Published in final edited form as:
Cell. 1994 September 23; 78(6): 1051–1061.

DNA Replication Triggered by Double-Stranded Breaks in E. coli: Dependence on Homologous Recombination Functions

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Summary

Homologous recombination-dependent DNA replication (RDR) of a λ *cos* site-carrying plasmid is demonstrated in E. coli cells when the cells express λ terminase that introduces a double-stranded break into the cos site. RDR occurs in normal wild-type cells if the plasmid also contains the recombination hotspot χ. χ is dispensable when cells are induced for the SOS response or contain a *recD* mutation. *recBC sbcA* mutant cells are also capable of RDR induction. A *recN* mutation greatly reduces RDR in normal cells, but not in SOS-induced cells. RDR proceeds by the θ mode or rolling circle mode of DNA synthesis, yielding covalently closed circular plasmid monomers or linear plasmid multimers, respectively. Previously described inducible stable DNA replication is considered to be a special type of RDR that starts exclusively from specific sites (*or/M*s) on the chromosome.

Introduction

The major homologous recombination pathway in wild-type Escherichia coli, the RecBCD pathway, requires RecBCD enzyme (exonuclease V), which consists of the products of the *recB, recC,* and *recD* genes (for reviews see Smith, 1988; Kowalczykowski et al., 1994). The enzyme binds a flush or nearly flush end of duplex DNA and unwinds it by its helicase activity. When the enzyme encounters a χ sequence (5′-GCTGGTGG-3′) from the 3′ side, it frequently nicks the χ-containing strand 4–6 nt to the 3′ side of χ (Ponticelli et al., 1985). The single-stranded DNA (ssDNA) tail with a 3′ end produced by subsequent unwinding would then be assimilated into a homolog by the action of RecA, yielding a recombination intermediate, i.e., a D loop (Kowalczykowski et al., 1994). Thus, properly oriented χ stimulates homologous recombination. The RecBCD enzyme also possesses a nonspecific nuclease activity that can be attenuated by the interaction between the enzyme and χ (Dixon and Kowalczykowski, 1991, 1993; Taylor and Smith, 1992; Dabert et al., 1992). Subsequent translocation of the nuclease-attenuated RecBCD enzyme could produce ssDNA that remains undigested and can be used by RecA for D loop formation.

The attenuation of the nuclease activity is due either to removal or functional inactivation of the RecD subunit (Dixon et al., 1994). This suggests that the RecBC enzyme found in *recD* mutant cells may be functionally similar to the wild-type enzyme that has encountered χ (Kowalczykowski et al., 1994). This is consistent with the observations that, while *recB* and

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recC mutants are recombination deficient, *recD* mutants are recombination proficient (Chaudhury and Smith, 1984; Amundsen et al., 1986; Thaler et al., 1989) and that the χ stimulation is not detected in *recD* mutants (Chaudhury and Smith, 1984). The nuclease activity of RecBCD enzyme is undetectable in cell-free extracts prepared from *recD* mutants (Chaudhury and Smith, 1984). Furthermore, purified or reconstituted RecBC enzyme possesses a significant level of helicase activity but little nuclease activity (Palas and Kushner, 1990; Boehmer and Emmerson, 1991; Masterson et al., 1992). In vivo experiments also suggest that RecBC enzyme in *recD* mutant cells is capable of DNA unwinding, but not DNA degradation (Chaudhury and Smith, 1984; Rinken et al., 1992). The χ stimulation of homologous recombination is partially suppressed in SOS-induced cells, although overall recombination levels are not affected (Rinken and Wackernagel, 1992). Since overproduction of the RecD subunit alleviates the suppression, it is proposed that an SOS-inducible factor modifies RecBCD via interaction with the RecD subunit to produce a χ-independent enzyme. The nuclease activity of RecBCD is also attenuated after SOS induction (Kannan and Dharmalingam, 1990, and references therein).

Activation of one of the other two pathways of homologous recombination, i.e., the RecE and RecF pathways, suppresses recombination deficiencies in the RecBCD pathway of *recB* and *recC* mutants (Clark, 1991). The RecE pathway is activated by an *sbcA* mutation and requires the product of the *recE* gene, exonuclease VIII. Exonuclease VIII degrades one strand of linear duplex DNA in the 5′ to 3′ direction (Joseph and Kolodner, 1983), and thus the enzyme yields a ssDNA tail having a 3′ end that is utilized by RecA for D loop formation. The RecF pathway is activated by mutations in the *sbcB* and *sbcC* genes. In this pathway, RecQ helicase (Umezu and Nakayama, 1993) and RecJ nuclease (5′ to 3′ ssDNA exonuclease; Lovett and Kolodner, 1989) are proposed to play important roles in the production of ssDNA (for review see Clark, 1991).

Chromosome replication in E. coli is initiated by interactions between DnaA protein and its binding sites, which results in duplex unwinding of the AT-rich region within the minimal *oriC* (Kornberg and Baker, 1992). Every round of initiation at *oriC* requires transcription and translation. Homologous recombination functions are normally not essential for chromosome replication. When E. coli cells are induced for the SOS response by DNA damage or DNA replication inhibition, a different type of DNA replication, called inducible stable DNA replication (iSDR), is activated (for review see Asai and Kogoma, 1994). iSDR replicates the entire genome semiconservatively. Major origins for iSDR map in the *oriC* and *terC* regions of the chromosome (Magee et al., 1992) and are termed *oriM1* and *oriM2*, respectively. The active *oriC* site, however, is not necessary tor *oriM1* functions (Asai et al., 1994). The initiation mechanism of iSDR is radically different from that of the *oriC* system; it is independent of normally required DnaA protein, transcription, and translation, but absolutely requires homologous recombination functions. The D loop model for iSDR initiation (Asai et al., 1993) postulates that upon induction of the SOS response, an endonuclease is induced and introduces a double-stranded break (DSB) at or near *oriM* on a circular DNA molecule, yielding a linear duplex. The subsequent action of a helicase (e.g., RecBC, RecQ) or a nuclease (e.g., RecBCD, exonuclease VIII) or both at the end of the duplex should, in the presence of RecA, produce a D loop, provided that an intact homolog is available. This reaction, i.e., D loop formation, could accomplish duplex unwinding for iSDR initiation (Asai and Kogoma, 1994).

The D loop model predicts that DSBs trigger a novel type of DNA replication that is independent of transcription and translation. We have tested this prediction by placing a λ *cos* site on plasmid DNA and generating an artificial DSB at the site with λ terminase, which introduces two staggered nicks, 12 bp apart, one on each strand of the duplex (for review see Feiss and Becker, 1983). We have found that regulated expression of the terminase indeed

allows replication of the *cos*-carrying plasmid in the absence of transcription and translation. The replication requires E. coli homologous recombination functions. Unlike iSDR, it occurs in the complete absence of SOS induction, provided that the *cos*-carrying plasmid also contains a properly oriented χ site. We designate this replication RDR, for homologous recombination-dependent DNA replication. We discuss possible roles of RDR in DSB repair and homologous recombination.

