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Role of bacteriophage T7 DNA primase in the initiation of DNA strand synthesis

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ABSTRACT

Bacteriophage T7 DNA primase (gene-4 protein, 66,000 daltons) enables T7 DNA polymerase to initiate the synthesis of DNA chains on single-stranded templates. An initial step in the process of chain initiation is the formation of an oligoribonucleotide primer by T7 primase. The enzyme, in the presence of natural SS DNA, Mg^{++} (or Mn^{++}), ATP and CTP (or a mixture of all 4 rNTPs), catalyzes the synthesis of di-, tri-, and tetraribonucleotides all starting at the 5' terminus with pppA. In a subsequent step requiring both T7 DNA polymerase and primase, the short oligoribonucleotides (predominantly pppA-C-C-A_{OH}) are extended by covalent addition of deoxyribonucleotides. With the aid of primase, T7 DNA polymerase can also utilize efficiently a variety of synthetic tri-, tetra-, or pentanucleotides as chain initiators. T7 primase apparently plays an active role in primer extension by stabilizing the short primer segments in a duplex state on the template DNA.

INTRODUCTION

The synthesis of phage T7 DNA proceeds to a large extent independently of the DNA synthesizing apparatus of the host (1). Of the viral gene products known to be required for T7 DNA replication, the proteins encoded by the genes 4 and 5 play a major role in fork propagation (2). The gene-5 protein combines with E. coli thioredoxin to become a subunit of the T7-induced DNA polymerase (3). The gene-4 protein, which we refer to as T7 DNA primase or T7 primase, has been isolated from T7-infected E. coli with the aid of a complementation assay (4 - 7). The multiple functions associated with the purified enzyme include: (i) SS DNA stimulated hydrolysis of rNTPs and dNTPs to NDPs and P_i (8); (ii) participation in strand separation during replication of duplex DNA (7,8); and (iii) cooperation with T7 DNA polymerase in the initiation of DNA strand synthesis (7,9). In this communi-

cation we present a detailed analysis of the mechanism by which T7 primase enables T7 DNA polymerase to initiate the synthesis of a polynucleotide chain. It is shown that T7 primase is not only a novel type of a DNA-dependent oligoribonucleotide synthetase but that the enzyme is also actively engaged in the process of primer extension.

MATERIALS AND METHODS

Enzymes and proteins

T7 DNA polymerase (DNA nucleotidyltransferase, deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7) and T7 DNA binding protein were isolated as previously described (5). T7 DNA primase was purified according to a published procedure (7), except that the enzyme was chromatographed on phosphocellulose in place of DEAE-Sephadex A25. The preparation contained two polypeptides of molecular weights 66,000 and 58,000 (mass ratio of 1:1) as judged by NaDodSO$_4$/gel electrophoresis. Both these polypeptides have been identified previously as products of the T7 gene 4 (6,7). *E. coli* alkaline phosphatase (EC 3.1.3.1; phosphodiesterase free) was prepared according to the method of Torriani (10). Endonuclease HpaI of *Haemophilus parainfluenzae* (15 units/µg) was obtained from BioLabs (U.S.A.).

Nucleotides, oligonucleotides and DNAs

Ribo- and deoxyribonucleotides were obtained from Boehringer Mannheim GmbH. [^{3}H]TTP (30 Ci/mmol) and [α-^{32}P]-labeled dCTP and rCTP (200-250 Ci/mmol) were from Radiochemicals (Amersham, U.K.). dNTPs were treated with KIO_4, and rNTPs were further purified by preparative paper chromatography (7). Oligoribonucleotides were synthesized using polyribonucleotide nucleotidyltransferase of *Micrococcus luteus* (EC 2.7.7.8) according to a modification (11) of the procedure of Thach (12). Phage T7 and øX174 DNA were prepared as previously described (7).

Assay of oligonucleotide-primed DNA synthesis

Reaction mixtures (50 µl) contained 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 20 mM NaCl, 5 mM DTT, 0.2 mg BSA/ml, 50 µM each of dATP, dGTP, dCTP, and [^{3}H]TTP (400 cpm/pmol), 0.6 nmol (as nucleotide) of øX174 template DNA, 1.6 µg T7 DNA binding protein,

0.15 µg T7 DNA polymerase, 0.35 µg T7 DNA primase and oligonucleotide primer additions as specified. The incubation mixtures were prewarmed to 30°C before the reaction was started by addition of the enzymes. [^{3}H]TMP incorporation into trichloroacetic acid-insoluble material was determined after incubation at 30°C for 5 min.

