Mutations in the Escherichia coli Tus Protein Define a Domain Positioned Close to the DNA in the Tus-Ter Complex*

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Aikaterini Skokotast, Hiroshi Hiasa†, Kenneth J. Marians†, Leslie O’Donnell‡, and Thomas M. Hill§

From the †Department of Bioscience and Biotechnology, Drexel University, Philadelphia, Pennsylvania 19104, the ¶Molecular Biology Program, Sloan-Kettering Memorial Cancer Center, New York, New York 10021, and the §Department of Microbiology and Immunology, University of North Dakota School of Medicine, Grand Forks, North Dakota 58202-9037

A new genetic screen for mutations in the tus gene of Escherichia coli has been devised that selects for Tus proteins with altered ability to arrest DNA replication. We report here the characterization of three such mutants: TusP42S, TusE49K, and TusH50Y. TusP42S and TusE49K formed a more stable protein-DNA complex than wild-type Tus whereas TusH50Y functions at 78% efficiency. The loss of replication arrest activity did not correlate with changes in the stability of the Tus-TerB complexes formed by the mutant proteins. TusE49K formed a more stable protein-DNA complex than wild-type Tus (t½ of 178 versus 149 min, respectively) and TusP42 had a 9-min half-life, yet these two mutants showed identical efficiencies for replication arrest. When tested in vitro using a helicase assay or an oriC replication system, we observed a general, but imperfect, correlation between the in vivo and in vitro assays. Finally, the half-lives of the mutant protein-DNA complexes suggested that the domain of Tus where these mutations are located is positioned close to the DNA in the Tus-Ter complex.

DNA replication forks traversing the chromosome of Escherichia coli are halted at specific protein-DNA complexes composed of the Tus protein bound to a Ter site (for reviews, see Hill (1992), Yoshikawa and Wake (1993), and Baker (1995)). Tus binds a Ter site as a monomer (Sista et al., 1991; Coskun-Ari et al., 1994) and forms an asymmetric protein-DNA complex that arrests replication forks in an orientation-dependent fashion (Gottlieb et al., 1992). Thus, the Ter sites that have been identified in the E. coli chromosome (TerA-TerF) are oriented to permit DNA replication in the origin to terminus direction, but block DNA replication forks moving in the terminus to origin direction (deMassy et al., 1987; Hill et al., 1987; Hidaka et al., 1988).

The Tus protein contains 309 amino acids and binds the TerB site with high affinity, yet does not contain a recognizable DNA binding motif (Hill et al., 1989). Furthermore, it has not been possible to identify a DNA-binding fragment by proteolysis and the protein appears to be fully protected from trypsin or chymotrypsin when bound to a Ter site (Coskun-Ari et al., 1994). Tus is believed to arrest DNA replication by inhibiting the action of DnaB (Khatri et al., 1989; Lee et al., 1989; Hiasa and Marians, 1992), the major replicative helicase of E. coli (Lebowitz and McMacken, 1986). The mechanism for Tus function is proposed to occur through protein-protein interactions between a domain of Tus and DnaB. The first evidence for this type of mechanism was the apparent specificity of the interaction between Tus and DnaB (Khatri et al., 1989). More compelling evidence for a protein–protein interaction between Tus and the replisome came from studies where the length of the oligomer on a helicase substrate affected the ability of Tus to block DnaB helicase (Hiasa and Marians, 1992). When a short 60-nucleotide long oligomeric substrate was used, Tus was able to block DnaB progression in an orientation-dependent manner. But when a longer, 250-nucleotide long substrate was used, Tus was unable to block DnaB. These observations suggested that on a short substrate, one measures strand displacement rather than helicase unwinding. Therefore, on the long substrate where true helicase unwinding is occurring, Tus was not able to block DnaB progression, suggesting that binding of Tus to Ter alone does not inhibit DnaB and that protein–protein interactions must also be involved.

This observation is supported by the recently characterized mutant TusA173V (Skokotast et al., 1994), TusA173V has a much lower affinity for the TerB site than wild-type Tus (Kobs of 4.4 × 10⁻¹¹ M versus 7.8 × 10⁻¹³ M, respectively, in KG200 buffer) and forms a less stable protein-DNA complex, yet still arrests DNA replication in vivo at approximately 75% the efficiency of wild-type Tus. This result indicated that a large decrease in the affinity of TusA173V for TerB did not lead to a corresponding decrease in replication arrest activity, as would be expected if binding alone was responsible for the inhibition of DNA replication.

