Interaction of Ribonucleoside Triphosphates with the Gene 4 Primase of Bacteriophage T7

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

| Citation       | Frick, David N., Shiv Kumar, and Charles C. Richardson. 1999. “Interaction of Ribonucleoside Triphosphates with the Gene 4 Primase of Bacteriophage T7.” Journal of Biological Chemistry 274 (50): 35899–907. https://doi.org/10.1074/jbc.274.50.35899. |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Citable link   | http://nrs.harvard.edu/urn-3:HUL.InstRepos:41483372                                                                                                                                             |
| Terms of Use   | This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA |
Interaction of Ribonucleoside Triphosphates with the Gene 4 Primase of Bacteriophage T7*

(Received for publication, June 9, 1999, and in revised form, September 3, 1999)

David N. Frick‡§, Shiv Kumar¶, and Charles C. Richardson‡‡

From the ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and the ¶Nucleic Acid Chemistry, Amersham Pharmacia Biotech, Piscataway, New Jersey 08855

The primase fragment of bacteriophage T7 gene 4 protein catalyzes the synthesis of oligoribonucleotides in the presence of ATP, CTP, Mg2+ (or Mn2+), and DNA containing a primase recognition site. During chain initiation, ATP binds with a \( K_m \) of 0.32 mM, and CTP binds with a \( K_m \) of 0.85 mM. Synthesis of the dinucleotides proceeds at a rate of 3.8/s. The dinucleotide either dissociates or is extended to a tetraderulic acid. The primase preferentially inserts ribonucleotides forming Watson-Crick base pairs with the DNA template, and proceeds at a rate of 3.8/s. The primase recognition site preferentially inserts ribonucleotides forming Watson-Crick base pairs with the DNA template and proceeds at a rate of 3.8/s. The primase recognition site prefers to be extended to a tetraderulic acid.

The product of gene 4 of bacteriophage T7 (1, 2) synthesizes the oligoribonucleotides used as primers by T7 DNA polymerase. Gene 4 encodes a multifunctional protein that provides both primase and helicase activity required for the replication of the phage DNA. The gene 4 protein is a hexamer (3–5) comprised of two co-linear proteins translated from separate in-frame translational start sites (6). The larger 63-kDa protein has both primase and helicase activities. As a helicase, the 63-kDa gene 4 protein translocates 5'-to-3' on ssDNA (7) and unwinds duplex DNA in a reaction coupled to the hydrolysis of nucleoside triphosphates (8–10). The smaller 56-kDa protein lacks a 7-kDa Cys4 zinc ribbon motif as well as the ability to unwind duplex DNA in a reaction coupled to the hydrolysis of nucleoside triphosphates (8–10). The smaller 56-kDa protein has enabled detailed kinetic studies to be conducted using the T7 helicase (11–13). However, the competitive effects of multiple activities have complicated characterization of T7 DNA primase activity. For example, although the T7 helicase preferentially hydrolyzes dTTP as an energy source to unwind double-stranded DNA, the enzyme also hydrolyzes ATP (9). Hence, this activity would dramatically lower the concentration of ATP in primase assays and therefore artificially decrease rates of primer synthesis measured.

We have recently isolated the DNA primase from bacteriophage T7 as a 30-kDa peptide fragment containing only the N-terminal 271 amino acids (14). This primase fragment lacks dTTPase and helicase activities but retains primase activity (14). We showed in the accompanying paper that at saturating concentrations of DNA, the primase fragment catalyzes oligoribonucleotide synthesis at rates even higher than those of the 63-kDa gene 4 protein (16). This system, requiring only a 30-kDa protein, a 7-nucleotide DNA template, NTPs, and Mg2+加快发展，provides an elegant model system to study template-directed nucleic acid polymerases. Here, we use the primase fragment to examine the rates of each step in oligoribonucleotide synthesis and to examine the binding of the NTP precursors required for the oligoribonucleotide initiation. Such studies would be impossible using the full-length gene 4 protein since NTPs are rapidly hydrolyzed as the energy source for translocation of the helicase domain.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized and purified by Integrated DNA Technologies (Corvalis, IA). Their sequences are listed in the Results or Fig. Legends. Primase recognition sites are underlined. The concentration of each DNA oligonucleotide (moles 3-ends/L) was calculated from \( A_{260} \) and its extinction coefficient. The primase fragment contains residues 1–271 of the 63-kDa gene 4 protein and was purified as described (14). The gene 4 protein, a 63-kDa gene 4 protein encoded by a T7 gene 4 gene, was isolated from Escherichia coli smalls (17). This mutant allele supports the growth of phage lacking gene 4, and the enzymatic activities of the M64G protein are indistinguishable from wild-type 63-kDa gene 4 protein (17). The 63-kDa M64G allele was modified to code for a protein containing only the N-terminal 271 amino acids of the T7 gene 4 protein. We refer to this peptide as the primase fragment because it lacks dTTPase and helicase activities but retains primase activity (14). NTPs and radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. The ATP analogues \( \alpha,\beta\)-methylene ATP, \( \beta,\gamma\)-methylene ATP, and \( \beta,\gamma\)-imidodiphosphate were purchased from Sigma.

The CTP analogue 3'-dCTP was synthesized from 3'-deoxyoctosine as follows. Phosphorus oxychloride (230 mg, 1.5 mmol) was added dropwise to a stirred, cool (0–5 °C) solution of 3'-deoxyctydine (227 mg, 1 mmol) in a mixture of trimethyl phosphate and triethylamine (1:1, 6 ml). After stirring for 3 h, the reaction mixture was added dropwise to a stirred, cool (0–5 °C) solution of tri-n-butyl ammonium pyrophosphate (5 mmol) and tributylamine (5 mmol). After stirring for 10 min, the reaction mixture was quenched with a 1 M triethylammonium bicarbonate solution (20 ml, pH 7.5) and stirred at room temperature for 2 h. The crude mixture was purified on Sephadex A-25 column using a linear gradient from 0 to 1 M triethylammonium bicarbonate buffer.

This paper is available online at http://www.jbc.org.
(pH 7.5). The triphosphate peak fractions were collected, concentrated in vacuo, and purified by reserve phase high pressure liquid chromatography (Waters Delta-Pak™ 15 micron C-18 column (1.9 x 30 cm)) using a gradient of 0–100% buffer A (0.1 M triethylammonium bicarbonate) and buffer B (25% acetonitrile in 0.1 M TEAB) at 12 ml/min. Yield was 150 mg of 3′-dCTP. Analysis of the product by 32P NMR (ppm) (D2O/EDTA) gave three peaks: 10.17 (dimer), 10.65 (dimer), and 22.71 (trimer).

