A DNA translocation motif in the bacterial transcription–repair coupling factor, Mfd

A. L. Chambers, A. J. Smith and N. J. Savery*

University of Bristol, Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK

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ABSTRACT

The bacterial transcription–repair coupling factor, Mfd, is a superfamily II helicase that releases transcription elongation complexes stalled by DNA damage or other obstacles. Transcription complex displacement is an ATP-dependent reaction that is thought to involve DNA translocation without the strand separation associated with classical helicase activity. We have identified single amino acid substitutions within Mfd that disrupt the ability of Mfd to displace RNA polymerase but do not prevent ATP hydrolysis or binding to DNA. These substitutions, or deletion of the C-terminal 209 residues of Mfd, abrogate the ability of Mfd to increase the efficiency of roadblock repression in vivo. The substitutions fall in a region of Mfd that is homologous to the ‘TRG’ motif of RecG, a protein that catalyses ATP-dependent translocation of Holliday junctions. Our results define a translocation motif in Mfd and suggest that Mfd and RecG couple ATP hydrolysis to translocation of DNA in a similar manner.

INTRODUCTION

DNA is subjected to a continuous barrage of assaults from endogenous and exogenous sources, and cells employ a wide range of enzyme activities to recognise and repair DNA damage and thus ensure the integrity of the genetic material. DNA damage within genes that are being transcribed is prioritised by transcription–repair coupling pathways: lesions that occur in the transcribed strand and cause RNA polymerase (RNAP) to stall are repaired ~10-fold more rapidly than similar damage in the bulk DNA (1,2). The process is mediated by transcription–repair coupling factors that displace the stalled RNAP from the site of damage, and interact with repair proteins (3,4).

The bacterial transcription–repair coupling factor is the Mfd protein, which binds to stalled transcription elongation complexes in vitro and catalyses their dissociation from template DNA in an ATP-dependent manner (5). Sequence analysis indicates that Mfd contains superfamily II helicase motifs (5). These are essential for the RNAP displacement activity of Mfd (6), but Mfd does not possess conventional strand-separating helicase activity (7). In order to displace RNAP, Mfd requires ~20 bp of accessible DNA upstream of the region bound by RNAP but little or no DNA downstream of RNAP (8). Mfd is also able to ‘rescue’ elongation complexes that have become backtracked, allowing them to resume transcription. In backtracked complexes, the 3’ end of the nascent transcript has been extruded from the active site through backwards translocation of RNAP (8). Transcription elongation factors GreA and GreB rescue backtracked complexes by causing the extruded 3’ end of the transcript to be cleaved (9), but Mfd appears to directly reverse the backtracking process by causing the stalled RNAP to move forward (8).

These results support a model in which ATP hydrolysis by Mfd is coupled to dissociation of RNAP by translocation of double-stranded DNA (8). At stalled elongation complexes, an Mfd molecule interacts with RNAP and with the region of DNA immediately upstream of the complex. If transcription is blocked by a DNA lesion, or by a protein bound to the DNA in front of the polymerase, translocation by Mfd results in dissociation of RNAP. In the case of backtracked complexes, the translocation results in realignment of the 3’ end of the transcript and resumption of transcription.

Double-stranded DNA translocation is thought to be important for a range of processes including chromatin remodelling, DNA replication and DNA cleavage by type I restriction enzymes (10–12). One of the best characterised DNA translocases is the bacterial RecG protein, which is involved in the rescue of stalled replication forks and can catalyse branch migration of a Holliday junction structure in vitro in an ATP-dependent manner (13). The region of Mfd encompassing the helicase motifs shares a high degree of sequence identity with the helicase motifs of RecG (38% identity between 378 residues of Escherichia coli Mfd and the corresponding region of E.coli RecG) (5,14). The crystal structure of a Thermatoga maritima RecG-ADP–DNA complex (12) reveals that RecG consists of three domains: an N-terminal domain, which binds to the replication fork, and two C-terminal domains, which contain superfamily II helicase motifs. It is proposed that ATP hydrolysis by RecG results in translocation of double-stranded DNA by domains 2 and 3 (12), and it is these domains that contain the region of homology to Mfd.

Substitutions that prevent DNA translocation by RecG have recently been identified in the region immediately downstream of helicase motif VI (14). These substitutions fall in a helical...
hairpin motif and an adjacent surface-exposed loop that was not defined in the RecG crystal structure. This region was termed the TRG (translocation in RecG) motif. On the basis of Mfd:RecG sequence alignments, it was proposed that Mfd also contains a TRG motif (14) (Fig. 1). In this study, we show that the putative TRG motif in Mfd is essential for Mfd-mediated displacement of RNAP, but is not essential for ATP-dependent DNA binding. We also show that Mfd increases the efficiency of roadblock repression of transcription in vivo, and that residues within the TRG motif are required for this function.

