

# Formation of dAMP-glycerol and dAMP-Tris Derivatives by *Thermococcus kodakaraensis* DNA Primase\*

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**Background:** Archaeal primase catalyzed the DNA-independent synthesis of oligonucleotide-like products from dNTPs.

**Results:** Mass spectrometry, NMR, and biochemical analyses identified these products as dNMP-glycerol and dNMP-Tris derivatives.

**Conclusion:** Archaeal primase catalyzes the formation of phosphodiester bonds between dNMPs and various acceptors.

**Significance:** Archaeal primase forms DNA-independent covalent adducts between deoxynucleotides and acceptors that mimic *de novo* aborted initiation reactions.

In the presence of dATP, glycerol, and Tris buffer, the DNA primase isolated from *Thermococcus kodakaraensis* catalyzed the formation of dAMP and two products that were identified as dAMP-glycerol and dAMP-Tris. These products were formed by the *T. kodakaraensis* p41 catalytic subunit alone and the *T. kodakaraensis* p41-p46 complex in the absence of a DNA template. They were not formed with preparations containing the catalytically inactive p41 subunit. Similar glycerol and Tris derivatives as well as dNMPs were also formed with dGTP, dCTP, or dTTP. The mechanism contributing to the formation of these products and its implications in the initiation reaction catalyzed by the *T. kodakaraensis* primase are discussed.

In the accompanying article (1), the isolation and characterization of the DNA primase from the archaeon *Thermococcus kodakaraensis* were described. Similar to eukaryotic DNA primases, the *T. kodakaraensis* primase is a two-subunit complex (p41-p46) in which the catalytic activity resides within the small subunit. The large subunit of both primases (*T. kodakaraensis* p46 and eukaryotic p58) contains a Fe-S cluster (2, 3), and sequence alignments between the two enzymes reveal homologies between both small and large subunits (4). In the small subunit, the region around the catalytic aspartate residues of the eukaryotic primase closely resemble the archaeal primase, suggesting that it is biologically important. The interaction of the small subunit with the large subunit stabilizes and modulates the catalytic activity of both primases (5–7).

In eukaryotes, the p48 catalytic subunit and the p48-p58 complex initiate short oligonucleotide synthesis only in the presence of rNTPs,<sup>2</sup> and the size of these chains is influenced by

the larger subunit (8). The eukaryotic complex and the catalytic subunit support dNTP incorporation only after *de novo* initiation with rNTPs. In archaea, the catalytic subunit initiates and extends DNA chains with dNTPs but fails to form or weakly forms RNA chains with rNTPs (1, 6, 7). Although the archaeal heterodimeric complex retains its ability to initiate and form DNA, the sizes of the DNA products synthesized are significantly shorter than those formed by the catalytic subunit (0.3–0.5 kb when compared with 3–7 kb). Furthermore, the archaeal complex synthesizes RNA chains with rNTPs (1, 6).

It has been reported that archaeal fragments formed *in vivo* contain oligoribonucleotides at their 5' ends (9), suggesting that the archaeal primases initiate primer synthesis with rNTPs. *In vitro* studies with the *Sulfolobus acidocaldarius* DNA primase complex indicated that the apparent  $K_m$  for rNTPs was 3 orders greater than the  $K_m$  for dNTPs (10). However, studies with other archaeal primases isolated from *Pyrococcus abyssi* (7) and *T. kodakaraensis* (1) noted considerably less disparity in their  $K_m$  values for rNTPs and dNTPs. Because archaea appears to lack DNA polymerase  $\alpha$  activity, it has been suggested that the DNA synthetic activity of the archaeal primases may mimic the activity of this DNA polymerase (8). Thus, at present, the *in vivo* significance of dNTP initiation events observed with the archaeal DNA primase *in vitro* are unclear.

In the accompanying article (1), we noted the formation of two <sup>32</sup>P-labeled products following incubation of the *T. kodakaraensis* primase complex or the *T. kodakaraensis* p41 catalytic subunit with [ $\alpha$ -<sup>32</sup>P]dNTPs that migrated as <sup>32</sup>P-labeled ~16- and ~25-nt bands following urea-PAGE separation. Their formation required a catalytically active p41 subunit but was independent of a DNA template. In this study, we identify these products as dAMP-glycerol (~16-nt band) and dAMP-Tris (~25-nt band). We show that each of the four common dNTPs support formation of these products, whereas rNTPs are considerably less active. In addition to these derivatives, dNMP is

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<sup>2</sup> The abbreviations used are: rNTP, ribonucleoside 5'-triphosphate; rATP, riboadenosine 5'-triphosphate; ClP, calf intestinal phosphates; PPase, inor-

ganic pyrophosphatase; nt, nucleotide; TE, Tris-EDTA; dAp, 3'-deoxyadenosine phosphate; pdAp, 5',3'-deoxyadenosine diphosphate.

also produced in the reaction, indicating that H<sub>2</sub>O can replace glycerol and Tris in this reaction. Although the mechanism governing formation of these products is unclear, we discuss its possible significance in the DNA primase initiation and aborted initiation-elongation reactions.

## EXPERIMENTAL PROCEDURES

**Preparation of Proteins**—The *T. kodakaraensis* primase proteins were isolated as described in the accompanying article (1). All enzymes were stored at  $-80^{\circ}\text{C}$  and stable to repeated freezing and thawing.

**Commercial Enzymes**—The enzymes and their sources included: spleen phosphodiesterase (46 units/mg, Amersham Biosciences); yeast inorganic pyrophosphatase and micrococcal nuclease (Worthington Biochemical); muscle myokinase, snake venom phosphodiesterase, yeast hexokinase, and calf intestinal phosphatase (CIP) (Sigma-Aldrich); and 3-phosphoglycerate dehydrogenase and 3-phosphoglycerate kinase (Roche Applied Science).

**Assays for Production of dNMP Derivatives**—Unless indicated, reaction mixtures (20  $\mu\text{l}$ ) containing 40 mM Tris-HCl, pH 8.0, 2 mM DTT, 10 mM magnesium acetate, 100  $\mu\text{M}$  labeled dNTP or rNTP, 50  $\mu\text{g/ml}$  BSA, and enzyme (as indicated) were incubated at  $70^{\circ}\text{C}$  for a specified time. Unless noted, primase preparations were diluted with a solution containing 50% glycerol, 0.02 M Tris-HCl, pH 8.0, 1 mM DTT, 10  $\mu\text{M}$  EDTA, and 50  $\mu\text{g/ml}$  BSA (glycerol diluent). Aliquots of reactions were subjected to ascending thin layer chromatography (TLC) in the indicated solvents until the solvent front reached  $\sim 1$  cm from the top of the polyethyleneimine (PEI) cellular plate strips (Brinkmann Instruments); the strips were air-dried and subjected to autoradiography or phosphorimaging. Aliquots of reactions were also subjected to 8 M urea-20% PAGE separation for 2–3 h at 300 V in  $1\times$  Tris-borate-EDTA, as described (1). Gels were usually subjected to autoradiography wet. Frozen gels were wrapped in paper towels to prevent moisture condensation.

**Preparations of dAMP-glycerol and dAMP-Tris Products Using [<sup>13</sup>C,<sup>15</sup>N]dATP and Deuterated *d*-Tris for Analysis by High Mass Accuracy LC-MS and High Resolution NMR**—Reaction mixtures (0.25 ml) containing 40 mM deuterated *d*-Tris-HCl (or light normal Tris-HCl), pH 8.0, 2 mM DTT, 10 mM magnesium acetate, 80  $\mu\text{M}$  [<sup>13</sup>C,<sup>15</sup>N]dATP (or light ATP), 50  $\mu\text{g/ml}$  BSA, and 5.84  $\mu\text{M}$  p41 subunit (containing 0.64 M glycerol following dilution in the glycerol diluent described above) were incubated for 30 min at  $70^{\circ}\text{C}$ . An identical reaction (25  $\mu\text{l}$ ) containing [ $\alpha$ -<sup>32</sup>P]dATP revealed that following incubation with the p41 subunit and TLC analysis on PEI plates,  $>60\%$  of the <sup>32</sup>P was recovered in the fast migrating dAMP derivatives,  $>30\%$  in dAMP, and  $<10\%$  in ATP. This same distribution was observed in reactions containing *d*-Tris-HCl, pH 8.0, [<sup>13</sup>C,<sup>15</sup>N]dATP, or normally light reagents. Thus, under the conditions used, the use of heavy isotopes did not alter the utilization of dATP in the reaction. Samples from reaction mixtures were analyzed using a capillary reverse phase liquid chromatography system coupled to an Orbitrap Discovery mass spectrometer in negative mode (11). Negative mode nanospray mode was set to  $-1.5$  kV, and chromatographic flow rate was

$<20$  nl/min. Fragmentation was achieved using high collision dissociation at 45% energy. Isotope-labeled samples were run both individually and combined. By analyzing the mass difference between heavy and light fragmentation pattern, the identity of each fragmentation peak was established.