**Primase Assays—Oligoribonucleotide synthesis was measured as described in the accompanying paper (16). Except for the experiment described in Fig. 1, all reactions were carried out in the presence of 10 mM free Mg2+. Assuming each mol of NTP binds 1 mol of Mg2+ tightly, NTPs were premixed with equal molar concentrations of MgCl2 before use, and 10 mM additional MgCl2 was added to each reaction. Oligoribonucleotide extension reactions were performed under conditions similar to those described for the oligoribonucleotide synthesis assay (16) except that the extension of the ribonucleotide 5′-ACC-3′ was monitored. The primer fragment was incubated with 0.44 mM of 5′-ACC-3′, 50 mM DNA template, and 1 mM of [α-32P]NTP. Reactions contained 50 mM template, 40 mM Tris-Cl, pH 7.5, 50 mM potassium glutamate, 10 mM MgCl2, 10 mM dithiothreitol, 50 μM bovine serum albumin, 50 mM potassium glutamate, and the indicated concentrations of primer fragment.

After incubation at 23 °C for 30 min, the reactions were stopped by the addition of 10 μl of stop solution (98% formamide, 10 mM EDTA, pH 8.0, 0.1% xylene cyanol FF, and 0.1% bromphenol blue). Samples were heated to 95 °C for 5 min, the products were separated by electrophoresis on a 25% polyacrylamide gel containing 3 M urea, and amounts of products were measured using phosphorimaging analysis with a Fuji BAS 1000 Bio-imaging analyzer.

**RESULTS**

The synthesis of oligoribonucleotides by the T7 DNA primase requires ATP and CTP, divalent cations, and a ssDNA template (1). On natural ssDNA templates, most of these primers are synthesized at specific DNA sequences designated “primase recognition sites,” the majority of which contain the sequence 5′-GTGTC-3′ or 5′-GTGTGC-3′ (7). Oligoribonucleotide synthesis begins opposite the T in the recognition site, and all primers have the triphosphate moiety of ATP retained at the 5′-terminus (1, 18). The essential 3′-C in the recognition site is not copied into the primer and is designated as “cryptic” (7, 19).

The primase makes contact with the DNA via interactions with the two initiating NTPs, the cryptic C, and the two bases flanking the 3′-end of the cryptic C. As a consequence, although a 5-nucleotide DNA template (i.e. 5′-GGGTC-3′) is sufficient to support oligoribonucleotide synthesis, a 7-nucleotide template such as 5′-GGGTCAA-3′ provides for optimal synthesis (16). DNA binds more tightly to the helicase domain of the 63-kDa gene 4 protein that lacks helicase and NTPase activities but retains the ability to synthesize oligoribonucleotides.

**Synthesis of Oligoribonucleotides on Templates Containing Truncated Primase Recognition Sites**—The primase fragment requires only DNA containing a primase recognition site in addition to ATP, CTP, and divalent metal ions to catalyze the synthesis of short template-directed oligoribonucleotides (16). All known T7 primase recognition sites on natural DNA templates share the core trinucleotide sequence 5′-GTTC-3′, and in the presence of synthetic templates containing this sequence, the T7 primase catalyzes the synthesis of the dinucleotide 5′ppAC from precursors ATP and CTP (20). Because this trinucleotide sequence occurs much more frequently in DNA than longer primase recognition sites such as 5′-GGGTTC-3′, it was of interest to determine the relative efficiency with which the primase synthesizes oligoribonucleotides when given only this trinucleotide sequence.

Oligoribonucleotide synthesis reactions were performed using three different DNA templates composed of the sequences 5′-TGGTCAA-3′, 5′-GGTCAA-3′, and 5′-GTTCAA-3′, which differ only in the length of the primase recognition site. Reactions contained 1 mM ATP, 1 mM [32P]CTP (100 Ci/mmol), 50 mM primer fragment (determined using the monomer molecular weight) and 80 μM DNA template. After 30 min, the products were separated from [α-32P]CTP by electrophoresis on 25% PAGE containing 3 M urea. A, products of reactions containing the template 5′-GGTCAA-3′. B, products of reactions containing the template 5′-GGTCAA-3′. C, products of reactions containing the template 5′-GTTCAA-3′. The gels in A–C show the products of reactions containing either 1 mM EDTA (lane 1), 0.1 mM MgCl2 (lane 2), 0.4 mM MgCl2 (lane 3), 1.2 mM MgCl2 (lane 4), 4 mM MgCl2 (lane 5), 8 mM MgCl2 (lane 6), or 16 mM MgCl2 (lane 7). D, the amount of each product in A–C was measured by phosphorimaging analysis, and the velocity of oligoribonucleotide synthesis (pmol of NMP incorporated/min) is reported for reactions containing the template 5′-GGGTCAA-3′ (△), 5′-GGTTCAA-3′ (□), or 5′-GTTCAA-3′ (○).
Both the rate and the length of the products synthesized are dependent on the length of the template. In the presence of the template 5'-TGGTCAA-3' (Fig. 1A), the primase fragment catalyzes the synthesis of di-, tri-, and tetraribonucleotides, and oligoribonucleotide synthesis extends to the end of the DNA template. The oligoribonucleotides are truncated when shorter DNA templates are used. In the presence of 5'-GGGTCAA-3', the primase fragment synthesizes only di- and trinucleotides (Fig. 1B); in the presence of 5'-GTCAA-3', only dimers are produced (Fig. 1C). The data indicate that when there is no other option, the primase fragment synthesizes dinucleotides quite rapidly. In the presence of longer DNA template sequences, the primase can either extend the dinucleotide or reinitiate the synthesis of another dinucleotide. As a result, under conditions allowing for dinucleotide extension, the overall rate of oligoribonucleotide synthesis is actually lower than rate of synthesis of the dinucleotide.

Effect of Divalent Metal Cations on Oligoribonucleotide Synthesis—Previous reports have suggested that the 63-kDa gene 4 protein catalyzes the synthesis of dinucleotides in the absence of an added DNA template, albeit at a rate much lower than that observed in the presence of a template (21). This DNA-independent dinucleotide synthesis is enhanced in the presence of MnCl₂ (22). Because only dinucleotides are synthesized by the gene 4 protein in the absence of DNA and the rate of this reaction is dependent on the nature of the divalent cation, divalent cations may play a different role in the synthesis of dinucleotides than in the extension of dinucleotides. Consequently, we examined the rates of di-, tri-, and tetraribonucleotide synthesis in reactions containing the primase fragment and various concentrations of divalent metal cations.