MATERIALS AND METHODS

Strains and plasmids

The mfd+ E.coli K-12 strain AB1157 was obtained from the E.coli Genetic Stock Centre, Yale University. UNCNOMFD is an mfd− derivative of AB1157 in which a 2317 bp NsiI–NsiI fragment of the chromosomal mfd gene has been replaced by a cassette encoding kanamycin resistance (6). Standard methods for isolation and manipulation of DNA fragments were used throughout (15). All constructs were confirmed by sequencing. Details of the primers used for directed and random mutagenesis are available on request.

The roadblock repression reporter vector pRCB-CAT1 (pRK2 ori, Tet') was constructed in several steps, as follows. The HindIII cassette carrying the chloramphenicol acetyltransferase gene (cat) from pCM7 (Pharmacia) was cloned into the HindIII site of pSRgalPcon6 [a pBR322 derivative carrying the constitutive galPcon6 promoter (16) upstream of a loop transcription terminator]. A self-complementary oligonucleotide containing the optimal lac operator sequence 5'–GAATTGTTGACGTGATC–3' (17) was cloned into the HindIII sites on either side of the cat gene to generate plasmid pCSB1. The Nhel–HindIII lacZ/A fragment was removed from the broad-host range lacZ reporter plasmid pRW50 (18), blunt ends were generated using Klenow polymerase, and the vector was religated to create plasmid pRCB. The EcoRI–BamHI fragment of pCSB1, which carried the galPcon6 promoter, the cat gene flanked by lac operators, and the transcription terminator, was inserted between the EcoRI and BamHI sites of pRCB to generate pRCB-CAT1.

The Mfd expression plasmids pETMfd and pETTrunc carry the mfd gene (or a derivative) cloned into the backbone of pET21a (Novagen). Neither pETMfd nor pETTrunc contain the T7 promoter of pET21a, but both retain the lacI0 gene that encodes the Lac repressor protein necessary for repression of the cat gene in pRCB-CAT1. Expression of Mfd from pETMfd and pETTrunc is controlled by the wild-type mfd promoter (19), and the Mfd proteins are not histidine tagged. pETMfd was constructed by ligating an EcoRI–SphI fragment of pMFD19 (5) containing the mfd gene and promoter with an EcoRI–SphI fragment of pET21a containing the ori, bla and lacI0 genes. Derivatives of pETMfd encoding Mfd with RA929, RA953 and QA963 substitutions were constructed by site-directed mutagenesis (15). pETMfd encoding Mfd with an HL948 substitution was isolated from a library of pETMfd derivatives in which codons 913–995 of mfd had been randomly mutated by error-prone PCR using Taq DNA polymerase (20).

pETTrunc encodes residues 1–939 of Mfd (Mfdtrunc) and was constructed in several stages, as follows. A HindIII–Styl PCR product carrying the loop transcription terminator from pSRlacUV5 (21) was inserted between the HindIII and Styl sites of pET21a to create pET21term. A stop codon and a HindIII site were introduced downstream of mfd codon 939 by PCR, using pMFD19 as a template. The resulting PCR product, which carried the mfd promoter and truncated mfd gene, was cut with BamHI and HindIII and ligated with pET21term that had been cut with BglII and HindIII, to create pET21term. The latter was cut with BamHI and HindIII and ligated with pET21term that had been cut with BglII and HindIII, generating pETTrunc.

CAT assays

AB1157 or UNCNOMFD cells transformed with pRCB-CAT1 and pETMfd, pETTrunc or a derivative were grown at 37°C in M9 medium containing 80 μg/ml ampicillin and 20 μg/ml tetracycline (plus 25 μg/ml kanamycin for UNCNOMFD). Where indicated, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture prior to growth. At OD600 of ~0.5, 1.5 ml of culture was harvested by centrifugation into the HindIII site of pET21a to create pET21term. A stop codon and a HindIII site were introduced downstream of mfd codon 939 by PCR, using pMFD19 as a template. The resulting PCR product, which carried the mfd promoter and truncated mfd gene, was cut with BamHI and HindIII and ligated with pET21term that had been cut with BglII and HindIII, generating pETTrunc.

CAT assays

Escherichia coli RNAP holoenzyme was purchased from Epicentre Technologies (Madison, WI). Wild-type and mutant Mfd proteins were purified from UNCNOMFD cells transformed with the appropriate pETMfd derivative. Cells were grown at 37°C in 1 l of 2X YT medium containing 80 μg/ml ampicillin and 25 μg/ml kanamycin. At an OD600 of ~1.7, the culture was harvested by centrifugation, the pellet was resuspended in 20 ml of lysis buffer [50 mM Tris pH 7.5, 5% glycerol (v/v), 120 mM NaCl, 10 mM EDTA, 5 mM dithiothreitol (DTT)] and the cells were lysed by sonication. Mfd was purified from the lysate using a 5 ml HiTrap Blue Sepharose column, a 5 ml HiTrap heparin column and a 1 ml Mono Q column (Amersham). Protein was eluted from each column with a 0.05–1.0 M KCl gradient in TGED buffer [10 mM Tris pH 7.5, 5% glycerol (v/v), 1 mM EDTA, 1 mM DTT], and fractions containing Mfd were diluted 10-fold in TGED, and fractions containing Mfd were diluted 10-fold in TGED before being loaded onto the next column. Purified
protein was dialysed against storage buffer [10 mM Tris pH 8.0, 50% glycerol (v/v), 1 mM EDTA, 2 mM DTT, 200 mM KCl] and stored at −20°C. Protein concentrations were determined using the Bradford assay.

