Gene 4 DNA Primase of Bacteriophage T7 Mediates the Annealing and Extension of Ribo-oligonucleotides at Primase Recognition Sites

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Citation
Kusakabe, Takahiro, and Charles C. Richardson. 1997. “Gene 4 DNA Primase of Bacteriophage T7 Mediates the Annealing and Extension of Ribo-Oligonucleotides at Primase Recognition Sites.” Journal of Biological Chemistry 272 (19): 12446–53. https://doi.org/10.1074/jbc.272.19.12446.

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The 63-kDa gene 4 primase of bacteriophage T7 recognizes a core trinucleotide sequence, 5′-GTC-3′, on single-stranded DNA at which it catalyzes the synthesis of the ribodinucleotide pppAC. The dinucleotide is extended to a tetranucleotide primer at the sites 5′-(G/T)GGTC-3′ and 5′-GTGGTC-3′. In the presence of T7 primase, T7 DNA polymerase extends the synthetic ribotetranucleotide pppACCA (1 μM), but not pCACA, on M13 DNA templates. The reaction is specific for T7 DNA polymerase and depends on dTTP and translocation of the gene 4 protein. T7 primase extends the dinucleotide AC and trinucleotide ACC to ACCC in the presence of CTP and an appropriate template, whereas other dinucleotides are extended less efficiently; the deoxyribodinucleotide dAC is not extended. The deoxyribodinucleotide dAC is not extended. The Cys₄ zinc motif of the primase is essential for extension of the dinucleotides. The 5′-cryptic cytidine of the recognition sequence is essential for extension of the dinucleotide AC to tri- and tetranucleotides. At a preformed replication fork, the dinucleotide AC provides for primer synthesis on the lagging strand. The synthesis of all Okazaki fragments is initiated by primers arising from the recognition sequence 5′-GGGTC-3′; none arise at an adjacent 5′-GGGTT-3′ sequence. If ADP or AMP replaces ATP in the primase reaction, primers terminating in di- or monophosphate, respectively, are synthesized.

DNA primases catalyze the template-directed synthesis of oligonucleotides for use as primers by the lagging strand DNA polymerase. The primases of T7, T4, and Escherichia coli all initiate oligonucleotide synthesis on single-stranded DNA (ssDNA)⁴ at basic trinucleotide recognition sites unique to each system (1–5). The 3′-nucleotide of the recognition sequence, in each case, is cryptic in that it is essential for recognition, but is not copied into the product oligonucleotide. Although the basic recognition sequence is a trinucleotide, with the potential to generate a dinucleotide, the oligonucleotides that actually function as primers for the T7, T4, and E. coli primases are tetra-, penta-, and 10–12-mers, respectively (6–10). The stringency for these subsequent nucleotide additions is less than that observed for the first two nucleotides polymerized at the basic trinucleotide sequence (1, 3, 4, 7, 8, 11).

The molecular mechanism by which prokaryotic DNA primases recognize a trinucleotide sequence on ssDNA is not known. However, the T7, T4, and E. coli primases have a potential metal-binding site, as do all known DNA primases (12, 13); the T7 and E. coli primases have been shown to be zinc metalloproteins (13, 14). In the case of the T7 primase, the Cys₄ zinc motif is located at the amino terminus of the protein; the substitution of a serine for any one of the four cysteines destroys its ability to catalyze the synthesis of oligonucleotides in a sequence-specific manner (13). However, altered T7 primases containing these single amino acid changes or even lacking the entire zinc motif still catalyze the DNA-independent formation of random dinucleotides, albeit at a greatly reduced rate, demonstrating that the site for phosphodiester bond formation is located in another domain of the protein (13, 15, 16). The zinc motif, however, is not the sole determinant of primase site recognition since we have shown that substituting the zinc motifs of the T4 and E. coli primases for that of the T7 primase does not lead to the creation of a chimeric primase that now uses the T4 or E. coli recognition sequence, but rather to one that recognizes an entirely new trinucleotide sequence (17).

Although the T7 primase shares many properties with the primases of phage T4 and E. coli, it has a number of distinguishing properties. A unique feature of the T7 DNA primase is the presence of a helicase domain, which allows the 63-kDa gene 4 protein, a protein composed of a single polypeptide chain, to catalyze both helicase and primase activities. (15, 18). In contrast, in the T4 and E. coli systems, helicase and primase activities reside within separate polypeptides (8, 19–22). Physical association of primases with a helicase in a functional complex is important because the translocation activity of the helicase provides a mechanism for the primase to reach its recognition sites on ssDNA (2, 9, 20, 21, 23). The presence of both helicase and primase activities in the same T7 protein arises from the presence of two co-linear gene 4 proteins, a 56- and a 63-kDa protein, in phage-infected cells (24). The two proteins are expressed from two in-frame translation initiation sites 189 bases apart on the gene 4 transcript. The 56-kDa gene 4 protein lacks the 63 N-terminal amino acid residues of the 63-kDa gene 4 protein, and is this domain that contains the zinc motif essential for primase activity. Thus, the 56-kDa gene 4 protein has only helicase activity, whereas the 63-kDa gene 4 protein has both helicase and primase activities (16, 18). In this report, we refer to the 63-kDa gene 4 protein as DNA primase even though it has helicase activity.

