Replication fork blockage by transcription factor-DNA complexes in *Escherichia coli*

Bryony T. I. Payne, Ingeborg C. van Knippenberg, Hazel Bell, Sergio R. Filipe\(^1\), David J. Sherratt\(^1\) and Peter McGlynn*\(^*\)

School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK and \(^1\)Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Received August 23, 2006; Accepted September 5, 2006

ABSTRACT

All organisms require mechanisms that resuscitate replication forks when they break down, reflecting the complex intracellular environments within which DNA replication occurs. Here we show that as few as three *lac* repressor-operator complexes block *Escherichia coli* replication forks *in vitro* regardless of the topological state of the DNA. Blockage with tandem repressor-operator complexes was also observed *in vivo*, demonstrating that replisomes have a limited ability to translocate through high affinity protein–DNA complexes. However, cells could tolerate tandem repressor-bound operators within the chromosome that were sufficient to block all forks *in vitro*. This discrepancy between *in vitro* and *in vivo* observations was at least partly explained by the ability of RecA, RecBCD and RecG to abrogate the effects of repressor-operator complexes on cell viability. However, neither RuvABC nor RecF were needed for normal cell growth in the face of such complexes. Holliday junction resolution by RuvABC and facilitated loading of RecA by RecF were not therefore critical for tolerance of protein–DNA blocks. We conclude that there is a trade-off between efficient genome duplication and other aspects of DNA metabolism such as transcriptional control, and that recombination enzymes, either directly or indirectly, provide the means to tolerate such conflicts.

INTRODUCTION

Genome duplication must occur concurrently with many other essential metabolic processes. Chemical damage to the DNA template caused, in part, by the byproducts of normal metabolism is known to block replication (1), but the consequences of duplicating template DNA unavoidably bound by a wide-range of proteins is largely unknown. Both prokaryotic and eukaryotic replication forks can be paused by specialized protein–DNA complexes that have evolved to act as preprogrammed blocks to replication (2,3). However, the majority of proteins associated with chromosomal DNA function in DNA packaging and transcriptional control and their impact on replication fork movement is unclear, although large tandem arrays of transcription factor-operator complexes do provide potent blocks to *Escherichia coli* chromosomal duplication (4).

Regardless of the source of replication blockage, any block must be removed or bypassed for replication to be restarted. Recombination enzymes play pivotal roles in the repair and restart of blocked replication forks both in prokaryotes and eukaryotes, facilitating bypass of the original block and/or the formation of intermediates upon which the replication machinery can be reloaded (5–7). Indeed, the enhanced recombination noted at both preprogrammed replicative blocks (8–10) and at transcription factor–DNA complexes (11,12) point to the potential problems of duplicating protein-bound DNA.

Studies with *E.coli* have suggested that unwinding of the nascent DNA at blocked replication forks to form four-stranded Holliday junctions plays an important role in the repair of damaged forks (13). Such junctions may be targeted by RecBCD helicase/nuclease, allowing degradation of the spooled out DNA end and resetting of the fork for subsequent replisome reloading by PriA (14). However, it could also be cleaved by the Holliday junction-specific helicase/endonuclease RuvABC. This cleavage would result in chromosome breakage, release of a free double-stranded DNA (dsDNA) end and a requirement for RecA-catalysed strand exchange with the sister chromosome to form a D-loop onto which the replication apparatus can be reloaded (15,16). The situations in which these processing events might take place are unclear at present, and evidence has been presented that cleavage of reversed forks by RuvABC may be a pathological event occurring only in the absence of replisomes.

*To whom correspondence should be addressed. Tel: +44 0 1224 555183; Fax: +44 0 1224 555844; Email: p.mcglynn@abdn.ac.uk

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

© 2006 The Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
of RecBCD (7). One problem is that how recombination enzymes act at blocked or otherwise damaged replication forks may depend on the context. Different types of replication block will result in different DNA structures at the block, dictating how the blocked fork will be processed by recombination enzymes, all of which display distinct polarities of action on DNA. Indeed, RecA in conjunction with RecFOR may stabilize replication forks blocked by ultraviolet (UV) light-induced DNA damage to inhibit subsequent processing, rather than to promote processing by catalysing strand exchange (17).

In this study, we have analysed the movement of replication forks through tandem lac repressor-operator complexes and have demonstrated that such high affinity protein–DNA complexes present additive barriers to fork movement in vitro. Lac repressor–operator complexes also inhibited fork movement in vivo. Blockage of fork movement by such barriers lead to a requirement for wild-type recA, recB and recG genes to maintain cell division. However, there was no such requirement for ranABC, recF, rep or uvrD. We conclude that RecA, RecBCD and RecG act to facilitate cell proliferation in the face of protein–DNA complexes that hinder replisomes, but that these tolerance mechanism(s) do not rely on Holliday junction cleavage by RuvABC or the loading of RecA by RecFOR. Moreover, Rep and UvrD, proposed to act as accessory replicative helicases during chromosomal duplication, do not appear to underpin replication through protein–DNA roadblocks.

