Proc. Nat. Acad. Sci. USA
Vol. 73, No. 3, pp. 752–756, March 1976
Biochemistry

Isolation of an intermediate which precedes *dnaG* RNA polymerase participation in enzymatic replication of bacteriophage φX174 DNA

(***dnaB* protein/*dnaC* protein/protein i/protein n/DNA unwinding protein)**

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Contributed by Arthur Kornberg, December 11, 1975

ABSTRACT **Conversion of φX174 single-stranded DNA to the duplex replicative form (RF) *in vitro* requires at least 10 purified proteins. Three stages—strand initiation, elongation, and termination—comprise this conversion. We now identify a separate stage in strand initiation which precedes *dnaG* RNA polymerase participation. Incubation of five proteins—protein i, protein n, DNA unwinding protein, *dnaB* protein, and *dnaC* protein—with ATP and φX174 DNA forms an intermediate which enables subsequent stages measured by DNA synthesis to proceed 20 times faster. The intermediate can be isolated in quantitative yield by gel filtration or by ultracentrifugation. Protein i and protein n are required in less than stoichiometric amounts and appear to be absent from the isolated intermediate. Whereas formation of the intermediate is sensitive to antibody to protein i and to *N*-ethylmaleimide (an inhibitor of protein n and *dnaC* protein), the intermediate itself is resistant to these reagents. DNA unwinding protein complexes the DNA in a ratio of 60 molecules per circle. Synthesis of the intermediate appears to require stoichiometric quantities of *dnaB* protein and *dnaC* protein but their presence in the intermediate has not been established as yet.**

Ten proteins required for the conversion of bacteriophage φX174 single-stranded DNA to the duplex replicative form (RF) have been partially purified from extracts of gently lysed *Escherichia coli* (1, 2). Among these are several proteins which are known to participate in the replication of the host chromosome. The *dnaG* protein, a rifampicin-resistant RNA polymerase (3, 4), catalyzes the synthesis of a short RNA primer on the DNA of φX174-like phage G4 and is presumed to serve in the initiation of a DNA strand; the DNA polymerase III holoenzyme functions in elongation of the DNA strand (5); DNA unwinding protein is required for RNA synthesis by *dnaG* protein (3, 6) and also stimulates the activity of DNA polymerase III holoenzyme (6); and DNA polymerase I and DNA ligase are necessary in termination of the DNA strand by converting the gapped duplex product (RF II) to the closed circular duplex (RF I) (7).

In this report we show that five of the proteins serve in the formation of an intermediate in a stage which precedes the RNA priming. Proteins i and n, and *dnaB* and *dnaC* protein interact with φX174 DNA (complexed with DNA unwinding protein) to produce an isolatable intermediate. The intermediate is then replicated at a very rapid rate in the presence of the four ribo- and four deoxyribonucleoside triphosphates, *dnaG* protein, and the DNA polymerase III holoenzyme.

MATERIALS

Materials were from sources previously described (2). Buffer A is: 50 mM Tris·HCl (pH 7.5), 10 mM dithiothreitol, and 200 μg/ml of bovine serum albumin; Buffer B is: 50 mM potassium phosphate (pH 6.8), 10% sucrose, 10 mM dithiothreitol, 200 μg/ml of bovine serum albumin, 0.5 mM ATP, and 1 mM $MgCl_2$.

Proteins, assayed as previously described (2), were: DNA unwinding protein [Fraction 3b, 0.62 mg/ml (6)]; *dnaG* protein [Fraction V, 27,000 units/ml, 500,000 units/mg (3)]; DNA polymerase III holoenzyme [50,000 units/ml, 30,000–200,000 units/mg (5)]; *dnaB* protein [Fraction IV, 110,000 units/ml, 10^5–10^6 units/mg (2)]; protein n [Fraction VI, 5000 units/ml, 26,000 units/mg (2)]; protein i (Fraction IV, 80,000 units/ml, 210,000 units/mg, purification to be reported elsewhere); and *dnaC* protein (Fraction VI, 10,000 units/ml, 130,000 units/mg, purification to be reported elsewhere).

