Chromosome duplication is regulated by the recruitment of the replication machinery to specific initiation sites (origins) where two forks are established and move in opposite directions until they meet either an opposing fork or the end of a chromosome. In *Escherichia coli*, DnaA protein controls replication initiation of the circular chromosome at oriC (1). Two replisomes are recruited and proceed in opposite directions until they meet within a specialized termination zone opposite oriC. This zone is flanked by ter sequences (terA to J), which, if bound by Tus protein, form polar traps that restrict fork movement (2). This divides the chromosome into two replication forks—one replicated by the fork moving clockwise, the other by the fork moving counterclockwise (3). The majority of highly transcribed genes within each replication fork are oriented in the same direction as replication, thereby minimizing head-on collisions between the transcription and replication machineries, which were suggested to be particularly problematic (4–7) (Fig. 1A).

Replication can initiate independently of DnaA and oriC. This origin-independent stable DNA replication (SDR) is observed in cells with defects in nucleic acid metabolism. In cells lacking RNase HI (encoded by the rnhA gene), SDR is sufficiently robust to sustain growth independently of oriC firing, especially on
minimal agar (8–10) (Fig. 1B). Thus, rnhA cells carrying a temperature-sensitive dnaA46 allele (referred to as dnaA in the rest of the text) grow at a restrictive temperature of 42°C (Fig. 1B). Because RNase HI removes RNA from DNA:RNA hybrid duplexes (11), Kogoma and coworkers suggested that SDR initiates at R-loops (8). Similarly, RecG protein can unwind the RNA from R-loops (12, 13), and recG cells also show increased levels of SDR (14, 15), suggesting that SDR in both backgrounds might have a common underlying mechanism (8, 16).

Recently we published results indicating that RecG helicase is a key player for processing intermediates arising from the fusion of two replication forks (10, 15, 17, 18). Replication profiles determined by high-resolution marker frequency analysis (19, 20) revealed that the vast majority of SDR in exponentially growing recG single mutants occurs in the termination area (10). Our genetic and cell biology data are consistent with the idea that fusion of two approaching replisomes can result in the formation of a 3° flap. 3° flaps are normally processed by RecG or 3° exonucleases. How-
ever, in the absence of RecG, a fraction of these 3' flaps persist and are processed instead by PriA, which triggers the formation of additional replication forks, which then start to resequence the already replicated DNA (10). Normally, this overreplication is contained by the ter/Tus replication fork trap, explaining why temperature-sensitive dnaA [dnaA(Ts)] recG cells are unable to grow at restrictive temperature (Fig. 1B). Upon inactivation of the replication fork trap by deletion of tus, synthesis can proceed, but it does so against the normal orientation of replication, resulting in conflicts with ongoing transcription (10). These conflicts can be alleviated by an rpoB*35 point mutation, which reduces the ability of transcribing RNA polymerase complexes to pause and backtrack (21), thereby reducing the conflicts between replication and transcription (21, 22). Indeed, recG tus rpoB*35 cells show robust growth in the absence of origin firing and can tolerate deletion of the entire oriC region (10).

In this study, we present evidence demonstrating that the mechanism for origin-independent synthesis in cells lacking RNase HI differs from that operating in cells lacking RecG. Our data support the suggestion that SDR in cells lacking RNase HI is mostly initiated at R-loops located at various sites along the chromosome. In contrast, R-loops do not appear to play a major role in the origin-independent synthesis in recG cells, where the majority of synthesis appears to be triggered by the formation of 3’ flaps at replication fork fusion sites. However, regardless of how origin-independent synthesis is initiated in both backgrounds, a significant number of the resulting forks proceed in the opposite direction from that normally dictated by the replichole arrangement, leading to more frequent head-on encounters with ongoing transcription complexes. These replication-transcription encounters threaten cell viability. Thus, both RecG and RNase HI are important factors for maintaining control of replication initiation, replication orientation, and limitation of fork collision events, and the absence of either causes serious problems in cell cycle control, chromosome dynamics, and genomic stability.

RESULTS

Initiation of chromosome replication is normally precisely regulated by the DnaA initiator protein and restricted to the oriC area (23). However, in the absence of RNase HI, a number of additional initiation sites away from oriC were shown by Kogoma’s laboratory, including a cluster in the termination area (8, 24). Even though termed orikS, these areas are not origins in the conventional sense, as they are not able to maintain plasmid replication if cloned into a plasmid without a functional origin in an rnhA background (25). Recently Maduike et al. published high-resolution marker frequency analyses by deep sequencing in cells lacking RNase HI (9). We have conducted similar experiments (Fig. 1C). For each strain examined, we established a replication profile based on the ratio of uniquely mapped sequence reads in a replicating sample to a nonreplicating control sample (stationary-phase wild-type cells) sequenced in parallel. The peaks observed in our profiles are more pronounced but otherwise in good agreement with those from previous studies (9, 24), suggesting that initiation of synthesis takes place in at least five chromosomal locations, as suggested in reference 9. A least-squares regression curve (see Text S1 in the supplemental material) suggests 5 main initiation sites at 0.4, 1.47, 2.24, 2.60, and 4.54 Mbp besides oriC, with potentially minor additional initiation sites at 1.98, 3.2, and 3.5 Mbp (Fig. 1C). This increased number of initiation sites is likely to be responsible for the replication profile of rnhA cells being flatter than the profile of wild-type cells. Indeed, it was shown before that recombination-dependent replication initiation in multiple locations in Haloferax volcanii cells lacking all replication origins led to a completely flat replication profile (26).

