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# Localization of the *nic* Site of IncN Conjugative Plasmid pCU1 through Formation of a Hybrid *oriT*

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**The N-type *oriT* of plasmid pMUR274 was cloned on a 474-bp *Rsa*I-*Ssp*I fragment, and the nucleotide sequence was determined. A comparison of the pMUR274 *oriT* sequence and the sequence of the *oriT*s of IncN plasmid pCU1 and IncW plasmid R388 demonstrated 57 and 28% identity, respectively. Intramolecular, site-specific recombination between the pCU1 *oriT* and the *oriT* of pMUR274 resulted in the formation of a hybrid *oriT* containing one half of each parental sequence. The junction point of the hybrid occurred within a 10-bp sequence, GCTATACACC, present in both parental sequences and represents the *nic* site of each *oriT*. Mutation of the first A or second T residue within the 10-bp junction sequence reduced transfer less than 20-fold, while mutation of either the second or third A residue reduced transfer over 1,000-fold. Site-specific recombination between a wild-type pCU1 *oriT* and these four mutant pCU1 *oriT*s demonstrated that *nic* lies between the second T and second A residues of the 10-bp junction sequence. Site-specific recombination between wild-type and mutant pCU1 *oriT*s also demonstrated that point mutations to the right of *nic* reduced both initiation and termination of transfer while point mutations to the left of *nic* reduced termination but had little or no effect on initiation. A 28-bp deletion within the AT-rich region 39 bases to the right of *nic* reduced both initiation and termination, while deletion of a 6-bp inverted repeat sequence at the right-most boundary of the minimal *oriT* region reduced initiation but not termination.**

During conjugal DNA transfer in gram-negative eubacteria, plasmid-encoded Mob proteins interact with a small *cis*-acting region called the origin of transfer (*oriT*) to form a DNA-protein complex called the relaxosome (40). Transfer is initiated by the binding of one or more small Mob proteins to the *oriT* region, following which a site-specific, single-stranded nick is made in the *oriT* by a second larger Mob protein which remains covalently bound to the 5′ end of the nicked strand. The nicked strand is unwound in the 5′-3′ direction, and complementary strand synthesis is initiated from the 3′ end of the nicked strand. Once a complete strand has been unwound, a second nick is made in the reconstituted *oriT* and the 5′ and 3′ ends of the unwound strand are sealed. Both nicking and sealing are carried out by the same enzyme (3, 28). The nucleotide sequences of *oriT*s of different plasmids have only limited identity, even among plasmids encoding similar transfer systems. This lack of sequence identity is reflected in the plasmid specificity exhibited by the Mob proteins (8, 41).

Brasch and Meyer (4) demonstrated that when two identical *oriT* sequences are placed on the same plasmid, transfer can be initiated at one *oriT* and terminated at the other, resulting in an intramolecular site-specific recombination event in which the DNA between the second and first *oriT* is deleted. This recombination event can be detected by the loss of a selectable marker on the deleted DNA. By placing mutations in either the *oriT* which is nicked first or that which is nicked second, this technique has been used to distinguish between sequences required for nicking (initiation of transfer) from those required for ligation (termination of transfer) (4). Site-specific recombination between one wild-type and one mutated *oriT* has also been used to locate the exact site of *nic* of IncQ plasmid R1162. Because the resultant hybrid *oriT* contains one half of each of the two parental *oriT*s (with *nic* representing the junction point), only mutations located to one side of *nic* are found in the recombinant *oriT* (1, 4).

As with the formation of a single *oriT* by site-specific recombination between two identical *oriT*s, it is possible to predict that a similar event between two different *oriT*s could result in the formation of a hybrid *oriT* with sequences from either parental *oriT* joined at the nick site. While production of such a hybrid *oriT* would require sufficient sequence identity between the two parental regions to allow interaction of the Mob proteins with both *oriT*s, some sequence variation is also necessary to allow identification of the junction in the hybrid. The *oriT* of pCU1 lies on a 325-bp sequence (32) which has 30% sequence identity to the *oriT* region of IncW plasmid R388 (18). Unsuccessful attempts to detect site-specific recombination between the *oriT*s of pCU1 and R388 (19, 31) suggest that these two plasmids do not have sufficient sequence identity to allow such an event to occur. This incompatibility is also seen in the inability of R388 to mobilize a plasmid carrying the *oriT* of pCU1 (31).

Conjugative plasmid pMUR274 encodes an N-type transfer system as determined by sensitivity to IKe phage. Its ability to mobilize a plasmid carrying the *mob* region of pCU1 but not the *oriT* region alone indicates that its *oriT* sequence is not identical to that of pCU1 but that it is probably more similar than the sequence of the R388 *oriT* (32). In this paper, we describe the formation of a hybrid *oriT* by in vivo site-specific recombination between the *oriT*s of pCU1 and pMUR274 in an effort to localize the *nic* site of pCU1. Sequences required for initiation and termination of transfer are also investigated.

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TABLE 1. Plasmids used in this study

| Plasmid | Description | Source |
|---|---|---|
| pUC18 | Ap$^r$ | 27 |
| pUC128 | Ap$^r$ | 15 |
| pNH-Kan/oriT | Ap$^r$ Km$^r$ (pUC1318 + Tn*5* Km) | 11 |
| pSPKm6 | Km$^r$ (pUC18 + Tn*5* Km) | This study |
| pCU1 | Sp$^r$ Ap$^r$ Sm$^r$ | 36 |
| pCU101 | Cm$^r$ (pACYC184), pCU1 *tra* | 36 |
| pBP1 | Ap$^r$ Km$^r$ (pMK2004), pCU1 *oriT* (1–325 bp) | 32 |
| pSP9 | Km$^r$ (pSPKm6), pCU1 *oriT* (1–266 bp) | This study |
| pSP10 | Km$^r$ (pSPKm6), pCU1 *oriT* (1–243 bp) | This study |
| pSP24 | Km$^r$ (pSPKm6), pCU1 *oriT* (1–288 bp) | This study |
| pSP28Km | Ap$^r$ (pUC18), Km$^r$ (Tn*5*), pCU1 *oriT* (1–325 bp) | This study |
| pSP29 | Km$^r$ (pSPKm6), pCU1 *oriT* (1–160 and 189–288 bp) | This study |
| pSP274 | Tc$^r$ (Tn*10*), pMUR274 *tra* | 32 |
| pMO2 | Ap$^r$ (pUC128), pMUR274 *oriT* (1.3-kb *Dra*I) | This study |
| pMO2R | Ap$^r$ (pUC128), pMUR274 *oriT* (474-bp *Rsa*I-*Ssp*I) | This study |
| pMKN | Ap$^r$ Km$^r$, pMUR274 and pCU1 *oriT*s | This study (Fig. 1) |
| pNKN | Ap$^r$ Km$^r$, wild-type and mutant pCU1 *oriT*s | This study (Fig. 4) |

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *E. coli* strains used in this study are DH5α [*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169*] (34) and HB101rif (F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-15 mtl-1* [spontaneous Rif$^r$ mutant of HB101]) (34).

