

Single-strand interruptions in replicating chromosomes cause double-strand breaks

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Replication-dependent chromosomal breakage suggests that replication forks occasionally run into nicks in template DNA and collapse, generating double-strand ends. To model replication fork collapse *in vivo*, I constructed phage λ chromosomes carrying the nicking site of M13 bacteriophage and infected with these substrates *Escherichia coli* cells, producing M13 nicking enzyme. I detected double-strand breaks at the nicking sites in λ DNA purified from these cells. The double-strand breakage depends on (i) the presence of the nicking site; (ii) the production of the nicking enzyme; and (iii) replication of the nick-containing chromosome. Replication fork collapse at nicks in template DNA explains diverse phenomena, including eukaryotic cell killing by DNA topoisomerase inhibitors and inviability of recombination-deficient vertebrate cell lines.

replication fork collapse | single-strand DNA breaks | double-strand DNA breaks

DNA-damaging treatments are more lethal if they are administered during chromosomal replication (1–4), suggesting that encounters of replication forks with DNA lesions aggravate these lesions. Most types of one-strand DNA damage are removed by excision repair, which proceeds via a common intermediate, single-strand DNA interruption (reviewed in ref. 5). What happens when, during an ongoing excision repair, a replication fork runs into a single-strand interruption in template DNA? There may be a natural safeguard mechanism preventing such an eventuality, because replication fork progress appears to depend on the presence of negative supercoiling in the DNA segment to be replicated. Indeed, partial loss of DNA supercoiling because of coumermycin inhibition of DNA gyrase activity in *Escherichia coli* parallels severe inhibition of DNA synthesis (6). Because the nick in the downstream template relieves the negative supercoiling in front of the replication fork, the loss of supercoiling should inhibit fork progress.

Quite a different idea is that a replication fork reaches the nick and collapses, producing a double-strand end (Fig. 1 *A–C*) (2, 7–10). If two replication forks converge on a single nick, a double-strand break will form (Fig. 1 *B–D*). This idea is based on two observations: (i) replication-induced chromosomal fragmentation (double-strand breakage) in mutants or in conditions that elevate the number of single-strand interruptions in DNA (11–14) and (ii) preferential degradation of the newly synthesized DNA in these conditions (2, 15). The most economical interpretation of these observations is that persistent nicks in replicating DNA *in vivo* are converted into double-strand ends by replication forks (reviewed in ref. 16). The relevance of this process to the formation of lethal DNA damage is underscored by the uncontrollable fragmentation of chromosomes in Rad51-deficient vertebrate cell lines (17, 18), which apparently underlies the inviability of *rad51* mutant mice (17).

The idea of replication fork collapse at single-strand interruptions is not new, but its experimental testing was delayed by difficulties with induction of persistent and site-specific single-

strand scissions in replicating chromosomes *in vivo*. A class of DNA-nicking enzymes that collaborate with the DNA replication machinery comprises proteins initiating rolling-circle DNA replication in conjugative plasmids and filamentous bacteriophages: gpII of phages M13, f1 or fd, or TraI of the F factor (19). Intriguingly, there is genetic evidence that the transfer origin (the nicking site) of F factor is a site of double-strand breakage *in vivo* (7, 20). I decided to use a cloned M13 nickase (gpII) to deliver controlled nicks in substrate chromosomes replicating in *E. coli* cells.

Materials and Methods

Media, Growth Conditions, and General Methods. Cells were grown in LB broth or on LB plates (21). When cells were carrying plasmids, the media were supplemented with 100 μ g/ml of ampicillin. For the wild-type gpII induction off pCL475, the temperature shift-up from 28 to 42°C was used; for the mutant gpII induction off pK125 or pK133, 0.5 mM isopropyl β -D-thiogalactoside (IPTG) was used.

Small-scale and preparative plasmid DNA isolation was done as described (22). λ phages were isolated from plate lysates by two rounds of polyethylene glycol-3400 precipitation (21). TM buffer is 10 mM Tris-HCl, pH 8.0/10 mM MgSO₄; TE buffer is 10 mM Tris-HCl, pH 8.0/1 mM EDTA.

