Double-stranded DNA ends, often from replication, drive genomic instability, yet their origin in non-replicating cells is unknown. Here we show that transcriptional RNA/DNA hybrids (R-loops) generate DNA ends that underlie stress-induced mutation and amplification. Depleting RNA/DNA hybrids with overproduced RNase HI reduces both genomic changes, indicating RNA/DNA hybrids as intermediates in both. An Mfd requirement and inhibition by translation implicate transcriptional R-loops. R-loops promote instability by generating DNA ends, shown by their dispensability when ends are provided by I-SceI endonuclease. Both R-loops and single-stranded endonuclease TraI are required for end formation, visualized as foci of a fluorescent end-binding protein. The data suggest that R-loops prime replication forks that collapse at single-stranded nicks, producing ends that instigate genomic instability. The results illuminate how DNA ends form in non-replicating cells, identify R-loops as the earliest known mutation/amplification intermediate, and suggest that genomic instability during stress could be targeted to transcribed regions, accelerating adaptation.
DNA double-strand breaks and ends (DSBs/DSEs) are the single most potent inducers of genomic instability in cells from bacteria to human. Imperfectly accurate repair of DSBs/DSEs causes genomic rearrangements and small mutations that underlie breast1 and other cancers2, many human genetic diseases3, evolution of pathogenic bacterial biofilms4, mutations that cause bacterial antibiotic resistance5, bacterial evasion of host defenses6, and stress-induced mutations (SIMs) that may accelerate evolution preferentially when cells are maladapted to their environments7,8. Although DNA breaks result from exogenous DNA-damaging agents, endogenous or spontaneous DSBs/DSEs are thought to be the commonest instigators of genomic instability mechanisms in all cells (for example, Vilenchik and Knudson10). However, despite their paramount importance to genomic instability that drives evolution and disease, and although their rates of formation are being quantified9, mechanisms of spontaneous DNA breakage are poorly understood.

Some mechanisms that produce spontaneous DSBs/DSEs are associated with DNA replication. First, when replication forks encounter single-stranded (ssDNA) breaks in the DNA template, they can ‘collapse’ producing a single DSE12 (illustrated Fig. 1e,f). Second, paused replication forks can be cleaved by endonucleases that cleave recombination intermediates creating DSEs13,14. Third, when converging replication and transcription complexes collide ‘head on’ on DNA, DSBs/DSEs can result15,16, though the precise mechanism of breakage is not known17. Fourth, DSBs/DSEs also result from co-directional collisions of the replisome with RNA polymerase (RNAP), when RNAP has ‘back-tracked’ on the DNA template17,18. DSEs produced by co-directional collisions are prevented by Mfd helicase, which dislodges the back-tracked RNAP18. Formation of these DSEs requires an RNA/DNA hybrid18 and appears to result from the DNA polymerase stopping at the RNA/DNA hybrid ‘under’ the back-tracked RNAP. This leaves a ssDNA nick or gap in the new DNA strand18,19, which then becomes a DSE by fork collapse when a second round of replication encounters the nick (per Fig. 1e,f). Thus, DSEs generated by co-directional collisions of the replisome with RNAP are: (1) prevented by Mfd helicase, and (2) caused by RNA/DNA hybrids plus replication.

Whereas the mechanisms of DSB/DSE generation listed above require replicating DNA, DSBs/DSEs also provoke SIM pathways in starvation-stressed cells that are unlikely to be replicating DNA. DSBs/DSEs initiate two mechanisms of SIM in starved Escherichia coli cells: stress-induced point mutation9,20,21 and gene amplification21,22 (reviewed in refs 7–9). Both mechanisms occur via repair of DSBs/DSEs that becomes mutagenic upon activation of the RpoS-controlled general stress response23. The point mutation mechanism is an RpoS-controlled switch to mutagenic DSE repair in which replication primed from DSEs uses error-prone DNA polymerases when RpoS is activated, causing indel and base-substitution mutations9,21,24,25 (illustrated Fig. 1g,k). The amplification mechanism causes genome rearrangements22,26,27 (reviewed in Hastings et al.28) is also thought to result from DSE-initiated replication21,22 (Fig. 1g–j) and requires RpoS23. Thus, parts g–k of Fig. 1 represent current

