Large Scale Purification and Characterization of TraI Endonuclease Encoded by Sex Factor Plasmid R100*

(Received for publication, December 23, 1994, and in revised form, May 25, 1995)

Hirokazu Fukuda and Eiichi Ohtsubo‡
From the Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

The TraI protein encoded by plasmid R100 was purified in a large scale by monitoring the strand- and site-specific nicking activity at the origin of transfer, oriT. The N-terminal amino acid sequence of the purified protein was identical to that deduced from the DNA sequence of an open reading frame encoding TraI. The TraI protein is a DNA helicase which is highly processive and unwinds DNA in the 5’ to 3’ direction. The Stokes radius and the sedimentation coefficient for the TraI protein in 200 mM NaCl indicate that the protein is a rod-shaped monomer, whose native molecular weight is 186,000. Chemical cross-linking analysis revealed that there exist more dimers of TraI under the low salt conditions, under which both nicking and unwinding reactions catalyzed by TraI are the most efficient, indicating that the TraI protein is functionally active in a dimer form. TraI hardly introduced a nick into the linearized plasmid DNA and only slightly into the relaxed closed circular DNA, indicating that TraI requires superhelical structure of substrate DNA for the nicking reaction. Delletion analysis in the oriT region revealed that a particular region of 54 base pairs containing oriT is required for the nicking reaction.

Conjugation is the process in which DNA is transferred from one bacterial cell harboring a sex factor plasmid, such as F or R100, to another by cell-to-cell contact (for the most recent review, see Frost et al., 1994). One of the initial events in DNA transfer is the strand- and site-specific nicking at the origin of transfer, oriT, by the plasmid-specific endonuclease that is the tral gene product whose molecular mass is estimated to be about 192 kDa from the nucleotide sequence (Yoshioka et al., 1990; Bradshaw et al., 1990). The TraI protein encoded by plasmid F or R100 has been purified by monitoring its ATPase activity or by its molecular size, respectively. The TraI proteins purified introduce the strand- and site-specific nick, such that it is covalently linked with the 5’ end of the nick (Inamoto et al., 1991, 1994; Reygers et al., 1991; Matson and Morton, 1991). The TraI proteins have been known to be DNA helicase (Abdel-Monem and Hoffman-Berling, 1976; Inamoto et al., 1994), which is supposed to unwind the duplex DNA from the nick introduced in the plasmid to provide the single-stranded DNA (Abdel-Monem et al., 1983), which is known to be transferred to the recipient cell (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968).

In this paper, we purified the TraI protein encoded by R100 by monitoring the site- and strand-specific endonuclease activity and extensively analyzed the nicking and unwinding reactions catalyzed by TraI. We show that a dimer form of the TraI protein is active for both nicking and unwinding reactions, and that the superhelical plasmid DNA molecules with a specific region of 54 bp are required for the nicking reaction.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Escherichia coli K12 strains used were E. coli M109 (Yanisch-Perron et al., 1985), CSR603 (Sancar et al., 1985), MV1184 (Vieira and Messing, 1987), and BW313 (Casadaban and Cohen, 1980).

Plasmids, pUC119 (Vieira and Messing, 1987), pS187-XE1 (Inamoto et al., 1988), and pYY35-1 (Inamoto et al., 1991), were derivatives of pUC18 or pUC19 (Yanisch-Perron et al., 1985) with a DNA segment of plasmid R100. Plasmid pHF11 was constructed by inserting a 236-bp BamHI fragment containing oriT of R100 into pUC18. The BamHI fragment was synthesized by polymerase chain reaction using a pair of primers with a BamHI site which flank the oriT region, followed by digestion of the polymerase chain reaction-amplified fragments with BamHI. The other pHF plasmids used were constructed as described under “Results.”

Media—Culture media used were L broth, L-rich broth, 2 × YT medium, and 0.5–medium (Yoshioka et al., 1987). O.5–Medium was used for transformation of plasmid DNA (Yoshioka et al., 1987).

Reagents and Enzymes—Deoxyribonucleoside 5’-triphosphate and ATP were purchased from Yamasai TRNA (type XX; Sigma) and bovine serum albumin (Seikagaku Kogyo) were used. [γ-32P]ATP (>5000 Ci/mmol) and [α-32P]dATP (6000 Ci/mmol) were purchased from Amer sham. Molecular size standards for proteins were obtained from BioRad or Pharmacia Biotech. SDS and dithiothreitol were purchased from Nakarai. Glutaraldehyde solution 10% (electron microscope grade) was purchased from Wako.

