Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6289–6293, December 1979
Biochemistry

DNA gyrase: Purification and catalytic properties of a fragment of gyrase B protein

(*Escherichia coli*/topoisomerase/site-specific DNA breakage/oxolinic acid)

MARTIN GELLERT, L. MARK FISHER, AND MARY H. O'DEA

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

Communicated by David R. Davies, October 1, 1979

ABSTRACT **A protein isolated from *Escherichia coli* complements the DNA gyrase A (NalA) protein to generate an activity that relaxes supercoiled DNA. Oxolinic acid, a known inhibitor of DNA gyrase, blocks this activity and causes double-strand cleavage of DNA at the same sites as are attacked by DNA gyrase. The protein, of molecular weight 50,000, appears to be a fragment of the DNA gyrase B (Cou) protein (molecular weight, 90,000) as judged by the identical sizes of numerous peptides produced by partial proteolytic digestion. The complex of this fragment and the gyrase A protein lacks both the DNA-supercoiling and DNA-dependent ATPase activities of DNA gyrase.**

DNA gyrase catalyzes the supercoiling of DNA in an ATP-dependent reaction (1). The enzyme is a complex of two proteins, the products of the *gyr*A (formerly *nal*A) and *gyr*B (formerly *cou*) genes (2–6). Mutations in either of these genes lead to the production of drug-resistant or temperature-sensitive DNA gyrase activity (refs. 2–4, and 7, and see below). The gyrase A and B proteins have been purified separately and shown to reconstitute gyrase activity when mixed (3, 5, 8).

In addition to catalyzing DNA supercoiling and DNA-stimulated hydrolysis of ATP, DNA gyrase has other related enzyme activities. In the absence of ATP, the enzyme causes the relaxation of supercoiled DNA (2, 3). When the normal activity is blocked by the inhibitor oxolinic acid, binding of DNA gyrase to DNA leads to double-strand breakage at specific sites (2, 3, 9).

During our studies on DNA gyrase we found a protein fraction from *Escherichia coli* that complemented the gyrase A protein to produce a DNA-relaxing (topoisomerase) activity. We have purified this protein to near homogeneity and shown that it has a molecular weight of 50,000 and is apparently a fragment of the gyrase B protein. The gyrase A protein–B fragment complex is able to carry out the oxolinic acid-mediated DNA cleavage reaction characteristic of DNA gyrase. However, the complex does not catalyze DNA supercoiling or exhibit a DNA-stimulated ATPase activity.

Recently, brief reports have appeared describing a protein fraction with apparently similar properties to those discussed here (*, †).

MATERIALS AND METHODS

Chemicals. DEAE-Sepharose was obtained from Pharmacia. Valine-Sepharose (10) was a gift from N. Nossal. Sources of other chemicals have been described (1, 2, 4, 6).

Substrates and Proteins. The supercoiled DNA of plasmid pBR322 (11) and its relaxed form were prepared by standard methods (1, 12). Linear pBR322 DNA was generated by cleavage with endonuclease *Eco*RI. Gyrase A and B proteins were purified separately to homogeneity (>99%) from *E. coli* strains in which the corresponding *gyr*A and *gyr*B genes had been cloned on plasmids (unpublished data). Each protein had a specific activity in the supercoiling assay of about 1×10^6 units/mg in the presence of an excess of the other.

Methods. The reaction conditions for assay of DNA supercoiling (6) and DNA relaxation (2) by DNA gyrase have been described. Activities given for the gyrase A and B proteins are defined by the supercoiling assay. DNA relaxation by the gyrase A protein–B fragment complex was assayed under somewhat different conditions. The reaction mixture (70 μl) contained 35 mM Tris·HCl (pH 7.5), 12 mM $MgCl_2$, 0.14 mM Na_3EDTA, 18 mM potassium phosphate (pH 7.5), 9 mM KCl, 5 mM dithiothreitol, 6.5% (wt/vol) glycerol, 90 μg of *E. coli* tRNA per ml, 0.36 mg of bovine serum albumin per ml, 50 ng of gyrase A protein, and 0.4 μg of supercoiled pBR322 DNA. Incubation was for 1 hr at 25°C. Proteins were diluted into the diluent previously described. The assay procedure was as described (1); 1 unit of activity is defined as the amount that brings 50% of the supercoiled DNA to the relaxed position in agarose gel electrophoresis.