MATERIALS AND METHODS

DNAs and proteins

DNA PolIII*, β, DnaB, DnaC, DnaG, HU and SSB were purified as described (18,19). LacI was purified as indicated (20). DnaA was purified with an N-terminal hexahistidine tag on a nickel NTA superflow column (Qiagen). DNA gyrase was purified as described (18,19). LacI was purified as indicated (20).

Replication plasmids were based on pBROTB535-I (21). pPM308 was constructed from pBROTB535-I by replacement of terB site 1 with EcoRV and XbaI sites. pPM437, pD506 and pIK02 were constructed by ligation of a Klenow-treated Xhol–Sall fragment from a derivative of pFX92 carrying 3, 6 and 22 tandem lacO repeats, respectively (22) into the EcoRV site of pPM308. pPM374, pPM378, pPM393 and pPM397 were constructed by cloning an apramycin resistance cassette (23) linked to 0, 3, 6 or 22 tandem lacO sites between the EcoRI and XbaI sites of pPM308. pPM306 was constructed by amplification of the lacI gene from E. coli MG1655 and cloning into pBAD/HisB (Invitrogen) as an Ncol–HindIII fragment.

In vitro replication assays

Standard oriC DNA replication reactions (15 µl) using DNA gyrase were performed as described (18) except incubation was at 37°C for 10 min. Electrophoretic analysis of replication products under denaturing conditions was performed in 0.7% alkaline agarose gels as described (18) using a 5'-labelled λ HindIII digest as a size marker. Fork blockage was quantified in standard replication assays by measuring the amount of replication product above the blocked 4 kb leading strand using reactions lacking LacI as the baseline.

Determination of replication fork movement along template DNA relaxed by cleavage with a restriction enzyme was based on a previous pulse-chase approach (24). All reaction components except the restriction enzyme and [α-32P]dCTP (LacI at 100 nM tetramers final concentration as indicated, the standard concentrations of DnaB, DnaC, SSB, β, PolIII*, HU and DnAG plus DnaA at 300 nM) were assembled on ice in reaction volumes of 14 µl and incubated at 37°C for 3 min. A total of 1 µl of a mixture of Smal (64 U/µl, Promega) and [α-32P]dCTP (0.3 pmol; 200 kBq/pmol) was then added to each reaction and incubation continued for 2 min at 37°C before reactions were stopped by addition of 1 µl of 0.5 M EDTA. This concentration of Smal was sufficient to cleave all the template plasmid within 20 s. Reactions were then analysed under denaturing conditions as described above. The degree of fork blockage using this approach was estimated by comparing the 2 kb leading strand peak heights and setting the blockage as 1 for 22 lacO sites—blockage was taken to be complete at lacO22 based on the absence of leading strand product above 4 kb in the presence of LacI (Figure 3B, lane 9).

Strain construction

To integrate lac operators into argEC, the same 22 tandem lac operators used to construct oriC replication plasmids described above and a fragment containing 34 tandem lac operators were each cloned downstream of an apramycin resistance cassette (23) and then subcloned between the HindIII and EcoRV sites of a plasmid carrying argE and the S' end of argC (25). The apramycin resistance gene was also cloned in the same manner but with no associated lacO sites. The apramycin cassette ± lacO together with flanking argEC sequences was then amplified and integrated into DY330 as described (26). These loci were then moved by P1 vir transduction from DY330 into PM203 (MG1655 Δara714). The resultant strains were BP38 (lacO0), PM222 (lacO22) and BP41 (lacO34) (see Table 1). Successful integration was confirmed by arginine auxotrophy, Southern blotting, diagnostic PCR and DNA sequencing of PCR products. Derivatives of PM222 and BP41 with mutations in recombination and replication genes were made by P1 vir transduction (see Table 1).

Growth experiments

Strains were transformed with pPM306 and selected on LB ampicillin plates overnight at 37°C. Single colonies were grown in 10 ml Luria–Bertani (LB) ampicillin in 50 ml conical flasks at 37°C with shaking to mid log phase and then inoculated into fresh 10 ml LB ampicillin, together with glucose, arabinose and isopropyl-β-d-thiogalactopyranoside (IPTG) as indicated, to an A600 of 0.01. Growth was continued at 37°C and samples removed at 0, 1, 2, 3 and 4 h after subculturing and viable cell counts performed on LB ampicillin IPTG plates at 37°C overnight. Concentrations of ampicillin, apramycin, glucose, arabinose and IPTG were 100 µg/ml, 50 µg/ml, 0.1%, 0.02% and 1 mM, respectively. Comparison of the number of cell divisions was performed using the increase in the number of viable cells between 0 and 4 h in
the above growth experiments. Growth experiments were performed between two and eight times for each strain.

