Localization of an accessory helicase at the replisome is critical in sustaining efficient genome duplication

John Atkinson1, Milind K. Gupta1, Christian J. Rudolph2, Hazel Bell1, Robert G. Lloyd2 and Peter McGlynn1,*

1School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD and 2Institute of Genetics, University of Nottingham, Queen’s Medical Centre, Nottingham NG7 2UH, UK

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ABSTRACT

Genome duplication requires accessory helicases to displace proteins ahead of advancing replication forks. *Escherichia coli* contains three helicases, Rep, UvrD and DinG, that might promote replication of protein-bound DNA. One of these helicases, Rep, also interacts with the replicative helicase DnaB. We demonstrate that Rep is the only putative accessory helicase whose absence results in an increased chromosome duplication time. We show also that the interaction between Rep and DnaB is required for Rep to maintain rapid genome duplication. Furthermore, this Rep–DnaB interaction is critical in minimizing the need for both recombinational processing of blocked replication forks and replisome reassembly, indicating that colocalization of Rep and DnaB minimizes stalling and subsequent inactivation of replication forks. These data indicate that *E. coli* contains only one helicase that acts as an accessory motor at the fork in wild-type cells, that such an activity is critical for the maintenance of rapid genome duplication and that colocalization with the replisome is crucial for this function. Given that the only other characterized accessory motor, *Saccharomyces cerevisiae* Rrm3p, associates physically with the replisome, our demonstration of the functional importance of such an association indicates that colocalization may be a conserved feature of accessory replicative motors.

INTRODUCTION

Duplication of DNA requires not only the breakage of hydrogen bonds between the parental strands but also the displacement of proteins bound to the template, both of which are catalysed by the replicative helicase at the fork. This requirement for displacement of proteins ahead of the fork is illustrated by high affinity nucleoprotein complexes such as those associated with transcription, centromeres and inactive replication origins presenting obstacles to movement of the replicative helicase and thus of the fork (1–3). Occasional failure of the replicative helicase to displace such proteins from the template results in fork blockage and the attendant risks of incomplete genome duplication or of genome instability associated with blocked fork repair (4).

Blockage of replication forks by nucleoprotein complexes suggests that recruitment of additional helicases to such forks might facilitate clearance of the block and resumption of replication (5). Rrm3p promotes movement of forks through high affinity non-histone protein–DNA complexes in *Saccharomyces cerevisiae*, indicating that Rrm3p acts as an accessory replicative helicase (3,6). Lack of Rrm3p is also associated with elevated recombination rates (7), a signature of recombinational repair of blocked forks. However, whether other *S. cerevisiae* helicases can act in such a capacity is unknown. Indeed, in *Escherichia coli* multiple helicases can perform this accessory helicase function. Rep and UvrD can each promote movement of *E. coli* replication forks along protein-bound DNA, a redundancy of function reflected in the inviability on rich medium of cells lacking both Rep and UvrD (8–10). This inviability is associated with nucleoprotein complexes associated with gene expression since RNA polymerase mutations can suppress the rich medium inviability of Δ*rep ΔuvrD* cells (9–11). A third *E. coli* helicase, DinG, may also promote fork movement along transcribed DNA. DinG is critical in promoting survival of strains bearing chromosomal inversions encompassing highly transcribed *rrn* operons and is

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

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also essential for the survival of Δrep ΔuvrD cells bearing RNA polymerase suppressor mutations (10).

Whether Rep, UvrD and DinG are redundant or whether only one of these helicas is primarily responsible for underpinning fork movement in wild-type cells is unclear. As with ΔdinG cells, Δrep cells are sensitive to inversion of rrn operons but ΔuvrD cells are not (10). Promotion of fork movement along transcribed DNA may be underpinned therefore by Rep and/or DinG rather than UvrD. Mean replication fork speed may be underpinned therefore by Rep and/or DinG rather than UvrD. Mean replication fork speed in vivo may also be reduced 2-fold in the absence of Rep (12), although whether this reduction reflects Rep-directed clearance of proteins ahead of blocked forks or the participation of Rep in PriC-directed replisome reassembly away from oriC is unknown (13). The C-terminus of Rep also interacts physically with the E. coli replicative helicase, DnaB, an interaction that promotes fork movement along protein-bound DNA in vitro and facilitates complementation of Δrep ΔuvrD lethality by plasmid-encoded Rep (9). In contrast, there is no detectable interaction between UvrD and DnaB (9). Thus Rep may be an integral component of the replisome, implying a critical role for Rep in promoting fork movement along protein-bound DNA. Saccharomyces cerevisiae Rrm3p also localizes to replisomes via Pol2p and/or PCNA (14,15), although the functional importance of this colocalization in S. cerevisiae is unknown.

