Characterization of the F Plasmid TraJ Protein Synthesized in F' and Hfr Strains of Escherichia coli K-12*

(Received for publication, July 8, 1985)

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Using purified F plasmid TraJ protein (Cuozzo, M., Silverman, P., and Minkley, E. (1984) J. Biol. Chem. 259, 6659–6666), we prepared rabbit anti-TraJ protein antibodies to analyze for the first time the TraJ protein as it is synthesized in normal F' and Hfr conjugal donor strains. Using affinity-purified antibody, we identified the protein on immuno-overlay blots of whole cell proteins separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. In contrast to Silverman, P., and Minkley, E. (1984) J. Biol. Chem. 259, 6659–6666), we prepared rabbit anti-TraJ protein antibodies to analyze for the first time the TraJ protein synthesized in large quantity by heat-induced λ (traJ) lysogens, the TraJ protein synthesized in normal donor cells was soluble, even after sedimentation at 100,000 × g. The soluble protein was found with the cytoplasmic fraction after separation of cytoplasmic and periplasmic proteins. Velocity sedimentation analysis indicated an s20,w of 3.5 for the single molecular species composed of or including all the TraJ polypeptide in crude extracts. Quantitative analyses showed that conjugal donor strains normally contain 2000–4000 TraJ monomers/cell. However, that level depended on other plasmid and chromosomal genes.

The F plasmid TraJ protein plays a key role in the establishment and maintenance of conjugal DNA donor activity of F' strains of Escherichia coli K-12. In the absence of the TraJ protein, the remaining tra genes are not transcribed at levels adequate to maintain donor activity (Willetts, 1977). In vivo studies indicate that the TraJ protein stimulates transcription from at least two tra promoters (Gaffney et al., 1983; Fowler et al., 1983). TraJ protein synthesis or function is in turn controlled by host cellular functions (Silverman, 1985). TraJ protein synthesized in abnormal experimental conditions (Kennedy et al., 1977; Achtman et al., 1979; Manning and Achtman, 1979; Johnson and Willetts, 1983; Cuozzo et al., 1984) appears to be a component of the E. coli outer membrane, a location not readily compatible with a direct genetic regulatory role. However, there are no studies of the protein as it exists in normal amounts in healthy F' or Hfr strains. We previously purified the TraJ protein from λ (traJ) lysogens that overproduced the protein after heat induction (Cuozzo et al., 1984), with the principal goal of preparing an antiserum to detect and characterize the protein from physiologically normal DNA donors. We report here the first results of such studies. They show that the TraJ protein in crude extracts of normal donor cells is soluble, a property incompatible with an outer membrane location. Moreover, we find that the cellular level of the TraJ protein is unexpectedly high for a genetic regulatory protein and that the level is reduced in certain chromosomal mutants.

EXPERIMENTAL PROCEDURES

RESULTS

Immunologic Detection of the TraJ Protein—The immunochemical detection of the TraJ protein in a normal F' strain is shown in Fig. 1. The electrophoretic mobility of purified TraJ protein is shown to the left of lane 1; the TraJ protein was detected in extracts of cells containing the F' traJlacZ + plasmid JCFLO (lane 2), but not in extracts of F' cells (lane 1) or of cells containing JCFLO (lane 3), the traJ90 [Am] derivative of JCFLO.

F' transfer from a strain containing both F and R100, an F-like R plasmid, is repressed. The mechanism responsible for this effect appears primarily on traJ expression (Finnegan and Willetts, 1973; Sambucetti et al., 1982; Gaffney et al., 1983) and requires two trans-acting components: the plasmid-specific traJ gene product encoded by F and the traJ gene product supplied by R100 (Finnegan and Willetts, 1971; see Willetts and Skurray (1980) for a review). No TraJ protein could in fact be detected in cells containing JCFLO and R100 (Fig. 1, lane 6). Moreover, this effect of R100 depended on its finO locus, since a normal level of TraJ protein accumulated in cells containing JCFLO and R100 000 1, a derepressed, finO mutant of R100 (Fig. 1, lane 7). Note that the presence of R100, like the traJ90 mutation, affected only a single immunoreactive species, the TraJ polypeptide itself.

