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The Role of the Zinc Motif in Sequence Recognition by DNA Primases*

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The DNA primase of bacteriophage T7 has a zinc-binding motif that is essential for the recognition of the sequence 3'-CTG-5'. The T7 primase also catalyzes helicase activity, a reaction coupled to nucleotide hydrolysis. We have replaced the zinc motif of the T7 primase with those found in the gene 61 primase of phage T4 and the DnaG primase of Escherichia coli. The T4 and E. coli primases recognize the sequences 3'-T(C/T)G-5' and 3'-GTC-5', respectively. Both chimeric proteins can partially replace T7 primase in vivo. The two chimeric primases catalyze the synthesis of oligoribonucleotides albeit at a reduced rate and DNA dependent dTTPase activity is reduced by 3-10-fold. Both chimeric proteins recognize 3'-(A/G)CG-5' sites on single-stranded DNA, a site that differs from those recognized by the T7, T4, or E. coli primases, indicating that the zinc motif is the major determinant in site-specific recognition.

DNA primases catalyze the template-directed de novo synthesis of oligoribonucleotides on single-stranded DNA (ssDNA) for use as primers for DNA polymerases to initiate DNA synthesis at origins of replication and on the lagging strand side of the replication fork. In addition to their interaction with DNA polymerases, DNA primases are also physically and functionally associated with DNA helicases, in part, to make use of the unidirectional translocation activity of the helicase to access recognition sites for primer synthesis.

DNA primases from at least four sources, bacteriophage T7 (2, 3), bacteriophage T4 (4, 5), Escherichia coli (6-8), and herpes simplex virus (9) all initiate oligoribonucleotides at specific recognition sequences. Three of these primases, the T7 gene 4 protein, the T4 gene 61 protein, and the E. coli DnaG protein initiate synthesis from trinucleotide recognition sequences, 3'-CTG-5', 3'-T(C/T)G-5', and 3'-GTC-5', respectively. In all three cases, the primases initiate synthesis at trinucleotide sequences in which the 3'-nucleotide of that sequence is essential for recognition but is not copied into the product oligoribonucleotide. The T7 primase catalyzes the synthesis of dimers, trimers, and tetraters at its recognition sites but only the tetraters function as primers for T7 DNA polymerase (2, 3, 10, 11). In the case of the T4 and E. coli primases the major functional species are pentamers and 10-12mers, respectively (12-19).

All DNA primases, whether from bacteriophage, viral, prokaryotic, or eukaryotic sources, have a potential metal binding site (20, 21). In the bacteriophage and prokaryotic primases, the potential metal binding site is located in the N terminus of the polypeptide. Two DNA primases, the phage T7 gene 4 primase (21) and the E. coli DnaG primase (22) have been shown to be zinc metalloproteins. It is reasonable to postulate that the zinc motif of primases is important in the recognition of primase sites since the recognition of specific sequences in DNA is known to occur in several other biological processes. For example, the Cys$_2$His$_2$ zinc fingers of the Zif268 (23) and the human oncogene product GLI (24) make contacts with three bases. An alternative metal-binding motif, the Cys$_4$ zinc motif, is found in many proteins such as the human elongation factor TFIIS and interacts with both DNA and RNA (25, 26). In one instance, gene 4 proteins with single amino acid substitutions of Ser at each of the four conserved Cys residues cannot support phage DNA replication and growth, and the altered proteins cannot catalyze template-directed synthesis of oligoribonucleotides (21).

The mechanism by which the prokaryotic DNA primases recognize a trinucleotide sequence on ssDNA is at present unknown as is the precise role of the zinc motif in this process. One approach to defining the role of the zinc motif in sequence recognition is to construct chimeric primases in which the zinc motif of one is substituted for another. The gene 4 primase of phage T7 has utility in this approach in that it is also a helicase, thus circumventing the dependence of the primase reaction on the presence of a separate specific helicase, as is the case for the phage T4 and E. coli priming systems (12, 27-30). Gene 4 actually encodes two polypeptides, a 56-kDa protein and a 63-kDa protein, the former protein arising as the result of an internal translation initiation sequence in the gene 4 transcript (31). The 56-kDa gene 4 protein is a helicase that translocates 5' to 3' on ssDNA, a reaction coupled to the hydrolysis of nucleotides, and catalyzes the unwinding of duplex DNA that it encounters (32). The 63-kDa gene 4 protein contains an additional 63 amino acids at its N terminus, and it is within this sequence that the zinc motif is located. Consequently, the 63-kDa gene 4 protein has primase activity in addition to the helicase activity found in both molecular mass species of the protein (3, 32). Since the 63-kDa protein can provide both primase and helicase activities, it is both necessary and sufficient for productive infection by T7 phage (33, 34). However, gene 4 protein functions as a hexamer (35-37), and most likely hexamers found in wild-type phage infected cells contain both molecular forms of gene 4 protein (38).