The Klenow fragment of DNA polymerase I and T4 polynucleotide kinase were obtained from Takara. Restriction endonucleases, BamHI and HindIII (New England Biolabs), were used. Single-stranded DNA-binding protein (SSB) was obtained from Promega. RNase A and proteinase K were obtained from Sigma and Boehringer Mannheim, respectively. They were used as recommended by their suppliers.

DNA Preparation—Covalently closed circular plasmid DNA was used for in vitro nicking experiments were isolated from large-scale cultures, as described (Ohtsubo et al., 1978). An alkaline lysis method (Yoshioka et al., 1987; Maniatis et al., 1982) was used to prepare a small amount of plasmid DNA from large numbers of cell cultures. Single-stranded DNA of pUC119 or its derivative was prepared as described previously (Vieira and Messing, 1987).

Oligonucleotides—Oligonucleotides listed in Table I were synthesized using Applied Biosystems 392 DNA/RNA Synthesizer and purified by reversed-phase chromatography or by elution from a denaturing polyacrylamide gel after electrophoresis.

Nicking Reaction—The standard nicking reaction was performed as follows: 2 μl of the covalently closed circular pHF11 DNA (37 fmol) in a solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) was mixed with 3 μl of 5 × buffer N (150 mM Tris-HCl (pH 7.4), 25 mM MgCl2, 5 mM dithiothreitol, 50 μg of bovine serum albumin/ml) and 8 μl of Milli-Q water. After preincubation for 5 min at 37 °C, the reaction was initiated by adding 2 μl of the TraI protein in buffer A containing 200 mM NaCl and 20% glycerol. The mixture was incubated at 37 °C for 30 min, and the

* This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Tel.: 81-3-3812-2111; Fax: 81-3-3812-3269.

† The abbreviations used are: bp, base pair(s); SSB, single-stranded DNA-binding protein; IHF, integration host factor; nt, nucleotide(s).
reaction was stopped by adding 5 μl of the stop solution (2% SDS, 33 mM EDTA). After addition of 1 μl of proteinase K (20 mg/ml), the mixture was further incubated at 30°C for 30 min. DNA in the mixture was then extracted with phenol/chloroform and precipitated with ethanol. After digestion of the sample DNA with BamHI, the 5' ends of the DNA fragments were labeled with exchange reaction using T4 polynucleotide kinase and [γ-32P]ATP (see Fig. 1) using the Megalabel kit (Takara). The 32P-labeled fragments were denatured and electrophoresed in a 6% polyacrylamide sequencing gel containing 7 M urea. The radioactivity of the proteins were pooled and dialyzed against buffer A containing 50 mM NaCl.

After addition of 1 mM P11 column (2.5 cm × 7.6 cm; Whatman) equilibrated with buffer A, and the column was washed with 3 column volumes of the same buffer. Proteins were eluted with a 400 ml of 0–1.0 M NaCl linear gradient in buffer A. The fractions (0.19–0.31 M NaCl) with the TraI endonuclease activity were pooled and dialyzed against buffer A containing 50 mM NaCl. The dialysate (Fraction V) was applied to a HiTrap Heparin (10 ml; Pharmacia)columnequilibrated with buffer A containing 200 mM NaCl, and the column was further incubated at 30°C for 30 min. DNA in the mixture was obtained and stored at −20°C.

Determination of Stokes Radius—Stokes radius was determined by electrophoresis in SDS-8% polyacrylamide gels according to Laemmli (1970). The protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as standard.

TABLE 1

| Primer | Sequencea | Positionb |
|--------|-----------|-----------|
| P-mcs  | 5'-GAATTCGAGCTCGTACCCGCCCAGTCTAGAGGCGAGGCAATGAC-3' | +396 ~ +446 |
| P-52   | 5'-GTTTCTCTGACCAAAAGGACC-3' | -24 ~ -1 |
| P-53   | 5'-GTTGCTTCTGAGTTGAGGCAACC-3' | -24 ~ -24 |
| P-54   | 5'-GTTGCTTCTACCAAAAGGACCACACACTACGCAAAAACA-3' | -24 ~ +18' |
| P-55   | 5'-caccctcacagacacgtGTTGCACTTGTGGTGAAGACCAC-3' | +18 ~ -24 |

a Small letters indicate transition mutations such that G, A, T, and C were converted to T, C, G, and A, respectively, in the original sequence in the region +18 to +1.

b Coordinates to P-mcs are of pUC119 (Vieira and Messing, 1987). Coordinates to the other primers are of R100 (see Fig. 8).

