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A DNA-Binding Protein Induced by Bacteriophage T7

(DNA cellulose/T7 DNA polymerase/stimulation of synthesis)

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ABSTRACT **A DNA-binding protein has been purified from *Escherichia coli* infected with bacteriophage T7 by DNA–cellulose chromatography. The protein is absent in uninfected cells. The purified protein has a molecular weight of 31,000 and binds strongly and preferentially to single-stranded DNA. *In vitro* studies show that this protein can stimulate the rate of polymerization catalyzed by the T7-induced DNA polymerase 10–15 times under conditions where the polymerase is unable to effectively use a single-stranded template. The degree of stimulation is dependent upon the ratio of binding protein to DNA template and is independent of polymerase concentration.**

The observed stimulation is specific for the T7 DNA polymerase in that addition of the protein to reactions catalyzed by *E. coli* DNA polymerases I, II, or III or T4 DNA polymerase is without effect.

Genetic analyses of bacteria and bacteriophages have demonstrated the existence of genes coding for unidentified proteins involved in DNA replication (1, 2). Without a biochemical assay, however, it is difficult to identify and purify these proteins by standard techniques of protein purification. The technique of DNA–cellulose chromatography was developed assuming that proteins involved in DNA metabolism would bind to the DNA, whereas unrelated proteins would show no affinity for the column matrix (3, 4). After purification of such DNA-binding proteins, one might then attempt to elucidate their *in vivo* function by characterization *in vitro*.

This technique led to the purification of the gene-32 protein of phage T4 (3), a protein known through genetic results to be involved in both DNA replication and recombination (5). The demonstration of its ability to promote both denaturation and renaturation of double-helical DNA (5), the subsequent demonstration of a specific stimulatory effect upon the T4 DNA polymerase (6), as well as the requirement for stoichiometric rather than catalytic amounts of the protein (7), led Alberts to propose that such "unwinding" proteins might be required as an essential component of the "replication apparatus" because the protein-DNA complex would provide an optimal template configuration for the corresponding DNA polymerase (8).

A search for a similar protein in *Escherichia coli* led to the purification of an *E. coli* "unwinding" protein. In addition to its DNA-binding properties, this protein was capable of specifically stimulating synthesis catalyzed by *E. coli* DNA polymerase II (9).

Since phage T7 had been shown to code for its own DNA polymerase (10, 11), the question arose as to whether it might also code for a corresponding "unwinding" protein. A search was undertaken to identify and purify such a protein and to determine its *in vitro* effect upon the activity of the purified T7 DNA polymerase. Preliminary results of Dr. B. Alberts and Dr. F. W. Studier suggested that such a protein did exist in T7-infected cells. In screening the total DNA-binding proteins induced by T7 infection, they found a major protein species that bound tightly to single-stranded DNA–cellulose but did not bind to double-stranded DNA–cellulose. While no T7 bacteriophage mutants for this protein could be found, the time course of its synthesis suggested a map location amoung the genes involved in DNA metabolism (B. Alberts & F. W. Studier, personal communication).

MATERIALS AND METHODS

Bacterial and Viral Strains. E. coli C600 (su^+) was used as host for preparation of phage stocks with amber mutations. *E. coli* B (su^-) was used as host for preparation of phage-infected cells. T7 am 147 mutant in gene 6 (coding for an exonuclease) was obtained from Dr. W. Summers.

Media. LB broth (10 g of Bacto-tryptone, 5 g of yeast extract, 10 g of NaCl per liter of H_2O) was used for preparation of phage stocks, uninfected, and T7-infected cells.

Preparation of Phage Stocks. E. coli C600 was grown to a cell density of 9×10^8 cells per ml and infected with phage T7 am 147 at a multiplicity of 0.1. After complete lysis (2–3 hr after infection), cell debris was removed by centrifugation and the lysate was made 1 M in NaCl to improve phage stability. Titers of 3×10^{10} phage per ml were obtained. After infection, the culture was poured over crushed ice 0.15 M in NaCl, harvested by centrifugation, and stored at −70°. Uninfected cells were prepared in an identical manner except for infection with phage.