In parallel, isotope-labeled products of the reaction mixtures were analyzed directly using high resolution <sup>31</sup>P NMR after the addition of <sup>2</sup>H<sub>2</sub>O to the mixture, leading to a volume-to-volume percentage ratio of 90:10. Data were collected on a Bruker DRX 500-MHz spectrometer, equipped with a Broadband probe at  $30^{\circ}\text{C}$ . Spectra were processed and analyzed using Topspin 1.3 on a Linux work station. One-dimensional <sup>31</sup>P spectra were acquired using a carrier frequency of 5 ppm, sweep widths of 6000 Hz, 4097 complex points, and 4096 scans. <sup>31</sup>P resonance assignments were made by comparison with one-dimensional <sup>31</sup>P spectra of standard samples (dATP, dAMP, inorganic pyrophosphate (PP<sub>i</sub>), and inorganic phosphate (P<sub>i</sub>)) and using two-dimensional <sup>1</sup>H-<sup>31</sup>P hetero-COSY and <sup>1</sup>H-<sup>13</sup>C HMQC correlated experiments applied to the isotope-labeled products.

**Preparation of [ $\gamma$ -<sup>32</sup>P]dATP**—The procedure used for this synthesis was a modified version of that described by Glynn and Chappell (12). Reaction mixtures (120  $\mu\text{l}$ ) containing 50 mM Tris-HCl, pH 8.0, 6 mM magnesium acetate, 2.5 mM cysteine, 10 mM 3-phosphoglycerate, 40  $\mu\text{M}$  NAD, 0.2 mM dATP, 2  $\mu\text{g}$  of 3-phosphoglycerate kinase, 40  $\mu\text{g}$  of 3-phosphoglycerate dehydrogenase, and <sup>32</sup>P<sub>i</sub> ( $7.68 \times 10^7$  cpm) were incubated for 60 min at  $37^{\circ}\text{C}$ . Mixtures were treated with 100  $\mu\text{l}$  of 10% charcoal slurry (previously washed with 1 N HCl and H<sub>2</sub>O until neutral), 50  $\mu\text{l}$  of 1 N HCl, and 50  $\mu\text{l}$  of 10 mM sodium phosphate, pH 7.5. Charcoal was collected by centrifugation, washed three times with 0.3 ml of 10 mM sodium phosphate buffer, pH 7.5, and then washed twice with 0.3 ml of H<sub>2</sub>O. Charcoal adsorbed material was eluted with three washes of 100  $\mu\text{l}$  of H<sub>2</sub>O-ethanol-concentrated NH<sub>4</sub>OH mixture (1:1, 0.08), which were pooled and concentrated *in vacuo*. The dried material was suspended in 0.2 ml of  $1\times$  TE and centrifuged at 20,000 rpm in an Eppendorf centrifuge at  $4^{\circ}\text{C}$ . Approximately 45% of the input <sup>32</sup>P was recovered in the eluted material. Assuming quantitative incorporation of <sup>32</sup>P<sub>i</sub> into dATP, [ $\gamma$ -<sup>32</sup>P]dATP would be expected to contain 3200 cpm/pmol. The <sup>32</sup>P recovery ( $3.5 \times 10^7$  cpm) suggested that  $\sim 11$  nmol of dATP was isolated. TLC on PEI cellular plates in 0.5 M ammonium formate, pH 3.5, indicated that the preparation contained 94% [ $\gamma$ -<sup>32</sup>P]dATP and 6% <sup>32</sup>P<sub>i</sub>. Incubation with excess glucose and hexokinase resulted in the transfer of the <sup>32</sup>P present in [ $\gamma$ -<sup>32</sup>P]dATP quantitatively to glucose 6-phosphate.

**Preparation of [ $\beta$ -<sup>32</sup>P]dATP**—Reaction mixtures (120  $\mu\text{l}$ ) containing 20 mM Tris-HCl, pH 8.0, 10 mM magnesium acetate, 0.4 mM cysteine, 2 mM 3-phosphoglycerate, 18 nM NAD, <sup>32</sup>P<sub>i</sub> ( $6 \times 10^8$  cpm), 0.25 mM dATP, 25 nM dAMP, 20  $\mu\text{g}$  of 3-phosphoglycerate kinase, 30  $\mu\text{g}$  of 3-phosphoglycerate dehydrogenase, and 5  $\mu\text{g}$  of muscle myokinase were incubated at  $37^{\circ}\text{C}$  for 60 min. Following incubation, 100  $\mu\text{l}$  of 20% charcoal and 220  $\mu\text{l}$  of 1 N HCl were added. After 20 min on ice, the mixture was centrifuged, washed four times with 0.4 ml of 5 mM sodium phosphate buffer, pH 7.5, and then washed once with 0.5 ml of H<sub>2</sub>O. The adsorbed material was eluted with a freshly prepared mixture of H<sub>2</sub>O-ethanol-NH<sub>4</sub>OH (400  $\mu\text{l}$ , six times).

## Formation of nt Adducts by *T. kodakaraensis* DNA Primase

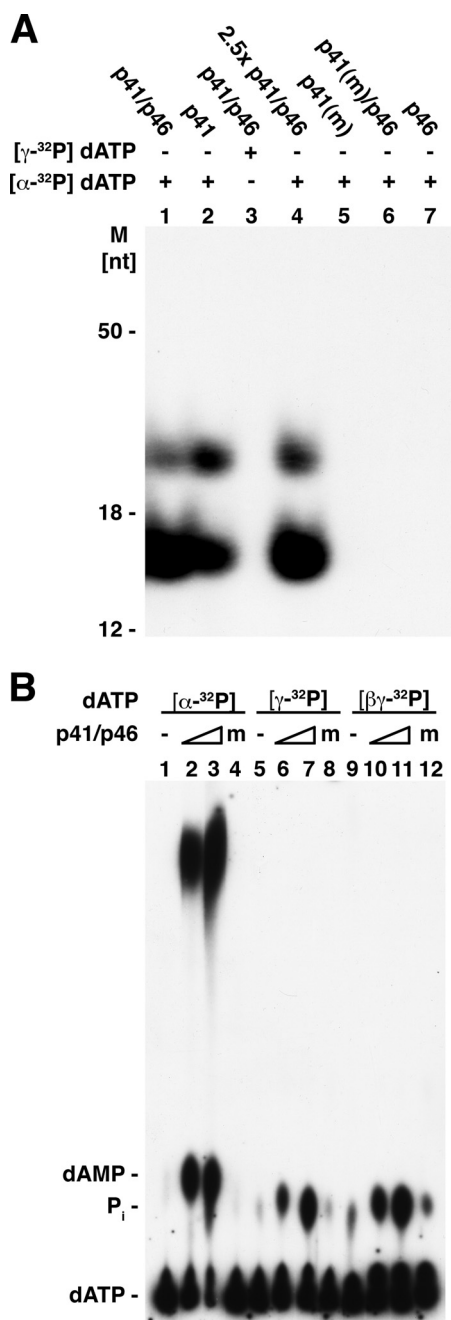
The washings were combined, vacuum-dried, and suspended in 0.2 ml of 1× TE. Approximately 40% of the  $^{32}\text{P}_i$  added to the reaction was recovered ( $2.36 \times 10^8$  cpm). Based on the level of dATP added and assuming complete utilization of the  $^{32}\text{P}$ , the isolated dATP had a specific activity of  $\sim 20,000$  cpm/pmol with  $^{32}\text{P}$  distributed equally between the  $\beta$  and  $\gamma$  phosphate residues of dATP. TLC on PEI plates in 0.5 M  $\text{NH}_4$  formate, pH 3.5, indicated that the preparation contained 92% [ $^{32}\text{P}$ ]dATP and 8% [ $^{32}\text{P}$ ]dADP; treatment with hexokinase and glucose resulted in the quantitative disappearance of [ $\gamma$ - $^{32}\text{P}$ ]dATP and formation of equally labeled dADP and glucose 6-phosphate, in keeping with the equal distribution of  $^{32}\text{P}$  between the  $\beta$  and  $\gamma$  phosphate residues of dATP.

