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# Specific Binding of the NikA Protein to One Arm of 17-Base-Pair Inverted Repeat Sequences within the *oriT* Region of Plasmid R64

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**Products of the *nikA* and *nikB* genes of plasmid R64 have been shown to form a relaxation complex with R64 *oriT* DNA and to function together as an *oriT*-specific nickase. We purified the protein product of the *nikA* gene. The purified NikA protein bound specifically to the *oriT* region of R64 DNA. Gel retardation assays and DNase I footprinting analyses indicated that the NikA protein bound only to the right arm of 17-bp inverted repeat sequences; the right arm differed from the left arm by a single nucleotide. The binding site is proximal to the nick site and within the 44-bp *oriT* core sequence. Binding of the NikA protein induced DNA bending within the R64 *oriT* sequence.**

Bacterial conjugation is a unique process that allows plasmid DNA in donor cells to be transferred to recipient cells through cell-to-cell contact (for reviews, see references 1, 31, and 32). The DNA transfer in conjugation is associated with the site- and strand-specific cleavage of a phosphodiester bond (nicking) at the origin of transfer, *oriT*, in donor cells (30). Upon conjugation, the nicked strand of DNA is then transferred into the recipient cell with the 5′ terminus leading. In the donor cells, replacement DNA synthesis occurs to make a substitute for the transferred strand. In the recipient cells, the DNA strand complementary to the transferred strand is synthesized, and both the strands make a circle to form an intact plasmid.

Nicking at the *oriT* site is usually mediated by the products of specific transfer genes. In IncF plasmids, such as F and R100, the products of the essential transfer genes, *traY* and *traI*, are involved in nicking at the *oriT* site (31). Purified F and R100 TraY proteins have been shown to bind specifically to F and R100 *oriT* DNA, respectively (8, 14). The F plasmid TraI protein was initially identified as DNA helicase I and was subsequently shown to have *oriT* nicking activity (16, 25). Purified R100 TraI and TraY proteins have been shown to relax supercoiled plasmid DNA containing the R100 *oriT* region, in vitro, in the presence of *Escherichia coli* integration host factor (IHF) (9).

In the IncP plasmid RP4, both the *traI* and *traJ* genes are essential for specific nicking at the *oriT* site (3, 23, 24). The RP4 TraJ protein was shown to bind specifically to the right arm of the 19-bp inverted repeat with three nucleotide mismatches located within the RP4 *oriT* sequence (34). Purified TraI and TraJ proteins, in conjunction with the TraH accessory protein, formed a DNA-protein complex in vitro at the RP4 *oriT* region on the supercoiled plasmid DNA (19). Treatment of the complex, called a relaxosome, with protein-denaturing agents resulted in the relaxation of the plasmid DNA via introduction of a specific nick within the *oriT* site (19). After relaxation, the 5′ end of the nicked DNA strand became covalently attached to tyrosine residue 22 of the RP4 TraI protein (21, 24). Both the TraI and TraJ proteins were required for the nicking process. The TraH protein was not essential for the nicking process but was required to stabilize the relaxosome (19). Recently, the RP4 TraI protein was shown to catalyze the cleaving-joining reaction of single-stranded DNA containing the nick region of RP4 (21). In IncQ plasmid RSF1010, the relaxosome formed on RSF1010 *oriT* was shown to contain a cleaved DNA strand that is tightly held by the RSF1010 Mob proteins so that superhelical tension of the plasmid DNA is retained (26).