Here we show that the optimal rate of genome duplication in E. coli requires a single accessory helicase, Rep. We show also that interaction between Rep and DnaB is critical for the maintenance of rapid genome duplication and that this interaction reduces the need both for blocked fork processing by recombination enzymes and for replisome reassembly. However, whilst physical interaction with the replisome is critical for the function of Rep in vivo, interaction with Rep has no allosteric effect on the integral speed of the replisome. The only function of the Rep–DnaB interaction is to increase therefore the local concentration of an accessory motor at the fork. Our demonstration of the functional importance of interaction of an accessory helicase with the replisome in E. coli implies that this colocalization may be a key signature of such helicas.

MATERIALS AND METHODS

Strains and plasmids

Strains are listed in Supplementary Table S1. A rep allele bearing a deletion of the final 33 codons, repAC33, was generated using the primers MKG64 (TTTTTTGCTGGA GCTGGCGAGGTAGCTGATTTTGTAAGTGTA GGCTGGAGCTGCTTC) and MKG63 (CCGATGCG ATGCTAGCCTTTTTCCGCTTTACATATG AATATCCTCCTAC) to amplify the kanamycin resistance cassette from pKD4 (16). The resulting PCR product was then inserted into the chromosome of DY330 via λRed integration (17) to generate repAC33 linked to a kanamycin resistance gene immediately downstream of the repAC33 stop codon. An otherwise isogenic rep+ strain was generated in a similar manner except that MKG62 (CTGAAAGCGATGCGCCGCAAAACG AGGAAATAAGTGTACTGCGAGCTGCTTC) was used in combination with MKG63. Both alleles were sequenced to ensure accurate integration and to confirm the absence of unwanted mutations in either rep allele.

pAM374, 403 and 407 are pRC7 derivatives encoding priA, rep and uvrD, respectively (9,18).

Flow cytometry

For measurement of chromosome duplication time in dnaA46 and dnaA5 strains, the indicated strains were grown in 5 ml of LB at 30°C with shaking until an A650 of 0.3 was reached. Cultures were then transferred to a 42°C shaking waterbath for 2 h. 5 ml of LB pre-cooled to 18°C was then added directly to the 42°C cultures to alter the culture temperature rapidly to 30°C and the mixture then placed in a 30°C shaking waterbath for 10 min. This 10-min period at the permissive temperature was the minimum needed to allow the majority of cells to initiate chromosome replication. 10 ml of LB pre-heated to 55°C was then added to the 30°C cultures, achieving a temperature of 42°C rapidly, and the mixture placed into a 42°C shaking waterbath for a further 70 min. Culture samples were removed immediately prior to the temperature downshift to 30°C and designated as time zero. Samples were then removed immediately prior to the temperature upshift to 42°C and at 10-min intervals thereafter. Culture samples were fixed in methanol and DNA stained with Sytox Green (Invitrogen). Flow cytometry was performed on a FACSCalibur Benchtop Cytometry Analyser (Becton Dickinson).