A strain containing R100 or R100 000 1, without an F plasmid, did not contain a protein cross-reactive with anti-TraJ protein antibodies, other than those detected in the corresponding strain without any plasmid (Fig. 1, lanes 4 and 5). This observation is consistent with genetic complementation data indicating that if R100 (or its very close relative R6-5) encodes a TraJ protein that molecule cannot substitute for the F plasmid TraJ protein (Willetts, 1971; Achtman et al.,

*This work was supported by Grants CA13330, GM11301, and HD07154 from the National Institutes of Health and Grant PCM8014274 from the National Science Foundation. 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 1754 solely to indicate this fact.

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with DNA-DNA hybridization data, indicating that R100 and F DNA lack sufficient homology in the region containing the F plasmid tral gene to form stable heteroduplexes, even under conditions of hybridization stringency that would tolerate up to 35% base pair mismatches (Sharp et al., 1973; see Achmann et al., 1978) for a summary.

Fractionation of the TraJ Protein of HfrH—As we previously reported, TraJ protein in an extract of a heat-induced λ(tral) lysogen quantitatively sedimented at 100,000 × g for 60 min, with outer membrane proteins (Cuozzo et al., 1984). However, the TraJ protein is not actually associated with the outer membrane. Thus, most of the TraJ protein also sedimented at 3,000 × g for 15 min, conditions which left nearly all the outer membrane protein in the supernatant fraction (data not shown). As noted under “Experimental Procedures,” low speed sedimentation is in fact an excellent purification step for TraJ protein from the overproducing strain. Sedimentation of the TraJ protein at very low centrifugal forces is especially efficient if the cells are concentrated before they are broken, conditions which probably favor the formation of very large aggregates. In fact, low speed pellet fractions enriched in TraJ protein, negatively stained, and examined under the electron microscope showed the absence of amorphous material, with no evidence for the presence of regular structures. The ρ subunit of RNA polymerase exhibits much the same property when it is overproduced (Gribskov and Burgess, 1983).

Once isolated in the pellet fraction, the TraJ protein was difficult to solubilize (12,000 × g for 10 min). Among a variety of agents tested, only 6 M guanidine HCl or 1% SDS were completely effective. Nonidet P-40 (2%), 3-[3-cholamidopropyl]dimethylammoni]1-propanesulfonate (10 mM), octylglucoside (30 mM), LiCl or NaCl (chloride salts, 2 M), 2-mercaptoethanol (5% v/v), deoxycholate (2%), or urea (6 M) were all ineffective.

In contrast to that produced after induction of λ(tral) lysogens, TraJ protein synthesized in strain HfrH in the exponential phase of growth was soluble. When HfrH cells were broken in the French Press and subsequently fractionated by differential sedimentation and detergent extraction as described by Cuozzo et al. (1984) for λ(tral) lysogens, the bulk of the TraJ protein (>90%) remained soluble after high speed sedimentation (data not shown). When extracts were prepared in 0.5 M NaCl, no TraJ polypeptide at all was detected in the high speed pellet fraction (Fig. 2A). The high speed supernatant fraction, containing all the TraJ polypeptide, was then analyzed by zone sedimentation as described under “Experimental Procedures.” That analysis showed that soluble TraJ protein sedimented as a single species (Fig. 2B).