Fig. 1. Specific nicking by the TraI protein. A, schematic representation of the structure of the BamHI fragment (236 bp) with or without a nick. Asterisks indicate the 32P-labeled 5' ends of DNA strands (thick lines). The fragment was obtained by digestion with BamHI of the pHF11 DNA after treatment of the DNA with the TraI protein under the conditions described under "Materials and Methods." Note that the 5' end of the nick is attached by the TraI protein (open circle) and thus cannot be labeled with 32P. B, a 6% polyacrylamide sequencing gel showing single-stranded DNA fragments generated from the BamHI fragment after denaturation of the sample DNA with heat. Lane 1, the sample DNA treated with TraI; lane 2, the sample DNA not treated with TraI. Lanes marked with M indicate sequencing ladders used as size markers. Note in lane 1 that a fragment of 143 nt is generated due to nicking by TraI at oriT (see A).

Five-drop fractions were collected, and the concentration of proteins was determined by SDS-polyacrylamide gel electrophoresis or by the Bio-Rad protein assay.

Chemical Cross-linking—Chemical cross-linking was carried out as described (Runyon et al., 1993) with the following modification: cross-linking of TraI (2.4 μM monomer) was carried out at 24°C in buffer A or in the nicking reaction buffer containing 10–275 mM NaCl after preincubation for 10 min at 24°C. Cross-linking was initiated by addition of 1% glutaraldehyde to 0.1% and quenched after 30 min by addition of 100 mM lysine chloride to 10 mM. Products were analyzed by SDS-5% polyacrylamide gel electrophoresis.

Helicase Assay—The substrate used for the helicase assay (see Fig. 1).
A 4A oligonucleotide (P-mcs) labeled with 32P was prepared as follows. Two times molar excess of the 51-mer oligonucleotide (P-mcs) labeled with 32P at the 5'-end was mixed with single-stranded DNA of pUC19 in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and the mixture was heated at 65°C for 5 min, cooled down slowly (−1°C/min) to room temperature, and applied to QIAGEN tip20 (QIAGEN Inc.) to remove non-annealed oligonucleotides. The DNA sample was dissolved in TE buffer.

The standard helicase reaction was performed as follows: 0.5 μl of the substrate (50 fmol) in TE buffer was mixed with 2 μl of 5× buffer N, 0.7 μl of 20 mM ATP, and 5.8 μl of Milli-Q water. After preincubation for 5 min at 30°C, the reaction was initiated by adding 1 μl of the TraI protein in buffer A containing 200 mM NaCl and 20% glycerol. The mixture was incubated at 30°C for 10 or 5 min, and the reaction was stopped by adding 2 μl of the stop solution (50 mM EDTA, 0.6% SDS, 40% glycerol, 0.12% bromphenol blue). The reaction mixture was electrophoresed on a nondenaturing 8% polyacrylamide gel in TAE buffer (40% glycerol, 0.12% bromphenol blue). The reaction mixture was electrophoresed on a nondenaturing 8% polyacrylamide gel in TAE buffer.

TABLE II
Purification of the TraI protein

| Property               | cell lysate | Phosphocellulose | Mono Q | Superox 200pg | HiTrap Heparin |
|------------------------|-------------|------------------|--------|---------------|---------------|
| Protein (mg/ml)        | 1.0         | 3.5              | 3.8    | 3.9           | 4.1           |
| Activity (units/mg)    | 100         | 100              | 100    | 100           | 100           |
| Specific activity      | 1.0         | 1.0              | 1.0    | 1.0           | 1.0           |

a One unit was defined as the amount of the enzyme required to produce 1 fmol of the nicked molecules in 30 min.
b A, relative values calculated from specific activities; B, relative values calculated from the amount of proteins which was determined by scanning SDS-polyacrylamide gels.
c A, relative values based on the nicking activity; B, relative values calculated from the amount of TraI protein.

The TraI covalently linked to its 5' end (see Fig. 1). It should be noted that nicking results in the generation of a single strand of the plasmid (Inamoto et al., 1994). Plasmid pHF11 carries a 236-bp BamHI fragment containing oriT on a specific strand of the plasmid. TraI introduces the nick at oriT in the BamHI fragment in this plasmid, resulting in generation of the single-stranded DNA of 143 nt. Therefore, the TraI protein was subsequently digested with BamHI, labeled with 32P at the 5'-end, and denatured (see Fig. 1). It should be noted that nicking results in generation of another fragment of 92 nt that also could not be labeled with 32P because of the presence of the TraI covalently linked to its 5'-end (see Fig. 1).

