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DNA Binding by the Xis Protein of the Conjugative Transposon Tn*916*

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We purified the Xis protein of the conjugative transposon Tn*916* and showed by nuclease protection experiments that Xis bound specifically to sites close to each end of Tn*916*. These specific binding sites are close to, and in the same relative orientation to, binding sites for the N-terminal domain of Tn*916* integrase protein. These results suggest that Xis is involved in the formation of nucleoprotein structures at the ends of Tn*916* that help to correctly align the ends so that excision can occur.

Conjugative transposons (7, 9, 29, 30, 32) have a very broad host range; they can transfer between bacteria of different species and genera by using the conjugation functions that they encode. During this process, they transpose. Most conjugative transposons encode tetracycline resistance, and the larger ones encode additional antibiotic resistance determinants. Because they usually cause drug resistance and because of their promiscuity, conjugative transposons are an important problem in bacterial pathogens, especially gram-positive ones. The best-studied conjugative transposons are Tn*916* (which encodes resistance to tetracycline) (12, 13) and its very close relative Tn*1545* (which encodes resistance to tetracycline, kanamycin, and erythromycin) (5, 10), as well as the lantibiotic-producing element Tn*5276* (26).

When conjugative transposons transpose to a new site they do not cause duplications of the target sequence. Rather, a 6-base sequence adjacent to the transposon in the donor strain is inserted into the target along with the transposon (6, 8). Transposition occurs via a covalently closed circular intermediate (33) that is produced by staggered cleavages 6 bp apart at the ends of the transposon (6, 22, 28). The intermediate contains a 6-bp segment between the transposon ends that, depending upon the sequences that flanked the transposon before excision, is usually heteroduplex DNA (6, 22). Integration of the transposon into a new target site occurs by staggered cleavage of the intermediate as well as the target sequence, followed by joining of the cleaved DNA strands (6, 31). This mechanism of transposition is more similar to phage lambda integration and excision than to transposition of other types of bacterial transposons.

There is an imperfect inverted repeat at each end of Tn*916* (8). In addition, there are three copies of an 11-bp sequence, termed DR-2, located in a segment of DNA beginning 150 bp from the left end of the transposon (8). Two copies are directly repeated and one copy is inverted. There are also two directly repeated copies of the DR-2 sequence in a segment of DNA beginning 100 bp from the right end of the transposon (8).

For Tn*916*, as well as for Tn*1545* and Tn*5276*, approximately 2 kb at one end of the transposon and 250 bp at the other end are required for transposition (3, 24, 25, 27, 34, 35). The longer segment, at the left end of Tn*916*, contains two genes, *int* and *xis*, whose products are involved in excision and integration. The *int* gene product is similar in structure and function to phage lambda integrase and is required for excision and integration (2, 3, 24, 34, 35). Like lambda integrase, the Int protein of Tn*916* is a bivalent DNA binding protein (20). The C-terminal domain of Tn*916* Int contains the conserved Arg, His, and Tyr residues characteristic of the integrase family of site-specific recombinases.

The N-terminal DNA binding domain of Tn*916* Int binds the regions near the ends of the element that contain the DR-2 repeats (20). The C-terminal domain binds specifically to part of the terminal inverted repeat of Tn*916* and flanking DNA sequences (20), as well as to sites that serve as targets for Tn*916* integration (21). This pattern of interactions is similar to that which occurs between the N- and C-terminal domains of lambda integrase and the integrated prophage (19). The C-terminal domain of lambda Int binds to the ends of the prophage (core type sites) and flanking DNA, while the N-terminal domain of lambda Int binds to sequences within the prophage (arm type sites).

Tn*916* Int cleaves DNA with the same polarity as lambda integrase and makes staggered cleavages at the ends of Tn*916*, leaving a free 5′-OH group (36). The protein remains attached to the 3′ end, presumably by a phosphotyrosine linkage. However, despite the similarities between Tn*916* and phage lambda, there are important differences. While integration of Tn*916* does not occur at random, this transposon does not insert into a specific sequence analogous to the single attachment site (*att*B) for phage lambda in the chromosome of *Escherichia coli*. Instead, preferred target sequences (16, 31) for Tn*916* integration appear to consist of bent DNA (21). This structural deformation of the DNA may be important for recognition of the target by Tn*916* Int.

