate of primer destruction by about 3-fold (Figure 2), which was not a large enough effect for our purposes. Equally unsuitable was the addition of dGMP, an inhibitor of holoenzyme's exonuclease activity (Fersht & Knill-Jones, 1983; Scheuermann & Echols, 1984). Even though dGMP, in the presence of the two dNTPs, was very effective at preventing hydrolysis of the DNA primer (Figure 2), dGMP caused misincorporation in up to 75% of the primer termini (data not shown).

In order to determine the fate of the radioactive label, we ran a PEI–cellulose thin-layer chromatogram of the reaction products for both the 5′- and 3′-[^{32}P]...dCdA primers (Figure 3). The only product generated from the degradation of 3′-[^{32}P]...dCdA was dAMP. A small amount of product generated from 5′-[^{32}P]...dCdA, probably a dinucleotide, migrated between dATP and dAMP. These data provided evidence that the potent 3′-5′ exonuclease activity of holoenzyme excises nucleotides as monophosphates and is capable of completely digesting a 25-nucleotide DNA primer.

Reduction of the Exonuclease Activity through the Use of Template-Primer Analaogues. The strategy that we employed to investigate the exonuclease activity involved the use of template-primer analogues (Tables I and II). A preliminary test of several analogues (Figure 4A) indicated that 3′-[^{35}S]...rCdAαS exhibited a moderate rate of hydrolysis suitable for convenient monitoring of a titration of the exonuclease

Table I: Summary of 3′-End Labeled Template-Primer Analogues[a]

template-primer analogue[b]	oligomer[c]	enzyme	nucleotide[d]
3′-[^{32}P]...dCdA	...dC	T7[e]	[α-^{32}P]dATP
3′-[^{35}S]...dCdAαS	...dC	T7	[α-^{35}S]dATPαS
3′-[^{32}P]...dCddA	...dC	T7	[α-^{32}P]ddATP
3′-[^{35}S]...dCddAαS	...dC	T7	[α-^{35}S]ddATPαS
3′-[^{32}P]...rCdA	...rC	T7	[α-^{32}P]dATP
3′-[^{35}S]...rCdAαS	...rC	T7	[α-^{35}S]dATPαS
3′-[^{32}P]...rCddA	...rC	T7	[α-^{32}P]ddATP
3′-[^{35}S]...rCddAαS	...rC	T7	[α-^{35}S]ddATPαS
3′-[^{32}P]...dCcordycepin	...dC	TdT[f]	[α-^{32}P]cordycepin
3′-[^{32}P]...rCcordycepin	...rC	TdT	[α-^{32}P]cordycepin

[a] All template-primer analogues were synthesized and labeled as described under Materials and Methods. The 3′-[^{32}P] or 3′-[^{35}S] designation indicates that the 25-mer primer is radioactively labeled at the 3′-terminus with either α-^{32}P or α-^{35}S in the phosphodiester or phosphorothioate linkage between the terminal and penultimate nucleotide. [b] The template-primer analogues were 25-mers hybridized to M13mp18. Each of the substrates are distinguished from one another by the nomenclature 3′-[^{32}P]...NN or 3′-[^{35}S]...NN. The symbol "..." represents the 23 nucleotides preceding the two 3′-terminal nucleotides. The 3′-penultimate and 3′-terminal nucleotide residues, NN, are distinguished from one another by the nucleotide nomenclature described above. [c] Oligonucleotides (24-mers) extended by addition of a single radioactive nucleotide analogue. [d] Nucleotide analogues used for 3′-end labeling reactions to generate the 25-nucleotide primer analogues. [e] T7 (Sequenase) DNA polymerase was used to extend the preannealed 24-mer by a single nucleotide residue. [f] TdT (terminal deoxynucleotidyl transferase) was used to catalyze a template-independent addition of a single nucleotide residue to the 3′-hydroxyl terminus of the 24-mer.