**In vitro analysis of RNAP displacement**

Stalled transcription elongation complexes were formed by nucleotide starvation on a 529 bp Rsal–SmaI fragment of plasmid pAR1707 (22). This fragment encodes a transcript in which UTP must be incorporated at +2 and +21. Transcription elongation complexes stalled at +20 could therefore be formed by using the dinucleotide ApU to initiate transcription and excluding UTP from the transcription reaction. Complexes were analysed by electrophoretic mobility shift assays (EMSAs) using 4.5% acrylamide/1× TBE gels run at 4°C, and radiolabelled bands were detected using a phosphor screen and quantified using Imagequant software (Molecular Dynamics).

For experiments to determine the time course of Mfd-mediated displacement of RNAP, the fragment of pAR1707 was end labelled using T4 polynucleotide kinase and [γ-32P]ATP (15). Initiation complexes were formed by incubating 0.4 nM labelled fragment with 5 nM RNAP holoenzyme in repair assay buffer [40 mM HEPEs, pH 8.0, 100 mM KCl, 8 mM MgCl2, 4% glycerol (v/v), 5 mM DTT, 100 μg/ml bovine serum albumin (BSA)] for 15 min at 37°C. Unstable complexes were removed by the addition of 10 μg/ml heparin, then ApU, ATP, GTP and CTP were added at final concentrations of 80 μM, 1.7 mM, 8 μM and 8 μM, respectively, to initiate transcription. The reaction mixtures were then incubated at 37°C for 15 min. Mfd was added at a final concentration of 250 nM and, after the indicated incubation periods at 37°C, aliquots were withdrawn and loaded onto an acrylamide gel under tension.

In experiments to analyse binding of mutant Mfd to transcription elongation complexes, either the fragment of pAR1707 was end-labelled as above, or the nascent transcripts were labelled by incorporation of [α-32P]CTP. For reactions using labelled template, initiation complexes were formed as described above. Where indicated, ApU, ATP, CTP and GTP were added at final concentrations of 80 μM, 0.8 mM, 8 μM and 8 μM, respectively. The reaction mixtures were incubated at 37°C for 15 min and then 5.5 μg/ml rifampicin was added. Mfd was added at a final concentration of 400 nM and samples were incubated at 37°C for 15 min before being analysed by gel electrophoresis. For reactions in which the nascent transcripts were labelled, initiation complexes were formed by incubating 2 nM unlabelled pAR1707 fragment with 10 nM RNAP holoenzyme in repair assay buffer at 37°C for 15 min. Rifampicin and then ApU, ATP and GTP were added at final concentrations of 20 μg/ml, 100 μM, 2 mM and 10 μM, respectively. Unlabelled CTP was added at a final concentration of 2 μM, together with 5 μCi of [α-32P]CTP per reaction. The reaction mixtures were incubated at 37°C for 15 min. Mfd was then added at a final concentration of 400 nM and the samples incubated at 37°C for 15 min before being analysed by gel electrophoresis.

**DNA binding assays**

Binding of Mfd to DNA was assayed by EMSAs (23). A 248 bp EcoRI–BamHI fragment from pSRlacUV5 (21) was end labelled using T4 polynucleotide kinase and [γ-32P]ATP (15). Reactions contained 100 nM purified Mfd and 0.4 nM labelled fragment in 15 μl of repair assay buffer. Where indicated, the reactions also contained 2 mM ATP or ATPγS. Reactions were incubated at 37°C for 25 min and analysed by electrophoresis at 4°C through 5% acrylamide/1× TAE/8 mM magnesium acetate gels. Bands were detected using a phosphor screen.

**ATPase assay**

Rates of ATP hydrolysis were determined by measuring the release of 32P-labelled inorganic phosphate from [γ-32P]ATP, essentially as described by Tombline and Fishel (24). Reaction mixtures contained 1 μM Mfd and 2 mM ATP in repair buffer at 37°C.