In order to understand further the molecular basis of replication termination, we have mutagenized the tus gene to produce proteins with altered function. Potential tus mutants identified by a new genetic screen were characterized on the basis of their ability to bind to Ter sites and by their ability to halt DNA replication in a plasmid containing a Ter site. Here we report the isolation and characterization of three new Tus mutants that bind Ter sites, yet have reduced ability to halt DNA replication forks.

MATERIALS AND METHODS

Plasmids and Bacterial Strains—Plasmid pBAD18 (from J. Beckwith, Harvard) contains the araC gene, pBAD promoter, and pBR322 origin of replication; it confers ampicillin resistance and is 4.6 kb1 in size. Plasmid pACMts was a generous gift from B. Michel (INRA, J ouy-en-ès-As) in which the araC gene, pBAD promoter, and tus gene of pBADts were cloned into pACYC184. It is 5.4 kb in size and confers chloramphenicol resistance.

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§ To whom correspondence should be addressed. Current address: The Wistar Institute, Philadelphia, PA 19104.

* The abbreviation used is: kb, kilobase pair.
resistance. pAXtus describes pACMtus plasmids that have been passed through XL-1 Red cells. Plasmid pHV750T2 is a derivative of pHV750 (Biene et al., 1991) and contains a TerB site inserted in an active orientation approximately 900 base pairs from the pBR322 unidirectional origin of replication.

Epicurian coli strain XL-1 Red is deficient in the primary DNA repair pathway; it was purchased from Stratagene (La Jolla, CA). E. coli strain TH460 was constructed by transforming TH320 (Atus, recA56 sr3100-Tn5, InvTsrpc1”/pPC143 aureus (Sharma and Hill, 1995) with plasmid pBAD18. Strain TH325 was constructed by transforming TH205 (Atuskan”) with plasmid pHV750T2.

Mutagenesis of pACMtus—Mutagenesis was performed as recommended by Stratagene. Briefly, to 1000 ml of E. coli DH10B (a generous gift from Roger McMacken) growing in LB medium supplemented with 50 mg/ml ampicillin, 0.4% arabinose, 100 mg/ml glucose, and 45 mg/ml chloramphenicol was added 20 mg of pBAD18. Strain TH325 was constructed by transforming the mixture with plasmid pHV750T2. Mutagenic colonies were picked from the plasmid-containing colony and purified as described in Gottlieb et al. (1994). Mutagenesis was performed as recom- mendated by McCann et al. (1992). Two overnight cultures of TH325 (pAXtus) were cut out of the total volume of 750 ml and incubated for 3 h at 25°C. Two 300-µl aliquots were washed through nitrocellulose filters as described previously in Gottlieb et al. (1992). The amount of TerB bound at each protein concentration was then used to determine the fraction of protein that is active for binding. The activities of the proteins were: TusP42S is 20% active, TusE49K is 25% active, and TusH50Y is 50% active.

Several Tus mutants and, for each mutant, dilution and incubation in the total volume of 750 ml were assayed for Tus protein. TusP42S, TusE49K, and TusH50Y were 10, 45.5, and 90% active, respectively. TusP42S is 20% active, TusE49K is 25% active, and TusH50Y is 50% active.

Assay for DNA Binding Activity—To assay the Tus protein for binding activity, a Cell-Porator with Voltage Booster (Life Technologies Inc., Gaithersburg, MD) was used, according to the manufacturer’s recommendations. Cells were plated onto LB media containing 50 μg/ml chloramphenicol, 50 μg/ml ampicillin, and 0.4% arabinose. Plates were incubated for 18 h at 37°C. Colonies that grew to normal size were then restreaked onto the same type of plates to screen for false positives. To reduce the frequency of siblings, no more than five colonies were characterized from each individual pool. Mutants that had the same mutation and which were collected from the same pool were considered to be siblings; mutants that had the same mutation and which were collected from independent pools were considered independent mutants.

Mutagenesis and Identification of Mutations in tus—E. coli Tus mutants were identified by conferring a phenotype on an osmotic stress assay. Tus mutant plasmids were transfected into strain RLM1038 (a generous gift from Roger McMacken) using the procedure described by Abarzuza et al. (1994).