METHODS

Intermediate Formation and Assay. Components were added at 0°, to make 6.5 μl, in the following order: 17 units of *dnaB* protein, 12 units of *dnaC* protein, 10 units of protein i, 6 units of protein n, 1 μg of DNA unwinding protein, 20 nmol of ATP, 40 nmol of spermidine·HCl, 120 nmol of $MgCl_2$, 220 pmol (as nucleotide) of φX174 DNA; the mixture was incubated at 30° for 20 min. To this "first-stage mixture" was added 7 μl of a "second-stage mixture" which contained: 25 units of DNA polymerase III holoenzyme, 27 units of *dnaG* protein, 2 μl of anti-protein i gamma globulin (6 mg of protein per ml), 2.5 nmol each of GTP, CTP, and UTP, 1.2 nmol each of dGTP, dCTP, and dATP, 400 pmol of dTTP (^3H-labeled, 200 cpm/pmol), and 120 nmol of $MgCl_2$. Buffer A was added to make the total volume 25 μl. The mixture was incubated 2 min at 30° and the reaction was chilled to 0° and stopped with three drops of 0.2 M sodium pyrophosphate and 0.5 ml of 10% trichloroacetic acid. The precipitate was collected, washed, and counted as previously described (6). The incorporation of deoxynucleotides during the second-stage incubation is a measure of the amount of intermediate formed.

^{125}I-Labeled DNA unwinding protein was prepared as previously described (6, 8). Antibody to protein i was prepared in New Zealand white rabbits by injecting 200 μg of homogeneous protein near the dorsal and ventral lymph nodes; intramuscular booster injections of 75 μg were administered 3 and 5 weeks later. Blood was collected 1 week after the second booster, and the gamma globulin was purified to homogeneity by the procedure of Wickner (9).

RESULTS

A stage which precedes DNA synthesis

When all the components necessary for the replication of φX174 single-stranded DNA, except for the deoxynucleoside triphosphates, were first incubated for 20 min (first stage), and then the triphosphates were added (second stage), there was a 20-fold stimulation of the initial rate of DNA synthesis (Fig. 1A). The initial rate of DNA synthesis was dependent on the length of the first-stage incubation, approaching a

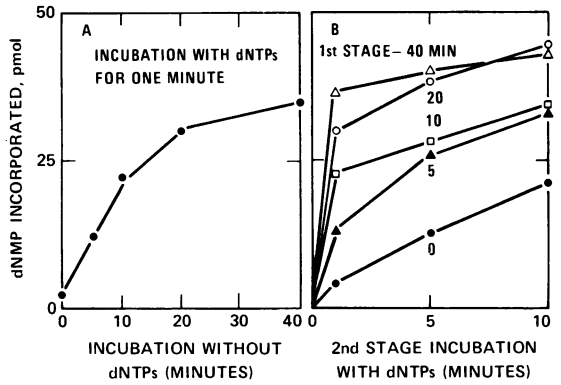

FIG. 1. (A) Stimulation of DNA synthesis by a first-stage incubation without dNTPs. Components were added at 0° (to a final volume of 250 μl) as follows: 170 units of *dnaB* protein, 120 units of *dnaC* protein, 100 units of protein i, 60 units of protein n, 10 μg of DNA unwinding protein, 200 nmol of ATP, 400 nmol of spermidine·HCl, 1.2 μmol of $MgCl_2$, 2200 pmol of φX174 DNA, 250 units of DNA polymerase III holoenzyme, 270 units of *dnaG* protein, 25 nmol each of GTP, CTP, and UTP, and Buffer A to volume. The mixture was incubated at 30° and 25 μl portions were removed at times indicated. To each was added 1.2 nmol each of dGTP, dCTP, and dATP and 400 pmol of [^{3}H]dTTP (200 cpm/pmol); DNA synthesis (second stage) was measured for 1 min at 30°. (B) Influence of duration of the first-stage incubation on time course of DNA synthesis (second stage). To each tube, 10 times the amount of "first-stage mixture" (*Methods*) was added, and the tube was incubated at 30° for the times specified and then transferred to 0°. Subsequently, 10 times the amount of "second-stage mixture" (minus anti-protein i gamma globulin) was added and the volume was brought to 250 μl with Buffer A. The tube was reincubated at 30°, and 25 μl portions were removed at indicated times, precipitated, and treated as described in *Methods*.

