JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 499–507
0021-9193/92/020499-09$02.00/0

Vol. 174, No. 2

The *oriT* Region of the Conjugative Transfer System of Plasmid pCU1 and Specificity between It and the *mob* Region of Other N *tra* Plasmids

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The *oriT* region of the conjugative IncN plasmid pCU1 has been localized to a 669-bp sequence extending from pCU1 coordinates 8.48 to 9.15 kb. The nucleotide sequence of this region was determined. The region is AT-rich (69% AT residues), with one 19-bp and one 81-bp sequence containing 79% or more AT residues. Prominent sequence features include one set of thirteen 11-bp direct repeats, a second set of two 14-bp direct repeats, six different inverted repeat sequences ranging from 6 to 10 bp in size, and two sequences showing 12 of 13 nucleotides identical to the consensus integration host factor binding sequence. Specificity between this *oriT* and mobilization (*mob*) functions encoded by the N *tra* system was demonstrated. This specificity is encoded by the region lying clockwise of the *Bgl*II site at coordinate 3.3 on the pCU1 map. Two N *tra* plasmids isolated in the preantibiotic era were unable to mobilize recombinant plasmids carrying the *oriT* region of pCU1 or to complement transposon Tn*5* mutations in the *mob* region of the closely related plasmid pKM101.

The origin of conjugative DNA transfer, *oriT*, of conjugative plasmids of the gram-negative eubacteria is a *cis*-acting region that is the focus of several coordinated events, resulting in the acquisition by the recipient of one plasmid duplicate and the retention in the donor of the other (31). There are several transfer (*tra*) systems, each with its own *oriT* and *oriT* specificity (25). One such distinct system is the N *tra* system, so-called because it is usually associated with plasmids of the incompatibility group N, such as plasmid pCU1 (11).

Previous studies have mapped a *tra* region of approximately 17 kb on the plasmid pCU1. The region is composed of a segment sufficient for the syntheses and assembly of an N pilus that can function to confer sensitivity to N pilus-specific bacteriophages and a second segment believed to specify functions related to conjugative DNA metabolism and plasmid DNA transfer (23, 24). This second segment includes its *oriT* region. Plasmid *trans*-acting elements that are believed to act at *oriT* are collectively referred to as *mob* (mobilization) elements. It is believed that specificity between *oriT* and *mob* can determine the difference in specificity between different *tra* systems.

To begin to study interactions between *oriT* and *mob* elements or host factors, we have delineated the *oriT* of pCU1, determined its nucleotide sequence, and constructed and analyzed deletions into and within it. This enables speculations on the possible functions of different domains of *oriT*. To study the specificity of *oriT* and *mob* of the N *tra* system, we have examined the ability of different conjugative plasmids to mobilize derivatives containing different parts of the *mob* region of pCU1.

(The studies reported here form part of the graduate dissertation of S. Paterson.)

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MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The strains, plasmids, and phages used are listed with their sources and relevant properties in Tables 1 and 2.

Growth, bacterial matings, and selection media. Cultures were grown either in Penassay broth (Difco Laboratories) or LB (18) or the same media solidified with 1.5% agar (Difco). Antibiotics or inhibitors were added from filter-sterilized stock solutions to solid media to yield the following concentrations (in micrograms per milliliter): ampicillin, 30; chloramphenicol, 30; kanamycin, 50; nalidixic acid, 50; rifampin, 100; spectinomycin, 50; and tetracycline, 10. For ampicillin, chloramphenicol, kanamycin, and spectinomycin, one-half of these concentrations were used in liquid media. Cultures were grown at 37°C. Bacterial matings for the determination of transfer and mobilization frequencies were done on membrane filters as described previously (13). When the donor carried two plasmids (mobilization experiments), transconjugants carrying the marker of each potential donor plasmid were selected separately and also tested for the presence of

TABLE 1. *E. coli* strains

Strain	Genotype or phenotype	Reference
HB101	*hsdS*$_B$*20 recA ara-14 endA galK12* Δ(*gpt-proA*)*62 leuB6 thi-1 lacY1 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44 mcrB*$_B$	2
C600Nal	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 mcrA* Nal^r	This laboratory
JM101	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15/*supE thi* Δ(*lac-proAB*)	17
DH5αF′	F′/*endA1 hsdR17* (r_K^- m_K^+) *gyrA* (Nal^r) *thi-1 recA1 relA1 supE44* Δ(*lacZYA-argF*)*U169* (ϕ80*lacZ*ΔM15)	15

TABLE 2. Plasmids and bacteriophages

Plasmid or bacteriophage	Relevant phenotype	Comments or map coordinates[a]	Reference or source
pCU1	Sp^r Ap^r Sm^r Tra^+ Rep(pCU1)		
pCU29	Sp^r Ap^r Sm^r Km^r Tra^+ Rep(pCU1)	Tn*5* at 9.55	23
pCU101	Cm^r Tra^+ Rep(p15A)	27.5–9.55	23
pCU109	Cm^r Tra^+ Rep(p15A)	27.5–11.2	23
pCU56	Cm^r Tra^- Mob^+ Rep(p15A)	7–11.5	23
pCU404	Ap^r Km^r Tra^- Mob^+ Rep(pMB1)	7–11.5	23
pCU57	Cm^r Tra^- Mob^+ Rep(p15A)	0–9.55	23
pVT30	Cm^r Km^r Tra^+ Rep(p15A)	Tn*5* at 9.15 in pCU109	23
pBGL	Ap^r Km^r Tra^- Mob^+ Rep(pMB1)	3.3–7, 8.5–9	This study
R46	Ap^r Sp^r Sm^r Tc^r Tra^+	N-type *tra*	T. Kato
R113	Tc^r Tra^+	N-type *tra*	J. Jorgensen
R199	Tc^r Tra^+	N-type *tra*	N. Datta
R45	Ap^r Tc^r Tra^+	N-type *tra*	N. Datta
pTM4245	Sm^r Tc^r Tra^+	N-type *tra*	C. Monti-Bragadin
pMUR274	Tra^+	N-type *tra*	10
pSP274	Tc^r Tra^+	pMUR274::Tn*10*	This study
pMUR545	Tra^+	N-type *tra*	10
pSP545	Tc^r Tra^+	pMUR545::Tn*10*	This study
pKM101 *traH1143*	Ap^r Km^r Tra^- Mob^+	N-type *tra*	29
pKM101 *traI1136*	Ap^r Km^r Tra^- Mob^+	N-type *tra*	29
pKM101 *traJ35*	Ap^r Km^r Tra^- Mob^+	N-type *tra*	29
pKM101 *traK1217*	Ap^r Km^r Tra^- Mob^+	N-type *tra*	29
pMK2004	Ap^r Km^r Tc^r Mob^- Rep(pMB1)		12
pIBI76	Ap^r Mob^- Rep(pMB1)		IBI[b]
pSP1	Ap^r Km^r	1–692	This study
pSP3	Ap^r Km^r	1–692	This study
pSP12	Km^r	401–692	This study
pSP13	Ap^r	1–400	This study
pSP19	Km^r	437–692	This study
pSP20	Km^r	487–692	This study
pSP22	Km^r	1–436	This study
pSP23	Km^r	1–486	This study
pSP25	Ap^r Km^r	369–692	This study
pBP1	Ap^r Km^r	1–692	This study
pBP11	Ap^r Km^r	1–551	This study
pBS47	Ap^r	1–419	This study
pBE42	Ap^r Km^r	562–692	This study
pBE60	Km^r	1–692	This study
M13mp18			17
M13mp19			17
M13K07	Km^r		26

[a] The coordinates shown are either those of the parental plasmid pCU1 (pCU29 to pBGL) or of its *oriT* region (pSP1 to pBE60). Coordinates 23 to 692 of the *oriT* region correspond to coordinates 9.15 to 8.48 of pCU1.

