JOURNAL OF BACTERIOLOGY, Oct. 1992, p. 6659–6665
0021-9193/92/206659-07$02.00/0

Vol. 174, No. 20

Role of the Origin of Transfer in Termination of Strand Transfer during Bacterial Conjugation

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Received 8 May 1992/Accepted 7 August 1992

Conjugal transfer of the broad-host-range plasmid R1162 is initiated and terminated at the *nic* site within the 38-bp origin of transfer (*oriT*). Termination involves ligation of the transferred single strand by the plasmid-encoded MobA protein. Several different assays were used to identify the *oriT* DNA required for termination. For plasmids containing two *oriT*s, with transfer initiated at one and terminated at the other, the inverted repeat within *oriT* is important for termination. Deletion of the outer arm reduces the termination frequency; those terminations that do occur probably depend upon nicking at this *oriT* prior to transfer. The locations of second-site suppressor mutations indicate that base pairing between the arms of the inverted repeat is important for termination. In vitro, the inverted repeat is not required for specific cleavage of single-stranded DNA at *nic*, but competition experiments indicate that *oriT*s with the inverted repeat are preferentially cleaved. We propose that the function of the *oriT* inverted repeat is to trap the plasmid-encoded MobA protein at the end of a round of strand transfer, thus ensuring that the protein is available for the ligation step.

The basic steps in DNA processing during conjugal transfer are probably similar for many different plasmids in gram-negative bacteria. One of the DNA strands is first nicked within the origin of transfer (*oriT*), which is required in *cis*. The nicked strand is then linearly transferred into a recipient cell in the 5′-to-3′ direction and subsequently recircularized. In both donor and recipient cells, the complementary strand of DNA is resynthesized to restore the duplex plasmid molecule. Many different plasmids can be isolated in the form of a relaxosome, a complex of proteins and supercoiled plasmid DNA, which can be induced to undergo site- and strand-specific nicking within *oriT* (for example, see references 5, 6, 12, and 16). This nicking models the initial DNA-processing step in plasmid transfer.

R1162 (nearly identical to RSF1010) is a broad-host-range, IncQ plasmid that is efficiently mobilized during the conjugal transfer of IncP1 group plasmids (15, 20). The R1162 *oriT* consists of no more than 38 bp of DNA (Fig. 1) (4). The cleavage site within *oriT* has been mapped previously (1, 4) and is designated *nic* in Fig. 1. RSF1010 DNA has been isolated as a relaxosome (16); more recently, this complex has been reconstructed from plasmid DNA and purified proteins (18). The recircularization of plasmid DNA after transfer is modeled by the recombination of directly repeated *oriT* copies on replicating M13 phage DNA (1, 14). In this system of recombination, it is likely that the single-stranded *oriT* copies are treated as intermediates in transfer and are therefore cleaved and ligated at the *nic* site. MobA*, encoded by a segment of the R1162 *mobA* gene, is required for this recombination and can carry out these reactions in vitro (2).

The effects of mutations indicate that *oriT* consists of more than one functional domain (10). Two simultaneous C-to-T transitions at bp 30 and 32 (Fig. 1, line 2) affect the initiation of transfer but not the termination step modeled by the recombination of *oriT* copies in M13 (10, 14). Deletion of bp 1 to 8, which make up the outer arm of the inverted repeat in *oriT* (Fig. 1, line 3), has the opposite effect (1, 10). This mutation does not affect initiation but does interfere with termination and phage recombination.

We have examined in greater detail than previously the structural requirements within *oriT* for the termination of conjugal transfer of R1162 DNA. Our results indicate that the inverted repeat within *oriT* is essential for efficient termination in vivo. However, an intact inverted repeat is not required for specific cleavage in vitro of single-stranded DNA within *oriT* by the MobA* protein. We propose that during transfer, the inverted repeat acts to initiate termination by trapping the protein required for ligation of the transferring strand.

MATERIALS AND METHODS

Strains and plasmids. The *Escherichia coli* K-12 strains used in this study are MV12 (*recA56 thr-1 leuB6 lacY1 thi-1 rfbD1 supE44 fhuA ΔtrpE5*) (7), the nalidixic acid-resistant derivative MV12 Nal^r, derived by spontaneous mutation, and JM103 [Δ(*lac pro*) *strA thi supE endA sbcB hsdR* F′ *traD36 proAB lacI^q lacZ*ΔM15] (13). Bacteria were grown in 1% Bacto Tryptone–0.5% Bacto-yeast extract–0.5% NaCl, supplemented as required with carbenicillin (200 μg/ml), trimethoprim (200 μg/ml), streptomycin (37.5 μg/ml), or nalidixic acid (25 μg/ml).

