Specific DNA Recognition by F Factor TraY Involves β-Sheet Residues*

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The F Factor TraY protein is a sequence-specific DNA-binding protein required for efficient conjugal transfer. Genetic and biochemical studies indicate that TraY has two functional roles in conjugation. TraY binds to the Py promoter to up-regulate transcription of tra genes. TraY also binds to the plasmid origin of transfer (oriT), serving as an accessory protein in the nicking of F Factor in preparation for transfer. TraY is thought to belong to the ribbon-helix-helix family of transcription factors. These proteins contact DNA using residues of an antiparallel β-sheet. We engineered and characterized six TraY mutants each having a single potential β-sheet DNA contact residue replaced with Ala. Most TraY mutants had significantly reduced affinity for the TraY oriT binding site while possessing near wild-type stability and nonspecific DNA recognition. These results indicate that TraY β-sheet residues participate in DNA recognition, and support inclusion of TraY in the ribbon-helix-helix family.

Bacterial conjugation is the process by which a conjugative plasmid directs transfer of a copy of itself from donor to recipient bacterium (for review, see Ref. 1). The TraY protein of Escherichia coli K12 Sex Factor F (F Factor or F plasmid) is required for efficient F Factor conjugal transfer (2). TraY, a sequence-specific DNA-binding protein, plays two distinct roles in F Factor conjugation. First, TraY binds to the Py operon promoter (3), up-regulating transcription of the tra operon (4, 5). Second, TraY participates in the "relaxosome," a complex of three proteins that assembles at the F plasmid origin of transfer (oriT). In addition to TraY, the relaxosome includes F-Factor-encoded TraI, and the host-genome-encoded integration host factor. Through an undefined mechanism, TraY and integration host factor enhance the nucleolytic activity of TraI as it cleaves one DNA strand in preparation for transfer of the cut strand to the recipient bacterium (6, 7).

Based on a shared pattern of mainly hydrophobic amino acids (Fig. 1A), Bowie and Sauer (8) assigned TraY to the ribbon-helix-helix family of transcription factors. This family includes three proteins of known three-dimensional structure: Arc (9) and Mnt (10) repressors of phage P22, and the Met repressor of E. coli (11). The family name derives from a structural motif that includes a β-sheet region (1), which contains the sequence bound and nicked by TraI (18). In addition, a 20-bp DNA fragment was used to assess the sequence-specific DNA binding of TraY and TraY mutants that is 5'-TAGTTTCTCTTACTCTCTTT-3' and its complement. This sequence, bp 203–222 of the F Factor tra region (1), is within the TraY DNAse I footprint (3), and will be referred to as the specific oriT binding site oligonucleotide. For radiolabeling, the strand was 5'-end-labeled using [γ-32P]ATP (Amersham) and T4 polynucleotide kinase under conditions suggested by Stratagene. Unincorporated [γ-32P]ATP and labeled oligonucleotide were separated using a Sephadex G-25 Quick Spin column (Roche Molecular Biochemicals). The labeled strand was annealed to its unlabeled complement by slow cooling after heating to 90 °C.

The 22-bp DNA fragment was used to assess nonspecific DNA recognition by TraY. The sequence is 5'--AAAGCCACCAACCCAGCGAAA-3' and its complement. This sequence corresponds to bp 133–154 of the F Factor tra region (1), which includes the sequence bound and nicked by TraI (18). In addition, a 20-bp DNA fragment lacking 1 bp on each end was used in some assays. These are referred to as the 22- and 20-bp nonspecific binding site oligonucleotides, respectively.

Cloning of traY—The traY gene was PCR-amplified using genomic DNA of F' E. coli strain JM109 as template. The amplification reaction and conditions were as described (14) except Taq2000 DNA polymerase was used. Primers (5'-CGGGAGTGTCATATGAAAGATTTGTA- CAGGTCTC-3', complementary to the antisense strand, and 5'-CGTC TCGAGCTAGGTGTAAAAGTGTATAC-3', complementary to

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the sense strand) encoded NdcI and XhoI sites (underlined) to facilitate cloning. The PCR-amplified tomato fragment was gel-purified, and recovered using the Wizard PCR Preps Kit (Promega). The DNA fragment was digested with NdcI and XhoI, and ligated into NdeI/XhoI-digested pET-21a(+) or pET-23a(+) isolated from E. coli BL21(DE3) cells. Competent E. coli BL21 cells were transformed using a PCR-based procedure. PCR primers also encoded the desired amino acid substitutions. To facilitate screening, PCR primers also encoded a unique restriction site introduced through silent mutations.