A second distinguishing property of the T7 primase involves the selectivity in the particular nucleotides incorporated into the primers after the invariant dinucleotide is synthesized at the basic primase recognition site. T7 primase greatly prefers...
AMP and CMP in the third and fourth positions of the primer, whereas the T4 and \textit{E. coli} enzymes are less restrictive in the nucleotides incorporated (25, 26). We have shown that this specificity is, at least in part, due to the inherent translocation activity of the T7 primase, which does not allow the enzyme to pause for sufficient time to incorporate unfavorable nucleotides (11).

A third distinguishing property, and the subject of this study, is the ability of the T7 primase to synthesize relatively large amounts of the dinucleotide pppAC together with the functional primer species, a tetranucleotide. The actual RNA primers found at the 5′-termini of Okazaki fragments synthesized in cells infected with phage T7 (6) or in reactions containing the T7 primase and T7 DNA polymerase are tetranucleotides, predominantly pppACC, pppACCA, and pppACAC (7, 26). These tetranucleotides arise from the general recognition sites 5′-GGGTTC-3′, 5′-TGGTTC-3′, and 5′-GTGGTTC-3′, respectively, all containing the core recognition sequence for the T7 primase, 5′-GTC-3′ (7). On naturally occurring ssDNA templates such as phage M13 DNA or on synthetic oligonucleotides containing one of the general recognition sites, the T7 primase, in the presence or absence of coupled DNA synthesis, catalyzes the synthesis of di- and tri- nucleotides as well as the functional tetranucleotide primers (18). In fact, on templates containing only the core tetranucleotide sequence 5′-GTC-3′, the enzyme catalyzes exclusively the synthesis of the dinucleotide pppAC (18). Although small amounts of dinucleotide have been observed with the T4 and \textit{E. coli} primases under some conditions, their abundance is far less than that seen with T7 primase (2, 27).

The ability of the T7 primase to catalyze the exclusive synthesis of dinucleotides at core recognition sites (5′-GTC-3′) not contained within a general recognition site is intriguing and raises the possibility that dinucleotides may serve some functional role in the priming reaction. One possibility is that, although the dinucleotides cannot be extended at these sites, they may remain associated with the primase during its translocation to a general recognition site, where extension to a functional primer could occur. In early studies on the gene 4 protein, Scherzinger et al. (28) reported that certain tri- and tetranucleotides could stimulate DNA synthesis by T7 DNA polymerase on ssDNA templates in a reaction dependent on the presence of the T7 gene 4 protein. Interestingly, of the tetranucleotides tested, ACCA was the most effective, and priming appeared to be initiated at specific sites on dX174 DNA; in this study, dinucleotides were without effect. Subsequent studies revealed that the 63-kDa gene 4 protein alone could stimulate T7 DNA polymerase in the presence of a tetranucleotide (15). In this report, we show that the ribodinucleotide AC can be extended to a tetranucleotide by the 63-kDa gene 4 protein and that the reaction occurs only at a general primase recognition site, 5′-NNGTC-3′, containing the essential but cryptic cytidine residue. In addition, we examine the use of dinucleotides in the priming reaction at a replication fork, where leading and lagging strand DNA synthesis is carried out by T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the gene 2.5 ssDNA-binding protein.

**EXPERIMENTAL PROCEDURES**

\textbf{DNA, Nucleotides, Enzymes, and Biochemicals—M13mp18 ssDNA was purified as described (29). All nucleotides were purchased from Amersham Corp. Synthetic dinucleotides (AC, AA, CC, CA, and dAC) and the trinucleotide ACC were purchased from Sigma. Oligonucleotide templates for the assay of ribo-oligonucleotide synthesis were chemically synthesized by C. Dahl (Harvard Medical School). The nucleotide sequences of the oligonucleotide templates are as follows: T7-01, 5′-C-C\footnotesize{TTCTCACAC}CTAGGGATCTACGACA-3′; T7-02, 5′-C\footnotesize{CTCTCTCTCT}CAAGGAGTCCATATACGACA-3′; T7-03, 5′-C\footnotesize{AGTGGATGAC}TGATACGACA-3′; T7-04, 5′-C\footnotesize{TGATGAGCAC}TGATACGACA-3′; T7-05, 5′-C\footnotesize{CTCTCTCTCT}CAAGGAGTCCATATACGACA-3′; T7-06, 5′-C\footnotesize{CAGTGGATGAC}TGATACGACA-3′; T7-07, 5′-C\footnotesize{AGTGGATGAC}TGATACGACA-3′. They were used as described (30). The T7 56-kDa gene 4 protein was purified as described by B. Beauchamp (Harvard Medical School) as described (18). T7 gene 5 protein-\textit{E. coli} thioredoxin (1:1 complex) was purified and kindly provided by S. Tabor (Harvard Medical School) as described (31). We refer to the 1:1 complex of the gene 5 protein with thioredoxin as T7 DNA polymerase unless otherwise indicated. The T7 gene 2.5 ssDNA-binding protein was purified as described (32). T4 DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase, and other enzymes were purchased from Amersham Corp.**

