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Initiation and Termination of DNA Transfer during Conjugation of IncI1 Plasmid R64: Roles of Two Sets of Inverted Repeat Sequences within *oriT* in Termination of R64 Transfer

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Intercellular transfer of plasmid DNA during bacterial conjugation initiates and terminates at a specific origin of transfer, *oriT*. We have investigated the *oriT* structure of conjugative plasmid R64 with regard to the initiation and termination of DNA transfer. Using recombinant plasmids containing two tandemly repeated R64 *oriT* sequences with or without mutations, the subregions required for initiation and termination were determined by examining conjugation-mediated deletion between the repeated *oriT*s. The *oriT* subregion required for initiation was found to be identical to the 44-bp *oriT* core sequence consisting of two units, the conserved nick region sequence and the 17-bp repeat A sequence, that are recognized by R64 relaxosome proteins NikB and NikA, respectively. In contrast, the nick region sequence and two sets of inverted repeat sequences within the 92-bp minimal *oriT* sequence were required for efficient termination. Mutant repeat A sequences lacking NikA-binding ability were found to be sufficient for termination, suggesting that the inverted repeat structures are involved in the termination process. A duplication of the DNA segment between the repeated *oriT*s was also found after mobilization of the plasmid carrying initiation-deficient but termination-proficient *oriT* and initiation-proficient but termination-deficient *oriT*, suggesting that the 3′ terminus of the transferred strand is elongated by rolling-circle-DNA synthesis.

Intercellular transfer of plasmid DNA during bacterial conjugation is accomplished by the function of transfer genes borne on each conjugative plasmid (for reviews, see references 4 and 15). All conjugative and mobilizable plasmids, such as R64, F, RP4, and R1162, contain *oriT* sites as *cis* elements which function as the origin of transfer of plasmid DNA. At the initiation stage of DNA transfer, a site- and strand-specific nick is introduced into the *oriT* site with a covalent attachment of the cognate relaxase protein to the 5′ terminus of the nicked strand. The nicked strand is transferred from donor to recipient cells with the 5′ terminus leading through a putative channel. In the donor cells, replacement strand DNA synthesis reconstitutes the double-stranded plasmid DNA. After one round of DNA transfer, the relaxase-attached 5′ terminus of the transferred-strand DNA is religated to its 3′ terminus to reconstitute the circular structure, and complementary-strand synthesis establishes double-stranded plasmid DNA in the recipient cells. Among these steps, the mechanisms by which initiation and termination of conjugative DNA transfer occur are important issues which remain to be elucidated.

Initiation and termination of conjugative DNA transfer at the *oriT* site were extensively studied using a small mobilizable plasmid, R1162 (RSF1010). R1162 *oriT* consists of a specific nick site and a 10-bp inverted repeat with one mismatch, which is situated 8 bp from the nick site (3, 27). Three R1162 proteins, MobA, MobB, and MobC, form a protein-DNA complex called the relaxosome at *oriT* (27). From the mobilization experiments with recombinant plasmids containing two tandemly repeated *oriT* sequences with or without mutations, the 10-bp inverted repeat structure as well as the sequence around the nick site was shown to be required for termination, while the nick site-distal arm of the inverted repeat was not required for initiation (1). From the analyses of mutations introduced into the 10-bp repeat, it has been postulated that during termination of DNA transfer, formation of a hairpin loop structure by the inverted repeat is required for the resealing of the transferred DNA by the R1162 MobA relaxase (32).

The initiation and termination of DNA transfer at *oriT* of the F plasmid, the fertility factor of *Escherichia coli*, were also studied. The F *oriT* sequence is located within an approximately 250-bp segment at one end of the transfer region (5, 10). Within the *oriT* sequence, the binding sites for F-encoded TraY and TraM and for integration host factor (IHF) are situated near the nick site that is recognized by F TraI protein. F TraI has both *oriT*-specific nicking activity and DNA helicase activity (17, 24). Although the 250-bp F *oriT* sequence is required for efficient conjugation, an approximately 100-bp sequence that contains the nick region sequence and IHF- and TraY-binding sites is required for efficient nicking in vivo by the TraI nickase activity (10). From the mobilization experiments with recombinant plasmids containing two tandemly repeated *oriT* sequences of various lengths, the 100-bp *oriT* sequence required for the efficient nicking was found to be essential for the initiation of DNA transfer (10). However, only a 36-bp F *oriT* sequence containing the nick site but missing all of the IHF-, TraY-, and TraM-binding sites was sufficient for termination (10).