Gel electrophoresis

7.5% or 20% (wt/vol) polyacrylamide / 7 M urea denaturing gels (slab dimensions 0.15 cm x 20 cm x 36 cm) were prepared and run in 50 mM Tris-borate, pH 8.3 / 1 mM EDTA according to Maxam and Gilbert (13). After electrophoresis gels were frozen with solid CO_2 and analyzed by autoradiography at -20°C.

RESULTS

Oligoribonucleotide synthesis by T7 DNA primase

Products and templates. A combination of T7 DNA polymerase and primase can initiate DNA synthesis on various natural DNA templates by a mechanism involving the rNTP-dependent synthesis of a short RNA primer (7,9). The primer formed and extended in a coupled RNA priming-øX174 DNA replication reaction was found to be primarily a tetraribonucleotide of the sequence pppA-C-C-A-(DNA) (7). The enzyme responsible for primer generation is T7 primase. To demonstrate this, T7 primase was incubated with ATP and [α-^{32}P]CTP in the presence of øX174 DNA, and the products formed were fractionated by electrophoresis on a 20% polyacrylamide gel (Fig. 1). Label from [α-^{32}P]CTP was incorporated into a small number of oligoribonucleotides (lane b), which upon removal of the 5'-terminal phosphate groups were separated into 3 major spots (lane c). These were identified by re-electrophoresis on DEAE-paper along with authentic marker oligonucleotides as A-C (I), a mixture of A-C-C and A-C-A (II), and A-C-C-A (III). Some minor components (<3%) migrating as pentanucleotides were also detected. Note that the dephosphorylated oligonucleotides were fractionated by charge and not by molecular sieving in the gel system used. Qualitatively the same products were synthesized when øX174 DNA was replaced by denatured T7 DNA (lane d) or fd DNA (not shown). With native T7 DNA as template, oligoribonucleotide formation occurred only under conditions of active DNA synthesis

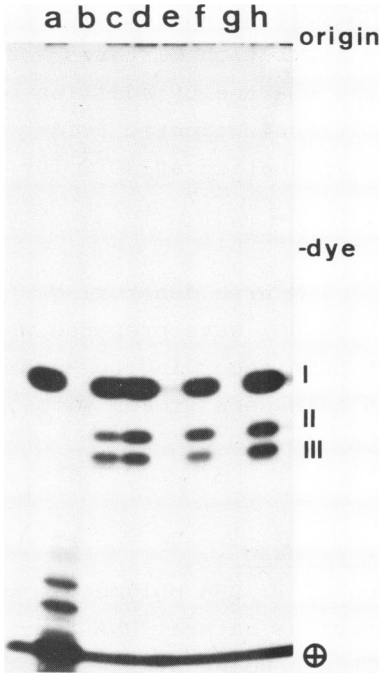

Fig. 1 Autoradiograph of fractionated oligoribonucleotides synthesized by T7 DNA primase. The complete reaction mixture (20 µl) contained 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.2 mg BSA/ml, 100 µM ATP, 10 µM [α-^{32}P] CTP (10^4 cpm/pmol), 0.6 nmol of øX174 DNA, and 0.7 µg T7 primase. Incubation was at 30°C for 5 min. The reaction was terminated by heating at 100°C for 1 min. To remove terminal phosphate groups, 1 µl 0.5 M Tris-base containing 2 mM $ZnCl_2$ and 0.5 µl alkaline phosphatase (5 mg/ml) were added followed by incubation at 37°C for 60 min. Reactions were stopped by addition of 20 µl 10 M urea/ 20 mM EDTA / 0.1% xylene cyanol and heating at 100°C for 30 sec. Samples were subsequently subjected to electrophoresis at 25 V/cm on a 20% polyacrylamide / urea slab gel. (a) Complete, RNase A digested; (b) complete, no phosphatase treatment; (c) complete; (d-f) øX174 DNA replaced by T7 DNA: (d) heat-denatured T7 DNA, (e) native T7 DNA, (f) native T7 DNA, T7 DNA polymerase (0.3 µg) and 4 dNTPs (50 µM each) present; (g) øX174 DNA omitted; and (h) GTP + UTP (10µM each) present. Spots marked I, II and III correspond to A-C, A-C-C/A and A-C-C-A, respectively.