The plasmids used in the bacterial matings have the general structure shown in Fig. 2. These plasmids are derivatives of pBR322 (3) and contain two copies of *oriT*, one on either side of a 230-bp *lacO* fragment. The construction of pUT1183, which contains unmutated copies of *oriT*, will be described in detail elsewhere (submitted for publication); the other members of this family were derived from pUT1183 by replacement of the normal *oriT* in position 1 with one of the mutated *oriT*s shown in Fig. 1.

Mutagenesis of *oriT*. The isolation of mutated *oriT*s containing C-to-T transitions at bp 30 and 32 or deleted for bp 1 to 8 (Fig. 1, lines 2 and 3) has been described previously (4). An *oriT* fragment lacking bp 1 to 19 (Fig. 1, line 4) was generated by digestion of *oriT* DNA with *Age*I. An *Hpa*I site (GTTAAC) was introduced into *oriT* DNA at the location shown in Fig. 1, line 5, by oligonucleotide-directed mutagen-

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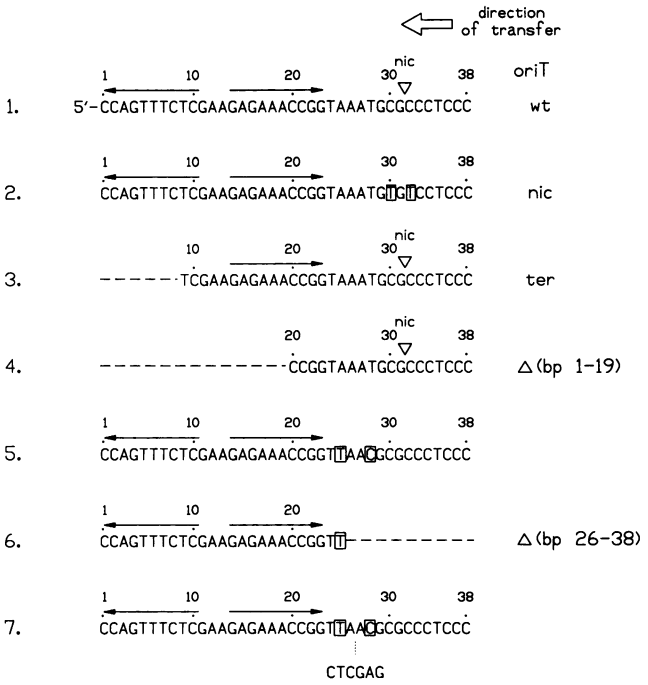

FIG. 1. Base sequences of R1162 *oriT* (line 1) and different mutated derivatives (lines 2 to 7). wt, wild type; other abbreviations are defined in the text.

esis (11). The resulting DNA was then digested with *Hpa*I to delete bp 26 to 38 (Fig. 1, line 6). The *Hpa*I site was also used to clone a chemically synthesized, 6-bp fragment between bp 26 and 27 (Fig. 1, line 7).

Assay for *oriT* recombination in M13 phage DNA. Recombination at *oriT* was assayed with derivatives of M13mp19 (17). These derivatives contain two directly repeated copies of *oriT* in the vector-cloning region (see Fig. 5). The standard conditions for the assay are described in detail elsewhere (14). The *oriT* copies interrupt the *lacZ*α-coding sequence, and phage plaques are therefore white on lawns of JM103 in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). When the phage is propagated in a JM103 strain that encodes MobA*, cleavage and rejoining at the *nic* site of each *oriT* result in molecules with a single *oriT* insertion. Translation through this remaining *oriT* results in an altered *lacZ*α polypeptide that nevertheless complements the product of the *lacZ*ΔM15 gene in JM103. Thus, recombinant phage forms blue plaques on JM103 lawns. The overall recombination frequency was enumerated after propagation of the phage by scoring the percentage of these blue plaques.

Bacterial conjugation. Donor MV12 strains contained the IncP1 plasmid R751 (8) as the mobilizing vector, R1162 (to provide the plasmid-encoded proteins necessary for mobilization), and the test plasmid containing copies of the R1162 *oriT*. Matings were carried out by separately growing donor and recipient cells (MV12 Nal^r) to mid-log phase (approximately 5×10^8 cells per ml). Donor and recipient cells were then mixed at a 1:10 ratio (final volume, 110 μl) and immediately pelleted in a microcentrifuge. The cells were resuspended in 40 μl of broth, placed on broth agar medium, and incubated at 37°C for 90 min. The mating mixture was then resuspended in 1 ml of broth and 100 μl of various dilutions, mixed with 50 μl of 2% X-Gal, and plated on medium containing nalidixic acid and carbenicillin supplemented with methicillin (2 mg/ml) (21). Donor cells were enumerated by plating the mating mixture on medium containing carbenicillin.

