Mutational Analysis of the R64 oriT Region: Requirement for Precise Location of the NikA-Binding Sequence

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Conjugative DNA transfer of IncI1 plasmid R64 is initiated by the introduction of a site- and strand-specific nick into the origin of transfer (oriT). In R64 oriT, 17-bp (repeat A and B) and 8-bp inverted-repeat sequences with mismatches are located 8 bp away from the nick site. The nicking is mediated by R64 NikA and NikB proteins. To analyze the functional organization of the R64 oriT region, various deletion, insertion, and substitution mutations were introduced into a 92-bp minimal R64 oriT sequence and their effects on oriT function were investigated. This detailed analysis confirms our previous prediction that the R64 oriT region consists of an oriT core sequence and additional sequences necessary for full oriT activity. The oriT core sequence consists of the repeat A sequence, which is recognized by R64 NikA protein, and the nick region sequence, which is conserved among various origins of transfer and is most probably recognized by NikB protein. The oriT core sequence is sufficient for NikAB-mediated oriT-specific nicking. Furthermore, it was shown that the repeat A sequence is essential for localization to a precise position relative to the nick site for oriT function. This seems to be required for the formation of a functional ternary complex consisting of NikA and NikB proteins and oriT DNA. The repeat B sequence and 8-bp inverted repeat sequences are suggested to be required for the termination of DNA transfer.

During conjugation, DNA transfer is initiated at the origin of transfer (oriT) of plasmid DNA, into which a site- and strand-specific nick is introduced by a nicking enzyme (for reviews, see references 3, 17, and 32). The nicked strand of DNA is transferred from donor to recipient cells with the 5’ end associated with the nicking enzyme. DNA transfer is terminated with the ligation of the ends of the transferred strand at oriT, resulting in a covalently closed-circle formation.

The structure and length of oriT sequences differ widely among various conjugative and mobilizable plasmids. Three major groups (IncF, IncI1-IncP, and IncQ) of oriT sequences have been identified by their respective nick region sequences (17). The oriT sequence of IncQ plasmid R1162 (RSF1010) has a simple structure and is short (38 bp) (2), while the structures of various plasmids appear to depend mainly upon the number and properties of proteins required for the overall oriT function.

Each conjugative or mobilizable plasmid encodes genes for its oriT-specific nicking-ligation enzymes required for the initiation and termination of DNA transfer. These proteins form a DNA-protein complex, called the relaxation complex or relaxosome, at oriT (17). Following sodium dodecyl sulfate (SDS) or proteinase treatment of the relaxation complex, introduction of a site- and strand-specific nick at the oriT site was observed.

The oriT sequence of the mobilizable plasmid R1162 (RSF1010) is located within a mere 38-bp segment, in which 10-bp inverted-repeat sequences with one mismatch are found 8 bp away from the nick site (2). Three R1162 proteins, MobA, MobB, and MobC, are required to form a relaxation complex, in which the MobA protein functions as a nicking-ligation enzyme (27, 34). In the IncF plasmids F and R100, the TraI protein, originally identified as DNA helicase I, introduces the oriT-specific nick (6, 18, 24). In addition to TraI, two F proteins, TraY and TraM, and the host protein, integration host factor (IHF), bind to the F oriT sequence. The binding sites of these proteins have been mapped within approximately 250 bp of the F oriT sequence (4, 19, 29).

The IncI1 and IncP plasmids, including R64, RP4, R751, and the Thiobacillus ferrooxidans mobilizable plasmid pTF-FC2, carry globally similar oriT structures (7, 8, 25). Inverted-repeat sequences of 17 to 19 bp with a mismatch(es) are located about 8 bp away from the nick site (for R64 oriT, see Fig. 1B). Short inverted-repeat sequences are also present further upstream. The sequences of the long and short inverted repeats are not similar to each other. However, a 6-bp sequence from the 3’ end of the nick site is completely conserved among these systems together with the T-DNA border sequences of Ti and Ri plasmids (21). At least two proteins, NikA and NikB in R64 and TraJ and TraI in RP4 or R751, are required to form a relaxation complex (7, 11, 20). NikA and TraJ proteins have amino acid sequence homology, as do NikB and TraI proteins. The RP4 TraI protein has been demonstrated to exhibit an oriT-specific nicking-ligation activity (22).

