Sequence-specific Interactions in the Tus-Ter Complex and the Effect of Base Pair Substitutions on Arrest of DNA Replication in Escherichia coli*

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Arrest of DNA replication in Escherichia coli is mediated by specific interactions between the Tus protein and terminator (Ter) sequences. Binding of Tus to a Ter site forms an asymmetric protein-DNA complex that arrests DNA replication in an orientation-dependent fashion. In this study, mutant Ter sites carrying single base pair substitutions at 16 different positions were examined for their ability to bind purified Tus protein and arrest DNA replication. In vitro competition assays demonstrated that base pair substitutions at positions 8–19 had significant effects on the free energy of Tus binding (ΔΔG° of 1.5 to >4.0 kcal/mol). Concomitant with loss of binding affinity, mutations at these positions also showed significantly lower or undetectable replication arrest activities in vivo. Substitutions at positions 6, 20, and 21 had moderate effects on Tus-Ter interactions, suggesting that these base pairs contribute to Tus binding. Even though the effects on binding were minimal, these Ter mutants were not as efficient as wild type Tus-TerB complexes at arresting replication forks. Three new potential Ter sites, referred to as TerH, TerI, and TerF, were identified by searching the E. coli genome for sequence similarity to a consensus Ter site sequence.

The Tus protein of Escherichia coli binds to specific chromosomal sequences, called Ter sites, and arrests DNA replication in an orientation-dependent fashion (for review, see Ref. 1). Replication forks approaching from the non-permissive side of the Tus-Ter complex are arrested, but replisomes approaching from the permissive side can pass through the complex. The Ter sites are oriented in the chromosome to permit DNA replication fork trapping and prevent DNA replication in regions other than in the chromosomal terminus.

The functional polarity demonstrated by Tus is reflected in the asymmetry of the protein-DNA complex, whose crystal structure has been recently solved (2). Tus binds as a monomer (3, 4) and contacts both strands of the Ter site on the nonpermissive side of the complex, but only a single strand on the permissive side (2, 5). The Ter site DNA is nestled into a cleft formed by the two primary domains of Tus (amino and carboxyl domains) and the interstrand β-sheets that connect the two domains (2). The primary determinants of base pair recognition and binding are mediated by the main two interstrand β-sheets, which penetrate deeply into the major groove of the Ter site, making both polar and hydrophobic contacts with the bases. Binding is also enhanced by extensive contacts between Tus and the phosphates in the DNA backbone. A total of 42 amino acid residues stretched along the length of the protein make contacts with the DNA.

Tus binds to the chromosomal Ter sites with a very high affinity. The K_{obs} for Tus binding to the TerB site ranges between 3.4 \times 10^{-13} M and 7.5 \times 10^{-13} M, depending on the buffer conditions used (5, 6). Half-lives (t_{1/2}) of the protein-DNA complex were determined to be 550 to 149 min, respectively, in these studies. The high affinity of the Tus-Ter interaction in conjunction with the distribution of protein-DNA contacts has been used to suggest that Tus can arrest DNA replication by functioning as a clamp on the DNA and preventing the unwinding activity of the DnaB helicase (2, 5, 7, 8). Alternately, protein-protein interactions between Tus and the DnaB helicase have been postulated to mediate replication arrest. This latter model is based upon the specificity of Tus function (9, 10), differential ability of Tus to halt helicase unwinding when presented with different templates (11), and mutational studies on Tus (6, 12).

Ter sequences were originally identified as 22–23 base pairs in length, based on sequence identity between TerA, TerB, and TerC (13, 14). As additional sites were identified both in the chromosome and in plasmid replicons, it became apparent that the essential conserved elements of the Ter site were an 11-base pair “core” sequence (positions 9–19) and an upstream G-C base pair at position 6 (Fig. 1). Nucleoside analogs have been used to partially map the determinants of Tus binding (15, 16) and it was shown that (i) the G residues at positions 10, 13, and 17 within the core sequence contributed both major and minor groove interactions, (ii) the conserved G residue at position 6 contributed little to the overall stability of the Tus-Ter complex, and (iii) hydrophobic interactions with thymine methyl groups occurred at positions 8, 9, 12, 14, 16, and 19.

The solution of the co-crystal structure has identified specific interactions between Tus and the Ter site, but provides no information about the relative contribution of each contact to the free energy of binding. Likewise, the nucleotide analog studies can measure the energetic contributions of individual bases, but cannot determine the effect of natural base pair substitutions on binding. In addition, neither technique can
address the role that specific protein-DNA interactions play in the ability of the Tus-Ter complex to arrest DNA replication. With this in mind, we undertook a systematic mutational analysis of the TerB site to determine which natural base pair substitutions affect Tus binding in vitro and what effect base pair substitutions have on replication arrest function in vivo.

