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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 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 *oriT* sequences of IncF plasmid F and IncP plasmid RP4 are complex and long (up to 250 bp) (5, 31). The differences in the *oriT* 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 I1 (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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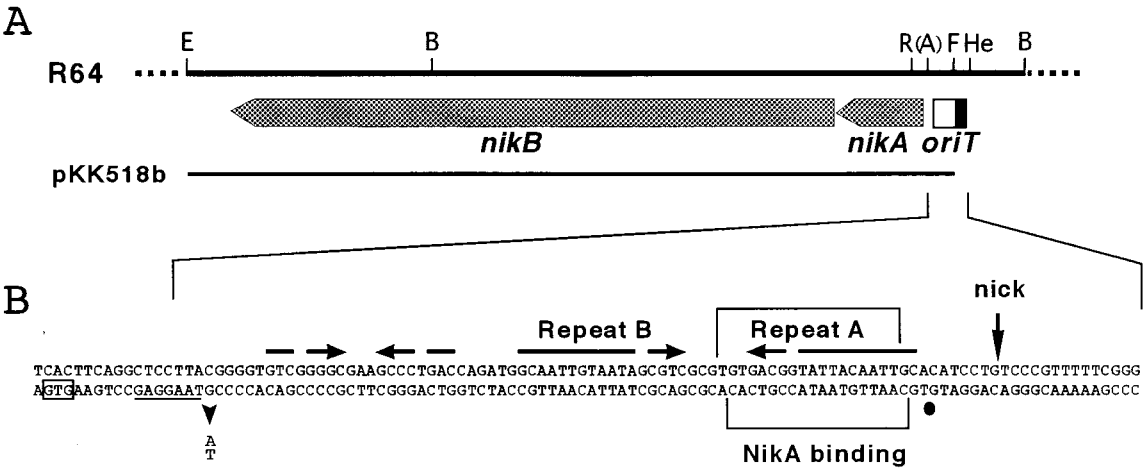

FIG. 1. (A) Structure of the R64 *oriT* operon. A restriction map of the R64 *oriT* region is shown at the top. The left-hand *Eco*RI site and right-hand *Bgl*II site correspond to R64 coordinates kb 53.3 and 56.9, respectively (15). B, *Bgl*II; E, *Eco*RI. Only relevant restriction sites are shown for *Hae*III (He), *Fnu*DII (F), and *Rsa*I (R). The *Afl*II (A) site introduced by site-directed mutagenesis is indicated in parentheses. Short and long wedges indicate coding sequences of the *nikA* and *nikB* genes, respectively. The solid and open bars indicate *oriT* core sequence and additional sequence required for full *oriT* activity, respectively (16; see the text). The solid line represents a DNA segment present in pKK518b (11), whose vector is pTK219 (pSC101 replicon). (B) Nucleotide sequence of the R64 *oriT* region (GenBank accession no. D90273). Repeat A and B sequences are indicated. Brackets and the solid circle mark the protected sequences and hypersensitive site upon binding of NikA protein, respectively, in DNase I footprint experiments (9). The initiation codon of the R64 *nikA* gene is boxed. Nucleotides replaced to generate an *Afl*II site are indicated by a downward arrowhead.

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 JM83 (33) was used for cloning. *E. coli* NF83 (Sm^r *recA*) (10) and TN102 (Nal^r strain of W3110) (15) were used for conjugation experiments.

The cloning vectors pHSG398, pHSG399 (28), and pTK219 (15) were used. The mini-R64*drd-11* plasmid pKK607 has been described previously (15).

Luria-Bertani and M9 glucose media were prepared as described previously (26). The agar medium contained 1.5% agar. The following antibiotics and concentrations were added to liquid or solid medium: chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml; and streptomycin, 200 μg/ml.

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 *Afl*II site was introduced by site-directed mutagenesis, as shown in Fig. 1B, to give pKK530. Then pKK531 was constructed by cloning the 96-bp *Afl*II-*Hae*III DNA fragment containing the 92-bp R64 *oriT* sequence of pKK530 into the *Hin*cII site of pHSG398 (see Fig. 2). pKK531-a through pKK531-g were generated from pKK531 by site-directed mutagenesis (see Fig. 2).