The IncI1 plasmid R64 is a 122-kb self-transmissible plasmid originally isolated from *Salmonella typhimurium* (4). The R64 *oriT* sequence is located at one end of the 54-kb transfer region (11). A 141-bp *Rsa*I-*Hae*III DNA fragment carrying the full *oriT* activity of R64 contains 17- and 8-bp inverted repeat sequences with one-nucleotide mismatches (12) (see Fig. 1 and 6). A 44-bp portion of the 141-bp fragment, containing only the right arm of the 17-bp inverted repeat, possessed approximately 1/10 of the *oriT* activity and was called the *oriT* core sequence (12). The R64 *nikA* and *nikB* genes, organized into an *oriT* operon transcribed from the *oriT* region, were shown to be involved in *oriT* DNA nicking (7). The *nikA* and *nikB* gene products formed a relaxation complex with *oriT* DNA. Treatment of the relaxation complex isolated by the mild lysis method from *E. coli* cells with sodium dodecyl sulfate (SDS) resulted in the introduction of a site- and strand-specific nick in the *oriT* region (5). The nick was introduced within the 44-bp *oriT* core sequence of R64 and was located 8 bp apart from the 17-bp inverted repeat sequence. A protein, probably the *nikB* gene product, was covalently attached to the 5′ end of the nicked DNA strand. These results suggest that the NikA and/or NikB proteins recognize a specific DNA sequence in the 44-bp *oriT* core region. The 17-bp inverted repeats in the R64 *oriT* region have a one-nucleotide mismatch; therefore, in the present paper, the inverted repeats, located proximal and distal to the nick site, were designated repeats A and B, respectively (see Fig. 6).

In the present study, we isolated and purified the NikA protein from *E. coli* cells overexpressing the *nikA* gene. Purified NikA protein was found to bind specifically to R64 *oriT* DNA. NikA-induced bending of *oriT* DNA was observed.

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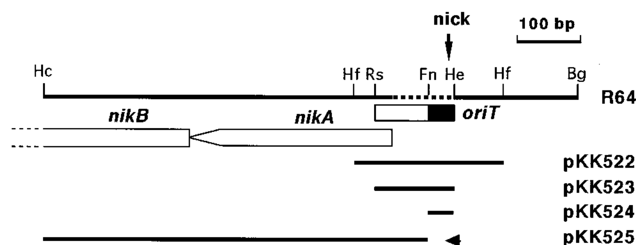


FIG. 1. Plasmid structures. The solid line at the top represents the restriction map and gene organization of the *oriT*-proximal segment of the R64 transfer region (for greater detail, see reference 7). Bg, *Bgl*II; Fn, *Fnu*DII; Hc, *Hin*cII; He, *Hae*III. Only the relevant restriction sites are shown for *Hin*fI (Hf) and *Rsa*I (Rs). The broken portion of the line represents the DNA sequence shown in Fig. 6, and the downward arrow indicates the *oriT* nick site. The solid and open bars indicate the 44-bp *oriT* core sequence, indispensable for *oriT* activity, and the remaining sequence required for full *oriT* activity, respectively. The open bars pointing leftward represent the coding sequences of the *nikA* and *nikB* genes, and the solid lines below them represent the fragments of DNA present in various plasmids. The arrowhead on pKK525 indicates the position of the T7 promoter used to express the *nikA* gene.

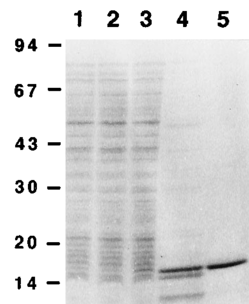


FIG. 2. Overexpression and purification of the NikA protein. The NikA protein was overproduced by IPTG induction of JM109(DE3) cells harboring pKK525 and was purified by column chromatography. Proteins from each purification step were separated by SDS-PAGE. Lane 1, crude cell lysate of IPTG-induced JM109(DE3) harboring pBluescript II KS+; lanes 2 and 3, crude cell lysate of uninduced and IPTG-induced JM109(DE3) harboring pKK525, respectively; lanes 4 and 5, pooled NikA protein-containing fractions obtained by phosphocellulose and DNA-cellulose column chromatography, respectively. The locations of the molecular mass markers (in kilodaltons) are indicated at the left.

## MATERIALS AND METHODS

**Bacteria and media.** *E. coli* K-12 strains JM83 and JM109(DE3) (27, 33) were used to clone and express the *nikA* gene, respectively. Luria-Bertani medium was prepared as described by Miller (18), and the solid medium contained 1.5% agar. Either ampicillin or chloramphenicol was added to the liquid and solid media at a concentration of 100 μg/ml or 25 μg/ml, respectively, to select *E. coli* cells harboring plasmids.