Results

A Model System for DSB-Triggered DNA Replication

pFM123 (Figure 1; Murialdo, 1988) is a derivative of pBR322, which carries the genes *Nu1* and *A* encoding λ terminase. Expression of the genes is regulated by the λ P_R and P_L promoters and a mutant repressor gene, *c1857*, which encodes a temperature-sensitive repressor. This plasmid does not contain *cos* sites. pHK-cos (Figure 1) was constructed by inserting a *cos*-containing fragment into the multiple cloning site of pHK (Asai et al., 1994), a derivative of pSC101. When cells harboring both pHK-cos and pFM123 are incubated at intermediate temperatures, a small amount of terminase could be produced. The terminase is expected to linearize some of the pHK-cos molecules by cleaving *cos* sites (Figure 2, step A; Kuzminov et al., 1994), while the rest of the molecules remain intact. The left end of λ DNA contains a strong terminase-binding site (for review see Becker and Murialdo, 1990). Thus, terminase, after cutting at *cos*, remains bound to the left end and blocks access to the end by nucleases and helicases, such as RecBCD and exonuclease VIII (Kuzminov et al., 1994). It is, therefore, expected that the terminase also protects one end of the linearized pHK-cos and that ssDNA tails are produced by a helicase, a nuclease, or both, preferentially from the other end, which contains the right end of λ DNA (Figure 2, step B). The ssDNA would be assimilated into an uncut pHK-cos molecule by RecA, generating a D loop (Figure 2, step C). A DNA molecule containing a Holliday junction could then be formed from the D loop (Figure 2, step D). Resolution of the junction (Figure 2, step E or F) leads to the formation of one of the two types of templates for rolling circle replication, depending on cutting sites (e or f, respectively). DNA replication from the D loop should lead to the formation of a template for either rolling circle mode (Figure 2, steps G and H) or θ mode (Figure 2, steps I and J) of DNA synthesis (see below for details). Therefore, DSBs could trigger extensive DNA synthesis that is independent of the plasmid replication origin.

DSBs Induce Origin-Independent Plasmid Replication in SOS-Induced *rec*$^+$ *sbc*$^+$ Cells

A *thyA* strain, AQ5004 (*rec*$^+$ *sbc*$^+$; Table 1), carrying both pHK-cos and pFM123 was grown at 30°C in M9-CAA medium containing [^{3}H]thymine (see Experimental Procedures). At this temperature, only a small amount of terminase would be produced. The exponentially growing cells were collected by filtration, washed, and resuspended in M9-CAA medium lacking required thymine. The cell suspension was then incubated at 30°C for a duration of time equivalent to two doublings (80 min) to induce the SOS response by thymine deprivation. A mixture of thymine, rifampicin (Rif), and chloramphenicol (Cm) was added (time 0) to the suspension at the end of thymine deprivation to inhibit subsequent origin-dependent plasmid replication (Asai et al., 1994). After the addition of Rif and Cm, the suspension was incubated at 37°C. Samples were withdrawn at the indicated times, and total DNA was prepared from each sample. Amounts of DNA samples normalized to give equal ^{3}H counts were loaded for agarose gel electrophoresis. pHK-cos plasmid DNA was detected by Southern blot hybridization using a ^{32}P-labeled plasmid-specific probe.

An autoradiogram of this experiment is shown in Figure 3A. Plasmid DNA steadily increased despite the presence of Rif and Cm. The major product of this origin-independent replication of pHK-cos was covalently closed circular (ccc) monomers, although a small amount of linear

plasmid multimers (LPMs), which migrate in agarose gel with a mobility similar to that of chromosome DNA fragments (Cohen and Clark, 1986), was also detected (see below). The intensity of ^{32}P radioactivity of each band corresponding to ccc monomers was quantified, and the relative copy number increase was determined. The result (Figure 3B) shows that the ccc form of pHK-cos increased more than 9-fold after a 5 hr incubation in the presence of Rif and Cm.

When pHK-cos or pFM123 was replaced with the corresponding parental plasmid, i.e., pHK or pBR322, respectively, plasmid copy number increase was limited to ~2-fold (Figure 3B), indicating that origin-independent replication requires both *cos* and terminase. The 2-fold increase in copy number during hour 1 is due most likely to replication from the plasmid origin. Initiation potential, which allows a single round of plasmid replication in the presence of Rif and Cm, would have been accumulated prior to the addition of the drugs.

The copy number increase of pHK-cos in the presence of Rif and Cm was also monitored without SOS-inducing treatment. In this case, cells carrying pHK-cos and pFM123 were grown and treated as described above except that a mixture of thymine, Rif, and Cm was added (time 0) to cell suspension with no period of thymine deprivation. As shown in Figure 3B, the copy number of pHK-cos increased during hour 1 and then remained constant. The result suggests that some factor(s) that is essential for the origin-independent replication of pHK-cos is not present in normal (uninduced) *rec*$^+$ *sbc*$^+$ cells and that the factor(s) can be induced by SOS induction. The expected increase of RecA amount after SOS induction is not the factor because SOS-inducing treatment was still required even when *recAo* (Con) mutant cells, in which RecA is constitutively overexpressed, were used for host cells (data not shown).

Attenuation of the Nuclease Activity of RecBCD Is Required for Origin-Independent Plasmid Replication in Uninduced *rec*$^+$ *sbc*$^+$ Cells

We considered the possibility that the failure to observe origin-independent plasmid replication in normal (uninduced) cells is due to the nuclease activity of RecBCD, which is expected to degrade linear duplexes created by *cos* cutting (Kuzminov et al., 1994). The SOS induction would be necessary to attenuate the nuclease activity of RecBCD (see Introduction). In normal *recB*$^+$ *recC*$^+$ *recD*$^+$ cells, the nuclease activity can be attenuated by a χ site, but the pHK-cos plasmid contains no χ sequence (data not shown). χ is effective only when RecBCD enzyme encounters it from the 3′ side (Taylor et al., 1985). In our system, the enzyme can only bind to one end of linearized pHK-cos that contains the right end of λ DNA (see Figure 2). This allowed us to analyze the orientation-dependent effect of χ. pHKχ1-cos and pHKχ3-cos (see Figure 1) contain a χ site downstream of the RecBCD entry site in the active and inactive orientations, respectively. When pHKχ1-cos along with pFM123 was present in *rec*$^+$ *sbc*$^+$ cells, the copy number of the plasmid increased in the presence of Rif and Cm without SOS-inducing treatment (Figures 4A and 4B). In contrast, the level of the copy number increase of pHKχ3-cos was similar to that of pHK-cos (Figure 4B). The basal level of SOS induction in *rec*$^+$ *sbc*$^+$ cells (AQ9789; a LacZ$^-$ derivative of AQ5004 carrying an *sfiA::lacZ*$^+$ operon fusion) and the same cells containing pHKχ1-cos and pFM123 (AQ9833) was analyzed by measuring the activity of β-galactosidase expressed by an *sfiA::lacZ*$^+$ operon fusion. The results are 56.3 ± 1.4 and 57.1 ± 0.4 Miller units for AQ9789 and AQ9833, respectively. This implies that χ-dependent plasmid replication occurs in the complete absence of SOS induction.

The result described above supports the notion that the attenuation of the nuclease activity of RecBCD is necessary for origin-independent plasmid replication. To test the notion further, we analyzed the copy number increase of pHK-cos (without χ) in *recD* mutant cells in the presence of pFM123. As shown in Figure 4B, pHK-cos continued replication in this mutant in the presence of Rif and Cm without SOS-inducing treatment. Thus, the attenuation of the nuclease activity of RecBCD is a prerequisite for origin-independent plasmid replication in

rec$^+$ *sbc*$^+$ cells. The copy number increase of pHKχ1-cos in *rec*$^+$ *sbc*$^+$ cells was ~50% of that of pHK-cos in *recD* mutant cells (Figure 4B). This could be explained by the in vivo and in vitro observations that RecBCD has a less than 50% chance of recognizing a particular χ site placed in the active orientation (Yagil and Shtromas, 1985; Stahl et al., 1990; Taylor and Smith, 1992).

χ-Dependent Plasmid Replication Requires Homologous Recombination Functions

The dispensability of SOS induction for origin-independent replication of pHKχ1-cos allowed us to analyze the role of the recombinase activity of RecA in this type of DNA replication. *recA* mutant cells harboring pFM123 are viable at 30°C (Murialdo, 1988). When pHKχ1-cos and pFM123 were introduced into *recA* mutant cells, the origin-independent replication of pHKχ1-cos was not observed (Figure 4C). Similarly, the *recB* and *recC* mutations blocked the replication (Figure 4C). These results indicate that χ-dependent plasmid replication observed in *rec*$^+$ *sbc*$^+$ cells requires the function of the RecBCD pathway of homologous recombination. We designate this type of origin-independent replication RDR.

