DNA Recognition by F Factor TraI36: Highly Sequence-Specific Binding of Single-Stranded DNA†

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ABSTRACT: The TraI protein has two essential roles in transfer of conjugative plasmid F Factor. As part of a complex of DNA-binding proteins, TraI introduces a site- and strand-specific nick at the plasmid origin of transfer (*oriT*), cutting the DNA strand that is transferred to the recipient cell. TraI also acts as a helicase, presumably unwinding the plasmid strands prior to transfer. As an essential feature of its nicking activity, TraI is capable of binding and cleaving single-stranded DNA oligonucleotides containing an *oriT* sequence. The specificity of TraI DNA recognition was examined by measuring the binding of *oriT* oligonucleotide variants to TraI36, a 36-kD amino-terminal domain of TraI that retains the sequence-specific nucleolytic activity. TraI36 recognition is highly sequence-specific for an 11-base region of *oriT*, with single base changes reducing affinity by as much as 8000-fold. The binding data correlate with plasmid mobilization efficiencies: plasmids containing sequences bound with lower affinities by TraI36 are transferred between cells at reduced frequencies. In addition to the requirement for high affinity binding to *oriT*, efficient in vitro nicking and in vivo plasmid mobilization requires a pyrimidine immediately 5′ of the nick site. The high sequence specificity of TraI single-stranded DNA recognition suggests that despite its recognition of single-stranded DNA, TraI is capable of playing a major regulatory role in initiation and/or termination of plasmid transfer.

During bacterial conjugation, a single strand of plasmid DNA is transferred from donor bacterium (containing a conjugative plasmid) to recipient (reviewed in refs *1* and *2*). Transfer of conjugative plasmid F Factor utilizes proteins encoded within its *tra* (transfer) region. The TraI protein is essential for F Factor transfer (*3*, *4*). F TraI possesses an N-terminal sequence-specific single-stranded DNA (ssDNA)[1] nuclease, or "relaxase," activity and a C-terminal helicase activity (*3*−*6*). Other bifunctional relaxases include TraI from the F-like R100 plasmid (*7*) and TrwC from the more distantly related R388 plasmid (*8*). This bifunctionality is not universal, as RP4 TraI and many other relaxases lack helicase activity. F TraI binds specifically to the plasmid *oriT* (origin of transfer) as part of the relaxosome complex (*9*, *10*). The other relaxosome members, integration host factor (IHF) and F TraY, facilitate TraI binding (*9*), possibly by generating a DNA distortion that TraI recognizes (*9*, *11*−*13*).

TraI nicks DNA site- and strand-specifically through a Mg^{2+}-dependent transesterification reaction (*5*, *10*, *14*, *15*). The reaction involves nucleophilic attack at the plasmid nick site (termed *nic*) by a tyrosyl hydroxyl oxygen on a DNA phosphate (*16*; see Results). This mechanism is analogous to that of RP4 TraI (*17*) and R388 TrwC (*18*). TraI forms a stable, covalent intermediate consisting of a phosphotyrosyl linkage to DNA 3′ to *nic* (*14*, *15*). Upon formation of a stable donor and recipient "mating pair", TraI apparently responds to an unidentified signal by initiating unwinding of the plasmid DNA strands (*16*). The nicked strand is then transferred to the recipient in the 5′-to-3′ direction. The cleavage reaction is readily reversible in vitro, consistent with TraI serving as a ligase to join the ends of the transferred strand when unwinding is complete (*2*, *19*).

Deletion analysis (*20*, *21*) and analysis of transfer-deficient *oriT* mutants (*15*, *21*, *22*) indicate that TraI must recognize its *oriT* binding site for F to transfer. Results from mobilization of plasmids containing chimeric *oriT* regions (*23*) and selection of transfer-deficient *oriT* mutants (*22*) confirm that one or two base substitutions in the F TraI binding site can dramatically reduce transfer efficiency. Similarly, insertions or substitutions in oligonucleotides can greatly reduce their in vitro cleavage by R100 TraI (*24*). These results suggest that TraI DNA recognition is sequence specific.

To explore the sequence specificity of TraI DNA recognition, we measured the affinity of TraI for *oriT* binding site variants. DNA binding was observed through changes in fluorescence anisotropy and intensity of a fluorophore-labeled oligonucleotide. A single-stranded oligonucleotide was used, precluding use of other relaxosome proteins. Binding measurements used TraI36, a TraI fragment possessing specific ssDNA nicking and binding activities but lacking helicase

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[1] Abbreviations: kD, kilodalton; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TAMRA, carboxytetramethylrhodamine; bp, base pair; kbp, kilo base pair; LB, Luria-Burtani; *oriT*, plasmid origin of transfer; F-TAM, 3′-TAMRA-labeled 22-base single-stranded F TraI binding site oligonucleotide; CPG, controlled pore glass; TAE, 40 mM Tris-acetate, 1 mM EDTA; TEBP, *Oxytricha nova* telomere end binding protein; hnrA1, heterogeneous nuclear ribonucleoprotein A1; IHF, integration host factor.

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activity (Street et al., in preparation). No Mg^{2+} is present, preventing DNA cleavage without interfering with binding. Our results indicate that TraI36 ssDNA binding is highly specific for an 11-base region. Binding is required but not sufficient for cleavage, however, as in vitro assessments of nicking of variant *oriT* sequences identified an additional sequence requirement for nicking. Variant *oriT* sequences were also tested for their ability to facilitate plasmid mobilization between cells. These results correlated well with our binding and nicking data, supporting the relevance of the in vitro results.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *Escherichia coli* strains used for mobilization studies were TB1 (F$^-$ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [*ϕ80 dlac*Δ(*lacZ*)*M15*] *thi hsdR* ($r_K^-m_K^+$)) and ER2738 (F′ *proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15 zzf::Tn10(Tet*^{R}*)/ fhuA2glnV* Δ*(lac-proAB) thi-1* Δ*(hsdS-mcrB)5)*. Vector pNEB193 (New England Biolabs) is a pUC19 derivative.

Oligonucleotide Synthesis and Purification. Oligonucleotides were purchased from Integrated DNA Technologies. The 3′-TAMRA-labeled oligonucleotide F-TAM (5′-TTTGCGTGGGGTGTGGTGCTTT-3′) containing the TraI *oriT* binding site (*19*) was synthesized using a TAMRA CPG column. F-TAM was gel-purified using a 16% polyacrylamide-urea gel (Sequagel; National Diagnostics) and isolated as described (*25*). Other oligonucleotides were used with no additional purification.

Protein Engineering and Purification. TraI36, a 36-kDa N-terminal relaxase domain of F Factor TraI, was used in these experiments. TraI36 expression and purification is described elsewhere (Street et al., in preparation). TraI36 mutant Y16F was engineered using PCR as described (*26*). Primers (5′-AAGGATAATTACTACGTACTGGGC-3′ and 5′-CAGTACGTAGTAATTATCCTTGTCGGTATAAAAGTTCCCGGC-3′) encoded a *SnaB*I site (underlined).

Steady-State Fluorescence Anisotropy and Intensity Studies. Data were collected using an SLM 48000 spectrafluorometer configured in T format. Excitation wavelength was 520 nm. Excitation slit widths were 4 nm. Two Corning 524 nm cutoff filters (cs 3−67) were used in the emission beam. A Neslab refrigerated circulator maintained temperature (25 °C). A magnetic stirrer continuously stirred samples.

