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Impairment of lagging strand synthesis triggers the formation of a RuvABC substrate at replication forks

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The *holD* gene codes for the ψ subunit of the *Escherichia coli* DNA polymerase III holoenzyme, a component of the γ complex clamp loader. A *holD* mutant was isolated for the first time in a screen for mutations that increase the frequency of tandem repeat deletions. In contrast to tandem repeat deletions in wild-type strains, deletion events stimulated by the *holD* mutation require RecA. They do not require RecF, and hence do not result from the recombinational repair of gaps, arguing against uncoupling of the leading and lagging strand polymerases in the *holD* mutant. The *holD recBC* combination of mutations is lethal and *holD recBts recCts* strains suffer DNA double-strand breaks (DSBs) at restrictive temperature. DSBs require the presence of the Holliday junction-specific enzymes RuvABC and are prevented in the presence of RecBCD. We propose that impairment of replication due to the *holD* mutation causes the arrest of the entire replisome; consequently, Holliday junctions are formed by replication fork reversal, and unequal crossing over during RecA- and RecBCD-mediated re-incorporation of reversed forks causes the hyper-recombination phenotype.

Keywords: deletion/DNA polymerase/double-strand breaks/homologous recombination/repair

Introduction

Alterations in the length of repetitive sequences within or near genes can alter their expression or function. In humans, expansion of repeated sequences has been found associated with several genetic degenerative disorders (reviewed in Ashley and Warren, 1995; Djian, 1998). Two classes of pattern of tract instability have been described: small changes as little as a few nucleotides in length and rearrangements that involve long sequences. With the aim of understanding better the circumstances in which recombination between long repeated sequences is stimulated, we isolated and characterized mutants where the frequency of this type of rearrangement was increased (Bierne *et al*., 1997b). This revealed that different recombination processes are specifically stimulated by mutations in DNA repair or DNA replication genes.

In wild-type *Escherichia coli* chromosomes, the frequency of recombination between long tandem repeats is unaffected by the inactivation of RecA, the main homologous recombination protein, and is also independent of proteins involved in the pre-synaptic (RecBCD, RecFOR) and post-synaptic (RuvABC) steps of homologous recombination (Lovett *et al*., 1993; Bierne *et al*., 1997b). This suggests that such deletions can occur independently of the classical homologous recombination pathways. A systematic determination of recombination frequencies in mutants affected for DNA replication showed that mutations in most of the *E.coli* genes required for chromosomal replication affect tandem repeat recombination (Saveson and Lovett, 1997). The mechanism involved was studied in a *dnaBts* mutant, impaired for the main replicative helicase. Stimulation of deletions by *dnaBts* mutation was dependent on RecA and also on RecBCD, the complex that specifically initiates recombinational repair of DNA double-strand breaks (DSBs) (Saveson and Lovett, 1999). The RecBCD complex binds to double-stranded ends and promotes DNA degradation by its helicase and exonuclease activities (reviewed in Kowalczykowski, 1994; Kuzminov, 1999). Upon the encounter of a specific sequence, CHI, the activity of the complex is modified to generate a single-stranded 3′ tail on which RecBCD facilitates the loading of RecA and thus promotes the invasion of a homologous sequence (Anderson and Kowalczykowski, 1997; Churchill *et al*., 1999). The RecBCD dependence of tandem repeat deletions in *dnaBts* strains is in agreement with the observation that double-strand ends are produced in the bacterial chromosome upon replication arrest by inactivation of this helicase (Michel *et al*., 1997; Seigneur *et al*., 1998).

Using random mutagenesis, we screened for mutations that stimulate tandem repeat recombination in *E.coli*. Two such mutations were previously shown to map in *uvrD* and *dnaE* (Bierne *et al*., 1997a,b). Hyper-recombination in *uvrD* mutants, defective for the repair of mismatches and for the excision repair of UV lesions, was dependent on RecA and RecFOR. The RecFOR proteins are thought to catalyze recombination at single-stranded gaps by facilitating the action of RecA on gapped DNA (Umezu *et al*., 1993; Webb *et al*., 1997; reviewed in Kuzminov, 1999). We proposed that the absence of UvrD resulted in the formation of gaps and that unequal exchanges during gap repair by RecFOR-mediated recombination caused the hyper-recombination phenotype (Bierne *et al*., 1997a,b). DnaE is the catalytic subunit (α) of the *E.coli* DNA polymerase III holoenzyme (Pol III HE), the main *E.coli* polymerase. In contrast to *uvrD* and *dnaBts* mutations, mutations in the *dnaE* gene led to a similar increase in the frequency of tandem repeat deletions in the presence and

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absence of RecA (Bierne *et al*., 1997a,b). Consequently, deletions were proposed to occur by replication slippage, a RecA-independent mechanism. The *dnaE* mutation would cause a pause in the replication progression, leading to dissociation of the 3′ end of the growing strand from the template. Re-association on the second homologous repeat and continuation of replication would lead to the loss of one of the repeats in the newly synthesized genome.

In the present work, we describe the characterization of a third hyper-recombination mutation isolated by random mutagenesis. This mutation affects one of the *E.coli* Pol III HE subunits, ψ, encoded by the *holD* gene. *Escherichia coli* Pol III HE is composed of 10 different subunits, assembled in two catalytic cores, two sliding clamps and a clamp loader (reviewed in Kelman and O'Donnell, 1995). The polymerase core contains the catalytic subunit α (*dnaE*), the proofreading activity ε (*dnaQ*) and a polypeptide of unknown function, θ (*holE*). The two cores are held together by a dimer of the τ subunit (*dnaX*), which binds the clamp loader and DnaB, allowing the two cores to function in a coordinated fashion, one on the leading strand and the other on the lagging strand. Each core polymerase is tethered to the DNA by a β clamp (*dnaN*). The clamp loader (γ complex) contains five different polypeptides: γ (*dnaX*), δ (*holA*), δ′ (*holB*), χ (*holC*) and ψ (*holD*). The function of the γ complex during replication has been studied extensively *in vitro*. This complex was proposed to be required for loading and unloading β after completion of each Okazaki fragment, thus allowing rapid cycling of the lagging strand polymerase (reviewed in Kelman and O'Donnell, 1995; Naktinis *et al*., 1996; Turner *et al*., 1999). ψ is a small protein of 15.2 kDa that acts as a bridge between γ and χ (Kelman and O'Donnell, 1995; Glover and McHenry, 1998, 2000; Kelman *et al*., 1998).

In contrast to extensive studies of replication *in vitro*, little is known about the individual role of each Pol III HE subunit *in vivo*. *dnaE*, *dnaX*, *holA* and *holB* are essential genes. Knockout of the *dnaQ* or *holC* genes, although viable, confers a severe growth defect. Only deletion of *holE* has no noticeable phenotype (reviewed in Kelman and O'Donnell, 1995). The *holD* mutant isolated in the present work is the first *holD* mutant described. The mutation increases the frequency of recombination between tandemly repeated sequences ~20-fold in a RecA-dependent way. In the *holD* mutant, *recBC* inactivation is lethal and causes the accumulation of DSBs. We propose a model to account for these observations that implies the arrest of the entire replisome when HolD fails to function.

Results

Isolation of a hyper-recombination mutant that maps in the holD gene

To isolate mutants in which the frequency of recombination between tandemly repeated sequences is increased, an *E.coli* strain that carries a tandem repeat of 624 bp in the *lacZ* gene was used (Bierne *et al*., 1997b). The duplication inactivates *lacZ* and deletion of one of the repeats restores a Lac$^+$ phenotype. One clone in which deletion frequency was increased ~20-fold was studied (JJC616; Tables I and II). The mutation was mapped by Hfr conjugation and P1 transduction at min 99 on the genetic map of *E.coli* and P1 transduced in the wild-type isogenic strain JJC520, using the linked *mdoB* or *thr* markers and screening for the hyper-recombination phenotype (JJC867, JJC947 and JJC1303; Tables I and II). Among genes that map at min 99, we tested *holD*, which encodes the ψ subunit of the Pol III HE. Introduction of the wild-type *holD* gene, carried on the vector pAM34, in the hyper-recombination mutant decreased the level of tandem repeat deletion to that observed in the wild-type strain, whereas the vector had no significant effect (Table II). We conclude that the mutation responsible for the hyper-recombination phenotype of this strain is located in the *holD* gene.

The holD gene of the hyper-recombination strain carries an amber mutation, suppressed by an unknown suppressor

The nucleotide sequence of the *holD* gene was determined in JJC520 (wild type) and JJC867 (*holD*). The only change in JJC867 compared with the wild-type strain was a C to T mutation at position 28, which changes the tenth amino acid, a glutamine, to an amber nonsense codon. The presence of an amber suppressor mutation in JJC520 was shown previously with the use of a plasmid carrying an amber mutant reporter gene (Bierne *et al*., 1997b) and was confirmed here by the observation that JJC520 supported the growth of λ^{am} phages (not shown). In spite of our efforts, we could not identify the suppressor mutation carried in this strain (see Materials and methods). Nevertheless, the presence of an amber suppressor in the *holD*^{am} strain suggests that a mutated full-size protein is synthesized.