The IncI1 plasmid R64 carries a 54-kbp transfer region containing genes required for several steps of the conjugation process, such as the formation of two kinds of conjugative pili and processing of DNA during DNA transfer (12–14, 31). Located at one end of the R64 transfer region is an *oriT* operon consisting of an *oriT* site and two genes, *nikA* and *nikB*, encoding an *oriT*-binding protein and a putative relaxase, respectively (9). Deletion experiments have identified a 92-bp minimal *oriT* sequence (*oriT92* [see Fig. 2]) which consists of a specific nick site, repeat A, repeat B, and 8-bp inverted repeat

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sequences and displays full *oriT* activity (8). The repeat A and repeat B sequences form a 17-bp inverted repeat with a 1-bp mismatch. The ATCCTG sequence from the 3′ end of the nick site is precisely conserved among the *oriT* sequences of various conjugative and mobilizable plasmids, such as RP4 (RK2), R751, and pTF-FC2, and the T-DNA border sequences of Ti and Ri plasmids (19, 29, 30). The specific relaxases, R64 NikB, RP4 and R751 TraI, and Ti VirD2, share three conserved amino acid sequence motifs that recognize the conserved nick region sequence (23). RP4 TraI and Ti VirD2 were actually shown to cleave and religate single-stranded oligonucleotides containing their respective nick region sequences (21–23), suggesting that the analogous R64 protein, NikB, also shares these activities.

To analyze the relationship between the structure and function of the R64 *oriT* sequence, various deletion, insertion, and substitution mutants were constructed (see Fig. 2) (8). Removal of the 8-bp inverted repeats from the minimal *oriT* sequence resulted in a slight decrease in mobilization (*oriT64* [see Fig. 2]). A 44-bp *oriT* core sequence containing the nick region and repeat A sequences (*oriT44*) exhibited a mobilization frequency 1/25 that of the minimal *oriT* (*oriT92*). The NikA protein was shown to specifically bind to the repeat A sequence but not to the repeat B sequence (see Fig. 2) (7). The NikA and NikB proteins form a relaxosome at the minimal or core *oriT* sequence. Upon sodium dodecyl sulfate or proteinase treatment of the relaxosome, a strand- and site-specific nick was introduced at the *oriT* nick site. The failure to form a functional relaxosome (*oriT32*, *oriT92-G21C*, and *oriT*Δ*28*) results in an incapability to mobilize. The NikA-binding sequence was required to be localized to a precise position relative to the nick site (*oriT44-*Ω*11T*).

To explain the differences in mobilization frequencies of the minimal *oriT* sequence and the *oriT* core sequence, we have previously predicted that the *oriT* core sequence is essential for the initiation of R64 DNA transfer and that the remaining sequence of the minimal *oriT* is involved in the termination (8). Here we present results which strongly support our previous prediction.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains NF83 and NF84 are streptomycin-resistant (Sm^r) and nalidixic acid-resistant (Nal^r) derivatives of *E. coli* CL83 *recA* (16), respectively. *E. coli* K-12 strain TN102 is a nalidixic acid-resistant derivative of the wild-type strain W3110 (13). *E. coli* K-12 strain JC7623 *recB21 recC22 sbcB15 thr thi leu his pro arg rpsL* (18) was used for the construction of mini-R64 plasmid pKK610a. Vector plasmid pHSG398 (28) was used for the construction of dual *oriT* plasmids.

Medium. Luria-Bertani broth was prepared as previously described (26). The solid medium contained 1.5% agar. Antibiotics were added to liquid or solid medium at the following concentrations: chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 25 μg/ml; and streptomycin, 200 μg/ml.