[b] IBI, International Biotechnologies, Inc.

the marker of the other plasmid by replication on appropriate media.

To avoid misunderstandings, we define explicitly the terms mobilization, donation, and conduction as used in this article (3). Mobilization is the conjugative transfer of a plasmid derivative that is itself not self-transmissible but which can be mobilized and transferred with the help of a self-transmissible plasmid. The term does not connote mechanisms. Donation is used to imply that the plasmid is transferred without covalent linkage to the helper plasmid, and conduction is used to imply that such linkage occurs.

DNA manipulations. Standard procedures (6, 9, 20, 21, 26) were used for all DNA manipulations, including plasmid DNA extractions, restriction mapping, molecular cloning, and nucleotide sequencing.

Nucleotide sequence accession number. The sequence discussed here has been assigned GenBank accession number M81668.

RESULTS

Position of *oriT* on the pCU1 map. In the course of this study, we found that the position of the transposon Tn*5* insert in pCU29 had to be revised from that reported earlier (24). The corrected position, which is consistent with all of the relevant restriction analyses and the sequence reported in this article, is shown in Fig. 1 and is located 0.4 kb further from the end of *tra* than the Tn*5* insert in pVT30. pVT30 is a Tra^+ Tn*5* derivative of pCU109 (24) which carries the entire *tra* region cloned into pACYC184. This, together with the information (23) that pCU404 and pCU56 carry all *cis*-acting functions, confined *oriT* to the region between the coordinates 7 and 9.15 on the pCU1 map (Fig. 1).

Construction of pSP3, a small plasmid mobilized efficiently by donation. The strategy used to localize *oriT* was to subclone the smallest known fragment required in *cis* for efficient transfer and then create deletions into the region with Bal31 nuclease. pMK2004 is a cloning vector which

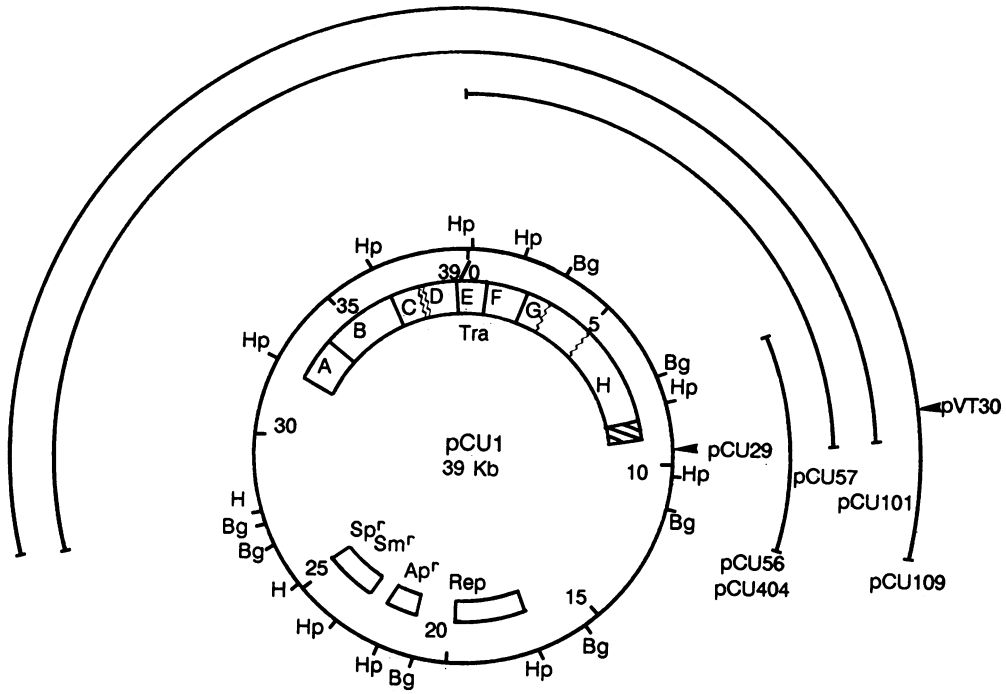

FIG. 1. Derivatives of plasmid pCU1 used in this study. Outer arcs represent regions cloned into the vector DNA indicated in parentheses: pCU404 (pMK2004), pCU56 (pACYC184), pCU57 (pACYC184), pCU101 (pACYC184), and pCU109 (pACYC184). Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Sm^r, streptomycin resistance; Rep, replication region; Tra, transfer region. Hatched portion of the *tra* region indicates the *oriT* region as determined in this study. Complementation groups of *tra*, designated by letters A to H, were defined by complementation tests between Tn*5* insertion derivatives of pCU109 (23). Wavy borders indicate uncertainty in the extent of the complementation groups due to the lack of Tn*5* insertions within the closed segment of DNA. Arrowheads identify the Tn*5* insertions, accompanied by the plasmid designation of the insertion derivative. In the case of pCU29 and pVT30, the placements of the Tn*5* inserts represent corrected locations as determined in this study. Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I. Numbers inside the circle are kilobase coordinates on the pCU1 map.

encodes a ColE1-type replicon and which cannot be mobilized by donation, as it does not contain the site required for strand nicking (*nic* site) at its transfer origin (2, 12). Insertions into the unique *Bam*HI site of pMK2004 result in the inactivation of its tetracycline resistance (Tc^r) gene.

pVT30 plasmid DNA, digested with *Bgl*II, was ligated with pMK2004 plasmid DNA linearized at the unique *Bam*HI site (Fig. 2). The ligation mixture was transformed into *Escherichia coli* HB101, and kanamycin-resistant (Km^r) transformants were screened for inactivation of the Tc^r gene. pCU1 was introduced into Km^r Tc^s transformants by matings with C600(pCU1). Spectinomycin-resistant (Sp^r) Km^r transconjugants were purified, and mobilization experiments were conducted by a second mating with C600Nal. One isolate, pSP1, demonstrating mobilization of the Km^r marker at frequencies comparable to those with pCU404 was examined by restriction analysis. The *Bgl*II fragment subcloned in this manner from pVT30 contained a 1.55-kb *Hpa*I fragment comparable to one of the bands in the pVT30 *Hpa*I digest. This placed the Tn*5* insert at 9.15 kb on the pCU1 map. pSP1 was partially digested with *Hpa*I and *Pvu*II, self-ligated, and transformed into HB101. pCU1 was introduced into transformants by matings, and the resultant Sp^r Km^r transconjugants were tested for mobilization of Km^r with C600Nal. Restriction analysis on transconjugants demonstrating mobilization frequencies comparable to those with pCU404 identified a clone (pSP3) in which the Tn*5*-derived $HpaI_2$-$PvuII_2$ fragment had been deleted, placing the pCU1-derived DNA closer to the unique *Sal*I site. The pCU1-derived *Pvu*II-*Hpa*I fragment in pSP3 is inverted in comparison to that in pSP1 (Fig. 2).