We recently constructed a T3/T7 chimeric primase in which the zinc motif of the phage T3 primase, a primase whose recognition site is not known, was substituted for the zinc motif of...
the T7 primase (39). The recognition sites used by the chimeric primase were identical to those used by the T7 primase, and the chimeric primase was functional in vivo, not a surprising result since T3 and T7 are closely related and their gene 4 proteins are highly conserved (40, 41). The ability of the T3/T7 chimeric primase to function both in vivo and in vitro provided the incentive for us to examine chimeric proteins in which the zinc motif of the primase of either phage T4 or E. coli is substituted for that of the phage T7 primase. Since the trinucleotide recognition sequences for these three primases differ from one another, the identity of any functioning chimeric protein will provide definitive information on the role of the zinc motif in sequence recognition and will perhaps yield insight into the importance of specific recognition sequences in a particular replication system. In this report we show that the zinc motifs of both the T4 and E. coli primases can replace the zinc motif of the T7 gene 4 protein to yield a functional primase but that the zinc motif alone does not dictate the sequence specificity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain of Bacteriophage—** E. coli DH5α (Life Technologies, Inc.) was used for cloning of DNA, and E. coli C600 was used for complementation analysis. E. coli HMS 174 and bacteriophage M13 were used for expression of recombinant genes. Wild-type bacteriophage M13 mp18 DNA containing a deletion of the entire gene 4 were kindly provided by S. Notarnicola (Harvard Medical School). Bacteriophage T4 was obtained from American Type Culture Collection.

DNA and Nucleotides—T4 DNA was purified from T4 phage as described previously (42) and used as a template for PCR. Expression plasmids, pGP4–5, pGP4–6G64510, pGP4–6G6410C36.39S have been described (21, 32, 33). M13mp18 DNA was purified as described elsewhere (42). All nucleotides were purchased from Amersham Corp. Oligonucleotide primers for PCR and oligonucleotide templates for assay of DNA primase were chemically synthesized. The nucleotide sequences of oligonucleotide templates are as follows: oligo-GCG, 5'-TTTACAGAATTTCCGATCGACATCTTAACTGCTGCTCTAG-3'; oligo-GCA, 5'-TTTACAGAATTTCCGATCGACATCTTAACTGCTGCTCTAG-3'; oligo-GCT, 5'-TTTACAGAATTTCCGATCGACATCTTAACTGCTGCTCTAG-3'; oligo-GCC, 5'-TTTACAGAATTTCCGATCGACATCTTAACTGCTGCTCTAG-3'; and oligo-GCGACCAGAACCCGGTCCCGACGACGAGAAAACCCCGTCCTT-5'.

**Construction of Expression Plasmids for Chimeric and Mutant Primases—** To construct the chimeric proteins, Fsp1 and Spfi recognition sites were introduced into the loop region of the zinc finger motif of pGP4–664c. The zinc loop region of T7 primase was amplified by PCR with the primers T7–1 (5'-ACATCGACAGACAACTCTCCCTCC-3') and T7–2 (5'-CGACTCTGACATCTTGTCACGACGACGAGAAAACCCCGTCCTT-3'). The amplified fragments were digested with Fstl and Sba1 restriction enzymes and the resulting fragments were cloned into the Fstl and Sba1 sites of pGP4–664c (pGP4–664–C). The regions of T4 primase and E. coli primase were amplified from T4 DNA and E. coli genomic DNA using PCR. The primers used for amplification of the T4 fragment were 5'-GGGACATTCATCTGGACATCTTAACTGCTGCTCTAG-3' and 5'-CCGTTGAAATTTCCGATCGACATCTTAACTGCTGCTCTAG-3'. The amplified fragments were then incubated with T4 DNA polymerase to remove the additional T residue at the 3'-end. The fragments were inserted into the blunt-ended Fsp1 and Spfi sites of pGP4–664–C. Nucleotide sequences of the resulting plasmids (pGP4–664–T4, pGP4–664–E) were confirmed by DNA sequencing. pGP4–664–T4-C36.39S, pGP4–664–E–C36.39S, pGP4–64–T4, pGP4–64–E, pGP4–664–C were constructed by cloning the Fstl and Sba1 fragment from pGP4–664–T4 or pGP4–664–E into pGP4–664–C. The amplified fragments were purified by gel electrophoresis. The purified primase was prepared as described previously by Notarnicola et al. (37). Three liters of E. coli cells carrying the appropriate expression plasmids were cultured in 2X YT medium (16 g/liter Bacto-Tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl) at 37°C until A600 was 1.0 and infected with bacteriophage M13 which encode RNA polymerase of bacteriophage T7 at a multiplicity of infection of 10. The cells were cultured for an additional 3 h and harvested. The cells were suspended in buffer L (50 mM Tris-CI (pH 7.4), 5 mM EDTA, 100 mM NaCl) containing 50 mg/ml lysozyme and 400 μM phenylmethylsulfonfluride and incubated on ice for 1 h. The suspension was frozen, thawed twice, and centrifuged at 30,000 × g for 1 h at 4°C, and the supernatant was collected. The precipitate was reextracted twice with buffer L-II (50 mM Tris-CI (pH 7.4), 5 mM EDTA, 500 mM NaCl), and the supernatant obtained after centrifugation (15,000 × g for 15 min at 4°C) was combined with the first supernatant. The combined lyase (fraction I) was adjusted to a NaCl concentration of 0.5 M, and the protein was precipitated by the addition of polyethylene glycol 4000 to a final concentration of 10%. The precipitate was dissolved in buffer P (20 mM KPO4 (pH 6.8), 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 20 mM KCl (fraction II) and loaded onto a phosphocellulose (Whatman P11) column (3.2 cm2 × 10 cm) equilibrated with buffer P containing 20 mM KCl. The column was washed with 2 column volumes of buffer P containing 20 mM KCl, and the protein was eluted with a gradient of 20 to 1000 mM KCl. The amount of gene 4 protein in fraction II was determined by SDS-PAGE. The fractions containing gene 4 protein were pooled, and MglCl2 and KCl were added to a final concentration of 10 mM and 0.5 M, respectively (fraction II). Fraction III was loaded onto an agarose-hexane-ATP type-3 (Sigma) affinity column (0.8 cm2 × 1.5 cm) equilibrated with buffer T (20 mM KPO4 (pH 6.8), 0.5 mM EDTA, 0.5 mM DTT) and washed with 2 column volumes of buffer T containing 10 mM MglCl2. The column was eluted with buffer T containing 20 mM EDTA, and the fractions containing gene 4 protein were pooled and dialyzed at 4°C against 20 mM KPO4 (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol (fraction IV).