The 50-µl reaction mixtures contained reaction buffer, 200 µM dNTPs, 250 ng of each primer, from 5 to 400 ng (optimized for each primer pair) of the plasmid template, and either 5 units of Taq2000 or 2.5 units of Pfu DNA polymerase. The reaction involved denaturation at 94 °C for 30 s, 2 cycles of denaturation for 30 s at 94 °C, annealing for 60 s at 45 °C, and extension for 8 min at 68 °C, and 16 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 68 °C for 8 min. PCR products were treated with DpnI to digest the parental DNA template. The mutated, amplified linear vector was then religated restriction sitegenerated cohesive ends, and the ends of the plasmid were ligated. E. coli BL21 cells were transformed with the mutated plasmid. Plasmid purified from transformants was screened by restriction enzyme digestion. Both strands of mutant tomato genes were fully sequenced prior to protein expression and functional analysis. Plasmids were transformed into E. coli BL21(DE3)/pLySE cells for protein production.

Expression and Purification of Wild-type and Mutant TraY Proteins—For protein expression, cells were grown in LB broth with 50 µg/ml ampicillin and (for E. coli strain BL21(DE3)/pLySE) 34 µg/ml chloramphenicol. Cells were tested for protein expression and cell pellets prepared for protein purification as described (14). Cells harboring the wild-type tomato gene cloned into pET-21a(+) were used for protein purification as they grow faster and expressed more protein than those with the gene in pET-23a(—). Mutant Tomato proteins expressed well from pET-23a(—) constructs.

For protein purification, cell pellets were thawed at 4 °C and resuspended in 50 ml of ice-cold buffer A (50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 5 mM dithiothreitol) plus 100 mM NaCl. Phenylmethylsulfonyl fluoride was added to a final concentration of 200 µM. Cells were lysed by sonication, and the lysate DNAwas separated by electrophoresis on a 1X-TBE, 7% polyacrylamide gel. Following electrophoresis and gel drying, radioactive bands were quantified as described above. Data were converted to fractions of labeled DNA bound in the absence of unlabeled competitor DNA, and were plotted versus inhibitor DNA concentration. Multiple experiments were fit simultaneously with an equation of the form \[ \theta = 1 + \frac{[\text{COMP}]}{[\text{IC}_{50}]}, \] where \( \theta \) is the fraction of labeled oligonucleotide bound in the absence of competitor, \([\text{IC}_{50}] \) is the concentration of unlabeled oligonucleotide required to compete away 50% of the binding of the labeled oligonucleotide, and \([\text{COMP}] \) is the concentration of the unlabeled competitor oligonucleotide. Fits were performed using Kaleidagraph 3.0.

Stoichiometry of TraY Binding—Stoichiometry of TraY binding to the specific 20-bp oriT oligonucleotide was determined using a modified direct binding assay. TraY at final concentrations of 225 nM to 4 µM was incubated with a 207 µM solution of Ca2+-CHS-720. The complex was separated by electrophoresis, and bands of an annealed oligonucleotide, and solutions were incubated for 90 min. Bound and unbound oligonucleotides were separated by electrophoresis and bands quantified as described above. Data were converted to total concentration of DNA bound. Results from three experiments were plotted as concentration of DNA bound versus TraY concentration. Data corresponding to TraY concentrations from 0 to 1 µM, and >2 µM were separately fit by linear regression. The TraY concentration corresponding to the intersection of these two lines was used to calculate the binding stoichiometry.

Equilibrium Unfolding by Guanidine Hydrochloride (GdnHCl) Denaturation—GdnHCl unfolding of TraY and TraY mutants was monitored by the change in circular dichroism ellipticity at 234 nm. Experiments were performed and analyzed as described (14) except the instrument used was a Jasco J-710 spectropolarimeter, protein concentrations of 2 or 2.5 µM were used, and the denaturant starting solution contained 6 M GdnHCl (Mallinkrodt) rather than 9.5 M urea. Data from multiple experiments were normalized and combined into a single fit.

RESULTS

Expression and Purification of Wild-type and Mutant TraY Proteins—The gene encoding F Factor TraY was amplified by PCR and cloned into expression vectors pET-21a(+) and pET-23a(+) (see “Experimental Procedures”). The gene cloned into pET-23a(+) served as the template for PCR-based mutagenesis of TraY. Six mutants, each having a single Ala substituted for a residue within the TraY β-sheet, were generated. Sites of amino acid substitutions were selected based upon the sequence alignment of TraY with other members of the ribbon-helix-helix family (Fig. 1A). The sites of amino acid substitutions within the ribbon-helix-helix fold are depicted in Fig. 1B. The residues in the x-ray crystal structure of the E. coli methylonidine opsonin repressor, that correspond to the sites of engineered substitutions in TraY are highlighted. Wild-type and mutant TraY proteins were expressed and purified. The wild-type and variants demonstrated similar chromatographic characteristics, simplifying purification.

