5194–5202 *Nucleic Acids Research, 2006, Vol. 34, No. 18* *Published online 25 September 2006*
doi:10.1093/nar/gkl682

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

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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

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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 *ruvABC, 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 supplied by A. Maxwell (John Innes Centre, Norwich, UK).

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 XhoI–SalI 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 pPM379 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 NcoI–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-^{32}P]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 SmaI (64 U/μl, Promega) and [α-^{32}P]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 SmaI 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 $lacO_{22}$ 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 5′ 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 Δ*ara714*). The resultant strains were BP38 ($lacO_0$), PM222 ($lacO_{22}$) and BP41 ($lacO_{34}$) (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 A_{650} 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

Table 1. Strains used in this study

Strain	Relevant genotype	Source or derivation
MG1655	Wild-type	(38)
LMG194	KS272 Δ*ara714 leu*::Tn*10*	(39)
BP01	MG1655 Δ*ara714 leu*::Tn*10*	P1 (LMG194) × MG1655 Tc^r ara^- leu^-
PM203	MG1655 Δ*ara714*	P1 (MG1655) × BP01 Tc^s ara^- leu^+
DY330	W3110 Δ*lacU169 gal490* λ*cI857* Δ*(cro-bio)*	(26)
BP38	MG1655 Δ*ara714 argEC::[apra^r]*	This study
PM222	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$]*	This study
BP13	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$] recA269::*Tn*10*	P1 (N4279) × PM222 to Tc^r
BP22	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$] recB268::*Tn*10*	P1 (N4278) × PM222 to Tc^r
BP60	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$] recF143 tna::*Tn*10*	P1 (N5540) × PM222 to Tc^r
BP33	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$] ΔrecG263::kan*	P1 (N4256) × PM222 to Kan^r
BP19	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$] ruvABC::cat*	P1 (N4583) × PM222 to Cm^r
BP16	MG1655 Δ*ara714 argEC::[apra^r lacO$_{22}$] rep::cat*	P1 (N4982) × PM222 to Cm^r
BP41	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$]*	This study
BP43	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$] recA269::*Tn*10*	P1 (N4279) × BP41 to Tc^r
BP45	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$] recB268::*Tn*10*	P1 (N4278) × BP41 to Tc^r
BP44	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$] recF143 tna::*Tn*10*	P1 (N5540) × BP41 to Tc^r
BP54	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$] ΔrecG263::kan*	P1 (N4256) × BP41 to Kan^r
BP52	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$] ruvABC::cat*	P1 (N4583) × BP41 to Cm^r
BP47	MG1655 Δ*ara714 argEC::[apra^r lacO$_{34}$] rep::cat*	P1 (N4982) × BP41 to Cm^r
BP55	MG1655 Δ*ara714 argEC::[aprar lacO34] ΔuvrD::Tc*	P1 (CS5431) × BP41 to Tc^r
N4279	MG1655 *recA269::*Tn*10*	(25)
N4278	MG1655 *recB268::*Tn*10*	(25)
N5540	MG1655 *recF143 tna::*Tn*10*	R. G. Lloyd
N4256	MG1655 Δ*recG263::kan*	(15)
N4583	MG1655 *ruvABC::cat*	R. G. Lloyd
N4982	MG1655 *rep::cat*	R. G. Lloyd
CS5431	KMBL1001 Δ*uvrD::Tc*	(40)

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 A_{650} 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 1× 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 [α-^{32}P]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 ≈0.5 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 $lacO_{22}$ 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 $lacO_{22}$.

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 strands expected if forks stopped at the LacI–*lacO* complexes (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 $lacO_3$ and $lacO_6$ 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

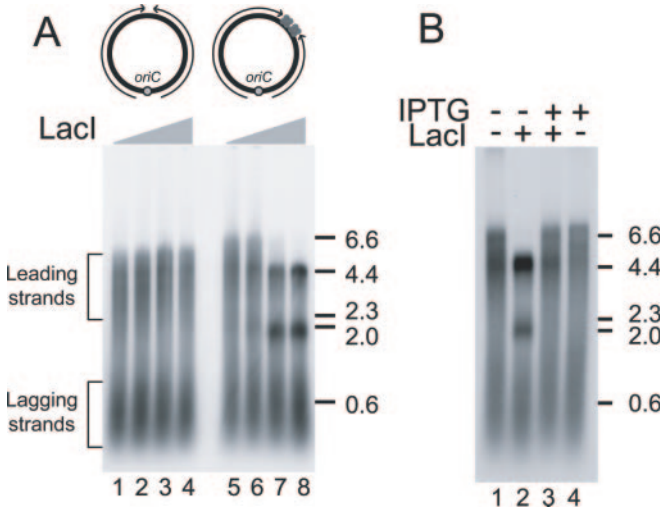

Figure 1. *lac* repressor–operator complexes block replisome movement *in vitro*. (**A**) Replication of plasmid templates with no *lac* operator sequences (pPM308, lanes 1–4) and with 22 tandem *lacO* sequences (pIK02, lanes 5–8) in the presence of 0, 5, 25 and 100 nM LacI tetramers. Products of replication 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 ($lacO_{22}$) bound by LacI is abrogated by addition of IPTG. LacI (100 nM tetramers) and IPTG (1 mM) were present as indicated.