**Nucleotide Hydrolysis Assays—** The assay for measuring nucleotide hydrolysis by the primases was performed essentially as described previously (37). The reactions (20 μl) contained 40 mM Tris-CI (pH 7.5), 10 mM MgCl2, 10 mM DTT, 50 μg/ml bovine serum albumin, 50 mM NaCl, 5 mM ATP, and varying concentrations of primase. After incubation at 30°C for 10 min in the presence or absence of 50 μM M13 ssDNA (nucleotide phosphorothioate), the reaction was stopped by the addition of 5 μl of 150 mM EDTA and 10 μl DTPD. The products of the reactions were separated by CHEM 3000 polyethyleneimine/UV thin layer chromatography (Brinkmann Instrument Inc.), and the amount of radioactivity in the dTDP spot was determined by scintillation counting.

Oligonucleotide Synthesis Assay—Oligonucleotide synthesis by DNA primase was determined by measuring the amount of radioactivity labeled oligonucleotide synthesized after electrophoretic separation of the products. The reactions (20 μl) contained 40 mM Tris-CI (pH 7.5), 10 mM MgCl2, 10 mM DTT, 50 μg/ml bovine serum albumin, 50 μM potassium glutamate, 0.6 mM dTTP, 21 nM M13 ssDNA or 100 nM synthetic oligonucleotides, and 320 nM (monomer) primase. After incubation at 20°C for 10 min in the absence of rNTPs, 0.3 mM rNTPs at final concentration were added, incubated at 37°C for 60 min, and processed as described previously (3). RNA-primed DNA Synthesis—Analysis of primase stimulation of DNA synthesis on ssDNA by T7 DNA polymerase was performed using a modification of the method described previously (43). The reactions (20 μl) contained 40 mM Tris-CI (pH 7.5), 10 mM MgCl2, 10 mM DTT, 50 mM potassium glutamate, 0.3 mM rNTP, 0.3 mM dTTP, 0.3 mM ([α-32P]dTTP, and varying concentrations of primase for 20 min at 37°C. The reactions were stopped by the addition of 5 μl of 0.2 mM EDTA (pH 8.0) and spotted onto DE81 filters. The filters were washed with 0.3 M ammonium formate (pH 8.0) four times and then washed with 95% ethanol once. After drying, the radioactivity retained on the filter was determined by scintillation counting.

**RESULTS**

**Generation of Chimeric Primases—** The DNA primases of phage T7, phage T4, and E. coli all contain a Cys₂Zn₂-type zinc motif, and each primase recognizes a different trinucleotide sequence (see Introduction). We have replaced the zinc motif of the T7 63-kDa gene 4 primase with those found in the T4 gene 41 primase and the E. coli DNA primase in order to examine the properties of the resulting chimeric proteins in vivo and in vitro.
Zinc Motif of DNA Primases

Amino acid residues of the T7 zinc loop region are replaced by 17-amino acid residues from the loop region of T4 gene 61 primase. Amino acid sequence of the zinc binding domain of wild-type 63-kDa gene 4 protein of T7 bacteriophage which lack the both of gene 4 proteins.