\textbf{Ribotetranucleotide Use by T7 Gene 4 Primase—The ability of tetranucleotides to stimulate T7 DNA polymerase in the presence of the T7 primase was measured using an M13mp18 ssDNA template. The reaction mixtures contained 20 mM Tris-Cl (pH 7.5), 10 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, 50 \textmu M bovine serum albumin, 50 mM potassium glutamate, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM [\textsuperscript{32}P]dCTP, 10 mM T7 primase (hexamer), 20 nM T7 or T4 DNA polymerase, 10 mM M13 ssDNA, and the indicated amount of pACC or pCAC tetranucleotides. After incubation at 37 °C for 10 min, the reactions were stopped by the addition of 2 \mu l of 0.2 M EDTA (pH 8.0). The reaction mixtures were spotted onto Whatman DE81 filters, and the filters were washed four times for 10 min with 0.3 M ammonium formate (pH 8.0) followed by 98% ethanol. After drying, the amount of [\textsuperscript{32}P]dTMP incorporated into DNA was measured as the radioactivity remaining on the filters by scintillation spectrometry.**

\textbf{Ribo-oligonucleotide Synthesis Assay—Oligonucleotide synthesis assays using synthetic oligonucleotide templates were performed as described (18). The standard reactions (10 \mu l) contained 40 mM Tris-Cl (pH 7.5), 10 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, 50 mg/ml bovine serum albumin, 50 mM potassium glutamate, 0.5 mM dATP, 0.1 mM [\textsuperscript{32}P]dCTP, 20 nM T7 or T4 DNA polymerase, 10 mM M13 ssDNA, and 100 nM oligonucleotides, and either 0.1 mM ATP or 0.1 mM di- or trinucleotide. After incubation at 37 °C for 60 min, the reactions were stopped by the addition of 20 \mu l of sequencing dye (98% formamide, 50 mM EDTA (pH 8.0), 0.1% xylene cyanol FF, and 0.1% bromphenol blue). The reaction mixtures were then heated at 95 °C for 5 min, and the labeled products were separated by electrophoresis through 25% polyacrylamide gels containing 3.8 \mu M urea.**

\textbf{DNA and Primer Synthesis at a Minimal Replication Fork—Formation of a minimal replication fork was performed as described (11). The minimal replication fork consists of a 71-nt TK-03 circular DNA to which a 71-nt TK-01 linear DNA molecule has been annealed through a 36-nt complementary to TK-03. The nucleotide sequences of the TK-01 and TK-03 oligonucleotides used for construction of the replication fork are presented above. DNA synthesis reactions (20 \mu l) using the minimal replication fork contained 40 mM Tris-Cl (pH 7.5), 10 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, 50 \mu M bovine serum albumin, 50 mM potassium glutamate, 0.7 mM dTTP, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.1 mM [\textsuperscript{32}P]dCTP, 50 nM T7 DNA polymerase, 500 nM gene 2.5 protein, and 5 nM T7 primase (hexamer). Either 0.1 mM ATP or ApC ribodinucleotide was present to measure de novo primer synthesis or the extension of ApC to primers, respectively. The reactions were started by the addition of 50 nM replication fork and incubated at 37 °C for 5 min. The reaction was stopped by the addition of 5 \mu l of 0.2 M EDTA. 5 \mu l of the reaction was mixed with 15 \mu l of sequencing dye and applied directly to a 25% urea gel to measure ribotetranucleotide synthesis. The remainder of the reaction (20 \mu l) was deproteinated, and the DNA was digested with \textit{BamH}I and loaded onto 4% urea gels. After electrophoresis, the DNA fragments bearing ribonucleotide primers were visualized by autoradiography.**