Bacterial Strains. Bacterial strains are *E. coli* K-12; they are derivatives of AB1157 (23), except AK98 and AK99, which are derivatives of R594 (23). *recBCD* mutants were confirmed by their ability to plate T4 2 mutant phage (=ExoV⁻) (24); *rep* mutants were confirmed by their inability to plate M13 phage (25).

recB270 recC271 is SK129 (26); *recB268* is N2101 (27); BT125 and WA800 are described (28), as well as JJC213 and JJC735 (29). Δ *rep::kan recD1011* is AK92 (BT125 \times P1 JJC213, selection for kanamycin resistance, inability to support M13 replication). Δ *rep::cam recD1011* is AK93 (BT125 \times P1 JJC735, selection for chloramphenicol resistance, inability to support M13 replication). Δ *rep::cam recB270 recC271* is AK95 (SK129 \times P1 JJC735, selection for chloramphenicol resistance, inability to support M13 replication, inviability at 42°C). AK98 is Su⁻ Δ *rep::cam* (R594 \times P1 JJC735, selection for chloramphenicol resistance, inability to support M13 replication). Su⁻ Δ *rep::cam recC1010* is AK99 (AK98 \times P1 WA800, selection for tetracycline resistance, T4 2 permissivity).

Plasmids. pK107 is a pBR322 derivative carrying the λ cI and *PaeR7I* restriction-modification system (*XhoI* specificity) (30). pCL475 is an M13 wild-type gpII-producing plasmid (31). pK125

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Abbreviations: IPTG, isopropyl β -D-thiogalactoside; moi, multiplicity of infection.

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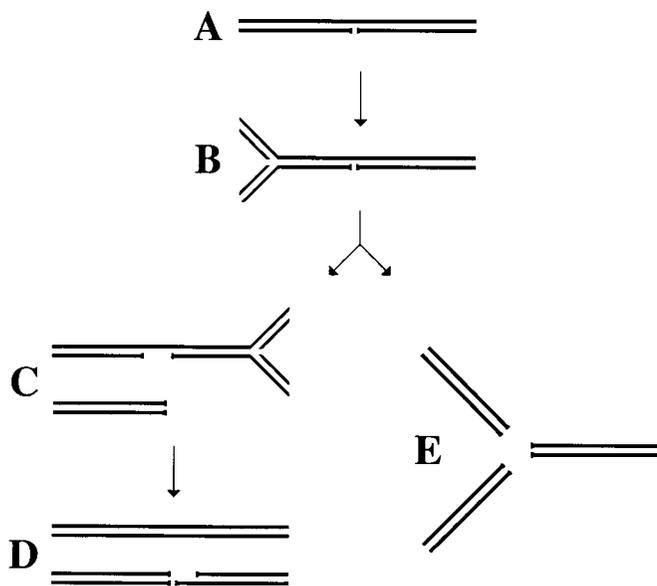


Fig. 1. The idea of replication fork collapse at a single-strand interruption in template DNA. (A) A DNA segment with a long-lived nick. (B) A replication fork is approaching the nick. (C) The replication fork has reached the nick and collapsed; another replication fork is approaching the remaining single-strand interruption from the opposite direction. (D) The second replication fork has reached the interruption and collapsed; as a result, one of the daughter chromosomes has a double-strand break. (E) An alternative scenario of double-strand breakage at replication forks: replication fork "explosion" at the nick.

carries G73A mutant gpII under the control of a strong *tacl* promoter and *lacI^q* gene; gpII synthesis is, therefore, IPTG-inducible. pK125 was constructed by combining three fragments: the 1,580-bp-long *EcoRI-EcoRI* from pDG117IIA.H11 carrying *Ptacl*-gpII G73A (32), the 1,260-bp-long *EcoRI-HindIII* from pAH3 carrying *lacI^q* (32), and the 4,328-bp-long *HindIII-EcoRI* backbone of pBR322. pK133 is a shrink of pK125, from which the 882-bp-long *NgoMI* fragment of nonessential DNA has been deleted.

Bacteriophages. λ phages MMS2660 and MMS2663 are described (33). Because they carry the *Psus80* mutation, they can replicate in AB1157 (SuII) background, but cannot replicate in R594 (Su⁻) background. The sequence of the nicking site-containing oligo corresponds to "complex II" of ref. 34:

5'-TCGAGTTCCTT^ÅATAGTGGACTCTTGTTC^ÅCAACTGGAACAACA
CAAGAAATTATCACCTGAGAACAAGGTTTGACCTTGTGTAGCT-5'.