**Figure 1 | Model for transcription-promoted R-loop initiation of DSEs.** (a–c) R-loops form by incorporation of the transcript (red) into supercoiled DNA (blue/black) behind the site of transcription41. R-loop formation is usually initiated by ribosomes or the R-loop removed by RNase H. (b) Stalled RNAP (circle) (c) is removed by Mfd38. (d) The R-loop can then form a replication fork35. (e) If the replication fork encounters a nick in a template DNA strand, the fork will collapse (f), forming a single DSE12. In stressed cells, the collapsed fork might be repaired and restarted by microhomology-mediated break-induced replication (g–j)26 producing genome rearrangements (duplication shown here). (g) DSE degradation and 5’-end resection by RecBCD might be followed by h annealing of the overhanging 3’-end to ssDNA at a site of microhomology (vertical lines), shown here in the lagging-strand template of another replication fork (blue). (ij) This replication restart is shown at a position behind where the initial fork collapsed, so that a segment of the genome including the lac region becomes duplicated. The duplication can be expanded into an amplified array by unequal crossing-over (not illustrated). (k) Alternatively, point mutation is proposed to occur when the DSE is repaired by homologous recombination-mediated replication-fork restart using error-prone polymerase Pol IV during restart due to licensing of Pol IV (and also Pols II and V) by the RpoS and SOS stress responses9,21,24.
understanding based on much previous work. Stress-induced point mutation occurs both in a Lac plasmid-based mutation assay, and in chromosomes of plasmid-free cells, and underlies half of spontaneous base-substitution and frameshift mutagenesis in the chromosomes of starving E. coli, and so is likely to be important to evolution. Amplification is an important model for mechanisms of human copy-number variation.

The origin of DSBs/DSEs in non-replicating cells, including the DSEs that that underlie SIM, is poorly understood. At sites in an F plasmid, SIM requires TraI ssDNA endonuclease, which makes ssDNA nicks in the F plasmid. As TraI is not needed when DSBs are delivered to the F using double-stranded endonuclease I-SceI, ssDNA nicks are inferred to promote SIM by causing DSBs/DSEs, for example via fork collapse (Fig. 1e,f). Cells that express a phage ssDNA endonuclease and carry its cleavage site in the F do not require TraI for point mutation, implying that any ssDNA nick will allow SIM. However, spontaneous DSBs/DSEs also promote mutation in starving plasmid-free E. coli, indicating that DSBs/DSEs also form independently of TraI. Given that replication is repressed during starvation, that at least some of the DSBs/DSEs probably arise from fork collapses, and that most models for the origins of spontaneous DSEs involve replication, how spontaneous DSEs form in starvation-stressed non-growing cells is unclear. If fork collapse is the mechanism, then how and where do the forks originate? If not, then how are the ssDNA nicks converted into the DSEs that underlie SIM? How spontaneous DSEs are formed in non-replicating cells, and how replication-dependent processes (for example, Fig. 1) can occur in starved, non-replicating cells are important and fundamental problems, the solution to which is likely to pertain to many different organisms and circumstances.

DNA replication in growing cells is initiated at fixed sites (origins). Origin activity is repressed in stationary-phase cells. However, origin-independent replication can be initiated by RNA paired with unbroken DNA (R-loops) and at D-loops (similar structures made solely of DNA) under some circumstances. Here, we provide evidence that DSE production is promoted by R-loops in starving cells.

We show that RNA/DNA hybrids produced by transcription (R-loops) are precursors to the DSBs/DSEs that initiate stress-induced amplification (genome rearrangement) and point mutation, making R-loops the earliest known molecular intermediate for both processes. We show that R-loops provoke DSBs/DSEs by a two-step mechanism that is unlike the co-directional collision mechanism. We suggest that R-loop-primed replication forks collapse at independent ssDNA nicks to generate DSEs (Fig. 1a–f). This mechanism may explain DSE formation in many circumstances, particularly in non-replicating cells in which firing of standard replication origins is suppressed.