To measure TraI36 affinity for F-TAM, TraI36 in binding buffer [100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA] was titrated into a 4 nM solution of the oligonucleotide in binding buffer. After each addition of protein, the solution equilibrated for at least 3 min. This was sufficient to ensure equilibrium as indicated by the stability of anisotropy and intensity readings. Furthermore, use of equilibration periods greater than 3 min yielded similar results. Binding data were fit using the program SPECTRABIND (*27*), which calculates binding parameters from anisotropy data by weighting for changes in the total fluorescence intensity. For SPECTRABIND fits, standard deviations of anisotropy, and intensity measurements were set at 0.001, and standard deviations of protein and oligonucleotide concentrations were 1%.

Sequence specificity of TraI36 ssDNA recognition was assessed by competition assay. F-TAM (4 nM in binding buffer) was brought to 5 nM TraI36 via titration, yielding approximately half-maximal F-TAM binding. Unlabeled competitor oligonucleotide in binding buffer was then titrated into the solution. On average, three points per log of unlabeled oligonucleotide concentration were taken. After each addition of protein or oligonucleotide, the solution equilibrated for at least 3 min, which is sufficient to reach equilibrium. Intensity data were fitted using Kaleidagraph 3.0 (Synergy Software) with the formula:

$$IC_{50} = \frac{[u_i + (u_S)(\log(\mathrm{comp}))] - [l_i + (l_S)(\log(\mathrm{comp}))]}{1 + (IC_{50}/\mathrm{comp})} + [l_i + (l_S)(\log(\mathrm{comp}))]$$

where comp is the concentration of unlabeled competitor oligonucleotide, IC_{50} is the competitor concentration giving 50% inhibition of binding, u_i is the upper intercept, u_s is the slope of the upper baseline, l_i is the lower intercept, and l_s is the slope of the lower baseline. The baseline slope terms account for a small increase in intensity and anisotropy that appears linear with the log of the competitor oligonucleotide concentration. Incorporating these terms significantly improved the fits. When the lower baseline was insufficiently defined due to incomplete inhibition by a low affinity competitor oligonucleotide, the lower intercept was held constant at the initial intensity of the labeled oligonucleotide in the absence of protein.

oriT Plasmid and Mutant Plasmid Construction. F Factor *oriT* was PCR-amplified using F′ *E. coli* strain JM109 DNA as template. *oriT* is defined as the region between the *Bgl*II site at nucleotide 1 and the *Sal*I site at nucleotide 530 [numbered as defined by Frost and colleagues (*20*)], with primed numbers indicating bases on the "bottom" (nicked) strand. Primers (5′-CCTGAGATCTCATTTATAAACATCAGG-3′ and 5′-TTCCTGTCGACGCTTCTCAAT-3′) included *Bgl*II and *Sal*I sites (underlined), respectively. The PCR reaction (100 μL) contained 1 μM of each primer, 1 mM total dNTPs, and 5 units of *Taq2000* DNA polymerase (Stratagene). The PCR reaction included initial denaturation at 94 °C (4 min); 3 cycles of denaturation at 94 °C (1 min), annealing at 45 °C (45 s), and extension at 70 °C (45 s); and 21 cycles of denaturation at 94 °C (1 min), annealing at 65 °C (45 s), and extension at 70 °C (45 s). Product was digested with *Sal*I and *Bgl*II, ligated into *Sal*I−*Bam*HI digested pNEB193, and transformed into *E. coli* strain TB1. Plasmid DNA was purified from ampicillin-resistant transformants. Plasmid inserts were verified by DNA sequencing (Johns Hopkins University School of Medicine DNA Analysis Facility). The resulting plasmid (pNEB193-*oriT*) was used as the template to create *oriT* mutants.

oriT mutant plasmids were created using a one- or two-step PCR protocol. For mutants containing substitutions at positions 141′ or 144′, the two-step procedure was employed. Primers *oriT*-reverse (5′-TTGGTGGTGTTAACCACCAACCTGTT-3′, *Hpa*I site underlined), Amp-forward (5′-GACTGGTGAGTACTCAACCAA-3′, *Sca*I site underlined), and Amp-reverse (5′-ACCTGTTGAGTACTCAGGAGTC-3′, *Sca*I site underlined) were used, along with a mutational primer encoding the desired base substitution and an *Ecl136*II site. PCR amplification using primers Amp-forward and *oriT*-reverse with pNEB193-*oriT* as template yielded a 1-kb product. After *Sca*I and *Hpa*I digestion, the product extended

from the *Sca*I site in the *bla* gene through *oriT* base 133. A second amplification used primer Amp-reverse, which primes from the *Sca*I site in *bla*, and an *oriT* mutational primer, yielding a 2-kb product. Following *Sca*I and *Ecl136*II digestion, the product was ligated to the *Sca*I−*Hpa*I PCR product of the PCR amplification reaction of primer Amp-forward and *oriT*-reverse to generate a mutant pNEB193-*oriT* containing a single bp substitution. Ligating the digested *Hpa*I (GTT/AAC) and *Ecl136*II (GAG/CTC) fragments yielded the wild-type GTTCTC *oriT* sequence starting at base 120.

Mutant plasmids containing substitutions at positions 139′ or 149′, or the R100 sequence were generated using the one-step PCR process. The mutational primer and the *oriT*-reverse primer were included in a reaction with pNEB193-*oriT* as template. The PCR product was digested with *Hpa*I and *Ecl136*II. Mutational primers, with underlined *Ecl136*II sites, were C139′G (5′-CAAGGTGGAGCTCACCACCAAAAGCAGCACACCCCACGC-3′), A141′T (5′-CAAGGTGGAGCTCACCACCAAAAGCACCTCACCCCACGC-3′), C144′G (5′-CAAGGTGGAGCTCACCACCAAAAGCACCACACAGCCCACGC-3′), C149′T (5′-CAAGGTGGAGCTCACCACCAAAAGCACCACACCCCATGCAAA-3′), and R100 *oriT* (5′-CAAGGTGGAGCTCACCACCAAAAGCACCACACACTACGCAAA-3′), which encodes substitutions C145′A and C147′T found in *oriT* of F-like plasmid R100.

Ligations used Ampligase (Epicentre Technologies) as described (*28*). Bridge oligonucleotides were *oriT*-bridge (5′-AACAGGTTGGTGGTTCTCACCACCAAAAGC-3′; to join the *Ecl136*II end of the Amp-reverse/mutant fragment and the *Hpa*I end of the *oriT*-reverse/Amp-forward fragment) and Amp-bridge (5′-CTGTGACTGGTGAGTACTCAACCAAGTCAT-3′; to join the *Sca*I ends of both fragments together). One-step PCR mutants used only the *oriT*-bridge oligonucleotide. Maximum ligation efficiency required 100 nM total fragment DNA and 50 nM total bridge oligonucleotide DNA. Ten microliters of the 50 μL ligation reaction were run on a 1% agarose TAE gel to confirm ligation. $CaCl_2$-competent TB1 cells were transformed with the remainder. Ampicillin- and streptomycin-resistant transformants were selected, plasmid DNA isolated, and *oriT* inserts sequenced.