**Plasmid construction.** Plasmid vectors pUC9 and pHSG399 (28, 33) were used for cloning, and pBluescript II KS+ (Stratagene) was used as the expression vector. In addition, the DNA bending vector, pBend2 (10), was used to analyze protein-induced DNA bending.

The plasmid pKK522 was described previously (5). The 141-bp *Rsa*I-*Hae*III DNA fragment containing the R64 *oriT* sequence was inserted between the *Bam*HI-*Hin*dIII sites of pHSG399, generating pKK523 (Fig. 1). A 70-bp *Xba*I-*Sal*I DNA fragment containing the 44-bp R64 *oriT* core sequence was inserted between the *Xba*I-*Sal*I sites of pBend2, generating pKK524 (Fig. 1; see also Fig. 7A). The 642-bp *Fnu*DII-*Hin*cII fragment of R64 containing the *nikA* gene was inserted into the *Hin*cII site of pBluescript II KS+ in the same orientation as the T7 promoter of the plasmid vector, generating pKK525 (Fig. 1).

**Overproduction of NikA protein.** An overnight culture of JM109(DE3) cells harboring pKK525 was diluted 50-fold in Luria-Bertani medium containing ampicillin and incubated at 37°C with shaking. When the $A_{620}$ of the culture reached approximately 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 3 h, the cells were harvested by centrifugation and used to obtain the NikA protein.

**Isolation and purification of NikA protein.** All of the purification steps were carried out at 4°C. First, the IPTG-induced cells (ca. 5 g [wet weight]) of JM109(DE3) harboring pKK525 were resuspended in 10 ml of a combination of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10 mM 2-mercaptoethanol, and 200 mM NaCl. Second, 10 ml of a detergent mixture containing 1% Brij 58, 0.1% sodium deoxycholate, 50 mM Tris-HCl (pH 8.0), and 10 mM EDTA was added to the cell suspension with gentle mixing. Then, 5 mg of egg white lysozyme was added, and the mixture was incubated on ice for 10 min, which resulted in a viscous lysate. The lysate was centrifuged at 100,000 × *g* for 30 min, and the supernatant was loaded onto a 10-ml phosphocellulose P11 (Whatman) column equilibrated with buffer A (10 mM Tris-HCl [pH 8.0]–0.1 mM EDTA–5 mM 2-mercaptoethanol) containing 200 mM NaCl. The column was then washed with 20 ml of buffer A containing 200 mM NaCl, and the bound proteins were eluted with 100 ml of buffer A with a linear gradient (from 200 to 1,000 mM) of NaCl. Each column was analyzed for specific binding to R64 *oriT* DNA by a gel retardation assay (see below). The NikA-containing fractions were pooled (fraction I) and dialyzed against buffer A containing 100 mM NaCl.

Fraction I was then loaded onto a 3-ml DNA cellulose column equilibrated with buffer A containing 100 mM NaCl. The column was washed with 20 ml of buffer A containing 100 mM NaCl and eluted with 50 ml of buffer A with a linear gradient (from 100 to 750 mM) of NaCl. NikA-containing fractions were pooled, dialyzed against buffer A containing 50 mM NaCl and 50% glycerol, and stored at −20°C (fraction II).

Proteins from each purification step were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. The purity of the NikA protein was determined by densitometry with a Helena Quick Scan R&D densitometer.

To achieve the level of purity necessary for protein sequencing, fraction II was further purified by reversed-phase chromatography with a Hitachi gel 3013 column. The N-terminal amino acid sequence of the purified NikA protein was then determined by Edman degradation in a model 477A/120A protein sequencer (Applied Biosystems).

**Gel retardation assay.** Purified NikA protein 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, the reaction mixtures were loaded onto a 4% polyacrylamide gel (acrylamide-to-bisarylamide weight ratio of 29:1) in E buffer (40 mM Tris-acetate [pH 7.8]–1 mM EDTA) and electrophoresed at 10 V/cm and 4°C. Following electrophoresis, DNA fragments in the gel were stained with ethidium bromide and visualized under a long-wave UV light.

