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Replication of duplex DNA by bacteriophage T7 DNA polymerase and gene *4* protein is accompanied by hydrolysis of nucleoside 5′-triphosphates*

(nucleotide hydrolysis/DNA nucleotidyltransferase/nucleoside triphosphatase)

RICHARD KOLODNER AND CHARLES C. RICHARDSON

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **Homogeneous preparations of phage T7 gene *4* protein catalyze the hydrolysis of dNTPs and rNTPs to NDPs and P_i in the presence of single-stranded DNA. Synthesis on single-stranded DNA by T7 DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) does not affect the hydrolysis of NTPs by the gene *4* protein. Gene *4* protein does not catalyze the hydrolysis of NTPs in the presence of duplex DNA, nor can T7 DNA polymerase use duplex DNA as a template. However, the two proteins together can replicate duplex DNA and, under these conditions, synthesis is accompanied by hydrolysis of NTPs. During synthesis on duplex templates in the presence of T7 DNA polymerase, gene *4* protein, dNTPs, and rNTPs, 4.2 NTPs are hydrolyzed for each dNMP polymerized. 2′3′-Dideoxy-TTP, an inhibitor of DNA synthesis, inhibits hydrolysis by the gene *4* protein during synthesis on duplex DNA, and β,γ-methylene-dTTP, an inhibitor of hydrolysis by the gene *4* protein, stops DNA synthesis on duplex DNA. The multiple activities of gene *4* protein are shown to reside in a single protein molecule.**

For the past several years we have been studying the replication of phage T7 DNA as a model for elucidating the molecular mechanisms by which a linear duplex DNA molecule is replicated. From *in vivo* and *in vitro* studies it is clear that two proteins, the phage T7-induced DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) and the protein encoded by gene *4* of phage T7, play a major role in replication. Genetic (2–4) and biochemical (5, 6) analyses show these two proteins to be essential for T7 DNA replication. The T7-induced DNA polymerase (7, 8) is composed of *Escherichia coli* thioredoxin and the gene 5 protein of the phage (1, 9, 10). The gene *4* protein has been purified with the aid of a complementation assay (11–13) to near homogeneity (Fig. 2).

Whereas neither the T7 DNA polymerase nor the gene *4* protein alone can synthesize DNA on duplex templates, the two proteins together catalyze extensive DNA synthesis (12). The reaction is specific for T7 DNA polymerase; neither T4 DNA polymerase nor *E. coli* DNA polymerases I, II, or III are effective (12). The reaction is stimulated by, but is not dependent on, the presence of rNTPs (12), as is replication in the *in vitro* system (5, 6). Although earlier studies (12, 14) with gene *4* protein suggested that the stimulation of DNA synthesis on duplex DNA resulted from a role of the gene *4* protein in initiation of DNA strands, our recent studies do not support such an interpretation. Our results (unpublished data) show that, in reaction mixtures containing the two proteins, synthesis initiates at single-strand breaks in duplex molecules with elongation of the polynucleotide chain proceeding in a 5′ → 3′ direction, displacing the parental DNA strands. The number of replicative forks approximates the number of polymerase molecules, with polymerization proceeding at rates up to 6000 nucleotides per min per fork. At later times in the reaction the polymerase is displaced from the template by branch migration (15, 16) allowing it, along with part of the newly synthesized strand, to jump to the displaced strand and copy it in a gene *4* independent reaction. In this way T7 DNA polymerase and gene *4* protein synthesize self-complementary product molecules up to 120,000 nucleotides in length.

Because the replication of duplex DNA by T7 DNA polymerase and gene *4* protein requires only 10 gene *4* protein molecules per polymerase molecule, it seems plausible that the gene *4* protein acts catalytically in conjunction with the polymerase to effect unwinding of the duplex DNA template. Inasmuch as this is likely to be an energy-requiring reaction, we looked for the hydrolysis of nucleoside 5′-triphosphates during the course of synthesis on duplex DNA. In this communication we show that the gene *4* protein is not only a single-strand-DNA-dependent nucleoside triphosphatase (NTPase; unspecific diphosphate phosphohydrolase, EC 3.6.1.15) but that hydrolysis of NTPs by gene *4* protein is coupled to DNA synthesis on duplex DNA.