Plasmid Mobilization Assays. To assess mobilization efficiency of plasmids containing mutant *oriT* sequences, F′ strain ER2738 was transformed with wild-type or mutant pNEB193-*oriT*. Tetracycline- and ampicillin-resistant transformants were selected. Overnight cultures of TB1 (recipient) and ER2738/pNEB193-*oriT* (donor) were diluted and grown to $OD_{600} \sim 0.5$, approximately 10^8 cells/mL. Approximately 10^8 cells of each of the donor and recipient were pelleted by centrifugation in separate microfuge tubes and washed twice with LB broth. Each pellet was resuspended in 0.5 mL of LB broth, and donor and recipients combined (1 mL total volume). The conjugative mixture was incubated at 37 °C without agitation for 30 min. After incubation, cells were vortexed and immediately serially diluted in LB broth on ice. A total of 50 μL of each dilution were plated onto LB agar plates supplemented with tetracycline (34 μg/mL) and ampicillin (100 μg/mL) to detect donor cells, streptomycin (50 μg/mL) to detect recipient cells, and streptomycin and ampicillin to detect transconjugants (TB1 cells containing wild-type or mutant pNEB193-*oriT*). Transfer efficiency was calculated as number of transconjugant colonies per donor colony. Under these conditions, transfer efficiency of the ER2738 F′ is comparable to that of pNEB193-*oriT*. The presence of pNEB193-*oriT* does not significantly affect the transfer efficiency of F′.

Nicking and Ligation Assays. Nicking of wild-type and mutant *oriT* sequences by TraI36 was tested using a cleavage assay (*19*) with modifications. Single-stranded 22-base oligonucleotides were 5′ labeled with γ-^{32}P dATP (Amersham) using T4 polynucleotide kinase (New England Biolabs) under conditions suggested by the supplier. Oligonucleotide and unincorporated label were separated using a Sephadex G-25 Quick Spin column (Roche Molecular Biochemicals). The 15 μL nicking reaction contained 4 nM labeled oligonucleotide and TraI36 concentrations ranging from 1 nM to 1 μM. Reaction buffer was 100 mM NaCl, 50 mM Tris (pH 7.5), and 5 mM $MgCl_2$. Reactions were incubated at 37 °C for 30 min, then stopped by addition of sodium dodecyl sulfate (0.1% final concentration), and incubated for 10 min at 37 °C. Samples were mixed with 16.5 μL of loading buffer (80% formamide (v/v) in 225 mM Tris-borate, 5 mM EDTA), heated for 5 min at 90 °C, snap-cooled on ice, and run on a 16% polyacrylamide-urea gel at 300V for 2.5 h. Bands were visualized using an SF Phosphorimager (Molecular Dynamics).

The ability of TraI36 to use mutant *oriT* sequences as ligation substrates was tested using a recombination assay (*19*) with modifications. Wild type or variant *oriT* 22-base single-stranded oligonucleotide was 3′ labeled with α-^{32}P ddATP using terminal transferase (Boehringer Mannheim) under conditions suggested by the supplier. Unincorporated label and oligonucleotide were separated as above. A 10 μL reaction containing 5 μM TraI36 and 4 nM 3′-labeled oligonucleotide in reaction buffer was incubated at 37 °C for 30 min. Unlabeled wild type or mutant oligonucleotides consisting of the 22 bases 5′ of the *oriT* nick site were then added to the reactions to final concentrations ranging from 0.5 to 5 μM. Reactions were incubated at 37 °C for 30 min. The reaction was stopped and results analyzed as for the nicking assay.

RESULTS

Assessment of TraI36 Binding Affinity. To measure the in vitro affinity of TraI36 for its ssDNA substrate, we used a fluorescence-based equilibrium assay. Adding TraI36 to a solution of F-TAM, a fluorophore-labeled *oriT*-derived oligonucleotide, increases fluorescence anisotropy (Figure 1a) and intensity (Figure 1b), indicating binding of TraI36 to F-TAM. Half-maximal binding was reached at 2−3 nM TraI36, and binding was saturated at 10 nM TraI36. Using SPECTRABIND to fit data from 50 pM to 100 nM TraI36 with a model assuming 1:1 stoichiometry yields a 0.5 (± 0.2) nM K_D. This represents the K_D for sequence-specific binding, as indicated by competition experiments (see below).

A second increase in both anisotropy and intensity began at 300 nM TraI36. We conclude that this rise represents nonspecific binding for several reasons. The increase is unlikely to result from direct protein−protein interactions because TraI36 is monomeric even at concentrations exceeding 50 μM (Street et al., in preparation). Emission spectra

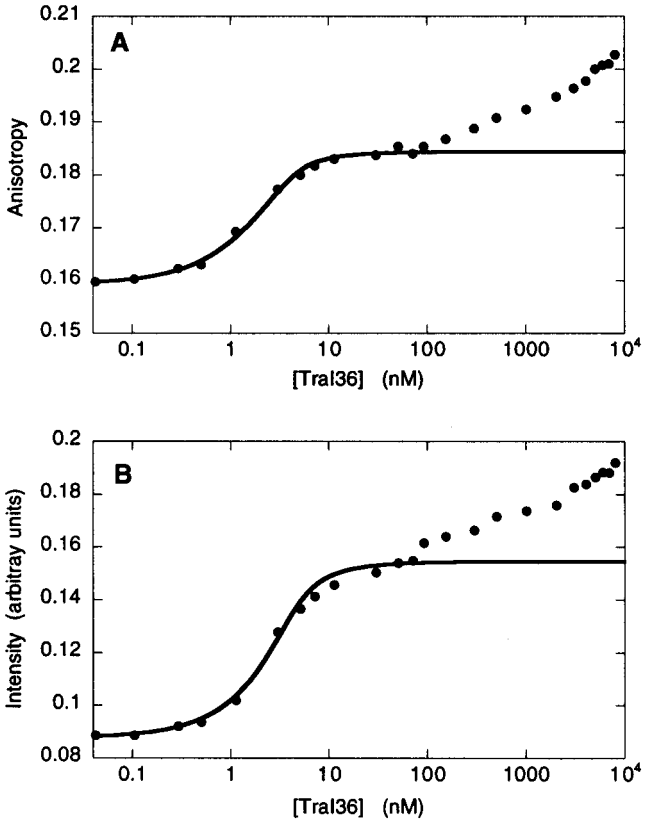

FIGURE 1: Affinity titration using F-TAM oligonucleotide. Fluorescence anistropy (A) and total fluorescence intensity (B) are plotted against total TraI36 concentration. Curves shown represent a simultaneous fit to both anisotropy and intensity data, truncated at 100 nM, using the program SPECTRABIND (*27*). The calculated K_D for the data shown is 0.7 nM. The average and standard error for four measurements of K_D is 0.5 ± 0.2 nM.