**DNase I footprinting analysis.** Purified NikA protein and $^{32}P$-labeled DNA fragments were mixed in a buffer (100 μl) containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 10 μg of salmon testis DNA. The mixtures were incubated at 37°C for 10 min, and then 0.05 U of DNase I was added to each sample. After incubation at room temperature for 10 min, DNase I was inactivated by adding 5 μl of 0.4 M EDTA. Following phenol extraction and ethanol precipitation, samples were separated by electrophoresis on a sequencing gel. The cleavage pattern was visualized by autoradiography.

**Materials.** Restriction endonucleases, T4 polynucleotide kinase, and Sequenase were obtained from Takara Shuzo, Nippon Gene, Toyobo, and United States Biochemical Corp. [α-$^{32}P$]dCTP (800 Ci/mmol) and [γ-$^{32}P$]ATP (3,000 Ci/mmol) were from Du Pont, NEN Research Products.

## RESULTS

**Overproduction of NikA protein.** The product of the *nikA* gene of plasmid R64 was previously identified as a 15-kDa protein by the maxicell procedure (7). To overproduce the NikA protein, we inserted the *nikA* coding sequence into an expression vector, pBluescript II KS+, generating pKK525 (Fig. 1). Then, we transformed *E. coli* JM109(DE3) cells, carrying an IPTG-inducible T7 RNA polymerase gene, with pKK525. Because the *nikA* gene on pKK525 is located downstream of the T7 promoter, the addition of IPTG induced expression of the *nikA* gene. IPTG induction resulted in the overproduction of NikA protein as observed by SDS-PAGE (Fig. 2). A discernible 15-kDa protein band was detected in IPTG-induced *E. coli* cells carrying pKK525 (Fig. 2, lane 3) but was not observed in cells with the vector (lane 1) or in uninduced cells (lane 2), indicating that overexpression of the *nikA* gene resulted from the T7 RNA polymerase-promoter system.

**Purification of NikA protein.** The NikA protein was purified from IPTG-induced JM109(DE3) cells harboring pKK525. When the highly viscous lysate of *E. coli* cells was ultracentrifuged, the NikA protein was retained in the supernatant. The NikA protein was purified by phosphocellulose and DNA-cellulose column chromatography. After each chromatography procedure, the NikA protein in various fractions was detected

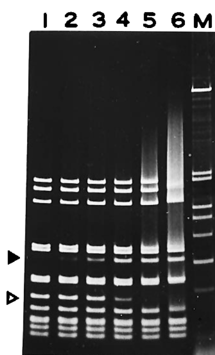


FIG. 3. Specific binding of the NikA protein to the R64 *oriT* DNA fragment. Plasmid pKK522 DNA (1 μg) was digested with *Hae*III and *Rsa*I and incubated at 37°C for 10 min with various amounts of NikA protein. The DNA-protein mixtures were separated by electrophoresis on a 4% polyacrylamide gel. Lane 1, without NikA protein; lanes 2, 3, 4, 5, and 6, NikA protein at 2.5, 5, 10, 20, and 40 ng, respectively (7 ng of NikA protein to 1 μg of pKK522 DNA corresponds to a molar ratio of about 1:1); lane M, *Hin*fI-digested pBR322 DNA used as a size marker. The open and solid triangles point to the free and NikA protein-bound 141-bp *Hae*III-*Rsa*I *oriT* DNA fragments, respectively.

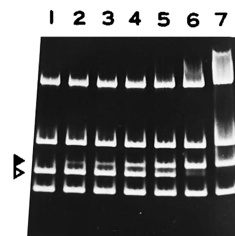


FIG. 4. Specific binding of the NikA protein to the 403-bp DNA fragment containing the 44-bp R64 *oriT* sequence. Plasmid pKK523 DNA (1 μg) was digested with *Fnu*DII and incubated at 37°C for 10 min with various amounts of NikA protein. The mixtures were separated by electrophoresis on a 4% polyacrylamide gel. Lane 1, without NikA protein; lanes 2, 3, 4, 5, 6, and 7, NikA protein at 2.5, 5, 10, 20, 40, and 80 ng, respectively. The open and solid triangles point to the free and NikA protein-bound 403-bp DNA fragments containing the 44-bp *oriT* core sequence, respectively.

by SDS-PAGE. The pooled fraction obtained by DNA-cellulose column chromatography contained NikA protein that ran as a single band on the gel (Fig. 2, lane 5). Densitometric analysis of the gel indicated that the purity of the NikA protein (fraction 2) was about 90%. Approximately 300 μg of purified NikA protein was obtained from 5 g (wet weight) of induced cells. The purified NikA protein remained stable at −20°C for more than 1 year.

