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Homologous Recombination by the RecBCD and RecF Pathways.

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Abstract

In all cells, genetic recombination is used to repair DNA breaks and, as a result, genetic information is exchanged between homologous DNA molecules. Discontinuities in DNA strands, specifically double-strand DNA breaks and single-strand DNA gaps, attract the enzymes responsible for the initiation of homologous recombination. In wild-type *Escherichia coli*, two distinct pathways are responsible for the repair of DNA by recombination: the RecBCD- and the RecF-pathways. The RecBCD-pathway is specific to recombination initiated at double-strand DNA breaks, whereas the RecF-pathway is primarily responsible for recombination initiated at single-strand DNA gaps, although it can also act at double-strand breaks. This review summarizes the biochemical mechanisms of homologous recombination in *E. coli*.

Recombinational repair of DNA damage.

Homologous, or general, recombination is a crucial biological process that involves the pairing and transfer of strands between DNA molecules that share a region of significant sequence homology. Since its discovery in 1946 by Lederberg and Tatum (48), homologous recombination in bacteria was associated with the sexual process of conjugation and was viewed as an evolutionary mechanism both for shuffling the genome and for spreading favorable alleles. However, more recently, a more immediate function of homologous recombination was recognized: namely, it is a mechanism for the maintenance of chromosomal integrity that acts to repair DNA lesions, both double-strand DNA breaks (DSBs) and single-strand DNA gaps (SSGs), generated during the course of DNA replication. The intimate connection between the processes of replication and recombination was initially appreciated in the life-cycle of bacteriophage T4 (56) and then later recognized as an important determinant of viability in bacteria (39, 45). In T4 phage, recombination is linked to replication to produce a high yield of phage DNA; in *E. coli,* recombination is linked to replication to permit its completion when interrupted by DNA damage, and also to initiate DNA replication in the absence of origin function. This view of recombination as an integral part of efficient chromosome duplication also reconciled the high level of inviability (up to 95%) of recombination-deficient cells (12).

Generation of a double-strand break in DNA.

A significant fraction of DNA damage affects only one strand of the DNA duplex. Such lesions can often be repaired by one of the repair systems that is specific for a particular DNA lesion (see (28) for review). These repair systems use the intact complementary strand as a template to restore the damaged DNA molecule to its original state. Occasionally both strands of

the dsDNA can be broken opposite to each other, resulting in a double-strand break. DSBs can be produced, for example, as a direct consequence of ionizing radiation (Figure 1a). However, the bulk of the DSBs in bacteria are generated indirectly as the result of DNA replication through an unrepaired break in just a single-strand of DNA (Figure 1b). Replication of DNA containing a single-strand nick or a gap in the leading-strand results in the dissociation of the DNA polymerase holoenzyme complex, and the generation of one blunt DSB (Figure 1b), whereas replication of DNA with a nick in lagging-strand produces a DSB with an 3'-terminated ssDNA tail.

A DSB can also be produced when the DNA replication process is halted by anything that might block progress of the replisome. Upon dissociation of the replisome, the stalled replication fork can regress, to produce a Holliday junction. Such a structure contains both a dsDNA end and an intermediate of recombination and, hence, attracts recombination machinery in a manner similar to the simple DSB. In addition, the Holliday junction can be cleaved to provide yet another path for DSB formation (Figure 2). Left unrepaired, DSBs are lethal, and *E. coli* can repair only few DSBs per chromosome without dying (43). Thus, DNA replication is a major source of endogenous DSBs that, in turn, are repaired by homologous recombination. This relationship between DNA replication and recombinational repair of DSBs is summarized in Figure 1.

Recombinational repair, however, requires a homologous DNA molecule to be used as a template from which to restore, by DNA synthesis, the genetic content of the damaged DNA molecule. These homologous DNA sequences are generated by DNA replication and, therefore, the meridiploid character of bacterial chromosomes provides the necessary templates for recombinational repair.

Generation of a single-strand gap in DNA.

When synthesis of a DNA strand is blocked by a non-coding lesion (for example, an abasic site or an intra-strand crosslink, such as a thymine dimer), continued replication of the flawless strand beyond the lesion produces a single-strand DNA gap (Figure 1c) (42, 54). SSGs are also known as daughter-strand gaps, since they appear on one of the newly synthesized daughter strands during semi-conservative DNA replication.

The inter-strand DNA crosslink is a special class of chemical damage to DNA, since it blocks DNA replication completely by preventing DNA strand separation (Figure 3) (28). Repair of this special class of DNA damage is absolutely dependent on homologous recombination (65). Even a single crosslink is lethal for recombination-deficient cells, while wild-type *E. coli* cells can tolerate up to 70 crosslinks per chromosome (65). DNA crosslinks are repaired through incisional-recombinational mechanism. An incision at both sides of cross-link is made on one DNA strand, and displacement of the incised oligonucleotide results in a SSG that can be repaired through a SSG-repair pathway. Subsequent incision on the second strand releases the crosslinked strands, producing a second SSG that is repair by a second round of recombinational repair.

For all SSGs, if the ssDNA in the gap is cleaved before the completion of SSG-repair, then the lesion is converted into a DSB. Thus, some fraction of SSGs is converted into DSBs and is repaired through the DSB-repair pathway.

Conjugation, transduction, and transformation create DSBs and SSGs.

DSBs arise not only as a consequence of DNA damage; in fact, DSBs are formed as a natural step in several normal cellular processes. The molecular feature common to each of these

processes is the cellular acquisition of a linear segment of dsDNA. The end of this linear DNA molecule is seen as a DSB, and the recombinational repair of the DSB is initiated. Bacteria can acquire linear DNA by any of three different routes. First, during conjugation, a copy of the chromosome is transferred from one bacterium, which possesses a fertility factor (the F' plasmid) to another. Genetic studies of this process led to isolation of the first recombination-deficient mutants (21). Transformation is a second route by which some bacteria take-up a segment of DNA from the environment; unrelated in process, but not in the form of the DNA involved, artificial transformation is widely used as a laboratory technique to introduce foreign dsDNA into cells. Finally, infection by bacteriophage also results in the introduction of linear DNA into bacteria. Note, however, that in addition to DSBs, the processes of conjugation and natural transformation also produce SSGs due to incomplete replication of the DNA intermediates that form during these processes.

Homologous pairing and DNA strand exchange mediated by RecA protein.

A step common to all pathways of recombinational repair discussed below is the homologous pairing of DNA (see Figure 1). In bacteria, the ubiquitous RecA protein catalyzes an invasion of ssDNA into homologous duplex DNA, and the exchange of DNA strands. Regardless of whether the DNA lesion is a DSB or SSG, pathway-specific processing of the break produces an extensive region of ssDNA that serves as the substrate for assembly of the RecA nucleoprotein filament. The process of finding DNA sequence homology and exchanging DNA strands occurs in three defined stages: (i) presynapsis, during which the RecA nucleoprotein is assembled; (ii) synapsis, during which the homology search and exchange of

DNA strands occur; and (iii) postsynapsis, during which branch migration can occur. Afterward, resolution of the resulting recombination intermediate produces a recombinant molecule.

In *E. coli*, *recA* null mutations reduce conjugational recombination by 100,000-fold (21). The active form of RecA protein is a nucleoprotein filament formed by the cooperative binding of RecA protein to the ssDNA tails of the processed DSB, or to the ssDNA in the SSG (see (6) for review). Thus, ssDNA is an essential, although transient, intermediate in the process of homologous recombination. Most of the SSGs, whose average length is minimally 200 nucleotides (78) (but can be as large as 800 nucleotides (37), or half the average size of an Okazaki fragment (66)), are sufficiently large for RecA nucleoprotein filament assembly; however, DSBs are not the normal direct targets of RecA protein binding. Therefore, DSBs and short SSGs are processed to produce longer regions of ssDNA. This processing requires either a nuclease, helicase, or both (see below).