Deletions from pSP3 or its derivatives. pSP3 DNA was digested to completion by *Eco*RI, *Sal*I, or *Pvu*II in separate reactions, and each sample of linear DNA was digested with Bal31 at 30°C over a range of incubation times between 2 and 10 min. The overhanging ends were made flush with Klenow polymerase, and *Bam*HI linkers were ligated to the ends. The DNA was recircularized and transformed into HB101. The resulting transformants were designated pBP, pBE, and pBS, depending on the restriction site at which pSP3 was linearized prior to Bal31 digestion digestion (Fig. 3). pBE transformants were selected on kanamycin plates, while pBS and pBP transformants were selected on ampicillin plates. The extent of deletions in each of a series of deletion derivatives was determined by screening *Eco*RI-*Bam*HI or *Pvu*II-*Bam*HI double digests and confirmed by *Hin*dIII-*Bam*HI double digests. Chosen derivatives were then tested for the efficiency with which they were mobilized by pCU101, a Tra$^+$ derivative of pCU1 (*Hin*dIII fragment from pCU29 cloned into the *Hin*dIII site of plasmid vector pACYC184 [24]).

pBP1 and pBE60 (Fig. 3, top) represented the plasmids with the largest deletions extending into either end of the *oriT* region which were still mobilized by pCU101 at frequencies comparable to those with pCU404 (Table 3). By aligning the pBP1 and pBE60 deletion derivatives, it can be concluded that the region required in *cis* for transfer is encoded within the overlap of the two derivatives (delineated by

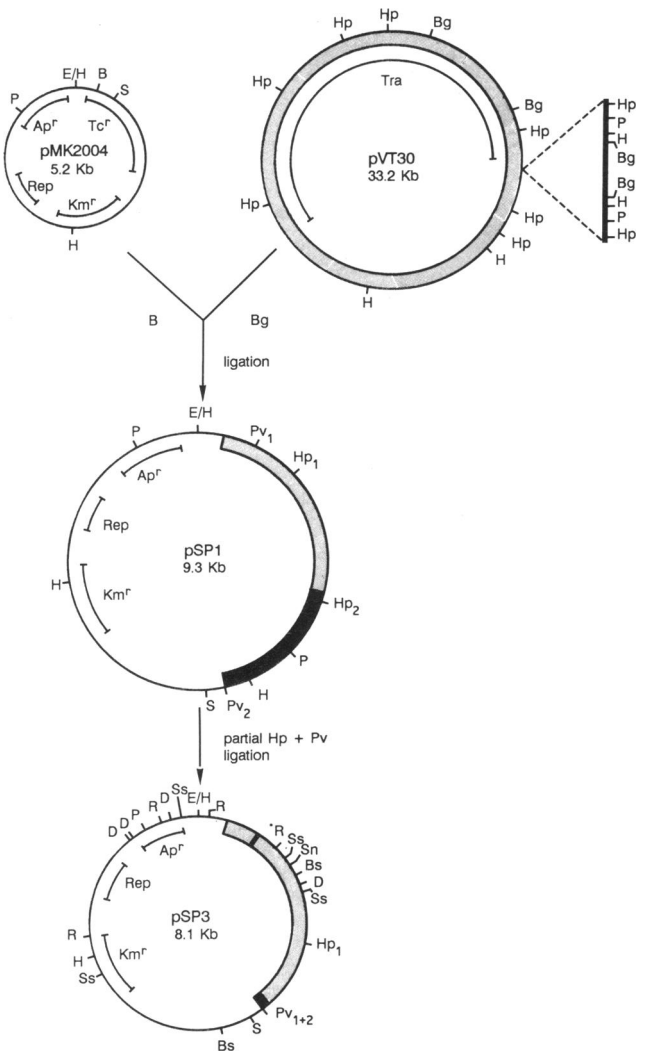

FIG. 2. Construction of pSP1 and pSP3. The 3.5-kb *Bgl*II fragment containing the right-hand end of pCU1 *tra* was subcloned from pVT30 into the unique *Bam*HI site of vector pMK2004. The Tn*5*-derived $HpaI_2$-$PvuII_2$ fragment was removed from pSP1 via partial digestion with both enzymes and recircularized to produce pSP3. In pSP3, the pCU1-derived $PvuII_1$-$HpaI_2$ fragment is inverted with respect to its position in pSP1. pMK2004 DNA is signified by a single line, pCU1 DNA from pVT30 is shown by a thick lightly shaded line, and Tn*5* DNA from pVT30 is shown by a thick solid line. Plasmids have not been drawn to scale relative to each other; however, regions within each plasmid are drawn to scale within the limitations of restriction mapping. Regions conferring phenotypic characteristics are indicated by arcs within the circles: Tra, transfer region; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Rep, replicon. Restriction site designations: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bsp*MI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; Pv, *Pvu*II; P, *Pst*I; R, *Rsa*I; S, *Sal*I; Ss, *Ssp*I. *R site in pSP3 represents seven closely spaced *Rsa*I sites which can be seen in the expanded view in Fig. 3. *Pvu*II sites of pVT30 are numerous, and therefore they have only been identified in pSP1 and pSP3 derivatives.