Assay for relaxosome-induced plasmid nicking. Cleared lysates were prepared from 100-ml cultures grown to approximately 5×10^8 cells per ml by the method of Katz et al. (9). The lysates were treated first by the addition of sodium dodecyl sulfate (SDS) (0.5%) and incubated at 65°C for 10 min and then by the addition of proteinase K (50 μg/ml) and incubated at 37°C for 45 min. The nucleic acids were precipitated and subjected to dye-buoyant density ultracentrifugation (19). The DNA bands were removed from the centrifugation tube, extracted with CsCl-saturated isopropanol, and dialyzed against 0.05 M Tris-HCl (pH 8.0)–0.005 M Na_2-EDTA–0.05 M NaCl. The plasmids in each band and their relative amounts were estimated by digestion of the DNA with *Eco*RI followed by agarose gel electrophoresis.

Cleavage of single-stranded *oriT* DNA in vitro. The assay for cleavage of single-stranded *oriT* by purified MobA*–β-galactosidase hybrid protein was as previously described (2), except that reactions were carried out for 2 h at pH 8.0. Each reaction mixture contained 0.1 μg of M13 viral strand DNA, end labeled at the *Bsu*36I site (2) and having the cloned test *oriT*, and an equal amount of end-labeled viral strand DNA with the unmutated *oriT* as an internal control. The control DNA (φrm290) contains a 42-base *Eco*RI fragment, derived from M13mp7 (13), inserted at the *Eco*RI site between *oriT* and the *Bsu*36I site, so that the cleavage product could be distinguished by its mobility during electrophoresis. After being digested with MobA*, the reaction mixture was further incubated with 1 μg of proteinase K at 37°C for 30 min. The DNA was then denatured, subjected to electrophoresis on an 8% polyacrylamide gel containing 8 M urea, and visualized by autoradiography.

RESULTS

Frequency of termination of conjugal transfer within *oriT*s containing different deletions. We constructed pBR322 derivatives containing a copy of *oriT* on each side of the *lac* operator, *lacO* (Fig. 2). Conjugal transfer of the intact plasmid into the *lacZ*$^+$ *recA* recipient MV12 Nal^r resulted in transconjugant colonies that were light blue on medium containing X-Gal, because the high *lacO* gene dosage titrated

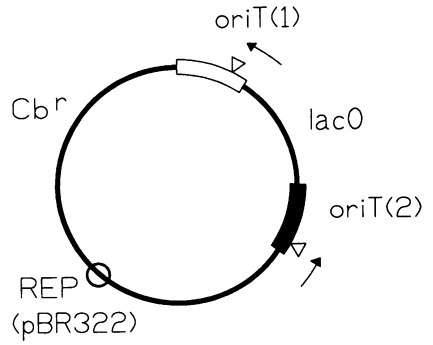

FIG. 2. General structure of plasmids for determining termination frequency during conjugal mobilization. The arrows show the direction of transfer for each of the cloned R1162 *oriT*s, and the triangles represent the *nic* sites. Regions of the plasmid that encode the replicator and resistance to carbenicillin are indicated by REP-(pBR322) and Cb^r, respectively.

TABLE 1. Effects of *oriT* mutations on termination

Plasmid	Transfer frequency[a]	Mutation in $oriT_1$	White transconjugant colonies (%)[b]
pUT1183	9.8×10^{-3}	None	56
pUT1216	1.2×10^{-3}	*nic* (C to T, bp 30 and 32)	53
pUT1238	1.3×10^{-3}	*ter* [Δ(bp 1 to 8)]	0.12
pUT1254	7.0×10^{-3}	Δ(bp 1 to 19)	<0.004
pUT1214	4.1×10^{-3}	A to T, bp 25; Δ(bp 26 to 38)	<0.005
pUT1250	1.8×10^{-3}	*ter* + *nic*	0.009

[a] Transfer frequency is defined as the number of transconjugants per donor cell.
[b] When the frequency is less than 1%, at least 10,000 transconjugant colonies were examined.

the chromosomally encoded *lac* repressor. However, the *oriT*s were oriented so that when transfer was initiated at $oriT_2$ and terminated at $oriT_1$, the *lacO* segment was deleted and the transconjugant colony was white. Thus, the proportion of white colonies in a mating reflects the efficiency of termination at $oriT_1$. These plasmids are similar in design to others described earlier (10) that contain a kanamycin resistance gene instead of *lacO*. The advantage of the molecules used here is that colonies of cells containing the plasmid products of rare terminations can be easily identified and enumerated.

When the *lacO* plasmid contained two copies of unmutated *oriT*, the frequency of white colonies after conjugation was 56% (Table 1). We confirmed that deletion of *lacO* took place only during conjugation: when plasmid DNA was instead transferred from the donor strain to the recipient by transformation, only 5 of 10,456 colonies were white, a frequency of 0.05%. C-to-T transitions at bp 30 and 32 in $oriT_1$ (the *nic* mutation; Fig. 1, line 2) did not significantly reduce the termination frequency (Table 1), which was consistent with the effect of this mutation on initiation only (10). The *ter* mutation, an 8-bp deletion of the outer arm of the inverted repeat within the R1162 *oriT* (Fig. 1, line 3), inhibits the termination but not the initiation of conjugal DNA transfer (10). When $oriT_1$ contained this mutation, the frequency of termination was much lower, as expected (Table 1). However, white transconjugant colonies still occurred at a frequency of 0.12% and were readily detectable among the majority of blue colonies.