Plasmid R64 is a self-transmissible plasmid belonging to incompatibility group I (14, 15). The R64 transfer genes are encoded within a 54-kb region, with the oriT sequence located at one end (15, 16). The genes encoding the R64 oriT-specific nicking enzyme, nikA and nikB, are adjacent to oriT, forming the R64 oriT operon (Fig. 1A). When a recombinant plasmid carrying the R64 oriT operon was introduced into Escherichia coli cells, a relaxation complex was formed at the oriT site (11). Purified NikA protein was demonstrated to specifically bind to the nick site-proximal arm (repeat A) of the 17-bp inverted-repeat (single-mismatch) sequences (Fig. 1B) (9).

In this study, various substitution, deletion, and insertion mutations were introduced into the R64 oriT sequence to analyze its functional organization. We have identified three

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Functional units: a nick region sequence, the nikA-binding sequence, and inverted-repeat sequences.

MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *E. coli* K-12 strain JM109 (33) was used for cloning. *E. coli* NF83 (Sm<sup>·</sup>rec4) (10) and TN102 (Na<sup>+</sup> strain of *W3110*) (15) were used for conjugation experiments.

The cloning vectors pHSG398, pHSG399 (28), and pTK219 (15) were used. The mini-R6<sup>kd</sup>-oriT plasmid pKK607 has been described previously (26).

**Construction of plasmids.** Recombinant DNA techniques were carried out as described previously (26). Mutations were introduced by PCR-mediated site-directed mutagenesis (13). To construct a minimal oriT plasmid, an AFII site was introduced by site-directed mutagenesis, as shown in Fig. 1B, to give pKK530. Then pKK531 was cloned by growing the 92-bp AFII-HaeIII DNA fragment containing the 92-bp R6<sup>kd</sup> oriT sequence of pKK530 into the HaeIII site of pHSG398 (see Fig. 2). pKK531-a through pKK531-g were generated from pKK531 by site-directed mutagenesis (see Fig. 2).

**Materials and Methods** (Fig. 1B). pKK531 was efficiently mobilized by R6<sup>kd</sup> at a frequency similar to that of R6<sup>kd</sup> transfer. Within the 141-bp oriT sequence, characteristic sequences such as 17-bp (repeat A and B) and 8-bp inverted repeats and a specific nick site are found (Fig. 1B). However, the 141-bp fragment includes the N-terminal portion of the nikA gene (11). To further define the boundary of the R6<sup>kd</sup> oriT sequence, pKK531 carrying a 92-bp R6<sup>kd</sup> oriT sequence (without nikA coding sequence) was constructed as described in Materials and Methods (Fig. 1B). pKK531 was efficiently mobilized by the R6<sup>kd</sup>-drd-11 derivative plasmid pKK607 (Fig. 2), indicating that the 92-bp sequence carries the entire oriT function.

**Gel retardation assay.** Cleared lysates were prepared from *E. coli* NF83 cells harboring oriT plasmids and pKK531b as described previously (11), except that M<sub>9</sub> glucose containing 0.5% Bacto Tryptone (Difco) was used as the medium. SDS treatment was conducted by adding 3 g/15% SDS in 5× TES buffer (0.25 M Na<sub>2</sub>C<sub>2</sub>, 25 mM EDTA, 0.25 M Tris-HCl [pH 8.0]) to 12 g/10 ml of cleared lysate followed by incubation at 37°C for 5 min. SDS-treated samples were analyzed by agarose gel electrophoresis (0.7% agarose).

**RESULTS**

Construction of a minimal oriT plasmid of R6<sup>kd</sup>. The R6<sup>kd</sup> oriT sequence was previously shown to be located within the 141-bp RsaI-HaeIII DNA fragment (16) (Fig. 1A). The recombinant plasmid pKK508 carrying the 141-bp DNA sequence was mobilized by R6<sup>kd</sup> at a frequency similar to that of R6<sup>kd</sup> transfer. Within the 141-bp oriT sequence, characteristic sequences such as 17-bp (repeat A and B) and 8-bp inverted repeats and a specific nick site are found (Fig. 1B). However, the 141-bp fragment includes the N-terminal portion of the nikA gene (11). To further define the boundary of the R6<sup>kd</sup> oriT sequence, pKK531 carrying a 92-bp R6<sup>kd</sup> oriT sequence (without nikA coding sequence) was constructed as described in Materials and Methods (Fig. 1B), pKK531 was efficiently mobilized by the R6<sup>kd</sup>-drd-11 derivative plasmid pKK607 (Fig. 2), indicating that the 92-bp sequence carries the entire oriT function.