Determination of Binding Constants—The activity of the mutant Tus proteins was determined by titrating a fixed amount of labeled TerB (1 × 10^14 m) with increasing protein concentrations (3.72 × 10^{-15} m to 1.86 × 10^{-14} m) in KG200 binding buffer. Mutant Tus protein and TerB were combined in a final reaction volume of 750 μl and incubated for 3 h at 25°C. Two 300-µl aliquots were washed through nitrocellulose filters as described previously in Gottlieb et al. (1992). The amount of TerB bound at each protein concentration was then used to determine the fraction of protein that is active for binding. The activities of the proteins were: TusP42S is 20% active, TusE49K is 25% active, and TusH50Y is 50% active.

The complete gene sequence of each mutant was determined using the following reverse sequence primers: 730 (17-mer) TGTAAATTC, 1344 (17-mer) AATCGGCTGCACCTTCA, 1480 (17-mer) TGATAAGCTGTCAAACATGAACTTTAGTTACAACATACTTATTT-3’. The data from the K_{obs} and K_{ex} experiments were analyzed and plotted as described in Gottlieb et al. (1992).

Helicase Assay—DNA helicase assay was performed as described in Skokotas et al. (1994). The complete Tus protein was added to the reaction mixture before the addition of DnaA. The reaction was performed at 30°C for 15 min before 25 mM EDTA was added to halt the reaction. Reaction products were analyzed by gel electrophoresis to determine the extent of replication inhibition by the mutant Tus proteins.
1.6-kb region between the mosome is completed. The blocked replication forks leave a clockwise moving replication fork are arrested before the chromosome would trigger the SOS response, but less cumbersome than in our previous mutants screen (Skokotas et al., 1994).

The starting point for the screen is plasmid pACMtus, which contains the araC gene and the tus gene under control of a pBAD promoter. In this plasmid, expression of tus can be induced by the addition of 0.4% arabinose to the growth medium. Prior to selection, pACMtus was mutagenized by passing the plasmid through the DNA repair-deficient strain XL-1 Red for several days. The mutagenized plasmid, called pAXtus, was then extracted and used to transform the selection strain, TH460. TH460 has a tus gene knockout, carries a recA56 allele, and also contains an InvTer::spc' cassette (Sharma and Hill, 1995). The InvTer::spc' cassette is inserted into the terminus region of the E. coli chromosome and contains two Ter sites oriented in a “blocking” configuration, such that both clockwise and counterclockwise moving replication forks are arrested before the chromosome is completed. The blocked replication forks leave a 1.6-kb region between the Ter sites unreplicated. Normally, the incomplete chromosome would trigger the SOS response, but the recA56 allele prevents this. The absence of the SOS response allows cell division to proceed even though the chromosome is partially replicated. It is speculated that loss of the SOS response causes the chromosome to be severed and leads to cell death (Sharma and Hill, 1995). Consequently, in the presence of wild-type Tus, these cells grow very slowly and form small colonies on plates. However, if no Tus is present or a mutant Tus is produced, the chromosome is replicated normally, allowing rapid cell growth and the production of normal sized colonies.

When TH460 was transformed with pACMtus and tus gene expression was induced on LB-arabinose plates, the colonies formed after 18 h of incubation were extremely small, while colonies on plates without arabinose were normal in size (Fig. 1). We determined that the frequency of a normal sized colony appearing on an LB-arabinose plate was approximately 1 in 2500 when wild-type Tus was expressed. In comparison, when the pAXtus plasmid was transformed into TH460, many normal sized colonies appeared on the plates containing arabinose, indicating that these colonies carried potential tus mutants. The frequency of normal sized colonies on LB-arabinose plates was 1 in 22 after tus mutagenesis.

Candidates producing normal sized colonies are expected to result from mutations in the tus gene that either affect the binding of Tus to Ter or influence the interaction of Tus with DnaB. However, normal sized colonies on arabinose plates could also result from a mutation in the promoter region of the tus gene, which would prevent tus gene expression. Likewise, a mutation in the araC gene of pAXtus would yield a similar phenotype, preventing induction of the tus gene. To compensate for the araC mutations, strain TH460 also carried the plasmid pBAD18, which contains the araC gene. This ensured that a wild-type copy of araC was always present in the cell.

Identification of tus Mutants—First, to determine if candidate tus mutants retained Ter binding activity, crude cell extracts were prepared, incubated with 32P-labeled TerB oligomer, and passed through nitrocellulose filters. The relative Ter binding activity of each mutant tus extract was then compared to the activity of extracts prepared from cells carrying pACMtus that had been grown: 1) in the presence arabinose, which provided the basis for full Ter binding activity; or 2) in the absence of arabinose, which should be devoid of Tus. A total of 77 potential tus mutants were examined for their DNA binding activity. Of these, 62 displayed no DNA binding activity (<10% of wild type activity), five displayed intermediate levels of DNA binding activity (10–60%), and 10 displayed high levels (>60%).

