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The role of *oriT* in *tra*-dependent enhanced recombination between mini-F-*lac*-*oriT* and λp*lac5*

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Summary

Recombination between F42*lac* and λp*lac5* is typically 20- to 50-fold more efficient than recombination between chromosomal *lac* and λp*lac5*. This enhancement of recombination is *recBCD*-dependent and requires the expression of genes from the *tra* regulon of the F factor. Also required is *oriT*, the origin of F factor conjugational transfer, which must be located in-*cis* to the cellular copy of *lac*. In this study we show that enhanced recombination is not supported by an *oriT* point mutant that reduces *oriT* function in conjugation. We also present evidence that the activation of *oriT* for recombination enhancement involves the same strand-specific nick that is required for conjugal DNA transfer. Although it is thought that the role of *oriT* in recombination enhancement is related to the facilitated entry of RecBCD enzyme into the DNA duplex, we were unable to detect any double-strand breakage at *oriT*.

1. Introduction

F42*lac* × λp*lac5* transductional crosses typically demonstrate levels of recombination 20- to 50-fold higher than levels seen in λp*lac5* × chromosomal *lac* crosses (Porter *et al.* 1978, 1981). This enhanced transductional recombination requires constitutive expression of *traY* and *traI* from the F factor transfer (*tra*) operon (Carter & Porter, 1991), and the presence of *oriT*, the origin of F conjugal transfer, on the same plasmid as the resident *lac* gene copy that will undergo recombination with λp*lac5* (Seifert & Porter, 1984). Additional requirements include a functional *recA* gene product (Porter *et al.* 1978, 1981) and a functional RecBCD enzyme (Porter *et al.* 1978, 1982). Enhancement does not occur in strains that are dependent on the RecE or RecF recombination pathways (Porter *et al.* 1978; Porter, 1983).

Enhanced recombination between two different *lac* alleles has been monitored by two assays. In the first assay, viable Lac$^+$ transductants are scored as a measure of those recombination events which proceeded to completion. The second assay measures β-galactosidase enzyme units per colony-forming unit as an indication of recombination events which proceeded to a stage where a wild-type sequence of *lacZ* could be transcribed and translated; i.e. a transcribable intermediate (Birge & Low, 1974). Enhancement of recombination typically results in 20- to 50-fold elevation of β-galactosidase level, while the increase in Lac$^+$ transductants is usually less dramatic.

In this report, we have further investigated the role of *oriT* in recombination enhancement. Evidence is presented that the *tra*-mediated nick at *oriT* is instrumental in allowing enhancement to occur. It has been shown that an *oriT* point mutant that is not believed to be nicked in a *tra*-dependent manner demonstrates both a markedly reduced ability to promote conjugal DNA transfer and a lack of ability to support recombination enhancement. We also show that the expected *tra*-dependent strand-specific nick at *oriT* occurs at high frequency under conditions associated with recombination enhancement. It has been hypothesized that the *cis*-acting role of *oriT* in recombination enhancement is to permit entry of the RecBCD enzyme into the *lac*-containing DNA molecule in the recipient cell (Seifert and Porter, 1984). As entry of the RecBCD enzyme into duplex DNA *in vitro* requires flush or nearly flush double-strand ends (Taylor & Smith, 1985), we also explored the possibility that the nick at *oriT* is converted to a double-strand break. We have been unable to find any evidence for the occurrence of such a double-strand break.

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Table 1. *Strains of* Escherichia coli

Strain	Relevant genotype and plasmids	Source/reference
AB2463	F⁻ *thr1 leuB6 argE3 his4 proA2 rpsL recA13 thi*	Bachmann (1972)
KL528	F⁻ Δ(*lac-pro*) *supF trp pyrE his rps thi*	Porter *et al.* (1981)
KL695	F⁻ Δ(*lac-pro*) Δ(*tonB*) *rpsL* (*ϕ*80d*lacI3*)	Porter (1982)
KL791	F⁻ Δ(*lac-pro*) *met his trp rpsL thi* (*λind*)	Porter (1981)
KL765	F⁻ *lacZ813 lacI3 pro met his trp rpsL thi* (*λind*)	Porter *et al.* (1981)
RDP102	F⁻ Δ(*lac-pro*) *lev th*: *acrA*(?)*sup E44* (*λc*I857 *Sam* 7p *lac 5I⁻Z118Y⁻*)	Porter *et al.* (1981)
RDP146	F⁻ Δ(*lac-pro*) *recA1 rpsE thi λ⁻*	Yancey & Porter (1985)
RDP195	KL791 (pRPZ118)	Seifert & Porter (1984)
RDP196	RDP195 (pRPZ117)	Seifert & Porter (1984)
RDP206	RDP146 (pRPZ121)	Seifert (1984)
RDP312	KL791 (pRPZ147)	This work
RDP313	KL791 (pRPZ117, pRPZ147)	This work
RDP314	RDP206 (pRPZ144)	This work
RDP315	RDP206 (pRPZ145)	This work