The bold Å in the top strand marks the 5'-end of the nick. Note that, when inserted into a *XhoI*-site as written, this sequence will restore the *XhoI* site on its left. Insertion of this oligo as written into the natural *XhoI* site of MMS2660 gives λ AK2 (nick in the top strand), whereas its insertion in the opposite orientation gives λ AK3 (nick in the bottom strand). Likewise, insertion of this oligo, as written, into the *SstII::XhoI* site of MMS2663 gives λ AK4 (nick in the top strand), whereas its insertion in the opposite orientation gives λ AK5 (nick in the bottom strand).

Isolation of Phage λ DNA. For isolation of big quantities of λ DNA, concentrated phage stocks are prepared from 8–10 plates by the standard technique (see above), but with pure agarose instead of agar. To 450 μ l of an agarose λ stock, 50 μ l of 10% SDS are added, and the aqueous phase is extracted once with 500 μ l of phenol, once with 500 μ l of phenol/chloroform (1:1), and once with 500 μ l of chloroform. Then 20 μ l of 4 M KCl is added, and

the phage DNA is precipitated with 1 ml of ethanol and dissolved in 200 μ l of TE. After addition of 300 μ l of 6 M LiCl, the tube is vortexed vigorously and, after chilling on ice for 15 min and spinning for 2 min in an Eppendorf centrifuge, the supernatant is transferred into a fresh tube. Nucleic acids are precipitated with 1 ml of ethanol, dissolved in 500 μ l of TE, and, after addition of 20 μ l of 4 M KCl, reprecipitated with 1 ml of ethanol and dissolved in 500 μ l of TE.

The Standard Cross. A 2-ml LB culture, supplemented with 100 μ g/ml of ampicillin, is inoculated with a fresh colony and incubated with shaking at 28°C overnight. The next morning, 200–400 μ l of the saturated culture is used to inoculate 20 ml of LB, supplemented with 100 μ g/ml of ampicillin; the diluted culture is grown with shaking at 28°C to mid-logarithmic phase. Cells are collected by centrifugation, resuspended in 1 ml of TM, and counted under the microscope in a Petroff-Houser chamber. In a 10-ml glass tube on ice, 4×10^8 cells in 200 μ l of TM are mixed with the appropriate quantity of phage in 50 μ l of TM to produce the indicated multiplicity of infection (moi); the adsorption mixture is vortexed gently and chilled on ice for 15 min. The glass tube is placed at 37°C for 3 min to effect phage injection, and the whole adsorption mixture is transferred into 2 ml of prewarmed LB culture, supplemented or not with 0.5 mM IPTG. Incubation is at the indicated temperatures. At the indicated time points, a 1.5 ml aliquot is taken for DNA isolation.

Preparation of Total DNA from *E. coli*. The cells are pelleted in a microcentrifuge, the supernatant is removed with a pipet, and the cells are resuspended in 50 μ l of 30% sucrose in TE buffer. The cells are lysed by adding 350 μ l of 2% SDS in TE, mixing by inversion and incubating at 70°C for 5 min. To degrade the protein, 20 μ l of 10 mg/ml Proteinase K is added, and the lysates are incubated for 15 min at 70°C. After chilling on ice, the lysates are extracted once with 200 μ l of phenol, once with 200 μ l of phenol/chloroform (1:1), and once with 300 μ l of chloroform. The aqueous phase is transferred into a fresh tube, and the nucleic acids are precipitated, after thorough mixing with 20 μ l of 4 M KCl and 1 ml of ethanol, by centrifugation in a microcentrifuge. The supernatant is discarded, the pellet is dissolved in 500 μ l of TE, and nucleic acids are reprecipitated as above. The pellet is eventually dissolved in 200 μ l of TE; 1–50 μ l of this solution is used for subsequent analysis.

DNA Analysis. DNA is cut with specified restriction endonucleases, separated in 0.7% agarose gels, either in 40 mM Tris-acetate, pH 8.5/2 mM EDTA (neutral gels) or in 30 mM NaOH, 1 mM EDTA (denaturing gels), vacuum transferred to Zeta-Bind nylon membrane (Cuno), and probed with λ -specific probes, as described (33). Probe 1 covers both sides of the *XhoI* site in phages MMS2660, λ AK2, and λ AK3, and is λ *NruI-NheI* fragment (32409–34679). Probe 2 covers both sides of the *SstII::XhoI* site in phages MMS2663, λ AK4, and λ AK5, and is λ *HincII* fragment (39836–40942).

