JOURNAL OF BACTERIOLOGY, Oct. 1996, p. 5787–5789 Vol. 178, No. 19
0021-9193/96/$04.00+0

NOTES

Effect of *traY* Amber Mutations on F-Plasmid *traY* Promoter Activity In Vivo

PHILIP M. SILVERMAN* AND APRIL SHOLL

Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Received 17 May 1996/Accepted 4 August 1996

We have examined the effect of the F plasmid TraY protein on *tra* gene expression in vivo. Expression was assayed as alkaline phosphatase activity in cells containing a *traY* Φ(*traA′-′phoA*)hyb operon under *traY* promoter control. Amber mutations in *traY* significantly reduced alkaline phosphatase activity. Since nonsense polarity effects were minimal, if they occurred at all, these data provide the first direct evidence that TraY regulates *tra* gene expression.

F plasmid DNA transfer (*tra*) gene expression is controlled by a network of host (*Escherichia coli*)- and plasmid-encoded proteins (25, 26). This network is surprisingly complex, and its physiological basis and molecular mechanisms have yet to be elucidated.

Recent reports have suggested yet another unexpected regulatory feature of *tra* gene expression. The TraY proteins encoded by the F-like R factor R100 (14) and by F itself (20) bind in vitro to DNA sites very close to their cognate *traY* promoters. These observations suggested that TraY regulates its own synthesis and that of other genes under *traY* promoter control.

Such a function would not be surprising on structural grounds. TraY belongs to a family of genetic regulatory proteins that are distinguished by DNA-binding domains composed of antiparallel β-sheets and including the Arc, Mnt, and MetJ repressor proteins (4, 5, 23, 27). However, direct evidence that TraY has a genetic regulatory function is lacking. Frost et al. (10), citing unpublished results, noted that insertion of an antibiotic resistance cassette near the end of the F plasmid *traY* gene reduced F-pilus expression when the *tra* genes were under *traY* promoter control but not when they were under control of a bacteriophage T7 promoter. We show here that *traY* amber mutations reduce expression of a reporter gene under *traY* promoter control.

TraY also regulates *oriT* nicking, an early reaction in conjugal DNA transfer (13, 18, 19, 24). A regulatory function for TraY at *oriT* does not, of course, preclude a function at the *traY* promoter.

Construction and properties of pAE5000. For the present studies, we constructed pAE5000 (Fig. 1) from three DNA fragments: a 1.6-kb, *Sal*I-*Pst*I *tra* fragment (see pLA101 in reference 26), a 2.6-kb *Pst*I-*Xho*I *traA′-′phoA* fragment from pWP501 (21), and the pSC101 replicon pRW300 (26). The first two fragments were isolated by agarose gel electrophoresis after digestion of the appropriate plasmid with *Sal*I or *Xho*I, dephosphorylation with alkaline phosphatase, and *Pst*I digestion. The three fragments were mixed in equimolar amounts, ligated, and then incubated with polynucleotide kinase and ATP to rephosphorylate the *Sal*I and *Xho*I termini. The fragment mixture was then ligated into pRW300 digested with *Sal*I and alkaline phosphatase (*Sal*I and *Xho*I termini are compatible). Transformation and selection on Luria-Bertani plates containing kanamycin and the chromogenic alkaline phosphatase substrate XP yielded 3 blue colonies from a total of 1,500 Kan^r transformants. One isolate, which was designated pAE5000, was chosen for the experiments reported herein.

The Φ(*traA′-′phoA*)hyb-57 fusion gene of pAE5000 encodes the 51-amino-acid TraA leader peptide and six amino acids of mature F pilin fused to alkaline phosphatase. The leader peptide serves to deliver alkaline phosphatase to the periplasm, where the enzyme is active (21). This posttranslational process involves only host components; no other *tra* gene products are required (22).

Expression of the pAE5000 Φ(*traA′-′phoA*)hyb-57 gene depended entirely on *traY* promoter activity, as shown by the effect of the TraJ protein. The only function of TraJ in conjugal DNA donor activity is to regulate *traY* promoter activity (12, 16). The TraJ level in a pAE5000 transformant of strain AE2388 was, therefore, reduced by introduction of the $finO^+$ plasmid R100. In concert with the $finP_F$ anti-sense RNA (encoded by pAE5000), the R100 *finO* gene product effectively blocks TraJ synthesis (see reference 10 for a complete discussion and references). Control strains contained pAE5000 and the *finO* mutant R100-1 or no plasmids at all. While still in exponential growth, the pAE5000/R100-1 strain generally contained 1,500 to 2,000 U of alkaline phosphatase activity per culture optical density unit. This was reduced to ≤100 U in cells containing R100, which was the same amount as that in cells with no plasmids at all. This result establishes that pAE5000 Φ(*traA′-′phoA*)hyb-57 gene expression is under *traY* promoter control. The data argue against the suggestion by Anthony et al. (2) that there is a promoter in the intergenic region between *traY* and *traA*, unless such a promoter is also TraJ dependent.