The N-terminal amino acid sequence of the purified NikA protein was determined to be $NH_2$-Ser-Asp-Ser-Ala-Val-Arg-Lys-Lys-Ser-Glu-Val-Arg-Gln-Lys-Thr-Val-Val-Arg-Thr-Leu. The amino acid sequence was identical to the sequence predicted from the nucleotide sequence of the R64 *nikA* gene (7) with the exception of an initial methionine. The sequence identity confirmed that the purified protein was actually the product of the *nikA* gene. The molecular weight of NikA protein is calculated to be 12,482, which is slightly lower than the observed molecular mass of 15 kDa (Fig. 2).

**Specific binding of NikA protein to the R64 *oriT* sequence.** Previous studies indicated that the *nikA* gene was essential for R64 transfer and required for the formation of the R64 *oriT* relaxation complex, suggesting a specific association of the NikA protein with the *oriT* DNA sequence (7). To investigate the *oriT*-NikA association, we performed a gel retardation assay with the purified NikA protein. DNA fragments produced by *Hae*III and *Rsa*I digestion of pKK522 DNA were incubated with various amounts of NikA protein and analyzed by PAGE (Fig. 3). As the amount of NikA protein added to the reaction mixture was increased, the 141-bp *Hae*III-*Rsa*I DNA fragment containing the R64 *oriT* sequence shifted to a new position (compare the DNA bands labeled with the open and solid triangles in Fig. 3), suggesting that the NikA protein bound specifically to the *oriT* sequence. The specific binding of the NikA protein to the R64 *oriT* DNA fragment was observed even in the absence of $Mg^{2+}$ (data not shown).

To determine whether the NikA protein binds to the 44-bp *oriT* core sequence, we digested pKK523 DNA with *Fnu*DII. The digestion of pKK523 with *Fnu*DII divides the 141-bp *oriT* DNA sequence into two portions (Fig. 1), the 44-bp *oriT* core sequence (containing repeat A) and the 97-bp remaining sequence (containing repeat B), and generates a total of four DNA fragments (Fig. 4, lane 1). A gel retardation assay was performed on mixtures of *Fnu*DII-generated pKK523 DNA fragments and NikA protein. As the amount of NikA protein added to the reaction mixture was increased, there was a mobility shift of the 403-bp DNA fragment containing the 44-bp *oriT* core sequence (open and solid triangles in Fig. 4). In contrast, the mobility of the 315-bp DNA fragment containing the remaining 97 bp of the *oriT* sequence remained unchanged even at high concentrations of the NikA protein. Therefore, these results indicate that the NikA protein binds specifically to the 44-bp *oriT* core sequence.

**NikA protein binds specifically to the repeat A sequence.** We performed DNase I footprinting analyses with pKK522 DNA to precisely define the NikA protein-binding sequence of R64 *oriT* DNA (Fig. 5). In the case of the bottom strand, the NikA protein protected the DNA region over the repeat A sequence of the 17-bp inverted repeat from DNase I cleavage (Fig. 5A). However, the DNA region over the repeat B sequence was not protected. An enhanced sensitivity to DNase I, caused by NikA binding, was observed immediately from the 5′ end to the protected region. The nick site did not appear to be protected. In the case of the top strand, the repeat A sequence was protected, but no enhancement of sensitivity to DNase I was detected near the protected region (Fig. 5B). Therefore, the results of DNase I footprinting experiments indicate that the NikA protein binds to the repeat A sequence of the 17-bp inverted repeat, although the protected sequence is slightly shifted to the left (Fig. 6). However, the NikA protein did not bind to the repeat B sequence, indicating that the single mismatched nucleotide between the repeat A and B sequences plays a critical role in the binding of NikA to *oriT* DNA.