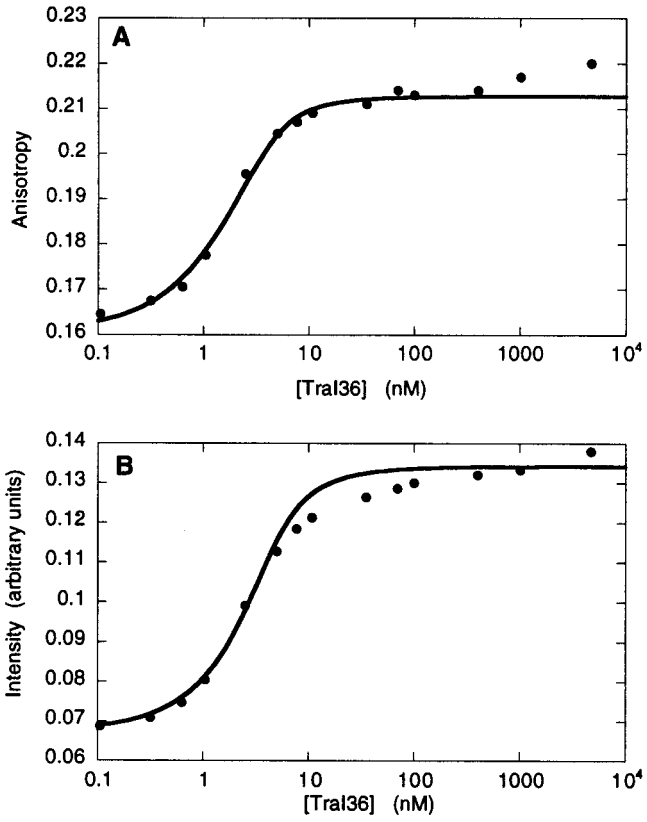

FIGURE 2: Affinity titration using a 17-base 3′-TAMRA-labeled oligonucleotide (5-bases removed from the 3′ end of F-TAM). Fluorescence anistropy (A) and total fluorescence intensity (B) are plotted against total TraI36 concentration. Curves shown represent a simultaneous fit to both anisotropy and intensity data over the entire concentration range using the program SPECTRABIND (*27*). The calculated K_D for the data shown is 0.7 nM. The average and standard error for two measurements of K_D is 0.6 ± 0.2 nM.

of F-TAM with various protein concentrations exclude the possibility that light scattering at high protein concentrations causes the increase. The fluorescence spectra have identical shapes at all protein concentrations, with only relative intensity differing between them (data not shown). Finally, a 17-base 3′ TAMRA-labeled oligonucleotide lacking the five 3′-bases of F-TAM exhibits a similar first transition, but the second increase in intensity and anisotropy is considerably smaller in magnitude and occurs at higher protein concentrations (Figure 2). The calculated dissociation constant for the 17-base oligonucleotide is 0.6 (± 0.2) nM. These results suggest that one or more of the 3′ bases of F-TAM are involved in a nonspecific reaction at high protein concentrations. Because this interaction did not interfere with subsequent measurements, and because the 22-base sequence of F-TAM had been used previously in TraI studies (*19*), we used the F-TAM oligonucleotide for our studies.

During the course of these experiments, we realized that F-TAM, which contains four consecutive guanines, may form oligonucleotide tetraplexes (*29*). Analytical ultracentrifugation experiments confirmed that up to 20% of the 22-base oligonucleotide was involved in higher order structures (data not shown). To test whether affinity measurements were affected, F-TAM was heated for 1 h at 90 °C and snap cooled on ice. After this treatment, no higher order oligonucleotide complexes were detected by ultracentrifugation (data not shown). The affinity measurement was repeated, and the results did not differ measurably from the initial experiments.

All binding data were collected in the absence of Mg^{2+} to prevent TraI36 from cleaving the substrate. The TraI transesterification reaction is Mg^{2+}-dependent (*5*). To test whether Mg^{2+} alters TraI36 affinity for F-TAM, the affinity of a noncatalytic TraI36 mutant was measured. In the presence of Mg^{2+}, and using conditions under which TraI36 readily nicks the single-stranded *oriT* oligonucleotide, the Y16F mutant, which has Phe substituted for Tyr at position 16, fails to cleave the substrate (Street et al., in preparation). This loss of activity is similar to that seen when Phe is substituted for Tyr22 of RP4 TraI (*17*) and for Tyr18 of R388 TrwC (*18*). In the absence of Mg^{2+}, TraI36 Y16F binds F-TAM with the same affinity as wild-type TraI36. In the presence of 5 mM Mg^{2+}, which is sufficient for cleavage, the Y16F K_D is unaltered (data not shown).

TraI36 Binding Specificity. We used a competition assay to determine sequence requirements for TraI36 recognition of the *oriT* ssDNA oligonucleotide. TraI36 (5 nM final concentration) is added to 4 nM F-TAM, increasing fluorescence anisotropy and intensity. Addition of unlabeled oligonucleotide, which competes for binding with labeled oligonucleotide, decreases fluorescence anisotropy and intensity. The concentration dependence of the decrease reflects the affinity of TraI36 for the competitor sequence (Figure 3). Using a competitor oligonucleotide with the same sequence as F-TAM, the measured IC_{50}, the concentration required for 50% inhibition, is 4 nM. In this assay, we used

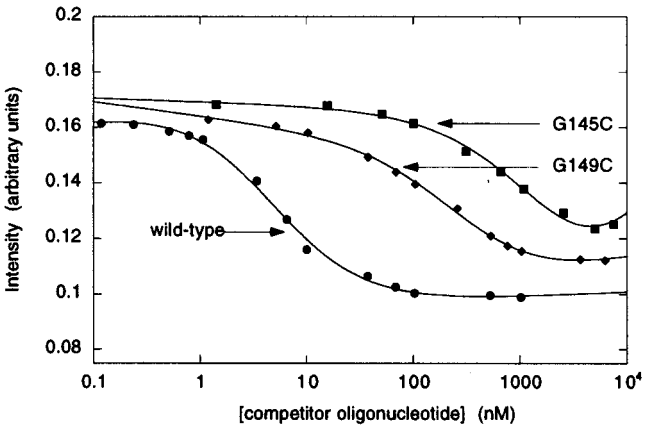

FIGURE 3: Fluorescence intensity plots of competitor oligonucleotide titrations to assess binding specificity. Competition of binding of the F-TAM oligonucleotide to TraI36 by competitor oligonucleotides with wild-type sequence (●, IC_{50} = 4 nM), or the variant sequences sequences G145′C (■, IC_{50} = 950 nM) and G149′C (♦, IC_{50} = 290 nM). Curves shown represent fits to the data performed as described in Materials and Methods.

a concentration of labeled ligand (4 nM F-TAM) greater than the K_D of the interaction (0.5 nM K_D of TraI36 for F-TAM) to ensure sufficient signal. These conditions should yield IC_{50} values 9-fold higher than the K_I, the dissociation constant for the inhibitor (*30*). Therefore, the 4 nM IC_{50} value correlates well with the 0.5 nM K_D measured in the affinity titrations. This correlation confirms that the first transition seen in the affinity titrations corresponds to specific binding and indicates that the TAMRA label does not significantly affect binding.

To define the *oriT* region recognized by TraI36, we tested binding of competitor oligonucleotides containing overlapping 22-base *oriT* sequences. The oligonucleotides, including *nic* (the nicking site, between bases 140′ and 141′), and their binding are shown in Table 1. The highest affinity interactions required at least two bases 3′ to *nic* (compare oligonucleotide 161′ to 160′) and at least 11 bases 5′ to *nic* (compare oligonucleotide 154′ to 150′).