MATERIALS AND METHODS

**Bacteria and Plasmid Strains**—E. coli TH396 strain, the isogenic ΔterB derivative strain TH396 ΔterB (lac::proAB, P proAB lacI lac2ΔM15) was used to select plasmids carrying single base-substituted Ter sites. E. coli TH463 strain (ts) was constructed by transforming strain PK457 (13) with plasmid pACtet.

pSPORT1 (Life Technologies Inc.) contains a unidirectional origin of replication from the pUC vectors and confers ampicillin resistance. This 4.1-kilobase plasmid carries a multiple cloning site within the α-peptide of the lacI gene. Plasmids p5Ter were constructed by cloning mutated TerB oligomers into pSPORT1 in an active orientation approximately 1000 base pairs from the origin of replication. The plasmids were then transformed into TH463.

Plasmid pACtet, a generous gift from Bénédicte Michel (INRA, Jouy-en-Josas, France), was used as an internal control in *in vivo* experiments. This plasmid was constructed by deleting the CmR gene of pACYC184 (18). pACtet is 2.4 kilobase pairs in size and confers tetracycline resistance (19).

**Preparation of Oligomers with Substituted Ter Sites**—Two complementary 33-base oligodeoxyribonucleotides were synthesized such that base pair substitutions were introduced at two adjacent positions in the TerB sequence (for example, the first synthesis reaction introduced substitutions at positions 6 and 7). This process was repeated for each pair of substitutions from positions 6 to 21 (Fig. 1). Using this method of oligomer synthesis, it was possible to collect all single base substitutions at positions 6 and 7 only. This process was repeated for each DNA sample.

**Cloning of Mutated Ter Sites into the Plasmid pSPORT1**—The substituted 33-mer duplexes were cloned between the EcoRI and BamHI sites of the plasmid pSPORT1 in the "blocking" orientation so that functional Ter sites would arrest plasmid replication in the *in vivo* replication termination assay.

**Purification of Tus**—Tus protein, Fraction 6A, was purified from strain TH241 as described previously by Hill and Marians (20) with an additional fractionation step using Sephacyr S-100. Protein concentrations were determined using either Tus protein or bovine serum albumin as a standard.

**Activity of Tus Protein**—The activity of the Tus protein preparation was determined using the filter binding assay described previously by Gottlieb et al. (5). Binding of Tus to a 32P-labeled oligomer containing the TerB site was measured by incubating varying concentrations of Tus with a fixed amount of TerB (1 × 10⁻¹³ m) in KG200 binding buffer containing 50 mM Tris-Cl, pH 7.5 (at 25 °C), 200 mM potassium glutamate, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 100 μg/ml bovine serum albumin. Aliquots from each sample were applied to 25-mm nitrocellulose filters (Whatman HAWP) that had been presoaked in KG200 filter buffer. The filters were then washed with 1 ml of KG200 filter buffer, dried, and counted. The amount of TerB bound at each protein concentration was then used to determine the fraction of active Tus protein. The preparation of Tus used in this study was 50% active.

**Equilibrium Competition Assay**—The binding affinities (K<sub>0.5</sub>) of Tus to wild-type TerB and to all single base-pair-substituted mutant Ter sequences were determined. The quantitative competition assay described by Lin and Riggs (21, 22) with some modifications.

Intact plasmids carrying the wild-type and mutant Ter sites were used as competing DNA for Tus binding instead of the short oligomers used in previous competition studies (15). Bacterial cultures of the TH396 (ΔM101 [tsu::kan]) strain containing the appropriate plasmids were grown in 5 ml of standard LB medium at 37 °C for 24 h, and 500 μl of cells were then used to inoculate a 500 ml of LB medium. Following 24 h of shaking at 37 °C, cells were harvested and plasmid DNAs for each sample were isolated using the Wizard maxi-prep DNA purification system (Promega) according to the manufacturer's recommendations.

To prepare radiolabeled DNA for the competition experiments, 0.2 μg of double-stranded DNA (37-mer) containing the wild-type TerB sequence was labeled by a fill-in reaction with [α-³²P]dATP and [α-³²P]dTTP using Sequenase (U. S. Biochemical Corp.). Following incubation at room temperature for 15 min, the reaction mixture was passed through a Sephadex G-50 column to separate unincorporated nucleotides from the labeled DNAs. Because approximately 30% of the starting material was lost during this step, the final DNA concentration was adjusted accordingly.