However, production of ssDNA is not sufficient for RecA nucleoprotein filament assembly *in vivo.* Within the cell, ssDNA rapidly forms a complex with the ssDNA-binding (SSB) protein. SSB protein binding has the beneficial consequences of protecting ssDNA from degradation by nucleases and of disrupting inhibitory DNA secondary structures. Unfortunately, SSB protein blocks assembly of the RecA nucleoprotein filament by competing directly with RecA protein for ssDNA binding. Thus, the SSB protein-ssDNA complex is a kinetic barrier for the assembly of a RecA-ssDNA nucleoprotein filament, and its formation is inhibitory for the subsequent steps of homologous recombination. However, to counteract this inhibitory effect of SSB protein, there is a class of the recombination/replication mediator proteins (RMPs) whose function is to facilitate assembly of RecA protein (and its homologs in other organisms) on ssDNA, thereby alleviating the kinetic barrier imposed by SSB protein (the role of RMPs is

reviewed in (5)). Thus, in addition to the production of a single-stranded region within dsDNA, recombination requires the "loading" of RecA protein onto this ssDNA.

In wild-type *E. coli*, the processing of a broken DNA molecule, and the subsequent delivery of RecA protein to this ssDNA, occurs by either of two pathways: RecBCD- and RecF-pathways. The pathway names reflect critical and unique enzymes acting in each of the two pathways. The RecBCD-pathway is used primarily to initiate recombination at a DSB, whereas the RecF-pathway is used for recombinational repair at SSGs. The concept of recombination pathways was initially postulated by Clark (20). In both pathways, the Holliday junctions that result are resolved by the RuvABC enzyme complex (see (46) for review) into the recombinant progeny.

Double strand break repair by recombination: the RecBCD-pathway.

The RecBCD-pathway comprises the RecBCD, SSB, RecA, and RuvABC proteins; in addition, a specific DNA locus called χ (Chi = crossover hotspot instigator, 5'-GCTGGTGG-3') is required in the dsDNA that is broken.

In wild-type *E. coli*, RecBCD enzyme is required for approximately 99% of the recombination events associated with conjugation and transduction, *i.e.*, the processes that involve linear DNA and, hence, a DSB. Genetic studies revealed that the products of *recB* and *recC* genes are necessary for the repair of DSBs and for conjugational recombination. Deletion of either *recB* or *recC* genes reduces the levels of sexual recombination 100- to 1000-fold and increases sensitivity to DNA-damaging agents, such as UV-irradiation and mitomycin C (26, 36, 84). This effect, however, is significantly smaller then the 100,000-fold decrease in homologous recombination in the *recA*$^-$ mutants. In contrast, cells deleted for *recD* are not only proficient in

homologous recombination and DSB repair, but they catalyze homologous recombination at high rates (1).

The RecBCD enzyme is a heterotrimer consisting of the three non-identical polypeptides, RecB (134 kDa), RecC (129 kDa), and RecD (67 kDa) (1, 58). The three subunits comprise a complex, multifunctional enzyme that possesses a number of seemingly disparate catalytic activities, which include DNA-dependent ATPase, DNA helicase, ssDNA endo- and exonuclease, and dsDNA exonuclease. *In vivo*, these activities enable RecBCD enzyme to carry out a highly coordinated set of biochemical reactions resulting in conversion of the broken dsDNA into the active species in homologous recombination: the RecA nucleoprotein filament assembled on the ssDNA containing χ.

Among DNA helicases, an unusual feature of the RecBCD enzyme is its ability to initiate unwinding at a blunt or nearly blunt dsDNA end. RecBCD enzyme binds to blunt DNA ends with a very high affinity (≈ 1 nM), forming an initiation complex (30) that has a footprint of about 20 – 21 nucleotides of the 5'-terminated strand and 16 – 17 nucleotides of the 3'-terminated strand.

RecB protein has a modular organization. The N-terminal domain of RecB subunit contains motifs characteristic for the Superfamily I (SF1) DNA helicases. Moreover, the purified RecB protein is an ssDNA-dependent ATPase and a DNA helicase (10). Its behavior is related to the well characterized Rep, UvrD, and PcrA helicases. Similar to these enzymes, RecB protein displays 3'→5' helicase activity, requiring a 3'-terminated ssDNA region flanking the duplex DNA that is to be unwound. The C-terminal domain of this same subunit contains a nuclease motif resembling the nuclease domain of some type II restriction enzymes (such as *Fok*I and *Bam*HI) (77, 85). Besides being responsible for the complicated nuclease activity of RecBCD

enzyme, the C-terminal domain of RecB subunit has another crucial function: it harbors a site for interaction with RecA protein (19).

The amino acid sequence of RecC protein provides no clue as to its role in the holoenzyme. However, the existence of the RecC mutants that enable the holoenzyme to recognize an altered χ-sequence suggests a significant role in χ-recognition (3). Interaction with RecC subunit greatly stimulates the weak nuclease activity of the RecB helicase, and increases its affinity for dsDNA ends (61). The resulting RecBC enzyme is a fast, processive helicase that can initiate homologous recombination. However, RecBC enzyme displays negligible nuclease activity. As result, homologous recombination in the *recD*$^-$ cells strongly depends on the function of RecJ nuclease (51).

The RecD subunit also contains SF1 helicase motifs. The purified RecD protein is a DNA-dependent ATPase that was recently shown to be a 5'→3' helicase (23) similar to the closely related TraI and Dda helicases. Like these other helicases, RecD protein unwinds substrates containing a 5'-terminated ssDNA flanking the duplex DNA. Interaction with the RecD subunit further stimulates both the helicase activity and the dsDNA end-binding affinity of RecBC enzyme. Given that the RecB subunit is bound to the 3'-terminated strand; the RecD subunit is bound to the 5'-terminated strand (27); and each motor subunit moves with an opposite polarity, the resulting bipolar RecBCD enzyme translocates with each motor subunit moving in the same direction relative to the DSB. In addition, the resulting RecBCD holoenzyme now manifests its vigorous nuclease activity, implying that the RecD subunit also activates the nuclease contained within the RecB subunit (41).

The activities described above permit the following description for the mechanism of action of the RecBCD helicase/nuclease (Figure 3). Upon binding to the DNA end (Figure 4

(a.)), the enzyme uses energy of ATP hydrolysis to translocate along and unwind the dsDNA molecule, consuming approximately two to three molecules of ATP per 1 base pair unwound (63) (Figure 4 (c.)). RecBCD enzyme unwinds, on average, 30,000 bps of dsDNA per binding event (62) at a rate of approximately 1,000-1,300 bps/sec (at 37 ºC). DNA unwinding by RecBCD enzyme is accompanied by endonucleolytic cleavage of newly produced ssDNA. The nuclease activity of RecBCD enzyme is asymmetric, with digestion occurring preferentially on the 3'-terminated strand relative to the DSB (25).

The recombination hotspot, Chi.

Originally, χ was identified as a *cis*-acting mutation in bacteriophage λ that allowed its more efficient growth in *E. coli* by stimulating the host's recombination system. Stimulation of recombination is approximately 10-fold (47), and it is confined to loci that are downstream of χ (70): stimulation is highest at χ and then decreases exponentially (17, 47). The χ sequence is over-represented in *E. coli*; there are 1009 χ sequences found in the 4.6 Mb genome of the MG1655 strain (9). Furthermore, over 60% of χ sequences are oriented towards the replication origin. Such an orientation would facilitate RecBCD-mediated recombination repair of DSBs created during DNA replication (11).