DSBs Trigger RDR in *recBC sbcA* Mutant Cells

The RecE pathway of homologous recombination is active in *recBC sbcA* mutant cells. Since RecBCD is inactive in this strain, attenuation of the nuclease activity of RecBCD by χ or SOS induction was expected to be dispensable for origin-independent plasmid replication. Thus, the copy number increase of pHK-cos in the presence of Rif and Cm was monitored in this strain without SOS-inducing treatment. An autoradiogram of this experiment is shown in Figure 5A. The copy number of ccc monomers increased ~6-fold after a 5 hr incubation (Figure 5C). The increase depends on the presence of pFM123 (Figure 5C). In *recBC* (*sbcA*$^+$) (Figure 5D) and *recBC sbcA recE* (Figure 5B and 5D) mutant cells, in which neither the RecBCD nor RecE pathway of homologous recombination is active, the origin-independent replication of the ccc molecules of pHK-cos was not observed. A *recA* mutation also greatly reduced the copy number increase of the ccc molecules in *recBC sbcA* mutant cells (Figure 5D). The residual replication seen in this strain could be accounted for by the activity of RecT protein. RecT is derepressed by *sbcA* mutations, and some recombination events occurring in *recBC sbcA recA* mutant cells depend on it (Clark et al., 1993; Hall and Kolodner, 1994, and references therein).

In addition to ccc monomers, LPMs of pHK-cos are also obvious in this strain; ~60% of total plasmid DNA is made up of LPMs (Figure 5A). The copy number of the LPMs increased ~3-fold after a 5 hr incubation in Rif and Cm (Figure 5C). The amount of LPMs was greatly reduced in *recBC* (data not shown) and *recBC sbcA recE* (Figure 5B) mutant cells. These results suggest that the efficient formation of templates for rolling circle replication in *recBC sbcA* mutant cells requires the function of the RecE pathway of homologous recombination. A template for rolling circle replication could also be produced from a circular molecule by introducing a nick (Cohen and Clark, 1986). This mechanism, which does not require homologous recombination functions, could account for the formation of a small amount of LPMs in recombination-deficient cells.

From these results, we conclude that the major part of origin-independent plasmid replication (both the θ mode and the rolling circle mode of DNA synthesis) observed in *recBC sbcA* mutant cells is also RDR, which depends on the function of the RecE pathway of homologous recombination.

Requirement of RecN for RDR

As shown in Figure 6A, the copy number increase of pHKχ1-cos was greatly reduced by a *recN::Tn5* mutation. This indicates that RecN is required for DSB-triggered RDR in *recB*$^+$

recC$^+$ *sbcA*$^+$ cells. In contrast, the *recN* mutation had no effect on RDR when cells were induced for the SOS response (Figure 6B). In this case, induction of some factor(s) might have rendered RDR independent of RecN.

Discussion

Homologous recombination-dependent DNA replication is essential for bacteriophage T4 genome replication (Mosig, 1983). When replication forks started from origins reach the ends of T4 linear DNA, the 3′ ends of parental DNA remain unreplicated. The resulting ssDNA end invades a homologous region of another or the same T4 DNA molecule by the action of T4 recombinase, UvsX, and primes the leading-strand synthesis. A similar reaction has been reconstituted in vitro using purified T4 recombination and replication proteins (Formosa and Alberts, 1986; for review see Kreuzer and Morrical, 1994). In this study we have demonstrated that plasmid DNA replication can be initiated in normal wild-type E. coli cells in a manner that is independent of plasmid replication origin but is absolutely dependent on E. coli homologous recombination functions. This RDR is promoted by either the RecBCD or RecE homologous recombination pathway (for the RecF pathway, see below). RDR is observed only when a plasmid contains a λ *cos* site and cells express λ terminase. The in vivo generation of DSBs at the *cos* sites by λ terminase has clearly been demonstrated in a similar system (Murialdo and Fife, 1984). Thus, RDR is most likely to require a duplex DNA end. This is further supported by the result that, in *recBC sbcA* mutant cells, initiation of RDR depends on the *recE* gene product exonuclease VIII (Figure 5), which preferentially acts on duplex DNA ends and appears to be unable to initiate degradation from single-stranded breaks (Joseph and Kolodner, 1983). We conclude that DSBs can trigger extensive DNA replication that depends on homologous recombination functions.

The major product of RDR in *rec*$^+$ *sbc*$^+$ cells is ccc monomers (Figure 3A and Figure 4A). This suggests that RDR proceeds preferentially by the θ mode of DNA synthesis. As predicted from our model (Figure 2), LPMs produced by the rolling circle mode of DNA synthesis are also detected in samples prepared from SOS-induced cells (Figure 3A). In contrast, LPMs are undetectable in uninduced cells (Figure 4A). This is probably because terminase molecules eventually leave the cleaved *cos* sites. χ sites on the LPMs cannot protect them from digestion when RecBCD molecules enter from the ends of the multimers (see Figure 2). In *recBC sbcA* mutant cells, a significant amount of plasmid DNA replicates as LPMs (Figures 5A and 5C) owing to the absence of the RecBCD nuclease activity. Importantly, the formation of LPMs largely depends on the RecE pathway of homologous recombination (Figures 5B and 5D). Thus, the rolling circle mode of DNA synthesis observed in this mutant is also likely to be RDR. It has been demonstrated that LPMs are efficiently generated in *recBC sbcBC* mutant cells in a manner dependent on the RecF pathway of homologous recombination (Silberstein and Cohen, 1987; Kusano et al., 1989). The multimer formation does not require the activity of plasmid replication origin (Silberstein and Cohen, 1987), suggesting that RDR is also induced in these mutant cells.

A Model for the Initiation Mechanism of DSB-Triggered RDR

When a DSB is introduced into a circular DNA molecule in normal wild-type cells, RecBCD enzyme would bind to each end of the linearized molecule and begin unwinding it, with simultaneous degradation of the unwound ssDNA. When the enzyme encounters a properly oriented χ site on the linear molecule, the nuclease activity is attenuated, and thus ssDNA produced by the subsequent helicase action of the enzyme remains undigested. This ssDNA would be assimilated into an intact homolog by the action of RecA recombinase. This reaction, i.e., D loop formation, could accomplish initial duplex opening, which is a prerequisite for replication initiation of duplex DNA molecules (Figure 7A). In normal wild-type cells,

therefore, DSB-triggered DNA replication, RDR, could occur preferentially at or downstream of χ sites. When a DSB occurs in *recD* mutant or SOS-induced wild-type cells, in which the nuclease activity of RecBCD is inactivated, ssDNA generated by RecBC(D) helicase would remain intact unless other ssDNA-specific nucleases destroy it. Therefore, in these cells, D loop formation and subsequent initiation of DNA replication could occur at sites independent of χ. In place of RecBC(D) helicase, RecQ helicase, together with RecJ exonuclease, might also produce invasive ssDNA independent of χ. In *recBC sbcA* mutant cells, exonuclease VIII would recognize the ends of linear DNA and produce ssDNA with 3′ ends that are utilized by RecA. RDR thus could also occur in this mutant in a manner independent of χ.

