Acidic Residues in the Nucleotide-binding Site of the Bacteriophage T7 DNA Primase*

Seung-Joo Lee and Charles C. Richardson‡
From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

DNA primases catalyze the synthesis of oligoribonucleotides to initiate lagging strand DNA synthesis during DNA replication. Like other prokaryotic homologs, the primase domain of the gene 4 helicase-primase of bacteriophage T7 contains a zinc motif and a catalytic core. Upon recognition of the sequence, 5′-GTC-3′ by the zinc motif, the catalytic site condenses the cognate nucleotides to produce a primer. The TOPRIM domain in the catalytic site contains several charged residues presumably involved in catalysis. Each of eight acidic residues in this region was replaced with alanine, and the properties of the altered primases were examined. Six of the eight residues (Glu-157, Glu-159, Asp-161, Asp-207, Asp-209, and Asp-237) are essential in that altered gene 4 proteins containing these mutations cannot complement T7 phage lacking gene 4 for T7 growth. These six altered gene 4 proteins can neither synthesize primers de novo nor extend an oligoribonucleotide. Despite the inability to catalyze phosphodiester bond formation, the altered proteins recognize the sequence 5′-GTC-3′ in the template and deliver preformed primer to T7 DNA polymerase. The alterations in the TOPRIM domain result in the loss of binding affinity for ATP as measured by surface plasmon resonance assay together with ATP-agarose affinity chromatography.

DNA primase is an essential component of most replisomes in that it catalyzes the synthesis of oligoribonucleotides that serve as primers for the lagging strand DNA polymerase. In the bacteriophage T7 replication system, the DNA primase is encoded by gene 4 of the phage (1). The multifunctional gene 4 protein contains both a helicase and a primase domain located in the carboxyl- and amino-terminal halves, respectively, of the polypeptide (3). The gene 4 helicase-primase, together with the gene 5 DNA polymerase, the processivity factor Escherichia coli thioredoxin, and the gene 2.5 single-stranded DNA erase. The alterations in the TOPRIM domain result in the loss of binding affinity for ATP as measured by surface plasmon resonance assay together with ATP-agarose affinity chromatography.

A crystal structure of the T7 DNA primase domain reveals a distinct physical separation of the amino-terminal zinc motif from the catalytic core in that the two are linked via an extended 11-residue polypeptide (10). Such a flexible alignment of these two motifs could modulate primase activity and provide a mechanism by which primers are transferred to the lagging strand DNA polymerase. The flexibility of the linker is illustrated by the observation that the zinc motif of one gene 4 protein subunit within a hexamer can functionally interact with the catalytic core of an adjacent subunit (14). The RNA polymerase core can be further divided into two substructures. One subdomain that encompasses motifs II and III consists of an antiparallel four-stranded β-sheet. The other subdomain (motifs IV–VI) contains a TOPRIM fold also found in topoisomerases (15). A shallow cleft separates the amino-terminal subdomain of the RNA polymerase core from the TOPRIM fold. E. coli DnaG primase also has a cleft between these two subdomains (16, 17), and both clefts contain a number of basic residues. It has been proposed that the basic residues within this cleft electrostatically bind the single-strand DNA and that the polymerization of ribonucleotides occurs at a neighboring metal-binding center within the TOPRIM subdomain. We have described previously two lysines (Lys-122 and Lys-128) located in the basic cleft near motif III that are critical for the catalytic activity of T7 DNA primase (18).

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† To whom correspondence should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1864; Fax: 617-432-3362; E-mail: ccr@hms.harvard.edu.
‡ The abbreviations used are: ssDNA, single-stranded DNA; DTT, dithiothreitol.
The TOPRIM fold found in prokaryotic DNA primases (motifs IV–VI) of both *E. coli* DnaG primase and T7 DNA primase are quite similar, both structurally and with regard to conserved residues (10, 16). Both have a number of acidic residues that cluster on the TOPRIM side of the cleft formed by the amino-terminal portion of RNA polymerase core (motifs II and III) and the TOPRIM subdomain. In the T7 DNA primase structure, five acidic residues create the acidic patch and bind two metal ions, presumably Mg$^{2+}$ (8) (Fig. 1B). One of the metal-binding sites consists of a conserved triad of acidic residues (Glu-157, Asp-207, and Asp-209) similar to that found in DnaG primase as well as in the active site of RNA polymerases (19, 20). The second metal-binding site contains Asp-237 that coordinates another Mg$^{2+}$.