Biochemically, the activities of RecBCD enzyme are altered upon recognition of χ. Alteration of RecBCD enzyme activity is manifest only when the enzyme approaches χ, 5'-GCTGGTGG-3', from its 3'-side. In the schematic depiction (Figure 4), χ, which is the sequence in the "top strand" (7), is recognized by an enzyme moving only from the right to the left. *In vivo*, interaction with χ results in the stimulation of homologous recombination downstream of χ (69, 71). *In vitro*, recognition of the χ-sequence causes RecBCD enzyme to switch the polarity

of its nuclease activity (Figure 4 (d.)): upon interaction with χ, degradation of the 3'-terminated strand is down-regulated, while degradation of the 5'-terminated strand is up-regulated (24, 25). Consequently, the enzyme produces a lengthy ssDNA tail with χ at the 3'-terminated end. As determined from production of the χ-specific ssDNA fragments, the probability of recognizing a single χ is about 30 – 40% (3, 72). Interaction with χ also affects the helicase activity of RecBCD enzyme. Recognition of χ causes the enzyme to pause briefly (typically, a few seconds) at χ and to resume translocation after the χ site, but at a rate that is reduced by approximately 2-fold (68). Another consequence of χ-modification is that the RecBCD enzyme gains the ability to "load" RecA protein onto the newly produced ssDNA (2) (Figure 4 (e.)). Thus, in response to χ, the RecBCD enzyme accomplishes both tasks essential for initiation of homologous recombination: 1) it recesses the DSB to produce an ssDNA-tailed duplex DNA with χ at its terminus; and 2) it catalyzes formation of the RecA nucleoprotein filament on the ssDNA produced. This RecA nucleoprotein filament now can search for homology, promote invasion of the homologous recipient, and exchange the DNA strands (Figure 4f.).

The exact molecular mechanism, by which χ-recognition is translated into the observed changes in the activities of the RecBCD enzyme, remains unknown. Most models propose either dissociation or inactivation of RecD subunit (2, 19, 57, 73). Indeed, as mentioned previously, the RecBC enzyme (lacking the RecD subunit) is recombinationally proficient both *in vivo* (13) and *in vitro* (18) (Figure 4a'). The RecBC enzyme is a processive helicase but with little or no nuclease activity (41), which is in contrast to the χ-activated form of RecBCD enzyme; however, this distinction is consistent with the requirement for RecJ nuclease activity in *recD*$^-$ cells *in vivo* (51). Similar to RecBCD enzyme, RecBC helicase facilitates asymmetric assembly of RecA protein only onto ssDNA that is 3'-terminal at the enzyme entry site (Figure 4 (c')). The RecBC-

mediated loading of RecA protein is constitutive and is independent of χ, consistent with the phenotypic behavior of *recD*$^-$ cells *in vivo*.

Regulated helicases/nucleases in other bacterial species.

For decades, the interaction between RecBCD enzyme and χ was known to exist only in the species of enteric bacteria closely related to *E. coli*. But relatively recently, short (5 – 8 bps) sequences, which protect linear dsDNA from degradation by attenuation of the nuclease activity of RecBCD-like helicase/nuclease enzymes, were found in several distantly related bacteria (8, 16, 67). This finding indicates that, although apparently not universal, the regulation of recombinational helicases/nucleases by specific DNA sequences is widely spread among prokaryotes. While some bacteria possess clear homologues of RecBCD enzyme, other species contain its functional equivalent, the AddAB enzyme (reviewed in (15)). AddAB helicase/nuclease is constituted of two subunits encoded by *addA* and *addB* genes. The sole motor subunit of AddAB enzyme, AddA protein, contains an SF1-helicase and a nuclease domain, which display high degree of similarity to those in RecB protein (32). Also similar to RecB protein, AddA is a 3'→5' helicase, and its 3'→5' nuclease activity is down-regulated upon interaction with the cognate recombination hotspot of *Bacillus subtilis*, χ_{Bs} (5'-AGCGG-3') (14, 16). The AddB subunit has no substantial similarity to either the RecC or RecD subunits, but it does contain a putative ATPase motif and a second nuclease site similar to that of the AddA protein. The AddB subunit is responsible for the degradation of the 5'-terminated strand. In spite of the limited sequence similarity to the RecBCD enzyme, AddAB enzyme is functional in *E. coli*: its expression overcomes the recombination and repair defects of *recBC*-deficient cells (40). Similar to RecBCD enzyme, AddAB enzyme binds to the blunt-ended dsDNA, and uses the energy of ATP hydrolysis to translocate along and unwind dsDNA. However, where RecBCD

enzyme degrades the dsDNA asymmetrically, the AddAB enzyme degrades both strands of the DNA duplex equally. Interaction of the translocating AddAB enzyme with a correctly oriented χ_B results in down-regulation of only the 3'→5' nuclease activity of the enzyme. The outcome, therefore, is the same as that occurring for the *E. coli* enzyme: namely, the production of ssDNA-tailed dsDNA with χ at the 3'-terminus. Homologues of AddAB enzyme are found in 12 different species of gram-positive bacteria, and χ-homologs were identified in several bacterial species (reviewed in (15)).

Single-strand gap repair by recombination: the RecF-pathway.

The conjugal recombination deficiency of *recB*$^-$ or *recC*$^-$ mutants can be overcome by the combined effect of two extragenic suppressor mutations, *sbcB* and either *sbcC* or *sbcD* (suppressor of *recBC*). The *sbcB* mutation disables the nuclease activity of Exonuclease I (44), while *sbcC* (31) disable one of the two subunits of the SbcCD nuclease which, in wild-type *E. coli,* cleaves DNA hairpin and cruciform structures formed during replication of palindromic sequences (22, 49). The combined effect of these mutations is the full activation of an alternative pathway of sexual homologous recombination, referred to as the RecF-pathway. Interestingly, the efficiency of conjugational and transductional recombination by the RecF-pathway in the *recBC sbcBC* cells is similar to that of the RecBCD-pathway in wild-type cells, showing that the machinery of this pathway can be as productive as that of the RecBCD-pathway. Moreover, some bacterial species, whose survival depends on homologous recombination (such as *Deinococcus radiodurans*), do not possess obvious RecBCD or AddAB enzymes, implying that a RecF-like pathway is the wild-type pathway in those bacteria.