Table 2. *Plasmids*

Plasmid	Parent replicon	Relevant *tra* genes	Useful markers	Source/reference
pMF3	F	None	Ap^r	Manis & Kline (1977)
pRPZ114	R1	None	Km^r	Seifert & Porter (1984)
pRPZ117	pRPZ114	*JYALEKBPVSTDI(Z)*	Km^r	Seifert & Porter (1984)
pRPZ118	pMF3	*oriT*	Lac, Ap^r	Seifert & Porter (1984)
pRPZ121	pSC101	all	Km^r	Seifert (1984)
pRPZ144	pRPZ118	*oriT*	Ap^r	This work
pRPZ145	pRPZ144	*oriT* (mutant)	Ap^r	This work
pRPZ147	pRPZ145	*oriT* (mutant)	Lac, Ap^r	This work
pRPZ148	pUC8	*oriT*	Ap^r	This work

2. Materials and methods

(i) *Bacterial strains, phages and plasmids*

The *Escherichia coli* K-12 strains used herein are listed in Table 1. A *lacZ118* version of λp*lac5* was prepared by heat induction of RDP102. Phage titers were done with KL528 as previously described (Porter *et al.* 1981). All plasmids used in this study are listed in Table 2.

(ii) *Cell growth and transformation*

Cells were routinely grown in LB medium (Miller, 1972). Transformations were performed according to the method of Kushner (1978), or by a laboratory protocol for preparing and transforming frozen competent cells. Transformed cells were plated onto LB agar supplemented with the appropriate antibiotic(s). The antibiotic concentrations used for counterselection or plasmid selection and maintenance were as follows: streptomycin at 100 μg ml^{-1}; ampicillin and neomycin/kanamycin at 50 μg ml^{-1}.

(iii) *Plasmid DNA extraction and purification*

Large-scale plasmid extractions were performed by the alkaline-SDS method of Birnboim & Doly (1979). Plasmid DNA extracts generally were subjected to one or two rounds of CsCl-ethidium bromide centrifugation. Ethidium bromide was extracted with NaCl-saturated isopropanol and the DNA-containing fractions were dialysed extensively against TE (10 mM-Tris, pH 8, 1 mM-EDTA) at 4 °C. Small scale extractions were performed using a scaled-down version of the alkaline-SDS lysis method.

(iv) *Total cellular DNA isolation*

Cells were grown to mid-exponential phase in minimal medium 56/2 (Low, 1973) supplemented as previously described (Porter, 1981). After centrifugation, cell pellets were resuspended in 50 mM-Tris pH 8 containing 25% sucrose; 0·4 volumes of 0·25 M-EDTA was then added. After a 5 min incubation on ice, egg white lysozyme, RNase and SDS were added to final concentrations of 0·5 mg ml^{-1}, 0·5 μg ml^{-1} and 1·0%, respectively. After 2 h on ice, a fresh solution of proteinase K was added to a final concentration of 100 μg ml^{-1}. After overnight incubation at 37 °C, the lysate was phenol/chloroform extracted several times and processed through one 55% (w/v) CsCl gradient. DNA samples from these gradients were dialysed against TE.

(v) In vitro *DNA manipulations and electrophoresis*

Restriction enzyme digestions, ligations and agarose gel electrophoresis were done as previously described (Carter & Porter, 1991).

(vi) *Plasmid constructions*

The mini-F-*oriT* plasmid, pRPZ144, was constructed by deleting the 27-kb *lac*-containing *Hin*d III fragment from the mini-F-*lac-oriT* plasmid pRPZ118. To construct a version of pRPZ144 which contained a mutant *oriT*, the 529 bp *oriT*-containing *Bgl* II-*Sal* I fragment of pRPZ144 was replaced with the corresponding fragment of pED833 (Everett & Willetts, 1982), which contains an *oriT* point mutation. This replacement generated pRPZ145. To create an *oriT* mutant plasmid analogous to pRPZ118, the original 27-kb *lac*-containing *Hin*d III DNA fragment was inserted into pRPZ145 in the same orientation as in pRPZ118 to create pRPZ147. pRPZ148 was constructed by cloning the 529 bp *oriT*-containing *Bgl* II-*Sal* I fragment from pRPZ144 into pUC8 that had been digested with *Bam*H I and *Sal* I.