**Results**

RNA/DNA hybrids underlie stress-induced genomic changes. We used the E. coli Lac assay for stress-induced point mutation, and gene amplification. In this assay, cells carrying a lac+1 bp frameshift allele in an F plasmid are grown to saturation in liquid, then spread on solid lactose minimal medium on which they starve. Lac+ revertant colonies are of three types (for example, Fig. 2a): generation-dependent mutants that occurred during the liquid growth of the culture before starvation on lactose appear as colonies on day 2 (ref. 29); and stress-induced point-mutant and lac-amplified clones, formation of which requires activation of the RpoS stress response, are visible as Lac+ colonies from day 3 onward. Point mutants dominate early (for example, Fig. 2a), and amplified clones rise from ~5% of Lac+ colonies on day 5 to ~40% by day 8 (ref. 36). Point-mutant

![Figure 2](https://example.com/figure2.png)
and amplified colonies are distinguished by appearance on color-indicator medium (Methods).

We find that overproduction of RNase HI, which degrades the RNA from RNA/DNA hybrids, reduces stress-induced amplification and point mutation (Fig. 2a). Preliminary experiments showed that overproduction of RNase HI, encoded by rnhA, from the arabinose-inducible pBAD promoter in plasmid pBAD18-rnhA, inhibits growth. We used the highest concentration of arabinose that did not inhibit growth of cells containing this plasmid (5 × 10^{-8} (w/v)). Overproduction of RNase HI-reduced amplification and point mutation to about 60% (representative experiments, Fig. 2a), presumably by reducing levels of RNA/DNA hybrids. In three experiments, amplification was reduced to 56 ± 8%, days 2–7 (P = 0.017, Student’s t-test). For point mutation, the decrease was to 57 ± 14%, days 2 to 7, (P = 0.047, Student’s t-test). There was no loss of pBAD18-rnhA or loss of cell viability during the experiment (Fig. 2b). Amplification and point mutation rates were unaffected by the empty vector with or without arabinose, and were the same as in cells uninduced for pBAD18-rnhA (Fig. 2a). These data imply that about half of amplification and point mutagenesis requires an RNA/DNA hybrid intermediate, and that removal of the RNA/DNA hybrid by overproduced RNase HI curtails both processes.

**Loss of RNase HI increases amplification and mutation.** We find that knock out of RNase HI increases both amplification and point mutation ~3-fold (Fig. 3). In three experiments, amplification was increased by 2.9 ± 0.01-fold, days 2–7 (P = 0.003, Student’s t-test), and point mutation by 3.1 ± 0.3-fold, days 2–5, (P = 0.006, Student’s t-test). Thus, failure to remove RNA/DNA hybrids promotes both processes. Cell viability was unaffected. These data support the conclusion that RNA/DNA hybrids promote both amplification and point mutation.

**Instability promoted by RNA/DNA hybrids requires Mfd.** Mfd helicase is a transcription accessory protein that associates with RNAP, couples nucleotide-excision repair to transcription in transcription-coupled repair, and, when a replication fork collides with active RNAP, mediates the conflict by removing RNA from RNA/DNA hybrids 37, reduces stress-induced mutagenesis, and amplified colonies are distinguished by appearance on color-indicator medium (Methods).

We find that overproduction of RNase HI, which degrades the RNA from RNA/DNA hybrids 37, reduces stress-induced amplification and point mutation (Fig. 2a). Preliminary experiments showed that overproduction of RNase HI, encoded by rnhA, from the arabinose-inducible pBAD promoter in plasmid pBAD18-rnhA, inhibits growth. We used the highest concentration of arabinose that did not inhibit growth of cells containing this plasmid (5 × 10^{-8} (w/v)). Overproduction of RNase HI-reduced amplification and point mutation to about 60% (representative experiments, Fig. 2a), presumably by reducing levels of RNA/DNA hybrids. In three experiments, amplification was reduced to 56 ± 8%, days 2–7 (P = 0.017, Student’s t-test). For point mutation, the decrease was to 57 ± 14%, days 2 to 7, (P = 0.047, Student’s t-test). There was no loss of pBAD18-rnhA or loss of cell viability during the experiment (Fig. 2b). Amplification and point mutation rates were unaffected by the empty vector with or without arabinose, and were the same as in cells uninduced for pBAD18-rnhA (Fig. 2a). These data imply that about half of amplification and point mutagenesis requires an RNA/DNA hybrid intermediate, and that removal of the RNA/DNA hybrid by overproduced RNase HI curtails both processes.