To define TraI36 sequence specificity, we tested binding of 22-base oligonucleotides with single substitutions, mostly transversions, from 11 bases 5′ to *nic* to 2 bases 3′ to *nic* (Table 2 and Figure 3). Substitutions immediately 5′ to *nic* had the greatest effect, with some substitutions at positions 142′, 143′, and 144′ causing at least 5000-fold reductions in affinity. Substitutions elsewhere also had measurable effects. Eight-fold or greater increases in IC_{50} values were observed for substitutions from 2 bases 3′ to *nic* (G139′C) to 9 bases 5′ to *nic* (G149′C and G149′A). Substitutions 10 or 11 bases 5′ to *nic* (C150′G and G151′C) had little effect (Table 2). However, an oligonucleotide lacking G151′-G154′ has reduced binding (150′; Table 1), suggesting that one or more of these bases contributes, specifically or nonspecifically, to binding.

Both transversion (G → C) and transition (G → A) substitutions were made at positions 144′ and 149′. The transitions represent *oriT* sequences previously identified as transfer deficient (*15*, *21*, *22*). G144′A and G149′A were bound with 900- and 100-fold higher IC_{50} values, respectively. At position 149′, substitutions of C or A for G reduced affinity similarly, while G144′C had a significantly lower affinity than G144′A.

The TraI *oriT* binding site from plasmid R100 was also used as a competitor oligonucleotide. In vivo studies indicate that F Factor TraI is specific for F *oriT* and fails to act efficiently on the R100 sequence (*23*, *31*). R100 and F differ by two bases near *nic*. We tested each substitution individually as well as the pair. The G147′A substitution (R100A) reduced binding by 90-fold, G145′T (R100T) reduced binding by 1400-fold, and the combination (R100) reduced binding by 1800-fold. As was observed for position 144′, substitution of C for G147′ had a greater effect than substitution of A.

Several substitutions at position 141′ were tested (Table 3) after a pyrimidine at this location was shown to be critical for the cleavage reaction (see below). All substitutions for T141′ reduced affinity, although substitution of uridine caused only an 8-fold increase in IC_{50}. Substitution of other pyrimidines (cytidine, IC_{50} = 520 nM; 5-methylcytidine, IC_{50} = 1.6 μM) caused greater losses in affinity. Substitution of purines for T141′ increased IC_{50} values at least 600-fold, relative to wild type. Presence of a dSpacer, which has a methyl group replacing the base, reduced binding by 50-fold, probably reflecting loss of energetically favorable interactions between T141′ and the protein.

Variant oriT Sequences as Cleavage and Ligation Substrates for TraI36. To relate in vitro binding to cleavage, nicking assays were performed on some *oriT* variants. To assess cleavage, selected single-stranded 22-base oligonucleotides were 5′ labeled with γ-^{32}P ATP. Cleavage of a 22-base oligonucleotide generated a 14-base 5′-labeled product that could be separated from uncut oligonucleotide electro-

Table 1: Binding Data for Competitor Oligonucleotides Containing Overlapping 22-Base Sequences along the TraI *oriT* Binding Site[a,b]

oligonucleotide		IC_{50} (nM)
163′	AAACTTGTTTTTGCGTGGGGTG	13000 ± 1700
162′	AACTTGTTTTTGCGTGGGGTGT	49 ± 18
161′	ACTTGTTTTTGCGTGGGGTGTG	20 ± 20
160′	CTTGTTTTTGCGTGGGGTGTGG	1.7 ± 0.3
157′	GTTTTTGCGTGGGGTGTGGTGC	1.7 ± 0.3
154′	**TTTGCGTGGGGTGTGGTGCTTT**	**4.2 ± 1.1**
150′	CGTGGGGTGTGGTGCTTTTGGT	160 ± 49
147′	GGGGTGTGGTGCTTTTTGGTGGT	2600 ± 340
143′	TGTGGTGCTTTTTGGTGGTGAGA	8400 ± 780

[a] The numbers listed under oligonucleotide indicate the *oriT* position of the 5′ base of the oligonucleotide. Numbering is as defined by Frost and colleagues (*2*), with the primes referring to bases in the "bottom" (nicked) strand. The oligonucleotide in bold contains the F-TAM sequence. The vertical line indicates the nick site between bases 141′ and 140′. [b] IC_{50} values, the concentrations required to inhibit 50% of binding of the labeled oligonucleotide, are given as an average of at least three measurements, with the standard error of the averaged values.

Table 2: Competition Binding Data for Competitor Oligonucleotides Containing Individual Base Substitutions in the TraI *oriT* Binding Site[a,b]

oligonucleotide		IC_{50} (nM)
wild-type	T T T G C G T G G G G T G T | G G T G C T T T	4.2 ± 1.1
G139′C	- - - - - - - - - - - - - | - **C** - - - - - -	40 ± 18
G140′C	- - - - - - - - - - - - - | **C** - - - - - - -	310 ± 45
G142′C	- - - - - - - - - - - - **C** | - - - - - - - -	21,000 ± 920
T143′A	- - - - - - - - - - - - **A** | - - - - - - - -	20,000 ± 490
G144′C	- - - - - - - - - - - **C** - | - - - - - - - -	35,000 ± 13,000
G144′A	- - - - - - - - - - - **A** - | - - - - - - - -	3,900 ± 550
G145′C	- - - - - - - - - - **C** - - | - - - - - - - -	950 ± 150
G146′C	- - - - - - - - - **C** - - - | - - - - - - - -	5,200 ± 1,900
G147′C	- - - - - - - - **C** - - - - | - - - - - - - -	5,800 ± 3,200
T148′A	- - - - - - - **A** - - - - - | - - - - - - - -	31 ± 4.0
G149′C	- - - - - - **C** - - - - - - | - - - - - - - -	290 ± 120
G149′A	- - - - - - **A** - - - - - - | - - - - - - - -	490 ± 330
C150′G	- - - - - **G** - - - - - - - | - - - - - - - -	13 ± 3.5
G151′C	- - - - **C** - - - - - - - - | - - - - - - - -	7.5 ± 5.1
R100	- - - - - - - - **A** - **T** - - - | - - - - - - - -	7,500 ± 1,200
R100A	- - - - - - - - **A** - - - - - | - - - - - - - -	370 ± 59
R100T	- - - - - - - - - **T** - - - - | - - - - - - - -	5,900 ± 930

[a] The vertical line in the sequence indicates the nick site. Dashes indicate no sequence difference from wild type. [b] IC_{50} values, the concentrations required to inhibit 50% of binding of the labeled oligonucleotide, are given as an average of at least three measurements, with the standard error of the averaged values.

Table 3: Binding Data for Competitor Oligonucleotides with Substitutions at Position T141′[a,b]

oligonucleotide		IC_{50} (nM)
wild type	T T T G C G T G G G G T G T | G G T G C T T T	4.2 ± 1.1
T141′C	- - - - - - - - - - - - - | **C** - - - - - - -	520 ± 130
T141′U	- - - - - - - - - - - - - | **U** - - - - - - -	33 ± 5.3
T141′5MeC	- - - - - - - - - - - - - | **M** - - - - - - -	1,600 ± 400
T141′A	- - - - - - - - - - - - - | **A** - - - - - - -	2,800 ± 430
T141′G	- - - - - - - - - - - - - | **G** - - - - - - -	2,500 ± 660
T141′I	- - - - - - - - - - - - - | **I** - - - - - - -	3,500 ± 670
T141′dSpacer	- - - - - - - - - - - - - | **d** - - - - - - -	190 ± 40