(vii) *Recombination/β-galactosidase assays*

Recombination assays were performed as previously described (Porter, 1981). Briefly, cells containing a *lacI3 lacZ813* version of the appropriate *lac*-containing plasmid were grown exponentially in supplemented 56/2 minimal salts medium at 37 °C and infected with λ*placZ118* at a MOI of 5. Samples were taken 1 h after infection and plated onto minimal lactose plates fro viable Lac^+ transductants and onto LB for total colony-forming units. Three hours after infection, samples were taken to assay for β-galactosidase enzyme units and colony-forming units. One enzyme unit is the amount of enzyme activity needed to hydrolyse 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 1 min at 28 °C (Birge & Low, 1974).

(viii) *Southern blotting*

Blotting of agarose gels (Southern, 1975) onto Gene Screen nylon membrane (New England Nuclear) was performed according to Maniatis *et al.* (1982); prehybridization and hybridization of the blot were done according to the Gene Screen instructions provided by the manufacturer. Blots were probed with a 529-bp DNA fragment containing *oriT* that had been radiolabeled using the Random Primed DNA Labeling kit from Boehringer Mannheim Biochemicals and 50 μCi of [α-^{32}P]dATP (3000 Ci mmol^{-1}, 50 μCi μl^{-1}) purchased from New England Nuclear.

(ix) *Primer extensions and DNA sequencing*

For the primer extension reactions, total genomic DNA from RDP195 or RDP196 was first digested with either *Dra* I or *Bgl* II. Aliquots of these digests were removed to 1·5 ml screw-cap tubes to create a series of tubes having two-fold increases in total DNA; the absolute amount of DNA added to each tube was not determined. The primers used were primer A:

5′CAGGCAGATGGCTAACATCC3′

and primer B:

5′CAATAAAGAGAGTAAGAGAAAC3′.

Approximately 50 nmol of primer, kinased in the presence of [γ-^{32}P]ATP, was added to each tube. After precipitation and resuspension in 5 × Sequenase reaction buffer, the tubes were incubated in a 95–98 °C water bath for 30 min. After slow cooling to the desired annealing temperature (58 °C for primer A and 50 °C for primer B), the annealing of primers was allowed to continue for at least 8 h. After annealing, 6 μl of reaction mix (Sequenase enzyme from United States Biochemical Corporation plus dATP, dCTP, dGTP, and dTTP, each at 1 mM, and DTT at 20 mM) was added to 10 μl of annealed primer and template, and the reactions were incubated at 45 °C for 30 min; reactions were stopped by incubation at 90 °C for 15 min. After further processing, the reaction products were resuspended in Sequenase stop solution diluted by 60% with H_2O. Each primer extension sample was incubated in a boiling water bath for several minutes before gel loading.

For reference purposes, sequencing ladders of pRPZ148 (pUC8*oriT*) primed with primers A or B were generated using Sequenase by the dideoxy chain termination method (Sanger *et al.* 1977) on denatured double-strand plasmid DNA (Hattori & Sakaki, 1986). The primer extension and DNA sequencing reactions were electrophoresed through a 6% polyacrylamide gel (acrylamide ratio of 19:1 and made using 1 × TBE with 8 M urea) at 55 W of constant power for approximately 2·5 h. Following electrophoresis, the gel was transferred to a piece of Whatman 3 mm paper and dried under vacuum at 80 °C. The dried gel was then incubated next to Kodak X-Omat AR 5 film for between 6 h and 3 days.

3. Results

tra-dependent recombination enhancement requires that *oriT* be located in-*cis* to the cellular copy of *lac* (Seifert and Porter, 1984) and that the TraY and TraI proteins act in-*trans* (Carter & Porter, 1991). The fact that TraY and TraI are believed to constitute an *oriT*-specific nicking complex (Everett & Willetts, 1980; Traxler & Minkley, 1987) is consistent with a model wherein the *tra*-mediated nick at *oriT* required for F

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Table 3. *Recombination assays with mini-F-lac-*oriT *plasmids*

Strain	Relevant plasmids	Relative Lac$^+$ transductants	Relative EUs
RDP195	pRPZ118	1·0	1·0
RDP196	pRPZ118 & pRPZ117	9·9	17·8
RDP312	pRPZ147	0·8	1·2
RDP313	pRPZ147 & pRPZ117	1·1	1·4

Strains RDP195 and RDP196 contain the mini-F-*lac-oriT* plasmid pRPZ118 (Seifert & Porter, 1984). RDP196 also contains pRPZ117, which provides all of the *tra* genes required for enhancement (Seifert & Porter, 1984). Strains RDP312 and RDP313 contain the mini-F-*lac-oriT*$^-$ plasmid pRPZ147. RDP313 also contains pRPZ117. Recombination assays involve infecting each strain with *λcI857Sam7placZ118* at a multiplicity of five followed by platings for Lac$^+$ recombinants and assays for β-galactosidase activity as described in Materials and methods. EU refers to β-galactosidase enzyme units. Values for all stains have been normalized to those for RDP195; the actual values for RDP195 are $6{\cdot}4 \pm 0{\cdot}4$ Lac$^+$ transductants per 10^4 c.f.u. and $5{\cdot}7 \pm 1{\cdot}6$ EU per 10^{10} c.f.u. The ratio of the standard deviation to the average for RDP195 is typical of each of the other strains represented. Each average is based on data obtained from three experiments.