Estimation of intracellular Rep concentrations

To estimate the number of Rep molecules in wild-type cells (TB28), cells were grown in 10 ml of LB at 37°C with shaking until an A650 of 0.4 was reached. Serial dilutions were plated onto LB agar and the number of viable cells per ml estimated after incubation overnight at 37°C. The remaining 10 ml was chilled on ice and cells harvested by centrifugation at 4°C. Cell pellets were resuspended in 400 μl of 50 mM Tris.HCl (pH 8.4), 150 mM NaCl, 0.2 mg/ml lysozyme and 20 mM EDTA and incubated on ice for 10 min before addition of Brij 58 to a final concentration of 0.1%. Incubation was continued on ice for another 20 min. Protein concentration was determined using Bio-Rad protein assay reagent to give an estimate of the total protein content per ml of culture. Combining this value with the number of viable cells per millilitre of culture was used to estimate the number of viable cells required to obtain 10 μg of total cell protein. 10 μg of extract was then loaded onto a 8% SDS polyacrylamide gel along with 0.5, 1, 2.5, 5 and 10 ng of Rep, purified as described (9). After electrophoresis at 180 V for 80 min in an Invitrogen XCell Surelock gel tank, proteins were transferred onto Hybond-P (GE Healthcare) in transfer buffer (0.48 mM Tris base, 3.84 mM glycerol and 20% methanol). The filter was then blocked overnight at 4°C in 25 ml of PBS/T (100 mM NaCl, 80 mM disodium hydrogen phosphate, 20 mM sodium dihydrogen...
phosphate, 0.1% Tween 20 and 5% dried milk). The filter was then rinsed twice in 25 ml PBS/T before being incubated for 1 h at room temperature in 25 ml PBS/T containing rabbit anti-Rep serum, raised against purified Rep (19) by Eurogentec, at a dilution of 1:6250. The blot was then rinsed briefly in 2 × 25 ml PBS/T, once in 25 ml PBS/T for 15 min and then 3 × 5 min in 25 ml PBS/T at room temperature. The blot was then incubated for 1 h at room temperature in 25 ml PBS/T containing goat anti-rabbit/peroxidase conjugate (Sigma) at a dilution of 1:50000. The blot was then rinsed as described above. Antibody was detected using the ECL Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer’s recommendations. Amounts of Rep were quantified using a Bio-Rad Fluor-S MultiImager. The fluorescence intensity obtained with each known quantity of purified Rep was plotted against the amount of Rep to obtain a standard curve for the blot. Fluorescence intensity corresponding to the Rep signal in lanes containing 10 μg whole-cell extracts was then used in conjunction with this standard curve to estimate the number of Rep molecules within 10 μg of whole cell extract. The number of Rep molecules per cell was then calculated by dividing the number of Rep molecules per 10 μg total cell protein by the number of viable cells required to obtain 10 μg of total cell protein, as estimated above from the viable cell count. Rep concentrations in MKG08, MKG10, HB159, HB252 and HB254 were estimated in a similar manner using TB28 whole cell extracts as standards. Strains containing dnaA46 were grown at 30°C rather than 37°C.

Viability, cell length and growth rate assays

The ability of strains to form colonies in the absence of pRC7 derivatives was monitored as described (9). Growth rates of ΔuvrD strains bearing rep<kan>, repΔC33<kan> or Δrep were monitored by initially plating pAM407-containing strains onto minimal agar containing Xgal and IPTG. After 4 days at 37°C, plasmid-free segregants were identified and inoculated into 10 ml of liquid minimal medium. Cultures were grown at 37°C to an A650 of 0.4 and then 125 μl of each culture was inoculated into 10 ml of LB. Incubation was continued at 37°C. Aliquots were removed at the indicated times after inoculation of the LB cultures, serial dilutions made in ice-cold 56/2 salts and the number of viable cells estimated after spotting of these dilutions onto minimal agar and incubation at 37°C for 3 days. The median length of cells was measured in minimal medium when an A650 of 0.4 was reached and then after 3 h in LB medium using a Zeiss Axioskop mot plus microscope and QCapture Pro v5.1 software.

RESULTS

Interaction of Rep with DnaB is required to minimize chromosome duplication time

The mean speed of replication fork movement in cells lacking Rep is half that in wild-type cells (12), implying an important role for Rep in underpinning genome duplication. However, the importance of localizing Rep at the replisome in maintaining replication fork speed is unknown. To measure the time required for chromosome duplication, synchronization of replication initiation was achieved using the temperature-sensitive dnaA46 allele (20). Synchronization was performed by shifting cells to the restrictive temperature for 2 h, generating cells with a single chromosome. Chromosome duplication was then initiated by shifting the cells to the permissive temperature for 10 min, before a return to 42°C to inhibit continued initiation. DNA content per cell was monitored by flow cytometry at 10-min intervals, with time zero being the point at which cells were shifted to the permissive temperature.

