Proc. Nat. Acad. Sci. USA
Vol. 72, No. 10, pp. 3907–3911, October 1975
Biochemistry

ϕX174 DNA-dependent DNA synthesis *in vitro:* Requirement for P1 *ban* protein in *dnaB* mutant extracts of *Escherichia coli*

(DNA replication/P1*bac* and P1*bacban* mutants/ATP/rifampicin)

HEINZ SCHUSTER, MARIA MIKOLAJCZYK, JEAN ROHRSCHNEIDER*, AND BRIGITTE GESCHKE

Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, Germany

Communicated by Arthur Kornberg, July 18, 1975

ABSTRACT **Ammonium sulfate fractionation of crude extracts of *E. coli* yields a soluble enzyme fraction (about 25-fold purification) that catalyzes the conversion of ϕX174 single-stranded DNA to duplex DNA. The reaction is rifampicin-resistant, requires single-stranded DNA, Mg^{++}, deoxynucleoside triphosphates, and ATP, and is stimulated by KCl. Such soluble enzyme fractions were prepared from *E. coli* strains carrying the prophage mutant P1*bac,* in which the viral *dnaB* analog (*ban*) protein is expressed constitutively, or P1*bacban,* in which the expression of *ban* protein is prevented. DNA-synthesizing activity of *ban* protein containing fractions from wild-type or *dnaB*(P1*bac*) lysogens was more temperature-resistant than that from *E. coli* containing only wild-type *dnaB* protein, whereas that from *dnaB*(P1*bacban*) lysogens or *dnaB* cells was extremely thermolabile. It is suggested that the temperature-resistant DNA synthesis with fractions from P1*bac* lysogens is mediated by the P1 *ban* protein.**

Infection of *Escherichia coli dnaB* mutants at 41° by phage P1 not only leads to phage production but also to a transient recovery of bacterial DNA replication (1, 2). It was shown that P1 codes for a *dnaB* analog (*ban*) protein, which is repressed in P1 wild type and expressed constitutively in P1*bac* (*dnaB* analog control) mutants (3). *E. coli dnaB*(P1 *bac*) lysogens are able to grow at temperatures that arrest DNA synthesis in the nonlysogen (3). In addition, P1*bacban* mutants were isolated in which the expression of the viral *dnaB* analog is prevented. These mutants do not suppress the *dnaB* character (3).

We were interested in the question whether the suppressing action of P1*ban* protein could be imitated by using an *in vitro* system for DNA replication. Such a system was described for the conversion of ϕX174 single-stranded DNA to its duplex form (4). Using *E. coli* mutants thermosensitive (ts) in DNA synthesis, it was shown that ϕX174 DNA duplex formation is dependent on the products of the *E. coli* genes *dnaB, dnaC(D), dnaE,* and *dnaG.* In addition, other proteins are needed for this reaction (5, 6, †).

The product of gene *dnaB* has been purified (4, 7, 8). In performing initial purification steps (7) using extracts of *E. coli dnaB*(P1*bac*) lysogens, we found that an ammonium sulfate fractionated enzyme system contains a sufficient level of replication proteins to replicate ϕX174 DNA. In such enzyme systems from wild-type or *dnaB* lysogens the P1 *ban* protein dominates DNA synthesis, as measured by its temperature resistance.

Abbreviations: ts, temperature-sensitive; AS, ammonium sulfate.

* Present address: Institut für Human-Virologie, Universität Giessen 63 Giessen, Germany.

† The reader is referred to the most recent papers (5, 6) in which further references can be found.

MATERIALS AND METHODS

Chemicals. Ribonucleoside triphosphates, deoxyribonucleoside triphosphates, lysozyme, streptomycin sulfate (Boehringer GmbH, Mannheim); [*methyl*-^{3}H]thymidine-5′-triphosphate, 25–50 Ci/mmol (The Radiochemical Centre, Amersham); chloramphenicol, rifampicin (Calbiochem, Switzerland); spermidine·HCl, dithioerythritol (Serva GmbH, Heidelberg).

