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χ sites in combination with RecA protein increase the survival of linear DNA in *Escherichia coli* by inactivating exoV activity of RecBCD nuclease

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In *Escherichia coli*, unprotected linear DNA is degraded by exoV activity of the RecBCD nuclease, a protein that plays a central role in the repair of double-strand breaks. Specific short asymmetric sequences, called χ sites, are hotspots for RecBCD-promoted recombination and are shown *in vitro* to attenuate exoV activity. To study RecBCD–χ site interactions *in vivo* we used phage λ's terminase to introduce a site-specific double-strand break at λ's *cos* site inserted into a plasmid. We show that after terminase has cut *cos in vivo*, nucleases degrade linearized DNA only from the end that does not have a strong terminase binding site. Linearized cosmid DNA containing χ sites in the proper orientation to the unprotected end is degraded more slowly in *rec*$^+$ *E.coli* than is χ-less DNA. Increased survival of χ-containing DNA is a result of partial inactivation of exoV activity and is dependent on RecA and SSB proteins. The linearization of χ-containing DNA molecules leads to RecA-dependent formation of branched structures which have been proposed as intermediates in the RecBCD pathway of double-strand break repair.

Key words: chi sites/*cos*-cutting/double-strand break/RecA/RecBCD enzyme

Introduction

Prokaryotic cells have a uniquely powerful nuclease activity, exoV (Telander-Muskavitch and Linn, 1981), which enables them to destroy foreign DNA that has been cut by endogenous restriction endonucleases (Simmon and Lederberg, 1972; Oliver and Goldberg, 1977). Because of exoV, wild-type (WT) *Escherichia coli*, unlike yeast, is unable to do recombinational repair of double-strand breaks that have only several kilobases of terminal homology to an intact DNA molecule (Clyman and Belfort, 1992; Eddy and Gold, 1992). The failure of repair is due, apparently, to the rapid destruction of the terminal homologous sequences. In *E.coli*, exoV activity is embodied in the RecBCD protein, a heterotrimeric enzyme encoded by the *recB, recC* and *recD* genes [see Taylor (1988) for review]. Paradoxically, the same RecBCD enzyme plays a central role in *E.coli*'s principal pathway for recombinational repair of double-strand breaks in chromosomal DNA [see Smith (1988) for review]. Somehow, cells manage to use this destructive machine for constructive purposes when a double-strand break in the chromosomal DNA needs to be repaired.

RecBCD enzyme was shown to interact with the asymmetric octanucleotide sequence called χ when approaching it from one side, but not from the other (Faulds *et al.*, 1979; Yagil *et al.*, 1980; Smith *et al.*, 1981; Kobayashi *et al.*, 1982; Taylor *et al.*, 1985). χ sites were discovered in phage λ as mutations creating hotspots for RecBCD-promoted recombination (Lam *et al.*, 1974; Henderson and Weil, 1975; Stahl *et al.*, 1975; Stahl and Stahl, 1977). A solution to the paradox of a single enzyme carrying out two mutually exclusive activities on linear DNA (complete destruction versus preservation through recombination) was suggested on the basis of genetic data. It was proposed that when RecBCD enzyme encounters a properly oriented χ site, its exoV activity is temporarily inactivated, turning the nuclease into a 'recombinase' (Thaler *et al.*, 1988, 1989; Stahl *et al.*, 1990). This idea has recently received support from *in vitro* experiments (Dixon and Kowalczykowski, 1991, 1993). We have studied the consequences of RecBCD and χ site interaction *in vivo* with a complementary approach, inducing synchronous double-strand breaks at a specific site in an intracellular plasmid and monitoring degradation of the linearized DNA. To do this, we inserted the phage λ packaging signal, *cos*, into specifically designed plasmids, introduced the resulting cosmids into *E.coli* cells, and induced the synthesis of the phage-encoded enzyme, terminase, that makes a double-strand break at *cos*. We report here that, after linearization, χ-less DNA is quickly degraded by RecBCD. This degradation proceeds primarily from only one end of an opened molecule, since the other end is blocked by bound terminase. χ sites in the proper orientation to the unprotected end increase the survival of linear DNA in WT background. This χ-mediated increase in survival is a result of cells being converted to partial exoV$^-$ phenocopies. Nevertheless, the χ-mediated increase in survival is dependent on RecA and SSB. Moreover, linearization leads to RecA-dependent formation of branched structures in χ-containing cosmid DNA. Our results are consistent with a recent report of protection of linear DNA by χ sites from RecBCD-degradation both *in vitro* and *in vivo* (Dixon and Kowalczykowski, 1991, 1993; Dabert *et al.*, 1992). On the basis of these findings we entertain a model for double-strand break repair in chromosomal DNA of *E.coli* in which χ sites stop the DNA degradation by inactivating exoV activity of RecBCD nuclease, and the resulting frayed end, with the aid of RecA protein, invades an intact homologous sequence to form a structure that may be converted into a replication fork.

Results

exoV degrades cosmid DNA opened at cos

It was shown previously that terminase produced from a plasmid can cleave a cosmid at its *cos* site *in vivo* (Murialdo

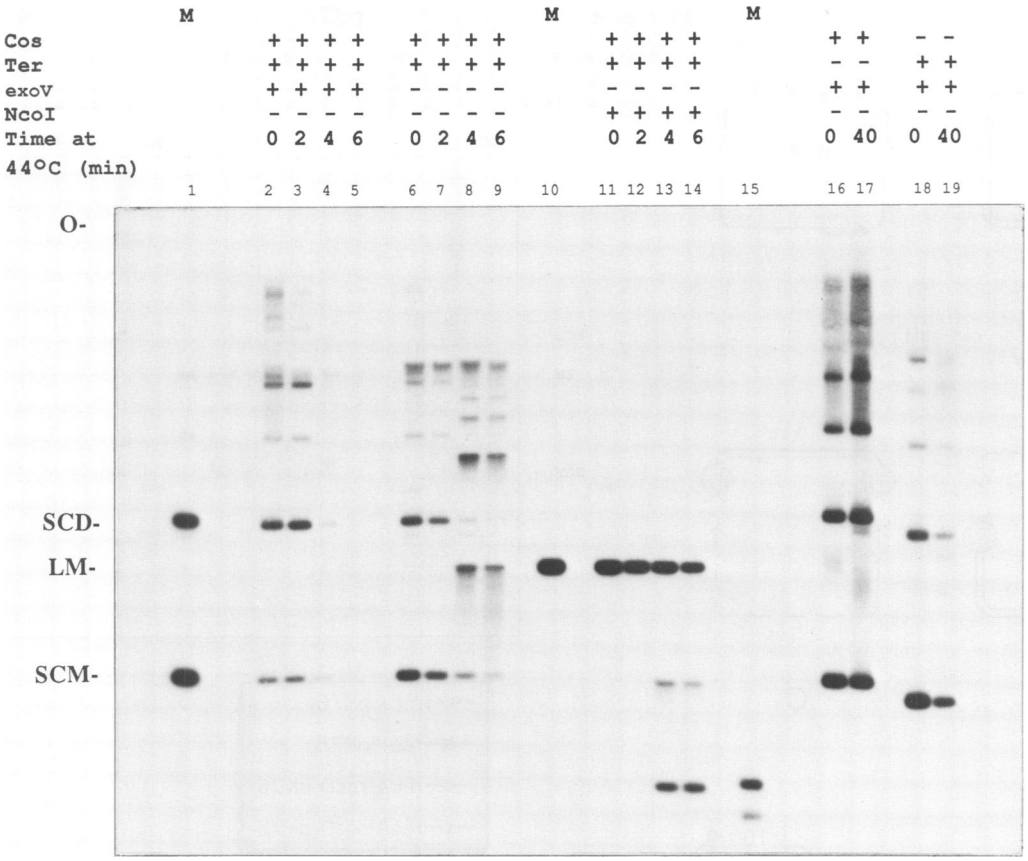

Fig. 1. exoV degrades cosmid DNA opened at *cos*. M, marker; O, origin of the gel; SCD, supercoiled dimeric plasmid; LM, linear monomeric plasmid; SCM, supercoiled monomeric plasmid. Cos, the presence (+) or the absence (−) of λ's *cos* site; Ter, terminase is produced (+) or not produced (−) upon shifting of the incubation temperature from 24°C to 44°C; exoV, strain is exoV$^+$ (WT) or exoV$^-$ (*recD*); NcoI, after isolation, total DNA was treated (+) or not treated (−) with restriction endonuclease *Nco*I. Lane 1: marker of intact cosmid pK51, featuring mostly supercoiled monomers and dimers; lanes 2−5: DNA of pK51, purified from WT strain at different time points after terminase synthesis induction; lanes 6−9: pK51 DNA, purified from *recD1011* strain at different time points after terminase synthesis induction; lane 10: marker of linear plasmid pK51 (3830 bp); lanes 11−14: DNA of pK51, purified from a *recD1011* strain at different time points after induction of terminase synthesis and cut *in vitro* with *Nco*I (this should generate two fragments of 2230 and 1600 bp in length; lane 15: molecular weight markers, 1622 bp and 1262 bp (pK11 cut with *Eco*O109I, which cuts near the *cos*, and *Nco*I; the difference in signal intensities is due to a non-homology in the smaller fragment to the probe used (pK22 cut with *Eco*RI)); lanes 16 and 17: DNA of pK51, purified from WT strain in the absence of terminase-producing plasmid after incubation at 24°C or 40 min after shifting the culture to 44°C; lanes 18 and 19: DNA of pK59 (no *cos* site) from WT strain that harbors the terminase-producing plasmid, purified after incubation at 24°C or 40 min after shifting the culture to 44°C.

and Fife, 1984). When cells harboring pCM101 (Chow *et al.*, 1987) are shifted from 24°C to 44°C, they produce phage λ terminase. Crude extracts prepared from these cells cleave cosmids *in vitro* with at least 80% efficiency (Chow *et al.*, 1987). We used the *cos* site in combination with inducible terminase to introduce synchronous double-strand breaks in cosmid DNA to study the fate of linearized cosmid DNA *in vivo*. Since our terminase-producing plasmid is pBR322-based, our cosmids were constructed from other, compatible plasmids.