DNA–Cellulose. DNA–cellulose was prepared according to Alberts and Herrick (4). Single-stranded DNA–cellulose contained 1.3 mg of DNA per 1 ml of packed volume of cellulose; double-stranded DNA–cellulose contained 0.9 mg/ml. Calf-thymus DNA was purchased from Worthington Biochemicals, cellulose (Munktell 410) from Bio-Rad.

Preparation of DNA. Phage λ DNA was prepared by phenol extraction of the purified phage (a gift of Dr. G. Zubay). The DNA was heated for 5 min at 100°, followed by quick cooling,

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immediately before addition to reaction mixtures. Double-stranded DNA with "gaps" was prepared as reported (12).

Phage T7 DNA Polymerase and DNA Polymerase Assay. Phage T7 DNA polymerase was purified according to Grippo and Richardson (10). The polymerase used in these experiments had a specific activity of 3400 units/mg as assayed according to these authors. Our preparations, however, were routinely assayed under the following conditions. The reaction mixture (0.3 ml) contained 20 μmol of Tris·acetate (pH 7.5), 2 μmol of $MgCl_2$, 1 μmol of 2-mercaptoethanol, 10 nmol each of dCTP, dGTP, dATP, and 10 nmol of [^{3}H]TTP (200 cpm/pmol), and 40 nmol of "gapped" calf-thymus DNA. Incubation was for 5 min at 30° and nucleotide incorporation into acid-insoluble product was determined. For this manuscript, 1 unit of enzyme activity is the amount catalyzing the incorporation of 1 nmol of total nucleotide into acid-insoluble product in 5 min at 30°. This corresponds to about 0.2 units as assayed according to Grippo and Richardson.

Nuclease Assay. Nuclease activity was determined by measurement of the release of acid-soluble material from radioactively labeled DNA. The reaction mixture (0.3 ml) contained 20 μmol of Tris·HCl (pH 7.5), 2 μmol of $MgCl_2$, 1 μmol of 2-mercaptoethanol, and 8 nmol of nucleotides in ^{3}H-labeled *E. coli* DNA (10 cpm/pmol). Incubations were for 30 min at 37°. After the incubation period, the reaction mixture was chilled to 0° and 0.1 mg of bovine serum albumin and 20 μl of 50% Cl_3CCOOH were added. The mixture was allowed to stand for 5 min at 0°. Insoluble material was removed by centrifugation (10 min at 8000 × *g*), and the radioactivity in the supernatant was determined.

E. coli and Phage T4 DNA Polymerases. DNA polymerase I (fraction VII, 18,000 units/mg) was a gift from D. Brutlag. DNA polymerase II (fraction V, 133 units/mg) (13) and DNA polymerase III (fraction V, 12,000 units/mg) (12), were prepared as previously described. T4 DNA polymerase (fraction VII, 30,000 units/mg) was a gift of Dr. C. Harvey, Hoffman-La Roche, Inc.

Phage T7 Binding Protein Assay. The standard reaction mixture was the same as the DNA polymerase assay except that "gapped" calf-thymus DNA was replaced by 1.6 μg of denatured phage λ DNA. 0.12 units of T7 DNA polymerase were added before the addition of between 1 and 40 μg of T7 binding protein. Incubation was for 10 min at 12°, and nucleotide incorporation into acid-insoluble product was determined. Incorporation was linear in the range of 1–4 μg of binding protein.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 10% gels according to Shapiro *et al.* (14).

Protein Determination. Protein was determined according to Bücher, with bovine-serum albumin as the standard (15). Protein concentration throughout the purification was estimated by measurement of the ratio $A_{280/260}$.