Bacteria and Bacteriophages. *E. coli* BT1000 (*polA1 end thy*) and *E. coli* BT1071 *dnaB* (9) were obtained from F. Bonhoeffer. Phages P1Cm1*bac*-1 and P1Cm1*bac*-1*ban*-1 (3) were obtained from A. Jaffé-Brachet and D. Touati-Schwartz and are abbreviated P1*bac* and P1 *bacban* for convenience. The chloramphenicol-resistance of P1Cm1 (10) serves as a convenient selective indicator of prophage (3). P1*bac*- and P1*bacban*-lysogenic derivatives of BT1000 and BT1071 were isolated in this laboratory. ϕX174am3, a lysis deficient mutant of ϕX174 (11), was used as a source of ϕX174 DNA. Phages were purified by CsCl density centrifugation, and the DNA was extracted by hot phenol (12).

Medium. TY: 10 g of Difco Bacto Tryptone, 5 g of Difco yeast extract, 5 g of NaCl, and 1 g of glucose per liter, supplemented with thymine and thiamine (25 μg/ml each).

Buffers. A: 20 mM Tris·HCl (pH 7.5)–10 mM $MgCl_2$–2 mM EDTA–1 mM dithioerythritol. B: 20 mM Tris·HCl (pH 7.5)–2 mM EDTA–1 mM dithioerythritol–40 mM KCl.

Ammonium Sulfate. A saturated solution (AS-100) is prepared by adding 70 g of ammonium sulfate to 100 ml of 50 mM Tris·HCl (pH 8.5). For extraction of proteins the following dilutions were prepared:

40 ml of AS-100 + 60 ml of buffer B (AS-40),
30 ml of AS-100 + 70 ml of buffer B (AS-30),
20 ml of AS-100 + 80 ml of buffer B (AS-20).

When proteins were precipitated by the addition of solid ammonium sulfate, 0.1 ml of 0.5 M NaOH/10 g of ammonium sulfate was added in order to prevent the pH from dropping below 7.

Growth of Cells. Bacteria were grown in TY medium in a 12-liter New Brunswick Fermenter at 30° or 40°. For growth of P1Cm1-lysogenic strains, 200 ml of culture of exponentially growing cells in TY medium containing 25 μg/ml of chloramphenicol were diluted into 10 liters of TY medium (without chloramphenicol) at the same temperature. After the cells reached a concentration of about 2×10^8/ml, ammonia was added to prevent the pH from dropping below 7. Bacteria grown at 30° were usually harvested at 5×10^8 cells per ml, those grown at 40° at 8×10^8 cells per ml. The pellet obtained after centrifugation was resus-

pended in 50 mM Tris·HCl (pH 7.5)–10% sucrose (1 ml/g of wet cell paste) and quickly frozen in liquid nitrogen. The yield was about 2 g (30° culture) and about 2.5 g (40° culture) of wet cells per liter of growth medium.

Preparation of Extract. Cells were opened with lysozyme as described elsewhere (4). After incubation at 0°, the tubes were transferred into a 30° water bath and stirred very gently until the temperature reached 26°. After 2 min at 26°, the lysate was cooled and centrifuged at 100,000 × *g* for 90 min at 4°. The supernatant was immediately used for further operations or was frozen in liquid nitrogen and kept at −70°. Usually 35–40 mg of protein per g of wet cell paste were obtained. Protein was measured by the method of Miller (13).

Streptomycin Sulfate and Ammonium Sulfate Precipitation. All operations were done at 0–4°. To the crude extract 0.25 volume of streptomycin sulfate (20% in buffer A) was added, stirred for 30 min, and centrifuged at 60,000 × *g* for 30 min. The supernatant was adjusted to 67% saturation with solid ammonium sulfate (42 g/100 ml), stirred for 20 min, and centrifuged at 15,000 × *g* for 20 min.

Fractionation by Ammonium Sulfate. The pellet (obtained from 350 to 400 mg of crude extract protein) was successively resuspended and stirred in 4 ml of AS-40 (20 min), 2 ml of AS-30 (10 min) and 1 ml of AS-20 (10 min). After each resuspension the extract was centrifuged at 15,000 × *g* for 20 min. To the supernatant obtained after extraction with AS-20, an equal volume of AS-100 was added. After stirring for 20 min and centrifugation, the pellet was dissolved in about 0.7 ml of buffer B. The solution was dialyzed twice for 90 min against at least a 50-fold volume of buffer B, frozen in dry ice, and kept at −70° or immediately used.