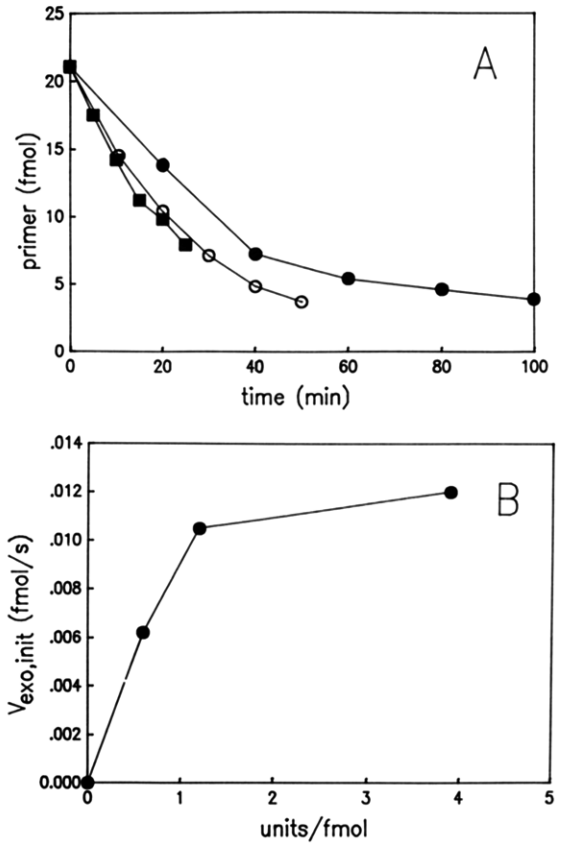

FIGURE 4: Determination of saturating levels of holoenzyme exonuclease suitable for use in pseudo-first-order kinetic determinations. (A) The exonuclease reaction was monitored as described under Materials and Methods with the primer 3′-[^{35}S]...rCdAαS annealed to the template. Holoenzyme was added at 0.6 (●), 1.2 (○), and 3.9 (■) units/fmol of primer-template. (B) Replot of the exonuclease initial velocity versus the ratio of holoenzyme to template-primer.

Table II: Summary of 5′-End Labeled Template-Primer Analogues[a]

template-primer analogue[b]	oligomer	enzyme	nucleotide[c]
5′-[^{32}P]...dCrA	...dCrA		
5′-[^{32}P]...rCrA	...rCrA		
5′-[^{32}P]...dCdA	...dC	T7[d]	dATP
5′-[^{32}P]...rCdA	...rC	T7	dATP
5′-[^{32}P]...rCdAαS	...rC	T7	dATPαS

[a] All template-primer analogues were synthesized and labeled as described under Materials and Methods. The 5′-[^{32}P] designation indicates that the 25-mer primer is 5′ radioactively labeled with ^{32}P. [b] The template-primer analogues were 25-mers hybridized to M13mp18. Each of the substrates are distinguished from one another by the nomenclature 5′-[^{32}P]...NN. The symbol "..." represents the 23 nucleotides preceding the unique 3′-terminus. The 3′-penultimate and 3′-terminal nucleotide residues, NN, are distinguished from one another by the appropriate nucleotide nomenclature. [c] Nucleotide analogues used to extend the 24-mers by one residue. [d] T7 (Sequenase) was used to extend the preannealed 24-mers by a single nucleotide residue.

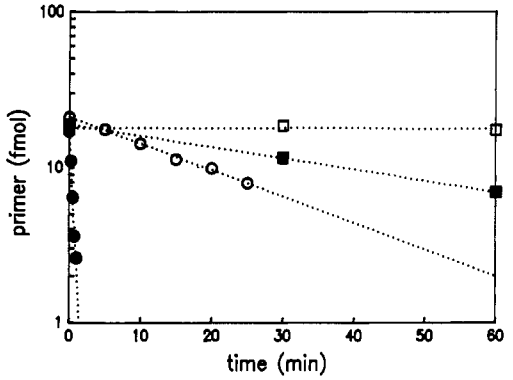

FIGURE 5: Semilogarithmic plot of primer 3′-termini remaining versus time to determine k_{exo}. Exonuclease assays were as described under Materials and Methods. The template-primers shown are 3′-[^{32}P]...rCdA (●), 3′-[^{35}S]...rCdAαS (○), 3′-[^{35}S]...dCddAαS (■), and 3′-[^{35}S]...rCddAαS (□). The initial concentration of primer was 2 nM (25 fmol/12.5 μL).