Purification of Phage T7 DNA-Binding Protein. Thawed T7-infected or uninfected cells were disrupted by sonication and cell debris was removed by centrifugation. Nucleic acids were precipitated by 10% polyethylene glycol (final concentration) in 2.0 M NaCl. After overnight dialysis, the extract was centrifuged at 100,000 × *g* for 45 min and the supernatant was applied to a double-stranded DNA–cellulose column. The material not adhering to the column was applied to a single-stranded DNA–cellulose column and the bound protein was eluted by stepwide increases in NaCl concentration. Buffers that were 0.4 M, 0.6 M, 1.0 M, and 2.0 M in NaCl were used.

The 1.0 M eluate was further fractionated by gel filtration on Sephadex G-75. The activity eluted at a position corresponding to a protein with a molecular weight of 31,000. Fractions containing this activity were pooled and concentrated by adsorption to and elution from a second single-stranded DNA–cellulose column. The protein used for these experiments was pure as judged by electrophoresis in polyacrylamide gels. No polymerization or nucleolytic activity could be detected when as much as 20 μg of binding protein were incubated up to 120 min in the standard polymerase or nuclease assay reaction mixtures.

RESULTS

Identification of phage T7 DNA-binding protein

In order to determine if a "DNA-binding" protein was present in T7-infected cells, crude extracts from both infected and uninfected cells were prepared. Each extract was passed through a double-stranded DNA–cellulose column to remove proteins binding to native DNA. The material not adhering to the column was applied to a single-stranded DNA–cellulose column and the bound proteins were eluted with steps of increasing salt concentration. The A_{280} of the eluate was monitored and the peak protein fractions from each step elution were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A protein of molecular weight 31,000, present in high concentration, was found in the 1.0 M eluate of the single-stranded column. This protein was absent from uninfected cells (Fig. 1). At this stage this protein was greater than 50% pure, as judged by gel electrophoresis. It was further purified to apparent homogeneity as described in *Methods*.

The 2.0 M eluate of both infected and uninfected preparations contained the *E. coli* "unwinding" protein of molecular weight 22,000 and was active in stimulating *E. coli* DNA polymerase II under appropriate conditions (9).

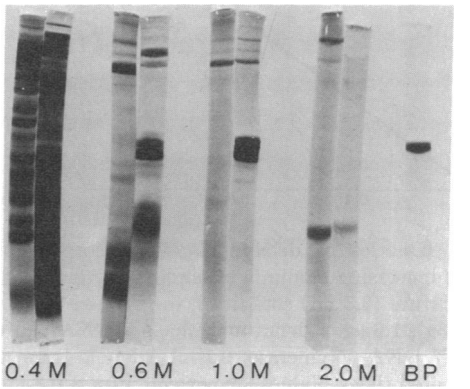

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gels of denatured DNA–cellulose eluates. In each pair, the sample from uninfected cells is on the *left*, the sample from phage T7-infected cells is on the *right*. Labels refer to material eluted at various salt concentrations. The single gel on the *right* is the purified phage T7 DNA-binding protein.

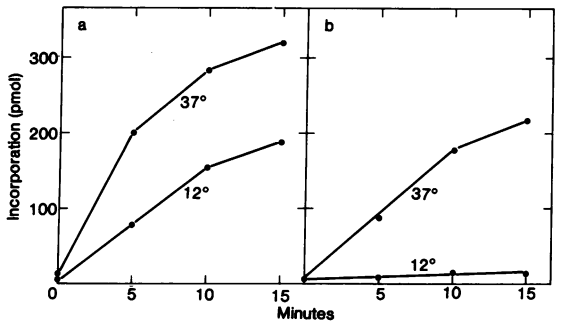

FIG. 2. Kinetics of deoxynucleotide incorporation in phage T7 DNA polymerase-catalyzed reactions at 37° and 12°. (*a*) Double-stranded DNA with "gaps" as template (20 μg), (*b*) single-stranded DNA as template (7.5 μg). The reaction (0.3 ml) was incubated at the temperature indicated under standard conditions. 0.1-ml Aliquots were withdrawn at fixed intervals and acid-precipitable radioactivity was determined. Values are corrected for total incorporation.