*E. coli* DnaG primase also contains two metal-binding sites along with the same conserved acidic amino acids.

In the present study we have investigated eight conserved acidic residues located in the TOPRIM fold based on sequence homology (8, 15). From the crystal structure of T7 DNA primase, four of these residues appear to be involved in the coordination of metal in the active site (10). In order to determine the role of these residues in primase function, we used *in vitro* mutagenesis to change each residue to alanine. Genetic and biochemical studies of the altered proteins show that six of these residues are indeed involved in the binding of nucleoside triphosphate.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were obtained from the Biopolymer Laboratory at Harvard Medical School. Restriction endonucleases, alkaline phosphatase, and Deep Vent® polymerase were purchased from New England Biolabs. T4 polynucleotide kinase, T4 DNA ligase, radio-labeled nucleotides, and high molecular weight protein markers were purchased from Amersham Biosciences. Agarose and PCR amplification products were kindly provided by shrimp. Polyethyleneimine cellulose TLC plates were from J. T. Baker. NTP-agarose resins and biotin were purchased from Sigma. T7 DNA polymerase (T7 gene 5 protein) was purchased from Amersham Biosciences. Agarose and RNA-primed DNA synthesis by DNA polymerase) used a reaction product that appeared on the plate were determined. Autoradiography was performed after washing the membrane with a T7 promoter (pET24-gp4) was grown at 37 °C. Gene 4 proteins were overproduced by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Reaction product was analyzed by repeated injection of 1 M NaCl for 1 min at a flow rate of 50 μl/min.

**Surface Plasmon Resonance Analysis—**The binding of proteins to NTPs was examined using surface plasmon resonance on the Biacore instrument. Bio-17-ATP and Bio-11-CTP are analogs of ATP and CTP, respectively, which have a biotin attached to the base of the NTP through the indicated number of atoms. The biotinylated NTPs were immobilized on the surface of a sensor Chip SA through a streptavidin-biotin interaction by manually injecting 10 μl of 10 μM in a buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM MgCl₂, and 10% glycerol). Each aliquot from the initial protein pool, flow-through, wash, and elution fraction was analyzed on 10% SDS-PAGE and visualized by staining with Coomassie Blue.

**RESULTS**

A sequence alignment of bacterial and bacteriophage DNA primases revealed six conserved motifs in the DnaG family of primases (Fig. 1A) (8). Motifs IV and V have homologies to motifs found in proteins that bind nucleotides such as ATPases, DNA, and RNA polymerases. An iterative profile search combined with structural modeling proposed that motifs IV–VI form a unique fold, the TOPRIM fold, also found in topoisomerases (15). Within these motifs, a number of acidic residues were considered candidates for metal-mediated binding of NTP and for catalysis (23). The crystal structures of T7 DNA primase and *E. coli* DnaG primase confirmed that motifs IV and V are...
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Table I

| Alteration in gene 4 protein | Δ4 | WT |
|-----------------------------|----|----|
| WT                          | 1.0| 1.0|
| E157A                      | <10⁻⁵| 0.67|
| E159A                      | <10⁻⁵| 1.7|
| D161A                      | <10⁻⁵| 1.7|
| D207A                      | <10⁻⁵| 1.0|
| D209A                      | <10⁻⁵| 3.3|
| E210A                      | 2.9| 2.7|
| D237A                      | <10⁻⁵| 0.83|
| E240A                      | 2.0| 2.7|

a WT, wild type.
Relative efficiency of plating.

Growth of altered gene 4 proteins to complement growth of T7 phage

Gene 4 proteins containing the indicated amino acid substitution were expressed in E. coli DH5α. After infection with either T7Δ4 or wild-type (WT) T7 phage, the number of plaques were counted and normalized to the value obtained with wild-type gene 4 protein. Data were obtained from at least duplicated experiments.

Fig. 1. Primase domain of T7 gene 4 protein. A, the schematic presentation shows the primase domain located in the amino-terminal half of the gene 4 protein. The boxes, labeled with roman numerals, represent conserved motifs in the primase superfamily. The TOPRIM fold is composed of motifs IV–VI and the RNA polymerase-basic region is located between motifs III and IV. Based on sequence homology among prokaryotic DNA primases, conserved amino acid residues (underlined) were identified. The eight acidic residues investigated in this study are numbered. B, crystal structure of the catalytic subdomain is shown as a ribbon model (10). The eight acidic residues as well as Lys-122 are labeled, and their side chains are shown. Two magnesium ions are depicted by spheres with their electron density and labeled as A and B.