As in lambda, the *xis* genes of Tn*916*, Tn*1545*, and Tn*5276* are immediately upstream of *int* (24, 27, 35). Like the lambda Xis protein, the Tn*916* Xis protein is small (67 amino acids) and highly basic (pI = 9). In lambda, Xis is required for prophage excision under physiological conditions in vivo and under most conditions in vitro (1). The C-terminal end of lambda Xis interacts with lambda Int and the N-terminal end interacts with DNA by binding to two sites at the right end of the lambda prophage (4, 23, 38). The binding of lambda Xis bends the DNA (37), and lambda Xis participates with Int and the *E. coli* protein IHF in the formation of a complex structure required for prophage excision (17, 18). In addition, the binding of lambda Xis to its specific binding sites inhibits the binding of Int to a nearby site (11) and thus inhibits the integration of lambda prophage into *att*B.

The role of Tn*916* Xis has not yet been defined. A Tn*5*

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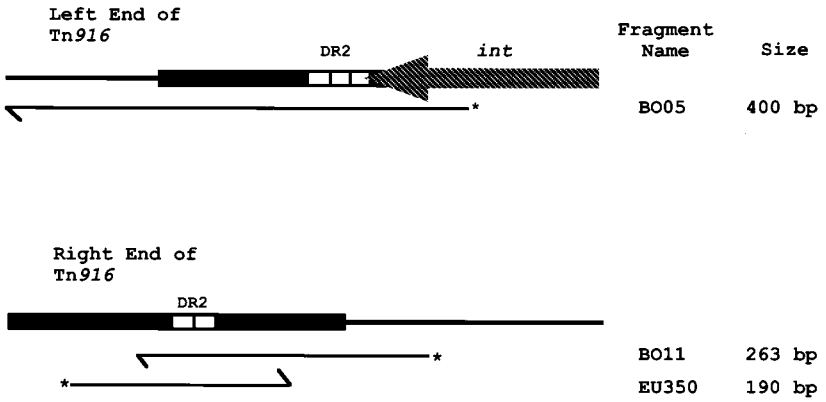

FIG. 1. Left and right ends of Tn*916* and PCR fragments used in the DNase I protection assays. The thick black lines represent transposon sequences, and the thin lines represent flanking bacterial sequences. The white boxes labeled DR2 represent the 11-bp repeats. The full (hatched) arrow indicates the 3′ end of the *int* gene. Lines with half arrowheads show the DNA fragments used in the DNase I protection assays. The fragments' names and sizes are indicated to the right. The stars indicate the end labeled with ^{32}P.

insertion mutant of Tn*916* that did not produce a functional Xis protein was unable to excise in *E. coli* (35), as measured by loss of a transposon marker, suggesting that Xis is needed for excision in this system. However, when a more sensitive electrophoresis assay was used to detect excision of a Tn*1545* derivative lacking *int* and *xis* from a plasmid in *E. coli*, excision was observed when Int alone was provided in *trans* (25). Nevertheless, more excisant plasmid was present when Xis as well as Int was supplied in *trans*, suggesting that Xis is not essential for excision but stimulates it. Similar observations were made for Tn*5276* (27). There have been no reports of the role of Xis in conjugative transposition in gram-positive bacteria or of its in vitro activity. Therefore, to learn more about the role of Xis, we studied its DNA binding properties.