**RESULTS**

**A roadblock repression assay for Mfd function**

Proteins bound to DNA in the path of a transcription elongation complex can cause RNAP to pause or stall, thus decreasing transcription of sequences downstream of the protein-binding site. Regulated formation of protein roadblocks is involved in carbon catabolite repression of the hut and gnt operons in Bacillus subtilis (25,26), and catabolite repression of these operons is partially relieved in mfd− cells (27). It has been proposed that the Mfd dependence of roadblock repression in B.subtilis is the result of Mfd-mediated displacement of RNAP paused at roadblock sites (27). We established an in vivo assay to test this model in E.coli and to provide an Mfd-dependent phenotype that could be readily monitored.

The rationale for the assay is shown in Figure 2. Lac repressor–operator interactions are sufficient to cause roadblock repression both in vivo and in vitro, although roadblock repression is less efficient than repression of the lacP1 promoter by promoter occlusion (28–31). We constructed a reporter vector, rRCB-CAT1, in which roadblock repression by Lac repressor protein controlled expression of the cat gene. In order to maximise the level of roadblock repression, we placed one optimal lac operator (17) upstream of the cat gene, and a second optimal lac operator downstream of the cat gene. The two operators were separated by 815 bp (centre-to-centre), a distance at which simultaneous binding of the two operators by a single Lac repressor tetramer would be expected to increase occupancy of the upstream operator ~2-fold (32) without introducing a second roadblock between the promoter and the reporter gene. Expression of the cat gene was driven by a constitutive derivative of the galP1 promoter, located 64 bp upstream of the centre of the upstream lac operator. Lac repressor bound to the upstream lac operator was expected to block the progress of RNAP and decrease transcription of the cat gene. If Mfd displaces transcription complexes stalled by protein roadblocks in vivo, as it does in vitro (7), it should increase the likelihood that the stalled RNAP dissociates from the DNA before Lac repressor does, decreasing transcription of the cat gene still further. The Lac repressor roadblock should therefore repress transcription more effectively when Mfd is present than when Mfd is absent.
To determine the effectiveness of this roadblock repression system in the absence of Mfd, an mfd" strain, UNCNOMFD, was transformed with the roadblock repression reporter vector and a plasmid carrying the lacI\textsuperscript{R} gene encoding Lac repressor. Cultures of this strain were grown to mid-exponential phase in the presence or absence of IPTG, and CAT activities were measured (Table 1). The CAT activity in the absence of IPTG (when Lac repressor should be bound to operator DNA) was 9.5-fold lower than that in the presence of IPTG (when Lac repressor should not be bound to operator DNA). Lac repressor bound downstream of the transcription start site therefore repressed transcription of the cat gene 9.5-fold in this system, presumably by acting as a block to transcription elongation.

To determine the effect of Mfd on roadblock repression in this system, the experiment was repeated using UNCNOMFD (mfd") cells transformed with the roadblock repression reporter vector and a plasmid carrying the wild-type mfd gene in addition to lacI\textsuperscript{R} (Table 1). In these cells, the CAT activity in the absence of IPTG was 61-fold lower than that in its presence (Table 1). Both the CAT activity in the absence of IPTG was 61-fold lower than that in its presence (Table 1). Both the UNCNOMFD (mfd") cells transformed with the roadblock repression reporter vector and a control plasmid (pET21a) that did not encode Mfd. Cultures were grown to OD\textsubscript{600} ~0.5 in M9 medium + antibiotics \( \pm 0.5 \text{ mM IPTG} \) as indicated. CAT activities shown (with SD) are the average of three independent experiments and are expressed in units of nmol of chloramphenicol acetylated/min/mg of protein.

### Table 1. Effect of Mfd on roadblock repression \textit{in vivo}

<table>
<thead>
<tr>
<th>Mfd</th>
<th>+IPTG (induced)</th>
<th>−IPTG (repressed)</th>
<th>Repression (+IPTG/−IPTG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24.0 ± 2.1</td>
<td>2.5 ± 0.5</td>
<td>9.5-fold</td>
</tr>
<tr>
<td>Wild-type</td>
<td>15.2 ± 2.0</td>
<td>0.3 ± 0.1</td>
<td>61-fold</td>
</tr>
<tr>
<td>Mfd\textsubscript{trunc}</td>
<td>20.2 ± 2.3</td>
<td>3.1 ± 0.5</td>
<td>6.5-fold</td>
</tr>
</tbody>
</table>

To determine the effect of Mfd on roadblock repression reporter vector and a control plasmid (pET21a) that did not encode Mfd grew readily on M9 minimal medium agar containing 5 μg/ml chloramphenicol in the absence of IPTG. Identical cells transformed with a plasmid that encoded wild-type Mfd (pETMfd) were unable to grow under the same conditions. A chloramphenicol-resistant phenotype in this assay is therefore an indication of Mfd activity. Mutants that are defective in the pathway by which Mfd affects roadblock repression should confer a chloramphenicol-resistant phenotype and thus be readily selectable. Several mutants with such a phenotype are described below.