Assay of DNA Replication. The assay measured incorporation of [^{3}H]dTMP into acid-insoluble material. Unless otherwise indicated, each assay mixture (50 μl) contained 15 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, 1 mM dithioerythritol, 2.5 mM spermidine·HCl, 25 μg/ml of rifampicin, 2 mM ATP, 125 μM each of the four ribonucleoside triphosphates, 12.5 μM each of the four deoxynucleoside triphosphates, [*methyl*-^{3}H]thymidine 5′-triphosphate, specific activity = 1.6–3.2 Ci/mmol (about 900 cpm/pmol), φX174 DNA (400 pmol of nucleotide), and KCl in amounts indicated. Protein fraction was added last. Incubation was for 30 min at 25°. The reaction was stopped by the addition of 0.7 ml of sodium dodecyl sulfate (7%)–0.4 M NaOH–0.03 M sodium pyrophosphate containing 200 μg of calf thymus DNA. After 15 min at 80°, samples were cooled, 4 ml of trichloroacetic acid (10%) was added, and the acid-insoluble material was collected on membrane filters. Filters were washed with trichloroacetic acid (5%, containing 0.05 M sodium pyrophosphate) and with acetic acid (2%), and dried. Radioactivity was determined in a toluene-based scintillation fluid.

RESULTS

Growth of cells and the preparation of crude extracts

In most of the *E. coli dnaB* ts mutants a temperature shift from 30° to 40° leads to an abrupt cessation of DNA synthesis due to the inactivation of the *dnaB* protein (14). When these mutants carry a P1*bac* prophage, the *dnaB* character is suppressed (3). However, it is not yet known whether in a particular *dnaB* mutant the P1 *ban* protein prevents inactivation of the *dnaB* protein or simply replaces it at elevated temperatures. Therefore, in order to study the P1 *ban* protein function, the choice of the growth temperature may be important. In the following experiments P1*bac* lysogens as well as wild-type cells were grown in TY medium at 40°. The *dnaB* mutant and the *dnaB*(P1*bacban*) lysogen were grown at 30°. We observed that the presence of the P1 prophage retards bacterial growth at 40° (40-min generation time instead of 30 min for the wild type), but not at 30° (50 min for all strains used).

DNA synthesis in crude extracts from BT1000 and BT1071(P1*bac*) was completely dependent upon the addition of φX174 DNA. On the contrary, in most of the extracts from BT1071 and BT1071(P1*bacban*) endogenous DNA-synthesizing activity interfered with φX174 DNA stimulated synthesis. The extracts were kept frozen at −70° or in liquid nitrogen without loss of activity for at least several weeks.

Streptomycin sulfate precipitation and fractionation of the proteins by ammonium sulfate

Nucleic acids were removed from crude extracts by streptomycin sulfate, as described in *Materials and Methods*. We suspected that the P1 *ban* protein would behave similarly to the *dnaB* protein in its properties. Therefore, we followed the ammonium sulfate fractionation scheme described for the isolation of *dnaB* protein (7) with minor modifications. After protein precipitation by ammonium sulfate (67%) and extraction of the protein pellet (see *Materials and Methods*) the ammonium sulfate solutions AS-40, AS-30, and AS-20 contained between 30 and 44, 32 and 53, and 2 and 4% of the proteins of the crude extract. When tested for their capacity to synthesize DNA at 25°, only the redissolved protein pellet (containing more than 80% of the total proteins) and the AS-20 fractions did incorporate dTMP. In no case could activity be detected in fractions AS-40 and AS-30. Furthermore, addition of proteins from AS-40 and AS-30 to the AS-20 fraction did not stimulate the activity of the latter. None of the ammonium sulfate fractions had any endogenous template DNA activity.

We conclude from these results that after at least a 25-fold purification, all replication proteins are concentrated in the AS-20 fraction in sufficient amounts to catalyze φX174 DNA-dependent DNA synthesis. It is this particular fraction with which all the following experiments were done. AS-20 fractions (10–30 mg of protein per ml of buffer B) from strains BT1000, BT1000(P1*bac*), BT1071, and BT1071(P1*bac*) were kept frozen at −70° without loss of activity for several weeks. Repeated freezing and thawing, however, reduced the activity. All experiments with AS-20 fractions from BT1071(P1*bacban*), on the other hand, had to be done with unfrozen samples, since freezing only one time was sufficient to destroy more than 80% of the DNA synthesizing activity.