Table 4. *Conjugation properties of mini-F-*oriT *plasmids*

Donor strain	*oriT* plasmid	Transconjugants per recipient cell	
		Km^r ($\times 10$)	Ap^r ($\times 10^4$)
RDP314	pRPZ144	$2{\cdot}3 \pm 0{\cdot}6$	340 ± 140
RDP315	pRPZ145	$2{\cdot}1 \pm 0{\cdot}8$	$3 \pm 1{\cdot}3$

The recipient strain in these experiments was AB2463; all donor and recipient strains are *recA*$^-$. Donor and recipient strains were grown at 37 °C with vigorous aeration in LB media to a density of about $1{\cdot}5 \times 10^8$ cells per ml. Cells were mixed at a donor to recipient ratio of 1:5, and 37 °C incubation with moderate aeration was continued for an additional 30 min. Conjugal transfer of the pRPZ121 helper plasmid (pSC101-*tra*) was determined by interrupted platings (Low & Wood, 1965) on LB agar plates supplemented with kanamycin and streptomycin; transfer of the two mini-F-*oriT* plasmids was monitored by platings on LB agar plates supplemented with ampicillin and streptomycin. Total recipient cells present were determined by platings on LB plates supplemented with streptomycin. The numbers shown are based on data from three experiments.

factor conjugal DNA transfer is also required for recombination enhancement. To test this theory, a point mutant of *oriT* that is not subject to appreciable *tra*-dependent nicking (Everett & Willetts, 1982) was tested for the ability to support enhancement. This *oriT* point mutant from pED833 (Everett & Willetts, 1982) was used to create the mini-F-*oriT*$^-$ plasmid pRPZ147 (see Materials and Methods).

The results of the recombination assays involving pRPZ147 are presented in Table 3. RDP195 is the negative control strain which contains only the original mini-F-*lac-oriT* plasmid, pRPZ118. RDP196 is the positive control strain that contains pRPZ118 and pRPZ117, a plasmid which provides all of the *trans*-acting *tra* genes required for enhancement (Seifert & Porter, 1984). RDP312 contains only pRPZ147 (mini-F-*lac-oriT*$^-$) while RDP313 contains both pRPZ147 and pRPZ117. The recombination levels obtained with RDP313 therefore provide an indication of the degree to which the point mutant of *oriT* is able to support enhancement. From the data shown in Table 3, it is evident that this mutant of *oriT* totally fails to support enhancement.

The *oriT* point mutant was also tested for its in-*cis* ability to permit conjugal DNA transfer; these data are presented in Table 4. It can be seen from this data that the mini-F plasmid carrying the *oriT* point mutant transfers approximately 60-fold less efficiently than the wild-type *oriT* analog. Thus, this *oriT* mutation results in a deficiency with regard to both its ability to support enhancement and its ability to promote conjugal transfer.

In the working model for recombination enhancement (Seifert & Porter, 1984; Carter & Porter, 1991),

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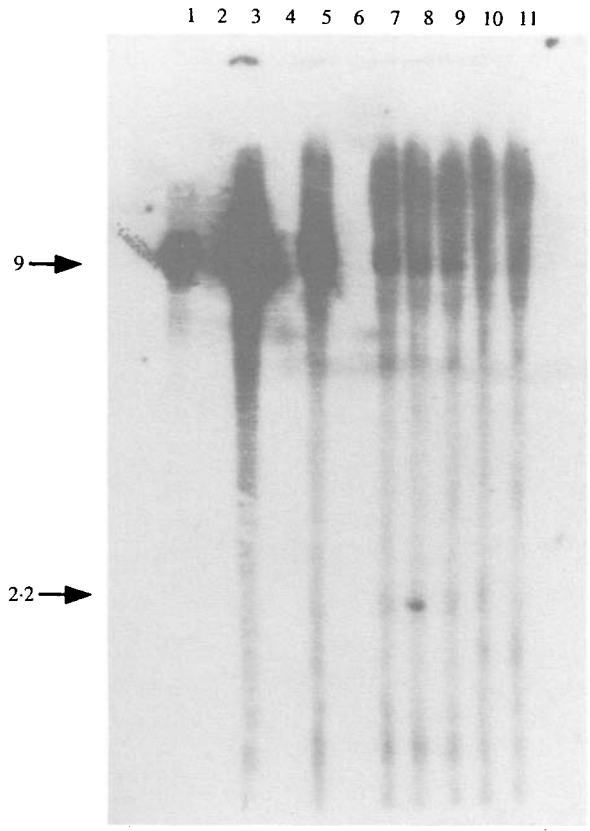