In the competition assay, a constant amount of ³²P-labeled wild-type TerB (1 × 10⁻¹³ m) was mixed with the Tus protein (2.1 × 10⁻¹⁴ m) and varying amounts of unlabeled competitor plasmid DNA in a total volume of 800 μl. All DNA and protein dilutions used in this assay were prepared using KG200 binding buffer. The reaction mixtures were incubated for 5 h at room temperature (25 °C), and 230-μl aliquots of each sample were filtered through nitrocellulose filters presoaked in KG200 filter buffer. The filters were washed with 1 ml of KG200 filter buffer, dried, and counted in 5 ml of Ultima Gold scintillation mixture (Packard) to determine the amount of radioactivity retained on the filters. Each bound and unbound sample was determined by incubating labeled wild-type TerB DNA (1 × 10⁻¹³ m) with competitor plasmid DNA (1 × 10⁻⁹ m) in a total reaction volume of 800 μl in the absence of Tus protein. The maximum retention counts were also determined by mixing labeled wild-type TerB DNA (1 × 10⁻¹³ m) with saturating quantities of Tus protein (2 × 10⁻¹⁰ m). The competition assays were performed twice for each DNA sample.

The data from these experiments were evaluated using a nonlinear regression analysis method by applying the SIGMAPLOT software program (Jandel Scientific Inc.) and the following equation as described in Duggan et al. (15).

\[ RO = \frac{[K_0 + [K_{RC}]}{[K_{RC}]} C_t + R_t + O_t \]  

where R<sub>t</sub> is the total Tus protein, K<sub>0.5</sub> is equilibrium dissociation constant for wild-type TerB, K<sub>RC</sub> is equilibrium dissociation constant for competitor DNA (mutant Ter), C<sub>t</sub> is total competitor DNA, and O<sub>t</sub> is total labeled wild-type TerB DNA.

**Estimation of Binding Free Energy Changes**—To estimate how each base pair energetically contributes to Tus-Ter binding, the free energy changes relative to wild-type TerB were determined. Briefly, the observed binding constants (K<sub>0.5</sub>) of Tus for the mutated Ter sites were converted to ΔΔG° values using the equation from Takeda et al. (23),

\[ \Delta \Delta G° = -RT \ln \left( \frac{K_{0.5 \text{mutant}}}{K_{0.5 \text{wild-type}}} \right) \]  

where, ΔΔG° is free energy change, T is temperature at which the experiments were performed (298 K), and R equals the gas constant (1.987 cal/mol K).

**Replication Intermediate Assay**—Single base-substituted mutant Ter sites were examined for their ability to arrest replication *in vivo* using the replication intermediate or "Y-fork" assay described by Horiuchi et al. (24), with some modifications. Isolated plasmids carrying the substituted mutant Ter sites (p5Ter) were transformed first into a Tus<sup>−</sup> strain (TH463) containing a secondary plasmid, pACtet, which served as an internal control for copy number of plasmid p5Ter. A 100-μl aliquot from an overnight culture of TH463/p5Ter was used to inoculate 7 ml of LB medium containing ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml), and the cells were grown to an A<sub>600</sub> of 1.0. After pelleting the cells by centrifugation, the total cell DNA was isolated by treating the cells with 50 mg/ml lysozyme in TE buffer for 5 min, then adding 10 μg/ml Proteinase K and 1% SDS. Following overnight incubation at room temperature, the DNA was extracted with phenol and phenol/CHCl<sub>3</sub> precipitated with EtOH, and resuspended in 300 μl of TE buffer. The DNA was then digested with EcoRV, which cuts the p5Ter plasmid once in the region between the origin and the Ter site, producing linear and Y-fork replication intermediate fragments. The EcoRV digestion also linearizes the internal control plasmid pACtet. The digested DNA samples were loaded onto a 0.8% agarose gel.
and electrophoresed for 4 h at 150 volts, blotted onto nitrocellulose membrane, and then probed with $^{32}$P-labeled pSPORT1 and pACtet plasmids. After autoradiography, the bands corresponding to the linear and replication intermediates of pS/Ter and to the internal control plasmid (pACtet) were excised from the nitrocellulose membrane and counted. The data from this in vivo assay was evaluated using the following equation,

$$\text{Activity} = \frac{(\text{fraction of pSPORT1 in Y-fork form})}{(\text{fraction of pSPORT1 in linear form})}$$  \hspace{1cm} (Eq. 3)

RESULTS

Sixteen different base pairs of the TerB site, between positions 6 and 21, were subjected to substitution analysis (Fig. 1). It was possible to collect all three single base-substitutions at each position, except for positions 7 and 19, where only two substitutions were isolated. Therefore, a total of 46 single base pair-substituted Ter mutants were isolated and examined further using two different assays. An equilibrium competition assay was used to determine the binding affinity changes of Tus to the mutant Ter sites and a replication intermediate assay was performed to examine the ability of the mutant Ter sequences to arrest DNA replication in vivo.

