Volume 11 Number 6 1983 Nucleic Acids Research

Cloning of the gene *top*A encoding for DNA topoisomerase I and the physical mapping of the *cys*B-*top*A-*trp* region of *Escherichia coli*

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Received 5 January 1983; Revised and Accepted 10 February 1983

ABSTRACT

The gene topA of Escherichia coli that encodes for DNA topoisomerase I has been cloned by a combination of genetic and radioimmunal screening. The gene has been mapped to be within a 3.4 Kb segment of the bacterial genome. The intracellular level of the enzyme in strains harboring extrachromosomal copies of topA gene increases with increasing copy number of the gene and the introduction of extrachromosomal copies of the topA gene truncated at its 3' side into a topA strain of E. coli does not significantly influence the expression of the chromosomal copy of topA. These results suggest that the expression of topA is not tightly regulated. Strains in which DNA topoisomerase I is overproduced grow significantly slower in broth and give smaller size colonies on agar plates. Physical mapping of a 20 Kb region containing cysB; topA and trp has also been carried out with a number of restriction enzymes; topA is found to be immediately adjacent to cysB and is separated from trp by a 7 Kb segment where no known gene resides.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes that catalyze the breakage and rejoining of DNA phosphodiester bonds, and evidence for their functional importance in vivo is rapidly accumulating (for recent reviews, see references 1-5). The enzyme Escherichia coli DNA topoisomerase I has been studied extensively in this laboratory since its discovery over a decade ago. It has been purified to homogeneity as a single subunit protein with a molecular weight of 105,000 (6), and the various DNA topoisomerization reactions it catalyzes, including the relaxation of negatively supercoiled DNA (7), the intertwining of single-stranded DNA rings of complementary sequences into duplex rings (8), the interconversion of single-stranded DNA rings with and without topological knots, and the catenation and decatenation of nicked duplex DNA rings (9,10), are well-characterized. The locus of the structural gene topA encoding for this enzyme has been mapped genetically (11,12). Studies on the physiological effects resulting from mutations in this gene have been initiated (13), and evidence has been presented that the supX

mutants of Salmonella typhimuriun, identified originally by their phenotypical suppression of a mutation leu500 that reduces the level of transcription of the leu operon (14-16), represent mutations in the structural gene for Salmonella typhimuriun DNA topoisomerase I (13,17). This identification of supX and topA implicates a strong effect of the topoisomerase on gene expression.

There is still little information, however, on the chemistry of catalysis of DNA phosphodiester bond breakage and rejoining by this enzyme or by any other DNA topoisomerase. It has been shown that a DNA topoisomerase catalyzed reaction involves the formation of a covalent protein-DNA complex (6,18,20), and in several cases the covalent linkage has been identified as a phosphodiester bond between a tyrosyl group of the protein and a phosphoryl group of the DNA (21,22). The location of the particular tyrosine involved in this covalent bond formation and the nature of the active sites for covalent catalysis and for DNA binding are unknown, however. Clearly, a deeper understanding of the mechanistic aspects of catalysis by DNA topoisomerases can not be achieved without knowing the structural details of these enzymes.

It is well-recognized that the cloning of the structural gene encoding for an enzyme provides a powerful approach in the elucidation of the structural and functional aspects of the enzyme. Cloning permits the determination of the primary sequence of the enzyme through DNA sequencing, and facilitates the isolation of the enzyme in large quantities for physicochemical and biochemical studies and the application of in vitro mutagenesis techniques to introduce particular modifications of the enzyme. In addition, cloning makes it possible to apply the methods of chemical genetics for studying the physiological roles of the enzyme. In view of the biochemical novelty and the functional importance of DNA topoisomerases, we have undertaken the cloning and sequencing of the structural genes of a number of these enzymes. In this communication, we report our results on the cloning and physical mapping of the structural gene topA encoding for the enzyme Escherichia coli DNA topoisomerase I. The construction of low and high copy number plasmids containing the entire topA gene and its 5' part has also made it straightforward to test whether the expression of the gene is under tight cellular control; evidence in favor of a lack of strict regulation is presented. During the course of this work, we have also mapped physically a 20 Kb region of the E. coli genome spanning the genes cysB, topA and trp, including a 7 Kb region where no known gene has been located.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli K12 strain DM700 ΔcysB, and its prototrophic parent W3110 were from Dr. D. Mascarenhas. DM700 has been identified as ΔtopA recently (13). Strain AB1369 F^- thi-1 argE3 cysB38 proA2 his-4 galK2 lacY1 mtl-1 xyl-5 tsx-5 tsx-29 supE44 was from the collection of Dr. B. Bachmann. Strain MM294 was from the collection of Dr. M. Meselson.