**Preparation of [ $^{32}\text{P}$ ]dAMP—**[ $^{32}\text{P}$ ]dAMP was synthesized from [ $\alpha$ - $^{32}\text{P}$ ]dATP by the combined action of hexokinase and myokinase. Reaction mixtures (20  $\mu\text{l}$ ) containing 40 mM Tris-HCl, pH 8.0, 10 mM magnesium acetate, 0.5 mM DTT, 50  $\mu\text{g}/\text{ml}$  BSA, 50 mM glucose, 25  $\mu\text{M}$  [ $\alpha$ - $^{32}\text{P}$ ]dATP ( $2 \times 10^4$  cpm/pmol), 2  $\mu\text{g}$  of yeast hexokinase, and 6  $\mu\text{g}$  of muscle myokinase were incubated at 37 °C. The mixture was extracted with phenol/ $\text{CHCl}_3$ /isoamyl/alcohol mixture, the aqueous phase was adsorbed to activated charcoal (50  $\mu\text{l}$  of 10% solution in 0.1 N HCl), which was collected by centrifugation and washed with 100  $\mu\text{l}$  of  $\text{H}_2\text{O}$  three times, and the bound nucleotide was eluted with  $\text{H}_2\text{O}$ /ethanol/ $\text{NH}_4\text{OH}$  mixture (twice). The pooled eluate were vacuum-concentrated and suspended in 30  $\mu\text{l}$  of TE buffer. Based on the  $^{32}\text{P}$  recovered, approximately 50% of the input [ $\alpha$ - $^{32}\text{P}$ ]dATP was recovered as [ $^{32}\text{P}$ ]dAMP. TLC on PEI plates indicated that this material contained 92% [ $^{32}\text{P}$ ]dAMP, 7% dADP, and 1% dATP.

## RESULTS

**Requirements for Synthesis of [ $^{32}\text{P}$ ]dAMP Derivatives—**Both the *T. kodakaraensis* primase complex and the p41 subunit catalyzed the synthesis of two distinct labeled products from [ $\alpha$ - $^{32}\text{P}$ ]dATP that migrated as  $\sim 16$ - and  $\sim 25$ -nt bands following urea-PAGE separation (Fig. 1A). These derivatives were formed in the absence of a DNA template with any one of the [ $\alpha$ - $^{32}\text{P}$ ]dNTPs but were not readily detected with [ $\alpha$ - $^{32}\text{P}$ ]rNTPs (1). Their production required the catalytically active p41 subunit (compare lanes 1 and 2 with lanes 5 and 6) and were not observed when [ $\alpha$ - $^{32}\text{P}$ ]dATP was replaced by [ $\gamma$ - $^{32}\text{P}$ ]dATP (Fig. 1A, lane 3) or following incubation with the p46 subunit alone (lane 7). Incubation of [ $\alpha$ - $^{32}\text{P}$ ]dATP with higher levels of the p41-p46 complex increased the amount of products formed (lane 4). These findings indicated that the products contained the  $\alpha$ -phosphate moiety but not the  $\gamma$ -phosphate residue of dNTPs.

Products formed by the *T. kodakaraensis* primase complex with dATP were also analyzed by TLC on PEI plates (Fig. 1B). Incubation with [ $\alpha$ - $^{32}\text{P}$ ]dATP resulted in the production of a labeled fast migrating material and [ $^{32}\text{P}$ ]dAMP (Fig. 1B, lane 2). The amount of these products formed increased in the presence of increasing levels of the primase complex (Fig. 1B, lanes 2 and 3). They were not formed when the catalytically inactive primase complex (mutant p41-p46 complex) was used in place of the active complex (lane 4). When [ $\gamma$ - $^{32}\text{P}$ ]dATP (lanes 5–8) or [ $\beta\gamma$ - $^{32}\text{P}$ ]dATP (lanes 9–12) was added in lieu of



**FIGURE 1.** A, urea-PAGE analysis of products formed by *T. kodakaraensis* primase preparations following incubation with [ $\alpha$ - $^{32}\text{P}$ ]dATP or [ $\gamma$ - $^{32}\text{P}$ ]dATP. Reaction mixtures (20  $\mu\text{l}$ ) containing 40 mM Tris-HCl, pH 8.0, 10 mM magnesium acetate, 2 mM  $\text{MnCl}_2$ , 50  $\mu\text{g}/\text{ml}$  BSA, 2 mM DTT, 100  $\mu\text{M}$  [ $\alpha$ - $^{32}\text{P}$ ]dATP (4250 cpm/pmol) or 100  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]dATP (2430 cpm/pmol), where specified, and 0.5  $\mu\text{M}$  (or 1.25  $\mu\text{M}$ ) of the indicated *T. kodakaraensis* primase preparations were incubated for 30 min at 70 °C. Aliquots (5  $\mu\text{l}$ ) from reactions were separated by urea-PAGE and subjected to autoradiography. *m*, mutant; *M*, molecular mass markers. B, TLC analysis of products formed following incubation of *T. kodakaraensis* primase preparations with labeled dATP. Reactions were as described in panel A with the exception that  $\text{MnCl}_2$  was omitted. Where indicated, mixtures contained [ $\alpha$ - $^{32}\text{P}$ ]dATP ( $4 \times 10^3$  cpm/pmol), [ $\gamma$ - $^{32}\text{P}$ ]dATP ( $3.4 \times 10^3$  cpm/pmol), or [ $\beta\gamma$ - $^{32}\text{P}$ ]dATP ( $5.1 \times 10^3$  cpm/pmol) and 0.25 or 0.75  $\mu\text{M}$  *T. kodakaraensis* primase complex or 0.75  $\mu\text{M}$  mutant p41-p46 complex (indicated as *m*). Aliquots (1  $\mu\text{l}$ ) of each reaction were analyzed by TLC on a PEI plate developed in 0.3 M LiCl.

[ $\alpha$ - $^{32}\text{P}$ ]dATP, the rapid migrating labeled product was not detected. However,  $^{32}\text{P}_i$  was formed, and its production required an active primase complex. As discussed in more



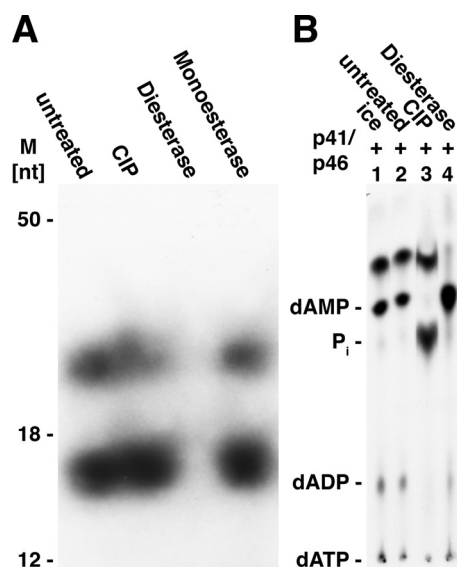


FIGURE 2. *A*, treatment of products with CIP, venom phosphodiesterase, and spleen phosphodiesterase. Aliquots of reactions following incubation of [ $\alpha$ - $^{32}$ P]dATP and the *T. kodakaraensis* primase complex (as described in the legend for Fig. 1) were subjected to the following treatment. Reactions (10  $\mu$ l) contained 5  $\mu$ l of the above reaction products, 40 mM Tris-HCl, pH 8.5, 2 mM magnesium acetate, CIP (1 unit), or snake venom phosphodiesterase (0.1 unit). Reactions with spleen phosphodiesterase (0.04 unit) contained 40 mM Tris-HCl, pH 7.0, and 1 mM magnesium acetate. All mixtures were incubated at 37  $^{\circ}$ C for 30 min and then subjected to urea-PAGE analysis and autoradiography. *M*, molecular mass markers. *B*, TLC analysis of products formed after treatment with CIP and snake venom phosphodiesterase. Reaction mixtures (5  $\mu$ l) containing 1  $\mu$ l of the products formed from [ $\alpha$ - $^{32}$ P]dATP (as described in panel *A*), 40 mM Tris-HCl, pH 8.5, 2 mM magnesium acetate, and CIP (0.5 unit) or snake venom phosphodiesterase (0.1 unit) were incubated for 20 min at 37  $^{\circ}$ C. Mixtures devoid of these enzymes were incubated on ice (lane 1) or at 37  $^{\circ}$ C (untreated, lane 2). Following incubation, aliquots (1  $\mu$ l) were plated on a PEI plate and developed in 0.5 M ammonium formate, pH 3.5. Markers indicating the migration of standards are shown on the left.

detail below, PP<sub>i</sub> is produced in the primase reaction, which is partially hydrolyzed to P<sub>i</sub> by the inorganic pyrophosphatase activity present in the primase preparations. We infer from these findings that *T. kodakaraensis* primase preparations convert [ $\alpha$ - $^{32}$ P]dATP to multiple labeled products, including the 16- and 25-nt bands detected following urea-PAGE separation and dAMP and a rapid migrating material (which as described below includes the ~16- and ~25-nt products) following TLC separation.