Alkaline phosphatase activity in *traY* mutants of pAE5000. We constructed three *traY* mutants of pAE5000 (Fig. 2). One had a single amber mutation at *traY* codon 38 [*traY38*(S38 Am)], another had tandem amber mutations at codons 114 and 115 [*traY114* (S114 Am) and *traY115*(E115 Am)], and the last contained *traY38*, *traY114*, and *traY115* amber mutations. The *traY38* plasmid also contained a +1 frameshift mutation at

* Corresponding author. Phone: (405) 271-7663. Fax: (405) 271-3153. Electronic mail address: SILVERMANP@OMRF.UOKHSC.EDU.

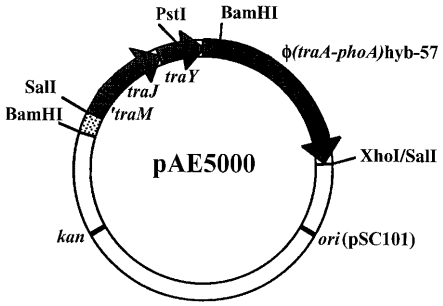

FIG. 1. The structure of pAE5000. Heavily shaded segments, *tra* genes; arrowheads, directions of transcription when these genes are expressed. Also shown are the *Sal*I-*Pst*I and *Pst*I-*Xho*I fragments used to construct pAE5000 and the *Bam*HI fragment used to construct pAE7000; the small amount of vector DNA included with the fragment is indicated by stippling.

codon 42, which was distal to the amber mutation and therefore irrelevant in the sup^0 strain used in these experiments.

The mutant pAE5000 derivatives were constructed by recombinant circle PCR (15). A 2.1-kb *Bam*HI fragment, including the *traY* gene, was first transferred from pAE5000 to pUC181 (pUC18 lacking its *Pst*I site) to yield pAE7000. The remaining pAE5000 *Bam*HI fragment was circularized with DNA ligase and used for transformation to yield pAE6000. Mutagenesis was then carried out on the smaller pAE7000, the mutation was confirmed by restriction analysis and DNA sequencing (Fig. 2), and the altered *Bam*HI fragment was transferred back into pAE6000. DNA sequence analysis also confirmed that no other *traY* mutations had occurred, except as indicated in Fig. 2.

The *traY38* mutation reduced alkaline phosphatase activity by 7.4-fold, whereas the *traY114* and *traY115* mutations reduced this activity by 4.3-fold (Table 1). Introduction of R100 into the *traY38* mutant further reduced its activity to the control (no plasmid) level of ≤100 U, indicating that the residual activity was from the *traY* promoter.

The effect of the amber mutations cannot be attributed to polarity. The *traY115* amber mutation is only 73 bp away from the *traA* ribosome-binding site, which is only 50 bp further than the normal translation termination codon (10). It is very unlikely that 50 bp would lead to a detectable polarity gradient (29). Moreover, the activity in *traY38* mutant cells was restored to the wild-type level by introducing the F′ *lac* plasmid JCFL0 (data not shown), whereas the polarity effects would be *cis* dominant. TraY also acts in *trans* with respect to its function at *oriT* (7, 9).

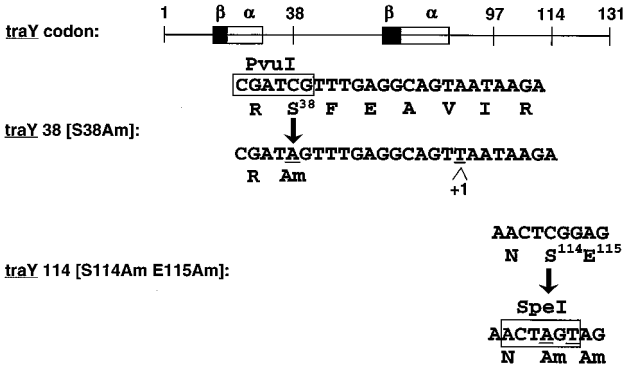

FIG. 2. *traY* mutants of pAE5000. The DNA and protein sequences of each mutant are shown below a linear schematic of the 131-codon *traY* gene. Segments encoding putative β-sheet DNA-binding domains which are followed immediately by α-helical segments, as proposed by Nelson et al. (20), are indicated. Base pairs altered by mutagenic oligonucleotides are underlined. Note that the *traY38* allele removed a *Pvu*I site and that the *traY114* and *traY115* alleles together created an *Spe*I site. These changes were used to screen transformants for appropriate mutations. Note also the +1 frameshift in the *traY38* gene.