MATERIALS AND METHODS

Nucleotides and DNAs. Unlabeled nucleotides were from P. L. Biochemicals. Labeled NTPs, P_i, and PP_i were from New England Nuclear. dNTPs were treated with $NaIO_4$, and rNTP and dNTPs were chromatographed on DEAE-Sephadex A25 (17). 2′,3′-Dideoxythymidine 5′-triphosphate (ddTTP) was the gift of A. Kornberg. Deoxythymidine 5′-(β,γ-methylene)triphosphate was from P. L. Biochemicals. T7 DNA and phage φX174 DNA were prepared as previously described (16).

Enzymes. The T7 DNA polymerase (Fraction VII; 5600 units/mg) was that previously described (7, 10). T7 gene *4* protein (Fraction VI; 10,000 units/mg) was purified by a modification of a previous procedure (12). The stimulation of T7 DNA polymerase by gene *4* protein was assayed as described (12) except that the NTPs were present at 0.075 mM and the Tris at 40 mM.

Assay of NTP Hydrolysis. The hydrolysis of NTPs was assayed by measuring the formation of either NDP and NMP or P_i and PP_i from [α-^{32}P]- and [γ-^{32}P]NTPs, respectively. Reactions containing an [α-^{32}P]NTP were stopped by the addition of 0.1 M EDTA (pH 8.0) to a final concentration of 8 mM greater than the $MgCl_2$ concentration. An aliquot was chromatographed along with marker nucleotides with 0.8 M LiCl/1 M HCOOH on PEI-cellulose thin-layer plates (18). The amount of NMP, NDP, and NTP was determined by measuring the

Abbreviations: ddTTP, 2′,3′-dideoxy-TTP; NTPase, nucleoside 5′-triphosphatase.

* This is paper VIII in a series entitled "Bacteriophage T7 deoxyribonucleic acid replication *in vitro*." The previous paper is ref. 1.

Table 1. Hydrolysis of dATP in the presence of single-stranded DNA

Reaction components	dADP formed, pmol
Complete	231
- Gene *4* protein	<5
- Mg^{2+}	<5
- Denatured T7 DNA	11
- Denatured T7 DNA + ϕX174 viral DNA	228
- Denatured T7 DNA + Native T7 DNA	20

The complete reaction mixture (100 μl) contained 40 mM Tris·HCl buffer (pH 7.5), 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.075 mM [α-^{32}P]dATP (50 cpm/pmol), 6 nmol of heat-denatured T7 DNA, and 2.0 units of gene *4* protein. Incubation was for 20 min at 30°. ϕX174 DNA (6 nmol) or native T7 DNA (6 nmol) was substituted for denatured T7 DNA as noted. dADP formation was measured as described in *Materials and Methods*.

radioactivity in 0.5 cm strips. The amount of DNA synthesis was determined by measuring the acid-insoluble radioactivity in a second aliquot. When the hydrolysis of an rNTP was measured, [^{3}H]dTTP was used to measure DNA synthesis.

Reactions containing a [γ-^{32}P]NTP were stopped by the addition of 0.7 ml of 1 M HCl, 0.1 ml of 1 mM $NaPP_i$/1 mM KPO_4 (pH 7.0), and 0.2 ml of 25% Norit charcoal, and the amount of radioactivity in P_i and PP_i was determined (19). DNA synthesis was measured in parallel reaction mixtures containing [^{3}H]dTTP.