Results

The Rational and Experimental Systems. To test ideas about replication fork encounters with persistent single-strand breaks in template DNA *in vivo*, one needs a substrate chromosome (i) that can be efficiently introduced into cells; (ii) that can be nicked at will at a specific location once inside the cell; and (iii) whose replication can be regulated *in vivo*. Because phage λ already proved itself in this "in vivo biochemistry" approach (30, 33), I have constructed λ phages carrying the minimal nicking site of the filamentous bacteriophage M13. Although the "functional origin" of M13 covers some 140 bp, increased production of the cognate nicking enzyme reduces the sequence requirements of the reaction to 40 bp (35). I inserted this 40-bp

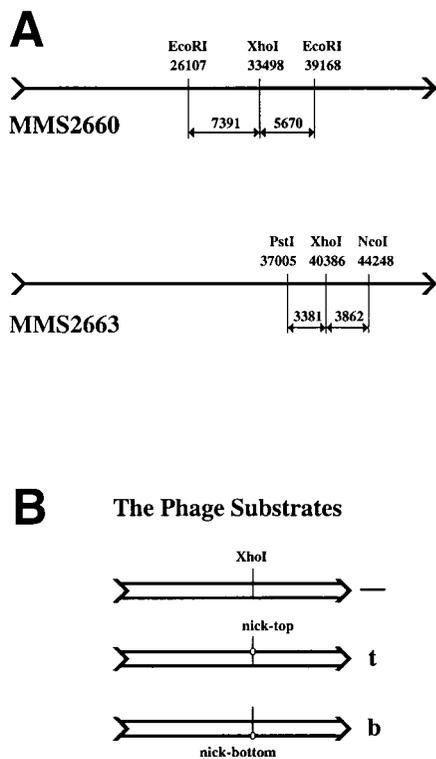


Fig. 2. Experimental substrates. (A) The two λ phages used to construct the nicking site-containing phages. The *XhoI*-sites at which the nicking sites were inserted and the restriction sites used in Southern analysis are shown, together with their coordinates on wild-type λ chromosome and the sizes of the generated fragments. (Upper) MMS2660, the progenitor of λ AK2 (nick in the top strand) and λ AK3 (nick in the bottom strand). These three phages are used in Figs. 3 and 4. (Lower) MMS2663, the progenitor of λ AK4 (nick in the top strand) and λ AK5 (nick in the bottom strand). These three phages are used in Fig. 5. (B) A set of three phage substrates (either MMS2660, λ AK2, and λ AK3 or MMS2663, λ AK4, and λ AK5) run in parallel in one experiment. All three phages within a set have a unique *XhoI* site at approximately the same location. The control phage (marked “—”) has nothing else; the middle phage carries the nicking site in the top strand and is therefore marked “t;” the bottom phage carries the nicking site in the bottom strand and is therefore marked “b.”

double-stranded fragment (see *Materials and Methods*) in either orientation at the unique *XhoI* site in the λ chromosome (Fig. 2A Upper), generating three phages (Fig. 2B): the original control phage without the nicking site, and the two experimental phages, carrying the nicking site in either the top or the bottom strand. Because the phages with the nicking site retain the *XhoI* site, I used *in vivo* cutting with *XhoI* (injecting the phages into *XhoI*-producing cells) to generate molecular weight markers for the double-strand break at the nicking site (Figs. 3B and 4B, lanes a–c).

In the experiment reported in Fig. 3B, the wild-type gene for the nicking enzyme of the phage M13 is under the control of a temperature-sensitive λ repressor, so both the production of the nicking enzyme and λ replication are effected by temperature shift-up (31). When I wanted to produce the nicking enzyme without λ replication, cells were preinduced at 42°C for 30 min before phage injection but kept at 28°C after phage injection.