The three plasmids pLC41-15, pLC4-6 and pLC-23 from the Clarke-Carbon library (23) were obtained from Dr. R. Sternglanz. These have been previously identified as ColE1 plasmids carrying trpE and cysB genes (23). Restriction mapping of pLC4-6 and pLC5-23 DNA shows that the two are identical twins. As will be described later, pLC4-6 contains the topA gene whereas pLC41-15 has a small deletion in the topA region and is therefore $TopA^-$. Plasmid pBR322 was originally constructed by Bolivar et al. (24). Plasmid pJW231 was one of a set of plasmids originally constructed by us for the overproduction of certain proteins. This set of plasmids were all derived from pJW200, a pBR322 derivative in which the section of pBR322 between the two HaeII sites at positions 235 and 2351 of the Sutcliffe map (25) was deleted. To give pJW231, the short segment in between the EcoR1 and HindIII sites of pJW200 was replaced by a segment containing the UV5 promoter of the lac operon derived from pTR161 (26), a plasmid constructed for the overproduction of phage λ cro protein, as follows. The plasmid pTR161 was first digested with BglII, and the ends were trimmed with Bal31 nuclease. Dodecameric HindIII linkers d(CCAAGCTT) were then ligated to the trimmed ends, and after the removal of the excess linkers the DNA was digested with HindIII and EcoR1. The small fragment containing the lac UV5 promoter was then inserted in between the EcoR1 and HindIII sites of pJW200. Individual isolates were grown to give a set of plasmids, of which pJW231 is a member. Several plasmids derived from pBR322 were also constructed for the cloning of promoter sequences. The parent pBR322 was first digested with HindIII and trimmed briefly with Bal31 nuclease to remove the promoter region of the tetracycline resistance determinants. HindIII linkers were then ligated to the ends of the DNA. Following the removal of excess linker molecules, the DNA was digested with EcoR1 and HindIII, and ligated with the short (about 200 bp) EcoR1-HindIII fragment of pJW231 that contains the lac UV5 promoter. The ligated sample is used for transforming MM294i^+z^+, and a tetracycline resistant colony that is blue on an indicator plate containing Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), which signifies the presence of multicopies of the lac UV5 promoter, was picked. The plasmid isolated from this

colony was designated pJW260. This plasmid was digested with EcoR1 and HindIII, and the protruding ends were repaired with E. coli DNA polymerase I in the presence of all four deoxynucleoside triphosphates. An octameric BglII linker d(CAGATCTG) was inserted in between the repaired ends by incubation with T4 DNA ligase to give the plasmid pJW261, which is tetracycline sensitive but is converted to tet^r when a promoter of the correct orientation is inserted into the single BglII or EcoR1 site. Another promoter selection plasmid, pJW262, was constructed by digesting pJW260 with EcoR1 and then briefly with Bal31 nuclease; HindIII linkers were then added to the ends, and the DNA was ligated after digestion with HindIII. The low copy number (1-2 copies per cell) plasmid pDF41 (27) was kindly provided to us by Dr. D. Helinski.

Materials. E. coli DNA used in the cloning of the topA gene was purified from strain MM294. Plasmid DNAs were purified by a procedure based on the published works of Hirt (28), Guerry et al. (29), and Humphreys et al. (30). In a typical preparation, cells from 2 liters of chloramphenicol-amplified or unamplified culture were pelleted by centrifugation at 4°C and resuspended in 32 ml of 25% sucrose in 0.04 M Tris·HCl, pH8. The suspension was transferred to two Oak Ridge type centrifuge tubes, and 2 ml of 10 mg/ml lysozyme and 0.6 ml of 0.2 M Na_3EDTA were added to each. After mixing and standing for three minutes on ice, approximately 1/10 volume of 10% sodium dodecyl sulfate was added to effect lysis. The total volume of the tube content was estimated and 1/4 volume of 5 M NaCl was added. The tubes were inverted back and forth a number of times and kept at 4°C overnight. They were then spun at 22 Krpm for 30 minutes in a No. 30 rotor (Beckman). The supernatant from each tube was poured into another centrifuge tube and 1/4 volume of 50% polyethylene glycol (Carbowax 6000) was added. After mixing and standing for two hours or longer on ice, the tubes were spun at 5 Krpm for five minutes. The supernatant was discarded and the pellet was dissolved in 5-10 ml of 10 mM Tris·HCl, pH8, 0.1 mM Na_3EDTA. Solid CsCl, 1.14 g for each gram of this crude DNA solution, was added. After the dissolution of CsCl, 50 µl of a 10 mg/ml ethidium bromide solution was added for each gram of CsCl added. Upon mixing, the solution was spun for five minutes at 35 Krpm in an SW50.1 rotor (Beckman). A red precipitate and a dark red floating cake were visible after this step and the solution in each tube was gently decanted into another centrifuge tube to remove both. The clear solution was spun at 35 Krpm in the same rotor for 48-60 hours, and the denser covalently closed DNA from each tube was collected, butanol extracted, and

dialyzed as described previously (31).