RESULTS

Hydrolysis of Nucleoside 5'-Triphosphates by the Gene *4* Protein in the Presence of Single-Stranded DNA. Homogeneous preparations of gene *4* protein hydrolyze dNTPs and rNTPs to NDPs and P_i in the presence of single-stranded DNA. During a 20 min incubation with denatured T7 DNA the gene *4* protein (2 units) hydrolyzes [α-^{32}P]dTTP (228 pmol), dATP (231 pmol), dGTP (41 pmol) dCTP (16 pmol), rATP (77 pmol), rUTP (25 pmol), and rCTP (10 pmol) when each is present alone at 0.075 mM; rGTP is not hydrolyzed. When all four dNTPs or rNTPs are present together, the amount of hydrolysis of each is increased by 25%. The products of hydrolysis are exclusively NDPs and P_i.

The hydrolysis of dATP is dependent on the presence of gene *4* protein and Mg^{2+} (Table 1). If denatured DNA is omitted or replaced by native T7 DNA, hydrolysis is reduced more than 20-fold. Since phage ϕX174 DNA is as effective as denatured T7 DNA (Table 1), termini are not required. Similar results are observed with the other NTPs. Addition of P_i to the reaction at concentrations of up to 40 mM had no effect (<5%) on the rate of hydrolysis; there is no exchange of $^{32}P_i$ (<1%) into NTPs. Thus, the gene *4* protein is a single-stranded-DNA-dependent NTPase.

T7 DNA polymerase synthesizes DNA in a reaction mixture containing single-stranded T7 DNA and the four dNTPs (7, 8); gene *4* protein does not stimulate this synthesis (12). The hydrolysis of each dNTP by gene *4* protein under conditions of synthesis on single-stranded T7 DNA (all four dNTPs present) is identical to that observed in the absence of T7 DNA polymerase. Similarly, if one or all four rNTPs are added to this synthetic reaction mixture, the amount of hydrolysis of each rNTP is the same as in the absence of polymerase. Thus, hydrolysis of NTPs by the gene *4* protein in the presence of single-stranded DNA is not affected by DNA synthesis.

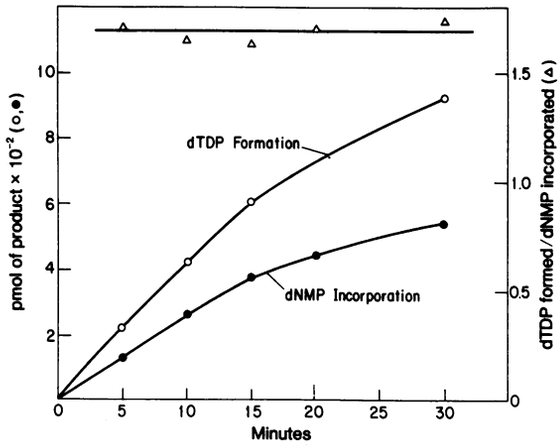

FIG. 1. Time course of DNA synthesis and dTTP hydrolysis by gene *4* protein and T7 DNA polymerase. The incubation mixture was that described in the legend to Table 2 except that, in addition to the four dNTPs, the four rNTPs were present at a concentration of 0.075 mM each and the time of incubation was varied as indicated. [α-^{32}P]dTTP was the radioactively labeled NTP. [^{32}P]dTDP and acid-insoluble [^{32}P]dNMP were measured as described in *Materials and Methods*. ○, dTDP formed; ●, dNMP incorporated; △, dTDP formed per dNMP incorporated.