The plasmids used in this study are listed in Table 1. pMO2 contains the pMUR274 *oriT* on a 1.3-kb *Dra*I fragment in the *Sma*I site of pUC128. pMO2R contains the internal *Rsa*I-*Ssp*I *oriT* fragment from pMO2. pMKN1A (Fig. 1) was constructed by cloning the pCU1 *oriT* from pBP1 on a *Cla*I-*Xho*I fragment into the *Cla*I-*Xho*I sites of pMO2Km (pMO2 with a *Sma*I fragment from pKan/oriT, carrying the Tn*5* Km gene, in the *Eco*RV site). pMKN2A (Fig. 1) was constructed from pMKN1A by inverting the *Eco*RI fragment carrying the pMUR274 *oriT*. To create pMKN1B (Fig. 1), the pMUR274 *oriT* was cloned on a *Bam*HI-*Hin*dIII fragment from pMO2 together with the Tn*5* Km gene on a *Bam*HI fragment from pNH-Kan/oriT into the *Bam*HI-*Hin*dIII sites of pSP28 (pUC18 carrying the 325-bp *oriT* fragment from pCU1 in the *Eco*RI and *Bam*HI sites). pMKN2B (Fig. 1) was constructed from pMKN1B by inverting the *Hin*dIII fragment carrying the pMUR274 *oriT*. pNKN#T derivatives (Fig. 2) were constructed by cloning the Tn*5* Km gene from pNH-Kan/oriT on a *Bam*HI-*Sma*I fragment together with the mutated pCU1 *oriT* on a *Pvu*II-*Hin*dIII fragment into pSP28 cut with *Hin*dIII and *Bam*HI. pNKN#I (Fig. 2) derivatives were constructed by cloning the Tn*5* Km gene from plasmid pNKN9T on an *Eco*RI-*Hin*dIII fragment together with the mutated pCU1 *oriT* on an *Eco*RI-*Hin*dIII fragment into the *Eco*RI site of pSP28.

**Growth, bacterial matings, and selective media.** Cultures were grown in LB medium (10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, each per liter) or on LBA (LB medium solidified with 1.5% agar). The following antibiotics were added to LBA from filter-sterilized stock solutions to yield the indicated concentrations (in micrograms per milliliter of medium): ampicillin, 30; chloramphenicol, 30; tetracycline, 10; kanamycin, 50; rifampin, 100. For all but the tetracycline, the antibiotics were used at one-half these concentrations in LB medium. Bacterial matings were conducted by filtering 1 ml of overnight recipient culture with 1 ml of 3- to 4-h donor culture through a 0.45-μm-pore-size filter. After 2 h of incubation at 37°C on an LBA plate, the filters were washed in 1 ml of saline and dilutions prepared in saline were plated on LBA containing antibiotics to select for the recipient and one of the plasmids. For spot matings, donor cultures were grown overnight on an LBA plate containing antibiotics and transferred by toothpick to 20 μl of a combination of overnight recipient culture and 3-h helper culture spotted onto the bottom of a sterile petri dish. Following mixing, 5 μl of the mixture was spotted onto a sterile 45-μm-pore-size filter on an LBA plate. A maximum of 15 spot matings were conducted on one 47-mm-diameter filter disc. The filter was incubated for 2 h at 37°C and then transferred to an LBA plate containing antibiotics selective for both the recipient and the plasmid marker.

**DNA manipulations.** Standard procedures were used for all DNA manipulations, including plasmid DNA extractions, restriction mapping, and molecular cloning. For sequencing double-stranded DNA, 7 μl of Wizard miniprep DNA and 20 pmol of primer were boiled for 5 min and quick chilled. Sequenase buffer, dithiothreitol, label mix, and [α-$^{32}$P]dATP were added as described in the manufacturer's instructions. After the addition of 1.5 U of Sequenase, the reactions were mixed, quick spun, and immediately added to the termination mixtures to incubate for 5 min at 37°C. Single-strand phagemid DNA was extracted with M13K07 helper phage as described by Vieira and Messing (38) and sequenced by the Sequenase protocol. Sequencing was done with reverse and forward pUC primers as well as primers specific for the pMUR274 *oriT* sequence.

**PCR with degenerate oligonucleotides.** Degenerate oligonucleotides were synthesized on a PCR mate synthesizer with chemicals from Applied Biosystems, Inc. In ORIT4 and ORIT4op, degeneracy was introduced at targeted A sites by use of a mixture of 90% A phosphoramidite and 3.3% of each of the other three phosphoramidites. Approximately 33% of the oligonucleotides were expected to contain a single mutation (34). The oligonucleotides were cleaved from the column, deprotected, and purified on an oligonucleotide purification column (Applied Biosystems, Inc.) as described in the manufacturer's instructions. Degeneracy of the T residue in ORIT4a was introduced by an equal mixture of A, G, and C phosphoramidite. The oligonucleotide was cleaved from the column and deprotected as described in the manufacturer's instructions.

The PCR was conducted in two sequential reactions. Separate halves of the pCU1 *oriT*, overlapping at the 10-bp junction sequence, were amplified in two 100-μl reaction mixtures containing 0.1 μg of pSP24 DNA, 2 U of Promega *Taq*, 1× reaction buffer supplied by the manufacturer, 0.2 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate, and 67 pmol of both a pUC and ORIT primer. The pUC forward (CCCAGTCACGACGTTG) primer was used together with either the ORIT4 (ATCCCTTAACCTGCTATACACCTAACGCA) or ORIT4a (CCTGCTATACACCTAACGC) primer, while the pUC reverse (TAACAATT TCACACAGGG) primer was used with the ORIT4op (CTGCGTTAGGTGT ATAGCAGGTTAAGGGAT) primer (degenerate bases are underlined). The reaction conditions consisted of an initial 1-min denaturation step at 94°C followed by 5 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. A final 5-min elongation period at 72°C completed the reaction. The PCR products of the first reactions were purified with a Centricon 100 column (Amicon) as described in the manufacturer's instructions. Forty to 50 μl of purified PCR product was collected. The entire pCU1 *oriT* was amplified in a second round of PCR containing the same components as the first, except that the pSP24 target DNA was replaced with a mixture of 10 μl of the ORIT4op and either ORIT4 or ORIT4a Centricon-purified PCR products from the first-round PCR and only 10

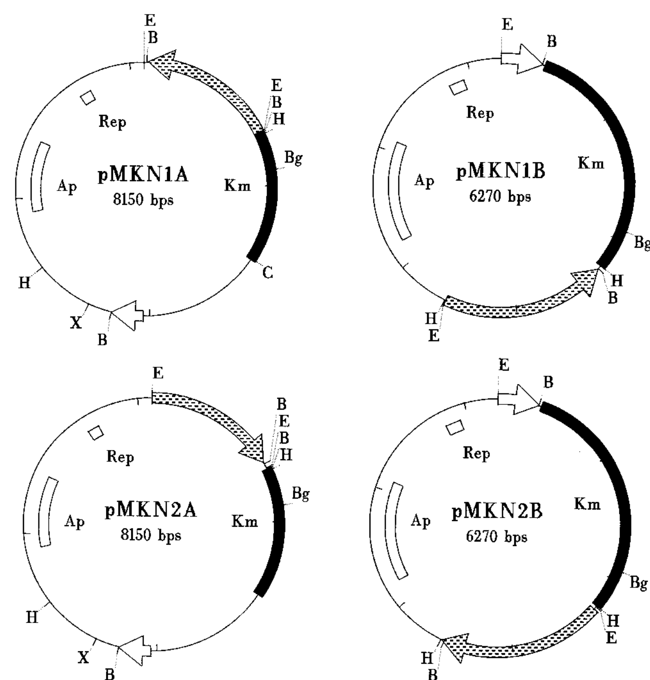


FIG. 1. Plasmid maps of pMKN double *oriT* constructs. Each plasmid is drawn to scale, but the scale varies with each plasmid. Open arrows represent the pCU1 *oriT* (325-bp *Rsa*I-*Bam*HI fragment), and dotted arrows represent the pMUR274 *oriT* sequence (1.3-kb *Dra*I fragment). The direction of the arrows corresponds to the orientation of the *oriT* sequence presented in the nucleotide alignment of Fig. 3. Solid regions represent the Km (kanamycin) gene from Tn*5*. Rep, plasmid replicon; Ap, ampicillin gene. Restriction sites relevant to construction of the plasmid (see Materials and Methods) are indicated by the following abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; C, *Cla*I; X, *Xho*I.