Wild-type cells took ~40–50 min for the DNA content to increase from 1 to 2 chromosome equivalents (Figure 1A) consistent with a 40-min chromosome duplication time given the 10 min spent at the permissive conditions.
temperature. Only a small fraction of cells underwent subsequent cell division, as indicated by the small increase between 40 and 80 min in the fraction of cells having 1 chromosome equivalent (Figure 1A). Absence of cell division after a single round of chromosome replication has been observed previously (20,21) and likely reflects the inability of chromosome duplication in itself to trigger cell division (22).

In contrast to wild-type cells, chromosome duplication in Δrep cells was incomplete after 40 min (Figure 1B). Even after 80 min, a significant fraction of Δrep cells had not completed chromosome duplication (Figure 1B). Thus the time taken to replicate chromosomes in Δrep cells is at least twice that in rep+ cells. These data are consistent with absence of Rep causing a 2-fold reduction in mean replication fork speed (12), demonstrating the validity of this assay in monitoring chromosome duplication time.

To establish the importance of the Rep–DnaB interaction in maintaining chromosome duplication time, we exploited a mutant version of Rep lacking the C-terminal 33 amino acids that fails to interact with DnaB but retains helicase activity (9). The wild-type rep allele was replaced with a rep gene lacking the final 33 codons together with a downstream antibiotic resistance cassette. An otherwise isogenic strain was also constructed in which the antibiotic marker was placed in an identical position downstream of the wild-type rep gene. This rep− strain again took ~40–50 min for the number of chromosome equivalents to increase from 1 to 2 (Figure 1C). However, as with Δrep cells, chromosome duplication in the repAC33 strain was incomplete after 40 min (Figure 1, compare parts B with D). This delay in completion of chromosome duplication in repAC33 was not due to either decreased expression of repAC33 or to instability of mutant as compared with wild-type Rep, as the number of molecules of RepAC33 per cell were comparable with those of the wild-type protein (Table 1). We conclude that interaction of Rep with DnaB is required for minimization of chromosome duplication time.

### Table 1. Intracellular concentrations of Rep and RepAC33

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Rep molecules per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB28</td>
<td>rep+ dnaA+</td>
<td>128 ± 16</td>
</tr>
<tr>
<td>MKG08</td>
<td>rep−&lt;kan&gt; dnaA+</td>
<td>120 ± 21</td>
</tr>
<tr>
<td>MKG10</td>
<td>repAC33&lt;kan&gt; dnaA+</td>
<td>123 ± 16</td>
</tr>
<tr>
<td>HB159</td>
<td>rep+ dnaA46</td>
<td>122 ± 11</td>
</tr>
<tr>
<td>HB252</td>
<td>rep−&lt;kan&gt; dnaA46</td>
<td>117 ± 10</td>
</tr>
<tr>
<td>HB254</td>
<td>repAC33&lt;kan&gt; dnaA46</td>
<td>145 ± 8</td>
</tr>
</tbody>
</table>

**Maintenance of chromosome duplication time is specific to Rep**

UvrD, like Rep, promotes replisome movement along protein-bound DNA in vitro and in vivo whilst DinG is also needed to facilitate fork movement through highly transcribed sequences (9,10). We tested therefore whether UvrD or DinG were necessary for maintaining wild-type rates of chromosome duplication. However, as with rep− uvrD− dinG− cells, chromosome duplication in ΔuvrD and ΔdinG cells took approximately 40 min (Figure 1, compare parts E and F with A). Rep but not UvrD or DinG is critical therefore in maintaining wild-type rates of chromosome duplication in vivo.

**Minimization of chromosome duplication time by Rep is not effected by PriC-directed replisome reassembly**

The increased chromosome duplication time in Δrep cells could be explained by absence of Rep-catalysed promotion of replisome movement along protein-bound DNA. However, the possible involvement of Rep in PriC-directed replisome reassembly away from oriC could also explain reduced mean fork speed in Δrep cells (13). If forks stall and become inactive, creating a need for replisome reloading, then a reduced capacity for such reloading might increase the time needed to replicate the chromosome.