When both the inner and outer arms of the inverted repeat were deleted (Fig. 1, line 4), no termination within $oriT_1$ was observed (Table 1). Thus, for $oriT_{ter}$, the inner arm of the inverted repeat is important for the remaining termination activity. The intact inverted repeat was not sufficient for termination: when $oriT_1$ was deleted for bp 26 to 38 and contained an A-to-T mutation at bp 25 (Fig. 1, line 6), no white transconjugant colonies were formed (Table 1).

Properties of termination within $oriT_{ter}$. Termination within $oriT_{ter}$ occurs at less than 1% of the normal frequency but at a rate that is still well above background (Table 1). To determine whether cleavage and ligation were nevertheless occurring at the normal position during these rare events, we constructed pUT1250, which contains both the *ter* and *nic* mutations at $oriT_1$. This *oriT* contains C-to-T transitions at bp 30 and 32, which bracket the *nic* site. Thus, if cleavage and rejoining of the transferred strand were occurring at the normal position, the mutation at bp 30 would be retained and the other mutation would be lost in the recombinant *oriT*. We extracted the plasmid DNA from three transconjugant colonies, each white on X-Gal and each derived from an independent mating, and determined the base sequences of the hybrid *oriT*. For each, the sequence around *nic* was GTGC for bp 29 to 33 (Fig. 3), indicating that recombination had taken place at the normal site.

Although termination in $oriT_{ter+nic}$ occurs at the normal position, it does so at a frequency that is approximately 10-fold lower than that in $oriT_{ter}$ (Table 1). In order to understand this decrease, we first examined the effect of the *nic* mutation on the DNA processing required for transfer. Since the *nic* mutation affects an early step in transfer and is located at the *nic* site, we thought it likely that it interferes

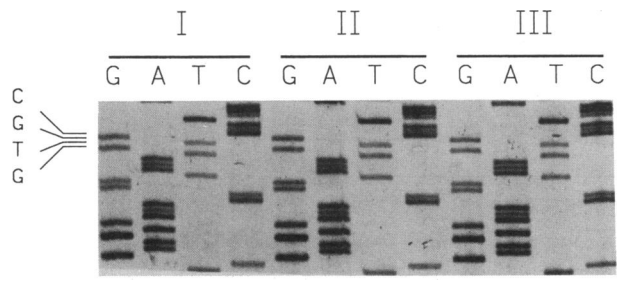

FIG. 3. Base sequences around *nic* sites for three hybrid *oriT*s formed after conjugal transfer of pUT1250 (Table 1) and deletion of *lacO*.

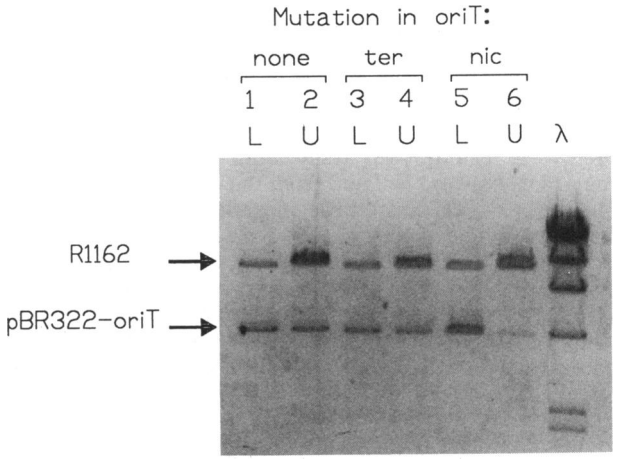

FIG. 4. Agarose gel electrophoresis of plasmid DNAs isolated from the lower (L) and upper (U) bands of a CsCl-ethidium bromide gradient and linearized by digestion with *Eco*RI. The molecular size marker is *Hin*dIII-digested λ DNA.