In contrast, we demonstrated recently that in cells lacking RecG, the majority of origin-independent synthesis is initiated in the termination area (10, 17), and as described in detail in the introduction, we suggested that this overreplication is the result of intermediates accumulating when replication forks fuse (10). This overreplication is efficiently blocked by the ter/Tus replication fork trap but can proceed into the replicholes upon deletion of tus. However, it does so against the normal orientation of replication, which results in conflicts with ongoing transcription (10). These conflicts can be alleviated by an rpoB*35 point mutation (10), which reduces the stability of transcribing RNA polymerase complexes (6, 21).

As synthesis is also initiated within the termination area of rnhA cells, we investigated the effect of a tus deletion. The absence of Tus in dnaA rnhA cells caused a mild reduction of growth at both 30°C and 42°C (Fig. 1D), suggesting perhaps that the release of synthesis from the termination area causes more harm than good. In contrast, introduction of an rpo* point mutation improved growth quite significantly in both dnaA rnhA and dnaA rnhA tus cells (Fig. 1D) and allowed the deletion of the entire oriC region (Fig. 1E). Marker frequency analysis of dnaA rnhA tus rpo* cells grown at the restrictive temperature revealed a relatively flat but effectively inverted replication profile (Fig. 1F), in which the major peaks observed in dnaA rnhA cells (Fig. 1C) are fused to a broadly elevated region roughly between 0.5 and 2.6 Mbp, while the oriC region shows a distinctly low marker frequency level (described below). These results are in line with the idea that a proportion of replication forks in dnaA rnhA cells initiated away from oriC will proceed in an orientation opposite to normal, thereby inducing conflicts with transcription. From these results, we conclude that the reported broth sensitivity of dnaA rnhA cells (Fig. 1B and D) (8) is, at least in part, caused by replication-transcription conflicts.

The genetic requirements for origin-independent synthesis in the termination area differ in cells lacking RecG and RNase HI. Both cells lacking RecG and those lacking RNase HI show a peak of synthesis in the termination area (Fig. 1C) (10), opening the question whether this synthesis arises by a similar mechanism (16). We showed before that initiation of origin-independent synthesis in recG cells has distinct genetic requirements, as it was dependent on PriA helicase activity (10). Even more specifically, it was entirely dependent on the specific ability of PriA helicase to process 3' flap structures (10). The srgA1 allele of priA encodes a mutant protein (PriA L557P) with a very specific alteration in its biochemical substrate specificity. It unwinds a replication fork with both a leading strand and a lagging strand at the branch point as efficiently as wild-type PriA, but it has lost the ability to unwind a fork in which the leading strand is missing (27), the equivalent of a 3’ flap. This led us to suggest that a 3’ flap structure persists in the absence of RecG, a result supported by the finding that the lack of 3' exonucleases also results in overreplication in the termination area (10, 28).

We introduced the srgA1 allele into a dnaA rnhA tus rpo* background and found that the cells grow robustly at 42°C, much in contrast to dnaA recG tus rpo* srgA1 cells (Fig. 2A). Thus, the
specific substrate activity of PriA helicase is essential for the initiation of synthesis in cells lacking RecG but not in cells lacking RNase HI, suggesting that different structures accumulate in the absence of each of these proteins. Introduction of a priA300 allele, which encodes the helicase-deficient PriA K230R (29), into a dnaA rnhA tus rpo* background reduced growth at 42°C on LB agar substantially, as observed previously (8, 30) and similar to the reduced growth seen in dnaA recG tus rpo* priA300 cells (Fig. 2A) (10). However, the replication profile of rnhA priA300 cells grown in LB broth revealed that the overreplication in the termination area was not totally abolished but only mildly reduced (Fig. 2B), much in contrast to recG priA300 cells where no trace of overreplication can be found (Fig. 2C) (10). In line with this result, we were able to demonstrate growth of dnaA rnhA tus rpo* priA300 cells on minimal medium, in contrast to dnaA recG tus rpo* priA300 cells (Fig. 2D). Thus, it appears that the helicase activity of PriA is not absolutely essential for origin-independent synthesis in cells lacking RNase HI, in line with the idea that the DNA intermediates accumulating in cells lacking RecG and RNase HI are different.

In addition, we found that chromosome linearization, which prevents the collision of replisomes, has different effects in cells lacking RecG or RNase HI. Linearization is achieved by introducing the tos linearization sequence from bacteriophage N15 near the chromosome dimer resolution site dif into the chromosome. Subsequent lysogenic infection with N15 results in the expression of the telomerase TelN, which will process the tos linearization sequence (31). We found that insertion of the tos site in rnhA cells resulted in a mild reduction of the overreplication in the termination area, a result not observed in recG cells (cf. Fig. 3Ai and 3Bi). Thus, it appears that integration of the tos linearization cassette interferes with initiation of overreplication in the termination area in rnhA but not recG cells. Linearization of the chromosome, which is clearly visible as a discontinuity in the replication profile (Fig. 3A and B; see Fig. S1 in the supplemental material) (19), caused a reduction of overreplication, both in cells lacking RecG and those lacking RNase HI. However, the resulting profiles look different. Upon linearization, the profile of rnhA cells becomes asymmetric, with no overreplication observed between tos and terA, while some overreplication is observed between terA/tos and tos, which appears abruptly cut off at the linearization site (Fig. 3Ai). In contrast, recG cells with a linearized chromosome show elevated marker frequency levels on both sides of the linearization site (Fig. 3Bi).