In reactions lacking template, and containing from 0 to 16 mM of either MgCl₂ or MnCl₂, no oligoribonucleotide synthesis was detected (data not shown). We conclude that all oligoribonucleotide synthesis catalyzed by the primase fragment requires the presence of a DNA template. Perhaps the template-independent dinucleotide synthesis catalyzed by the full-length gene 4 protein results from an activity of the helicase domain. In support of this notion, the 56-kDa protein that lacks primase activity retains the Mn²⁺-dependent ability to synthesize dinucleotides (23). It is also possible that a slight contamination of the 63-kDa gene 4 protein with DNA led to these earlier observations. Unlike the primase fragment, which is readily separated from cellular DNA (14), the full-length gene 4 protein binds tightly to DNA (24).

In reactions containing template, it is clear that the rate of oligoribonucleotide synthesis is dependent on the Mg²⁺ concentration (Fig. 1). Similar rates were observed when MnCl₂ was substituted for MgCl₂ (data not shown). The amount of each product from each reaction shown in Fig. 1 (A—C) was measured by phosphorimaging analysis by comparing the densities of the autoradiographs generated from each reaction with the appropriate standard. The total amount of NMPs incorporated into each product was divided by the total amount of NMPs incorporated into all of the products detected by each reaction. These values were averaged and multiplied by 100 to determine the percentage of reactions that terminate after the synthesis of a particular product. The ratios were averaged for each reaction. The results are reported from the leftmost product (left) to the rightmost (right) for each reaction.

Frequency of Abortive Oligoribonucleotide Synthesis—In the presence of the template 5'-GTCAA-3', 100% of the products detected are dinucleotides (Fig. 1C). In the presence of 5'-GGGTCAA-3', about half of the products are dinucleotides, and half are trinucleotides (Fig. 1B). However, in the presence of the template 5'-GGGGGTCAA-3', most of the products are tetraribonucleotides and trinucleotides, whereas only a small amount are trinucleotides (Fig. 1A). Because of the striking difference in the relative amounts of the di- and trinucleotide products, we next examined the ratios of the various products formed on longer DNA templates. On a natural DNA template such as M13 ssDNA, the gene 4 synthesized primers used by the T7 polymerase are mainly tetraribonucleotides (1). With synthetic DNA templates, T7 primase can synthesize longer oligoribonucleotides on templates containing long stretches of dG residues 5' to the recognition sequence (25). To reexamine this reaction in the absence of the helicase domain, the activity of the primase fragment was examined in the presence of longer DNA templates in which the template was extended on the 5' end by the addition of dG residues.

In the experiment shown in Fig. 2, the primase fragment was incubated in the presence of [α-32P]CTP, ATP, and five different DNA templates in which the 5'-end was extended by additional dG residues. Aliquots of each reaction were quenched after 0.5, 1, 1.5, 3, and 5 min, and the products were separated from [α-32P]CTP by gel electrophoresis. Indeed, the primase fragment retains the ability to synthesize longer oligoribonucleotides on templates containing stretches of guanosine residues 5' to the primase recognition site. Moreover, the total amount of all the products observed was linear over the time course of the experiment, and the overall rate of oligoribonucleotide synthesis on all of the templates varied less than 2-fold. The amount of NMPs incorporated into each product was di-
Fig. 3. Kinetic analysis of dinucleotide synthesis. Oligonucleotide synthesis reactions were performed on a template that supported the synthesis of only dinucleotides. The T7 primase fragment (12.5 nM) was incubated with the DNA template 5'-GTC-GAA-3' (65 μM) at six different concentrations of ATP ranging from 0.033 to 3 mM, and six different concentrations of α-32P-CTP (100 Ci/mol) from 0.1 to 3.2 mM. Under these conditions, the only product (pppAC) was separated from α-32P(CTP) using PAGE and then measured by phosphorimaging analysis. A, plot of velocity versus ATP at 0.1 mM (+), 0.2 mM (○), 0.4 mM (♦), and 0.8 mM (×), 1.6 mM (◇), 3.2 mM (□) CTP. Data were fit to the equation: 

\[ v = V_{\text{max}}a_1 + \frac{V_{\text{max}}K_{\text{m(A TP)}}}{K_{\text{m(CTP)}} + [\text{ATP}]} + \frac{V_{\text{max}}}{K_{\text{m(CTP)}}} \] 

by nonlinear regression analysis (26). B, kinetic scheme for T7 primase fragment catalyzed dinucleotide synthesis from ATP and CTP.

Provided by the total amount of NMPs incorporated into all of the products detected. This value gives an estimate of the frequency with which synthesis is aborted after the synthesis of that particular oligoribonucleotide product. For example, in most reactions, approximately 30% of the NMPs are incorporated into dinucleotides, indicating that 30% of reactions stop after synthesis of the dimer. This value for the various products remained constant over the time course of the experiment, indicating that the shorter products are not synthesized first and then extended, which confirms that the shorter products result from abortive primer synthesis. To more concisely report the results of the data from this experiment, the percentages of each product at each time point were averaged, and the averages are reported in Fig. 2.

These data show that, although the primase fragment will synthesize oligoribonucleotides to the end of DNA containing a primase recognition site, the frequency of abortive primers increases with the length of the template. In reactions containing the DNA template 5'-GGGTCAA-3', 68% of the NMPs are incorporated into the tetramer pppACACC, and 20% are incorporated into the dinucleotide. Less than 6% of the NMPs are incorporated into the trimer and a small amount (4%) of NMPs are incorporated into pentamers pppACCCA and pppACCCCA. When the DNA template is lengthened by a single nucleotide, the most obvious trend in the data is that fewer full-length products are synthesized on longer DNA templates (Fig. 2), suggesting that the ultimate length of oligoribonucleotides synthesized by the primase is limited. Secondary, of all the abortive products observed, the dimers are always the most common and account for 30 ± 10% of the products regardless of the length of the DNA template. This accumulation of dinucleotides provides evidence that the dinucleotide is synthesized more rapidly than it is extended.

