The *traE* Gene of Plasmid RP4 Encodes a Homologue of *Escherichia coli* DNA Topoisomerase III*

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The polypeptide encoded by the plasmid RP4 *traE* gene shows extensive protein sequence similarity to *Escherichia coli* topB, the gene encoding DNA topoisomerase III (Topo III). The *traE* gene product has been cloned into a bacteriophage T7-based transient expression system, and the polypeptide has been expressed and purified. The TraE protein exhibits topoisomerase activity similar to that of Topo III. Relaxation is stimulated by high temperature and low concentrations of Mg²⁺. In addition, similar to *E. coli* Topo III, the TraE protein is a potent decatenase and can substitute for Topo III activity in vivo. The biochemical properties of the TraE protein in vitro suggest that the protein may be involved in the resolution of plasmid DNA replication intermediates either during vegetative replication or in conjugative DNA transfer. Putative homologues of Topo III have been found to be encoded by other broad host range, conjugative plasmids isolated from both Gram-negative and Gram-positive organisms, suggesting that Topo III-like polypeptides may have an essential role in the propagation of many promiscuous plasmids.

Plasmids are autonomously replicating extrachromosomal elements. In addition to replicating within a single organism, many of these elements possess the ability of interspecies and interspecies transfer. Hence, many plasmids can be classified as mobile genetic elements. These mobile genetic elements are often clinically relevant as many of these “promiscuous” plasmids can encode polypeptide(s) that confer resistance to single/multiple antibiotic(s) and heavy metal ion(s). These resistance genes are often contained within a transposon that has integrated into the plasmid molecule (for reviews, see Refs. 1–4).

Plasmid RP4 is a 60,099-base pair broad host range, transmissible genetic element that belongs to the IncP incompatibility group. The RP4 plasmid genome has been completely sequenced and revealed the presence of 74 genes (5). The majority of DNA metabolic enzymes used by plasmid RP4 are host-encoded; however, the plasmid itself encodes several specialty enzymes. In addition to replicating within a single organism, plasmid RP4 DNA metabolism may shed light on the role of Topo III in cellular DNA metabolism.

**MATERIALS AND METHODS**

**DNA and Nucleotides—** dX174 replicative form I DNA (covalently closed, negatively supercoiled DNA molecules) was purchased from Life Technologies, Inc. DNA oligonucleotides were prepared by the University of Maryland Biopolymorph Laboratory. Radiolabeled nucleoside triphosphate was purchased from Amersham Corp.

**Enzymes and Reagents—** Acrylamide, restriction enzymes, and agarose were from Life Technologies, Inc. DE52 and P-11 cellulose were from Whatman. Trypsin inhibitor-agarose and single-stranded DNA-cellulose was from Sigma. Sephaeryl S-200 was from Pharmacia Biotech Inc.

**Protein Determination—** Protein concentration was determined by the method of Bradford (15) using a Bio-Rad protein assay kit.

**Radiolabeling of Oligonucleotides—** Oligonucleotides were 5’-end-labeled using bacteriophage T4 polynucleotide kinase (Life Sciences, Inc.) and [γ-³²P]ATP as per the manufacturer’s recommendations. The labeled oligonucleotides were fractionated on a polyacylamide gel. The region containing the labeled oligonucleotide was excised, and the DNA was isolated by direct elution of the fragment into 10 mCi Tris-HCl (pH 7.5 at 22 °C) and 1 mCi EDTA. The radiolabeled oligonucleotides were diluted to a specific activity of 5000 cpm/pmol by the addition of excess unlabeled oligonucleotide.

**Construction of the TraE Expression Vector—** The gene encoding the TraE protein was amplified by the polymerase chain reaction using the following primer sequences: 5’-GAGGGTAGGGGGAATCATGCAATT-GGAAGGTTGGTG-3’ (amino-terminal) and 5’-GAGGGTAGGGGGAATCATGCAATT-GGAAGGTTGGTG-3’ (carboxyl-terminal). The amplified product contained an NdeI restriction site (shown in boldface) encompassing the initiation codon of the *traE* open reading frame and a BamHI site (shown in boldface) after the termination codon of the *traE* open reading frame. The amplified product was digested with NdeI and BamHI and ligated with NdeI/BamHI-digested plasmid vector pET-3c (pTraE). To ensure that no mutations occurred during the polymerase chain reaction amplification process, the expression vector was also constructed.

