strain lacking the wild-type Ste5 and Pbs2 scaffolds but expressing this diverter scaffold survived on 1 M KCl only in the presence of α-factor (Fig. 3D). This conditional osmoresistance was independent of the osmosensor Sho1, indicating that α-factor is the only required input. This designed pathway was extremely specific: Cells bearing the diverter scaffold were sterile (mating efficiency $< 10^{-5}$; fig. S2) and did not yield the osmoresponse (Hog1 phosphorylation) upon salt stimulation. Instead, Hog1 phosphorylation was only observed upon stimulation with α-factor (Fig. 3E). The magnitude of the diverted response, as measured by Hog1 phosphorylation, was comparable to the normal osmoresponse. Microarray analysis revealed that the global transcriptional response elicited by the new pathway was nearly identical to that of the wild-type osmolarity response, but clearly distinct from the wild-type mating response (Fig. 3F). Thus, rewiring by the diverter scaffold is efficient and specific.

Mutagenesis revealed that the diverter-mediated response was dependent on the specific set of interactions consistent with pathway connectivity (Fig. 4). Mutation of the Ste11 binding site on the Ste5 fragment of the diverter destroyed its function, whereas mutation of binding sites for kinases downstream of Ste11 (Ste7 and Fus3) did not. Similarly, on the Pbs2 fragment, mutation of binding sites for components upstream of Ste11 (Sho1) had no effect on function, although mutation of activities downstream from Ste11 (Pbs2 kinase activity) destroyed function (mutations that selectively block Ste11 and Hog1 binding to Pbs2 have not been identified). In addition, covalent linkage between the Ste5 and Pbs2 fragments of the diverter scaffold was absolutely required. These specific requirements are inconsistent with indirect mechanisms of Hog1 activation, including simple α-factor–dependent targeting of Pbs2 to the membrane or buildup of high steady-state levels of activated Ste11 caused by disruption of negative feedback (*21*).

Although signaling by the diverter scaffold was efficient and specific, when wild-type Ste5 and the diverter scaffold were coexpressed, only a slightly attenuated mating response was observed (*18*). The apparent dominance of wild-type Ste5 over the diverter may result in part because Ste5 functions as an oligomer, or because of cross-pathway negative feedback. Wild-type efficiency may require more fine-tuned evolutionary refinement.

Our findings indicate that scaffolds such as Ste5 are conceptually similar to promoters: Both are modular and flexible organizing centers that can control the flow of information in signaling or transcription, respectively. Similarly, the regulation of a transcriptional response can be modulated by simple alterations in the presence or arrangement of diverse transcription factor docking sites (*22, 23*). Both of these organizing structures thus appear to be optimized for evolvability, a property that may provide increased fitness in the face of constantly changing environmental challenges and signaling needs. Conversely, just as promoter engineering can be used to control cellular behavior and to create useful tools (e.g., yeast two-hybrid systems) (*24*), these and other related results (*25*) indicate that scaffold engineering may allow for systematic manipulation of cytoplasmic signaling pathways.

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DNA Damage–Induced Replication Fork Regression and Processing in *Escherichia coli*

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DNA lesions that block replication are a primary cause of rearrangements, mutations, and lethality in all cells. After ultraviolet (UV)-induced DNA damage in *Escherichia coli*, replication recovery requires RecA and several other *recF* pathway proteins. To characterize the mechanism by which lesion-blocked replication forks recover, we used two-dimensional agarose gel electrophoresis to show that replication-blocking DNA lesions induce a transient reversal of the replication fork in vivo. The reversed replication fork intermediate is stabilized by RecA and RecF and is degraded by the RecQ-RecJ helicase-nuclease when these proteins are absent. We propose that fork regression allows repair enzymes to gain access to the replication-blocking lesion, allowing processive replication to resume once the blocking lesion is removed.

Irradiation of cells with near-UV light induces DNA lesions that block replication (*1*). In *E. coli*, replication is transiently inhibited after a moderate dose of UV irradiation, but it recovers efficiently at a time that correlates with the removal of the lesions from the genome by the nucleotide excision repair proteins (*1–3*). Cells deficient in lesion removal are severely impaired in their ability to recover replication and exhibit elevated levels of recombination, genomic rearrangements, and cell lethality (*4–7*).

