A COMPLEX MECHANISM DETERMINES POLARITY OF DNA REPLICATION FORK ARREST BY THE REPLACEMENT TERMINATOR COMPLEX OF *BACILLUS SUBTILIS* *

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Running Title: Polarity of DNA replication fork arrest

**SUMMARY**

Two dimers of the replication terminator protein (RTP) of *Bacillus subtilis* bind to a chromosomal DNA terminator site to effect polar replication fork arrest. Cooperative binding of the dimers to overlapping half-sites within the terminator is essential for arrest. It was previously suggested that polarity of fork arrest is the result of the RTP dimer at the blocking (proximal) side within the complex binding very tightly and the permissive-side RTP dimer binding relatively weakly. In order to investigate this “differential binding affinity” model, we have constructed a series of mutant terminators that contain half-sites of widely different RTP binding affinities in various combinations. While there appeared to be a correlation between binding affinity at the proximal half-site and fork arrest efficiency *in vivo* for some terminators, several deviated significantly from this correlation. Some terminators exhibited greatly reduced binding cooperativity (and therefore have reduced affinity at each half-site) but were highly efficient in fork arrest, while one terminator had normal affinity over the proximal half-site yet had low fork arrest efficiency. The results show clearly that there is no direct correlation between the RTP binding affinity (either within the full complex or at the proximal half-site within the full complex) and the efficiency of replication fork arrest *in vivo*. Thus, the differential binding affinity over the proximal and distal half-sites cannot be solely responsible for functional polarity of fork arrest. Furthermore, efficient fork arrest relies on features in addition to the tight binding of RTP to terminator DNA.

**INTRODUCTION**

Replication terminator proteins are a diverse group of DNA-binding proteins that cause DNA replication fork arrest (or pausing) at designated DNA terminator sites. They have roles in a variety of tasks including the coordination of DNA replication and transcription at eukaryotic rDNA loci (1, 2), regulation of mating-type switching in yeast by strand-specific imprinting during replication (3, 4), and participation in the final stages of bacterial chromosome replication and partitioning (5). These functions require the DNA replication fork to be blocked at the terminator site from only one direction of approach. Thus, a terminator protein-DNA complex exhibits functional polarity. An understanding of the mechanism of polar replication fork arrest has remained elusive.

Considerable progress in characterization of replication termination systems has been made with those from *Bacillus subtilis* and *Escherichia coli* (6, 7), and this has recently aided the study of analogous eukaryotic systems (4, 8, 9). In both bacteria, the numerous terminator (*Ter*) sites are clustered in the terminus region of the chromosome and are arranged so as to control the
location of replication fork fusion at the end of a round of replication (for a review, see Ref. 10). Despite the same apparent function of these two bacterial systems, the terminator proteins from *E. coli* and *B. subtilis* share no sequence or 3D structural similarity and may act through quite different mechanisms.

The *B. subtilis* replication terminator protein (RTP) binds as two 29 kDa dimers to two overlapping half-sites within each ~30 bp Ter site (11-13). The overall structural symmetry of the RTP dimer in both its free and DNA-bound forms (14, 15) means that polarity of the whole (two-dimer) complex must somehow be generated by the significantly different DNA sequences of the two adjacent half-sites. The polar Ter sites contain low- and high-affinity half sites (A and B sites, respectively) that each bind one dimer, and the level of protein-DNA contact is significantly higher at the B site (13). A replication fork is arrested only when it approaches the B-site side of the complex, although a single RTP dimer bound to an isolated B site cannot cause arrest (16). It is clear that interaction between the two bound RTP dimers (13, 17, 18), possibly involving direct protein-protein contacts (14), is essential for fork arrest at Ter sites. Structural models of the complete RTP-Ter complex have been developed with the aid of the crystal structures of RTP (15) and an RTP-DNA complex (14), together with other less direct structural studies (13, 18-20). Nevertheless, how the RTP dimer-dimer interaction activates the B-site side of the terminator complex is still unresolved.

Kralicek et al. (21) put forward two non-mutually exclusive models to explain how the chromosomal terminators generate functional polarity. The differential binding affinity (DBA) model proposes that the different affinities of RTP for the A and B sites within the RTP-Ter complex gives rise to the functional polarity, while in the induced conformational change (ICC) model, polarity derives from a different conformation of each RTP dimer positioned at each half-site. The current study directly addresses the mechanism of polarity of the RTP-Ter complex, by thorough testing of the DBA model. Our data point to a more complex and intricate mechanism than previously thought, possibly involving features of both the DBA and ICC models.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains** — *E. coli* DH5α was used for plasmid construction and purification. *B. subtilis* SU230, a constitutive over-producer of RTP, was used for *in vivo* replication fork-arrest assays (16).

**Plasmids** — pID1 was constructed by ligating the annealed oligonucleotides, 5′-AATTCCTATGAAACATAATTGTTCC and 5′-GATCCTATGAAACATTATTGTTCC, to the large EcoRI-BamHI fragment of pWS64-1 (16). pID6 was constructed by ligating the annealed oligonucleotides, 5′-AATCCGCCTGCAGAAATGAAACTATG AACATAATTGTTCC and 5′-GATCCTATGAAACATTATTGTTCC, to the large EcoRI-BamHI fragment of pWS64-1. pID2 was constructed by ligating the annealed oligonucleotides, 5′-AATCCGCCTGCAGAAACTATG AACATAATTGTTCC and 5′-GATCCTATGAAACATTATTGTTCC, to the large EcoRI-BamHI fragment of pWS64-1.

Fifteen plasmids with different combinations of variations of A and B sites, as listed in Fig. 3C, were constructed as follows. Two synthetic double-stranded oligonucleotides (one for the “distal” and one for the “proximal” half-site, listed respectively after the pTer moiety of the plasmid name; see Fig. 3) were ligated in vitro to form the full-length sequence, and the product was ligated to the large BamHI-SalI fragment of pWS64-1 (16). After transformation and purification from *E. coli*, all plasmids were sequence-verified over the region of the cloned terminator sequences.