The expression plasmids used to produce the chimeric primases were constructed by replacing the DNA sequence encoding a 15-amino acid sequence of the T7 primase with the corresponding regions derived from T4 gene 61 or E. coli DnaG (Fig. 1). The N-terminal fragments derived from T4 and E. coli encode 24 or 17 amino acids, respectively, and constitute the zinc motifs of these primases. The T4 and E. coli DNA fragments were obtained by PCR amplification of the appropriate sequences from T4 and E. coli DNA. The resulting DNA fragments were then inserted into the T7 gene 4 expression vector pGP4-6G64 replacing a segment of T7 gene 4 encoding a 15-amino acid residue found in the loop region of the zinc motif. The sequences were confirmed by DNA sequence analysis. The N-terminal amino acid sequences, derived from the DNA sequence, of the chimeric primases are shown in Fig. 1.

Complementation Analysis of Chimeric Primases—In order to determine if the chimeric primases function in vivo, plating efficiencies of T7 phage lacking gene 4 (T7Δ4-1) were compared on E. coli cells containing the expression vectors for T7, T4/T7, or E. coli/T7 chimeric proteins (Table I). T7Δ4-1 cannot grow on E. coli in the absence of exogenous gene 4. In agreement with earlier studies (33, 34) E. coli cells containing plasmids encoding both the 56- and 63-kDa gene 4 protein or the 63-kDa gene 4 protein alone support the growth of T7Δ4-1. However, a plasmid encoding the 56-kDa helicase does so less efficiently (0.8 × 10^9 p.f.u./ml). A mechanism allowing T7Δ4-1 to grow at all in cells expressing only the 56-kDa helicase is presently unknown. The essential function of the zinc motif is illustrated by the reduction of growth of T7Δ4-1 in cells harboring a plasmid that encodes a 63-kDa gene 4 protein in which two of the four cysteines of the zinc motif have been replaced with serine, finding in agreement with earlier studies (21). As shown in Table I a plasmid expressing the chimeric protein in which the zinc motif of the T4 primase is substituted for that of the gene 4 primase can support the growth of T7Δ4-1 phage, albeit at a lower efficiency (2.4 × 10^9 p.f.u./ml). On the other hand, the E. coli/T7 chimeric primase appears to support the growth of T7Δ4-1 at an efficiency similar to that observed with the plasmid expressing only the 56-kDa helicase (1.8 × 10^9 p.f.u./ml).

In order to determine unequivocally if the zinc motifs of the T4 and E. coli primases of the chimeric primases can support T7 growth, we replaced Cys-36 and Cys-39 in each primase with serine, an alternation that inactivates T7 primase (Table I). The mutant T7 bacteriophage which lack the both of gene 4 proteins.

As shown in Table I the mutation in the plasmids encoding each of the chimeric proteins reduced greatly the ability of the chimeric protein to support the growth of T7Δ4-1. These results show that the chimeric primases can function in vivo, although at greatly reduced efficiency. The apparent inability of the E. coli/T7 primase to support T7Δ4-1 growth (Table I) is most likely due to our finding that the chimeric protein has significantly lower dTTPase activity for translocation (see below). Hence, a direct comparison between T7Δ4-1 growth on cells expressing the T7 helicase alone and the chimeric primase can not be made.

Purification of Chimeric Primases—The reduced ability of the T4/T7 and E. coli/T7 chimeric primase to support T7 growth could arise from several defects in the proteins. One simple interpretation is that the conformation of the zinc motif in the protein is altered in such a way that its interaction with the recognition sites is weakened. More interesting would be a requirement for recognition of the T7 primase recognition sequence 3′-CTG-5′ for efficient DNA replication, a sequence different from that recognized by the T4 or E. coli primases. Alternately, the defect could reside in the helicase portion of the protein, limiting translocation to primase sites and perhaps even reducing helicase activity. Finally the chimeric protein could be defective in its interaction with other replication proteins such as the T7 DNA polymerase or gene 2.5 protein. In order to explore these possibilities we have purified the two chimeric proteins and characterized them biochemically.

The two chimeric proteins were purified to apparent homogeneity from cells expressing each of the recombinant genes.
The purification procedure was essentially that previously described for wild-type gene 4 protein with the exception that the cell pellet was extracted with high salt since the overexpressed chimeric proteins were less soluble than the wild-type gene 4 proteins.

Oligoribonucleotide Synthesis—The ability of the two chimeric primases to function even to a limited extent in vivo suggests that they catalyze the synthesis of oligoribonucleotides that can be used as primers by T7 DNA polymerase. The oligoribonucleotide synthesis assay provides a direct analysis of RNA primer synthesis (3, 10). As shown in Fig. 3, the wild-type gene 4 primase catalyzed the synthesis of di-, tri-, and tetraribonucleotides on M13 DNA. In this assay, triphosphate was removed from 5′-termini by phosphatase, and the resulting products were separated by denaturing PAGE. Oligoribonucleotide products were visualized by [α-32P]NTP incorporation and autoradiography.