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The recovery of replication also involves several *recF* pathway proteins, including RecA, RecF, RecO, RecR, RecJ, and RecQ (*2*, *8*–*11*). In the absence of RecA, RecF, RecO, or RecR, replication forks blocked at UV-induced DNA damage fail to recover and the nascent DNA is degraded, suggesting that these proteins are required to maintain the integrity of replication forks blocked at DNA lesions (*2*, *9*, *10*, *12*). In vitro, RecA, RecF, RecO, and RecR promote pairing between single-stranded DNA and homologous duplex DNA, an activity that is critical for bringing together homologous pieces of DNA during recombination (*13*–*15*). In the presence of DNA damage, cellular assays have shown that this same enzymatic activity is also required to maintain the homologous strands of the replication fork until replication can resume [reviewed in (*16*)]. Other *recF* pathway proteins, RecQ and RecJ, selectively degrade the nascent lagging strand at blocked replication forks (*10*) and play a role in suppressing the frequency with which illegitimate recombination occurs, perhaps by enhancing the ability of RecA and RecF, -O, and -R to bind and stabilize the blocked replication fork (*10*, *17*, *18*). These observations have led to the general model that when replication encounters a replication-blocking DNA lesion, several of the *rec* gene products act to maintain and process the replication fork so that repair enzymes or alternative DNA polymerases can gain access to the lesion and effect repair (*2*, *9*, *10*, *16*). In this way, processive replication would be maintained while avoiding the potentially dangerous consequences of strand exchanges and recombination.

Although several gene products are known to be involved in the recovery of replication, little is known about the structural characteristics or intermediates that occur during this process. Two-dimensional (2D) gel electrophoresis is a technique that allows one to differentiate and identify the structural properties of replicating DNA fragments (*19*). With the use of this technique, we characterized the structural intermediates that occur during the recovery of replication on the plasmid pBR322 after UV irradiation in vivo. pBR322 maintains a moderate copy number, uses the *E. coli* replication proteins for reproduction, and has been previously characterized by 2D agarose gel analysis, making it a useful model for examining a rare event such as replication through a specific DNA sequence (*20*). To examine the structural properties of replication forks after UV irradiation, we irradiated growing *E. coli* cultures containing pBR322 with 50 J/m^2. Under these conditions, 0.5 lesions are produced per plasmid strand and 97% of our parental cells survive to form colonies (Fig. 1A) (*21*). At this dose, the UV-induced DNA lesions are repaired and robust replication resumes approximately 30 min after treatment (Fig. 1, A and D).

To visualize the replication intermediates during this period, total genomic DNA (chromosomal and plasmid) was purified at various times after irradiation, digested with Pvu II, and analyzed by 2D agarose gel electrophoresis (*21*). Pvu II cuts pBR322 just downstream of the unidirectional origin of replication. This produces a migration pattern of replicating molecules that approximates a simple Y-arc pattern (Fig. 1; fig. S1). In the absence of DNA damage or at times immediately after UV irradiation, only Y-shaped replication intermediates are observed (Fig. 1; fig. S1). At later times, a transient increase in the amount of replicating Y-shaped intermediates occurs, as replication forks become blocked and accumulate at UV-induced lesions. Additionally, a transient intermediate, migrating in the cone region, also accumulates as a result of the presence of molecules that contain a double Y– or X-shaped structure (Fig. 1; fig. S1). The amount of DNA migrating in the cone region peaks around 30 min after UV irradiation and begins to wane at a time correlating to when the lesions are repaired and replication recovers (Fig. 1, B and D). The formation of the cone region intermediates depends on active replication because it is significantly reduced when nonreplicating, stationary phase cells are irradiated (Fig. 2, C and D). Hence, after UV-irradiation, replication intermediates are maintained and processed through a transient X-shaped structure during the period when the replication forks are blocked and the UV-induced lesions are repaired.