[a] T141′I contains an inosine, T141′U contains a uridine, and T141′5MeC contains a 5-methyl cytidine. The dSpacer modification places a methyl group at carbon 1′ of the deoxyribose in place of the thymidine. The nick site is indicated by the vertical line in the sequence. Dashes indicate no sequence difference from wild type. [b] IC_{50} values are given as an average of at least three measurements, with the standard error of the averaged values.

phoretically. Results showed a general correlation between affinity of TraI36 for a particular sequence and ability of TraI36 to nick an oligonucleotide containing that sequence. For example, under assay conditions, TraI36 was unable to cleave the G144′C single-stranded oligonucleotide (IC_{50} = 35 μM; Figure 4), while TraI36 could cleave the G140′C (IC_{50} = 310 nM) and G145′C (IC_{50} = 950 nM) sequences (Figure 4). Consistent with their IC_{50} values, G140′C and G145′C required relatively more TraI36 to generate product than did the wild-type sequence. The R100 *oriT* sequence (G145′T, G147′A; IC_{50} = 7.5 μM) was not cleaved by TraI36, while variants G139′C (IC_{50} = 40 nM), G145′T (R100T, IC_{50} = 5.9 μM), G147′A (R100A, IC_{50} = 370 nM), and G149′A (IC_{50} = 490 nM) were cleaved (not shown). Again reflecting IC_{50} values, greater concentrations of TraI36 were required to yield product from these oligonucleotides than from the wild-type sequence.

Because T141′A (IC_{50} = 2.8 μM) was not cleaved by TraI36, despite being bound with an affinity similar to that of G145′C and G145′T, which were cleaved, we examined the panel of T141′ variants (Table 3) as TraI36 substrates. The results indicate that a pyrimidine is required at this position for cleavage. Variants T141′C, T141′U, and T141′5MeC ($IC_{50} \leq$ 1.6 μM) were all cleaved (data not shown). Variants with purine substitutions (T141′A, T141′G, T141′I; $IC_{50} \sim$ 3 μM) were not cleaved. T141′dSpacer, which has a methyl group replacing the base, was not cleaved despite being bound with relatively high affinity (IC_{50} = 190 nM).

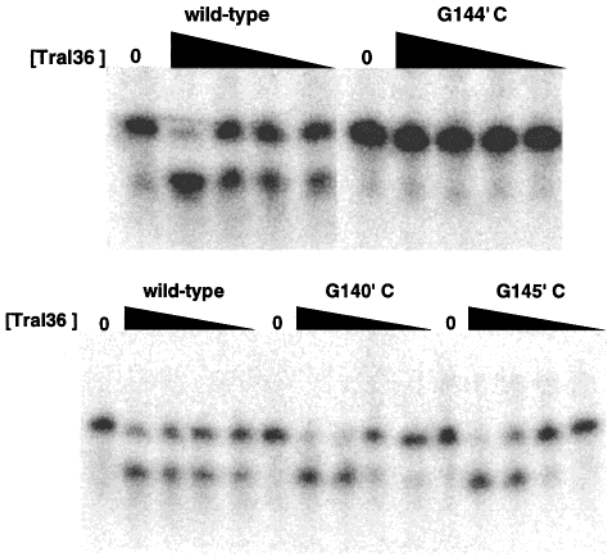

FIGURE 4: Cleavage of wild type and variant *oriT* 22-base oligonucleotides by TraI36. Top, cleavage of wild-type F (left) and G144′C (right) sequences. For each oligonucleotide, the TraI36 concentrations were, starting from the left lane, 0 nM (no protein control), 1 μM, 100 nM, 10 nM, and 1 nM. Bottom, cleavage of (left) wild-type F, (middle) G140′C, and (right) G145′C oligonucleotides by TraI36. TraI36 concentrations were as for top.

Because TraI may ligate the ends of the transferred strand to end the transfer process (*2*, *19*), we tested *oriT* variants as substrates in the ligation reaction. Variant G149′A is at least comparable to wild type in this assay, while T141′C and G140′C generated reduced levels of ligation product relative to the wild-type sequence (Figures 5 and 6, Table 3). Variants T141′A and G144′C generated no observable ligation product (not shown). The reduced ligation efficiencies of T141′C, T141′A, and G144′C relative to wild type may reflect their lower affinities for TraI36, at least in part. The results for T141′C and G140′C, however, suggest that factors other than binding affinity contribute to the ligation reaction. G149′A and T141′C bind TraI36 with similar affinities, yet G149′A is a substantially more efficient ligation substrate than T141′C. The G140′C substitution lies 3′ to *nic* and is therefore on the oligonucleotide segment covalently linked to TraI36 after cleavage. The apparently reduced ligation efficiency of G140′C cannot then be explained by a reduced affinity of TraI36 for the ligation substrate.

Efficiency of Mobilization of the oriT Mutants. We used a plasmid mobilization assay to relate in vitro affinities of *oriT* sequences to in vivo function. Plasmid pNEB193-*oriT*, which contains the wild-type *oriT* sequence, and its mutant counterparts were tested for their ability to be mobilized by F Factor *in trans*. F′ *E. coli* strain ER2738 was transformed with wild type or mutant pNEB193-*oriT*, then mated with the F^- TB1 strain. The transfer mixture was plated on selective media and mobilization efficiency was calculated as number of transconjugant colonies per donor colony. Transconjugants result from mobilization of pNEB193-*oriT* into TB1.

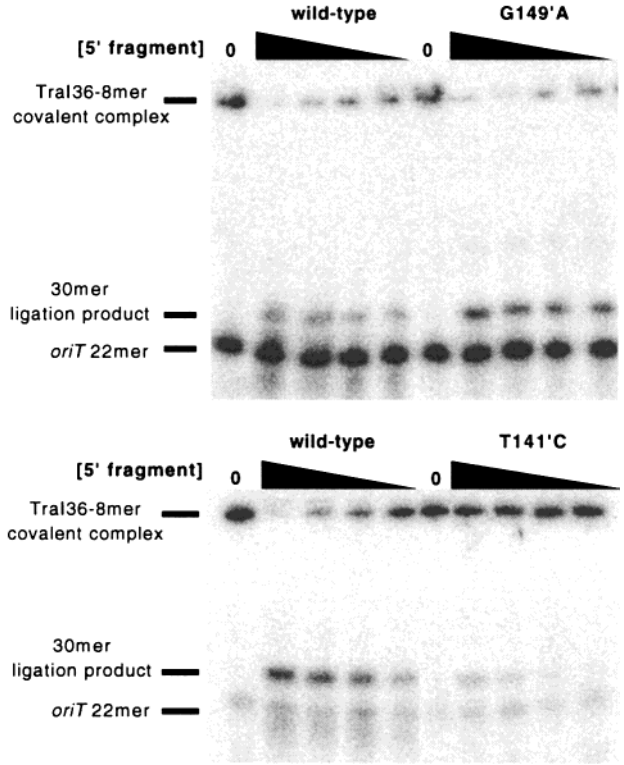

FIGURE 5: TraI36 ligation of *oriT* variant sequences as compared to the wild-type sequence. Top, following reaction of 5 μM TraI36 with 4 nM 3′-radioactively labeled oligonucleotide and the resulting formation of a covalent TraI36−8-base-oligonucleotide covalent complex, an excess of ligation substrate, an oligonucleotide representing the sequence 5′ to *nic* for wild-type *oriT* or the G149′A variant, is added. The reaction is incubated, and the products separated electrophoretically. For each oligonucleotide, the concentrations of the ligation substrate are, from left, 0, 5 μM, 2.5 μM, 1 μM, and 500 nM. Bottom, as for top, except the T141′C ligation substrate is used in the place of G149′A.