Recombinant DNA techniques. Recombinant DNA techniques and Southern blot hybridization were performed as previously described (26). Mini-R64 plasmid pKK610a, which contains the replication region and transfer region without the *oriT* core sequence of plasmid R64*drd-11*, as well as the kanamycin resistance (Km^r) gene, was constructed from pKK610 by the in vivo recombination method (13). Most of the mutant *oriT* sequences have been described previously (8). The *oriT92-*Ω*55* and *oriT92-*Ω*465* mutations were constructed by inserting 55- and 465-bp *Nde*I fragments, respectively, into an *Nde*I site generated between the 8- and 17-bp inverted repeat sequences by the PCR-mediated site-directed mutagenesis method (11). The inserted DNA fragments were obtained from the R64 *traABCD* region (nucleotide numbers 239 to 293 and 1358 to 1822 of the sequence under GenBank accession number AB027308 for the 55- and 465-bp fragments, respectively) (12). Dual *oriT* plasmids, pKK541 through pKK555 (see Fig. 1B), were constructed by inserting various mutant *oriT* sequences (see Fig. 2) and the tetracycline resistance (Tc^r) gene cassette within the multicloning sites of pHSG398 as indicated in Fig. 1A.

Conjugal transfer. To determine the mobilization frequency of plasmids with mutant *oriT*s, liquid mating was performed as described previously (12). Donor *E. coli* NF83 cells carrying both mini-R64 plasmid pKK607 and one of the mutant *oriT* plasmids were mated with recipient *E. coli* TN102 cells for 90 min at 37°C. The mobilization frequency was expressed as the ratio of the transfer frequency of the *oriT* plasmid to that of helper plasmid pKK607. For each mutant *oriT* plasmid, mobilization frequencies were determined from at least five independent experiments and their mean value was calculated.

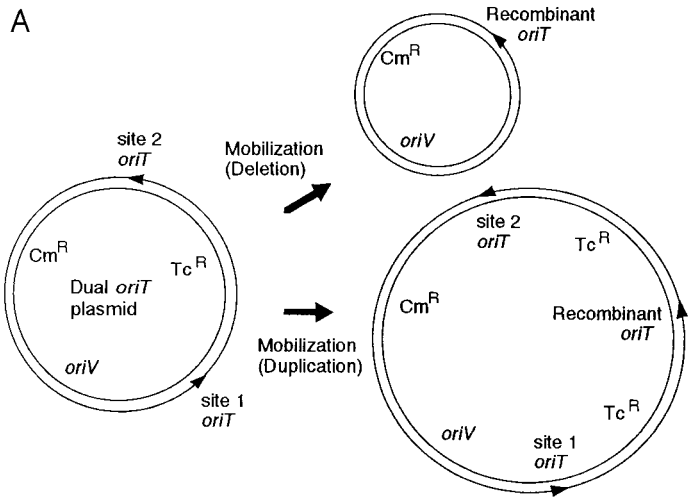
To determine mobilization-mediated deletion and duplication, *E. coli* NF84 (Nal^r) donor cells carrying both mini-R64 plasmid pKK610a and one of the dual *oriT* plasmids were mated with recipient *E. coli* NF83 (Sm^r) cells for 90 min at 37°C by the surface mating method (12). The mating mixture was directly inoculated into Luria-Bertani medium containing chloramphenicol and streptomycin for the selection of transconjugants and incubated overnight. DNAs were extracted from the transconjugants by the alkaline lysis method (26). After linearization at the unique *Eco*RI site, they were analyzed by 0.7% agarose gel electrophoresis. The DNA bands were visualized by staining with ethidium bromide. The relative amounts of DNA bands were measured by Southern blot analysis and probing with ^{32}P-labeled pHSG398 DNA. The intensity of radiolabeled DNA bands was measured by the BAS-2000 bioimaging analyzer system (Fuji).