To investigate the effect of chromosome linearization on the ability of rnhA and recG cells to grow in the absence of origin firing, we linearized the chromosome in dnaA recG tus rpo* and dnaA rnhA tus rpo* cells. As shown in Fig. 3C, chromosome linearization showed no detectable effect on the growth of dnaA rnhA tus rpo* cells, much in contrast to dnaA recG tus rpo* cells, as reported previously (10). This suggests that the overreplication in the termination area in recG cells is responsible for the ability of dnaA recG tus rpo* cells to grow at 42°C. In contrast, for growth of
in the termination area, is observed in all chromosomal areas at a low level (17), and while synthesis at the terminus is reduced upon chromosome linearization, synthesis elsewhere is not (10). Thus, origin-independent synthesis in recG cells might arise by two independent mechanisms. The absence of growth of dnaA recG tus rpo* cells with a linearized chromosome already indicates that the synthesis observed outside the termination area is not strong enough to allow the formation of visible colonies. However, it might allow abortive growth.

To investigate whether synthesis outside the termination area can contribute to cell duplication, we directly followed the viable titer of dnaA, dnaA recG, and dnaA rnhA cells following shift to 42°C. Both dnaA and dnaA recG cells showed approximately 2 cell division events in LB broth before growth arrest (Fig. 4A), with no hint of further divisions. In contrast, dnaA rnhA cells showed continuous growth over several hours (Fig. 4A). Growth is linear rather than exponential, which is likely to be caused by the broth sensitivity of dnaA rnhA cells (Fig. 1B). (8), and dnaA rnhA cells showed robust levels of bromodeoxyuridine (BrdU) incorporation in all chromosomal areas (see Fig. S2A in the supplemental material). Thus, synthesis in dnaA rnhA cells can contribute toward successful cell duplication for significant periods of time, whereas synthesis outside the termination area in recG cells cannot.

We used fluorescent repressor-operator arrays in the origin and terminus area of the chromosome to follow chromosome dynamics in dnaA cells lacking either RecG or RNase HI (Fig. 4B). We previously reported that a dnaA background forms cells with single origin and terminus foci 120 min after shift to restrictive temperature (15). We analyzed focus counts in the previously obtained images (15) and found that 60 min following the shift to 42°C, the majority of cells showed a single origin and terminus focus (65%), indicative of a single copy of a fully replicated chromosome. An additional 18% showed 2 origin and 2 terminus foci, suggesting that the presence of two fully replicated chromosomes without septation having taken place yet.

In line with the observed arrest in cell growth (Fig. 4A), 75% of dnaA recG cells showed either a single origin and terminus focus or 2 separated chromosomes 60 min following a shift to 42°C (Fig. 4B). However, we observed an increased number of cells with aberrant focus counts. Twenty percent of dnaA recG cells showed 3 or more terminus foci (6%) in dnaA single mutants, and 7% showed 8 or more origin foci (2.5% in dnaA single mutants). This uncontrolled amplification of chromosomal areas is typical for recG cells suffering from genotoxic damage (15).

The situation proved different in cells lacking RNase HI (Fig. 4B). While the focus distribution in dnaA rnhA cells growing at 30°C was overall similar to that in dnaA cells, we observed an increased number of dnaA rnhA cells in which the number of terminus foci exceeded the number of origin foci (15%), in line with synthesis initiating at multiple sites in and near the terminus region (Fig. 1C) (9, 24). Sixty minutes after the shift to 42°C, only a small fraction of cells showed a single origin and terminus focus (6%). Instead, the number of cells in which the number of terminus foci exceeded the number of origin foci was increased to 37%, with 34% showing more than 3 terminus foci per cell (Fig. 4B).

Thus, the majority of dnaA and dnaA recG cells arrest growth upon the shift to 42°C, with a fraction of cells showing a significant amplification of limited chromosomal areas, presumably because they suffer from some form of spontaneous DNA damage (17). To

**FIG 3** Effect of chromosome linearization on origin-independent synthesis in recG and rnhA cells. (A and B) Marker frequency analysis of the termination area of E. coli cells in the exponential phase. The number of reads (normalized against the reads for a stationary-phase wild-type control) is plotted against the chromosomal location. Positions of ter sites in the area as well as the integration site of the tos linearization sequence are highlighted. In panels Bi and Cl, data sets for recG and recG tos as well as rnhA and rnhA tos are plotted together for direct comparison. Sequencing templates were isolated from AM1975 (dnaA rnhA tos), and RCe608 (dnaA rnhA tus rpo* N15 lysogen). Data for recG (NR226), recG tos (RCe391), and recG tos N15 lysogen (RCe399) were replotted from reference 10. (C) Spot dilution assays to evaluate DnaA-independent growth in dnaA rnhA cells with a linearized chromosome. The strains used were RCE309 (dnaA recG tus rpo*), JD1160 (dnaA recG tus tus rpo* tus), JD1168 (dnaA recG tus tus rpo* N15 lysogen), JD1169 (dnaA recG tus tus rpo* tus N15 lysogen), RCE268 (dnaA recG tus tus rpo* tus), RCE385 (dnaA recG tus tus rpo* tus), RCE384 (dnaA recG tus tus rpo* N15 lysogen), and RCE387 (dnaA rnhA tus tus rpo* tus N15 lysogen).

dnaA rnhA tus rpo* cells, synthesis in the termination area is completely dispensable.