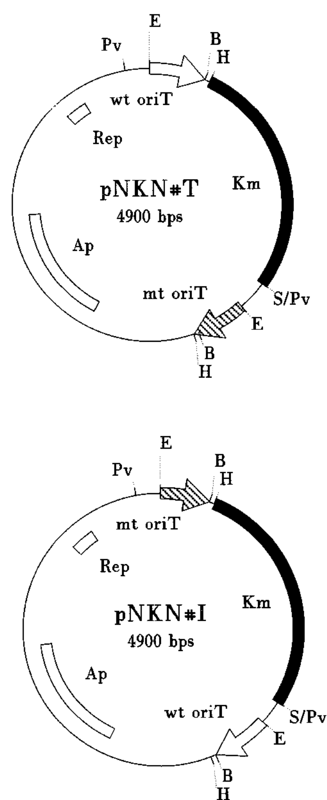


FIG. 2. Plasmid maps of pNKN double *oriT* derivatives. Wild-type (wt) pCU1 *oriT* is depicted by open arrows; mutant (mt) pCU1 *oriT* is represented by hatched arrows. Solid regions represent the kanamycin (Km) gene from Tn*5*. Rep, plasmid replicon; Ap, ampicillin gene. Restriction sites relevant to construction of the plasmid (see Materials and Methods) are indicated by the following abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sma*I; Pv, *Pvu*II.

pmol of each of the pUC forward and reverse primers in 50-μl reaction mixtures. The conditions of this PCR consisted of an initial 1-min denaturation step at 94°C, followed by 15 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. A single 5-min elongation step at 72°C finished the reaction.

**Nucleotide sequence accession number.** The sequence of the *Dra*I fragment containing the *oriT* sequence of pMUR274 has been assigned GenBank accession no. AF011924.

## RESULTS

**Cloning and sequencing the *oriT* region of pMUR274.** Prior to this study, there were no restriction or functional maps of plasmid pMUR274. Digestion of pSP274 (a Tra$^+$ Tn*10* derivative of pMUR274) with several restriction enzymes known to cut pCU1 demonstrated no comparable restriction fragments (data not shown). This is in contrast to the well-studied IncN plasmid R46 (and deletion derivative pKM101) which shares many restriction fragments with pCU1, particularly within the *tra* region (13). The size of the pMUR274 plasmid is approximately 40 kb.

Hybridization experiments using the 325-bp pCU1 *oriT* region to probe pSP274 restriction digests under low-stringency conditions produced a weak signal with a 1.3-kb *Dra*I fragment (data not shown). This fragment was cloned into the *Sma*I site of phagemid vector pUC128 to produce pMO2. pMO2 is mobilized efficiently by pSP274 (Table 2). Deletions into the 1.3-kb region cloned in pMO2 by use of internal restriction sites produced a clone, pMO2R, which carries a 474-bp *Rsa*I-*Ssp*I fragment from pMUR274. pMO2R is mobilized by pSP274 as efficiently as pMO2 (see Table 2), indicating that the 474 bp represents the minimal pMUR274 *oriT* region.

The nucleotide sequence of the 474-bp pMUR274 *oriT* region was determined and aligned with the *oriT* sequences of pCU1 and R388 (Fig. 3). The identity between the pCU1 and R388 *oriT* sequences has been described previously (18). The overall sequence identity between the pCU1 and pMUR274 *oriT* regions is 57% for the 266-bp sequence carried by the deletion derivative pSP9 found to carry the minimal pCU1 *oriT* region (Table 2). The identity for this region is slightly higher between pCU1 and R388, at 34.5%, than between pMUR274 and R388, at 28%. As described below, the *nic* sites of pCU1 and pMUR274 lie within a 30-bp sequence with over 80% identity between the three sequences.

All three plasmids contain a 10-bp inverted repeat (IR) at the left-most end of the sequence which shows some sequence conservation in pCU1 and pMUR274 (7 bases) but little in R388 (2 or 4 bases with the pCU1 and pMUR274 sequences, respectively). Each of the three plasmids has additional IRs to the right of *nic*; however, these IRs show little organizational or sequence conservation. In addition to the IR sequences, all three plasmids contain two directly repeated sequences, 9 bp long in pCU1 and 8 bp long in both pMUR274 and R388 with the conserved sequence CCTTAA. While these two direct repeats are in comparable locations in R388 and pMUR274, only the second repeat, directly to the left of *nic*, aligns in all three plasmids.

A major difference between the IncN and IncW *oriT* sequences is the presence of a large AT-rich region in both the pCU1 and pMUR274 sequences (99 bp in pCU1 and 70 bp in pMUR274) which is missing in the R388 *oriT*. Not only is there no corresponding AT-rich sequence in the IncW *oriT*, it was necessary to introduce a large gap in the R388 sequence in this region to maintain the sequence alignment.

One final observation which can be made with respect to the pMUR274 sequence is the lack of iterons directly to the left of the *oriT* sequence shown in Fig. 3. Both pCU1 and R388 have a series of 11-bp iterons (13 in pCU1 and 5 in R388) to the left of the *oriT* sequences shown in Fig. 3; however, the sequence of these iterons does not have any identity between the two plasmids (18). The lack of iterons to the left of the pMUR274 *oriT* supports an earlier observation that the iterons do not play a significant role in the transfer of pCU1 (32).

**Formation of a hybrid pCU1-pMUR274 *oriT*.** For the formation of a hybrid pCU1-pMUR274 *oriT*, four plasmids (designated pMKN) were constructed, each containing one copy of each of the pCU1 and pMUR274 *oriT* regions separated by the kanamycin gene from Tn*5* as shown in Fig. 1. The 1 and 2 versions of the plasmid are identical except that the two *oriT* regions are in opposite orientations relative to each other (based on sequence identity). The A and B versions of the

TABLE 2. Mobilization of pCU1 and pMUR274 *oriT* derivatives

| *oriT* plasmid | Conjugative plasmid | Mobilization frequency[a] |
|---|---|---|
| pMO2 | pSP274 | $1.0 \times 10^{0}$ |
| pMO2R | pSP274 | $1.2 \times 10^{0}$ |
| pSP24 | pCU101 | $1.1 \times 10^{-1}$ |
| pSP9 | pCU101 | $8.3 \times 10^{-2}$ |
| pSP10 | pCU101 | $1.5 \times 10^{-5}$ |
| pSP29 | pCU101 | $1.1 \times 10^{-6}$ |

[a] Expressed as the number of transconjugants receiving the *oriT* plasmid per transconjugant receiving the conjugative plasmid. Results are the average of at least two trials.