Replication of Duplex DNA by T7 DNA Polymerase and Gene *4* Protein Is Accompanied by Hydrolysis of NTPs. T7 DNA polymerase cannot use duplex DNA as a template. However, a combination of gene *4* protein and T7 DNA polymerase can initiate synthesis at single-strand interruptions in duplex DNA. Whereas gene *4* protein alone does not catalyze a hydrolysis of NTPs in the presence of duplex DNA, it does catalyze hydrolysis under conditions of DNA synthesis by T7 DNA polymerase and gene *4* protein (Fig. 1). Omission of polymerase, gene *4* protein, or DNA reduces the amount of hydrolysis more than 10-fold (Table 2). Similar results are ob-

Table 2. Hydrolysis of dATP and rATP under conditions of DNA synthesis

Reaction components	dNMP incorporated, pmol	dADP formed, pmol	rADP formed, pmol
Complete	362	673	464
- Polymerase	0	28	23
- Gene *4*	8	<5	<10
- DNA	0	46	<10
- Polymerase - DNA	0	15	<10
- Gene *4* - DNA	0	<5	<10
- Gene *4* - Polymerase	0	<5	<10

The complete reaction mixture (100 μl) contained 40 mM Tris·HCl buffer (pH 7.5), 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.075 mM each of dCTP, dGTP, dATP, and dTTP, 6 nmol of native T7 DNA, 2.0 units gene *4* protein, and 0.16 unit T7 DNA polymerase. [α-^{32}P] dATP replaced the unlabeled dATP, or [γ-^{32}P]rATP (0.075 mM) was added when rADP formation was measured. Incubation was for 20 min at 30°. [^{32}P]NDP and P_i were measured as described in *Materials and Methods*.

Table 3. Hydrolysis of dNTPs under conditions of DNA synthesis

Reaction components	dNMP incorporated, pmol	dTDP formed, pmol	dADP formed, pmol	dGDP formed, pmol	dCDP formed, pmol
Complete	362	774	673	138	63
− 3 dNTPs	<1	116	270	36	16
+ 4 rNTPs	495	810	559	81	31

The reactions, incubations, and analyses were carried out as described in the legend to Table 2 except that an individual [α-^{32}P]-dNTP replaced the corresponding unlabeled dNTP as indicated. All NTPs, when present, were at a concentration of 0.075 mM.

served with all NTPs. In all experiments gene *4* protein was present in amounts that give optimal stimulation of T7 DNA polymerase on duplex DNA in the standard assay.

(*i*) *Hydrolysis of dNTPs.* All four dNTPs are hydrolyzed to dNDPs during the course of the reaction, with dTTP and dATP exhibiting the most extensive hydrolysis (Table 3). When [γ-^{32}P]dNTPs are present in the reaction, both $^{32}P_i$ and PP_i are formed, corresponding to the amount of dNDP formed and the amount of dNMP incorporated into DNA, respectively. A total of 4.6 dNTPs are hydrolyzed to dNDPs for each dNMP polymerized in the absence of the four rNTPs, and a total of 3.0 in the presence of the four rNTPs. When only one dNTP is present in the reaction, DNA synthesis is eliminated and the rate of hydrolysis is decreased (to 10–40%). Thus, although DNA polymerase is required for hydrolysis to occur in the presence of double-stranded DNA, extensive DNA synthesis is not.

(*ii*) *rNTPs are hydrolyzed.* All of the rNTPs except rGTP are hydrolyzed to rNDP and P_i during DNA synthesis on duplex DNA (Table 4). The rate of hydrolysis of an individual rNTP is greatest when all four dNTPs and no other rNTPs are present. In the absence of the four dNTPs there is no DNA synthesis, and the amount of rNTP hydrolysis is reduced (to 10–20%). The total number of rNTPs hydrolyzed per dNMP incorporated in the presence of the four dNTPs and rNTPs is 1.2. The total hydrolysis of dNTPs and rNTPs per dNMP incorporated under these conditions is 4.2.