φX174 DNA directed DNA synthesis with a soluble enzyme fraction (AS-20)

Requirements. When DNA synthesis was followed as described in *Materials and Methods*, the reaction was found to be dependent on φX174 DNA, Mg^{++}, and ATP (Table 1). Omission of one or more of the four deoxyribonucleoside triphosphates still allowed an appreciable synthesis. For 300 μg of protein, 400–800 pmol of φX174 DNA was sufficient to optimize DNA synthesis. A further increase in template DNA concentration decreased dTMP incorporation. Omission of rifampicin had no effect on dTMP incorporation. However, it was included to prevent any RNA-polymerase-

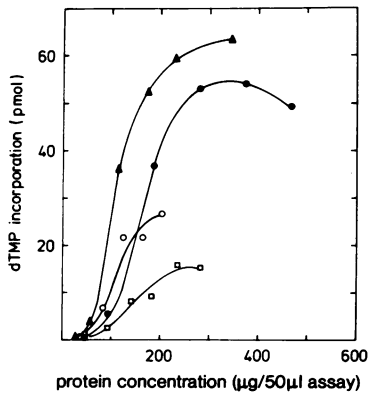

FIG. 1. Dependency on the protein concentration of DNA synthesis with AS-20 fractions from BT1000, BT1071(P1*bac*), BT1071(P1*bacban*), and BT1071. Synthesis was carried out as described in *Materials and Methods* with 100 mM KCl in the assay. In Figs. 1–5, the following symbols are used. *Open symbols:* strain grown at 30°; (○) BT1071; (□) BT1071(P1*bacban*). *Closed symbols:* strain grown at 40°: (●) BT1071(P1*bac*); (▲) BT1000.

initiated DNA synthesis. Addition of ribonucleoside triphosphates sometimes stimulated DNA synthesis slightly, but this was difficult to reproduce from one AS-20 fraction to another. DNA synthesis with AS-20 fractions from both BT1000 and BT1071(P1*bac*) was sensitive to glycerol; with 8% (final concentration) glycerol, inhibition was more than 80%.

Protein Concentration, pH Optimum, and Stimulation by Salt. The dependency on protein concentration was very similar among the AS-20 fractions from different mutants. DNA synthesis was optimal when the assay mixture contained at least 200–300 μg of protein of the AS-20 fraction (Fig. 1). DNA synthesis had a rather broad pH optimum around pH 7.5 (data not shown) and was stimulated considerably by KCl (Fig. 2). Optimal synthesis was observed in the range of 80–180 mM KCl. NaCl replacing KCl had the same effect.

Characterization of the Product DNA. DNA synthesized with AS-20 fractions from BT1000 and BT1071(P1*bac*) for 30 min at 25° (27% and 32% conversion, respectively, of φX174 DNA to duplex DNA) was analyzed by sucrose gradient centrifugation with φX174 DNA added as a marker

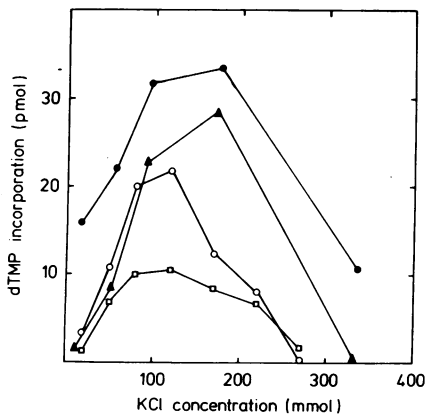

FIG. 2. Stimulation by KCl of DNA synthesis with AS-20 fractions from BT1000, BT1071(P1*bac*), BT1071(P1*bacban*), and BT1071. Synthesis was carried out as described in *Materials and Methods* with amounts of KCl as indicated. Protein concentrations in μg: 180 (BT1000), 240 [BT1071(P1*bac*)], 230 [BT1071(P1*bacban*)], and 210 (BT1071). For explanation of symbols, see legend to Fig. 1.