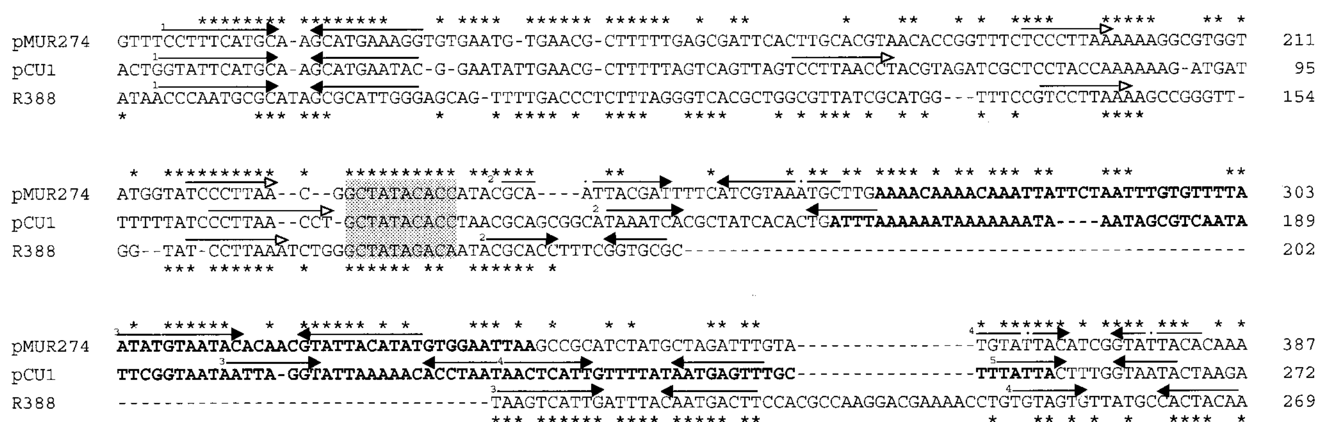


FIG. 3. Nucleotide sequence alignment of the *oriT* region of pCU1 with that of pMUR274 and R388. Numbering is such that nucleotide 1 is the *Rsa*I site at the left end of the pCU1 *oriT*, the *Ssp*I site at the left end of the pMUR274 *oriT*, and bp 1 of the R388 *oriT* sequence designated by Llosa et al. (18). Matches between pCU1 and pMUR274 are indicated by asterisks above the sequences, while matches between pCU1 and R388 are indicated by asterisks below the sequences. IRs are indicated by closed arrows above each sequence. IRs are numbered to the left of each repeat. Direct repeats are indicated by open arrows. AT-rich sequences of pCU1 and pMUR274 are in bold type. The shaded sequence indicates the 10-bp junction sequence found in pCU1-pMUR274 hybrid *oriT*s.

plasmid are similar except that the two *oriT*s are in opposite orientations with respect to the plasmid replicon and ampicillin gene.

Each of the four pMKN derivatives were mobilized by either pCU101, pSP274, or both conjugative systems together into recipient HB101rif. The resultant Rif$^r$ Ap$^r$ transconjugants were screened for sensitivity to kanamycin to detect those transconjugants carrying a plasmid which had undergone a site-specific recombination event between the two *oriT*s during transfer. The results presented in Table 3 indicate that Ap$^r$ Km$^s$ transconjugants were obtained with both constructs in which the pCU1 and pMUR274 *oriT* regions are in the same orientation relative to each other (pMKN2A and pMKN2B) but not in the constructs in which the *oriT*s are in opposite orientation relative to each other (pMKN1A and pMKN1B).

While pMKN2A produced equal numbers of Km$^s$ recombinants in the presence of either conjugative plasmid and twice the number when both were present, pMKN2B produced a much greater number of Km$^s$ derivatives when mobilized by pCU101 alone than when both plasmids were present in the donor. No Km$^s$ recombinants were produced when pMKN2B was mobilized by pSP274. These results are discussed below in terms of the mechanism of hybrid formation.

To determine the location of the site-specific recombination event, the *oriT* regions of 11 pMKN2AKm$^s$ and 8 pMKN2BKm$^s$ derivatives were sequenced. The isolates were randomly selected from nine independent matings involving each of the five different donors that produced Km$^s$ transconjugants. All of the pMKN2AKm$^s$ *oriT*s demonstrated the same sequence as did all of the pMKN2BKm$^s$ *oriT*s. Both the 2AKm$^s$ and the 2BKm$^s$ *oriT* regions were hybrids of the two parental *oriT*s; however, each contained opposite halves of the parental *oriT*s due to their position relative to the Ap gene and plasmid replicon. In both cases, the junction between the two sequences occurred within the same 10-bp sequence, GCTATACACC, found in both parental sequences (Fig. 3). We conclude that *nic* lies within or at either end of this 10-bp junction.

**Mobilization of the hybrid N *oriT*.** As indicated in Table 4, neither the pCU1 nor the pMUR274 transfer system is able to mobilize a plasmid carrying the *oriT* of the other, suggesting that the Mob proteins from one plasmid are unable to interact with the *oriT* of the other. To test whether either parental plasmid is capable of mobilizing the hybrid *oriT* plasmids, representatives of both the pMKN2AKm$^s$ and pMKN2BKm$^s$ hybrid *oriT* derivatives were mobilized by either pCU101, pSP274, or both conjugative plasmids together. The results shown in Table 4 indicate that each hybrid is mobilized efficiently only by the transfer system corresponding to the right-hand portion of the hybrid *oriT*.

**Mutagenesis of the *nic* junction.** In an attempt to identify nucleotides within the sequence surrounding *nic* that are important for transfer to occur, a 20-bp region encompassing the 10-bp junction sequence was mutagenized with degenerate oligonucleotides and PCR as described in Materials and Methods. The PCR product containing the mutagenized *oriT* region was cloned into the *Eco*RI-*Bam*HI sites of pSPKm6 (a Km$^r$ pUC18 derivative), and recombinant derivatives were tested for their ability to be mobilized by pCU1.

Fifteen unique mutations were isolated, six within the 10-bp junction sequence (Fig. 4). Mobilization experiments demonstrated that of the six mutations within the 10-bp junction, two (*nic3* and *nic16*) reduced transfer more than 1,000-fold, three

TABLE 3. Formation of Km$^s$ transconjugants during mobilization of pMKN derivatives by pCU101 and pSP274

| *oriT* plasmid | Conjugative plasmid(s) | Transconjugants[a] | |
|---|---|---|---|
| | | No. of Km$^s$/ no. of Ap$^s$ | % Km$^s$ |
| pMKN1A | pCU101 | 0/200 | <0.5 |
| | pSP274 | 0/200 | <0.5 |
| | pCU101, pSP274 | 0/200 | <0.5 |
| pMKN2A | pCU101 | 6/300 | 2.0 |
| | pSP274 | 4/200 | 2.0 |
| | pCU101, pSP274 | 14/300 | 4.7 |
| pMKN1B | pCU101 | 0/200 | <0.5 |
| | pSP274 | 0/200 | <0.5 |
| | pCU101, pSP274 | 0/200 | <0.5 |
| pMKN2B | pCU101 | 50/200 | 25.0 |
| | pSP274 | 0/200 | <0.5 |
| | pCU101, pSP274 | 10/200 | 5.0 |

[a] Results are the average of at least two trials.