(polymerase and dNTPs present) (lane f). Since T7 DNA polymerase in conjunction with T7 primase is known to effect unwinding of duplex DNA (7,8), it seems plausible that primer formation on this template reflects the liberation of single strands via strand displacement during DNA synthesis. In a separate analysis we have found that under these conditions a small fraction of the oligonucleotide primers is extended with deoxyribonucleotides (unpublished results). The pattern of radioactive products synthesized on øX174 DNA was not visibly changed by addition of GTP and UTP to the reaction mixture (lane h), indicating that the enzyme does not copy large stretches of the template. No oligomer synthesis was detected in the absence of DNA (lane g) or when rRNA was substituted for DNA (not shown). T7 primase is thus a DNA-dependent oligoribonucleotide synthetase which generates the

same set of di-, tri-, and tetranucleotides on all natural templates tested.

Table 1 Influence of various substances on primer synthesis

Additions	CMP incorporated into oligoribonucleotide
	pmol / 5 min
None	2.8
— Mg^{2+}, + EDTA (1 mM)	< 0.1
— Mg^{2+}, + Mn^{2+} (1 mM)	13.6
KCl (25 mM)	1.7
KCl (100 mM)	0.2
— DTT	1.6
— DTT, + N-ethylmaleimide (10 mM)	< 0.1
Rifampicin (50 µg/ml)	2.6
Nalidixic acid (100 µg/ml)	2.5
β,γ-methylene-dTTP (100 µM)	17.3
T7 DNA binding protein (0.8 µg)	2.0
T7 DNA binding protein (2.4 µg)	0.1

Conditions of synthesis and fractionation of the products were as described in the legend to Fig. 1 (complete reaction, 0.2 µg ØX174 DNA), except that Mg^{2+} was present at 2.5 mM. The amount of CMP incorporated was determined by measuring the radioactivity of oligoribonucleotide spots I, II and III.

Effect of various substances on oligoribonucleotide formation. The synthesis of oligoribonucleotides by T7 primase was dependent on the presence of a divalent cation (Table 1). Maximal activation was achieved with 1 mM Mn^{2+} or 2.5 mM Mg^{2+}. Mn^{2+} was about 5 times more effective than Mg^{2+}. The pH-optimum determined in 20 mM potassium phosphate or Tris-HCl was in the range of 7.0 to 7.5. Salt (KCl or NaCl) > 25 mM was strongly inhibitory. The enzyme was completely inhibited by N-ethylmaleimide, but not by rifampicin or by the potent inhibitor of T7 DNA replication *in vivo* nalidixic acid (14). β,γ-methylene-dTTP, an inhibitor of the NTPase activity associated with T7 primase (8), effected a 6-fold increase in oligomer synthesis. The stimulatory effect of this analogue was less pronounced if higher concentrations of ATP and CTP were used, suggesting that it acts protecting the triphosphates from cleavage. On the other hand, the experiment shows that primer synthesis on SS DNA is not directly coupled to NTP hydrolysis as is DNA synthesis by strand displacement on duplex DNA (8). T7 DNA binding protein, when added in amounts sufficient

to cover completely the template, caused a strong reduction in primer synthesis, indicating that the binding protein competes with T7 primase for DNA. Nevertheless, in a coupled RNA priming-øX174 DNA synthetic reaction the rate of DNA synthesis is increased in the presence of DNA binding protein (see also ref. 7).

Primer extension by T7 DNA polymerase and primase

Stimulation of oligonucleotide-primed DNA synthesis by T7 DNA primase. The process of primer extension has been uncoupled from that of primer synthesis by the use of exogenous oligonucleotides as initiating substrates. For this purpose DNA synthesis primed by a particular oligonucleotide was measured at 30°C either in the presence or absence of T7 primase using øX174 DNA

Table 2 Effect of T7 DNA primase on oligonucleotide-primed DNA synthesis

Oligonucleotide additions	dTMP incorporated	
	T7 DNA polymerase	T7 DNA polymerase plus primase
	pmol / 5 min	
none	6	17
A - C	5	17
C - A	5	18
A - C - C	4	73
C - C - A	17	112
A - C - C - A	80	234
A - C - C - G	14	132
A - C - C - U	7	102
A - C - C - C	15	123
A - C - A - A	8	160
A - A - C - A	11	185
C - C - C - A	44	178
C - C - A - A - A	37	146
A - C - C - A - A	82	179
U - U - U - U	4	34
C - C - C - C	4	91
A - A - A - A	6	188
pA - A - A - A	7	196
dA - dA - dA - dA	6	147

Reactions were carried out as described in Materials and Methods with the indicated oligonucleotides present at a concentration of 50 µM.

as template. T7 primase caused a dramatic stimulation of oligonucleotide-mediated DNA synthesis with all tri-, tetra-, and pentanucleotides examined (Table 2). These results provide evidence that the enzyme has a functional role in primer extension. At the relatively high primer concentration (50 μM) used, some of the oligomer primers, particularly those containing the C-C-A sequence, could be extended by T7 DNA polymerase alone. This may be due to a special affinity of the polymerase to primers bearing a C-C-A sequence. Alternatively, the possibility exists that these oligonucleotides have a higher annealing efficiency to complementary sequences in the template DNA.