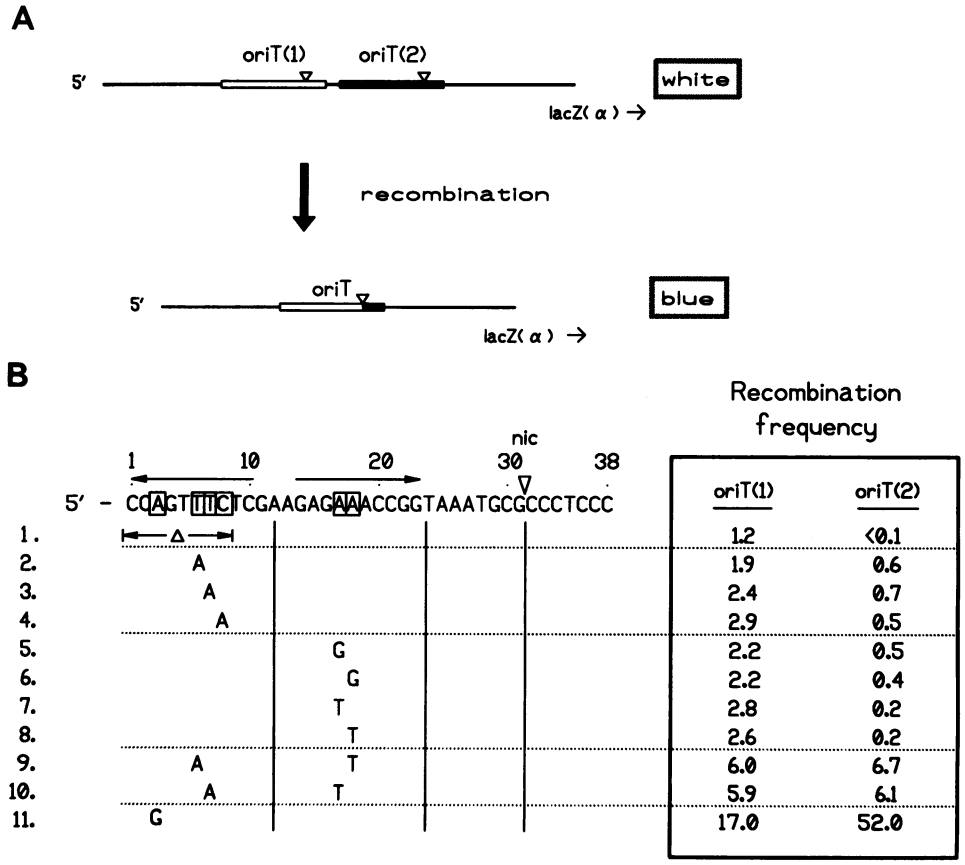

FIG. 5. (A) Recombination assay for directly repeated copies of *oriT* cloned in M13mp19; (B) mutations within the *oriT* inverted repeat and their effects on recombination frequency.

with initial nicking of the plasmid DNA. This is shown in Fig. 4.

Complexes of supercoiled plasmid DNA and Mob nicking proteins (the relaxation complex or relaxosome) were isolated from cells. Cells contained both a pBR322 derivative with a cloned *oriT* having the test mutation and R1162 to provide the Mob proteins and to serve as an internal control. The DNA was then relaxed by treatment with SDS and heat, and the supercoiled (unnicked) and open circular forms were separated by dye-buoyant density ultracentrifugation. The amount of plasmid DNA in each form was determined by gel electrophoresis after the DNA was linearized with *Eco*RI.

When plasmids containing either the unmutated *oriT* or $oriT_{ter}$ were tested in our assay, both supercoiled and open circular DNAs were isolated from the density gradient (Fig. 4, lanes 1 to 4). Thus, these plasmids are susceptible to relaxosome-induced nicking. Active nicking of $oriT_{ter}$ is in agreement with its ability to initiate transfer. In contrast, substantially less of the plasmid DNA containing $oriT_{nic}$ than R1162 DNA from the same preparation was converted to the nicked form (Fig. 4, lanes 5 and 6). We estimate from several experiments that the $oriT_{nic}$ mutation decreases relaxation complex activity by at least 90%. We conclude that the initiation defect for the *nic* mutation is at the nicking step of the closed circular duplex DNA.

Our results with the *nic* mutation suggest that it reduces termination within $oriT_{ter}$ because it interferes with nicking of duplex *oriT* at this site. Alternatively, it is possible that although the *nic* mutation itself has no effect on termination (Table 1), it exacerbates by some mechanism the effect of the *ter* mutation when present in the same *oriT*. We used the phage recombination assay for termination (14) to distinguish between these possibilities. Two directly repeated copies of *oriT*, cloned into the bacteriophage vector M13mp19, recombine during propagation of the phage DNA to result in a single, hybrid *oriT* (Fig. 5A). This recombination models the termination step in transfer, probably because the single-stranded viral DNA resembles DNA that has been conjugally transferred (1). Relaxosome activity is not involved in the recombination, which requires only the MobA* gene product.

When $oriT_1$ contained the *ter* mutation, the phage recombination frequency was reduced to about 10% of the normal

TABLE 2. Effect of the *nic* mutation on the activity of $oriT_{ter}$ in the phage recombination assay

Mutation no.	Mutation in:		Blue plaques (%)[b]
	$oriT_1$[a]	$oriT_2$[a]	
1	*wt*[c]	*wt*	11
2	*ter*	*wt*	1.4
3	*nic*	*wt*	17
4	*ter* + *nic*	*wt*	0.7

[a] See Fig. 5.
[b] Averages of two to four independent experiments.
[c] *wt*, wild type.