Next, we characterized the replication intermediates in mutants that are known to have an impaired ability to recover replication after DNA damage. *uvrA* mutants are unable to excise and remove UV-induced lesions from DNA (*2*, *3*). In the absence of lesion removal, the recovery of replication is severely inhibited, and both Y-shaped replication forks and cone region intermediates accumulate and persist throughout the time course (Fig. 2, A and D), indicating that the presence of DNA lesions during replication directly produces the X-shaped intermediates. It is also interesting to note that higher, multibranched molecules accumulate over time in the *uvrA* mutants as well, consistent with previous studies in

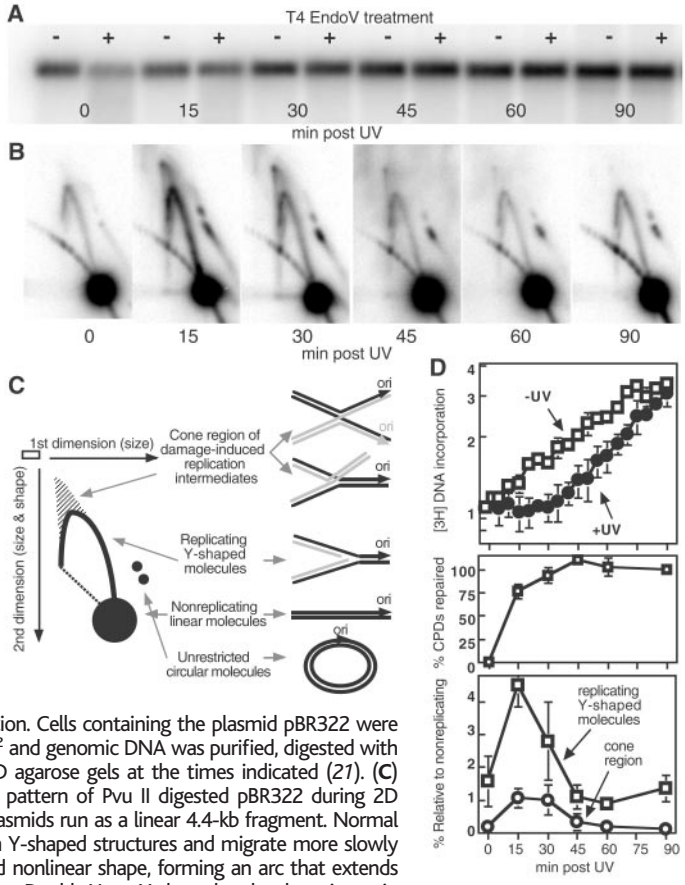
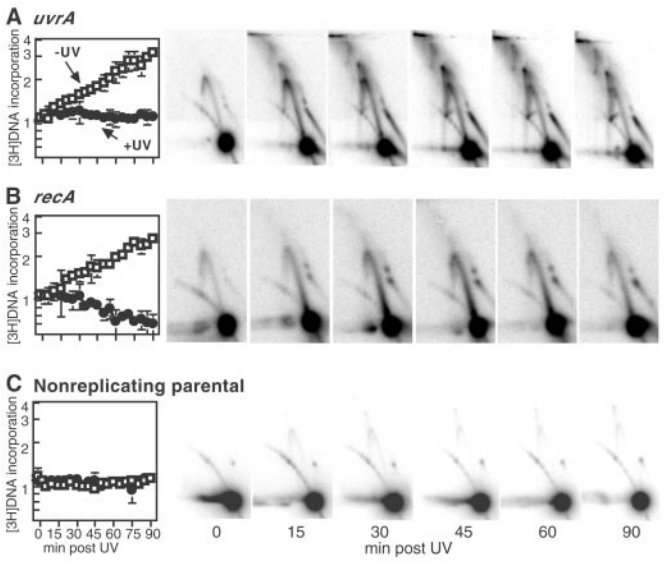