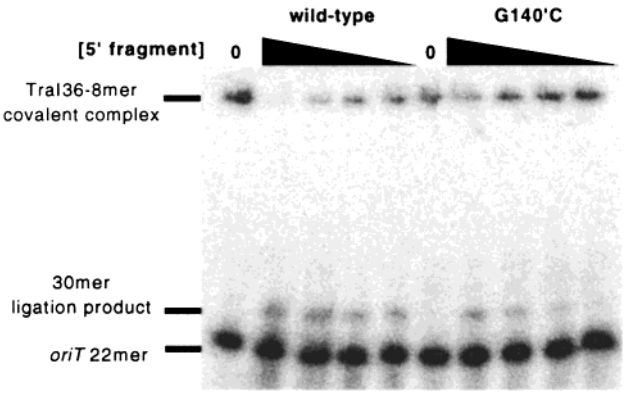

FIGURE 6: TraI36 ligation of *oriT* sequence 5′ to *nic* to wild-type *oriT* and G140′C sequences. Following reaction of 5 μM TraI36 with 4 nM 3′-radioactively labeled wild-type or G140′C oligonucleotide and the resulting formation of a covalent TraI36−8-base-oligonucleotide covalent complex, an excess of ligation substrate, an oligonucleotide representing the sequence 5′ to *nic* for wild-type *oriT*, is added. The reaction is incubated, and the products separated electrophoretically. For each reaction, the concentrations of the ligation substrate are, from left, 0 μM, 5 μM, 2.5 μM, 1 μM, and 500 nM.

Assay results are given in Table 4, with a summary of the in vitro activities of the *oriT* variants. The mutant plasmid containing the substitution G139′C was mobilized at approximately wild-type levels. The plasmid containing G149′A was mobilized with 20% of the efficiency of wild type.

Table 4: Comparison of in Vitro and in Vivo Sequence Specificity[a]

		in vitro		in vivo
substitution	IC_{50} (nM)	cleavage	ligation	mobilization efficiency
wild-type F	4	+	+	0.5
G139′C	40	+	ND	0.5
T141′A	2800	−	−	3×10^{-5}
G144′C	35000	−	−	7×10^{-5}
G149′A	490	++	+	0.1
R100	7500	−	ND	7×10^{-4}

[a] (−) indicates no visible nicking or ligation in these assays. (+) indicates cleavage or ligation product at wild-type levels at 5 μM TraI36. (++) indicates cleavage or ligation product at greater than wild-type levels at 5 μM TraI36. ND indicates not done.

Mobilization efficiencies of mutant plasmids containing the substitutions T141′A and G144′C were reduced over 4 logs relative to wild type. The mutant plasmid containing the R100 TraI *oriT* binding site was mobilized 3 orders of magnitude less efficiently than one containing F *oriT*. These results are consistent with the in vitro affinities, and with the finding that efficient cleavage requires a pyrimidine at position 141′. Plasmids containing *oriT* sequences that are not cleaved detectably in the in vitro assay are mobilized, albeit at greatly reduced efficiencies, suggesting that the mobilization assay is a more sensitive measure of activity.

DISCUSSION

Experimental observations, in vivo (*21*−*23*, *31*, *32*) and in vitro (*5*, *7*, *14*, *15*, *19*), indicate that F TraI specifically recognizes *oriT* as part of its function. We demonstrate here that TraI36 in vitro DNA recognition is of both high affinity and high sequence specificity. We also show that *oriT* variants bound by TraI36 with reduced affinity have altered in vitro cleavage and ligation characteristics and altered plasmid transfer efficiencies.

Specificity of TraI36 Recognition. The sequence specificity exhibited by TraI36 is remarkable. Single base changes at three consecutive positions (142′-144′; Table 2) in the TraI binding site reduce affinity by 5000-fold ($\Delta\Delta G \sim 5$ kcal/mol). TraI binds ssDNA instead of dsDNA, which may facilitate this strong discrimination between sequences. Because the DNA sequence is not base-paired, the DNA bases have considerably more surface area available for interaction with the protein. If the protein−ssDNA interface extends over much of the surface of a particular base, a substitution for that base could easily cause unfavorable steric or charge clashes, reducing affinity for the variant sequence. Structures of the sequence-specific ssDNA binding proteins *Oxytricha nova* telomere end binding protein (TEBP) (*33*, *34*) and heterogeneous nuclear ribonucleoprotein A1 (hnrA1) (*35*) in complex with ssDNA show excellent complementarity between DNA and protein, with bases partially or completely buried in the complexes. This complementarity may not always translate into specificity, however, as hnr A1 binds RNA oligonucleotides promiscuously (*36*, *37*). We do not know whether these or other ssDNA-binding proteins show the same level of sequence specificity for ssDNA as TraI36, because to our knowledge extensive specificity studies have not been performed.

TraI36 recognizes an 11-base length sequence-specifically, showing reduced binding for variants with substitutions from 9 bases 5′ to *nic* to 2 bases 3′ to *nic* (Tables 2 and 3). This

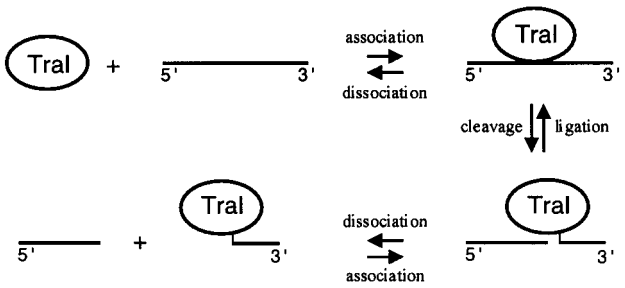

FIGURE 7: Schematic of TraI36 cleavage reaction.

stretch is similar to the 9-base region over which substitutions can reduce cleavage by R100 TraI (*24*) but is longer than that recognized by, for example, individual zinc fingers (*38*) or ribbon−helix−helix binding domains (*39*). Proteins hnrA1 and TEBP bind 12-base ssDNA sequences but do so by using multiple RNA-recognition motifs (*35*) or oligonucleotide/oligosaccharide binding folds (*33*, *34*), respectively. There is no evidence that TraI36 uses repeated motifs to bind DNA. Hydrodynamic analysis by analytical ultracentrifugation (Street et al., in preparation), however, suggests that TraI36 is a highly asymmetric prolate ellipsoid ($f/f_o = 1.48$). If so, TraI36 could easily accommodate even a fully extended 11-base ssDNA sequence by binding to the ssDNA along the protein's major axis.

Asymmetry of Recognition. TraI36 *oriT* recognition is highly asymmetric relative to the cleavage site, with most bases recognized being located 5′ to *nic*. This asymmetry may contribute to stability of the protein−DNA complex. After nicking, the protein is covalently linked to the DNA 3′ to *nic*. Most energetically important protein contacts are made to bases located 5′ to *nic*. This arrangement should slow or prevent the dissociation of the DNA both 5′ and 3′ to *nic* from TraI. The resulting tight association explains the observation that while TraI can form the covalent complex with DNA readily, TraI relaxes supercoiled plasmid optimally only upon protease and detergent treatment to disrupt the very stable protein−DNA interaction (*5*, *14*, *40*).