Kinetic Analysis of Dinucleotide Synthesis—To determine the kinetic constants describing the binding of the initiating NTPs to the enzyme, the T7 primase fragment (12.5 nM) was incubated with the DNA template 5'-GTC-GAA-3' (65 μM) at six different concentrations of ATP ranging from 0.033 to 3 mM, and six different concentrations of α-32P(CTP) (100 Ci/mol) from 0.1 to 3.2 mM. Under these conditions, the only product of the reaction, pppAC, can be separated from α-32P(CTP) using PAGE and then measured by phosphorimaging analysis. The rate of dinucleotide synthesis is clearly hyperbolically dependent on ATP or CTP concentration (Fig. 3A). To distinguish between the three basic bisubstrate kinetic mechanisms, the data were fit to the equations describing ping-pong and both ordered and random bisubstrate reactions. To obtain initial estimates of the kinetic constants needed for regression analysis, the data in Fig. 3A was initially fit to the Michaelis-Menten equation: 

\[ v = \frac{V_{\text{max}}[\text{NTP}]}{K_{\text{m}} + [\text{NTP}]} \]

This analysis yields an apparent \( K_m \) and \( V_{\text{max}} \) for each concentration ATP and CTP, and secondary plots of the apparent \( K_m \) values and \( V_{\text{max}} \) values provide estimates of the kinetic constants. Because the \( K_m \) values of ATP or CTP can be defined as the \( K_m \) of one NTP at infinite concentrations of the other NTP (Fig. 3B), these values were determined by plotting apparent \( V_{\text{max}} \) values for one NTP at specific concentrations of the other NTP. This method yields a \( K_m \) (CTP) of 1.1 mM, a \( K_m \) (ATP) of 0.63 mM, and a \( k_{\text{cat}} \) for dimer synthesis of 5.2 s⁻¹ (assuming one active site/protein monomer). A linear extrapolation of a secondary plot showing the 1/\( K_m \) (app) of ATP at different CTP concentrations yields a \( K_m \) (ATP) of 0.1 mM, and a plot of 1/\( K_m \) (app) of CTP versus ATP yields a \( K_m \) (CTP) of 0.2 mM.

Using these initial estimates, all of the data in Fig. 3 were fit by nonlinear regression analysis (26) to the appropriate equations describing ping-pong, ordered, and random bisubstrate
measured by phosphorimage analysis. A 3.2 mM (at 9-GGGTC-3') was incubated at 23 °C for 30 min with the DNA template 5'-GGGTCAAAAA-3' (65 μmol) at seven different concentrations of ATP ranging from 0.033 to 3 mM, and seven different concentrations (α-32P)CTP (100 Ci/mmol) from 0.1 to 6.4 mM. Under these conditions, the two major products are a dimer (pppA) and a tetramer (pppACCC). The products were separated from (α-32P)CTP using PAGE and measured by phosphorimage analysis. A, rates of dimer (□), trimer (△), and tetramer (○) formation at 2 mM ATP and seven different concentrations of CTP ranging from 0.1 to 6.4 mM. B, rates of dimer (□), trimer (△), and tetramer (○) formation at 0.2 mM CTP and seven different concentrations of ATP ranging from 0.033 to 3.0 mM. C, ratio of tetramer synthesis to dimer synthesis at seven different concentrations of ATP ranging from 0.033 to 3.0 mM and concentrations of CTP of 6.4 mM (○), 3.2 mM (□), 1.6 mM (△), 0.8 mM (△), 0.4 mM (◇), 0.2 mM (○), and 0.1 mM (+).

The data fit best to an equation describing a random bimolecular reaction as judged by minimal least squares and variances of fit, a χ² test of one, and minimal random positive and negative residuals. The refined constants are shown on the kinetic scheme shown in Fig. 3B, and curves generated using these constants are shown along with the data in Fig. 3A. The enzyme binds ATP 2.6-fold more tightly than CTP, suggesting that at equal molar concentration of ATP and CTP, ATP will bind first. Moreover, there is a small 2.7-fold decrease in the affinity of the primase for the second substrate if the first NTP is already bound. The biological significance of this kinetic mechanism remains unclear, but at the high physiological concentrations of ATP (>5 mM), ATP may be perpetually bound to the primase, making primer initiation particularly sensitive to cellular CTP concentrations.

The Rate of Dinucleotide Extension Is Dependent on the Concentration of Both ATP and CTP—After the synthesis of the dinucleotide, the next step in primer synthesis involves the extension of the dinucleotide to the tetranucleotide, which functions as a primer for T7 DNA polymerase (1). To examine the regulation of dinucleotide extension, the synthesis of oligoribonucleotides on templates containing the recognition sequence 5'-GGGTCT-3' was examined under various conditions. The fidelity of oligoribonucleotide synthesis reactions were performed on a template containing the primer recognition site 5'-GGGTCT-3' at various concentrations of ATP and CTP. The T7 primase fragment (50 nm) was incubated at 23 °C for 30 min with the DNA template 5'-GGGTCTCAAAAAA-3' (65 μmol) at seven different concentrations of ATP ranging from 0.033 to 3 mM, and seven different concentrations (α-32P)CTP (100 Ci/mmol) from 0.1 to 6.4 mM. Under these conditions, the two major products are a dimer (pppA) and a tetramer (pppACCC). The products were separated from (α-32P)CTP using PAGE and measured by phosphorimage analysis. A, rates of dimer (□), trimer (△), and tetramer (○) formation at 2 mM ATP and seven different concentrations of CTP ranging from 0.033 to 3.0 mM, and seven different concentrations of ATP ranging from 0.033 to 3.0 mM. C, ratio of tetramer synthesis to dimer synthesis at seven different concentrations of ATP ranging from 0.033 to 3.0 mM and concentrations of CTP of 6.4 mM (○), 3.2 mM (□), 1.6 mM (△), 0.8 mM (△), 0.4 mM (◇), 0.2 mM (○), and 0.1 mM (+).

Fig. 4. Effect of ATP and CTP concentrations on dinucleotide extension. Oligoribonucleotide synthesis reactions were performed on a template containing the primer recognition site 5'-GGGTCT-3' at various concentrations of ATP and CTP. The T7 primase fragment (50 nm) was incubated at 23 °C for 30 min with the DNA template 5'-GGGTCTCAAAAAA-3' (65 μmol) at seven different concentrations of ATP ranging from 0.033 to 3 mM, and seven different concentrations (α-32P)CTP (100 Ci/mmol) from 0.1 to 6.4 mM. Under these conditions, the two major products are a dimer (pppA) and a tetramer (pppACCC). The products were separated from (α-32P)CTP using PAGE and measured by phosphorimage analysis. A, rates of dimer (□), trimer (△), and tetramer (○) formation at 2 mM ATP and seven different concentrations of CTP ranging from 0.033 to 3.0 mM, and seven different concentrations of ATP ranging from 0.033 to 3.0 mM. C, ratio of tetramer synthesis to dimer synthesis at seven different concentrations of ATP ranging from 0.033 to 3.0 mM and concentrations of CTP of 6.4 mM (○), 3.2 mM (□), 1.6 mM (△), 0.8 mM (△), 0.4 mM (◇), 0.2 mM (○), and 0.1 mM (+).