RESULTS

Experimental design. To determine the *oriT* subregion(s) required for the initiation and termination of R64 DNA transfer, the dual *oriT* plasmid method originally developed by Bhattacharjee and collaborators was used (1). We have constructed plasmids carrying two *oriT* sequences in the same direction and a Tc^r segment between the two *oriT*s (Fig. 1A). The dual *oriT* plasmids were mobilized from donor to recipient cells by mini-R64 plasmid pKK610a. Since pKK610a contains all the transfer genes except the *oriT* core sequence, it is able to mobilize the dual *oriT* plasmids but unable to transfer itself. If the initiation of DNA transfer occurs at site 1 *oriT* on the dual *oriT* plasmid and the termination occurs at site 2 *oriT*, the plasmids recovered from the transconjugants are predicted to lose the Tc^r segment between the site 1 and site 2 *oriT*s (Fig. 1A). Such a deletion event is predicted to occur if site 1 and site 2 *oriT*s are initiation and termination proficient, respectively, whereas it may not occur if the site 2 *oriT* is termination deficient or the site 1 *oriT* is initiation deficient. Thus, it is possible to separately assess the initiation and termination abilities of mutant *oriT* sequences by examining the mobilization-mediated deletion of the dual *oriT* plasmids. Mutant *oriT* sequences used to construct dual *oriT* plasmids (Fig. 1B) are summarized in Fig. 2. Their mobilization frequencies when present once in each plasmid are also indicated in Fig. 2.

The R64 *oriT* segment required for the initiation of DNA transfer corresponds to the *oriT* core sequence. The minimal *oriT* sequence with full activity has been located within a 92-bp sequence of R64, as described in the introduction (*oriT92* in Fig. 2). When pKK541 with dual *oriT92* sequences was mobilized by pKK610a, transfer-mediated deletion was observed (Fig. 3). The fact that this deletion occurred precisely between the site 1 and site 2 *oriT*s was confirmed by restriction analysis and DNA sequencing. It was estimated that 64% of the mobilized plasmid carried the deletion (Fig. 2).

The minimal R64 *oriT* sequence consists of four structural units: a nick region, repeat A, repeat B, and 8-bp inverted repeat sequences (8). To determine the *oriT* subregion(s) required for the initiation of DNA transfer, various lengths of *oriT* segments were introduced into site 1 with the *oriT92* sequence retained at site 2, and then mobilization-mediated deletion was examined. When the *oriT* core sequence (*oriT44*) was used as a site 1 *oriT*, mobilization-mediated deletion was not affected (pKK542 in Fig. 3), indicating that the inverted repeat structures are not required for the initiation. However, when *oriT32* and *oriT44-*Ω*11T* sequences without *oriT* activity were used as a site 1 *oriT*, mobilization-mediated deletion was not observed (pKK543 and pKK544 in Fig. 3). In pKK543 and pKK544, mobilization is thought to initiate and terminate at site 2 *oriT*. These results indicate that the R64 *oriT* segment



B

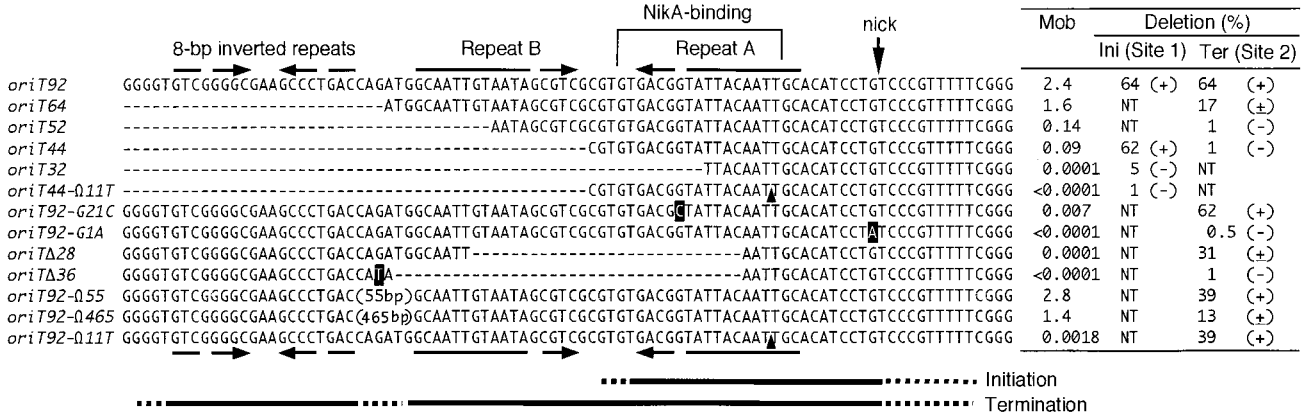
Plasmid	site 1 *oriT* (Initiation)	site 2 *oriT* (Termination)
pKK541	*oriT92*	*oriT92*
pKK542	*oriT44*	*oriT92*
pKK543	*oriT32*	*oriT92*
pKK544	*oriT44-Ω11T*	*oriT92*
pKK545	*oriT92*	*oriT64*
pKK546	*oriT92*	*oriT52*
pKK547	*oriT92*	*oriT44*
pKK548	*oriT92*	*oriT92-G21C*
pKK549	*oriT92*	*oriT92-Ω11T*
pKK550	*oriT92*	*oriTΔ28*
pKK551	*oriT92*	*oriTΔ36*
pKK552	*oriT92*	*oriT92-Ω55*
pKK553	*oriT92*	*oriT92-Ω465*
pKK554	*oriT92*	*oriT92-G1A*
pKK555	*oriTΔ28*	*oriT52*