Table 1. Requirements for DNA synthesis by AS-20 fractions from BT1000 and BT1071 (P1*bac*)

Composition of assay mixture	dTMP incorporation (pmol) BT1000	BT1071 (P1*bac*)
Complete	33.6	52.3
−φX174 DNA	<0.2	<0.2
−Mg	<0.3	<0.5
−ATP	<0.7	<0.3
−dCTP	6.7	10.1
−dCTP, dATP	4.5	10.2
−dCTP, dATP, dGTP	2.7	5.3

The complete assay mixture contained the substances described in *Materials and Methods* in 100 mM KCl [+180 μg of protein of BT1000 or 280 μg of protein of BT1071(P1*bac*)], except that ribonucleoside triphosphates were omitted. Incubation was for 30 min at 25°.

(Fig. 3). In both cases, about 70% of the trichloroacetic acid-precipitable radioactivity sedimented in neutral sucrose with a velocity similar to φX174 duplex DNA. In alkaline sucrose the product DNA was more heterogeneous in size, but the majority of the material sedimented near full-length linear molecules.

Kinetics. The kinetics of DNA synthesis with AS-20 fractions from BT1000 and BT1071(P1*bac*) cells showed the following characteristics (Fig. 4). After a lag of about 4 min,

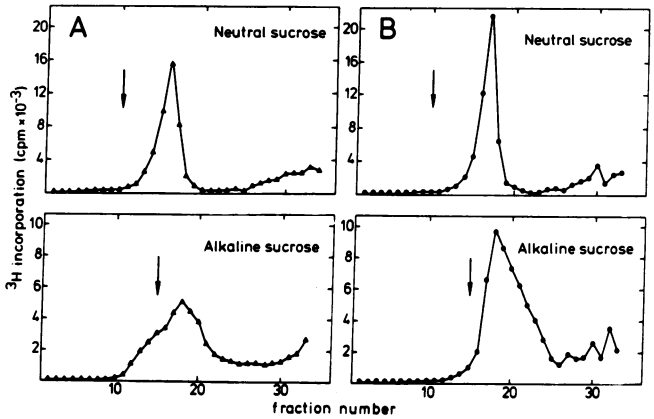

FIG. 3. Characterization of the product DNA by sucrose gradient centrifugation. From each assay mixture in 170 mM KCl, 80 μl [containing 1 mg of protein of BT1000 or 0.3 mg of protein of BT1071(P1*bac*)] were removed after 30 min at 25°. The reaction was terminated by addition of (*i*) 80 μl of 2% sodium dodecyl sulfate–1 M NaCl–10 mM EDTA (neutral sucrose gradient), and (*ii*) 80 μl of 2% sodium dodecyl sulfate–0.8 M NaCl–10 mM EDTA–0.2 M NaOH (alkaline sucrose gradient). Each solution contained about 40 μg of φX174 DNA as carrier. The mixtures were layered onto 5-ml sucrose gradients, 5–20%, in 0.05 M Tris·HCl, pH 7.6–1 M NaCl–5 mM EDTA, or in 0.8 M NaCl–0.2 M NaOH–1 mM EDTA. φX174 DNA (about 75 μg) was mixed with 80 μl of either solution *i* or *ii*, layered on separate neutral and alkaline sucrose gradients, and run as A_{260} marker. After centrifugation, 0.16-ml fractions were collected onto glass filters that had been moistened with trichloroacetic acid (5%, containing 0.05 M sodium pyrophosphate). All further operations were done as described (16). Fractions containing the A_{260} marker were diluted 5-fold with distilled water before reading. DNA synthesized by AS-20 fraction of BT1000 (A), and BT1071(P1*bac*) (B). Arrow: φX174 DNA as A_{260} marker. Centrifugation was in an SW 50.1 rotor for 4 hr at 50,000 rpm at 4°. Sedimentation was from right to left.