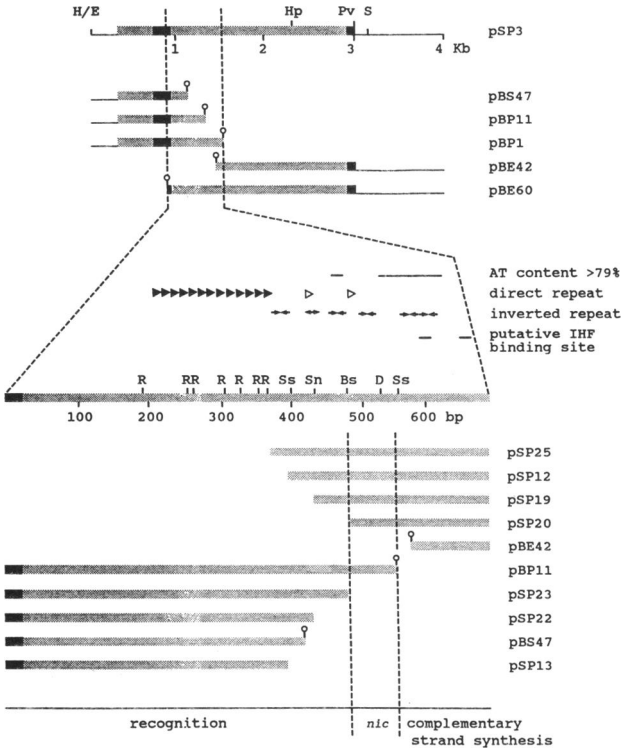

FIG. 3. Deletion derivatives of pSP3. The upper composite line represents the portion of pSP3 from 0 to 4 kb, composed of pMK2004 vector DNA (thin line), pCU1 DNA (thick, lightly shaded line), and Tn*5* DNA (thick, heavily shaded line). Bal31 deletions initiated at the *Sal*I, *Pvu*II, and *Eco*RI sites, designated with the prefixes pBS, pBP, and pBE, respectively, are shown directly below. Gaps indicate regions present in pSP3 that have been deleted. The broken vertical line indicates the segment that is present in all plasmids (e.g., pBP1 and pBE60) that are mobilized with high efficiency by pCU101 and inferred to be donated. The region contained between these broken vertical lines represents the 692 bp that was sequenced (see Fig. 4) and is expanded in the central horizontal bar. The numbers below this bar indicate nucleotides, with nucleotides 1 and 692 corresponding to pCU1 coordinates 9.17 and 8.48, respectively. Sequence features are shown above the central horizontal bar. The lower portion of the figure (below the central bar) shows additional deletion derivatives that were isolated from pBP1 and pBE60. A speculative delineation of different domains of the *oriT* region is indicated by the bar at the bottom of the figure. Restriction site abbreviations: B, *Bam*HI (linker added during construction); Bs, *Bsp*MI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; Pv, *Pvu*II; P, *Pst*I; R, *Rsa*I; S, *Sal*I; Sn, *Sna*BI; Ss, *Ssp*I. ♀, *Bam*HI linker added during construction.

vertical dashed lines in Fig. 3). The deletions in three other constructs, pBP11, pBS47, and pBE42, can be seen to extend into but not beyond this overlap region. The frequency of mobilization for each of these three constructs is significantly reduced with respect to the frequency for pBP1 and pBE60 (Table 3). These five plasmids were subsequently used to sequence the overlap in pBE60 and pBP1 that contains the *oriT* region. DNA sequence analysis, which will be described below, identified several useful restriction sites within the *oriT* region. In particular, two *Ssp*I sites, single *Sna*BI, *Bsp*MI, and *Dra*I sites, and the innermost *Rsa*I site were used to construct additional deletions within the *oriT* region carried by pBP1 and pBE60. These additional derivatives are shown in the expanded view of the *oriT* region in the lower portion of Fig. 3.

Mobilization of *oriT* plasmid constructs. The mobilization frequencies of each of the above plasmid derivatives were determined in mating experiments in which pCU101 was

TABLE 3. Mobilization frequency of pCU1 *oriT* derivatives by conjugative plasmid pCU101

oriT plasmid[a] (Km^r or Ap^r)	pCU101 (Cm^r) transfer frequency[b] (10^{-1})	Mobilization frequency[c]	Cm^s/Km^r[d]	*oriT* plasmid detected in randomly selected Km^r or Ap^r transconjugants
pCU404	2.1	8.5×10^{-2}	27/200	ND[e]
pBP1	2.7	4.8×10^{-2}	165/309	13/13
pBE60	9.0	1.0×10^{0}	59/110	9/9
pSP25	2.5	7.4×10^{-1}	ND	ND
pSP12	7.3	2.2×10^{-2}	84/321	13/13
pSP19	2.7	2.4×10^{-3}	149/200	7/12
pSP20	2.6	3.3×10^{-4}	5/109	3/38
pBP11	1.8	4.1×10^{-6}	11/106	27/27[f]
pBE42	2.9	5.9×10^{-6}	0/156	0/9
pBS47	5.4	5.2×10^{-6}	ND	0/15
pSP13	6.1	$<6.6 \times 10^{-6}$	0/22	0/30
pSP22	2.8	1.5×10^{-5}	0/200	0/9
pSP23	4.0	9.4×10^{-5}	0/200	0/8
pMK2004	3.3	8.0×10^{-6}	0/126	0/13

[a] *oriT* derivatives were maintained in HB101 that also carried pCU101.
[b] Ratio of recipients receiving conjugative plasmid per donor cell.
[c] Ratio of recipients receiving the *oriT* derivative per recipient receiving pCU101.
[d] Number of transconjugants receiving the *oriT* derivative (Km^r or Ap^r) which upon replication did not coinherit pCU101 (Cm^r)/number tested.
[e] ND, not done.
[f] Nine of 27 showed size alterations.

used as the helper plasmid in biparental matings. It should be noted that pCU101 is segregationally unstable in $RecA^+$ *E. coli*. The inheritance of an *oriT* plasmid marker without the inheritance of the marker of the helper plasmid provided evidence for mobilization by donation. The results are shown in Table 3. A first group of plasmids (pBP1, pBE60, pSP25, pSP12, pSP19, and pSP20) were mobilized at progressively reduced frequencies, which were, however, still substantially greater than that of the vector pMK2004 (Table 3). These relatively high mobilization frequencies were consistent with these plasmids being donated. This is supported by the observation that transconjugants carrying the *oriT* plasmid marker but not the helper plasmid marker were recovered (Table 3, Cm^s/Km^r). Further evidence that transfer of the nonconjugative plasmid occurred without cointegration with the conjugative plasmid is that at least some of the transconjugants carrying the *oriT* marker were found to contain a plasmid comparable in size to the *oriT* plasmid in the donor (Table 3, last column). The fraction of transconjugants carrying independent, unaltered *oriT* plasmids was reduced with pSP19 and pSP20.

The frequency of mobilization of the remaining plasmids (pBP11, pBE42, pBS47, pSP13, pSP22, and pSP23) approaches or is less than that of the vector pMK2004, suggesting that they are mobilized by conduction. In matings involving the last five of these *oriT* plasmids, transconjugants carrying the *oriT* plasmid marker demonstrated 100% coinheritance of the helper plasmid marker, and none of those tested could be shown to contain a plasmid comparable in size to the *oriT* plasmid in the donor. These results are consistent with conduction. By contrast, in matings involving pBP11, some of the transconjugants carrying the pBP11 marker lacked the helper plasmid marker, and all those tested carried a small plasmid (independent of the larger helper plasmid if present). In 18 of 27 transconjugants tested, the size of this smaller plasmid was unchanged from that of the original pBP11, suggesting that pBP11 was donated at a very low frequency. The remaining nine *oriT* plasmids from pBP11 transconjugants demonstrated alterations in size consistent with conduction.