The *oriT* deletion plasmids pKK533, pKK534, pKK535, pKK537, pKK538, pKK539, and pKK540 were constructed by cloning various restriction fragments of pKK531 DNA into the multiple-cloning sites of pHSG398 or pHSG399 (see Fig. 5). pKK536 was constructed by inserting a 31-bp synthetic double-stranded DNA fragment into the *Xba*I-*Sal*I sites of pHSG398. pKK532 was generated by self-ligation of *Mun*I-digested pKK531 DNA which was blunt-ended by DNA polymerase I Klenow enzyme treatment.

pKK535-a, pKK535-b, pKK535-e, and pKK535-f were generated from pKK535 by site-directed mutagenesis (see Fig. 6). pKK535-c and pKK535-g were generated by self-ligation of *Mun*I-digested pKK535 DNA following S1 nuclease or Klenow enzyme treatment, respectively. pKK535-d was accidentally obtained by S1 nuclease treatment of *Mun*I-digested pKK535 DNA. pKK535-h and pKK535-i were generated by inserting a 10-bp linker (5′-GGCGCCAATT-3′) into the *Mun*I site of pKK535.

Conjugal transfer. Liquid mating was performed as described previously (15). *E. coli* NF83 and TN102 were used as the donor and recipient cells, respectively. A culture of log-phase donor cells was mixed with an overnight culture of recipient cells. The mixture was incubated for 90 min at 37°C.

Gel retardation assay. The NikA protein was purified as described previously (9). Purified NikA protein (ca. 0.2 μg) and DNA fragments (ca. 1 μg) were mixed in a buffer solution (10 μl) containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 7.5% glycerol. After incubation at 37°C for 10 min, reaction mixtures were loaded onto a 4% polyacrylamide gel in E buffer (40 mM Tris-acetate [pH 7.8], 1 mM EDTA) and electrophoresed at 10 V/cm at 4°C. Following electrophoresis, DNA fragments in the gel were stained with ethidium bromide and visualized under long-wave UV light.

Relaxation assay. Cleared lysates were prepared from *E. coli* NF83 cells harboring *oriT* plasmids and pKK518b as described previously (11), except that M9 glucose containing 0.5% Bacto Tryptone (Difco) was used as the medium. SDS treatment was conducted by adding 3 μl of 15% SDS in 5× TES buffer (0.25 M NaCl, 25 mM EDTA, 0.25 M Tris-HCl [pH 8.0]) to 12 μl 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 R64. The R64 *oriT* sequence was previously shown to be located within the 141-bp *Rsa*I-*Hae*III DNA fragment (16) (Fig. 1A). The recombinant plasmid pKK508 carrying the 141-bp DNA sequence was mobilized by R64 at a frequency similar to that of R64 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 R64 *oriT* sequence, pKK531 carrying a 92-bp R64 *oriT* sequence (without *nikA* coding sequence) was constructed as described in Materials and Methods (Fig. 1B). pKK531 was efficiently mobilized by the R64*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,