The M13 nicking enzyme is the only phage protein required to start rolling-circle DNA replication from the nicking site (36). The enzyme does this by attracting the host Rep helicase, which then unwinds the 5'-side of the nick and assembles a regular replication fork on the unwound intermediate (Fig. 3A) (37). In *rep* mutants, the nicking still occurs, but no rolling-circle DNA

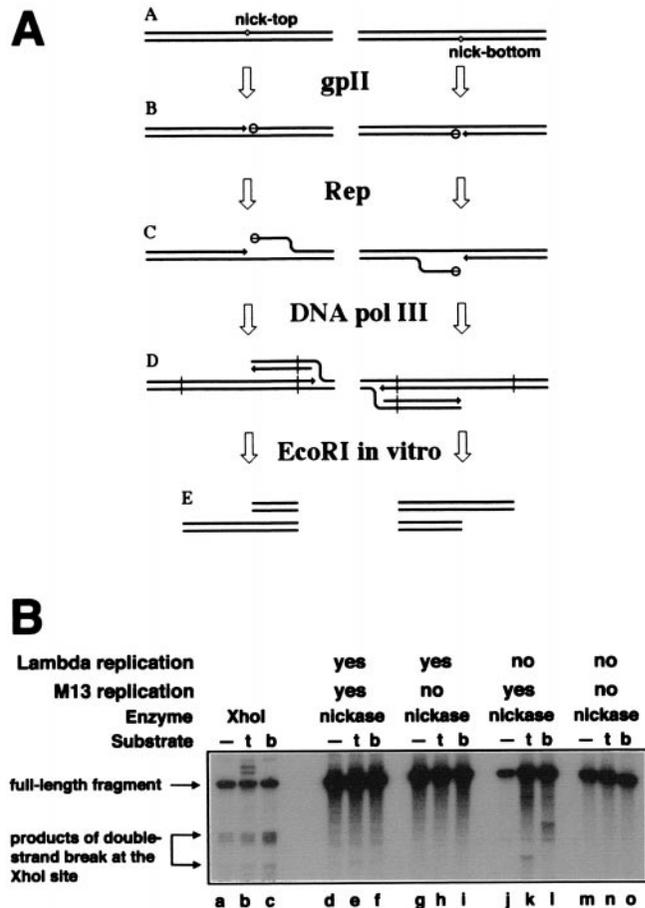


Fig. 3. The interplay between λ and M13 DNA replication. (A) Sigma replication in *Rep*⁺ cells is predicted to generate the “half-break” pattern at the nicking sites. 3'-ends are indicated by arrows. (A) The substrate chromosomes carrying nicking sites at the same location, either in the top or in the bottom strand. (B) The nicking sites are nicked by gpII (open circles attached to the 5'-sides of the nicks). (C) The Rep helicase unwinds the 5'-ends of the nicks, attracting replisomes to the replication fork structures. (D) Sigma replication from the nicks. (E) Restriction cutting *in vitro* (at sites indicated by vertical lines in D) reveals the “half-break” pattern. (B) Interference between λ DNA replication and nicking by the wild-type nicking enzyme. Phages MMS2660, λ AK2, or λ AK3, indicated in the entry “substrate” as “—,” “t,” or “b,” respectively, were infected at *moi* = 6 into the strains described below; the cells were incubated as indicated, and the phage DNA was extracted and analyzed by restriction digestion with *EcoRI* and blot hybridization with probe 1. Molecular weight markers for double-strand break at the natural *XhoI* site were generated *in vivo* (lanes a–c). The strains and conditions are as follows: lanes a–c, *recB268* pK107, 10 min at 28°C; lanes d–f, *recB270 recC271* pCL475, 30 min at 42°C; lanes g–i, Δ *rep::cam recB270 recC271* pCL475, 30 min at 42°C; lanes j–l, *recB270 recC271* pCL475, incubated for 30 min at 42°C before phage injection and 60 min at 28°C after phage injection; lanes m–o, Δ *rep::cam recB270 recC271* pCL475, incubated for 30 min at 42°C before phage injection and 60 min at 28°C after phage injection.

replication follows (25). Thus, in *Rep*⁺ strains, the nicking sites in λ were expected to stimulate rolling-circle DNA replication, producing a pattern of “half-breaks,” with a breakage product on one side of the nick only, and the complementary breakage product generated by the substrate with the nick in the other DNA strand (Fig. 3A). Indeed, the “half-break” pattern was observed in *Rep*⁺ cells and was especially clear when λ 's own DNA replication was disallowed (Fig. 3B, compare lanes d–f with j–l). As expected, this half-break pattern, indicative of rolling-circle DNA replication, was completely suppressed in *rep* mutants (Fig. 3B, lanes g–i and m–o).