Homologous recombination in the *recBC*$^-$ *sbcB sbcC*$^-$ background depends on RecF, RecJ, RecN, RecO, RecQ, RecR, and SSB proteins. The processing of a DSB is likely achieved

by the combined action of the RecQ helicase and RecJ nuclease. Although RecQ helicase is responsible for about 75% of conjugal recombination events occurring in *recBC sbcB sbcC*$^-$ cells, the remaining 25% require either UvrD (helicase II) or HelD (helicase IV) (53). The RecJ protein is an exonuclease that degrades ssDNA in the 5'→ 3' direction (Figure 5 (a – c)). RecQ protein is an SF2 DNA helicase with a 3'→ 5' polarity. Similar to RecBCD enzyme, RecQ helicase can unwind blunt ended dsDNA (76). The helicase activity of RecQ protein is not limited to blunt dsDNA ends: the enzyme can also unwind ssDNA-dsDNA junction with a 3'-ssDNA overhang (76), and even internal regions of dsDNA (33). It is hypothesized that DNA unwinding by RecQ helicase is coupled to the degradation of the 5'-terminated strand by RecJ nuclease, resulting in the production of the 3'-terminated ssDNA overhang, which can then be used as a substrate for RecA nucleoprotein assembly (Figure 5 (c.)). In contrast to RecBCD enzyme, RecQ does not facilitate RecA nucleoprotein filament assembly. Therefore, the ssDNA produced by RecQ is bound by SSB protein and must be protected from degradation by nucleases, which explains the requirement for the *sbcB* mutation, since the preferred substrate for Exonuclease I is SSB-complexed ssDNA. Intriguingly, two major classes of mutations in the structural gene for Exonuclease I were found. One class of mutations, referred to as *sbcB*, restores both recombination and the UV-resistance of *recBC* cells. In contrast, the other class, known as *xonA* mutants, suppresses only the UV-sensitivity but not the recombination deficiency of the *recBC*$^-$ bacteria (44). Exonuclease I activity is significantly reduced in both classes of mutants; moreover UV-sensitivity directly correlates with the amount of residual activity of the enzyme (60). The *sbcB* mutations are not the same as null mutations and, therefore, likely represent gain of function mutations. The molecular mechanism distinguishing the two types of

mutations still remains to be elucidated, but protection of the 3'-end of ssDNA by the *sbcB* mutations is envisioned.

The loading of RecA protein is an essential aspect of recombination in the RecBCD-pathway (4). Not unexpectedly, the RecF-pathway provides a RecA-loading activity in the form of the RecFOR complex. The genetic data had suggested that the loading of RecA protein onto SSB-coated ssDNA depends on the concerted action of RecF, RecO, and RecR proteins. Firstly, mutant RecA proteins that suppress the UV-sensitivity of *recF* mutations (such as the RecA803 protein) displace SSB protein from ssDNA much faster and more extensively than wild-type RecA protein (52), implying that RecF protein plays a role in SSB-displacement. Secondly, a number of mutant *recA* alleles co-suppress mutations in *recF*, *recO*, and *recR* genes. Finally, suppression of mutations in any of these three genes is dependent on *recJ* function, suggesting that RecF, RecO, and RecR proteins may function together as a complex (79). This view is strongly supported by biochemical observations showing that these proteins interact with one another (34, 74).

The RecFOR proteins form a number of complexes with different activities: RecO protein interacts with both RecR and SSB proteins (34), and facilitates RecA nucleoprotein filament assembly on SSB-coated ssDNA (75). RecO protein also promotes the annealing of ssDNA, and of SSB-ssDNA complexes (38). Finally, RecR protein interacts with RecF protein to form a complex (34, 81) that will bind to an ssDNA-dsDNA junction (55). Biochemical analysis revealed that RecF protein binds preferentially to the ssDNA-dsDNA junction (35), and that DNA binding by RecF protein is controlled by ATP hydrolysis (80). The RecFOR complex will bind to an ssDNA-dsDNA junction with a base-paired 5'-terminus at the junction region, and it will facilitate assembly of RecA protein onto ssDNA adjacent to the junction (55). RecF protein

(or RecFR complex) recognizes an ssDNA-dsDNA junction with a 5'-end, which is the structure that should be produced by RecQ and RecJ proteins. The RecOR complex (or RecO protein) binds to the DNA-RecF(R) complex, which alters SSB-ssDNA complex nearby and allows RecA protein nucleation; subsequent nucleoprotein filament extension permits assembly of RecA protein on the entire ssDNA tail.

Another component of the RecF-pathway, RecN protein, has not yet been assigned any biochemical function. However, the slight recombination deficiency and mild UV sensitivity of *recJ recN* double mutant, combined with severe recombination defect (50- to 100-fold reduction) of *recD recJ recN* mutant, suggest that RecN protein might be a functional equivalent of the RecJ nuclease (50).

In spite of its apparent complexity, the enzymatic machinery of the RecF pathway is as functional in DSB repair as RecBCD enzyme. Moreover, the components of the RecF pathway have functional homologs or paralogs in all organisms from bacteriophage to human (5).

Single-strand gap repair.

Because conjugation (and transduction) involve a DSB as the initiating site for recombination, it should not be surprising that the genes which emerged from a screen for mutants defective in sexual recombination, are essential for the repair of DSBs. However, the consequence of this nearly singular focus on conjugational recombination events led to the erroneous conclusion that the RecF-pathway serves only a minor function in recombination in the wild-type cells. Similarly incorrect, the discovery of the RecF pathway as the set of genes that permitted recombination in the absence of the primary RecBCD-pathway implied that the RecF-pathway was a cryptic recombination pathway that could be activated to compensate for the loss of RecBCD enzyme function. Rather, in wild-type cells, the RecF-pathway is

responsible for the repair of all SSGs. This conclusion emerged from many studies, but was made clearest from recombination assays that did not employ sexual events.

A genetic assay that measures recombination between direct repeats of a chromosomal segment allows detection of sister-chromosome exchanges that are required for the repair of DSBs (29). This assay was developed to approximate the function of homologous recombination in the repair of the DNA lesions produced during replication. The recombination events occurring between direct chromosomal repeats are detected in the colony-sectoring recombination assay, since the detected recombination events eliminate the joint-point markers located between the repeats. Recombination events detected in this assay, are absolutely dependent on the RecA protein function. The *recF* and *recJ* mutants display a rec^+ phenotype; *recB* mutants show only slight defect; and *recB recJ* double mutants are capable of supporting duplication segregation. On the other hand, *recB recF* double mutants are deficient in the recombination between chromosomal direct repeats, suggesting that both RecBCD and RecF pathways play major roles in recombination.

Initiation of homologous recombination on SSGs is presented in Figure 6. First, RecFOR complex assembles at the ssDNA-dsDNA junction at the end of SSG containing an complementary 5'-end (Figure 6 (a.)) and facilitates RecA nucleoprotein assembly in the ssDNA region of the gap (55) (Figure 6 (b.)). RecFR complex can bind to the 3'-containing end of SSG limiting RecA filament extension into the dsDNA region (82) (Figure 6 (c.)). The resulting RecA nucleoprotein filament formed on the SSG can then invade a homologous dsDNA molecule (Figure 6 (d.)). DNA strand exchange followed by heteroduplex extension results in the formation of two Holliday junctions. To complete the repair, RecA protein must be removed from the junctions and the Holliday junctions themselves need to be resolved. The processing of

a Holliday junction into mature recombinant molecules is achieved by the RuvA, RuvB, and RuvC proteins (see (64, 83) for review). The RuvA tetramer is a 4-way junction-specific recognition protein that binds to the Holliday junction and induces the square planar conformation of this junction. The RuvB protein is a specialized translocation protein that can "pump" or move DNA through its circular hexameric active form. To catalyze branch migration, two hexameric rings of RuvB protein bind to opposing arms of the Holliday junction that was recognized by the RuvA proteins, and then they translocate the dsDNA outward through the center of each ring, resulting in the relative movement of the junction (Figure 6 (h.)). RuvC protein is a 4-way junction specific endonuclease that resolves a Holliday junction by symmetrically cleaving opposite arm of the junction to produce the recombinant DNA product molecules (Figure 6 (i.)).