**Electrophoretic analysis of in vivo replication intermediates**

Strains were grown as described above for the growth experiments except that the culture volumes were increased to 40 ml in 250 ml conical flasks. Upon addition of arabinose plus or minus IPTG, growth was continued at 37°C with shaking until the A560 reached 0.4 (~3 h after subculturing). Genomic DNA plugs were made and processed as described (25) except that digestion was with PvuII. Electrophoresis was performed through 0.8% agarose gels in 1x TBE for 6 h at 3.5 V/cm, the gels were Southern blotted and then probed using the complete apramycin resistance gene labelled by random priming with [³²P]dCTP. Gene ruler 1 kb DNA ladder (Fermentas) was used as a marker.

**RESULTS**

**Movement of replisomes in vitro is inhibited by LacI–lacO complexes**

To assess the ability of the replication apparatus of *E. coli* to duplicate protein-bound DNA, the impact of lac repressor-operator complexes on replisome movement was analysed *in vitro*. DnaA-directed bidirectional replication of oriC-containing plasmids resulted in generation of ~5 kb lagging strands plus ~3 kb leading strands formed by convergence of the two forks in a region opposite oriC (Figure 1A, lane 1) (18). The sizes of leading and lagging strands generated on a plasmid lacking lacO sequences was not altered upon addition of lac repressor (LacI) (Figure 1A, lanes 1–4). Non-sequence-specific binding of LacI was not therefore a major impediment to replisome movement. However, addition of LacI to plasmid encoding 22 lacO sites located 2 and 4 kb from oriC lead to accumulation of leading strands of 2 and 4 kb in size (Figure 1A, lanes 5–8). Addition of the inducer IPTG to reactions with LacI on template DNA containing lacO₂₂ precluded appearance of 2 and 4 kb leading strand products (Figure 1B). These data demonstrate that LacI–lacO complexes inhibit replisome movement, allowing convergence of the two forks at lacO₂₂.

To determine the efficacy of replisome blockage by LacI–lacO complexes a series of plasmids were constructed bearing 0, 3, 6 and 22 lacO sites 2 and 4 kb away from oriC. Replication of each template in the presence of LacI resulted in accumulation of the 2 and 4 kb leading strand products (Figure 2A). At 100 nM LacI tetramers, 24, 43 and 90% of replisomes were blocked at 3, 6 and 22 lacO sites, respectively (Figure 2B). The lacO₄ and lacO₈ data suggest that the effects of LacI–lacO complexes on replisome movement are additive with one lacO site blocking ~5–10% of forks when bound by LacI.

**Inhibition of replication by repressor–operator complexes occurs on relaxed as well as supercoiled template**

The above data suggest that the energy input required to disrupt lac repressor–operator interactions presents a barrier to replication. However, the binding of repressor to tandem operators may have induced topological alterations within the plasmid template leading to replication fork stalling,
possibly by inhibiting access of DNA gyrase to the template DNA ahead of the replisome. To determine whether altered topology was the primary determinant in replication blockage, the effect of lac repressor–operator complexes on fork movement along a topologically unconstrained DNA template was analysed.

Omission of a topoisomerase from replication reactions still allows initiation of replication at oriC in negatively supercoiled plasmid template (24). However, only one of the two forks initiates DNA synthesis and the fork that does initiate stalls after about 1 kb of DNA synthesis due to accumulation of positive torsional strain (27). This inhibition of fork movement can be relieved by addition of a restriction enzyme (SmaI) that cleaves near oriC thus relieving any topological constraint by linearization of the template (24) [Figure 3A (i–iii)]. Inclusion of radiolabelled dCTP at the time of SmaI addition allows fork progression to be monitored.

To determine whether replication forks were blocked by repressor–operator complexes on linear template DNA, plasmids containing 0, 3, 6 or 22 lacO sites were employed. Forks moving rightwards [Figure 3A (iv and v)] would generate 4 and 6 kb leading strands upon addition of SmaI, depending on whether blockage occurred at lacO. Similarly, forks moving leftward would generate 2 or 6 kb leading strands [Figure 3A (vi and vii)].

2 and 4 kb leading strands were formed in the presence of LacI on template with 3, 6 or 22 lacO sites but were absent

Figure 1. lac repressor–operator complexes block replisome movement in vitro. (A) Replication of plasmid templates with no lac operator sequences (pPM308, lanes 1–4) and with 22 tandem lacO sequences (pIK02, lanes 5–8) in the presence of 0, 5, 25 and 100 nM LacI tetramers. Products of replication were analysed on a denaturing agarose gel, with DNA size markers shown in kb. (B) Production of distinct 2 and 4 kb leading strand products on template pIK02 (lacO22) bound by LacI is abrogated by addition of IPTG. LacI (100 nM tetramers) and IPTG (1 mM) were present as indicated.