Fig. 1. *oriT* probing of total DNA from RDP195 and RDP196. Total DNA from each strain was digested with *Pst* I. The DNA from RDP196 was diluted incrementally before loading onto the gel. Dilutions lower than tenfold were mixed with *Pst* I digested total DNA from KL791 before loading. The blot was probed with a radiolabelled 529 bp *oriT*-containing fragment from pRPZ148. The lanes are labelled as follows: lane 1, RDP195, DNA; lane 2, empty; lane 3, undiluted RDP196 DNA; lane 4, empty; lane 5, 1/10 diluted RDP196 DNA; lane 6, empty; lane 7 1/100 diluted RDP196 DNA; lane 8, 1/250 diluted RDP196 DNA; lane 9. 1/500 diluted RDP196 DNA; lane 10. 1/1000 diluted RDP196 DNA; lane 11, 1/2000 diluted RDP196 DNA. Positions corresponding to 9 and 2·2 kb are indicated. The actual amount of DNA loaded per lane was not determined.

nicking at *oriT* leads to facilitated entry of the RecBCD enzyme into the DNA duplex; the RecBCD enzyme then can process to the *lac* region in helicase mode. The most economical version of this model is that RecBCD enters the duplex directly at *oriT*. A large body of *in vitro* data has been accumulated which demonstrates that the helicase activity of the RecBCD enzyme requires entry at flush ends or small overhangs, whereas nicked, gapped or covalently closed circles are essentially refractory to this enzyme (Taylor & Smith, 1985; see Smith, 1988 for review). We therefore carried out experiments designed to detect a double-strand break at *oriT* that occurs under conditions permitting recombination enhancement.

Total cellular DNA was isolated from RDP195 and RDP196 in order to permit the use of Southern blot hybridization to probe for a *tra*-dependent break at *oriT*. RDP195 contains the mini-F-*lac*-*oriT* plasmid pRPZ118, the plasmid which undergoes recombination with λ*plac5*. This strain typically is used as the negative control strain in recombination enhancement experiments. RDP196 contains pRPZ118 and pRPZ117, a plasmid which provides all of the *tra* genes required in-*trans* for enhancement (Seifert & Porter, 1984). To verify that the cells from which DNA was purified were behaving as expected, a portion of each cell culture was used in a recombination assay. As expected, in both the β-galactosidase enzyme assay and the Lac^+ c.f.u. assay, only RDP196 demonstrated enhanced levels of recombination (data not shown). Everett & Willetts (1980) have reported that the strand-specific nick occurs at *oriT* at a frequency of 5–15% in a λ*oriT* nicking assay, and it was deemed necessary to be able to detect a double-strand break that occurs at a frequency much lower than that observed for nicking. Additionally, the level of Lac^+ recombinants per c.f.u. generally varies over a range of 1 per 160 (see Table 3) to 1 per 25 (Carter & Porter, 1991), so detection limits need to exceed 1 break per 160 molecules.

A *Pst* I digest of total DNA from RDP195 and RDP196 produces a 9 kb *oriT*-containing DNA fragment from pRPZ118 (Wehlmann & Eichenlaub, 1980) that would give bands of approximately 6·8 and 2·2 kb as the result of a double-strand break at *oriT*. The actual Southern blot experiment involved dilutions of *Pst* I-treated DNA from RDP196. To provide consistency in sample handling and in background due to non-specific hybridization, dilutions of greater than 10-fold were mixed with *Pst* I-treated genomic DNA from KL791, the parent strain of RDP195. The blot thus generated was hybridized with a radiolabelled *oriT* probe; this blot is shown in Fig. 1.

As the 9 to 6·8 kb range showed smearing at the higher concentrations of RDP196 DNA, we have used the anticipated 2·2 kb *oriT* breakage product as being indicative of breakage. This hypothetical 2·2 kb band is not present in the lane with undiluted RDP196 DNA (lane 3 in Fig. 1) while we can clearly detect the 9 kb band up to a dilution factor of 500 (lane 9 in Fig. 1). Thus, it is concluded that a double-strand break does not occur at or near *oriT* at a frequency of greater than 1 in 500.