Phenotype of the holD mutant

The phenotype of the *holD* mutant strain was analyzed. The *holD* mutation conferred sensitivity to rich medium since *holD* liquid cultures or *holD* colonies grown on minimal medium had a reduced plating efficiency on LB (Table II). The ~1% of bacteria that could form colonies on LB plates had lost the hyper-recombination phenotype and may have lost the *holD* mutation or acquired an additional amber suppressor. Sensitivity to rich medium is a property shared by several replication mutants in *E.coli* and is generally attributed to the difficulty in coping with an increased number of replication forks during propagation in rich medium. The sensitivity to rich medium was fully suppressed by introduction of the *holD* wild-type allele on a plasmid (Table II). In addition, the *holD* mutation conferred a mild sensitivity to high doses of UV irradiation (Figure 1) and a slight mutator phenotype, as the proportion of Rif^R cells in liquid cultures was increased nearly 7-fold by the *holD* mutation (3.3×10^{-7} in *holD* versus 4.8×10^{-8} in wild-type cells).

Hyper-recombination in holD cells depends on the homologous recombination function of RecA

Tandem repeat deletions can be increased in *E.coli* replication mutants by RecA-dependent or -independent mechanisms (Bierne *et al*., 1997b; Saveson and Lovett, 1999). A *ΔrecA*::Tn*10* mutation was introduced into the *holD* strain to determine whether RecA is required for the hyper-recombination phenotype. *holD recA* clones were obtained at the expected frequency and accordingly the

Table I. Strains and plasmids

Strain	Genotype	Origin or reference
CAG12079	*fuc*::Tn*10*	Singer *et al.* (1989); Nichols *et al.* (1998)
CAG12135	*recD1901*::Tn*10*	Singer *et al.* (1989); Nichols *et al.* (1998)
CAG18430	*mdoB*::Tn*10* (*zjj202*::Tn*10*)	Singer *et al.* (1989); Nichols *et al.* (1998)
CAG18442	*thr*-34::Tn*10*	Singer *et al.* (1989); Nichols *et al.* (1998)
CAG18619	*mdoB*::Tn*10kan* (*zjj3188*::Tn*10kan*)	Singer *et al.* (1989); Nichols *et al.* (1998)
GY9701	*ΔrecA938::cm* (p-RecA)	R.Devoret; Michel *et al.* (1997)
LN2926	Hfr PK191 *rpsL Δ(recBC)Ap*	Cornet *et al.* (1994)
JJC330 (SK129)	*recB270 recC271*	S.Kushner; Michel *et al.* (1997)
WA576	*recF400*::Tn*5*	W.Wackernagel
JJC40	AB1157 *hsdR*	laboratory collection
JJC99	wild type	Bierne *et al.* (1997b)
JJC443	*lexAind3 mal*::Tn*10*	Bierne *et al.* (1997a)
JJC754	*ΔruvABC::cm*	Seigneur *et al.* (1998)
JJC942	*trpA9605am his-29am*	laboratory collection
JJC1144	*holD*^{Q10am} *mdoB*::Tn*10kan*	P1 JJC867 * JJC99 selection Kan^R, screening for LB^S
JJC1159	*holD*^{Q10am} *mdoB*::Tn*10kan* [pAM-holD]	JJC1144 transformed by pAM-holD
JJC1220	*recB270 recC271 fuc*::Tn*10*	P1 CAG12079 * JJC330, selection Tet^R, screening for exo$^-$, UV^S
JJC1288	*holD*^{Q10am} *mdoB*::Tn*10kan* [pAM-holD] *ΔruvABC::cm*	P1 JJC754 * JJC1159, selection Cm^R
JJC1291	*holD*^{Q10am} *mdoB*::Tn*10kan* [pAM-holD] *ΔruvABC::cm Δ(recA-srl)*::Tn*10*	P1 JJC557 * JJC1288, selection Tet^R
JJC1294	*holD*^{Q10am} *mdoB*::Tn*10kan ΔruvABC::cm Δ(recA-srl)*::Tn*10*	JJC1291 cured of pAM-holD
JJC1298	Hfr PK191 *rpsL recD1901*::Tn*10*	P1 CAG12135 * LN2926, selection Tet^R
JJC1299	Hfr PK191 *rpsL recD1901*::Tn*10 ΔrecA938::cm*	P1 GY9701 * JJC1298, selection Cm^R
JJC1366	*ΔruvABC::cm*	P1 JJC754 * JJC99, selection Cm^R
JJC1367	*ΔruvABC::cm Δ(recA-srl)*::Tn*10*	P1 JJC557 * JJC1366, selection Tet^R
JJC520 derivatives. All JJC520 derivatives carry a 624 bp duplication in *lacZ* (*lacZ*$_{624dup}$) adjacent to a Cm^R marker		
JJC520	*lacZ*$_{624dup}$ Cm^R	Bierne *et al.* (1997b)
JJC557	*Δ(recA-srl)*::Tn*10*	Bierne *et al.* (1997b)
JJC561	*recF400*::Tn*5*	Bierne *et al.* (1997b)
JJC616	*holD*^{Q10am}	ethylmethane sulfonate mutagenesis of JJC520
JJC617	*holD*^{Q10am} *mdoB*::Tn*10kan*	P1 CAG18619 * JJC616, selection Kan^R, screening for LB^S
JJC867	*holD*^{Q10am} *mdoB*::Tn*10kan*	P1 JJC617 * JJC520 selection Kan^R
JJC868	*holD*^{Q10am} *mdoB*::Tn*10kan Δ(recA-srl)*::Tn*10*	P1 JJC557 * JJC867 selection Tet^R
JJC947	*holD*^{Q10am} *mdoB*::Tn*10*	P1 JJC616-*mdoB*::Tn*10* * JJC520, selection Tet^R, screening for LB^S
JJC1096	*holD*^{Q10am} *mdoB*::Tn*10kan* [pAM-holD]	JJC867 transformed by pAM-holD
JJC1097	*lexAind3 mal*::Tn*10*	P1 JJC443 * JJC867, selection Tet^R, screening for UV^S
JJC1157	*holD*^{Q10am} *mdoB*::Tn*10* [pAM-holD]	JJC947 transformed by pAM-holD
JJC1163	*holD*^{Q10am} *mdoB*::Tn*10* [pAM-holD] *recF400*::Tn*5*	P1 WA576 * JJC1157, selection Kan^R
JJC1164	*holD*^{Q10am} *mdoB*::Tn*10 recF400*::Tn*5*	JJC1163 cured of pAM-holD
JJC1218	*holD*^{Q10am} *mdoB*::Tn*10kan* [pAM-holD] *recB270 recC271 fuc*::Tn*10*	P1 JJC1220 * JJC1096 selection Tet^R
JJC1219	*holD*^{Q10am} *mdoB*::Tn*10kan recB270 recC271 fuc*::Tn*10*	JJC1218 cured of pAM-holD
JJC1297	*thr*-34::Tn*10*	P1 CAG18442 * JJC520, selection Tet^R
JJC1300	*holD*^{Q10am} *mdoB*::Tn*10kan* [pAM-holD] *recD1901*::Tn*10 ΔrecA938::cm*	conjugation from JJC1299 to JJC1096, selection Tet^R Cm^R; screening for UV^S
JJC1303	*holD*^{Q10am}	P1 JJC867 * JJC1297, selection Thr$^+$, screening LB^S
JJC1364	*holD*^{Q10am} *recD1901*::Tn*10*	conjugation from JJC1298 to JJC1303, selection Tet^R Cm^R

Table II. Mutation in the *holD* gene stimulates deletion of tandem repeats

Strain	Genotype	Recombinant proportion × 10^4	Relative values	C.f.u. LB/MM
JJC520	wild type	1.9 ± 0.7 (21)	1	0.9 ± 0.2 (12)
JJC616	*holD*	25 ± 13 (7)	13	ND
JJC867[a]	*holD*	50 ± 4 (33)	26	0.02 ± 0.013 (15)
JJC867 [pAM-holD][a]	*holD* HolD$^+$	2.9 ± 0.3 (3)	1.5	0.97 ± 0.4 (7)
JJC867 [pAM34][a]	*holD*	32 ± 6 (3)	17	0.0025 ± 0.0016 (5)