Figure 2. Additive blockage of replisome movement by repressor–operator complexes. (A) Replication of plasmid templates bearing 0, 3, 6 and 22 lacO sites (pPM374, 378, 393 and 379) in the presence of 0, 5, 25 and 100 nM LacI tetramers was monitored by denaturing agarose gel electrophoresis. DNA size markers are shown in kb. (B) Replication of pPM templates bearing 0, 3, 6 and 22 lacO sites (pPM308, lanes 1–4) and with 22 tandem lacO sequences (pIK02, lanes 5–8) in the presence of 0, 5, 25 and 100 nM LacI tetramers. Products of replication were analysed on a denaturing agarose gel, with DNA size markers shown in kb. (A) Replication of plasmid templates with no lac operator sequences (pPM308, lanes 1–4) and with 22 tandem lacO sequences (pIK02, lanes 5–8) in the presence of 0, 5, 25 and 100 nM LacI tetramers. Products of replication were analysed on a denaturing agarose gel, with DNA size markers shown in kb. (B) Production of distinct 2 and 4 kb leading strand products on template pIK02 (lacO22) bound by LacI is abrogated by addition of IPTG. LacI (100 nM tetramers) and IPTG (1 mM) were present as indicated.

Figure 3. Replication blockage occurs on linearized as well as supercoiled template DNA. (A) In the absence of a topoisomerase, replication can initiate at oriC but is inhibited after about 1 kb of synthesis (i). Addition of SmaI results in cleavage of the DNA near to oriC (ii and iii) and allows one of the two forks to progress around the now-linearized template. Unimpeded replication would generate leading strands of 6 kb for both forks (v and vi) whereas 4 and 2 kb leading strands would result from blockage of the forks at lacO (iv and vii). (B) Replication of pPM templates bearing 0, 3, 6 and 22 lacO sites (pPM308, lanes 1–4) and with 22 tandem lacO sequences (pIK02, lanes 5–8) in the absence of a topoisomerase but with LacI and SmaI as indicated, monitored by denaturing agarose gel electrophoresis. DNA size markers are shown in kb. The position of early replication intermediate (ERI) (37), which accumulated in the absence of SmaI, is shown using pPM308 as template (lane 1).
when LacI was absent, or when there were no lacO sites within the template (Figure 3B). Thus replication fork movement was inhibited by repressor–operator complexes on linearized template DNA. The reduction (lacO22) or absence (lacO34) of 6 kb leading strand products supported this conclusion (Figure 3B, lanes 7 and 9). The degree of blockage was ~30 and 70% for 3 and 6 lacO sites, respectively, similar to the inhibition of replication estimated on covalently closed supercoiled template (Figure 2). We conclude that blockage of replication forks by repressor–operator complexes occurs to a similar extent regardless of the topological state of the DNA template.

**LacI–lacO complexes present barriers to DNA replication in vivo**

Two hundred and forty tandem lac operators bound by repressor on the E.coli chromosome provide a complete block to replication and cell proliferation in vivo (22). The *in vitro* data in Figures 1–3 suggest that far fewer repressor–operator complexes are needed for effective replication blockage. We therefore analyzed the ability of replisomes to traverse 22 and 34 lacO sites integrated within the chromosome of E.coli (Figure 4A). To ensure that the lacO sites were saturated with repressor a plasmid (pPM306) encoding lacI under the control of an arabinose-inducible promoter was introduced into the strains. Upon addition of arabinose to the growth medium this plasmid generated an increase in levels of repressor from approximately 300 tetramers per cell to 10–20000 tetramers per cell, with significant overexpression beginning 1 h after addition of arabinose (data not shown).

In the absence of lacO sites, no slowly migrating DNA species indicative of replication intermediates could be detected as judged by Southern blot analysis of chromosomal DNA digests (Figure 4B, lanes 1 and 2). Replication intermediates did accumulate at 22 and 34 lacO sites, with more intermediates accumulating at lacO34 as compared with lacO22 (Figure 4B, compare lanes 3 and 5). These slowly migrating species were absent when cells were grown in medium supplemented with IPTG (Figure 4B, lanes 4 and 6). The requirement for lacO sites to observe these slowly migrating species, and the effect of IPTG, suggest that these DNA species were replication intermediates caused by blockage of replisomes by repressor–operator complexes. Similar slowly migrating DNA species have been observed upon blockage of replication by Tus–ter complexes (28,29). However, growth of lacO22 and lacO34 strains did not display any major defect in the presence of elevated lac repressor expression (Figure 4C) although there was a small decrease in growth of the lacO34 strain in arabinose as compared with glucose (Figures 4C, 6A and B).

Given the blockage of DNA replication by tandem lac repressor–operator complexes observed in vitro (Figures 1 and 2) it was surprising there was little effect on growth upon induction of lac repressor expression. A trivial explanation for this discrepancy was that neither the lacO22 nor the lacO34 loci were saturated with repressor, even at the high concentrations of repressor achieved upon induction of overexpression. Levels of replication intermediates detectable by gel electrophoresis at the lacO22 and lacO34 loci were compared therefore in the presence of native and overexpressed levels of repressor. Replication intermediates detected at lacO34 increased from 1.1% (±0) as a proportion of the total DNA signal with wild-type levels of repressor to 5.5% (±1.5) upon induction of repressor overexpression. This increase demonstrates that tandem lacO sites were accessible to enhanced levels of repressor. In contrast, the levels of replication intermediates detected at lacO22 were similar in the presence of wild-type or overexpressed levels of repressor (0.7% [±0.5] and 1.2% [±0.6], respectively). The lack of enhanced blockage at the lacO22 locus, in spite of the accessibility of tandem lacO sites to enhanced levels of repressor, indicates that lacO22 was saturated at wild-type repressor levels. Concerning lacO34, these data cannot unambiguously establish whether the locus was saturated with repressor but, given the 30- to 60-fold enhancement of repressor concentration obtained upon induction of overexpression, it is likely that lacO34 was also saturated in the presence of elevated levels of repressor.