Conditions for oxolinic acid-dependent cleavage of DNA by gyrase A protein and B fragment were the same as those of the relaxation assay, with linear pBR322 DNA substituted for supercoiled DNA, and with 50 μg of oxolinic acid per ml added. When added, ATP was present at 0.3 mM. After 75 min at 25°C, the reaction was stopped by the addition of 4 μl of 5% sodium dodecyl sulfate. Proteinase K (8 μl of 0.2 mg/ml solution) was then added and the solution was incubated at 37°C for 35 min. After the second incubation, samples were shaken with chloroform/isoamyl alcohol and prepared for agarose gel electrophoresis as described (1).

DNA cleavage assay by gyrase A and B proteins was carried out similarly but with the following modifications. The KCl concentration was 24 mM, the $MgCl_2$ concentration was 6 mM, and the potassium phosphate and glycerol were omitted.

Other methods were as described (1, 2, 6).

Purification of Gyrase B Fragment. A culture of *E. coli* N3048 (2) was grown and the cells were stored as described (1). All purification steps (Table 1) were carried out at 0–4°C. Centrifugation was at 15,000 × *g* for 10 min unless otherwise specified. TGED buffer is 50 mM Tris·HCl, pH 7.5/1 mM Na_3EDTA/5 mM dithiothreitol/10% (wt/vol) glycerol. Fractions 1 and 2 were not assayed for DNA relaxation.

Step 1. The frozen cell suspension (212 ml) was thawed in a 25°C water bath, chilled to 0°C, and distributed into four centrifuge tubes for the Beckman 45 Ti rotor (53 ml each). To each tube were added sequentially 0.65 ml of 0.2 M di-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Kreuzer, K. N., Brown, P. O., Peebles, C. L. & Cozzarelli, N. R. (1979) *Abstracts XIth International Congress of Biochemistry*, 43.

† Morrison, A., Brown, P. O. & Cozzarelli, N. R. (1979) *Abstracts XIth International Congress of Biochemistry*, 43.

Table 1. Purification of DNA gyrase B fragment

		Volume, ml	Protein, mg/ml	Specific activity, units/mg	Total activity, units
1.	Extract	112	64.6	—	—
2.	Streptomycin/ ammonium sulfate	33.5	29.5	—	—
3.	DEAE-Sepharose	63	0.61	800	30,000
4.	Hydroxyapatite	12	0.070	29,000	24,000
5.*	Valine-Sepharose	6	0.060	50,000	18,000

* This step was carried out on one-quarter the scale of the others. Values in the table have been multiplied by 4.

thiothreitol, 2.6 ml of 0.5 M Na_3EDTA, 6.5 ml of 1 M KCl, 0.65 ml of a freshly made solution (20 mg/ml) of lysozyme (Worthington) in 0.05 M Tris·HCl (pH 7.5), and 1.3 ml of a 10% solution of Brij-58. After gentle mixing, the solution was incubated at 0°C for 30 min and centrifuged at 35,000 rpm for 1 hr. The supernatant extract (fraction 1; 112 ml, 7.2 g of protein) was frozen in liquid nitrogen and stored at −70°C.

Step 2. Fraction 1 was thawed and diluted to a protein concentration of 20 mg/ml with 50 mM Tris·HCl, pH 7.5/1 mM Na_3EDTA/2 mM dithiothreitol (final volume, 362 ml). A 20% (wt/vol) solution of streptomycin sulfate was added dropwise, with stirring, to a final concentration of 4%. After stirring for an additional 15 min, the mixture was centrifuged and the supernatant solution was retained. Solid ammonium sulfate (0.31 g/g of supernatant) was added with stirring. After 15-min stirring, the precipitate was collected by centrifugation and redissolved in 22 ml of TGED buffer (fraction 2; 33.5 ml, 990 mg of protein).