\textbf{RESULTS}**

\textbf{T7 Primase-dependent Stimulation of T7 DNA Polymerase by Ribotetranucleotides—As discussed in the Introduction, earlier studies had indicated that ribotetranucleotides could stimulate DNA synthesis on ssDNA templates catalyzed by T7 DNA polymerase.**
polymerase, provided that the T7 primase was present (15, 25, 28). In these preliminary studies, the concentration of tetranucleotides, 100–1000 μM, far exceeded the concentration of NTPs normally used in the primase reaction. As a result, it was difficult to evaluate the effect of nucleotide sequence on the efficiency with which the oligonucleotides were extended. In the experiment presented in Table I, we examined the ability of two ribotetranucleotides, pACCA and pCACA, to prime DNA synthesis catalyzed by either phage T7 or T4 DNA polymerase on M13 ssDNA; ATP and CTP, the precursors for primer synthesis, were omitted from the reaction. The tetranucleotide pACCA is similar to the pppACCA primers synthesized by T7 primase at the recognition site 5'-TTGGTC-3'; five such sites are present on M13mp18 ssDNA (33). The tetranucleotide pCACA is not synthesized by T7 primase (7, 18), although the complementary sequence 5'-TGTG-3' is present on M13mp18 ssDNA (33). The tetranucleotide pCACA is not synthesized by T7 primase (7, 18), although the complementary sequence 5'-TGTG-3' is present on M13mp18 ssDNA (33). The tetranucleotide pCACA is in part due to the extent of DNA synthesis. The 298 pmol of dTMP incorporated represents nearly complete replication of the 0.2 pmol of M13 ssDNA template (351 pmol of dTMP incorporation sites) present in the reaction.

The T7 primase-dependent stimulation by the tetranucleotide is specific for T7 DNA polymerase; no significant stimulation of T4 DNA polymerase was observed. It should be noted that the use of the primer pCACA by T7 DNA polymerase is dependent on the presence of T7 primase, although this tetranucleotide is not synthesized by the enzyme in the normal priming reaction.

**ADP and AMP Can Be Incorporated into the First Position of Primers Synthesized by the T7 Primase**—Oligonucleotides synthesized by T7 primase contain a 5'-terminal ATP since ATP is the first nucleotide incorporated (11). In the studies presented in this report, we used oligonucleotides lacking a 5'-terminal triphosphate. Consequently, we examined the ability of the T7 primase to incorporate adenosine, AMP, and ADP into the dinucleotide precursor and to extend them to tri-, tetra-, and pentanucleotides (Fig. 1).

In the presence of ATP and CTP, the T7 primase catalyzes the synthesis of pppAC, pppACC, pppACCA, and pppACCAA on a synthetic oligonucleotide containing the known recognition site 5'-TTGGTC-3'. Both AMP and ADP are about equally effective in supporting oligonucleotide synthesis, but the trinucleotide cannot be extended beyond pppACC or pACC since ATP is not available in the reaction. Although incorporation of adenosine was greatly reduced compared with AMP and ADP, there was detectable synthesis of AC (Fig. 1). No oligonucleotide synthesis was observed with dAMP, dADP, or dATP.

**T7 Primase Catalyzes the Extension of Synthetic Dinucleotides and Trinucleotides**—To study the use of oligonucleotides by T7 primase uncoupled from DNA synthesis, we examined the extension of synthetic ribodinucleotides by the enzyme. In the experiment presented in Fig. 2A, we examined the extension of the dinucleotide AC to ACC and ACCC on a synthetic template containing the recognition sequence 5'-GGGTC-3' by measuring the incorporation of [α-32P]CMP. It is clear that the dinucleotide is efficiently extended to ACC and ACCC (Fig. 2A, lane 2). The extent of synthesis is approximately the same as that observed with ATP and [α-32P]CTP (lane 1), and reactions containing both ATP and AC synthesize approximately equal amounts of pppACCA and ACCC (lane 3). Therefore, the T7 primase appears to have the same affinity for ATP and AC.

Not all dinucleotides can be extended by T7 primase. As shown in Fig. 2B, the ribodinucleotide AC is extended far more efficiently than is AA, CC, or CA on either M13 ssDNA or synthetic oligonucleotide templates, each containing the complementary sequence to the dinucleotide sequence. Each of the sequences complementary to the extended dinucleotide is followed by a 5'-guanosine residue for labeling with [α-32P]CMP, and each contains the 3'-cryptic cytidine required by the primase for recognition: 5'-GGGTC-3' for AC, ACC, and dAC; 5'-GGGTC-3' for AA; 5'-GGGCC-3' for CC; and 5'-GTTGAC-3' for CA. The dinucleotide AA is not extended, whereas CC and CA are extended to CCCC, CCCAC, and CACCC, albeit at reduced

**Table I**

| Ribonucleotides | Conc (μM) | T7 DNA polymerase | T4 DNA polymerase |
|-----------------|----------|-------------------|-------------------|
| pCACA           | 1        | 181               | 1.0               |
|                 | 10       | 67                | 1.6               |
| pACCA           | 1        | 7.2                | 0.6               |
|                 | 10       | 67                | 1.6               |
| None            | <0.1     | <0.1              | <0.1              |

* DNA synthesis catalyzed by either T7 or T4 DNA polymerase on M13 ssDNA in the presence of ribotetranucleotides and in the presence or absence of the T7 63-kDa gene 4 protein was measured as described under "Experimental Procedures."
Ribodinucleotide Use by T7 Gene 4 Primase

**FIG. 2.** T7 primase catalyzes the extension of synthetic di- and trinucleotides. A, the ability of the T7 primase to extend the dinucleotide AC was measured in a primer synthesis assay using a synthetic oligonucleotide template. The oligonucleotide synthesis reactions contained 10 nM T7 primase (hexamer), 0.1 mM [α-32P]CTP, 100 nM MR-04 oligonucleotide as a template, and 0.1 mM ATP and CTP. Lane 1 contains the primase reaction with the indicated sequences. The complete nucleotide sequences for the template and the products are indicated. Lane M, pAC molecular marker.