[a]Similar results were obtained in JJC947 and JJC1303 in which the hyper-recombination phenotype was co-transduced with *mdoB*::Tn*10* and Thr+, respectively. In these mutants, the presence of the *holD*-carrying plasmid also restored a wild-type level of recombination and LB resistance (not shown).
Figures in parentheses indicate the number of determinations.

strain presented no growth defect at 37°C compared with the *holD* single mutant. The *holD recA* cells were sensitive to rich medium (JJC868; Table III). The frequency of recombination was similar in the *holD recA* strain to that of wild-type cells (Table III). Introduction of the wild-type *recA* allele on a plasmid restored the hyper-recombination

Table III. Tandem repeat deletions in *holD* mutants are dependent on RecA and independent of SOS induction, RecF and RecD

Strain	Genotype	Recombinant proportion $\times 10^4$	Relative values	C.f.u. LB/MM
JJC520	wild type	1.9 ± 0.7 (21)	1	0.9 ± 0.2 (12)
JJC867	*holD*	50 ± 4 (33)	26	0.02 ± 0.013 (15)
JJC868	*holD recA*	4.8 ± 3 (15)	2.5	0.00014 ± 0.00006 (3)
JJC868 [pGB-recA]	*holD recA* RecA$^+$	45 ± 20 (4)	23	0.0015 ± 0.0009 (5)
JJC1097	*holD lexAind3*	22 ± 10 (10)	11	0.0077 ± 0.0044 (6)
JJC1164	*holD recF*	30 ± 15 (8)	16	0.0023 ± 0.002 (7)
JJC1164 [pAM-holD]	*holD recF* HolD$^+$	3.1 ± 1 (2)	1.6	0.9 ± 0.1 (4)
JJC1364	*holD recD*	44 ± 21 (8)	23	ND

As reported previously (Bierne *et al.*, 1997a,b), recombination frequency was not significantly modified by the following mutations: *ΔrecA* (1.8×10^{-4}), *lexAind3* (0.9×10^{-4}), *recF400*::Tn*5* (1.1×10^{-4}), *recD1901*::Tn*10* (2.9×10^{-4}).
Figures in parentheses indicate the number of determinations.

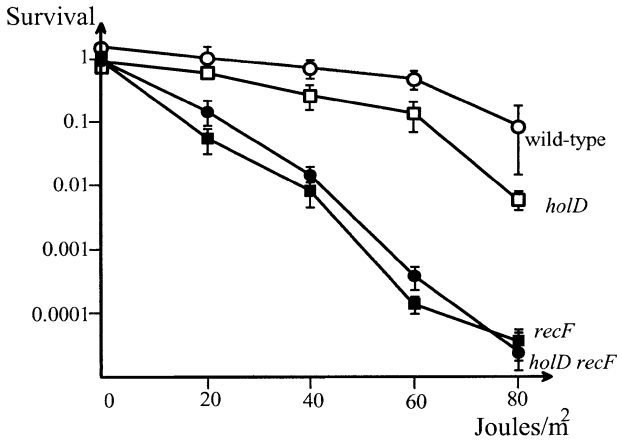

Fig. 1. UV sensitivity of *recF* mutants is not significantly affected by the *holD* mutation. Exponential cultures of cells grown to OD 0.5 were plated at appropriate dilutions on minimal medium plates that were irradiated at 2 J/m^2 for various times and incubated for 48 h at 37°C. Ratios of c.f.u. (colony-forming units) on irradiated versus non-irradiated plates are shown. Open circles, JJC520 (wild type); closed circles, JJC561 (*recF400*::Tn*5*); open squares, JJC947 (*holD*); closed squares, JJC1164 (*holD recF400*::Tn*5*). Results are the average of 3–6 independent determinations.

phenotype (JJC868 [pGB-RecA]; Table III). This shows that the hyper-recombination phenotype of the *holD* mutant requires RecA.

RecA is essential in *E.coli* for homologous recombination and for the induction of the SOS response, a set of repair genes induced by DNA damage (reviewed in Walker, 1996). In order to determine whether SOS induction is required for the hyper-recombination phenotype of the *holD* mutant, a *lexAind3* mutation was used. This mutation prevents cleavage of the LexA repressor, which precludes SOS induction without affecting homologous recombination. The frequency of recombination was not modified significantly by the presence of the *lexAind3* allele, which indicates that the hyper-recombination phenotype conferred by the *holD* allele does not require SOS induction (Table III). These results suggest that the stimulation of recombination between tandemly repeated sequences by the *holD* mutation requires the homologous recombination function of RecA. We therefore tested the role of proteins involved in the pre-synaptic steps of either of the two recombination pathways, RecFOR and RecBCD.

Hyper-recombination in holD cells is independent of RecF

The *holD* mutation affects a subunit of the γ complex of DNA Pol III HE responsible *in vitro* for the recycling of the lagging strand polymerase (reviewed in Kelman and O'Donnell, 1995). Impairment of the onset of Okasaki fragment synthesis could result in the formation of gaps on the lagging strand during replication. Since gap repair is mediated by the RecFOR pathway of recombination, if the hyper-recombination phenotype resulted from gap repair, it would require the presence of functional RecF protein. A *recF* null mutation did not modify the frequency of recombination in the *holD* mutant (Table III). Introduction of a plasmid carrying the wild-type *holD* allele in the *holD recF* strain decreased the frequency of recombination to the level observed in wild-type or *recF* strains, confirming that the hyper-recombination phenotype of the *holD recF* mutant is due to the *holD* mutation (Table III). These results indicate that the RecF protein is not required for the recombination events induced by the *holD* mutation. To test further whether the *holD* mutation caused the formation of gaps in the bacterial chromosome, sensitivity to UV irradiation was compared in *recF* and *holD recF* strains. Effectively, UV lesions on gapped DNA cannot be repaired by nucleotide excision repair (reviewed in Lin and Sancar, 1992) and therefore are mainly repaired by RecF-mediated recombination. The *holD* mutation did not increase the UV sensitivity of a *recF* strain (Figure 1) significantly, indicating that the *holD* mutation did not result in the accumulation of gaps.

The holD mutant requires RecBC for viability

Since the hyper-recombination phenotype of the *holD* mutant is dependent on RecA and independent of RecF, it presumably occurs by RecBCD-mediated recombination. However, this hypothesis could not be tested directly since attempts to introduce the null *recB*::Tn*10* allele in the *holD* strain failed, suggesting that the *holD recBC* strain might not be viable. A conditional mutant was constructed, which carries *recBts recCts* mutations. The *holD recBts recCts* strain (JJC1219; Table I) was viable at low temperature and did not grow at high temperature, hence in conditions where RecBC was inactive. This result shows that the inactivation of RecBC is lethal in a *holD* strain, indicating the formation of double-strand ends in this strain.

Table IV. The *holD* mutation causes the formation of RuvABC-dependent DSBs

Strain	Genotype	Percentage of linear DNA		*N*
		30°C	42°C	
JJC40	wild type	4.7 ± 0.4	4.4 ± 1.2	2/3
JJC330	*recBts recCts*	6.1 ± 0.6	23.1 ± 2	2/2
JJC1219	*holD recBts recCts*	32.8 ± 6.4	75.8 ± 6.9	4/4
JJC557 [p-Gam]	*recA* Gam$^+$	18.8 ± 3.5	33.2 ± 3.5	2/3
JJC868 [p-Gam]	*holD recA* Gam$^+$	13.7 ± 4.9	53.3 ± 3.7	4/6
JJC1367 [p-Gam]	*recA ruvABC* Gam$^+$	18.2 ± 0.6	33.6 ± 0.8	2/3
JJC1294 [p-Gam]	*holD recA ruvABC* Gam$^+$	15.5 ± 0.2	28 ± 3.9	2/5
JJC1294 [p-Gam] [pRuvABC]	*holD recA ruvABC* Gam$^+$ RuvABC$^+$	20.2 ± 7.2	47.3 ± 3.3	4/8

N is the number of determinations at 30°C/42°C. Results in JJC40 were published previously (Seigneur *et al*., 1998). Results in JJC330 are confirmatory of previous results (Seigneur *et al*., 1998).

The RecBCD complex is both a recombinase and an exonuclease. Whereas mutations in *recC* or *recB* inactivate all functions, mutations in *recD* inactivate only the exonuclease V activity while preserving the capacity to perform homologous recombination (Amundsen *et al*., 2000 and references therein). A *recD*::Tn*10* null mutation could be introduced in the *holD* mutant, indicating that the exonuclease action of the RecBCD enzyme is not essential in this mutant. Recombination between tandemly repeated sequences was as high in the *recD holD* strains as in the *holD* single mutant, indicating that exonuclease V is not required for the hyper-recombination phenotype of the *holD* mutant (Table III).