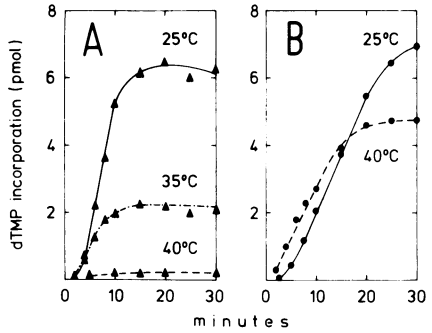

FIG. 4. Kinetics of DNA synthesis with AS-20 fractions from BT1000 and BT1071(P1*bac*). DNA synthesis with 420 μg of protein of BT1000 (A), and with 190 μg of protein of BT1071(P1*bac*) (B), at different temperatures. Assays in 90 mM KCl. At the indicated times 10 μl of assay mixture was precipitated with trichloroacetic acid.

dTMP incorporation was linear with time for about 10–20 min at 25°. Synthesis leveled off when about 30% of the φX174 DNA was converted to duplex DNA. In the most active AS-20 preparations, not more than 75% of the input DNA was replicated. AS-20 fractions from BT1071(P1*bac*) differed from the corresponding fraction of BT1000 in the temperature dependency of its DNA synthesis. At 40°, the fraction from wild-type cells catalyzed no synthesis (Fig. 4A), but with the fraction from BT1071(P1*bac*) synthesis proceeded linearly with time for about 15 min with almost no lag phase (Fig. 4B).

Temperature Dependency. DNA synthesis with AS-20 fractions from different mutants was studied in more detail. φX174 DNA was incubated with AS-20 fractions in 90 mmol of KCl for 30 min at temperatures ranging from 20 to 45°. The relative amounts of DNA that were made at different temperatures (25° = 1.0) are shown in Fig. 5. Fractions from BT1000(P1*bac*) and BT1071(P1*bac*) were more temperature-resistant than those from BT1000, which were, in turn, considerably more temperature-resistant than BT1071. Whereas AS-20 fractions from the P1*bac* lysogen were still active in DNA synthesis at 43°, the corresponding fraction from the P1*bacban* lysogen was about as temperature-sensitive as that from BT1071. Similar results were obtained when DNA synthesis was followed in 170 mM KCl at different temperatures (data not shown).

DISCUSSION

φX174 DNA-dependent DNA synthesis with a soluble enzyme fraction (AS-20) is similar in several respects to the φX174 DNA duplex formation using purified replication proteins (5, 6). It requires a single-stranded DNA as template. No synthesis is observed with P1 DNA. DNA synthesis is dependent on *dnaB* protein (and/or *ban* protein). The reaction is resistant to rifampicin and requires ATP. It differs from the one with purified replication proteins mainly in its requirements for a rather high salt concentration to optimize DNA synthesis. The reason for this is not clear.

φX174 does not replicate in *E. coli dnaB* mutants at 42° (15). However, phage replication is restored in strain BT1071(P1*bac*), but not in BT1071(P1*bacban*) at the restrictive temperature (unpublished results). In accordance with these findings, soluble enzyme fractions from *E. coli dnaB*(P1*bac*) lysogens convert φX174 DNA to its duplex form at temperatures at which corresponding fractions from *dnaB* cells or *dnaB*(P1*bacban*) lysogens are completely inactive. Thus, P1 *ban* protein not only suppresses the *dnaB* character *in vivo* but also does it *in vitro*.

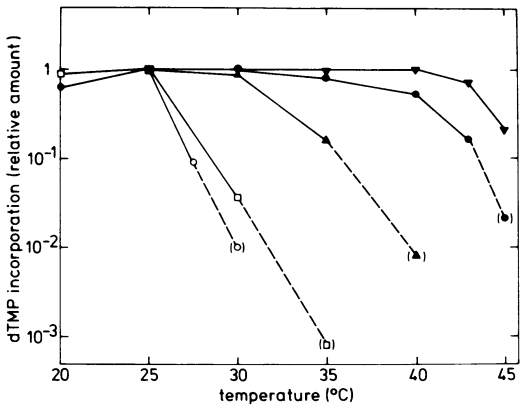

FIG. 5. DNA synthesis with AS-20 fractions from BT1000, BT1071, and its P1-lysogenic derivatives at different temperatures. dTMP incorporation was measured as described in *Materials and Methods*. Incubation was for 30 min at the indicated temperatures in 90 mM KCl. Incorporation at 25° = 1.0. Symbols in parentheses: Radioactivity incorporated is too close to background cpm to be accurate. For explanation of symbols see legend to Fig. 1. Additional symbol: ▼ = BT1000(P1*bac*).