Both of the chimeric primases catalyzed the synthesis of oligoribonucleotides, but the amount of protein required to detect oligoribonucleotides was considerably more than that required for wild-type gene 4 protein (Fig. 3). Under the condition of this assay the T4/T7 chimeric primase was 2.5-fold more active than the E. coli/T7 primase. However, the amounts of products with 320 nM T4/T7 primase and E. coli/T7 primase were only 0.78 and 0.29%, respectively, of that obtained with wild-type gene 4 protein, as measured by a Bio-Imaging analyzer. In this assay trimers and tetramers were not detected, but as will be shown below, these products are synthesized under appropriate conditions.

dTTP Hydrolysis—The 63-kDa gene 4 primase, like the 56-kDa gene 4 protein, catalyzes the hydrolysis of nucleotides, a reaction stimulated greatly by the presence of ssDNA (44). The energy of nucleotide hydrolysis drives the unidirectional translocation of the protein on ssDNA. As shown in Fig. 4A, the two chimeric primases catalyzed the hydrolysis of dTTP, the nucleotide preferred by T7 gene 4 protein, in the absence of ssDNA at rates similar to those catalyzed by the wild-type T7 primase. However, the hydrolysis of dTTP catalyzed by the two chimeric proteins was not stimulated by ssDNA to the same extent as that observed with the wild-type T7 primase (Fig. 4B). Whereas the rate of hydrolysis catalyzed by the wild-type T7 primase was stimulated approximately 38-fold by ssDNA, the T4/T7 and E. coli/T7 chimeric primases were stimulated only 16- and 5-fold, respectively.

Stimulation of Oligoribonucleotide Synthesis by 56-kDa Gene 4 Helicase—The reduced dTTPase activity observed with the chimeric primase proteins observed in the presence of ssDNA indicates either a defect in the binding of the proteins to ssDNA, a reaction that require the presence of a NTP but not its hydrolysis (45), or a diminished ability to translocate 5′ to 3′ on ssDNA. In either case, the decreased primase activity on M13 DNA observed with the chimeric proteins could result from an inability of the proteins to translocate to primase recognition sites. For example, mutations in the dTTP binding site of the 63-kDa primase eliminate dTTP hydrolysis, helicase activity, and primer synthesis on ssDNA (46). The latter activity can be recovered by wild-type 56-kDa helicase.

The addition of wild-type 56-kDa gene 4, a protein that has helicase activity but no primase activity, to reactions containing either T4/T7 or E. coli/T7 chimeric primase stimulated the synthesis of oligoribonucleotides 2.4- or 3.1-fold, respectively (Fig. 5), whereas the wild-type T7 primase was stimulated by only 1.3-fold. Not only were the amounts of dimer increased but the presence of tri- and tetranucleotide products were now observed. The latter are the only species that can serve as primers for T7 DNA polymerase either in vivo or in vitro (11, 47). These results show that the chimeric proteins can still interact physically with the 56-kDa helicase, presumably to form hexamers and to provide translocation activity for the primase chimera in the complex. The addition of T7 DNA polymerase to the reaction mixtures had no effect on oligoribonucleotide synthesis (data not shown).

5′-Terminal Sequence of Oligoribonucleotides—As presented above (Figs. 3 and 5), the T4/T7 and E. coli/T7 chimeric pri-

### Table II

| Gene 4 proteins* | T7 Δ4-1 |
|------------------|---------|
| T4/T7 primase    | 2.0 x 10^3 |
| T4/T7 primase C-36,39-S | 2.0 x 10^4 |
| E. coli/T7 primase | 1.5 x 10^3 |
| E. coli/T7 primase C-36,39-S | 1.0 x 10^4 |

* Plasmid-encoded chimeric and mutant chimeric gene 4 proteins expressed in the E. coli C600 cells.
primases (data not shown). Apparently the chimeric pro-
coliplates containing the recognition sequences for the T7, T4, or
alyzed the synthesis of oligoribonucleotides on synthetic tem-
DNA. However, to our surprise neither chimeric primase cat-
mases catalyze the synthesis of oligoribonucleotides on M13
63-kDa: T7 + + + +
56-kDa gene 4 protein: T7 + T4/T7 + E. coli/T7 + + + +
Dimer + + + +
Trimer + + + +
Tetramer + + + +

Fig. 5. Stimulation of oligoribonucleotides synthesis by 56-
63-kDa gene 4 helicase. Reaction mixtures (10 µl) contained 0.3 mM
(rG,A,U,T)TPs, 0.3 mM [α-32P]CTP, 0.6 mM dTTP, 23 nM M13 ssDNA,
and 320 nM chimeric primases in primer synthesis buffer. Reactions
were incubated in the presence or absence of 320 nM 56-kDa gene 4
protein as indicated. The reactions were carried out and the products
were analyzed as described under “Experimental Procedures.” An au-
toradiogram of radioactive labeled products are shown above. The ident-
ity of the oligoribonucleotide species are indicated.

mases in the absence (A) or presence (B) of 50 µM M13 ssDNA
were determined as described under “Experimental Procedures” using
the indicated amounts of protein. The product of hydrolysis, dTDP, was
measured after isolation by thin layer chromatography.