Pancreatic DNase I (DPFF grade, about 1700 units/mg) was purchased from Worthington. Restriction enzymes, phage T4 DNA ligase and polynucleotide kinase were purchased from New England Biolabs. Calf intestine alkaline phosphatase and E. coli DNA polymerase I were purchased from Boehringer/ Mannheim. The E. coli DNA methyltransferase that methylates the EcoR1 restriction sites was the generous gift of Drs. R. Reuben and P. Modrich.

Cloning of the topA gene. Several separate cloning experiments were carried out. In one, a partial Sau3A digest of E. coli DNA was cloned into the BamH1 site of pJW231. The E. coli DNA sample was first methylated with the methylase that modifies EcoR1 sites, and then digested partially with Sau3A to give an average size of about 10 Kb. Upon phenol extraction and exhaustive dialysis against 10 mM Tris·HCl, pH8, 0.1 mM Na_3EDTA (TE), this DNA was mixed with pJW231 DNA that had been cleaved with BamH1 and treated with alkaline phosphatase to remove the terminal phosphates. Forty-seven µl of the solution containing a total of approximately 10 µg of DNA was mixed with 2.5 µl of a ligation buffer containing 200 mM each of Tris·HCl, pH7.6, $MgCl_2$ and 2-mercaptoethanol, and 10 mM neturalized ATP. T4 DNA ligase was then added and the mixture was kept at 4°C overnight. The solution was heated at 65° for 10 minutes to inactivate ligase, and 5.25 µl of 1 M Tris· HCl, pH7.4, and an appropriate amount of EcoR1 restriction enzyme were added in succession. The mixture was then incubated at 37°C. Since all EcoR1 sites in the E. coli DNA had been methylated, cleavages by EcoR1 could occur only in the vector pJW231 DNA. The digested DNA was diluted fourfold with TE, phenol extracted, and dialyzed exhaustively against TE. The treatment with T4 ligase is then repeated as before, and 50 µl of each ligated sample was used directly for the transformation of AB1369 cysB. Cys^+ transformants were selected by spreading the cells on minimal agar plates.

The logic for the two cycles of restriction and ligation is that the conditions for the first ligation step can be chosen to favor the formation of concatemers. After the second restriction and dilution, the formation of monomeric circular molecules is favored during the subsequent ligation step. DNA from lysates of about 150 individual Cys^+ colonies was then used to transform DM700 ΔcysB ΔtopA and $TopA^+$ transformants were identified by the antibody sandwiching technique of Broome and Gilbert (32). When the first cycle of transformation with AB1369 cysB was omitted and transformation was carried out directly with DM700, very few colonies were obtained on minimal plates; they all turned out to be $topA^-$. This low efficiency of transfor-

mation with strain DM700 could probably be improved by modifying the procedure used for the preparation of competent cells for transformation, but we have not pursued this aspect.

One of the transformants was identified by the antibody sandwiching method as TopA$^+$, and digestion of the plasmid DNA from a lysate of this transformant with several restriction enzymes showed that there are no SalI, HindIII and AvaI sites. This transformant was converted to CysB$^+$ TopA$^-$, however, during subsequent passage. The instability of the TopA$^+$ plasmids will be discussed in a later section. In all subsequent cloning experiments, passage of transformants was minimized by isolating and storing the DNA from the desired transformants first, and retransforming cells with the DNA when needed.

The apparent lack of SalI and HindIII sites in the cysB-topA region led to the cloning of pJW242, in which an E. coli DNA segment bounded by a SalI and a HindIII site replaces a segment bounded by the same sites in a region of pBR322 encoding for tetracycline resistance. A mixture containing approximately equal weight of E. coli and pBR322 DNA was subject to two cycles of cutting and ligation, with HindIII and SalI present in the first and second cutting step respectively, in a manner similar to that described at the beginning of this section. Several Cys$^+$ transformants of E. coli AB1369 cysB were picked and screened immunologically for the production of DNA topoisomerase I, which led to the isolation of pJW242. Another plasmid, pJW243, was obtained in the same manner except that PstI was used in the place of HindIII in the first cutting step.

A number of topA-containing plasmids were obtained by subcloning of pLC4-6 by the Sau3A partial digestion procedure described earlier. Because of the high frequency of TopA$^+$ transformation in such subcloning, transformation was carried out directly with DM700 ΔtopA, and TopA$^+$ colonies were identified by antibody sandwiching screening of the amp^r transformants.