The ability of each of the genetically altered gene 4 proteins to support T7 phage growth was examined by using a complementation assay. In this assay, growth of phage T7Δ4 lacking gene 4 is dependent on gene 4 protein exogenously expressed from a plasmid harbored in the T7-infected E. coli. Defects in the function of gene 4 protein do not allow the phage to propagate, and no plaques are formed. Substitution at all 8 positions except for Glu-210 and Glu-240 resulted in loss of the ability of the protein to support growth of phage T7Δ4 (Table 1). These results suggest that the six residues, Glu-157, Glu-159, Asp-161, Asp-207, Asp-209, and Asp-237, are essential for gene 4 protein function. When the cells expressing each of the altered gene 4 proteins were infected with wild-type T7 phage, no significant difference between wild-type and altered gene 4 proteins was found. Thus, none of the altered proteins have a dominant negative effect on the function of the wild-type protein expressed by the phage.

Overproduction and Purification of Altered Gene 4 Proteins

In order to examine the biochemical properties of the altered gene 4 proteins, the proteins were purified to apparent homogeneity. The altered gene 4 proteins are designated gp4-E157A, E159A, D161A, D207A, D209A, E210A, D237A, and E240A. Although gp4-E210A and gp4-E240A could replace wild-type gene 4 protein in vivo, both of these altered proteins were also purified and characterized.

Initially we attempted to purify all of the gene 4 proteins by a standard purification procedure that utilizes both adsorption and elution from a phosphocellulose and an ATP-agarose resin (18). Whereas gp4-E210A and gp4-E240A demonstrated similar affinity for both resins, the other altered proteins displayed reduced affinity for both resins. For example, only 50% of gp4-D209A and gp4-D237A adsorbed to the phosphocellulose resin and less than 10% of gp4-E159A and gp4-D161A adsorbed. Gp4-E157A and gp4-D207A behaved similar to the wild-type protein on phosphocellulose column chromatography. More interestingly, all of these altered proteins except for gp4-E210A and gp4-E240A showed greatly reduced affinity for the ATP-agarose resin. As shown in Fig. 2, the majority of gp4-E157A and gp4-D207A applied to the column (lane 1) did not bind to the resin (lane 2), and consequently only a small percentage of the protein applied to the resin could be recovered (lane 3). This behavior is precisely opposite that observed with wild-type gene 4 protein irrespective of whether the proteins are monitored by staining with Coomassie Blue (Fig. 2A) or by a Western blot (Fig. 2B). These results suggest that the altered proteins are defective in their ability to bind nucleoside triphosphate, a property that we address below.

In view of the altered behavior of these proteins on phosphocellulose and ATP-agarose, it was necessary to design an alternative purification procedure. Basically, chromatography on DEAE-Sepharose, a purification step that has been used previously (18). Consistent with the ability of gp4-E210A and gp4-E240A to support T7Δ4 growth, these two proteins behaved similarly to wild-type gene 4 protein on both phosphocellulose and ATP-agarose, and hence these two proteins were purified following the normal procedure for wild-type protein. The purity of all of the gene 4 proteins, regardless of the purification procedure, was greater than 90% as measured by SDS-PAGE and staining with Coomassie Blue.

Biochemical Assays

Catalytic Activity of Primase—The single amino acid substitution introduced into the TOPRIM region of primase domain could potentially affect its catalytic function. In the template-directed de novo synthesis of primers, a zinc motif in T7 primase first recognizes a specific sequence (5'−GTC-3') on the ssDNA template. Subsequently, the catalytic RNA polymerase...
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domain polymerizes nucleotide cognate to the template. T7 primase is also able to extend a preformed ribonucleotide annealed to the primase recognition sequence in a template-dependent manner. In addition to template-directed synthesis, the catalytic center can also catalyze the synthesis of random diribonucleotides in the absence of template. We have examined these three catalytic activities of the altered gene 4 proteins and compared them to the activities of the wild-type gene 4 protein.