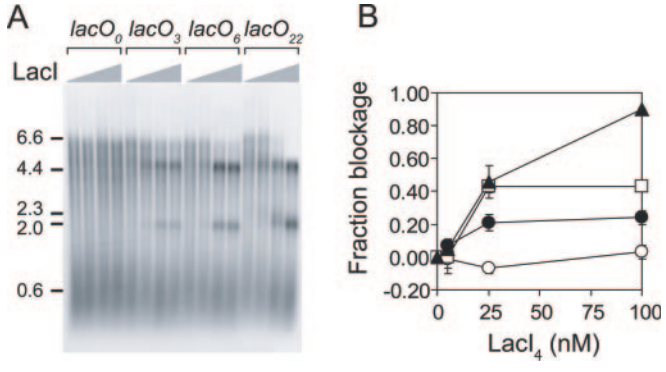

Figure 2. Additive blockage of replisome movement by repressor–operator complexes. (**A**) Replication of plasmid templates bearing 0, 3, 6 and 22 *lacO* sites (pPM374, 378, 393 and 379) in the presence of 0, 5, 25 and 100 nM LacI tetramers was monitored by denaturing agarose gel electrophoresis. DNA size markers are shown in kb. (**B**) Replisome blockage as a function of LacI concentration. Open circles, $lacO_0$; filled circles, $lacO_3$; open squares, $lacO_6$; filled triangles, $lacO_{22}$.

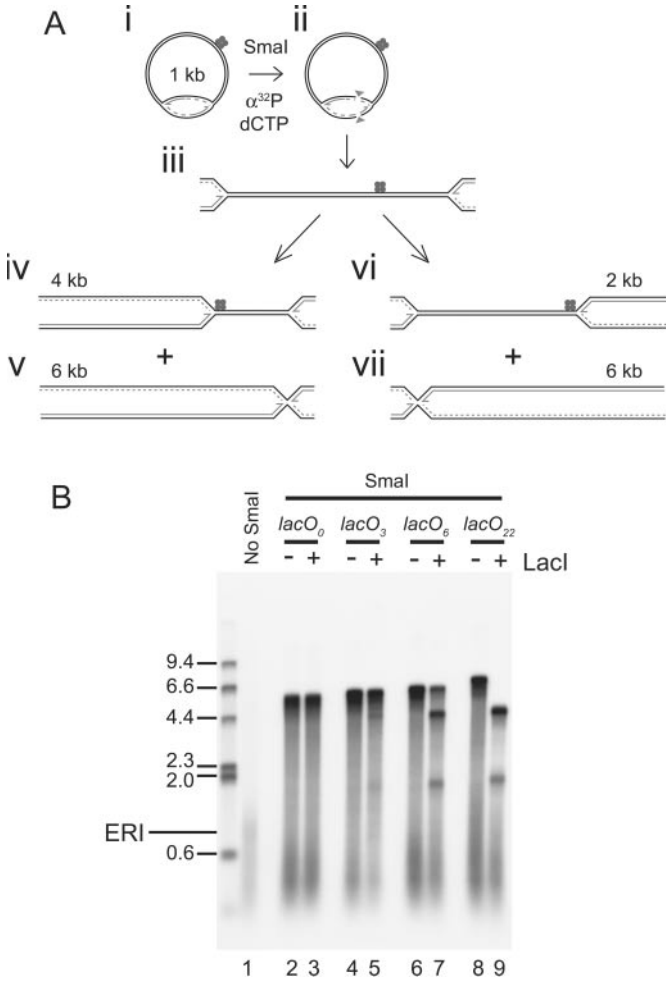

Figure 3. Replication blockage occurs on linearized as well as supercoiled template DNA. (**A**) In the absence of a topoisomerase, replication can initiate at *oriC* but is inhibited after about 1 kb of synthesis (i). Addition of SmaI results in cleavage of the DNA near to *oriC* (ii and iii) and allows 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 vii) whereas 4 and 2 kb leading strands would result from blockage of the forks at *lacO* (iv and vi). (**B**) Replication of pPM308 ($lacO_0$), pPM437 ($lacO_3$), pD506 ($lacO_6$) and pIK02 ($lacO_{22}$) 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 ($lacO_6$) or absence ($lacO_{22}$) 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 analysed 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 10–20 000 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 $lacO_{34}$ as compared with $lacO_{22}$ (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 $lacO_{22}$ and $lacO_{34}$ 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 $lacO_{34}$ 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 $lacO_{22}$ nor the $lacO_{34}$ 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 $lacO_{22}$ and $lacO_{34}$ loci were compared therefore in the presence of native and overexpressed levels of repressor. Replication intermediates detected at