Step 3. Fraction 2 was dialyzed for 4 hr against 2 liters of TGED buffer and then diluted 1:3 with TGED buffer to reduce its conductivity to that of the starting buffer in the following chromatography. The sample was applied to a DEAE-Sepharose column (bed volume, 60 ml) previously equilibrated with TGED buffer containing 25 mM NaCl. The column was washed with 200 ml of the same solution, and the protein was eluted with an 1800-ml linear gradient of 0.025–0.5 M NaCl in TGED buffer. Active fractions, which eluted around 0.1 M NaCl, were pooled (fraction 3; 63 ml, 38.4 mg of protein).

Step 4. Fraction 3 was dialyzed for 2 hr against 2 liters of 20 mM potassium phosphate, pH 6.8/5 mM dithiothreitol/10% (wt/vol) glycerol and loaded onto a column (bed volume, 10 ml) of hydroxyapatite (Bio-Gel HTP, Bio-Rad) previously equilibrated with the same buffer. The column was washed with 60 ml of the same buffer, and the protein was eluted with a 300-ml linear gradient of 0.02–0.5 M potassium phosphate (pH 6.8) containing 5 mM dithiothreitol and 10% (wt/vol) glycerol. The activity was eluted around 0.12 M potassium phosphate (fraction 4; 12 ml, 0.84 mg of protein).

Step 5. One-quarter of fraction 4 was diluted with 3 vol of 2 M potassium phosphate (pH 7.5) and loaded onto a column (bed volume, 0.25 ml) of valine-Sepharose equilibrated with 1.5 M potassium phosphate in TGED buffer. The column was washed with 1 ml of this buffer and the protein was eluted with an 8-ml linear gradient of 1.5–0.0 M potassium phosphate (pH 7.5) in TGED buffer. The activity was eluted around 1.25 M potassium phosphate. Active fractions were frozen in liquid nitrogen and stored at −70°C (fraction 5; 1.5 ml, 90 μg of protein). This fraction and all preceding ones were stable in storage at −70°C for at least 1 month.

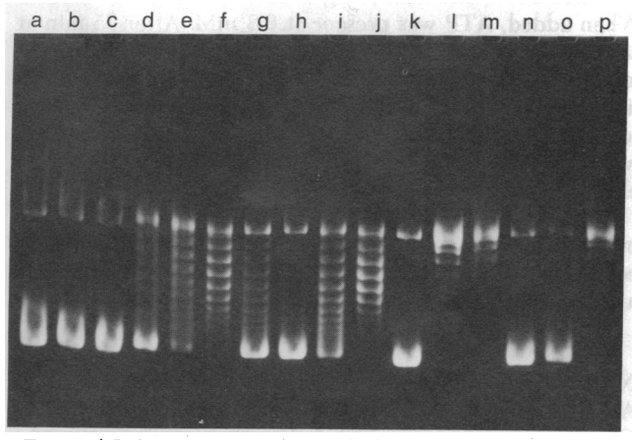

FIG. 1. Relaxation of DNA (and lack of supercoiling) by DNA gyrase A protein–B fragment complex. Lanes a–k, supercoiled pBR322 DNA with the following additions: lane a, no addition; lane b, 30 units of A protein; lane c, 2 units of B fragment; lanes d–f, 30 units of A protein and 0.5, 1, and 2 units of B fragment, respectively; lanes g and h, same as lane e with oxolinic acid added at 5 or 20 μg/ml; lane i, same as lane e with 1.4 mM ATP added; lanes j and k, 120 units of A protein and 45 units of B protein, without (j) and with (k) 1.4 mM ATP added. Lanes l–p, relaxed pBR322 DNA with the following additions: lanes l and m, 30 units of A protein, 2 units of B fragment, and 1.4 mM ATP; lanes n and o, 30 units of A protein, 3 units of B protein, and 1.4 mM ATP; lane p, no addition. Relaxation assay conditions were used except that in lanes m and o supercoiling assay conditions (6) were used.