activity of holoenzyme. ^{35}S was incorporated at the 3′-phosphorothioate linkage so that hydrolysis of this bond by exonuclease reflected removal of only a single nucleotide. The rate of excision of dAMPαS from 2 nM template-primer became maximal at approximately 1.5 units of holoenzyme/fmol of primer-template (Figure 4B).[2] The maximal velocity of holoenzyme on this primer analogue was 0.014 fmol of nucleotide/s, which corresponded to a first-order rate of 6.5 $\times$ 10^{-4} s^{-1} (Figure 5). When the primer-template concentration was reduced to 0.5 nM with saturating holoenzyme (data not shown), the first-order exonuclease rate was the same within experimental error (6.4 $\times$ 10^{-4} s^{-1}; $t_{1/2}$ = 1080 s). This verified that we were working within the correct substrate and enzyme concentration range to measure pseudo-first-order rate constants. Consequently, all exonuclease rates were determined with 2 nM primer-template and 3–4 units of holoenzyme/fmol of primer termini.

Having established the appropriate conditions needed to obtain first-order kinetics, we synthesized and tested other primer analogues (Figure 5; Tables III and IV). The 3′-penultimate and 3′-terminal nucleotide positions were the two sites we chose to manipulate within the primer sequence in our search for a template-primer analogue that would inhibit the 3′-5′ exonuclease activity of holoenzyme. Our strategy was to combine various nucleotide moieties to decrease the rate of hydrolysis of the primer within the holoenzyme–primer-template complex. The analogues consisted of variations in the 2′- and 3′-hydroxyls of the terminal nucleotide, a phosphorothioate or phosphodiester linkage, and either a ribose or 2′-deoxyribose sugar analogue as the penultimate nucleotide. The most slowly hydrolyzed primer-template was 3′-[^{35}S]...rCddAαS (Table III). This primer was not significantly hydrolyzed during 6 h of incubation. Thus, the three modifications of this primer analogue, the penultimate ribonucleotide, the phosphorothioate linkage, and the dideoxynucleotide terminus, acted synergistically to reduce the potent exonuclease rate. Of the primer analogues that carried a 3′-hydroxyl on the terminal nucleotide, 3′-[^{35}S]...rCdAαS was the slowest to be hydrolyzed (Table III). This primer analogue shared two of the features of the most slowly hydrolyzed primer analogue, the penultimate ribonucleotide and the phosphorothioate diester linkage, but had a terminal nucleotide that could be elongated.

Further analysis of substituent effects that decreased the exonuclease rate (Table III) revealed that nucleotides linked to a ribonucleotide were excised more slowly than those linked to deoxyribonucleotides. Although the substituent effects exhibit significant scatter, the general trend observed was that, as the first-order rate decreased, the penultimate ribonucleotide effect became greater.

The exonuclease first-order rate for 3′-[^{35}S]...dCddAαS was 200-fold slower than that for 3′-[^{32}P]...dCddA. The ratio of the rate constants (k_{P-O}/k_{P-S}) for these two analogues, which differ only in a phosphorothioate versus a phosphodiester linkage, is referred to as an elemental effect. The elemental effect results from the substitution of a sulfur for a nonbridging oxygen. The element effect for 3′-[^{32}P]...dCdA/3′-[^{35}S]...dCdAαS (Table III) was only 1.5 for holoenzyme, which was considerably less than the ratio of 120 found for the Klenow fragment of DNA polymerase I but close to the ratio of 1 obtained for T4 DNA polymerase (Gupta et al., 1984).