**TABLE II**

| Nucleotide | Total CMP incorporation |
|------------|------------------------|
|            | +dTTP | –dTTP |
| M13 DNA    | 361   | 1.7   |
| T7-01 DNA  | 620   | 37    |
| M13 DNA    | 203   | 2.0   |
| T7-01 DNA  | 190   | 24    |

a Nucleotide extension reactions were performed as described under "Experimental Procedures." The 63-kDa gene 4 protein was incubated with AC or ACC oligonucleotides, [α-32P]CTP, and M13 ssDNA or T7-01 oligonucleotide (5'-N3GGGTGTC-3') as a template in the presence or absence of dTTP.

Ribodinucleotide Use by T7 Gene 4 Primase

 efficiency. The trinucleotide ACC is extended to the tetranucleotide ACCC on either template. The reaction is specific for ribodinucleotides as evidenced by the fact that dAC is not extended on either template.

**Role of Helicase Activity in the Extension of Oligonucleotides by the T7 Primase**—In general, DNA primases rely upon DNA helicases for translocation to primer recognition sites (34). The T7 63-kDa gene 4 primase has an inherent helicase activity (15, 24) and hence does not have to interact physically with a separate helicase. The 5' to 3' translocation activity of T7 primase is dependent on the hybridization of dTTP to dTDP and P1 (35, 36). As shown in Table II, omission of dTTP from the di- or trinucleotide extension reaction reduces the extension of AC on M13 ssDNA to <1% of that observed in its presence. On the synthetic oligonucleotide T7-01 containing a primase recognition site, extension is reduced to ~5% in the absence of dTTP. The greater amount of extension on the short oligonucleotide is in keeping with earlier evidence that random diffusion to primer sites accounts for a significant amount of primer synthesis on short oligonucleotides (37).

**Requirement for the Primase Recognition Site for Oligonucleotide Extension by the T7 primase**—The dinucleotide normally synthesized in the primer reaction is AC. The preference for AC in the extension reaction catalyzed by T7 primase suggests either that the primase can accumulate only AC in its active site or that the reaction occurs only at primer recognition sites on the template. If the latter supposition is correct, then extension of AC to a tri- and tetranucleotide should occur only at template sites having the recognition sequence 5'-GTC-3', containing the cryptic deoxycytidine.

To determine if the 5'-GTC-3' site is required for dinucleotide extension, we examined the ability of the T7 primase to extend AC and ACC on two synthetic templates, one containing the sequence 5'-GTC-3' and the other 5'-GTT-3' (Fig. 3A). The two templates are identical except for the substitution of T for cryptic C in the latter template. A comparison of the extension of AC and ACC to ACC and ACCC on the two templates shows unequivocally that the cryptic cytidine is required. Essentially no extension of AC and ACC is observed on the template in which the cryptic cytidine is replaced by thymidine. Some trinucleotide extension can be observed on the latter template, but it is only 3% of that found with the template containing the 5'-GTC-3' site. The extension of AC and ACC on the template containing the primase recognition sequence is even more efficient than the synthesis of the tri- and tetranucleotides from ATP and CTP.

As shown in Fig. 2B (lanes 3 and 9), a reduced but significant amount of tri- and tetranucleotides was synthesized on the M13 ssDNA and on synthetic oligonucleotide templates in the presence of the dinucleotide CC. Therefore, we also examined the requirement of the 3'-cryptic cytidine for CC dinucleotide extension. Three templates containing the complementary sequence to CC, 5'-GGGC-3', 5'-GGGTGTC-3', and 5'-GGGTGTT-3', were used in the dinucleotide extension assay. As shown in Fig. 3B, the 5'-GGGTGTC-3' site cannot support the extension of CC, whereas the 5'-GGGTGTT-3' site does so fairly efficiently. Inserting a thymidine residue between the cryptic cytidine and guanosine diminishes significantly the ability of the T7 primase to extend the dinucleotide. Nonetheless, the fact that the 5'-GGGTGTC-3' site does support some extension suggests that the enzyme may recognize the 5'-GTC-3' site even in the absence of ATP.