Therefore, allowing λ replication in *rep* mutants should

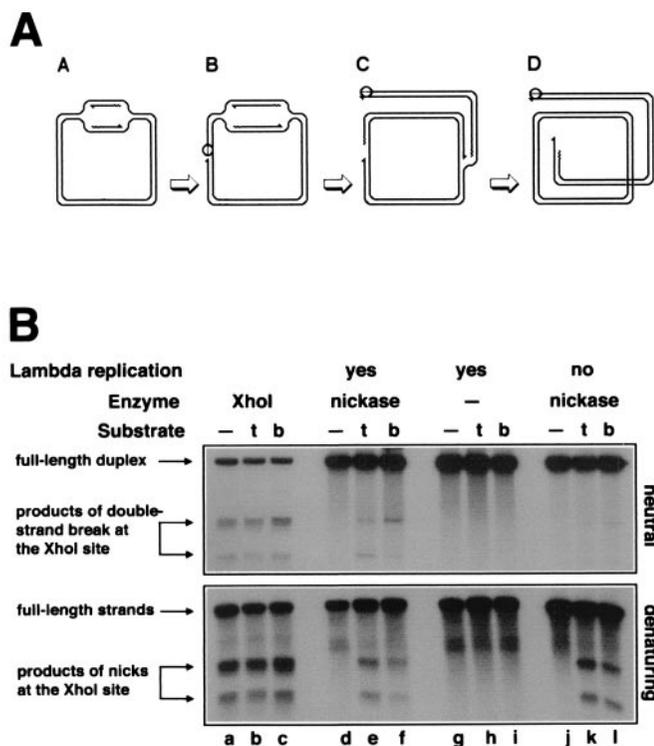


Fig. 4. Replication-induced double-strand breaks at persistent nicks at the natural *XhoI*-site. (A) Replication of a circular λ chromosome containing a nick is predicted to generate one circular chromosome and one linear chromosome with a double-strand break at the nick. Nicking enzyme is shown as an open circle attached to the 5'-end of the nick; 3'-ends are indicated by half-arrows, and RNA primers are marked by wavy lines. (A) Initiation of theta replication in the circular λ chromosome. (B) The nicking site is nicked while the theta replication continues. (C) The first replication fork runs into the nick and collapses, switching the chromosome to sigma replication. (D) The second replication fork reaches the single-strand interruption, resulting in a double-strand break in one of the replicated chromosomes. (B) Phages MMS2660, λ AK2, or λ AK3, indicated in the entry "substrate" as "—", "t," or "b," respectively, were infected at moi = 10 into the strains described below; the cells were incubated at 37°C for 40 min (unless indicated otherwise), and the phage DNA was extracted and analyzed by restriction digestion with *EcoRI* and blot hybridization with probe 1 under both neutral (Upper gel) and denaturing (Lower gel) conditions. Molecular weight markers for double-strand break at the *XhoI* site were generated *in vivo* (lanes a–c). The strains and conditions are as follows: lanes a–c, *recB268* pK107, incubated for 10 min at 37°C; lanes d–f, Δ *rep::kan recD1011* pK125 + IPTG; lanes g–i, Δ *rep::kan recD1011* pK125, no induction; lanes j–l, $Su^- \Delta$ *rep::cam recC1010* pK125 + IPTG. To compensate for the smaller amount of λ DNA because of the inhibition of λ replication, 25 times more total DNA has been loaded in the case of Su^- samples (lanes j–l).

reveal double-strand breakage because of replication fork encounters with nicks (Fig. 4A). However, no double-strand breakage was detected in this case (Fig. 3B, lanes g–i), suggesting either that replication forks never reach the nicks or that λ DNA replication interferes with the nicking. The nicking by the wild-type nicking enzyme requires DNA supercoiling (19); because replicating λ DNA loses supercoiling, the nicking reaction should be inhibited by λ 's replication. And yet, for the purpose of this experiment, it was critical to have an efficient nicking of replicating chromosomes. To increase the efficiency of nicking of replicating λ substrates, I used G73A mutant nickase, which shows a reduced superhelicity requirement (32). To further increase sensitivity of the system, I conducted all subsequent experiments in *rep* mutant cells, thus preventing rolling-circle DNA replication from the nicking sites.

Replication-Dependent Double-Strand Breaks. I found that, in the presence of the supercoiling-insensitive nickase, replication of λ DNA carrying the cognate nicking site causes double-strand breakage at the site (Fig. 4B Upper, lanes e and f). Similar patterns of double-strand breaks form with either orientation of the nicking site. Importantly, no double-strand breaks are observed in the parental phage lacking the nicking site (Fig. 4B Upper, lane d).