**Equilibrium Gel Mobility Shift Assays** — Binding buffer was 50 mM TrisCl (pH 7.8), 50 mM KCl, 10 mM MgCl2, 10 % (v/v) glycerol, 100 µg/mL BSA; BSA solutions were filter sterilized and heated at 65 °C for two hours prior to use. Plasmid DNA of known concentration (assuming A260 = 50 µg/mL and an average molecular mass per base pair of 649) was cut with EcoRI and then end-labeled with α-32P-dATP and Klenow, followed by cutting with another restriction enzyme to release the sequence to be analyzed within a fragment of 50–100 bp. This DNA was

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then diluted in binding buffer. RTP was purified using the published method (12). Samples of a range of concentrations in binding buffer (assuming \( \varepsilon_{280} \) (monomer) = 7680 M\(^{-1}\) cm\(^{-1}\)) were mixed with equal volumes of the DNA (final DNA concentration was 0.25 or 0.5 pM for each sample; 20 \( \mu \)L total volume). Each mixture was equilibrated at 25 °C for 30 min (at 1 min increments), and then 10 \( \mu \)L samples were withdrawn and loaded directly onto a running polyacrylamide gel (at 1 min increments). Control experiments on the RTP-TerI interaction confirmed that equilibrium was reached within 15–20 min. After electrophoresis (18), a phosphorimage acquired from a 1–7 day exposure of the fixed and dried gel was quantified using ImageQuant software (Molecular Dynamics) to give equilibrium concentration data for bound and unbound DNA. For determination of equilibrium constants, quantified data were analyzed by nonlinear least-squares regression analysis with the program EQUIL (22). For the isolated RTP dimer-half-site interactions, the reactions and notation for EQUIL were \( LL = L + L \) and \( ALL = A + L + L \) (where \( LL \) represents RTP dimer, \( L \) represents RTP monomer, \( ALL \) represents the RTP dimer-DNA complex and \( A \) represents the unbound DNA fragment). For each of the full-length terminators, the following reaction scheme was used: \( LL = L + L \), \( ALL = A + L + L \), \( LLALL = A + L + L + L \) and \( LLALL = A + L + L + L + L \) (where \( ALL \) and \( LLA \) represent the two different isolated half-site complexes and \( LLALL \) represents the complete complex containing four monomers of RTP).

**DNA Replication Fork-Arrest Assays**

The plasmid-based \textit{in vivo} fork-arrest assays were carried out as described (16).

**Analytical Ultracentrifugation**

Sedimentation equilibrium experiments made use of a Beckman Optima XL-A analytical ultracentrifuge fitted with an An-60Ti rotor. RTP was dialyzed against binding buffer containing no BSA, and then two samples (at 0.5 and 1.5 g/L) were centrifuged at 25 °C until equilibrium was attained at 27000, 32000 and 36000 rpm. Absorbance profiles were recorded at 230, 280 and 360 nm and those from the dialysate were subtracted. The data were recorded in 0.001 cm increments and ten scans were averaged to produce each profile. The equilibrium data were analyzed using non-linear least squares regression with the program NONLIN (23), with the partial specific volume of RTP (0.758 mL/g) and density of the buffer (1.031 g/mL) calculated using the program Sednterp (24). The model best describing the data was determined by examination of the magnitude and distribution of the residuals for each test fit.

**Isothermal Titration Calorimetry**

Duplex A-site DNA was prepared by annealing the purified oligonucleotides: 5'-GGATCCGAACTAAGAAAACAATGTT and 5'-AACATTGTTCCTTCGAGATCC. The B\* oligonucleotides were 5'-AACATGTTTCAATGTTCAGTGA and 5'-TCGACTGACATTTGAAAACATTGTT. A 21 bp sRB duplex was also used (14). RTP and DNA samples were extensively dialyzed against binding buffer without BSA or glycerol. (Glycerol was omitted due to solution mixing problems encountered in preliminary experiments.) A sample of RTP was then taken for concentration measurement by quantitative amino acid analysis. The DNA concentration of each sample was determined by spectrophotometry (as above). Titration experiments were performed in duplicate using a Microcal Omega VP-ITC at 25 °C with a mixing rate of 310 rpm, reference power of 10 \( \mu \)cal/sec and injections of 5–10 \( \mu \)L at intervals of 5 min. A-site (65 \( \mu \)M) or B\* DNA (68 \( \mu \)M) samples were titrated into an RTP sample (8.6 \( \mu \)M, as monomer) to give an ~1.5 molar excess of DNA over RTP monomer. Binding isotherms were corrected for DNA dilution and mixing effects by subtracting isotherms from DNA titrations into buffer. Data were fit to obtain thermodynamic parameters by the single set of sites model using the program Origin (Microcal).

**Dissociation Rate Gel Mobility Shift Assays**

These were carried out as previously described (25). Quantified data for percent of labeled DNA in complex II (two dimers bound) versus time were plotted and a first-order exponential regression analysis performed using Origin to yield dissociation rate constants.
RESULTS

The Differential Binding Affinity (DBA) Model for Polar Replication Fork Arrest by the RTP-Ter Complex — The DBA model is shown in Fig. 1. The current study describes all equilibria in terms of dissociation constants. The RTP monomer-dimer equilibrium in free solution is governed by $K_{MD}$. The model specifies that either the A or the B site of the terminator can bind a dimer of RTP with intrinsic equilibrium constants $K_2$ and $K_3$, respectively. The B site-RTP complex is much tighter than the A site complex; thus $K_4 << K_2$. A second dimer of RTP binds with high cooperativity to the remaining half-site (B or A; $K_4$ and $K_3$, respectively) yielding the complete terminator complex. Since $K_1 << K_2$, in other words, the affinity of RTP for the B site within the complete complex is much greater than it is for either the A site in the complete complex or the B site alone. Fork arrest is achieved only when the replisome approaches the B site end (from the right in Fig. 1); the DBA model proposes that this is because $K_4$ is the only affinity sufficient to prevent removal of RTP from the DNA. When the replisome approaches from the other direction (towards the A site), the two RTP dimers can be sequentially stripped from the DNA. RTP is removed first from the A site because the affinity is lower, and this is immediately followed by its removal from the B site, which has now lost the contribution of cooperativity to its affinity.