Solubility and zone sedimentation of the TraJ protein in 0.3 M NaCl. A, a crude extract of HfrH was prepared and fractionated by differential sedimentation as described under “Experimental Procedures.” A portion of each fraction corresponding to 1 ml of the original culture was analyzed by immuno-overlay blot for the TraJ polypeptide. Lane 1, whole cell extract; lane 2, low speed supernatant fraction; lane 3, low speed pellet fraction; lane 4, high speed supernatant fraction; lane 5, high speed pellet fraction. B, the high speed supernatant fraction was subjected to zone sedimentation, as described under “Experimental Procedures.” One portion of each fraction (120 μl) was analyzed by SDS/polyacrylamide gel electrophoresis followed by immuno-overlay blot for TraJ and SSB polypeptides. These are shown for the top half of the gradient (fractions 10-19); no TraJ polypeptide was observed in fractions 1-9. RNA polymerase (s20w = 12.7; Burgess, 1969), identified by its β and β′ subunits, peaked in fraction 10. The s20w of the native SSB tetramer is 4.6 (Williams et al., 1983). The gradient containing the standard proteins was analyzed by SDS/polyacrylamide gel electrophoresis. Myoglobin (s20w = 4.2; Sober, 1968) peaked in fractions 15 and 16; human γ-globulin (s20w = 7.1; Sober, 1968) peaked in fractions 13 and 14.

DNA binding (SSB) protein of E. coli K-12. We chose this protein to validate our measurement because the sbb gene product (177 amino acids; Sancar et al., 1981) is in the same size range as the TraJ protein (229 amino acids; Fowler et al., 1983), a polyclonal antiserum is available (Chase et al., 1984), and its cellular level has been measured at 1000-1200 polypeptides/cell (Weiner et al., 1975; Williams et al., 1984). Fig. 3C shows an autoradiogram from an immuno-overlay blot containing proteins from 5 × 10^7 F' cells (lane 1) and purified SSB protein in amounts from 1-8 ng (lanes 2-5). Fig. 3D shows the peak areas as a function of the amount of SSB protein; the arrowhead indicates the peak area for SSB protein in lane 1. Using this information and a molecular weight of 19,000 for an SSB protein monomer (Sancar et al., 1981), we calculated a value of 1400 SSB protein monomers/cell, a value entirely consistent with previous estimates.

With the same approach (Fig. 3, A and B), we estimated the number of TraJ protein monomers/cell to be 1700, using a value of 27,000 for the molecular weight of the TraJ polypeptide (Fowler et al., 1983). In an independent assay using the same strain, we obtained a value of 2300. We also determined the TraJ contents of two Hfr strains to be 2800 monomers/cell (strain AE1031) and 3800 monomers/cell (strain HfrH).

Effect of Chromosomal Mutations on the TraJ Protein Content of Donor Strains—Several chromosomal mutations have been identified as affecting F plasmid-dependent DNA donor activity (for a review see Silverman, 1985). The cpxA2[Ts]
Proteins were subjected to SDS/polyacrylamide gel electrophoresis. Containing 0.2 mg/ml bovine serum albumin as carrier and with purified proteins were diluted from stock solutions through 2% SDS of cells. The blot was incubated with affinity-purified anti-TraJ protein antibodies. Exposure was for 6 days on Cronex film. Panel B, peak areas as a function of the amount of purified TraJ protein from panel A; the autoradiogram of the immunoblot was scanned at two sensitivities to capture a linear densitometer response over the entire range of SSB protein in panel C. The blot was incubated with affinity-purified anti-TraJ protein antibodies. Exposure was for 6 days on Cronex film. Panel D, peak areas as a function of the amount of purified SSB from panel C; the arrowhead indicates the peak area of SSB protein in lane 1 of Panel C.

Fig. 3. Quantitative analysis of the TraJ protein in an F' strain. Proteins in a whole cell extract of strain JC3272/JCFLO (culture optical density = 0.57) and increasing amounts of purified proteins were subjected to SDS/polyacrylamide gel electrophoresis. Purified proteins were diluted from stock solutions through 2% SDS containing 0.2 mg/ml bovine serum albumin as carrier and with siliconized tubes and pipette tips to minimize losses. Immunoblots were prepared as described under "Experimental Procedures." Autoradiograms (panels A and C) were scanned with a laser densitometer and peak areas were determined with a Numonics digital planimeter (Panels B and D). Panel A, lane 1 contained protein from 1 ml of cells. The blot was incubated with affinity-purified anti-TraJ protein antibodies. Exposure was for 6 days on Cronex film. Panel B, peak areas as a function of the amount of purified TraJ protein from panel A; the autoradiogram of the immunoblot was scanned at two sensitivities to capture a linear densitometer response over the entire range of TraJ protein used. Arrowheads indicate the peak area for the TraJ protein in lane 1 of panel A. Panel C, lane 1 contained protein from 0.2 ml of cells from the same culture used for panel A. The blot was incubated with rabbit (anti-SSB)-IgG (1:1000 dilution). Exposure was 18 h on Cronex film. Panel D, peak areas as a function of the amount of purified SSB from panel C; the arrowhead indicates the peak area of SSB protein in lane 1 of Panel C.