Using this method of detection of the site- and strand-specific endonuclease activity of the TraI protein, we tried to purify the TraI protein in a large scale by column chromatography from the crude lysate of cells harboring plasmid pYY35–1 (traI+), overproducing the TraI protein. The crude cell lysate contained the TraI protein in an amount greater than 20% of total protein as estimated by SDS-polyacrylamide gel electrophoresis, but did not show any nicking activity (Table II). However, a fraction obtained from phosphocellulose column showed the nicking activity (Table II), suggesting that the initial crude lysate contained either an inhibitor(s) or a nuclease(s) causing degradation of the substrate DNA. The TraI protein in the phosphocellulose column fraction could be purified to homogeneity through MonoQ FPLC, Superdex 200pg, and HiTrap Heparin columns (see Table II). The specific activity of the TraI protein in the phosphocellulose column fraction could be purified to homogeneity (Table II), suggesting that the phosphocellulose fraction still contained an inhibitor(s) or a nuclease(s). In phosphocellulose, Superdex 200pg, and HiTrap Heparin columns, the specific activity of the TraI protein was determined as described under “Materials and Methods.”

![Table II](image)

**TABLE II**

Purification of the TraI protein

| Property               | mg | units | units/mg | Specific activity | Purification | Yield |
|------------------------|----|-------|----------|------------------|--------------|-------|
| Total protein          | 250| 1.0   | 1.0      | 1.0              |              | 100   |
| Total activitya        | 1.0| 1.0   | 1.0      | 1.0              | 1.0          | 100   |
| Specific activity      | 1.0| 1.0   | 1.0      | 1.0              | 1.0          | 100   |

a One unit was defined as the amount of the enzyme required to produce 1 fmol of the nicked molecules in 30 min.
b A, relative values calculated from specific activities; B, relative values calculated from the amount of proteins which was determined by scanning SDS-polyacrylamide gels.
c A, relative values based on the nicking activity; B, relative values calculated from the amount of TraI protein.

**RESULTS**

Purification of the TraI Protein—The TraI protein of plasmid R100 has been shown to introduce a nick at oriT on a specific strand of the plasmid (Inamoto et al., 1994). Plasmid pHiF11 carries a 236-bp BamHI fragment containing oriT of R100 (see Fig. 1). TraI introduces the nick at oriT in the BamHI fragment in this plasmid, resulting in generation of the single-stranded DNA of 143 nt long when the plasmid DNA was subsequently digested with BamHI, labeled with 32P at the 5'-end, and denatured (see Fig. 1). It should be noted that nicking results in generation of another fragment of 92 nt also could not be labeled with 32P because of the presence of the TraI covalently linked to its 5'-end (see Fig. 1).

Using this method of detection of the site- and strand-specific endonuclease activity of the TraI protein, we tried to purify the TraI protein in a large scale by column chromatography from the crude lysate of cells harboring plasmid pYY35–1 (traI+) overproducing the TraI protein. The crude cell lysate contained the TraI protein in an amount greater than 20% of total protein as estimated by SDS-polyacrylamide gel electrophoresis, but did not show any nicking activity (Table II). However, a fraction obtained from phosphocellulose column showed the nicking activity (Table II), suggesting that the initial crude lysate contained either an inhibitor(s) or a nuclease(s) causing degradation of the substrate DNA. The TraI protein in the phosphocellulose column fraction could be purified to homogeneity through MonoQ FPLC, Superdex 200pg, and HiTrap Heparin columns (see Table II). The specific activity of the TraI protein increased 6.2 times through the purification (Table II).

In these purification steps, the specific activity of the fraction obtained using MonoQ column was 4.6 times greater than that of the fraction obtained from phosphocellulose column. However, the relative amount of TraI estimated by scanning SDS-polyacrylamide gels increased only 1.1 times (Table II). This suggests that the phosphocellulose fraction still contained an inhibitor(s) or a nuclease(s). In phosphocellulose, Superdex 200pg or MonoQ column chromatography, the nicking activity corresponded to the single peak of protein mass (see Fig. 2A for the result of Superdex 200pg column chromatography). In HiTrap Heparin column chromatography, however, the profile of the nicking activity showed two peaks, which corresponded to those in the profile of protein concentration (Fig. 2B). We assume that this is due to multimerization of the TraI protein, as will be discussed later.

We carried out amino acid sequencing analysis of the purified TraI protein and found that the N-terminal amino acid sequence was MLSFSVV... which is identical to that deduced from the nucleotide sequence of an open reading frame for the tral gene (Yoshioka et al., 1990). This in turn indicates that the N terminus of the TraI protein is neither blocked nor processed.