Selection of assay

Experiments conducted in this laboratory with *E. coli* DNA polymerases II and III with homopolymers as templates had shown that, as the temperature of incubation was decreased below 12°, polymerization rates decreased markedly. On "gapped" DNA, however, these polymerases were still active, although at reduced rates. That this loss of activity below 12° was probably due to inhibitory secondary structure present in the homopolymer template was suggested by the marked stimulation of synthesis catalyzed by *E. coli* DNA polymerase II when the *E. coli* "unwinding" protein was included in the reaction mixture.

It had also been shown that the stimulation of synthesis catalyzed by phage T4 DNA polymerase by gene-32 protein was greater under conditions where one would expect a more highly folded template, i.e., low temperature or high ionic strength (6). Because of these observations the activity of phage T7 DNA polymerase on several templates, over a range of temperatures, was investigated. It was found that T7 DNA polymerase can effectively use, at both 37° and 12°, a double-stranded template with "gaps," although the rate at 12° is only one-third that at 37°. This polymerase can also use a single-stranded DNA template at 37°; however, with this template, the rate at 12° is only one-tenth the rate at 37° (Fig. 2). If this inhibition is due to the secondary structure of the single-stranded DNA at low temperatures, it might be expected that the addition of a DNA-binding protein would result in a marked stimulation of rate. This was found to be the case. Addition of the T7 DNA-binding protein to a reaction catalyzed by T7 DNA polymerase using a single-stranded DNA as template-primer at 12° results in a 10- to 15-fold stimulation of the rate of reaction (Fig. 3). This rate is comparable to that observed on "gapped" DNA at 12° or on single-stranded DNA at 37° with equivalent amounts of DNA polymerase.

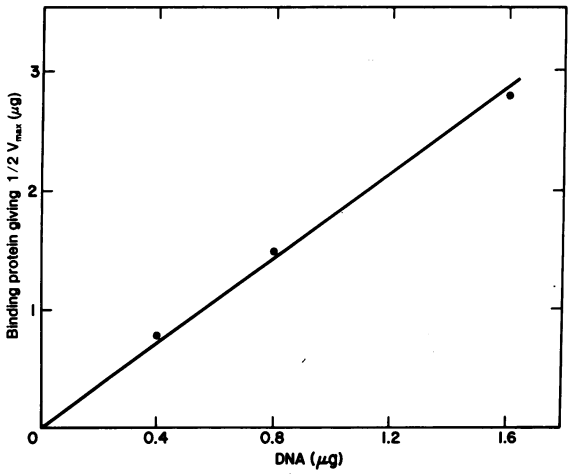

FIG. 3. Kinetics of deoxynucleotide incorporation in the presence of increasing amounts of binding protein. The standard reaction mixture (0.3 ml) contained, in addition to binding protein (0–40 μg), 1.6 μg of denatured phage λ DNA and 0.12 units of phage T7 DNA polymerase. 0.1-ml Aliquots were withdrawn at the indicated times. Values are corrected for a 0.3-ml reaction. The weight ratios of protein to DNA are indicated on the appropriate curves. The *insert* is a plot of the initial rate of reaction as a function of binding protein concentration. The standard reaction (0.3 ml) included 0.12 units of phage T7 DNA polymerase and 1.6 μg of denatured phage λ DNA. Incubation was for 10 min at 12°. Binding protein was varied from 0.8 to 40 μg.

Characteristics of the DNA-binding protein

Table 1 summarizes the properties of the stimulatory activity of the binding protein. The protein itself had no polymerase activity. All incorporation was absolutely dependent upon the addition of both T7 DNA polymerase and DNA template; omission of either resulted in complete loss of activity. Although polymerase was routinely added before binding protein, reversal of the order of addition had no effect, indicating that saturating amounts of binding protein did not interfere with polymerase binding. Furthermore, the binding protein had no irreversible effect upon the DNA; incubation of binding protein with DNA followed by heat denaturation of the protein destroyed stimulatory activity in a subsequent incubation with polymerase.