Fig. 4. dTTP hydrolysis activity of T7 and chimeric primases
in the presence and absence of ssDNA. The rate of hydrolysis of
[α-32P]dTTP catalyzed by wild-type T7 primase and the two chimeric
primases were determined as described under “Experimental Procedures.”
An autoradiogram of radioactively labeled products are shown above. The ident-
ity of the oligoribonucleotide species are indicated.

In order to identify the sites at which the chimeric primases
initiate the synthesis of oligoribonucleotides we have deter-
minalized the sequence of the oligoribonucleotides synthesized on
M13 DNA. We first identified the 3'-nucleotide of the dimers
(Fig. 6A) synthesized by the primases by measuring the incor-
poration of radioactivity from each of the four [α-32P]rNTPs in
the presence of the other three unlabeled rNTPs into dimers
using the gel assay procedure described above. Only [32P]phos-
phate incorporated into a phosphodiester bridge is expected to
appear in the dimer, since the 5'-triphosphate was removed by
treatment with phosphatase prior to gel analysis. As expected
from the known sequence of dimers, pppApC, synthesized by
the T7 gene 4 primase, rCMP was the predominant nucleotide
in the second position of the dimer. As shown in Fig. 6A, rCMP
was also the predominant nucleotide found in this position
of the dimers synthesized by the two chimeric primases.

The knowledge that the sequence of the chimeric dimers was
pppNpC permitted the determination of the first nucleotide by
oligoribonucleotide synthesis in the presence of each of the four
ribonucleoside 5'-triphosphate in the presence of [α-32P]rCTP
(Fig. 6B). Again, as expected from the known sequence of
dimers synthesized by T7 primase, synthesis occurred predom-
inantly in the presence of rATP and rCTP. Results with the
chimeric primases were equally unambiguous, synthesis of
dimers by both the T4/T7 and E. coli/T7 primases occurring in
the presence of rGTP and rCTP. The presence of rGTP as the
first nucleotide of the dimers synthesized by the chimeric
primases was confirmed by carrying out synthesis in the presence of
[α-32P]rCTP and two of the remaining three rNTPs (Fig. 6C).
In the case of the T7 primase, omission of rATP eliminated
oligoribonucleotide synthesis and the omission of rGTP elim-
nated synthesis by the two chimeric proteins. We conclude that
both chimeric primases synthesize the dimers pppGpC.

Template Recognition Site—As discussed in the Intro-
troduction, T7, T4, and E. coli primases each catalyze the synthesis
of oligoribonucleotides at trinucleotide sequences unique for each
primase. The 3'-nucleotide of the recognition sequence is cryp-
tic in that it is not copied into the primer whereas the other two
nucleotides are copied in a template-mediated reaction. It
seemed likely therefore that oligoribonucleotide synthesis catal-
yzed by the chimeric primases was occurring at a trinucle-
otide sequence, 3'NCG-5', in which N is the cryptic nucleotide.
In order to confirm this hypothesis and identify the cryptic
nucleotide, we synthesized four 33-mer oligoribonucleotides,
each containing the trinucleotide sequence 3'NCG-5'; each
oligonucleotide contained one of the four deoxynucleotides in
the N position. The length of the flanking sequences exceeded
those required by the T7 primase (3). As shown in Fig. 6D, both
chimeric primases preferred the purines A or G in the 3'-
position but the stringency was not as great as that found for
the wild-type T7 primase. For example, quantitation of the
activity on each templates (see the legend to Fig. 6) showed
that primer synthesis was only reduced by half even T was
present at the 3'-position.

Interactions with T7 DNA Polymerase—Specific interactions
between the T7 56- and 63-kDa gene 4 proteins and T7 DNA
polymerase are necessary for efficient and coordinated DNA
synthesis at the T7 replication fork (48, 49), and a physical
interaction between the two proteins has been demonstrated
(50). In addition to catalyzing the synthesis of oligonucleotides,
the gene 4 protein stabilizes them on ssDNA (50) and facilitates
their use as primers by T7 DNA polymerase (10, 32). In order to
determine if the oligoribonucleotides synthesized by the
chimeric DNA primases can be stabilized and used as primers,
we have used an assay that couples oligoribonucleotide synthe-
sis to DNA synthesis catalyzed by T7 DNA polymerase. This
assay measures both the ability of the proteins to synthesize
oligoribonucleotides and to provide functional primers for the
DNA polymerase. With wild-type T7 DNA primase, maximal
stimulation of DNA synthesis occurred at approximately 60 nM
protein (Fig. 7). Neither of the two chimeric proteins stimulated
nearly the same level of DNA synthesis, the T4/T7 protein was
0.71% as efficient as the wild-type T7 primase and the E.
coli/T7 only 0.39%. Under the conditions of this assay, the
concentration of primases, T7 DNA polymerase, and M13 ssDNA were 60 nM, 5 nM, and 10 nM, respectively. Therefore the molar ratio of primase (hexamer), DNA polymerase (monomer), and M13 ssDNA is 2:1:2. We do not know the basis of the biphasic curve obtained in Fig. 7. Such a curve is often seen with wild-type enzyme albeit at lower concentrations. The reaction is difficult to characterize kinetically since it involves at least three activities, helicase, primase, and DNA polymerase. Although the efficiency of priming of DNA synthesis was considerably lower than that observed with wild-type T7 primase, our results demonstrate that both of the chimeric primases can provide functional primers for T7 DNA polymerase.