Eleven of the candidates with no Ter binding activity have been sequenced in their entirety. Two, tusP95S and tusP238L, had mutations which probably affected the secondary structure of the protein. Similarly, the tusL159P mutation also probably perturbed the structure of the protein. One mutant, tusR9opal, produced a truncated protein and six tus mutants had frame-shift mutations, due to the insertion of an additional adenine residue at different positions within the coding sequence. Last, one mutant without significant Ter binding activity had a wild-type tus sequence; we speculate that a mutation is present within the promoter region of this gene. Because all of the mutants without DNA binding activity were not completely sequenced, it should be noted that some of the 62 mutants may be siblings, even though steps were taken to reduce this possibility. Furthermore, some of these mutants may be expression mutants or stability mutants. Therefore, we cannot be certain of the actual number of independent mutations that produce Tus proteins with undetectable DNA binding activity.

Of the five independent tus mutant candidates with intermediate counts, only one mutant was sequenced in its entirety and it had a wild-type sequence.

Of the 10 independent mutant candidates that displayed high DNA binding activity, four had the tusP42S mutation; four had the tusP49K mutation; and one had the tusS50Y mutation. The last candidate had a wild-type sequence, indicating that it had bypassed our selection screen. Since all three mutant proteins TusP42S, TusP49K, and TusS50Y had high binding activity and the amino acid substitutions were proximal (see Fig. 6), these mutants were examined further for their ability to arrest DNA replication.

Plasmids expressing these three mutant Tus proteins were transformed into strain TH325, a tus mutant strain that also contains plasmid pHV750T2', the test plasmid for replication arrest activity. pHV750T2' has a TerB site located 900 bases away from the unidirectional origin of replication. DNA replication is halted at the Ter site when it is bound by an active Tus protein. Furthermore, the ColE1 origin of pHV750T2' is compatible with the p15a origin of the pAXtus plasmid, allowing both plasmids to coexist in the same cell. After growth in the presence of 0.4% arabinose to induce expression of the mutant tus gene, whole cell DNA was isolated under conditions that leave replication intermediates intact. The DNA was then digested with NdeI, which cuts pHV750T2' between the replication origin and TerB. If DNA replication is not arrested at the TerB site, only a linear pHV750T2' DNA fragment will be produced.
Tus Mutants

Fig. 2. Replication arrest activities of mutant Tus proteins in vivo. Whole cell DNA was isolated under nondenaturing conditions and digested with NdeI and SstII prior to electrophoresis on a 0.8% agarose gel. The Southern blot of the gel was then hybridized to a pBR322 probe to visualize the pHV750T2 plasmid. The linear pHV750T2 band is 5.2 kb in length; arrest of DNA replication produces two slower-migrating bands (~6.6 kb) containing a single or double Y-fork. The two, faint lower bands (2.4 and 3.0 kb) are due cross-hybridization between the probe and the pACMtus plasmid. These bands are used as an internal control for plasmid copy number. However, if DNA replication is arrested at TerB, the NdeI digest produces a double-Y structure which migrates slower on a gel than the linear fragment (Skokotas et al., 1994).

As can be seen in Fig. 2, TusP42S, TusE49K, and TusH50Y all show replication arrest activity in vivo, as judged by the appearance of a slower-migrating plasmid band when the mutant proteins are expressed. However, two distinct differences in the pattern of linear and Y-fork plasmid bands is also apparent: the ratio of linear plasmid DNA to plasmid containing a replication intermediate differs from one mutant to the other, as does the total amount of plasmid DNA (i.e. plasmid copy number). These observations corroborate the conclusions of Bierne et al. (1994) who have shown that the placement of a Ter site in a plasmid affects the copy number of the plasmid and also affects the accumulation of replication intermediates relative to the amount of linear plasmid. Consequently, in assessing the efficiency of replication arrest in vivo, both of these effects had to be factored in when making quantitative comparisons between mutants and wild-type protein. To this end, bands corresponding to the linear and Y-fork forms of pHV750T2 were excised and counted to determine the relative amounts of the two forms. Also, bands corresponding to the pACMtus plasmid, which cross-hybridized to the probe, were also excised and counted to normalize for the amount of DNA loaded in each lane. We then used the following equation to derive a relative measure of Tus activity in vivo.