Similar comparisons can be made for the exonuclease rates concerning the number and location of the hydroxyls on the terminal ribose. The terminal nucleotide analogues may effect the excision rate by the way they fit into the active site of the polymerase or exonuclease subunits. The exonuclease most readily digested the 3′-terminal nucleotides in the following order: ribose ~ 2′-deoxyribose > 2′,3′-dideoxyribose > 3′-deoxyribose. Thus, a hydroxyl at the 2′-position conferred only a modest effect on the rate. The largest effects were observed with 2′,3′-dideoxyribose and cordycepin, indicating that the lack of a 3′-hydroxyl had the greatest influence on the excision rate.

Template-Primer Analogues with 5′-End Labels. Proof that the exonuclease activity was acting in the 3′ → 5′ direction, rather than 5′ → 3′, came from studies with 5′-^{32}P-labeled primer analogues. The primers, 5′-[^{32}P]...dCdA, 5′-[^{32}P]...dCrA, 5′-[^{32}P]...rCdA, and 5′-[^{32}P]...rCrA were half degraded in 19, 10, 45, and 50 s, respectively (Table IV). These results suggested that it was the 3′-end analogue which limited the rate of oligomer degradation. This was more clearly evident when the first-order rate of radiolabel released from 5′-[^{32}P]...rCdAαS (Table IV) was found to be the same as for its 3′-end labeled analogue (Table III). Since the nucleotide analogue resides at the 3′-end for both of these primers, the exonuclease must proceed in the 3′ → 5′ direction for the rates of radiolabel released from the 3′- and 5′-ends to be the same.

Stoichiometry and Intereaction of Polymerase and Exonuclease Subunits. The template-primer analogue 3′-[^{35}S]...rCdAαS was effectively inert to exonuclease on the time

[2] Multiple experiments with different template-primer analogues indicated the value was 2.0 ± 0.5 units.

Table III: Summary of Exonuclease Rates on Template-Primer Analogues Labeled at the 3′-Terminus[a]

template-primers	k_{exo} (s^{-1})	half-life (s)	k_{P-O}/k_{P-S}[b]	k_{dC-N}/k_{rC-N}[c]
3′-[^{32}P]...dCdA	0.35	2		
3′-[^{35}S]...dCdAαS	0.23	3	1.5	
3′-[^{32}P]...dCddA	0.057	12		
3′-[^{32}P]...dCcordycepin	0.036	19		
3′-[^{35}S]...dCddAαS	2.9×10^{-4}	2400 (40 min)	200	
3′-[^{32}P]...rCdA	0.032	22		11
3′-[^{35}S]...rCdAαS	6.5×10^{-4}	1060 (18 min)	48	353
3′-[^{32}P]...rCddA	4.0×10^{-4}	1750 (29 min)		146
3′-[^{32}P]...rCcordycepin	8.0×10^{-5}	8640 (2.4 h)		450
3′-[^{35}S]...rCddAαS	1.2×10^{-5}	57600 (16 h)	33	24

[a] All rates and half-lives were determined as described under Materials and Methods. The half-life for the 3′-end labeled primers represents the first-order rate of removal of a single nucleotide at the 3′-terminus. [b] The ratio of the exonuclease rates of a primer with a terminal phosphodiester linkage versus that for a phosphorothioate linkage. [c] The ratio of the exonuclease rates of a primer with a 3′-penultimate deoxyribonucleotide versus that for a 3′-enultimate ribonucleotide.

Table IV: Summary of Exonuclease Rates on Template-Primer Analogues Labeled at the 5′-Terminus[a]

template-primers	k_{exo} (s^{-1})	half-life (s)
5′-[^{32}P]...dCrA	0.069	10
5′-[^{32}P]...dCdA	0.036	19
5′-[^{32}P]...rCdA	0.015	45
5′-[^{32}P]...rCrA	0.014	50
5′-[^{32}P]...rCdAαS	6.2×10^{-4}	1060 (18 min)

[a] All rates and half-lives were determined as described under Materials and Methods. The half-life for the 5′-end labeled primers represents the first-order rate of hydrolysis of the full-length primer.