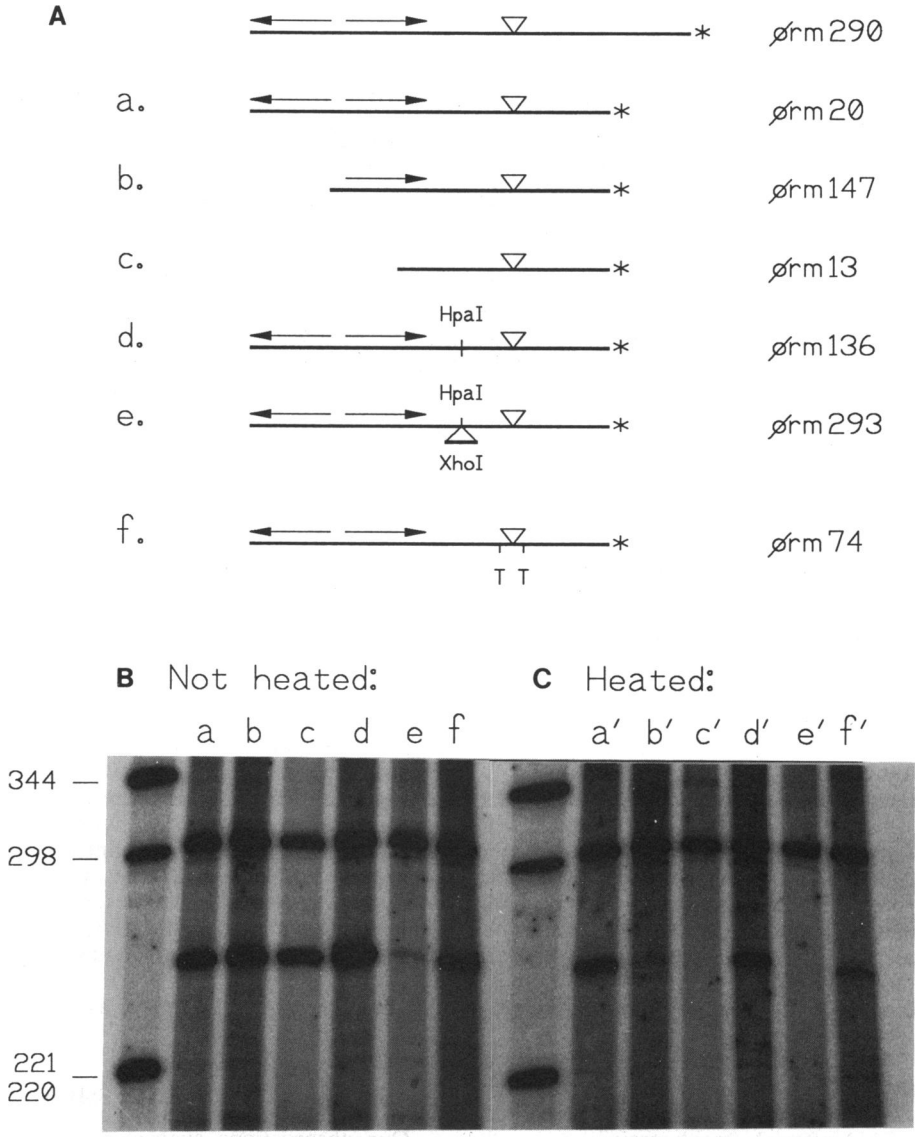

FIG. 6. Specific cleavage of mutated *oriT*s, present in M13 viral strand DNA, by MobA* fusion protein. Prior to being digested, the DNA was end labeled at the *Bsu*36I site (asterisks) in the viral DNA (2). The structure of each *oriT* and the name of the phage containing this *oriT* are indicated in panel A. An equimolar amount of end-labeled ϕrm290 phage DNA, which contains the unmutated *oriT*, was also present in each reaction mixture. In panel B, native protein was used; in panel C, the protein was heated at 70°C for 10 min prior to being digested. In addition to ϕrm290, each reaction mixture contained ϕrm20 (lanes a and a'), ϕrm147 (lanes b and b'), ϕrm13 (lanes c and c'), ϕrm136 (lanes d and d'), ϕrm293 (lanes e and e'), or ϕrm74 (lanes f and f'). The molecular size marker was *Hin*fI-digested, denatured pBR322 DNA.

value (Table 2, mutations 1 and 2). There was only a slight decrease in the recombination frequency when $oriT_1$ also contained the *nic* mutation (Table 2, mutation 4). Thus, the *nic* mutation, either alone (Table 2, mutation 3) or together with *ter*, has little effect on the cleavage of single-stranded DNA. It is likely, therefore, that the cleavage within $oriT_{ter}$ required for the termination of conjugal transfer is generated primarily by nicking of the duplex DNA.