For confirmation of the requirement of a primase recognition site containing a cryptic cytidine for the extension of AC by T7 primase, we identified the sites on M13 ssDNA at which extension occurs. The procedure we used is based on the fact that extension of the dinucleotide to a tetra- or pentanucleotide is required for its function as a primer for T7 DNA polymerase (7). The primers were labeled with [α-32P]CTP in the extension reaction, and the Okazaki fragments that were generated by T7 DNA polymerase were digested with EcoRI, which cut M13 DNA at one site. The resulting fragments, labeled with 32P at their 5'-termini, were of unique lengths depending on the site at which the primer initiated DNA synthesis. A knowledge of the M13 DNA sequence allows the identification of the sequence at which dinucleotide extension occurred (33).

In the presence of ATP and [α-32P]CTP, the precursors for primer synthesis, T7 primase and T7 DNA polymerase mediate the synthesis of Okazaki fragments that, after cleavage with EcoRI and denaturation, give rise to the bands shown in Fig. 4 (lane 1). Five major DNA fragments are observed whose lengths (919, 1123, 1469, 2073, and 2217 nt) indicate that they arose at positions 7150, 105, 451, 1055, and 1199 on the M13 DNA sequence allow...
Ribodinucleotide Use by T7 Gene 4 Primase

FIG. 3. Requirement for primase recognition site for oligonucleotide extension by T7 primase. A, template requirements for extension of AC and ACC oligonucleotides. The oligonucleotide synthesis reactions were performed as described under “Experimental Procedures” using 0.1 mM CC and dinucleotide. Extension reactions were performed as described under “Experimental Procedures.” B, template requirements for extension of the CC dinucleotide. Extension reactions were performed as described under “Experimental Procedures” using 0.1 mM CC and [α-32P]CTP. The complete nucleotide sequences for T7-01 with the 5’-GGGTC-3’ primer recognition site and T7-02 with a 5’-GGGT-3’ site are given under “Experimental Procedures.” The reaction products were resolved by electrophoresis on a 3% polyacrylamide gel containing 8.3 M urea and subjected to autoradiography. The identities of the oligonucleotide species are indicated.

circular DNA template. The 919- and 2217-nt fragments arise from the two 5’-GGGTC-3’ recognition sites found on M13 DNA, and the 1123-, 1469-, and 2073-nt fragments from 5’-TGGTC-3’ sites. There are a number of additional fragments that either arise from other minor recognition sites on the M13 DNA molecule (7) or result from fragments derived from molecules in which multiple priming events occurred. The latter give rise to fragments whose 3’-terminus is not derived from an EcoRI cut, but rather from termination of DNA synthesis at an adjacent Okazaki fragment.

When the coupled DNA synthesis reaction is carried out with AC in place of ATP (Fig. 4, lane 2), both the 919- and 2217-nt fragments are still observed, but not the 1123- and 1469-nt fragments. The recognition site 5’-TGGTC-3’ found at these two latter positions cannot give rise to a functional primer since only the trinucleotide ACC can be synthesized from the dinucleotide in the absence of ATP, which is needed to complete the tetranucleotide pppACCA. As was observed with the synthetic oligonucleotide template discussed above, no fragments are observed that would have arisen from 5’-GGGT-3’ sites or 5’-GGGTA-3’ as evidenced by the absence of fragments 404, 759, and 612 nt in length.

The amount of 919- and 2217-nt fragments found in the reaction using AC is considerably less than that observed with the primase reaction using ATP and CTP. This result could reflect a requirement for a terminal triphosphate on the primer for efficient extension by T7 DNA polymerase.

Role of the T7 Primase Zinc Motif in the Extension of Dinucleotides—The T7 gene 4 protein also exists in vivo as a 56-kDa species that has full helicase activity, but lacks template-dependent primase activity (15, 16). The 56-kDa helicase does, however, contain the active site for phosphodiester bond formation in that it can synthesize random dinucleotides at low efficiency (15). As shown in Fig. 5A, the 56-kDa helicase can catalyze the extension of AC and ACC on an M13 ssDNA template, but the amount of the extension is reduced greatly, 29- and 2.9-fold, respectively, compared with the extension by the 63-kDa gene 4 protein. We also examined the ability of an altered 63-kDa gene 4 protein, in which serine has been substituted for cysteine 36 in the zinc motif, to catalyze the extension of oligonucleotides. This altered 63-kDa protein lacks the ability to synthesize oligonucleotides in a sequence-dependent manner, and it is unable to support T7 growth (13). As shown in Fig. 5A, the C36S mutant gene 4 protein is unable to extend dinucleotides.