Equilibrium Chemical Denaturation—Denaturation of TraY
and TraY mutants by GdnHCl was monitored by change in circular dichroism ellipticity at 234 nm. As noted previously for urea denaturation of TraY (14), GdnHCl denaturation of TraY is fit well by a two-state reaction model (N → D) with no significantly populated intermediates. Denaturation of the TraY mutants also fit well with a two-state reaction model. Table I lists the values for the free energy of denaturation in the absence of GdnHCl ($\Delta G^{\circ}$) and the change in free energy with GdnHCl (m) obtained from nonlinear least squares fits of multiple denaturation experiments. Four of the six mutants have $\Delta G^{\circ}$ values within 0.3 kcal/mol of wild-type TraY. Of the other two mutants, T71A shows a reduction in stability of 0.8 kcal/mol, while the stability of K17A is enhanced by 0.7 kcal/mol.

Sequence-specific DNA Recognition—The affinities of TraY and TraY mutants for a specifically bound oriT DNA sequence were measured by electrophoretic mobility shift assay. The sequence of the 20-bp double-stranded oligonucleotide used in the assay is taken from the DNase I footprint of TraY (3). As shown in Fig. 2, the measured stoichiometry of TraY binding to this oligonucleotide is 1:1. The dissociation constants for TraY and TraY mutants as measured using the direct binding assay are listed in Table II, and representative curves are shown in Fig. 3. Wild-type TraY has a $K_D$ of 7 nM, while the mutants show reductions in affinity ranging from 2-fold to over 100-fold.

The IC$_{50}$ values for TraY and TraY mutants for the specific binding site were also determined using a competition assay. The competition assays were performed in addition to the direct binding assays for two reasons. First, the results of the competition assay are less influenced by dissociation during the electrophoretic separation of bound and free DNA than the results of the direct binding assay, and can therefore potentially yield more accurate numbers for proteins with fast off-rates. Second, affinities for both specific and nonspecific sites (see below) may be readily measured using the competition assay, while accurately measuring nonspecific binding using a direct binding assay is difficult. Measuring affinities for both specific and nonspecific sites by the same assay facilitates direct comparison of these values. The results from competition assays are listed in Table III, and generally agree with the results obtained from the direct binding assay. Representative curves are shown in Fig. 3.

Nonspecific DNA Recognition—To ascertain whether the observed differences in $K_D$ represent loss of specific DNA recognition, the affinities of TraY and the mutants for nonspecific DNA were measured using a competition assay. In this assay, binding of the radiolabeled, specific oriT TraY site was competed away with increasing concentrations of TraY, and bound and free DNA separated by electrophoresis through a nondenaturing gel. Results from three experiments are shown. The stoichiometry of protein to DNA is calculated to be 1.2:1.

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values is blurred. Therefore, we report the nonspecific binding presumably contribute more to the overall affinity, and the test whether the amino acids of the TraY IC50 values decreases, nonspecific protein-DNA interactions 3-fold. As the difference between the specific and nonspecific for specific and nonspecific binding sites differ by only 2- or

| Protein          | IC50 for specific site | IC50 for nonspecific site |
|------------------|------------------------|---------------------------|
| Wild type TraY   | 17 ± 2 (n = 4)         | 603 ± 176 (n = 5)         |
| K12A             | 104 ± 19 (n = 4)       | 400 ± 126 (n = 3)         |
| K15A             | 260 ± 96 (n = 3)       | 1270 ± 351 (n = 3)       |
| K17A             | 100 ± 20 (n = 3)       | 870 ± 250 (n = 5)        |
| K68A             | 28 ± 4 (n = 3)         | 350 ± 97 (n = 4)         |
| T71A             | 276 ± 42 (n = 3)       | 825 ± 162 (n = 3)        |
| R73A             | 388 ± 81 (n = 3)       | 630 ± 154 (n = 3)        |

FIG. 3. Specific and nonspecific DNA recognition by TraY and T71A. Upper, various concentrations of TraY (filled circles) or TraY mutant T71A (filled squares) were incubated with radiolabeled specific oriT oligonucleotide, and bound and free DNA separated by electrophoresis through a nondenaturing gel. Results from multiple experiments are shown. The solid and dashed lines represent the fit to TraY and T71A data, respectively. Lower, various concentrations of unlabeled specific oriT or 22-bp nonspecific oligonucleotide compete for binding to TraY or T71A with radiolabeled specific oriT oligonucleotide. Data for TraY are shown in filled circles (specific oriT oligonucleotide) and open circles (nonspecific oligonucleotide). Data for T71A are shown in filled squares (specific) and open squares (nonspecific). The solid and dashed lines represent the fits to TraY and T71A data, respectively.

have similar affinities for nonspecific DNA, most mutants exhibited significantly reduced affinities for the specific oriT TraY binding site. We also examined the binding of TraY and
some of the mutants to a second specific TraY binding site at the \( P_Y \) promoter (results not shown). The proteins examined demonstrated similar affinities, relative to wild type, for both DNA binding sites. TraY, therefore, binds the two sites in a similar fashion.