TABLE 4. Mobilization of pCU1 and pMUR274 wild-type and hybrid *oriT* plasmids by pCU101 and pSP274

| Hybrid *oriT* plasmid | Conjugative plasmid | Mobilization frequency[a] |
|---|---|---|
| pSP28Km | pCU101 | $1.3 \times 10^{0}$ |
| | pSP274 | $8.2 \times 10^{-7}$ |
| pMO2Km | pCU101 | $1.4 \times 10^{-6}$ |
| | pSP274 | $1.0 \times 10^{0}$ |
| pMKN2AKm$^s$ | pCU101 | $2.2 \times 10^{0}$ |
| | pSP274 | $<3.2 \times 10^{-5}$ |
| | pCU101, pSP274 | $2.0 \times 10^{0}$, $8.8 \times 10^{-1}$ |
| pMKN2BKm$^s$ | pCU101 | $<4.5 \times 10^{-4}$ |
| | pSP274 | $5.0 \times 10^{-2}$ |
| | pCU101, pSP274 | $1.1 \times 10^{0}$, $1.5 \times 10^{-1}$ |

[a] Mobilization frequencies are given as the number of transconjugants receiving the hybrid *oriT* plasmid per transconjugant receiving the conjugative plasmid. Where two frequencies are given, they correspond to the conjugative plasmids shown in the preceding column, respectively. Results are the average of matings with three randomly selected Km$^s$ isolates.

(*nic109*, *nic238*, and *nic311*) did so by less than 30-fold, and one (*nic325*) increased transfer 5-fold. Although *nic16* and *nic238* are different mutations of the same A residue, the A→T transversion reduced transfer less than the A→G transition, suggesting that the AT content may be important in this region. Similarly, *nic311* and *nic325* are mutations of the same T residue where the T→C transition increased transfer slightly while the T→G transversion reduced transfer.

Mutations to either side of the 10-bp junction (*nic315*, *nic320*, *nic188*, *nic326*, *nic11*, and *nic211*) had little or no effect on transfer (less than 10-fold reduction). Mutations were also isolated to the right of the 10-bp junction outside the targeted region presumably as a result of incorporation errors during PCR (*nic59*, *nic121*, and *nic15*); however, none of these reduced transfer more than 10-fold. Loewen and Switala (22) demonstrated an increased incidence of misincorporation during PCR next to the 3′ end of the primer when a hairpin loop structure was located just downstream of the primer.

**Locating *nic* by intramolecular site-specific recombination.** Just as it was possible to localize *nic* to the 10-bp junction in the hybrid pCU1-pMUR274 *oriT* due to sequence differences between the two parental sequences directly to the left and right of the GCTATACACC junction, it was possible to further localize *nic* within the 10-bp sequence by examining the sequence of junctions created by site-specific recombination between one wild-type pCU1 *oriT* and a second pCU1 *oriT* carrying one of the mutations introduced into the 10-bp *nic* sequence by oligonucleotide PCR mutagenesis.

Double *oriT* derivatives containing one wild-type pCU1 *oriT* and one mutated pCU1 *oriT* separated by the Km gene were constructed (Fig. 2). Derivatives with the mutated *oriT* in the position in which transfer would be initiated were designated pNKN#I, while those with the mutation in the *oriT* in which transfer would be terminated were designated pNKN#T. The # in each case corresponds to the number of the *oriT* mutant derivative used in the construction. Km$^s$ hybrid *oriT*s formed from I derivatives but not T derivatives should carry the mutation if it lies to the left of *nic*. The opposite would be true of mutations which lie to the right of *nic*; i.e., Km$^s$ *oriT* hybrids from the T derivatives would carry the mutation while those from I derivatives would not.

pNKN3I and pNKN3T were constructed from the nic3 mutant, while pNKN311I and pNKN311T were constructed from the nic311 mutant. Following mobilization of the pNKN3I and T derivatives, Ap$^r$ Km$^s$ transconjugants (those carrying recombinant plasmids) were screened for the *nic3* mutations by triparental spot matings with helper plasmid pCU101. Since the

```
A
5'         **      * * *   **
    ATCCCTTAACCTGCTATACACCTAACGCA    ORIT4, ORIT4a
    TAGGGAATTGGACGATATGTGGATTGCGTC   ORIT4op
3'       **    *  * *     *

B
                                                Mutant    Mob Freq
5'
TTTTATCCCTTAACCTGCTATACACCTAACGCAGGCGCATAAATCA  pSP24     1.4x10^-1
         A.    .   ... .  ..    . .....         nic320    8.3x10^-2
          C    .   ... .  ..    . .....         nic315    9.9x10^-2
               G   ... .  ..    . .....         nic188    1.8x10^-1
                   G.. .  ..    . .....         nic109    2.7x10^-2
                    C. .  ..    . .....         nic325    9.1x10^-1
                    G. .  ..    . .....         nic311    8.9x10^-3
                     G .  ..    . .....         nic3      1.1x10^-4
                       G  ..    . .....         nic16     6.1x10^-6
                       T  ..    . .....         nic238    4.5x10^-3
                          A.    . .....         nic326    9.5x10^-2
                           G    . .....         nic11     1.5x10^-1
                           C    . .....         nic211    1.3x10^-2
                                . .A...         nic59     3.1x10^-2
                                C .A..G         nic121    2.5x10^-2
                                  TGCG          nic15     3.1x10^-2
```

FIG. 4. Point mutations within the pCU1 *oriT*. (A) Sequences for the complementary degenerate oligonucleotide primers ORIT4, ORIT4a (bold type), and ORIT4op used in the generation of the mutated *oriT* DNA. Adenine residues targeted for mutation in ORIT4 and ORIT4op are indicated by asterisks; the T residue targeted in ORIT4a is underlined. (B) Upper strand of the targeted and surrounding sequence. Above the sequence, the second direct repeat is indicated by an open arrow, and the left arm of $IR_2$ is indicated by a closed arrow. The 10-bp hybrid *oriT* junction sequence is underlined. Mutations are indicated below the sequence. Mutant designations together with the mobilization frequency of each mutant are indicated on the right. The frequency is the average of at least two trials and represents the number of transconjugants that received the antibiotic marker of the *nic* derivative per transconjugant receiving the antibiotic marker of the conjugative helper plasmid pCU1.

TABLE 5. Recombination frequencies of pNKN derivatives

| Double *oriT* pNKN derivative[a] | Transconjugants[b] | |
|---|---|---|
| | No. of Km$^s$/no. of Ap$^r$ | % Km$^s$ |
| 9T | 183/200 | 91.5 |
| 9I | 182/200 | 91.0 |
| 10T | 190/200 | 95.0 |
| 10I | 7/200 | 3.5 |
| 29T | 0/200 | <0.5 |
| 29I | 3/200 | 1.5 |
| 3T | 23/345 | 6.7 |
| 3I | 75/288 | 26.0 |
| 238T | 81/396 | 45.7 |
| 238I | 159/292 | 40.6 |
| 311T | 117/200 | 58.5 |
| 311I | 167/200 | 83.5 |
| 109I | 99/200 | 49.5 |

[a] T derivatives carry a mutation in the termination *oriT*; I derivatives carry a mutation in the initiation *oriT* (Fig. 2). pNKN9 contains two wild-type *oriT*s (Table 1); pNKN10 and pNKN29 contain one *oriT* with a deletion (Table 1); pNKN3, -238, -311, and -109 contain one *oriT* with a point mutation close to *nic* (Fig. 4).

[b] Results are from at least two independent matings.

*nic3* mutation reduces transfer significantly, those recombinants carrying the *nic3* mutation transferred at much lower frequencies than those which did not contain the *nic3* mutation. Ap$^r$ Km$^s$ transconjugants from the pNKN311I and T matings were screened for the *nic311* mutation by restriction digest since this mutation introduces an *Bfa*I site into the *nic* junction.