In the first assay shown in Fig. 3A, a short ssDNA template (5'-GGGTCAAA-3') containing the primase recognition site was incubated with various amounts of gene 4 protein in the presence of ATP and [α-32P]CTP. Reaction products were analyzed on a sequencing gel, and the amount of diribonucleotides synthesized was determined by measuring radioactivity incorporated into the major product, pppACCC. The results showed that both gp4-E210A and gp4-E240A catalyzed synthesis of diribonucleotides as efficiently as the wild-type gene 4 protein, whereas none of the other altered proteins were able to catalyze the synthesis of diribonucleotides.

In the second assay (Fig. 3B), the ability of the proteins to extend a preformed diribonucleotide was measured. In this assay, ATP was replaced with the diribonucleotide, 5'-AC-3', and the incorporation of [α-32P]CTP was measured as in the previous assay. None of the altered proteins except for gp4-E210A and gp4-E240A could incorporate CTP.

In the third assay, the gene 4 proteins were incubated with [α-32P]CTP in the absence of DNA template (Fig. 3C). Only gp4-E210A, gp4-E240A and wild-type protein were capable of synthesizing the diribonucleotide 5'-CC-3'.

Taken together, all three assays show that residues Glu-157, Glu-159, Asp-161, Asp-207, Asp-209, and Asp-237 are critical for the catalytic function of T7 primase, whereas residues Glu-210 and Glu-240 are not. Acidic residues in nucleolytic enzymes are known to be involved in Mg2+ -mediated binding of NTP. By assuming that the loss of catalytic activity by the amino acid substitutions described here is due to disruption of the metal ion-mediated event, we attempted to recover the enzymatic activity of the altered proteins by supplying excess Mg2+. The addition of 10-fold more Mg2+ than that present in the standard reaction assay did not result in any measurable de novo synthesis of oligoribonucleotides.

Reconstitution of Primase Activity—For template-directed primer synthesis, both the zinc motif and the catalytic center of gene 4 protein are required. We have shown previously that a defect in either of these motifs can be complemented by a functioning motif in an adjacent subunit within a hexamer of the gene 4 protein (14). For example, a hetero-complex between a gene 4 protein lacking the zinc motif (56-kDa gene 4 protein) and a gene 4 protein with an inactive catalytic site (gp4-K122A) catalyzes template-directed oligoribonucleotide synthesis, whereas neither subunit alone can (14). In order to examine such a reconstitution of primase activity, we prepared various combinations of gene 4 protein mixtures with the 56-kDa gene 4 protein, gp4-K122A, and the six altered primase-inactive gene 4 proteins identified from this study. After mixing the two proteins, the ability of the gene 4 protein mixture to synthesize template-directed oligoribonucleotides was determined by measuring the incorporation of [α-32P]CTP into oligoribonucleotides (Fig. 4). Similar to gp4-K122A, all the primase-defective proteins could catalyze the synthesis of oligoribonucleotides in
the presence of the 56-kDa protein. However, all combinations of inactive primases did not show any activity for oligoribonucleotides synthesis. The ability to reconstitute primase activity with the 56-kDa gene 4 protein demonstrates that the zinc motif of the altered proteins can function properly.

**RNA-primed DNA Synthesis by T7 DNA Polymerase**—The ultimate role of DNA primase during DNA replication is to supply oligoribonucleotides for use as a primer by DNA polymerase to initiate lagging strand synthesis. It has been shown previously that a gene 4 protein defective in primer synthesis due to an inability to catalyze phosphodiester bonds can nonetheless successfully deliver a preformed tetraribonucleotide annealed at a primase recognition site to DNA polymerase to initiate DNA synthesis (18). In order to examine this functional aspect of the altered proteins, RNA-primed DNA synthesis by DNA polymerase was carried out by using two different assays.

In the first assay, ATP and CTP were provided as precursors for *de novo* synthesis of oligoribonucleotides. Consistent with the previous results, under these conditions wild-type, gp4-E210A, and gp4-E240A were enabled by T7 DNA polymerase to initiate DNA synthesis on ssDNA (Fig. 5A). None of the other altered proteins could enhance T7 DNA polymerase activity because they are unable to synthesize oligoribonucleotides. However, when the preformed oligoribonucleotide 5'-pppACCA-3' replaced ATP and CTP, all the altered gene 4 proteins could deliver the primer to DNA polymerase to initiate DNA synthesis (Fig. 5B). Thus, despite the inability of the altered proteins to synthesize primers, the defective gene 4 proteins can recognize the preformed oligoribonucleotides and their recognition sequence and then deliver the primer to T7 DNA polymerase.