First, we verified that the production of the double-strand breaks depends on the presence of both terminase and *cos*. We found that in the absence of the terminase-producing plasmid there are no double-strand breaks in the cosmid DNA upon shifting the culture to 44°C (Figure 1, lanes 16 and 17). Similarly, the induction of terminase synthesis does not result in cleavage of a plasmid that lacks the *cos* site (Figure 1, lanes 18 and 19).

We then followed the fate of cosmid DNA upon induction of terminase synthesis. In the WT strain, most of the cosmid DNA vanishes within 5 min of terminase synthesis induction (Figure 1, lanes 2−5). The disappearance of the cosmid DNA is preceded by its linearization and is dependent on exoV, as is apparent from the relative stability of linearized DNA in a *recD* (exoV$^-$) strain (Figure 1, lanes 6−9). *In vitro* digestion of the linearized DNA from the *recD* strain with a unique restriction endonuclease generates two fragments of expected length, confirming that the double-strand break is at *cos* (Figure 1, lanes 11−14).

Cosmid DNA is degraded only from one end

If the cosmid DNA linearized *in vivo* by terminase in the *recD* background is additionally cut *in vitro* with a unique restriction nuclease, the signal from one of the two resulting fragments is sharp and intense, while the signal from the other fragment is weak and slightly smeared (Figure 1, lanes 13 and 14). The difference can be explained by the fact that the undegraded fragment contains a strong terminase binding site (Becker and Murialdo, 1990), so this end of the linearized molecule is inaccessible to nucleases due to the bound terminase. We employed two cosmids (pK76 and pK78), each of them containing two oppositely oriented *cos* sites (Figure 2A), to check whether two bound terminase complexes can protect a DNA fragment between them.

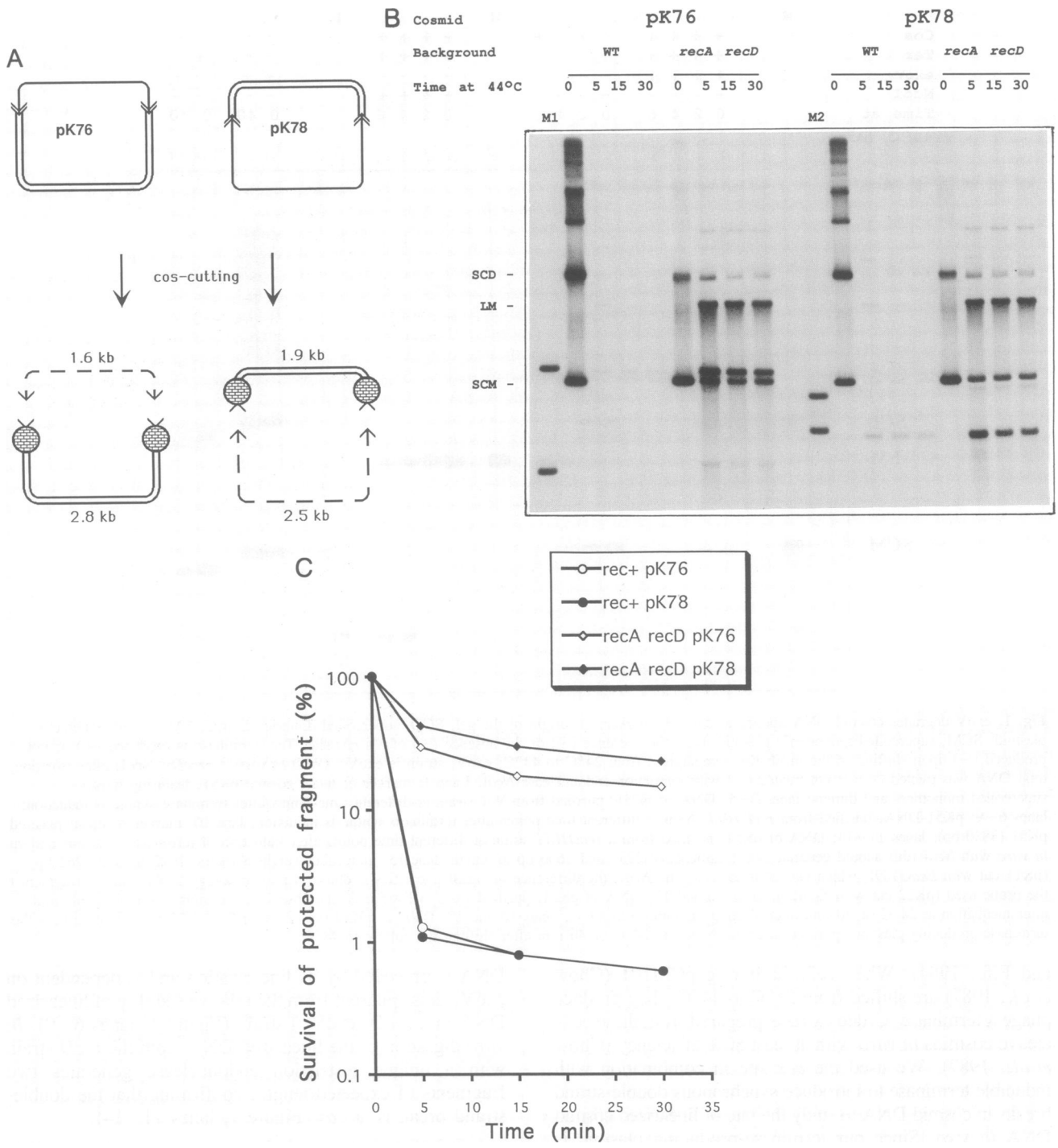

Fig. 2. Open cosmid DNA is degraded primarily from the end that is not protected by bound terminase. (**A**) A pair of double-*cos* cosmids used for the experiment and the fragments they give upon *cos* cutting. Double arrows represent *cos* sites and point in the direction of packaging. Circles with bricks inside designate the bound terminase. (**B**) Kinetics of the degradation of the fragments produced upon *cos* cutting of double-*cos* cosmids in WT and *recA recD* backgrounds. The time at 44°C is given, in minutes. M1, pK76 cut with *Eco*O109I; M2, pK78 cut with *Eco*O109I. *Eco*O109I cuts 23 nucleotides to the left of *cos*; the two fragments produced after cutting with this restriction endonuclease serve as approximate molecular markers for the cutting at both *cos* sites. SCD, supercoiled dimeric plasmid; LM, linear monomeric plasmid; SCM, supercoiled monomeric plasmid. As a probe, DNA of pK76 cut with *Bgl*II was used. (**C**) Semi-logarithmic plot showing the kinetics of survival of the fragment with two terminase binding sites in the WT and *recA recD* backgrounds. Each point is an average of three independent measurements.

Cutting of both *cos* sites in these cosmids generates two fragments of unequal length; only one of them should survive *in vivo* if terminase protects the end it binds.

In the WT strain, the fragment that has two terminase binding sites at its ends is only a small fraction of all the cosmid DNA that is linearized, but it is stable with time and fares much better than does the other fragment, which cannot be seen at all (Figure 2B). We presume that RecBCD quickly degrades the cosmid linearized at one *cos* site; thus, most of the DNA is degraded before it is protected by a cut at the second *cos* site. In agreement with this interpretation, the fragments with two terminase binding sites survive much