To confirm that the documented strand-specific nick at *oriT* can be detected in the same RDP196 DNA sample that was used in the attempt to detect a double-strand break two oligonucleotide primers were obtained that allow priming of DNA synthesis past the reported *oriT* nick site (Thompson *et al.* 1989). Primer A is a 20-base molecule designed to prime the strand of pRPZ118 DNA in which the nick was expected (the 'nicked' strand). As the 3′ end of this primer anneals 88 bases from the reported nick site, extension of this primer on *Dra* I digested pRPZ118 DNA was expected to yield a 108-base DNA extension product if *oriT* is nicked or a discrete DNA product of

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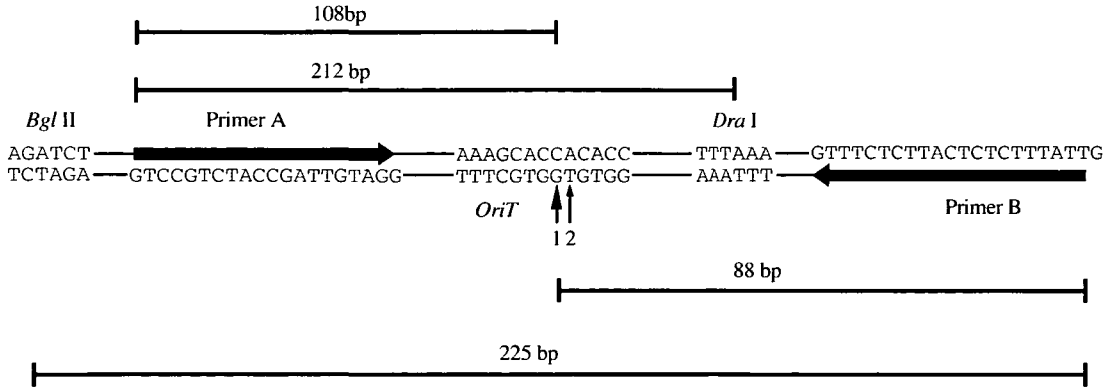

Fig. 2. Schematic representation of the primer extension experiment. The *Dra* I and *Bgl* II sites are as indicated. The sequences of primer A and primer B are depicted as long horizontal arrows which are the complement of the sequences to which these primers are shown to bind. Distances between the beginning of the primers and *oriT* or a relevant restriction site are shown; the diagram is not drawn to scale. The arrows indicate the 3′ end of the primers, and thus the direction of the primer extension. The sequence near *oriT* is shown, and the large arrow points to the base that is immediately 5′ to the *oriT* nick as determined from the data shown in Fig. 3 and as reported by Thompson *et al.* (1989).

212 bases ending at the *Dra*-I site if there is no nick at *oriT*. Primer B is a 21-base oligonucleotide used to prime the 'unnicked' strand of pRPZ118. The 3′ end of this primer is homologous to a region 67 bases from the base pair opposite the reported nick at *oriT* and 204 bp from a *Bgl* II site. Thus, primer B extensions on *Bgl* II digested pRPZ118 were expected to yield DNA fragments of 88 bases (nicked) and/or 225 bases (unnicked). The relationships among *oriT*, the two primers and the two relevant restriction sites are diagrammed in Fig. 2.

The actual primer extension results are presented in Fig. 3. Beside each set of primer extensions is a set of four sequencing reactions in which the plasmid pRPZ148 (pUC8*oriT*) was primed with the same primer that was employed in the corresponding extension reactions. These sequencing ladders served as size standards and allowed a direct identification of the base pair corresponding to the 3′ end of each extension product.

The unnicked template molecules from RDP195 (a negative control strain that contains no *tra* plasmids and does not demonstrate enhanced recombination) and RDP196 produce bands of the predicted sizes and whose 3′ ends correspond to the cleavage site of *Bgl* II or *Dra* I. Primer A extensions with total DNA from RDP196 produce two products corresponding to nicked molecules which differ in size by one base; the one-base-larger minor product is most likely a Sequenase artifact (see Discussion). Since these nick-associated products do not occur when primer A is used with RDP195 DNA, these species are *tra*-dependent. The absence of a product in the vicinity of 88 bases in the lanes with RDP196 DNA primed with primer B demonstrates that the 'unnicked' strand of *oriT* is not disrupted. It should be noted that the *Bgl* II or *Dra* I runoff products in the negative control lanes are more intense than the extension products in lanes 5–8 because they were exposed for longer times than the lanes containing RDP196 DNA primed with primer A. This was done to provide stronger evidence that no low-abundance extension products indicative of possible non-random, specific strand breakage exist in any of the negative control lanes. It is interesting to note that the products produced from nicked templates represent much more than half of the sum of all the extension products in lanes 5–8 of Fig. 3. Thus these primer extension experiments show extensive *tra*-dependent *oriT* nicking and are consistent with the conclusion that a double-strand break does not occur at or near *oriT* in RDP196.