FIG. 1. (A) Schematic representation of a plasmid for the investigation of initiation and termination functions of the *oriT* sequence. The deletion of the Tc^r segment occurs when the transfer is initiated and terminated at site 1 and site 2 *oriT*s, respectively, during mobilization. The duplication of the Tc^r segment occurs when the transfer is initiated at site 2 *oriT* and terminated at site 1 *oriT* after passing through an entire round of transfer. The arrowheads indicate the 5′ ends of the nicked strand at *oriT*. Cm^r, chloramphenicol resistance; *oriV*, origin of replication. (B) Structure of dual *oriT* plasmids. Mutant *oriT* sequences inserted into site 1 and site 2 of the dual *oriT* plasmids are described in Fig. 2.

required for the initiation of DNA transfer corresponds to the *oriT* core sequence.

The two inverted repeat structures within R64 *oriT* are required for efficient termination of DNA transfer. To determine the *oriT* subregion(s) required for the termination of DNA transfer, various mutant *oriT* sequences were introduced into site 2 with the minimal *oriT92* sequence retained at site 1, and then mobilization-mediated deletion was examined (Fig. 3). Deletion of the 8-bp inverted repeats (*oriT64* in Fig. 2) resulted in a decrease in mobilization-mediated deletion (pKK545 in Fig. 3), indicating an involvement of the 8-bp inverted repeats in termination. A further decrease in mobilization-mediated deletion was observed for the *oriT52* and *oriT44* mutants, in which a portion and all of the repeat B sequence were removed, respectively (pKK546 in Fig. 3).

On the other hand, several *oriT* mutants without *oriT* activity displayed termination activity. The *oriT92-G21C* mutant without *oriT* activity was found to display normal termination activity (pKK548 in Fig. 3), suggesting that NikA binding to the repeat A sequence is not essential for termination. The *oriT92-Ω11T* mutation, which severely affects *oriT* activity but not NikA binding (8), also yielded efficient termination activity (pKK549 in Fig. 3), indicating that this mutation diminishes initiation activity but does not affect termination activity. Furthermore, the *oriTΔ28* mutant, in which internal halves of the repeat A and B sequences were removed from the *oriT92*