TABLE 1. Effects of *traY* amber mutations on Φ(*traA′-′phoA*) hyb-57 expression in vivo

Mutation	Enzyme activity[a]
None	3,045
traY38	414
traY114 traY115	706
traY38 traY114 traY115	341

[a] Cells of strain AE2388 with the indicated pAE5000 derivative were grown to optical densities of 0.7 for measurements of alkaline phosphatase activities.

That the effect of the *traY38* allele was greater than that of the *traY114* allele could be the result of residual TraY activity, as suggested by Frost et al. (10); the gene product of the *traY114 traY115* mutant plasmid would retain both presumptive DNA-binding domains (Fig. 2). As expected if this were the case, the greater effect of the 5′ *traY38* mutation was epistatic to the lesser effect of the 3′ *traY114* and *traY115* mutations in cells with the triple-mutant plasmid (Table 1). However, at this point, we do not know whether the TraY amber fragments even accumulate in the cell.

The results described above provide the first direct evidence that TraY regulates *tra* gene expression. Regulation is positive, in that *traY* amber mutations significantly reduced expression in vivo. Presumably, TraY exerts its stimulatory effect by binding to DNA between the *traY* transcription and translation initiation sites (20), which would position TraY to interact directly with RNA polymerase or other regulatory components at the *traY* promoter. This would be consistent with the observation that neither TraY nor any other Tra protein affects the level of Φ(*traA′-′phoA*)hyb-57 expression from the *lac* promoter (22) and with observations reported by Frost et al. (10).

In view of the dual role of TraY in *oriT* nicking and in *tra* gene expression, it is worth noting that the same DNA strand that acts as a template for expression of the *traY* operon (11) is transferred during conjugation (see reference 28 for citations to original observations). Transfer, which occurs at a rate of approximately 47 kb/min (3), is much faster than transcription, which occurs at a rate of approximately 3 kb/min (8). Since F is 100 kb, transfer of the entire plasmid should require approximately 2 min, whereas RNA polymerase initiating transcription at the *traY* promoter should take 7 min to reach the next promoter, at *traS*, which is 20 kb from the *traY* promoter (see reference 10 for *tra* promoter sites). Owing to these different rates and the unusual length of the *tra* operon, the donor DNA pump would quickly run into elongating RNA polymerase, nascent *traY* operon mRNA, and nascent Tra polypeptides. In fact, since so many Tra proteins are membrane or periplasmic and since some are long enough to be cotranslationally secreted, DNA transfer could turn the donor cell inside out unless operon transcription is reduced once DNA transfer commences. TraY might play a role in coordinating operon transcription with the onset of DNA transfer.

We are indebted to Robbin Harris, whose early observations led to the present work.

This work was supported in part by NIH grant GM38657 and in part by the Oklahoma Medical Research Foundation. P.M.S. acknowledges support from the Marjorie Nichlos Chair in Medical Research.