Data from strains lacking the DNA replication initiation, dnaA64 priC cells are inviable (23), preventing replication synchronisation using this dnaA allele. However, we found that dnaA5, a priC-compatible allele (23), could also be used to synchronize replication initiation. rep+ priC+ dnaA5 cells took 40–50 min to complete chromosome duplication whereas Δrep priC+ dnaA5 cells took at least 80 min (Figure 2A and B), the same pattern as seen in dnaA46 cells (Figure 1A and B). These data confirm the duplication times seen in dnaA46-containing cells and demonstrate that the nature of the dnaA allele does not impact on the time required for genome duplication.

Chromosome duplication in rep+ ΔpriC dnaA5 took 40–50 min, a similar time as compared with rep+ priC+ dnaA5 (Figure 2, compare parts C with A). Thus PriC-directed replisome reloading does not have a
critical role in maintaining chromosome duplication time. The corollary of this observation is that the increased chromosome duplication time in Δrep and repΔC33 cells (Figure 1) is not due to a defect in PriC-directed replisome reloading.

The Rep–DnaB interaction facilitates resolution of conflicts between replication and transcription

Promotion of fork movement along protein-bound DNA by an accessory helicase is essential for viability under rapid but not restricted growth conditions (9,10). However, Δrep cells are viable on rich medium since UvrD, like Rep, can promote fork movement through nucleoprotein complexes (9). Inviability is only apparent therefore when both Rep and UvrD are absent.

To analyse the effect of absence of the Rep–DnaB interaction on the viability of ΔuvrD cells, repΔC33 and rep+ alleles were introduced into a ΔuvrD strain in which the uvrD mutation was complemented by a very low copy, highly unstable plasmid (pRC7) bearing wild-type uvrD (9,24). This plasmid also encodes lacZYA and so retention or loss of the plasmid can be monitored by blue/white colony colour in strains bearing a chromosomal deletion of the lac operon. As expected, colonies of plasmid-free segregants of the rep+ ΔuvrD strain formed readily on rich medium, with plasmid-free colonies being of similar size to those retaining the plasmid (Figure 3A(i)). The similar viability of cells with and without the uvrD+ plasmid was also reflected in the formation of mosaic blue and white colonies, formed only when plasmid-containing cells do not rapidly outgrow plasmid-free cells (18). The repΔC33 ΔuvrD strain also formed plasmid-free colonies on LB but these colonies were reduced in size as compared with those retaining the uvrD+ plasmid (Figure 3A, compare ii and i). The growth defect of repΔC33 ΔuvrD cells was reflected in the absence of mosaic colonies, reflecting the inability of plasmid-free cells to compete with uvrD+ plasmid-containing cells. Thus repΔC33 ΔuvrD cells displayed a viability defect under rapid growth conditions although this defect was not as extreme as that seen with Δrep ΔuvrD cells which fail to form any visible plasmid-less colonies on rich medium (9). In contrast, analysis of plasmid segregation under reduced growth conditions on minimal agar demonstrated that plasmid-free segregants of repΔC33 ΔuvrD formed with a colony size similar to that of rep+ ΔuvrD (Figure 3A). Limiting the growth rate thus restores the viability of repΔC33 ΔuvrD, as seen with Δrep ΔuvrD (9). The rich medium-dependent viability defect of repΔC33 ΔuvrD cells was confirmed by isolating plasmid-free segregants on minimal agar, subsequent growth in liquid minimal medium and then monitoring growth upon subculturing into LB. repΔC33 ΔuvrD cells did continue to produce viable progeny in LB, unlike Δrep ΔuvrD cells, but the growth rate was substantially lower than with rep+ ΔuvrD (Figure 3Bi). Indeed, although repΔC33 ΔuvrD cells could continue to divide in rich medium they were highly filamented as compared with rep+ ΔuvrD (Figure 3Bi).