Sequence analysis. The sequencing strategy and the sequence of the *oriT* region are shown in Fig. 4. Owing to the nature of the insertions in the sequencing vector, the strand sequenced in pBP1, pBP11, and pBS47 was different from that sequenced in pBE42 and pBE60. Nucleotides 1 to 358 were derived from one strand of a single clone, 359 to 379 and 563 to 692 were derived from different strands of two different clones, and 380 to 562 were derived from the same strand of two different clones. This sequence of the *oriT* region has been confirmed independently by V. Thatte in this laboratory from sequence analysis of both strands of independently constructed clones.

Structural features of the 692-bp sequence are shown diagrammatically in the middle portion of Fig. 3. The AT content of the sequence is about 63%. This percentage rises to 67% for the region carried by pSP25, where there is one string of 19 bp (453 to 471) containing only two GC residues and a second 23-bp string (524 to 546) completely composed of A and T residues. This latter AT string is within an 81-bp sequence (524 to 606) which has 79% AT. A striking feature of the 692 bp sequenced in pCU1 is the set of 13 11-bp direct repeats starting at nucleotide 202 and running to nucleotide 374. These direct repeats have a consensus sequence of TG(A/T)TGTA(A/C)TTT, the underlined nucleotides each varying in four of the repeats. In addition to this set of 11-bp direct repeats, a second 14-bp repeat, TTANNCCT TAACCT, where N is any nucleotide, is located at nucleotides 421 to 434 and 468 to 481. This repeated sequence is within the 87-bp region between the first *Ssp*I site and the *Bsp*MI site.

The sequenced region contains five inverted repeat sequences, three large (10 bp) and two small (6 and 7 bp), and one 12-bp region of imperfect twofold rotational symmetry. All of these structures lie within the region carried by pSP25. One 10-bp perfect inverted repeat lies just to the left of the first *Ssp*I site, outside the region carried by pSP12 (nucleotides 374 to 395). The remaining two large imperfect inverted repeats lie in the region between the second *Ssp*I site and the end of the Bal31 deletion found in pBP1, outside the region carried by pBP11 (nucleotides 566 to 588 and 589 to 612), while the smaller two inverted repeats and the region of imperfect twofold rotational symmetry lie within the internal *Ssp*I fragment (nucleotides 453 to 469, 504 to 528, and 431 to 442). The 6-bp inverted repeat at 453 to 469 overlaps the second 14-bp direct repeat. The region of imperfect twofold rotational symmetry overlaps the first 14-bp direct repeat.

To the right of the second *Ssp*I site at nucleotides 588 to 600 and 658 to 670, there are two sequences showing consensus with 11 of 12 nucleotides of the integration host factor (IHF) binding site of bacteriophage lambda [PyA ANNNNTTGAT(A/T), where Py is a pyrimidine (14)]. Both sites lie outside the region carried by pBP11, and the first site overlaps one of the two inverted repeat sequences in this region.

Demonstration of specificity. Eight conjugative plasmids encoding an N-type *tra* system were tested for their ability to mobilize each of three nonconjugative derivatives, pCU57, pCU56 (or pCU404, which carries the same pCU1 region as pCU56 but in a different vector), and pBP1, from HB101 to C600Nal. These plasmids and strains are described in Tables 1 and 2 and Fig. 5. It was believed that if pCU1-specific *mob*