**Origin-independent synthesis outside the termination area.** Origin-independent synthesis in dnaA recG cells, while prevalent

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establish how much this fraction of cells contributes to the overall synthesis visualized by BrdU incorporation, we visualized newly replicated DNA via pulse-labeling with 5-ethynyl-2′-deoxyuridine (EdU) (32). dnaA, dnaA recG, and dnaA rnhA cells were shifted to 42°C for 90 min to stop oriC firing while allowing ongoing forks to complete synthesis. Cells were then pulse-labeled with EdU for 15 min and visualized via high-resolution microscopy in flow with an Amnis ImageStream® Mark II. As shown in Fig. 4C and D, the majority of dnaA cells showed either no detectable signal (63%) or very low levels of fluorescence (33%), in line with our previous data showing that synthesis has mostly ceased in dnaA cells 90 min after the shift to the restrictive temperature (17, 23) (see Fig. S2A in the supplemental material). In contrast, 75% of dnaA rnhA cells showed robust levels of signal distributed over the entire length of the cell (Fig. 4C and D), suggesting robust levels of EdU incorporation even in the absence of oriC firing, in line with our fluorescence microscopy data (Fig. 4B), cell growth (Fig. 4A), and BrdU incorporation data (see Fig. S2A). Some of
these cells showed multiple “spots” of synthesis, which is likely to be explained by multiple replisomes generating multiple stretches of newly synthesized DNA (Fig. 4D). Only 4% of cells showed no signal. dnaA recG cells, however, showed a mixed population (Fig. 4C and D). The majority of cells showed either no fluorescence (44%) or a low signal (27%) (Fig. 4D). Thus, 71% of cells showed fluorescence levels comparable to the levels observed in a dnaA single mutant, in line with the observed 75% of cells showing either a single origin and terminus focus or two segregated chromosomal copies (Fig. 4B). It is tempting to speculate that the BrdU incorporation observed in chromosomal areas away from the termination zone stems only from a fraction of cells. Indeed, 29% of cells showed a fluorescence signal in either several distinct locations, likely caused by multiple replisomes, or distributed over the entire length of the cell (Fig. 4D), implying high levels of synthesis. These high levels of synthesis will contribute rather significantly to the BrdU signal observed outside the termination area (see Fig. S2A). We cannot exclude that some synthesis takes place away from the termination area in all recG cells. However, if this is the case the levels of synthesis must be so low that they cannot be detected in our EdU pulse-labeling assay, in line with the lack of growth observed (Fig. 4A).

Cells lacking RNase HI deal with genotoxic damage similar to wild-type cells. We demonstrated before that genotoxic stress triggers dramatic chromosomal overreplication, a filamentation phenotype, and severe segregation defects in recG cells (15, 17). To test what happens when damage-induced synthesis is triggered in cells lacking RNase HI, we determined the filamentation period after UV exposure. As shown in Fig. 5A, rnhA cells show a minor elongation of the filamentation period following UV exposure. However, this effect is moderate, and time-lapse microscopy confirmed that the filaments formed break down into normal-sized

**FIG 5** Complementation of rnhA and recG cells with E. coli RNase HI and yeast RNase H1. (A) Viable cell replication following irradiation (see Materials and Methods). The strain used was N4704 (rnhA). Data for the irradiated cells are means (± standard error [SE]) from three experiments. The data for the unirradiated cells are means from two experiments that gave almost identical values. Data for MG1655 and the recG strain were replotted from reference 15 for comparison. All experiments were performed under comparable conditions with identical equipment. (B) Filamentation of UV-irradiated recG cells with increased levels of rnhA expression. The strain used was AM2304 (recG proB::rnhA). This strain has, in addition to the functional rnhA gene, a second rnhA copy under control of the parA promoter integrated into the proB gene (33). Growth in the presence of arabinose will induce additional expression of RNase HI, whereas glucose will repress expression, resulting in native expression levels. Data for irradiated cells are means ± SE from three experiments. Data for unirradiated cells are means from two experiments with almost identical values. Data for MG1655 (wild type) and N4560 (recG) were replotted from reference 15 for comparison. Experiments were performed under comparable conditions. (C) Origin-independent synthesis in dnaA rnhA cells and dnaA recG tus rpo* cells expressing either Saccharomyces cerevisiae RNH1 (pECR22 [labeled “ScRNH1”]) or E. coli recG (pDIM104 [labeled “EcrecG”]) gene. Expression is either repressed by 0.2% glucose or stimulated by 0.05% arabinose, as indicated. All numbers represent the fraction of colonies relative to the 30°C control, which was set to 1 (dashed line). Data are means from at least 3 independent experiments (± standard deviation [SD]). Growth of the dnaA rnhA vector control is stronger in medium with arabinose, which most likely reflects the broth sensitivity of dnaA rnhA cells. The strains used were RCE552 (dnaA rnhA pECR22 [RCE557 for vector control]), SLM1008 (dnaA recG tus rpo* pECR22 [SLM1010 for vector control]), SLM1104 (dnaA rnhA pDIM104), and RCE326 (dnaA recG tus rpo* pDIM104).
cells (see Fig. S3 in the supplemental material), in contrast to recG cells (15, 17). In addition, dnaA rnhA cells show comparable levels of synthesis before and after UV irradiation (see Fig. S2A in the supplemental material), and an excessive accumulation of chromosomal areas is not observed (see Fig. S2B), highlighting that rnhA cells cope with UV-induced synthesis and the resulting increase in fork collision events similar to wild-type cells.