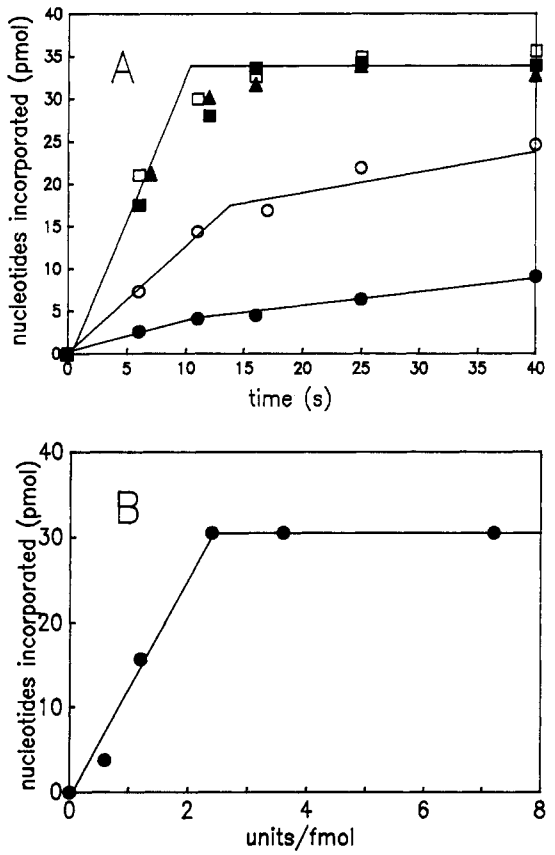

FIGURE 6: Use of elongation reaction to monitor titration of template-primer by holoenzyme. (A) The elongation reaction was monitored as described under Materials and Methods with primer 3′-[^{35}S]...rCdAαS except with the addition of holoenzyme at 0.6 (●), 1.2 (○), 2.4 (■), 3.6 (□), and 7.2 (▲) units/fmol of primer-template. (B) Replot of the amount of nucleotides incorporated in the elongation burst (inflection point in panel A) versus the ratio of holoenzyme to template-primer.

scale of this experiment and was efficiently elongated by the polymerase (Figure 6A). Only a single round of elongation

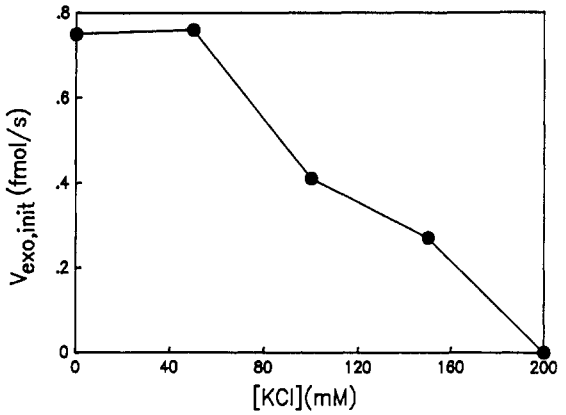

FIGURE 7: Salt inhibition of holoenzyme exonuclease activity. The exonuclease assays were performed on the primer-template 5′-[^{32}P]...dCrA as described under Materials and Methods.

should be observed on the time scale measured (Johanson & McHenry, 1982); thus, the moles of DNA circles replicated are proportional to the moles of holoenzyme available at the start of the reaction. A plot of nucleotide incorporation versus holoenzyme saturated at about 1.5 units of holoenzyme/fmol of primer-template (Figure 6B). This was the same amount of holoenzyme that saturated the exonuclease reaction (compare to Figure 4B), indicating that there were equivalent levels of the polymerase and exonuclease active sites.