RESULTS

Activities of the B Fragment. Characteristics of the DNA-relaxing activity are shown in Fig. 1. Neither the newly purified protein nor the gyrase A protein alone had any activity (Fig. 1, lanes b and c), but the combination efficiently relaxed supercoiled DNA (lanes d, e, and f). Oxolinic acid blocked relaxation (lanes g and h); some inhibition was seen at 5 μg/ml and inhibition was almost total at 20 μg/ml. These concentrations are similar to those needed to inhibit DNA gyrase. ATP did not interfere appreciably with DNA relaxation (lane i) but it abolished relaxation by the gyrase A + B protein complex (lanes j and k). There was no supercoiling activity in the presence of ATP, either with the reaction conditions used for relaxation or with normal DNA gyrase assay conditions (lanes l and m), again in contrast to DNA gyrase (lanes n and o). In parallel with DNA gyrase (2, 3), this activity also caused relaxation of positively supercoiled DNA (data not shown).

When DNA gyrase is incubated with DNA in the presence of oxolinic acid, a complex is formed which leads to the production of double-strand breaks at specific sites on the DNA on subsequent treatment with sodium dodecyl sulfate (2, 3, 9). The relative frequency of cleavage at sites was altered in the presence of ATP (13) as demonstrated in Fig. 2, lane b. The gyrase A protein–B fragment complex also carried out the oxolinic acid-dependent cleavage reaction, giving essentially the same