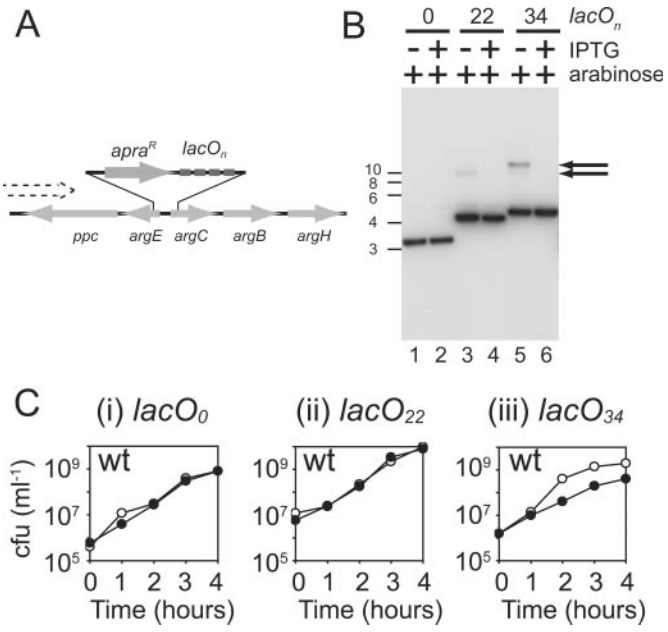

Figure 4. *lac* repressor–operator complexes present blocks to DNA replication *in vivo*. (**A**) Insertion of tandem *lac* operators within the chromosomal *arg* locus, linked to an apramycin resistance gene. The direction of replication fork movement is indicated with a dashed arrow. (**B**) Southern blot of a 1D agarose gel of PvuII 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.

$lacO_{34}$ 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 $lacO_{22}$ 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 $lacO_{22}$ locus, in spite of the accessibility of tandem *lacO* sites to enhanced levels of repressor, indicates that $lacO_{22}$ was saturated at wild-type repressor levels. Concerning $lacO_{34}$, 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 $lacO_{34}$ was also saturated in the presence of elevated levels of repressor.

Although the above electrophoretic analysis of replication intermediates did indicate saturation of $lacO_{22}$ with repressor, this analysis also suggested that replication fork blockage *in vivo* (~1% for $lacO_{22}$) was much less efficient than that seen at $lacO_{22}$ *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

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 $lacO_{34}$ (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 $lacO_{22}$ 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 $lacO_{34}$ 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), demonstrating that the observed inhibition of growth was dependent on formation of *lac* repressor–operator complexes. In contrast, *ruvABC* or *recF* mutations had only minor effects on growth rate upon repressor overexpression in the $lacO_{34}$ background (Figure 6A and B). Thus fork blockage at this artificial *lac* repressor-$lacO_{34}$ array presented a significant impediment to normal cell proliferation that required the activities of RecA, RecB and RecG to tolerate, but that did not require RuvABC, RecF, Rep or UvrD.

The accumulation of replication intermediates at the $lacO_{34}$ locus was analysed in *recA*, *recB* and *recG* strains upon induction of *lac* repressor overexpression. Slowly migrating DNA species were detected in all three mutant backgrounds but were absent when IPTG was present in the growth medium (Figure 6C). However, deletion of *recA*, *recB* or *recG* did not lead to a large increase in accumulation of replication intermediates as a proportion of the total DNA signal (Figure 6C, compare lanes 1, 3, 5 and 7) implying that, as expected, these gene products acted downstream of the initial blockage event.