Current theories of the Klenow fragment 3′→5′ exonuclease function indicate that the primary DNA binding site is the polymerase active site (Joyce & Steitz, 1987). It is only when the polymerase stalls that the less active exonuclease has a significant opportunity to act (Kuchta et al., 1988). One way that we can show that the holoenzyme subunits are acting as a complex is by the salt dependence of exonuclease activity. The KCl inhibition of exonuclease activity (Figure 7) was similar to that observed for polymerase activity [see Figure 3 in Griep and McHenry (1989)]. The addition of up to 50 mM KCl had only a slight effect on the exonuclease rate while higher amounts had an inhibitory effect. The KCl concentration at 50% inhibition was about 100 mM. Salt inhibits holoenzyme binding to the primer-template by effecting the binding constant (Griep & McHenry, 1989). Since salt inhibition of holoenzyme binding affects both the exonuclease and polymerase activities in a similar fashion, these activities probably reside within the same DNA-binding complex. Previous work has shown that the ϵ subunit of holoenzyme is tightly associated with the α subunit; this also supports the idea that the activities reside within the same DNA-binding complex (Livingston & Richardson, 1975; McHenry & Crow, 1979; Scheuermann & Echols, 1984; Maki & Kornberg, 1987;

Studwell & O'Donnell, 1990).

Discussion

DNA polymerase III holoenzyme forms an exceedingly stable initiation complex with DnaG primase synthesized primer on a template coated with single-stranded DNA-binding protein (Johanson & McHenry, 1982). A number of laboratories have used model templates containing synthetic deoxyoligonucleotides to investigate the mechanism of the DNA polymerase III holoenzyme reaction (Burgers & Kornberg, 1983; O'Donnell & Kornberg, 1985; Kwon-Shin et al., 1987; McHenry et al., 1989; O'Donnell, 1987). Our studies using deoxyoligonucleotide primers in the presence of saturating holoenzyme have been frustrated by a marked instability of the resulting isolated initiation complexes. Instability has thwarted our obtaining precise quantitative results. To properly address these issues, we embarked on a study of the stability of these primers to the 3′→5′ exonucleolytic activity of the DNA polymerase III holoenzyme.

Our initial assessment of the stability of primers indicated that a 5′-end labeled 25-mer was completely degraded in less than 1 min. Due to the placement of the label on the 5′-terminus, loss of radioactivity from the DEAE filters required that extensive degradation of the primer must occur. We checked whether the addition of two deoxynucleoside triphosphates was adequate to stabilize the primer. The half-life for degradation of the entire primer increased from 19 s to 1 min under these conditions, an inadequate length of time to permit experimental manipulations with isolated initiation complexes. dGMP, an inhibitor of the 3′→5′ exonuclease of the holoenzyme, together with two dNTPs did stabilize the primer. However, dGMP also led to incorporation of extra nucleotides noncomplementary to the template in 75% of the primer termini. dGMP, an inhibitor of the proofreading exonuclease, has been shown to be mutagenic by Fersht and Knill-Jones (1983).

From these preliminary experiments, we realized that we would not be able to adequately stabilize conventional deoxyoligonucleotides for rigorous mechanistic studies of the holoenzyme. We embarked on a systematic search for primer analogues that would permit us to form stable initiation complexes without significant primer degradation during the coarse of the experiments. We decided to limit our investigation to substituent effects in the 3′-terminal and 3′-penultimate nucleotide positions. Where possible, we radiolabeled the 3′-terminal phosphodiester linkage so that our assay could detect removal of the initial nucleotide rather than degradation of the entire primer.

As one would expect for a 3′→5′ exonuclease, the rate of removal of a single nucleotide from the 3′-terminus is significantly faster than the rate of degradation of the entire primer. We observed a half-life of approximately 2 s for release of dAMP from the 3′-end of a 25-mer. In studies of other enzymes, the rate of exonucleolytic cleavage of a phosphorothioate linkage is often up to 100-fold slower (Mizrahi et al., 1985; Gupta et al., 1984; Kuchta et al., 1988; Kunkel et al., 1981; Eckstein, 1985). Upon substituting dAMPαS for the terminal dAMP in our synthetic model primer, we observed only a modest (~1.5-fold) increase in primer stability. We have previously demonstrated that holoenzyme incorporates 2′,3′-dideoxynucleotides and becomes stably attached to the resulting primer termini (Johanson & McHenry, 1982). Thus, we next checked the stability of primers terminated with 2′,3′-ddAMP and 3′-dAMP (cordycepin) and found that the stability was only enhanced 6–10-fold. After this series of disappointingly modest effects, we were surprised that a combination of a dideoxynucleotide and a phosphorothioate linkage was enormously synergistic with an increase in primer stability of 1200-fold ($t_{1/2}$ = 40 min). Thus, primers terminated with ddAMPαS were sufficiently stable for many experimental manipulations but were limited in that they cannot be elongated.