**Physicochemical Properties of TraI**—The molecular mass of

![Figure 2](image)
the TraI protein encoded by R100 has been estimated to be 192 kDa from the nucleotide sequence of the trAI gene (180 kDa) and by SDS-polyacrylamide gel electrophoresis of the protein purified previously by monitoring its molecular size. Positions of monomers (186 kDa), dimers (372 kDa), and multimers of TraI are indicated on the left side of the gel. Molecular size standards used were ferritin (440 kDa), catalase (230 kDa), aldolase (158 kDa), and bovine serum albumin (66.2 kDa). B, relative amounts of monomers, dimers, and multimers of TraI under the various concentrations of NaCl, as determined by densitometric scan of the gel shown in A using EPSON GT-6000 scanner and NIH image.

FIG. 3. Cross-linking analysis of the TraI protein. A, an SDS-5% polyacrylamide gel stained with Coomassie Brilliant Blue, showing the TraI protein chemically cross-linked under the various concentrations of NaCl. Lane 1, TraI not cross-linked; lanes 2-8, cross-linked TraI at the salt concentration indicated at the bottom. Cross-linking was carried out as described under “Materials and Methods.” Positions of monomers (186 kDa), dimers (372 kDa), and multimers of TraI are indicated on the left side of the gel. Molecular size standards used were ferritin (440 kDa), catalase (230 kDa), aldolase (158 kDa), and bovine serum albumin (66.2 kDa). B, relative amounts of monomers, dimers, and multimers of TraI under the various concentrations of NaCl, as determined by densitometric scan of the gel shown in A using EPSON GT-6000 scanner and NIH image.

The TraI protein encoded by R100 has been estimated to be 192 kDa from the nucleotide sequence of the trAI gene and 180 kDa by SDS-polyacrylamide gel electrophoresis of the protein purified previously by monitoring its molecular size. Positions of monomers (186 kDa), dimers (372 kDa), and multimers of TraI are indicated on the left side of the gel. Molecular size standards used were ferritin (440 kDa), catalase (230 kDa), aldolase (158 kDa), and bovine serum albumin (66.2 kDa). B, relative amounts of monomers, dimers, and multimers of TraI under the various concentrations of NaCl, as determined by densitometric scan of the gel shown in A using EPSON GT-6000 scanner and NIH image.

Unwinding Reaction by TraI—We have recently demonstrated that TraI encoded by R100 is a DNA helicase (Inamoto et al., 1994). We quantitatively analyzed the helicase activity of TraI purified as described above using the single-stranded pUC119 DNA annealed with the 51-mer oligonucleotide labeled with 32P at its 5' end as substrate (see Fig. 4). The unwinding reaction by TraI was found to reach a plateau in several minutes (Fig. 5A, a). Eighty percent of the substrate added were unwound by addition of 2.2 TraI molecules per substrate DNA molecule (Fig. 5A, b). The optimal NaCl concentration was 20 mM, and the efficiency of the reaction was reduced to one-hundredth in 100 mM NaCl (Fig. 5A, c). These results combined with the data shown in Fig. 3 suggest that the TraI helicase is functionally active in a dimer form.

Lineweaver-Burk plotting of the data gave Vmax of 0.47 nM/min and K1/2 of 6.6 nM at 30°C, indicating efficient binding of TraI to the substrate DNA. The value of Vmax corresponds to an unwinding velocity of 290 bp/s, which means that the unwinding reaction is immediately completed once the reaction starts. From these values, we calculated kcat (turnover) to be 0.12/min and kcat/K1/2 to be 1.9 × 107 M−1 min−1. The values indicate that TraI does not rapidly turn over. These results show that the TraI helicase is highly processive.

We examined several parameters for the unwinding reaction. The unwinding reaction occurred most efficiently at 37°C. Optimal pH was 7.2. The unwinding reaction absolutely required Mg2+ and ATP. The optimal concentration of Mg2+ is 5

![FIG. 4. Helicase assay for the TraI protein. A, schematic representation of the substrate DNA used for the helicase assay. The asterisk indicates the 5' end of the 51-nt oligonucleotide labeled with 32P. B, an 8% polyacrylamide gel, showing the DNA unwinding activity of the TraI protein. The activity was assayed by monitoring production of the 32P-labeled oligonucleotides by the conditions described under “Materials and Methods.” Positions of the substrate and the product are indicated. Lane 1 shows the substrate DNA denatured at 95°C. Lanes 2-9 show the sample DNA treated with increasing amounts of the TraI protein (0, 15, 30, 60, 120, 240, 480, and 960 fmol, respectively).](image-url)
TraI Endonuclease Encoded by Plasmid R100

Concentration of NaCl over 100 mM (Fig. 5).