A Completely Symmetrical Mutant B Site — We firstly examined the importance of DNA sequence symmetry within an individual B site to the tight DNA binding of an RTP dimer (naturally occurring B sites are only partially symmetric). We carried out a gel mobility shift assay in which two DNA fragments of differing size were mixed; one fragment contained a B site derived from the wild-type terminator TerI, while the other contained a completely symmetrical variant B site named sRB. As shown in Fig. 2B, both fragments bound to RTP equally well, indicating that sRB and the isolated B site from TerI have indistinguishable binding affinities for RTP. This result raises the possibility that the conserved bases in the B site that were changed in sRB (Fig. 2A) may be important in the function of a complete terminator that binds two RTP dimers, rather than affecting the binding of just a single RTP dimer.

To examine the replication fork arrest efficiency of a mutant terminator containing sRB in place of the normal B site in TerI, a sequence containing the A and sRB sites (TerA/sRB) was firstly cloned into pWS64-1 (16), resulting in pID6. A control TerI fragment (containing the wild-type A and B site combination) with the same sequence context was also cloned (pID2). In B. subtilis, pWS64-1-based plasmids carry out DNA replication unidirectionally from the origin towards the cloned terminator. DNA replication intermediates indicative of arrest at the cloned terminator can be detected and quantified after plasmid isolation from the B. subtilis strain SU230 (16), which overproduces RTP and saturates the plasmid terminator sequences. The three plasmids pWS64-1 (no terminator, control), pID2 (TerI, bp 1–30) and pID6 (TerA/sRB, bp 1–30) were extracted from exponential phase cultures and analyzed by restriction enzyme digestion and Southern blotting to detect replication intermediates. As can be seen in Fig. 2C, the slower-migrating forked DNA species indicative of arrest at the terminator (16, 26) was clearly present for both pID2 and pID6, but not for the control pWS64-1. The linear DNA species results from plasmids not engaged in replication through the probed region at the time of extraction (16). Quantification of the ratio of specific forked to linear DNA provides a measure of the efficiency of fork arrest. The average ratio for TerA/sRB in pID6 was ~44% of that of TerI in pID2 (Fig. 2C). Thus, TerA/sRB is a significantly poorer terminator than is TerI, despite our finding that the isolated B and sRB sites have indistinguishable affinities for RTP. This suggested that the interaction between the two RTP dimers could be perturbed in TerA/sRB, resulting in less efficient activation of the complex compared to TerI.

Cloning of a Series of Synthetic Terminators — We next sought to test the DBA model, by determining whether a general correlation exists between the efficiency of fork arrest and the binding affinity at the “proximal” half of the complex, which faces the replisome during the replication fork arrest assay (both the intrinsic affinity of the RTP dimer for the half-site and the dimer-dimer cooperativity factor contribute to the affinity at each half-site). For
these studies, a number of mutant terminators expected to have reduced binding affinity and cooperativity were synthesized and cloned (Fig. 3A). The terminators were based on two half-sites flanking a symmetrical central AAT trinucleotide. Three mutant half-site sequences, as well as the normal A and B sites, were used in various combinations to produce the terminators (Fig. 3B). One of the mutant half-sites was an sRB site, which was predicted to reduce the effectiveness of the dimer-dimer interaction. Another mutant half-site, named the B* site, was designed to have intrinsically lower affinity for RTP than the B site.

It contains sequence features from both the B and A sites and is based on the “SymB-GAAA” mutations (bp 17–20) made previously in the Terl B site (18). Finally, site “Y” was designed so that it would not bind RTP specifically and was used to isolate the various adjacent half-sites, to provide a consistent sequence context for separate measurements of their binding affinities.

The series of mutant terminators is best described as four sub-series (Fig. 3C). In the first, the distal half-site with respect to replication fork approach in the plasmids is the A site, while the proximal half-site contains either the normal B site or the mutant sRB, B* or Y sites. This series, designated the “distal A-site series”, contains pTerA.B (wild-type site combination, active orientation), pTerA.sRB, pTerA.B* and pTerA.Y. For the second series, the B site was present in the distal position and the proximal half-sites were likewise varied (pTerB.B, pTerB.sRB, pTerB.B* and pTerB.Y). Similarly, the distal sRB-site series comprised pTersRB.B, pTersRB.sRB, pTersRB.B* and pTersRB.Y, and the final series contained the dummy Y site in the distal position to allow measurement of the affinity of RTP binding to the various proximal sites in isolation (pTerY.B, pTerY.sRB and pTerY.B*).

**Isolated Half-Site-RTP Interactions** — In analyzing these terminators, it was important to quantify the binding of a single RTP dimer to the various “proximal” and “distal” half-sites in isolation. Firstly, we used analytical ultracentrifugation to assess the oligomeric state of RTP in the same buffer conditions used for the binding assays. The combined sedimentation equilibrium data for a number of speeds and initial RTP concentrations were best described by a simple monomer-dimer equilibrium with $K_{MD} = 6.3 \times 10^{-7}$ M (95% confidence limits of $3.5 \times 10^{-7}$ and $1.1 \times 10^{-6}$ M). No further association to tetramer was detected, suggesting that the RTP dimer-dimer interaction is negligible in the absence of DNA. The results confirm that under the dilute conditions of most DNA binding assays, RTP would exist largely as the monomer. Therefore, to avoid making assumptions about whether it is the preformed RTP dimer that binds DNA in gel mobility shift assays or whether the complex is assembled by sequential binding of monomers, the data below refer to “overall” equilibrium constants: the equilibrium constants for two RTP monomers plus one DNA molecule forming a ternary complex. That is:

$$\text{(RTP)}_2:\text{DNA} \rightleftharpoons K_D 2\text{RTP} + \text{DNA}$$

Equilibrium gel mobility shift assays were carried out side-by-side for the normal B site in pTerY.B and the mutant sRB site in pTerY.sRB (Fig. 4A). We analyzed the data using the non-linear least squares fitting program EQUIL (22). Fig. 4B shows the fitted data and Table 1 shows the overall equilibrium constants for these RTP dimer-half-site complexes, determined from simultaneous fitting of data from replicate experiments. Both the RTP dimer-B site complex (from pTerY.B) and the RTP dimer-sRB complex (from pTerY.sRB) had very high overall affinities, with the sRB site being slightly higher. This difference was not observed with the earlier constructs (Fig. 2B), possibly because of sequence differences at the edges of those sites. When the B and sRB sites were in the distal position (in pTerB.Y and pTersRB.Y, respectively), the overall affinities were very similar, but were significantly weaker than the otherwise equivalent proximal sites (Table 1). This is almost certainly due to the 1 bp shorter terminator consensus agreement at the distal ends (see Fig. 3B). The data emphasize the importance of using equivalent DNA sequence contexts for reliable comparisons of binding affinities.

**Low Affinity Half-Site-RTP Interactions** — The affinities of the A and B* sites for RTP were too low to be accurately determined by gel mobility shift analysis, although qualitative analysis of gel mobility shift data (not shown) indicated that the affinity of RTP for the B* site...
was significantly higher than for the A site. Isothermal titration calorimetry (ITC) analysis of RTP binding to DNA containing either the A or B* site (in distal and proximal sequence contexts, respectively) indicated a stoichiometry of one RTP dimer bound per site in both cases, as expected (Fig. 5 and Table 2), and dissociation constants of the order of \(10^{-7} \text{ M}\). In the analysis of ITC data, it was assumed that free RTP existed as the dimer. This assumption was supported by gel filtration and multiangle laser light scattering data (not shown). While data from the sedimentation equilibrium analysis indicated that \(20-30\%\) of the RTP should be monomeric at the concentrations used in ITC experiments, differences in buffer composition and temperature are most likely accountable for the minor difference. The ITC data analysis indicated that the B* site had several fold greater intrinsic affinity than the A site (Table 2). An RTP-sRB site complex (14) was formed with the same stoichiometry but with a dissociation constant at least an order of magnitude smaller (data not shown); the sharp transition in the binding isotherm prevented reliable determination of the dissociation constant.

In order to make comparisons with the data from gel mobility shift assays, the overall equilibrium constants for the A and B* sites were estimated by obtaining the product of the \(K_D\) for each (Table 2) and \(K_{MD}\) for the solution RTP monomer-dimer association measured above. The values obtained were \(K\) (overall, A site) = \(3.7 (\pm 1) \times 10^{-13} \text{ M}^2\) and \(K\) (overall, B* site) = \(1.0 (\pm 0.4) \times 10^{-13} \text{ M}^2\). In the presence of glycerol (i.e., under the conditions of the gel mobility shift assays), the overall dissociation constants would be expected to be 10-100 fold lower (11). Nevertheless, it is clear that both the A and B* sites had very much lower RTP-binding affinities than did the normal B site. Thus, there is a great differential binding affinity between the A and B sites within the normal RTP-Ter complex, as predicted for the DBA model.

**Binding Affinities and Cooperativity of the Full-Length RTP-Ter Complexes** — Having measured the overall affinities of the individual half-site-RTP dimer complexes, we next sought to determine the overall affinities and degrees of cooperativity for the complete terminator complexes containing two RTP dimers. Fig. 6A shows representative data from equilibrium gel mobility shift assays that compare the normal terminator sequence in pTerA.B to the mutant terminator in pTerA.sRB. Each terminator bound RTP in the expected manner; as the concentration of RTP was increased, an intermediate complex containing one bound RTP dimer (complex I) was observed followed by a second shifted band (complex II) resulting from two dimers bound to the terminator (11, 13). The quantified data and EQUIL best-fit curves are shown in Fig. 6B and the derived overall equilibrium constants and cooperativity factors are shown in Table 3. These parameters were essentially the same for the A.sRB and A.B sequences; a slightly higher affinity for the first bound RTP was consistent with the slightly higher affinity of RTP for sRB over B in Table 1. This effect was also observed when data for pID2 (containing the wild-type TerI A and B site combination) and pID6 (containing the earlier A and sRB site combination) were compared (not shown). We also carried out dissociation rate experiments using pID2 and pID6 DNA. These experiments are highly sensitive to cooperativity effects and are subject to very little experimental error (25). The dissociation of complex II followed first-order kinetics in both cases. Its half-life with TerI (in pID2) was 226 (\(\pm 28\)) min (\(R^2 > 0.99, N = 9\)) and that with TerA/sRB (in pID6) was 39 (\(\pm 1\)) min (\(R^2 > 0.99, N = 7\)). Since the A sites of these terminators are identical, the degree of cooperativity appears to be slightly reduced in the TerA/sRB sequence, even though this difference was not detectable in the equilibrium binding studies.

The final terminator in the distal A-site series, A.B*, bound RTP quite efficiently despite the relatively low overall affinity of the individual half-sites (Tables 2 and 3). Complex I was not observed in the raw binding data for A.B*; only free DNA and complex II were observed (not shown). This observation is in accord with the very high degree of cooperativity exhibited by this terminator (Table 3).

Equilibrium binding experiments were also carried out for all the terminators of the distal B-site and distal sRB-site series (Table 3). Interestingly, B.B exhibited a similar overall affinity to A.B, despite the presence of two high-affinity B sites. This is accounted for by the unexpected large reduction in the degree of
cooperativity in the B.B complex compared to A.B (Table 3). All combinations involving just the high-affinity B and/or sRB sites exhibited similarly high overall affinities and low degrees of cooperativity. Finally, the B.B* and sRB.B* sequences showed similar overall affinities, and in both cases the degree of cooperativity was slightly reduced compared to A.B. These results are described in more detail when comparing the fork arrest efficiencies of the mutant terminators, below.