**Properties of Products Formed from [ $\alpha$ - $^{32}$ P]dATP**—Products formed from [ $\alpha$ - $^{32}$ P]dATP by the *T. kodakaraensis* primase complex were subjected, without isolation, to digestion with calf intestinal phosphatase (CIP), snake venom phosphodiesterase, or spleen phosphodiesterase. Urea-PAGE analysis indicated that both labeled products were hydrolyzed quantitatively by snake venom phosphodiesterase but unaffected by CIP or spleen phosphodiesterase (Fig. 2*A*). It should be noted that spleen phosphodiesterase requires the presence of a 5'-hydroxyl group for its action (13). The influence of CIP and venom phosphodiesterase was also examined by TLC separation using PEI plates (Fig. 2*B*). CIP treatment did not alter the level of the fast migrating dAMP derivatives, but hydrolyzed the residual dATP (and dADP) remaining after primase action to P<sub>i</sub> (lane 3). Venom phosphodiesterase converted the fast migrating labeled derivatives to dAMP almost quantitatively (lane 4). Incubation

of the  $^{32}$ P-labeled fast migrating labeled material with both venom phosphodiesterase and CIP converted the  $^{32}$ P present in this derivative to  $^{32}$ P<sub>i</sub>. These findings indicated that the dAMP present in this product contained a phosphodiester linkage.

**Analysis of Products Formed by Capillary HPLC-MS—TLC and urea-PAGE separation indicated that the dAMP derivatives were not pdApdA, dApdA, pdApdApA, or dApdApdA (data not presented). We next turned to HPLC-MS analysis to identify the nature of the products. For this purpose, the dAMP derivatives formed from [ $C^{12},N^{14}$ ]dATP, [ $C^{13},N^{15}$ ]dATP, and *d*-Tris buffer were prepared as described under "Experimental Procedures," analyzed by high mass accuracy LC-MS, and compared with dATP. Three chromatographic peaks were prominent in product samples with molecular weights of 330, 404, and 433 (the structure of the 330 and 404 peaks is shown in Fig. 3*A*). The peak with a molecular weight of 330 was identified as dAMP based on exact mass and fragmentation pattern matching the METLIN database. To further investigate the identity of peaks 404 and 433,  $^{13}C,^{15}N$  (heavy) stable isotopes of dATP were used in conjunction with naturally occurring isotopes  $^{12}C,^{14}N$  (light) of dATP. Heavy and light dATP were incubated in separate reactions with enzyme, and the products were analyzed for exact mass and fragmentation pattern. Subsequently, both heavy and light samples were mixed 1:1 to ensure proper correlation by chromatographic co-elution. As expected, peak 330 (light) yielded a corresponding peak 345 (heavy). Fragmentation of both these compounds and the products formed is shown in Fig. 3, *B* and *C*. The first major fragments formed (200/195) indicate the loss of the adenine base, which contains 10 heavy isotopes. A mass of 79 is indicative of a phosphate group. These findings establish the identity of the 330 *m/z* peaks as dAMP. To identify the unknown compound 404 *m/z* (structure shown in Fig. 3*A*), a similar analysis was undertaken. The 419/404 (heavy/light) *m/z* derivatives were thought to be a modified dAMP based on dATP being the sole substrate of the reaction and its mobility on TLC plate. The 419/404 *m/z* derivatives yielded fragmentation patterns similar to those of dAMP (345/330 *m/z*) with the exception of a 79 *m/z* peak. The difference between 419/404 and their major fragment peaks of 274/269 *m/z* was the exact mass and isotope composition of an adenine base. This confirms that 419/404 *m/z* is derived from dATP and that the unknown modification is not attached directly to the adenine base. The mass difference between dAMP and 404 *m/z* is 74.03676. The exact mass indicates that such a modification is C<sub>3</sub>H<sub>6</sub>O<sub>2</sub>. Fig. 3, *D* and *E*, show that peak 171 appears in both the 419 *m/z* and the 404 *m/z* samples. Subtracting a mass of 74.03676 from 171 *m/z* and accounting for water loss, which is common during MS fragmentation, suggests that 171.00615 *m/z* is phosphate + C<sub>3</sub>H<sub>6</sub>O<sub>2</sub>. This indicates that the modification is attached to the phosphate. As 171 *m/z* is found in both heavy and light samples, the 74.03676 *m/z* cannot be derived from the original dATP as all the dATP carbons in the heavy sample are labeled with  $^{13}C$ . Examination of the exact mass of 171 *m/z* and factoring in the dehydration reaction necessary to bond to phosphate identified the modification as glycerol, which is the sole match in the database. This modification is likely as the enzyme was diluted in 50% glycerol. Peak 448/433, examined in a similar manner, identified Tris**

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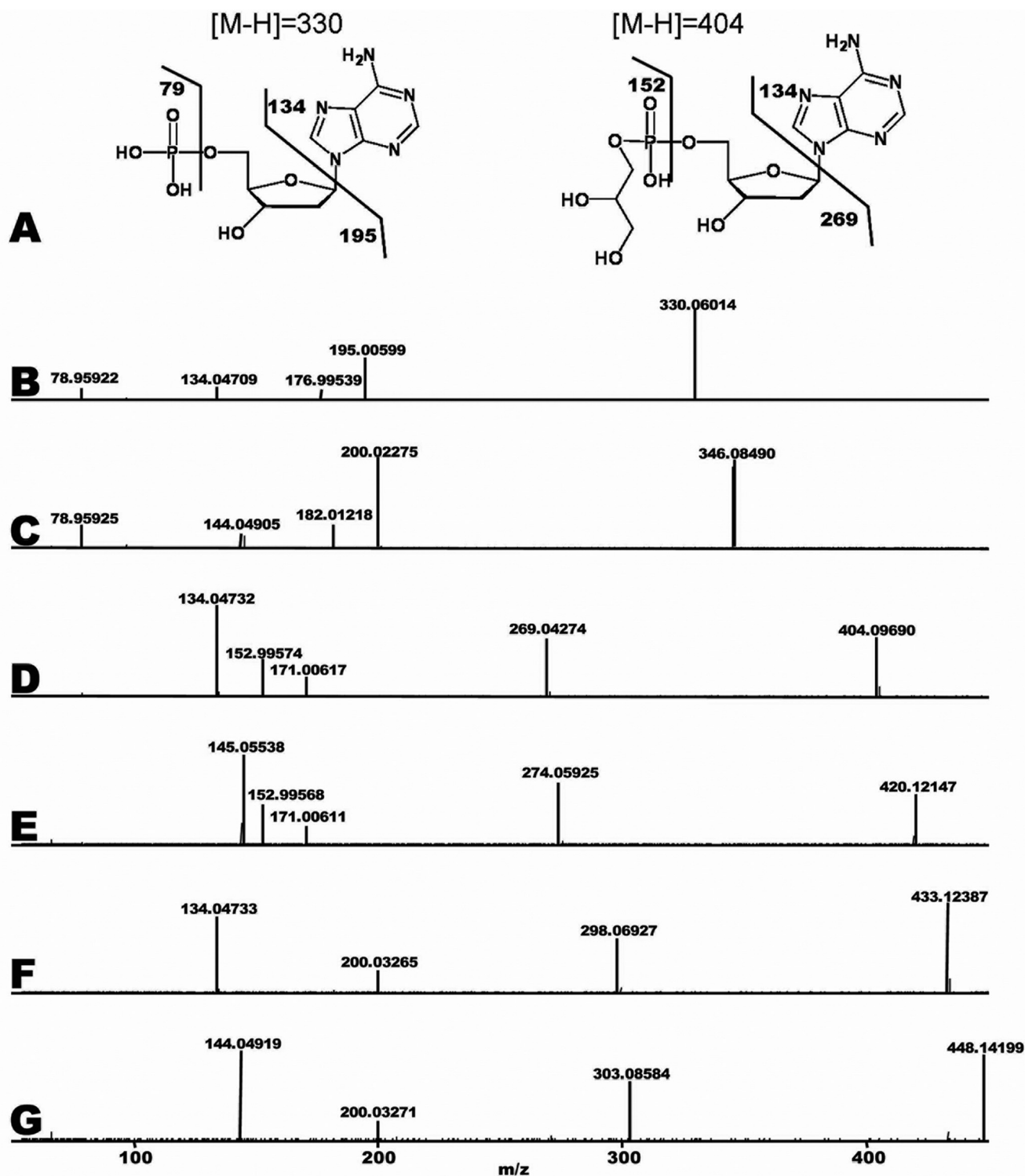
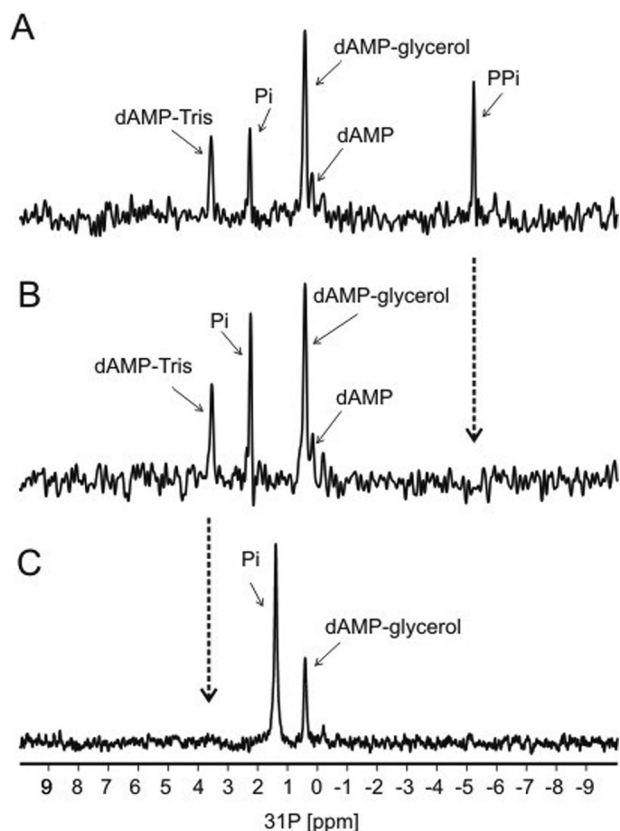