Since dinucleotide synthesis catalyzed by the 56-kDa gene 4 protein is not template-dependent (15), the cryptic cytidine residue found in the core recognition site should not play a role in extension by the 56-kDa gene 4 protein. The synthetic oligonucleotides T7-01 and T7-02, containing the sequences 5’-GGGTC-3’ and 5’-GGGT-3’, respectively, were used as templates. As shown in Fig. 5B, whereas the 63-kDa gene 4 protein could efficiently extend both the di- and trinucleotides on the template T7-01, containing the cryptic cytidine, the 56-kDa gene 4 protein was able to extend only the trinucleotide. In the absence of the cryptic cytidine (template T7-02), neither the 63-kDa nor the 56-kDa gene 4 protein could extend the dinucleotide, but there was significant extension of the trinucleotide. Hence, the zinc motif plays a far more significant role in the extension of dinucleotide than in the extension of trinucleotide.
Ribodinucleotide Use by T7 Gene 4 Primase

Dinucleotide Utilization at a Replication Fork—In the experiments described above, we examined the dinucleotide-dependent priming of DNA synthesis by T7 primase on ssDNA templates. Replication of the duplex T7 chromosome involves simultaneous DNA synthesis on both the leading and lagging strands at a replication fork. The economy of T7 replication is such that four proteins suffice to mediate the multiple reactions required for this process (38). T7 DNA polymerase (gene 5 protein complexed to E. coli thioredoxin) accounts for the processive polymerization of nucleotides on both strands, and T7 primase, the 63-kDa protein, provides helicase activity as well as primase activity. The fourth protein, the gene 2.5 protein, is a ssDNA-binding protein that interacts with both the DNA polymerase and the gene 4 protein and enhances their activity (39, 40).

To examine the use of dinucleotides under conditions that mimic replication in vivo, we used a preformed DNA replication fork, where leading and lagging strand syntheses occur simultaneously. The preformed replication fork depicted in Fig. 6a consists of a 71-nt circular ssDNA to which a 71-nt linear ssDNA has been annealed through a 36-nt complementary region. The resulting molecule is a partially duplex circle bearing a 5'-single-stranded tail of 35 nt. The sequence for a BamHI site is located on the ssDNA circle, and DNA synthesis through this sequence generates a functional BamHI restriction site (Fig. 6a). In addition, the ssDNA circle contains two sequences (5'-AACC-3' and 5'-GACCC-3') that, when copied by DNA polymerase, generate the sequences 5'-GGGTT-3' and 5'-GGGTC-3'. The latter sequence is the primase recognition sequence, whereas the former lacks the cryptic cytidine residue required for recognition. If DNA synthesis is initiated from tetranucleotide primers generated at the 5'-GGGTC-3' site, cleavage with BamHI will generate a 60-nt fragment. Any dinucleotide extended to a functional primer at the 5'-GGGTT-3' recognition site, 5'-GACCC-3', is strongly preferred. Our data further show that the extension of the dinucleotide to a tetranucleotide occurs only at a primase recognition site such as 5'-GGGTC-3'. Thus, the cryptic but essential cytidine residue that composes a portion of the recognition sequence is essential; no extension of AC occurs on templates containing the sequence 5'-GGGTT-3'.

We also examined the relative efficiency with which ATP and AC are used in the T7 primase reaction in the presence of [α-32P]CTP (Fig. 6c). When ATP, AC, and CTP are added together to a reaction mixture containing the replication fork, ACC, ACCC, ppACC, and pppACC are synthesized at comparable rates. Thus, it appears that the T7 primase has very similar affinities for ATP and AC.

DISCUSSION

Early studies of the gene 4 proteins encoded by bacteriophage T7 showed that they stimulate the use of ribotetrancleotide as primers by T7 DNA polymerase (15, 25). In this study, we extend these findings to show that T7 DNA polymerase uses ribotetrancleotide as primers far more efficiently in the presence of the T7 primase than in its absence. Furthermore, ribodinucleotide can be extended by the primase in the presence of the appropriate nucleoside triphosphate, CTP, to yield functional tetranucleotide primers. Remarkably, the use of dinucleotide is dictated by their sequence; the dinucleotide 5'-AC-3', the dinucleotide synthesized by the T7 primase at its basic recognition site, 5'-GACCC-3', is strongly preferred. Our data further show that the extension of the dinucleotide to a tetranucleotide occurs only at a primase recognition site such as 5'-GGGTC-3'. Thus, the cryptic but essential cytidine residue that composes a portion of the recognition sequence is essential; no extension of AC occurs on templates containing the sequence 5'-GGGTT-3'.

The precise mechanism by which the dinucleotide AC is selected by the T7 primase and extended at a primase recognition site is not known. It is clear from our studies that the Cys4 zinc motif of the T7 primase is essential for the extension of the AC dinucleotide in that neither the 56-kDa gene 4 helicase, which lacks this domain, nor a genetically altered T7 primase in which one of the cysteines of the zinc motif has been replaced by serine can catalyze the extension. However, these studies do not allow us to distinguish between two possibilities regarding the mechanism by which the dinucleotide arrives at the primase recognition site. In one scheme, the AC dinucleotide transiently binds to all GT sequences on the template, but is stably bound only when the GT sequence lies within a pri-
mase recognition site that is already occupied by a primase molecule. In the second scenario, the T7 primase binds the dinucleotide; the complex then translocates until it reaches a primase recognition site, where it is extended. In both instances, the translocation of the primase along ssDNA plays a crucial role; in fact, we find that omission of dTTP, the nucleotide required for translocation, drastically reduces extension of the dinucleotide.