Fig. 1. UV-induced DNA replication intermediates observed during the recovery of replication. (**A**) UV-induced lesions are repaired from the plasmid within 30 min after UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50 J/m^2, and genomic DNA was purified, digested with Pvu II, and analyzed at the times indicated to measure the rate that the predominant UV-induced lesion, the cyclobutane pyrimidine dimer (CPD), was removed from the plasmid. Lesion removal was determined by fragment sensitivity to T4 endonuclease V (TEV), which cleaves DNA containing CPDs (*21*). (**B**) Blocked replication forks and cone region intermediates transiently accumulate after UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50 J/m^2 and genomic DNA was purified, digested with Pvu II, and analyzed by 2D agarose gels at the times indicated (*21*). (**C**) Diagram of the migration pattern of Pvu II digested pBR322 during 2D analysis. Nonreplicating plasmids run as a linear 4.4-kb fragment. Normal replicating fragments form Y-shaped structures and migrate more slowly due to their larger size and nonlinear shape, forming an arc that extends out from the linear fragment. Double Y– or X-shaped molecules migrate in the cone region (fig. S1) (*21*). (**D**) The replication intermediates persist until a time correlating with replication recovery and lesion removal. Replication recovery, lesion repair, and the relative amount of replicating fragments (squares) and cone region intermediates (circles) are plotted. Replication recovery was assayed by [^{3}H]thymine incorporation for UV-irradiated (solid symbols) or mock-irradiated (open symbols) cultures (*21*). Error bars indicate 1 SD.



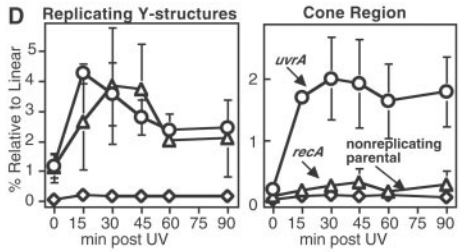


Fig. 2. Formation of the cone region intermediates depends on the presence of UV-induced lesions, RecA, and active replication. Replication recovery was assayed by [^{3}H]thymine incorporation, as in Fig. 1, and the replication intermediates observed during the normal recovery period were monitored by 2D gel analysis. (**A**) *uvrA* mutants fail to recover replication after UV-induced DNA damage and the cone region intermediates persist and accumulate. UV-irradiated cultures, solid circles; mock-irradiated cultures, open squares. (**B**) *recA* mutants fail to recover replication after UV-induced DNA damage and the cone region intermediates do not accumulate. Symbols are as in (A). (**C**) Nonreplicating stationary phase cultures do not accumulate damage-induced replication intermediates. Symbols are as in (A). (**D**) The relative amount of plasmid molecules migrating in the replication arc and cone region is plotted for *uvrA* (circles), *recA* (triangles), and nonreplicating, parental cells (diamonds).