AS-20 fraction	μg protein	pmol dTMP incorporated at 25°
BT1000	170–420	45(4)
BT1000(P1*bac*)	230	24(2)
BT1071	210	25(2)
BT1071(P1*bac*)	180–280	37(3)
BT1071(P1*bacban*)	230	31(1)

Number in parentheses = number of experiments. Each point of the corresponding curve shown above represents an average value.

The molecular mechanism of this suppression is still not clear. φX174 DNA duplex formation may take place with *ban* molecules replacing thermolabile *dnaB* molecules, or a *ban*-mediated DNA synthesis requires formation of a complex between *ban* and *dnaB* molecules. In both cases, *ban* protein could mediate the temperature-resistant DNA synthesis observed. Replacement of *dnaB* by *ban* molecules presupposes that both molecules can compete, for example, for a particular binding site. In that case, DNA synthesis with a *ban* and *dnaB*$^+$ protein containing enzyme system [from BT1000(P1*bac*)] presumably would be less temperature-resistant than with *ban* protein alone. That this is not the case (Fig. 5) could be explained by an excess of *ban* over *dnaB*$^+$ molecules. This, indeed, would follow from genetic data according to which *ban* protein is expressed constitutively in P1*bac* lysogens (3). On the other side, the slightly less thermostable DNA synthesis with the enzyme system from BT1071(P1*bac*), when compared with BT1000(P1*bac*), may indicate the presence of thermolabile *dnaB* protein in a complex of *dnaB* and *ban* molecules.

Note Added in Proof. A *dnaB*-like function of P1 prophage has also been found by T. Ogawa (1975) *J. Mol. biol.* **94**, 327–340.

We are much indebted to Aline Jaffé-Brachet and Danièle Touati-Schwartz for supplying us with the P1 mutants. We also thank them, R. D'Ari, and M.B. Yarmolinsky for making available to us their results prior to publication. We appreciate very much helpful suggestions by E. Scherzinger.

1. Lanka, E. & Schuster, H. (1970) *Mol. Gen. Genet.* **106,** 274–285.
2. Beyersmann, D. & Schuster, H. (1971) *Mol. Gen. Genet.* **114,** 173–176.
3. D'Ari, R., Jaffé-Brachet, A., Touati-Schwartz, D. & Yarmolinsky, M. B. (1975) *J. Mol. Biol.* **94,** 341–366.
4. Wickner, W., Brutlag, D., Schekman, R. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 965–969.
5. Schekman, R., Weiner, A. & Kornberg, A. (1974) *Science* **186,** 987–993.
6. Wickner, S. & Hurwitz, J. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 4120–4124.
7. Wright, M., Wickner, S. & Hurwitz, J. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 3120–3124.
8. Kohiyama, M. & Milewski, E. (1973) *Biochem. Biophys. Res. Commun.* **53,** 858–868.
9. Wechsler, J. A., Nüsslein, V., Otto, B., Klein, A., Bonhoeffer, F., Herrmann, R., Gloger, L. & Schaller, H. (1973) *J. Bacteriol.* **113,** 1381–1388.
10. Kondo, E. & Mitsuhashi, S. (1964) *J. Bacteriol.* **88,** 1266–1276.
11. Hutchison, C. A. & Sinsheimer, R. L. (1966) *J. Mol. Biol.* **18,** 429–447.
12. Sinsheimer, R. L. (1967) in *Procedures in Nucleic Acid Research,* eds. Cantoni, G. L. & Davies, D. R. (Harper & Row, New York), pp. 569–576.
13. Miller, G. L. (1959) *Anal. Chem.* **31,** 964.
14. Wechsler, J. A. & Gross, J. D. (1971) *Mol. Gen. Genet.* **113,** 273–284.
15. Steinberg, R. A. & Denhardt, D. T. (1968) *J. Mol. Biol.* **37,** 525–528.
16. Mikolajczyk, M. & Schuster, H. (1971) *Mol. Gen. Genet.* **110,** 272–288.