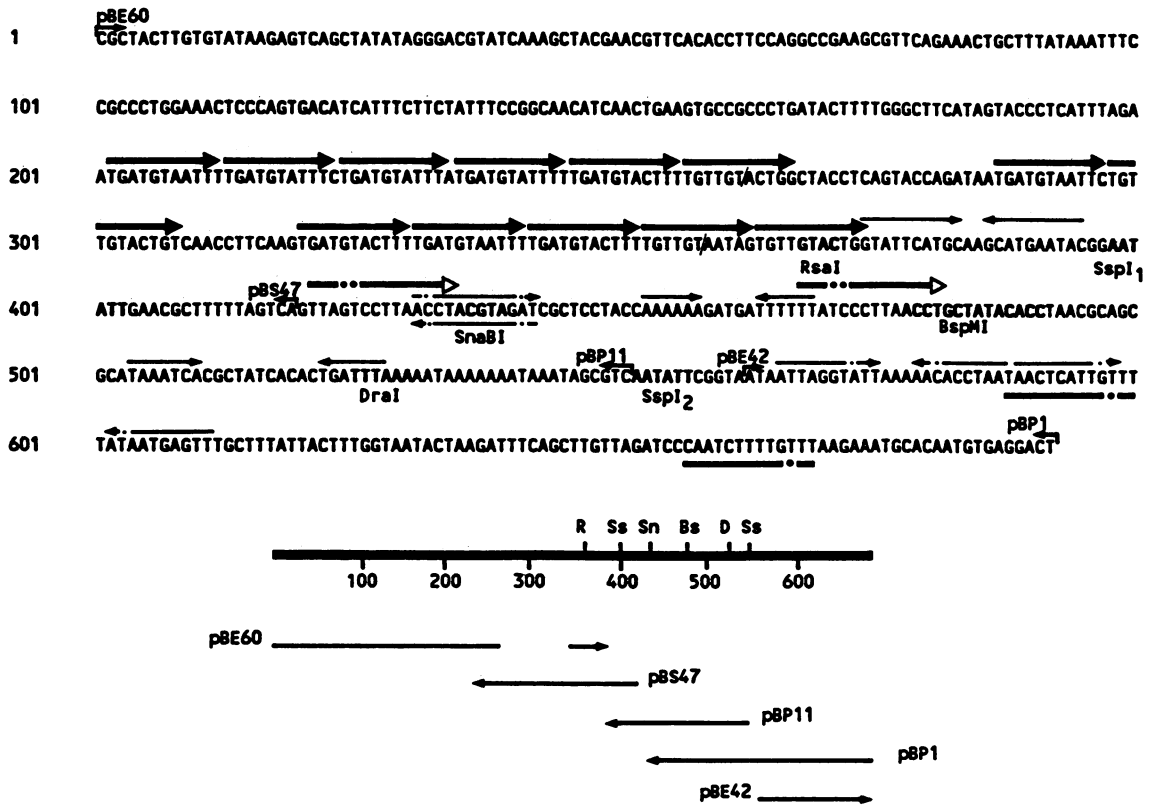

FIG. 4. Nucleotide sequence of *oriT* and strategy for sequencing. The 692-bp sequence was determined from the five *oriT* derivatives shown in the sequencing strategy (lower portion). The first 23 nucleotides correspond to Tn*5* sequences. The end of the deletion in each of the five *oriT* derivatives used in the sequencing is indicated by ↱ or ↰, with the plasmid designation above. The region between the two slashes (nucleotides 262/263 and 358/359) indicates the region deleted from the pBE60 derivative used for sequencing. The two *Ssp*I (Ss) and single *Dra*I (D), *Bsp*MI (Bs), *Rsa*I (R), and *Sna*BI (Sn) restriction sites used for subcloning are indicated in boldface lettering. There are six additional *Rsa*I sites not shown at nucleotide 186 and in the first set of direct repeats at nucleotides 250, 261, 303, 325, and 347. Directly repeated sequences are indicated by the heavy arrows above the sequence. The first set of 13 11-bp direct repeats extends from nucleotides 202 to 374. The second set of two 14-bp repeats are located at nucleotides 421 to 434 and 468 to 481. Bases showing lack of consensus are indicated by a dot. Inverted repeats and regions of twofold rotational symmetry are indicated by light arrows above the sequence and are located at nucleotides 374 to 395, 431 to 442, 453 to 469, 504 to 528, 566 to 588, and 589 to 612. Mismatched nucleotides are indicated by a dot. Sequences showing consensus to the IHF binding site are indicated by heavy lines below the sequence at nucleotides 588 to 600 and 658 to 670.

functions existed, they would definitely be encoded by pCU57 but possibly not by pCU56 or pCU404 and almost certainly not by pBP1, which contains only a minimal *oriT* region. A difference in mobilization frequencies of the three plasmids by different N conjugative systems would indicate specificity in *mob* functions.

As shown in Table 4, six of the eight plasmids tested were able to mobilize pCU57, pCU56, and pBP1 at frequencies comparable to mobilization by pCU1. The exceptions were pSP274 and pSP545, plasmids derived by Tn*10* insertion from a collection of plasmids called the pMUR plasmids, isolated in the preantibiotic era (10). These two plasmids mobilized pCU57 as efficiently as the other conjugative plasmids but demonstrated a reduced ability to mobilize pCU56 (10^{-4}) and negligible ability to mobilize pBP1 ($<10^{-6}$). The results show that as the amount of pCU1 DNA extending from the *oriT* end of *tra* decreased on the nonconjugative test plasmid (Fig. 5), the ability of the pMUR derivatives pSP274 and pSP545 to mobilize the test plasmid also decreased. The results indicate the existence of plasmid specificity among plasmids encoding the N *tra* systems and that this specificity is associated with *mob* functions. In particular, the region present in pCU57 but absent in pCU56 (or pCU404) and pBP1 is contributing to this specificity.

Localization of the *mob* specificity functions. The reduced mobility of pCU56 by the pMUR plasmids combined with the knowledge that N pili are encoded by the region counterclockwise from the 2.7-kb coordinate (23) suggested that the region carried on the *Bgl*II fragment extending from 3.3 to 7 kb on the pCU1 map (Fig. 5) may encode at least some of the plasmid-specific *mob* functions. A derivative, pBGL, was constructed by subcloning this *Bgl*II fragment into the *oriT* derivative pBP1 so that the 3.3-kb coordinate was adjacent to the *oriT* sequence and the 7.0-kb coordinate was adjacent to the Km^r gene. Plasmid pBP1 was linearized at the unique *Bam*HI site (at the end of the pCU1-derived DNA) and ligated to pCU57 DNA digested with *Bgl*II. DNA from potential plasmid recombinants was digested with both *Kpn*I and *Pvu*I to identify those clones carrying the desired *Bgl*II fragment. The mobilization frequency of pBGL by the pMUR derivatives was no greater than that of pBP1. However, when pCU56 and pBGL were combined in the same donor cell, the pMUR plasmids were capable of mobilizing both efficiently (Table 5).

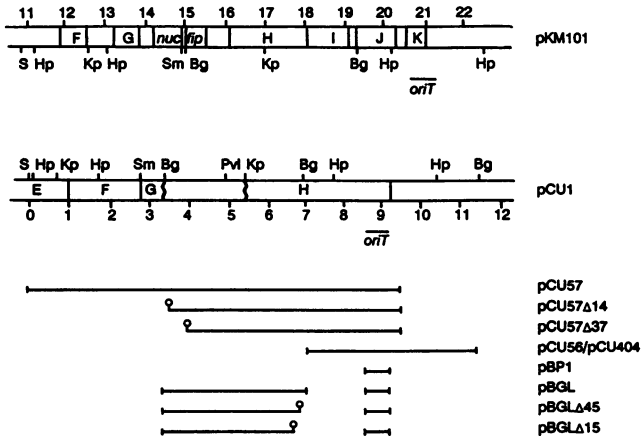

FIG. 5. *mob-oriT* region of pCU1 and pKM101. The upper portion of this figure is a comparison of these regions in the two plasmids. Double lines represent pKM101 and pCU1 regions extending from the *Sal*I sites located at coordinates 10.8 and 0 kb of the respective plasmids. Complementation groups are indicated within the boxes, and the *oriT* region is indicated below. The *nuc* and *fip* regions of pKM101 determine an endonuclease and fertility inhibition of P group plasmids, respectively. Information for pKM101 is from reference 29, and that for pCU1 is from reference 23. Restriction sites: Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; PvI, *Pvu*I. ♀, *Bam*HI linker was added during construction. The lower portion of the figure illustrates the pCU1 *mob* derivatives used in complementation tests with pSP274 and pSP545. pCU56/pCU404, pCU57, pBP1, and pBGL are indicated along with deletion derivatives of pCU57 and pBGL. The extent of the deletions into pCU57 was determined by *Bam*HI-*Bgl*II digests. Deletions into pBGL were determined by *Kpn*I-*Bam*HI digests. The fragment sizes generated by these deletions are: pCU57, 3.5 kb; pCU57Δ14, 3.4 kb; pCU57Δ37, 2.9 kb; pBGL, 1.5 kb; pBGLΔ45, 1.4 kb; pBGLΔ15, 1.2 kb.

These results provide two important pieces of information. First, they demonstrate that at least some of the pCU1-specific *mob* functions are encoded on the region clockwise from the *Bgl*II site at 3.3 kb on the pCU1 map (Fig. 5). Second, the fact that complementation between the two plasmids in *trans* allowed efficient mobilization indicates that this region contains at least two complementation groups, at least one on each side of the *Bgl*II site at 7 kb on the pCU1 map. This agrees with the complementation map of pKM101.