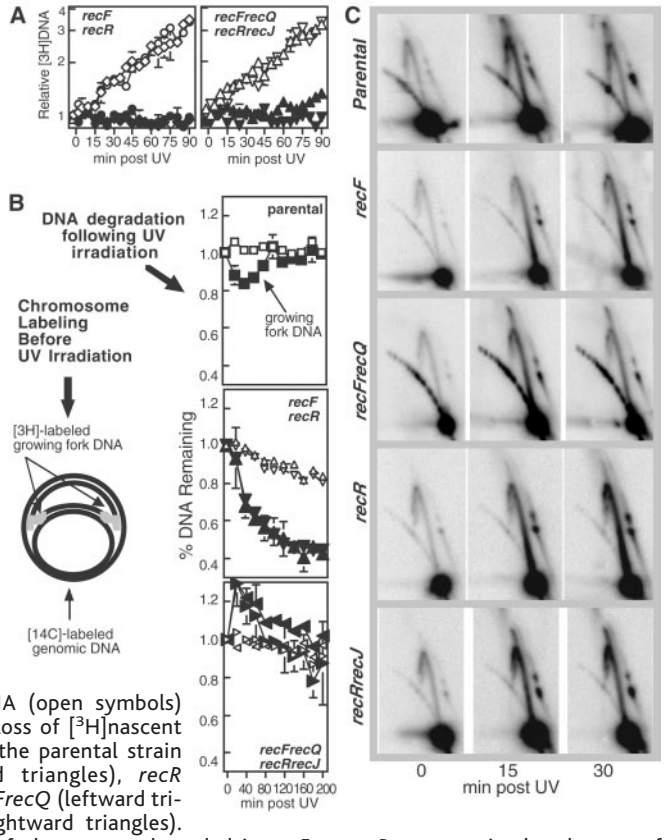


Fig. 3. RecF and RecR are required to protect regressed replication fork structures from degradation by the RecQ RecJ, helicase-nuclease. (**A**) *recF*, *recR*, *recFrecQ*, and *recRrecJ* fail to recover replication after UV irradiation. Replication recovery was assayed as before for UV-irradiated (solid symbols) or mock-irradiated (open symbols) cultures. Circles, *recF*; diamonds, *recR*; upward triangles, *recFrecQ*; downward triangles, *recRecJ*. (**B**) RecF and RecR are required to protect the nascent DNA from degradation by RecJ and RecQ. The fraction of the radioactivity remaining in the DNA after irradiation is plotted over time. The loss of [^{14}C]genomic DNA (open symbols) can be compared to the loss of [^{3}H]nascent DNA (solid symbols) for the parental strain (squares), *recF* (upward triangles), *recR* (downward triangles), *recFrecQ* (leftward triangles), and *recRrecJ* (rightward triangles). (**C**) Regressed replication forks are not degraded in *recF* or *recR* mutants in the absence of either RecQ or RecJ. Cells containing the plasmid pBR322 were UV-irradiated and analyzed by 2D gels as described. In *recF* or *recR* mutants, the nascent DNA is degraded after UV irradiation, and the cone region intermediate does not accumulate. However, in *recFrecQ* or *recRrecJ* mutants, the nascent DNA is not degraded and the damage-induced intermediate is restored.

both *E. coli* and humans demonstrating that the inhibition of replication recovery in excision repair mutants results in elevated levels of strand exchange and recombination (*5*, *22*, *23*).

In *recA* mutants, the recovery of replication is also severely impaired (Fig. 2B), and the failure to recover replication is associated with the degradation of the replication fork and genomic DNA (*9*, *12*). Consistent with these observations, the concentration of X-shaped intermediates after UV irradiation is significantly reduced in *recA* cultures (Fig. 2, B and D). Additionally, although Y-structures also accumulate, they are primarily concentrated in the initial portion of the Y arc (seen as a smear closest to the linear fragment), indicating that degradation at the replication forks is occurring.

These observations suggest that RecA is required to protect the regressed replication forks structures from degradation. However, because *recA* mutants are also deficient in recombination and strand exchange, it remains possible that the X-shaped molecules are entirely a product of RecA-mediated strand exchanges rather than a product of RecA-maintained regressed replication forks. To determine whether replication fork regression was occurring, we examined mutants in the *recF* pathway that are known to process the nascent DNA at blocked replication forks. RecF and RecR are both required to protect and maintain the replication forks that are blocked at UV-induced DNA damage (*9*). In mutants lacking *recF* or *recR*, replication fails to recover and the nascent DNA at the replication fork is degraded by the combined action of the RecQ helicase and RecJ nuclease (Fig. 3, A and B) (*2*, *9*, *10*). Similar to *recA* and consistent with a role in pro-

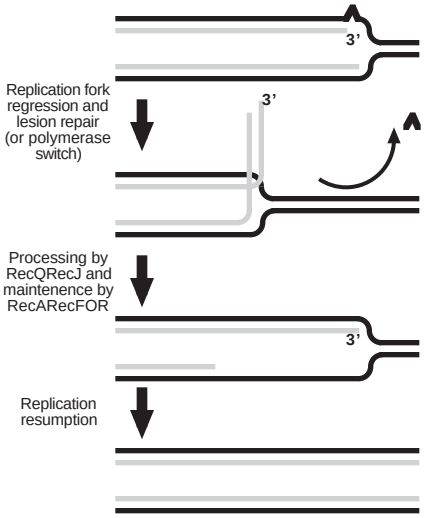


Fig. 4. Model for the structural intermediates associated with the recovery of replication blocked at a DNA lesion (^).