Replacement of the mismatched bases of the repeat A and B sequences. While the repeat A and B sequences differ by only one base (Fig. 1B), gel retardation and DNase I footprinting analyses revealed that purified NikA protein specifically bound to the repeat A sequence but not to the repeat B sequence (9). This suggests that the mismatched base in the repeat A and B sequences is critical for recognition by the NikA protein. To confirm the specificity of NikA binding to the repeat A and B sequences, their respective mismatched bases were replaced by site-directed mutagenesis to generate pKK531-a, pKK531-b, and pKK531-c (Fig. 2). In pKK531-a, the left-arm repeat sequence was replaced by the repeat A sequence; in pKK531-b,
the right-arm repeat sequence was replaced by repeat B sequence; and in pKK531-c, the positions of the repeat A and B sequences were exchanged. The effects of these mutations on NikA binding were examined by gel retardation analysis (Fig. 3). The plasmid DNAs were digested with FnuDII to separate the two arms of the repeat sequences into distinct DNA fragments, fragment 1 and fragment 2 (Fig. 2). In wild-type oriT plasmid pKK531, the gel mobility of fragment 2 containing the repeat A sequence was shifted in the presence of NikA protein but the gel mobility of fragment 1 containing the repeat B was unaffected (Fig. 3, lane 2). In pKK531-a, a mobility shift of both fragments was observed, indicating that the repeat A sequences in the two fragments were equally recognized by NikA (lane 3). In pKK531-b, in which both fragments contained the repeat B sequence, no mobility shift was observed (lane 4). In pKK531-c, fragment 1 containing the repeat A sequence was shifted but fragment 2 was not (lane 5). These results indicate that the repeat A sequence is recognized by the NikA protein regardless of its position. When the mismatched base (G) of the repeat A sequence within fragment 2 of pKK531 was replaced by A or T (pKK531-d and pKK531-e, respectively, in Fig. 2), binding of NikA to the modified repeat sequences was not detected (Fig. 3, lanes 6 and 7). These results suggest that the guanine residue at the mismatched position of the repeat A sequence is critical for NikA binding.

Effects of replacements of repeat A and B sequences on oriT activity. To examine the effects of mutations replacing the mismatched bases within repeat A and/or repeat B on oriT activity, mobilization frequencies of the mutant plasmids were determined with the mini-R64 plasmid pKK607 as a helper plasmid (Fig. 2). For each of the mutant plasmids except pKK531-a, various degrees of reduction of mobilization frequencies were observed. pKK531-a containing two repeat A sequences exhibited a mobilization frequency slightly higher than that of pKK531, suggesting that NikA binding to both arms of the repeat sequences appears to have a slightly positive effect on oriT activity. Mobilization frequencies of pKK531-b and pKK531-e lacking NikA binding sequence were less than 1/100 of that of pKK531. In pKK531-c, in which the locations of repeat A and B sequences were exchanged, the mobilization frequency was also reduced to about 1/250 of that of pKK531. The mobilization frequency of pKK531-d with the G-to-A mismatched bases within repeat A and/or repeat B on oriT sequence and a repeat B sequence (Fig. 4, lane 2). However, no relaxation was observed for any of the other plasmids, suggesting that these mutations affect the introduction of a specific nick mediated by the NikA and NikB proteins because of the absence (pKK531-b, pKK531-d, and pKK531-e) or incorrect location (pKK531-c) of the NikA-binding sequence.

Effects of mutations introduced into the nick region sequence. The DNA sequence between the R64 nick site and the repeat A sequence is highly conserved among the oriT sequences. Each plasmid DNA digested with FnuDII was incubated with purified NikA protein and electrophoresed on a 4% polyacrylamide gel. NikA-bound DNA fragments 1 and 2 are indicated by arrows on the left and right, respectively. Free DNA fragments 1 and 2 are indicated by arrowheads on the left and right, respectively.

![Figure 2](https://example.com/figure2.png)

**FIG. 2.** Mobilization frequencies and relaxation of mutant oriT plasmids. The R64 oriT sequence in pKK531 is shown at the top. For mutant plasmids pKK531-a through pKK531-g, only the replaced bases are shown. The repeat sequences, FnuDII site, and nick site are indicated. The mobilization frequency is expressed as the ratio of transfer frequency of the oriT plasmid to that of helper plasmid pKK607. The relaxation ability was summarized from Fig. 4: +, relaxation observed; –, relaxation not observed. Half-brackets indicate the DNA regions of fragments 1 and 2 produced by the FnuDII digestion (see Fig. 3).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Repeat A</th>
<th>Repeat B</th>
<th>nick</th>
<th>Mobilization</th>
<th>Relaxation</th>
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<td></td>
<td></td>
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<td>+</td>
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![Figure 3](https://example.com/figure3.png)

**FIG. 3.** Specific binding of the NikA protein to mutant oriT sequences. Each plasmid DNA digested with FnuDII was incubated with purified NikA protein and electrophoresed on a 4% polyacrylamide gel. NikA-bound DNA fragments 1 and 2 are indicated by arrowheads on the left and right, respectively.