maximum at about 20 min. DNA synthesis in the second stage was essentially complete in 1 min (Fig. 1B). With shorter times of first-stage incubation, DNA synthesis persisted beyond 1 min. This is due to an ongoing first-stage reaction during the DNA-synthesis stage. When the first-stage reaction was terminated by neutralizing antibody (see below), the second stage was complete within 2 min. These results indicate that intermediate formation is the rate-limiting step for DNA synthesis on a φX174 DNA template.

The first stage precedes RNA synthesis

The components necessary for the first-stage reaction were protein i, protein n, *dnaB* protein, *dnaC* protein, DNA unwinding protein, $MgCl_2$, ATP, and φX174 DNA (Table 1). Spermidine stimulated about 4-fold. The *dnaG* protein, DNA polymerase III holoenzyme, GTP, CTP, and UTP were not required. As will be shown below, the rapid rate of DNA synthesis in the second stage was due to formation of an isolatable replication intermediate in the first stage.

Two additional proteins were required for subsequent DNA synthesis (second stage) by the replication intermediate—*dnaG* protein and DNA polymerase III holoenzyme (Table 1). Addition of *dnaG* protein and the four ribonucleoside triphosphates to the replication intermediate resulted in the synthesis of short RNA fragments hybridized to the φX174 DNA which serve as primers for DNA synthesis by DNA polymerase III holoenzyme.*

Incubation of all five proteins with DNA was essential for production of intermediate. Incubation of any combination

* R. McMacken, unpublished observations.

Table 1. Components required in the first-stage reaction monitored by subsequent very rapid DNA synthesis

Component omitted	DNA synthesis, pmol
None	35.0
dnaB protein	1.3
dnaC protein	1.0
dnaG protein	33.0*
DNA polymerase III holoenzyme	34.0*
Protein i	2.0
Protein n	2.9
DNA unwinding protein	4.0
ATP	0.9
GTP, CTP, and UTP	35.0
Spermidine	8.0
$MgCl_2$	1.7
φX174 DNA	0.0

Components were mixed as described in Fig. 1A at one-tenth the scale. The DNA synthesis achieved after the 20-min incubation at 30° was measured by a 2-min incubation at 30° after addition of the four deoxyribonucleoside triphosphates and the omitted component to sustain the DNA synthesis reaction (as in Fig. 1A).

* When either *dnaG* protein or DNA polymerase III holoenzyme was omitted from the second stage mixture, no DNA synthesis was observed.

of four proteins with or without DNA did not abbreviate the incubation time required for the first-stage reaction when the omitted component was added subsequently (data not shown).

Intermediate formation was dependent on the temperature of incubation (Fig. 2); it was most rapid at 37° and did not proceed at 0°. Once formed at 30°, the intermediate was stable at 0° for at least 4 hr.