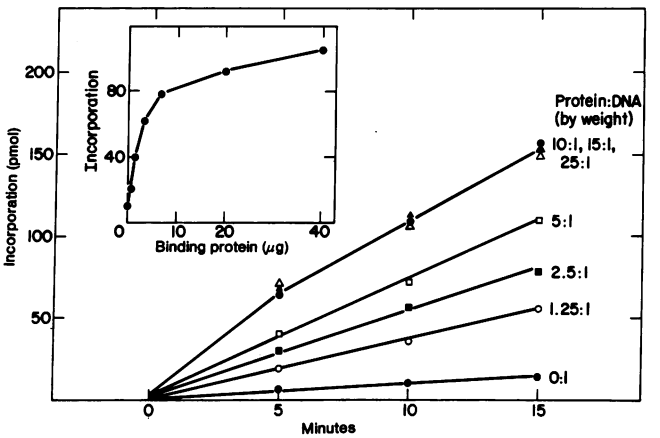

FIG. 4. Stoichiometry of binding protein action. Experiments were similar to that presented in Fig. 3, only titrations of binding protein were performed at different DNA concentrations. For each DNA concentration, the results were plotted as the reciprocal of the initial rate against the reciprocal of the binding protein concentration. The extrapolation of these curves gave values (negative reciprocal of the x-intercept) for the concentration of binding protein required to give one-half maximal stimulation. These values are plotted against DNA concentration.

The characteristics of binding protein-dependent stimulation were further examined. The effect of binding-protein concentration on the rate of reaction is shown in Fig. 3. At low binding protein to DNA ratios, stimulation is directly proportional to binding-protein concentration. A maximal stimulation is achieved when the weight ratio of protein to DNA is about 10 to 1. No further stimulation is observed beyond this point.

Accordingly, the absolute amount of binding protein required to achieve maximal stimulation is dictated by the amount of DNA template used. In experiments similar to that described above, the amount of binding protein required to achieve maximal stimulation was determined at three different DNA concentrations. A reciprocal plot was made of the effect of binding protein on the rates of reaction. Extrapolation of these curves gave the values for the concentration of binding protein required to achieve half-maximal stimulation. This value was found to be directly proportional to the DNA concentration used (see Fig. 4).

Independent of the binding protein to DNA ratio, the rate of reaction is directly proportional to DNA polymerase concentration. At all polymerase concentrations used (0.012–0.24 units), the fractional stimulation observed upon addition of binding protein is the same (Fig. 5).

Specificity

The effect of phage T7 binding protein on the rates of reaction catalyzed by *E. coli* DNA polymerases I, II, and III and phage T4-induced DNA polymerase was studied (Table 2). Equivalent polymerase activities measured on "gapped" DNA at 30° were used. All polymerases were shown to be active on "gapped" DNA at 12°, although to differing extents. As previously noted, phage T7 binding protein stimulates the T7 DNA polymerase-catalyzed reaction such that the rate observed on single-stranded DNA is comparable to that observed on gapped DNA. This was not observed with any of the other DNA polymerases. Thus, there appears to be specificity in the stimulation observed.

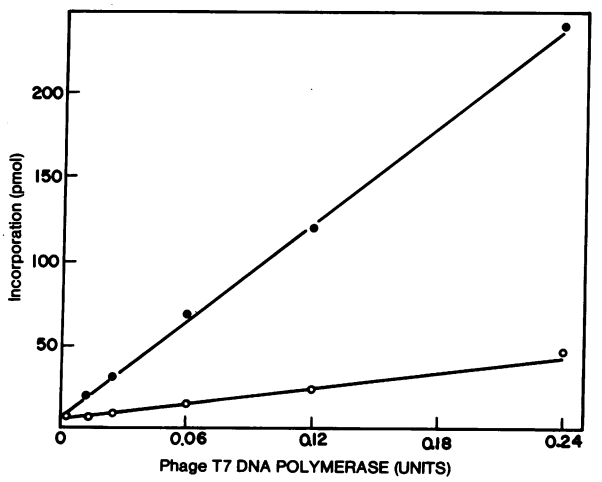

FIG. 5. Effect of increasing phage T7 DNA polymerase concentration at a fixed ratio of binding protein to DNA. Each 0.3-ml reaction contained 1.6 μg of denatured phage λ DNA. Binding protein (20 μg) was added to one (●——●), and no binding protein to the other (○——○). Polymerase content was varied from 0.012 units to 0.24 units as indicated. Incubation was for 10 min at 12°.