The relaxation experiments were also performed. Cleared lysate was prepared by the mild-lysis method from *E. coli* cells harboring mutant oriT plasmids together with pKK518b into which the R64 nikA and nikB genes were cloned (11). Each cleared lysate was treated with 3% SDS and electrophoresed in a 0.7% agarose gel (Fig. 4). For wild-type oriT plasmid pKK531, a DNA band corresponding to the open-circular form of pKK531 appeared in addition to its closed-circular form (Fig. 4, lane 1). The open-circular DNA was generated by an introduction of a specific nick into oriT (Fig. 1B), mediated by NikA and NikB proteins (8, 11). Relaxation also occurred for pKK531-a containing two repeat A sequences instead of a single repeat A sequence and a repeat B sequence (Fig. 4, lane 2). However, no relaxation was observed for any of the other plasmids, suggesting that these mutations affect the introduction of a specific nick mediated by the NikA and NikB proteins because of the absence (pKK531-b, pKK531-d, and pKK531-e) or incorrect location (pKK531-c) of the NikA-binding sequence.

Effects of mutations introduced into the nick region sequence. The DNA sequence between the R64 nick site and the repeat A sequence is highly conserved among the oriT sequences.
sequences of many plasmids (21, 31). To test for the requirement of a specific nick region sequence, substitution mutations were introduced into this region. Transition mutations were introduced into the first and sixth nucleotides from the 3’ end of the nick site to generate pKK531-f and pKK531-g, respectively, and the mobilization frequencies of these plasmids were determined (Fig. 2). Both mutations resulted in severely decreased mobilization frequencies. In particular, no mobilization was detected for pKK531-f, indicating the strict requirement for a guanine residue at the 3’ end of the orIT nick site. Relaxation was not detected for either mutant orIT plasmid and pKK531 was observed. In contrast, NikA binding was not detected for orIT DNA fragments from plasmids pKK537 and pKK538, in which the repeat A sequence is deleted. These observations confirm the indispensability of NikA binding for orIT function.

To examine NikA binding to these deletion mutants, gel retardation assays were carried out with DNA fragments derived from the orIT deletion plasmids (Fig. 5). Efficient binding of NikA protein to the orIT fragments of plasmids pKK533, pKK534, pKK535, and pKK536, all of which contain the repeat A sequence, was observed. In contrast, NikA binding was not detected for orIT DNA fragments from plasmids pKK537 and pKK538, in which the repeat A sequence is deleted. These observations indicate that the orIT core sequence was sufficient for the formation of a functional relaxation complex.

**Precise location of the NikA-binding sequence relative to the nick site is essential for orIT function.** The repeat A sequence is located 8 bp away from the nick site. Since a double mutation in pKK531-c, in which the positions of repeat A and B sequences were exchanged, resulted in a severe decrease in mobilization frequency, it was inferred that NikA binding to the correct position of the orIT sequence is necessary for orIT activity. To test this hypothesis, a series of insertion and deletion mutations was introduced into the nick site-proximal side of the repeat A sequence (Fig. 6). pKK535-a through pKK535-d are derivatives of pKK535 carrying 1- to 13-bp deletions within or around the repeat A sequence, and pKK536-i carried 1- to 20-bp insertions at the nick site-proximal side of the repeat A sequence (Fig. 6). pKK535-a through pKK535-i carried 1- to 20-bp insertions at the nick site-proximal side of the repeat A sequence (Fig. 6). pKK535-a through pKK535-i carried 1- to 20-bp insertions at the nick site-proximal side of the repeat A sequence (Fig. 6). pKK535-a through pKK535-i carried 1- to 20-bp insertions at the nick site-proximal side of the repeat A sequence (Fig. 6).
tardation assays revealed that NikA binding was similar to that observed for the wild-type repeat A sequence with the exception of pKK535-c and pKK535-d (Fig. 6). However, neither mobilization nor relaxation was detected for any of these deletion or insertion mutant plasmids. Furthermore, a mere single-base deletion (pKK535-a and pKK535-b) or insertion (pKK535-e) completely abolished oriT activity. In addition, oriT activity was not recoverable for pKK535-h and pKK535-i in which 10- and 20-bp sequences, corresponding to one and two helical turns, respectively, were inserted to maintain the NikA binding site and the nick site in the same phase. These results indicate that NikA binding to the precise location of the oriT sequence is essential for specific nicking and, consequently, for oriT activity.