and cpxB1 alleles together reduce donor activity 30-fold at 34 °C and 300-fold at 41 °C (McEwen and Silverman, 1980a). As Fig. 4 shows, the same mutations result in a 4-fold reduction in the TraJ protein level at 34 °C and a 10-fold reduction at 41 °C, as measured by densitometry.

We also examined three sfrA alleles. Two alleles, sfrA4 and sfrA5, reduced F' donor activity to about 5% and 0.06% of the control sfrA* strain, respectively (Beutin and Achtman, 1979; Buxton and Drury, 1983). The third sfrA allele is a deletion (Roeder and Somerville, 1979); very similar deletion alleles reduced F' donor activity nearly 6 orders of magnitude (Buxton and Drury, 1983). All three mutant alleles led to a reduced level of TraJ protein (Fig. 5). However, whereas the three alleles reduced donor activity to vastly different degrees, their respective effects on the TraJ protein level in the cell were not that much different; even the sfrA deletion did not entirely eliminate the accumulation of apparently intact TraJ protein (compare lanes 1 and 7).

Discussion

These are the first studies of the TraJ polypeptide as it normally exists in conjugal DNA donor strains. The results show that the protein is soluble and fractionates with cytoplasmic rather than periplasmic or cell envelope proteins. Moreover, we previously showed that the TraJ polypeptide lacks a cleaved signal peptide characteristic of outer membrane proteins (Cuozzo et al., 1984). These properties are more compatible with an interior cellular location for the TraJ protein than one in the outer membrane and, hence, with a direct genetic regulatory role. This is not to argue that the cell envelope plays no role in the cellular function of the TraJ protein, but only that the TraJ polypeptide is not itself an intrinsic cell envelope component.

Zone sedimentation of HfrH extracts indicated that no TraJ protein co-sediments with RNA polymerase, arguing against the possibility that the TraJ protein acts in association with core RNA polymerase in a manner analogous to the chromosomal rpoD or rpoH gene products (Grossman et al., 1984). The TraJ protein could independently activate transcription initiation at specific tra DNA sequences (Raibaud and Schwartz, 1984) or it could act at a later stage of transcription to stimulate production of full length tra mRNA. In either case, the soluble 3.5 S species composed of or at least containing all the TraJ polypeptide in the cell is the first candidate for biologically active TraJ protein, so that these hypotheses are now testable in vitro.

The amount of TraJ polypeptide we measure, 2000-4000 TraJ polypeptides/donor cell, seems excessive on the hypothesis that the TraJ protein functions exclusively as a transcriptional activator. Other studies suggest that positive regulators of gene expression in E. coli are usually present at levels corresponding to only a few hundred polypeptides/cell at most (Raibaud and Schwartz, 1984), just high enough to provide maximal expression of their target operons under the appropriate physiological conditions (Hofnung et al., 1971; Haggerty et al., 1978). The crp gene product, like the TraJ protein, is present at several thousand polypeptides/cell; however, that level may reflect in part the large number of operons subject to crp protein activation. The TraJ protein, in contrast, should need to act at a small number of sites, perhaps only two (Gaffney et al., 1983; Fowler et al., 1983). Our quantitative experiments support earlier observations of Achtman et al. (1980), who suggested on the basis of inefficient suppression of traJ[Am]* alleles that donor cells require large amounts of the TraJ polypeptide, and of Kennedy et al. (1977), who noted the unexpectedly high rate of TraJ protein synthesis in vitro and in minicells. Possibly, the TraJ polypeptide has a function in donor activity in addition to its genetic regulatory role that would account for its unexpectedly high level.