**Observed Equilibrium Dissociation Constants, (K_{app})**—The observed equilibrium dissociation constant (K_{app}) for each single base pair substitution was determined by measuring the ability of the mutant Ter sites to compete with a $^{32}$P-labeled oligomeric wild-type TerB site for Tus binding under equilibrium conditions. Because the competition assays utilized mutant Ter sites embedded in a plasmid, the possibility of non-specific binding of Tus to the vector sequences of the plasmid was first estimated using the following equation (25),

$$K_{app} = K_S + N_{NS}K_{NS}$$  \hspace{1cm} (Eq. 4)

where $K_{app}$ is the observed equilibrium association constant, $K_S$ is the true equilibrium association constant, $K_{NS}$ is the equilibrium association constant for nonspecific DNA (previously determined to be in the range of 2.4 x 10^{-7} M for Tus binding to a 33-base pair non-Ter oligomer),\(^1\) and $N_{NS}$ is the number of nonspecific binding sites in the plasmid (approximately 300 in pSPORT1). Based on this equation, we would expect non-specific binding to become problematic when the binding equilibrium constant exceeds $1 \times 10^{-9}$ M.

Non-specific binding of Tus to DNA was then empirically measured in a competition assay by mixing increasing concentrations of pSPORT1 DNA (without a Ter site) with a fixed amount of labeled TerB DNA and determining the decrease in binding to the labeled DNA. Only at DNA concentrations higher than $1 \times 10^{-9}$ M was significant non-specific binding of Tus to pSPORT1 observed (Fig. 2). Consequently, for all subsequent competition experiments, competitor DNA concentrations were kept in the range of $1 \times 10^{-9}$ to $1 \times 10^{-13}$ M. Additionally, to determine if the plasmid contained any other sequences that might affect Tus-Ter binding (such as the presence of pseudosites), unlabeled TerB oligomer and plasmid DNA containing the TerB site were individually assayed in competition reactions. The $K_{app}$ for oligomeric TerB was found to be almost identical to the $K_{app}$ of a TerB site embedded in plasmid DNA ($8 \times 10^{-13}$ M and $8.6 \times 10^{-13}$ M, respectively), indicating a lack of pseudosites or other non-specific interactions with the plasmid DNA.

To calculate the observed equilibrium dissociation constants, the data obtained from the competition assays were fitted to a binding curve using nonlinear regression analysis. The $K_{app}$ for all single base pair-substituted Ter sequences are listed in Table I and the data from these measurements are presented in Fig. 3. The relative affinity changes for Tus binding to the mutant Ter sites were also calculated in terms of free energy changes ($\Delta G^0$) for each base pair substitution.

The results indicate that the greatest energetic penalties ($\Delta G^0 > 4.0$ kcal/mol) are exacted by substitutions located in the 11-base pair core region, at positions 10 and 12–19. The magnitude of the free energy change obtained for substitutions in this region demonstrates that these positions are absolutely critical for Tus binding. A second class of important base pairs, where all substitutions resulted in a $\Delta G^0$ of 1.5–3 kcal/mol, are located at the front of the Tus-Ter complex and include positions 8, 9, and 11. The third class of substitutions were located at the edges of the complex and included the highly conserved GC base pair at position 6, as well as bases 7, 20, and 21. All substitutions but one in this third class imposed energetic penalties of less than 1.5 kcal.

**Examination of in Vivo Replication Arrest Ability of Mutant Ter Sites**—The mutant Ter sites were also assayed in vivo to determine the effect base pair substitutions had on the ability of the altered Tus-Ter complexes to arrest DNA replication. For this purpose, an assay was used that identifies replication intermediates arising from arrested DNA replication. First, plasmids containing the mutant Ter sites (pS/Ter) were transformed into the tus+ strain TH463, which also contains a compatible internal control plasmid, pACtet. Cells were grown to mid-log phase and whole-cell DNA was isolated under conditions that leave replicative intermediates intact. The extracted DNA samples were then digested with EcoRV, which cuts pS/Ter between the origin and the Ter site, and subjected to electrophoresis. If DNA replication was not arrested at the mutant Ter site, only a linear pS/Ter DNA was produced by EcoRV digestion. However, if DNA replication was arrested at