4. Discussion

This study was undertaken to further investigate the role of *oriT* in enhanced transductional recombination. The break produced at *oriT* during conjugal transfer is believed to be a strand-specific nick, and we have previously hypothesized that the *tra*-dependent nick at *oriT* constitutes the first step in allowing the RecBCD enzyme to gain access to the DNA duplex (Seifert & Porter, 1984; Carter & Porter, 1991). To test the requirement for *oriT* nicking *in vivo*, recombination assays were performed using pRPZ147, a plasmid in which the *oriT* point mutant from pED833 was cloned in-*cis* to the cellular copy of *lacZ813*. This *oriT* point mutant, in which no *tra*-dependent nick at *oriT* can be detected (Everett & Willetts, 1982), does not support enhancement (see Table 3). Further, a mini-F plasmid containing the *oriT* point mutation is 60-fold reduced in its ability to participate in conjugal DNA transfer as compared to an otherwise isogenic plasmid carrying wild-type *oriT* (see Table 4). Given these results, we conclude that the same strand-specific nicking that is required at *oriT* to allow conjugal DNA transfer is also required for recombination enhancement.

We also used primer extension experiments to carry

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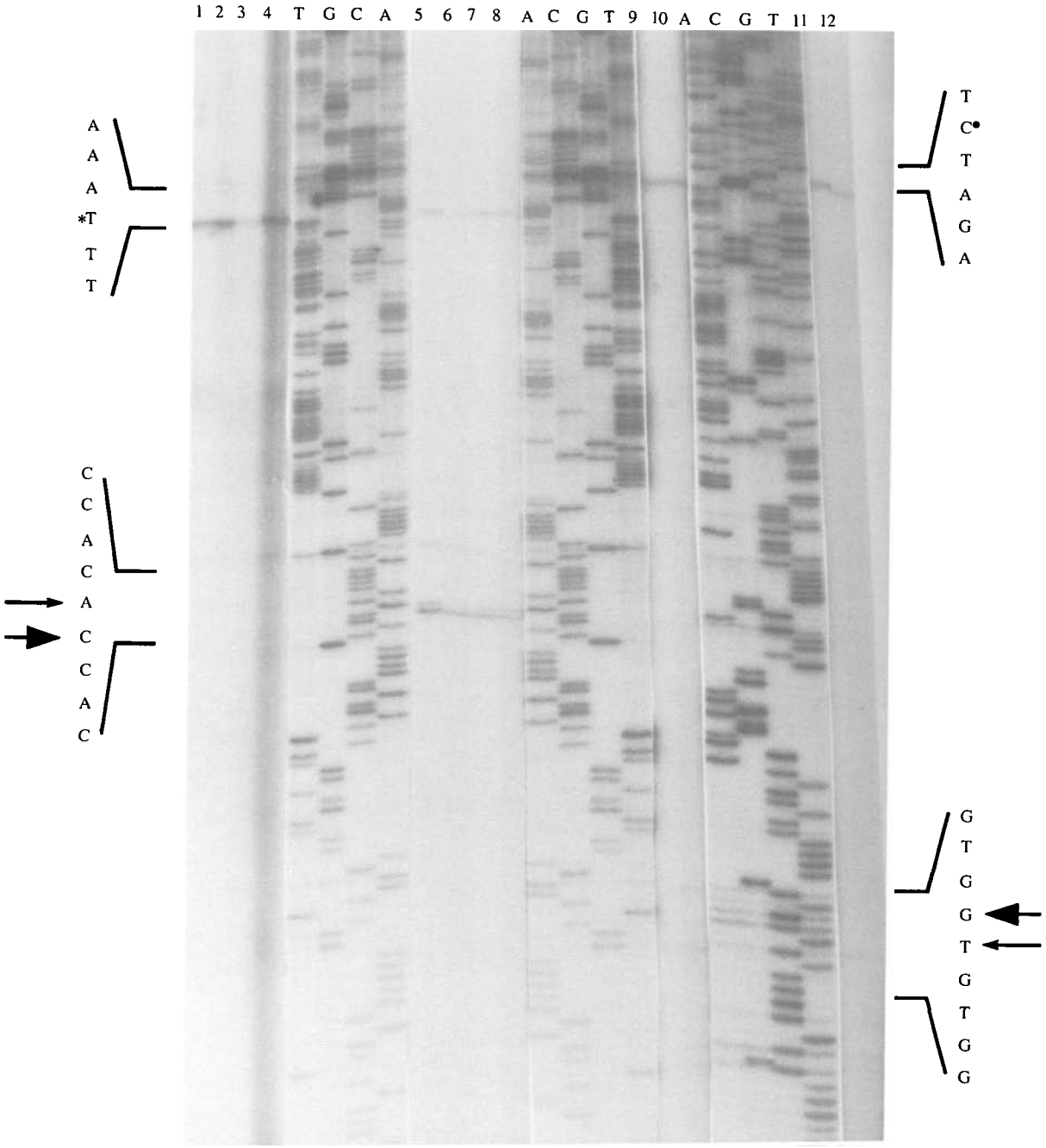