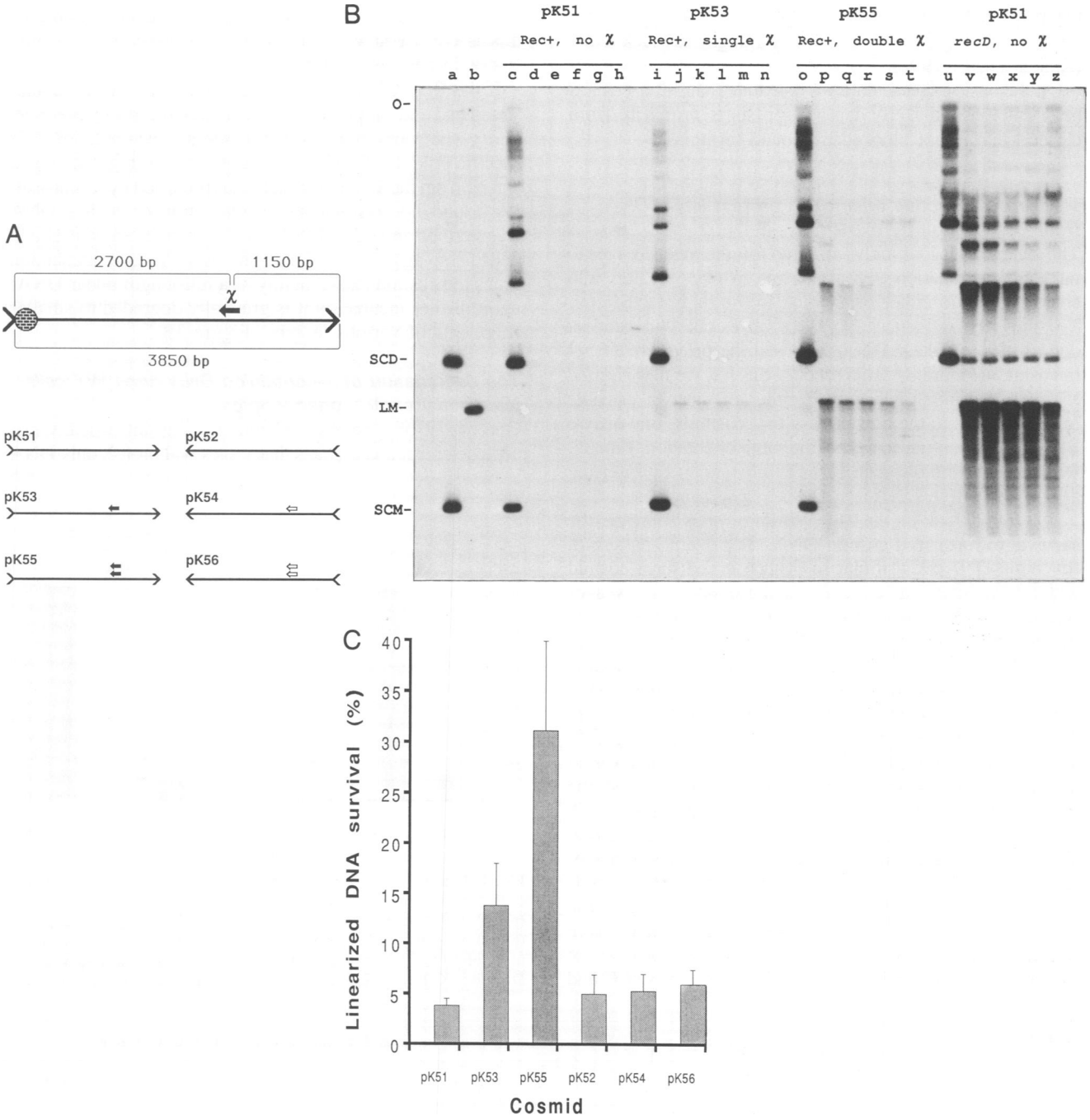

Fig. 3. χ sites protect linearized cosmid DNA from RecBCD degradation. (**A**) Top, overall view of the linearized cosmid of pK51−pK56 series with terminase bound to its left end and the distances from both ends to χ site(s). Bottom, schemes of the cosmids with relative orientation of *cos* and χ sites. Small solid arrows: active χ sites; small open arrows: inactive χ sites. (**B**) Kinetics of degradation of χ-less and χ-containing cosmids in WT background. O, origin of the gel; SCD, supercoiled dimeric plasmid; LM, linear monomeric plasmid; SCM, supercoiled monomeric plasmid. Lane a: a marker featuring supercoiled plasmid species, mostly monomers and dimers; lane b: linear monomeric plasmid marker; lanes c−h: DNA of pK51, purified from WT strain at 0, 10, 20, 30, 40 and 50 min after terminase synthesis induction; lanes i−n: DNA of pK53, purified from WT strain at 0, 10, 20, 30, 40 and 50 min after terminase synthesis induction; lanes o−t: DNA of pK55, purified from WT strain at 0, 10, 20, 30, 40 and 50 min after terminase synthesis induction; lanes u−z: DNA of pK51, purified from *recD1011* strain at 0, 10, 20, 30, 40 and 50 min after terminase synthesis induction. (**C**) Survival of linearized cosmid DNA in the WT strain. Each value is a mean of 10−23 independent measurements ± standard deviation.

better in *recD* and *recA recD* mutants (lacking exoV activity) than do the unprotected fragments, which can barely be seen on the gel (Figure 2B).

We have calculated the survival of the fragments protected by bound terminase. In our WT strain, the survival is ~2% at 5 min after the beginning of terminase synthesis induction and is still ~1% at 30 min (Figure 2C). In *recA recD*, the survival is 30−43% at 5 min after the beginning of the induction of terminase synthesis and 15−23% at 30 min (Figure 2C). Survival in the *recD* strain is somewhat higher than in *recA recD* (A.Kuzminov and F.Stahl, manuscript in preparation). The survival of the fragment that does not contain strong terminase binding sites in the *recA recD* and in the *recD* strains is at least 10 times lower than that of

the protected fragment. Apparently, there is appreciable nuclease activity in *recD* cells even though they lack exoV (Rinken *et al.*, 1992).

On the basis of these data we conclude that, after *cos* has been cut, cosmid DNA degradation starts primarily from the end that is not blocked by bound terminase.

χ sites in the active orientation protect linear DNA from degradation by exoV

The accessibility of only one end of the opened *cos* to nuclease degradation encouraged us to place χ sites into cosmid DNA in either orientation to the unprotected end. To study the influence of χ sites on RecBCD-dependent degradation of linear DNA, we used the pK51–pK56 set of cosmids (Figure 3A). To produce this set, we first inserted λ's *cos* sites into a plasmid in either of the two possible orientations. Into the pair of resulting cosmids, one or two χ sites were then introduced in the same orientation. Finally, we increased the distance between *cos* and χ by inserting a piece of χ-free DNA.

We found that in the *rec*$^+$ strain χ sites increase the survival of linearized cosmid DNA. The overall pattern of surviving DNA was similar to that observed for a cosmid without χ sites in a *recD* background, but weaker (Figure 3B). We quantified the degree of survival as the fraction of linearized cosmid DNA that can survive 11 min after the start of induction of terminase synthesis (Figure 3C). Without χ sites, <4% of cosmid DNA survives the treatment. The single χ site in pK53 improves the survival to almost 14%. Adding a second χ site (pK55) increases the survival to >30%. However, if *cos* is inverted (as in pK52, pK54 and pK56), χ sites do not increase the survival (Figure 3C). Since it is known that χ sites work in only one orientation relative to *cos* (Faulds *et al.*, 1979; Yagil *et al.*, 1980; Kobayashi *et al.*, 1982), the absence of an effect of χ on survival of pK54 and pK56 cosmids corroborates the preceding observation that terminase protects the left end of a linearized cosmid molecule from RecBCD entry. There is no increase in survival of χ-containing cosmids versus χ-less cosmids in *recBC* and *recD* cells, two known types of exoV$^-$ mutants (Table I); this is consistent with the observed interaction of χ with RecBCD nuclease *in vitro*.

If the RecBCD nuclease degrades DNA from a double-strand end and simply loses its degradation capacity precisely at a χ site, one might expect to see an accumulation of a specific product of such degradation. Such a fragment would extend from the χ site to the left end (protected by terminase). We found no conspicuous accumulation of such a DNA fragment or any other DNA species using both neutral and denaturing agarose gel electrophoresis. At early incubation times the cosmid exists mainly as a full-length linear DNA; upon further incubation it is gradually degraded to smaller fragments of various lengths (Figure 3B).

The degradation of χ-containing DNA turns WT cells into partial exoV$^-$ phenocopies

One model of χ action envisions it as an intrinsically *cis*-acting element and predicts that χ sites will protect only DNA

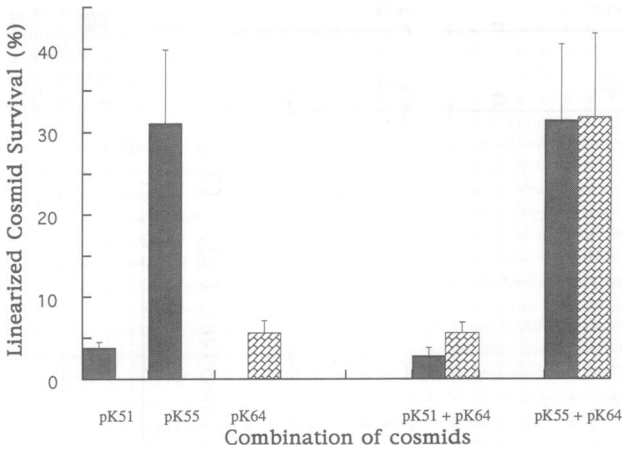

Fig. 4. Linearization of χ-containing DNA turns WT cells into partial exoV$^-$ phenocopies. Survival of the χ-less linearized indicator cosmid (pK64) in the presence or absence of the heterologous χ-containing (pK55) or χ-less (pK51) cosmids in the WT strain. Each value is a mean of 10 to 23 independent measurements ± standard deviation. Data for pK51 and pK55 in the absence of pK64 are from Figure 3C.