**DISCUSSION**

In the present study, we have introduced various mutations into the R64 oriT sequence to reveal significant features of the functional organization of the R64 oriT sequence that are required in cis for the initiation and termination of conjugation. Our previous prediction (8) that the functional R64 oriT sequence consists of a core oriT sequence as well as requisite additional sequence for full oriT activity has been confirmed by the present detailed analysis. The oriT core sequence is located within a 31-bp sequence containing the repeat A sequence and nick region sequence. pKK536 carrying the 31-bp R64 sequence was mobilized at a frequency 1/50 of that of the wild-type oriT plasmid.

The R64 oriT core sequence was found to consist of two elements: the nick region sequence and the repeat A sequence. From two mutations introduced into the nick region sequence of R64 oriT (pKK531-f and pKK531-g), requirement of a conserved nick region sequence for oriT activity was demonstrated, as in the case of RP4 and R1162 (2, 31). From two mutations introduced into the nick region sequence, homologous proteins, NikA and TraJ, respectively, bind only to the nick site-proximal arm of imperfect inverted-repeat sequences (9, 35). Removal of the nick site-distal arm of the inverted repeat from RP4 oriT caused a 10^3-fold reduction

| pKK535    | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-a  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-b  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-c  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-d  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-e  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-f  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-g  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-h  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |
| pKK535-i  | CGTGGCAGCTTATTACAAAATGACATCTGCTCCCCTTTTTCGGG |

**FIG. 6.** Effects on oriT activity of deletion and insertion mutations introduced into the oriT core sequence. The nucleotide sequence of oriT regions in pKK535 and its deletion and insertion derivatives are indicated. Deleted nucleotides are indicated by dashes. Inserted nucleotides are boxed. The mobilization frequency, NikA binding, and relaxation are as in Fig. 5. Weak binding of NikA is indicated by ±.
of transfer frequency, as was found in R64 oriT (30). Analyses of the mobilization of recombinant plasmids containing two R1162 oriT sequences have shown that the 10-bp inverted-repeat sequence, as well as the nick site sequence of R1162 oriT, is required for the termination of DNA transfer, while the nick site-distal arm of the 10-bp inverted repeat sequences is not required for initiation (1). It has been postulated that during termination of DNA transfer, formation of a hairpin loop by the inverted repeats is required for the ligation of transfer strand DNA by the R1162 MobA protein (34). These results strongly suggest that in addition to the oriT core sequence, the repeat B sequence and 8-bp inverted repeats are required for the termination of R64 DNA transfer. In contrast to IncQ and IncI1-IncP oriT, the F oriT sequence required for the termination of F transfer is only 36 bp long, while a region spanning at least approximately 100 bp from the nick site is required for the initiation of F transfer (12).

At the stage of termination of DNA transfer, the transferring strand is likely to form a hairpin loop structure between the repeat A and B sequences. This structure may function as a signal for the termination. To determine whether NikA protein binds to such a heteroduplex hairpin, we have performed a gel retardation assay by synthesizing oligonucleotides containing the repeat A and B sequences with or without mutations. However, NikA protein was shown to bind neither to the hairpin nor to the double-stranded heteroduplex DNA (data not shown). The mismatched nucleotide present in the repeat B sequence may function to ensure that the NikA protein binds only to the repeat A sequence. However, mutation of the repeat B sequence to repeat A had little effect on oriT activity, suggesting that the mismatch is not necessary.

It is remarkable that, in contrast to the global similarities between R64 and RP4 oriT sequences, the leader sequence adjacent to R64 oriT is not essential for DNA transfer, in contrast to RP4. The RP4 oriT leader region containing an approximately 200-bp intrinsic DNA-bending sequence was bound by TraK protein (36). Electron microscopic observations of TraK-oriT complexes suggest that DNA is wrapped around a core of TraK molecules (36). Deletion of the leader sequence from RP4 oriT resulted in a 102- to 104-fold decrease in transfer frequency (7, 30). It is postulated that binding of TraK may influence the local oriT topology to stimulate the unwinding of the neighboring nick region sequence for recognition by TraI protein. No such severe requirement for the leader sequence was detected for R64 oriT, which is analogous to IncQ plasmid R1162, in which the oriT sequence is located within a 38-bp sequence containing a specific nick site and a 10-bp inverted-repeat sequence (2).

For further elucidation of R64 oriT function, in vitro studies with purified NikA and NikB proteins are essential. Purification of NikB protein is in progress in our laboratory.

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REFERENCES


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