### The C-terminal region of Mfd is required for optimal roadblock repression

A truncated version of Mfd (Mfd\textsubscript{trunc}) lacking the C-terminal 209 amino acids has previously been shown to be specifically defective in RNAP displacement, but to retain the ability to bind to both RNAP and DNA (6). If the effect of Mfd on roadblock repression is dependent on its ability to dissociate stalled transcription elongation complexes, Mfd\textsubscript{trunc} should be unable to enhance roadblock repression \textit{in vivo}. We tested this prediction using the CAT reporter assay described above. UNCNOMFD (mfd") cells transformed with the roadblock repression reporter vector and a plasmid encoding Mfd\textsubscript{trunc} and lacI\textsuperscript{R}. The level of cat gene expression was monitored by testing the ability of the transformants to grow on M9 agar plates containing 5 μg/ml chloramphenicol and by assaying CAT activity in liquid cultures. The transformants were resistant to 5 μg/ml chloramphenicol (data not shown), and the CAT activity in the absence of IPTG was 6.5-fold lower than that in its presence (Table 1). Both the
chloramphenicol resistance phenotype and the efficiency of roadblock repression in these cells were therefore comparable with results obtained with cells that lacked Mfd, indicating that Mfd truncated (Mfd\textsubscript{trunc}) did not increase the efficiency of roadblock repression in vivo.

The effect of Mfd\textsubscript{trunc} expression was also determined in cells carrying an intact chromosomal mfd gene (Fig. 3A). The repressed level of CAT activity in mfd\textsuperscript{+} cells transformed with a plasmid that encoded Mfd\textsubscript{trunc} was higher than in those transformed with a control plasmid that did not encode Mfd. In control experiments, the repressed level of CAT activity in mfd\textsuperscript{+} cells transformed with a plasmid that encoded wild-type Mfd was ~3-fold lower than in those transformed with the control plasmid. The ability of Mfd\textsubscript{trunc} to exert a trans-dominant effect over wild-type Mfd confirmed that Mfd\textsubscript{trunc} was expressed, and suggests that despite being unable to dissociate the RNAP, it was able to compete with wild-type Mfd for binding sites on the stalled RNAP complexes. The increased repression in cells that contained plasmid-borne mfd genes in addition to the chromosomal gene indicates that wild-type levels of Mfd were not saturating.

The results obtained with Mfd\textsubscript{trunc} confirm that the ability of Mfd to increase the efficiency of roadblock repression is dependent on its ability to displace RNAP from DNA. Expression of genes that are controlled via RNAP displacement should therefore be sensitive to mutations that abolish the ability of Mfd to displace RNAP from DNA. Within the mfd gene, such mutations could affect Mfd–RNAP binding, Mfd–DNA binding, the ATPase activity of Mfd, or the mechanism by which ATP hydrolysis is linked to RNAP displacement.

Substitutions in the TRG motif affect Mfd function in vivo

To determine whether the putative TRG motif plays a functional role in RNAP displacement by Mfd, we generated Mfd derivatives carrying single amino acid substitutions at positions that correspond to key residues in the TRG motif of E.coli RecG (14) (Fig. 1). Site-directed mutagenesis was used to introduce single alanine substitutions at codons 913–995 of mfd (a region encompassing the putative TRG motif). The library of mutants was introduced into UNCNOMFD (mfd\textsuperscript{−}) cells carrying the roadblock repression reporter vector, and transformants were screened for mutants that were defective in RNAP displacement, as judged by their ability to grow on M9 minimal agar containing 5 \(\mu\)g/ml chloramphenicol. A nonsaturating screen yielded a chloramphenicol-resistant mutant encoding a leucine substitution at position H948 of Mfd.

The ability of the mutant Mfd proteins to displace stalled transcription complexes in vivo was assayed by monitoring their effect on roadblock repression using the CAT reporter system described above. UNCNOMFD (mfd\textsuperscript{−}) and AB1157 (mfd\textsuperscript{+}) cells were transformed with the roadblock repression reporter vector and pETMfd derivatives encoding Mfd RA929, RA953 and QA963. The effect of the Mfd derivatives on roadblock repression was monitored by directly assaying CAT activity in liquid cultures (Fig. 3) and by testing the ability of the transformants to grow on M9 agar plates containing 5 \(\mu\)g/ml chloramphenicol (data not shown).

UNCNOMFD (mfd\textsuperscript{−}) cells transformed with the roadblock repression reporter vector and with plasmids encoding Mfd RA953 and QA963 grew on M9 agar plates containing 5 \(\mu\)g/ml chloramphenicol. The repressed level of CAT activity measured during growth of these cells in liquid culture was similar to that observed in the absence of Mfd (Fig. 3B). Both Mfd RA953 and Mfd QA963 could be purified from UNCNOMFD transformants with a yield similar to wild-type (data not shown), demonstrating that they were stably expressed in this system. These results indicate that Mfd RA953 and Mfd QA963 did not affect the efficiency of roadblock repression in vivo and that the substitutions have affected a function of Mfd that is essential for displacement of RNAP. Mfd RA953 conferred a trans-dominant increase in CAT activity in AB1157 (mfd\textsuperscript{+}) cells (Fig. 3A), suggesting that, like Mfd\textsubscript{trunc}, the protein was able to compete with wild-type Mfd for binding sites on the stalled RNAP complexes.