FIGURE 3. **Identification of dAMP modifications by LC-MS/MS.** Reaction mixtures, as described under "Experimental Procedures" with dATP, resulted in products dAMP (330  $m/z$ ) and glycerol-dAMP (404  $m/z$ ) (structures consistent with the MS data are shown in A). B–G, reactions contained 0.75  $\mu\text{M}$  *T. kodakaraensis* primase preparation diluted in 50% glycerol with [ $^{12}\text{C},^{14}\text{N}$ ]dATP (B, D, and F) or [ $^{13}\text{C},^{15}\text{N}$ ]dATP (C, E, and G) as substrates. Samples were analyzed by capillary LC-MS/MS. B, high resolution fragmentation of 330  $m/z$  U- $^{12}\text{C},^{14}\text{N}$ ; C, 345  $m/z$ , the U- $^{13}\text{C},^{15}\text{N}$  counterpart to 330  $m/z$ ; D, 419  $m/z$  U- $^{12}\text{C},^{14}\text{N}$ ; E, 404  $m/z$  U- $^{13}\text{C},^{15}\text{N}$  counterpart to 404  $m/z$ ; F, 433  $m/z$  U- $^{12}\text{C},^{14}\text{N}$ ; G, 448  $m/z$ , the U- $^{13}\text{C},^{15}\text{N}$  counterpart to 433  $m/z$ . See "Results" for analyses.

base as the modifying conjugate (Fig. 3F, 433  $m/z$   $^{12}\text{C},^{14}\text{N}$ , and Fig. 3G, 448  $m/z$   $^{13}\text{C},^{15}\text{N}$ ).

**Analysis of Products Formed by  $^{31}\text{P}$  NMR**—One-dimensional  $^{31}\text{P}$  NMR spectral analysis was used to confirm the nature of the phosphorylated products formed in the reaction. For this pur-

pose, the dAMP derivatives prepared with [ $^{12}\text{C},^{14}\text{N}$ ]dATP, [ $^{13}\text{C},^{15}\text{N}$ ]dATP, and *d*-Tris buffer were again used. Initial one-dimensional  $^{31}\text{P}$  spectra of the reaction mixture (Fig. 4A) containing [ $^{13}\text{C},^{15}\text{N}$ ]ATP, glycerol, and *d*-Tris revealed four main  $^{31}\text{P}$  resonance peaks that could be assigned to phosphorus



**FIGURE 4.  $^{31}\text{P}$  NMR analysis of reaction products.** Reaction mixtures were as described in the legend for Fig. 3. *A*, initial one-dimensional  $^{31}\text{P}$  spectrum of the reaction mixture containing [ $^{13}\text{C}$ , $^{15}\text{N}$ ]ATP, glycerol, and *d*-Tris showing five  $^{31}\text{P}$  resonance peaks;  $^{31}\text{P}$  resonances in the spectra assigned to dAMP-Tris, dAMP-glycerol, dAMP,  $\text{P}_i$ , and  $\text{PP}_i$  are labeled. Note that there is no observable signal from dATP, indicating the complete conversion of the input dATP to products. *B*, one-dimensional  $^{31}\text{P}$  spectrum of the reaction mixture (after a few days) showing four  $^{31}\text{P}$  peaks;  $^{31}\text{P}$  resonances in the spectrum assigned to dAMP-Tris, dAMP-glycerol, dAMP, and  $\text{P}_i$  are labeled. The  $\text{PP}_i$  peak disappeared (noted by arrows) and the  $\text{P}_i$  peak increased, indicating a breakdown of  $\text{PP}_i$  to  $\text{P}_i$ . *C*, one-dimensional  $^{31}\text{P}$  spectrum of the reaction mixture containing [ $^{13}\text{C}$ , $^{15}\text{N}$ ]ATP, glycerol, and glycine, substituted for *d*-Tris, as buffer showing two  $^{31}\text{P}$  peaks;  $^{31}\text{P}$  resonances in the spectrum assigned to dAMP-glycerol and  $\text{P}_i$  are labeled. The  $^{31}\text{P}$  resonances in the spectrum assigned to dAMP-Tris are not observed. Note that the resonance of  $\text{P}_i$  in this reaction was slightly shifted when compared with the  $\text{P}_i$  signal from the Tris-buffered reactions containing Tris buffer due to sensitivity of the inorganic phosphate to differences in the pH of the reactions.

atoms contained in the dAMP-Tris and dAMP-glycerol adducts, as well as signals from  $\text{P}_i$  and  $\text{PP}_i$ . In addition, a minor peak was also observed that could be assigned to dAMP. Note that in the  $^{31}\text{P}$  spectra, no signals were observed from the starting substrate dATP, indicating that the input dATP was converted quantitatively to products. After a few days at room temperature, the one-dimensional  $^{31}\text{P}$  spectra of the same reaction mixture (Fig. 4*B*) revealed only three main  $^{31}\text{P}$  peaks that were assigned to dAMP-Tris, dAMP-glycerol, dAMP, and  $\text{P}_i$ . In this spectrum, the  $\text{PP}_i$  signal “disappeared” concomitant with an increased  $\text{P}_i$  peak, indicating that  $\text{PP}_i$  was hydrolyzed to  $\text{P}_i$ . This finding is in keeping with the presence of inorganic pyrophosphatase in *T. kodakaraensis* primase preparations as described below. To confirm the formation of the dAMP-Tris adduct, a one-dimensional  $^{31}\text{P}$  spectrum of the reaction mixture containing [ $^{13}\text{C}$ , $^{15}\text{N}$ ]ATP, glycerol and glycine, substituted for *d*-Tris as the buffer, was examined. In this spectrum (Fig. 4*C*),  $^{31}\text{P}$