**Initiation of synthesis at R-loops.** The fact that both RecG and RNase HI can process R-loops in vitro (11–13) has led to the idea that origin-independent synthesis in dnaA and recG cells might be initiated at R-loops (8, 14). To investigate whether R-loops are responsible for some of the phenotypes of recG cells, we tested whether increased levels of native E. coli RNase HI can suppress the extended filamentation period in UV-irradiated recG cells (15). E. coli rnhA cannot be expressed at high levels from a multi-copy plasmid, as this results in cellular toxicity (33). We therefore used a strain in which a second functional copy of the E. coli rnhA gene under control of the parABAD arabinose-controlled promoter was integrated into the chromosome (33). This allowed us to compare the filamentation period in UV-irradiated recG cells with native (promoter repressed by glucose) or increased (arabinose-induced) levels of RNase HI. As shown in Fig. 5B, increased levels of RNase HI had no effect on the filamentation period of cells lacking RecG.

Furthermore, we investigated whether the expression of S. cerevisiae RNase HI (encoded by RNH1), which hydrolyzes the RNA from DNA:DNA hybrids (34), can suppress origin-independent synthesis in dnaA and recG cells. It was shown before that some of the phenotypes of recG cells can be complemented by the expression of yeast RNase H from a high-copy-number plasmid (35). We cloned the RNH1 gene into pBAD24 to allow arabinose-controlled transcription. Expression of yeast RNH1 indeed reduced growth of dnaA rnhA cells at 42°C over 20-fold (Fig. 5C). In contrast, it did not show any suppression of growth of dnaA recG tus rpoA* cells at 42°C (Fig. 5C). Thus, neither the expression of yeast RNH1 nor the overexpression of E. coli RNase HI shows any effect on the phenotype of recG cells, suggesting that R-loops are either not accessible to RNase HI or are not responsible for the phenotypes of recG cells. In line with this, expression of E. coli recG reduced the ability of dnaA recG tus rpoA* cells to grow at 42°C 20-fold, while growth of dnaA dnaA cells at 42°C was unaffected (Fig. 5C).

**Head-on collisions of replication and transcription severely impede cell growth.** Overreplication in recG cells depends critically on RecA recombinase, as was shown before for dnaA cells (8). We confirmed this for our dnaA dnaA tus rpoA* background (Fig. 6A). Furthermore, overreplication in recG cells required the recombinase activity of RecBCD (recB) but not the ExoV activity (recD) (10), and growth of dnaA dnaA tus rpoA* cells was completely abolished in the absence of the RuvABC Holliday junction resolvase (10). We wanted to perform a similar investigation in dnaA dnaA cells, which proved surprisingly difficult and required us to use a synthetic lethality assay. This assay employs the unstable prC7 plasmid, which is rapidly lost, with cloned genes of interest to cover for chromosomal deletions (36, 37). prC7 carries a functional lac operon, and its loss can be revealed in a lac mutant background on plates containing the β-galactosidase indicator X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) by the formation of white colonies or white sectors within blue (lac+) colonies, depending on whether plasmid loss occurred before or after plating (Fig. 6Bi).

Synthetic lethality of rnhA recB cells was reported before (38), and we confirmed that rnhA recB cells were unable to grow without an rnhA+ covering plasmid, resulting in the formation of only blue colonies (Fig. 6Bii). In contrast, rnhA recA cells proved viable (Fig. 6C). Thus, cells lacking RNase HI rely on RecB, but not RecA, for survival, much in contrast to recG recB cells, which are viable (39). Some white colonies were observed on minimal medium (Fig. 6Biv), but these were very small, indicating a strong growth defect. Origin-independent synthesis was shown to continue in rnhA(Ts) recB cells upon shift to 42°C in the absence of origin firing (40), suggesting that origin-independent synthesis is initiated, but forks have problems progressing. The fact that the broth sensitivity of dnaA rnhA cells at 42°C is suppressed by an rpoA* point mutation indicates that origin-independent synthesis in dnaA cells triggers replication-transcription conflicts. Indeed, we found that dnaA recB rpoA* cells, even though exhibiting a severe growth defect, are viable without a covering plasmid (Fig. 6Bv), in line with the idea that RecBCD is vitally important to process intermediates resulting from replication-transcription encounters, as was shown for both E. coli and Bacillus subtilis (41, 42). As dnaA recB rpoA* cells are viable, we attempted to generate a dnaA dnaA rnhA rpoA* construct to investigate growth at 42°C. However, the sickness of dnaA recB rpoA* cells led to the rapid accumulation of suppressor mutations, which made generation of this construct impossible.