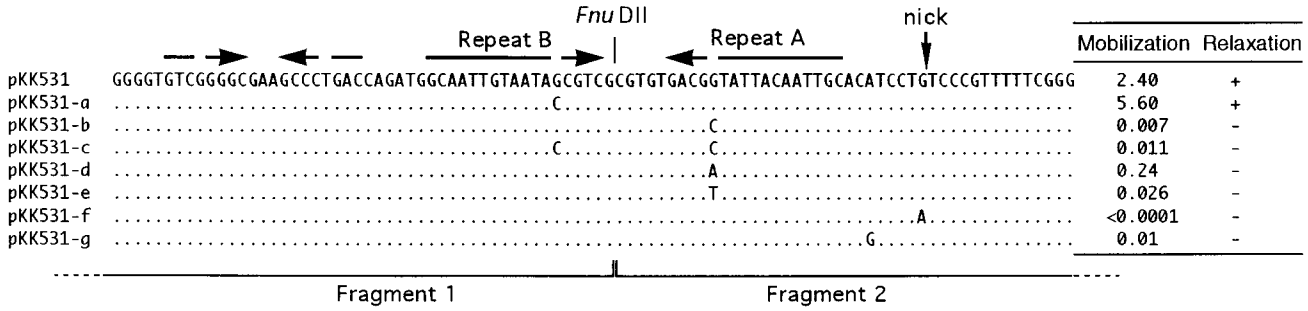

FIG. 2. Mobilization frequencies and relaxation of mutant *oriT* plasmids. The R64 *oriT* sequence in pKK531 is shown at the top. For mutant plasmids pKK531-a through pKK531-g, only the replaced bases are shown. The repeat sequences, *Fnu*DII site, and nick site are indicated. The mobilization frequency 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 *Fnu*DII digestion (see Fig. 3).

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 *Fnu*DII 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

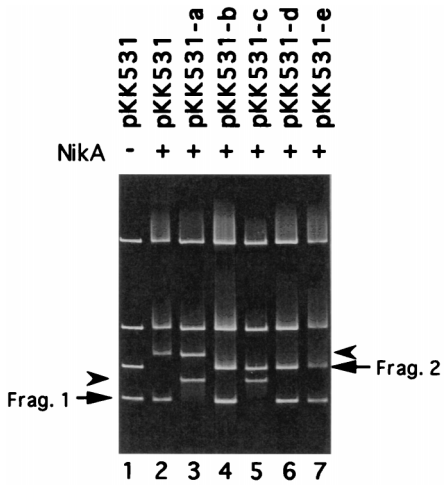

FIG. 3. Specific binding of the NikA protein to mutant *oriT* sequences. Each plasmid DNA digested with *Fnu*DII was incubated with purified NikA protein and electrophoresed on a 4% polyacrylamide gel. NikA-bound DNA fragments 1 and 2 are indicated by arrowheads on the left and right, respectively. Free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

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 mutation at the mismatched position in the repeat A sequence was not as greatly reduced as that of the other mutants (Fig. 2), although NikA binding to fragment 2 of pKK531-d was undetected (Fig. 3, lane 6). These results indicate that NikA binding to the nick site-proximal arm of the 17-bp inverted repeat sequences is required for *oriT* activity.

To investigate the effects of these mutations on the introduction of a specific nick into the *oriT* sequence, in vitro 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* se-

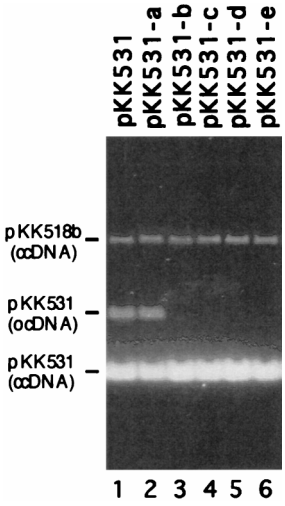

FIG. 4. Relaxation of mutant *oriT* plasmids by formation of relaxosome mediated by *nikA* and *nikB*. Cleared lysates prepared from *E. coli* cells harboring each mutant *oriT* plasmid and pKK518b were treated with 3% SDS and analyzed by agarose gel electrophoresis (0.7% agarose). The positions of closed-circular DNA (ccDNA) and open-circular DNA (ocDNA) of pKK518b or pKK531 and its derivative plasmids are indicated on the left.

quences 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 (Fig. 2), while NikA binding to both plasmids was observed (data not shown).