tecting the nascent DNA from degradation, the amount of X-shaped intermediates is severely reduced in *recF* and *recR* mutants (Fig. 3C). Furthermore, although *recF* and *recR* mutants still fail to recover replication in the absence of either the RecQ helicase or the RecJ nuclease, the nascent DNA at the replication fork remains intact (*10*) and, in these mutants, the X-shaped intermediates are clearly restored (Fig. 3; fig. S2), indicating that a portion of the intermediates in the cone region are formed through the regression of the replication fork and the extrusion of the nascent DNA (Fig. 4). Thus, DNA lesions that block replication fork progression induce a regressed intermediate that is maintained by RecA and RecF, -O, and -R. In the absence of these proteins, the intermediate is degraded by RecQ and RecJ, which have been previously shown to process the nascent lagging strand before the resumption of replication (*10*). Recently, *rad53* checkpoint mutants in *Saccharomyces cerevisiae* have been shown to contain nascent lagging strand abnormalities and accumulate a similar intermediate after hydroxyurea treatment (*24*, *25*), suggesting that similar mechanisms may operate in both prokaryotes and eukaryotes. It should be emphasized that not all lesions may block replication or be processed by the same mechanisms, and it is likely that a portion of the X-shaped intermediates in wild-type cells are produced through recombinational exchanges. This is especially true in the case of *uvrA* mutants in which multibranched molecules accumulate. It remains to be determined whether fork regression in vivo is promoted enzymatically or occurs spontaneously via positive supercoiling in front of the replication fork (*26*, *27*). We propose that the regressed intermediate allows repair enzymes or alternative polymerases to gain access to the replication blocking lesions, thereby allowing processive replication to resume once the block to replication has been removed or bypassed. By analogy, the recovery of transcription has been shown to require the removal of the RNA polymerase and nascent transcript before repair enzymes can effect repair (*28*, *29*). Consistent with this idea, the appearance and duration of the regressed replication fork intermediate coincides with the time it takes for the DNA lesions to be removed and robust replication to resume.

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Taming of a Poison: Biosynthesis of the NiFe-Hydrogenase Cyanide Ligands

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NiFe-hydrogenases have an Ni-Fe site in which the iron has one CO and two CN groups as ligands. Synthesis of the CN ligands requires the activity of two hydrogenase maturation proteins: HypF and HypE. HypF is a carbamoyltransferase that transfers the carbamoyl moiety of carbamoyladenylate to the COOH-terminal cysteine of HypE and thus forms an enzyme-thiocarbamate. HypE dehydrates the *S*-carbamoyl moiety in an adenosine triphosphate–dependent process to yield the enzyme thiocyanate. Chemical model reactions corroborate the feasibility of this unprecedented biosynthetic route and show that thiocyanates can donate CN to iron. This finding underscores a striking parallel between biochemistry and organometallic chemistry in the formation of an iron-cyano complex.

Hydrogenases catalyze the reversible oxidation of molecular hydrogen into protons and electrons. They are widely distributed among microorganisms, and they provide them with the capacity either to use hydrogen as an energy source or to dissipate excess reducing equivalents in the form of molecular hydrogen. These enzymes have attracted considerable attention, not only because of the distinctive chemical nature of their substrate and the reaction mechanism but also because of their potential biotechnological applications (*1*).

Two major classes of hydrogenases can be differentiated according to the metal content of the active site cofactor: Fe-hydrogenases and NiFe-hydrogenases. Although the overall structure of their metal centers differs, they share one unusual feature: diatomic, nonproteinaceous iron ligands, namely, carbon monoxide and cyanide. In NiFe-hydrogenases, the iron of the center carries two cyanide and one carbon monoxide moieties (*2*). The presence of these ligands stabilizes iron in a low oxidation and spin state.

In metal center synthesis and incorporation into proteins, important issues are con-

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