**Translation inhibition increases instability.** Formation of transcriptional R-loops in bacteria is inhibited by ribosomes on the nascent transcript 44 such that inhibiting protein synthesis increases levels of transcription-generated R-loops. We find that pulse-inhibition of ribosome progression increases amplification and point mutation (Fig. 4), supporting the hypothesis that both are promoted by transcriptional R-loops. We treated stationary-phase cultures with spectinomycin, an inhibitor of ribosome translocation 42, for 3 h before plating on lactose medium and observed a ~2-fold increase in amplification (2.2 ± 0.4-fold, three experiments, P = 0.048, Student’s t-test). Point mutation increased less but still significantly (1.23 ± 0.06-fold, three experiments, P = 0.024, Student’s t-test) (Fig. 4a,b). These increases require Mfd (Fig. 4a,b), supporting the transcriptional origin of the spectinomycin-induced increases. The increased point mutation caused by translational inhibition (Fig. 4c) and loss of RNase HI (Fig. 3c) both require DinB/Pol IV, indicating that it occurs by the usual Pol IV-dependent pathway.

Supporting the hypothesis that spectinomycin increases mutation by increasing R-loop formation, we find that overproduction of RNase HI suppresses spectinomycin-enhanced
mutagenesis (Fig. 4d). Spectinomycin increased mutation in the control-plasmid-carrying strain $1.34 \pm 0.09$-fold (mean $\pm$ s.e.m. of four experiments) but not the RNase HI-overproducing strain (Fig. 4d), which, with or without spectinomycin treatment, showed $0.58 \pm 0.23$ of the level of mutation in the untreated control-plasmid strain (Fig. 4d, mean $\pm$ s.e.m. of four experiments). We conclude that the mutation-promoting effect of spectinomycin is prevented by removal of RNA/DNA hybrids. Although inhibition of translation might affect cells in various ways, both the Mfd-dependence (Fig. 4a,b) and the dependence on RNA/DNA hybrids (Fig. 4d) support the interpretation that translation inhibition promotes amplification and mutagenesis by stabilizing transcriptional R-loops.

Endonuclease-induced breaks substitute for R-loops. In principle, R-loops might promote SIM by promoting DSB/DSEs (Fig. 1b–f) or by promoting some stage of the SIM mechanisms downstream of DSE formation (Fig. 1g–k). Stress-induced point mutation and amplification mechanisms can be separated experimentally into the stages that occur before or after formation of DSEs (Fig. 1g–k, reviewed in Rosenberg et al.29 and Hastings et al.28). Proteins or DNA intermediates that promote SIM because they promote DSE formation are not required if DSBs are created near lac with I-SceI site-specific endonuclease21, as shown for TraI ssDNA endonuclease21 and the major role of the $\sigma^E$ response44. By contrast, proteins that promote SIM at stages after creation of DSEs remain required when DSBs are provided by I-SceI (near lac21, or near chromosomal mutation-reporter genes24,25,30). We used I-SceI to address whether R-loops promote SIM by acting before or after DSE creation. Although I-SceI decreases viability, the RNase HI plasmid does not (Fig. 5c), allowing us to compare the rates of Lac reversion with and without the RNase HI plasmid.

Figure 5b shows that when DSBs are made by I-SceI, Lac$^+$ reversion is unaffected by overproduction of RNase HI. This contrasts with reduction of Lac reversion caused by RNase HI overproduction in cells without I-SceI-induced DSBs, either when no I-SceI is present (Fig. 2a) or in the presence of the I-SceI enzyme but no cutsite (Fig. 5a). We conclude that R-loops become superfluous for mutagenesis when a DSB is provided independently.

In three experiments, in the absence of I-SceI cutting (‘enzyme-only’ control), RNase HI overproduction reduced the Lac$^+$ reversion rate by a significant $1.7 \pm 0.2$-fold (mean $\pm$ s.e.m.) relative to the control plasmid (Lac$^+$ colonies per day, day 3 to day 5). In contrast, when DSBs were induced, RNase HI overproduction caused $1.23 \pm 0.06$ times more Lac$^+$ reversion than seen with the control plasmid. The lack of effect of RNase H overproduction in strains with I-SceI cuts is significantly different from the effect with a DSB