1 The abbreviations used are: Topo III, topoisomerase III; Topo I, topoisomerase I.
using site-specific mutagenesis. A 3348-base pair SpH1 fragment of plasmid RP4 that encompasses taeE was subcloned into M13mp19 replicative form DNA. Uracil-containing single-stranded DNA was isolated using this construct, and oligonucleotide-directed site-specific mutagenesis (16) was used to create the NdeI and BamHI sites at the beginning and end of the gene, respectively. The mp19 DNA (replicative form I) that contained the altered taeE gene was cleaved with NdeI and BamHI, isolated, purified, and ligated with NdeI/BamHI-digested pET-3c. The biochemical properties of the purified TaeE protein obtained from either construct were indistinguishable.

### Purification of the TaeE Protein
The induction of the TaeE polypeptide was initiated by infection of the expression strain, harboring the pTaeE plasmid DNA, with bacteriophage λ CE6 (17). Chromatography on trypsin inhibitor-agarose was included in the purification to reduce proteolysis (18). To prevent any contamination of the TaeE protein with endogenous Topo III, it was purified from E. coli strain BL21 in which the gene encoding Topo III (topB) had been disrupted (18). The TaeE protein was purified by a modification of a previously described protocol that included DE52, Bio-Gel HTP, single-stranded DNA-cellulose, and Sephacryl S-200 chromatography (19).

#### Superhelical DNA Relaxation Assays
Superhelical DNA relaxation reactions (25 mU) contained 40 mU Hepes-KOH buffer (pH 8.0 at 22 °C), 1 mM magnesium acetate (pH 7.0), 1 mM bovine serum albumin, 40% (v/v) glycerol, 200 ng of dX174 replicative form I DNA, and 5 pmol of the indicated amount of topoisomerase (20). Reaction mixtures were incubated at 52 °C for 10 min, and the reaction products were separated on an agarose gel and visualized by staining with ethidium bromide as described previously (19).

#### oriC DNA Replication Assay
The replication of oriC-containing DNA in vitro was performed as described previously (18). The replication products were separated by agarose gel electrophoresis and visualized by autoradiography (21). The percentage of replication products existing as form II molecules (nicked or gapped circular DNA molecules) was quantified using a Fuji BAS 1000 phosphomager.

### Plasmid-encoded Topoisomerase
Topoisomerase-induced DNA Cleavage Assay—Reaction mixtures (5 mU) contained 40 mU Hepes-KOH buffer (pH 8.0 at 22 °C), 1 mM bovine serum albumin, 1 mM magnesium acetate (pH 7.0), and 5 pmol of a radiolabeled 45-base oligonucleotide. The oligonucleotide was 5'-CAGATCGAGGCTGCCGCAACT' that contains the site of Topo III cleavage. The indicated amounts of Topo III and TaeE were incubated for 3 min at 37 °C, and the reaction was stopped by the addition of SDS to 2%. The indicated amounts of TaeE and Topo III, isolated, purified, and ligated with NdeI/BamHI-digested pET-3c. The biochemical properties of the purified TaeE protein obtained from either construct were indistinguishable.

### RESULTS

#### The taeE Gene Shows Extensive Protein Sequence Similarity to E. coli topB
A search of the National Center for Biotechnology Information (NCBI) nonredundant data base has revealed that E. coli topB shows extensive protein sequence similarity to the plasmid RP4 taeE gene (Fig. 1A). The taeE open reading frame is capable of encoding an 82-kDa polypeptide. The similarity between the polypeptides encoded by taeE and topB extends through their first 600 amino acid residues and then diverges in the carboxy-terminal residues. The region of the similarity is identical to that observed between E. coli topB and topA (the gene encoding DNA topoisomerase I (Topo I)) (14, 22). The carboxy-terminal residues of the putative TaeE protein contain two potential zinc-finger motifs that show protein sequence similarity to one of the zinc-finger motifs of Topo I (23) and to each other (Fig. 1B).

#### The Plasmid RP4 TaeE Protein Exhibits Superhelical DNA Relaxation Activity
To assess whether the taeE gene encoded a DNA topoisomerase activity, the gene was subcloned from plasmid RP4 into plasmid pET-3c, a bacteriophage T7 RNA polymerase-based transient expression plasmid (17). Cells containing the taeE expression plasmid pTaeE were induced by the addition of bacteriophage λ CE6, which contains the gene encoding T7 RNA polymerase under the control of the bacteriophage λ P2 promoter. The TaeE protein was then purified from the induced cells using the same purification protocol as that used for E. coli Topo III (19). The final preparation contained a polypeptide with the expected molecular mass of 82 kDa and two minor proteolysis products (Fig. 2).
The purified TraE polypeptide was assayed for superhelical DNA relaxation activity. A titration of TraE revealed that the protein possessed DNA relaxation activity comparable to that of Topo III (Fig. 3). In addition, the superhelical DNA relaxation activity of TraE exhibited virtually the same biochemical properties as that of Topo III. TraE-catalyzed relaxation of superhelical DNA was stimulated by increasing temperatures (Fig. 4, lanes 2–6 and 7–11) and inhibited by increasing concentrations of Mg2+ (compare lanes 2–6 and 7–11).