We had no difficulty in generating dnaA recD cells (Fig. 6D). However, when we tried to generate a dnaA dnaA recD construct, we found cells to be extremely sick on LB broth at the permissive temperature of 30°C (Fig. 6Dii). It appears that the ExoV activity of RecBCD is dispensable in dnaA cells but becomes vital, even at permissive temperature, if a dnaA(Ts) allele is introduced, suggesting that the dnaA46 allele might have a defect even at permissive temperature, as reported previously (43, 44). In line with this hypothesis, we noted that a dnaA dnaA construct in which the dnaA46 allele is covered by a wild-type copy of dnaA on the covering plasmid showed noticeably smaller white colonies at 30°C on LB but not on minimal medium (cf. Fig. S4Ai and S4Aii in the supplemental material), while dnaA recG cells show no such difference (see Fig. S4Aiii and S4Aiv). Thus, it appears that dnaA recD cells are viable as long as oriC activity is unperturbed. As an rpoA* mutation greatly improved the viability of dnaA dnaA recD cells on LB broth at 30°C (Fig. 6D), it appears that replication-transcription encounters are responsible for the problems observed.

As dnaA dnaA recD cells are viable on minimal medium (Fig. 6Div), we were able to investigate growth at 42°C. The absence of RecD slowed growth of dnaA dnaA cells, as indicated by the slow colony formation (Fig. 6E; see Fig. S4B in the supplemental material), much in contrast to recG cells, where quicker growth was observed (10). However, colony numbers of dnaA dnaA recD cells at 42°C were only mildly reduced, supporting the idea that origin-independent synthesis in dnaA cells is able to continue in the absence of RecD, as suggested (8).

Generation of a dnaA dnaA ruvABC background also proved difficult. Surprisingly, even in the presence of a ruvABC+ covering plasmid, dnaA dnaA ruvABC cells grew very slowly (see Fig. S4Av in the supplemental material). The total absence of white colonies suggests that dnaA dnaA ruvABC cells are synthetically lethal. In
contrast to the robust viability of *rnhA* recD cells (Fig. 6D), we reported previously that *rnhA* ruvABC cells show a growth defect (18). As *dnaA* *rnhA* cells also showed a growth defect, the extremely slow growth of *dnaA* *rnhA* ruvABC cells is likely to be due to multiple harmful genetic interactions. As for recB and recD, it was reported that a deletion of ruvABC did not affect initiation of origin-independent synthesis in *rnhA* cells (8). Thus, Holliday junction resolution appears to be important in cells lacking RNase HI, especially so if *oriC* firing is impeded, and it seems to be important for the processing of replication forks, rather than being required for the initiation of origin-independent synthesis. In line with this hypothesis, we found that an rpo* point mutation improved the viability of *dnaA* *rnhA* ruvABC cells enough to allow us to generate a *dnaA* *rnhA* ruvABC rpo* *rnhA* construct, which showed small but clearly visible white colonies on LB and larger white colonies on minimal medium (Fig. 6F). Taken together, our results support the idea that the problems observed in the absence of RecB, RecD, and RuvABC...
are induced by replication-transcription conflicts, as multiple replication forks are traversing the chromosome in a direction opposite to normal.

**DISCUSSION**

Cells lacking either RNase HI or RecG show substantial levels of origin-independent replication of the chromosome, a form of replication that is independent of the DnaA initiator protein and which was originally described by Kogoma and coworkers as stable DNA replication (SDR) (8). Since both RNase HI and RecG eliminate R-loops in vitro, the former by degrading the RNA (11), the latter by unwinding RNA:DNA hybrids (12, 13), it was assumed that an accumulation of R-loops might be the trigger for SDR in both cases (8, 14). However, the data presented in this study demonstrate that this is not so. They also reveal that SDR increases conflicts between replication and transcription, thereby threatening the stability of the genome and cell viability (Fig. 7).

We found that, whereas cells lacking RecG show a pronounced initiation of SDR at a single location in the termination area (10), cells lacking RNase HI show initiation at multiple sites around the chromosome (Fig. 1), as reported previously (9, 24). One of these is located in the termination area, but the effect of chromosome linearization on synthesis in this region (Fig. 3) suggests that the mechanism responsible for this synthesis is different from that operating in the absence of RecG.

This idea is further supported by our PriA results. Although initiation of SDR depends in both cases on the ability of PriA helicase to load the replicative helicase, DnaB, at a branched DNA substrate and thereby to prime replisome assembly, we demonstrated that the substrates exploited for this purpose are different. In a previous study, it was shown that the ability of SDR to promote growth of dnaA recG tus rpo* cells at 42°C specifically requires the ability of PriA to unwind a replication fork with a lagging strand only, the equivalent of a 3' flap (10). It was suggested that 3' flaps are generated when replication forks fuse in the terminus area and that these flaps accumulate in the absence of RecG, providing a suitable template for Dnab loading. We found that this particular activity of PriA is dispensable for the initiation of SDR in dnaA rnhA tus rpo* cells (Fig. 2). Further confirmation that PriA exploits different substrates to initiate SDR in rnhA and recG cells came when we found that yeast RNH1 reduces growth of dnaA rnhA cells at 42°C 20-fold, whereas it does not reduce the viability of dnaA recG tus rpo* cells at all (Fig. 5).