**DNA Replication Fork Arrest Efficiency of the Terminators in vivo** — To assess a possible correlation between the RTP binding affinity of the mutant terminators and the efficiency of replication fork arrest, all plasmids containing full-length terminator sequences from Fig. 3C were transferred into *B. subtilis* SU230 for *in vivo* replication fork-arrest assays (Fig. 7). The normal terminator in pTerA.B (lane 1) displayed a level of fork arrest consistent with other wild-type terminators (see Fig. 2 and Ref. 27).

When the fork-arrest efficiencies of the mutant terminators are compared, clear trends are apparent. The A.sRB mutant terminator exhibited a significant reduction in fork arrest (Fig. 7, lanes 1 and 2), as observed in earlier experiments (Fig. 2C). This is significant because the overall affinity of RTP at the proximal sRB site was slightly higher than the B site within the respective complete terminator complexes (Table 3). This is inconsistent with the hypothesis that polarity is uniquely described by the DBA model as presented in Fig. 1. Furthermore, it is clear that high binding affinity at the proximal half-site within the terminator complex is not the only factor controlling the mechanism of replication fork blocking. The final terminator sequence in the distal A-site series, A.B* (lane 3), was able to effect fork arrest but it was substantially less effective than either A.B or A.sRB (efficiency was reduced by ~70 % compared to A.B). Thus, the B* mutant site has a strong negative influence on the efficiency of fork arrest. The B* site had a low affinity for RTP compared to the B site within their respective terminator complexes (Table 3), which probably causes the observed reduction in fork arrest efficiency. However, this effect could also be caused by other features of the complexes that were not examined directly (*e.g.*, RTP-DNA conformational differences).

A similar trend of relative fork arrest efficiencies was seen throughout the distal B-site series (*i.e.*, pTerB.B, pTerB.sRB and pTerB.B*). Thus, a decrease in fork arrest efficiency was observed for B.sRB compared to B.B, and there was an even greater decrease for B.B* (Fig. 7, lanes 4–6). This general trend was also retained in the distal sRB-site series (lanes 7–9) described further below. Remarkably, B.B and B.sRB from the distal B-site series had much greater fork arrest efficiencies than the respective terminators from the distal A-site series (~100 % increase in each case), while A.B* and B.B* showed similar efficiencies to one another (compare lane 1 with 4, 2 with 5, and 3 with 6 in Fig. 7). This indicates that the identity of the distal site can have a very significant influence on terminator function. Importantly, since the distal B-site series exhibited a significantly lower cooperativity compared to the distal A-site series (Table 3), it is clear that maximal cooperativity is not required for efficient terminator function. Furthermore, it is clear that, for complexes containing the same proximal site, a higher degree of cooperativity does not necessarily lead to increased activation of an RTP-Ter complex.

The distal sRB-site terminators also revealed important information about terminator function. Firstly, a comparison of B.B, B.sRB and sRB.B (lanes 4, 5 and 7; Fig. 7) indicates that the sRB site had a similar negative influence on fork arrest irrespective of whether it was present in the proximal or the distal position. This was probably due to perturbation of the interaction between the two RTP dimers, resulting in less efficient activation of the complex. In this regard it is worth noting that sRB.sRB showed a further reduction in fork arrest activity (compare lane 8 with lanes 4, 5 and 7), indicating that the negative influence of each sRB site was compounded in this terminator. Also, sRB.sRB was the only terminator that contained two high-affinity half-sites yet exhibited lower fork arrest efficiency than the low and high affinity half-site combination in A.B. Thus, the combined affinity of both RTP dimers for the full-length terminator DNA, regardless of the degree of cooperativity, is not the only factor that controls the efficiency of fork arrest. Finally, sRB.B* (lane 9) showed the least efficient fork arrest (~10-fold less efficient than A.B), presumably due to the combined effects of both mutant sites.
DISCUSSION

In this study, we tested predictions of the DBA model (Fig. 1) for polar replication fork arrest by the RTP-Ter complex in B. subtilis. Our initial studies focused on a mutant B site containing three single base-pair substitutions that resulted in a completely symmetrical half-site (named the sRB site). While the substitutions were at conserved positions, they had no negative effect on the overall affinity of binding to RTP. This can be explained by the fact that the substitutions reside on one side of the half-site, and create an inverted repeat of one of the wild-type sides (Fig. 2A). This would allow high-affinity binding of both RTP monomers within the complex. The structure of an RTP dimer-sRB oligonucleotide complex confirms the perfect symmetry of the complex (14). The conserved bases changed to make sRB may have another role aside from tight DNA binding of an RTP dimer. When we tested the effect of replacing the B site of the wild-type Terl terminator with the mutant sRB site, we observed a significant reduction in the efficiency of replication fork arrest. This indicates that the changed bases have some structural role in the complete RTP-Ter complex that is important for efficient fork arrest. These bases could affect the degree of cooperativity between the two RTP dimers and/or influence a potentially important conformation of the terminator complex; either or both of these effects could result in a reduced efficiency of replication fork arrest.

Using gel mobility shift assays and ITC, we measured the affinity of RTP for the isolated half-sites. In order to analyze these data, we needed to ascertain the oligomerization state of RTP. Analytical ultracentrifugation data demonstrated that under the dilute conditions needed for accurate gel mobility shift assays, the vast majority of RTP exists as monomer. Previous studies have assumed that only the RTP dimer is competent to bind DNA. However, there is no evidence against a second reversible pathway wherein RTP binds DNA as a monomer followed by highly cooperative binding of another monomer to form each dimer-half-site complex. This stepwise binding pathway could co-exist with the direct dimer-binding pathway and would be more significant at lower RTP concentrations. Both kinetic and equilibrium data support the existence of a stepwise pathway. Kinetic experiments indicate that chemical equilibrium is reached within 15–20 min for the RTP-Terl interaction at the low concentrations used for the equilibrium binding experiments (11). Under the same conditions, the half-life of the RTP-Terl complex is 182 min (25). These findings are not consistent with a model in which only preformed RTP dimer is competent to bind DNA because the effective concentration of the dimer would be too low to support the observed equilibration rate; the association rate constant would have to greatly exceed the diffusion-controlled limit for this type of reaction, estimated to be ~10^9 M^-1 s^-1 (28).