Of the two possibilities mentioned above, we favor the second, in which the dinucleotide is first bound to the enzyme and transported to the site. The competition experiments described here have shown that the AC dinucleotides are used for tetranucleotide synthesis equally as well as ATP when present together at identical concentrations. Furthermore, it seems unlikely that, at the low concentrations of dinucleotides used in these experiments, annealing to the template would occur to an extent that the rapidly translocating primase (300 nt/s) would ever encounter one bound at a recognition site. Finally, cross-linking studies with 

The 3'–cryptic cytidine of the 5'–GTC-3' recognition site clearly plays an important role in the specific extension of AC to the trinucleotide ACC, as is the case when ATP is the initiating dinucleotide. Although the significance of the 3’-cytidine residue in oligonucleotide extension depends on the length of the oligonucleotide to be extended, it is nonetheless very important in the extension of the trinucleotide ACC. For example, in the experiment shown in Fig. 3, the ability of the primase to extend ACC at the sequence 5'–GGGT-3’, lacking the cryptic cytidine residue, was only 3-fold greater than its ability to extend AC. Cross-linking studies have clearly shown that both the DnaG primase and the β-subunits of E. coli RNA polymerase maintain contact with the first nucleotide of the newly synthesized RNA during the polymerization of the first several nucleotides (41, 42). Thus, the association of the primase with the cryptic cytidine may increase the stability of the complex; the dependence on this association to stabilize the complex, however, may be reduced slightly as the length of the primer increases.

An unexpected finding is that both ADP and AMP could replace ATP as the first nucleotide incorporated by the T7 primase. This observation is not too surprising in light of cross-linking studies with E. coli DnaG primase in which the triphosphate of the first nucleotide was chemically modified and the modified ATP was incorporated by the enzyme (41). However, although the pAC and AC dinucleotides can be readily extended to the tetranucleotide, it is not yet clear if the absence of a triphosphate affects the subsequent extension of the primer by the DNA polymerase. In the experiments presented in Figs. 4A and 6B, the AC dinucleotide appeared less efficient than ATP in supporting lagging strand DNA synthesis. Whether or not triphosphates alone or a mixture of monophosphate, diphosphate, and triphosphates are found at the 5’-termini of Okazaki fragments synthesized in vitro is at present not known since the in vivo experiments on primer synthesis in T7-infected cells did not address this point (6). Our in vitro results suggest that the presence of these various groups would depend on the intracellular pool of AMP, ADP, and ATP. In any case, the 5’-terminal nucleotide along with the remainder of the RNA primase must be removed prior to ligation. Both the T7 gene 6 exonuclease and the 5’-to-3’ exonuclease activity of E. coli DNA polymerase.
I have no difficulty in removing such terminal RNA with a 5′-triphosphate (43).

The large amounts of dinucleotides relative to the functional tetranucleotide primers that arise during primer synthesis catalyzed by the T7 primase both on synthetic oligonucleotides and on M13 ssDNA remain puzzling. Clearly, some arise as intermediates in the synthesis of the tetranucleotide at primer recognition sites shown by their appearance on synthetic templates that have only a complete primer recognition site (18). On templates such as M13 DNA, however, the dinucleotides can arise from the many 5′-GTC-3′ sequences that represent the basic primer recognition site, but yet do not have the proper 5′-sequence that can support extension of the dinucleotide. Why dinucleotides are synthesized at sites where they cannot be extended to functional primers is not known. It is possible that mechanisms exist in vivo to curtail their synthesis at such sites. Another possibility is that the dinucleotides synthesized at the basic recognition sites remain bound to the primase, as we suggested above, and are thus properly positioned when the primase reaches a complete primer recognition site, where they can be extended to a tetranucleotide.

It is also possible that, during replication of the leading and lagging strands at the replication fork, the action of the helicase/primase hexamer is modulated by its interaction with T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. In this study, we used a preformed replication fork containing a single primase recognition site. In the presence of T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. Lagging strands at the replication fork, the action of the helicase/primase hexamer is modulated by its interaction with T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. In the presence of T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. In the presence of T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. In the presence of T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. In the presence of T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein. In the presence of T7 DNA polymerase, the 63-kDa gene 4 helicase/primase, and the T7 DNA polymerase and the T7 gene 2.5 ssDNA-binding protein.

Acknowledgments—We are grateful to Benjamin B. Beauchamp, Joonsoo Lee, Daochun Kong, and Stanley Tabor for providing purified proteins and helpful discussions. We are also very grateful to Ingrid Richardson and Khandan Baradaran for comments on and constructive criticisms of the manuscript.

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J. Biol. Chem. 1997, 272:12446-12453.
doi: 10.1074/jbc.272.19.12446

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