These data, when taken together with studies revealing that recombination mutations inactivating RecA or eliminating components of RecBCD or RuvABC have contrasting effects on the viability of rnhA and recG cells (Fig. 6) (8, 10, 40), reinforce the idea that the SDR detected in these cells is the result of very different events: the accumulation of R-loops in the former as opposed to persisting 3' flaps in the latter (Fig. 7).

However, regardless of this difference, our studies reveal that the SDR induced in both cases has one common feature. By compromising the normal replisome arrangement, SDR leads to increased conflicts between replication and transcription (Fig. 7). This is highlighted by the impact of an rpo* mutation that destabilizes ternary transcription complexes. It strongly improves the viability of dnaA rnhA and dnaA recG cells at 42°C (Fig. 1D) (10), although in the latter case, the effect depends on the deletion of tus. This is hardly surprising, as SDR in recG cells is initiated within the replication fork trap in the terminus region, thereby preventing forks from proceeding toward oriC in the presence of functional ter/Tus complexes. Multiple initiation sites in rnhA cells avoid this difficulty. Replication-transcription collisions are severe enough to threaten the viability of rnhA cells in the absence of factors such as RecBCD (Fig. 6). RecBCD was shown before to be vital for the processing of forks stalled at transcription complexes (41, 42, 45). Recent data from *B. subtilis* suggest that restart at replication-transcription conflict sites is dependent on RecA-driven D-loop formation initiated either by RecBCD homologues or RecFOR (42). While *rnhA recB* cells are synthetically lethal (Fig. 6), both *rnhA recA* (Fig. 6) and *rnhA recF* (data not shown) double mutants could be constructed without difficulty, highlighting perhaps the importance of RecBCD for replication restart at replication-transcription conflict sites in *E. coli*, as suggested (41). RecBCD is likely to be required for processing replication-transcription conflicts in recG cells, but as overreplication in the termination area in recG cells is strictly dependent on RecB recombinase activity (10), replication-transcription conflicts simply do not arise in recG recB double mutants, explaining why they are viable.

Our results indicate that replication-transcription conflicts are a major problem for cells lacking RNase HI, an effect exacerbated when DnaA activity is compromised (Fig. 1 and 6; see Fig. S4 in the
supplemental material). The broth sensitivity of dnaA rnhA cells was observed in an earlier study but never explained (8). Our finding that an rpo* mutation suppresses this effect (Fig. 1) makes it quite clear that it is most likely the result of replication-transcription conflicts.

In B. subtilis, rrr operons have been identified as a severe impediment to DNA replication. The high levels of transcription can interfere with replication even if both processes are proceeding codirectionally (46), a result that might also be true for E. coli (47). Head-on encounters are thought to be even more deleterious than codirecional encounters. Indeed, the most dramatic effect on replication in B. subtilis cells in which a part of the chromosome is replicated opposite to normal is observed at rrr operons, an effect much alleviated in minimal salts medium (48). A comparison of our rnhA and dnaA rnhA replication profiles reveals a significant “step” that coincides with the location of the rrnCABE operon cluster (3.94 to 4.21 Mbp) (see Fig. S5 in the supplemental material), a step that is much reduced in dnaA rnhA tss rpo* cells (cf. Fig. 1C and F; see Fig. S5). This observed “step” indicates a slowing of replication forks in this area, in line with head-on collisions between replication and transcription as forks coming from the initiation site at 4.54 Mbp will progress into this area in the wrong orientation. Indeed, we observed recently that replication forks initiated at an ectopic replication origin are significantly impeded at the rrr operons (47), as reported for Bacillus subtilis (48, 49). If replication forks are slowed or blocked at rrr operons, this would also explain the very low marker frequency at 4.54 and 0.4 Mbp, which is relatively close to oriC, a higher marker frequency level might be expected unless synthesis traversing toward oriC is blocked at rrnCABE.

To conclude, our data from this as well as our previous studies (10, 47) provide strong additional in vivo evidence that codirectionality of transcription and replication and their interaction is likely to be a particularly important driving force that has shaped the chromosomal architecture in bacteria. Regardless of the mechanism of initiation, replisomes traversing the chromosome in the wrong orientation induce collision events with transcription, which require subsequent processing by recombination proteins (Fig. 7), with RecB being of particular importance, as reported previously (41, 42). The threat to the integrity of the genome is likely to be substantial. However, our data also support the idea that fork fusion events are another important factor that has shaped the chromosomal architecture (Fig. 7) (10, 47, 50). The importance of dealing with fork fusion intermediates is highlighted by the multiple proteins involved in their processing, such as RecG, 3’ exonucleases, polymerase I, and RecBCD (10, 18, 28, 51). It is significant that recG rnhA is synthetically lethal (8, 52), and it remains to be established whether this synthetic lethality might be caused by the consequences of too many unprocessed replication fork fusions, replication-transcription conflicts, or both.