In an attempt to find stable deoxyoligonucleotide primers capable of being elongated, we next examined the effect of substituents at the penultimate position. Keeping in mind the stability of natural DnaG primase synthesized primers, we explored the effect of ribonucleotide substitution. The placement of a ribonucleotide at the 3′-penultimate position led to only a 10-fold increase in stability. Encouraged by this modest effect and keeping in mind the marked synergistic effects that we previously observed, we investigated each of the terminal nucleotide substituents described in the preceding paragraph in combination with a penultimate ribonucleotide. Three analogues, dAMPαS, 2′,3′-ddAMP, and 3′-dAMP, when placed after a ribonucleotide were between 150- and 450-fold more stable than their corresponding deoxynucleotide counterparts. 2′,3′-ddAMPαS, which already provided a very stable primer when inserted after a deoxynucleotide, became 24-fold more stable when inserted after a ribonucleotide ($t_{1/2}$ = 16 h). This was the most stable primer analogue that resulted from these studies. Of those primers that were capable of being elongated, ...rCdAαS provided a primer with an adequate stability for most purposes ($t_{1/2}$ = 18 min).

Recalling the high stability of DnaG-synthesized primers, we finally chose to explore the effect of ribonucleotide placement at 3′-terminal positions. We inserted these residues chemically during oligonucleotide synthesis; therefore, we had to place the radiolabel at the 5′-end. We found that oligonucleotides terminated with either one or two ribonucleotides were not significantly more stable than the corresponding deoxyoligonucleotides. Thus, the reason for the stability of the natural DnaG-synthesized primers at the phage G4 origin is either (i) due to ribonucleotides inserted further within the structure causing a shift of DNA structure from B-type to A-type, (ii) due to other structural or sequence specificities of the exonuclease, or, perhaps, (iii) due to the direct presence of the DnaG primase blocking exonuclease action.

Other than the practical experimental benefits of providing stable primer analogues, this study provided significant insight into the mechanism of the holoenzyme-catalyzed 3′→5′ exonucleolytic reaction. The substituent effects observed are particularly interesting. Benkovic and colleagues have used sulfur substitution or elemental effects to determine if the chemical step is rate limiting in these reactions. Insertion of improperly base-paired nucleotides exhibits up to a 65-fold elemental effect with the large fragment of DNA polymerase I (Kuchta et al., 1988) while insertion of properly base-paired nucleotides shows no effect. This result may indicate that a step other than phosphodiester bond formation is rate limiting for paired nucleotide insertion (for example, a conformational change), while the chemical step is rate limiting for incorrect nucleotide insertion. The large fragment of DNA polymerase I exhibits a 100-fold elemental effect in nucleotide removal, consistent with the chemical step of nucleotide excision being rate limiting (Gupta et al., 1984). Conversely, no elemental effect is observed for the T4 DNA polymerase catalyzed removal of nucleotides (Gupta et al., 1984).

We observed a wide range of elemental effects with the holoenzyme-catalyzed 3′→5′ exonuclease reaction. As a general rule, the slower the reaction was, the greater the elemental effect observed. With the excision of dAMPαS from

the primer ...dCdAαS, we observed only a 1.5-fold elemental effect, suggesting that, like the T4 reaction, a step other than the chemical step was rate limiting. When a ribonucleotide was placed at the penultimate position as in the primer ...rCdAαS, the rate of dAMPαS excision decreased over 300-fold, and a 50-fold elemental effect was observed. This was consistent with the chemical step, the step directly involving phosphodiester bond cleavage, becoming partially rate limiting.