The holD recA recD strain is not viable

holD recA and *holD recD* strains, in which either homologous recombination or exonuclease V action is inactivated, are viable whereas *holD recBC* strains, which lack both activities, are not viable. The non-viability of the *holD recBC* strain could result from the simultaneous inactivation of both exonuclease V and homologous recombination in this strain. To examine this possibility, we tested the viability of a *holD recA recD* strain. The pAM-holD plasmid was used. This plasmid carries a conditional replication origin, which functions only in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). In the absence of IPTG, the plasmid does not replicate and is lost during bacterial growth. Upon propagation of *holD* strains carrying the pAM-holD plasmid in the absence of IPTG, the plasmid was lost as expected and the *holD* mutant was recovered. A *holD recA recD* [pAM-holD] strain was constructed (JJC1300; Table I) and cells were propagated in the absence of IPTG to cure the plasmid (see Materials and methods). No *holD recA recD* cells could be isolated, indicating that this strain is not viable. Consequently, (i) the *holD recA* strain requires the exonuclease V activity of RecBCD for viability and (ii) the *holD recD* strain depends on the homologous recombination functions of RecA and RecBC for viability.

The holD mutant suffers DSBs in the absence of RecBC or in recA cells deficient for exonuclease V

The RecBCD complex is specifically required for the repair of DSBs since it initiates homologous recombination at double-stranded DNA ends. In order to determine whether the non-viability of the *holD recBC* strain results from DSB formation in this strain, the presence of linear DNA *in vivo* was directly tested by pulsed field gel electrophoresis (PFGE). The circular chromosome of *E.coli* does not enter gels in PFGE, in contrast to linear molecules. Therefore, the proportion of DNA that enters pulsed field gels reflects the extent of chromosome linearization. *In vivo* tritium labeling allows a quantitative determination of the proportion of DNA that migrates in gels, and gentle cell lysis in plugs ensures that the linear molecules detected are formed *in vivo* (Michel *et al*., 1997). Very little linear DNA can be detected in RecBCD$^+$ cells in which linear DNA is either degraded or repaired by homologous recombination (wild-type cells, Table IV; Michel *et al*., 1997; Seigneur *et al*., 1998). The proportion of linear DNA was compared in *recBts recCts* and *holD recBts recCts* strains at low (RecBC mainly active) and high (RecBC inactive) temperature. The presence of the *holD* mutation led to a strong increase in the proportion of linear DNA upon inactivation of RecBC (compare JJC330 to JJC1219 at 42°C; Table IV). An effect of the *holD* mutation could also be observed at 30°C, which probably results from a saturation of the residual RecBCD activity in the *holD recBts recCts* mutant at low temperature. These results show that the *holD* mutation leads to the accumulation of linear DNA in a *recBC* context, hence to the occurrence of DSBs.

Since the *holD recA recD* mutant is not viable, we tested whether simultaneous inactivation of exonuclease V and RecA in the *holD* mutant also leads to an increased level of linear DNA. We used the λ Gam protein to inhibit the exonuclease V action of RecBCD. Gam was carried on a plasmid, under the control of a promoter active only at high temperature (pSF117; Murphy, 1991; called p-Gam here). In *holD recA* cells carrying this plasmid, exonuclease V is active at low temperature, which allows propagation of the strain, whereas at high temperature exonuclease V is inactivated by the induction of Gam. PFGE analysis showed an increase in the level of linear DNA due to the *holD* mutation in *recA* [p-Gam] cells at 42°C (compare JJC557[p-Gam] and JJC868[p-Gam]; Table IV). These results confirm that *holD* mutants devoid of homologous recombination and exonuclease V suffer DSBs due to the *holD* mutation.

DSB formation is RuvABC dependent in holD strains

The DSBs caused by the *holD* mutation in *recBC* and *recA* Gam$^+$ cells may result from pauses of the replication forks

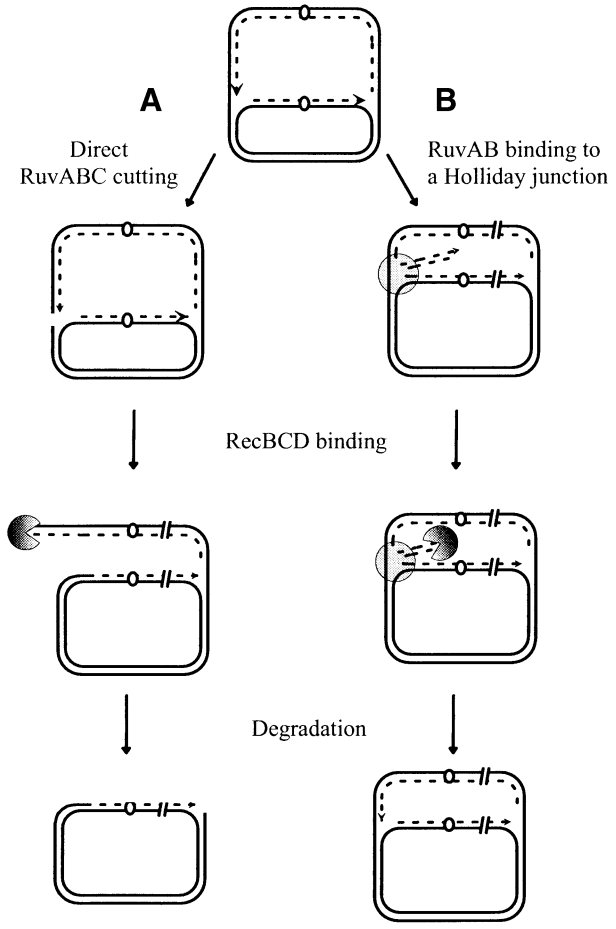

Fig. 2. In *recA* strains, extensive DNA degradation is expected if blocked replication forks are directly broken, not if they form a Holliday junction. (**A**) The fork is directly broken. (**B**) A Holliday junction is formed.

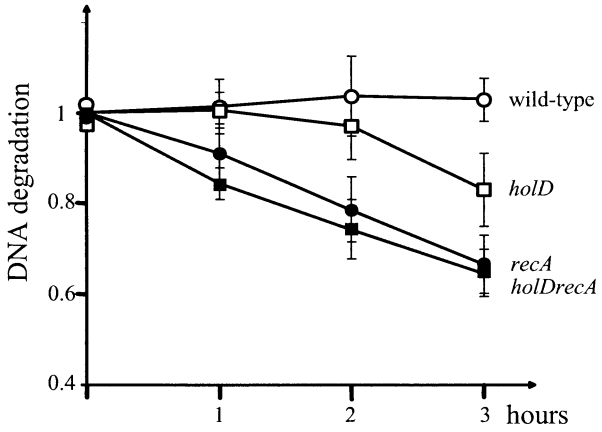

Fig. 3. DNA degradation in *recA* strains is not significantly affected by the *holD* mutation. DNA degradation was measured at different times as described previously (see Materials and methods; Seigneur *et al*., 1998). Open circles, JJC520 (wild type); closed circles, JJC557 (*recA*); open squares, JJC867 (*holD*); closed squares, JJC868 (*holD recA*). Results are the average of 3–5 independent determinations.

when the γ complex fails to promote the lagging strand synthesis. Replication fork arrest caused by the inhibition of a replicative helicase was previously shown to cause chromosomal DSBs that result from the action of a Holliday junction specific enzyme: the RuvABC complex (Michel *et al*., 1997; Seigneur *et al*., 1998; reviewed in Michel, 2000). The RuvAB complex binds specifically to Holliday junctions and promotes branch migration of recombination intermediates. The RuvC endonuclease binds to the RuvAB–DNA complex and catalyzes Holliday junction resolution (reviewed in West, 1997). We tested whether RuvABC plays a role in DSB formation in *holD* mutants. For unknown reasons, we could not introduce the *ruvABC* mutation into the *holD recBts recCts* strain. Therefore, we tested the effects of the *ruvABC* mutation in the *holD recA* Gam$^+$ cells. Inactivation of RuvABC led to a significant decrease in the level of DSB formation from 53 to 28% (compare JJC868[pGam] and JJC1294[pGam]; Table IV). Consequently, the *holD* mutation did not significantly modify the level of DSBs in a *ruvABC* context (compare JJC1294[pGam] and JJC1367[pGam]; Table IV). The amount of linear DNA was almost fully restored by the introduction of *ruvABC* wild-type genes on a plasmid (JJC1294[pGam][pRuvABC]; Table IV), whereas the presence of a plasmid carrying inactive *ruvABC* genes did not significantly modify the level of breakage (26% on average; data not shown). We conclude from these experiments that the occurrence of DSBs caused by the *holD* mutation requires functional RuvABC proteins.