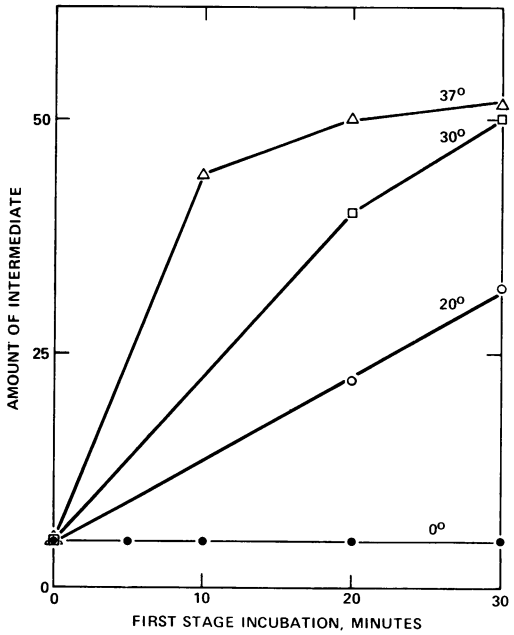

FIG. 2. Temperature dependence of intermediate formation. Ten times the amount of "first-stage mixture" was incubated at the indicated temperatures. At various times 6 μl samples were removed and mixed with 7 μl of "second-stage mixture" (minus anti-protein i gamma globulin) and 12 μl of Buffer A at 30°. The amount of intermediate was measured by the extent of deoxyribonucleotide incorporation (pmol) in 2 min at 30°.

Table 2. Inhibitors of the formation of the replication intermediate

Agent	Amount of intermediate; Inhibitor added before or after first-stage incubation	
	Before	After
None	28.1	29.5
Control γ-globulin	27.0	30.5
Anti-protein i γ-globulin	0.7	24.0
Anti-DNA unwinding protein γ-globulin	1.5	4.2
N-Ethylmaleimide, followed by dithiothreitol	3.0	24.0
Dithiothreitol control	21.4	23.1

Assays were performed as described in *Methods*, except that γ-globulin fractions (12 μg) were added to the first-stage mixture either before or after a 20-min incubation at 30°. *N*-Ethylmaleimide was added to 10 mM and was neutralized by 20 mM dithiothreitol prior to the second-stage incubation (as described in *Methods*). When *N*-ethylmaleimide was added after the first-stage incubation, a 10-min incubation at 30° preceded the addition of dithiothreitol prior to the second-stage incubation. The amount of intermediate was measured by the extent of deoxyribonucleoside triphosphate incorporation (pmol) during the second-stage.

The replication intermediate is resistant to antibody to protein i and to *N*-ethylmaleimide

Formation of the intermediate was blocked by antibody against protein i (Table 2). Once formed, the intermediate was resistant to the antibody. The onset of antibody resistance directly paralleled the rate of intermediate formation (data not shown). For this reason, the antibody was added routinely after the first-stage incubation to block further intermediate formation. The amount of DNA synthesis observed in 2 min during the second-stage incubation was a direct measure of the amount of intermediate formed. No further DNA synthesis was observed upon longer incubation.

Intermediate formation was also blocked by antibody directed against DNA unwinding protein. However, the intermediate remained sensitive to this antibody.

N-Ethylmaleimide, an inhibitor of protein n and *dnaC* protein (2, 10), blocked intermediate formation. This was true even when protein n was already bound to the single-stranded DNA. *N*-Ethylmaleimide had no effect on the intermediate once formed. This may be explained either by masking of the proteins [e.g., *dnaB* protein is known to protect *dnaC* protein from inactivation by *N*-ethylmaleimide (10)] or absence of the proteins from the intermediate.

Isolation of the replication intermediate

Filtration of the first-stage incubation mixture over Bio-Gel A-5m at 23° yielded a fraction in the void volume capable of rapid DNA synthesis (Fig. 3). The replication intermediate was thus separated from free proteins present in the mixture. No activity was found in the void volume if the first-stage incubation was carried out at 0° or if the mixture was filtered immediately without incubation at 30°. The intermediate was lost in filtration if ATP or $MgCl_2$ was omitted from the column equilibration buffer. Recovery of the intermediate from the column was nearly quantitative (>90%); when the Bio-Gel A-5m filtration was performed at 4°, only 40% of the activity was recovered. The isolated fraction was