Table I. Survival of linearized χ-less and χ-containing cosmid DNA in mutants deficient in recombination, SOS-induction and replication

Background	Survival		χ effect (*b* − *a*)
	No χ sites (*a*)	Two χ sites (*b*)	
rec$^+$ [a]	3.6 ± 0.9 (23)	31.1 ± 8.8 (23)	27.5
recA200^{ts} [b]	5.3 ± 1.2 (10)	10.6 ± 3.8 (9)	5.3
ssb-113	6.4 ± 2.1 (7)	9.6 ± 1.5 (7)	3.2
lexA3	23.0 ± 2.4 (9)	51.7 ± 20.2 (8)	28.7
recB21 recC22	174.5 ± 30.8 (9)	195.7 ± 52.1 (8)	NA
recD1013	308.7 ± 70.3 (10)	304.8 ± 64.2 (10)	NA
dnaE486^{ts}	3.7 ± 1.6 (10)	25.7 ± 7.7 (9)	22.0
dnaB22^{ts}	6.6 ± 1.4 (10)	54.5 ± 11.8 (8)	47.9
polA4109^{ts}	4.2 ± 0.7 (10)	18.5 ± 4.3 (12)	14.3

Survival was calculated as % of linearized cosmid DNA that can be detected 11 min after beginning of terminase synthesis induction (described in Materials and methods). Values are means ± standard deviations. The number of independent determinations of the value is given in parenthesis. 'χ effect' is defined as the difference between the survival value for the cosmid with χ sites (*b*) and the survival value for the cosmid without χ sites (*a*).

[a]Data from Figure 3C.

[b]χ sites do not increase linearized cosmid survival in *recA13* and *recA* deletion mutants as well (A.Kuzminov, E.Schabtach and F.Stahl, manuscript in preparation).

molecules on which they reside (Smith, 1991). The alternative model for the mechanism of χ action, although not stating that explicitly, raises the possibility that χ sites can protect DNA *in trans* by inhibiting the cellular exoV activity of RecBCD nuclease (Thaler *et al.*, 1988, 1989; Stahl *et al.*, 1990). Our approach would enhance any effect of χ *in trans* since the cells are exposed to multiple χ sites present on some 50 synchronously linearized cosmid molecules. To see whether χ protects only *in cis*, or can protect also *in trans*, we constructed a new cosmid, pK64, non-homologous to pK51 and pK55 except for ~50 nucleotides at the right end of the *cos* site and 200 nucleotides at the left end of the *cos* site (protected by bound terminase). The new cosmid is compatible with the previous cosmids as well as with the terminase-producing plasmid and does not have χ sites. To monitor the effect of χ *in trans*, we introduced the previous χ-containing cosmid (pK55) alone or together with the new cosmid into the WT cells, linearized cosmids simultaneously, and measured their survival after blot-hybridization with a probe complementary to either the old cosmid or the new one. As a control, we did the same manipulations, using the old χ-less cosmid (pK51) instead of the χ-containing one.

We found that in the absence of χ sites on the old cosmid, the survival values for both cosmids are not different from those which were obtained when each cosmid was alone (Figure 4). However, in the presence of χ sites on the old cosmid, the second, χ-less cosmid enjoys survival similar to that of the χ-containing cosmid, confirming that, in these cells, exoV activity is indeed partially inhibited.

Protection by χ sites depends on RecA and SSB proteins

The χ-dependent increase in cosmid DNA survival could be simply a result of the χ–RecBCD interaction, as seems to be the case *in vitro* (Taylor and Smith, 1992; Dixon and Kowalczykowski, 1993). However, the formation of phage λ recombinants along the RecBCD pathway requires (in addition to RecBCD enzyme) several other proteins, among them RecA and SSB (Ennis *et al.*, 1987). To see whether χ-dependent increase in cosmid survival is also dependent on these proteins, we quantified the survival of cosmid DNA with and without χ sites in strains carrying mutations in the corresponding genes (Table I). Perhaps unexpectedly, both *ssb* and *recA* mutant cells allow only weak χ-dependent survival of the linearized cosmids. We conclude that the χ-dependent survival cannot be explained solely by interaction of RecBCD with χ sites. RecA and SSB may protect DNA by somehow inactivating exoV, by binding to single strands and/or by promoting recombinational events (see below).

Linearization of χ-containing cosmid DNA might induce the SOS response. If so, the observed χ-dependent survival of linearized DNA could be due to the elevated level of proteins whose expression is increased as a part of the SOS response. However, in a strain where SOS induction is impossible because of an uncleavable SOS repressor (*lexA3*), χ sites increase the survival to the same extent as in the WT strain (Table I). The increased survival of the χ-less cosmid in *lexA3* mutant, as well as in unconditional *recA* mutants, is a consequence of chromosomal DNA degradation in these backgrounds (A.Kuzminov, E.Schabtach and F.W.Stahl, manuscript in preparation).

χ sites stimulate formation of branched structures

The participation of SSB and RecA proteins in the χ effect prompted us to look for evidence of RecA-promoted homologous pairing. In our case, the RecA-mediated invasion of the unprotected end of a linearized cosmid into an intact cosmid molecule would result in a σ-structure, a circular DNA molecule with a linear tail. Digestion with a restriction endonuclease that has a unique site in the circular domain of the σ-structure yields a linear piece of cosmid DNA of less than monomer length and a Y-shaped molecule with a molecular weight between linear monomer and linear dimer (Figure 5A). The configuration of this Y-shaped molecule is such that two of its arms are of the same length, while the length of the third arm plus one of the equal arms is equal to the length of the cosmid monomer. Because of its branched structure, it can be separated from linear molecules by 2D agarose gel electrophoresis, in which the first dimension separates DNA only on the basis of molecular weight, while the second dimension separates according to both molecular weight and molecular structure (Bell and Byers, 1983; Brewer *et al.*, 1988). In this 2D gel, linear DNA of different sizes forms a characteristic arc, while branched and circular DNA lags behind this arc (Figure 5B) (Brewer *et al.*, 1988).

At time zero, we found that there are a few intermediates of cosmid DNA replication lagging behind the linear arc; they disappear as a result of the terminase cutting (Figure 5C). In the WT background, the linearization of the χ-less cosmid does not produce new, slowly migrating species (Figure 5C, top panel). In contrast, the linearization of χ-containing cosmid does result in new, slowly migrating species (which represent 0.2–0.5% of all the surviving linearized DNA) running between linear monomer and linear dimer in the first dimension (Figure 5C, middle panel). To see their structure, we isolated χ-containing cosmid DNA linearized by terminase *in vivo* by a preparative procedure that eliminates most of the chromosomal DNA (see Materials and methods), separated it in 2D gels and extracted DNA from the position corresponding to the slowly migrating species (Figure 5B). Electron microscopy revealed that this DNA contains Y-shaped molecules (79 out of 200 molecules counted), most of them having the expected configuration (Figure 6). We also saw occasional σ-structures with a circular domain of monomer length as well as X-shaped molecules (Figure 6). DNA preparations extracted in parallel from the 'linear arc' have only a few branched molecules (three out of 200 molecules counted), and none of them is of the expected configuration. We conclude that, in a WT strain, cosmid DNA linearization leads to the χ-dependent formation of branched structures which, before cutting, may have been σ-structures.

The formation of branched structures requires RecA

The formation of branched species was not observed in a *recA200*^{ts} (temperature sensitive) strain (Figure 5C, bottom panel), suggesting that they have a recombinational origin. On the other hand, Dabert *et al.* (1992) proposed that χ sites act to protect the tails of rolling circles from RecBCD degradation; in their system, χ-protection was also dependent on RecA. Thus, the alternative interpretation of our results might be that *cos*-cutting somehow switches plasmid replication to σ-mode, and that χ sites with the help of RecA protein protect the linear tails from RecBCD degradation.