The inviability of Δrep ΔuvrD cells on rich medium can be suppressed by mutations in RNA polymerase, reflecting the importance of transcribing or stalled RNA polymerases as nucleoprotein blocks to replication (9–11). The ability of a mutation in rpoB, known to destabilize transcription complexes (25) and to suppress Δrep ΔuvrD inviability (9), was tested for suppression of the repΔC33 ΔuvrD viability defect. Loss of plasmid-encoded UvrD in repΔC33 ΔuvrD rpoB35 resulted in plasmid-less colonies of a size similar to those seen with strains bearing rep+.
Retention or loss of pRC7-encoded PriA in is an expanded view of blue and white colonies, illustrating the formation of micro-colonies alongside the large plasmid-containing blue colonies. (Figure 3A). Destabilization of transcription complexes can suppress therefore the viability defect observed in repΔC33 ΔuvrD cells.

For Δrep and repΔC33 the correlation between increased chromosomal replication time in ΔuvrD cells (Figure 1), growth defects in ΔuvrD cells (Figure 3) and suppression of these viability defects by rpoB*35 (Figure 3C) indicates that inefficient fork movement along transcribed DNA is responsible for decreased mean fork speed. Moreover, since the repΔC33 ΔuvrD growth defect on rich medium was not as pronounced as that seen with Δrep ΔuvrD cells (Figure 3Bii) (9), we conclude that Rep helicase can promote fork movement along protein-bound DNA in the absence of interaction with DnaB but that this promotion is restricted.

Rep localization with DnaB minimizes the need for blocked fork processing

Cells lacking both Rep and the helicase/exonuclease RecBCD are inviable (26) indicating that increased fork blockage in the absence of Rep creates a requirement for blocked fork processing by RecBCD (27). We tested if repΔC33 also rendered recB cells inviable by establishing whether recB strains bearing either rep+ or repΔC33 could survive in the absence of pRC7 encoding Rep. Plasmid-free colonies were formed by repΔC33 rec+ with a frequency and size comparable to rep+ rec+ (Figure 4Aii and i). However, repΔC33 recB formed large blue colonies but no white colonies of comparable size, in contrast to rep+ recB (Figure 4Aiv and ii). Micro-colonies did also form with repΔC33 recB (Figure 4Aiv, inset) but these could not be subcultured (data not shown). The absence of RecBCD caused therefore a major growth defect in repΔC33 cells, consistent with absence of the Rep–DnaB interaction increasing the need for blocked fork processing by RecBCD.

Processing of blocked replication forks results ultimately in a need to reload the replication machinery back onto the chromosome, either by PriA or by PriC (28–30). Cells lacking either PriA or PriC are viable whereas cells lacking both are inviable, indicating the importance of replisome reloading away from oriC (29). Cells lacking both PriA and Rep are also inviable which may reflect a requirement for Rep in PriC-directed restart (29) but might also be caused by absence of Rep increasing the frequency of fork blockage and a consequent increased need for PriA-dependent fork reassembly.

The ability of ΔpriA cells to survive in combination with repΔC33 was tested. As shown previously (18), rep+ ΔpriA cells bearing pRC7 encoding PriA could form plasmid-free colonies (Figure 4Bi). These plasmid-free segregants were much smaller than those obtained in rep+ priA+ cells (Figure 4B, compare ii with i), reflecting the decreased viability of ΔpriA cells (31). In contrast, whilst large plasmid-free segregants were formed by repΔC33 priA+ cells, no segregants were formed from repΔC33 ΔpriA cells (Figure 4B, compare iii and iv). PriA was essential therefore in repΔC33 cells, indicating that absence of the Rep–DnaB interaction increased the requirement for PriA-directed replication restart. We cannot exclude the possibility that the growth defects noted in repΔC33 ΔpriA cells are associated with defects in Rep/PriC-dependent replisome reloading (29) rather than Rep-promoted fork movement. However, the viability defects of repΔC33 recB cells (Figure 4A) support a model in which there is an elevated requirement for RecBCD-dependent blocked fork processing and consequent PriA-directed replication restart due to increased fork blockage in the absence of a Rep–DnaB interaction.