Mapping of restriction enzymes. Restriction sites were mapped by the partial digestion of a uniquely end-labeled plasmid DNA (33,34) as well as by other conventional methods of sizing a plasmid DNA digested with one or two restriction enzymes. For the large (25 Kb) plasmid pLC4-6, mapping of some of the sites was carried out by isolating first the individual fragments of a HindIII-EcoRl double digest. Electrophoresis of the double digest on a 1% agarose gel was first carried out. Upon staining with ethidium, the gel was viewed on a long wavelength UV source and the DNA bands were sliced out quickly. DNA was recovered by three cycles of freezing and thawing of the

gel slices (35), filtration of the resulting solution through a disposable pipet tip plugged with a porous polypropylene disk, and alcohol precipitation of the filtrate. It has been our experience that the DNA recovered by this procedure can be cut by other restriction enzymes, joined by T4 DNA ligase, or subcloned, but not with all batches of agarose from different suppliers. We used agarose purchased from FMC corporation, Marine Colloids Division, or from Bethesda Research Laboratories.

Other methods. The antibody sandwiching technique was performed as described by Broome and Gilbert (32).

The Southern blot-hybridization method (35) was performed as described by Davis et al. (37), using the alternative C of their hybridization procedure. Labeling of pLC4-6 DNA with ^{32}P was done by nick translation with E. coli DNA polymerase I. Digestion with pancreatice DNase I was first carried out with pBR322 DNA at 20°C in a medium containing 10 mM Tris·HCl, pH8, 0.1 M NaCl, 1 mM Na_3EDTA, 3 mM $MgCl_2$ and 50 µg/ml bovine serum albumin. Aliquots of the solution were digested with varying amounts of the nuclease and Na_3EDTA was added to 20 mM after 10 minutes at 20°C. The samples were examined by agarose gel electrophoresis, and the level of nuclease that gave one hit per DNA molecule was estimated from the Poisson relation that the fraction of the supercoiled form that remains is e^{-i} when there are an average of i hits per DNA molecule. For the particular lot of DNase I used, this corresponded to about 0.1 µg/ml of the enzyme. Plasmid pLC4-6 DNA was then digested with the nuclease at 20°C for 10 minutes at a nuclease level of about 1.8 µg/ml to give approximately one scission per 500 nucleotides. As a first approximation, the DNA concentration has no effect on the required DNase I concentration (38). Nick translation was then carried out as described (37). Sampling of aliquots for monitoring the reaction was done by dipping the tip of a finely drawn glass fiber into the reaction mixture, followed by rinsing the tip in 25 µl of a solution containing 0.02 M Na_3EDTA, 0.1 mM cold dATP and 100 µg/ml of calf thymus DNA. Two 5 µl aliquots of each rinse were delivered to two 1x1 cm pieces of DEAE paper that had been numbered with a pencil. One of the two was dried and counted for total counts, and the other dropped into a beaker containing about 100 ml of 0.3 M NH_4formate, pH7.8, for the determination of counts that had been incorporated into DNA (39). After sampling throughout the time course of nick translation, the NH_4formate solution was decanted and the filters were washed twice more with the same solution, once with 95% ethanol, and dried. The percent of label incorporated was calculated from the ratio of counts for each pair of DEAE squares.

The volume of solution sampled by this procedure is typically 0.01-0.03 µl. About 50% of the counts was incorporated after one hour at 14°C, and no change of this level was observed in the next five hours.

E. coli cell lysate for the assay of DNA topoisomerase I was obtained by the lysozyme treatment and freezing and thawing method (40). The supernatant after the high speed centrifugation step was used in assays in the medium described earlier (11).

Turbidity measurements of cell cultures were done in a Klett-Summerson colorimeter or a Bausch and Lomb Spectronic 710 spectrophotometer. Overnight cultures of E. coli strain W3110 harboring topA$^+$ and topA$^-$ plasmids were also plated after serial dilutions; no significant difference was observed for the ratios of turbidity to viable cells per ml among these cultures.

RESULTS

Cloning of Escherichia coli topA gene encoding for DNA topoisomerase I. Cloning of Escherichia coli topA gene was much facilitated by two recent findings: the close proximity of the genes topA and cysB (11,12), and the existence of mutants in which topA is deleted (13). Since cysB is an easily selectable auxotrophic marker, the initial cloning of topA can be achieved by selecting DNA fragments containing cysB, and screening for the presence of topA on the cloned fragments. The existence of ΔtopA mutants makes it straightforward to apply the antibody sandwiching technique (32) for the screening of transformants of such a mutant in which the production of DNA topoisomerase I is restored.

Fig. 1 illustrates the application of the antibody sandwiching technique. Plastic sheets coated with rabbit immunoglobulin G(IgG) directed against E. coli DNA topoisomerase I were placed over chloroform vapor lysed colonies of E. coli that had been grown on agar plates. After standing for several hours to permit the adsorption of the enzyme or fragments of the enzyme carrying antigenic determinants that are recognized by the antibodies, the sheets were lifted off, washed, stained with the same antibody labeled with ^{125}I, and imaged by autoradiography. Fig. 1a shows the pattern obtained with E. coli topA$^+$ colonies. Each and every colony registers as a positive image on the autoradiogram. Fig. 1b shows the pattern obtained with about the same number of colonies of E. coli strain DM700 ΔtopA. None of the colonies gives a positive imprint.