Coupling of DNA Synthesis to NTP Hydrolysis. Although maximal hydrolysis of NTPs occurs during extensive synthesis on duplex DNA, there is considerable hydrolysis in the absence of extensive synthesis when only one dNTP is present (Table 3). Furthermore, during extensive synthesis the polynucleotide

Table 4. Hydrolysis of rNTPs under conditions of DNA synthesis

Reaction components	dNMP incorporated, pmol	rADP formed, pmol	rUDP formed, pmol	rCDP formed, pmol	rGDP formed, pmol
Complete	362	464	222	54	<5
+ 3 rNTPs	495	450	122	27	<5
− dNTPs	<1	70	25	10	<5
− dNTPs + 3 rNTPs	<1		31	13	<5

The reactions, incubations, and analyses were carried out as described in the legend to Table 2 with the four dNTPs and one [α-^{32}P]rNTP present in the reaction mixture. All NTPs, when present, were at a concentration of 0.075 mM.

Table 5. Inhibition of DNA synthesis and triphosphate hydrolysis by β,γ-methylene-dTTP

Reaction components	dNMP incorporated, pmol	dADP formed, pmol
Polymerase, denatured DNA	30	<5
Polymerase, denatured DNA, β,γ-methylene-dTTP	30	<5
Gene *4*, denatured DNA	<1	132
Gene *4*, denatured DNA, β,γ-methylene-dTTP	<1	36
Polymerase, gene *4*, native DNA	324	376
Polymerase, gene *4*, native DNA, β,γ-methylene-dTTP	27	58
Polymerase, gene *4*, native DNA (stopped after 6 min)	122	120
Polymerase, gene *4*, native DNA, β,γ-methylene-dTTP (added after 6 min)	160	137

The reaction was carried out essentially as described in the legend to Fig. 1 with the four dNTPs and the four rNTPs present. The presence of T7 DNA polymerase (0.16 unit), gene *4* protein (2.0 units), native or denatured T7 DNA (6 nmol), and β,γ-methylene-dTTP (0.15 mM) is as indicated.

chain ahead of the growing chain is displaced, and these single strands could promote hydrolysis of NTPs by the gene *4* protein. Although these two possibilities can account for some of the observed NTP hydrolysis, several lines of evidence strongly suggest that hydrolysis of NTPs is necessary for DNA synthesis on duplex DNA.

(*i*) *Maximal hydrolysis occurs during DNA synthesis.* The hydrolysis of NTPs parallels DNA synthesis (Fig. 1), while the displaced single strands are only transient intermediates in this reaction (unpublished data). Furthermore, the amount of hydrolysis occurring during synthesis is 3- to 4-fold greater than that observed in reaction mixtures containing single-stranded DNA (*text;* Tables 1, 3, and 4).

(*ii*) *An inhibitor of DNA synthesis inhibits hydrolysis of nucleotides by gene 4 protein.* Under conditions of DNA synthesis as described in Fig. 1 using native T7 DNA, T7 DNA polymerase, and gene *4* protein, the presence of 0.05 mM ddTTP (20) inhibits both DNA synthesis and dATP hydrolysis (>85%). In contrast, hydrolysis of dATP by gene *4* protein using denatured T7 DNA is unaffected by the presence of 0.05 mM ddTTP, whereas synthesis by T7 DNA polymerase, using denatured T7 DNA, is abolished (>95%). If ddTTP (0.05 mM) is added to a reaction after 5 min of synthesis (85 pmol and 173 pmol of hydrolysis of dATP) and the reaction is allowed to continue for an additional 15 min, the amount of synthesis (87 pmol total, including the first 5 min) is not increased, and hydrolysis of dATP is much less (308 pmol total) than that observed if ddTTP has not been added (750 pmol total). The additional hydrolysis occurring in the absence of DNA synthesis probably reflects hydrolysis on the displaced single strands formed during the initial period of synthesis. Thus, an active T7 DNA polymerase and a 3′-OH terminated primer are required for hydrolysis of NTPs with duplex DNA.