MATERIALS AND METHODS

Expression and purification of Xis. The *xis* gene was cloned from the plasmid pTrcHisBX3 (supplied by Kathy Taylor) by digestion with *Hin*dIII and *Eco*RI and was ligated into the pMAL-c2 expression vector (NEB). The resulting plasmid was electroporated into *E. coli* SG22094 (Δ*lon clpP1*::*cat*) (15). Expression of the fused Xis-maltose binding protein (MBP) in bacteria growing in Luria broth was induced by incubation with 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. The cells were lysed in a French pressure cell, and the cell debris was removed by centrifugation at 10,000 rpm in an SS-34 rotor for 10 min. The protein concentration was measured in a Bio-Rad protein assay, and the sample was diluted to 2 mg/ml and spun at 20,000 rpm in an SS-34 rotor for 30 min to clarify the lysate. The diluted fused protein was purified by chromatography on an amylose column, eluted with 10 mM maltose in column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA), and concentrated by centrifugation through a Centricon 30 column (Amicon). The chimeric protein was cleaved with factor Xa (protein-to-enzyme ratio, 100:1) during incubation overnight at room temperature in column buffer. The cleaved Xis was highly insoluble and precipitated out of solution. The precipitate, containing Xis and various amounts of uncleaved chimeric protein and MBP, was washed once in water and then suspended in 0.5 M imidazole–0.5 M NaCl, pH 6.0. The size of the cleaved Xis was estimated from its mobility in a Tricine-sodium dodecyl sulfate-16% polyacrylamide gel.

DNA fragments and competitors. DNA fragments used in footprinting experiments and their sizes are shown in Fig. 1. Fragments of DNA containing sequences from each end of the transposon were amplified by PCR from pUC18::Tn*1545*del4 and cloned into pUC18 as described previously (20). Smaller fragments were then subcloned from these fragments by using PCR. Fragment EU350 was produced with primers 5′d(GCATAGAATTCTAGTTATCCGC) and 5′d(CGGGTAAAAATCTAGACACCTC). It was cloned into pBluescript KS$^-$. Fragments BO05 and BO11 were produced with primer pairs 5′d(CGCCAGGGTTTTCCCAGTCACGAC) and 5′d(GATCGGATCCGCTTGAATAAAGAGAAGC) and 5′d(TGTCAAAAGAATTCCCATTT) and 5′d(AAATATTCAAGCTTTATTGGAAGT), respectively. They were cloned into pUC18.

Double-stranded oligonucleotide competitors 53 (GCTCTAGAAGATACTTCACGTTTCTTTGCATATTTCCTCATGTCTTCTAGACG), 56 (CTAGATGGAAGTATCTTTATATCTTCACTTTTCAAGGATAAATCGTCGTATCAAAGTCGA), and DR23 (TAGCTGTCAGAAGTGGTAAATAAAGTAGTAAATTCATTTGTACTACTAAGCA) were made by synthesizing the two complementary strands on an Oligo 1000 DNA synthesizer (Beckman) and annealing the two strands by heating to 60°C and cooling slowly to room temperature over 1 h. Their concentrations were estimated by comparing them to standards of known DNA concentrations (NEB) that were run on an agarose gel.

Nuclease protection assays. Fragments were radiolabeled at the 3′ ends by using the Klenow fragment of DNA polymerase I. Some of this labeled DNA was subjected to A+G and T+C Maxam-Gilbert sequencing reactions. For DNase I protection assays, 3′-end-labeled DNA at 100 to 150 pM was incubated with various concentrations of Xis, ranging from 0.2 to 9 μM (0.2 to 8 μg), or with Int at 100 and 300 nM (1 to 3 μg) in a buffer containing 60 mM HEPES (pH 7.5), 60 mM KCl, 7 mM $MgCl_2$, 3 mM $CaCl_2$, 12% glycerol, 0.6 M dithiothreitol, and 1 mM EDTA (total volume, 0.1 ml) for 15 to 20 min at room temperature. DNase cleavage and competition assays were performed as described previously (20).

RESULTS

Production of Xis. Fused Xis-MBP was purified from *E. coli* by chromatography on amylose resin. The fused protein showed the mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis expected for a size of ~52 kDa (data not shown). Preliminary experiments showed that uncleaved, fused Xis-MBP had no DNA binding activity and that DNA binding was only observed after cleavage with factor Xa protease. Following cleavage, a small ~8 kDa protein, the size expected for Xis, was produced. The N terminus of the cleaved protein contained the initial methionine and subsequent amino acids of Xis, preceded by four additional amino acids (Ile Ser Glu Phe). Although the fused MBP-Xis protein was soluble in water or low-ionic-strength buffer at neutral pH, the cleaved Xis protein was insoluble. It was, however, soluble in 0.5 M imidazole–0.5 M NaCl at pH 6.0.