The effects of the cpx and sfrA mutations also need to be incorporated into hypotheses about TraJ protein function. As shown above, these chromosomal mutations lead to reduced levels of the TraJ protein. Their effects on donor activity and surface exclusion (McEwen and Silverman, 1980a; Beutin and Achtman, 1979) can be explained entirely by that reduction, especially in view of the evidence that fairly large amounts of TraJ protein are important for the full expression of donor activity (Achtman et al., 1980).

Beutin et al. (1981) proposed that sfrA mutations reduce transcription initiation at traJ. However, their proposal was based on DNA-mRNA hybridization data that did not clearly separate traJ transcription from transcription of other tra genes. Subsequent studies using traJ-lacZ fusion genes (Sambucetti et al., 1982; Gaffney et al., 1983) and UV-irradiated cells infected with λ(t)raJ bacteriophage (Sambucetti et al., 1982) indicated that traJ transcription initiation and trans-
lotion initiation from traJ mRNA sites are not reduced in either sfrA or cpxA mutant cells. In addition, the traJ promoter is active in vitro in the presence of purified RNA polymerase
(Fowler et al., 1981); no auxiliary proteins are required. Hence, while the SfrA and CpxA polypeptides both function, perhaps together (Silverman, 1985), in maintaining the cellular level of the TraJ protein, the mechanism(s) by which they exert their effect remain to be identified.

Acknowledgments—We thank Dr. John Chase for material essential to use SSb protein as a standard in several experiments.

REFERENCES


Preparation of an anti-TraJ Antibody — Purified TraJ protein (1 mg) was dialyzed against 0.1 M Tris-buffered saline (pH 8.0, 0.1 M NaCl, 0.1% gelatin, 0.02% sodium azide) and concentrated to 1 mg/ml by lyophilization.


EXPERIMENTAL PROCEDURES

Materials — Bacterial strains, plasmids, and DNA were described previously, respectively, for E. coli (Collet, 1975), P. aeruginosa (Messing and Sinskey, 1980), and B. subtilis (Chen and Shah, 1980). DNA was isolated from E. coli, P. aeruginosa, and B. subtilis by published procedures and was checked for bacterial contamination. DNA from E. coli was digested with restriction enzymes and separated on agarose gels.

Western Blot — The proteins were transferred from a nitrocellulose filter to a nitrocellulose filter using a semi-dry blotter apparatus (Schleicher & Schuell, Keene, NH). After transfer, the nitrocellulose was blocked with 5% milk and stored in Tris-buffered saline at 4°C. The filters were blocked in Tris-buffered saline with 0.1% Tween-20, 0.02% sodium azide, and 0.1% gelatin.

The filters were then washed for 15 min by incubation with 0.1% Tween-20 saline solution containing 1% non-fat dry milk and 0.05% sodium azide, and were incubated with the primary antibody for 1 h at room temperature. After washing the filter for a second time, the filter was exposed to a second antibody, visualized with X-O-ray film. The filters were then washed at 20°C and exposed to Kodak X-O-Mat film.
TraJ Protein in E. coli Donor Strains

Zone sedimentation - Strain D242 was grown to an optical density of 0.3 in 200 ml of LB medium at 37°C. The cells were collected by sedimentation and suspended in 1 ml of buffer A (10 mM Tris, pH 7.5). The cells were broken by passage through a French pressure cell at 16,000 psi (while cell extract). The extract was fractionated into low-speed supernatant and pellet fractions by sedimentation at 10,000 g for 10 min. The low-speed supernatant fraction was further separated into high-speed supernatant and pellet fractions by sedimentation at 100,000 g for 30 min. A portion of the supernatant fraction (0.8 ml) was layered on a linear 10% to 30% glycerol gradient (11 ml) in buffer A containing 0.5 M NaCl. A companion sample (0.5 ml) contained 10 µg each of spermine, spermidine, hexamethylene diamine, adenosine, guanosine, cytidine, and uridine 3H-ribonucleotides, 3H-5'-AMP, 3H-5'-cGMP, 3H-GTP, 3H-UTP, and 3H-tRNA. A density of 1.56,000 r.p.m./min in the Beckman SW25 rotor. Twenty fractions were collected from each gradient (0.6 ml fraction); these were analyzed by 4% polyacrylamide gel electrophoresis followed either by staining with Coomassie Blue R-250 (Searl et al., 1984) or by immunoelectrophoresis blot. Protein Determination - Protein was measured routinely as described by Lowry et al. (1951), with bovine serum albumin as standard. To confirm the accuracy of this determination for purified TraJ protein, we compared the value obtained by the lowry assay with the value obtained by acid hydrolysis and amino acid analysis, with two different samples; the two values agreed to within about 10.