(*iii*) *An inhibitor of nucleotide hydrolysis stops DNA synthesis.* T7 DNA polymerase does not incorporate β,γ-methylene-dTTP into DNA using single-stranded templates, and the presence of this analogue has no effect on the incorporation of the other dNTPs (Table 5). However, this analogue is a potent

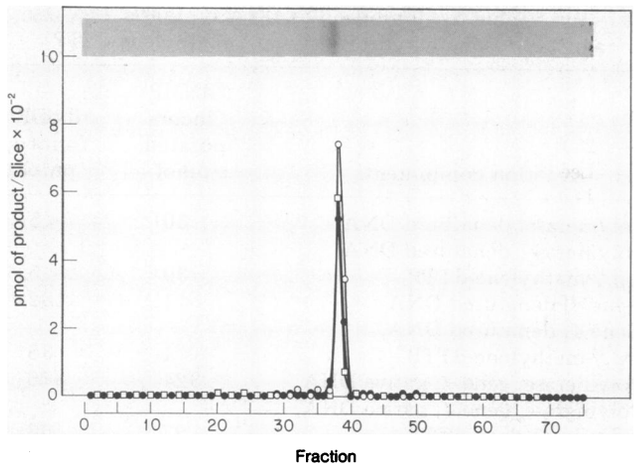

FIG. 2. Polyacrylamide gel electrophoresis of the native gene 4 protein. The electrophoresis was carried out as described (9) except that the gel slices were incubated in diluent for 4 hr to elute the protein. The T7 DNA polymerase stimulatory activity (●) and the hydrolysis of [α-^{32}P]dTTP (○) were assayed as described in the legend to Fig. 1. The hydrolysis of [γ-^{32}P]rATP (□) was assayed as described in the legend to Table 2. Recovery of activity from the gel was 25%. The upper part of the figure contains a photograph of an identical gel that was stained with Coomassie blue as described (9). Densitometry showed that the major protein band contained 90–95% of the Coomassie-positive material.

inhibitor of NTP hydrolysis by the gene 4 protein in the presence of single-stranded DNA. On the other hand, β,γ-methylene-dTTP completely inhibits synthesis on duplex DNA as well as the hydrolysis of NTPs. The gene 4 protein is required at all times during DNA synthesis on duplex DNA because the addition of the analogue during the reaction immediately stops DNA synthesis and NTP hydrolysis.

Identity of the Hydrolysis Activity with the Gene 4 Protein. The NTPase activity appears to reside in the gene 4 protein. First, the ratios of the gene 4 complementing activity (12), the T7 DNA polymerase stimulating activity, and the NTPase activity remain constant during the later stages of purification. Second, as shown in Fig. 2, the T7 DNA polymerase stimulating activity, the NTPase activity, and the major protein band all migrate together during electrophoresis through polyacrylamide gels. This major protein band contains both the gene 4 complementing activity and the T7 DNA polymerase stimulating activity (unpublished data). Finally, both the NTPase activity and the T7 DNA polymerase stimulating activity are inhibited by β,γ-methylene-dTTP.

DISCUSSION

Our earlier studies (12) showed that the gene 4 protein and T7 DNA polymerase carry out extensive synthesis of DNA on duplex templates. It seems likely that this property of the gene 4 protein can, in part, explain its essential role in DNA replication. More recent studies have shown that the stimulation of T7 DNA polymerase by the gene 4 protein on duplex DNA does not reflect initiation events, but reflects an enhancement of elongation of polynucleotide chains.

Our present results show the gene 4 protein to be a DNA-dependent nucleoside 5′-triphosphatase. In the presence of single-stranded DNA the gene 4 protein catalyzes the hydrolysis of NTPs. The more interesting question is whether this hydrolysis reflects a role of these nucleotides in the unwinding of duplex DNA during replication. For this reason it is of particular interest that hydrolysis of NTPs occurs during the synthesis of DNA on duplex templates catalyzed by T7 DNA polymerase and gene 4 protein. This raises two questions: (*i*) Is hydrolysis essential for the synthesis observed? (*ii*) Does hydrolysis reflect a role of the gene 4 protein in unwinding the DNA strands?