Although the above electrophoretic analysis of replication intermediates did indicate saturation of lacO22 with repressor, this analysis also suggested that replication fork blockage in vivo (~1% for lacO22) was much less efficient than that seen at lacO22 in vitro (100%). However, such direct comparisons are inappropriate since the ratios of replication intermediates to linear DNA fragments as observed by 1D and 2D gel techniques may not correlate directly with the actual levels of replication fork blockage—nicking or branch migration of DNA replication forks occurring in vivo or elsewhere in the genome. The data suggest that in vivo replication fork blockage can be estimated by the extent of blockage observed in vitro and by Southern blot analysis of chromosomal DNA digests.

Figure 4. lac repressor–operator complexes present blocks to DNA replication in vivo. (A) Insertion of tandem lac operators within the chromosomal *arg* locus, linked to an apramycin resistance gene. The direction of replication fork movement is indicated with a dashed arrow. (B) Southern blot of a 1D agarose gel of *Pvu*II digests from strains bearing 0 (BP38), 22 (PM222) and 34 (BP41) lacO repeats together with pPM306, a plasmid bearing an arabinose-inducible lacI gene, both with and without 1 mM IPTG. DNA size markers are shown in kb. The entire apramycin resistance gene was used to probe the blot. The positions of replication intermediates are marked with arrows. (C) Growth curves of strains BP38 (i), PM222 (ii) and BP41 (iii) containing pPM306. Open circles, medium supplemented with glucose (lacI expression repressed); filled circles, medium supplemented with arabinose (lacI expression induced). c.f.u.: colony forming units.
replication intermediates to form linear DNA structures during their isolation likely result in underestimates of fork blockage (4).

Enzymes of recombination facilitate tolerance of repressor–operator complexes in vivo

If the lack of any major growth defects in cells harbouring 22 and 34 lacO sites bound by repressor could not be explained by sub-saturating levels of repressor, what other factors could explain this discrepancy? Fork movement through protein–DNA complexes may be facilitated by accessory factors, such as Rep helicase in vivo (30), factors which were absent from the in vitro replication reactions. Alternatively, direct or indirect processing of blocked replication forks, possibly by recombination enzymes (5–7), may have ameliorated the effects of protein–DNA blocks to replication in vivo. Therefore we examined the genetic requirements for growth of strains bearing either 22 or 34 lacO sites by analysing growth in the absence or presence of elevated levels of lac repressor.

Deletion of rep had no major effect on growth with either 22 or 34 lacO sites (Figures 5 and 6). Rep did not therefore have a critical role in promotion of fork movement through these repressor–operator complexes. We also tested whether UvrD, implicated in a fork clearing role (31), aided cell growth in the face of repressor–operator complexes. However, a uvrD mutation had no effect on the ability of cells to grow in the face of lacO mutation (Figure 6A and B).

Analysis of recA, recB, recF, recG and ruvABC recombination mutations also did not reveal any significant impact on growth in the lacO background (Figure 5). Recombination enzymes appeared therefore not to be required for tolerance of this repressor–operator block. However, deletion of recA, recB or recG had major impacts on growth in the lacO background upon repressor overexpression (Figure 6A and B). These defects in cell growth became apparent between 1 and 2 h after addition of arabinose (Figure 6A) and correlated with the rise of lac repressor levels as detected by western blotting (data not shown). These effects were abolished if IPTG was present in the growth medium in addition to arabinose (Figure 6A) and correlated with the rise of lac repressor levels as detected by western blotting (data not shown). The data presented here suggest that collisions between the replisome and protein–DNA complexes have evolved to terminate replication lead to enhanced genome instability near the site of termination (10,29,32). The consequences of unavoidable encounters between the replisome and other protein–DNA complexes remain obscure. The data presented here suggest that collisions between the DNA replication apparatus and transcription factors may present barriers to replisome movement. The inhibition of replication observed in vitro occurred on both supercoiled (Figures 1 and 2) and linearized (Figure 3) template DNA indicating that blockage of forks was a direct consequence of collision between the replication apparatus and the repressor–operator complexes. Therefore displacement of transcription factor–DNA complexes by the replisome appears to present an energetic barrier to fork movement. The use of multiple repressor–operator complexes to hinder replication in vitro (Figure 1A) is clearly a situation never encountered in vivo. However, the additive nature of the blockage shown by 3 and 6 lacO sites bound by repressor (Figure 2) suggest that even single repressor–operator...