On the other hand, if RTP monomers can bind DNA, the much higher effective concentration of RTP monomer would enable equilibrium to be reached in the observed time. Control analyses for the present study also demonstrated that equilibrium gel mobility shift data are described with a significantly better fit by the ternary complex reaction scheme (two monomers on DNA) compared to a scheme in which it is assumed that only the RTP dimer binds DNA. While we have been unable to detect RTP monomer-DNA species directly, this is almost certainly due to a combination of high cooperativity in forming the dimer-DNA complex, resulting in low equilibrium concentrations of RTP monomer-DNA intermediates, together with their low electrophoretic stability. Our inability to measure affinities for each monomer-DNA complex means that it is not possible to measure the affinity of the cooperative RTP monomer-monomer interaction on the DNA or the relative contribution of the RTP monomer-monomer and RTP-DNA interactions to the overall equilibrium constants for the half-site complexes (29, 30). Therefore, we report only the overall equilibrium constants.

The overall equilibrium constants for the A and B sites were very different, as expected (13). This hinted that the differential binding affinity could play an important part in establishing functional polarity of the whole complex, in accord with the DBA model. Knowledge of the affinity of RTP for both the A and B sites allowed us to measure the degree of cooperativity associated with the dimer-dimer interaction at the full-length terminator (Fig. 6, Table 3). These analyses demonstrated that the
RTP dimer-dimer interaction at a normal terminator results in a very high degree of cooperativity compared to other DNA binding proteins (31, and Refs. therein). Interestingly, our sedimentation equilibrium experiments demonstrated that RTP does not have a strong propensity to form tetramers in free solution; fits to models incorporating tetrameric RTP were not significantly better than monomer-dimer models. While the entropic cost of RTP binding to DNA would contribute significantly to the affinity of a cooperative interaction between RTP dimers, it is also possible that a significant portion of the observed cooperativity is generated indirectly though DNA structural changes associated with RTP-DNA binding. The observation of a reorganization of terminator DNA upon RTP binding may be significant in this regard (21).

The very high cooperativity associated with the RTP dimer-dimer interaction initially suggested that the cooperativity could serve to activate the complex by elevating the effective affinity of RTP bound at the B site sufficiently to block a replication fork, as described by the DBA model. Our analysis of the series of mutant terminators revealed that some mutants displayed both reduced RTP-binding affinity at the proximal site and reduced fork-arrest efficiency (compare terminators containing the proximal \( B^* \) site to those with proximal B sites, Table 3 and Fig. 7). However, some of the mutant terminators had large reductions in cooperativity with accompanying large reductions in proximal-site affinity, yet retained high efficiency in fork arrest (compare B.B, B.sRB and sRB.sRB with A.B, Table 3 and Fig. 7). Also, one particular mutant terminator (A.sRB) retained high-affinity binding at the proximal site, yet exhibited low fork arrest efficiency. If the proximal-site binding affinity is the sole determinant of fork arrest efficiency, then A.sRB should have been the most efficient of all terminators shown in Fig. 7, and B.B should have been less efficient than all of the distal A-site series (B.B had \( \sim 10^5 \)-fold weaker proximal-site affinity than A.sRB). Neither is the case. Thus, the effective binding affinity of RTP at the proximal site of the mutant terminator complexes did not correlate consistently with efficiency of fork arrest. These findings suggest that the difference in affinity between the A and B sites of the normal polar terminator is not the only factor controlling polarity.

It appears possible that conformational changes associated with the RTP dimer-dimer interaction contribute significantly to activation of a dimer at a B site, and that specific interaction between RTP dimers at the terminator establishes functionally important differences in the RTP-DNA structure at the A and B sites. On the other hand, our observations of a very large differential binding affinity at the A and B sites and the partial correlation of fork arrest efficiency with proximal site affinity of some of the reduced-affinity terminator complexes argues that differential binding affinity may contribute to polarity to some degree. The relative importance of RTP-DNA conformation and affinity in establishing functional polarity remains a pertinent issue, but it is now clear that this is a more complex process than originally envisaged. There also remains the separate unresolved issue concerning the type of contact or interference between the RTP-Ter complex and the advancing replisome (14, 25, 32, 33). Mutational and structural analyses of the complete RTP-Ter complex, when it is associated with components of the arrested replication fork, present new challenges in this field.
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**FOOTNOTES**

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1 The abbreviations used are: Ter, terminator; kDa, kilodaltons; bp, base pair(s); DBA, differential binding affinity; ICC, induced conformational change; BSA, bovine serum albumen; ITC, isothermal titration calorimetry; kb, kilobases; MCS, multiple cloning site.

2 I. G. Duggin, unpublished data.
FIGURE LEGENDS

Fig. 1 The differential binding affinity (DBA) model for a polar RTP-Ter complex, indicating the notation used to specify the various dissociation equilibrium constants of the important reactions. Note that while the model shows that RTP binds as a dimer, it does not specify that RTP monomers cannot assemble cooperatively on the DNA in forming each dimer-DNA complex (see “Discussion”). In addition, the model does not specify the structural mechanism giving rise to cooperativity; this could be mediated through direct protein-protein interactions or by mechanisms associated with DNA structural changes.