These experiments do not reveal the precise roles of each of these \( \beta \)-sheet residues in DNA recognition, and further experimentation is underway to better define their contributions. Given the effects of the mutations on specific but not nonspecific DNA binding, it is likely that some \( \beta \)-sheet residues participate in base-specific contacts. Most of the Ala substitutions, however, were for positively charged Lys or Arg residues. These residues are capable of forming energetically favorable, but nonspecific, electrostatic interactions with the phosphate moieties of the DNA backbone. If, however, these amino acid side chains contribute to binding in a nonspecific fashion (for example, with the DNA backbone), the results suggest that they contribute preferentially within the context of sequence-specific recognition. Contribution of presumably nonspecific backbone contacts to DNA binding specificity has been observed previously (21–23).

Some \( \beta \)-sheet amino acid side chains may also contribute to specific DNA recognition indirectly, in addition to forming direct contacts with DNA. An extensive hydrogen bond network involving the side chains of DNA-contact residues is apparent in the crystal structure of the Arc repressor tetramer-operator complex (9, 19). These hydrogen bonds presumably orient the side chains for optimal DNA contact, and thereby contribute to DNA recognition. If an analogous series of interactions occurs between TraY contact residues, substitution of one of the involved amino acids could have a substantial effect on specific DNA recognition, even if that side chain makes only minor energetic contributions to DNA binding through side chain-DNA contacts. Loss of cooperative interactions between contact residues could explain why three different Ala substitutions reduce affinity for the \( oriT \) sequence to within 4-fold of the affinity for a nonspecific sequence (Table III).

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REFERENCES
1. Frost, L. S., Ippen-Ihler, K., and Skurray, R. A. (1994) Microbiol. Rev. 58, 162–210
2. Carter, J. R., and Porter, R. D. (1991) J. Bacteriol. 173, 1027–1034
3. Nelson, W. C., Morton, B. S., Lahue, E. E., and Matson, S. W. (1993) J. Bacteriol. 175, 2221–2228
4. Silverman, P. M., and Shull, A. (1996) J. Bacteriol. 178, 5787–5789
5. Maneewannakul, K., Kathir, P., Endley, S., Moore, D., Manchak, J., Frost, L., and Ippen-Ihler, K. (1996) Mol. Microbiol. 22, 197–205
6. Nelson, W. C., Howard, M. T., Sherman, J. A., and Matson, S. W. (1995) J. Biol. Chem. 270, 28374–28380
7. Howard, M. T., Nelson, W. C., and Matson, S. W. (1995) J. Biol. Chem. 270, 28381–28386
8. Bowie, J. U., and Sauer, R. T. (1990) J. Mol. Biol. 211, 5–6
9. Raumann, B. E., Roulid, M. A., Pabo, C. O., and Sauer, R. T. (1994) Nature 367, 754–757
10. Burgering, M. J., Boelens, R., Gilbert, D. E., Breg, J. N., Knight, K. L., Sauer, R. T., and Kaptein, R. (1994) Biochemistry 33, 15036–15045
11. Somers, W. S., and Phillips, S. E. (1992) Nature 359, 387–393
12. Raumann, B. E., Brown, B. M., and Sauer, R. T. (1994) Curr. Opin. Struct. Biol. 4, 36–43
13. Phillips, S. E. V. (1991) Curr. Opin. Struct. Biol. 1, 89–98
14. Schildbach, J. F., Robinson, C. R., and Sauer, R. T. (1998) J. Biol. Chem. 273, 1329–1333
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
17. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130
18. Matson, S. W., Nelson, W. C., and Morton, B. S. (1993) J. Bacteriol. 175, 2599–2606
19. Brown, B. M., Mills, M. E., Smith, T. L., and Sauer, R. T. (1994) Nat. Struct. Biol. 1, 164–168
20. Mills, M. E., Brown, B. M., and Sauer, R. T. (1994) Nat. Struct. Biol. 1, 518–523
21. Nelson, H. C., and Sauer, R. T. (1985) Cell 42, 549–558
22. Nelson, H. C., and Sauer, R. T. (1986) J. Mol. Biol. 192, 27–38
23. Schildbach, J. F., Karzai, A. W., Raumann, B. E., and Sauer, R. T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 811–817
24. Nelson, W. C., and Matson, S. W. (1996) Mol. Microbiol. 20, 1179–1187
25. Brown, B. M., Bowie, J. U., and Sauer, R. T. (1996) Biochemistry 29, 11189–11195
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