While none of the 16 pNKN3I transconjugants tested (from two separate matings) carried the *nic3* mutation, all 16 of the pNKN3T transconjugants tested (from two separate matings) did carry the mutation, indicating that *nic* lies to the left of the *nic3* mutation. The presence of the *nic311* mutation (*Bfa*I site) in all of the 18 pNKN311I transconjugants tested (from two separate matings) and its absence from all of 18 pNKN311T transconjugants tested (from two separate matings) indicate that *nic* lies to the right of this mutation. The presence or absence of the mutations in the plasmids from one transconjugant from each mating was confirmed by sequencing. Together, these results indicate that *nic* lies between the *nic311* and *nic3* mutations, between the second T and the second A residue in the 10-bp junction sequence GCTAT ACACC.

**Initiation and termination within *oriT*.** The frequency of site-specific recombination between mutant and wild-type *oriT* sequences in the double-*oriT* constructs described above was also used to indicate whether the mutation affects initiation of transfer or termination. Mutations that reduce recombination frequency when located in the *oriT* nicked first (as in constructs designated pNKN I) represent sites important for initiation, while those which reduce recombination when located in the *oriT* nicked second (as in constructs designated pNKN T) represent sites that affect termination. Point mutations within the *nic* region as well as a 28-bp deletion (*Dra*I-*Ssp*I deletion) within the AT-rich region (from pSP29) and deletion of the rightmost IR (IR$_5$) (from pSP10) were tested for their effect on initiation and termination.

The frequency of recombination observed with the various pNKN derivatives, as measured by the percentage of Km$^s$ transconjugants following mobilization by pCU101, is presented in Table 5. As evidenced by the pNKN9I and pNKN9T constructs in which both *oriT*s contain wild-type sequences, termination at the second *oriT* occurs in 91% of the transferred DNA strands. This is almost four times greater than the recombination frequency observed between the pCU1 and pMUR274 *oriT*s during mobilization by pCU101 (Table 3, pMKN2B). The difference reflects the effect of nonconserved sequences on termination.

Both of the point mutations to the left of *nic* (*nic109* and *nic311*) affected termination, reducing the number of Km$^s$ transconjugants by half; however, *nic311* had little effect on initiation (*nic109* was not tested). By contrast, both mutations to the right of *nic* (*nic3* and *nic238*) affected both termination and initiation. Of these two, the mutation closest to *nic* (*nic3*) had a more-pronounced effect than the mutation 2 bases further to the right (*nic238*). Also, while the *nic3* mutation had a more-pronounced effect on termination than on initiation, the *nic238* mutation affected initiation and termination equally.

As shown in Table 2, deletion of IR$_5$ (as in pSP10) or a 28-bp deletion of 28 bp within the AT-rich region (as in pSP29) results in a significant reduction in mobility. When these mutated *oriT*s were tested in the double-*oriT* constructs, deletion of IR$_5$ reduced site-specific recombination only when located in the termination *oriT* (pNKN10T), while the deletion in the AT-rich region affected both initiation and termination (pNKN29I and -T).

## DISCUSSION

The N-type *oriT* of plasmid pMUR274 was cloned on a 474-bp *Rsa*I-*Ssp*I fragment and sequenced. Alignment of this sequence with that of the pCU1 *oriT* demonstrated over 50% identity. Intramolecular, site-specific recombination between the pCU1 and pMUR274 *oriT*s resulted in the formation of a hybrid *oriT* containing opposite halves of each parental *oriT*. The junction between the pCU1 and pMUR274 *oriT* sequences, containing *nic*, occurred within or at either end of the 10-bp sequence GCTATACACC common to both parental sequences. Similar recombination events between directly repeated copies of wild-type and mutated pCU1 *oriT* sequences further located *nic* within the 10-bp junction to the right of the second T residue, between nucleotides 117 and 118 from the *Rsa*I site which defines the left end of *oriT*. The lack of any variation in the sequence of the junctions in any of the hybrid *oriT*s indicates that a unique site is nicked, that this site is the same in both pCU1 and pMUR274, and that mutations within the sequence surrounding *nic* do not alter its location. The use of a RecA$^-$ background, combined with the fact that no other junction sequences were observed despite extended sequences of homology between the two *oriT* sequences as well as other sequences outside *oriT*, supports the conclusion that this is a *recA*-independent, site-specific recombination event.

Consistent with the homology described between the Mob proteins of the IncF and IncW transfer systems (20), the 10-bp pCU1-pMUR274 junction sequence surrounding *nic* has sequence identity at 8 and 7 bases of the sequence surrounding the *nic* sites of R388 and F, respectively (Fig. 5). These se-

```
pCU1/pMUR274   G  G  T  G  T  A  T  A  G  C
                            ^
R388           t  G  T  c  T  A  T  A  G  C
                            ^
F              G  G  T  G  T  g  g  t  G  C
                            ^
```

FIG. 5. Alignment of pCU1, R388, and F sequences surrounding *nic*. The sequences represent the nicked strands (complementary to that presented in Fig. 3 in the case of pCU1 and pMUR274) in the 5′-3′ orientation. Bases in the R388 and F sequence that do not match the pCU1 or pMUR274 sequence are shown in lowercase letters. Carets indicate the location of *nic* in the IncN plasmids pCU1 and pMUR274 (as determined in this study) and the *nic* sites in the F plasmid (37) and the IncW plasmid R388 (21).

quences align such that the *nic* sites of F and R388 correspond to the putative pCU1 *nic* site identified in this study. The *tra* region of IncN plasmid R46 shows conserved restriction sites with the *tra* region of pCU1 (13) and has 100% sequence identity with the *oriT* region of pCU1 (6) and near 100% sequence identity with the *mob* region which has been sequenced to date (33). As shown for other conjugative plasmids, transfer of R46 occurs such that the *tra* genes enter the recipient last (5). Assuming that transfer occurs in the 5′-3′ direction as predicted for all other transfer systems studied to date (17), it would be the strand complementary to that shown in Fig. 3, as depicted in Fig. 5, that is nicked in the pCU1 *oriT*. This agrees with findings for both F and R388 plasmids (21, 37). While this supports the observation that the IncN *mob* region is closely related to the IncW and IncF regions, comparisons of the sequence required for mating pair formation during transfer demonstrate that the IncN and IncW regions are more closely related to the *vir* system of *Agrobacterium* (7, 14).

Of the point mutations generated within the sequence surrounding *nic*, only two to the right of *nic* (which represents the 3′ side of the nicked strand) reduced transfer significantly. Mutation of the base representing the 5′ end of the nicked strand resulted in less than a 20-fold reduction in transfer. Other more-distant mutations on both the 5′ and 3′ sides had little or no effect on transfer. That the two bases in the R388 sequence that do not match the pCU1 and pMUR274 sequences lie to the right of *nic* may explain the lack of success in attempts to obtain a R388-pCU1 hybrid *oriT* by site-specific recombination (19, 31). Considering the sequence conservation to the right of *nic* in pCU1 and the F plasmid, it may be possible to create a hybrid between these two *oriT*s.

Finding that the bases on the 3′ side of *nic* are more important to transfer than those on the 5′ side is consistent with results obtained with other systems. In the IncP and IncQ plasmids, mutations within the 8 bases of the 3′ end of the nicked strand between *nic* and an IR result in the greatest reduction in transfer. This region has been implicated in recognition by the nicking enzyme (30, 39, 42). Similarly, two point mutations 4 and 8 bp from the 3′ side of the F *nic* result in significant reductions, while several 6-bp substitutions in the 5′ side of *nic* had little or no effect on transfer (24). The fact that the *nic3* mutation affected termination more severely than initiation is also similar to results observed with an F point mutation 4 bases on the 3′ side of *nic* (10) and with point mutations in the IncQ plasmid R1162 (2).