Figure 5. Recombination enzymes are not required in vivo for tolerance of 22 lacO sites bound by repressor. (A) Growth of strains harbouring 22 lacO sites and the indicated mutations, plus the lacI plasmid pPM306. Open circles: cells grown in glucose (no lac repressor overexpression); filled circles: cells grown in arabinose (elevated lac repressor). lacO22 strains were PM222 (otherwise wild-type), BP13 (recA), BP22 (recB), BP60 (recF), BP33 (recG), BP19 (ruvABC), BP16 (rep). Individual growth curves are shown but each curve was performed between two and eight times with all curves yielding similar results. C.f.u.: colony forming units. (B) Mean number of cell divisions between 0 and 4 h of growth for strains bearing 22 lacO sites, determined from growth curves as represented in (A). Open bars: cells grown in glucose; filled bars: cells grown in arabinose. Strains are as described in (A).

DISCUSSION

Collisions between the replisome and protein–DNA complexes that have evolved to terminate replication lead to enhanced genome instability near the site of termination (10,29,32). The consequences of unavoidable encounters between the replisome and other protein–DNA complexes remain obscure. The data presented here suggest that collisions between the DNA replication apparatus and transcription factors may present barriers to replisome movement. The inhibition of replication observed in vitro occurred on both supercoiled (Figures 1 and 2) and linearized (Figure 3) template DNA indicating that blockage of forks was a direct consequence of collision between the replication apparatus and the repressor–operator complexes. Therefore displacement of transcription factor–DNA complexes by the replisome appears to present an energetic barrier to fork movement. The use of multiple repressor–operator complexes to hinder replication in vitro (Figure 1A) is clearly a situation never encountered in vivo. However, the additive nature of the blockage shown by 3 and 6 lacO sites bound by repressor (Figure 2) suggest that even single repressor–operator...
complexes will pose barriers to fork movement. Indeed, although we have failed to detect reproducible replication blockage with a single repressor–operator complex in vitro (data not shown), one lac repressor–operator complex does act as a recombination hotspot in vivo (11), a signature of replication fork problems.

Cells were capable of tolerating tandem protein–DNA complexes that were sufficient to block all replication in vitro (Figure 4), demonstrating that duplication of protein-bound DNA is enhanced in vivo, either directly or indirectly. Tolerance of 22 lacO sites within the chromosome did not depend on any of seven recombination enzyme genes tested (Figure 5A). Other mechanisms, such as enhancement of replicative helicase function by chromosomal topology (28), might therefore underpin this tolerance. However, mutations in recA, recB and recG did have major impacts on viable cell growth in the presence of 34 lacO sites (Figure 6A and B). Thus the strand exchange protein RecA, the helicase/exonuclease RecBCD and the branch migration helicase RecG all function to facilitate replication through these protein–DNA complexes, either directly or indirectly. In contrast, Rep helicase, previously implicated in removal of proteins ahead of replication forks (30,33), was not essential for this tolerance (Figure 6). The possible fork clearing role ascribed to UvrD (31) was also not required for tolerance of this block (Figure 6).

These data show that recombination enzymes somehow promote fork movement along protein-bound template, correlating with the enhanced recombination noted at a lac repressor–operator complex in vivo (11). However, given that any homologous recombination reaction between sister chromosomes will occur upstream of the protein–DNA array, it is difficult to envisage how such reactions would promote bypass of the block. Moreover, whilst recombination generates DNA structures onto which PriA can reload the replication machinery (34), any reassembled replications would still have to confront the protein–DNA barrier that caused the original fork to stall. However, given the stochastic nature of replication blockage seen in vivo (Figure 2) reinitiation of replication by recombination might provide another chance for the fork to translocate through the protein–DNA array. Such a mechanism would promote replication through barriers other than those presented by direct chemical damage to the template DNA. It would also explain how replication forks can eventually move through multiple Tus–ter complexes, given enough time (4).

If recombination via RecA-catalysed strand exchange is involved in tolerance of repressor–operator arrays then Holliday junctions would be expected to be formed. However, RuvABC was not critical for normal growth in the face of the lacO34 protein–DNA array (Figure 6) indicating that similar stabilization is unlikely to occur at forks blocked by protein–DNA barriers. Such a mechanism would promote replication through barriers other than those presented by direct chemical damage to the template DNA. It would also explain how replication forks can eventually move through multiple Tus–ter complexes, given enough time (4).

One proposed mechanism of replication fork processing that invokes RecA catalysis without a need to resolve Holliday junctions posits that RecA facilitates stabilization of reversed replication forks formed as a result of blockage by UV-induced DNA damage, thus protecting the forks from nucleolytic attack (17). However, this stabilization also requires RecF (35). The lack of any effect of a recF mutation on cell viability in the face of repressor–operator arrays (Figure 6) indicates that similar stabilization is unlikely to occur at forks blocked by protein–DNA complexes. Such differences likely reflect the diversity of fork repair mechanisms needed to face the different sources of replicative blocks that challenge all cells.