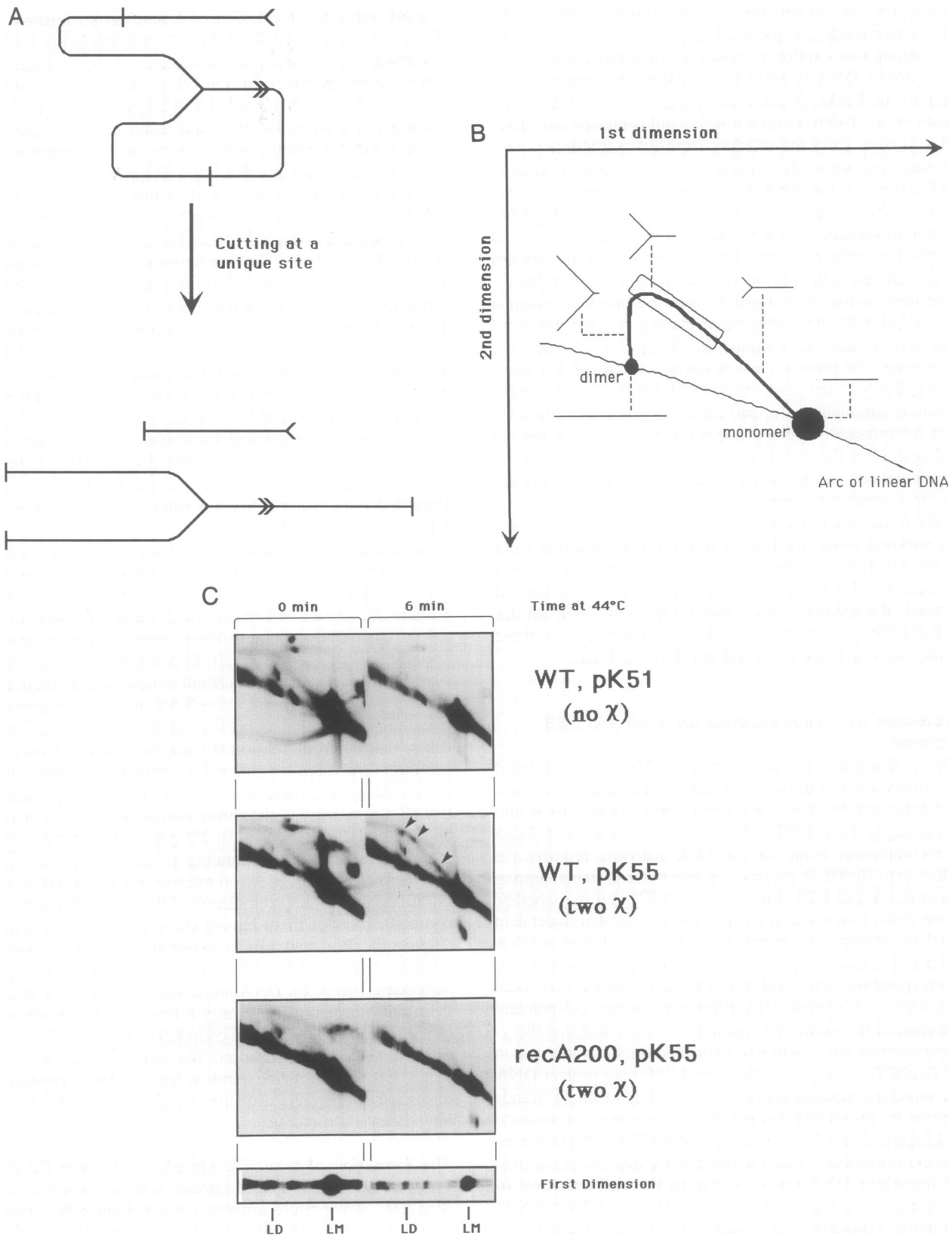

Fig. 5. Analysis of recombinational intermediates with 2D agarose gel electrophoresis. **(A)** Cutting with a unique restriction endonuclease converts a σ-structure into a Y-shaped molecule and a linear molecule less than monomer length. Double arrows represent intact *cos* sites which point in the direction of packaging. Vertical bar indicates a unique restriction site. **(B)** A scheme of a pattern of separation of linear and Y-shaped molecules in 2D agarose gel electrophoresis. The part of the gel from which the DNA structures shown in Figure 6 were purified is boxed. **(C)** Autoradiograms of separation of cosmid DNA in 2D gels, where 0 min (no *in vivo* cutting) and 6 min (experiment) DNA preparations were run in parallel on a single gel. Arrowheads in the case of χ-containing cosmid in WT background (middle panel) point to the spots of Y-shaped molecules.

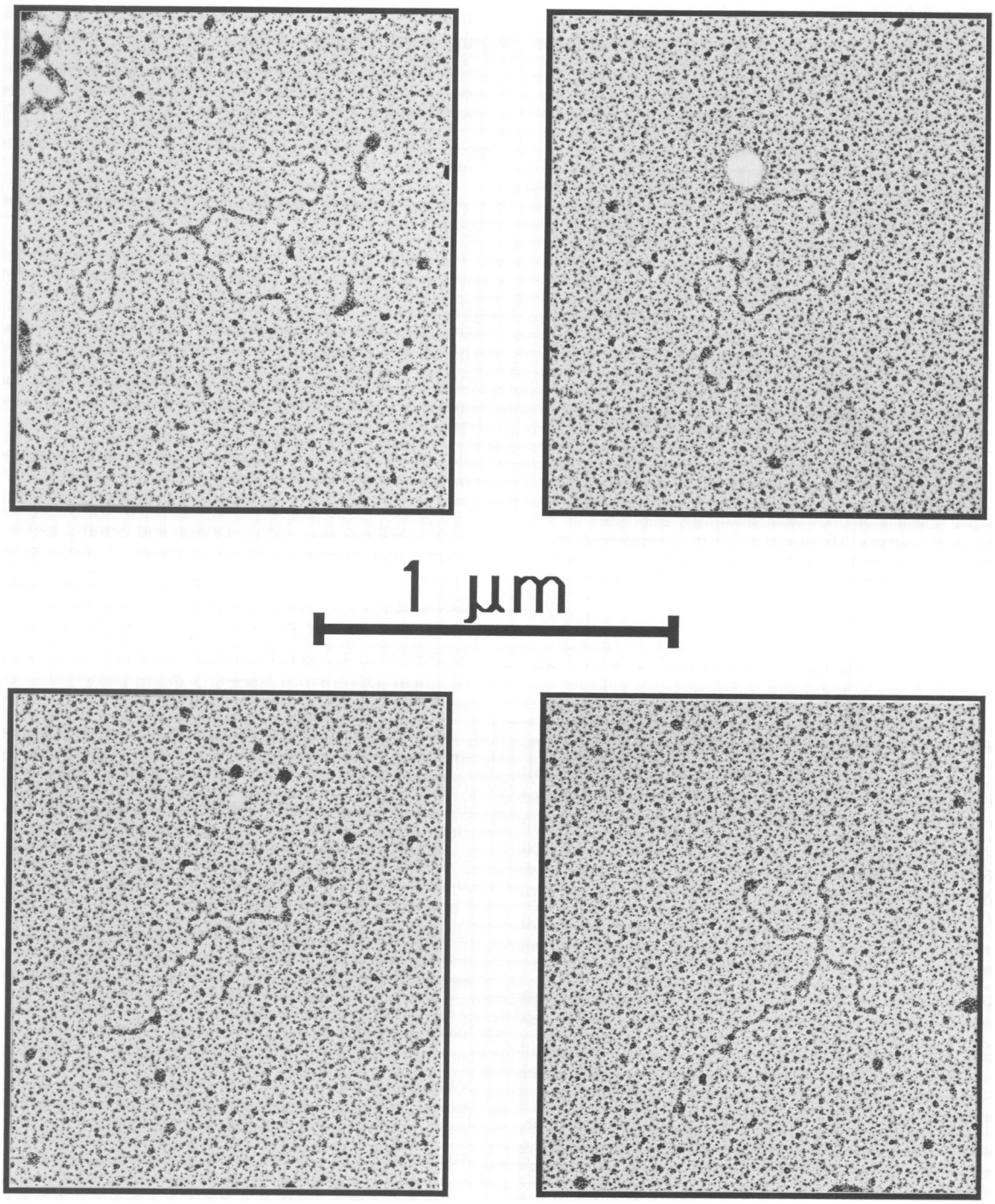

Fig. 6. Electron micrographs of DNA species that lag behind the 'linear arc'. These DNA species were purified from the position on a 2D gel shown as a rectangle in Figure 5B as described in Materials and methods.

To distinguish between the two interpretations, we examined the influence of a block to DNA replication on survival of the linearized χ-containing cosmid. In *E.coli*, DnaB protein is the primary DNA helicase of the replication fork, while DnaE (PolC) protein is a polymerizing subunit of the DNA polIII complex (McMacken *et al.*, 1987). Shifting *dnaB*^{ts} or *dnaE*^{ts} mutants to the non-permissive temperature shuts down most of the chromosomal DNA synthesis within 5–10 min, as measured by [^{3}H]thymidine incorporation into TCA-precipitable counts (Figure 7). Nevertheless, there is no decrease in χ-dependent cosmid DNA survival at 44°C in either background (Table I), indicating that the effect of χ is not a result of any considerable DNA synthesis. This result also shows that the newly formed branched structures are not used immediately by the *E.coli* replication machinery. Interestingly, χ-dependent cosmid DNA survival is slightly decreased by inactivating the polymerase subunit of DNA polymerase I (Table I).

Discussion

We introduced synchronous double-strand breaks into circular plasmid DNA molecules *in vivo* and then isolated and characterized the linearized DNA. This approach has

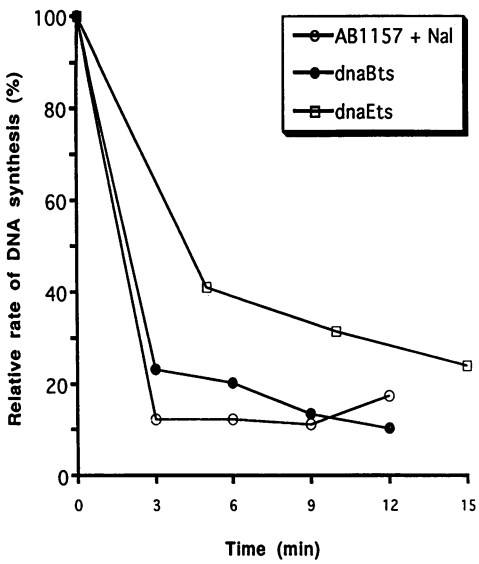

Fig. 7. Rapid inhibition of DNA synthesis at high temperature in *dnaB*^{ts} and *dnaE*^{ts} mutants. The procedure for the measurements of DNA synthesis is described in Materials and methods. As a control, the rapid inhibition of DNA synthesis in the WT cells by DNA gyrase inhibitor, nalidixic acid, is also shown. Time zero for the *dna*^{ts} strains corresponds to the point when the cultures were shifted from 28°C to 44°C; for the WT strain it corresponds to the moment of nalidixic acid addition. 100% is incorporation per 10^8 cells of 4.0×10^{-2} μCi for AB1157, 1.7×10^{-2} μCi for *dnaE*^{ts} and 2.3×10^{-2} μCi for *dnaB*^{ts}.

been widely used to study recombination in eukaryotic cells (White and Haber, 1990; Maryon and Carroll, 1991) but has not been practised with WT *E.coli*, probably because of the rapid destruction of unprotected linear molecules in bacterial cells by exoV activity. In WT cells, linearized plasmid DNA disappears by the time our simplified DNA isolation protocols allow us to disrupt the cells (within 5 min of DNA linearization). The relative stability of the linearized DNA in exoV$^-$ cells confirms that most of the degradation is carried out by RecBCD enzyme.