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**Figure 4 | Inhibition of translation increases amplification and point mutation.** Gene amplification (a) and point mutation (b) in WT (SMR4562) and mfd (PJH813) strains pulsed with spectinomycin (spec) (broken lines). WT + spec (■), WT (●), mfd + spec (○), mfd (▲). (c) The spectinomycin treatment-induced increase in point mutagenesis is DinB/Pol IV-dependent. Point mutation in WT (SMR4562) and dinB (SMR5830) cells pulsed with spec. WT + spec (■), WT (●), dinB + spec (○), dinB (▲). The curves for the spectinomycin-treated WT cells differ from those of the untreated WT cells significantly, WT and spec-treated WT differ (for three experiments, $P = 0.002$ for point mutation and $0.001$ for amplification, Student’s t-test); mfd and spec-treated mfd are not significantly different ($P = 0.6$ for point mutation, and 0.7 for amplification). (d) Spectinomycin treatment does not increase Lac$^+$ revertants in cells overproducing RNase HI. Vector pBAD18 (■), vector pBAD18 spec-treated (○), pBAD18-rnhA (●), pBAD18-rnhA spec-treated (▲); broken lines denote spectinomycin treatment. Error bars represent one s.e.m. of four parallel cultures. These experiments were performed three times with comparable results.

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**Figure 4d | Amplification**

-WT + Spec
-WT
-Δmfd
-Δmfd + Spec

**Figure 4b | Point mutation**

-WT + Spec
-WT
-Δmfd
-Δmfd + Spec

**Figure 4c | Point mutation**

-dinB + Spec
-dinB

**Figure 4d | Total**

-Vector + Spec
-Vector
-prnhA + Spec
-prnhA
from its effect without I-SceI (P = 0.013, Student’s t-test). We conclude that R-loops contribute to DSB or DSE generation and so are not needed if DSBs are provided.

We could not distinguish amplification from point mutation in these experiments because of the low proportion of lac-amplified colonies. lac-amplified colonies appear later than point mutants36, and so are more affected by loss of viability over time. Also, although amplification is stimulated by I-SceI cuts made near lac, point mutation is stimulated more than amplification37, because co-amplification of the cutsite with lac causes loss of the repeats21.

These results indicate that R-loops function in the mutagenesis pathway upstream of (leading to the production of) the DSBS or DSEs that initiate mutagenesis.

Formation of R-loop-promoted DSEs requires a ssDNA nick.

In this section and the next, we provide two additional, independent lines of support for the conclusion that R-loops promote SIM by creation of DSEs. We also show that they do so in a two-step process that additionally requires a ssDNA nick.

First, we produced the DSE-binding Gam protein from phage Mu in RNase HI-deletion cells. Gam binds to DSEs and prevents them from being processed by nuclease45, and thus prevents DSE-induced recombination46. We produced Gam from the chromosome controlled by a doxycycline-inducible promoter. Doxycycline induction of Gam reduces amplification and point mutation (Fig. 6), confirming that both mechanisms require a DSE that is blocked by Gam. Importantly, induction of Gam prevents the increase of amplification and point mutation seen otherwise in RNase HI-defective cells, in which R-loops accumulate. These results show that the SIM promoted by R-loops in RNase HI-defective cells requires DSEs, and does not result from a different, DSE-independent pathway.

Second, we suggest a two-step model in which R-loops generate DSEs in stationary cells by priming replication forks that become DSEs when they encounter a ssDNA nick (Fig. 1a–f, Introduction). R-loops can prime replication independently of standard origins in RNase H-defective cells34,35 and in solution with purified proteins19. In the F' plasmid, ssDNA nicks are made constitutively by TraI, a site-specific ssDNA endonuclease that nicks the F' transfer origin, and which is required for SIM at sites in the F'21. The antimutagenic effect of deleting traI is more than suppressed by providing I-SceI-generated DSEs near lac21, showing that TraI contributes to formation of DSEs that drive mutagenesis, presumably by fork collapse at the ssDNA nick (Fig. 1e,f)7,21. We suggest that R-loops cause DSEs by priming replication forks that collapse at a ssDNA nick (Fig. 1a–f). This model predicts that ssDNA nicks made by TraI will be required for the R-loop-mediated production of DSEs. Conversely, if R-loops promoted DSE formation independently of ssDNA nicks, we might find that although TraI is required for most mutagenesis in RNase HI+ cells21, it would not be required for the extra mutagenesis observed in RNase HI-defective cells. Supporting the two-step R-loop-plus-ssDNA-nick model (Fig. 1), Fig. 7 shows that most mutation induced in RNase HI knock-out cells requires TraI. Whereas ΔtraI reduced mutation rate in otherwise wild-type cells about 60-fold, it reduced the rate in RNase HI-defective cells 108-fold (Fig. 7, Supplementary Table S1), showing a very strong requirement for TraI in the mutagenesis promoted by R-loops. Thus, most DSEs generated by R-loops in the F' form in a process that also requires the ssDNA nick made by TraI.