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\(^1\) F. F. Coskun-Ari and T. M. Hill, unpublished results.
In a previous study using the replication intermediate assay to examine mutant Tus proteins (6), two factors were influenced by \textit{in vivo} replication arrest activity. First, the ratio of the replication intermediate band to the linear band differed from one mutant to the other, depending on the efficiency of the Tus-Ter complex. Second, the total amount of plasmid DNA, which is related to the plasmid copy number, varied in each mutant as well. These factors were expected to vary in the replication intermediate assay because it was previously shown that the presence of a functional Ter site affected the copy number of pBR322-type plasmids (14, 26) and also affected the accumulation of replication intermediates relative to the amount of linear plasmid (26). Thus, to determine the efficiency of replication arrest in this previous study, the copy number of the plasmid and the ratio of replication intermediate to linear plasmid forms were considered when making quantitative comparisons between the mutant and wild-type Tus proteins.

In this study, we also observed a general correlation between the efficiency of the Ter site and the plasmid copy number; however, we were unable to include this effect when quantitating the efficiency of replication arrest by the mutant Ter sites. This is because the correlation between the effectiveness of the Ter sites and the copy number of the plasmids was not always consistent with the pSPORT1 vector. The most obvious inconsistency was observed when comparing the pSPORT1 vector without a Ter site and the same plasmid with the wild type TerB site: the copy number of the two plasmids were almost identical (1.13 ratio; Fig. 4). In previous studies (6, 26) the copy of the Ter-less plasmid was always significantly higher (twice as much) than the same plasmid with the TerB site. We also observed a significant increase in the plasmid copy number when a weak Ter site was substituted for the TerB site. In several cases, such as substitutions at 12, 13, 14, and 15, the copy number of the Ter-containing plasmid was as much as four times higher than the copy number of the pSPORT1 alone (Fig. 4). At this point, we cannot explain these inconsistencies. Consequently, only the ratio of the Y-fork to linear band was used to quantitate Ter site efficiency in this study.

We also noted two distinct replication intermediate bands in samples of functional Ter mutants and in the WT \textsuperscript{4} TerB. We speculate that the doublet replication intermediate bands are generated from head-to-tail pSPORT dimers, which were found in significant quantities in our plasmid preparations (data not shown). Dimeric plasmids can contain either one or two replication bubbles, depending upon whether initiation has occurred at one or both of the replication origins. Subsequent digestion of the dimeric plasmids with EcoRV would produce replication intermediates with either a single or double Y. These two DNA species would migrate differently in agarose gel, presumably giving rise to the replication intermediate doublet. The appearance of doublet replication intermediate bands has been observed previously in studies of Tus mutants, in which plasmid pHV750T <sup>2</sup> was used to measure replication arrest function (6). Because the two bands in the doublet are believed to result from replication arrest, both bands were excised together when measuring the relative replication arrest activity of the substituted Ter sites.

A final observation regarding the \textit{in vivo} assay was that the relative amounts of the two replication arrest bands varied in samples according to the strength of the Ter site (Fig. 4, position 6 mutants). In the WT TerB and the 6TA mutant the banding pattern was the same: the slower-migrating band of the doublet, which was assumed to be the double Y form, was

| Table I |
|-----------------|-----------------|-----------------|-----------------|
| **WT BP** | **Position** | **Substitution** | **$K_{app}$** | **$\Delta G^\circ$** |
|  |  |  | $10^{-12}$ M | kcal/mol |
| A 1  | C  | A  | 9 ± 1 | —<sup>a</sup> |
| A 2  | C  | G  | 35 ± 4 | 0.83 |
| A 3  | G  | C  | 16 ± 4 | 0.36 |
| T 4  | C  | A  | 31 ± 12 | 0.97 |
| A 5  | G  | C  | 46 ± 6 | 0.99 |
| A 6  | G  | C  | 668 ± 13 | 2.57 |
| A 7  | G  | C  | 139 ± 4 | 1.65 |
| A 8  | G  | C  | 952 ± 4 | 2.79 |
| A 9  | G  | C  | 232 ± 33 | 1.95 |
| G 10 | A  | T  | 57 ± 5 | 0.80 |
| G 11 | A  | T  | 428 ± 16 | 2.31 |
| G 12 | A  | T  | 507 ± 22 | 2.41 |
| G 13 | A  | T  | 1204 ± 181 | 2.92 |
| G 14 | A  | T  | 454 ± 14 | 2.35 |
| G 15 | A  | T  | 1622 ± 56 | 3.10 |
| G 16 | A  | T  | 6015 ± 280 | 3.88 |
| G 17 | A  | T  | 6994 ± 68 | 3.97 |
| G 18 | A  | T  | 4561 ± 253 | 3.71 |
| G 19 | A  | T  | 538 ± 5 | 2.45 |
| G 20 | A  | T  | 4335 ± 315 | 3.68 |
| G 21 | A  | T  | 69 ± 17 | 1.23 |
| G 22 | A  | T  | 165 ± 9 | 1.75 |
| G 23 | A  | T  | 29 ± 5 | 0.72 |
| G 24 | A  | T  | 93 ± 18 | 1.41 |
| G 25 | A  | T  | 39 ± 16 | 0.89 |
| G 26 | A  | T  | 11 ± 1 | 0.15 |

<sup>a</sup> Not determined.