\[
\text{Activity} = \frac{\text{Fraction of pHV750T2 as linear}}{\text{Fraction of pHV750T2 as Y-fork}} \times \frac{\text{Fraction of Tus plasmid as linear}}{\text{Fraction of Tus plasmid as Y-fork}}
\]

(Eq. 1)

When this equation was applied to the in vivo data, we obtained the results shown in Table I. In the strain expressing wild-type Tus, the linear and Y-fork bands were present in roughly equal proportions and the total amount of pHV750T2 was reduced significantly compared to the amount produced in the absence of Tus. However, strains expressing TusP42S and TusE49K showed higher relative amounts of linear plasmid DNA and also increased total plasmid DNA, suggesting that they arrest DNA replication at only 36% of the efficiency of wild-type Tus. In the strain expressing TusH50Y, the amount of plasmid present in the Y-fork band was almost identical to wild-type Tus, but the total amount of pHV750T2 plasmid was greater, indicating that TusH50Y arrests DNA replication at 78% efficiency compared to wild-type Tus.

It should also be noted that we observed two distinct replication intermediate bands in each lane. This is the result of dimerization of the pHV750T2 plasmid. The dimerized plasmid will give rise to either single Y replication intermediates or double Y replication intermediates following NdeI digestion, depending upon whether initiation has occurred at one or both of the replication origins in the dimeric plasmid.

Dissociation Constant for the Equilibrium and Dissociation Rate Constants for the Tus-TerB Complexes—To compare directly the binding affinities of wild-type and mutant Tus proteins for TerB, the observed dissociation constants for the equilibrium, \( K_{eq} \), and the dissociation rate constant, \( k_{d} \), were measured in KG200 buffer. The use of this buffer accelerates the dissociation rate and makes the collection of data easier than the original KG buffer described in a previous paper (Gottlieb et al., 1992).

The data revealed that wild-type Tus, TusE49K, and TusH50Y had similar equilibrium binding constants (Table I). The differences in \( K_{eq} \) values obtained for the mutant proteins and wild-type Tus could be attributed to the rates of dissociation of the Tus-TerB complexes (Fig. 3). The dissociation rates of TusP42S and TusH50Y protein-DNA complexes were 16.5-fold and 5.7-fold faster, respectively, than the wild-type Tus-TerB complex, indicating that these mutant Tus-TerB complexes are inherently less stable. On the contrary, TusE49K, which arrests DNA replication in vivo less well than wild-type Tus, formed a tighter complex with TerB, with a measured half-life of 178 min compared to 149 min for wild-type Tus-TerB complexes.

Inhibition of DnaB-catalyzed Strand Displacement by Tus Mutant Proteins TusP42S, TusE49K, and TusH50Y—In the past, it was shown by several laboratories that Tus blocks the progression of DnaB helicase in an orientation-specific manner (Lee et al., 1989; Khatri et al., 1989; Hiasa and Marians, 1992). This was demonstrated using a helicase assay where a short oligomer (30–62 bases) was hybridized to a single-stranded circular substrate. We were interested in determining if TusP42S, TusE49K, and TusH50Y were able to inhibit DnaB-catalyzed strand displacement using a similar substrate. For these studies, two substrates were used, one containing Ter in an active orientation, M13YTBS, and the other containing Ter

| Protein          | Fraction as linear | Fraction as Y-fork | Ratio plasmid (mutant) to plasmid (wild type)* | Relative activity |
|------------------|--------------------|--------------------|-----------------------------------------------|------------------|
| None             | 1.00               | 0.00               | 2.42                                          | 0.00             |
| Wild type        | 0.49               | 0.51               | 1.0                                           | 1.04             |
| TusP42S          | 0.63               | 0.37               | 1.57                                          | 0.37             |
| TusE49K          | 0.64               | 0.36               | 1.48                                          | 0.38             |
| TusH50Y          | 0.53               | 0.47               | 1.1                                           | 0.81             |

* Calculated from (total cpm linear + total cpm Y-fork pHV750T2 in sample) / (total cpm linear + total cpm Y-fork pHV750T2 from wild-type Tus lane).

2 A. Skokotas and T. M. Hill, unpublished results.
in an inactive orientation, M13YT835. The single-strand circles were hybridized to a complementary 62- and 61-mer oligomer, respectively, containing a TerB site oriented to block progression of the DnaB helicase. The molar ratio of Tus to the substrate DNA is indicated; DnaB, when present, was in a 65:1 molar excess. Lane 1 shows the starting substrate; lane 2 shows the substrate following heat denaturation; all other lanes are self-explanatory.