A small fraction of the extra R-loop-promoted SIM in RNase HI null cells is TraI-independent, in that knock out of RNase HI increased mutation slightly but just significantly in TraI-defective cells (1.8-fold, mean of three experiments, P = 0.0376, Student’s t-test). This could result from spontaneous ssDNA nicks (Fig. 1a–f). We conclude that ssDNA nicks are required for most of the mutagenesis promoted by R-loops. This supports the hypothesis that R-loops generate the breaks in these stationary cells via the two-step mechanism in which the RNA primes replication that collapses at a ssDNA nick producing a DSE (Fig. 1a–f). This is unlike DSEs generated by co-directional collisions in replicating cells, in which the R-loop itself is thought to create a ssDNA nick18. In molecules other than F', spontaneous ssDNA nicks, which are ubiquitous and common47, are expected to play this role.

R-loops plus single-stranded nicks generate visible DSEs. We used a visual assay to show directly that R-loops generate DSEs, and that DSE formation requires both the R-loop and a ssDNA nick. We assayed DSEs in living cells by quantifying cells
Gam prevents R-loops from initiating amplification and point mutation. The increased amplification and mutation seen in cells with increased R-loops due to knock out of RNase HI (ΔrnhA) is prevented by doxycycline-induced production of Gam protein (Gam-On) (Methods). Gam binds DSEs, prevents them engaging in repair and reduces (Doxycycline-induced production of Gam protein (Gam-On) prevents the increase in (17.7%) is foci caused by DSEs in the chromosome, and in the F0 cells returned to growth upon entry to stationary phase. Fourth, most GamGFP foci in RNase HI-defective cells with an F0 are TraI-dependent. Whereas 73 ± 2% of F0 RNase HI-deletion cells show foci, only 50 ± 2% of F0 RNase HI-deletion cells lacking TraI do. This number is not significantly higher than the 38 ± 4% of F- RNase HI-deletion cells that show foci (Supplementary Table S2, Fig. 8a, Student’s t-test), suggesting that most of the increased DSBs caused by F are caused by TraI activity. Overall, the data confirm that first, DSEs, assayed directly as GamGFP foci, are promoted by R-loops, and second, that the R-loop-promoted DSE foci also require a second event, a ssDNA nick, which in the F0 is usually generated by TraI. This supports the two-step R-loop-plus-nick model in Fig. 1a–f, in which R-loops promote DSE formation by priming replication forks that collapse at a ssDNA nick.

Figure 6 | Gam prevents R-loops from initiating amplification and point mutation. The increased amplification and mutation seen in cells with increased R-loops due to knock out of RNase HI (ΔrnhA) is prevented by doxycycline-induced production of Gam protein (Gam-On) (Methods). Gam binds DSEs, prevents them engaging in repair and reduces amplification and point mutation, confirming that DSEs are required for both pathways. Doxycycline-induced production of Gam protein (Gam-On) prevents the increase in point mutation and amplification caused by knock out of RNase HI (ΔrnhA), demonstrating that the increased R-loops cause increased amplification and point mutation via DSEs. Strains: ΔrnhA tetR PJH2039 (●); ΔrnhA Ph2Stwogam tetR PJH2023 (○); Gam-On induction, broken lines. Gam-Off, the same strains as ‘Gam-On’ without doxycycline induction; mock-On, strains with the Tet repressor but without the gam gene treated with doxycycline. Error bars represent one s.e.m. of four parallel cultures. This experiment was performed three times with comparable results.

Figure 7 | R-loop-promoted mutagenesis requires ssDNA endonuclease TraI. The abundance of R-loops caused by deletion of mha is not sufficient, but also requires TraI-generated ssDNA nicks, to promote mutagenesis. Therefore generation of DSEs by R-loops requires both an R-loop and a ssDNA nick. Strains: WT, SMR4562; Δmha, PJH683; ΔtraI, PJH2134; ΔrnhA ΔtraI, PJH 963. Mean ± s.e.m. of three experiments.