Thenicking reaction efficiently occurred under low salt, but not under high salt; nicking could hardly be detected at the concentration of NaCl over 100 mM (Fig. 5). The helicase and nicking activities were assayed by the conditions described under “Materials and Methods.”

FIG. 5. Unwinding reaction (A) and nicking reaction (B) by TraI. a, time course of the unwinding or nicking reaction. Concentrations of TraI and substrate used were 11 and 5 nM, respectively. b, stoichiometry of the unwinding or nicking reaction. c, effect of the NaCl concentration on the unwinding or nicking reaction. Concentrations of TraI and substrate used were 22 and 5 nM, respectively. The helicase and nicking activities were assayed by the conditions described under “Materials and Methods.”

We also determined the direction of DNA unwinding by Tral using the substrate, α or β, which is a 24-bp duplex with the 5'- or 3'-overhang of 18 nt, respectively (Fig. 6A). TraI unwound substrate α with the 5'-overhang very efficiently, but unwound substrate β with the 3'-overhang very poorly (Fig. 6B). This indicates that TraI unwinds DNA in the 5' to 3' direction. This agrees with the direction of unwinding by TraI (Helicase I) encoded by F (Lahue and Matson, 1988).

Nicking Reaction Catalyzed by TraI—The nicking reaction catalyzed by TraI reached a plateau essentially in a relatively short time (Fig. 5B, a). However, nicking did not occur beyond 55% of substrate molecules even upon long incubation of the reaction mixture and/or addition of much TraI protein (Fig. 5B, b). The nicking reaction efficiently occurred under low salt, but not under high salt; nicking could hardly be detected at the concentration of NaCl over 100 mM (Fig. 5B, c). This indicates that the TraI nickase is active in a dimer form.

We then examined several parameters for the nicking reaction. The nicking reaction occurred most efficiently at 37 °C. Optimal pH was 7.6. The optimal concentration of Mg2+ was 10 mM. Unlike the unwinding reaction, the nicking reaction occurred in the absence of ATP. ATP rather inhibited the nicking activity, probably because ATP enhanced the helicase activity that leads to movement of TraI on DNA, resulting in inhibition of the nicking reaction. We also examined the effect of SSB on the nicking reaction. Interestingly, SSB inhibited the nicking reaction to 33% at 13 mM (5.4 times over substrate) and to 7% at 210 mM (87 times over substrate).

Superhelical Structure and DNA Sequence Required for the Nicking Reaction by TraI—To see whether TraI binds to a specific DNA sequence around oriT, we carried out gel-shift assay and filter-binding assay using the restriction fragments of plasmid DNA digested with several restriction enzymes. TraI was, however, found to bind not specifically to the fragment containing oriT (data not shown). Nicking did not occur when the plasmid DNA linearized with a restriction enzyme, such as HindIII which cleaves the DNA at a single site, was used as substrate (Fig. 7A). Nicking did not efficiently occur when the closed circular DNA was relaxed by treatment with topoisomerase I and used as substrate, while nicking occurred very efficiently when superhelical DNA was used as substrate (Fig. 7A). When the nicking reaction was carried out using the superhelical DNA in the presence of ethidium bromide, the nicking efficiency was decreased or increased in a manner dependent on the concentration of ethidium bromide (Fig. 7B). These results show that TraI requires superhelical DNA as substrate for the nicking reaction.

As described above, the sequence recognized by TraI could not be determined using the linear fragments of plasmid DNA. To define the region required for nicking by TraI, therefore, we constructed a set of plasmids with a deletion in the oriT region (see Fig. 8) and examined them to see whether TraI introduces

FIG. 6. Direction of DNA unwinding by TraI. A, substrate DNA, α and β, used to determine the direction of DNA unwinding by TraI. Asterisks indicate the 5' ends labeled with 32P. B, a 10% polyacrylamide gel, showing the unwinding activity of the TraI protein. Substrate α or β was incubated with TraI, and the products were analyzed using the conditions described under “Materials and Methods.” Note that substrate α was unwound, whereas substrate β was not unwound, demonstrating that TraI travels in the 5' to 3' direction on single-stranded portion of the substrate and unwinds the double-stranded DNA.
nicking at oriT indicates that this 54-bp region is recognized by TraI for the nicking reaction. The nicking activity was assayed as described under "Materials and Methods." B, nicking of superhelical pHF11 DNA in the presence of ethidium bromide. The nicking reaction was initiated by adding 2 µl of the TraI protein (477 fmol/µl) after addition of ethidium bromide to the reaction mixture and preincubation for 12 min.