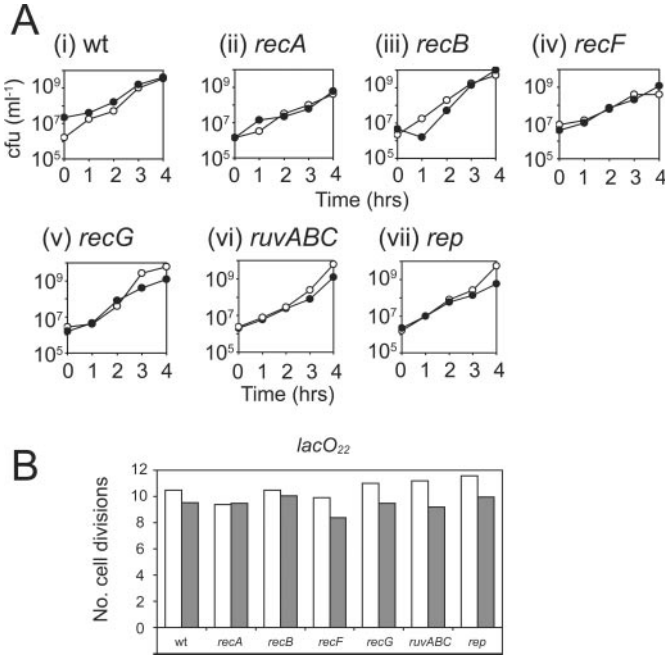

Figure 5. Recombination enzymes are not required *in vivo* for tolerance of 22 *lacO* sites bound by repressor. (**A**) Growth of strains harbouring 22 *lacO* sites and the indicated mutations, plus the *lacI* plasmid pPM306. Open circles: cells grown in glucose (no *lac* repressor overexpression); filled circles: cells grown in arabinose (elevated *lac* repressor). $lacO_{22}$ 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

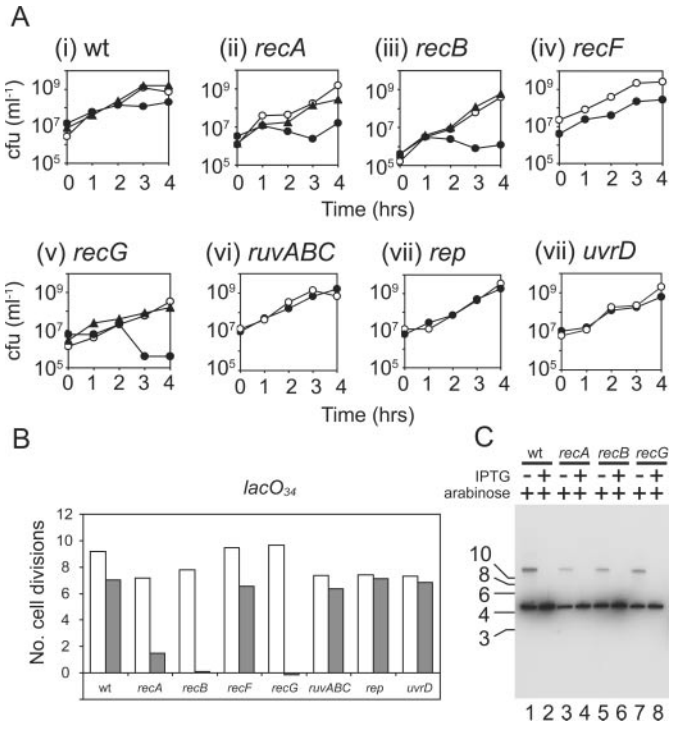

Figure 6. RecA, RecB and RecG are needed *in vivo* to tolerate 34 *lacO* sites bound by repressor. (**A**) Growth of strains harbouring 34 *lacO* sites and the indicated mutations, plus the *lacI* plasmid pPM306. Open circles: cells grown in glucose (no *lac* repressor overexpression); filled circles: cells grown in arabinose (elevated *lac* repressor); filled triangles: cells grown in arabinose plus IPTG. Strains were BP41 (otherwise wild-type), BP43 (*recA*), BP45 (*recB*), BP44 (*recF*), BP54 (*recG*), BP52 (*ruvABC*), BP47 (*rep*), BP55 (*uvrD*). Each curve was performed between two and six times with very similar results. (**B**) Mean number of cell divisions between 0 and 4 h of growth for strains bearing 34 *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). (**C**) Southern blot of a 1D agarose gel of PvuII digests of chromosomal DNA from strains bearing $lacO_{34}$, all containing pPM306, grown in the presence of arabinose with and without 1 mM IPTG. Strains were BP41 (otherwise wild-type), BP43 (*recA*), BP45 (*recB*) and BP54 (*recG*). The entire apramycin resistance cassette was used to generate the radiolabelled probe. Note also that the amounts of chromosomal DNA detected by the probe varied between strains and likely reflected variation between strains in the number of chromosomes per cell. However, the amount of replication intermediate as a proportion of the total DNA signal did not vary greatly (5 to 8%).

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 replisomes 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 vitro* (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 $lacO_{34}$ protein–DNA array (Figure 6) indicating that Holliday junction resolution by RuvABC-directed cleavage was not essential. The lack of any requirement for RuvABC in a $lacO_{34}$ background also suggests that cleavage of Holliday junctions formed by unwinding of stalled replication forks (13) was not essential for tolerance of the *lac* arrays.

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 $lacO_{34}$ 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 $lacO_{34}$–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.

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