Discussion

The results presented demonstrate a mechanism of generation of DSBs/DSEs by R-loops in starving, stationary-phase E. coli cells that requires Mfd translocase and also depends on ssDNA nicks. Because ssDNA nicks are common, spontaneously this mechanism is likely to be generally important. We suggest that replication forks initiated from R-loops in stationary cells collapse, generating DSEs at independently occurring ssDNA nicks (Fig. 1a–f). This model can explain how DSEs can occur in non-replicating cells, in which standard origin-dependent replication is inhibited, in contrast with most current models for spontaneous DSE production (reviewed in the Introduction).

R-loops were inferred previously to initiate replication in RNase HI- or RecG-defective cells, and origin-independent replication (not demonstrably primed by R-loops) was seen in RNase HI+ RecG+ cells returned to growth upon entry to stationary phase. This mechanism might be R-loop-independent and also does not resemble R-loop-promoted DNA breakage demonstrated here, in that it is RecB (DSB)-independent.
recombination and somatic hypermutation in the mammalian immune system (reviewed by Chaudhuri and Ali54). R-loops are associated with hyper-recombination, genomic instability and DSBs from bacteria to yeast and human (reviewed by Li and Manley55). R-loops underlie breakage at common fragile sites in human56. Some of these instances of breakage require replication as well as transcription58,59, and so might also result from R-loop-generated fork collapse at nicks.

Transcription was implicated previously in DSB-dependent mutagenesis in E. coli in that a strain with a partially-defective NusA transcription anti-termination factor shows reduced SIM, and might do so via reduced interactions with DinB error-prone DNA polymerase57. Our data suggest a possible alternative explanation that could unite their data and ours. R-loops form preferentially at particular DNA sequences, hypothesized to have special structural features57. When NusA is functional, some transcripts will be longer (anti-terminated). Perhaps, in the presence of functional NusA, a transcript that would otherwise not reach a site prone to stable R-loop formation reaches such a site, such that R-loops are formed or stabilized, promoting SIM. This model and others remain to be tested.

The association of transcriptional R-loops with DSEs opens the possibility that in addition to being targeted preferentially to times of stress, by their coupling to the stress responses7 and see Al Mamun et al.30), stress-induced point mutation and amplification could be targeted preferentially to regions of active transcription. DSE-dependent point mutagenesis occurs in strong hotspots maximally within two and up to 60 kb from a DSE25. This means that transcription-produced DSEs would be expected to cause hotspots of mutations near sites of transcription. The suggestion that DSBs cause the hotspots observed in cancer genomes and chemically damaged yeast cells50,51 suggests the possibility of transcriptional/R-loop targeting of mutations also in cancer genomes. Transcription also promotes local SIM in starving B. subtilis cells, though apparently independently of DSEs and recombination58. In stressed cells, actively transcribed regions are likely to harbour genes whose products counter the stress. Thus, stress-induced point mutations and genome rearrangements might occur preferentially where they are most likely to affect phenotypes immediately, including advantageously. Both regulation of mutagenesis in time, by stress responses, and in genomic space, by linkage with transcription and DSEs, could enhance the ability to evolve during stress for small populations in which mutation supply is limiting.

Methods

Strains. Strains and plasmids used in this study are listed in Supplementary Table S3 and are isogenic with SMR4562, an independent construction of FC40 (ref. 29). FC40/SMR4562 carry a +1 frameshift mutation in a lacI-lacZ fusion gene in F β plasmid F128. FC29 (ref. 29) is an F carrying non-revertible lac deletion strain that is used to scavenge extraneous carbon sources from minimal lactose plates. SMR4563, an ampicillin-resistant araR scavenger derived from FC29, was used in experiments involving pBAD constructs or plasmids. Δmfd and Δmha alleles were introduced into strains by P1 transduction.

SIM experiments. Lac. E. coli were grown in minimal glycerol medium to prolonged stationary phase, plated onto minimal lactose solid medium and incubated for 7 days at 37 °C. The Lac⁺ colonies that arise each day are counted and marked. They have acquired either a compensatory-framenew (-‘point’) mutation, or amplification of the leaky lac allele to 20 or more copies, which allows growth without a reversion mutation66. Amplification of lac is unstable under non-recessive conditions, so lac amplified clones can be identified by plating cells from Lac⁺ colonies onto nonselective medium containing 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) on which lac-amplified isolates show a sectored appearance. In spectinomycin-pulse experiments, 100 µg ml⁻¹ spectinomycin was added 3 h before plating and then washed from cells before plating on minimal lactose medium. All experiments were performed 3 times, and repeat experiments gave consistent results. Representative graphs were chosen for illustration and
summaries of mutation rate data from multiple experiments (mutations per cell per day) are given in the text.