Table II

| Protein             | $k_{diss}$ (s$^{-1}$) | $k_d$ (sec$^{-1}$) | $t_{1/2}$ (min) |
|---------------------|----------------------|-------------------|-----------------|
| Tus (wild type)     | $7.5 \times 10^{-13}$ | $7.7 \times 10^{-5}$ | 149             |
| TusP42S             | ND                   | $1.3 \times 10^{-3}$ | 9               |
| TusE49K             | $6.5 \times 10^{-13}$ | $6.5 \times 10^{-5}$ | 178             |
| TusH50Y             | $4.4 \times 10^{-12}$ | $4.4 \times 10^{-4}$ | 26              |

* ND, not determined.

Fig. 3. Dissociation rates of Tus-TerB complexes formed by mutant and wild-type proteins. Tus proteins (2.0 $\times$ 10$^{-16}$ M) were incubated with TerB (1 $\times$ 10$^{-12}$ M) for 30 min and a 400-fold excess of unlabeled TerB was then added to the mixture. All reactions were performed at the indicated times and filtered to determine the number of counts remaining as protein-DNA complex. A representative experiment for each protein type is shown.

Fig. 4. Inhibition of DnaB-catalyzed strand displacement activity by wild-type and mutant Tus proteins. The substrate for this figure was M13YT835, which contains a TerB site oriented to block progression of the DnaB helicase. The molar ratio of Tus to the substrate DNA is indicated; DnaB, when present, was in a 65:1 molar excess. Lane 1 shows the starting substrate; lane 2 shows the substrate following heat denaturation; all other lanes are self-explanatory.

DISCUSSION

Because loss of Tus function in E. coli does not correlate with an observable phenotype, one of the most powerful avenues for investigating structure-function relationships of this protein has been denied: a genetic screen for Tus mutants with altered function. We report here the development of a new screen for mutations in the tus gene that greatly facilitates the isolation and characterization of Tus mutants. The basis of this screen is the strain TH460, which has in its chromosomal terminus a r cassette, an artificial construct that contains two Ter sites arranged in a blocking orientation (Sharma and Hill, 1995). In the presence of Tus, the two replication forks arising from oriC are arrested at the InvTer::spc' cassette, preventing completion of chromosome replication. The arrested replication forks would normally induce the SOS response and prevent septum formation. However, the presence of a recA56 allele circumvents SOS induction and the cell proceeds with division, severing one of partially replicated chromosomes.

Thus, in the presence of wild-type Tus, these cells grow slowly and form very small colonies on LB plates, whereas tus mu-
All three mutants still displayed replication arrest activity, albeit at lower efficiencies than wild-type Tus. This conclusion was based on the presence of stable replication intermediates in the Y-fork assay and also by the reduced copy number of plasmid pHV750T2 in the presence of mutant Tus proteins, relative to the copy number when no Tus was present. TusE49K and TusP42S arrested DNA replication at only 36% of the efficiency of the wild-type Tus, while TusH50Y arrested DNA replication at almost 78% of wild type efficiency in the plasmid assay. We also assayed these mutants for replication arrest in the chromosome instead of in a plasmid. It was more difficult to quantitate activity in this case, due to the relatively weak signal for the arrested replication forks. However, we did observe a “copy-number” effect in the chromosome as well: the intensity of bands representing the linear DNA fragment increased as Tus activity decreased (data not shown), similar to the increase in the linear plasmid band. This confirms a previous observation that arrest of DNA replication at the InvTER::spc cassette leads to under-representation of the region containing the cassette relative to other parts of the chromosome (Sharma and Hill, 1995).

The binding affinities of the mutant proteins for the TerB site suggest that the region between amino acids 42 and 52 of the Tus protein are positioned close to the DNA in the Tus-Ter complex. Our rationale for drawing this conclusion is based on the observed changes in the stability of protein-DNA complexes formed by TusE49K and TusH50Y. The TusE49K mutant has a negatively charged amino acid, glutamic acid, converted to the positively charged amino acid lysine, which also has a longer side chain. This substitution increases the half-life of the TusE49K-TerB complex to 178 min compared to 149 min for wild-type Tus-TerB complexes. We speculate that the positively charged lysine residue is in position to form an additional electrostatic interaction between the Tus protein and the phosphate groups of the DNA backbone, stabilizing the protein-DNA complex. Likewise, in the TusH50Y mutant, one polar amino acid, histidine, is replaced by another, tyrosine, which is similar in size, but is less hydrophilic and uncharged at neutral pH. This substitution causes a 5.7-fold reduction in the half-life of the TusH50Y-TerB complex compared to a wild-type complex, indicating that this mutation destabilizes the protein-DNA complex, possibly through loss of a protein-DNA contact. The fact that substitutions at adjacent amino acids affect the stability of the protein-DNA complex and that one substitution actually increases the half-life of the complex argues strongly that these amino acids are proximal to the DNA. While it could also be argued that these amino acids are only altering the structure of the protein and not affecting DNA binding directly, it seems improbable that a structural perturbation resulting from the TusE49K substitution would favor DNA binding rather than decrease it, especially in light of the apparent sensitivity of Tus binding to structural alterations (discussed below).