<sup>b</sup> ND, relative arrest activity was not detectable.

<sup>2</sup> F. F. Coskun-Ari and T. M. Hill, unpublished data.

<sup>3</sup> The abbreviation used is: WT, wild type.
predominant over the faster-migrating band of the doublet, or the single Y-fork form. However, in the 6C-G mutant the single Y-fork form was found to be the most predominant band. Finally, in the 6A-T mutant sample the two Y-fork forms were present in equal amounts. The observation that the double Y was predominant in strong Ter sites and the single Y was predominant in weak Ter sites was consistent and reproducible, and seen with several other Ter mutants (positions 8, 9, and 18; Fig. 4). The reason for such a banding pattern is currently unknown.

The efficiency of replication arrest in each of Ter mutant is presented in Table I. Base pair substitutions in the core region of the Ter site had the most significant effects on replication arrest activity and correlated with loss of DNA binding (Fig. 5).

In general, \( \Delta \Delta G^0 \) values greater than 2.5 kcal completely abolished the ability of the Tus-Ter complex to halt DNA replication in vitro (Table I). Substitutions that resulted in low levels of replication arrest activity (from 20 to 1% of wild type levels) were typically associated with \( \Delta \Delta G^0 \) values of 1.7 to 2.5 kcal, with the notable exception of the 6C-G substitution, which had a \( \Delta \Delta G^0 \) value of only 0.35 kcal. Intermediate levels of replication arrest activity (80–20%) also showed a strong correlation with loss of binding activity, with \( \Delta \Delta G^0 \) values ranging from about 1 to 1.75. Again, notable deviations occurred with position 6 substitutions (A-T and T-A), and with the 20T-A substitution, all of which resulted in \( \Delta \Delta G^0 \) values of less than 1.
A systematic mutational analysis of the E. coli Ter sequence was undertaken to investigate the contribution of individual base pairs to the binding and function of the Tus protein. Substitutions were made at positions 6–21, which were identified previously as highly conserved bases associated with Tus binding (13, 14). It is now known that position 5 is also contacted by Tus, through residue 198 (2). However, this information was unavailable to us at the time this project was initiated and substitutions at this position were not investigated.

Effects of Substitutions at Positions 6 and 7—The G-C base pair at position 6 is conserved in all known Ter sites, however, the results reported here and in studies with nucleoside analogs (15) suggest that this position contributes little to the stability of the Tus-Ter complex. The minimal change associated with the free energy of binding (ΔΔG0 values of less than 1) may indicate that the Arg198 residue of Tus, which normally contacts the N-3 atom of G6 and the N-3 atom of A5 (Fig. 6), remains anchored to position 5 when substitutions are made at position 6. Alternatively, contact between Arg198 and the DNA could be completely lost when substitutions are made at position 6. In either scenario, it is possible that the position 6 substitutions do not have significant effects on Tus binding because the primary contacts for binding of this domain of Tus are made by other amino acids in the L3 loop and αVII domain (Ser193, Val200, and Trp208), with the phosphate group and deoxyribose moieties around base pair 6. With this in mind, it is of some interest to note that the tus gene of Salmonella has an alanine substituted for arginine at position 198, whereas the residues at positions 193, 200, and 208 are identical.4

Even though the effects of position 6 substitutions on DNA binding were minimal, the in vivo replication intermediate assay indicated a strong preference for the G-C base pair at this position. Replication arrest activities of the mutants were found to be significantly reduced (Fig. 4), with the C-G substitution showing the greatest effect (7% of the efficiency of the WT TerB). Furthermore, these substitutions showed the greatest deviation from the correlation between loss of DNA binding and loss of replication arrest activity (Fig. 5).

In contrast to position 6, position 7 is not well conserved in Ter sites (Fig. 7) and no amino acid residues of Tus contact this base pair (2). The two substitutions made at position 7 did not significantly affect the binding affinity of Tus, since the ΔΔG0 values were less than 1.0 kcal/mol and similar to the changes associated with substitutions at position 6. Because no contacts are made between protein and DNA at this position, we speculate that the minor changes associated with position 7 substitutions reflect a perturbation of the protein-DNA interface, which destabilizes the Tus-Ter complex.