REFERENCES

1. **Achtman, M., N. Willetts, and A. Clark.** 1971. Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterization of transfer-deficient mutants. J. Bacteriol. **106:**529–538.
2. **Anthony, K., C. Sherburne, R. Sherburne, and L. Frost.** 1994. The role of the pilus in recipient cell recognition during bacterial conjugation mediated by F-like plasmids. Mol. Microbiol. **13:**939–953.
3. **Bachmann, B.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54:**130–197.
4. **Bowie, J., and R. Sauer.** 1990. TraY proteins of F and related episomes are members of the Arc and Mnt repressor family. J. Mol. Biol. **211:**5–6.
5. **Breg, J., J. van Opheusden, M. Bugering, R. Boelens, and R. Kaptein.** 1990. Structure of the Arc repressor in solution: evidence for a family of β-sheet DNA-binding proteins. Nature (London) **346:**586–589.
6. **Brickman, H., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96:**307–316.
7. **Carter, J. R., and D. R. Porter.** 1991. *traY* and *traI* are required for *oriT*-dependent enhanced recombination between *lac*-containing plasmids and λp*lac5*. J. Bacteriol. **173:**1027–1034.
8. **Chamberlin, M., and T. Ryan.** 1982. Bacteriophage DNA-dependent RNA polymerases. Enzymes **15B:**87–108.
9. **Everett, R., and N. Willetts.** 1980. Characterization of an *in vivo* system for nicking at the origin of conjugal DNA transfer of the sex factor F. J. Mol. Biol. **136:**129–150.
10. **Frost, L., K. Ippen-Ihler, and R. Skurray.** 1994. Analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol. Rev. **58:**162–210.
11. **Gaffney, D., R. Skurray, and N. Willetts.** 1983. Regulation of the F conjugation genes studied by hybridization and *tra-lacZ* fusions. J. Mol. Biol. **168:**103–122.
12. **Grossman, T., and P. Silverman.** 1989. Structure and function of conjugative pili: inducible synthesis of functional F-pili by *Escherichia coli* K-12 containing a *lac-tra* operon fusion. J. Bacteriol. **171:**650–656.
13. **Howard, M., W. Nelson, and S. Matson.** 1995. Stepwise assembly of a relaxosome at the F plasmid origin of transfer. J. Biol. Chem. **270:**28381–28386.
14. **Inamoto, S., and E. Ohtsubo.** 1990. Specific binding of the TraY protein to *oriT* and the promoter region for the traY gene of plasmid R100. J. Biol. Chem. **265:**6461–6466.
15. **Jones, D., and B. Howard.** 1991. A rapid method for recombination and site-specific mutagenesis by placing homologous ends on DNA using polymerase chain reaction. BioTechniques **10:**62–66.
16. **Maneewannakul, K., S. Maneewannakul, and K. Ippen-Ihler.** 1992. Sequence alterations affecting F plasmid transfer gene expression: a conjugation system dependent on transcription by the RNA polymerase of phage T7. Mol. Microbiol. **6:**2961–2973.
17. **Matson, S., and B. Morton.** 1991. *Escherichia coli* DNA helicase I catalyzes a site- and strand-specific nicking reaction at the F plasmid *oriT*. J. Biol. Chem. **266:**16232–16237.
18. **Matson, S., W. Nelson, and B. Morton.** 1993. Characterization of the reaction product of the *oriT* nicking reaction catalyzed by DNA helicase I. J. Bacteriol. **174:**2599–2606.
19. **Nelson, W., M. Howard, J. Sherman, and S. Matson.** 1995. The *traY* gene product and integration host factor stimulate *Escherichia coli* DNA helicase I-catalyzed nicking at the F plasmid *oriT*. J. Biol. Chem. **270:**28374–28380.
20. **Nelson, W., B. Morton, E. Lahue, and S. Matson.** 1993. Characterization of the *Escherichia coli* F factor *traY* gene product and its binding sites. J. Biol. Chem. **175:**2221–2228.
21. **Paiva, W., T. Grossman, and P. Silverman.** 1992. Characterization of F-pilin as an inner membrane component of *Escherichia coli* K-12. J. Biol. Chem. **267:**26191–26197.
22. **Paiva, W., and P. Silverman.** Effects of F-encoded components and F-pilin domains on the synthesis and membrane insertion of TraA′-′PhoA fusion proteins. Mol. Microbiol., in press.
23. **Raumann, B., M. Rould, C. Pabo, and R. Sauer.** 1994. DNA recognition by β-sheets in the Arc repressor-operator crystal structure. Nature (London) **367:**754–757.
24. **Reygers, U., R. Wessel, H. Muller, and H. Hoffman-Berling.** 1991. Endonuclease activity of *Escherichia coli* DNA helicase I directed against the transfer origin of the F factor. EMBO J. **10:**2689–2694.
25. **Silverman, P., L. Tran, R. Harris, and H. Gaudin.** 1993. Accumulation of the F plasmid TraJ protein in *cpx* mutants of *Escherichia coli*. J. Bacteriol. **175:**921–925.
26. **Silverman, P., E. Wickersham, and R. Harris.** 1991. Regulation of the F plasmid *traY* promoter in *Escherichia coli* by host and plasmid factors. J. Mol. Biol. **218:**119–128.
27. **Somers, W., and S. Phillips.** Crystal structure of the *met* repressor-operator complex at 2.8A resolution reveals DNA recognition by β-strands. Nature (London) **359:**387–393.
28. **Willetts, N., and R. Skurray.** 1987. Structure and function of the F plasmid and mechanism of conjugation, p. 1110–1133. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
29. **Yanofsky, C., V. Horn, M. Bonner, and S. Stasiowski.** 1971. Polarity and enzyme functions in mutants of the first three genes of the tryptophan operon of *Escherichia coli*. Genetics **69:**409–433.