As described in detail in the Materials and Methods section, a number of

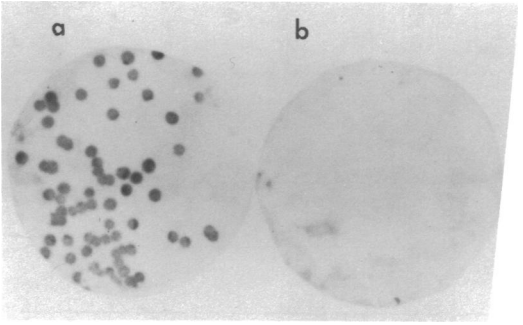

Fig. 1. Radioimmunal imaging of colonies of E. coli strain W3110 topA$^+$ (a), and strain DM700 ΔtopA derived from W3110 (b). The agar plates had about equal number of colonies grown to roughly the same size.

topA-containing plasmids have been obtained. Characterization of these plasmids is described below.

Restriction Mapping and the Identification of the CysB, TopA and Trp Genes. Fig. 2 depicts the locations of the cleavage sites by a number of restriction enzymes over a 20 Kb region of the E. coli genome, and the boundaries of the cloned E. coli DNA fragments in several of the plasmids constructed.

Identification of the structural gene topA encoding for the topoisomerase was accomplished as follows. The plasmid pJW240 was selected as a TopA$^+$ transformant of strain DM700 ΔtopA by the immunological screening procedure. As indicated in Fig. 2, pJW240 contains only 2.3 Kb of E. coli DNA. The molecular weight of purified E. coli DNA topoisomerase I, about 105,000, predicts a gene size considerably larger. Therefore it appears that the E. coli fragment in pJW240 contains part of topA such that a protein fragment that is recognized by antibodies directed against the topoisomerase is produced.

There is the possibility, however, that strain DM700 ΔtopA is not ΔtopA, but is missing a gene that activates topA. If this is the case, then pJW240 might contain this regulatory element rather than part of topA. To distinguish these two possibilities, DM700 cells transformed with pJW240 were grown, lysed, and assayed for the presence of DNA topoisomerase I activity that can relax negatively supercoiled DNA. No activity is detectable. This presence of antigenic determinants of DNA topoisomerase I and absence of catalytic activity of the enzyme indicates that the E. coli DNA segment in pJW240 is derived from the region containing the structural gene topA itself.

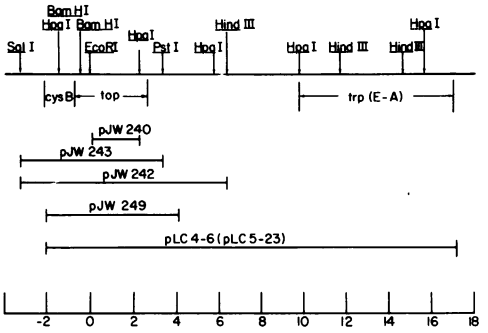

Fig. 2. A map of the cysB-topA-trp region of E. coli. The bottom scale gives distance in Kb, with the position of the EcoRl site arbitrarily taken as zero. One of the two BamHl sites shown is very close to an adjacent HpaI site. The boundaries of several cloned E. coli DNA fragments are indicated. Plasmids pJW240 and pJW249 were obtained from subcloning of an Sau3A partial digest of pLC4-6 into the BamHl site of pJW231, and pJW242 and pJW243 were obtained by cloning E. coli DNA into pBR322 as described in the Materials and Methods section. The SalI to HindIII fragment shown for pJW242 has also been cloned into a low copy number plasmid pDF41. In addition, the segment from the SalI site to the single SphI site (not shown) at position 2.37 has been cloned into pBR322 to give pJW80. Plasmid pJW83 is identical to pJW80 except that the E. coli DNA used in its construction carried a nonsense mutation in the topA gene that was identified as a supX mutation (P. Margolin, personal communication). A more detailed description of the pair of plasmids pJW80 and 83 will be published elsewhere.

In agreement with the above conclusion, DM700 transformed with pJW242, pJW243 and pJW249 becomes TopA$^+$ by either the immunological or the enzymatic assays. This delimits the topA gene to within the region from -2 Kb, the left boundary of pJW249, to +3.3 Kb, the right boundary of pJW243 (see Fig. 2). Further refinement of the boundaries of topA was carried out by subcloning of pJW249. In one experiment, the HindIII site in the pBR322 part of pJW249, which is located near the left boundary of the E. coli part of the plasmid shown in Fig. 2, was cleaved and the linearized DNA was trimmed to different extents by the exonucleolytic action of Bal31 nuclease (41). The DNA was then ligated with a dodecameric HindIII linker, and the small HindIII-BamHl fragment from this DNA was used to substitute the region in between the HindIII site and the BamHl site of pJW249 at around position -0.4 Kb in Fig. 2. Assaying for topoisomerase I activity in DM700 transformed with these substituents locates the left boundary of topA around -0.7 Kb. Similar experiments indicate that the right boundary of topA does not go beyond +2.7 Kb. These positions are indicated in Fig. 2.