UNCNOMFD (mfd\textsuperscript{−}) cells transformed with the roadblock repression reporter vector and with a plasmid encoding Mfd RA929 did not grow on M9 agar plates containing 5 \(\mu\)g/ml chloramphenicol, and the repressed level of CAT activity measured during growth of these cells in liquid culture was similar to that observed with cells transformed with a plasmid
encoding wild-type Mfd (Fig. 3B). These results indicate that Mfd RA929 was able to increase the efficiency of roadblock repression in vivo and must therefore be capable of displacing stalled transcription elongation complexes.

UNCONMFD (mfd') cells transformed with the roadblock repression reporter vector and with a plasmid encoding Mfd HL948 grew on M9 agar plates containing 5 μg/ml chloramphenicol, but the repressed level of CAT activity measured during growth of these cells in liquid culture was similar to that observed with cells transformed with a plasmid encoding wild-type Mfd (Fig. 3B). These results are apparently contradictory, as growth on plates containing 5 μg/ml chloramphenicol requires a higher level of CAT expression than occurs in cells containing wild-type Mfd. The discrepancy presumably reflects a difference in physiology between cells grown in liquid media and cells grown on solid media. The result of the CAT assay indicates that Mfd HL948 was able to increase the efficiency of roadblock repression in vivo and must therefore be capable of displacing stalled transcription elongation complexes, at least under certain conditions.

As in vitro analysis revealed that the RNAP displacement activity of Mfd HL948 was functional but impaired (see below), it appears that the chloramphenicol resistance phenotype on solid media is a more sensitive assay of Mfd function than CAT activity in liquid culture.

**DNA and ATP binding by purified mutant Mfd proteins**

The Mfd derivatives carrying substitutions in the TRG motif were purified, and their properties analysed in vitro. Mfd has been shown to possess non-specific ATP-dependent DNA binding activity (5,6), and we tested the DNA binding properties of the purified mutant Mfd proteins in the presence of ATP or ATPγS (a poorly hydrolysable ATP analogue) and in the absence of nucleotides (Fig. 4). In accordance with previously published work (6), wild-type Mfd only formed a stable complex in the presence of ATP. Mfd RA953, HL948 and QA963 also formed stable complexes in the presence of ATPγS but, in contrast to wild-type Mfd, these mutant proteins formed a significant amount of stable complex in the presence of ATP. This ATP-induced complex had a higher electrophoretic mobility than the complex formed in the presence of ATPγS, suggesting that the two complexes differ in character. Mfd RA929 did not form a stable complex in the presence of ATP, and the complex formed by Mfd RA929 in the presence of ATPγS resembled that formed by the other mutants in the presence of ATP.

The observation that all four Mfd derivatives carrying substitutions in the TRG motif bound DNA stably in the presence of ATPγS, but not in the absence of nucleotides, confirmed that they were able to bind ATP and suggested that the substitutions did not grossly affect the overall fold of the protein. We assessed the ATPase activity of our purified Mfd proteins at saturating ATP concentration in the absence of DNA. Under these conditions, all four of the Mfd derivatives carrying substitutions in the TRG motif hydrolysed ATP at the same rate, within the error of the experiment (data not shown). Wild-type Mfd presumably does not bind DNA stably in the presence of ATP because hydrolysis of ATP causes Mfd to be lost from the fragment. This might occur either by a simple conformational change induced by ATP hydrolysis or product release, or through ATP-driven DNA translocation. The ability of Mfd RA953, HL948, and QA963 to form stable complexes in the presence of ATP might therefore result either from defects in the ATP hydrolysis cycle that were too subtle to be detected in our ATPase assay, or an inability to couple ATP hydrolysis to DNA translocation or release.

**Substitutions in the TRG motif disrupt Mfd-mediated displacement of RNAP in vitro**

The ability of purified mutant Mfd proteins to displace stalled transcription complexes in vitro was assayed by EMSAs (Fig. 5). Plasmid pAR1707 contains the T7 A1 promoter, and elongation complexes initiating from this promoter can be stalled at +20 by excluding UTP from a reaction mixture that contains all other components necessary for transcription (22,33). Experiments using 32P-labelled template DNA were used to detect transcription initiation complexes, transcription elongation complexes and DNA to which RNAP had not bound (Fig. 5A), and experiments in which 32P-labelled nucleotides were incorporated into the nascent transcript were used to detect transcription elongation complexes specifically (Fig. 5B). Initiation complexes and stalled elongation complexes were distinguished on the basis of their differing mobility during acrylamide gel electrophoresis (e.g. compare