The crucial step in replication initiation following duplex unwinding is the loading of the replication fork helicase, a DnaB hexamer, onto ssDNA in the unwound region (for reviews see Kornberg and Baker, 1992; Marians, 1992). In RDR, D loop formation could expose a signal for DnaB loading (e.g., n′-pas) on the displaced ssDNA (Figure 7A). DnaG primase would then interact with DnaB and synthesize an RNA primer on the ssDNA (Figure 7B). DNA synthesis from the primer by DNA polymerase III holoenzyme would reach to the end of the unwound region (Figure 7B). Translocation of DnaB in the 5′ to 3′ direction on the ssDNA would result in the formation of a replication fork that leads to unidirectional replication (Figure 7C). For bidirectional replication, loading of another DnaB hexamer on the other strand is necessary. The priming mechanism at D loops formed at *oriM* for iSDR is most likely to involve PriA protein, which binds to n′-*pas* and delivers DnaB through the formation of the φ X-type primosome (H. Masai, T. A., Y. Kubota, K. Arai, and T. K., submitted). The extreme similarities between the two replication systems (see Introduction and below) make it likely that a PriA-dependent priming mechanism might also accomplish DnaB loading at D loops for RDR.

iSDR as a Special Type of DSB-Triggered RDR

Genetic requirements for the initiation of iSDR, which replicates the entire E. coli genome, are very similar to those for RDR induction described here in the plasmid system. Thus, the initiation mechanisms of these replication systems are most likely to share common features. iSDR induction in *recD* and *recBC sbcA* mutant cells requires an SOS-inducing treatment (Asai et al., 1993), although the nuclease activity of RecBCD is absent in these mutants. In this study we have shown that, when DSBs are artificially provided in these mutant cells, RDR is initiated without SOS-inducing treatments. It follows, therefore, that SOS induction is necessary for iSDR not only to attenuate the RecBCD nuclease but also to generate DSBs at *oriMs*. Although initiated in similar manners, RDR and iSDR are distinct from each other in that the former starts from any site on the chromosome where a D loop can be formed, whereas the latter is restricted to specific sites on the chromosome. We envisage that iSDR is a special type of DSB-triggered RDR that occurs exclusively at *oriMs*.

Possible Roles of RDR in DSB Repair

Repair of DSBs is critical for cell survival (see Smith, 1991; Cox, 1993, and references therein). DSBs in intracellular DNA are created by the action of various physical and chemical agents. DSBs also result from enzymatic activities during the repair of DNA damage. Moreover, a single-stranded break can readily be converted to a DSB when a replication fork encounters it (see below). Since single-stranded breaks can be generated by reactive oxygen species, the chromosome of aerobically growing cells would suffer DSBs constantly, even in the absence of external DNA-damaging agents. DSB repair may be also involved in meiotic recombination in yeast. Various observations obtained with yeast strongly suggest that meiosis-specific DSBs occurring transiently at many locations in the genome initiate meiotic gene conversion and crossing over (Sun et al., 1989; Bishop et al., 1992; Wu and Lichten, 1994). DSB repair requires homologous recombination functions (Krasin and Hutchinson, 1977; Bishop et al., 1992;

Shinohara et al., 1992). In fact, in the absence of the functions, a single DSB in the E. coli chromosome is lethal (Murialdo, 1988). The current leading models for DSB repair proposed for both yeast and E. coli systems (Thaler and Stahl, 1988; Sun et al., 1991; Kobayashi, 1992), therefore, emphasize the role of homologous recombination in the process. The possible involvement of extensive DNA replication in DSB repair has not been addressed.

The minimal requirement for RDR in wild-type cells, besides homologous recombination functions, is a DSB (or a duplex end), a homolog, and attenuation of the RecBCD nuclease. Since the χ sequence is frequently found in the E. coli chromosome (about once every 5 kb on the average; Faulds et al., 1979) and actively growing cells normally contain more than one replicating chromosome, it is quite reasonable to expect that RDR can be manifested when a DSB is introduced into the chromosome. This expectation immediately suggests one function of RDR: DSB repair. We wish to propose the following mechanism for DSB repair, which involves extensive DNA replication. When a chromosome suffers a DSB (Figure 8, step B), RecBCD enzyme begins to degrade DNA from both ends, converting the DSB to a double-stranded gap (step C). Upon encountering χ, the enzyme starts producing ssDNA (Figure 8, step D). Invasion of 3′-ended ssDNA strands in the respective regions of an intact homolog yields a structure (Figure 8, step E) that contains two D loops. Loading of a DnaB hexamer and subsequent priming at each D loop produce two replication forks that proceed toward each other to repair the gap. When the two replication forks meet and replication is completed, the two homologs are now joined by two Holliday junctions (Figure 8, step F). Resolution of the junctions yields two monomers (Figure 8, step A) or one cointegrate (step G), depending on whether cutting occurs in the same sense (i.e., a north–south or east–west cutting at both junctions) or in the opposite sense, respectively. The cointegrate can be resolved into two monomers. The speculation that the ends generated by DSBs may trigger DNA replication leading to DSB repair has been presented (Thaler and Stahl, 1988; Smith, 1991).

The efficiency of DSB repair in wild-type cells is dramatically reduced by *recN* mutations (Picksley et al., 1984; Sargentini and Smith, 1986). Consistent with our proposal that RDR is involved in DSB repair, we have found that a *recN* mutation greatly reduces RDR in rec^+ sbc^+ cells. In contrast, double-stranded gap repair in *recBC sbcA* mutant cells studied by Kobayashi and coworkers does not require RecN (Kusano et al., 1993). The requirement of RecN for RDR in *recBC sbcA* mutant cells remains to be tested.

The feature described in the model seems ideally suited for restoration of a replication fork that is destroyed because of a DSB (Figure 9). When a replication fork encounters a single-stranded break, a DSB should result and the replication fork would be destroyed (Figure 9A). However, the end of the chromosome arm that is broken off could be recognized by RecBCD and the arm would be degraded up to the nearest χ site (Figure 9B). Formation of a D loop at or downstream of the χ site and subsequent loading of DnaB helicase followed by replication protein assembly should effectively restore the replication fork (Figures 9C and 9D). Finally, resolution of a Holliday junction would regenerate the original chromosome structure (Figure 9E). A consideration on the distribution and orientation of χ sites on the E. coli chromosome has led to speculation about the possible involvement of χ sites in the repair of collapsed replication forks (Kuzminov et al., 1994).

Is RDR Involved in Homologous Recombination?

Reciprocal recombination between a recipient chromosome and a linear duplex fragment introduced into a recipient cell by Hfr conjugation or P1 transduction would regenerate a duplex fragment that could recombine with the resultant recombinant chromosome. This endless recombination event, as proposed by Smith (1991), can be prevented if the ends of the initially introduced fragment prime DNA replication on the recipient chromosome, yielding a “recombinant” chromosome. Our results strongly support this novel mechanism of

homologous recombination. We have recently found that a *priA::kan* mutation reduces the frequency of P1 transduction drastically and the recombination frequency after Hfr conjugation moderately both in wild-type and *recBC sbcA* mutant cells (T. K., G. W. Cad-well, and T. A., unpublished data). These results are also consistent with the idea that RDR is involved in homologous recombination.

Experimental Procedures

Media and Growth Conditions

Unless otherwise stated, cells were grown at 30°C, with aeration by shaking, in M9 salts–glucose medium (Miller, 1972) supplemented with Casamino acids (0.2%; Difco Laboratories, Detroit, Ml), required amino acids (50 μg/ml), and thiamine hydrochloride (2 μg/ml) (M9-CAA medium). For *thyA deoB* (or *deoC*) mutant cells, thymine (8 μg/ml) was also added. For the selection of plasmid-containing cells, antibiotics were added at the following concentrations: ampicillin, 40 μg/ml; kanamycin, 50 μg/ml: spectinomycin, 40 μg/ml.

E. coli Strains and Phage

The E. coli strains used in this study are listed in Table 1. Strains were constructed by phage P1-mediated transduction. The *ΔrecA306*, *lacZU118*, and *thyA* mutations were cotransduced with the *srlC::Tn10, lacl42::Tn10*, and *zga::Tn10* mutations, respectively. Transductants carrying the *lacZU118* mutation were detected as white colonies on plates containing X-Gal and IPTG. A "low thymine" strain (AQ9717) was obtained from a *thyA* mutant by a spontaneous mutation in the *deo* operon (Miller, 1972). λp[*sfiA::lacZ*$^{+}$ *cl*(*lnd*$^{-}$)] was previously described (Huisman and D'Ari, 1983) and provided by S. Sommer.