When the expression of the nicking enzyme is not induced, double-strand breaks do not form in replicating λ phages (Fig. 4B Upper, lanes g–i), indicating dependence of the double-strand breakage on the nickase. *In vitro*, the nicking enzyme of filamentous phages catalyzes a low level of double-strand breakage at the nicking site when Mg^{2+} is replaced with Mn^{2+} (19). Therefore, the double-strand breaks (Fig. 4B Upper, lanes e and f) might have been because of an infrequent double-strand cutting by the nickase *in vivo*. However, preventing λ DNA replication (in a Su^- host) eliminates almost all double-strand breakage (Fig. 4B Upper, lanes k and l), although the nicks, as monitored by alkaline agarose gel electrophoresis, still form efficiently in these cells (Fig. 4B Lower, compare lanes e and f with k and l). The alkaline gel also shows no nicking when the nicking enzyme is not produced (Fig. 4B Lower, lanes h and i). I conclude that, at this particular location in the λ chromosome, nicking sites are prone to double-strand breakage in the presence of the cognate nicking enzyme if the chromosome is undergoing replication (Fig. 4A).

Locus Independence of the Breaks. To make sure that the observed phenomenon is not restricted to a particular location on the λ chromosome, I inserted the nicking site at the unique *SstI::XhoI* site in a different λ phage (Fig. 2A Lower). Because λ has bidirectional DNA replication, it was important to place the nicking site on the other side of the λ replication origin [*ori* is around position 39100 on the λ chromosome (38)] to see whether the findings at the first location would hold. In this experiment, *in vivo* molecular weight markers are generated by using sigma replication of the same set of substrates in Rep^+ cells; therefore, the markers are "half-breaks" (compare Fig. 5, lanes a–c, with Fig. 3B, lanes j–l) rather than regular double-strand breaks.

A similar pattern of breakage was obtained at the new location, that is, double-strand breaks formed efficiently in replicating λ carrying the nicking sites in the presence of the nicking enzyme (Fig. 5 Upper, lanes e and f). No breaks were formed if the enzyme was not produced (Fig. 5 Upper, lanes h and i), or if the nicking sites were absent (in the original control phage) (Fig. 5 Upper, lanes d, g, and j), or if λ 's DNA replication was inhibited (Fig. 5 Upper, lanes k and l). The alkaline counterpart indicated an efficient cleavage of the nicking sites whenever the nicking enzyme was produced (Fig. 5 Lower, lanes b and c, e and f, and k and l). Therefore, the overall phenomenon looks similar at two separate locations on opposite sides of the λ replication origin. I conclude that, when a replication fork runs into a persistent single-strand break in template DNA, a double-strand break at the nick results.

Discussion

The main purpose of these experiments was to test ideas about replication fork interaction with persistent nicks in template DNA. One idea predicted that a nick relaxes DNA supercoiling and therefore would inhibit the replication fork progress in its vicinity. Another idea predicted that the replication fork would run into the nick and collapse, generating a double-strand end (Fig. 1 A \rightarrow B \rightarrow C). In the case of two replication forks converging on the same nick, a double-strand break would be generated (Fig. 1 C \rightarrow D). My observations are consistent with the replication fork collapse scenario: persistent nicks, introduced by a nicking enzyme in the replicating λ chromosome,