**DNA bending of *oriT* DNA results from NikA protein binding.** To determine the influence of NikA protein binding on the bending of *oriT* DNA, we used a permutated set of DNA fragments in which the position of the *oriT* sequence varied. The DNA bending vector pBend2 (10), a derivative of pBR322 containing a set of tandemly repeated 109-bp sequences, was used to generate a permutated set of *oriT* DNA fragments. Cloning sites are located between the repeated sequences in the pBend2 vector, and the R64 *oriT* core sequence was cloned between the *Xba*I and *Sal*I sites, generating pKK524 (Fig. 7A). The digestion of pKK524 DNA with *Mlu*I, *Spe*I, *Pvu*II, *Stu*I, *Kpn*I, or *Bam*HI produced a set of six 188-bp permutated DNA fragments that migrated to identical positions on a 4% polyacrylamide gel, indicating that there is no intrinsic DNA bending at the R64 *oriT* sequence (data not shown). However, when the NikA protein was bound to each fragment, the mobilities of the NikA protein-bound fragments differed from one another (Fig. 7B). The mobility shift patterns of the NikA pro-

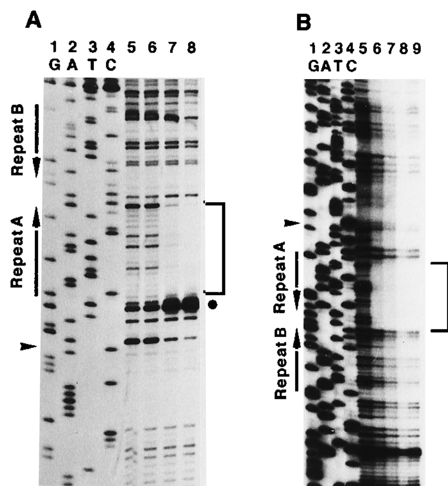


FIG. 5. DNase I footprint analyses of *oriT* DNA-NikA protein complexes. (A) Protection of the bottom strand by NikA protein binding to the *oriT* sequence. The 5′ end of pKK522 DNA, linearized by *Eco*RI digestion, was dephosphorylated with calf intestinal alkaline phosphatase and labeled with [$\gamma$-$^{32}$P]ATP by means of T4 polynucleotide kinase. The labeled DNA was digested with *Hin*dIII, and the resulting 278-bp DNA fragment was used as a probe. Lanes 1, 2, 3, and 4, sequencing ladders prepared by the dideoxy chain-termination method in conjunction with a 35-nucleotide DNA primer identical to the first 35 nucleotides of the bottom strand; lanes 5, 6, 7, and 8, DNase I-treated probe DNAs in the presence of 0, 0.16, 0.5, and 2.0 $\mu$g of the NikA protein, respectively. The DNase I-sensitive site resulting from NikA protein binding is indicated by the solid circle on the right. (B) Protection of the top strand by NikA protein binding to the *oriT* sequence. The 278-bp *Eco*RI-*Hin*dIII *oriT* DNA fragment labeled with [$\gamma$-$^{32}$P]ATP at the *Hin*dIII site was obtained in the same manner as that described for the bottom strand and was used as a probe. Lanes 1, 2, 3, and 4, sequencing ladders for the top strand; lanes 5, 6, 7, 8, and 9, DNase I-treated probe DNAs in the presence of 0, 0.4, 0.8, 2.0, and 4.0 $\mu$g of the NikA protein, respectively. In both panels A and B, the protected nucleotides are indicated by brackets to the right of the final lane, the repeat A and B sequences are indicated by vertical arrows on the left, and the position of the nick site is indicated by arrowheads on the left.

tein-bound DNA fragments depended on the location of the NikA protein binding site on each fragment. The repeat A sequence was located at the center of the *Pvu*II-generated fragment, and the *Pvu*II-generated fragment showed the least amount of migration when bound to the NikA protein. In contrast, the *Mlu*I-generated fragment had the repeat A sequence located near the right end and migrated the fastest when bound to the NikA protein. These results indicate that binding of the NikA protein to the repeat A sequence induces DNA bending.