Double-strand breaks were introduced by phage λ terminase cutting at a *cos* site in the plasmid. Terminase binding at *cos* is asymmetrical, with a strong binding site on one side, and a possible weak binding site on the other side of *cos* (Becker and Murialdo, 1990). Earlier, on the basis of genetic data it was suggested that, after *cos* cutting, terminase stays bound to the strong binding site, protecting this end from entry by RecBCD (Feiss *et al.*, 1983; Kobayashi *et al.*, 1984). We show that this assumption is correct. We cut a cosmid containing two *cos* sites in inverted orientation and saw that the fragment bracketed by strong terminase binding sites is protected relative to the fragment bracketed by weak sites. The slow disappearance of the protected fragment (50% of what was detectable at 5 min is still present at 30 min) indicates that terminase leaves the strong binding site (exposing one of the ends to a nuclease attack) with a probability of ~25% in 25 min. The protection of one of the two DNA ends resulting from *in vivo* cutting of the phage P1 packaging site by phage-encoded protein(s) has also been reported (Sternberg and Coulby, 1987).

The differences in survival of protected fragments in two double-*cos* cosmids indicate that the rate of degradation from a double-stranded end in a *recA recD* background is quite uniform. Indeed, the relative survival of the protected fragment in these cosmids is proportional to the length of the complementary, unprotected fragment (which must be degraded by a nuclease to prevent the cutting at the second *cos*). The ratio of lengths of unprotected fragments for pK78/pK76 (Figure 2A) is 2.5/1.6 = 1.56, while the mean of the survival ratios of the protected fragments (Figure 2C) is 1.55.

χ sites increase the survival of linearized DNA in WT cells

Terminase protection of one end of a linearized cosmid allowed us to see the orientation-dependent effect of χ sites on survival of linearized DNA. χ sites in the active orientation to the unprotected end increase the survival of linearized DNA in WT cells while χ sites in the opposite orientation do not. Two χ sites are more effective than one, and the χ effect is absent in both *recBC* and *recD* mutants. This confirms a unique role of χ sites in the RecBCD recombinational pathway and corroborates earlier *in vivo* and *in vitro* observations that a RecBCD molecule has a <50% chance of 'seeing' a particular χ site (Yagil and Shtromas, 1985; Stahl *et al.*, 1990; Taylor and Smith, 1992). It also shows that terminase alone (without a prohead) prevents entry of nucleases through the end of linearized λ and cosmid DNA bearing a strong terminase binding site, and confirms that the χ–RecBCD interactions studied here are relevant to those seen in λ genetic crosses. Recently, *in vivo* protection of linear DNA from RecBCD degradation by χ sites in a different system was reported (Dabert *et al.*, 1992).

Several possibilities can account for the absence of a specific DNA fragment corresponding to products of degradation from *cos* to χ: (i) χ does not stop the degradation by RecBCD *in vivo* precisely; (ii) a product of discrete χ-dependent length is further degraded by some other enzyme(s); (iii) the predicted fragment loses its identity by becoming a part of a recombination intermediate. The 'further degradation' notion will be tested in *E.coli* strains deficient in several minor nucleases. On the other hand, the formation of branched structures, which could be recombinational intermediates, does happen, but with low yield (<1% of all the survived χ-containing cosmid DNA is in branched structures). However, in this study, we did not take special steps (cross-linking with psoralen, for example) to stabilize the branched structures during DNA purification and separation. Therefore, the apparent low yield cannot be taken as evidence against them being the major reason for the absence of the expected fragment.

In *recBC* and *recD* backgrounds, the survival values of >100% indicate an increase in amount of cosmid DNA after linearization. The experiments addressing this issue will be reported elsewhere.

Degradation of χ-containing DNA results in RecA-dependent inactivation of exoV

In the presence of linearized χ-containing DNA, a non-homologous χ-less cosmid exhibits the same level of survival as does the χ-containing cosmid, indicating that χ protects both *in cis* and *in trans*. Thus, the interaction of RecBCD with χ leads to temporary inactivation of its exoV activity. Transient χ-mediated loss of exoV activity by RecBCD enzyme was proposed, but it was tacitly assumed that RecBCD regains exoV activity as soon as it leaves the χ-containing molecule (Stahl *et al.*, 1990). In this respect, our observation that RecBCD molecules with disabled exoV

activity are not confined to the χ-containing DNA is somewhat unexpected. On the other hand, it is in agreement with the well established fact that exoV activity is inhibited in *E.coli* cells with damaged chromosomes (Pollard and Randal, 1973; Marsden *et al.*, 1974; Day, 1977; Dharmalingam and Goldberg, 1980; Thoms and Wackernagel, 1982; Kannan and Dharmalingam, 1990; Brcich-Kostich *et al.*, 1991). In these cells, RecBCD nuclease is exposed to chromosomal DNA which contains numerous χ sites (see below).

It should be noted, though, that in our experimental setting, we have not excluded the possibility that χ-modified RecBCD molecules may be simply trapped at the left end of the linearized cosmid DNA by the terminase complex bound there. One way to address this question is to see whether RecBCD enzyme modified by χ is able to promote recombination of other, χ-less molecules.

In vitro, exoV activity of RecBCD enzyme is down-modulated by χ-containing DNA in the absence of any other protein or in the presence of SSB (Taylor and Smith, 1992; Dixon and Kowalczykowski, 1993). In contrast, we found that strains deficient in RecA or SSB protein show no χ-dependent increase in survival of linear DNA *in vivo*. Our favored interpretation of these results is that the inactivation of exoV *in vivo* is effected by direct interactions of RecA (+SSB) with RecBCD. However, the possibility of such interactions is yet to be tested; moreover, the idea is in conflict with the *in vitro* observations mentioned earlier (Dixon and Kowalczykowski, 1993). *In vitro*, both RecA and SSB proteins inhibit nuclease activity of RecBCD enzyme on single-stranded DNA (MacKay and Linn, 1976; Williams *et al.*, 1981). *In vivo*, SSB and RecA proteins apparently produce the χ effect by reacting with χ-containing DNA that was acted upon by RecBCD nuclease. In fact, protection *in trans* can be explained if RecA filaments assembled on frayed ends of χ-containing molecules non-specifically capture other DNA molecules (Radding, 1988), protecting some of them from RecBCD degradation. Another plausible explanation for the RecA-dependence of the χ effect is inhibition of RecBCD nuclease by a protein whose induction is RecA-dependent (Kannan and Dharmalingam, 1990). The absence of DNA protection by χ sites in *recA* and *ssb* mutants is in agreement with the 'reckless' DNA degradation in these backgrounds after DNA damage (Willetts and Clark, 1969; Lieberman and Witkin, 1983; Whittier and Chase, 1983). In a different system, χ-dependent protection of linear DNA *in vivo* also depended on RecA (Dabert *et al.*, 1992).

χ-dependent RecA-dependent formation of branched structures

We showed that cosmid DNA linearization resulted in a RecA-dependent formation of branched structures only if the cosmid had χ sites. Y-shaped intermediates of plasmid replication in yeast form a continuous arc in 2D gels (Brewer *et al.*, 1988). In contrast, Y-structures formed as a result of plasmid linearization are represented by several discrete species (Figure 4C). The non-random distribution of the lengths of linear 'tails' in branched structures might be due to the particular lengths of the linear molecules that recombined with intact circles to form branched structures. This can be true only if, once formed, the branched structures do not immediately prime DNA synthesis, which otherwise would elongate their linear 'tails'. In agreement with this interpretation, χ-dependent survival in WT background is not lowered by inactivation of either of the two major components of the *E.coli* replication machinery, DnaB or DnaE (PolC) proteins.

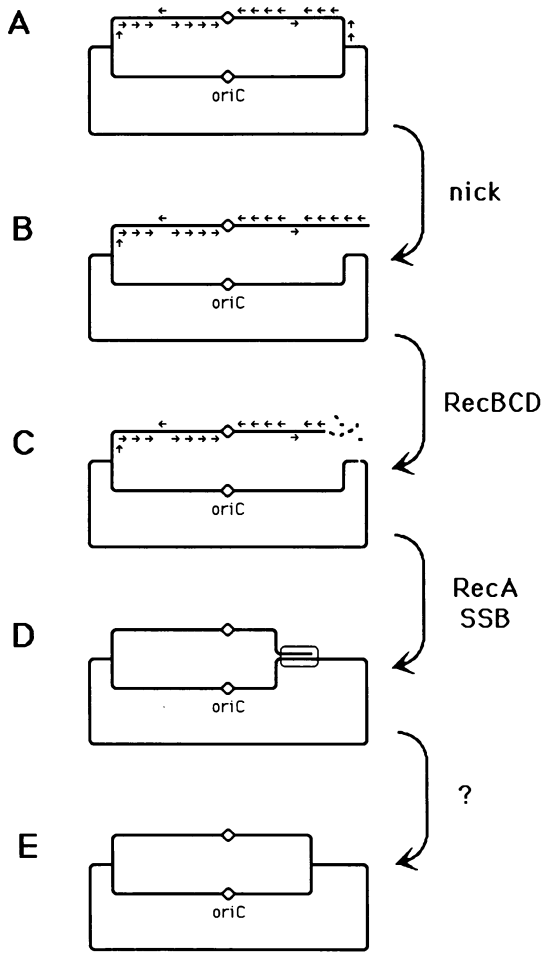

Fig. 8. A mechanism for the restoration of a collapsed replication fork in *E.coli* chromosome. (**A**) A replication intermediate of the *E.coli* chromosome (θ-structure) with the orientation of χ sites shown as little arrows along one of the branches. (**B**) One of the replication forks has run into a nick and collapsed. (**C**) RecBCD nuclease degrades the resulting double-strand end until it is inactivated at one of the numerous χ sites. (**D**) RecA and SSB proteins help the frayed end to invade the intact branch of θ-structure. (**E**) The intact θ-structure is restored. This stage may require DNA gyrase, DNA polymerase I and DNA ligase, all of which are needed for recombinant formation along the RecBCD pathway (Ennis *et al.*, 1987).