Fig. 2 Analysis of the mutant sRB site. A, DNA sequence alignment of the consensus regions of the B site from TerI and the sRB site. Arrows indicate DNA sequence symmetry, dots mark the centers of the sequences and vertical bars indicate sequence differences. Bold text in the B-site sequence indicates the nucleotide positions completely conserved among the nine B. subtilis chromosomal terminators. B, An equilibrium gel mobility shift assay simultaneously comparing the affinities of the isolated B site from TerI (in pWS69-1 (16)) and the sRB site (in pID1) within different restriction fragments. The RTP subunit concentration (expressed as monomer, pM) in each sample is indicated above each lane. Overall DNA concentration was 0.5 pM in each sample (0.25 pM each). C, Southern blot of replication fork arrest assays comparing TerI (pID2) and TerAs/sRB (pID6) in B. subtilis SU230. Bands representing the levels of specific forked DNA replication intermediate (indicative of fork arrest) and the non-replicating linear DNA for the probed restriction fragment are marked Fork and Linear respectively. The average level of the forked DNA band (F %) from at least two independent experiments is shown below each lane together with the standard error of the mean (SEM).

Fig. 3 Cloning of the series of terminators for RTP-binding and replication fork arrest assays. A, Demonstration of the plasmid nomenclature used, which indicates the RTP binding sites positioned proximal and distal to the advancing replisome generated at the unidirectional origin in these pWS64-1-based plasmids. In this example of pTerA.B, the replisome approaches the B-site side of the terminator sequence during the B. subtilis in vivo replication fork arrest assay. MCS, Multiple cloning site. oriB, B. subtilis origin of replication. B, Sequences of the various half-sites used in either distal or proximal positions in pWS64-1, illustrating the central join sites (the “P” represents a 5’ phosphate to allow ligation) and the BamHI and SalI overhangs used for directional cloning into pWS64-1. Sequence shading over the proximal half-sites represents base pairs that differ from the B site. C, Systematic list of the plasmids constructed in the four series of mutant terminators.

Fig. 4 A, Equilibrium gel mobility shift assays that compare RTP binding to the half-sites in pTerY.B and pTerY.sRB. The RTP subunit concentration (expressed as monomer, pM) for each sample is shown above each lane. The overall plasmid concentration was 0.25 pM in each sample. 32P-end-labeled DNA bands containing the Y.B or Y.sRB sequences are marked as either unbound (0) or bound to one RTP dimer (I). B, Plots of DNA fragment concentration at equilibrium versus total RTP monomer concentration for both data sets. Filled symbols, Y.B; open symbols, Y.sRB; square symbols, unbound DNA; circular symbols, complex I DNA. Best-fit curves are superimposed (Table 1 shows the relevant equilibrium constants). Solid lines, Y.B; dashed lines, Y.sRB.

Fig. 5 Isothermal calorimetric titrations of half-site DNA into RTP. A, A site DNA. B, B* site DNA. The upper panels show the incremental heat evolution during sequential injections, and the lower panels show the integrated heat data fitted by a single set of sites binding model.

Fig. 6 A, Equilibrium gel mobility shift assays comparing RTP binding to full-length terminators in pTerA.B and pTerA.sRB. The RTP subunit concentration (expressed as monomer, pM) used for each sample is shown above each lane. The same RTP dilution series was used for both assays. The total DNA
concentration for both plasmids was 0.25 pM in each sample. $^{32}$P-end-labeled DNA bands, containing the A.B (upper panels) or A.sRB (lower panels) sequences are marked as either unbound (0) or bound to one (I) or two (II) RTP dimers. B, Plots of DNA fragment concentration at equilibrium versus total RTP monomer concentration for both data sets. Filled symbols, A.B; open symbols, A.sRB; square symbols, unbound DNA; circular symbols, complex I DNA; triangular symbols, complex II DNA. Best-fit curves are superimposed (Table 3 contains the relevant equilibrium constants). Solid lines, A.B; dashed lines, A.sRB.

Fig. 7 Southern blot of DNA replication fork arrest assays. Plasmids containing the various terminator sequences (shown above each lane) were extracted from respective exponential phase cultures of B. subtilis SU230 (16) and the specific forked DNA indicative of arrest at the cloned terminator was detected using gel electrophoresis and Southern blotting. Note that the unidirectional replication fork in these plasmids approaches the cloned terminators towards the second half-site listed in the name of each; see Fig. 3A. One of three replicate experiments is shown. The control lane contains DNA from an equivalent culture containing the base vector pWS64-1. Below each lane is a quantitative analysis from three independent sets of experiments; the percent of total probed radioactivity in the arrested-fork band (F %, ± SEM) and normalized fork arrest activity (FAA %) are shown.
Table 1. Binding constants obtained from gel mobility shift assays of isolated RTP dimer-half-site interactions.

| Plasmid (half-site) | Overall $K_D (M^2)^{(a)}$ |
|---------------------|---------------------------|
| $pTer B . Y$ (B site, distal) | $1.8 \pm 0.2 \times 10^{-19}$ |
| $pTer RB . Y$ (sRB site, distal) | $1.9 \pm 0.3 \times 10^{-19}$ |
| $pTer Y . B$ (B site, proximal) | $1.3 \pm 0.1 \times 10^{-20}$ |
| $pTer Y . sRB$ (sRB site, proximal) | $6.1 \pm 0.7 \times 10^{-21}$ |

(a) The overall $K_D$ for the half-site complexes represents the dissociation equilibrium constant for the reaction: $AL_2 = A + 2L$, where $A$ is the half-site DNA and $L$ is RTP monomer.
Table 2. Calorimetric analysis of isolated RTP dimer-half-site interactions.

| Parameter                        | A site            | B* site           |
|----------------------------------|-------------------|-------------------|
| \( n \) \(^{(a)} \)              | 0.45 (± 0.01)     | 0.46 (± 0.01)     |
| \( \Delta H_{\text{cal},298\text{ K}} \) \(^{(b)} \) | 9.7 (± 0.2)       | 11.9 (± 0.2)      |
| \( \Delta G_{\text{cal},298\text{ K}} \) \(^{(b)} \) | -8.5 (± 0.2)      | -9.3 (± 0.2)      |
| \( K_D \) (M) \(^{(b)} \)       | \( 5.9 (± 0.1) \times 10^{-7} \) | \( 1.6 (± 0.7) \times 10^{-7} \) |

(a) The molar ratio of DNA:RTP monomer at the inflection point of the fitted curve (see Fig. 5), indicating the stoichiometry of the observed interaction.