		Mob	Deletion (%) Ini (Site 1)		Ter (Site 2)	
oriT92	GGGGTGTCGGGGCGAAGCCCTGACCAGATGGCAATTGTAATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	2.4	64	(+)	64	(+)
oriT64	----------ATGGCAATTGTAATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	1.6	NT		17	(±)
oriT52	----------AATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	0.14	NT		1	(−)
oriT44	----------CGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	0.09	62	(+)	1	(−)
oriT32	----------TTACAATTGCACATCCTGTCCCGTTTTTCGGG	0.0001	5	(−)	NT	
oriT44-Ω11T	----------CGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	<0.0001	1	(−)	NT	
oriT92-G21C	GGGGTGTCGGGGCGAAGCCCTGACCAGATGGCAATTGTAATAGCGTCGCGTGTGACGCTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	0.007	NT		62	(+)
oriT92-G1A	GGGGTGTCGGGGCGAAGCCCTGACCAGATGGCAATTGTAATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTATCCCGTTTTTCGGG	<0.0001	NT		0.5	(−)
oriTΔ28	GGGGTGTCGGGGCGAAGCCCTGACCAGATGGCAATT----------AATTGCACATCCTGTCCCGTTTTTCGGG	0.0001	NT		31	(±)
oriTΔ36	GGGGTGTCGGGGCGAAGCCCTGACCATA----------AATTGCACATCCTGTCCCGTTTTTCGGG	<0.0001	NT		1	(−)
oriT92-Ω55	GGGGTGTCGGGGCGAAGCCCTGAC(55bp)GCAATTGTAATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	2.8	NT		39	(+)
oriT92-Ω465	GGGGTGTCGGGGCGAAGCCCTGAC(465bp)GCAATTGTAATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	1.4	NT		13	(±)
oriT92-Ω11T	GGGGTGTCGGGGCGAAGCCCTGACCAGATGGCAATTGTAATAGCGTCGCGTGTGACGGTATTACAATTGCACATCCTGTCCCGTTTTTCGGG	0.0018	NT		39	(+)

FIG. 2. Effects of various deletion, insertion, and substitution mutations on mobilization and on initiation or termination of DNA transfer. DNA segments deleted from the original sequence are indicated by dashes. Substitution mutations are indicated by white letters. One-base insertions of T in *oriT44-Ω11T* and *oriT92-Ω11T* are indicated by arrowheads. Insertions in *oriT92-Ω55* and *oriT92-Ω465* are indicated by "(55bp)" and "(465bp)," respectively. Mobilization frequencies (Mob) of plasmids carrying a single copy of the mutant *oriT* sequences are expressed as the ratio of transfer frequencies of the *oriT* plasmids to that of pKK607. Some data for mobilization frequencies were taken from reference 8. Initiation (Ini) or termination (Ter) activities of various *oriT* mutants are expressed as the ratio (percentage) of DNA of the plasmid that has been subjected to mobilization-mediated deletion to total mobilized plasmid DNA from the dual *oriT* plasmids carrying mutant *oriT* at site 1 (pKK542 to pKK544) or mutant *oriT* at site 2 (pKK545 to pKK554), respectively. The ratio was determined by measuring intensity of DNA bands in Fig. 3 using Southern hybridization with ^{32}P-labeled pHSG398 DNA as a probe. NT, not tested. Inferred initiation or termination activities are indicated in parentheses (+, the efficiency is similar to that of *oriT92*; ±, weak activity; −, no activity). Solid lines at the bottom indicate the regions required for initiation and termination. Broken lines indicate the junctions bordering the essential sequences.

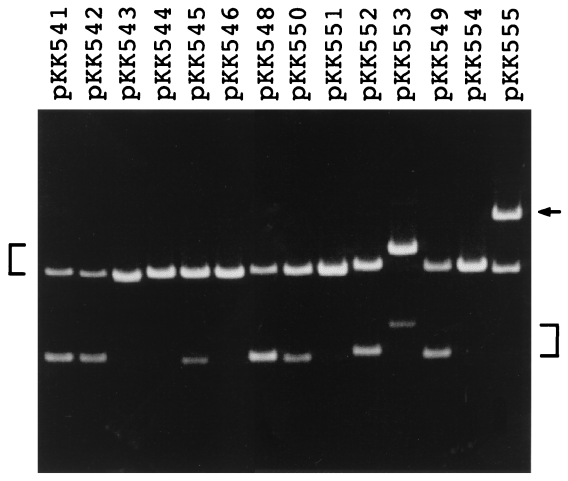


FIG. 3. Mobilization-mediated recombination of dual *oriT* plasmids. Each plasmid was mobilized from donor cells by the mini-R64 plasmid pKK610a. Mobilized plasmids were recovered from the transconjugants, linearized at the unique *Eco*RI site, and subjected to agarose gel electrophoresis followed by staining with ethidium bromide. Plasmid bands of original size are indicated by a bracket on the left. Plasmid bands with the deletion or duplication of the Tc^r segment are indicated by a bracket or an arrow, respectively, on the right.

sequence, was found to have less efficient but still significant termination activity (pKK550 in Fig. 3). In contrast, termination activity was severely reduced for *oriTΔ36*, in which the repeat B sequence was completely removed from *oriTΔ28* (pKK551 in Fig. 3). These results indicate that both the 8- and 17-bp inverted repeat sequences are required for efficient termination of *oriT*-mediated DNA transfer, whereas NikA binding to repeat A is not essential for termination.