Alternatively, an ssDNA endonuclease can convert an SSG into a DSB, which can be repaired through a DSB repair mechanism (Figure 6 (b.)). If the region of ssDNA in the gap is too small, then it can be expanded by the combined action of RecQ helicase and RecJ nuclease. Similar to the DSB repair situation, RecQ helicase function can be substituted by UvrD helicase or Helicase IV (53) (Figure 6 (c.)). The SSG, on which the RecA nucleoprotein filament is assembled, is not necessarily repaired only by homologous recombination. The error-prone UmuD'C DNA polymerase can be attracted to such a RecA filament assembled on the SSG to catalyze translesion DNA synthesis (59) (Figure 6 (j.)).

Conclusion

Homologous recombination can be initiated either at DSBs or SSGs in duplex DNA. Two major pathways are responsible for homologous recombination in wild-type *E. coli*: the RecBCD- and RecF-pathways. The RecBCD-pathway is specific for the recombinational repair of DSBs, and in the wild-type cells, the RecF-pathway is primarily used for recombination that initiates at SSGs. However, with appropriate suppressor mutation in *E. coli*, and presumably in bacteria that lack a RecBCD-pathway, the RecF-pathway can efficiently act at DSBs as well. Despite the different initiating lesions, both pathways have the same subsequent step: conversion of the broken DNA molecule into a central intermediate of recombination, which is the RecA protein nucleoprotein filament assembled along the ssDNA. In the RecBCD pathway, this process is carried out by a combined helicase/nuclease activity of RecBCD enzyme, and depends on the presence of the recombination hotspot, χ. In the RecF-pathway, the combined efforts of RecQ helicase and RecJ ssDNA nuclease are needed in combination with the RecFOR complex. In the RecBCD pathway, RecBCD enzyme facilitates assembly of the RecA protein onto SSB-coated ssDNA; while in the RecF pathway, this task is accomplished by RecF, RecO, and RecR proteins. RecA nucleoprotein filament can then initiate invasion of ssDNA into homologous dsDNA, progressing into the final stage of homologous recombination which is resolution by RuvABC proteins.

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Figure Legends

Figure 1. ***Recombinational repair of DNA damage.*** The DNA strands that are used as templates for the leading strand synthesis are shown in black, and the strands used as templates for the lagging strand synthesis are shown in gray. Arrows indicate the direction of DNA synthesis.

A) Recombinational repair of directly induced DSBs. A DSB, occuring in newly synthesized DNA, can be repaired by completion of the following steps: First, the DSB is processed to produce 3'-terminated ssDNA. Then, one of the ssDNA tails can invade the homologous dsDNA daughter, displacing one of the resident strands to form a D-loop. This structure can be used as a template for DNA synthesis, ultimately resulting in the formation of a Holliday junction. Upon resolution of the Holliday junction, the replication fork is restored to its original form. When a DSB occurs in a part of the chromosome that has not yet replicated, there is no homologous DNA to serve as a template, and the DSB can be lethal.

B) Recombinational repair of DSBs. DSBs can be produced by replication through a single-strand DNA break. The source of the replication-dependent DSBs is a nick or an ssDNA gap present in one of the strands of the replicated DNA molecule. Replication through the strand discontinuity results in the formation of one intact and one broken DNA molecule. The end of the broken chromosome is processed to form a 3'-terminated ssDNA tail, which invades the intact homologous DNA to form a D-loop, which can then be used to restore a normal replication fork.

C) Recombinational repair of SSGs. SSGs can be produced when synthesis of only one DNA strand is halted by an encounter of a non-coding lesion in that DNA strand. After DNA strand exchange of the ssDNA in the gap with a strand in the intact daughter homolog, the

displaced DNA strand can be used as a template for DNA synthesis resulting in restoration of the replication fork. Upon completion of replication, one of the DNA molecules will still contain the original DNA damage. If this damage is not repaired by the appropriate repair system, then an SSG will be formed again by the next round of DNA replication.

Figure 2. ***DSBs can be produced as a result of Holliday junction cleavage.*** Reversal of a stalled DNA replication fork results in the formation of a regressed replication fork, which is a four-way junction (Holliday junction) that contains a dsDNA end. There are two potential fates for this regressed replication fork: it can be degraded by the recombination machinery to produce a 3-way junction that resembles a replication fork, or the Holliday junction can be cleaved to produce a DSB that is repaired by a DSB-repair pathway.

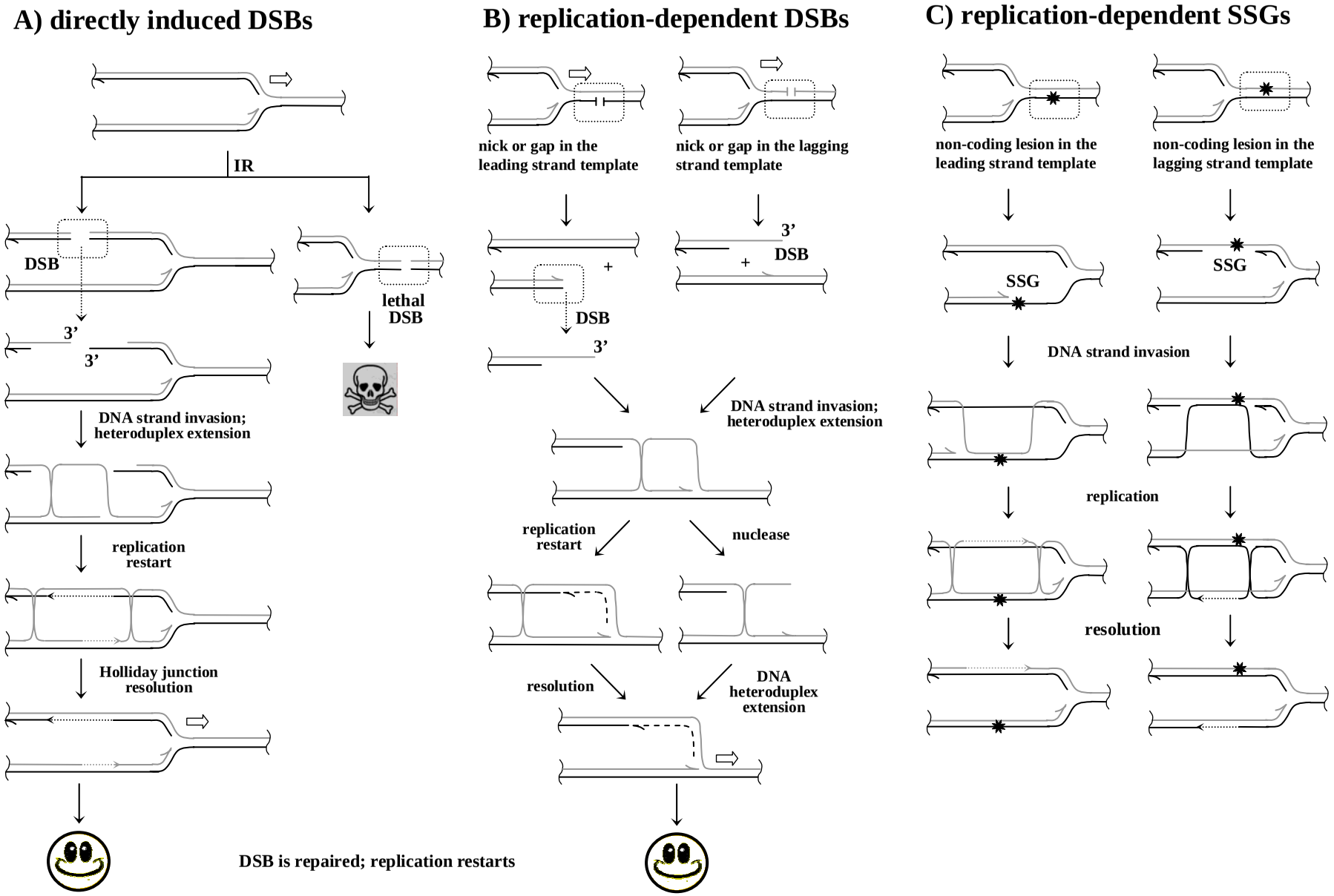
Figure 3. ***Inter-strand crosslinks are converted into DSBs and SSGs.*** Schematic representation for the incision-recombination mechanism of inter-strand DNA crosslink repair. The repair of an inter-strand crosslink depends on both the nucleotide excision repair (NER) and recombination machineries. The NER enzymes make an incision on either side of the lesion on one DNA strand and also displace the crosslink-containing oligonucleotide to produce an SSG; this SSG can be repaired by an SSG-repair pathway. However, the incised oligonucleotide remains crosslinked to the second DNA strand. If this lesion is recognised by NER machinery prior to the completion of SSG repair of the top strand, then the subsequent incisions on the second strand converts the inter-strand crosslink into a DSB, which is then repaired by a DSB-repair pathway.