The fidelity of oligoribonucleotide extension—The role of the template in NTP selection was next examined by comparing the rate of incorporation of each of the four canonical NTPs on defined DNA templates. To estimate the fidelity of oligoribonucleotide synthesis by the primase fragment, a minimal DNA template was used in the presence of a synthetic triribonucleotide ACC. In addition to synthesizing oligoribonucleotides de novo, the T7 gene 4 protein also catalyzes the extension of di- and trinucleotides to functional tetranucleotide primers at specific primer recognition sites starting with a cryptic C (27). The primase fragment also catalyzes oligoribonucleotide extension (14). Hence, the trimer is extended to a tetramer in the presence of a template containing the recognition sequence (Fig. 5). The rate of insertion of each of the four α-32P-labeled NTPs was measured in the presence of two different DNA templates 5'-GGGTCTA-3' and 5'-CTCTCA-3' (Fig. 5). When the amount of enzyme routinely used for rate determination was used (0.05–0.1 μmol enzyme), no misincorporation was detected. However, when 100 times more enzyme was used, misincorporation was detectable. Not surprisingly, the insertion of nucleotides by the primase is template-directed. When the primase is presented with a template dT, the enzyme preferentially inserts AMP (Fig. 5A), and when the enzyme is presented with a template dG, the enzyme inserts CMP (Fig. 5B). The fidelity of the enzyme was calculated by dividing the turnover rate of the enzyme when the correct nucleotide is inserted by the tested, the amount of trimer formed was substantially less than the amount of either dimer or tetramer observed. It is noteworthy that the rate of tetramer formation was lower than the rate of dimer formation at concentrations of CTP less than 1 mM. The rate of tetramer formation increases with increasing CTP concentration until it exceeds the rate of dimer formation. The opposite pattern is seen when the concentration of ATP is varied, whereas the concentration of CTP is held constant at 0.2 mM. In Fig. 4B, the rates of appearance of the dimer, the trimer, and the tetramer are plotted versus ATP concentration. Again, under these conditions, the amount of trimer produced is less than 1/10 that of either the dimer or the tetramer. The amount of tetramer synthesis decreases at concentrations of ATP above 1 mM. Nevertheless, under the same conditions, the amount of dimer produced steadily increases with the concentration of ATP. In summary, frequency of dinucleotide extension, which can be simply defined as the rate of tetramer formation divided by the rate of dimer formation, increases with increasing CTP concentrations (at 1 mM ATP, Fig. 4A) and decreases with increasing concentration of ATP (at 0.2 mM ATP, Fig. 4B).

To further examine the effect of NTP concentration on the length of products formed, all data from this experiment were analyzed comparing the rate of formation of dinucleotides and tetranucleotides. The amount of tetramers formed at each concentration of ATP and CTP was divided by the amount of dimers formed. This value compares the relative rate of dinucleotide extension with dinucleotide formation. At high concentrations of ATP, the enzyme less frequently extends dinucleotides, and the production of dimers is favored. Moreover, this pattern is seen at all concentrations of CTP tested. Other, more complex trends are apparent in the plot in Fig. 4C. For example, in contrast with Fig. 4A, which was performed at 1 mM ATP, at lower concentrations of ATP (<0.3 mM), the dinucleotide extension decreases with increasing concentrations of CTP (>0.8 mM). These trends hamper the fitting of these data to a single equation. However, in general, dinucleotide synthesis is favored over extension at high concentrations of ATP or CTP, and dinucleotide extension is favored at low concentrations of ATP.
turnover rate of the enzyme for the insertion of the incorrect nucleotide. The T7 primase is 200–900 times more likely to insert a “correct” nucleotide than a nucleotide that could form non-Watson-Crick base pair.

In most of the trinucleotide extension reactions (Fig. 5) only a single product, a tetranucleotide, was detected. However a small amount of a second product was observed in reactions containing [α-32P]ATP and high concentrations of enzyme. This product migrates in the gel at the same location as a dinucleotide pppAA. The formation of a dinucleotide only in reactions containing ATP is in agreement with the previous observation that all primers synthesized by gene 4 protein initiate with ATP (1, 28). It is possible that this dinucleotide is synthesized in a template-independent manner or is due to the misincorporation of AMP opposite the dG at the primase recognition site 5'-GTC-3'. The experiments described in the next section were designed to differentiate between these two possibilities.

The Fidelity of Dinucleotide Synthesis—It has been suggested that the dinucleotide pppAC can be synthesized in a template-independent manner and that this dimer, bound tightly by the enzyme, can be used to locate the recognition sequence 5'-GTC-3' in DNA (22, 25). The lack of oligoribonucleotide synthesis by the primase fragment in the absence of template (14) argues against this hypothesis. However, the observed formation of a dimer pppAA (Fig. 6A, lane 1) again raises the possibility of template-independent dinucleotide synthesis. To determine the role of the template in dinucleotide synthesis by the primase fragment, oligoribonucleotide synthesis reactions were performed at various concentrations of CTP, [γ-32P]ATP, and a template containing the primase recognition site 5'-GGGTC-3'. The pppAA dimer forms in the presence of a DNA template containing a primase recognition site 5'-GGGTC-3' (Fig. 6A, lane 1), suggesting that an AMP is actually misincorporated against a template dG. In the absence of DNA no dinucleotide is synthesized (data not shown). When CTP, which forms a Watson-Crick base pair with the template dG, is added to the reaction mixture, the production of a pppAA dimer is inhibited, and virtually no pppAA is detected even at the lowest concentration (0.06 mM) of CTP tested (Fig. 6A, lane 2). The formation of a pppAC dimer, as well as the dinucleotide extension to the tetranucleotide pppACCC is also observed, and the rate of these reactions is clearly dependent on the concentration of CTP (Fig. 6A, lanes 2–6).

The fidelity of dinucleotide synthesis was examined in more detail by repeating the experiment shown in Fig. 6A substituting other canonical nucleoside triphosphates for CTP. No additional oligonucleotides (besides pppAA) were observed in the presence of ATP, and GTP, UTP, dGTP, dATP, or dCTP. Hence, the T7 primase clearly prefers to synthesize the dinucleotide pppAC at the recognition site 5'-GTC-3', although AMP may be misincorporated versus a template dG at a low rate. The misincorporation of AMP occurs at a rate 1/100 that of the incorporation of CMP. Furthermore, the primase specifically incorporates ribonucleotides; 2'-deoxyribonucleotides cannot substitute.