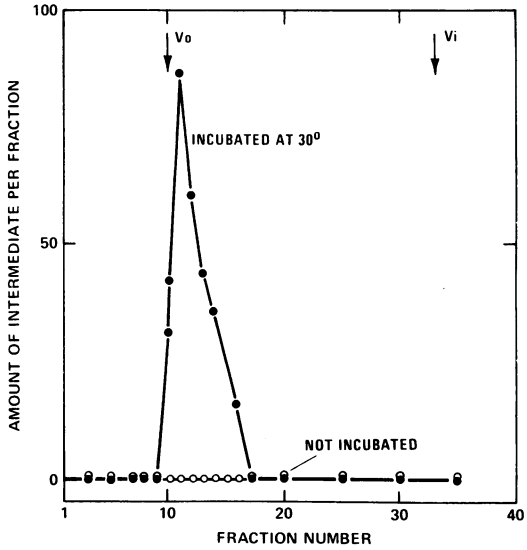

FIG. 3. Bio-Gel A-5m filtration of the intermediate. A 10 times "first-stage mixture" was incubated for 20 min at 30°. The mixture (capable of incorporating 250 pmol of deoxyribonucleoside triphosphate) was passed over a 3 ml (0.7 × 8.5 cm) column of Bio-Gel A-5m, 100–200 mesh, equilibrated in Buffer B (*Materials*) at 23°. Forty fractions of 90 μl were collected; 13 μl portions were assayed for intermediate activity by adding 7 μl of "second-stage mixture" and incubating for 2 min at 30°. On a separate column, a 10 times "first-stage mixture" which had not been incubated was filtered and assayed as above. V_0 is the column void volume and V_i is the included volume. The amount of intermediate was measured by the extent of deoxyribonucleotide incorporation (pmol).

unstable; 50% of the activity was lost after 50 min at 0° compared to no loss in activity of the unfiltered material after 4 hr at 0°; this instability was not prevented by the addition of components of the first-stage incubation mixture.

Sedimentation of the first-stage incubation mixture after incubation at 30° for 20 min separated the intermediate from unassociated proteins. The intermediate was collected in a 60% sucrose shelf in an air-driven ultracentrifuge (Fig. 4). Little or no activity was found in the shelf when the incubation was carried out at 0°. The isolated, active fractions were as stable as the unsedimented intermediate.

The supernatant fluid after sedimentation (fractions 8 to 13 in Fig. 4) were assayed for the levels of each of the proteins; the mixture incubated at 30° was compared with a control incubated at 0°. The protein i and protein n levels were identical in both incubations, indicating that little or none of these proteins were removed upon sedimentation of the intermediate (Table 3). The levels of *dnaC* protein and *dnaB* protein were reduced by about 40% by the incubation at 30°, suggesting that these proteins were either associated with the sedimented material or removed in some other way.

Presence of DNA unwinding protein in the isolated intermediate

Availability of active ^{125}I-labeled DNA unwinding protein (6) permitted an estimation of the amount of this protein associated with the isolated intermediate. With DNA unwinding protein alone, binding to φX174 single-stranded DNA was at a level of about 2.3 μg/nmol of nucleotide, in agreement with previous studies (6). Addition of all the components necessary for intermediate formation reduced bound unwinding protein to 1.0 μg/nmol; this lower level was observed with or without incubation at 30° and corresponds to about 60 molecules of the native tetramer per DNA circle.

Table 3. Levels of replication proteins after formation of the replication intermediate

Protein measured	Protein levels, units/μl	
	Incubated at 0°	Incubated at 30°
dnaB protein	0.8	0.5
dnaC protein	0.75	0.5
Protein i	1.6	1.5
Protein n	0.9	0.9

After sedimentation of the replication intermediate, supernatant fractions 8 to 13 (Fig. 4) were assayed for each of the protein activities by partial reconstitution assays (2).

Kinetics and stoichiometries in formation of the intermediate

The kinetics of intermediate formation were examined at several concentrations of each of four of the required proteins (Fig. 5). The amount of intermediate formed was directly proportional to the amount of *dnaB* protein and *dnaC* protein added. For *dnaB* protein, the rate of intermediate formation did not appear to be directly dependent on the level of protein added. The behavior of *dnaC* protein was more complex. Both the rate and extent of intermediate formation were dependent on the level of *dnaC* protein.