It should be noted that the reported experiments do not rule out the possibility that some of the branched species are pre-existing (or formed as a result of *cos*-cutting) replicating molecules protected, along with the linearized cosmid DNA, from RecBCD degradation by χ sites. Proof of the recombinational nature of the branched species requires a demonstration of their formation between two genetically distinct molecules. Also, whether χ sites are required for the frayed end to invade an intact homologous molecule is yet to be determined.

How RecBCD nuclease is used both for the degradation of foreign DNA and for the repair of chromosomal DNA

The RecBCD-dependent degradation of damaged chromosomal DNA in *E.coli* is so rapid that up to one-third of the

Table II. *Escherichia coli* strains used in this study

Strain designation	Relevant genotype[a]	Source or reference
AB1157	*rec*$^+$	Bachmann (1971)
JC10287	*Δ(srlR-recA)304*	Czonka and Clark (1979)
JC2926	*recA13*	Clark and Margulies (1965)
JC9941	*recA200*^{ts} [b]	Alvin J.Clark
JC5519	*recB21 recC22*	Willetts *et al.* (1969)
RDK1309	*ssb-113*	Kolodner *et al.* (1985)
BT125	*recD1011*[c]	W.Wackernagel
RDK1792	*recD1013*	Lovett *et al.* (1988)
DM49	*lexA3*	Mount *et al.* (1972)
AK9	*polA4109*^{ts}	this study[d]
AK12	*dnaE486*^{ts}	this study[e]
AK18	*dnaB22*^{ts}	this study[f]

[a]Other mutations: *thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1.*
[b]This strain is also *srlD50.*
[c]This strain is also *argE*$^+$.
[d]AB1157 transduced to Tc^r UV^r ts by P1 lysate from 837 (BT4109 (Olivera and Bonhoeffer, 1974) with *tet* 50% linked to the *polA*^{ts} mutation (Gordon Lark, personal communication)).
[e]CR34 derivative E486 (James A.Wechsler) was P1-transduced to Km^r grown on CAG18580 (*zae3095*::Tn*10* Kan) (Singer *et al.*, 1989). Then AB1157 was then transduced to ts-growth Kn^r with P1 grown on this strain.
[f]AB1157 was transduced to ts-growth Cm^r with P1 grown on FS1906 (*malB*::Tn*9 dnaB22*^{ts}) to yield AK14. The Cm^r phenotype of this strain was then changed to Kn^r by transduction with P1 grown on CAG12119 (*malE*::Tn*10* Kan) [Singer *et al.* (1989); William Walter, personal communication] to yield AK18.

whole genome may be converted to an acid-soluble form in less than an hour (Stuy, 1960; Clark *et al.*, 1966; Brcich-Kostich *et al.*, 1991). Only SOS-amplified levels of RecA protein save the chromosomal DNA from the complete degradation that is observed in *recA* or *lexA3* mutants (Willetts and Clark, 1969; Marsden *et al.*, 1974; Brcich-Kostich *et al.*, 1991). The apparent way to stop the RecBCD degradation early is to enrich the DNA with χ sites. The theoretical frequency of appearance of a χ site (or any other octanucleotide) is one per ~33 kb of double-stranded DNA for which G+C = A+T. By multiplying the genomic frequencies of trinucleotides which make up the χ octanucleotide one is able to calculate a statistical weight of χ sequence in a given genome. *E.coli* bacteriophages T4 and λ inactivate RecBCD with specific proteins. Although the calculated weights of χ sequence in T4 and λ (trinucleotide frequencies in their genomes were kindly provided by Samuel Karlin, Stanford University) are the same as the χ weight for *E.coli*, T4 has only three χ sites in its >180 kb genome, while λ (48.5 kb) has none. The *E.coli* chromosome, in contrast, has one χ site per 4−5 kb (Faulds *et al.*, 1979; Blaisdell *et al.*, 1993; Burland *et al.*, 1993).

A peculiar feature of χ site orientation throughout the *E.coli* genome makes the argument for their adaptive presence in the chromosomal DNA even stronger. Nine out of 10 χ sites in the *E.coli* chromosome are oriented such that they would protect against the degradation of DNA proceeding towards the origin of replication (Burland *et al.*, 1993; Medigue *et al.*, 1993). This non-random orientation of χ sites indicates that the majority of the DNA damage to the chromosome comes in the form of one double-strand end (oriented toward the origin), rather than a double-strand break. We propose that this double-strand end is a result of a replication fork running into a single-strand interruption in a template DNA. The following steps in the repair of the collapsed replication fork can be imagined on the basis of our findings (Figure 8): RecBCD degrades the double-strand end until it is inactivated by one of the properly oriented χ sites, the frayed end with the help of RecA and SSB proteins invades the intact homologous branch of the broken θ-structure, and the covalently connected replication fork is restored.

The progress made in recent years (Stahl *et al.*, 1990; Dixon and Kowalczykowski, 1991, 1993; Smith, 1991; Dabert *et al.*, 1992; Taylor and Smith, 1992; this work) offers a clue to how *E.coli* cells use RecBCD nuclease both for the degradation of foreign DNA and for the recombinational repair of its own chromosome. Linearized DNA that lacks χ sites is degraded by RecBCD. Linearized DNA with frequent χ sites inactivates exoV activity of RecBCD and 'seeks' an intact homologous chromosome to repair its damage via recombination. However, if the intact homologue is absent, the linearized DNA is eventually fully degraded.

Materials and methods

Bacterial strains and plasmids, media and growth conditions

E.coli strains, listed in Table II, are all derivatives of AB1157 (Bachmann, 1971). Cells were grown in M9 medium (Miller, 1972) supplemented with necessary amino acids to 20 μg/ml and vitamin B_1 to 10 μg/ml, or in LB medium or on LB plates. The media were supplemented when necessary with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, 10 μg/ml tetracycline, 20 μg/ml kanamycin or 100 μg/ml spectinomycin. Small-scale and preparative purification of plasmid DNA were as described (Birnboim, 1983). Nalidixic acid (Sigma) was prepared freshly before each experiment by dissolving 5 mg in 1 ml of 25 mM NaOH; the concentration of the drug in the medium was 50 μg/ml. *recA*, *lexA* and *ssb* mutants were confirmed by their characteristic sensitivities to UV. The absence of exoV activity was verified by the increased efficiency of plating of T4 *2*$^-$ (Oliver and Goldberg, 1977). The *recBC* mutants were verified as exoV$^-$ UV-sensitive strains; *recD* mutants were verified as exoV$^-$ UV-resistant.

Plasmid constructions

Standard techniques for plasmid construction were used (Maniatis *et al.*, 1982). pK2 was constructed by cloning the 322 bp *Pvu*II−*Pvu*II fragment with the polylinker from pUC18 into the *Pvu*II site of pBR322 so that the *Hin*dIII site of the polylinker is proximal to the *tet* gene. pK47 is a deletion derivative of pK2, missing 425 bp between the *Xma*III site and the distal *Pfl*MI site.

The *Pst*I−*Pst*I fragment (~450 bp) containing the λ *cos* site from

pMUA10 (Meyerowitz *et al.*, 1980) was inserted into the *Pst*I site of pSU19 ((Martinez *et al.*, 1988); Eduardo Martinez, personal communication) in both orientations to give pK11 and pK12. In pK11 the *lacZ'* gene is proximal to the right end of linearized *cos*. In pK12 the *cos* site has the opposite orientation. pK35 and pK36 were constructed from pK11 and pK12, respectively, by introducing into them the χ-containing 12 bp sequence:

5'-GCTGGTGGAGCT-3'
3'-TCGACGACCACC-5'

The fragment was inserted into the *Sac*I site of the polylinker so that the 3' end of the upper strand as written is proximal to *cos*. pK37 and pK38 were constructed from pK35 and pK36, respectively, by introducing into them the second χ-containing 12 bp sequence:

5'-CTAGGCTGGTGG-3'
3'-CGACCACCGATC-5'

This fragment was inserted into the *Xba*I site of the polylinker in the same orientation as the χ site at *Sac*I. The distance between the two χ sites is 21 nucleotides.

pK22 was constructed by inserting the *Sal*I−*Sal*I fragment (1400 bp) from pK2 into the *Sal*I site of pK12 so that the 'polylinker end' of the fragment is proximal to the *cos*. pK48 was constructed by insertion of the *cos*-containing *Sph*I−*Sph*I fragment (~460 bp) from pK22 into the *Sph*I site of pMTL22 (Chambers *et al.*, 1988) so that the right end of the open *cos* is proximal to the *Sma*I site of the polylinker. pK50 was constructed from pK48 by deleting ~160 bp between the *Sma*I site of the polylinker and the *Bsa*BI site near the right end of the open *cos*.