In experiments with plasmid pBAD18-mRNA producing RNase HI, 100 μg ml⁻¹ ampicillin was added to the minimal lactose plates to maintain the plasmid. The arabinose is not catabolized (the cells still starve) because the cells are ara⁻. Plugs of agar from these plates were taken with capillary pipettes periodically during the experiment, resuspended and plated for colony-forming units on plates with and without ampicillin to verify that cells retained the plasmid. No significant plasmid loss was observed. To produce RNase HI, 5 × 10⁻⁸ (w/v) arabinose was added to the minimal lactose plates. This concentration was chosen because we found that higher concentrations caused inhibition of growth. The same concentrations of ampicillin and arabinose were used in experiments with I-scl and overproduction of RNase HI. The arabinose induces production of both RNase HI and I-scl. In I-scl experiments, the number of ampicillin-resistant cells carrying the plasmid was similar for the control and RNase HI-producing plasmids (0.77 ± 0.09 versus 0.70 ± 0.16, respectively, in cells recovered from lactose plates after 1 day, mean ± s.e.m.), and did not change significantly during the experiment. The proportion of cells showing resistance to arabinose (indicating loss of the I-scl cut site) was not different between the DSB-inducing strains with and without the RNase HI plasmid (0.19 ± 0.02 versus 0.22 ± 0.02, respectively, in the experiment shown).

For experiments in Fig. 6, Gam was induced by doxycycline at 100 ng ml⁻¹ applied for 48 h from early stationary phase.

Inducible Gam and GAMGFP of phage Mu. Details of construction and validation of the chromosomally expressed inducible Gam and GamGFP will be published separately. In brief, the EcoRI fragment of the bacteriophage Mu gam gene from plasmid pA2125 was sub-cloned downstream from the P_{SctO} promoter20, a promoter that can be induced by tetracycline or doxycycline, in cells carrying P_{SctO}tetR, encoding a constitutive Tet repressor, which represses transcription from P_{SctO} tetO in non-inducible conditions20. P_{SctO} tetO was placed in the E. coli chromosome. Similarly, the gam-gfp fusion gene, to be described elsewhere, was placed downstream of the P_{SctO} tetO.

For experiments in Fig. 8, production of GamGFP was induced in log-phase with 100 ng ml⁻¹ doxycycline for 3 h. Cells were concentrated and placed on slides and photographed in bright field and phase contrast. At least ten fields of ~10–100 cells were counted for each sample. Samples were scored blindly as focus-containing or not.

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Author contributions
P.J.H. conceived the study. H.W., S.M.R. and P.J.H. conceived the experiments. P.J.H. directed the experiments. H.W., C.S., P.C.T. and P.S. constructed strains, performed experiments and analysed data. S.M.R. and P.J.H. also interpreted data. H.W., S.M.R and P.J.H. wrote the paper.

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Corrigendum: R-loops and nicks initiate DNA breakage and genome instability in non-growing Escherichia coli

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In Supplementary Table S3 of this Article, some of the genotype entries are incorrect, as follows:

For the SMR6280 strain, SMR4562 ΔaraBAD567 [F’ mhpA32miniTn7Kan(1-SceI cutsite)] should be SMR4562 ΔaraBAD567 ΔattBc::P_BAD1-SceI [F’ mhpA32::miniTn7Kan(1-SceI cutsite)]

For the PJH813 strain, SMR4562 Δmfd::FRTKanFRT should be SMR4562 Δmfd::FRT.

For the PJH2023 strain, SMR4562 ΔattTn7::PN25tetOgam·gfpFRT Δzfd2509.2::PN25tetRFRT should be SMR4562 ΔattTn7::PN25tetOgam·FRT Δzfd2509.2::PN25tetRFRT.

For the PJH2443 strain, SMR4562 ΔattTn7::PN25tetOgam·gfpFRT ΔrnhA::FRTKanFRT Δzfd2509.2::PN25tetRFRT should be SMR4562 ΔattTn7::PN25tetOgam·FRT ΔrnhA::FRTKanFRT Δzfd2509.2::PN25tetRFRT.