Despite the similarities noted between positions 6 and 7 substitutions at the level of DNA binding, Ter sites substituted at position 7 retained near normal levels of replication arrest activity. The difference in the effect of substitutions on replication arrest activities at positions 6 and 7 suggests that Tus retains its proper configuration for replication arrest when position 7 substitutions are introduced, but not when substitutions are made at position 6. This differential effect may explain the conservation of position 6 in all Ter sites. We speculate that position 6 substitutions alter the geometry of the Tus-helicase interaction and allow the helicase to invade the Tus-Ter-binding domain, thereby displacing Tus more efficiently. If so, it follows that the L3 loop or αVII domain might be involved in a protein-protein interaction with the helicase. Site-directed mutagenesis of this region to test this hypothesis is currently underway.

Effects of Substitutions at Position 8—The A-T base pair at position 8 is conserved in all known chromosomal Ter sites, but several plasmid Ter sequences contain a G-C base pair at this position. Because of the sequence substitution in plasmids,
position 8 has not been considered part of the core region. These plasmid Ter sites have been shown to bind Tus less well than the chromosomal TerB sequence and arrest DNA replication less efficiently (5, 13, 14). However, because the plasmid Ter sites also have substitutions at other positions, the impact of position 8 substitution alone could not be determined. The data obtained from the substitution studies presented here suggests that this base pair does play a significant role in Tus binding and represents a critical base pair for both Tus binding and replication arrest activity.

Tus makes two contacts with the thymidine residue at position 8: Lys 89 makes a minor groove interaction with the O-2 group and Thr 139 makes a major groove contact with the methyl group (Fig. 6). As might be expected from this pattern of contacts, we observed that the energetic penalty was the lowest (1.65 kcal/mol) with the G$^z$C substitution. This substitution retains the Lys 89 contact (which also interacts with the O-2 group of the position 9 thymidine), but the Thr 136 contact is lost. The two other base pair substitutions, C$^z$G and T$^z$A, eliminate both contacts and hence showed relatively large free energy changes for Tus binding (>2.5 kcal/mol). In accordance with the effects of substitutions on DNA binding, the replication arrest activity of the Tus-Ter complex was reduced the least with the G$^z$C substitution (27% compared with the activity of Tus-TerB complex) and the most with the T-A and C-G substitutions (only 6 and 1%, respectively, of the WT TerB efficiency). Unlike substitutions at position 6, the correlation between replication arrest and loss of DNA binding was good.

Effects of Substitutions at Positions 9 through 19—Positions 9 through 19 constitute the 11-base pair core sequence and make extensive contacts with multiple residues of the Tus protein (Fig. 6). With the notable exceptions discussed in the following paragraphs, substitutions in the core region imposed very severe energetic penalties on DNA binding and concomitant loss of replication arrest activity. In particular, all substitutions at positions 10, 12–16, and 19 destroyed both DNA binding and replication arrest activity. Single substitutions at positions 17 and 18, and multiple substitutions at positions 9 and 11 were tolerated, but these mutant Ter sites bound Tus far less well than wild-type sites, with ΔΔG$^0$ values ranging from 1.7 to 2.9 kcal/mol. The observed loss of DNA binding activity throughout the core region corroborates the results from nucleoside analog substitution studies performed previously (15).

One set of substitutions that did not completely eliminate Tus binding activity occurred at position 9. This A$^z$T base pair makes contacts with the Lys 89 and Arg 232 residues of Tus through the O-2 and methyl group moieties, respectively, of the thymidine residue. Each base pair substitution caused significantly reduced affinity for Tus binding, with free energy changes ranging from 1.9 to 2.4 kcal/mol (Table I). The ΔΔG$^0$ values associated with the A-T and C-G substitutions are comparable in magnitude to the G-C substitution at position 8; likewise, the in vivo replication arrest activity of the C-G sub-
The G-C substitution at position 18 is the only core substitution associated with a known Ter site, TerF. However, we noted that the \( K_{obs} \) measured for the G-C substitution (5.4 \( \times \) 10\(^{-11}\) M) is 50-fold higher than that measured previously for TerF (1 \( \times \) 10\(^{-12}\) M; Ref. 27). Upon checking the previous publication, we discovered that the oligomer used for the TerF binding studies had a wild type T-A base pair at position 18 rather than the G-C substitution. Thus, we believe that the value reported here more accurately reflects the effects of position 18 substitutions and that the binding of Tus to TerF and the replication arrest activity of this site is weaker than previously reported.