This finding also helps explain why TraI often does not completely cleave plasmid (*5*, *12*, *15*) or oligonucleotide substrates (*19*; Figure 4) in in vitro cleavage reactions, even when in great excess. In contrast, many of the variant oligonucleotides used here were cleaved completely at TraI36 concentrations at which the wild type *oriT* sequence was incompletely cleaved. This is seen in Figure 4b by comparing the ratio of 22-base substrate to 14-base product for the different oligonucleotides at 100 nM and 1 μM TraI36. More TraI36 is required to observe any cleavage of the variant oligonucleotides than the wild type *oriT* sequence, presumably reflecting their lower affinities for TraI36. These variant oligonucleotides, however, are completely cleaved at 1 μM TraI36, while the wild-type sequence is not. The in vitro cleavage reaction, schematized in Figure 7, has multiple steps. The incomplete cleavage of the wild type *oriT* sequence can be explained by noting the high affinity of TraI36 for the 5′ product (162′, Table 1) and the covalent link to the 3′ segment. If the ligation (reverse) reaction rate is comparable to the cleavage reaction rate and the dissociation of the 5′ product is slow, the apparent K_{eq} would be near 1 and incomplete cleavage of the substrate could be observed, even at protein excess. The increased cleavage of wild type *oriT* observed at high TraI36 concentrations could be due to trapping of the released 14-base product by excess TraI36, slowing or preventing reassociation and ligation.

The increased cleavage yield from some variant oligonucleotides, relative to wild type, may have several causes, including a reduced ligation (reverse) rate and an increased release of the 14-base product. The reduced affinity of TraI36 for T148′A, relative to wild type, is due to an increased off-rate (J.C.S. and J.F.S., unpublished observation). Assuming that all affinity reductions are due to increased off-rates, substrates such as G145′C that have reduced affinities for TraI36 resulting from substitutions 5′ to *nic* will have increased off-rates for the 14-base product. In this case, dissociation of the 14-base product will be favored relative to ligation, and a greater product yield will be observed. G140′C, like G145′C, yields more cleavage product than wild type *oriT*. The G140′C substitution, however, is 3′ to *nic* and should not affect the off-rate of the cleavage product. G140′C exhibits reduced ligation efficiency relative to the wild-type sequence (Figure 6), explaining the greater product yield for G140′C than wild type. The T141′C oligonucleotide, whose substitution is located 5′ to *nic*, has a reduced affinity relative to wild type. In addition, T141′C is less active in the ligation assay than G149′A, although both oligonucleotides are bound by TraI36 with similar affinities. For T141′C, an enhanced product off-rate and reduced ligation efficiency both contribute to higher product yield.

Role of T141′. Affinities of most *oriT* variants correlate with activity, with those sequences bound with lower affinity requiring higher concentrations of TraI36 for cleavage, and facilitating mobilization at a reduced efficiency. The base immediately 5′ to *nic* (141′), though, has an effect on cleavage and transfer that is disproportionate to its effect on binding. Pyrimidines at this position are favored for binding, and required for cleavage. The MobA protein, which performs the *oriT* cleavage/ligation reactions during conjugative transfer of plasmid R1162, also demonstrates stricter sequence requirements for cleavage than for binding, although the sequence specificities of MobA and TraI differ (*41*).

The role of T141′ in TraI catalysis is unclear. The cleavage reaction involves a nucleophilic attack by a tyrosyl hydroxyl oxygen on a DNA phosphate. For F Factor TraI, Tyr16 serves this purpose (see Results). T141′ may be directly interacting with Tyr16, other active site residues, or the required Mg^{2+}, contributing directly or indirectly to catalysis. Alternatively, pyrimidines at 141′ could permit the proper orientation of the DNA backbone phosphate to the catalytic Tyr side chain. Substitutions of purines or the dSpacer at this position could force local rearrangements of the protein and DNA, altering the position of the phosphate relative to the catalytic Tyr, preventing cleavage.

F and R100 TraI Specificity. F Factor and R100 *oriT* differ by only 2 bp near *nic*, yet the affinity of F TraI36 for the R100 sequence is reduced by almost 2000-fold (Table 2) and mobilization is reduced by 1500-fold (Table 4). This level of sequence specificity is not completely shared by R100 TraI, despite the high sequence identity between F and R100 TraI. While F TraI is apparently only active against F *oriT* in vivo, R100 TraI possesses some activity against both R100 and F *oriT* sequences in vivo, permitting transfer of chimeric plasmids (*23*). Because the rest of the TraI *oriT*

binding site is identical in F and R100, the activity of R100 TraI against both F and R100 *oriT* probably represents a relaxation, rather than a significant alteration, of specificity.

Oddly, F TraI36 failed to cleave the R100 *oriT* sequence in vitro, while oligonucleotides containing each of the two substitutions individually were cleaved (not shown). This came despite only a small difference in F TraI36 affinity for the R100 and R100T (G145′T) variant oligonucleotides (Table 2). As proposed for some substitutions at 141′, this result may represent adoption of an oligonucleotide conformation by the R100 sequence that prevents cleavage, rather than a loss of affinity of F TraI36 for the R100 sequence.

Correlation to in Vivo Activity. Our in vitro studies were performed with TraI36, while the mobilization studies were performed using an F′ strain that expresses full length TraI. We do not know how TraI regions beyond the identified relaxase domain might modulate ssDNA recognition and what the functional role of this modulation might be. Nevertheless, the correlation between our in vitro binding and cleavage/ligation assays and our in vivo mobilization assays suggests that the ssDNA recognition observed in vitro accurately reflects at least one step in the conjugative process. Consistent with this correlation, many labs have proposed that the binding of accessory proteins such as F TraY and IHF to *oriT* forces a unique, perhaps single-stranded, DNA structure on the TraI binding site, facilitating TraI binding (*9*, *11*−*13*).

The high level of sequence specificity of F Factor TraI36 is striking, given that TraI functions as part of a complex of DNA binding proteins. Both F TraY (*42*, *43*) and IHF (*44*, *45*) bind DNA with some sequence specificity. Because TraI binding is preceded by the binding of two other DNA binding proteins, we thought TraI might demonstrate lesser specificity, with the site of formation of the relaxosome dictated largely by the binding of TraY and IHF. The sequence specificity of TraI suggests that it is capable of serving as the final arbiter of relaxosome formation, interacting only with ssDNA with the appropriate *oriT* sequence. The specificity may be a byproduct of selection for high affinity TraI binding that would contribute to relaxosome stability. Alternatively, the specificity may play a greater role in steps following relaxosome formation, such as termination of the transfer through ligation of the ends of the single-stranded DNA. In this step, TraI might act without the assistance of other DNA binding proteins. A smaller segment of *oriT* is required for F transfer termination than for initiation (*21*), consistent with fewer proteins being involved at this stage. If so, TraI might be the sole determinant of specificity at this stage, possibly forcing TraI to maintain a high level of sequence specificity. Regardless of the details of its selection, the sequence specificity of TraI36 is remarkable, and its necessity can be easily rationalized. DNA sequences outside the TraY, IHF, and TraI binding sites are essential for proper initiation and termination of conjugative transfer (*21*, *46*). The sequence specificity of TraI may be required to ensure that relaxosome formation and transfer initiation occurs at only the appropriate plasmid site, so that the transferred plasmid possesses the necessary sequences for maintenance and subsequent transfer.

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