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**Figure 4.** Effect of single amino acid substitutions in Mfd on DNA binding in vitro. A 100 nM concentration of wild-type Mfd (Wt), or Mfd carrying the substitutions indicated, was incubated for 25 min at 37°C with 0.4 nM end-labelled EcoRI–BamHI DNA fragment ± 2 mM ATP or ATPγS, as indicated. The complexes were analysed using a 5% acrylamide gel.
lanes 2 and 4 in Fig. 5A). Initiation complexes migrated as a single band, and were less stable than elongation complexes under our electrophoresis conditions. Elongation complexes migrated faster than the initiation complex, and several minor species were detected in addition to the major band (e.g. lane 4 in Fig. 5A and lane 2 in Fig. 5B). Previous studies using this template indicate that the fainter, faster moving bands correspond to elongation complexes that contain transcripts shorter than 20 nucleotides (22,33).

As described previously (5,6,8), addition of wild-type Mfd to a reaction mixture containing stalled elongation complexes resulted in the complete dissociation of all elongation complexes (Fig. 5A, compare lanes 4 and 5; Fig. 5B, compare lanes 1 and 2). Analysis of samples taken at timed intervals after the addition of Mfd revealed that >90% of the elongation complexes were displaced from the DNA within 5 min (Fig. 5C). In contrast, Mfd RA953 and Mfd QA963 were unable to displace any elongation complexes, even after 20 min incubation (Fig. 5C). However, in reactions containing Mfd RA953 or Mfd QA963, the transcription elongation complex band was ‘supershifted’ (i.e. its mobility was decreased; compare lanes 2, 3 and 6 in Fig. 5B; supershifting by QA963 is also evident in Fig. 5A). We conclude that alanine substitution of R953 or R963 abolishes the ability of Mfd to displace RNAP from DNA, but that the mutant proteins retain the ability to bind to stalled elongation complexes. As both Mfd RA953 and QA963 bound naked DNA non-specifically under the conditions of these assays (note the shifting of the fragment band in Fig. 5A, and see Fig. 4), it is not clear whether the supershifted elongation complexes involved Mfd–RNAP–DNA interactions or solely Mfd–DNA interactions.

Mfd RA929 and Mfd HL948 were able to displace stalled elongation complexes, but did so at a rate that was considerably slower than wild-type Mfd (Fig. 5C). In samples taken before the elongation complex was displaced, Mfd HL948 caused a supershift of the elongation complex band (Fig. 5A, lane 17, and Fig. 5B, lane 5) but no supershifted intermediate was observed in reactions containing Mfd RA929. As these substitutions impaired, rather than abolished,
the ability of Mfd to displace RNAP from DNA, we conclude that Mfd residues R929 and H948 contribute to the displacement process but are not essential for it.

DISCUSSION

We have shown that Mfd increases the efficiency of roadblock repression in vivo in E.coli, as observed previously in B.subtilis (27). Mfd proteins in which the RNAP displacement activity was disabled by deletion or single substitutions were unable to increase the efficiency of roadblock repression, and in some cases could impair the ability of co-expressed wild-type Mfd to do so. The simplest explanation of these data is that in wild-type cells, Mfd displaces a high proportion of transcription complexes that are stalled by protein roadblocks before the repressor protein dissociates from the DNA. In addition to its role in roadblock repression, Mfd is able to revive backtrack elongation complexes (8), and is responsible for RNAP release during transcription termination by the phage λ Nun protein (34). It is increasingly apparent that Mfd plays a more general role in the regulation of transcription elongation than simply mediating transcription-coupled repair.

Mfd couples ATP hydrolysis to movement of DNA–RNAP complexes, and RecG couples ATP hydrolysis to movement of specific DNA structures. Both proteins therefore require a ‘motor’ domain. Sequence alignment of the two proteins revealed that, in addition to conserved superfamly II helicase motifs, they share an area of high sequence identity that includes a helical hairpin motif termed the TRG motif (14). As mutational analysis indicated that residues within this motif were important for DNA translocation by RecG (14), it was likely that the TRG motif would also be important for the function of Mfd. We tested this hypothesis by making single amino acid substitutions at the residues within Mfd that correspond to the key residues in the TRG motif of RecG (Fig. 1). All of these substitutions caused defects in Mfd.