In assaying the stimulatory activity of T7 primase, the size rather than the base composition of the primer is of major importance. Using oligo(A) or oligo(C) nucleotides of increasing chain length as initiators, T7 DNA polymerase combined with primase clearly preferred the tetranucleotide primers (Fig. 2). Similar results were obtained with fd DNA as template (data not shown). By comparison, T7 DNA polymerase alone exhibited a preference for

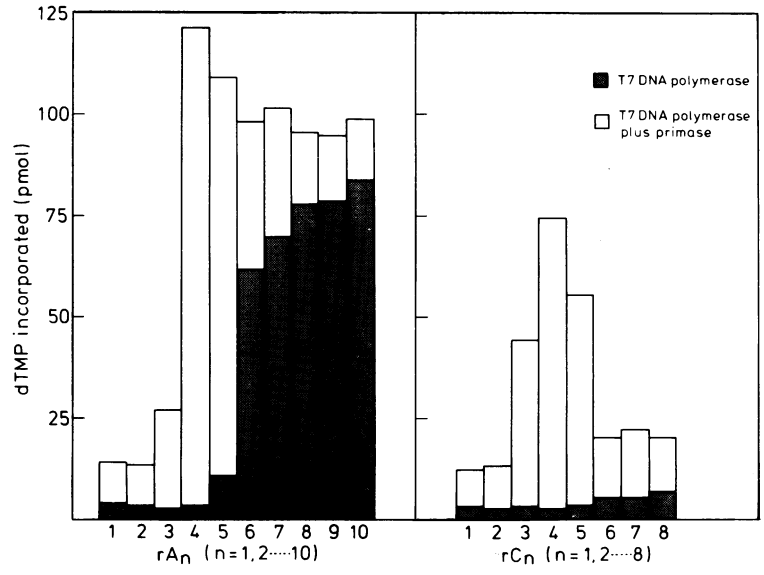

Fig. 2 Primer activity of oligoribonucleotides of various chain length. Reactions were carried out as described in Materials and Methods, using 5 μM oligo(A)$_n$ or 50 μM oligo(C)$_n$ as chain initiators as indicated.

primers of chain lengths > 5. The relatively poor primer activity of $(C)_{5-8}$ compared to $(A)_{5-8}$ can be explained by the fact that the longest complementary sequences in øX174 DNA are $(dG)_4$ and $(dT)_7$, respectively (15).

The extent to which T7 primase stimulated oligomer-primed DNA synthesis was markedly influenced by the conditions employed. Using A-C-C-A as initiator, the stimulatory effect of T7 primase was greatest at low primer concentration (Table 3) and high temperature (Table 4). Maximal stimulation is, therefore, achieved

Table 3 Influence of primer concentration on DNA synthesis

[A-C-C-A] (μM)	dTMP incorporated + T7 primase	dTMP incorporated - T7 primase	Ratio +/- primase
	pmol / 5 min	pmol / 5 min	
0.3	35	< 1	> 35
1	94	3	31
3	141	14	10
10	188	51	3.7
30	217	75	2.9
100	245	98	2.5

A-C-C-A at the indicated concentrations was used to prime øX174 DNA-directed synthesis. Background synthesis in the absence of primer was subtracted.

Table 4 Influence of temperature on oligomer-primed DNA synthesis

Temperature (°C)	dTMP incorporated + T7 primase	dTMP incorporated - T7 primase	Ratio +/- primase
	pmol / 5 min	pmol / 5 min	
10	17	11	1.5
20	88	49	1.8
30	222	66	3.4
40	220	21	10.5

Incubations were carried out at the indicated temperature using 50 μM A-C-C-A to prime øX174 DNA-directed synthesis.

under conditions which are unfavorable for the formation of transient primer-template duplexes.