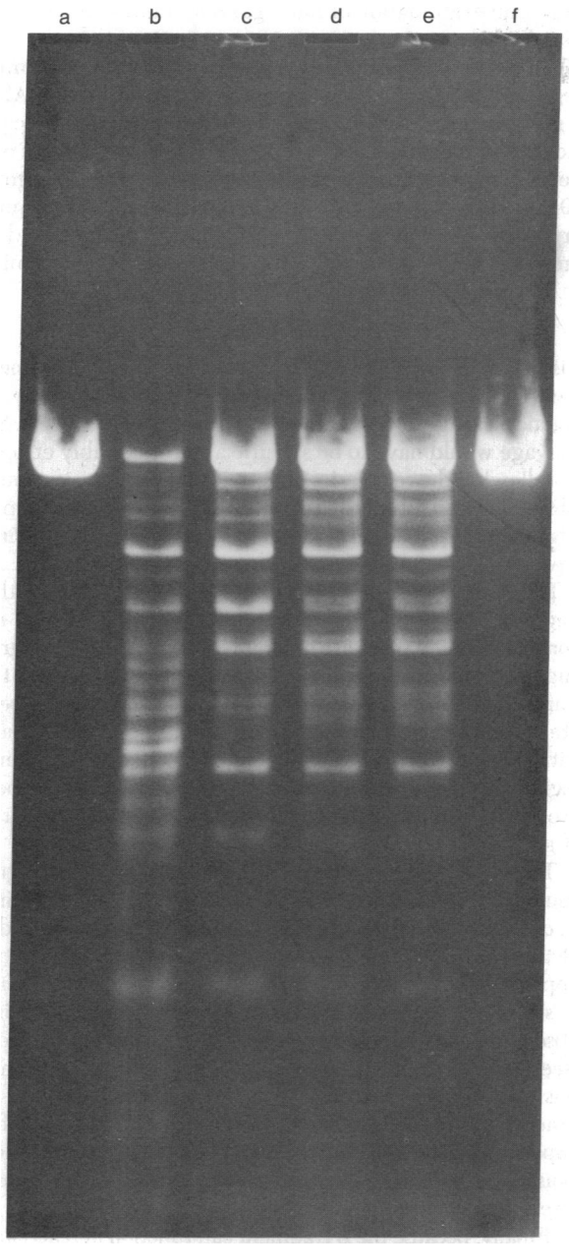

FIG. 2. Site-specific breakage of linear pBR322 DNA by gyrase proteins in the presence of oxolinic acid. Lanes: a, DNA alone; b–e, all contained A protein and oxolinic acid and, in addition, B protein plus ATP in b, B protein in c, B fragment in d, B fragment plus ATP in e, and A protein and B fragment added but no oxolinic acid in f. Additions were: A protein, 400 units; B protein, 300 units; and B fragment, 8 (relaxation) units.

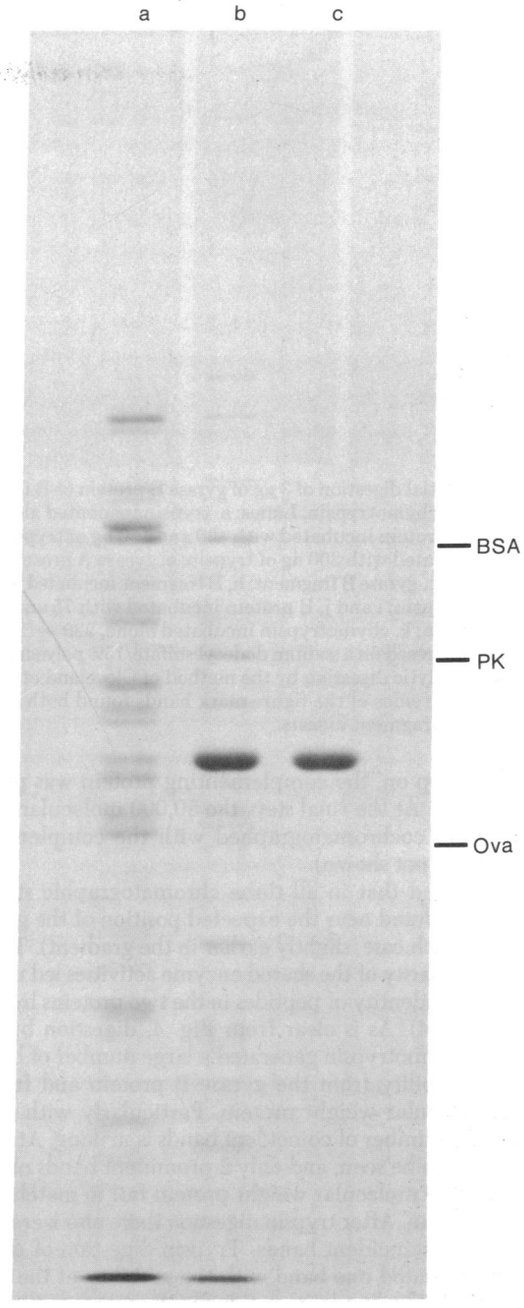

FIG. 3. Purification of gyrase B fragment. Sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis of successive fractions through the purification. Lanes: a, fraction 3; b, fraction 4; c, fraction 5. The lines indicate the positions of proteins used as size markers: BSA, bovine serum albumin; PK, pyruvate kinase; Ova, ovalbumin.

pattern of fragment sizes (although in a few cases in different amounts) from pBR322 DNA as did DNA gyrase. In this case, however, there was no redistribution in the presence of ATP (lane e). Cleavage was dependent on the presence of oxolinic acid (lane f) and was not induced by any of the three proteins alone. In a more detailed experiment (not shown), a restriction fragment of pBR322 DNA that contained one strong gyrase cleavage site and was labeled at one end with ^{32}P was subjected to the oxolinic acid-dependent cleavage reaction, and the exact length of the resulting DNA chain was determined by denaturing acrylamide gel electrophoresis. The break occurred at the same nucleotide position with DNA gyrase and with the gyrase A protein–B fragment complex.