Construction of Plasmids

The Pstl fragment (~450 bp) of pMUA10 (Meyerowitz et al., 1980) contains the λ Hincll fragment (Hincll [coordinate 48298]–*cosN* (48502/1]–Hincll [199]) carrying a *cos* site. pHK-cos (Figure 1) was constructed by inserting the Pstl fragment into the Pstl site of pHK (Asai et al., 1994).

The following oligonucleotides (TA103 and TA106) containing a χ sequence and their complementary fragments (TA104 and TA105, respectively) were used to construct χ-containing plasmids: TA103, 5′-AGCTTAGCGCTGGTGGCTGCA-3′; TA104, 5′-GCCACCAGCG-CTA-3′, TA105, 5′-AGCTTAGCCCACCAGCCTGCA-3′; TA106, 5′-GGC-TGGTGGGCTA-3′. Construction of pHKχ1-cos (Figure 1) was accomplished by first annealing TA103 and 104. One end of the resultant double-stranded DNA fragment carries the ssDNA tail compatible with a Hindlll site, and the other end is compatible with a Pstl site. The fragment was inserted between the Hindlll and the Pstl sites of pUC19, generating pUC19-χ. A Hindlll fragment (2014 bp) that contains the *Spc*′ gene of R100.1 (Ω fragment; Prentki and Krisch, 1984) was then inserted, after treatment with Klenow enzyme, into the Hincll site of pUC19-χ, generating pUC19-χΩ. The Ω fragment does not contain χ. Finally, the EcoRI-digested pHK-cos fragment and the Hindlll and BamHI fragment of pUC19-χΩ containing χ and Ω were ligated after treating the fragments with Klenow enzyme. One of the two possible constructs, which contains the χ site in the active orientation, was named pHKχ1-cos. pHKχ3-cos (Figure 1), carrying a χ site in the inactive orientation, was constructed by the same procedure with oligomers TA105 and 106.

Assay for Origin-Independent Plasmld Replication

The copy number increase of pHK and its derivatives in the presence of Rif and Cm were measured as described in the text and figure legends.

Extraction of total DNA and Southern blot hybridization were carried out as described previously (Magee et al., 1992; Asai et al., 1993). The BamHI fragment (1.7 kb) of pHK carrying the kanamycin gene was used to prepare ^{32}P-labeled probe for detection of pHK and its derivatives. The intensity of ^{32}P radioactivity in each DNA band was determined with a phosphorimager (425E-120, Molecular Dynamics, Sunnyvale, CA). Open circular plasmid molecules were generated in the course of preparation of total DNA. When a significant amount of open circular DNA was detected, the intensity of ^{32}P radioadivity in each open circular DNA band was also measured and added to that of the corresponding ccc DNA. The averages of standard error of means of copy number determinations at each sampling time were obtained from a number of independent experiments. They are as follows: time 0 sample, ~8%; time 1 sample, ~15%; time 2 and 3 samples, ~10%; time 4 and 5 samples, ~7%.

Acknowledgments

We are grateful to S. Sommer for preparing *λsfiA::lacZ*$^+$ phage, to N. Tsurushita for synthesizing DNA oligomers, and to A. J. Clark, R. D. Kolodner, H. Murialdo, and R. S. Myers for sending us E. coli strains and plasmids. We are also grateful to K. N. Kreuzer for helpful discussions and to E. Boye, S. C. Kowalczykowski, A. Kuzminov, T. R. Magee, R. S. Myers, G. R. Smith, and F. W. Stahl for critical reading of the manuscript. T. A. is especially thankful to C. L. Squires for her support in finishing this work. This work was supported by Public Health Service grant GM22092 from the National Institutes of Health and by National Science Foundation grant BIR-9218818.

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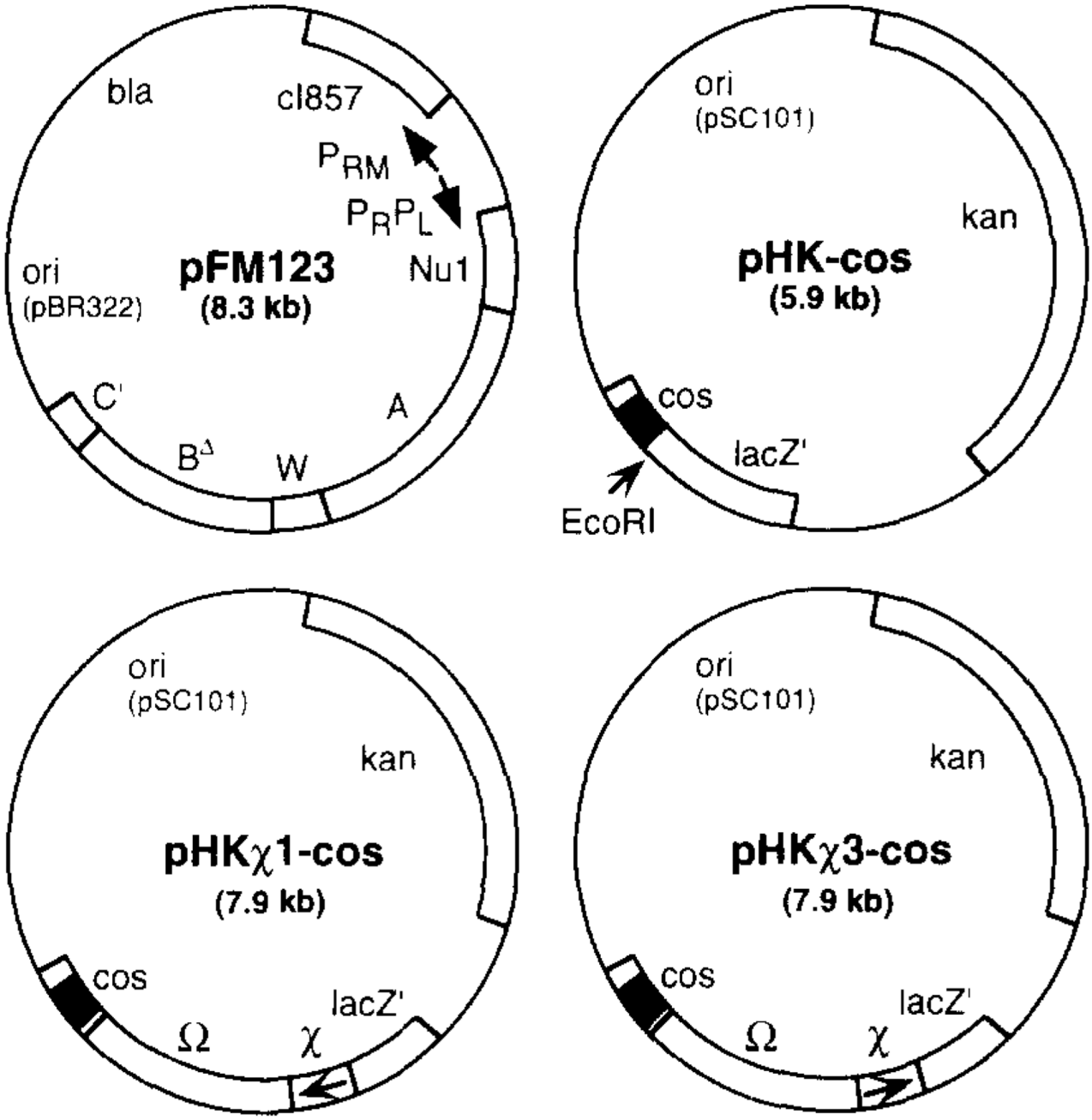

Figure 1. Maps of Plasmids Containing λ Terminase Genes or a *cos* Site

The position and direction of promoters on pFM123 relevant to the regulated expression of the terminase genes *Nu1* and *A* are shown by arrows. P_R and P_L are the λ early rightward and leftward promoters, respectively, and P_{RM} is the promoter for the gene *cI* necessary for the maintenance of a repressed prophage. *W*, *B*, and *C* are the genes located at the left end region of λ. *bla* is the β-lactamase gene. See Murialdo (1988) for details of the construction of pFM123. The unique EcoRI site in pHK-cos is shown on the map. DNA fragments containing the χ and Ω sequences were inserted into this site to generate pHKχ1-cos and pHKχ3-cos. The closed region in pHK-cos, pHKχ1-cos, and pHKχ3-cos represents the λ fragment (~450 bp) carrying

a *cos* site (see Experimental Procedures). The terminase-cutting site is located around the middle of the fragment, and in these plasmids the *lacZ′* gene is proximal to the right end sequence of λ. Therefore, RecBCD enzyme recognizing a DSB generated at these *cos* sites travels counterclockwise (see text). An arrow in pHKχ1-cos and pHKχ3-cos represents the χ sequence, and the arrowhead indicates the 3′ end of the sequence. Abbreviations and symbols: *kan*, the kanamycin resistance gene; *ori*, the plasmid replication origin; prime and capital delta, the truncation and internal deletion of genes, respectively. The figures are not drawn to scale.