To examine the specificity of the primase with regard to the nucleotide sugar in more detail, we have synthesized the nucleotide analogue 3'-dCTP. If the primase solely requires the presence of a 2'-OH functional group, then 3'-dCTP, which retains the 2'-OH, may be incorporated by the primase. If 3'-dCTP is incorporated efficiently by the primase, then it could be a potent primase inhibitor because the nucleotide lacks a 3'-OH necessary for the subsequent nucleotidyl transfer reaction. Fig. 6B compares the ability of the primase to synthesize oligonucleotides in the presence ribo, 2'-deoxy-, and 3'-deoxyCTP. The primase clearly prefers ribonucleoside triphosphates, and the elimination of either the 2' or 3' hydroxyl groups decreases the rate of oligonucleotide synthesis by over 100-fold. This observation demonstrates that the 2'-OH is not the sole determinant of primase sugar specificity. Although 2'-dCMP is not incorporated at any detectable rate, some oligonucleotide synthesis occurs in the presence of high levels of 3'-dCTP (1 mM). The products of oligonucleotide synthesis in the presence of CTP (Fig. 6C, lane 1) or 3'-dCTP (Fig. 6C, lane 2) are distinctively different. In the presence of ATP, CTP, and the primase recognition site 5'-GGGTC-3', the primase synthesizes di-, tri-, and tetranucleotides. However, when CTP is replaced with 3'-dCTP, only a single product is detected. This product...
NTP Binding by the T7 Primase

was not directly identified, but it is most likely a dinucleotide with $[\gamma^{32}P]ATP$ at the 5'-end and 3'-dCMP at the 3'-end. This conclusion is supported by the facts that the product retains the $\gamma$-phosphate from ATP and migrates in the gel more rapidly than pppAA or pppAC.

To determine the relative affinity of the primase for modified NTPs, we made several attempts to measure the $K_i$ of 3'-dCTP. Because the 3'-dCTP is incorporated as a chain terminator at a low rate relative to CTP (Fig. 6B), the analogue is a weak inhibitor of the oligoribonucleotide synthesis in reactions containing ATP and CTP with a $K_i$ of approximately 2 mM assuming competitive inhibition. One experiment comparing the relative affinity of the enzyme for CTP and 3'-dCTP is shown in Fig. 6D. The formation of a pppAA dimer is inhibited by the nucleotide analogue 3'-dCTP, which like CTP, can base pair with a template dG. When the primase fragment is titrated with either 3'-dCTP or CTP, the formation of the pppAA dimer is inhibited (Fig. 6D). In these conditions, 50% inhibition occurs as either 80 $\mu$M CTP or 120 $\mu$M 3'-dCTP, indicating that the two nucleotides bind the enzyme with similar affinities. We conclude that both nucleotides bind the enzyme opposite a dG residue similarly to inhibit the misincorporation of AMP versus a template dG (Fig. 6B). Although the two compounds bind the enzyme with similar affinities, 3'-dCMP is incorporated into dinucleotides at a much lower rate than CMP (Fig. 6B), suggesting a role of the nucleotide sugar in properly orienting the NTP in the active site of the enzyme.

**Effect of Modifications of the Triphosphate Moiety on Oligoribonucleotide Synthesis**—At the primase recognition site 5'-TG-GTC-3' the T7 primase incorporates ATP at the 5'-end of the oligoribonucleotides and AMP at the 3'-end of the oligoribonucleotides pppACC. The nucleotide analogue $\alpha,\beta$-methylene ATP contains a Cl$_2$ group between the first and second phosphates of ATP, which should block the nucleotide transfer reaction necessary for AMP incorporation at the 3'-end of the oligoribonucleotides. One would therefore expect $\alpha,\beta$-methylene ATP to be a potent inhibitor of the T7 primase. However, when reactions containing ATP, $[\gamma^{32}P]CTP$, the primase fragment, and the template 5'-TG-GTC-3' are titrated with $\alpha,\beta$-methylene ATP, little inhibition of oligoribonucleotide synthesis occurs. In fact, a slight stimulation (less than 2-fold) occurs in the presence of the highest concentrations of $\alpha,\beta$-methylene ATP. The products of four reactions containing increasing amounts of $\alpha,\beta$-methylene ATP are shown on the autoradiogram of the gel in Fig. 7A. When the experiment is repeated in the absence of ATP, the rate of oligoribonucleotide synthesis is now dependent on the concentration of $\alpha,\beta$-methylene ATP with no synthesis occurring in the presence of only CTP. The major products of the reaction are dinucleotides and trinucleotides. The tetranucleotide is not synthesized. A small amount of the tetranucleotide pppACC is seen resulting from the misincorporation of CMP. This finding indicates that $\alpha,\beta$-methylene ATP is readily incorporated at the 5'-end as the initiating nucleotide of the oligoribonucleotide but is not a substrate for the incorporation of AMP at the 3'-end of oligoribonucleotides.

When the methylene group is instead placed between the second and third phosphate of ATP, as in the nucleotide analogue $\beta,\gamma$-methylene ATP, this analogue is likewise incorporated only at the 5'-end of the of the RNA primer (Fig. 7B). When the experiment was repeated in the presence with the ATP analogue $\beta,\gamma$-imido ATP, the ATP analogue was incorporated at both ends of the oligoribonucleotide (Fig. 7C). However, the amount of tetramer produced relative to the amount of dimer is less than that in reactions containing ATP instead of $\beta,\gamma$-imido ATP. Hence, the majority of the products are the trinucleotides, suggesting that the imido group $\beta,\gamma$-imido ATP hampers the transfer of AMP to the 3'-end of the oligoribonucleotide.

**DISCUSSION**

Bacteriophage T7 provides a model system to study the biochemistry of proteins involved in DNA replication because of the relatively few required proteins (29). The coordinated synthesis of both leading and lagging DNA strands requires only four proteins, the T7 gene 2.5 protein, the T7 gene 4 helicase/primase, the T7 gene 5 DNA polymerase, and *Escherichia coli* thioredoxin. At the core of this complex is the product of T7 gene 5, a DNA polymerase (30) that also has a 3'-5' proofreading exonuclease (31). The host *E. coli* thioredoxin forms a tight complex with the T7 gene 5 protein, greatly enhancing the processivity of the polymerase (32, 33). The 63-kDa T7 gene 4 protein is a DNA helicase and, in addition, as a primase, catalyzes the template-directed synthesis of oligoribonucleotides (7, 9–11, 34). These short oligoribonucleotides serve as primers for T7 DNA polymerase (28). The final component of the T7 replisome, the gene 2.5 protein, is a single-stranded DNA-binding protein (35) that is important in the coordination of DNA synthesis (36). Of all the enzymatic reactions taking place at the T7 replication fork, the least well characterized is the synthesis of oligoribonucleotide primers by the DNA primase. Previously kinetic studies of the primase reaction were complicated by the fact that the gene 4 protein contains, besides primase activity, a helicase activity fueled by the hydrolysis of nucleoside triphosphates. Any activity affecting NTP pool levels would affect the rates of primer synthesis, because NTPs are the precursors of oligoribonucleotide synthesis. Moreover, the DNA binding and the 5'-3' tranlocation of the protein on ssDNA undoubtedly affects the frequency with which the primase encounters primase recognition sites. Although many gene 4 point mutants have been isolated or constructed that affect helicase or primase activities, the only way to unambiguously study primase function is to isolate primer apart from any helicase activity.