Unlike DNA gyrase (6, 13), the A protein–B fragment complex had no detectable DNA-dependent ATPase activity, either under the conditions of the relaxation assay above or under those used for DNA gyrase (6).

Identification of the Protein as a Fragment of the Gyrase B Protein. The purification resulted in an essentially homogeneous protein with a molecular weight of 50,000 in the denatured form, as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 3). Because the gyrase A protein is not separated from the complementing protein until the DEAE-Sepharose step, assay by complementation for DNA-relaxing activity of the earlier fractions was not feasible.

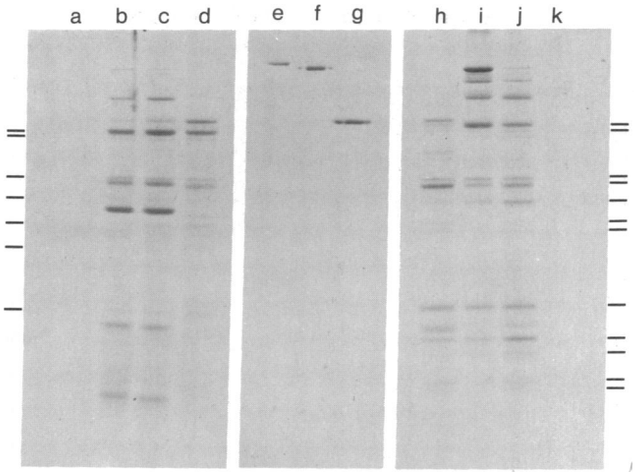

FIG. 4. Partial digestion of 3 μg of gyrase B protein or B fragment by trypsin and chymotrypsin. Lanes: a, trypsin incubated alone, 450 ng; b and c, B protein incubated with 450 and 150 ng of trypsin; d, B fragment incubated with 300 ng of trypsin; e, gyrase A protein; f, gyrase B protein; g, gyrase B fragment; h, B fragment incubated with 110 ng of chymotrypsin; i and j, B protein incubated with 75 and 220 ng of chymotrypsin; k, chymotrypsin incubated alone, 220 ng. Samples were electrophoresed on a sodium dodecyl sulfate/15% polyacrylamide gel after proteolytic digestion by the method of Cleveland *et al.* (14). The lines at the sides of the figure mark bands found both in the B protein and B fragment digests.

From that step on, the complementing protein was purified about 60-fold. At the final step, the 50,000 molecular weight protein band cochromatographed with the complementing activity (data not shown).

We observed that in all three chromatographic steps the activity was eluted near the expected position of the gyrase B protein (in each case, slightly earlier in the gradient). This and the close similarity of the shared enzyme activities led us to test for a possible identity of peptides in the two proteins by partial proteolysis (14). As is clear from Fig. 4, digestion by either trypsin or chymotrypsin generated a large number of bands of identical mobility from the gyrase B protein and from the 50,000 molecular weight protein. Particularly with chymotrypsin, the number of coincident bands is striking. At least 12 such pairs can be seen, and only 2 prominent bands of the digested 50,000 molecular weight protein fail to match a band of the B protein. After trypsin digestion there also were several examples of coincident bands. Trypsin digestion of gyrase B protein generated one band with the mobility of the smaller protein itself. The bands are not due to the proteases themselves (compare lanes a and k with the others). It is thus highly probable that the 50,000 molecular weight protein is a fragment of the same amino acid sequence contained in the gyrase B protein (molecular weight 90,000).

The protein structural evidence does not necessarily imply a common genetic origin for the gyrase B protein and B fragment. To test this question, we examined B fragment activity from a *gyr*B mutant strain that is temperature-sensitive for growth and is coumermycin-resistant at low temperature (unpublished results). This strain produced a temperature-sensitive gyrase B protein whose activity in supercoiling or in relaxation was less than 5% of that of a control strain when assayed at 42°C. The B fragment relaxing activity, however, was not temperature sensitive. Because the mutation was selected to affect that part of the B protein concerned with coumermycin sensitivity, and therefore with ATP binding and energy coupling (6, 13), the altered part of the protein may well have been lost from the B fragment. The experiment is thus equivocal; examination of more *gyr*B mutants will be needed to settle this question.