![Figure 4](http://nar.oxfordjournals.org/) The Rep–DnaB interaction minimizes the need for blocked fork processing and replication restart. (A) Retention or loss of pRC7-encoded Rep in rec+ and recB strains bearing chromosomal rep+ or repΔC33 alleles monitored on LB plates containing Xgal and IPTG. The inset panel in (iv) is an expanded view of blue and white colonies, illustrating the formation of micro-colonies alongside the large plasmid-containing blue colonies. (B) Retention or loss of pRC7-encoded PriA in priA+ and ΔpriA strains bearing chromosomal rep+ or repΔC33 alleles monitored on LB containing Xgal and IPTG.
with DnaB (9). As seen in
retains the C-terminal 33 amino acids known to interact likely to encode a helicase-defective Rep but one that helicase activity in many helicases (37). Thus shown to be required for ATP hydrolysis and hence motif I (32,33) was mutated to arginine. This lysine RepK28R in which the lysine residue within helicase cation time was measured in a independent of Rep helicase activity? Chromosome duplication time. The Rep–DnaB interaction does not alter the mean integral speed of the replisome. Chromosome duplication was monitored by flow cytometry in dnaA46 strains bearing rep alleles encoding (A) wild-type Rep and (B) RepK28R. Time zero indicates samples removed after 2h at 42°C immediately prior to shifting the cultures to 30°C. Positions of 1 and 2 chromosome equivalents are indicated.

**Interaction of DnaB with Rep does not impact directly on replisome movement**

The above data demonstrate that the Rep–DnaB interaction is critical for Rep helicase function in vivo. We also tested whether the Rep–DnaB interaction has an allosteric effect on replisome function. In other words, does the interaction of DnaB with Rep contribute to the maintenance of wild-type rates of replication fork movement independent of Rep helicase activity? Chromosome duplication time was measured in a rep2001 strain encoding RepK28R in which the lysine residue within helicase motif 1 (32,33) was mutated to arginine. This lysine residue interacts with the phosphate tail of ATP in Rep and other Superfamily 1 helicases (34–36) and has been shown to be required for ATP hydrolysis and hence helicase activity in many helicases (37). Thus rep2001 is likely to encode a helicase-defective Rep but one that retains the C-terminal 33 amino acids known to interact with DnaB (9). As seen in ∆rep cells (Figure 1B), chromosome duplication in rep2001 cells was incomplete after 40 min, with a significant fraction of cells having less than two chromosome equivalents even after 80 min (Figure 5, compare parts A and B). Thus the Rep–DnaB interaction per se has no detectable impact on mean replication fork speed, indicating the absence of any significant direct positive effect of this interaction on genome duplication time. The Rep–DnaB interaction is critical therefore only in terms of Rep helicase function, not in terms of the integral speed of the replisome.

**DISCUSSION**

We have shown that although absence of Rep increases the time required to replicate a chromosome, absence of either UvrD or DinG has no detectable effect (Figure 1). Moreover, this decrease in mean replication fork speed in ∆rep cells is not due to involvement of Rep in replication restart (Figure 2). Rapid genome duplication in *E. coli* requires therefore only one type of accessory helicase, Rep, and this underpinning occurs via promotion of fork movement along protein-bound DNA rather than replication restart.

Our data also demonstrate that interaction of Rep with DnaB is critical for the ability of wild-type levels of Rep to maintain wild-type rates of chromosome duplication (Figure 1) and to promote viability in the absence of UvrD (Figure 3), a phenotype associated with problems replicating protein-bound DNA (9,10). Moreover, suppression of the repAC33 ∆uvrD viability defect by an RNA polymerase mutation (Figure 3C) demonstrates that conflicts between replication and gene expression create the need for Rep colocalization with DnaB. Absence of the Rep–DnaB interaction also increases the requirement for RecBCD (Figure 4A) indicating an increased need for blocked fork processing by RecBCD (27). RepAC33 also cannot support colony formation in cells lacking the replication restart protein PriA (Figure 4B). Although we cannot distinguish whether repAC33 ΔpriA lethality is due to increased fork blockage or to defective PriC-catalysed replication restart (29), this lethality indicates that the Rep–DnaB interaction is important for all aspects of Rep function in vivo. In contrast, this interaction in itself did not have any major allosteric effect on replisome movement (Figure 5). The Rep–DnaB interaction appears therefore to modulate Rep function only.