We have isolated the primase function of the T7 gene 4 protein independent from the helicase function by expressing...
and purifying the N-terminal 271 residues of the T7 gene 4 protein (14). The ability to isolate the primase activity as a separate functional peptide confirms that the activity resides in a separate protein domain. Although this primase fragment binds DNA 100-fold less tightly than the full-length protein because of the absence of the helicase domain, the primase fragment synthesizes oligoribonucleotides as rapidly as the full-length protein. Under optimal conditions, the rate of RNA synthesis catalyzed by the primase fragment reflects an enzyme turnover rate of 1–5/s, a rate more than sufficient to support coupled simultaneous synthesis of both strands of DNA (16). If the synthesis of the leading strand proceeds in a continuous fashion, then the lagging strand must be synthesized in a discontinuous manner requiring the frequent initiation of new Okazaki fragments. Assuming a 3,000-nucleotide average length for Okazaki fragments (36–38), the rate of primer synthesis is sufficient to facilitate T7 DNA synthesis at the measured rate of 300 nucleotides/s (39). We have used the primase fragment to examine the kinetics of oligonucleotide synthesis by the T7 primase in the absence of helicase and NTPase activities. Several conclusions may be drawn involving the nucleotide binding events required to initiate dinucleotide synthesis and to extend the dinucleotide to a functional primer. We use the data to propose a model for oligoribonucleotide synthesis based heavily on the mechanism of RNA strand initiation of RNA polymerase (40, 41). The model is depicted in Fig. 8.

T7 primase catalyzes the synthesis of oligoribonucleotide primers in a minimum of four discrete steps: NTP binding, dinucleotide formation, extension of the dimer to a trimer, and trimer extension to the functional tetramer. The simple mechanism for primer synthesis outlined in Fig. 8 involves the utilization of only two nucleotide-binding sites during primer synthesis. Because all known polymerases add nucleotides in a 3′ → 5′ direction, we assume that the T7 primase is not different. Hence, the nucleotide-binding site where nucleotide incorporated at the 5′-end of the primer binds is designated the “initiation site.” Nucleotides that are added to elongate the 3′-end of the primer bind at a second site referred to as the “elongation site.” As has been previously demonstrated for E. coli RNA polymerase (42) and E. coli primase (43), the initiation site may be sufficiently flexible to either bind a single nucleotide or a short oligonucleotide. Hence, at each step of primer synthesis, the product (N+1) oligonucleotide may be transferred to the initiation site so that another NTP may bind to the elongation site. The length of the oligonucleotide that can bind in the initiation site limits the final length of the oligoribonucleotide synthesized by the primase. Hence the frequency of abortive primers increases with template length (Fig. 2).

During the first step in oligoribonucleotide synthesis, the first nucleotide incorporated, ATP, is bound by the primase at the initiation site. The second nucleotide to be incorporated binds the enzyme at the elongation site. The affinity of the enzyme for ATP is 2.6-fold higher than for CTP, suggesting that unlike the primase from calf thymus (46), the first nucleotide to bind the enzyme may be incorporated at the first position of the primer. Another important difference between the two nucleotide-binding sites is their specificity. The initiation site only binds ATP or a 5′-A terminated oligonucleotide, as evidenced by the fact that all oligoribonucleotides begin with ATP. In contrast the specificity of elongation site is template-directed (Fig. 5). Even during the synthesis of the dinucleotide, the binding of the NTP to the elongation site is template-directed (Fig. 6). A third difference between the two primase nucleotide-binding sites involves their binding of nucleotide analogues. The primase fragment does not incorporate methylene derivatives of ATP, whether between the α and β or the β and γ phosphates, at the 3′-end of the primer (Fig. 7). These modifications likely disrupt the ability of the enzyme to catalyze nucleotidyl transfer. In addition, the ATP analogues (Fig. 7) do not inhibit the incorporation of ATP at the 3′-end of the oligoribonucleotides, suggesting that they bind the initiation site more weakly than ATP. The same analogues are readily incorporated at the 5′-end of the primer, and hence ATP binding in the initiation site must be unaffected by the presence of modifications to the three phosphates (Fig. 7). The incorporation ATP analogues at the 5′-end of oligoribonucleotides, coupled with the previous observation that the 63-kDa gene 4 protein can begin oligoribonucleotides with AMP or ADP (27) suggests that the initiation site makes few contacts with the triphosphate end of the nucleotide. Such an open or flexible nucleotide-binding pocket would help explain the ability of the initiation site to bind either ATP or an oligoribonucleotide.

After NTP binding and dinucleotide formation, the next step in primer synthesis involves the extension of the dinucleotide to the tetrinucleotide. This reaction occurs in two steps. In the first step, the dinucleotide is extended to a trinucleotide, and in a second chemical reaction the trinucleotide is extended to a tetrinucleotide. The extension of the dinucleotide to a trinucleotide occurs more slowly than the extension of the trinucleotide to a tetranucleotide, and under steady state conditions, the amount of trimer observed is a small fraction of the amount of tetramer (Fig. 2). This result could be explained if the trimer is bound more tightly in the initiation site than the dimer or if the trimer is released more slowly as an abortive product. The added stability could result from either an extra set of hydrogen bonds formed with the template DNA or base stacking between the first two RNA nucleotides.

Abortive oligoribonucleotides (dimers and trimers) observed in previous studies using the full-length T7 gene 4 protein and natural ssDNA template such a M13 could arise for various indirect reasons. For example, there is a higher frequency of short primase recognition sites 5′-GTC-3′ (68 sites) compared with longer sites such as 5′-GGGTC-3′ (2 sites) in M13 ssDNA. In addition, the action of the helicase domain of gene 4 affects the ratio of oligoribonucleotide products (14). This study using
the primase fragment and short defined templates demonstrates that this abortive primer synthesis is an intrinsic characteristic of the T7 primase and not a secondary effect resulting from the sequence of the DNA template or the helicase. When the primase fragment is provided only a short DNA template containing a single primase recognition site (5′-GGGTCAA-3′), the amount of dimers formed is still substantial compared with tetramers (Fig. 1A). One simple explanation for this abortive synthesis is that ATP competes with pppAC for binding at the initiation nucleotide-binding site. Indeed high levels of ATP favor dinucleotide synthesis (Fig. 4B). Although the exact enzymatic mechanism regulating the length of oligonucleotide products remains elusive, small changes in the cellular NTP pools could greatly influence the rate at which full-length oligonucleotides are produced (Fig. 4C). Because the tetramers function both in vivo and in vitro to prime DNA synthesis, whereas dimers or trimers do not prime, such regulation may be biologically significant.