The binding characteristics of the TusP42S mutant, which most certainly creates a conformational change in the protein, also tells us something about this region of Tus. The perturbation in the secondary structure of Tus at this position only causes a 17-fold decrease in the half-life of the TusP42S-TerB complex, as compared to a complete loss of DNA binding activity in other Tus mutants in which proline has been substituted for another amino acid. This relatively mild effect on the stability of the Tus-TerB complex suggests that the amino acids between 42 and 52 contribute to, but are not absolutely critical for, Ter binding and may be positioned close to the DNA for another purpose.

Fig. 6 shows the locations of all single-site mutations that
Tus2 that failed to identify a distinct DNA binding domain of lytic digests (Coskun-Arimage domain" model of Tus structure is consistent with proteolytic to changes in protein conformation because structural that the binding characteristics of Tus are particularly sensi-
different regionsof the protein participate in DNA binding and TusP42S. Based on these observations, we proposethat several have very serious effects on mapping to a specific area, and that mutants that are typically observed have been identified in Tus and their relative effects on replication arrest efficiency. TusP42S, TusE49K, and TusH50Y were taken from Fig. 4. The data for TusA173V, TusA173T were taken from Fig. 8 of Skokotas et al. (1994). The value for wild type was comparable in the two assays (17 versus 13%). The data used to derive the least squares fit did not include the data point for TusE49K.

We do not see a correlation between the half-lives and replication arrest activities for each mutant. TusE49K has the longest half-life, 178 min, but has replication arrest activity that is only one-third of wild-type Tus. TusP42S has the shortest half-life of the three mutants, but it can arrest replication in vivo as well as the TusE49K mutant. TusH50Y has a half-life of 26 min, yet, it is relatively efficient in replication arrest activity, being 78% of wild-type Tus. Last, another mutant, TusA173V, which we have previously characterized (Skokotas et al., 1994) has a half-life of only 4.6 min, but it is able to arrest replication at 75% the efficiency of wild-type Tus.

One model that could explain the effect of these mutations on replication arrest is that the geometry of the interaction between Tus and the replication fork is changed. For example, TusE49K forms a tighter complex with TerB, but the mutant protein may not be positioned correctly on the DNA and is not able to recognize the replisome proteins and block their progression at 100% efficiency. Likewise, TusP42S may have structural changes that affect binding as well as disturb the interaction between replication arrest domain and the replisome. In the case of TusH50Y, the substitution may destabilize the protein-DNA complex without significantly affecting the position of the Tus domain required for replication arrest, allowing for efficient replisome inhibition. An alternate model is that the geometry of the Tus-replisome interaction remains intact in these mutants, but the observed decrease in replication arrest efficiency is because certain amino acids between positions 42 and 52 of Tus participate directly in replication fork inhibition. By this model, the glutamic acid at position 49 would form part of the active site of Tus, as a substitution at this position has a greater effect on replication arrest efficiency than a substitution at the adjacent histidine.