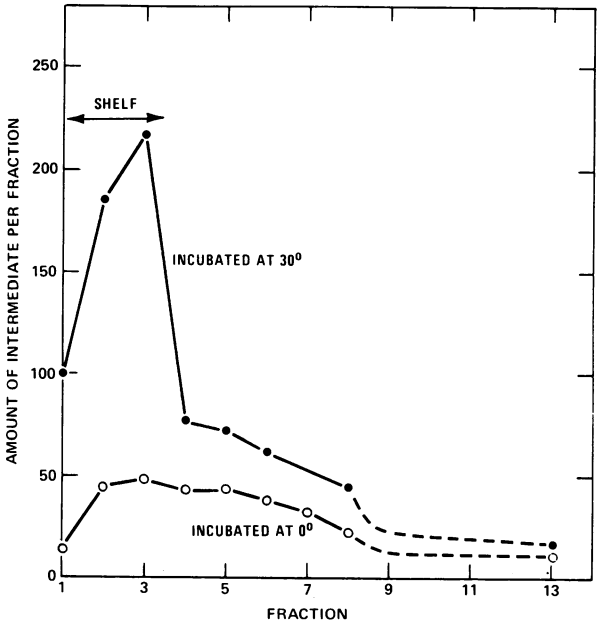

FIG. 4. Sedimentation of the intermediate. A 20 times "first-stage mixture" (capable of incorporating 460 pmol of deoxynucleoside triphosphate) was incubated for 20 min at 30°, diluted to 100 μl with 50 mM Tris·HCl (pH 7.5), and layered onto a 40 μl shelf of 60% (wt/vol) sucrose, 50 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM ATP in a 175 μl cellulose nitrate tube. The tube was centrifuged at 160,000 × *g* for 25 min at 23° in a Beckman air-driven ultracentrifuge. Thirteen portions of 10 μl were removed by inserting the tip of a 25-μl Hamilton syringe to the bottom of the tube and sequentially removing samples. One microliter was assayed for the amount of intermediate by adding 7 μl of "second-stage mixture" and 19 μl of Buffer A and incubating 2 min at 30°. A parallel sample which had been incubated at 0° was also run. Fractions 8 to 13 were pooled and assayed for intermediate and for individual protein components (see *text*). The amount of intermediate was measured by the extent of deoxynucleotide incorporation (pmol).

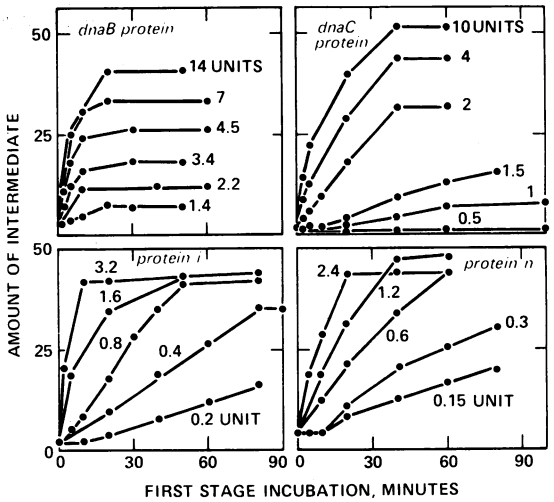

FIG. 5. Effect of varying the concentrations of required proteins on intermediate formation. A 10 times "first-stage mixture" (60 μl) was prepared except that the amount of one protein was varied as indicated. After various times of incubation at 30°, 6 μl was removed and added to 7 μl of "second-stage mixture" and 12 μl of Buffer A. The amount of intermediate was measured by the extent of deoxynucleotide incorporation (pmol).

From data previously published (2), the molecular weight of *dnaB* protein is 250,000, with a specific activity of 1×10^6 units/mg for homogeneous preparations†. Based on these values and the number of φX174 circles added (2.2×10^{10}), we estimate that approximately one molecule of *dnaB* protein was added per DNA circle at saturation. Similar calculation for *dnaC* protein (molecular weight 30,000; estimated specific activity of pure *dnaC* of 500,000 units/mg) indicates that about 10 molecules per circle were required.