The gyrase B fragment was roughly as active in complementation for DNA relaxation as the entire B protein. About 40 supercoiling units (40 ng) of B protein were needed to produce 50% relaxation in the standard assay in the presence of an excess of A protein. The specific activity of the B fragment (50,000 units/mg; see Table 1) implies that 20 ng is required for the same extent of reaction. Given the roughly 2-fold difference in molecular weight, the molar activities are similar.

DISCUSSION

It is not yet known how the B fragment is produced. One explanation that cannot yet be excluded is that it arises by proteolytic cleavage of the B protein on disruption of the cells. Such cleavage would have to be specific and remarkably efficient. By following the recoveries of activity through the two purifications, one can estimate that, at the DEAE-Sepharose step, the B fragment is present in severalfold molar excess over the intact B protein.

The second and more intriguing possibility is that the B fragment is an intracellular protein present normally *in vivo*. For this situation there are several ways in which the fragment might be produced. Again, a simple explanation is that there is an intracellular protease that cleaves the B protein at a specific site. Alternatively, the B fragment could be produced along with the B protein by dual translation from the *gyr*B genetic region. A third possibility is that the fragment might be the product of another gene closely related in structure to a part of *gyr*B.

The coexistence *in vivo* of the B protein and B fragment could have important consequences for DNA supercoiling in *E. coli*. This is because the principal activity of gyrase and that of the gyrase A protein–B fragment complex have mutually opposite effects. The DNA gyrase complex is much more active in supercoiling than in relaxation. Its relaxing activity (in the absence of ATP) is about 1/40th of its activity in supercoiling (see above). Because the gyrase A protein–B fragment complex has relaxing activity only, the presence of a relatively large amount of the B fragment in the cell could serve to modify the superhelical state of the DNA without the intervention of topoisomerases outside the DNA gyrase system. These questions need further examination.

Finally, because the B fragment can function in reactions that express the nicking/closing characteristics of gyrase but not the energy-transducing features, it should now be possible to isolate these reactions for further study. Thus, the availability of the B fragment should assist in determining the mechanism of DNA gyrase.

We thank Kiyoshi Mizuuchi for his thoughtful advice and for help with some experiments. Support for L.M.F. from a Damon Runyon–Walter Winchell Cancer Fund Postdoctoral Fellowship is gratefully acknowledged.

1. Gellert, M., Mizuuchi, K., O'Dea, M. H. & Nash, H. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3872–3876.
2. Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T. & Tomizawa, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4772–4776.
3. Sugino, A., Peebles, C. L., Kreuzer, K. N. & Cozzarelli, N. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4767–4771.
4. Gellert, M., O'Dea, M. H., Itoh, T. & Tomizawa, J. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4474–4478.
5. Higgins, N. P., Peebles, C. L., Sugino, A. & Cozzarelli, N. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1773–1777.

6. Mizuuchi, K., O'Dea, M. H. & Gellert, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5960–5963.
7. Kreuzer, K. N., McEntee, K., Geballe, A. P. & Cozzarelli, N. R. (1978) *Mol. Gen. Genet.* **167,** 129–137.
8. Liu, L. F. & Wang, J. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2098–2102.
9. Morrison, A. & Cozzarelli, N. R. (1979) *Cell* **17,** 175–184.
10. Rimerman, R. A. & Hatfield, A. W. (1973) *Science* **182,** 1268–1270.
11. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2,** 95–113.
12. Sakakibara, Y. & Tomizawa, J. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 802–806.
13. Sugino, A., Higgins, N. P., Brown, P. O., Peebles, C. L. & Cozzarelli, N. R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4838–4842.
14. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.