RESULTS

PLASMID: NONE JCP19 JCP58 RS1 RS5 RS160 RS161

Fig. 1. Immunologic detection of the TraJ protein in extracts of E. coli donors. Cell growth and the preparation of whole cell extracts and immunogens were as described in Experimental Procedures. Affinity-purified anti-TraJ antibodies were used as primary reagent and goat anti-rabbit IgG-125I, as secondary reagent. Protein corresponding to that in lane 1 of each culture (optical density 0.35 ± 0.05) applied to each lane. Exposure was for 20 days on Kodak XAR film. Lane 1: XH72; lane 2: XH72/CP10; lane 3: XH372/CP10; lane 4: AE2008/R100; lane 5: AE2098/R100; lane 6: AE2008/R100; lane 7: AE2008/R100. The electrophoretic mobility of purified TraJ protein is indicated to the left of lane 1.

The enhanced sensitivity with 125I-labeled secondary antibody revealed several immunoreactive species in addition to the TraJ protein. All of these species had a slower electrophoretic mobility than the TraJ protein and are therefore not TraJ protein fragments; nor can they be attributed to protein-bound lipopolysaccharide because we see no immunoactivity with free lipopolysaccharide, whose electrophoretic mobility is faster than the TraJ polypeptide (Kurlin et al., 1981). All but one of the other immunoreactive species were in the corresponding F' strain, and some of them could be correlated with the presence of the TraJ polypeptide or with TraJ-dependent expression of other F' plasmid tra genes. Two of them were detected with pBR-immune serum. A third, present only in the XCP10 and XCP40 strains (Fig. 1), was not detected in other donor strain backgrounds, e.g., that of A20811 (see below). Some was detected in the absence of primary antibody or in immunoperoxidase blots of purified TraJ protein. Some (or all) of these immunoreactive species may represent cellular proteins with antigenic homology to the F' plasmid TraJ protein; others may be abundant cellular proteins that we detect owing to a low level of contaminating antibodies and to the sensitivity of the immunoperoxidase assay. All of the immunoreactive species can be attributed to TraJ polypeptide fragments or aggregates. Figures 2-5 show only the region of immunoperoxidase blots containing the TraJ polypeptide.

Fig. 4. Effect of chromosomal mutations in gene gaaC and gaaE on the cellular level of TraJ protein. Whole cell extracts of Fif strains and immunogens were prepared as described in Experimental Procedures and the legend to Fig. 2. Exposures were seven days on Kodak XAR film. Aliquots of protein from 1 ml of each culture (optical density 0.35 ± 0.05) were applied per lane. The gaa genotype and growth temperature are indicated above each lane. Lanes 1 and 2: AE2101; lanes 2 and 3: A2105. The electrophoretic mobility of purified TraJ protein is indicated to the left of lane 1.

Fig. 5. Effect of chromosomal mutations in gene gaa on the cellular levels of the TraJ protein. See the legend to Fig. 6 for details. Exposure was 5 days on Kodak XR film. The gaa genotypes and F DNA contents are indicated above each lane. Lanes 1: XH72 (F'); lane 2: XH372/CP10; lane 3: XH372/CP10; lane 4: XH147/CP10; lane 5: XH176/CP10; lane 6: HR8 strain 37-1; lane 7: HR8 strain 122-1.