(b) These values were determined using the data in Fig. 5 with the single set of sites binding scheme. \( K_D \) represents the intrinsic affinity of the RTP dimer for each half-site.
Table 3. Binding constants obtained for full-length RTP-Ter interactions.

| Plasmid     | Overall $K_D$ (Ter) (M$^4$) ($^a$) | Cooperativity constant ($^b$) ($^c$) | Proximal site ($^c$) ($^d$) |
|-------------|----------------------------------|-------------------------------------|------------------------------|
| pTerA.B     | 3.7 ($\pm$ 0.5) $\times 10^{-42}$ | 2.1 ($\pm$ 0.6) $\times 10^{-8}$ – $10^{-7}$ ($^e$) | 1.0 ($\pm$ 0.2) $\times 10^{-28}$ – $10^{-27}$ ($^e$) |
| pTerA.sRB   | 1.9 ($\pm$ 0.2) $\times 10^{-42}$ | 3.2 ($\pm$ 0.9) $\times 10^{-8}$ – $10^{-7}$ ($^e$) | 5.2 ($\pm$ 0.8) $\times 10^{-29}$ – $10^{-28}$ ($^e$) |
| pTerA.B$^*$ | 1.1 ($\pm$ 0.2) $\times 10^{-38}$ | 3.0 ($\pm$ 2.1) $\times 10^{-11}$ – $10^{-9}$ ($^e$) | 3.0 ($\pm$ 0.6) $\times 10^{-25}$ – $10^{-24}$ ($^e$) |
| pTerB.B     | 8.5 ($\pm$ 2.1) $\times 10^{-42}$ | 4.3 ($\pm$ 3.3) $\times 10^{-3}$ | 4.7 ($\pm$ 1.7) $\times 10^{-23}$ |
| pTerB.sRB   | 2.0 ($\pm$ 0.5) $\times 10^{-42}$ | 2.7 ($\pm$ 1.8) $\times 10^{-3}$ | 1.1 ($\pm$ 0.4) $\times 10^{-23}$ |
| pTerB.B$^*$ | 3.0 ($\pm$ 0.7) $\times 10^{-40}$ | 1.7 ($\pm$ 1.5) $\times 10^{-7}$ – $10^{-6}$ ($^e$) | 1.7 ($\pm$ 0.6) $\times 10^{-21}$ |
| pTersRB.B   | 8.7 ($\pm$ 1.8) $\times 10^{-42}$ | 1.6 ($\pm$ 1.5) $\times 10^{-3}$ | 4.6 ($\pm$ 1.7) $\times 10^{-23}$ |
| pTersRB.sRB | 9.1 ($\pm$ 1.3) $\times 10^{-42}$ | 1.7 ($\pm$ 1.2) $\times 10^{-3}$ | 4.8 ($\pm$ 1.4) $\times 10^{-23}$ |
| pTersRB.B$^*$ | 8.6 ($\pm$ 1.5) $\times 10^{-41}$ | 4.5 ($\pm$ 4.0) $\times 10^{-8}$ – $10^{-7}$ ($^e$) | 4.5 ($\pm$ 1.5) $\times 10^{-22}$ |

(a) Overall equilibrium constant for the complete complex (i.e., for the reaction: AL$_4$ = A + 4L, where A is the DNA and L is RTP monomer).

(b) The cooperativity constant was determined as the overall equilibrium constant ($K_D$ (Ter)) divided by the product of the relevant individual half-site overall equilibrium constants. This is the inverse of the “omega” cooperativity parameter (34).

(c) Errors were propagated using standard methods (35).

(d) The proximal site affinity within the whole complex was determined as the overall equilibrium constant ($K_D$ (Ter)) divided by the overall equilibrium constant for the distal half-site-RTP dimer complex. (This is equal to the product of the cooperativity constant and the overall equilibrium constant for the proximal half-site-RTP dimer complex.)

(e) Due to the absence of glycerol in the binding buffer for ITC experiments, these constants are presented as a range based on an estimated 10–100 fold increase in overall binding affinity of the respective half-site complex(es) that would occur in the presence of glycerol (11). Note that each value was likely to be similarly affected by this discrepancy, suggesting that valid comparisons of these terminators can be made.
Fig. 1
A.  

\[ \text{B} \begin{array}{c}
14 \\
\text{TGTACCAATGTTC} \\
28 \\
\text{sRB} \\
\text{TGAACATAATGTTCA}
\end{array} \]

B.  

![Image of gel electrophoresis](http://example.com/image.png)

C.  

![Image of Western blot](http://example.com/image.png)

\[ \text{Fork} \text{ pWS64-1}\]
\[ \text{pID2}\text{ pID6} \]

\[ \text{F \% (SEM)} \begin{array}{c}
0 \\
13.0 (±1.5) \\
5.7 (±0.3)
\end{array} \]

Fig. 2
A. 

B. Distal sites

Proximal sites

C. 

Fig. 3
Fig. 4
Fig. 5
Fig. 6
| F (%) (SEM) | 10.5 ± 0.15 | 5.1 ± 0.05 | 3.2 ± 0.01 | 22.6 ± 0.50 | 10.9 ± 0.05 | 2.9 ± 0.10 | 11.9 ± 0.75 | 7.9 ± 0.40 | 0.9 ± 0.10 | 0.0 ± 0.00 |
|-------------|--------------|-------------|-------------|--------------|--------------|------------|-------------|-------------|------------|------------|
| FAA (%)     | 100 ± 40.00  | 49 ± 21.00  | 30 ± 11.00  | 215 ± 90.00  | 104 ± 40.00  | 28 ± 11.00 | 113 ± 42.00 | 75 ± 30.00  | 9 ± 4.00   | 0 ± 0.00   |

Fig. 7
A complex mechanism determines polarity of DNA replication fork arrest by the replication terminator complex of bacillus subtilis
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