TraE Protein-catalyzed Cleavage Sites Are Identical to Those of E. coli Topo III—Type I DNA topoisomerases act by binding to DNA, making a transient single-stranded break in the DNA, catalyzing a strand passage event, and rescaling the transient break (for a review, see Ref. 24). Although E. coli Topo I and Topo III possess a high degree of protein sequence similarity (14), the two enzymes cleave single-stranded DNA substrates at different sites (19, 25); therefore, the TraE protein was analyzed for its cleavage site specificity (Fig. 5). In contrast to Topo I (Fig. 5, lanes 8–10), TraE-catalyzed cleavage of a 45-bp oligonucleotide substrate (lanes 5–7) generated a pattern identical to that of Topo III (lanes 2–4). The 45-bp substrate contained one predominant Topo III cleavage site and multiple minor sites. TraE protein-catalyzed cleavage of the substrate occurred predominantly at the major Topo III cleavage site (Fig. 5, compare lanes 4 and 5). Since only a small number of Topo III cleavage sites are present on the substrate, it is possible that TraE protein-catalyzed cleavage of single-stranded DNA can occur at sites distinct from those of Topo III. However, at a minimum, the two enzymes do have a subset of cleavage sites in common. Cleavage of the substrate by the TraE protein and Topo I is more efficient than cleavage by Topo III. In addition, with increased levels of TraE protein, a cleavage product accumulates that is identical to that of Topo I (Fig. 5, compare lanes 7 and 8). This product has also been observed using large amounts of Topo III in a cleavage reaction (26).

The TraE Protein Exhibits Decatenation Activity—It has previously been shown that E. coli Topo III and Topo I catalyze distinctly different reactions in vitro (18). DNA topoisomerase I was very efficient in the relaxation of negatively supercoiled DNA (i.e. removing intramolecular linkages), whereas DNA relaxation assays were performed as described under “Materials and Methods.” Reactions (25 μl) contained no TraE protein (lanes 1 and 12) or 120 fmol of TraE protein (lanes 2–11). Reactions were incubated at 30 °C (lanes 2 and 7), 37 °C (lanes 3 and 8), 42 °C (lanes 4 and 9), 52 °C (lanes 5 and 10), and 65 °C (lanes 6 and 11) in the presence of either 5 mM (lanes 1–6) or 1 mM (lanes 7–12) magnesium acetate. The reaction products were electrophoresed on a 1% agarose gel, and the DNA was visualized by staining with ethidium bromide. oc, open circle nicked or gapped circular DNA; sc, negatively supercoiled circular DNA.

The purified TraE protein was purified as described “Materials and Methods.” 1.0 μg of the purified enzyme was electrophoresed on a 10% polyacrylamide gel in the presence of 0.1% SDS (41). The protein was visualized by staining the gel with Coomassie Blue. Lane 1, TraE protein; lane 2, molecular mass markers.

DNA relaxation assays were performed as described under “Materials and Methods.” Reactions (25 μl) contained 25 fmol (lanes 1 and 5), 5 fmol (lanes 2 and 6), 1 fmol (lanes 3 and 7), and 0.2 fmol (lanes 4 and 8) of either TraE protein (lanes 1–4) or Topo III (lanes 5–9) or no topoisomerase (lane 10). The reaction products were electrophoresed on a 1% agarose gel, and the DNA was visualized by staining with ethidium bromide. o.c., open circle nicked or gapped circular DNA; s.c., negatively supercoiled circular DNA.

Effect of magnesium concentration and temperature on TraE-catalyzed relaxation of negatively supercoiled DNA.