The selective ability of each parental plasmid to mobilize only one of the hybrid *oriT*s raises the question of how recombination between the *oriT* regions occurs in the presence of only one of the conjugative plasmids. Site-specific recombination in the presence of only one parental *tra* system would suggest that plasmid specificity is a function of the ability of the Mob protein(s) to bind to the *oriT* and not one of nicking-sealing ability. By comparison to other systems, the relaxosome protein(s) would bind to and initiate transfer from the analogous *oriT*. The nicking protein would remain bound to the 5′ end of the nicked DNA and unwind the nicked strand in the 5′-3′ direction (12, 25, 29). The bound protein would then nick the second, nonself *oriT* due to the similarity in the DNA sequence surrounding the *nic* site and seal the two ends to terminate the recombination event.

Production of a hybrid *oriT* from the pMKN derivatives can occur only when transfer is initiated at one *oriT* on one side of the plasmid replicon and the DNA is unwound through the replicon and ampicillin gene and then terminated in the second *oriT* on the other side of the replicon. Initiation of transfer in one *oriT* followed by unwinding of the DNA through the Km gene and termination in the other *oriT* would produce a nonviable molecule due to the lack of a functional replicon. This arrangement can help to explain the variation in recombination frequencies of the two pMKN derivatives in the presence of each parental plasmid. In the case of pMKN2B, viable recombinants would be observed when transfer initiated in the pCU1 *oriT* was followed by transfer in the counterclockwise direction and termination in the pMUR274 *oriT*. pCU101, a derivative of pACYC184, has a higher copy number than pSP274, a Tn*10* insertion derivative of naturally occurring pMUR274 (31). As a result, pCU101 would produce a higher concentration of Mob proteins than pSP274, and hence, transfer initiation could proceed at a higher rate. This would explain the high recombination frequency (25%) of pMKN2B in the presence of pCU101 (Table 3). In pMKN2A, recombinant formation would be initiated by nicking within the pMUR274 *oriT*, and due to the lower copy number of plasmid pSP274, fewer Mob proteins would be available for this initiation and hence site-specific recombination would occur at a lower frequency (2%) (Table 3).

This arrangement also explains why pMKN2A recombinants were observed in the presence of either parental transfer system alone but pMKN2B recombinants were observed only if pCU101 was present in the donor. If the Mob proteins of each transfer system are able to bind only with the analogous *oriT*, then pMKN2A recombinants should readily occur in the presence of the pMUR274 transfer system by initiation within the pMUR274 *oriT* and termination within the pCU1 *oriT*. For pMKN2AKm$^s$ recombinants to occur in the presence of pCU101 alone, the Mob proteins would first have to bind to the pCU1 *oriT*, nick it, unwind the DNA in the counterclockwise direction, terminate transfer by nicking the pMUR274 *oriT*, and produce a nonviable circular molecule containing the kanamycin gene and no replicon. The pCU101 Mob proteins could then continue unwinding the DNA from the nick in the pMUR274 *oriT* and proceed counterclockwise to terminate transfer within the pCU1 *oriT*, producing a viable recombinant plasmid containing the replicon and the ampicillin gene. Due to the increased chance of dissociation of the Mob protein from the plasmid, this double initiation event would presumably occur at a reduced frequency. It is assumed that the higher concentration of Mob protein molecules, due to the higher copy number of pCU101, compensates for the lower efficiency of the double initiation event, and hence we observe comparable recombination frequencies for pMKN2A in the presence of either conjugative plasmid. By comparison, in the case of pMKN2BKm$^s$, recombinant molecules would most easily be formed by the initiation of transfer within the pCU1 *oriT* followed by termination in the pMUR274 *oriT* by the pCU101 Mob proteins. In the presence of pMUR274, recombinant pMKN2B molecules would require the less-efficient double initiation process; however, due to the low copy number of pSP274, this event is not observed.

In all systems studied to date, the binding of the small Mob protein involved in nicking to the 3′ side of the nicked strand is plasmid specific (8, 41). The findings presented in this paper suggest that this is probably also the case for the IncN transfer systems. The ability of each of the pCU1 and pMUR274 transfer systems to mobilize only the hybrid *oriT* plasmid carrying the analogous right-hand portion of *oriT* indicates that plasmid specificity is dictated by the right-hand portion of *oriT*.

In each of the IncP, IncQ, and IncI1 *oriT*s, there is a large IR sequence 8 bp to the 3′ side of the nicked strand which has been shown to bind the smaller of the two Mob proteins involved in nicking (8, 9, 35, 43). However, while the pMUR274 plasmid contains an 11-bp imperfect IR 10 bases to the right of

*nic*, the closest IR to *nic* in pCU1 is $IR_2$, which is only 7 bp in length and a full 18 bp away from *nic*. A further argument against this IR acting as a protein-binding site is the fact that in the more closely related F plasmid, an IR sequence 10 bp from the 3′ end of *nic* has not been implicated in Mob protein binding.

A second candidate for a protein-binding site is $IR_5$ at the far right side of *oriT*, the deletion of which reduces transfer to background levels, indicating that it plays a significant role in transfer. Moreover, deletion of $IR_5$ affects initiation of transfer but not termination. Initiation involves both binding of the Mob proteins as well as nicking, while termination involves nicking and sealing but not binding, since the nicking enzyme presumably remains bound to the 5′ end of the nicked strand during unwinding.

A third candidate for a Mob protein-binding site is the AT-rich sequence of pCU1. Deletion of 28 bp within this region also reduces transfer to background levels. The TraY protein of the F plasmid binds at two sites at the distal end of the AT-rich region which begins 11 bases from the 3′ end of the nicked strand and extends approximately 80 bp (16, 23, 26). By comparison, the AT-rich sequence of the IncN *oriT* begins almost 40 bp from *nic* and extends to $IR_5$. In the F plasmid, deletion of the distal portion of the AT-rich sequence containing the TraY-binding sites affects initiation of transfer but not termination, while deletions up to 30 bases on the 3′ side of *nic* affect both functions (10). These results are similar to the situation in pCU1, where deletion of the last IR affects initiation only while deletion of part of the AT sequence closer to *nic* affects both initiation and termination.

Binding of Mob proteins in pCU1 presents an interesting question. The closer relationship of pCU1 to the F plasmid than to the IncP, -Q, or -I plasmids suggests that the AT-rich sequence and/or the $IR_5$ is a better candidate for Mob protein-binding sites than $IR_2$ next to *nic*. Should the binding site be located within the AT-rich sequence, the lack of such a sequence in the more closely related IncW plasmid R388 would indicate a significant difference between these two systems and might also help to explain the inability to isolate a R388-pCU1 hybrid *oriT*. On the other hand, should $IR_5$ be the binding site, its close proximity to the start codon of the *traK* gene (61 bp) might suggest that binding not only initiates transfer but also regulates expression of downstream transfer genes. Preliminary studies of this possibility suggest that the TraK protein does regulate expression of the *traK* gene.