Figure 4. ***Initiation of homologous recombination at DSBs by the RecBCD-pathway.*** Schematic representation of the early enzymatic steps of the RecBCD-pathway of recombinational repair. **a.** and **a')** RecBCD (or RecBC) enzyme binds to the blunt or nearly blunt dsDNA end. **b.)** RecBCD enzyme uses energy of ATP hydrolysis to translocate along and to unwind the dsDNA. The associated nuclease activity degrades the newly produced ssDNA. **c.)** Interaction with χ results in the attenuation of the RecBCD nuclease activity and production of ssDNA terminated with the χ sequence at its 3' end. **d.)** The χ-modified enzyme loads RecA protein onto the χ-containing ssDNA to the exclusion of SSB protein, forming a RecA nucleoprotein filament. **e.)** The RecA-ssDNA nucleoprotein filament invades homologous dsDNA. **b'.)** The RecBC enzyme (*i.e.*, lacking the RecD subunit) behaves as a constitutively χ-modified RecBCD enzyme that can load RecA protein without the need for χ-interaction. The RecBC enzyme lacks nuclease activity that is compensated by the action of the RecJ nuclease.

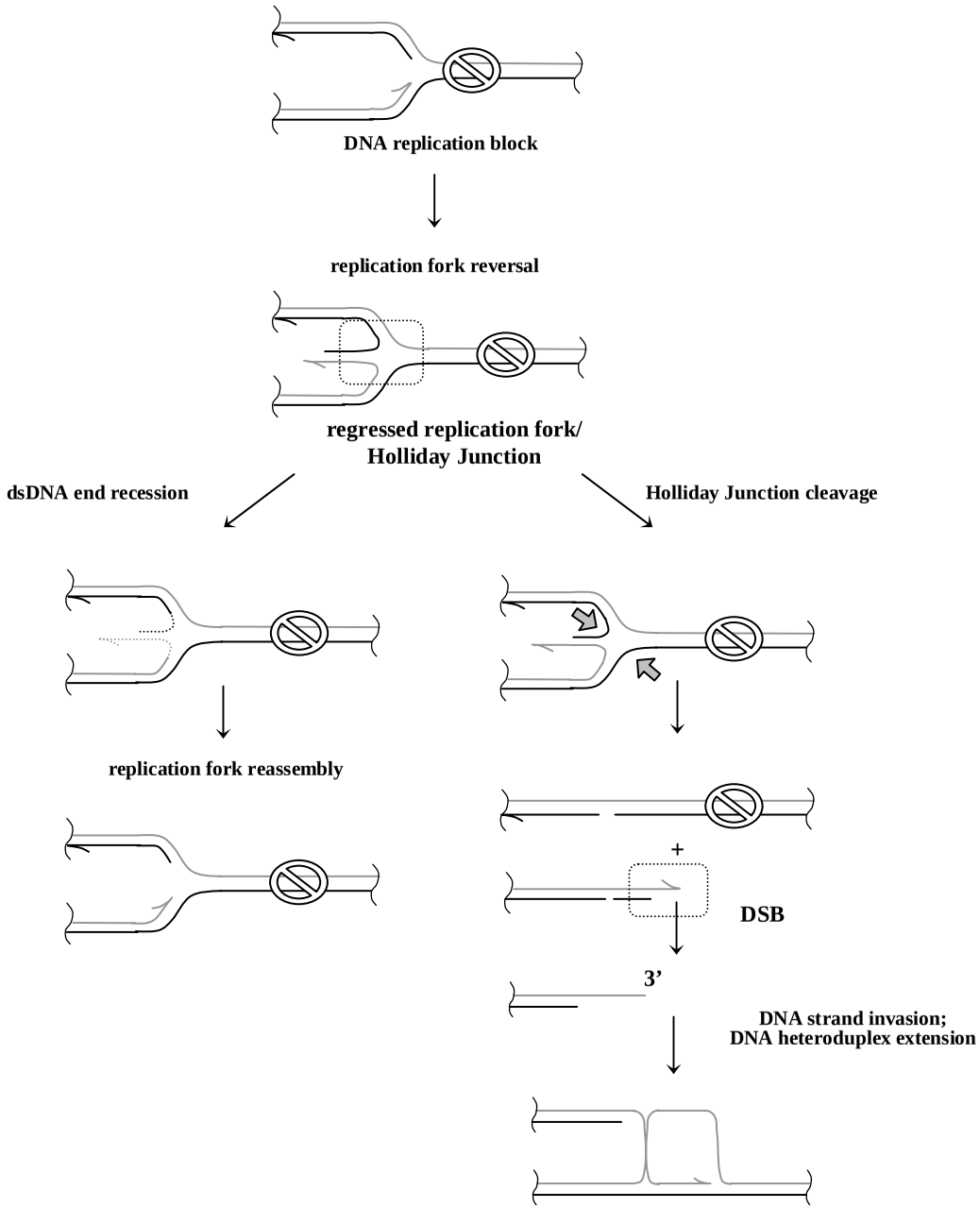
Figure 5. ***Initiation of homologous recombination at DSBs by the RecF- pathway.*** Schematic representation of the early enzymatic steps of RecF-pathway. **a., b.,** and **c.)** The combined action of RecQ helicase and RecJ nuclease converts a DSB into an ssDNA-dsDNA junction with 3'-terminated ssDNA overhang; this ssDNA is complexed with SSB protein. **d.** and **e)** The RecF, RecO, and RecR proteins form a complex at the junction and facilitate RecA nucleoprotein filament assembly on the SSB-coated ssDNA. **f.)** The RecA-ssDNA nucleoprotein filament invades homologous dsDNA.