The magnitude of the 3′→5′ exonuclease elemental effect could result from a variety of factors that effect enzyme-catalyzed phophoryl transfer reactions. Among the factors that control these types of enzymes are the steric environment, the stereochemical course of the reaction, metal binding, changes in hydrogen-bond donor–acceptor properties, and an associative, S_N2, or a dissociative, S_N1, mechanism (Benkovic & Schray, 1973; Mildvan, 1979; Knowles, 1980). Sterically, the van der Waals' radii of sulfur, 1.85 Å, and oxygen, 1.40 Å, are different as are the interatomic distances of P═S, 1.95 Å (Saenger & Eckstein, 1970), and P═O, 1.55 Å (Saenger, 1984). The P═S distance is characteristic of a fully double bond, so that the associated nonbridged P–O bond carries the full negative charge in phosphorothioates. In contrast, the P═O distance is intermediate between a single bond, 1.71 Å, and a double bond, 1.40 Å, so that the negative charge is delocalized equally to both nonbridging oxygens. The stereochemistry of the exonucleases from several different polymerases (for example, T7 DNA polymerase I) is tightly controlled (Eckstein, 1985) such that hydrolysis occurs with inversion of phosphate (or phosphorothioate) stereochemistry. Metal coordination may play a number of roles including orienting the substrate and enzyme catalytic groups, charge neutralization, etc. (Benkovic & Schray, 1973). The large range of effects by various metals on the ability of DNA polymerase I to incorporate diastereomeric phosphorothioate analogues of dATP into DNA (Burgers & Eckstein, 1979) illustrates the fine tuning that can be achieved with metal coordination.

Only a few nonenzymatic hydrolytic studies have been performed on phosphate compared to thiophosphate monoesters and triesters. The alkaline hydrolysis of phosphate (or thiophosphate) triesters proceeds by an associative mechanism and has a rate ratio k_{P-O}/k_{P-S} of 10–30 (Heath, 1956). Nucleophilic addition to form a pentacoordinate phosphate is followed by elimination. In contrast, monoesters are hydrolyzed by a dissociative mechanism and have a rate ratio k_{P-O}/k_{P-S} of 0.016 at pH 7 and 70 °C (Breslow & Katz, 1968). Elimination of alcohol produces metaphosphate, which has less localized charge than the initial or final states and is expected to be accelerated by metal coordination (Mildvan, 1979). The d orbital electrons of sulfur may lead to a stabilization of the intermediate and thus increase the nonenzymatic reaction rate of phosphorothioates compared to phosphates. However, because of the variety of parameters controlled by an enzyme, it is not possible to interpret the exact magnitude of an enzymatic elemental rate ratio solely in terms of the chemical step.

Other substituent effects were also observed in the holoenzyme 3′→5′ exonuclease reaction that became more significant as the reaction rate was decreased. In all cases, the replacement of dC with rC in the penultimate position decreased the rate of the reaction. Depending on the magnitude of the reaction rate decrease, the "penultimate ribonucleotide effect" varied from 11 to 450. The results were not directly interpretable on the basis of solution hydrolysis model studies and were probably derived in part by structural perturbations to the enzyme's active site rather than just electrophilicity effects. These results raise a caution in simple interpretations of elemental effects, since sulfur replacement of oxygen in a phosphodiester link is not a neutral change. The phosphorothioate compound may likely bind to an enzyme's active site in an altered conformation relative to the natural phosphate-containing compound. Nevertheless, given the consistency of the general trends observed for both sulfur and ribonucleotide substitutions, both series support the notion that there is a shift in the rate-limiting step as the reaction rate decreases. This shift is probably due to a change of the rate-limiting step to the exonucleolytic chemical step from either a conformational change or movement of the primer terminus from the polymerase active site to the exonuclease active site. With DNA polymerase I, the primer terminus must migrate from the polymerase active site to an exonuclease active site located ~27 Å away with an accompanying rupture of four base pairs (Joyce & Steitz, 1987).

Resolution of issues pertaining to the actual mechanism used by the ε subunit of the DNA polymerase III holoenzyme in its exonucleolytic editing of nascent primer termini must await a thorough pre-steady-state kinetic analysis. These studies should lead to an understanding of the coordination of the polymerase and editing functions within a true replicative complex.

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