Effect of base pairing between the arms of the *oriT* inverted repeat on termination. We also used the phage recombination assay to examine in greater detail than previously the requirement for the inverted repeat in termination. This assay is more sensitive than transfer, because many rounds of phage propagation amplify the effects of small differences in the activities of mutated *oriT*s.

We showed previously that point mutations in the outer arm of the inverted repeat were similar to the *ter* mutation in their abilities to reduce the rate of *oriT* recombination from the normal frequency of 10 to 15% (1) (Fig. 5B, lines 1 to 4). All of the mutations had the greatest effects in $oriT_2$. The results in Fig. 5B show that other point mutations, located within the inner arm of the inverted repeat, had quantitatively similar effects (Fig. 5B, lines 5 to 8). However, when the mutations in each arm were matched so that the potential for base pairing between the arms was restored, the deleterious effects of the mutations were partially suppressed (Fig. 5B, lines 9 and 10). These results suggest that base pairing per se is important for the activity of the inverted repeat in termination.

The arms of the *oriT* inverted repeat naturally contain a

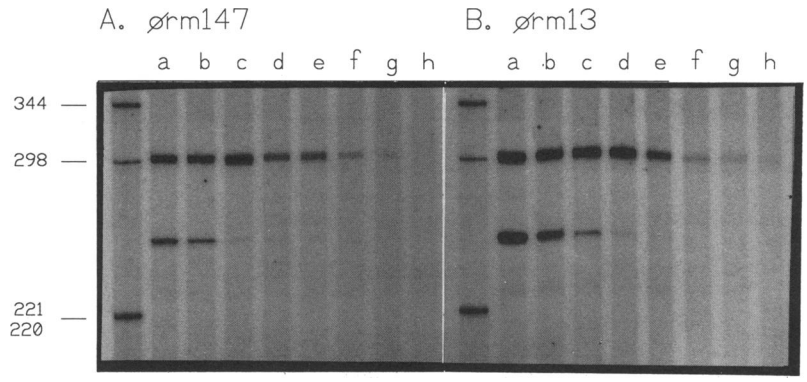

FIG. 7. Competition for cleavage by MobA* protein between ϕrm290 (unmutated *oriT*) and ϕrm147 (*oriT* deleted for outer arm of inverted repeat) (A) or ϕrm13 (*oriT* deleted for both arms of inverted repeat) (B). Each reaction mixture contained 0.1 μg of each labeled DNA and 0 (lanes a), 0.1 (lanes b), 0.2 (lanes c), 0.4 (lanes d), 0.8 (lanes e), 1.6 (lanes f), 3.2 (lanes g), and 6.4 (lanes h) μg of unlabeled ϕrm20 DNA (unmutated *oriT*). The molecular size marker was *Hin*fI-digested, denatured pBR322 DNA.

single mismatch, which is at bp 3 and 21 (Fig. 5B). We asked whether repairing this mismatch by introducing an A-to-G transition at bp 3 would affect the frequency of recombination of *oriT*. The results (Fig. 5B, line 11) show that there is a significant increase in the recombination frequency when the mutation is in the $oriT_2$ position.

Cleavage of mutated *oriT*s in vitro by MobA* fusion protein. MobA* is the amino-terminal segment of the large protein encoded by the R1162 *mobA* gene (2) and is the only R1162-encoded protein required for termination (14). In vitro, MobA*, as a β-galactosidase fusion protein, cleaves one of the two *oriT* single strands at *nic* and also slowly ligates this DNA (2). We tested different mutated *oriT*s for cleavage by this protein (Fig. 6). Linear M13mp19 viral strand DNA containing a mutated *oriT* was incubated with MobA* fusion protein. The DNA was end labeled so that cleavage at the *nic* site would generate a 245-base labeled fragment. As an internal control, each reaction mixture also contained M13 viral DNA with an unmutated *oriT* (ϕrm290). This DNA has a 42-base insertion between *oriT* and the labeled end, so that the labeled cleavage product, which comigrates with the 298-base marker fragment, is separated from that of the test DNA after electrophoresis.

The results of incubating different mutated *oriT* DNAs with fusion protein are shown in Fig. 6. We found that MobA* cleaved *oriT* deleted for either the outer arm of the inverted repeated, bases 1 to 8, or the entire inverted repeat, bases 1 to 19 (ϕrm147 and ϕrm13) (Fig. 6B, lanes b and c). Cleavage was specific and occurred at the site cleaved in the unmutated *oriT* in ϕrm20 (Fig. 6B, lane a). MobA* was also active on $oriT_{nic}$ in ϕrm74 (Fig. 6B, lane f), which was expected, since the mutations in this *oriT* do not affect termination.