Replication forks blocked at 240 tet operators bound by tet repressor have no requirement for RecA or RecB to restart replication upon induced dissociation of the repressor–operator complexes (4). This lack of requirement appears to conflict with the need for both RecA and RecB to allow cell proliferation in the face of lacO34 bound by lac repressor (Figure 6). However, recombination may not be needed for resumption of replication once a block has been removed by
addition of inducer. Stable association of the replisome with the template DNA at the site of blockage (4) would obviate the need for processing by recombination enzymes. In contrast, continued proliferation in the face of lacO33–repressor complexes may require active mechanisms to facilitate replication through these complexes. What these mechanisms are, and how they rely on RecA, RecBCD and RecG, remain to be elucidated. However, our data demonstrate that transcription factor–operator complexes can act to halt DNA replication, and that recombination enzymes are needed subsequently to endure such blocks. Indeed, occasional blockage of replisomes at transcription factor–DNA complexes in vivo may contribute to the decreased viability observed in recombination mutants (16,36). Our findings also imply that such collisions may be a factor in the coevolution of replication with other DNA metabolic processes.

ACKNOWLEDGEMENTS

The authors would like to thank Ken Marians, Bob Lloyd and Divijendra Sirigiri for plasmids, strains and proteins, Tom Meddows for advice on agarose gels and Tony Maxwell for DNA gyrase. This work was supported by the MRC and the Lister Institute of Preventive Medicine (P.M.) and by the Wellcome Trust (D.J.S.). Funding to pay the Open Access publication charges for this article was provided by the MRC.

Conflict of interest statement. None declared.