Selective initiation with oligonucleotide primers. ϕX174 DNA was converted to duplex forms in DNA synthetic reactions primed either by ATP and CTP or by oligonucleotides of various base composition. The DNA products labeled with ^{32}P in the complemen-

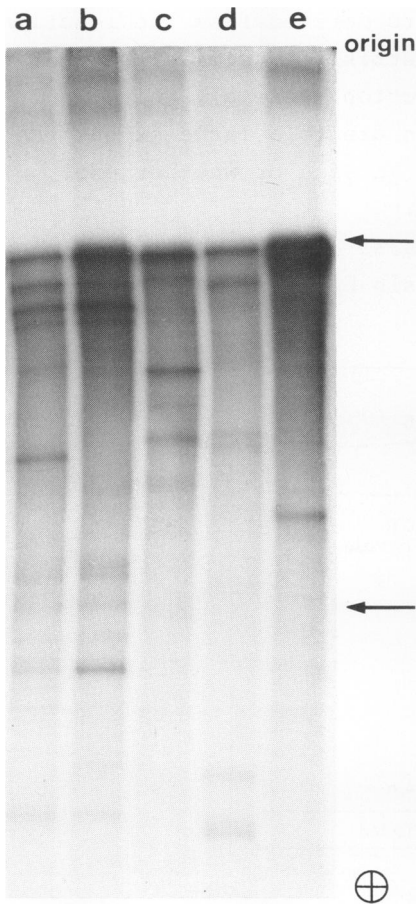

Fig. 3 Ribonucleotide-primed synthesis of ϕX174 DNA complementary strand and analysis of a restriction fragment by gel electrophoresis under denaturing conditions. ϕX174 duplex form DNA labeled in the complementary strand with [^{32}P]dCMP (2.5 x 10^3 cpm/pmol) was synthesized in 0.25 ml-reaction mixtures (without T7 DNA binding protein) as described in Materials and Methods. Either a mixture of ATP (0.5 mM) and CTP (0.1 mM) or various synthetic oligonucleotides (50 µM) were used to initiate synthesis as indicated below. After incubation at 30°C for 4 min, the reaction mixture was extracted with an equal volume of neutral phenol. Residual phenol was removed with ether, and DNA was precipitated together with 20 µg carrier tRNA in ethanol (13). For restriction enzyme digestion, the precipitate was dissolved in 20 µl of a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM KCl, 1 mM DTT, 0.1 mg BSA/ml and 5 units endonuclease HpaI, followed by incubation at 37°C for 1 h. Native DNA cleavage products were fractionated by electrophoresis on a 1.6% agarose flat gel (16), and the radioactive band corresponding to the position of HpaI fragment 2 was excised. The DNA fragment was eluted from the crushed gel slice, precipitated with ethanol, and redissolved in 20 µl of 0.1 M NaOH / 5 mM EDTA / 5 M urea / 0.05% xylene cyanol. After heating at 100°C for 15 s, the sample was subjected to electrophoresis (8 V/cm) at 45°C on a 7.5% polyacrylamide / urea slab gel. (a) A_C_C_A, (b) ATP plus CTP, (c) A_C_C_G, (d) A_C_A_A, and (e) A_A_A_A_A. Arrows indicate positions of full-length HpaI fragments 2 (1264 bases) and 3 (392 bases).

tary strand were then cleaved by the endonuclease HpaI to create duplex fragments with defined termini. HpaI fragment 2 was selected for an analysis by gel electrophoresis under denaturing conditions. As shown in Fig. 3, the labeled product DNA was fractionated into a series of discrete bands characteristic for each primer species used. This is the result expected if synthesis with an individual primer starts from distinctive sites (probably base complementary regions) within the fragment examined. Interestingly, the pattern of products derived from the reaction containing ATP and CTP (lane b) was strikingly similar to that obtained from the A_C_C_A-primed reaction (lane a). This result suggests that the sites of initiation are to a large extent the same whether the primer is generated in situ or whether exogenous A_C_C_A is utilized as primer.

Assay of T7 DNA primase in crude cell extracts. Stimulation of oligonucleotide-primed DNA synthesis by T7 primase provides a