The holD mutation does not cause DNA degradation in recA cells

Theoretically, RuvABC-mediated DSBs could occur either by direct breakage of the chromosome (Figure 2A), or after formation of a Holliday junction (Figure 2B). These models can be distinguished in *recA* RecBCD$^+$ cells (Figure 2). Direct breakage of the *E.coli* chromosome leads to the formation of a long linear chromosomal arm, which is a substrate for exonuclease V action of RecBCD in cells defective for homologous recombination, leading to extensive DNA degradation (reviewed in Kuzminov, 1995; Figure 2A). In contrast, replication fork reversal leads to the formation of a small double-stranded tail, therefore to a non-detectable amount of DNA degradation in *recA* RecBCD$^+$ cells (Seigneur *et al*., 1998; Figure 2B). To distinguish between these two models, DNA degradation was compared in *holD*$^+$ and *holD* mutants in either *recA*$^+$ or *ΔrecA* contexts (Figure 3). As reported previously, no DNA degradation was detected in wild-type cells whereas 30–40% of the chromosomal DNA was degraded in 3 h in the *recA* single mutant (Figure 3; Skarstad and Boye, 1993). The *holD* mutation caused a modest level of DNA degradation in *recA*$^+$ cells and no detectable DNA degradation in the *recA* strain (Figure 3). The absence of *holD*-induced DNA degradation in the *recA* context is not compatible with the hypothesis of a direct breakage of chromosomes (Figure 2A) and rather supports the hypothesis of replication fork reversal (Figure 2B).

Discussion

In this work, we characterized the properties of the first *holD* mutant isolated. We show that this mutation causes lethality in combination with *recBC* mutation and

stimulates homologous recombination between tandemly repeated sequences in a RecA-dependent way. The *holD* mutation induces the formation of RuvABC-dependent DSBs in the bacterial chromosome of *recA* strains lacking exonuclease V activity. Our results point to the arrest of the entire replication machinery upon HolD defect, causing replication fork reversal and, in turn, RecA- and RecBCD-mediated homologous recombination.

Simultaneous arrest of leading and lagging strand polymerases in holD mutants

The HolD subunit of Pol III HE, also called ψ, is part of the γ complex, thought to catalyze the recycling of the lagging strand polymerase at each Okazaki fragment (reviewed in Kelman and O'Donnell, 1995). The current model of γ complex action, based on *in vitro* experiments, is as follows. Loading of the sliding clamp β at the 3′ end of the RNA primer, which allows the binding of the lagging strand core polymerase, is catalyzed by the δ subunit (Turner *et al*., 1999). This loading requires the action of χ, which clears the 3′ end of the RNA primer by displacing the primase and perhaps SSB (Glover and McHenry, 1998; Kelman *et al*., 1998; Yuzhakov *et al*., 1999). The γ polypeptide binds and hydrolyzes ATP, inducing a conformational change in the γ complex that exposes δ (Turner *et al*., 1999; Ason *et al*., 2000 and references therein). ψ, encoded by *holD*, acts as a bridge between the ATPase γ and the protein χ (Xiao *et al*., 1993; Glover and McHenry, 2000). Although the precise role of ψ has remained elusive, it may contribute to the correct positioning of the γ and χ polypeptides for the energy-driven loading of the β clamp at the onset of Okazaki fragment synthesis. *In vitro*, the absence of χ decreases the processivity of Pol III HE, which indicates that χ is involved in efficient chain elongation (Kelman *et al*., 1998). *In vivo*, a mutation that affects the onset of Okasaki fragment synthesis could theoretically cause the formation of gaps in the lagging strand if lagging strand synthesis restarts after interruption, or the formation of a large single-stranded DNA region on the lagging strand at the fork if the synthesis on this strand does not restart while the leading strand synthesis goes on. The presence of numerous and/or large gaps is expected (i) to increase the UV sensitivity of the strain, since the nucleotide excision repair proteins do not act on single-stranded DNA, (ii) to increase the UV sensitivity of *recF* mutants, since lesions on gapped DNA are repaired mainly by RecF-mediated homologous recombination and (iii) to stimulate RecF-mediated homologous recombination. In contrast, our results show that (i) the *holD* mutant is only mildly sensitive to UV irradiation, (ii) the *holD* mutation does not increase the sensitivity to UV irradiation of a *recF* strain significantly and (iii) recombination between tandemly repeated sequences is not affected by a *recF* mutation. We conclude that the *holD* mutation characterized in this work does not lead to the accumulation *in vivo* of single-strand DNA on the chromosome, hence to the uncoupling of leading and lagging strand synthesis. This suggests that, *in vivo*, impairment of the recycling of the lagging strand polymerase by a mutation in the γ complex may lead to the arrest of the entire replication machinery.

A model for RuvABC-dependent DSBs in a holD recBC context

In contrast to the lack of effect of the *recF* mutation in the *holD* mutant, the RecBC complex is essential for viability in this strain. Therefore, for viability the strain requires a protein that acts at double-stranded DNA ends. However, the low level of DNA degradation in the *holD* single mutant and the observation that DNA degradation is not increased by the *holD* mutation in the *recA* strain argue against the formation of a high amount of linear DNA in *holD* or *holD recA* strains. Furthermore, previous studies have shown that to survive the occurrence of DSBs in the duplicated part of its chromosome, *E.coli* requires a functional RecA protein (Cromie *et al*., 2000; reviewed in Kuzminov, 1995). In contrast, we found that the *holD recA* strain is viable. Altogether, these observations suggest that most of the linear chromosomes detected in the *holD recBts recCts* strain do not form in *holD* single mutants, hence that RecBCD prevents breakage.

The DSBs caused by the *holD* mutation depend on the presence of the RuvABC proteins. These proteins have been shown previously to act at arrested replication forks blocked by the inactivation of a replicative helicase (Seigneur *et al*., 1998; reviewed in Michel, 2000). Based on genetic analysis of the *rep* mutant, defective for a helicase that participates in chromosome replication, a model was proposed for RuvABC-dependent DSB formation upon replication fork arrest (Figure 4). In a first step, a Holliday junction forms at blocked forks by annealing of neo-synthesized strands (Figure 4, A); then, binding of the RuvAB complex extends a double-stranded tail on which RecBCD can act (Figure 4, B); finally, RecBC- and RecA-mediated homologous recombination leads to re-integration of the tail into the chromosome, which, in turn, allows replication restart (Figure 4, C1–C2). Resolution of the Holliday junction by RuvC restores a viable chromosome (Figure 4, C3). Alternatively, RecBCD-mediated DNA degradation and removal of the RuvABC complex can lead to the restoration of a replication fork (Figure 4, D). In *recBC* or *recA recD* mutants, both the homologous recombination (Figure 4, C1–C3) and the degradation pathways (Figure 4, D) are inactive, and resolution of the Holliday junction by RuvC leads to a DSB (Figure 4, E). This model can remarkably explain our observations in the *holD* strain. It accounts for the lethality of the *holD recBC* and *holD recA recD* mutants, and for the formation of RuvABC-dependent DSBs (Figure 4, E). It can also explain the paradoxical observation that the *holD recA* mutant relies on exonuclease V activity for viability, whereas no DNA degradation caused by the *holD* mutation was detected in this strain. Indeed, degradation of a short DNA double-stranded tail would be sufficient for viability and would not be detectable (Figure 4, D). We could not test directly whether DSBs in the *holD recBts recCts* strain result from RuvABC action; however, the weak amount of DNA degradation in the *holD* mutant suggests that most of the linear DNA detected by PFGE may not be formed in the presence of RecBC. Therefore, we propose that most of the DSBs detected in the *holD recBts recCts* strain also result from the action of RuvABC on reversed replication forks. The similarity of the properties of helicase and *holD* mutants reinforces the idea that in *holD* cells the two DNA