**Fig. 4.** Determination of the cleavage site specificity of the TraE protein. Cleavage reactions were performed as described under “Materials and Methods.” Reactions contained no topoisomerase (lane 1); 5 pmol (lane 2), 10 pmol (lane 3), and 20 pmol (lane 4) of Topo III; 2.5 pmol (lane 5), 5 pmol (lane 6), and 10 pmol (lane 7) of TraE protein; and 2.5 pmol (lane 8), 5 pmol (lane 9), and 10 pmol (lane 10) of Topo I. Reaction products were separated by electrophoresis on a 25% polyacrylamide gel in the presence of 50% (w/v) urea, and the products were visualized by autoradiography. The position of the 45-base oligonucleotide is indicated (45 mer). The arrows indicate the positions of the major Topo III and Topo I cleavage products.
topoisomerase III was very efficient in the decatenation of multiply interlinked plasmid DNA dimers and the resolution of DNA replication intermediates (i.e. intermolecular linkages). To assess whether the TraE protein was capable of resolving DNA replication intermediates, the enzyme was included in an in vitro replication reaction. The products of the reaction were separated on an agarose gel, and the products were visualized by autoradiography (Fig. 6). Similar to Topo III, the TraE protein was capable of resolving plasmid replication intermediates (Fig. 6, lanes 2–5). The specific activity of the TraE protein was 30% of that of Topo III (Fig. 6, lanes 7–10).

The traE Gene Can Substitute for topB in Vivo—In the course of constructing E. coli strains that lack the activities of the two type 1 topoisomerases, Topo I and Topo III, it has been observed that it is extremely difficult to transduce a Topo B gene disruption into the DNA deletion strains that contain gyrA compensatory mutations (i.e. DM750 and DM800 (27, 28)) however, in the presence of a Topo III expression plasmid, pDE1 (14), the disruption of the chromosomal copy of Topo B in these strains is easily accomplished. 2 This is presumably because promiscuous transcription of Topo B from the expression plasmid provides the cell with sufficient amounts of the enzyme to remain viable in the absence of the chromosomal gene. Western blot analysis of cell extracts prepared from cells harboring plasmid pDE1 reveals detectable amounts of Topo III, whereas cell extracts prepared from cells harboring the expression plasmid do not contain the expression plasmid do not reveal detectable quantities of the enzyme (data not shown). This observation is consistent with the extremely low abundance of Topo III in E. coli (19).

To assess whether the TraE protein can substitute for Topo III in vivo, a P1 transduction experiment was performed in which the DM750 cells (ΔtopA) being transduced to TOPB (using a P1 lysate prepared from cells with a topB::kan R disruption) contained the traE expression vector pTraE (traE in vector pET-3c). As can be seen in Table I, cells harboring the TOPB expression plasmid pDE1 (TOPB in vector pET-3c (14)) and the traE expression plasmid pTraE could be transduced to topB::kan. DM750 cells harboring a topA expression plasmid, pTII

2 S. Malpure and R. J. DiGate, unpublished data.

Bacteriophage P1 transductions each were performed as described by Miller (42). The transduction frequencies of each plasmid-containing strain are expressed relative to the transduction frequency of the strain without the plasmid. The data represent the average ratio of three different experiments. The transduction frequency of cells containing the topB expression plasmid ranged from $1.8 \times 10^{-3}$ to $16.1 \times 10^{-3}$. The total numbers of transductants in the three experiments were 1137 (for the strain containing pDE1) and 568 (for the strain containing pTraE).

Table I

| Plasmid | None | pDE1 | pTraE | pTII | pDE2 | pF328 |
|--------|------|------|-------|------|------|-------|
| Relative transduction frequency | 1 | 340.5 | 119.6 | 7.7 | 1.9 | 2.5 |

DISCUSSION

The polypeptide encoded by the plasmid RP4 traE gene exhibits extensive protein sequence similarity to E. coli DNA topoisomerase III. The carboxyl-terminal amino acid residues of the TraE protein contain two potential zinc-finger motifs and more closely resemble E. coli DNA topoisomerase I. To ascertain whether the TraE protein was a topoisomerase, the traE gene was cloned into the bacteriophage T7 RNA polymerase-based transient expression vector. The polypeptide was induced, purified to apparent homogeneity, and shown to possess DNA topoisomerase activity in vitro.