Fig. 7. DNA substrate required for the nicking reaction. A, nicking at oriT on superhelical DNA (closed circles), relaxed closed circular DNA (open circles), or linearized DNA (closed squares) of plasmid pHF11. The nicking activity was assayed as described under "Materials and Methods." B, nicking of superhelical pHF11 DNA in the presence of ethidium bromide. The nicking reaction was initiated by adding 2 µl of the TraI protein (477 fmol/µl) after addition of ethidium bromide to the reaction mixture and preincubation for 12 min.

Fig. 8. The essential region for nicking by TraI. A critical portion of the oriT region is shown at the top. Numbers are nucleotide positions defining the 3' end of the nick at +1, and the 5' end of the nick as -1. Inverted repeat sequences are indicated by pairs of arrows between two DNA strands. The IHF-binding site, IhfA (Inamoto et al., 1990), and the TraI-binding site, sbyA (Inamoto and Ohtsubo, 1990), are shown by hatched and open boxes, respectively. The pHF plasmids carry a DNA segment from the oriT region of R100 (thick lines). These DNA segments were synthesized by polymerase chain reaction using oligonucleotide primers with a BamHI site. The polymerase chain reaction-amplified fragments were digested with BamHI and cloned into the BamHI site of pUC18. The results of nicking by TraI are shown on the right side of the panel.

a nick or not. As shown in Fig. 8, a particular region of 54 bp containing oriT was found to be essential for nicking. This indicates that this 54-bp region is recognized by TraI for the nicking at oriT.

DISCUSSION

In this paper, we have shown that the TraI protein of R100 is a rod-shaped molecule with molecular weight 186,000. This is consistent with the result obtained by electron microscopy that the protomer of TraI protein of plasmid F is rod-shaped (Abdel-Monem et al., 1977). We have also shown here that the TraI protein forms dimers under the low salt conditions and monomers under the high salt conditions, and that dimers of TraI are active in both nicking and unwinding reactions. Previous stoichiometric analysis has suggested that a multimer consisting of about 70 to 90 monomers of TraI of F shows the highest helicase activity (Kuhn et al., 1978; Benz and Muller, 1990), and that 10 molecules of TraI of F per DNA molecule show the highest helicase activity although substantial activity was detected in the reaction involving a 1:1 ratio of enzyme molecules to DNA molecules (Lahue and Matson, 1988). These results differ from ours obtained for TraI of R100. It is noted here, however, that several other helicases, except one, form either dimers or hexamers (Lohman, 1992). Rep helicase and helicase II (UvrD) have been shown to be dimerized upon binding to either single-stranded or duplex DNA (Wong et al., 1992; Runyon et al., 1993). This reminds us the result that in the purification procedure of TraI protein, chromatography using the HiTrap Heparin column showed two peaks, although chromatography using other columns showed a single peak (see Fig. 2). This may suggest that TraI was dimerized upon binding to heparin, a DNA analog.

We have shown here that the TraI protein introduces the nick into only a little over half of the substrate DNA even upon long incubation and/or addition of an excess amount of the TraI protein. It is possible that the nicking reaction requires some factors, which are not present in the reaction mixture. Such factors may include the tral gene product and the integration host factor IHF, which have been recently shown to stimulate the nicking reaction catalyzed by TraI (Inamoto et al., 1994) by binding to the sites (sbyA and ihfA) located immediately adjacent to the nicking site oriT (Inamoto and Ohtsubo, 1990; Inamoto et al., 1990). It might also be possible that TraI possesses the topoisomerase activity to convert superhelical DNA to relaxed closed circular DNA, which was shown in this paper to be poorly nicked by the TraI protein. However, the TraI protein has no topoisomerase activity, since the relaxed circular DNA molecules were not detected in an agarose gel containing chloroquine after electrophoresis of the sample treated with TraI (data not shown). We cannot, however, exclude the possibility that the nicked molecules with TraI covalently attached are still in a superhelical DNA form and can be readily converted to the superhelical DNA molecules without TraI, leading to an equilibrium state between two kinds of superhelical DNA molecules with and without TraI.

We have recently found that the TraI protein has the single-stranded DNA binding activity and cleaves the single-stranded DNA at oriT. This DNA cleaving activity is inhibited by SSB. These findings lead us to assume that the oriT region in the double-stranded DNA molecules melts locally, providing a single-stranded DNA portion which is cleaved by TraI. This assumption may be supported by our present result that the nicking reaction is strongly inhibited by SSB.