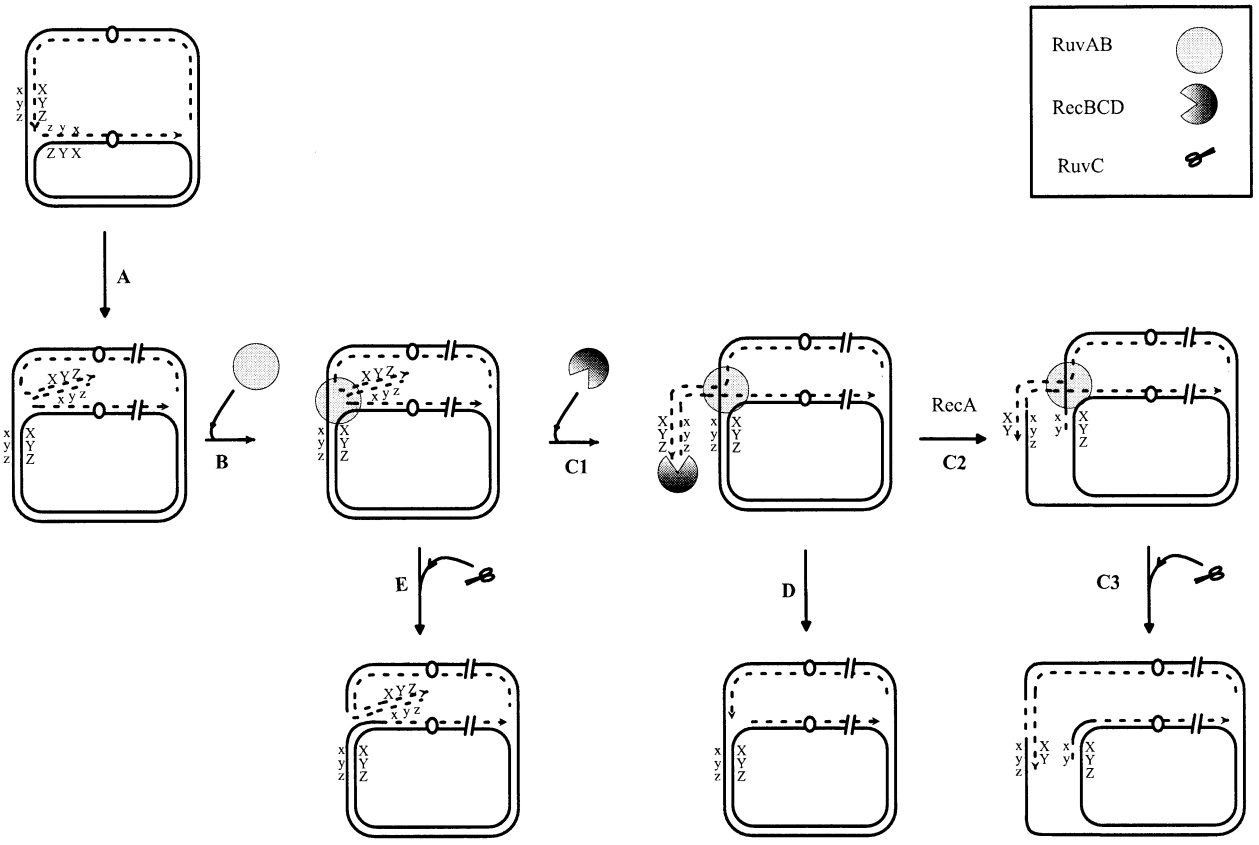

Fig. 4. Model for processing of arrested replication forks by recombination proteins in the *holD* mutants (adapted from Seigneur *et al*., 1998). In step A, the replication fork is blocked due to a defect in the lagging strand polymerase in the *holD* mutant. The two newly synthesized strands anneal, forming a Holliday junction that is stabilized by RuvAB binding (step B). Pathway C: RecBCD binds to the double-stranded tail (C1) and initiates a genetic exchange mediated by RecA (C2), RuvC resolves the first Holliday junction bound by RuvAB (C3). Pathway D: RecBCD-mediated degradation of the tail progresses up to the RuvAB-bound Holliday junction. Replication can restart when RecBCD has displaced the RuvAB complex. Pathway E: RuvC resolves the RuvAB-bound Holliday junction. Breakage at both forks results in the linear DNA detected by PFGE. Continuous and discontinuous lines represent the template and the newly synthesized strands of the chromosome, respectively; the arrow indicates the 3′ end of the growing strand.

polymerases are arrested in concert when the mutated HolD protein fails to function.

Alternative pathways for tandem repeat recombination

Stimulation of recombination between tandemly repeated sequences by mutations in the holoenzyme Pol III has been reported previously (Bierne *et al*., 1997b; Saveson and Lovett, 1997). In *dnaEts* strains, affected in the catalytic polymerase subunit α, stimulation of deletion was independent of recombination proteins and was therefore proposed to occur by replication slippage (Bierne *et al*., 1997b). In the *holD* strain described here, RecBCD-mediated recombination is taking place, since RecBC inactivation is lethal. We propose that RecBCD acts at reversed replication forks to restore a viable chromosome by re-incorporation of the double-stranded tail generated by fork reversal (Figure 4, C). Unequal crossing over during the fork repair process would cause the increased recombination between tandemly repeated sequences. Similarly to the *holD* mutation, *dnaBts* mutations increased tandem repeat deletion (Saveson and Lovett, 1997, 1999) and caused RuvABC-dependent DSBs (Michel *et al*., 1997; Seigneur *et al*., 1998). The hyper-recombination phenotype of *dnaBts* mutants was dependent on RecA and RecBCD, and could also result from the re-incorporation of a double-stranded tail formed by replication fork reversal. Our results show that impairment of different components of the replisome can stimulate recombination at arrested replication fork by different mechanisms. They support a new model of recombination between tandemly repeated sequences, which implies a replication arrest due to a DNA polymerase mutation, and erroneous repair of reversed replication forks.

Replication fork reversal

Recent studies suggest that the reversal of replication forks can occur by several different pathways in *E.coli*, depending on the cause of replication arrest. RecA is required for the formation of the RuvABC substrate in the *dnaBts* strain, whereas it is not required in a *rep* mutant (Seigneur *et al*., 2000). In the *holD* strain, a high level of RuvABC-catalyzed DSBs is observed in *recA* mutants (Table IV), indicating that RecA is not essential for their formation. The observation that RuvABC inactivation does not restore the viability of the *holD recBts recCts* strain at high temperature, whereas it renders the *rep recBts recCts* strain thermoresistant, may be due to

different mechanisms of replication fork reversal in *rep* and *holD* mutants. The RecG protein, which can bind to forked DNA and to Holliday junctions *in vitro*, was proposed to catalyze replication fork reversal at forks blocked by UV lesions (McGlynn and Lloyd, 2000). In contrast, RecG does not seem to act at forks blocked by helicase inactivation, since *recG* mutation has no effect on RuvABC-dependent DSBs in a *rep* mutant and only a minor effect in *dnaBts* strains (M.Seigneur and B.Michel, unpublished results). A putative role of RecG in replication fork reversal in *holD* strains remains to be tested.

We can imagine three pathways for the re-establishment of a functional replication fork after reversal: (i) re-integration by homologous recombination (Figure 4, C); (ii) degradation of the double-stranded tail by an exonuclease (Figure 4, D); and (iii) migration of the Holliday junction back to the original configuration by a branch migration enzyme (in Figure 4, step A is a reversible reaction). In *E.coli rep* and *holD* mutants, the latter pathway is either rare or does not lead to the formation of a viable chromosome since RecBCD is essential for viability. Homologous recombination may be favored because it facilitates the binding of the primosomal proteins, a set of proteins that assemble at recombination intermediates to load a new replisome (Kuzminov and Stahl, 1999; Motamedi *et al*., 1999; reviewed in Kogoma, 1997; Marians, 2000; Sandler and Marians, 2000). The distribution of the recombination hotspot CHI is not random on the *E.coli* chromosome (Blattner *et al*., 1997). The bias in the orientation of CHI sites is such that RecBCD progressing on the double-stranded tail formed by replication fork reversal encounters CHI sites in the active orientation very frequently (one every 5 kb on average; Blattner *et al*., 1997), which stimulates homologous recombination. Consequently, re-integration of a reversed fork by homologous recombination may be the major pathway in Rec$^+$ cells, causing the hyper-recombination phenotype of the *holD* strain. Nevertheless, this is not the only pathway since *holD recA* and *rep recA* strains (Seigneur *et al*., 1998) are viable. The lethality of *rep recD recA* and *holD recD recA* combinations indicates that DNA degradation is the alternative pathway. The mechanisms of re-establishment of a replication fork may depend on the enzymes that have catalyzed the formation of the Holliday junction. For example, RecG was proposed to promote both the formation of reversed forks and their branch migration back to the original configuration during UV repair (McGlynn and Lloyd, 2000).

In eukaryotes, reversed forks could presumably be reset either by homologous recombination or by backward branch migration. The former model is supported by the observation that replication can be initiated from a recombination intermediate formed by DSB repair (Chen *et al*., 1998; Holmes and Haber, 1999; reviewed in Haber, 2000). The latter model is supported by the properties of eukaryotic enzymes belonging to the RecQ subfamily of helicases, which have been proposed to catalyze such a reaction. Sgs1 in *Saccharomyces cerevisiae*, Rqh in *Schizosaccharomyces pombe* and the Bloom syndrome protein (BLM) in human were shown to act at Holliday junctions, and were proposed to participate in genome replication by disrupting Holliday junctions formed upon replication arrest (Doe *et al*., 2000; Gangloff *et al*., 2000; Karow *et al*., 2000).