The 8-bp inverted repeats still function in termination when positioned at a more distant location. The above-described results indicate that both the 8- and 17-bp inverted repeat structures are required for efficient termination. We next examined whether the distance between the two inverted repeats affects termination activity. The *oriT92-Ω55* and *oriT92-Ω465* mutants, containing 55- and 465-bp insertions between the two inverted repeats, respectively, were constructed (Fig. 2), and their termination activities were measured. pKK552, carrying *oriT92-Ω55* at site 2, exhibited half the level of termination activity of the wild type (Fig. 3), indicating that the 8-bp inverted repeat is able to function at a distant location. On the other hand, pKK553, carrying *oriT92-Ω465*, exhibited the same level of termination activity as *oriT64* lacking the 8-bp inverted repeat, indicating that the 8-bp repeat does not function beyond a certain distance. These results suggest that the 8-bp inverted repeat is functional in the termination of DNA transfer at a location separate from that of the 17-bp inverted repeat, but the effect gradually decreases with the length of distance between them.

The nick region sequence is required for the termination of DNA transfer. Mutations introduced into the conserved nick region sequence resulted in severely decreased *oriT* activity (8). We examined whether this type of mutation affects the termination efficiency at site 2 *oriT*. The termination efficiency of pKK554 (with the *oriT92-G1A* mutation at site 2) was very low (Fig. 3), indicating the importance of the conserved nick region sequence in termination, as was expected from the established importance of the nick region sequence for recognition by relaxases (23).

Mobilization-mediated duplication of the Tc^r segment. It was found that mutant *oriT* sequences, such as *oriT92Δ28* and *oriT92-G21C*, retain significant termination activities, although they do not display *oriT* activities due to the lack of a functional repeat A sequence. Such mutations are likely to be initiation deficient but termination proficient. When the initiation-deficient but termination-proficient *oriT92Δ28* sequence and the initiation-proficient but termination-deficient *oriT52* sequence are located at site 1 and site 2, respectively, transfer of the resultant plasmid, pKK555, is predicted to occur at *oriT52* at site 2, proceed through the entire plasmid, pass site 2 *oriT*, and finally terminate at *oriTΔ28* at site 1, resulting in the duplication of the Tc^r segment (Fig. 1A, duplication). pKK555 was found to actually generate 62% of a plasmid larger than the unit length through mobilization (Fig. 3). The Tc^r-duplicated structure of the large plasmid formed from pKK555 was confirmed by restriction analyses (data not shown). In addition, a low level (approximately 2%) of Tc^r-duplicated plasmid was produced after mobilization of dual *oriT* plasmids carrying initiation-proficient *oriT* at site 2 and termination-proficient *oriT* at site 1, including pKK541, -545, -546, -547, -552, and -553 (data not shown). These results confirm the discrete functions of the R64 *oriT* sequence for the initiation and termination of DNA transfer.

DISCUSSION

In this study, we have analyzed the subregions of the R64 *oriT* sequence required for the initiation and termination of DNA transfer during conjugation. The initiation and termination activities of each mutant *oriT* sequence inferred from the present work are summarized in Fig. 2. From these results, we conclude that different portions within the R64 *oriT* sequence are required for the initiation and termination of DNA transfer as illustrated at the bottom of Fig. 2.

The R64 *oriT* core sequence (8) turned out to be identical to the region required for the initiation of DNA transfer. However, the remaining region appears not to be required for the initiation process. It is likely that the decreased *oriT* activity of the R64 *oriT* core sequence compared to that of the minimum *oriT* sequence is due to deficiency of the termination activity.