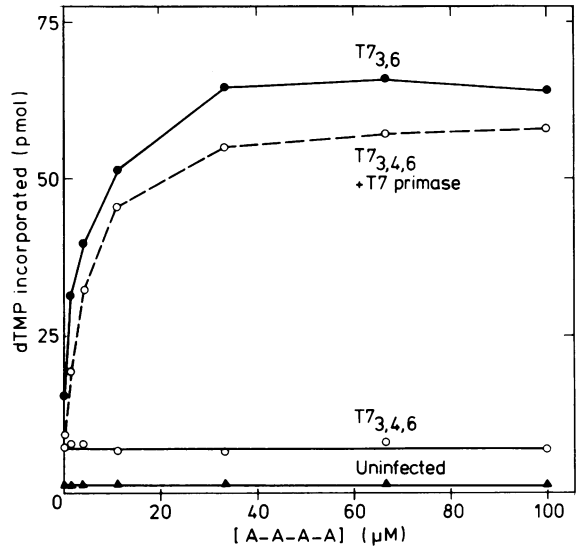

Fig. 4 Tetranucleotide-primed DNA synthesis in crude cell extracts. Reactions were essentially carried out as described in Materials and Methods using varying amounts of A_A_A_A as primer, ϕX174 DNA (1.2 nmol) as template, and extracts (0.1 mg protein) of uninfected or T7 mutant infected E. coli H559 (polA1, end, F⁻, su⁻) as source of enzymes. T7 carrying amber mutations in both genes 3 and 6 (nucleases) were used for infection. Cell extracts were prepared as previously described (5).

specific assay for the detection of the enzyme in crude extracts of T7-infected cells (Fig. 4). Addition of A_A_A_A to an extract of T7 gene 4^+-infected cells resulted in a clear stimulation of øX174 DNA-directed synthesis. No response was observed with extracts of uninfected or T7 gene 4^--infected cells. However, upon complementation of the gene 4 mutant extract with purified T7 primase, the tetranucleotide again served as a primer for øX174 DNA-dependent synthesis.

DISCUSSION

Synthesis of progeny strands during T7 DNA replication is discontinuous (17) and requires the function of the gene-4 protein. Our present results show this protein to be a DNA-dependent oligoribonucleotide synthetase which is apparently involved in initiation of DNA strand synthesis. It has, therefore, been given the descriptive designation "T7 DNA primase" (7). The requirement for DNA is best satisfied by natural SS DNA. The enzyme can recognize internal DNA regions and it displays base-specificity as judged from its inability to synthesize oligoribonucleotides containing A and C residues with homodeoxypolymer templates. ATP and CTP are the preferred substrates. In its specificity of nucleotide incorporation, T7 primase is reminiscent of tRNA nucleotidyltransferase which catalyzes the addition of 2 CMP and 1 AMP residues to tRNA lacking the _C_C_A_{OH} 3'-end (18). The enzyme has, in common with classical RNA polymerases, the unique triphosphate initiation site, but is sharply distinguished from them by its failure to make long copies from a template strand. Regardless of the DNA substrate used, T7 primase essentially stops chain growth once it has polymerized 4 nucleotide residues (Fig. 1).

For continuation of polymerization, T7 primase interacts with T7 DNA polymerase, thereby effecting a rapid and efficient transition from primer RNA to DNA synthesis. The interaction between the T7 enzymes is specific; neither *E. coli* polymerases I, II, or III nor T4 DNA polymerase can cooperate with T7 primase for the initiation of DNA synthesis (7). An active role of the enzyme in the process of primer extension has been established by its pronounced stimulatory effect on DNA synthesis primed by synthetic oligonucleotides (Fig. 2, Table 2,3 and 4). The determination of

conditions resulting in maximal stimulation suggests that T7 primase may act by stabilizing the short primer segments in a duplex state on the template prior to the covalent attachment of the first deoxyribonucleotide.

The T7 enzyme pair initiates the synthesis of polynucleotide chains at multiple but distinctive sites in a ØX174 DNA synthetic model reaction (Fig. 3). The sites of initiation appear to be mainly determined by the base sequence complementary to A_C_C_A. The finding that T7 primase generates A_C_C_A also on T7 DNA suggests that the mechanism of priming for T7 lagging strand synthesis may be similar to that found for ØX174 (-) strand synthesis.

Another enzyme serving a role as a primer-generating polymerase is dnaG protein of E. coli. In contrast to T7 primase, this enzyme has to rely on the guidance of several other replication proteins (at least DNA binding protein) for primer synthesis (19-21). Moreover, dnaG protein can polymerize in vitro either ribo- or deoxyribonucleotides with nearly equal efficiency (22). Reichard et al. (23) have reported that discontinuous synthesis of polyoma DNA in a nuclear system is primed by an RNA piece approximately 10 nucleotides in length. This observation should encourage a search for "primases" also in eukaryotic organisms.

ABBREVIATIONS: SS DNA, single-stranded DNA; rNTP, ribonucleoside triphosphate; dNTP, deoxyribonucleoside triphosphate; DTT, dithiothreitol; BSA, bovine serum albumin.

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