Several lines of evidence indicate that the transcription of topA proceeds in the direction of the PstI site to the EcoR1 site depicted in Fig. 2. Cloning of the 3.4 Kb PstI-EcoR1 fragment into the HindIII site of the promoter selection plasmid pJW262, for example, gives tetracycline resistant colonies only if the PstI to EcoR1 direction is the same as the direction of transcription of the tetracycline resistance region. In a separate experiment, the region of topA gene from the PstI site to an EcoRV site 1.3 Kb to the left was replaced by a lac UV5 promoter oriented with its direction of transcription from right to left. Assays of DNA topoisomerase I activity in extracts of strain DM700 ΔtopA carrying plasmids with the topA region so modified show that the expression of topA is under the control of the lac UV5 promoter (data not shown).

Plasmids pJW242, pJW243 and pJW249 all give the Cys^+ transformants of DM700 ΔcysB or AB1369 cysB. The left boundary of the gene cysB must be to the right of position -2 Kb, the left boundary of pJW249 shown in Fig. 2. Also, from the results discussed in the paragraph above, the right boundary of cysB is likely to be to the left of -0.7, assuming that the genes topA and cysB do not overlap. Further refinement of the boundaries awaits the complete sequencing of the cysB-topA region, which is being carried out in this laboratory (Y.-C. Tse, S. Swanberg and J.C. Wang, to be reported).

Identification of the trp operon is achieved by comparing the restriction sites shown in Fig. 2 with the published restriction maps and sequences of the trp operon (42-46). Excellent agreement was found for all of the restriction enzymes used.

The Lack of Tight Regulation of TopA. One of the most intriguing questions in regard to the physiological effects of DNA supercoiling in vivo is the regulation of the degree of supercoiling. As mentioned in the Introduction, DNA topoisomerase I is known to influence gene expression. It is generally thought that DNA topoisomerase I exerts this influence indirectly through its effect on the degree of DNA supercoiling in vivo (13,54-56): namely, there is a dynamic balance between the supercoiling action of the DNA gyrase and the relaxation action of DNA topoisomerase I and other relaxation activities. Since the rate of relaxation of a negatively supercoiled DNA by DNA topoisomerase I is strongly dependent on how supercoiled the DNA is (1), one plausible mechanism of supercoiling regulation is that the intracellular level of DNA topoisomerase I is autoregulated or is regulated by other regulated elements; this level of the enzyme in turn regulates the degree of supercoiling by a feedback loop based on the strong dependence of the rate of

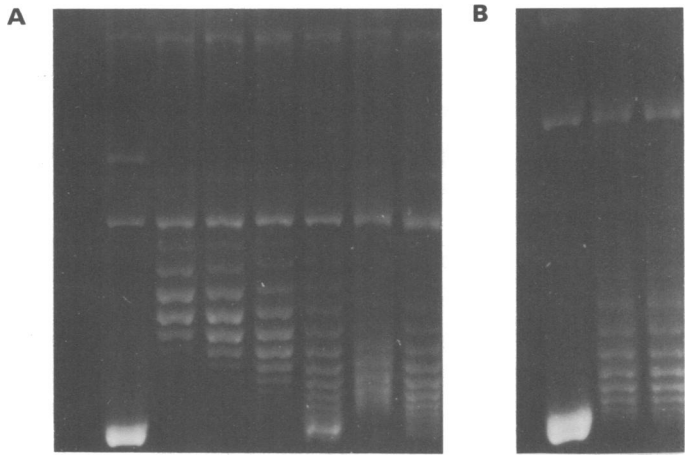

Fig. 3. Assays of DNA topoisomerase I activity in lysates of E. coli W3110 topA$^+$ cells carrying topA$^+$ or topA$^-$ plasmids. Cells were grown to a Klett-meter reading of 100 (corresponding to about 5x10^8 cells/ml), pelleted, washed, and lysed as described (40). DNA topoisomerase I activity is then assayed by its relaxation of supercoiled pBR322 DNA. (a) Agarose gel electrophoresis patterns of supercoiled DNA after incubation with extracts. From left to right, lane 1 is the control DNA incubated in the absence of extract; lanes 2-5 are DNA samples after incubation with 1 µl of various dilutions (6-, 12-, 24- and 48-fold, respectively) lanes 6 and 7 are, respectively, DNA samples after incubation with 1 µl of undiluted and two-fold diluted extract of W3110 topA$^+$ carrying pJW200 topA$^-$. (b) Agarose gel electrophoresis patterns of supercoiled pBR322 DNA (left-most lane) after incubation with 1 µl of an extract of W3110 topA$^+$ carrying pJW200, which does not contain any part of the topA gene (middle lane), and after incubation with the same amount of an extract of W3110 topA$^+$ carrying pJW249 ΔBam, a multi-copy plasmid that carries a topA gene with a deletion on the 3' side of the gene (right-most lane).

relaxation on negative supercoiling.