pK41−pK46 plasmids were constructed by replacing the *Pst*I−*Pst*I *cos*-containing fragment (~450 bp) in pK11, pK12 and pK35−pK38, respectively, with the *Pst*I−*Pst*I *cos*-containing fragment (~250 bp) from pK50 in the same orientation.

pK51−pK56 plasmids were constructed by inserting the *Sal*I−*Sal*I fragment (~1100 bp) from pK47 into the *Sal*I site of pK41−pK46 plasmids so that the 'polylinker end' of the fragment is proximal to *cos*.

pK57 was constructed by inserting the *Hin*dIII−*Sma*I fragment (~1300 bp) containing *neo* gene from pRLM5 (Wold *et al.*, 1982) into pMTL24 (Chambers *et al.*, 1988) at the *Hin*dIII and the *Mlu*I sites (the latter made blunt with mung bean nuclease).

pK59 was constructed by deleting the *cos*-containing *Pst*I−*Pst*I fragment (~250 bp) from pK51.

pK64 and pK65 were constructed by substituting the *Eco*RI−*Hin*dIII fragment of the polylinker in pGB2 (Churchward *et al.*, 1984) for the *Eco*RI−*Hin*dIII *cos*-containing fragments from pK11 and pK37, respectively.

pK76 was assembled by ligating together *Bam*HI-cut pSU19 and the *Eco*RI−*Eco*RI fragment from pK57 (~1400 bp) containing the *neo* gene with the aid of two *Eco*RI−*Bgl*II fragments (~250 bp) from pK50 containing the *cos* site of λ. pK78 was assembled similarly by ligating together *Eco*RI-cut pSU19 and the *Bam*HI−*Bam*HI fragment from pK57 (~1400 bp) containing the *neo* gene with the aid of the two *Eco*RI−*Bgl*II fragments (~250 bp) from pK50 containing the *cos* site of λ.

Measurements of DNA synthesis

The rate of DNA synthesis was determined as described (Drlica *et al.*, 1980). Cells were grown in supplemented minimal M9 medium. 0.2 ml of exponentially growing bacterial cultures (2×10^8 cells/ml) were transferred to tubes containing 1 μCi of [^{3}H]thymidine (sp. act. 67 Ci/mmol) and incubated at 37°C (44°C for *dna*^{ts} strains) for 2 min. Incorporation of radioactivity was terminated by addition of 2 ml of cold 5% (w/v) trichloroacetic acid. Acid precipitates were collected on Whatman GF/A filters, washed with cold 5% trichloroacetic acid, then with 95% ethanol, and then dried. Acid-precipitable radioactivity measured by scintillation counting (Beckman LS7500) was taken as a measure of the rate of DNA synthesis.

Quick preparation of total DNA

Competent cells of a strain transformed with pCM101 were prepared by suspending them in 50 mM $CaCl_2$; after addition of 0.2 vol. of glycerol, they were stored at −70°C. For each experiment these cells were freshly transformed with the appropriate cosmid and plated on LB plates supplemented with ampicillin and chloramphenicol. After 24 h incubation at 28°C, a single colony was picked to inoculate 3 ml of LB supplemented with the same antibiotics. Cultures were grown at 24°C with shaking to a density of $1-2 \times 10^8$ cells/ml. A 1 ml aliquot was withdrawn (zero point) while the rest of the culture was poured into a 150 ml flask prewarmed to 44°C and incubated with shaking. At 6 min, a second 1 ml aliquot of the culture was withdrawn. Total DNA from the 0 and 6 min points was purified as follows: cells were spun down in an Eppendorf centrifuge for 1 min, resuspended in 100 μl of 1% SDS in TE buffer and lysed by addition of 120 μl of phenol and vigorous vortexing. After centrifugation of the lysate and removal of the phenol phase, the aqueous phase was extracted once with an equal volume of phenol/chloroform (1:1) and once with chloroform. Alternatively, after the cells had been resuspended in 50 μl of 1% SDS in TE buffer, they were lysed by addition of 50 μl of 10% SDS. Proteinase K was added in 150 μl of 50 mM Tris−acetate pH 8.0 to a final concentration of 500 μg/ml, and the tubes were incubated at 65°C for 1.5 h. One-third of the total DNA preparation (0.5−1.0 μg) was usually used for each electrophoretic run.

For 2D gel electrophoresis, either method of DNA preparation was scaled up 10-fold, and the DNA was further purified by three ethanol precipitations. After being cut twice with restriction endonuclease *Nco*I, the DNA was used completely in a single 2D run.

Electrophoresis and blot-hybridization

One-dimensional electrophoresis was in 1.1% agarose gel in Tris−acetate (TAE) buffer, at 1.5 V/cm. For 2D agarose gel electrophoresis, the first dimension was in 0.7% agarose in Tris−borate (TBE) buffer, at 1.5 V/cm. The second dimension was perpendicular to the first one, 1.2% agarose in TBE buffer, at 6−7 V/cm. No ethidium bromide was added to the electrophoretic buffer. After completion of electrophoresis, gels were soaked for 40 min in 0.2 M HCl, then for 50 min in 0.5 M NaOH, then for 20 min in 1 M Tris−HCl pH 7.0. DNA was then vacuum-transferred to a positively charged nylon membrane (Zetabind, CUNO) in distilled water and was covalently linked to the membrane by UV light (using a UV-crosslinker-1000, Fisher Scientific). Radioactive probes (*Eco*RI-linearized DNAs of pK22 or pGB2, unless otherwise stated) were prepared by random primer labeling. Prehybridization and hybridization were at 65°C in 5% SDS, 500 mM sodium phosphate, pH 7.0. After hybridization the membrane was washed three times for 10 min each in distilled water at ambient temperature, dried and autoradiographed. Direct radioactivity scanning of the membrane was by AMBIS.

Quantification of linearized DNA survival using AMBIS

Each sample was represented by two lanes—0 min and *x* min of incubation at 44°C—that were compared pairwise. In the *x* min lane, linearized cosmid DNA and products of degradation were quantified separately from uncut supercoiled and relaxed circular DNA. In the 0 min lane total cosmid DNA was quantified. The amount of cosmid DNA linearized after heat-induction of terminase was calculated by subtracting total supercoiled and relaxed circular DNA at *x* min from total cosmid DNA at 0 min. 'Degree of survival' was calculated as the total amount of detectable linear DNA plus products of degradation divided by total cosmid DNA linearized. It is presented as the percent of linear cosmid DNA that was still detectable after *x* min of incubation at 44°C.

Preparation of DNA for electron microscopy

Total cosmid DNA substantially free of chromosomal DNA was purified by a modified cleared lysate technique (Clewell and Helinski, 1969). Each small freshly transformed colony was inoculated into 2 ml of LB supplemented with ampicillin and chloramphenicol and grown overnight at 24°C. After 12 h, the entire 2 ml culture was added to 400 ml LB with ampicillin and chloramphenicol and grown at 24°C to a cell density of 1×10^8/ml. The culture was then heated in a boiling water bath until its temperature reached 44°C, transferred to a 44°C bath and incubated for 3 min. Cells were pelleted (15 000 *g*, 5 min) and resuspended in 4 ml of 100 mM Tris−acetate pH 8.0, 10 mM EDTA. 1 ml of 2 mg/ml lysozyme in 100 mM Tris−acetate pH 8.0, 50 mM EDTA was added and cells were incubated for 1.5 min at room temperature. Cells were lysed by addition of 10 ml of 2% Triton X-100 in 10 mM EDTA followed by gentle inversion of the tube; the lysate was centrifuged (15 000 *g*, 20 min). The resulting cleared lysate was cautiously decanted, made 0.5% with SDS and 500 μg/ml with proteinase K and incubated at 65°C for 16 h. Then it was made 200 mM in NaCl, and nucleic acids were precipitated with ethanol and pelleted by centrifugation (13 000 *g*, 10 min). The nucleic acid pellets were gently resuspended in 200 μl of TE buffer, 300 μl of 6 M LiCl were added (Birnboim, 1983), and the contents of the tubes were mixed by vortexing. After 20 min on ice, polysaccharides and RNA were pelleted by centrifugation in an Eppendorf microcentrifuge (13 000 r.p.m., 2 min), supernatants were transferred to fresh tubes, and DNA was pelleted after addition of 900 μl of ethanol. DNA was reprecipitated with ethanol before cutting with restriction endonucleases. After hydrolysis with a restriction endonuclease, half of the DNA was run through standard 2D gel electrophoresis, gels were stained with ethidium bromide, and pieces of agarose were cut from positions of interest. The DNA was extracted by electroelution in 0.2 × TBE buffer on dialysis membrane (electroelution apparatus was from ISCO), concentrated two or three times under vacuum, precipitated with ethanol, dried and dissolved in TE buffer (10 mM Tris−HCl pH 8.0, 1 mM EDTA).

Electron microscopy

The grids with DNA were prepared by a modified droplet technique (Lang and Mitani, 1970). A 20 μl DNA mixture containing 0.07 mg/ml cytochrome *c*, 140 mM Tris−HCl pH 8.5, 14 mM EDTA, 250 mM NH_4Ac and 10 μg/ml ethidium bromide was incubated at room temperature for 30−60 min. The mixture was transferred as a droplet to a piece of Parafilm, and a protein film was allowed to form for 0.5−2 h. The electron microscopic grid coated with Parlodion and carbon was then touched to the surface of the droplet, agitated for 1 min in 50 μM uranyl acetate in 86% ethanol, washed in 86% ethanol, dried, rotary-shadowed with platinum and examined in a Philips CM12 electron microscope at 60 kV.

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