Figure 6. ***Repair of SSGs by the RecF-pathway.*** Schematic representation of SSG repair by the RecF-pathway. The SSG has several potential fates: **a.)** The RecFOR proteins can bind to

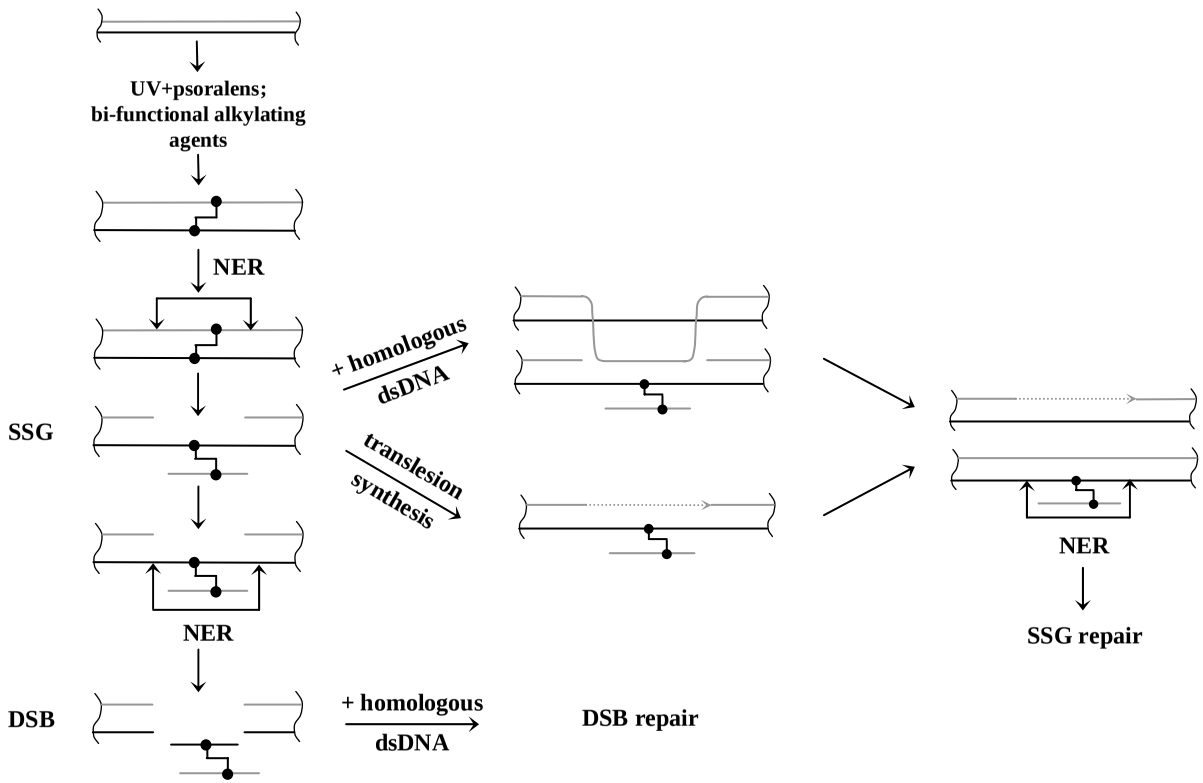
the 5'-end of the ssDNA-dsDNA junction; **b.)** the SSG can be cleaved to produce a DSB; **c.** and **d.)** or, the ssDNA region can be expanded by the combined activities of the RecQ helicase and RecJ nuclease. **f.)** RecFOR proteins facilitate RecA protein loading onto the SSB-coated ssDNA; growth of the RecA nucleoprotein filament beyond the ssDNA region is prevented by the RecFR complex bound to the ssDNA-dsDNA junction containing a free 3' end. **g.)** The RecA nucleoprotein filament invades homologous dsDNA and catalyses DNA strand exchange. **h.)** DNA heteroduplex expansion results in the formation of two Holliday junctions. **i.)** RuvABC proteins facilitate branch migration and Holliday junction resolution to produce repaired recombinant molecules.



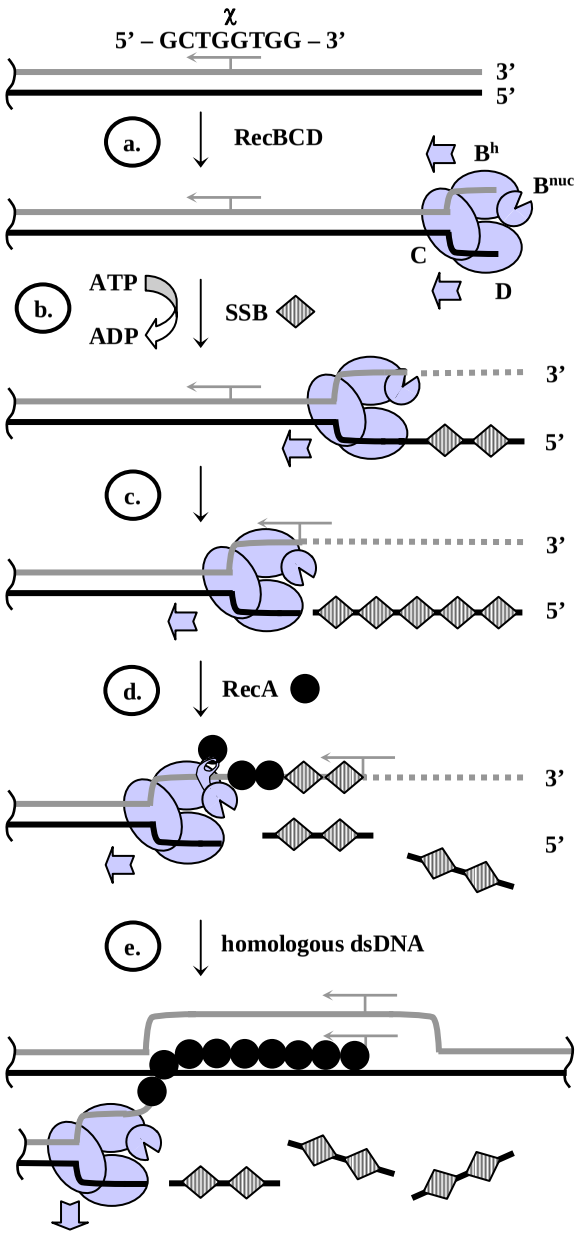
Spies and Kowalczykowski, Fig. 1 Recombinational repair of DNA damage



Spies and Kowalczykowski, Fig. 2 DSBs can be produced as a result of Holliday Junction cleavage



Spies and Kowalczykowski, Fig. 3 Inter-strand crosslinks are converted into DSBs and SSGs



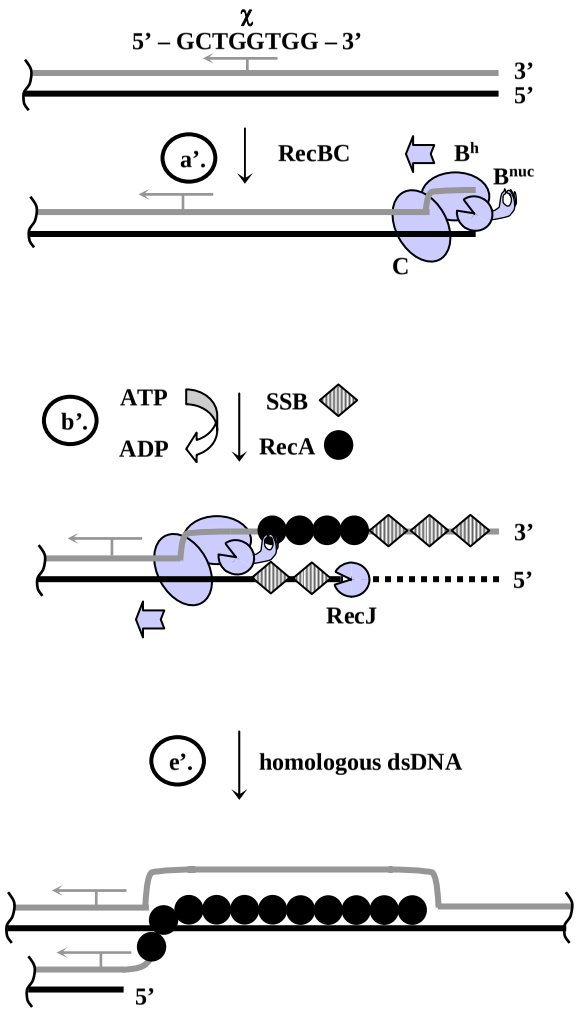
a) RecBC(D) binding to dsDNA end

b) RecBCD-mediated unwinding of dsDNA and preferential degradation of 3’-terminated strand
b’) RecBC-mediated unwinding of dsDNA and χ-independent RecA protein-loading; RecJ-dependent degradation of 5’-terminated strand