TABLE 4. Mobilization of pBP1, pCU57, and pCU56 (or pCU404) by N *tra* plasmids

Conjugative plasmid[a]	Conjugative plasmid transfer frequency[b]	Mobilization frequency[c] of nonconjugative plasmid		
		pBP1	pCU56/pCU404	pCU57
pCU1	6.8×10^{0}	5.4×10^{-1}	5.8×10^{-1}	5.1×10^{-1}
R113	3.9×10^{0}	7.0×10^{-1}	1.4×10^{-1}	1.1×10^{0}
R199	5.1×10^{-1}	1.5×10^{0}	8.3×10^{0}	6.0×10^{0}
R45	4.6×10^{0}	1.5×10^{0}	4.7×10^{-1}	2.3×10^{0}
R46	3.6×10^{0}	ND[d]	2.4×10^{0}	1.0×10^{0}
pTM4245	3.0×10^{0}	8.1×10^{-1}	3.0×10^{-1}	9.3×10^{-1}
pSP274[e]	3.3×10^{0}	8.8×10^{-7}	1.2×10^{-4}	2.5×10^{-1}
pSP545[e]	4.2×10^{0}	6.5×10^{-8}	1.8×10^{-4}	1.2×10^{-1}

[a] Plasmids were in the donor strain HB101. Recipients were C600Nal.
[b] Number of transconjugants receiving the marker of the respective plasmid per donor. Average results obtained for three strains carrying different nonconjugative plasmids.
[c] Ratio of transconjugants that carried the non-self-transmissible plasmid to transconjugants that carried the self-transmissible plasmid.
[d] ND, not done.
[e] pSP274 and pSP245 are pMUR plasmids marked by Tn*10*.

TABLE 5. Mobilization of pCU1 *mob* derivatives by pCU1 and the pMUR::Tn*10* derivatives pSP274 and pSP545

Plasmids in the donor[a]	Transfer frequency of conjugative plasmid[b]	Mobilization frequency[c] nonconjugative plasmid	
		Plasmid 2	Plasmid 3
pCU1/pBGL	4.0×10^{0}	1.1×10^{0}	
pCU1/pCU57Δ14	6.0×10^{1}	4.8×10^{-1}	
pCU1/pCU57Δ37	2.4×10^{0}	6.5×10^{-1}	
pSP274/pBGL	1.7×10^{0}	5.5×10^{-7}	
pSP274/pCU56/pBGL	6.4×10^{-1}	2.2×10^{-2}	3.6×10^{-1}
pSP274/pCU56/pBGLΔ45	2.5×10^{1}	4.3×10^{-5}	$<5.5 \times 10^{-5}$
pSP274/pCU56/pBGLΔ15	6.5×10^{0}	4.0×10^{-5}	$<8.1 \times 10^{-5}$
pSP274/pCU57Δ14	2.1×10^{0}	2.4×10^{-5}	
pSP274/pCU57Δ37	2.7×10^{0}	1.1×10^{-5}	
pSP545/pBGL	2.0×10^{-1}	9.1×10^{-7}	
pSP545/pCU56/pBGL	5.9×10^{0}	1.7×10^{-2}	1.9×10^{-3}
pSP545/pCU57Δ14	7.4×10^{0}	8.9×10^{-6}	
pSP545/pCU57Δ37	8.3×10^{-1}	3.0×10^{-6}	

[a] Plasmids were maintained in HB101.
[b] Number of transconjugants receiving conjugative plasmid antibiotic marker per donor.
[c] Number of transconjugants receiving the nonconjugative antibiotic marker per transconjugant receiving the conjugative plasmid marker. Plasmids 2 and 3 are the nonconjugative plasmids listed in that order in the first column.

To further localize the specificity functions, deletions were made into either end of the *Bgl*II fragment from coordinates 3.3 to 7 kb. pCU57 and pBGL DNA was linearized at *Sma*I (Fig. 5) and *Xho*I (within the adjacent Km^{r} gene), respectively, and digested with Bal31 nuclease for between 2 and 10 min. After the ends were made flush with Klenow enzyme and deoxynucleoside triphosphates and *Bam*HI linkers were ligated to the ends, the DNA was recircularized and transformed into HB101. The extent of the deletions in the pCU57 derivatives was determined by *Bgl*II-*Bam*HI double digests, while deletions in pBGL were deetermined by *Kpn*I-*Bam*HI digests (Fig. 5). These deletion derivatives were tested for their ability to be mobilized by the pMUR derivatives. These results are shown in Table 5. Additional derivatives with larger deletions showed similar results (data not shown).

The low mobilization frequencies for the Bal31 deletion derivatives of pCU57 indicate that any loss of DNA clockwise from the 3.3-kb *Bgl*II site (Fig. 5) reduces the mobilization frequency 10,000- to 100,000-fold. Similar reductions in mobilization are noted with deletions extending into the other end of this *Bgl*II fragment, as seen with the pBGL Bal31 deletion derivatives (Table 5).

Complementation of Tra^{-} pKM101::Tn*5* insertion mutants by pMUR plasmids. pKM101, a Tra^{+} deletion derivative of the IncN plasmid R46, possesses striking restriction pattern similarities to pCU1 within the *tra* region, particularly in the pCU1 region extending from 0 kb to the right-hand end of *tra* (Fig. 5). Sequence analysis presented in this article has also shown that the sequence of the pCU1 *oriT* region is essentially the same as that determined for the *oriT* region of pKM101. These similarities between pCU1 and pMK101

TABLE 6. Mobilization of pMK101::Tn*5* *tra* mutants[a] by the pMUR::Tn*10* derivatives pSP274 and pSP545

Plasmid(s)	pSP plasmid transfer frequency[b]	pKM101 *tra* plasmid cotransfer frequency[c]
pKM101 *traH1143*	N/A[d]	1.4×10^{-6}
pKM101 *traI136*	N/A	1.9×10^{-6}
pKM101 *traJ35*	N/A	2.8×10^{-8}
pKM101 *traK1217*	N/A	$<5.5 \times 10^{-8}$
pSP274/pKM101 *traH1143*	1.4×10^{0}	7.9×10^{-4}
pSP274/pKM101 *traI1136*	8.3×10^{-1}	2.1×10^{-4}
pSP274/pKM101 *traJ35*	7.8×10^{-3}	2.8×10^{1}
pSP274/pKM101 *traK1217*	2.6×10^{0}	1.9×10^{-6}
pSP545/pKM101 *traH1143*	2.5×10^{-1}	3.0×10^{-4}
pSP545/pKM101 *traI1136*	1.7×10^{-1}	7.0×10^{-4}
pSP545/pKM101 *traJ35*	8.5×10^{-1}	2.0×10^{0}
pSP545/pKM101 *traK1217*	4.8×10^{0}	7.6×10^{-5}

[a] Tn*5* derivatives of pKM101 are described by Winans and Walker (29).
[b] Number of recipients receiving pSP plasmid marker per donor.
[c] Number of recipients receiving pKM101 plasmid marker per donor (first four) or per recipient receiving pSP plasmid marker (last eight).
[d] N/A, not applicable.

prompted the use of pKM101::Tn*5* insertion mutants in the examination of *mob* function specificity among the IncN plasmids.

The upper part of Fig. 5 shows a map of the four complementation regions in the *oriT*-proximal end of the *tra* region of pKM101 which are not involved in pilus production. pCU57 has been shown in this laboratory to complement mutations in each of the four pKM101 *mob* complementation regions. As with pCU1, *oriT* is located at the far right end of the *tra* region. To examine the plasmid specificity of *mob* functions, complementation tests were conducted between the pMUR derivatives pSP274 and pSP545 and pKM101 mutants carrying a Tn*5* insertion in each of the four complementation regions. The results are shown in Table 6.