Alanine substitutions of R953 and Q963 abolished the ability of Mfd to displace stalled transcription elongation complexes in vitro, and produced Mfd derivatives that were unable to complement an mfd deletion mutant in vivo. These residues therefore appear to play essential roles in the mechanism by which Mfd dissociates RNAP from DNA. Substitution of R929 or H948 impaired the RNAP displacement activity of Mfd in vitro, but proteins containing these substitutions retained sufficient activity to complement an mfd deletion mutant in vivo. The DNA and ATP binding activities of all four mutants appeared to be intact. The ‘hierarchy’ of effects of TRG substitutions in Mfd mirrored that observed in RecG: substitutions of equivalent residues caused defects of equivalent magnitude in both proteins. In Mfd, alanine substitutions of R953 and Q963 caused greater defects than substitution of R929 in vivo and in vitro. In E.coli RecG, substitutions of R630 (equivalent to Mfd R953) and Q640 (equivalent to Mfd Q963) had a greater effect on the in vivo role of RecG in UV tolerance than substitutions of R609 (equivalent to Mfd R929), and substitutions of R630 caused greater defects in RecG helicase and ATPase activity in vitro than substitutions of R609 (the effects of RecG Q640 substitutions in vitro have not been reported) (14). We conclude that the TRG motif of Mfd is essential for Mfd-mediated displacement of stalled RNAP, and that Mfd couples ATP hydrolysis to RNAP displacement via a mechanism very similar to that used by RecG to drive migration of Holliday junctions. Our results therefore support a model for Mfd action in which dissociation of RNAP is dependent on translocation of double-stranded DNA by Mfd.

The primary sequences of the helicase motifs and TRG motif of Mfd and RecG are highly homologous, and we constructed a model of residues 541–983 of Mfd based on the crystal structure of residues 309–755 of T.maritima RecG (Fig. 6). Within this model, Mfd residues R929 and R953 are closely juxtaposed at the base of a helical hairpin, and Mfd residue Q963 falls in a surface-exposed loop that was not resolved in the RecG-ADP–DNA crystal structure. Mfd H948, which is not conserved in RecG or in Mfd sequences from different species, falls within the helical hairpin bounded by R929 and R953, and is adjacent to D949, a highly conserved residue that is implicated in TRG function in RecG (14). R953 is suitably located to form hydrogen bonds with D889 (a residue at the N-terminal end of a helix that extends into helicase motif VI) and with D949.

Mahdi and co-workers (14) suggested a plausible model for the generation of ATP-driven conformational change in the TRG motif, based on the crystal structure of RecG (12). Despite differences in the size of the TRG helical hairpin, and some differences in the residues involved, this model appears to be equally applicable to Mfd. The model, adapted for Mfd, can be summarised as follows: an arginine residue at the C-terminal end of helicase motif VI interacts with the γ phosphate of ATP. The orientation of the helix containing helicase motif VI changes during the cycle of ATP binding, hydrolysis and release, and consequently so does the pattern of hydrogen bonds made between D889 and the arginines within...
the TRG motif. This causes a change in the conformation of the helical hairpin structure bounded by R929 and R953, which is transmitted to the loop containing Q963. The path of DNA across the translocase region of RecG and Mfd is not well defined, the manner in which this series of conformational changes leads to DNA translocation remains speculative. Mahdi et al. (14) have suggested that the conserved glutamine (Q640 in E. coli RecG; Q963 in E. coli Mfd) might interact directly with DNA, and that the associated surface-exposed loop might drive DNA translocation by acting as a swinging arm. Mfd QA963 retains the ability to bind DNA and it is not currently possible to state whether or not Q963 interacts with DNA during the translocation process. Our structural model of Mfd and the mechanistic model of Mahdi et al. (14) offer explanations for the effects of the other Mfd substitutions tested in this work. The positively charged side chains of R929 and R953 are in close proximity, and the removal of electrostatic repulsion that would result from alanine substitution of either of these residues would probably disrupt the fold of the helical hairpin region as well as removing the hydrogen bonding link to ATP via helicase motif VI. The effect of HL948 may be direct, as it results in the removal of an ionisable side chain from the immediate vicinity of R929 and R953, or indirect, as the side chain is immediately adjacent to D949 which forms a hydrogen bond with R953 in the modelled Mfd structure. The identification of the Mfd TRG motif also explains the properties of the deletion mutant MfdΔtrg, which was isolated in early studies of Mfd and found to be unable to dissociate RNAP (6): the deletion removes the C-terminal helix of the TRG helical hairpin and the loop containing Q963.

Whilst the ATP-driven motors of Mfd and RecG share a common mechanism, they appear to differ in performance. RecG is an efficient, DNA-stimulated ATPase (kcat >100/s) (13), but Mfd is an inefficient ATPase (kcat ~3/min) whose activity is not enhanced by DNA or by a stalled RNAP complex (5). RecG is able to drive the translocation of Holliday junctions in vitro in the absence of other factors (13), but there is as yet no evidence for translocation of naked DNA by Mfd and, unlike some DNA translocating enzymes (10,11), RecG is an efficient, DNA-stimulated ATPase (12). The low kcat of RecG may act as a kinetic brake that ensures Mfd competes in vivo for translocating RNAP and/or translocase activities of Mfd are stimulated by auxiliary factors.

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