A comparison of the T7 primase with other DNA-dependent RNA polymerases reveals several apparent differences and similarities. Assuming that the T7 protein is active as a monomer, its measured $k_{\text{cat}}$ of 2–4 s$^{-1}$ is even higher than the rate of strand initiation by T7 RNA polymerase (0.83 s$^{-1}$) (44). Other DNA primases incorporate nucleotides much more slowly. For example, the E. coli DnaG primase synthesizes primers with a $k_{\text{cat}}$ of 0.00089 s$^{-1}$ (45), whereas the eukaryotic primase from calf thymus (46) has a $k_{\text{cat}}$ of 0.0075 s$^{-1}$ (46). Although the T7 primase fragment is more active than similar enzymes, the average length of its products is less than related primases with a

\[ k_{\text{cat}} \]

characteristic of the T7 primase and not a secondary effect resulting from the sequence of the DNA template or the helicase. The further structural and enzymatic analysis of the highly active primase fragment should provide a simple model to study the mechanism of DNA-directed RNA synthesis.

REFERENCES

1. Scherzinger, E., Lanka, E., and Hillenbrand, G. (1977) Nucleic Acids Res. 4, 4151–4163
2. Nakai, H., and Richardson, C. C. (1986) J. Biol. Chem. 261, 15208–15216
3. Patel, S. S., and Hingurani, M. M. (1993) J. Biol. Chem. 268, 10668–10675
4. Notarnicola, S. M., Park, K., Griffith, J. D., and Richardson, C. C. (1995) Biochemistry 34, 2585–2592
5. Helmlinger, H., Yu, X., Wild, R., Hingurani, M. M., and Patel, S. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3869–3873
6. Dunn, J., and Studier, F. W. (1983) J. Mol. Biol. 166, 477–535
7. Tabors, S. W., and Richardson, C. C. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 205–209
8. Koldobner, R., and Richardson, C. C. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1525–1529
9. Matson, S. W., and Richardson, C. C. (1983) J. Biol. Chem. 258, 14009–14016
10. Bernstein, J. A., and Richardson, C. C. (1989) J. Biol. Chem. 264, 13066–13073
11. Bernstein, J. A., and Richardson, C. C. (1988) J. Biol. Chem. 263, 14891–14899
12. Hacker, K. J., and Johnson, K. A. (1997) Biochemistry 36, 14080–14087
13. Washington, M. T., and Patel, S. S. (1998) J. Biol. Chem. 273, 7880–7887
14. Frick, D. N., Bandaran, K., and Richardson, C. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7957–7962
15. Ilivina, T. V., Gorabalenya, A. E., and Koonin, E. V. (1992) J. Mol. Biol. 232, 351–357
16. Matson, S. W., and Richardson, C. C. (1999) J. Biol. Chem. 274, 35889–35898
17. Mendelow, L. V., Notarnicola, S. M., and Richardson, C. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10638–10642
18. Romano, L. J., and Richardson, C. C. (1979) J. Biol. Chem. 254, 10476–10482
19. Mendelow, L. V., Kuimelis, R. G., McLaughlin, L. W., and Richardson, C. C. (1995) Biochemistry 34, 10187–10193
20. Mendelow, L. V., and Richardson, C. C. (1991) J. Biol. Chem. 266, 23240–23250
21. Mendelow, L. V., Beaucamp, B. B., and Richardson, C. C. (1994) EMBO J. 13, 3909–3916
22. Kusakabe, T., Hine, A. V., Hyberts, S. G., and Richardson, C. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4295–4300
23. Bernstein, J. A., and Richardson, C. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 396–400
24. Matson, S. W., and Richardson, C. C. (1985) J. Biol. Chem. 260, 2281–2287
25. Kusakabe, T., and Richardson, C. C. (1997) J. Biol. Chem. 272, 5943–5951
26. Hernandez, A., and Ruiz, M. T. (1996) Bioinformatics 14, 229–228
27. Kusakabe, T., and Richardson, C. C. (1997) J. Biol. Chem. 272, 12446–12453
28. Romano, L. J., and Richardson, C. C. (1979) J. Biol. Chem. 254, 10483–10489
29. Richardson, C. C. (1983) Cell 33, 315–317
30. Horii, K., Mark, D. F., and Richardson, C. C. (1979) J. Biol. Chem. 254, 11391–11397
31. Horii, K., Mark, D. F., and Richardson, C. C. (1979) J. Biol. Chem. 254, 11398–11404
32. Mustaev, A., and Richardson, C. C. (1975) J. Biol. Chem. 250, 5515–5522
33. Tabors, S., Huber, H. E., and Richardson, C. C. (1987) J. Biol. Chem. 262, 16212–16223
34. Matson, S. W., Tabors, S., and Richardson, C. C. (1983) J. Biol. Chem. 258, 14017–14024
35. Kim, Y. T., and Richardson, C. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10173–10177
36. Lee, J., Chastain, P. D., Kusakabe, T., Griffith, J. D., and Richardson, C. C. (1998) Mol. Cell 1, 1001–1010
37. Debyser, Z., Tabors, S., and Richardson, C. C. (1994) Cell 77, 157–166
38. Park, K., Debyser, Z., Tabors, S., Richardson, C. C., and Griffith, J. D. (1998) J. Biol. Chem. 273, 5260–5270
39. Lechner, R. L., and Richardson, C. C. (1983) J. Biol. Chem. 258, 11185–11196
40. Soua, R., Patra, D., and Lafer, E. M. (1992) J. Mol. Biol. 224, 319–334
41. Jia, Y., and Patel, S. S. (1997) Biochemistry 36, 4223–4232
42. Mustaev, A., Kashlev, M., Zaychikov, E., Grechov, M., and Goldfarb, A. (1993) J. Biol. Chem. 268, 19185–19187
43. Mustaev, A. A., and Godson, G. N. (1995) J. Biol. Chem. 270, 15711–15718
44. Martin, C. T., and Coleman, J. E. (1987) Biochemistry 26, 2690–2696
45. Sart, J. R., and Griep, M. A. (1995) Biochemistry 34, 16097–16106
46. Sheaff, R. J., and Kohn, R. D. (1993) Biochemistry 32, 3027–3037
47. Hinton, D. M., and Nossal, N. G. (1987) J. Biol. Chem. 262, 10873–10878
48. Martin, C. T., Muller, D. K., and Coleman, J. E. (1988) Biochemistry 27, 3966–3974