Of the four pKM101 *tra* mutants tested, only the *traJ* mutant was complemented by the pMUR plasmids, indicating that the pMUR plasmids produce a *traJ*-like product. By comparing the mobilization of these pKM101::Tn*5* derivatives (Table 6) with that of those obtained with the pCU1 *mob* deletion derivatives (Tables 4 and 5), it can be seen that the insertional inactivation or deletion of similar regions in the two plasmids has produced similar reductions in mobilization frequencies by the pMUR plasmids. For example, the pKM101 *traK* mutant has a mobilization frquency comparable to that of pBP1. This suggests that a pKM101 *traK*-like complementation group is not carried by the pCU1 derivative pBP1. Similar inferences can be drawn for the pKM101 *traH* and pKM101 *traI* mutants and the pCU57 and pBGL deletion derivatives that are complemented by pCU56.

All of the self-transmissible plasmids used in this study are transferred with comparable and high efficiency. We have also observed that none of the four Tra$^-$ Tn*5* insertion mutants (including *traJ*::Tn*5*) are complemented when the donor carries plasmids specifying *tra* systems of the groups F, I, P, and W (results not shown). Thus, the observed differences in specificity between the two pMUR plasmids on the one hand and pCU1 and R46 on the other relate to the *mob* regions defined by *traH*, *traI*, and *traK* of pKM101 but not the region defined by *traJ*.

DISCUSSION

A commonly observed feature of mobilizable plasmids is that mobilization by donation is much more efficient than mobilization by conduction and can be similar to the frequencies of transfer of the self-transmissible plasmids from which they derive. The mobilization frequencies of pSP3 and of its two deletion derivatives pBP1 and pBE60 (Table 3) are consistent with the conclusion that they are mobilized by donation. Sequence analysis has demonstrated that the region shared by pBP1 and pBE60 is 692 bp in size, with 23 bp at one end being derived from the terminus of Tn*5*. It is concluded that the *oriT* region is contained in the 669 bp between the 9.15 and 8.48 kb coordinates on the pCU1 map. The sequence of this *oriT* region of pCU1 is identical to the sequence of *oriT* of the closely related plasmid R46 (4) with two exceptions: nucleotides 276 and 277 are reversed, and the GCGC sequence at nucleotides 498 to 502 is GCGGC in the R46 *oriT* sequence (nucleotides 421 to 425 [4]).

Deletion of DNA from either end of the 669-bp *oriT* region of pCU1 reduces the frequency of mobilization. It is possible to identify particular structural features of this sequence whose deletion may be responsible for the reduced mobility. Loss of some structural features allows donation to occur at a detectable though reduced frequency; loss of other features exposes events in which mobilization occurs by conduction.

While the *oriT* regions of F, R100, R1, and RK2 contain two or three copies of directly repeated sequences and ColB4 contains 10 copies of an 8-bp sequence that is directly repeated and dispersed over the 400-bp *oriT* region (7, 8), the cluster of 13 11-bp direct repeats arranged in tandem is unique to the *oriT* regions of pCU1 and R46. Deletion of these repeated sequences (pSP25) results in only a two- to threefold reduction in transfer frequency, suggesting a minor role for these sequences in conjugation. The frequency of mobilization is reduced an additional fourfold with the further deletion of the 10-bp inverted repeat sequence directly adjacent to the direct repeats. Additional reductions in mobility with the deletion of DNA to the *Sna*BI site (pSP19) and the *Bsp*MI site (pSP20) suggest a role for either or both the second set of 14-bp direct repeats or the two regions of dyad symmetry, each deletion removing one direct repeat and one region of dyad symmetry. While this series of deletions from the left end up to *Bsp*MI has progressively reduced the efficiency of mobilization, the resulting *oriT* plasmids can still be mobilized by donation, as demonstrated by the independent loss of the pCU101 helper plasmid in the transconjugants (Table 3). This is consistent with a structural role for the deleted sequences in promoting donation by allowing access, recognition, or stability by *trans*-acting elements that act at *oriT*.

Assuming that donation is initiated at a *nic* site, as is the case for *oriT* of other *tra* systems, these observations indicate that the *nic* site of this *oriT* region is to the right of the *Bsp*MI site. Since pBP11 was also shown to be donated, albeit at very low frequencies (Table 3), and it ends at the second *Ssp*I site, the *nic* site can be inferred to be in the 65-bp sequence between the *Bsp*MI site and the second *Ssp*I site (shown in the lowermost portion of Fig. 3). This region is different from the one proposed for the *nic* site of *oriT* of R46 (4), which would correspond to nucleotides 605 and 606 of *oriT* of pCU1 in Fig. 3. The latter proposal was based on sequence homology in this region of R46 to *oriT* of pSC101 (19). In the *oriT* region of pCU1, there are also sequences showing partial homology to *oriT* of ColE1 and *oriT* of

CloDF13 (nucleotides 426 to 450 and 467 to 489, respectively) (Fig. 3 and 4) (1, 22). Within the 65-bp sequence that is proposed here as the location of *nic* of *oriT* of pCU1 (and by implication, *oriT* of R46), there is a 7-bp inverted repeat proximal to an 81-bp AT-rich region (Fig. 3 and 4). The *nic* site of *oriT* of the F *tra* system is also located next to an AT-rich region (7).

The lowermost part of Fig. 3 suggests that in addition to the 65 bp defining the site of *nic*, regions to both the left and the right of this site are needed to promote efficient donation. The possible structural role of the sequences to the left of the *nic* site has been discussed. Features of sequences to the right of the proposed *nic* site suggest that they may be involved in conjugative DNA replication. The two inverted-repeat structures in this region may contribute to secondary structures that are recognized by proteins for the initiation of DNA synthesis at *oriT*. Another feature in this region that may be relevant is the presence of sequences similar to those that bind to the bacteriophage lambda IHF and which have been implicated in conjugal transfer of plasmid R100 (5). The proposals made here are speculative and are intended to stimulate further studies on this *oriT* region.

Plasmid specificity for *mob* functions among plasmids encoding related *tra* systems has been demonstrated for both the F- and P-type *tra* systems (30, 33). In both cases, specificity can be shown to involve *mob* proteins which bind within the *oriT* region and therefore are assumed to be involved in nicking (8, 30). This study has demonstrated that specificity within the N *tra* system is also due to *mob* functions and involves at least three complementation groups. Further comparative structural and functional studies of *mob* regions of the pMUR, pCU1, and pKM101 plasmids are required to clarify the role of the plasmid-specific *mob* functions.

ACKNOWLEDGMENTS

This research has been supported by the Medical Research Council of Canada and by an equipment grant from the Natural Sciences and Engineering Research Council of Canada.

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