The enzyme, unlike E. coli DNA topoisomerase I, exhibits biochemical properties virtually identical to those Topo III. The TraE protein is stimulated by both low concentrations of Mg$^{2+}$ and high temperature. In addition, TraE protein-catalyzed cleavage of a single-stranded substrate occurs at sites identical to those of Topo III. Perhaps the most distinguishing biochemical property of E. coli Topo III is the ability of the enzyme to catalyse the resolution of DNA replication intermediates in vitro. Topo III and DNA topoisomerase IV are unique among the bacterial topoisomerases in their ability to efficiently decatenate multiply interlinked plasmid DNA dimers and resolve plasmid replication intermediates (19, 29–31). DNA gyrase is very inefficient at decatenating multiply interlinked plasmid DNA dimers (32), and Topo I has been shown to be incapable of resolving plasmid replication products in vitro (18). Since the TraE protein has features that are common to both Topo I and Topo III, it was of interest determine whether the enzyme was capable of resolving plasmid replication intermediates in vitro.

Analysis of the TraE protein using an oriC-based in vitro replication system indicated that the protein could efficiently resolve DNA replication intermediates and that the specific activity of the protein was one-third that of Topo III.

The TraE protein can also effectively substitute for Topo III.
the presence of a plasmid-encoded topoisomerase/decatenase would always guarantee the presence of a functional and compatible topoisomerase activity, regardless of the host organism. Alternatively, one could envision a role for these enzymes during conjugative transfer. The topoisomerase could serve as a swivel to facilitate the unwinding of the donor strand that is transferred to the recipient host. In either case, the presence of multiple examples of Topo III homologues contained on conjugative plasmids is compelling and suggests that the presence of topB-like genes may define a distinct subclass of these types of plasmids.

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TABLE II

| Organism                        | Source           | Gene     | p value | Ref.       |
|---------------------------------|------------------|----------|---------|------------|
| Haemophilus influenzae          | Chromosomal     | topB     | 2.5e-299| 43         |
| Pseudomonas spp.                | pRP4             | traE     | 1.0e-142| 5          |
| Enterobacter aerogenes          | pR751            | traE     | 2.1e-135| a          |
| Streptococcus pyogenes          | pSM19035         | hyp. p'  | 3.8e-2   | 45         |
| Enterococcus faecalis           | pAMjM           | hyp. p'  | 2.7e-23  | 46         |
| Staphylococcus aureus           | pGO1             | traF     | 9.0e-56  | 47         |
| Staphylococcus sp.              | pSK41            | traJ     | 9.0e-56  | 48         |
| Bacillus anthracis              | pXO1             | topX     | 1.1e-40  | 49         |
| Methanococcus                   | Chromosomal     | topA     | 1.4e-25  | 50         |
| jannaschii                      |                  |          |         |            |
| Bacillus firmus                 | Chromosomal     | topA     | 9.5e-34  | 51         |
| Streptococcus agalactica        | pIP501           | orf2     | 8.7e-24  | 52         |
| Escherichia coli                | Chromosomal     | topA     | 4.7e-25  | 44         |

a C. M. Thomas, submitted for publication.

b Partial sequence.

c Representing identical plasmids isolated from different sources.

in vivo. The presence of a plasmid that carries the traE or topB gene in cells that contain a deletion of topA is sufficient to allow the disruption of the chromosomal copy of topB. This cannot be efficiently accomplished in the presence of plasmids that encode Topo I or an inactive Topo III polypeptide. Taken together, these data strongly suggest that the TraE protein is a true homologue of Topo III.

The role of the TraE protein in plasmid RP4 DNA metabolism is unclear. The genes encoding the essential enzymes for plasmid replication have been mapped and do not include traE (33–36). The gene is located in the primase gene cluster (5) and is embedded in a region that contains genes essential for plasmid transfer; however, traE has not been found to be essential for this process (36–38). The finding that the TraE protein is a true homologue of E. coli Topo III may explain why no function has been ascribed to the traE gene. The majority of studies concerning both RP4 plasmid replication and conjugative transfer have used E. coli as the host organism. In addition, many organisms may possess Topo III-like activities. It is not surprising that the role of TraE remains undefined when the host(s) contains a homologous enzyme that possesses analogous properties; however, although the TraE protein can substitute for Topo III in vivo, it remains to be seen whether the converse is also true. To address this issue, our laboratory is in the process of constructing a RP4 plasmid in which only traE has been deleted. The replication and transfer properties of this plasmid will be examined in isogenic strains in which topB remains intact or has been disrupted.

In addition to the plasmid RP4 gene product, a search of the NCBI nonredundant data base using the BlastP program (39) revealed that there are 12 additional open reading frames that have been isolated from different sources. Given the high degree of similarity among these polypeptides (39), the gene symbol and references for each homologue are given. Protein sequence alignments of many of these polypeptides have been published previously (40). hyp.prt., hypothetical protein.
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