Deletion analyses of the R64 *oriT* sequence. To further analyze the relationship between DNA sequences and *oriT* activity, R64 *oriT* plasmids carrying deletions of various lengths were constructed and their mobilization frequencies were determined (Fig. 5). pKK533 lacking the leftmost 28-bp sequence, including the 8-bp inverted repeat sequences, was mobilized at a frequency slightly lower than that of pKK531. It is possible that the 8-bp repeat sequences is involved in *oriT* function. pKK534, pKK535, and pKK536, all of which lack the repeat B sequence, were mobilized at a frequency 1/20 to 1/50 of that of pKK531, indicative of the repeat B requirement for full *oriT* function (Fig. 5). pKK537, pKK538, and pKK539, carrying progressive deletions into the repeat A sequence, showed only trace *oriT* activity in comparison to wild-type *oriT*. pKK532, which lacks a 28-bp sequence spanning the central region of the 17-bp inverted repeat sequences, also exhibited trace *oriT* activity. In contrast, a 9-bp deletion at the rightmost region of pKK531 did not significantly affect the mobilization frequency (pKK540 in Fig. 5). These results indicate that the sequences essential for *oriT* core activity consist of the repeat A sequence and the nick region sequence.

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 confirm the indispensability of NikA binding for *oriT* function.

To examine the effects of the deletion mutations on the introduction of a specific nick into the *oriT* site, the relaxation of these mutant plasmids mediated by the NikA and NikB proteins was measured (Fig. 5). The repeat A-containing plasmids pKK533, pKK534, pKK535, pKK536, and pKK540 showed efficient relaxation, whereas the plasmids without repeat A, pKK532, pKK537, pKK538, and pKK539, showed no relaxation. These results 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 (Fig. 6). pKK535-e through pKK535-i carried 1- to 20-bp insertions at the nick site-proximal side of the repeat A sequence (Fig. 6).

Although these insertion and deletion mutations disrupted the nick site-proximal side of the repeat A sequence, gel re-

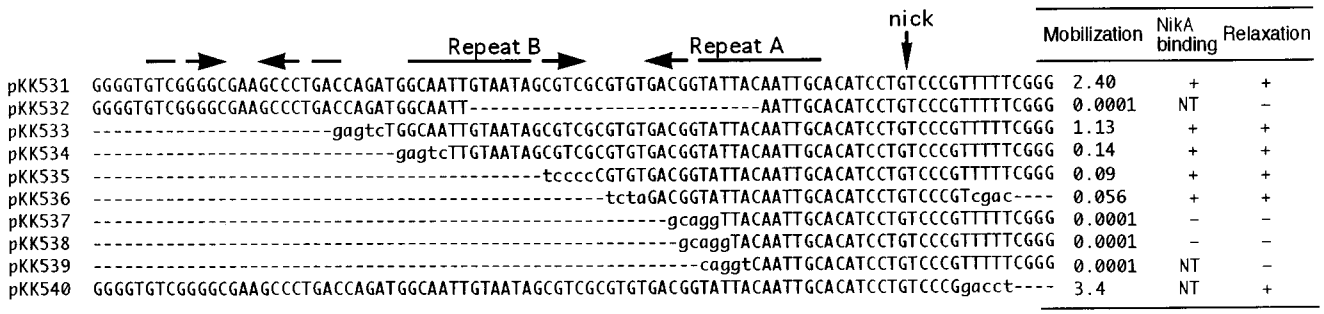

FIG. 5. Deletion analyses of the *oriT* sequence. Lowercase letters within the DNA sequences indicate bases from the vector. Deleted sequences are indicated by dashes. The mobilization frequency is expressed as the ratio of the transfer frequency of *oriT* plasmid to that of the helper plasmid pKK607. NikA binding and relaxation were summarized from gel retardation assays as in Fig. 3 and relaxation assays as in Fig. 4, respectively; +, detected; −, not detected; NT, not tested.