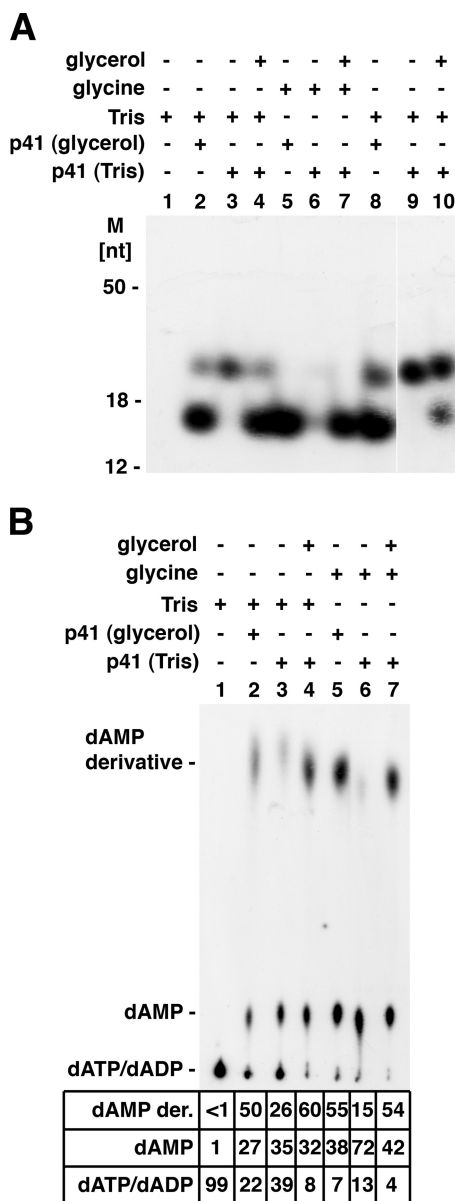
resonances assigned to dAMP-glycerol and  $\text{P}_i$  were observed, and as expected, the  $^{31}\text{P}$  resonance signal assigned to dAMP-Tris product was not observed. For both the dAMP-Tris and the dAMP-glycerol adducts, the  $^{31}\text{P}$  NMR confirms that these adducts formed via a 5'  $\alpha$ -phosphorus linkage to dAMP. In addition,  $^1\text{H}$  NMR indicated no other chemical modifications to the base or sugar of the dAMP moiety in these adducts.

**Formation of dAMP-glycerol and dAMP-Tris Products**—We next demonstrated that formation of the dAMP-Tris product (migrating as the  $\sim 25$ -nt product) and the dAMP-glycerol product (migrating as an  $\sim 16$ -nt product) required the addition of Tris buffer and glycerol, respectively, to primase reaction mixtures (Fig. 5). A glycerol diluent was added to primase reaction mixtures to dilute enzyme preparations (solutions containing 50% glycerol, equivalent to 5 M glycerol). For these reasons, reactions containing glycine buffer in place of Tris buffer were employed, and enzyme fractions were diluted with 10 mM Tris-HCl buffer, pH 8.0, and 1 mM EDTA ( $1\times$  TE) rather than the 50% glycerol buffer diluent. As shown in Fig. 5*A*, primase reactions containing both 40 mM Tris buffer and enzyme diluted with the glycerol diluent (p41 (glycerol)) yielded the dAMP-Tris and dAMP-glycerol products following urea-PAGE analysis (Fig. 5*A*, lane 2). Reactions containing Tris buffer and primase fractions diluted in TE buffer (p41 (Tris)) rather than the glycerol diluent yielded the dAMP-Tris product but no dAMP glycerol derivative (lane 3); glycerol addition to such reactions (0.6 M glycerol, final concentration) led to the formation of both dAMP derivatives (lane 4). The omission of glycerol in reactions containing Tris buffer resulted in an increased formation of the dAMP-Tris derivative (compare lane 3 with lanes 2 and 4), suggesting that glycerol and Tris may compete in the reaction. When reactions contained enzyme diluted with the glycerol diluent and glycine buffer in place of Tris buffer, the dAMP-glycerol derivative was the only product formed (lane 5). When both Tris and glycerol were omitted, neither derivative was produced (lane 6). Identical results were obtained when primase reactions were carried out in 10- $\mu\text{l}$  rather than 20- $\mu\text{l}$  reaction mixtures (lanes 8–10). In the presence of Tris and glycerol, the catalytic p41 subunit generated both products (lane 8); omission of glycerol resulted in the formation of the dAMP-Tris derivative (lane 9), and the addition of low levels of glycerol (0.12 M rather than 0.6 M) resulted in the formation of low levels of the dAMP-glycerol product (compare lane 8 and lane 10).

The influence of glycerol and Tris on the formation of the dAMP-glycerol and dAMP-Tris derivative was also examined by TLC on PEI plates (Fig. 5*B*). As these derivatives are not separated by TLC and the dAMP-glycerol product represents the major dAMP derivative formed, omission of Tris had little quantitative effect on the level of the fast migrating products formed, whereas glycerol addition (compare lanes 3 and 4 with lanes 6 and 7) stimulated formation of dAMP-glycerol. The production of dAMP was also increased substantially in the absence of Tris and glycerol (lane 6). It should be noted that dATP hydrolysis to dAMP required the catalytically active p41 subunit. Collectively, these findings are in accord with the mass spectrometric and NMR analyses and indicate that the prod-



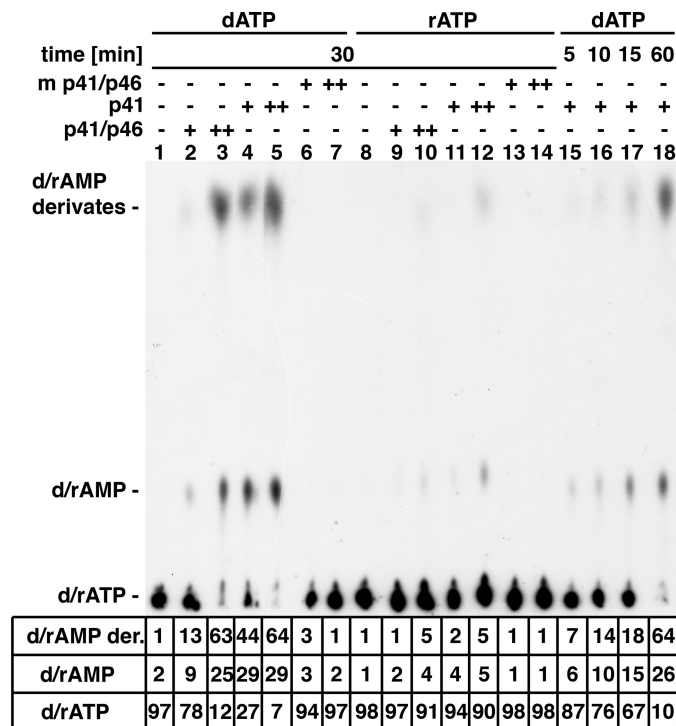
## Formation of nt Adducts by *T. kodakaraensis* DNA Primase



**FIGURE 5. Influence of glycerol and Tris on formation of dAMP-glycerol and dAMP-Tris derivative.** *A*, analysis of products by urea-PAGE separation. Reaction mixtures (lanes 1–7) were as described in the legend for Fig. 1 with [ $\alpha$ - $^{32}$ P]dATP and (where indicated) 40 mM glycine buffer, pH 8.8 (glycine), 40 mM Tris-HCl buffer, pH 8.0 (Tris), 0.5  $\mu$ M *T. kodakaraensis* p41 subunit diluted in 50% glycerol diluent (p41 (glycerol)), or 0.5  $\mu$ M *T. kodakaraensis* p41 subunit diluted in 1  $\times$  TE (p41 (Tris)). MnCl<sub>2</sub> was omitted in this experiment. Reactions described in lanes 8–10 were carried out in 10- $\mu$ l reactions. Reaction described in lanes 4 and 7 (p41 (glycerol)) contained 0.6 M glycerol, whereas the reaction in lane 10 contained 0.2 M glycerol. *M*, molecular mass markers. *B*, analysis of products by thin layer chromatography. Reaction mixtures were as described in panel *A*; aliquots (1  $\mu$ l) were analyzed on PEI plates developed in 0.3 M LiCl. The  $^{32}$ P distribution (in percentage) is presented. *dAMP der.*, dAMP derivative.

ucts formed are the dAMP-glycerol and dAMP-Tris derivatives.

We also examined whether RNA and DNA synthesis catalyzed by the *T. kodakaraensis* primase complex as well as the p41 subunit was affected by the presence or absence of glycerol and/or Tris. Their omission decreased DNA synthesis slightly (20–30%) with both primase preparations, whereas RNA synthesis catalyzed by the complex was unaffected (data not presented).