## DISCUSSION

In the present work, we purified the NikA protein of plasmid R64. Using gel retardation and DNase I footprinting assays, we found that the NikA protein bound specifically to the repeat A sequence within the 44-bp *oriT* core sequence of plasmid R64. The protected region of the sequence was shifted slightly to the left of the repeat A sequence (Fig. 6). Taking into account the possibility of overestimating the size of the sequence actually recognized by the NikA protein from the DNase I footprint pattern, the sequence recognized by the NikA protein may be restricted to a region within the repeat A sequence. In contrast, there was no indication that the repeat B sequence was protected by NikA protein binding. The results of the DNase I footprint assay are consistent with those of the gel retardation analysis, in which no mobility shift was observed for the DNA fragment carrying only the repeat B sequence when mixed with the NikA protein (Fig. 4), and there was no evidence of second-step binding of the NikA protein to the DNA fragment carrying both the repeat A and B sequences (Fig. 3). Since there is only a single nucleotide difference between the sequences of repeats A and B, the different nucleotide must play a critical role in the recognition of the DNA sequence by the NikA protein. Even though the repeat B sequence does not bind to the NikA protein, it may still play an important role in *oriT* function, inasmuch as the *oriT* core sequence lacking the repeat B sequence showed only 1/10 of the *oriT* activity of the complete *oriT* sequence (12).

The specific nick introduced during relaxation in vitro was located 8 bp away from the repeat A sequence of the R64 *oriT* region (5) (see Fig. 6). Because the nick site was not protected by the NikA protein, the sequence around the nick site is unlikely to interact with the NikA protein. Pansegrau and Lanka (20) indicated the presence of a conserved 12-bp DNA sequence motif in the *oriT* nick regions of various plasmids, including R64, RP4, R751, and pTF-FC2, and in the border sequences of the transfer DNA regions of various agrobacterial Ti and Ri plasmids. The conserved DNA sequence motif was also found in the replication origins of pC194, pUB110, and $\phi$X174-like bacteriophages (30). Three common amino acid sequence motifs were also found among the nickase proteins involved in various DNA transfer systems, including those in R64 (NikB), RP4 (TraI), R751 (TraI), pTF-FC2, pS194, pC221, pC225, and agrobacterial Ti and Ri plasmids (22). The conserved tyrosine residue in motif I of the RP4 TraI protein was identified as the active-site amino acid of the site-specific cleaving-joining reaction of single-stranded *oriT* DNA (22). Therefore, it is possible that a sequence near the nick site in the R64 *oriT* region is recognized by the NikB protein as single-stranded DNA. Our preliminary results indicated that mutations in the nick region resulted in a substantial loss of *oriT* function (6). Significant amino acid sequence similarity was also found among *oriT*-binding proteins, including R64 NikA, R388 TrwA, RP4 TraJ, R751 TraJ, and pTF-FC2 MobB (15).

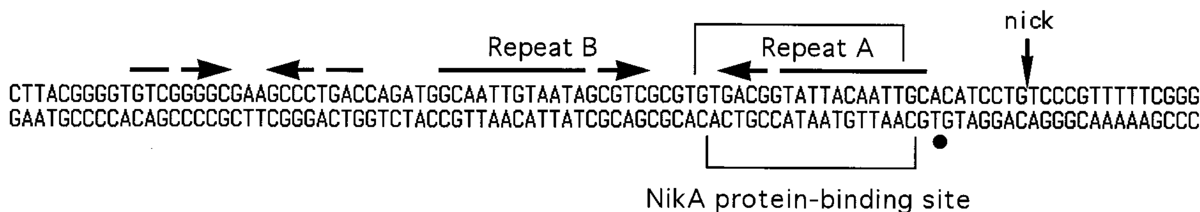


FIG. 6. Sequence of the NikA-protected region within the *oriT* sequence. The DNA sequence is taken from reference 7. Brackets mark the top and bottom strands of sequence protected from DNase I cleavage. The position of the phosphodiester bond hypersensitive to DNase I is indicated by the solid circle. The nick site is indicated by the downward arrow.