Comparison of the viability of cells bearing the repAC33 allele in combination with ∆uvrD, recB or ΔpriA (Figures 3 and 4) indicates that Rep helicase in the absence of interaction with DnaB can sustain cell division, albeit at a reduced efficiency, in the absence of UvrD but not of RecBCD or PriA. It is tempting to speculate that recombinational processing of blocked forks and replisome reassembly are more important therefore than clearance of protein blocks ahead of replication forks. However, the multiple blocked fork processing pathways existing in vivo, with the attendant possibility for off pathway reactions to occur in mutant strains that are never normally operative in wild-type cells, make such speculation meaningless. The severe viability defects seen with all the above mutant combinations suggest, though, that a combination of accessory helicase activity, blocked fork processing and replisome reloading is important in underpinning genome duplication.

Why might localization with DnaB be critical for Rep function? Functional cooperativity between helicases translocating along ssDNA may promote displacement of proteins from the DNA (38). Increasing the local concentration of Rep near the replisome may facilitate therefore the loading of multiple Rep monomers onto ssDNA at forks, aiding the clearance of proteins ahead of blocked forks and subsequent resumption of fork movement (9). In this view of the function of the Rep–DnaB interaction, this interaction does not impact directly on either DnaB or Rep catalysis, it merely increases the

![Figure 5](http://nar.oxfordjournals.org/)

**Figure 5.** The Rep–DnaB interaction does not alter the mean integral speed of the replisome. Chromosome duplication was monitored by flow cytometry in dnaA46 strains bearing rep alleles encoding (A) wild-type Rep and (B) RepK28R. Time zero indicates samples removed after 2h at 42°C immediately prior to shifting the cultures to 30°C. Positions of 1 and 2 chromosome equivalents are indicated.
effective concentration of Rep at the required site within the cell. This model also implies that absence of a UvrD–DnaB interaction renders the 500–900 UvrD molecules per cell (39) (and data not shown) incapable of sustaining wild-type rates of chromosome duplication, given the 2-fold increase in chromosome duplication time in uvrDΔrep cells (Figure 1). However, we cannot exclude the possibility that other differences between Rep and UvrD, in addition to the interaction with DnaB, may contribute to the ability of Rep but not UvrD to sustain wild-type rates of chromosome duplication.

Regardless of whether interaction with DnaB facilitates functional cooperativity between multiple Rep molecules, it remains unknown whether Rep is always present at replication forks or whether Rep associates only with blocked replisomes. The high affinity association between Rep and DnaB (9) might suggest that Rep is continuously associated with moving forks. However, it remains possible that other components within a moving replisome inhibit the Rep–DnaB interaction, possibly by masking the interaction domain on DnaB, and that such inhibition is relieved only within the context of a blocked fork.

If a high concentration of an accessory helicase at the fork is all that is required to underpin replisome movement, is it possible that other organisms rely on accessory helicases that operate at replisomes by virtue of high intracellular concentration rather than colocalization with replisomes? Such a system would imply non-specific targeting of accessory helicases to DNA. However, an emerging theme in regulation of helicase catalysis in vivo is that other factors target helicases to specific substrates, in effect conferring DNA substrate specificity on helicases. Untargeted and hence unregulated helicase activity may therefore be deleterious with respect to maintenance of genome stability. Note that although the absence of any significant impact of high level plasmid-based expression of Rep on viability suggests that unrestricted Rep activity is not toxic (9,40), more subtle detrimental effects cannot be excluded. DnaB could be viewed therefore as a substrate specificity factor that allows low concentrations of Rep to underpin genome duplication effectively in vivo.

Localization at the replisome may be a conserved feature of accessory helicases. The only other known accessory replicative helicase, S. cerevisiae Rrm3p, is also associated with the replisome although this association may be via the sliding clamp and/or the leading strand polymerase rather than the replicative helicase (14,15). Our work with Rep demonstrates that physical association with the replisome has an important functional impact on accessory helicase activity. The work presented here also suggests that the identity of the interacting partner within the replisome may not be critical so long as the local concentration of the accessory helicase at the fork is high.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.
physically interacts with proliferating cell nuclear antigen. 