**MATERIALS AND METHODS**

**Bacterial strains and general methods.** The Escherichia coli K-12 strains used are derivatives of MG1655 (see Table S1 in the supplemental material). Strains were constructed via P1vir transductions (53) or single-step gene disruptions (54). For the compositions of LB broth and 56/2 minimal medium, see Text S1 in the supplemental material. The dnaA46 allele encodes a thermosensitive DNA protein that is inactive at 42°C. For assessment of growth without DnaA initiation, cultures of dnaA46 constructed grown at 30°C to an A600 of 0.4 were diluted in 10-fold steps to 10−5 before spotting of 10-μl samples of each dilution on LB agar. Duplicate plates were incubated at 30°C and 42°C. Chromosome linearization was conducted as described previously (31). The synthetic lethality assay, fluorescence microscopy, BrdU labeling and detection via immunostaining, the determination of the multiplication of cells surviving UV irradiation, and the generation of a loess regression curve have been described before and are detailed in the supplemental material.

**Multiplication of dnaA cells following a shift to the restrictive temperature.** To quantify the number of cell divisions of dnaA46 strains following the shift to 42°C, strains were grown with vigorous aeration in LB broth to an A600 of 0.2 at 30°C. The samples were transferred into a water bath prewarmed at 42°C. Samples were removed as indicated and diluted in conditioned medium, which was created by growing the wild-type strain in fresh LB broth to an A600 of 0.2 with subsequent sterile filtration. Samples were mixed with 2.5 ml of molten 0.6% top agar kept at 42°C and plated on LB agar. Colonies were counted after incubation for 48 h at 30°C.

**Suppression of the rnhA phenotype by yeast RNH1.** For details of the generation of the yeast RNHI expression plasmid pECR22, see Text S1 in the supplemental material. Cells were grown in LB broth supplemented with ampicillin (50 μg/ml) to an A600 of 0.48 at permissive temperature (30°C). Samples were diluted in 10-fold steps from 10−1 to 10−5 before each dilution was spotted onto minimal medium supplemented with either 0.2% glucose or 0.05% arabinose. Two identical plates were generated, and one was incubated at 30°C, while the other was incubated at 42°C. Colonies were counted, and the viable titer at 30°C was set to 1. Titers at 42°C on medium containing either glucose or arabinose were calculated as a fraction of the viable titer measured at 30°C.

**Marker frequency analysis by deep sequencing.** Samples from cultures of a strain grown overnight in LB broth were diluted 100-fold in fresh broth and incubated at 37°C with vigorous aeration until an A600 reached 0.4. The culture was then diluted again 100-fold in prewarmed fresh broth and grown until an A600 of 0.4 was reached. Samples were flash-frozen in liquid nitrogen at this point for subsequent DNA extraction. Strains harboring the dnaA46 allele were grown overnight at 30°C. Upon dilution, cells were incubated for 60 min at 30°C and then shifted to 42°C for the remainder of the experiment. For exponentially growing wild-type cells, a culture was grown in parallel under the same conditions. For a stationary-phase sample, a wild-type culture was grown overnight until the culture had saturated and showed no further increase in the A600. DNA was extracted using the GenElute bacterial genomic DNA kit (Sigma-Alrich). Marker frequency analysis was performed using Illumina HiSeq 2000 sequencing (fast run) to measure sequence copy number. Enrichment of uniquely mapping sequence tags, in 1-kb windows, was calculated for an exponentially growing (replicating) sample relative to a nonreplicating stationary-phase wild-type sample to correct for differences in read depth across the genome and to allow presentation of the data as a marker frequency, as described previously (9, 19, 20).

**EdU click labeling of newly replicated DNA in E. coli.** EdU click labeling of newly replicated DNA in E. coli was performed essentially as described previously (32), using the Click-IT Plus EdU Alexa Fluor 488 kit from Life Technologies. Briefly, dnaA46 cells and derivatives were grown at 30°C to an A600 of 0.2 before shift to 42°C for 90 min to achieve a run-out of ongoing synthesis. EdU was added (30 μg/ml final concentration), and cells were labeled for 15 min; 2-ml sample aliquots were fixed by adding 13 ml of 90% methanol. The components of the click labeling reaction mixture were prepared according to the manufacturer’s de-
scription. The labeling reaction was performed as described previously (32).

**Imaging flow cytometry.** Imaging flow cytometry was conducted by using the Imagestream® Mark II system (Amnis, Inc., Seattle, WA). This permits image capture of single cells in flow using a maximum of six optical channels. Images of between 6,000 and 12,000 cells were acquired at a 60× magnification for bright-field (BF) microscopy and for Alexa Fluor 488, using the 488-nm excitation laser set to 50 mW. The side scatter was established with a 785-nm laser set to 2 mW. Fluorescence levels per background were analyzed with the IDEAS imaging flow cytometry software V6.1. Following gating to identify single and in-focus cells, fluorescence levels were compared to images of the relevant cells, identifying cells with no detectable fluorescence, low fluorescence levels, or high signal intensity, as presented.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01294-15/-/DCSupplemental

Text S1, DOXC file, 0.04 MB.
Text S2, DOXC file, 0.02 MB.
Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 1.6 MB.
Figure S3, PDF file, 1.4 MB.
Figure S4, PDF file, 0.7 MB.
Figure S5, PDF file, 0.4 MB.
Table S1, DOXC file, 0.05 MB.

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