A mutated *oriT* with two base changes outside the inverted repeat, at positions 25 and 28, was constructed (Fig. 1, line 5). This *oriT* was cleaved by MobA* (ϕrm136) (Fig. 6B, lane d). The mutations create an *Hpa*I site, and a 6-base insertion at this position to generate ϕrm293 (Fig. 1, line 7) prevents cleavage (Fig. 6B, lane e). We conclude that the inverted repeat is not required for specific cleavage of *oriT* single-stranded DNA at *nic*. However, disruption of *oriT* DNA adjacent between the inverted repeat and the *nic* site did affect cleavage.

Different results were obtained when the cleavage reactions were repeated with fusion protein that had first been heated at 70°C for 10 min. The heated protein still cleaved the unmutated *oriT* in ϕrm20 and ϕrm290 (Fig. 6C, lane a'), but cleavage of *oriT*s lacking part or all of the inverted repeat was strongly reduced (Fig. 6C, lanes b' and c'). Cleavage of either $oriT_{nic}$ or *oriT* with the *Hpa*I site was unaffected by the heat treatment (Fig. 6C, lanes d' and f'), and the *oriT* with the 6-base insertion remained inactive (Fig. 6C, lane e').

Although *oriT* DNAs lacking part or all of the inverted repeat are cleaved by unheated MobA* fusion protein, they compete poorly with unmutated *oriT* DNA for this protein. End-labeled ϕrm290 DNA, with an unmutated *oriT*, was mixed with an equimolar amount of similarly labeled ϕrm147 or ϕrm13 DNA (Fig. 7). The mixtures were then digested with MobA* protein in the presence of increasing amounts of unlabeled ϕrm20 phage DNA, which also contains the unmutated *oriT*. As the amount of unlabeled DNA was increased, ϕrm147 and ϕrm13 became progressively less able to compete with ϕrm290 as a substrate in the cleavage reaction (Fig. 7).

DISCUSSION

Our observations suggest the following model for the termination of conjugal R1162 DNA transfer. During mobilization, the MobA protein is covalently bound to the 5' end of the transferring strand (18). We propose that the protein is also positioned (in either the donor or the recipient cell) so that the DNA strand passes by MobA as it is being transferred. When the *oriT* inverted repeat moves near MobA, it traps the protein and termination is initiated. Because a mutation in the inverted repeat is suppressed by a second mutation restoring base complementarity between the arms, we believe that base pairing is important for trapping by the inverted repeat. Indeed, repair of the naturally occurring mismatch in the *oriT* arms results in a greater rate of termination, as measured by the phage recombination assay. Possibly, the *oriT* sequence has evolved so that in conjugation there is a balance between binding of the protein and its subsequent release after ligation of the transferred strand.

Although there is no termination in *oriT* deleted for the entire inverted repeat, there is a low rate of termination in $oriT_{ter}$, which lacks only the outer arm of this repeat. The termination frequency in $oriT_{ter}$ is greatly affected by the *nic* mutation, and therefore it probably requires prior nicking of the duplex $oriT_{ter}$ DNA. Since transfer can be initiated

within $oriT_{ter}$, and since $oriT_{ter}$ allows an active relaxation complex (Fig. 4), duplex $oriT_{ter}$ DNA can be nicked in vivo. Termination at $oriT_{ter}$ would then depend on molecules that had been nicked at both *oriT*s prior to transfer. The possibility of doubly nicked molecules is surprising, since one might expect that after being nicked at one site, the open circular DNA would not longer be susceptible to relaxosome activity. In this connection, it is interesting that Scherzinger et al. have shown that linear DNA is a substrate for relaxosome-dependent nicking in vitro (18).

In vitro, trapping of MobA protein by the inverted repeat is reflected in competition experiments by the intact *oriT* having a greater rate of cleavage than the *oriT* deletion derivatives lacking part or all of the inverted repeat. However, the inverted repeat is not itself required for specific cleavage of single-stranded *oriT* DNA at the *nic* site. Protein that has been heated to 70°C shows greater specificity, preferentially digesting those *oriT* molecules having the inverted repeat. One way of rationalizing these observations is by noting that under our reaction conditions, active protein is in excess. When the protein is either heated or titrated with excess unlabeled DNA, it becomes limiting in the reaction. Under these conditions, molecules with the inverted repeat are better able to trap the protein and are more likely to be cleaved. We cannot rule out other explanations for our observations, however. For example, it is possible that the protein molecules in our preparations are present in two forms, only one of which requires the inverted repeat for cleavage at *oriT*. In this case, titration with excess *oriT* DNA would have a greater effect on cleavage of molecules without the inverted repeat. Heating might promote refolding of the protein into the form requiring the inverted repeat, so that the overall specificity of the preparation would be increased.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institutes of Health (GM37462).

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