Fig. 3. Analysis of *oriT* nicking by primer extension. Primer extension and sequencing reactions were performed as described in Materials and methods. The left and centre sets of sequencing reactions were made using pRPZ148 primed with primer A; the right set involved priming with primer B. The letter label above each of these lanes indicates the dideoxyribonucleotide used. Lanes 1–4 contain *Dra* I-digested RDP195 DNA primed with primer A while lanes 5–8 contain *Dra* I-digested RDP196 DNA primed with primer A; in each case the relative amounts of DNA in these lanes are 1×, 2×, 4×, and 5×, respectively. Lanes 9–12 involve digestion with *Bgl* II followed by priming with primer B; RDP195 DNA was used in lanes 9 and 10 while RDP196 DNA was used in lanes 11 and 12. In both cases, the relative amounts of DNA in the two lanes are 4× and 2×, respectively. Relevant sequence derived from the first set of sequencing reactions is shown to the left. The large arrow indicates the 3′ base of the product of the primer extension of nicked pRPZ118 DNA that is seen in lanes 5–8. The small arrow indicates the weaker band in lanes 5–8 that has been attributed to the addition of one base to the 3′ end of the primer extension product (see text). The star indicates the 3′ end of the product of primer extension of unnicked pRPZ118, which corresponds to the end of *Dra* I-cleaved DNA. To the right is shown the sequence opposite *oriT*. A large and small arrow indicate bases opposite those which are at the end of the nicked strand. A *Bgl* II site derived from the rightmost set of set of sequencing reactions is also shown. The filled circle indicates the 3′ end of DNA derived from primer extension of pRPZ118 using primer B.

out *in vitro* testing for the presence of the strand-specific nick at *oriT* under conditions conducive to recombination enhancement. As shown in Fig. 3, strand-specific nicking was readily detected in total DNA from a strain demonstrating enhancement, RDP196, and not detected in the negative control

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strain, RDP195. The major primer extension product corresponds to a nick 137 bp from the middle of the *Bgl* II site, which is in agreement with the results reported by Thompson *et al.* (1989). The less abundant product which is one base larger probably results from the non-template-directed addition of a single nucleotide to the 3′ end of the extended DNA product. Such an activity is known to exist for Sequenase (pers. comm., United States Biochemical Technical Services) and for several other DNA polymerases (Clark, 1988). The extension product that is 10 bases larger than the 'nick' product (see Fig. 3) is also found in the sequencing ladders and in the lanes containing RDP195 DNA. This product probably is attributable to an artifact of the polymerization reaction, such as a Sequenase-specific pause site located at this region of the template.

The recombination results with the *oriT* point mutation demonstrate that *tra*-dependent *oriT* nicking is an essential step in recombination enhancement; this is supported by the primer extension experiments which show extensive nicking at *oriT* under enhancement conditions. As *in vitro* analysis has shown that the RecBCD enzyme may only enter duplex molecules that have flush or nearly flush ends, we have also sought to determine whether or not the nick at *oriT* is processed into a double-strand break. Southern blotting was used to attempt the detection of a 2·2 kb DNA fragment from pRPZ118 which would have been indicative of such a double-strand break, and it was determined that *oriT* is not subject to a double-strand break at a level of greater than 1 in 500 molecules. Although we had seen some indication that a double-strand break might be occurring near *oriT* in preliminary experiments (Smith, 1988), we were unable to reproduce those preliminary results. Lac$^+$ recombinants per potential transductant cell appear in a frequency range from 1 per 160 to 1 per 25 under enhancement conditions (see Table 3; Carter & Porter, 1991), and we therefore would have expected to detect any double-strand breaks that persist for a suitable fraction of a generation time. As the length of time for which such a hypothetical double-stranded break might actually persist is uncertain, we cannot rule out the possibility that short-lived double-strand breaks occur at a frequency below our limits of detection.

Since the RecBCD enzyme requires a flush or nearly flush end to enter duplex DNA *in vitro* (Taylor & Smith, 1985), the data in this paper bring into question how the RecBCD enzyme might enter an *oriT*-activated DNA duplex without the benefit of a flush end. In this regard it should be noted that the *in vivo* situation is necessarily more complex than the *in vitro* one, and the participation of other proteins such as the TraI and/or TraY proteins may permit RecBCD enzyme to enter duplex DNA at an activated *oriT* without double-strand breakage. Finally, the possibility exists that the nick at *oriT* causes an uncharacterized change in the *oriT*-containing plasmid which allows RecBCD enzyme to load at a location other than *oriT*.

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