**FIGURE 6. Analysis of products formed from [ $\alpha$ - $^{32}$ P]dATP and [ $\alpha$ - $^{32}$ P]rATP.** Reaction mixtures (20  $\mu$ l), as described in the legend for Fig. 1, containing 100  $\mu$ M [ $\alpha$ - $^{32}$ P]dATP (611 cpm/pmol) or 200  $\mu$ M [ $\alpha$ - $^{32}$ P]ATP (1090 cpm/pmol), 0.25 or 0.75  $\mu$ M *T. kodakaraensis* DNA primase complex (lanes 2, 3, 9, and 10), and p41 subunit (lanes 4, 5, 11, and 12) or mutant (*m*) p41 subunit (lanes 6, 7, 13, and 14) were incubated for 30 min at 70  $^{\circ}$ C. Reactions containing 0.25  $\mu$ M of the p41 subunit were incubated for 5 (lane 15), 10 (lane 16), 15 (lane 17), and 60 min (lane 18). Aliquots (1  $\mu$ l) from reactions were separated by TLC on PEI plates in 0.3 M LiCl, after which plates were dried and quantified by phosphorimaging. *d/rAMP der.*, dAMP/rAMP derivative; *rAMP*, 5'-riboadenosyl monophosphate.

**TLC Analysis of Products Synthesized in Presence of [ $\alpha$ - $^{32}$ P]dATP or [ $\alpha$ - $^{32}$ P]rATP**—The synthesis of the fast migrating derivatives by the primase complex, the p41 subunit, and the mutant p41 catalytic subunit with [ $\alpha$ - $^{32}$ P]dATP and [ $\alpha$ - $^{32}$ P]rATP was examined by TLC analysis (Fig. 6). Labeled dAMP derivatives were formed more extensively with the p41 subunit than with the primase complex (~3.5-fold, compare lane 2 and with lane 4). Their formation required the catalytically active p41 subunit as no derivatives were detected with the mutated p41 subunit (lanes 6 and 7). In addition to dAMP derivatives, primase preparations also generated dAMP from dATP. Identical levels of dAMP and the rapid migrating dAMP products were formed by the p41 subunit at early time points (5–15 min, lanes 15–17), but differed at later times. Approximately 90% of the input dATP was utilized in 30 min in the presence of 0.75  $\mu$ M p41 subunit; 60% of this material was converted to the dAMP derivatives, and ~30% was converted to dAMP (lane 5). When [ $\alpha$ - $^{32}$ P]rATP was used in place of dATP, significantly lower levels of the putative AMP products were formed (lanes 9–12) than observed with dATP. Like dATP, rATP was not utilized by the mutant catalytic subunit (lanes 13 and 14). Thus, under the conditions used, the *T. kodakaraensis* DNA primase preparations utilized rATP less effectively than dATP (>10%).

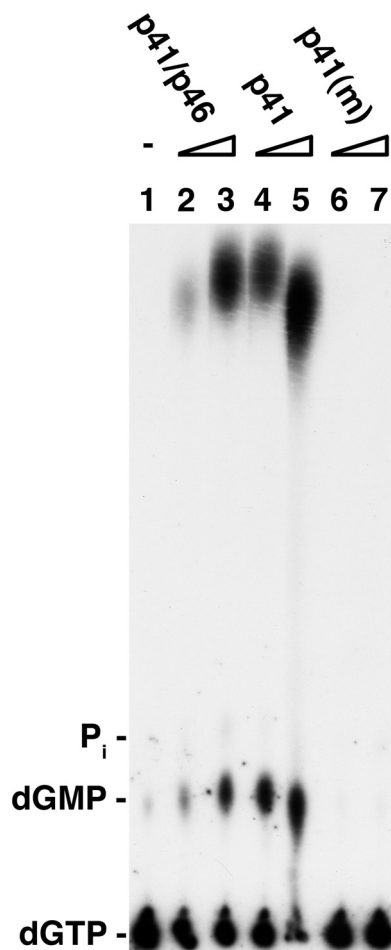


FIGURE 7. **Formation of dGMP-glycerol and dGMP-Tris products.** Reactions (20  $\mu$ l) were as described (Fig. 6) with  $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$  (9400 cpm/pmol) used in place of  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ . Aliquots of reactions (1  $\mu$ l) were subjected to TLC on PEI plates in PEI thin layer chromatography in 0.3 M LiCl. *m*, mutants.

**Formation of Glycerol and Tris Derivatives with Other dNTPs**—The *T. kodakaraensis* primase complex and p41 subunit converted the other  $[\alpha\text{-}^{32}\text{P}]\text{dNTPs}$  to the glycerol and Tris derivatives, and as observed with  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ , the mutant p41 subunit did not support these reactions. Products formed in reactions containing  $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ , analyzed by TLC on PEI plates, were dGMP and the dGMP derivatives (Fig. 7). Based on the  $^{32}\text{P}$  distribution, the level of the products formed equaled the amount of dGTP utilized. Maximally (with 0.75  $\mu\text{M}$  p41 subunit), 90% of the input dGTP was utilized, leading to the production of  $\sim 65\%$  dGMP-glycerol and Tris derivatives and 20% dGMP. As observed with  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ , low levels of the catalytic subunit were more active than the primase complex (Fig. 7, compare lanes 2 and 3 with lanes 4 and 5).  $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$  and  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  were also utilized in a manner similar to that observed with dGTP and dATP (data not presented); thus, all four dNTPs are converted to the dNMP-glycerol and Tris derivatives and dNMP. The products formed with each of the four  $[\alpha\text{-}^{32}\text{P}]\text{dNTPs}$ , separated by urea-PAGE analysis, yielded the identical  $\sim 16$ - and  $\sim 25$ -nt species, which were hydrolyzed by venom diesterase and resistant to CIP and spleen phosphodiesterase (data not presented).

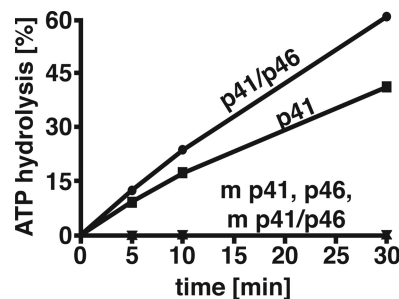


FIGURE 8. **Rate of  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  hydrolysis by *T. kodakaraensis* primase preparations.** Reaction mixtures (20  $\mu$ l) containing 40 mM Tris-HCl, pH 8.0, 2.5 mM DTT, 10 mM magnesium acetate, 100  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  (1900 cpm/pmol), and 0.5  $\mu\text{M}$  of *T. kodakaraensis* DNA primase preparation (as indicated) were incubated for 5, 10, and 30 min at 70  $^{\circ}\text{C}$ . Aliquots (1  $\mu$ l) were subjected to TLC on PEI plates developed with 0.3 M LiCl. After drying, the plate was subjected to phosphorimaging, and the  $^{32}\text{P}$  that migrated as  $\text{P}_i$  (in percentage) was determined. The only other  $^{32}\text{P}$  material detected migrated in the dATP region and was not separated from  $\text{PP}_i$ . Preparations labeled *m p41* and *m p41-p46* indicate the presence of mutated p41 subunit.

**Formation of  $\text{PP}_i$  and  $\text{P}_i$  from dNTPs**—Formation of dAMP-glycerol, dAMP-Tris, and dAMP by *T. kodakaraensis* DNA primase preparations suggested that  $\text{PP}_i$  or possibly  $\text{P}_i$  was produced as well. Incubation of  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  with *T. kodakaraensis* DNA primase preparations resulted in the time-dependent formation of  $^{32}\text{P}_i$  (Fig. 8). After 30 min of incubation,  $\sim 40$  and  $60\%$  of the input  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  was converted to  $^{32}\text{P}_i$  by the p41 subunit and primase complex, respectively, whereas  $^{32}\text{P}_i$  was not formed by mutant preparations or the p46 subunit alone. In the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , rather than  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ ,  $\text{P}_i$  formation was reduced  $\sim 10$ -fold (data not presented). Examination of the different primase preparations for inorganic pyrophosphatase (PPase) activity revealed that all preparations contained this activity, but their levels varied. The p41, mutant p41, wild-type complex, mutant p41-p46 complex, and p46 subunit hydrolyzed  $\text{PP}_i$  to  $\text{P}_i$  (pmol/min/pmol of preparation at 70  $^{\circ}\text{C}$ ) 0.7, 0.9, 12.1, 18, and 12.0, respectively, suggesting the  $\text{P}_i$  could arise by hydrolysis of  $\text{PP}_i$  generated by the action of primase. Preparations devoid of primase activity (mutant p41, mutant p41-p46, and p46 subunit), incapable of generating  $\text{PP}_i$  from  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ , did not form  $\text{P}_i$  (Fig. 8).