REFERENCES

1. Lindahl,T. (1996) The Croonian Lecture, 1996: endogenous damage to DNA. Philos. Trans. R. Soc. Lond. B. Biol. Sci., 351, 1529–1538.
2. Rothstein,R., Michel,B. and Ganagoloff,S. (2000) Replication fork pausing and recombination or ‘gimmick a break’. Genes Dev., 14, 1–10.
3. Mulcair,M.D., Schaeffer,P.M., Oakley,A.J., Cross,H.F., Neylon,C., Hill,T.M. and Dixon,N.E. (2006) A molecular mousetrap determines polarity of termination of DNA replication in E. coli. Cell, 125, 1309–1319.
4. Possoz,C., Filipe,S.R., Grainge,I. and Sherratt,D.J. (2006) Tracking of controlled Escherichia coli replication fork stalling and restart at repressor-bound DNA in vivo. EMBO J., 25, 2596–2604.
5. Cox,M.M., Goodman,M.F., Kreuzer,K.N., Sherratt,D.J., Sandler,S.J. and Marians,K.J. (2000) The importance of repairing stalled replication forks. Nature, 404, 37–41.
6. McGlynn,P. and Lloyd,R.G. (2002) Recombination repair and restart of damaged replication forks. Nature Rev. Mol. Cell. Biol., 3, 859–870.
7. Michel,B., Grompone,G., Flores,M.J. and Bidnenko,V. (2004) Multiple pathways process stalled replication forks. Proc. Natl Acad. Sci. USA, 101, 12783–12788.
8. Kobayashi,T., Heck,D.J., Nomura,M. and Horiuichi,T. (1998) Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. Genes Dev., 12, 3821–3830.
9. Bierne,H., Ehrlich,S.D. and Michel,B. (1991) The replication termination signal terB of the Escherichia coli chromosome is a deletion hot spot. EMBO J., 10, 2699–2705.
10. Lambert,S., Watson,A., Sheedy,D.M., Martin,B. and Carr,A.M. (2005) Gross chromosomal rearrangements and elevated recombination at an inducible site-specific replication fork barrier. Cell, 121, 689–702.
11. Vilette,D., Urest,M., Ehrlich,S.D. and Michel,B. (1992) DNA transcription and repressor binding affect deletion formation in Escherichia coli plasmids. EMBO J., 11, 3629–3634.
12. Fan,Q., Xu,F. and Petes,T.D. (1995) Meiosis-specific double-strand DNA breaks at the HIS4 recombination hot spot in the yeast Saccharomyces cerevisiae: control in cis and trans. Mol. Cell. Biol., 15, 1679–1688.
13. Seigneur,M., Bidnenko,V., Ehrlich,S.D. and Michel,B. (1998) RuvAB acts at arrested replication forks. Cell, 95, 419–430.
14. Flores,M.J., Ehrlich,S.D. and Michel,B. (2002) Primosome assembly requirement for replication restart in the Escherichia coli holdG10 replication mutant. Mol. Microbiol., 44, 783–792.
15. McGlynn,P. and Lloyd,R.G. (2000) Modulation of DNA polymerase by (ppGpp) reveals a RecG-dependent mechanism for replication fork progression. Cell, 101, 35–45.
16. Gregg,A.V., McGlynn,P., Jakatari,R.P. and Lloyd,R.G. (2002) Direct rescue of stalled DNA replication forks via the combined action of PriA and RecG helicase activities. Mol. Cell, 9, 241–251.
17. Courcelle,J., Donaldson,J.R., Chow,K.H. and Courcelle,C.T. (2003) DNA damage-induced replication fork regression and processing in Escherichia coli. Science, 299, 1064–1067.
18. Hiasa,H. and Marians,K.J. (1994) Primase couples leading- and lagging-strand DNA synthesis from oriC. J. Biol. Chem., 269, 6058–6063.
19. Marians,K.J. (1995) X174-type primosomal proteins: purification and assay. Meth. Enzymol., 262, 507–521.
20. Rosenberg,J.M., Khallai,O.B., Kopka,M.L., Dickerson,R.E. and Riggs,A.D. (1977) Lac repressor purification without inactivation of DNA binding activity. Nucleic Acids Res., 4, 567–572.
21. Hiasa,H. and Marians,K.J. (1994) Tus prevents overreplication of oriC plasmid DNA. J. Biol. Chem., 269, 26959–26968.
22. Lau,F., Filipe,S.R., Soballe,B., Okstad,O.A., Bar,F.X. and Sherratt,D.J. (2003) Spatial and temporal organization of replicating Escherichia coli chromosomes. Mol. Microbiol., 49, 731–743.
23. Wilkinson,C.J., Hughes-Thomas,Z.A., Martin,C., Bohm,L., Mironenko,T., Deacon,M., Wheatcroft,M., Wirtz,G., Staunton,J. and Leadlay,P.F. (2002) Increasing the efficiency of heterologous promoters in actinomycetes. J. Mol. Microbiol. Biotechnol., 4, 417–426.
24. Marians,K.J., Hiasa,H., Kim,D.R. and McHenry,C.S. (1998) Role of the core DNA polymerase III subunits at the replication fork. α is the only subunit required for processive replication. J. Biol. Chem., 273, 2452–2457.
25. Meddows,T.R., Savory,A.P. and Lloyd,R.G. (2004) RecG helicase promotes DNA double-strand break repair. Mol. Microbiol., 52, 119–132.
26. Yu,D., Ellis,H.M., Lee,E.C., Jenkins,N.A., Copeland,N.G. and Court,D.L. (2000) An efficient recombination system for chromosome engineering in Escherichia coli. Proc. Natl Acad. Sci. USA, 97, 5978–5983.
27. Smelkovka,N. and Marians,K.J. (2001) Timely release of both replication forks from oriC requires modulation of origin topology. J. Biol. Chem., 276, 39186–39191.
28. Valjavec-Gratian,M., Henderson,T.A. and Hill,T.M. (2005) Tus-mediated arrest of DNA replication in Escherichia coli is modulated by DNA supercoiling. Mol. Microbiol., 58, 758–773.
29. Bidnenko,V., Ehrlich,S.D. and Michel,B. (2002) Replication fork collapse at replication terminator sequences. EMBO J., 21, 3898–3907.
30. Lane,H.E. and Denhardt,D.T. (1975) The rep mutation IV. Slower movement of replication forks in Escherichia coli rep strains. J. Mol. Biol., 97, 99–112.
31. Flores,M.J., Sanchez,N. and Michel,B. (2005) A fork-clearing role for UvrD. Mol. Microbiol., 57, 1664–1675.
32. Horiiuchi,T., Fujimura,Y., Nishihami,H., Kobayashi,T. and Hidaka,M. (1994) The DNA replication fork blocked at the Ter site may be an entrance for the RecBCD enzyme into duplex DNA. J. Bacteriol., 176, 4656–4663.
33. Yancey-Wrona,J.E. and Matson,S.W. (1992) Bound Lac repressor protein differentially inhibits the unwinding reactions catalyzed by DNA helicases. Nucleic Acids Res., 20, 6713–6721.
34. Xu,L. and Marians,K.J. (2003) PriA mediates DNA replication pathway choice at recombination intermediates. Mol. Cell, 11, 817–826.
35. Courcelle,J., Carswell-Crompton,C. and Hanawalt,P.C. (1997) recF and recR are required for the resumption of replication at DNA replication forks in Escherichia coli. Proc. Natl Acad. Sci. USA, 94, 3714–3719.
36. Capaldo,F.N., Ramsey,G. and Barbour,S.D. (1974) Analysis of the lac repressor. J. Mol. Biol., 81, 242–249.
37. Hiasa,H. and Marians,K.J. (1994) Topoisomerase IV can support oriC DNA replication in vitro. J. Biol. Chem., 269, 16371–16375.
38. Bachmann, B.J. (1996) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In Neidhardt, F.C., Curtiss, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. and Umbarger, H.E. (eds), *Escherichia coli and Salmonella cellular and molecular biology, 2nd edn*. ASM Press, Washington, DC, pp. 2460-2488.

39. Guzman, L.M., Belin, D., Carson, M.J. and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P<sub>BAD</sub> promoter. *J. Bacteriol.*, 177, 4121-4130.

40. Moolenaar, G.F., Moorman, C. and Goosen, N. (2000) Role of the *Escherichia coli* nucleotide excision repair proteins in DNA replication. *J. Bacteriol.*, 182, 5706-5714.