To test whether the level of DNA topoisomerase I is under strict cellular control, we have examined the level of this activity in lysates of cells containing different number of copies of the topA gene. Fig. 3a illustrates one of such experiments. Assays of extracts of cells of strain W3110 harboring pJW80, a pBR322 derivative with one copy of the topA gene per plasmid, and comparable amounts of extracts of cells of W3110 harboring a control plasmid pJW200 indicate that the level of DNA topoisomerase I is more than 10 times higher in W3110 carrying the topA$^+$ multicopy plasmid pJW80 than in the same strain carrying the control plasmid, in which there is only the chromosomal topA gene (compare lanes 3 and 7, Fig. 3a). Similarly, lysates of W3110 transformed with a topA containing low copy number (1-2 copies per cell)

plasmid derived from pDF41 (27) is about two- to four-fold higher than that in lysates from untransformed W3110 (data not shown).

The results described above indicate that the cellular level of DNA topoisomerase I increases with increasing copy number of the topA gene. This in turn suggests that the expression of the gene is not under strict cellular regulation.

The above notion is also consistent with the observation that the level of DNA topoisomerase I activity in strain W3110 topA$^+$ carrying a multicopy plasmid pJW249 ΔBam, which is constructed from pJW249 by the deletion of the 1.1 Kb section in between the two BamHl sites (Fig. 2) and is therefore missing the 3' terminal part of the topA gene, is not significantly different from that of the untransformed strain (Fig. 3b). The truncated plasmid topA gene produces antigenic determinants that are recognized by rabbit antibodies specific for the gene product, but not functional DNA topoisomerase I (results not shown). If topA is regulated by positive or negative regulatory elements, or if it is autoregulated, then the presence of multicopies of a plasmid that carries the promoter region of topA but produces no functional DNA topoisomerase I would be expected to affect the expression of the chromosomal copy of the topA gene.

Overproduction of DNA Topoisomerase I Reduces Growth Rate. Strains of E. coli harboring multicopy plasmids that contain an intact topA gene exhibit a significantly slower growth rate. In one typical experiment, strain W3110 topA$^+$ carrying pJW80, an ampicillin resistant pBR322 derivative that contains the entire topA gene and the same strain transformed with pJW83, a plasmid that is identical to pJW80 except that it is topA$^-$ because of a nonsense mutation (see the legend to Fig. 2), were grown in broth under identical conditions. Turbidity measurements show that cells carrying the topA$^+$ multicopy plasmid grow slower by about 20%. The difference in growth rate is also reflected by the sizes of the colonies on Luria broth agar plates containing ampicillin. When the diameter of colonies of cells carrying pJW83 topA$^-$ is 2-3 mm, the diameter of colonies of cells carrying pJW80 topA$^+$ is half a mm or smaller. When cells harboring pJW80 topA$^+$ are plated on plates without ampicillin, colonies as large as those of cells carrying pJW80 topA$^-$ appear with a frequency of about 10^{-6}. All these larger colonies are found to be sensitive to ampicillin, and are most likely cells that have lost the plasmid.

The physical mapping of the cysB-topA-trp region in wild-type E. coli. During the cloning of topA in pBR322 or its derivatives pJW200 or pJW231, we have on occasions observed the loss of the TopA$^+$ phenotype in topA$^-$ cells

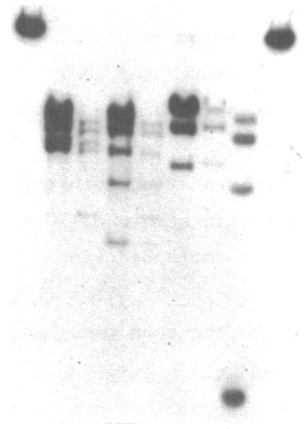

Fig. 4. A comparison of sizes of restriction fragments from E. coli genomic DNA and cloned E. coli DNA in the cysB-topA-trp region. The left-most and right-most lanes contained a marker DNA loaded five minutes before the termination of electrophoresis for the purpose of marking the top of the gel. The other seven lanes were loaded at the same time, and the duration of electrophoresis in a 20 cm x 20 cm x 0.4 cm 1% agarose slab was four hours at 80 volts. The right-most of the seven lanes contained sizing markers that are 8800 bp, 4360 bp, 2300 bp, and 450 bp; the other six lanes contained three pairs of samples digested with, from left to right, HpaI, HpaI and EcoR1, and HindIII. For each pair, the left contained 0.05 µg of pLC4-6 DNA and the right 1 µg of E. coli DNA. Blot-hybridization was carried out as described in the Materials and Methods section, using ^{32}P-labeled pLC4-6 as the probe.