TABLE 1. *Requirements of binding protein activity**

	Conditions	Incorporation (pmol)
1.	Standard reaction	120
2.	Reverse order of addition	118
3.	Omit polymerase + heat + polymerase	7
4.	Omit polymerase & BP† + heat + polymerase & BP	90
5.	Omit DNA	<1
6.	Omit polymerase	<1
7.	Omit DNA & polymerase	<1

* (*1*) The standard reaction, containing 20 μg of binding protein, was as described in *Methods*. (*2*) The binding protein was added before addition of the polymerase. (*3*) The binding protein was incubated in a standard reaction mixture for 10 min at 12°, except that polymerase was omitted. The reaction mixture was then heated for 5 min at 65°. After cooling, 0.12 units of phage T7 DNA polymerase were added; the mixture was then incubated for an additional 10 min at 12°. (*4*) The conditions were the same as in (*3*) except that both polymerase and binding protein were omitted and then added after heat treatment. (*5*) DNA was omitted from the standard reaction. (*6*) Phage T7 DNA polymerase was omitted from the standard reaction. (*7*) DNA and polymerase were omitted from the reaction mixture.

† Binding protein.

DISCUSSION

It has been suggested (B. Alberts, ref. 5) that proteins exhibiting a selective affinity for single-stranded DNA might function *in vivo* to unwind the double helix. Although it has not been demonstrated that such proteins exhibit any DNA base-sequence specificity, polymerase specificity has been observed. Since phage T7 DNA encodes its own DNA polymerase, it was of interest to see if a conjugate "unwinding"

TABLE 2. *Specificity of binding protein activity**

	Incorporation (pmol)			
	"Gapped" DNA			
DNA Polymerase	5 min at 30°	10 min at 12°	Single-stranded DNA − BP†	+ BP†
T7-induced	120	92	12	121
E. coli I	121	80	<1	5‡
E. coli II	116	25	<1	<1
E. coli III	117	38	<1	5‡
T4-induced	138	48	2	2

* Components of the reaction mixture and details of the assay for deoxynucleotide incorporation with gapped DNA as template were as described in *Methods*. Equivalent activities of DNA polymerases were determined by their rates on "gapped" DNA at 30°. The activity of each DNA polymerase at 12° was also determined on the same template. The conditions for assay of phage T7 binding protein activity were as described in *Methods*, except that phage T7 DNA polymerase was replaced by the DNA polymerases indicated.

† Binding protein.

‡ Although a slight stimulation was observed, this could not be enhanced by the addition of more polymerase.

protein was also encoded. A likely candidate was selected and purified to homogeneity. Our approach was to attempt to discern the *in vitro* biochemical properties of this protein and, in particular, its effect on the T7 DNA polymerase. It was found that this protein could stimulate polymerase activity 10- to 15-times when present in amounts stoichiometric with the DNA. The stimulator effect of a given amount of protein was shown to depend on the amount of template present in the reaction and to be independent of polymerase concentration. The protein has neither polymerization nor nucleolytic activity; its stimulatory activity is absolutely dependent upon the presence of both T7 DNA polymerase and template. It would appear, therefore, that this protein might play a structural role either in aligning the template or as part of a "replication apparatus," as has been proposed for phage T4 gene-32 protein (8).

The DNA-binding protein selected for purification is not altered by any of the known mutations in phage T7 (1). Our studies indicate that the *E. coli* "unwinding" protein can stimulate the T7 DNA polymerase to the same extent under our assay conditions (data not shown). One might expect, therefore, that a mutation affecting the T7-specified protein would not be lethal even if this protein is required for DNA replication, since the host protein might be able to substitute for the missing T7 protein *in vivo* as it does *in vitro*.

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