Position 19 is the last base pair of the consensus core sequence, but the crystal structure study did not report any protein-DNA contacts with either nucleotide of this A-T base pair (2). However, the two substitutions reported here resulted in \( \Delta G^0 \) values of \( \approx 4 \) kcal/mol and 3.68 kcal/mol, respectively, for the C-G and G-C substitutions, indicating that this base pair makes essential contacts with Tus. Nucleotide analog studies indicated that the methyl group of the thymidine residue is involved in binding Tus (15), but other contacts between protein and DNA are possible. The Gln248 residue of Tus appears involved in binding Tus (15), but other contacts between protein and DNA are possible. The Gln248 residue of Tus appears involved in binding Tus (15), but other contacts between protein and DNA are possible. The Gln248 residue of Tus appears involved in binding Tus (15), but other contacts between protein and DNA are possible. The Gln248 residue of Tus appears involved in binding Tus (15), but other contacts between protein and DNA are possible. The Gln248 residue of Tus appears involved in binding Tus (15), but other contacts between protein and DNA are possible.
complex (T19) and are positioned along the DNA axis at the protein-DNA interface.

We were also surprised to find that substitutions at position 21 affected Tus binding, because this position is not conserved in known Ter sites. \( \Delta \Delta G^0 \) values ranged from 0.15 to 1.41 kcal/mol, with in vitro replication arrest efficiencies falling into the 60% range. We speculate that the effects on Tus binding are indirect, since it is unlikely that Tus makes contact with this base pair. A more likely explanation is that the sequence context of the Ter site influences Tus binding along with replication arrest efficiency. Such an effect has been postulated for sequences upstream of Ter sites (26). It would be of some interest to examine the effect of substitutions at both the 5' and 3' end of the Ter site to determine if additional context effects can be demonstrated.

Correlation between Tus Binding and Tus Function—We observed a very good correlation between loss of Tus binding and loss of replication arrest activity (Fig. 5). Replication arrest activity became undetectable when a \( \Delta \Delta G^0 \) value of 2.7 kcal/mol was reached or exceeded, which corresponds to a \( K_{obs} \) of approximately \( 9 \times 10^{-11} \) M. If we assume that the primary effect of substitutions on Tus binding is to destabilize the complex, as has been shown previously in nucleoside analog substitutions (15), then it follows that the decrease in the stability of the Tus-Ter complex decreases results in a corresponding loss of replication arrest activity until a point is reached at which Tus does not occupy the Ter site for a length of time sufficient to arrest DNA replication. A rough calculation of the \( t_{50} \) of the complex at which replication arrest function is lost can be made. In the buffer conditions used for these studies, the half-life of wild type Tus bound to the TerB site is 149 min and the \( K_{obs} \) was \( 9 \times 10^{-13} \) M. Thus, a \( K_{obs} \) of \( 9 \times 10^{-11} \) M would correspond to a half-life of 1.5 min in vitro.

New Ter Sites Identified—The data obtained from these studies allowed us to derive a new consensus for Tus binding and identify allowable substitutions that permit Tus to bind and arrest DNA replication, albeit at reduced efficiency compared with the consensus sequence. Using this information, we searched for new Ter sites in the E. coli genome, Genbank™ accession number U00096. One strong site and two weak sites were recovered, TerH, TerI, and TerJ. A strong site with only a position 20 TA substitution, is located at 12.97 min on the E. coli genome (bases 601897–601912) near the pheP gene. TerI and TerJ both contain single A/T substitutions at position 9, and are relatively weak in function. TerI is located at 13.46 min (bases 624637–624652) in the coding region of the enC gene and TerJ is located at 55.49 min (bases 2574042–2574057) in the coding region of a 759-amino acid open reading frame located next to tktB. All of the new sites are oriented in the chromosome such that replication is permitted in the origin-to-terminus direction.

The identification of the new sites brings the total number of Ter sequences to 10, with five sites located on each half of the chromosome. All 10 of the sites are located in the terminal third of the chromosome. Four very strong sites (TerA-TerD) are located at the boundaries of the terminus region and two very strong sites (TerE and TerG) are located outside the terminus, as are the remaining moderately strong site (TerH) and the weak sites (TerF, TerI, and TerJ). In Bacillus subtilis, which contains an unrelated but functionally similar replication arrest system, at least six Ter sites are found in the terminal segment of the chromosome (three on each arm) and are oriented to form a replication fork trap (28). The striking symmetry in the distribution of the Ter sites on the chromosomal arms of both bacteria and their placement in the terminal region of the chromosome emphasizes (i) the need for multiple “backup” Ter sites to prevent replication forks from escaping the terminus region and (ii) the importance of restricting the point in the chromosome where replication forks meet.

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