The nicking reaction occurs most efficiently under the low salt conditions, but it hardly occurs at high salt (100 mM NaCl or more), as described in this paper. The cleavage reaction of the single-stranded DNA by TraI occurs most efficiently under the low salt conditions like the nicking reaction, but it still occurs even at the NaCl concentration 100 mM unlike the nicking reaction. It is possible that the superhelical DNA, that is the substrate for the nicking reaction, cannot be melted locally under the high salt conditions, thus not providing any single-stranded DNA portion which is bound and cleaved by the TraI protein. Alternatively, dimers of TraI formed under the low salt conditions may form a DNA-protein complex at the partially melted region around oriT which leads to the cleavage reaction, but monomers formed under the high salt conditions may not form such a complex.

Acknowledgments—We thank Dr. H. Maki for generous suggestion for purification of the TraI protein and analysis of the purified protein. We also thank Dr. K. Horiuchi for critical reading of the manuscript.

REFERENCES

Abdel-Monem, M., Durwald, H., and Hoffmann-Berling, H. (1976) Eur. J. Biochem. 65, 433–449.

Abdel-Monem, M., Duppe, H. F., Kartenbeck, J., Durwald, H., and Hoffmann-

2 H. Fukuda and E. Ohtsubo, unpublished data.
Tral Endonuclease Encoded by Plasmid R100

21325

Berling, H. (1977) J. Mol. Biol. 110, 667–685
Abdel-Monem, M., Taucher-Scholz, M. G., and Klinkert, M. Q. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4659–4663
Benz, I., and Muller, H. (1990) Eur. J. Biochem. 189, 267–276
Bradshaw, H. D., Jr., Trayler, B. A., Minkley, E. G., Jr., Nester, E. W., and Gordon, M. P. (1990) J. Bacteriol. 172, 4127–4131
Casadaban, M. J., and Cohen, S. N. (1980) J. Mol. Biol. 138, 179–207
Frost, L. S., Ippen-Ihler, K., and Skurray, R. A. (1994) Microbiol. Rev. 58, 162–210
Inamoto, S., and Ohtsubo, E. (1990) J. Biol. Chem. 265, 6461–6466
Inamoto, S., Yoshioka, Y., and Ohtsubo, E. (1988) J. Bacteriol. 170, 2749–2757
Inamoto, S., Abo, T., and Ohtsubo, E. (1990) J. Gen. Appl. Microbiol. 36, 287–293
Inamoto, S., Yoshioka, Y., and Ohtsubo, E. (1991) J. Biol. Chem. 266, 10086–10092
Inamoto, S., Fukuda, H., Abo, T., and Ohtsubo, E. (1994) J. Biochem. (Tokyo) 116, 834–844
Kuhn, B., Abdel-Monem, M., Krell, H., and Hoffmann-Berling, H. (1978) J. Biol. Chem. 254, 11343–11350
Laemmli, U. K. (1970) Nature 227, 680–685
Lahue, E. E., and Matson, S. W. (1988) J. Biol. Chem. 263, 3208–3215
Lohman, T. M. (1992) Mol. Microbiol. 6, 5–14
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 90–91, 156, and 466–467, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Matson, S. W., and Morton, B. S. (1991) J. Biol. Chem. 266, 16232–16237
Ohkii, M., and Tomizawa, J. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 631–638
Ohtsubo, E., Rosenbloom, M., Schremph, H., Goebel, W., and Rosen, J. (1978) Mol. & Gen. Genet. 159, 131–141
Reygers, U., Wessel, R., Muller, H., and Hoffman-Berling, H. (1991) EMBO J. 10, 2689–2694
Runyon, G. T., Wong, I., and Lohman, T. M. (1993) Biochemistry 32, 602–612
Rupp, W., and Ihler, G. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 647–650
Sancar, A., Hack, A. M., and Rupp, W. D. (1979) J. Bacteriol. 137, 692–693
Siegel, L. M., and Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362
Vieira, J., and Messing, J. (1987) Methods Enzymol. 153, 3–11
Wong, I., Chao, K. L., Bujalowski, W., and Lohman, T. M. (1992) J. Biol. Chem. 267, 7596–7610
Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
Yoshioka, Y., Fujita, Y., and Ohtsubo, E. (1990) J. Mol. Biol. 214, 39–53
Yoshioka, Y., Ohtsubo, H., and Ohtsubo, E. (1987) J. Bacteriol. 169, 619–623