**DISCUSSION**

All DNA primases must have a catalytic site at which phosphodiester bridges are formed between nucleotides. All primases identified to date contain a zinc motif. In the case of bacteriophage T7 the two molecular mass forms of gene 4 protein dramatically illustrate this point in that the catalytic site is located within the 56-kDa gene 4 protein. The 56-kDa helicase cannot synthesize functional tetranucleotide primers, but it does catalyze the template-independent synthesis of random dinucleotides. By contrast, the colinear 63-kDa gene 4 protein that has a zinc motif at its N terminus catalyzes a template-directed synthesis of oligoribonucleotides at specific primase recognition sites. As is the case for the T4 and E. coli primases, the recognition site is a trinucleotide sequence in which the 3'-nucleotide is cryptic, being required for recognition but not for incorporation into the primer.

What is the precise role of the zinc motif in sequence recognition and primer synthesis? The inability of the 56-kDa gene 4 protein to carry out template-directed synthesis of oligoribonucleotides, even at nonspecific sites, indicates that one role of the zinc motif is to mediate the interaction of the catalytic domain with the template. Such a role is also supported by the finding that substitution of a serine residue for any one of the four cysteine residues in the T7 primase abolishes template-
directed oligoribonucleotide synthesis but not random dinucleotide synthesis (21). An earlier approach to dissecting the multiple roles of the zinc motif involved the substitution of the zinc motif of the primase of the closely related phage T3 for that of the T7 primase (39). The chimeric primase functioned relatively normally in vivo and recognized the same trinucleotide sequence in vitro as did the wild-type T7 primase. This result was not surprising, since the zinc motifs of the two primases are 50% homologous.

In the present study we examined the properties of two T7 chimeric primases whose zinc motifs were derived from two primases, phage T4 and E. coli, that do not share a large degree of homology with the T7 primase and that are known to interact with template sequences that differ from those recognized by the T7 primase. Despite the difference in the sequences recognized by the three primases, they share certain molecular structures and biochemical properties that make a plausible case for the creation of functional chimeric primases. For example, the three primases contain six conserved regions (boxes 1 through 6) (20) and they all function as a complex with their cognate DNA helicase. More important, the phage T4 gene 61 and the E. coli DnaG primases have a C-X_2-C-X_2-3-C-X_2-C and a C-X_2-H-X_2-C-X_2-C, respectively, motifs that are strikingly similar to that of the T7 63-kDa gene 4 primase, C-X_2-C-X_15-C-X_2-C (Fig. 1) (31, 51, 52). However, as described in the Introduction, while the T7 primase recognizes the trinucleotide sequence 3'-CTG-5', the T4 and E. coli primases recognize the sequences 3'-T(C/T)G-5' and 3'-GTC-5', respectively. The similarity in size and shape of the zinc motifs of these three primases suggests that a substitution of only the zinc loop region of the T4 and E. coli primases for that of T7 primase would not drastically alter the conformation of the T7 enzyme.

The effect of changes in the zinc motif on the function of gene 4 protein can be monitored by the ability of the chimeric proteins to catalyze the hydrolysis of dTTP, an activity that resides in the helicase portion of the gene 4 protein (32, 38, 53). In the absence of DNA both chimeric primases hydrolyze dTTP at approximately equal rates, suggesting that no major alterations in the active site have occurred. In the presence of ssDNA the hydrolysis of dTTP by wild-type T7 primase is stimulated approximately 40-fold, indicative of its ability to translocate on ssDNA. The hydrolysis of dTTP catalyzed by the chimeric primases is also stimulated by ssDNA albeit to a lesser extent. This result confirms earlier studies suggesting that changes in the zinc motif of the T7 primase can affect helicase activity. For example, substitution of a serine for one of the cysteine residues in the zinc motif not only eliminates primase activity but also reduces helicase activity by 10-fold (21). These results, taken together, suggest that the zinc motif maintains intimate contacts with the helicase domain during translocation on ssDNA.

Although both chimeric primases are drastically impaired in their ability to catalyze template-dependent synthesis of oligoribonucleotides, they do function sufficiently to partially support T7 DNA replication and growth. The addition of wild-type helicase to the chimeric primases significantly enhances oligonucleotide synthesis in vitro, presumably by providing for translocation on ssDNA so that the primase gains access to primase recognition sites. Precedence for this rescuing of primase activity comes from studies with T7 primase defective in translocation due to a mutation in its nucleotide binding site (46). Wild-type 56 kDa gene 6 protein can form heterohexamers with 63 kDa protein (38) and thus provide translocation for the primase. However, coexpression of 56 kDa helicase with the chimeric primases did not increase its ability to provide for T7 growth in vivo (data not shown).