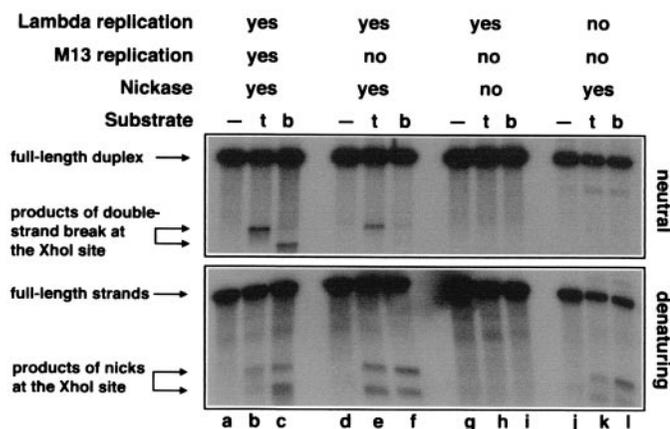


Fig. 5. Replication-induced double-strand breaks at persistent nicks at the *XhoI*-site on the other side of the λ replication origin. Phages MMS2663, λ AK4, or λ AK5, indicated in the entry "substrate" as "-", "t," or "b," respectively, were infected at $\text{moi} = 6$ into the strains described below; the cells were incubated at 28°C for 80 min, and the phage DNA was extracted and analyzed by restriction digestion with *Pst*I + *Nco*I and blot hybridization with probe 2 under both neutral (Upper gel) and denaturing (Bottom gel) conditions. Molecular weight markers for "half-breaks" at the *XhoI* site were generated *in vivo* (lanes a–c). The strains and conditions are as follows: lanes a–c, *recB268* pK133 + IPTG; lanes d–f, Δ *rep::cam recD1011* pK125 + IPTG; lanes g–i, Δ *rep::cam recD1011* pK125, no induction; lanes j–l, $\text{Su}^- \Delta$ *rep::cam recC1010* pK125 + IPTG. To compensate for the smaller amount of λ DNA because of the inhibition of λ replication, 25 times more total DNA has been loaded in the case of Su^- samples (lanes j–l).

cause the formation of double-strand breaks (Fig. 4A). It should be noted that these experiments were not designed to rule out the replication fork inhibition scenario; indeed the majority of replication forks could be inhibited by the nick-associated relaxation and never reach the nick.

The nicking enzymes of bacteriophages are related to type I DNA topoisomerases and, in this respect, the reported experiments have their predecessors. Camptothecin is a progenitor of a family of potent anticancer drugs (39, 40); camptothecin kills eukaryotic cells during S-phase (3) by preventing the closing part of the DNA topoisomerase I cycle (41). Persistent nicks, caused by camptothecin-inhibited DNA topoisomerase I, were proposed to cause double-strand breaks in replicating simian virus 40 (SV40) molecules (9, 14, 42), but because the sites of the original single-strand interruptions were difficult to map, the question remained as to the link between topoisomerase-induced nicks and detected double-strand ends. Electron microscopy studies of replicating SV40 DNA, extracted from camptothecin-treated cells, produced spectacular pictures of circular chromosomes with what looked like disintegrated replication forks (43), but the results were again only suggestive. In

my experiments, replication-dependent double-strand breaks form at preexisting single-strand scissions and nowhere else.

What could be the molecular mechanisms of this DNA replication-induced double-strand breakage at persistent nicks? One widely discussed possibility (2, 7–10, 39, 44–46) is that when a replication fork reached a single-strand interruption, it collapses, and if a converging replication fork soon comes to the same nick, a double-strand break results (Fig. 1A–D), exactly what is observed in the experiments reported here. An alternative explanation for the nick-induced double-strand breakage is that a replication fork encounters the nick and "explodes," generating two replicated branches and one unreplicated branch (40) (Fig. 1E). The reported *in vivo* results, as well as earlier *in vitro* results of others (47), do not distinguish between the "collapse" versus "explosion" ideas. Although the possibility of replication fork explosion is less specified, it will be a legitimate contender until distinct predictions of the two ideas are tested experimentally.

One limitation of the used experimental system is that nicks produced by specialized replicative enzymes are not generic single-strand interruptions of the type generated during excision repair. Rather, the 5'-end of the nick is likely to be bound by the M13 nicking enzyme [may be covalently (48)], which creates a certain "microenvironment" around the nick and likely even around the subsequent double-strand end. Therefore, nicks introduced by other means [for example, by "nicking" mutants of restriction endonucleases (49, 50)] could better approximate spontaneous single-strand interruptions encountered by replication forks *in vivo*.

Apart from inevitable limitations of the experimental system, this work illustrates that replication not only duplicates DNA strands but also "duplicates" any lesion in these strands. Fragmentation and loss of chromosomes in rapidly dividing *rad51* mutant vertebrate cells (17, 18), apparently because of normal levels of single-strand DNA damage, dramatically illustrate this point. As a result, one-strand DNA damage, which before replication would have been fixed by excision repair, is aggravated by DNA replication and now requires a more sophisticated repair system. There is good evidence that two-strand DNA damage repair is based on homologous recombination (16). Experiments to detect homologous exchange in response to the replication-dependent double-strand breaks in the λ chromosome should be the next step in characterization of two-strand damage formation and repair.

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