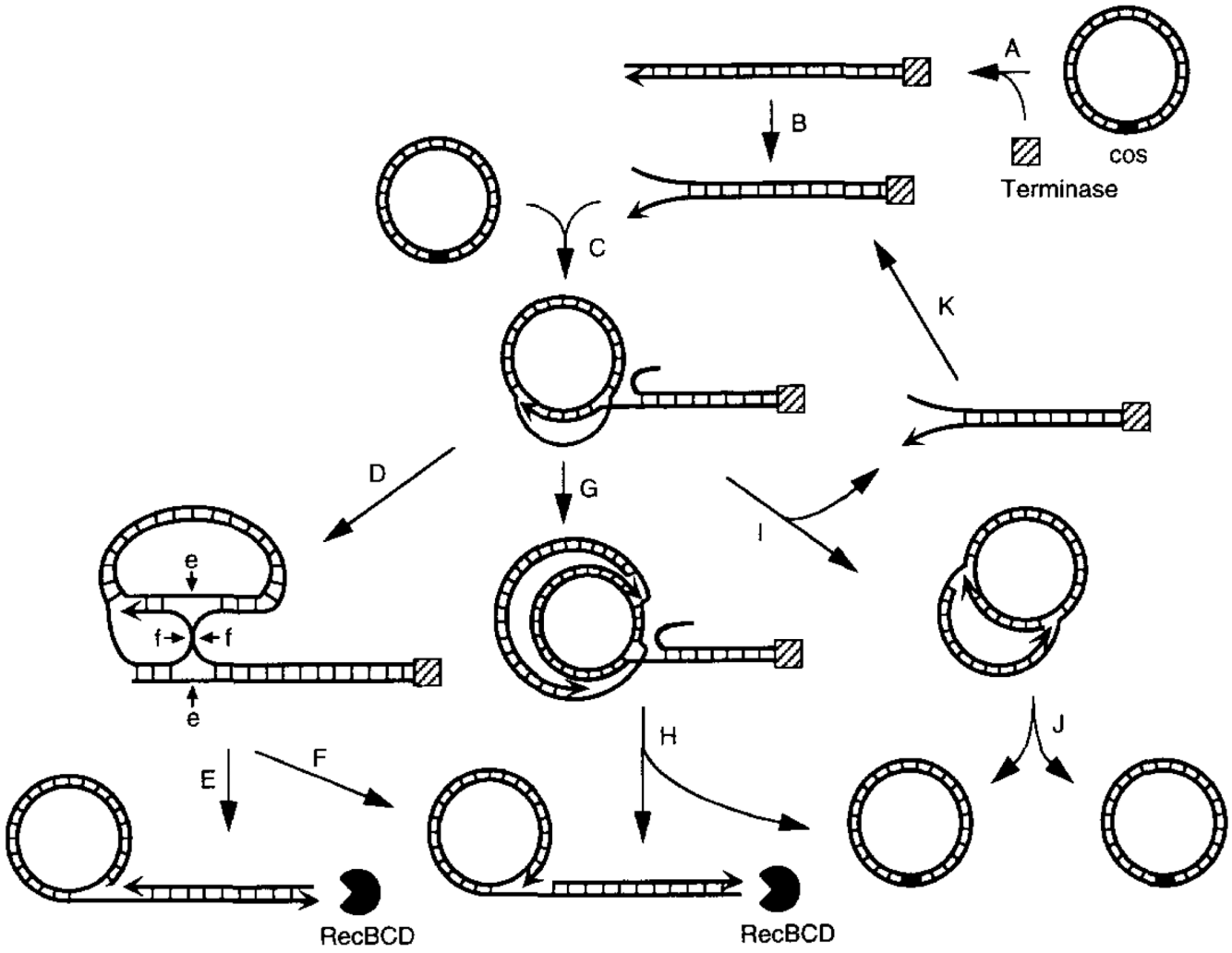

Figure 2. The Expected Reactions of DSB-Triggered DNA Replication

The closed region in circular plasmids represents a λ fragment carrying a *cos* site. λ terminase molecules are shown by hatched squares. Arrowheads represent the 3′ ends of DNA strands. Supercoiling of circular molecules is ignored. See text for details.

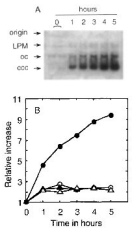

Figure 3. The Copy Number Increase of pHK-cos and pHK in the Presence of Rif and Cm in *rec*$^+$ *sbc*$^+$ Cells

(A) An autoradiogram of Southern blot hybridization to determine replication of pHK-cos after SOS induction. AQ5004 (*rec*$^+$ *sbc*$^+$ *thyA*) carrying pHK-cos and pFM123 was grown to 2.5 × 10^8 cells/ml in M9-CAA medium containing [^{3}H]thymine (2 μCi/ml) and treated as described in the text. A mixture of thymine (8 μg/ml), Rif (200 μg/ml), and Cm (150 μg/ml) was added at time 0. Origin, the origin of electrophoresis migration; oc, open circular plasmid molecules. Duplicated samples were withdrawn at time 0 and processed independently.

(B) The Southern blot hybridization filter shown in (A) was exposed to a screen for a phosphorimager, and the intensity of ^{32}P radioactivity in each ccc DNA band was determined. The relative increase obtained from the result is shown by closed circles. AQ5004 carrying pHK-cos and pBR322 or pHK and pFM123 was also grown as described above and was induced for the SOS response. The relative increase of ccc molecules of pHK-cos and pHK in the presence of Rif and Cm is shown by closed and open triangles, respectively. Open circles are the relative increase of pHK-cos in uninduced AQ5004 cells in the presence of pFM123.