The fact that the chimeric primases recognize a template sequence that differs from that recognized by the T7 primase establishes that the zinc motif of the gene 4 protein is required, at least in part, for the recognition of the 3'-CTG-5' sequence. The earlier finding that primase activity was abolished by substitution of serine for any of the four cysteines in the T7 zinc motif demonstrates the importance of this motif in primer synthesis (21). However, those results did not allow us to distinguish between a role of the zinc motif in mediating the binding of the protein to the template or in sequence recognition. Likewise, the finding that a T3/T7 chimeric primase recognized the same trinucleotide sequence as did the T7 primase left this question unresolved (39).

An important finding of the current study is that the T4/T7 and E. coli/T7 chimeric primases fail to use the recognition sequences of either the T7 primase (3-CTG-5'), the T4 (3'-T(C/T)G-5'), or the E. coli (3'-GTC-5') primase. Instead, they both recognize the trinucleotide sequence 3'-T(C/T)G-5' which differs from that recognized by T7, T4, or E. coli primase. For example, while all three of these wild-type primases have a high specificity for a single 3'-cryptic nucleotide in the template trinucleotide recognition site, the chimeric primases appear to have lost this specificity in that they can recognize either a G or A residue at this position. The only reservation in interpreting these results is the fact that, by necessity, we have, in part, deduced the template recognition sequence from the identity of the oligoribonucleotides synthesized by the chimeric primases. Hence, the slight possibility remains that the chimeric primases can bind to different recognition sequences but cannot catalyze the formation of phosphodiester bonds at these sites.

A major role of the zinc motifs found in proteins other than DNA primases, for instance transcription factors, is their stable binding to specific sequences in DNA. Unlike the DNA primases these factors bind, in turn, to other proteins that mediate the catalytic activity occurring at this site. In the case of DNA primases the same polypeptide that contains the zinc motif also contains the catalytic site for oligoribonucleotide synthesis. Our results suggest that the zinc motifs found in DNA primases function in two capacities. In one case the zinc motif serves to bind the protein stably to the DNA such that the catalytic domain is properly positioned for the template directed condensation of nucleotides. Second, the zinc motif plays a role in selecting the site of primer synthesis by selectively binding to primer recognition sites which are unique to each primase. In the case of the chimeric primases the exogenous zinc motif fulfills its first role but it fails to address its cognate recognition site. Consequently, the sequence of the oligoribonucleotides is dictated by other interactions between the protein and the template, perhaps involving the catalytic site. The fact that G is the 5'-residue of the trinucleotide sequence recognized by T7 primase and by both of the chimeric primases suggests that the catalytic site or at least some other region that was also not altered in the construction of the chimeric primases is involved in the recognition of this position. In fact, it has been shown recently that the nucleotide substrate for primer synthesis can be cross-linked to both a residue within the zinc motif of the E. coli DnaG primase and to residues in the vicinity of boxes 3 and 4 that most likely comprise the catalytic domain (54). The latter region (amino acids 205–268) is homologous to a region in the T7 primase comprising amino acids 105–160 (20). Furthermore, in the case of the DnaG primase it is known that protein-protein interaction can influence primer specificity. In one instance an interaction of the DnaG primase with the DnaB helicase loosens the recognition sequence of the primase from 3'-GTC-5' to the more general
sequence 3'-PuPyPy-5' (7).

Interestingly, the transcription factors Krox-20, Sp-1, and Zif268 contain zinc fingers that recognize specific trinucleotide sequences (55, 56). However, the zinc motifs of these proteins differ from those of the DNA primases described here in several respects: the zinc fingers consist of a Cys∗His2 motif, there are multiple zinc fingers that interact with a relatively long stretch of DNA, and recognition occurs on double-stranded DNA. The Cys∗His2 zinc motif contains both an α-helix and a β-sheet with the base contacts occurring through residues in the a-helix (23, 24, 55).

Structurally, the zinc motifs of the DNA primases more likely resemble the zinc ribbon sequences that contain three- or four-stranded antiparallel β-sheets around a single Cys∗gene (25). Examples of proteins that belong to this zinc ribbon family are TFIIIS, TFIIIE, RNA polymerase II large subunit, DNA polymerase α-subunit, and several bacterial repair proteins. Many members of this protein family bind to ssDNA as well as to dsDNA but, unlike the DNA primases, without sequence specificity. Similarly, proteins such as the T4 gene 32 protein and DNA repair proteins members of this protein family bind to ssDNA as well as to dsDNA but, unlike the DNA primases, without sequence specificity. Similarly, proteins such as the T4 gene 32 protein and DNA repair proteins.

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