Hyper-recombination associated with a mutation in a DNA polymerase gene or with the encounter of replication forks with a replication barrier was reported in yeast (Zou and Rothstein, 1997; Kobayashi *et al*., 1998; Defossez *et al*., 1999; reviewed in Rothstein *et al*., 2000). Furthermore, the existence of links between replication impairment and recombination was also described in higher eukaryotes (Sonoda *et al*., 1998; Karow *et al*., 2000). The present work is a new illustration of a role for DNA recombination proteins during replication progression. It extends the replication fork reversal model to cells affected in a DNA polymerase subunit involved in the synthesis of the lagging strand.

Materials and methods

Bacterial strains and plasmids

The bacterial strains used in this study are shown in Table I. The construction of strain JJC520, which carries a 624 bp duplication in the *lacZ* gene adjacent to a Cm^R gene, the mutagenesis of JJC520 to isolate hyper-recombination clones and the mapping of the mutation by Hfr crossing have been described previously (Bierne *et al*., 1997a,b). All *E.coli* strains used in this study were constructed by classical techniques of P1 transduction or Hfr conjugation, and grown on standard media, Luria broth supplemented with 25 mg/ml thymine and minimal medium M63 (Miller, 1992). The UV-sensitive phenotype of *lexAind* and *recA*, *recBC*, *recF* mutants and the exonuclease-deficient phenotype conferred by the *recBCD* mutation were verified as described previously (Seigneur *et al*., 1998). The sensitivity to rich medium of *holD* strains was systematically verified for each experiment. To measure deletion frequencies, isolated colonies were picked from minimal medium plates and resuspended in 1 ml of minimal medium. Dilutions of these bacterial suspensions were plated on several minimal medium plates containing X-gal. LacZ$^+$ and *lacZ* colonies were counted; the average of the proportion of LacZ$^+$ among *lacZ* clones in several colonies was calculated.

pGBara-recA and pSF117 (pBR322-Gam$^+$, called p-Gam here) were described previously (Michel *et al*., 1997). pAM-holD was constructed by cloning in the pAM34 vector (Gil and Bouché, 1991) a 937 bp PCR fragment carrying the *holD* gene. This plasmid carries an inducible replication origin that functions only in the presence of IPTG. Segregation of the plasmid was performed in minimal medium. Overnight cultures grown in the presence of 50 μg/ml IPTG and 40 μg/ml ampicillin were diluted 1000-fold in minimal medium devoid of IPTG and of ampicillin. These overnight cultures were diluted again 1000-fold in the same medium. The second cultures propagated in the absence of IPTG either did not grow, indicating that cured cells were not viable (e.g. *holD recA recD* strain), or contained >99% of cured bacteria that could be isolated by plating and tested (e.g. *holD* and *holD recA* strains). *holD recF* and *holD recBts recCts* strains were constructed by the introduction of *recF* or *recBts recCts* mutations in the *holD* [pAM-holD] strain, respectively, and curing of the plasmid by propagation in the absence of IPTG as described above.

Genetic tests to ascertain the presence of an amber suppressor mutation in JJC520 were a plasmid carrying an α-amylase amber mutant gene [in contrast to a previous mis-interpretation (Bierne *et al*., 1997b), this test does not allow identification of the amino acid incorporated; Declerck *et al*., 1990] and λ^{am} phages. The λ^{am} phages allowed it to be determined that the suppressor could not be *supG*, therefore the *lysT* gene was not sequenced.

Sequence analysis and research of a suppressor mutation

DNA preparation and sequence analysis were performed as described (Seigneur *et al*., 1998). The *holD* locus was amplified by PCR from JJC520 ($holD_{520}^+$) and JJC867 ($holD_{867}$) colonies. The sequence of two independent PCR products from each strain was determined entirely on one strand with the use of three primers: GTGGTGGAATTCCTG-GGTATG, TACAGCAACTGGGCATTACCCA and GAAGCACGG-AATTCCAGCCATA.

To identify the suppressor mutation carried in JJC520, known amber suppressor tRNA genes were sequenced. *glnUW*, *glnXV*, *leuX*, *serU*, *tyrT*,

tyrU, *trpT* were amplified by PCR. The sequence was determined entirely on both strands with the use of two primers. Sequences were compared with the DNA sequences in GenBank (Blattner *et al*., 1997) and found to be identical, showing the absence of any mutation in these tRNA genes. We then attempted to clone the suppressor gene from JJC520. Chromosomal DNA purified from JJC520 and partially digested with either *Sau*3A, *Eco*RI, *Hin*dIII or both *Eco*RI and *Hin*dIII was cloned in pBR322 or pGB2 vectors. These banks were used to transform an indicator strain, JJC942, that carried a *his* and a *trp* amber mutation, selecting for His^+ Trp^+ clones. No gene that conferred a His^+ Trp^+ phenotype could be isolated, whereas, as expected, the *his* and *trp* wild-type alleles were cloned. We conclude that the strain may carry a non-clonable or multigenic amber suppressor.

Measurement of DSBs by PFGE

Measurement of DSBs was performed as described previously (Seigneur *et al*., 1998). Briefly, for chromosome labeling, cells were grown in minimal medium in the presence of tritiated thymidine and deoxyadenosine. Cells were collected, washed and embedded in agarose plugs. Gentle lysis was performed in plugs. Plugs were used for PFGE and the proportion of DNA migrating was determined by cutting each lane into slices and counting the tritium present in the wells and in the gel slices (Seigneur *et al*., 1998). To avoid DNA damage during PFGE, the apparatus was routinely washed with 0.1% SDS.

Measurement of DNA degradation

Measurement of DNA degradation was as described previously (Seigneur *et al*., 1998). Briefly, cells were grown in the presence of tritiated thymidine and deoxyadenosine at 37°C until OD 0.4. Cells were then collected, washed, suspended at OD 0.15 in cold medium containing 40 μg/ml cold thymidine and incubated at 37°C. The amount of trichloroacetic acid (TCA)-precipitated tritium was determined at time 0 (transfer to cold medium) and every hour for 3 h. TCA-precipitated DNA was bound to Millipore filters, washed on a Millipore multi-filtration unit and counted in a Beckman counter.

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References

Amundsen,S.K., Taylor,A.F. and Smith,G.R. (2000) The RecD subunit of the *Escherichia coli* RecBCD enzyme inhibits RecA loading, homologous recombination and DNA repair. *Proc. Natl Acad. Sci. USA*, **97**, 7399–7404.

Anderson,D.G. and Kowalczykowski,S.C. (1997) The translocating RecBCD enzyme stimulates recombination by directing RecA protein onto ssDNA in a χ-regulated manner. *Cell*, **90**, 77–86.

Ashley,C.T.J. and Warren,S.T. (1995) Trinucleotide repeat expansion and human disease. *Annu. Rev. Genet.*, **29**, 703–728.

Ason,B., Bertram,J.G., Hingorani,M.M., Beechem,J.M., O'Donnell,M., Goodman,M.F. and Bloom,L.B. (2000) A model for *Escherichia coli* DNA polymerase III holoenzyme assembly at primer/template ends—DNA triggers a change in binding specificity of the γ complex clamp loader. *J. Biol. Chem.*, **275**, 3006–3015.

Bierne,H., Seigneur,M., Ehrlich,S.D. and Michel,B. (1997a) *uvrD* mutations enhance tandem repeat deletion in the *Escherichia coli* chromosome via SOS induction of the RecF recombination pathway. *Mol. Microbiol.*, **26**, 557–567.

Bierne,H., Vilette,D., Ehrlich,S.D. and Michel,B. (1997b) Isolation of a *dnaE* mutation which enhances RecA-independent homologous recombination in the *Escherichia coli* chromosome. *Mol. Microbiol.*, **24**, 1225–1234.

Blattner,F.R. *et al*. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science*, **277**, 1453–1474.

Chen,C., Umezu,K. and Kolodner,R.D. (1998) Chromosomal rearrangements occur in *S.cerevisiae rfa1* mutator mutants due to mutagenic lesions processed by double-strand-break repair. *Mol. Cell*, **2**, 9–22.

Churchill,J.J., Anderson,D.G. and Kowalczykowski,S.C. (1999) The RecBC enzyme loads RecA protein onto ssDNA asymmetrically and independently of χ, resulting in constitutive recombination activation. *Genes Dev.*, **13**, 901–911.

Cromie,G.A., Millar,C.B., Schmidt,K.H. and Leach,D.R.F. (2000) Palindromes as substrates for multiple pathways of recombination in *Escherichia coli*. *Genetics*, **154**, 513–522.

Declerck,N., Joyet,P., Gaillardin,C. and Masson,J.M. (1990) Use of amber suppressors to investigate the thermostability of *Bacillus licheniformis* α-amylase. Amino acid replacements at 6 histidine residues reveal a critical position at His-133. *J. Biol. Chem.*, **265**, 15481–15488.