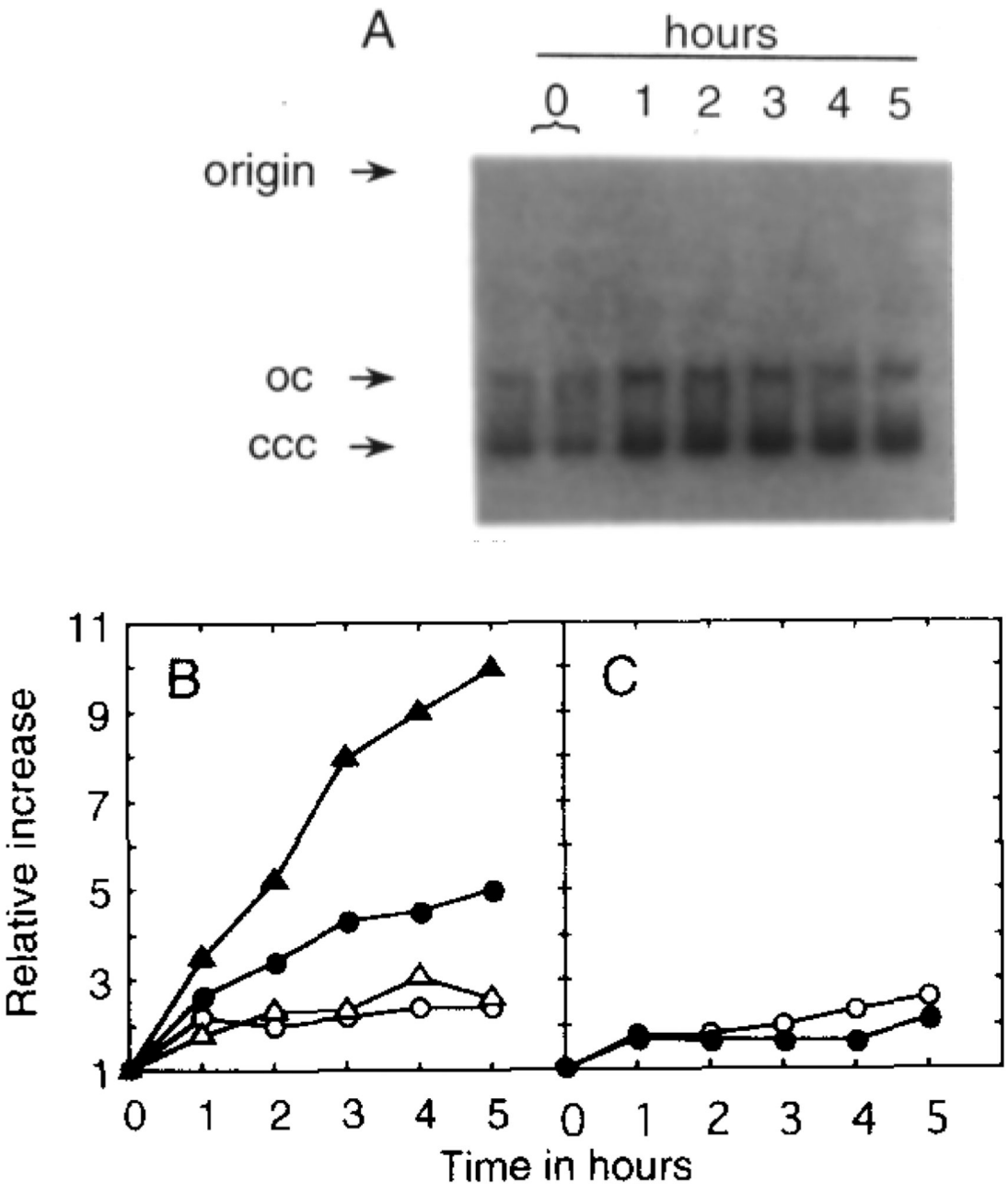

Figure 4. The Copy Number Increase of pHKχ1-cos, pHKχ3-cos, and pHK-cos in the Presence of pFM123 in Uninduced Wild-Type and *rec* Mutant Cells

Cells were grown and treated as described in the text and Figure 3.

(A) An autoradiogram of Southern blot hybridization to determine replication of pHKχ1-cos in AQ5004 (rec^+ sbc^+).

(B) The relative copy number increase of pHKχ1-cos (closed circles), pHKχ3-cos (open triangles), and pHK-cos (open circles) in AQ5004 and of pHK-cos (closed triangles) in AQ9601 (*recD*). The result of pHKχ1-cos was obtained from the experiment shown in (A).

(C) The relative copy number increase of pHKχ1-cos in AQ9450 (*recA*, open circles) and AQ9169 (*recBC*, closed circles).

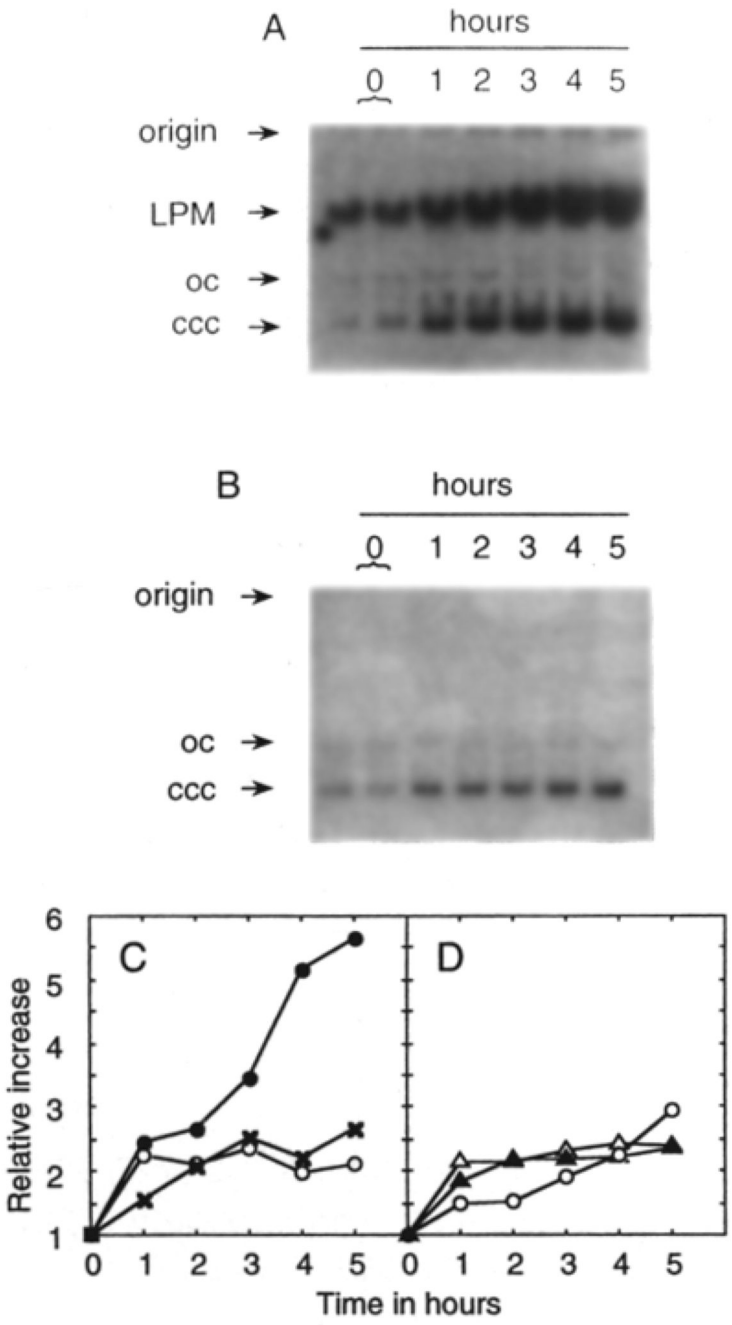

Figure 5. The Copy Number Increase of the ccc Molecules and LPMs of pHK-cos in Cells Carrying *recB* and *recC* Mutations

(A and B) Autoradiograms of Southern blot hybridization to determine replication of pHK-cos in *recBC sbcA* and *recBC sbcA recE* mutant cells. AQ3625 (A) and AQ3626 (B) carrying pHK-cos and pFM123 were grown to 2.5×10^8 cells/ml in the presence of [^{3}H]thymidine (2 μCi/ml), thymidine (10 μg/ml), and 2′-deoxyadenosine (300 μg/ml) and treated as described in the text. Rif and Cm were added at time 0.

(C) The relative increase of ccc molecules (closed circles) and LPMs (bold x's) of pHK-cos in AQ3625 (A). AQ3625 carrying pHK-cos and pBR322 was also grown as described above,

and the relative increase of ccc molecules of pHK-cos (open circles) was determined by the same method.

(D) The relative increase of ccc molecules of pHK-cos in the presence of pFM123 was determined as described above in the following strains: AQ9169 (*recBC*, open triangles), AQ3626 (*recBC sbcA recE*, closed triangles), AQ9561 (*recBC sbcA recA*, open circles). The result of AQ3626 was obtained from the experiment shown in (B).