**Affinity for NTP**—Although the defect in oligoribonucleotides synthesis created by the replacement of the six acidic residues in the TOPRIM fold could arise by any one of several mechanisms, several lines of evidence suggest that the alterations affect nucleotide binding. As discussed earlier, all of the altered proteins defective in primer synthesis had very low affinity for either AMP or ADP (Table II).

Based on the data just presented for wild-type gene 4 protein binding to ATP, we examined the ability of each of the eight altered gene 4 proteins to bind to an ATP affinity column where the ATP is linked to the resin via C-8 of the base. In contrast to wild-type gene 4 protein, none of the altered gene 4 proteins defective in primase activity could bind to the column (Table II and Fig. 2). However, gp4-E210A and gp4-E240A bound as well as wild-type gene 4 protein.

We have also examined the binding of wild-type and the altered gene 4 proteins to ATP by using surface plasmon resonance. In these experiments, biotinylated ATP or CTP was immobilized on the surface of a Biacore chip via a biotin-streptavidin interaction. Each of the gene 4 proteins were then flowed over the NTP linked to the chip, and the response units (RU) were measured. It was observed that gene 4 protein nonspecifically bound to streptavidin on the surface of the chip. To eliminate the contribution of this nonspecific binding, proteins were flowed over a chip where only biotin was immobilized to the streptavidin chip instead of the biotinylated NTP. Specific binding of gene 4 protein to immobilized NTP was expressed by the difference between a binding signal from an experimental lane and the one from the control lane. Although wild-type gene 4 protein displayed a rapid association with the bound ATP, neither gp4-E157A, gp4-D207A, nor gp4-D237A, all defective in primase activity, displayed any measurable binding (Fig. 6). We also examined gp4-K122A, which we had shown previously to be defective in catalyzing phosphodiester bond formation, and we found that it also did not bind to ATP (Fig. 6). When CTP, another preferred nucleotide by T7 primase, was immobilized to the chip, no binding signal to the wild-type protein was detected (data not shown).

**Helicase Domain Activity**—Although primase and helicase domains are separated in T7 gene 4 protein (Fig. 1A), the activity of one domain is often affected by the other domain. For instance, the efficiency of oligoribonucleotides synthesis on the long template is dependent on not only the primase domain but also the helicase domain (11). The enhancement of primase activity results from the ability of the helicase domain to transport the primase domain to recognition site. In order to determine whether the alterations in TOPRIM also affect the helicase domain, we examined the helicase activity of the altered proteins. As summarized in Table III, all activities of the altered proteins (oligomerization, DNA binding, ssDNA-dependent dTTP hydrolysis, and DNA unwinding) were similar to those of the wild-type protein. Therefore, we conclude that the alterations are confined within the primase domain.
is indicated. dTMP incorporated into DNA. The amount of DNA synthesis was determined by measuring the amount of radioactive 5 protein. After incubation for 5 min at 37 °C, the products of the reaction were spotted on DE81 membrane, and unincorporated dNTP was removed by extensively washing with ammonium formate.

A DNA sequence from which synthesis is initiated are shared with RNA polymerases that recognize, albeit much longer DNA length that can be used as a primer by the respective DNA polymerase (1). Thus, their synthesis of RNA and recognition of a trinucleotide sequence, on single-stranded DNA, is indicated. dTMP incorporated into DNA. The amount of DNA synthesis was determined by measuring the amount of radioactive 5 protein. After incubation for 5 min at 37 °C, the products of the reaction were spotted on DE81 membrane, and unincorporated dNTP was removed by extensively washing with ammonium formate. The amount of DNA synthesis was determined by measuring the amount of radioactive dTMP incorporated into DNA. WT, wild type; No prot, no protein.