Defossez,P.A., Prusty,R., Kaeberlein,M., Lin,S.J., Ferrigno,P., Silver,P.A., Keil,R.L. and Guarente,L. (1999) Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol. Cell*, **3**, 447–455.

Djian,P. (1998) Evolution of simple repeats in DNA and their relation to human disease. *Cell*, **94**, 155–160.

Doe,C.L., Dixon,J., Osman,F. and Whitby,M.C. (2000) Partial suppression of the fission yeast rqh1(–) phenotype by expression of a bacterial Holliday junction resolvase. *EMBO J.*, **19**, 2751–2762.

Gangloff,S., Soustelle,C. and Fabre,F. (2000) Homologous recombination is responsible for cell death in the absence of the Sgs1 and Srs2 helicases. *Nature Genet.*, **25**, 192–194.

Gil,D. and Bouché,J.P. (1991) ColE1-type vectors with fully repressible replication. *Gene*, **105**, 17–22.

Glover,B.P. and McHenry,C.S. (1998) The χ Ψ subunits of DNA polymerase III holoenzyme bind to single-stranded DNA-binding protein (SSB) and facilitate replication of an SSB-coated template. *J. Biol. Chem.*, **273**, 23476–23484.

Glover,B.P. and McHenry,C.S. (2000) The DnaX-binding subunits δ′ and Ψ are bound to γ and not τ in the DNA polymerase III holoenzyme. *J. Biol. Chem.*, **275**, 3017–3020.

Haber,J.E. (2000) Recombination: a frank view of exchanges and vice versa. *Curr. Opin. Cell Biol.*, **12**, 286–292.

Holmes,A.M. and Haber,J.E. (1999) Double-strand break repair in yeast requires both leading and lagging strand DNA polymerases. *Cell*, **96**, 415–424.

Karow,J.K., Constantinou,A., Li,J.L., West,S.C. and Hickson,I.D. (2000) The Bloom's syndrome gene product promotes branch migration of Holliday junctions. *Proc. Natl Acad. Sci. USA*, **97**, 6504–6508.

Kelman,Z. and O'Donnell,M. (1995) DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. *Annu. Rev. Biochem.*, **64**, 171–200.

Kelman,Z., Yuzhakov,A., Andjelkovic,J. and O'Donnell,M. (1998) Devoted to the lagging strand—the χ subunit of DNA polymerase III holoenzyme contacts SSB to promote processive elongation and sliding clamp assembly. *EMBO J.*, **17**, 2436–2449.

Kobayashi,T., Heck,D.J., Nomura,M. and Horiuchi,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.

Kogoma,T. (1997) Stable DNA replication: interplay between DNA replication, homologous recombination and transcription. *Microbiol. Mol. Biol. Rev.*, **61**, 212–238.

Kowalczykowski,S.C., DixonA.K., Eggleston,S.D., Lauder,W. and Rehauer,M. (1994) Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.*, **58**, 401–465.

Kuzminov,A. (1995) Collapse and repair of replication forks in *Escherichia coli*. *Mol. Microbiol.*, **16**, 373–384.

Kuzminov,A. (1999) Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage λ. *Microbiol. Mol. Biol. Rev.*, **63**, 751–813.

Kuzminov,A. and Stahl,F.W. (1999) Double-strand end repair via the RecBC pathway in *Escherichia coli* primes DNA replication. *Genes Dev.*, **13**, 345–356.

Lin,J.J. and Sancar,A. (1992) (A)BC excinuclease—the *Escherichia coli* nucleotide excision repair enzyme. *Mol. Microbiol.*, **6**, 2219–2224.

Lovett,S.T., Drapkin,P.T., Sutera,V.A. and Gluckman-Peskind,T.J. (1993) A sister-strand exchange mechanism for *recA*-independent deletion of repeated DNA sequences in *Escherichia coli*. *Genetics*, **135**, 631–642.

Marians,K.J. (2000) PriA-directed replication fork restart in *Escherichia coli*. *Trends Biochem. Sci.*, **25**, 185–189.

McGlynn,P. and Lloyd,R.G. (2000) Modulation of RNA polymerase by (P)ppGpp reveals a RecG-dependent mechanism for replication fork progression. *Cell*, **101**, 35–45.
Michel,B. (2000) Replication fork arrest and DNA recombination. *Trends Biochem. Sci.*, **25**, 173–178.
Michel,B., Ehrlich,S.D. and Uzest,M. (1997) DNA double strand breaks caused by replication arrest. *EMBO J.*, **16**, 430–438.
Miller,J.H. (1992) *A Short Course in Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Motamedi,M.R., Szigety,S.K. and Rosenberg,S.M. (1999) Double-strand-break repair recombination in *Escherichia coli*: physical evidence for a DNA replication mechanism in vivo. *Genes Dev.*, **13**, 2889–2903.
Murphy,K.C. (1991) λ-Gam protein inhibits the helicase and χ-stimulated recombination activities of *Escherichia coli* RecBCD enzyme. *J. Bacteriol.*, **173**, 5808–5821.
Naktinis,V., Turner,J. and O'Donnell,M. (1996) A molecular switch in a replication machine defined by an internal competition for protein rings. *Cell*, **84**, 137–145.
Nichols,B.P., Shafiq,O. and Meiners,V. (1998) Sequence analysis of Tn*10* insertion sites in a collection of *Escherichia coli* strains used for genetic mapping and strain construction. *J. Bacteriol.*, **180**, 6408–6411.
Rothstein,R., Michel,B. and Gangloff,S. (2000) Replication fork pausing and recombination or 'gimme a break'. *Genes Dev.*, **14**, 1–10.
Sandler,S.J. and Marians,K.J. (2000) Role of PriA in replication fork reactivation in *Escherichia coli*. *J. Bacteriol.*, **182**, 9–13.
Saveson,C.J. and Lovett,S.T. (1997) Enhanced deletion formation by aberrant DNA replication in *Escherichia coli*. *Genetics*, **146**, 457–470.
Saveson,C.J. and Lovett,S.T. (1999) Tandem repeat recombination induced by replication fork defects in *Escherichia coli* requires a novel factor, RadC. *Genetics*, **152**, 5–13.
Seigneur,M., Bidnenko,V., Ehrlich,S.D. and Michel,B. (1998) RuvAB acts at arrested replication forks. *Cell*, **95**, 419–430.
Seigneur,M., Ehrlich,D. and Michel,B. (2000) Resolution of Holliday junctions by RuvABC prevents dimer formation in *rep* mutants and UV-irradiated cells. *Mol. Microbiol.*, **37**, 180–191.
Singer,M. *et al*. (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.*, **53**, 1–24.
Skarstad,K. and Boye,E. (1993) Degradation of individual chromosomes in RecA mutants of *Escherichia coli*. *J. Bacteriol.*, **175**, 5505–5509.
Sonoda,E., Sasaki,M.S., Buerstedde,J.M., Bezzubova,O., Shinohara,A., Ogawa,H., Takata,M., YamaguchiIwai,Y. and Takeda,S. (1998) Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *EMBO J.*, **17**, 598–608.
Turner,J., Hingorani,M.M., Kelman,Z. and O'Donnell,M. (1999) The internal workings of a DNA polymerase clamp-loading machine. *EMBO J.*, **18**, 771–783.
Umezu,K., Chi,N.-W. and Kolodner,R. (1993) Biochemical interaction of the *Escherichia coli* RecF, RecO and RecR proteins with RecA protein and single-stranded DNA binding protein. *Proc. Natl Acad. Sci. USA*, **90**, 3875–3879.
Walker,G.C. (1996) The SOS response of *Escherichia coli*. In Neidhardt,F.C. *et al*. (eds), *Escherichia coli and Salmonella typhimurium*, *Cellular and Molecular Biology*, 2nd edn. American Society for Microbiology, Washington, DC, pp. 1400–1417.
Webb,B.L., Cox,M.M. and Inman,R.B. (1997) Recombinational DNA repair: the RecF and RecR proteins limit the extension of RecA filaments beyond single-strand DNA gaps. *Cell*, **91**, 347–356.
West,S.C. (1997) Processing of recombination intermediates by the RuvABC proteins. *Annu. Rev. Genet.*, **31**, 213–244.
Xiao,H., Crombie,R., Dong,Z., Onrust,R. and O'Donnell,M. (1993) DNA polymerase III accessory proteins. III. *holC* and *holD* encoding χ and Ψ. *J. Biol. Chem.*, **268**, 11773–11778.
Yuzhakov,A., Kelman,Z. and O'Donnell,M. (1999) Trading places on DNA—a three-point switch underlies primer handoff from primase to the replicative DNA polymerase. *Cell*, **96**, 153–163.
Zou,H. and Rothstein,R. (1997) Holliday junctions accumulate in replication mutants via a RecA homolog-independent mechanism. *Cell*, **90**, 87–96.

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