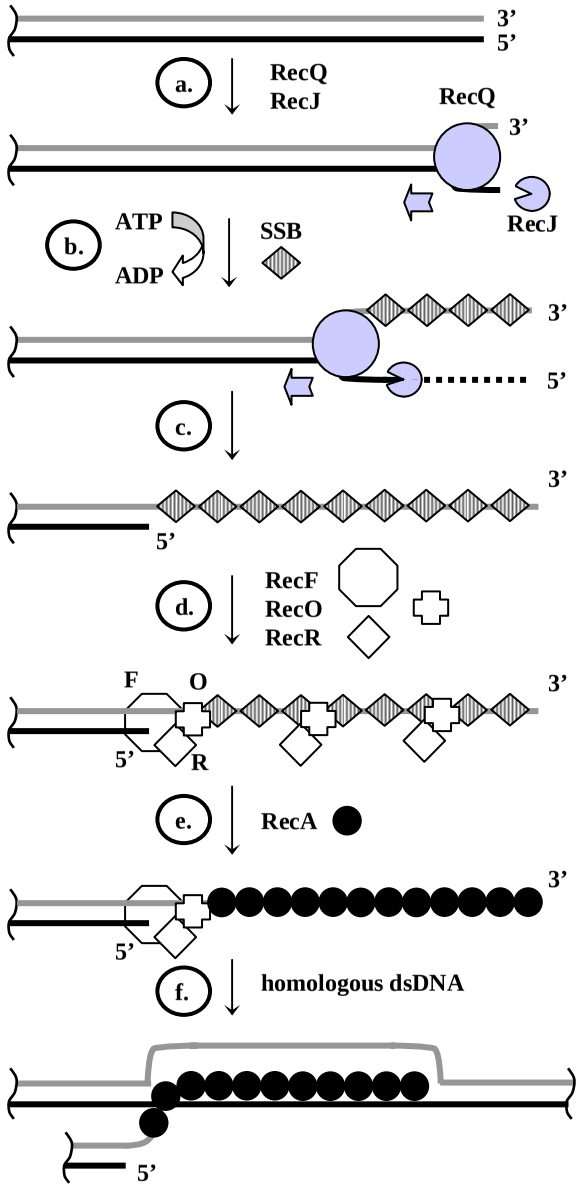
c) Recognition of χ: pause at χ; attenuation of nuclease activity (down-regulation of 3’-5’ nuclease, lesser up-regulation of 5’-3’ nuclease); reduction of translocation

d) RecA protein-loading

e) Homologous pairing and DNA strand exchange



Spies and Kowalczykowski, Fig. 4 Initiation of homologous recombination at DSBs by the RecBCD-pathway



a.) RecQ helicase binds to the dsDNA end

b.) RecQ unwinds dsDNA, RecJ nuclease degrades the 5’-terminated strand

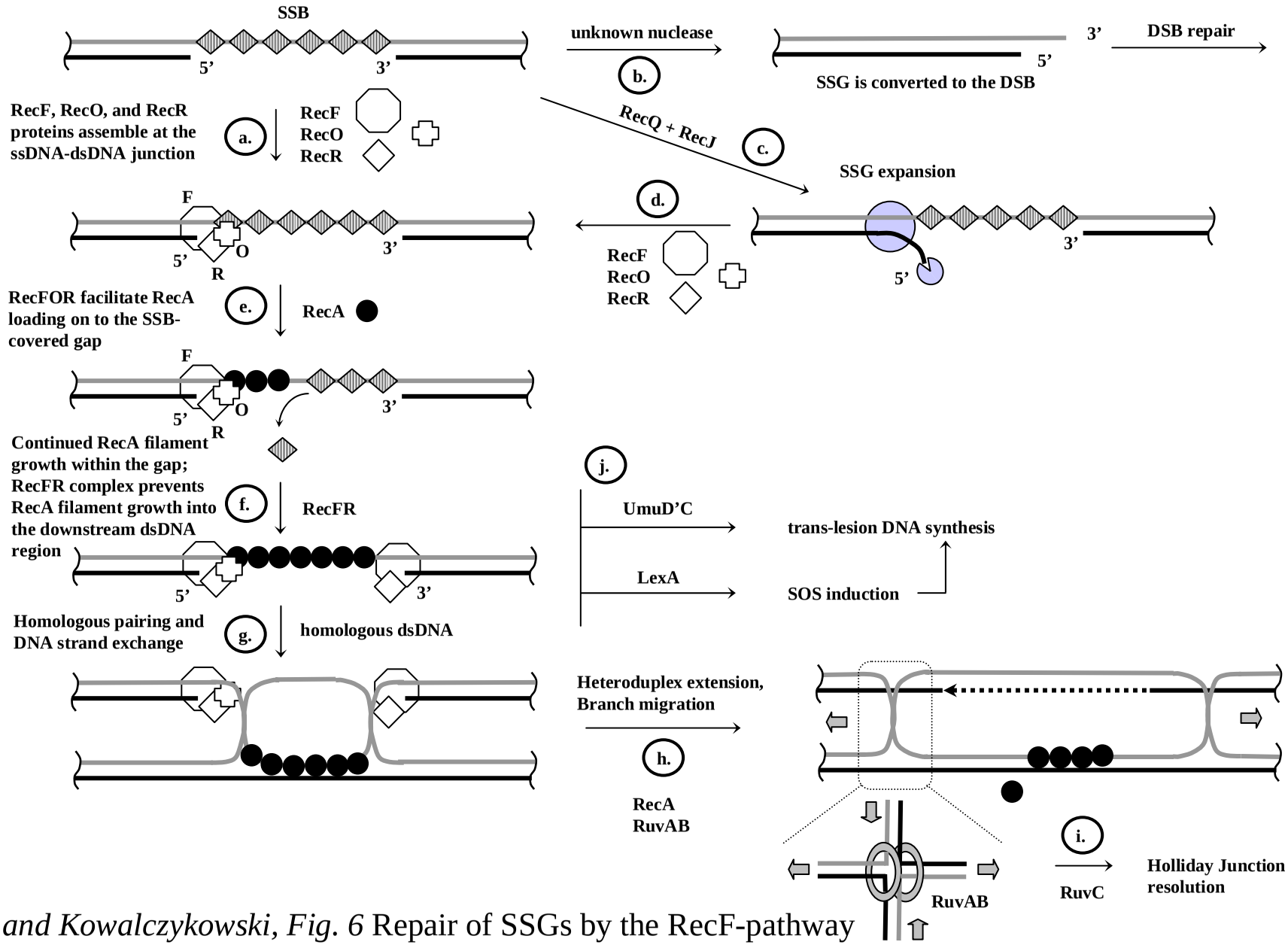
c.) Dissociation of RecQ and RecJ proteins

d.) Assembly of RecF, RecO, and RecR proteins at ssDNA-dsDNA junction

d.) RecA protein-loading

e.) Homologous pairing and DNA strand exchange

Spies and Kowalczykowski, Fig. 5 Initiation of homologous recombination at DSBs by the RecF-pathway



Spies and Kowalczykowski, Fig. 6 Repair of SSGs by the RecF-pathway