We next examined whether  $\text{PP}_i$  was formed during the primase reaction. TLC on PEI plates, using a number of different solvents, failed to adequately resolve dATP (and other dNTPs) from  $\text{PP}_i$ . To evaluate whether  $^{32}\text{PP}_i$  was formed during  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  hydrolysis, we used charcoal, which selectively binds nucleotides but not  $\text{PP}_i$  or  $\text{P}_i$ , subjected the charcoal non-adsorbed material to yeast inorganic PPase treatment, and measured  $^{32}\text{P}_i$  formation following TLC separation (Table 1). Under the conditions used, 95% of the input  $^{32}\text{P}$  of the added  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  was adsorbed to charcoal, whereas the low level of nonadsorbable material migrated as  $^{32}\text{P}_i$ . When  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  was incubated with the p41 subunit,  $\sim 20\%$  of the  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$  was hydrolyzed to  $\text{P}_i$ . TLC separation of the charcoal nonadsorbable material showed that 80% of this material was  $\text{PP}_i$ . When this material was incubated with yeast inorganic PPase, the  $^{32}\text{P}$  present in the fraction was converted quantitatively to  $\text{P}_i$  (Table 1). Similar analysis of the products formed by the *T. kodakaraensis* primase complex revealed that a major portion of the charcoal nonadsorbable material released from



## Formation of nt Adducts by *T. kodakaraensis* DNA Primase

**TABLE 1**

**Identification of PP<sub>i</sub> and P<sub>i</sub> as products formed by *T. kodakaraensis* DNA primase preparations**

Reaction mixtures, as described in the legend for Fig. 8, were incubated for 10 min at 70 °C. Charcoal (10 μl of 15% slurry) and 0.1 M HCl (10 μl) were added, and after 10 min on ice, the mixture was centrifuged. Aliquots of the supernatant (2 μl) were incubated in reactions (5 μl) containing 100 mM Tris-HCl, pH 7.5, and 2 mM magnesium acetate in the presence or absence of yeast inorganic PPase (1 μg) for 30 min at 37 °C. Aliquots (2 μl) were then subjected to TLC separation on PEI plates in 0.3 M LiCl and phosphorimaging.

Primase preparation added and treatment	Charcoal nonadsorbable product (% of distribution)	
	PP <sub>i</sub>	P <sub>i</sub>
p41	80.4	19.6
p41 + PPase	4.0	96.0
p41/p46	20.2	78.8
p41/p46 + PPase	4.2	95.8

[ $\alpha$ -<sup>32</sup>P]dATP was isolated at <sup>32</sup>P<sub>i</sub>; the <sup>32</sup>PP<sub>i</sub> in this fraction was converted to <sup>32</sup>P<sub>i</sub> following yeast PPase digestion. These findings suggest that the PP<sub>i</sub> is most likely formed by hydrolysis of dATP, which is then converted to P<sub>i</sub> by the PPase present in the enzyme fraction. A major source of PPase activity present in the primase complex appeared to be the p46 subunit. Because the PPase activity measurements were carried out at 70 °C, it is likely that this activity is associated with the archaeal proteins and not an *Escherichia coli* contaminant. The nature of this activity, however, remains to be explored.

### DISCUSSION

In the accompanying article, we demonstrated that the *T. kodakaraensis* primase initiated primer synthesis in reactions containing only dNTPs (1). In this study, we show that the *T. kodakaraensis* p41-p46 complex and the *T. kodakaraensis* p41 catalytic subunit hydrolyzed dNTPs to dNMP and PP<sub>i</sub>. When these preparations were incubated in reactions containing high levels of Tris buffer and/or glycerol, dNMP-Tris and/or dNMP-glycerol derivatives were also formed. Surprisingly, these reactions were unaffected by DNA. We suggest that these findings may reflect the mechanism by which the archaeal *T. kodakaraensis* primase initiates the synthesis of oligonucleotide chains because they resemble an aborted initiation-elongation reaction. Where examined, all primases generate substantial levels of short oligonucleotides, presumably reflecting their instability and/or inability to be extended. In some cases, primases can form dinucleotides even in the absence of DNA (summarized in Ref. 14).

Frick and Richardson (14) proposed a model for the synthesis of primers by primases that involves two NTP binding sites on the primase protein. These include a site at which a NTP is to be incorporated at the 5' end of the primer (initiation site) and a second site that binds the NTP added to the 3' end of the primer (elongation site). During each elongation step of primer synthesis, the product ( $n + 1$ ) oligonucleotide is transferred to the initiation site, leading to the binding of another NTP at the elongation site. To explain the formation of the dAMP derivatives described here, we propose that H<sub>2</sub>O, glycerol, or Tris occupy the initiation site and that dATP is loaded at the elongation site. Hydrolysis of dATP (to PP<sub>i</sub>) concomitant with dAMP transfer to the occupant at the initiation site could lead to the formation of dAMP, glycerol, or dAMP-Tris. In keeping

with this notion, the binding of NTPs by primase appears to occur initially at the elongation site (15). The crystal structure of the *Pyrococcus horikoshii* DNA primase complex containing a single UTP molecule was determined (16). This complex was obtained by soaking the primase crystals with UTP. We suggest that this nucleotide is bound in the elongation site and that a similar complex would be formed if dNTPs rather than rNTPs were used.

Primers synthesized by prokaryotic and eukaryotic primases contain 5'-triphosphate termini (5), whereas only a small percentage (5–10%) of the products formed by the *T. kodakaraensis* primase complex retained triphosphate ends (1). We noted that the amount of dNMP derivatives formed in the *T. kodakaraensis* primase reaction was hardly altered by the presence of DNA template (see experiments with synthetic oligonucleotide templates (1)). We speculate that the transfer of dNTPs (or possibly rNTPs) from the elongation nucleotide binding site to the initiation site (occupied with H<sub>2</sub>O, glycerol, or Tris) in the presence of a DNA template could lead to the formation of polynucleotide chains deficient in triphosphate ends. This idea is in keeping with our findings that a substantial amount of chains formed contained five monophosphate ends. Because the dNMP-glycerol and dNMP-Tris derivatives were produced at rates significantly higher than that observed with the corresponding rNTP derivatives, our proposed model would be expected to lead to the formation of a higher percentage of triphosphate-terminated RNA chains. Our findings, however, indicated that the level of RNA and DNA chains containing triphosphate ends produced were similar (both ~5–10%). Further studies on products synthesized with rNTPs in the *T. kodakaraensis* primase reaction may help explain this discrepancy.

In this study, we focused on the synthesis of the dNMP-glycerol and the dNMP-Tris derivatives because their production was more robust than those formed with rNTPs. However, we detected the production of various derivatives formed in reactions with rNTPs and the *T. kodakaraensis* primase complex that migrated as ~15- and ~5–8-nt bands (particularly with GTP) and small levels of fast migrating material with ATP following TLC on PEI plates. Similar to observations made with dNTPs, their formation did not require a DNA template and instead required an active p41 subunit or primase complex, glycerol, and Tris buffer. We also showed that other small molecule acceptors, including glucose, could be linked to dAMP, although they were not characterized in more detail. Because little is known about the physiology and metabolic activities of archaea, the biological role of these adducts remain to be explored.

As described here, *T. kodakaraensis* primase preparations formed oligonucleotides only in the presence of DNA templates, whereas production of the dNMP-glycerol and dNMP-Tris derivatives occurred in the absence of DNA. Because of their aberrant migration through polyacrylamide gels and cleavage by venom phosphodiesterase, these derivatives appeared to represent the *de novo* synthesis of oligonucleotides. A number of enzymes, including other archaeal primases, DNA polymerase  $\lambda$ , DNA polymerase  $\mu$ , and terminal deoxynucleotidyl transferase, have been reported to possess template-

dependent and template-independent polymerase activities that synthesize oligodeoxynucleotides *de novo* (17, 18). Based on our findings, these conclusions warrant a more careful characterization.

Formation of the dNMP adducts, described above, suggests that the *in vitro* initiation reactions catalyzed by the purified *T. kodakaraensis* primase *in vitro* lack specificity. Initiation sites detected with purified eukaryotic DNA primases also appear to be nonspecific as they are altered by the level of NTPs added to reactions (8). These findings raise the possibility that other replication proteins (perhaps at the fork) interact with eukaryotic DNA primases and influence the specificity of the initiation reaction.

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