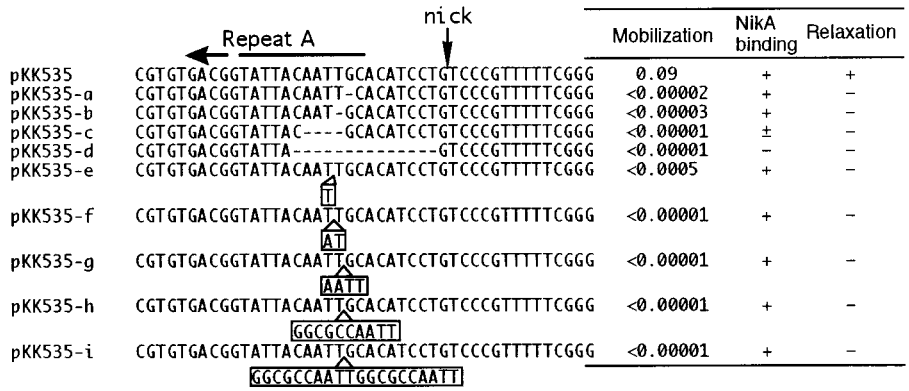

FIG. 6. Effects on *oriT* activity of deletion and insertion mutations introduced into the *oriT* core sequence. The nucleotide sequence of *oriT* regions in pKK535 and its deletion and insertion derivatives are indicated. Deleted nucleotides are indicated by dashes. Inserted nucleotides are boxed. The mobilization frequency, NikA binding, and relaxation are as in Fig. 5. Weak binding of NikA is indicated by ±.

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 the in vitro experiments with purified RP4 TraI and transfer strand oligonucleotides of the nick region, Pansegrau et al. (23) reported that the 6-bp sequence (ATCCTG) from the 3′ end of the nick site was essential for the nicking and ligation reaction by the TraI protein. The 5′ terminus of the nicked strand is covalently attached to TraI protein via tyrosine residue at the 22nd position (22). It is likely that the R64 nick region sequence is also required in a similar reaction by R64 NikB protein, since both R64 NikB and RP4 TraI proteins carry the relaxase motif (23).

The indispensability of NikA binding to the repeat A sequence for *oriT* function was demonstrated by constructing various deletion and substitution mutations of repeat A sequence. Decreases in the mobilization frequency of mutants carrying substitutions at the mismatched position of repeat A sequence may be the results of defective NikA binding to mutant repeat sequences. Our present observations indicate that the NikA recognition sequence is located in an approximately 10-bp nick site-distal portion of the repeat A sequence. Deletions at the nick site-proximal position of the repeat A sequence did not affect NikA-binding ability (pKK535-a and pKK535-b), whereas the *oriT* function was completely abolished. These results are in agreement with those of DNase I footprint analyses of the NikA-*oriT* complex (9), in which the nucleotides protected by NikA protein from DNase I digestion were shifted leftward from the actual repeat A sequence (Fig. 1B). TraJ protein was also shown to specifically bind to the nick site-proximal arm of the 19-bp RP4 inverted repeat sequences with three mismatches (35).

The strict positional requirement of the repeat A sequence relative to the nick region sequence (a putative NikB recognition sequence) is striking. Even a 1-bp deletion or insertion between the NikA-binding site and the nick region sequence is not permissible. It is likely that the NikB protein must interact simultaneously with both the NikA protein and the nick region sequence for relaxation complex formation. For this purpose, the repeat A sequence must be located at a precise position relative to the nick region sequence. NikA binding to the repeat A sequence resulted in protein-induced DNA bending at the repeat A sequence (9). Hence, it is possible that a specific change in DNA ternary structure is also required for recognition of the nick region sequence by NikB protein.

The core *oriT* sequence was found to correspond to the sequences required for specific nicking at *oriT*. All mutant *oriT* plasmids that could be relaxed in the presence of NikA and NikB proteins showed a mobilization frequency higher than that of the core *oriT* sequence. For F *oriT*, it was shown that an approximately 250-bp F *oriT* sequence is required for *oriT* function while an approximately 100-bp *oriT* sequence including the nick region and IHF- and TraY-binding sites is sufficient for specific nicking by TraI protein (5).

What is the function of the repeat B sequence and the 8-bp inverted-repeat sequence? In both R64 and RP4 *oriT* sequences, 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 10^4-fold reduction

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 structure, 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 10^2- to 10^4-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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