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## REFERENCES


1. **Barlett, M. M., M. J. Erickson, and R. J. Meyer.** 1990. Recombination between directly repeated origins of conjugative transfer cloned in M13 bacteriophage DNA models ligation of the transferred plasmid strand. Nucleic Acids Res. **18:**3579–3586.
2. **Bhattacharjee, M., X.-M. Rao, and R. J. Meyer.** 1992. Role of the origin of transfer in termination of strand transfer during bacterial conjugation. J. Bacteriol. **174:**6659–6665.
3. **Bhattacharjee, M. K., and R. J. Meyer.** 1993. Specific binding of MobA, a plasmid-encoded protein involved in the initiation and termination of conjugal DNA transfer, to single-stranded oriT DNA. Nucleic Acids Res. **21:** 4563–4568.
4. **Brasch, M. A., and R. J. Meyer.** 1987. A 38-base-pair segment of DNA is required in *cis* for conjugative mobilization of broad-host-range plasmid R1162. J. Mol. Biol. **198:**361–369.
5. **Coupland, G. M.** 1984. The conjugation system and insertion sequences of the IncN plasmid R46. Ph.D. thesis, University of Edinburgh, Edinburgh, United Kingdom.
6. **Coupland, G. M., A. M. C. Brown, and N. S. Willetts.** 1987. The origin of transfer (*oriT*) of the conjugative plasmid R46: characterization by deletion analysis and DNA sequencing. Mol. Gen. Genet. **208:**219–225.
7. **Farrand, S. K., I. Hwang, and D. Cook.** 1996. The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J. Bacteriol. **178:**4233–4247.
8. **Furste, J. P., W. Pansegrau, G. Ziegelin, M. Kroger, and E. Lanka.** 1989. Conjugative transfer of promiscuous IncP plasmids: interaction of plasmid-encoded products with the transfer origin. Proc. Natl. Acad. Sci. USA **86:** 1771–1775.
9. **Furuya, N., and T. Komano.** 1995. Specific binding of the NikA protein to one arm of 17-base-pair inverted repeat sequences within the *oriT* region of plasmid R64. J. Bacteriol. **177:**46–51.
10. **Gao, Q., Y. Luo, and R. C. Deonier.** 1994. Initiation and termination of DNA transfer at F plasmid *oriT*. Mol. Microbiol. **11:**449–458.
11. **Hengen, P. N., and V. N. Iyer.** 1992. DNA cassettes containing the origin of transfer (*oriT*) of two broad-host-range transfer systems. BioTechniques **13:**58–62.
12. **Inamoto, S., Y. Yoshioka, and E. Ohtsubo.** 1991. Site- and strand-specific nicking *in vitro* at *oriT* by the TraY-TraI endonuclease of plasmid R100. J. Biol. Chem. **266:**10086–10092.
13. **Iyer, V. N.** 1989. IncN group plasmids and their genetic systems, p. 165–183. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press Ltd., London, United Kingdom.
14. **Kado, C. I.** 1993. *Agrobacterium*-mediated transfer and stable incorporation of foreign genes in plants, p. 243–254. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
15. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Tollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
16. **Lahue, E. E., and S. W. Matson.** 1990. Purified *Escherichia coli* F-Factor TraY protein binds at *oriT*. J. Bacteriol. **172:**1385–1391.
17. **Lanka, E., and B. M. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64:**141–169.
18. **Llosa, M., S. Bolland, and F. de la Cruz.** 1991. Structural and functional analysis of the origin of conjugal transfer of the broad-host-range IncW plasmid R388 and comparison with the related IncN plasmid R46. Mol. Gen. Genet. **226:**467–472.
19. **Llosa, M., S. Bolland, and F. de la Cruz.** 1994. Conjugation-independent, site-specific recombination at the *oriT* of the IncW plasmid R388 mediated by TrwC. J. Bacteriol. **176:**3210–3217.
20. **Llosa, M., S. Bolland, and F. de la Cruz.** 1994. Genetic organization of the conjugal DNA processing region of the IncW plasmid R388. J. Mol. Biol. **235:**448–464.
21. **Llosa, M., G. Grandoso, and F. de la Cruz.** 1995. Nicking activity of TrwC directed against the origin of transfer of the IncW plasmid R388. J. Mol. Biol. **246:**54–62.
22. **Loewen, P. C. and J. Switala.** 1995. Template secondary structure can increase the error frequency of the DNA polymerase from *Thermus aquaticus*. Gene **164:**59–63.
23. **Luo, Y., Q. Gao, and R. C. Deonier.** 1994. Mutational and physical analysis of F plasmid *traY* protein binding to *oriT*. Mol. Microbiol. **11:**459–469.
24. **Luo, Y., Q. Gao, and R. C. Deonier.** 1995. Boundaries of the nicking region for the F plasmid transfer origin, *oriT*. Mol. Microbiol. **15:**829–837.
25. **Matson, S. W., W. C. Nelson, and B. S. Morton.** 1993. Characterization of the reaction product of the *oriT* nicking reaction catalyzed by *Escherichia coli* DNA helicase I. J. Bacteriol. **175:**2599–2606.
26. **Nelson, W. C., B. S. Morton, E. E. Lahue, and S. W. Matson.** 1993. Characterization of the *Escherichia coli* F factor *traY* gene product and its binding sites. J. Bacteriol. **175:**2221–2228.
27. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene **26:**101–106.
28. **Pansegrau, W., W. Schroder, and E. Lanka.** 1993. Relaxase (TraI) of IncPα plasmid RP4 catalyzes a site-specific cleaving-joining reaction of single-stranded DNA. Proc. Natl. Acad. Sci. USA **90:**2925–2929.
29. **Pansegrau, W., G. Ziegelin, and E. Lanka.** 1990. Covalent association of the *traI* gene product of plasmid RP4 with the 5′-terminal nucleotide at the relaxation nick site. J. Biol. Chem. **265:**10637–10644.
30. **Pansegrau, W., and E. Lanka.** 1996. Mechanisms of initiation and termination reactions in conjugative DNA processing. J. Biol. Chem. **271:**13068–13076.
31. **Paterson, E. S.** Unpublished observation.
32. **Paterson, E. S., and V. N. Iyer.** 1992. The *oriT* region of the conjugative transfer system of plasmid pCU1 and specificity between it and the *mob* region of other N *tra* plasmids. J. Bacteriol. **174:**499–507.
33. **Paterson, E. S., M. I. More, V. N. Iyer, and S. C. Winans.** Unpublished data.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

35. **Scherzinger, E., R. Lurz, S. Otto, and B. Dobrinski.** 1992. *In vitro* cleavage of double- and single-stranded DNA by plasmid RSF1010-encoded mobilization proteins. Nucleic Acids Res. **20:**41–48.
36. **Thatte, V., and V. N. Iyer.** 1983. Cloning of a plasmid region specifying the N transfer system of bacterial conjugation in *Escherichia coli*. Gene **21:**227–236.
37. **Thompson, T. L., M. B. Centola, and R. C. Deonier.** 1989. Location of the nick at *oriT* of the F plasmid. J. Mol. Biol. **207:**505–512.
38. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
39. **Waters, V. L., K. H. Hirata, W. Pansegrau, E. Lanka, and D. G. Guiney.** 1991. Sequence identity in the nick regions of IncP plasmid transfer origins and T-DNA borders of *Agrobacterium* Ti plasmids. Proc. Natl. Acad. Sci. USA **88:**1456–1460.
40. **Wilkins, B. M., and E. Lanka.** 1993. DNA processing and replication during plasmid transfer between Gram-negative bacteria, p. 105–136. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
41. **Willetts, N., and J. Maule.** 1985. Specificities of IncF plasmid conjugation genes. Genet. Res. **47:**1–11.
42. **Zhang, S., and R. J. Meyer.** 1995. Localized denaturation of *oriT* DNA within relaxosomes of broad host range plasmid R1162. Mol. Microbiol. **17:**727–735.
43. **Ziegelin, G., J. P. Furste, and E. Lanka.** 1989. TraJ protein of plasmid RP4 binds to a 19-base-pair invert sequence repetition with the transfer origin. J. Biol. Chem. **264:**11989–11994.