An obvious possibility is that the hydrolysis occurs as a result of the liberation of single strands via strand displacement during synthesis, an event known to occur. However, the amount of hydrolysis exceeds by 3- to 4-fold that observed with gene 4 protein and single-stranded DNA alone. In addition, the kinetics of hydrolysis are not compatible with the transient nature of the single strands; they are rapidly converted to duplex structures by branch migration and polymerase action. More important, however, is the fact that β,γ-methylene-dTTP, an inhibitor of hydrolysis of NTPs by the gene 4 protein, but not an inhibitor of T7 DNA polymerase, stops DNA synthesis on duplex DNA simultaneously with the cessation of NTP hydrolysis. The observation that hydrolysis of NTPs occurs when only a single dNTP is present in the synthetic reaction may reflect an abortive attempt to unwind in the absence of extensive synthesis. A 3′-hydroxyl-terminated primer is required, because ddTTP eliminates such termini and inhibits hydrolysis.

Under conditions of active DNA synthesis by the T7 DNA polymerase and gene 4 protein on duplex templates, approximately four NTPs are hydrolyzed for each dNMP polymerized. This number represents an upper limit, because some hydrolysis undoubtedly occurs on the displaced single strands. Experiments in which DNA synthesis was stopped with ddTTP at various times provide an estimate of this component of the reaction and suggest that only two or three NTPs are hydrolyzed for each dNMP polymerized at the replication fork. However, in this system two dNTPs are polymerized for each base pair disrupted at the replicative fork; therefore the number of NTPs hydrolyzed per base pair of template unwound is four to six.

In recent years several *E. coli* DNA-dependent NTPases have been implicated in DNA replication. In the *in vitro* replication of ϕX174 both the *dnaB* protein (21) and replication factor Y (22) are NTPases and ATP plays a role in the initiation of DNA synthesis on primed ϕX174 DNA molecules by polymerase III, *dnaZ* protein, and elongation factors I and III (23, 24). The products of genes *44* and *62* of phage T4 function as a complex to promote DNA synthesis on single-stranded templates by T4 DNA polymerase and gene *45* protein (25). It has been postulated that hydrolysis of ATP by the gene *44–62* complex serves as a "motive force" to drive the DNA polymerase along its template (25).

Two *E. coli* proteins have been postulated to use the energy of nucleotide hydrolysis to unwind DNA. The product of the *recB,C* genes, exonuclease V, may use ATP hydrolysis to unwind duplex DNA prior to degradation of the resulting single strands (26). A 180,000 dalton protein (27) hydrolyzes ATP in the presence of single-stranded DNA, and can promote the unwinding of duplex DNA in an ATP-dependent reaction (28). The latter protein differs from the T7 gene 4 protein in that approximately 85 protein molecules are required to unwind a 6000 nucleotide long duplex and the unwinding occurs in the absence of DNA synthesis.

The mechanism by which the gene 4 protein uses the energy of hydrolysis of NTPs to facilitate unwinding is not known. Considerably more information is needed to distinguish between such diverse mechanisms as, for example, an active role of the gene 4 protein in mechanically separating the parental DNA strands or a role in increasing the affinity of the polymerase for the template strand. In any case, one can estimate that the energy required to disrupt one base pair (29) could be

provided by the hydrolysis of approximately one NTP, a value not far removed from that observed here. Finally, it should be stressed that the orderly movement of the replication fork undoubtedly requires proteins and factors in addition to those described here.

Note Added in Proof. The *rep* protein of *E. coli* has recently been shown to catalyze the melting of the replicative form of phage ϕX174 DNA in the presence of the cistron *A* protein of ϕX174 and the *E. coli* DNA binding protein; in the process ATM is hydrolyzed (30). Recently Scherzinger *et al.* (31) have reported that the gene *4* protein is involved in the initiation of DNA synthesis on single-stranded templates.

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