### Table II

| Nucleotide | Position of attachment | Length of linker | Binding of proteins |
|------------|-----------------------|-----------------|--------------------|
| AMP        | N-6 of base           | 8               | -                  |
| ADP        | C-8 of base           | 8               | +                  |
| ATP        | N-6 of base           | 11              | -                  |
| ATP        | Hydroxyls of ribose   | 22              | +                  |

![Fig. 5. RNA-primed DNA synthesis by T7 DNA polymerase.](image1.png)

**Fig. 5. RNA-primed DNA synthesis by T7 DNA polymerase.** DNA synthesis on an M13 ssDNA template catalyzed by T7 DNA polymerase requires primers annealed to the template. The primer was either synthesized de novo from ATP and CTP by gene 4 protein (A) or supplied as 5’-ACCA-3’ (B). Reaction mixtures contained dGTP, dCTP, dATP, [α-32P]dTTP, M13 ssDNA, T7 DNA polymerase, and 100 nM of the indicated gene 4 protein. After incubation for 5 min at 37 °C, the products of the reaction were spotted on DE81 membrane, and unincorporated dNTP was removed by extensively washing with ammonium formate. The amount of DNA synthesis was determined by measuring the amount of radioactive dTMP incorporated into DNA. WT, wild type; No prot, no protein.

**DISCUSSION**

DNA primases are an essential component of the replisomes found in most replication systems. They have a dual role in that they must catalyze the template-directed synthesis of oligoribonucleotides, usually of a well defined length, and then interact with their cognate DNA polymerase to deliver the oligonucleotide for use as a primer to initiate DNA synthesis. In the first step of primer synthesis, the prokaryotic DNA primases recognize a trinucleotide sequence, on single-stranded DNA, that can be used as a primer by the respective DNA polymerase (1). Thus, their synthesis of RNA and recognition of a DNA sequence from which synthesis is initiated are shared with RNA polymerases that recognize, albeit much longer DNA sequences, promoters and catalyze the synthesis of RNA. The fact that the recognition sequence for DNA primases resides on single-stranded DNA serves as another distinguishing characteristic of the primases. Unlike classical RNA polymerases, the primases have low processivity of nucleotide polymerization and relatively low fidelity (1). In view of their ability to catalyze template-directed synthesis of oligoribonucleotides, it is not surprising that the DNA primases and polymerases share catalytic mechanisms and structural features. For example, two aspartic acid residues in a motif, DXD, are often conserved not only throughout many polymerases but also in DNA primases (1, 25). Those acidic residues form an acidic patch in the active sites of T7 DNA primase and E. coli DnaG primases and are thought to bind two divalent metal ions (10, 16, 26), reminiscent of the two metal ion-mediated mechanism of nucleotide condensation proposed for all polymerases by Steitz et al. (27). However, despite these similarities, the primases do differ significantly from the polymerases. The RNA polymerase domain of both T7 primase and DnaG primase contains a TOPRIM fold found in a variety of DNA topoisomerases but not polymerases (15). More interestingly, the DNA primases have a zinc-binding domain that, in the case of phage T7 primase, consists of a four-stranded anti-parallel β-sheet flanked by a carboxyl-terminal α-helix (10). The zinc in T7 primase is bound by two pairs of cysteines (28) and, like the zinc-binding domain of DnaG primase, is located at the amino terminus of the protein, tethered to the catalytic core of the primase domain by a highly flexible linker (10). This zinc-binding domain is involved in nucleotide sequence recognition as well as in the transfer of the primer to the polymerase (9, 10).

The conserved nature of the acidic residues in the TOPRIM fold and the involvement of several of these in the coordination of two metal ions in the crystal structures of E. coli DnaG primase and T7 DNA polymerase (10, 16) make a strong case that they play a critical role in nucleotide binding and/or catalysis. Indeed, a number of biochemical studies have implicated motifs IV, V, and VI in the coordination of nucleoside triphosphate substrates and divalent metal cations necessary for catalysis (29–31). In the case of T7 DNA primase, one metal ion (A in Fig. 1B) is coordinated with Glu-157 and Asp-207 and possibly with the side chain of Asp-209, although the latter in the crystal structure appears too distant to make direct contact (10). Nonetheless, these three acidic residues are conserved in all proteins containing the TOPRIM fold and constitute the acidic triad found in the catalytic site of RNA polymerase (19, 20). The second metal (B in Fig. 1B) site clearly has a contact with Asp-237, the equivalent residue in DnaG that was detected by Fe2+ affinity (31). Asp-161 is also a member of the...
cluster of five invariant acidic residues on the TOPRIM side of the cleft that constitutes the RNA polymerase domain but is somewhat remote for direct involvement with either of the two metals in the crystal structure.