Protein i and protein n appeared to act in a catalytic rather than stoichiometric fashion. The rate, but not the amount, of intermediate formation was dependent on protein concentration. Protein i functioned at a level of less than one molecule per added circle (molecular weight 80,000; estimated specific activity of pure protein i of 1×10^6 units/mg)‡. A similar calculation for protein n cannot be made due to uncertainties of the molecular weight and specific activity. This observation does not conflict with previous results which indicated that protein n is a DNA binding protein which dramatically constricts single-stranded DNA (2, 11). DNA binding may be modulated by one or more of the five proteins required in the first stage.

DISCUSSION

One of the immediate goals in the resolution and purification of the components of a multi-enzyme system is to determine the sequential steps in which each component participates. Among the 10 proteins responsible for the *in vitro* conversion of the φX174 viral single-stranded circle to the duplex circular form, six are thought to operate in an initiation step which precedes DNA synthesis (1). These include protein i, protein n, *dnaB* protein, *dnaC* protein, *dnaG* protein, and the DNA unwinding protein.

The functions of the *dnaG* protein and the DNA unwinding protein may be surmised from their actions in the initia-

† K. Ueda and R. McMacken, unpublished observations.

‡ The minimum molecular weight of native protein i is estimated to be 80,000. R. McMacken, unpublished observations.

tion of DNA synthesis on the circular DNA template of G4, a phage closely related to ϕX174 (12). In this case the *dnaG* protein serves as an RNA polymerase (3) to synthesize a short RNA transcript[§] which is covalently extended by DNA polymerase III holoenzyme (3). The role of the DNA unwinding protein may be, as with RNA polymerase recognition of phage M13 DNA (13, 14), to complex and mask all but the promoter region of the template recognized by the *dnaG* protein. The need for the other four proteins (i, n, *dnaB*, and *dnaC*) for ϕX174 replication might be explained by their converting the ϕX174 template to a form as suitable as that of G4 for *dnaG* protein action.

To this end we have sought and found an intermediate stage which precedes the RNA primer synthesis by the *dnaG* protein. Formation of the intermediate requires the participation of proteins i and n (whose genetic loci are still unknown), *dnaB* and *dnaC* proteins (required for *E. coli* chromosome replication), ϕX174 DNA, and the DNA unwinding protein complexed with it, and ATP (Table 1).

The intermediate can be isolated in quantitative yield by gel filtration or sedimentation (Figs. 3 and 4). It can be recognized by these criteria: (*i*) support of DNA synthesis (upon addition of the four ribo- and four deoxyribonucleoside triphosphates, *dnaG* protein, and DNA polymerase III holoenzyme) at 20 times the rate observed without prior formation of the intermediate, and (*ii*) resistance of the DNA synthesis to inhibitors of protein i, protein n, and *dnaC* protein.

The structure and composition of the intermediate are still undefined. The ϕX174 DNA is probably complexed with about 60 molecules of DNA unwinding protein. Proteins i and n appear to be absent because: (*i*) the kinetics of intermediate formation dependent on their individual concentrations suggest a catalytic participation, (*ii*) there is no depletion of them upon formation of the intermediate, and (*iii*) the intermediate is resistant to antibody to protein i and to *N*-ethylmaleimide an inhibitor of protein n. The *dnaB* and *dnaC* proteins may be part of the intermediate because: (*i*) the kinetics of intermediate formation indicate a stoichiometric participation of each of these proteins, (*ii*) formation of a complex of *dnaB* and *dnaC* proteins dependent on ATP has been shown by Wickner and Hurwitz (10), and (*iii*) there is a depletion of these proteins upon formation of the intermediate. None of the foregoing statements provides a strong argument for the composition of the intermediate. Clearly, a direct analysis of the isolated material is needed. Wickner and Hurwitz (10) have reported a two-stage reaction for the replication of ϕX174 DNA, the first stage of which may reflect the formation of the replication intermediate reported here.

Current studies* indicate that the intermediate supports the synthesis of very short RNA transcripts upon the addition of *dnaG* protein and the four ribonucleoside triphosphates. The nature of these RNAs and their priming of DNA synthesis require further study.

[§] J.-P. Bouché, S. L. Rowen, and A. Kornberg, unpublished observations.

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