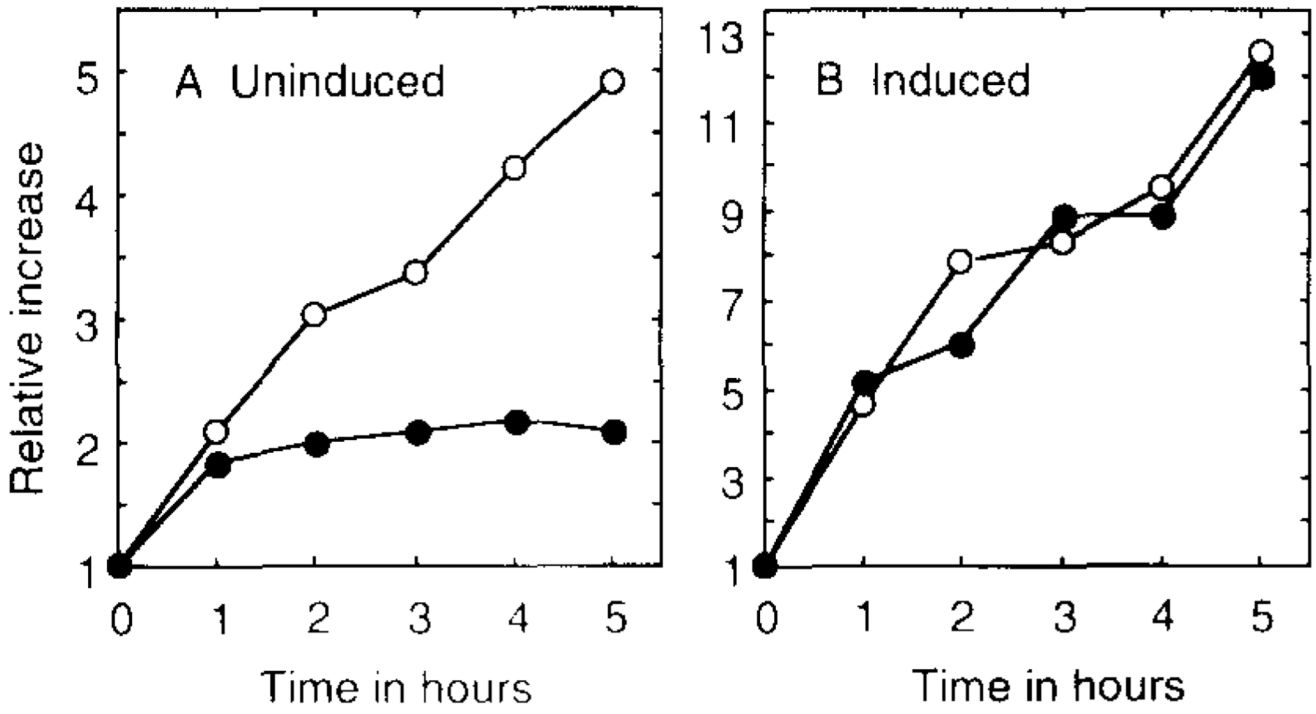

Figure 6. The Relative Copy Number Increase of ccc Molecules of pHKχ1-cos in Wild-Type and *recN* Mutant Cells

AQ5004 (*recN*$^+$ *thyA*) and AQ9717 (*recN thyA*) carrying pHKχ1-cos and pFM123 were grown and treated as described in the text and Figure 3. The results are the relative increase of ccc molecules of pHKχ1-cos in uninduced (A) and SOS-induced (B) cells. Open symbols, AQ5004; closed symbols, AQ9717.

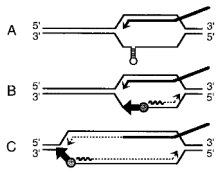

Figure 7. A Model for the Initiation Mechanism of RDR

A part of an intact circular DNA molecule (thin line), an invading ssDNA tail (thick line), a primer RNA molecule (wavy line), and a newly synthesized DNA fragment (broken line) are shown. A stem–loop structure on the displaced ssDNA in (A) represents a signal for DnaB loading. A DnaB hexamer and its direction of translocation on ssDNA are shown by a thick arrow. DnaG primase is represented by a stippled circle. Small arrowheads indicate the 3′ ends of DNA strands. See text for details.

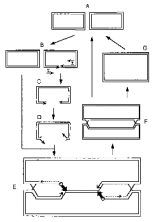

Figure 8. A Model for DSB Repair in Wild-Type Cells

The E. coli chromosome and a newly synthesized DNA fragment are shown by thin double lines and a broken line, respectively. A χ site is denoted by a small arrow. Arrowheads represent the 3′ ends of DNA strands and of χ sequences. DnaB helicase and DnaG primer are shown by the same symbols as in Figure 7. See text for details of the model.

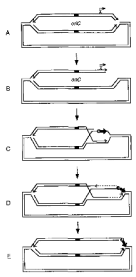

Figure 9. Restoration of a Destroyed Replication Fork by RDR

The E. coli chromosome and a newly synthesized DNA fragment are as in Figure 8. A small arrow represents a χ site. Arrowheads indicate the 3′ ends of DNA strands and of the χ sequence. DnaB helicase and DnaG primer are shown by the same symbols as in Figure 7. See text for details.

Table 1

E. coli K-12 Strains

Strain	Relevant Genotype	Source, Reference, or Construction
Strains of AB1157 background		
AB1157[a]	*thy*$^+$ *rec*$^+$ *sbc*$^+$	Bachmann, 1972
AQ3625	*recB21 recC22 sbcA23*	As JC8679; Gillen et al., 1981
AQ3626	*recB21 recC22 sbcA23 recE159*	As JC8691; Gillen et al., 1981
AQ5004	*thyA deoB* (or *deoC*)	R. Davern
AQ9169	*recB21 recC22*	As JC5519: Willetts and Clark, 1969
AQ9450	*ΔrecA306 srlC::Tn10*	AB1157 × P1.AQ7982 select Tc^r, UV^s
AQ9561	*recB21 recC22 sbcA23 ΔrecA306 srlC::Tn10*	AQ3625 × P1.AQ7982 select Tc^r, UV^s
AQ9601	*thyA deoB (or* deoC) *recD1903::mini-tet*	AQ5004 × P1.AQ8070 select Tc^r, T4.2^s
AQ9717	*recN1502::Tn5 thyA zga::Tn10 deoB (* or *deoC*)	RDK1540 × P1.AQ5786 select Tc^r, low thymide
AQ9747	*thyA deoB* (or *deoC) lacZU118 lacl42::Tn10*	AQ5004 × P1.CAG18439 select Tc^r, Lac$^-$
AQ9789	AQ9747 λp[*sfiA::lacZ*$^+$ *cI(Ind*$^-$)	AQ9747 lysogenized with λp[*sfiA lacZ*$^+$*cI(Ind*$^-$)]
AQ9833	AQ9789 carrying pHKχ1-cos and pFM123	AQ9789 transformed with pHKχ1-cos and pFM123
RDK1540	*recN1502::Tn5*	Lovett et al., 1988
Intermediates in strain constructions		
AQ5786	*thyA zga::Tn10 rec*$^+$ *sbc*$^+$ *deoB* (or *deoC*)	Laboratory stock
AQ7982	*ΔrecA306 srlC::Tn10*	As GY7313; Bailone et al., 1988
AQ8070	*recD1903::mini-tet recJ284::dTn10*	Asai et al., 1993
CAG18439	*lacZU118 lacl42::Tn10*	Singer et al., 1989

[a]The remaining genotypes are *F*$^-$*argE3 his-4 leuB-6 proA2 thr-1 thi-1 rpsL31 galK2 lacY1 ara-14 xyl-5 mtl-1 supE44*. UV^S, sensitivity to ultraviolet; T4.2^S, sensitivity to phage T4 gene 2 mutants (Chaudhury and Smith, 1984).