transformed with topA containing plasmids. One such case has been mentioned in the Materials and Methods section. Another example concerns the plasmid pLC41-15 (23). Although this plasmid is cysB$^+$ and trpE$^+$, and is therefore expected to contain the gene topA as well from the map shown in Fig. 2., DM700 ΔtopA transformed with this plasmid remains TopA$^-$ as evidenced by either the immunological or the enzymatic activity assay. Restriction mapping of the plasmid identifies a deletion of about 700 base pairs in the region between the EcoR1 site and the HincII site to its right.

Since the physical maps of the three independently derived plasmids pJW242, pJW243 and pJW249 agree in the cysB-topA region, it is unlikely that deletion or rearrangement has occurred in this region during the cloning of these plasmids. The physical map of the remaining region shown in Fig. 2 is

essentially derived from, however, a single clone of pLC4-6. In order to check whether deletions or rearrangements might have occured in pLC4-6, aliquots of E. coli DNA from strain K12 MM294 were digested separately with several restriction enzymes, sized by gel electrophoresis, and a Southern blot was obtained with pLC4-6 DNA labeled by nick translation as the hybridization probe. To facilitate comparison, samples of unlabeled pLC4-6 DNA digested with the same set of restriction enzymes were electrophoresed on the same gel. An autoradiogram of the nitrocellulose sheet after hybridization is shown in Fig. 4. Clearly, within the resolution of this experiment, there is no indication of deletion or rearrangement in pLC4-6. For the pair of samples doubly digested with HpaI and EcoR1 for example, the five fragments that should be present in both pLC4-6 (lane D) and genomic E. coli DNA (lane E) line up in pairs as expected.

DISCUSSION

We have cloned the structural gene topA that encodes for E. coli DNA topoisomerase I by a combination of genetic and immunostaining methods. The use of immunological screening in cloning has so far been overshadowed by hybridization screening with a labeled nucleic acid that is complementary to the cloned sequence. In several recent instances, however, the potential of the radioimmunal screening technique have been well-demonstrated (47,48).

Plasmids containing the cysB-trp region are among the earliest known episomes. The sex factor F ColV-ColB trp cys for example, happened to be the first example of a covalently closed, supercoiled plasmid DNA (49). It is only recently, however, that the gene topA encoding for DNA topoisomerase I has been located in this region (11,12). From the restriction map of the cloned gene and that of the genomic DNA obtained by blot-hybridization, it is clear that in E. coli there is a single copy of the gene in between cysB and trp. The topA gene is immediately adjacent to cysB and is more distal to trp.

Bouché (50) has recently mapped physically a 470 Kb segment of the E. coli chromosome around the terminus of DNA replication, including the cysB to trp region studied in this work. The restriction sites reported for EcoR1, HindIII and PstI in the cysB to trp region are in agreement with those depicted in Fig. 2. The 7 Kb DNA segment between topA and trp contains no known gene. Although earlier studies suggested that the gene opp that affects the transport of certain oligopeptides might be located in between cysB and trp (51,52), this conclusion has been disputed by the more recent

genetic analysis of opp mutants of both E. coli and S. typhimuriun (53). The availability of cloned plasmids containing this region should help the identification of new genes on this segment of DNA.

The observation that deletions in the topA region are generated sometimes during the passage of the cloned plasmids hints that there might be a selection against the expression of topA on a multicopy plasmid. This notion is supported by the finding that the overproduction of the type I topoisomerase reduces growth rate. We have suggested previously that the cellular level of DNA topoisomerase I may affect the degree of supercoiling of the DNA, which may in turn affect the expression of a multitude of genes (13,54). Recently, Pruss et al. (55) have shown that in several topA$^-$ cells the superhelicity of the E. coli chromosome is indeed increased, although a quantitative interpretation is complicated by the frequent acquisition of secondary mutations in topA$^-$ strains, some of which map in the gyrase structural genes (55,56 and R.E. Depew, personal communication). Thus it is most likely that alteration of the cellular level of DNA topoisomerase I may result in rather complex physiological changes, of which the slowing down of growth rate is one reflection of such changes. A more detailed dissection of the physiological effects that result from changes in the cellular level of DNA topoisomerase I is clearly needed in order to have a better understanding of the functional roles of this enzyme.

ACKNOWLEDGEMENT

We are most grateful to Stephanie Broome for her instructions on the antibody sandwiching method, to Rolf Sternglanz and Yuk-Ching Tse for helpful discussions, and to Louis Zumstein for his assistance in the assay of DNA topoisomerase I activity in cell lysates. This work has been supported by a grant from the U.S. Public Health Service (GM 24544).

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