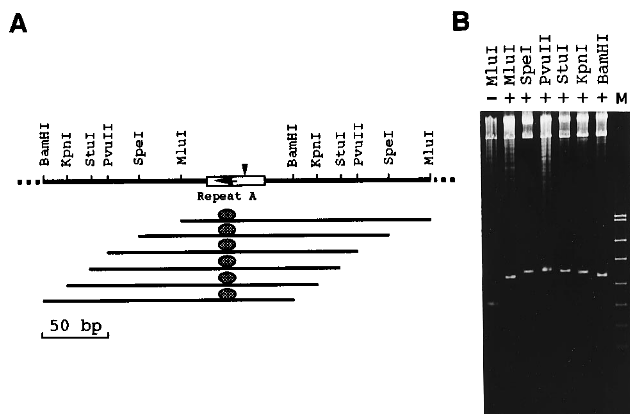


FIG. 7. DNA bending of the NikA protein-bound DNA fragment. (A) Structure of pKK524. The solid line at the top represents a restriction map of the region in pKK524 surrounding the cloned fragment. The cloned fragment, shown as an open bar, contains the 44-bp *oriT* core sequence. The horizontal arrow within the box represents the 17-bp repeat A sequence, and the downward arrowhead indicates the nick site. The solid lines beneath the map represent permutated DNA fragments derived from pKK524 DNA digested with six different restriction enzymes. The hatched ovals above the lines represent NikA protein bound to *oriT* DNA. (B) Gel retardation assay of the permutated DNA fragments. DNA from pKK524 was digested with *Mlu*I, *Spe*I, *Pvu*II, *Stu*I, *Kpn*I, or *Bam*HI and incubated with the NikA protein (20 ng) at 37°C. The mixtures were analyzed by 4% PAGE. −, without NikA protein; +, NikA protein added; lane M, pUC19 DNA digested with *Hpa*II used as a size marker.

The RP4 TraJ protein bound specifically to the nick site-proximal arm of the 19-bp inverted repeat sequence within the RP4 *oriT* region but not to the other arm (34). The 19-bp inverted repeat in the RP4 *oriT* region has three mismatched nucleotides; therefore, the RP4 TraJ protein probably recognizes one or more of the three mismatched nucleotides, just as the R64 NikA protein recognizes the mismatched nucleotide of the R64 repeat A sequence. We previously pointed out that the *oriT* structures of R64 and RP4 exhibit a global similarity: the nick sites of R64 and RP4 are situated 8 bp away from the 17-bp and 19-bp inverted repeats, respectively, although the actual sequences of the 17-bp and 19-bp repeats are different (5). The fact that both the R64 NikA and RP4 TraJ proteins bind to the nick site-proximal arms of the 17-bp and 19-bp inverted repeats, respectively, further confirms the similarities between the *oriT* structures of R64 and RP4. In addition, the R64 NikA and RP4 TraJ proteins share a 30% amino acid sequence similarity (7).

In the present study, we showed that binding of the NikA protein causes R64 *oriT* DNA to bend at the binding site. In addition, our DNase I footprinting analysis revealed that NikA protein binding gave rise to a DNase I-sensitive phosphodiester bond adjacent to the protected region. Thus, it appears that the NikA protein-induced bending of *oriT* DNA creates the DNase I-sensitive site and that this conformational change plays a role in nicking at the *oriT* region. In the F plasmid, there are two intrinsic DNA bending sequences and two IHF-binding sites in the *oriT* region (29). Binding of IHF to DNA is known to induce bending of the bound DNA (13). Two IHF-binding sites were also identified in the R100 *oriT* region, and IHF was a required host factor for the transfer of R100 (2, 17). The RP4 plasmid also has an intrinsic DNA-bending sequence situated in the leader region adjacent to the RP4 *oriT* nick site (24), and the sequence was found to be recognized by the product of another RP4 transfer gene, *traK* (35). Observation of the relaxosome consisting of the TraJ, TraI, and TraH proteins and RP4 *oriT* DNA under an electron microscope revealed a sharp bend in the DNA at the nucleoprotein complex site (19). Therefore, both intrinsic DNA bending and protein-induced DNA bending appear to play important roles in the formation of the relaxation complex and in the introduction of a specific nick at the *oriT* site. Bending of *oriT* DNA might expose single-stranded DNA at the nick region, which is recognized by DNA relaxases such as R64 NikB and RP4 TraI proteins.

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