The isolation of these mutants also afforded us the opportunity to examine the relative authenticity of in vitro assays that are currently available for measuring Tus activity. The standard in vitro assay for Tus function is the helicase assay, in which one measures the ability of DnaB to displace an oligomer annealed to a single-stranded substrate. When the results of the helicase assay and the in vivo assay are compared, we see a general but imperfect correspondence of Tus activity between the two assays. We find that TusH50Y is able to arrest replication less well than wild-type Tus in vivo and impede DnaB progression less well in the helicase assay, at 80 and 90% efficiency, respectively. Likewise, the TusE49K and TusP42S mutants displayed less activity than TusH50Y in both assay, consistent with in vivo observations. However, it should also be noted that we consistently observed a greater degree of inhibition by TusE49K than TusP42S in the helicase assay, whereas in vivo, both mutants showed virtualy identical replication arrest activities. Furthermore, the relative rates of Tus activity for these two mutants were almost twice as high in the helicase assay as in the in vivo assay. In part, this difference is due to the efficiency of replication fork arrest in the two assays. In the helicase assay we observe almost complete inhibition of DnaB strand displacement, suggesting that helicase activity is halted on virtually all substrate molecules. In contrast, only 50% of the pHV750T2 plasmid contains arrested replication forks in the in vivo assay, even in the presence of wild-type Tus. This observation, along with previously reported discrepancies between the two assays, suggests that the activity of Tus mutants in the helicase assay does not correlate directly to their in vivo activity; however, the helicase assay might be useful when describing the general pattern of replication inhibition among the mutant proteins in qualitative terms.

We also noted a general correlation between Tus activity in the helicase assay and the half-lives of the protein-DNA complexes. When the log of the half-life of wild-type and mutant Tus-TerB complexes in KG200 buffer (excluding TusE49K) were plotted against the percent of oligomer displaced in the helicase assay and a least-squares analysis was applied to the data points, the correlation coefficient was greater than 0.95 (Fig. 7). These results would seem to indicate a simple linear relationship between a decrease in the stability of the Tus-TerB complex and a corresponding reduction in the inhibition of oligomer displacement by DnaB in the helicase assay. Such a relationship does not exist when we attempt to compare in vivo Tus activity to the half-lives of the mutant Tus complexes. These results would suggest that the activity of a mutant Tus protein in the helicase assay is primarily related to
the stability of the protein-DNA complex, rather than the intrinsic replication arrest activity of mutant. We will be able to confirm or debunk this hypothesis once more mutants of Tus become available.

The other in vitro assay used to measure Tus activity utilizes a replication fork reconstituted from purified proteins. Replication forks assembled at oriC or a pBR322 origin of replication have been successfully used to examine replication inhibition by Tus (Lee et al., 1989; Hiasa and Marians, 1992; 1994; Hill and Marians, 1990). In theory, this assay should provide a more realistic measurement of Tus activity since the presumed target of Tus, the DNA helicase, functions as part of the replisome in this assay rather than as an independent protein, which it does in the helicase assay. The oriC assay has the added advantage that overreplication of a Ter-containing substrate is suppressed in the presence of Tus, a possible corollary to the reduction in copy number of Ter-containing plasmids in vivo. When the different Tus mutants were tested in the oriC system using buffer containing 200 mM potassium glutamate, we saw a good correspondence between the results in vivo and in the oriC system at lower concentrations of Tus (8:1 Tus to substrate ratio). Under these conditions, TusH50Y was as effective as wild type (only 5% form II accumulation compared to no Tus), TusE49K and TusP42S were less efficient than wild type (22–24% form II accumulation), TusA173V showed 80% of the amount of form II in the absence of Tus, and TusA173T was virtually nonfunctional, with 90% form II accumulation. However, at higher levels of protein (16:1), we noted that TusE49K and TusP42S became as effective as wild type. This differs from the helicase assay, where partial activity was observed even at ratios of 30:1 with these two mutants, and suggests that when Ter sites are saturated, these mutants effectively block replication forks in the oriC assay. The ability of these partially functional mutants to show full activity in the oriC assay may be related to the dissociation rate of the replisome under these assay conditions. Studies have demonstrated that when progression of the replication apparatus is blocked, the half-life of the replisome is approximately 5 min. It follows that any Tus mutant with a half-life greater than 5 min should effectively end DNA replication for the duration of the assay once it has stopped the replication apparatus at a Ter site. Thus, mutants with half-lives greater than 5 min, such as TusP42S, TusE49K, and TusH50Y, will prevent accumulation of form II products, whereas mutants with shorter half-lives will dissociate before the replication apparatus does and produce form II products.

Regardless of the actual mechanism of replication fork blockage by mutant Tus proteins in the oriC system, the results described in this paper suggest that even the sophisticated oriC assay does not discriminate between mutant Tus proteins as well as the in vivo assay. Possibly, as more becomes known about the mechanism of replication arrest by Tus, the oriC assay can be modified to accurately mimic the process as it occurs in vivo.

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Mutations in the *Escherichia coli* Tus Protein Define a Domain Positioned Close to the DNA in the Tus- Ter Complex
Aikaterini Skokotas, Hiroshi Hiasa, Kenneth J. Marians, Leslie O'Donnell and Thomas M. Hill

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