The R64 *oriT* region required for efficient termination of DNA transfer was found to consist of the following three sequences: (i) the nick region sequence, (ii) the 17-bp inverted repeat sequences, and (iii) the 8-bp GC-rich inverted repeat sequences. The nick region sequence is essential for the termination process of DNA transfer, since the *oriT92-G1A* mutation caused severe defects in termination. In RP4, the TraI relaxase was found to cleave and religate single-stranded DNA containing the nick region sequence (20). Therefore, the R64 nick region sequence may be required for rejoining the 5′ terminus of the transferred strand by R64 NikB relaxase to the 3′ terminus at the termination stage of R64 DNA transfer.

Although the importance of the repeat A sequence for NikA binding and subsequent *oriT* activity has been established (8), the inverted repeat structure itself within the 17-bp inverted repeat sequences is sufficient for the termination of DNA transfer, since the mutations preventing NikA binding to the repeat A sequence (e.g., *oriT92-G21C*) did not abolish termination activity. Significant termination activities were detected when a portion of the 17-bp inverted repeat structure was removed (*oriT92Δ28*) or when a one-base insertion was introduced between the nick region sequence and the 17-bp inverted repeat sequences (*oriT92-Ω11T*). Thus, NikA binding to a precise location of *oriT* is not necessary for termination, although it is essential for R64 relaxosome formation at the

initiation stage (8). The role of the 17-bp inverted repeat sequences in the termination of R64 DNA transfer is similar to that of the 10-bp inverted repeat sequences within R1162 *oriT*. In the case of R1162 *oriT*, it is postulated that, at the termination step of R1162 DNA transfer, a hairpin loop structure formed by the 10-bp inverted repeats located 8 bp upstream from the nick site is directly recognized by the R1162 relaxase protein MobA (2, 32).

In addition to the 17-bp inverted repeat sequences, the 8-bp inverted repeat sequences were also required for efficient termination. They might help the termination ability of 17-bp repeat sequences in an enhancer-like function. The 8-bp inverted repeats were still functional when moved to an upstream position within a certain distance (*oriT92-Ω55*). However, movement to a further location (*oriT92-Ω465*) diminished the stimulation effect.

It is noteworthy that the R64 core *oriT* sequence carries residual termination activity although the minimal *oriT* sequence is required for efficient termination, since most of pKK535 carrying a single copy of the R64 core *oriT* sequence was monomeric after mobilization (data not shown). On the other hand, the minimal *oriT* sequence did not exhibit 100% termination activity, since 36% of pKK541 was still in the original form after mobilization. The reasons for these phenomena are unknown.

There is a global similarity in the *oriT* structure between R64 and IncP plasmids RP4 and R751 (6). Such a similarity also exists within the *oriT* sequences of IncI2 plasmid R721 (data not shown) and the mobilizable plasmid pTF-FC2 (25). In these plasmids, there are long (17- to 19-bp) inverted repeats 8 bp apart from the nick site. The GC-rich short (6- to 8-bp) inverted repeats are situated 6 to 54 bp upstream of the long ones. Although the sequences of the inverted repeats themselves are not similar, the nick region sequence and most of the relaxosome proteins are conserved in these plasmids (R64 NikA and NikB, RP4 TraJ and TraI, and pTF-FC2 MobB and MobA). The RP4 TraJ protein was shown to specifically bind to the nick site-proximal arm of the 19-bp inverted repeat sequence (33). These observations suggest that similar termination mechanisms of DNA transfer exist among IncI, IncP, and pTF-FC2 plasmids. In particular, the termination proficiency of the R64 *oriT92-Ω55* mutant suggests that short GC-rich inverted repeats function in efficient termination of IncP and pTF-FC2 plasmids even at a separate location.

It is an interesting question whether or not the 3′ terminus of the transferred strand is elongated by DNA synthesis in the donor cells to produce a transfer intermediate with a length greater than the unit length (15). Our present results suggest that this may be the case during R64 DNA transfer, in which the 3′ terminus of the transferred strand is elongated by rolling-circle DNA synthesis, since a plasmid greater than unit length was produced after mobilization of pKK555. If the 3′ terminus of the nicked strand remained free until completion of one round of DNA transfer, continuation of DNA transfer beyond the initiation site could not occur. It is thus obvious that the 3′ terminus of the nicked strand can be elongated before one round of DNA transfer is completed. It is possible that the 3′ terminus could be used directly as a primer for conjugative DNA synthesis.

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