In the present study, we have determined the importance of eight of the acidic residues located in motifs IV–VI that compose the TOPRIM domain. Each of the eight acidic residues was individually substituted with alanine, and the altered protein was examined for its ability to support the growth of T7 phage lacking gene 4. Each of the altered proteins was then purified, and its biochemical properties were examined. Of the eight acidic residues, only two, Glu-210 and Glu-240, do not appear to be important for primase activity in vivo or in vitro. Although Glu-210 and Glu-240 are proximate to the critical acidic residues, Asp-209 and Asp-237, both gp4-E210A and gp-E240A support T734 growth, and no biochemical defects were detected in the battery of primase assays. This non-essential role is supported by the crystal structure of the primase domain; both Glu-210 and Glu-240 are exposed to solvent, and the side chain of Glu-210 is oriented opposite to the metal (10) (Fig. 1B). The side chain of Glu-240 does face the metal but at a distance of 6 Å.

We were reassured to find that four of the six acidic residues are essential for primase activity and for phage growth, either from inspection of the crystal structure of T7 primase or from homologous residues in other primases, in major roles in the catalytic cavity. The two residues (Glu-157 and Asp-207) observed to coordinate one of the two metal ions (A in Fig. 1B), in the structure were both found to be essential as well as Asp-209 whose side chain is in a position to complete the acidic triad. Asp-237, which coordinates the second metal ion (B in Fig. 1B), is likewise essential. Of the other two essential acidic residues, Glu-159 and Asp-161, the latter is a part of the cluster that forms the acidic patch that coordinates the second metal ion. Glu-159, on the other hand, is notably distant from the metal ions to be involved in coordination, and most interestingly, it is not one of the highly conserved residues among DNA primases. More likely, it is a critical determinant of the binding pocket for nucleotide via its interactions with other residues.

We find that all of the amino acid changes in the TOPRIM fold which affect primase activity also drastically reduce the affinity of gene 4 protein to bind to an ATP-affinity column. We have routinely used an ATP-affinity column to purify T7 gene 4 protein because the wild-type protein binds with high affinity in the presence of Mg2+ and Mg2+ -EGTA, necessitating elution with EDTA (18). Our initial attempts to purify the altered proteins described in this study revealed the defect in ATP binding, requiring the substitution of a DEAE-Sepharose chromatography step. The inability of the primase-defective gene 4 proteins to bind to ATP was confirmed using surface plasmon resonance to demonstrate their lack of binding to ATP immobilized to the surface of a Biacore sensor chip. Clearly, the essential acidic residues described in this study affect the ability of ATP to bind to the catalytic site of the primase, a defect either mediated through the inability to bind the essential metal ion or via the absence of side chains that interact directly with the nucleotide.

A surprising result has been the finding that the gene 4 protein binds to the ATP-affinity resin via the ATP-binding site of the primase domain. We had assumed previously that the binding of gene 4 protein to this resin was occurring during binding of the ATP at the nucleoside triphosphate site of the helicase domain, which binds and hydrolyzes both dTTP and ATP (32). The binding of ATP to the primase domain is relatively weak with a K_m of 0.32 mM as measured kinetically (33). Precisely why the nucleotide-binding site of the primase has such a high affinity for the ATP-agarose resin remains unclear.

As is the case for most polymerases, T7 DNA primase contains essential acidic residues on the floor of the active site. In the general mechanism of two metal ion-mediated catalysis by polymerases, one metal ion promotes the deprotonation of the 3'-hydroxyl of the primer strand, and the other facilitates the formation of the pentacovalent transition state of the α-phosphate of incoming (d)NTP and the departure of the PP_i-leaving group (34). Three conserved acidic residues (Glu-157, Asp-207, and Asp-209) clustered in one magnesium ion in T7 primase would play such a role as aspartates in the catalytic triad of an archaeal primase interacting with phosphates of UTP (35). From their proximity from the other metal ion, Asp-161 and Asp-237 are also considered to coordinate with the other metal ion. Therefore, the five metal-ion-coordinated residues appear to interact with the first nucleotide ATP in oligoribonucleotide synthesis, as determined by ATP-affinity chromatography and surface plasmon resonance assay. However, Glu-159, from this work, and Lys-122, from the earlier study, are distant from any metal ion in the crystal structure, yet both are critical to binding to ATP. More interestingly, those residues are noticeably close to one another in the structure, suggesting they have a common basis for the loss of ATP binding upon replacement with alanine (Fig. 1B).

As the catalytic site of T7 DNA primase, it is clear that a common basis exists for the loss of ATP binding upon replacement with alanine (Fig. 1B).
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