Xis binds to a specific sequence in the DNA on the left end of Tn*916*. To determine if Xis binds specifically to the left end of Tn*916*, fragment BO05, containing the left end and flanking bacterial sequences (Fig. 1), was labeled at the 3′ end and used in DNase I protection assays. As shown in Fig. 2, Xis protected a region including bases +93 to +133 from the left end of the transposon. In addition, two positions within the protected region showed an increased susceptibility to cleavage. The protected region lies between the left end of the transposon and the DR-2 repeats (see Fig. 5).

To determine whether the binding by Xis is specific, competition DNase I protection assays were done with a 1,000-fold excess of a specific competitor, the double-stranded region

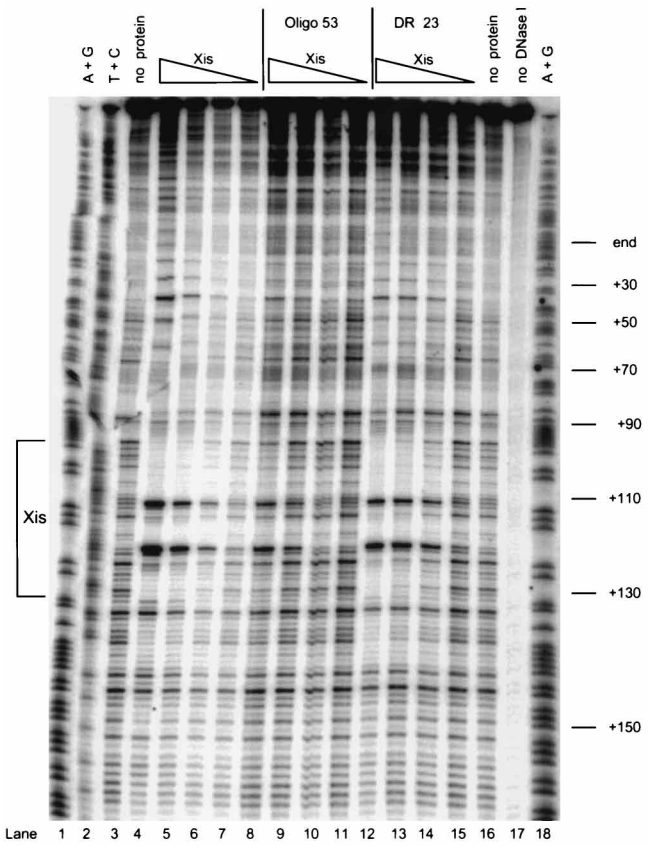

FIG. 2. DNase I protection of radiolabeled fragment BO05 and competition by specific and nonspecific competitor DNA. Lanes: 1, 2, and 18, Maxam-Gilbert sequences; 3 and 16, labeled DNA with no protein; 4 to 7, labeled DNA incubated, respectively, with 1.4, 0.7, 0.4, or 0.2 μg of Xis; 8 to 11, the same decreasing concentrations of Xis as in lanes 4 to 7 incubated with oligonucleotide 53 (a specific competitor); 12 to 15, the same decreasing concentrations of Xis as in lanes 4 to 7 incubated with DR23 (a nonspecific competitor); 17, labeled DNA with no DNase I.

found to be protected (oligonucleotide 53; see Materials and Methods), and a nonspecific competitor whose sequence includes the DR-2 repeat region bound by Int (the double-stranded oligonucleotide DR23; see Materials and Methods). Figure 2 shows that the footprint from Xis was abrogated by the specific competitor. In contrast, the nonspecific competitor had no effect on the footprint.

Xis binds to a related sequence in the DNA on the right end of Tn*916*. To determine if Xis binds to a specific sequence on the right end of Tn*916*, the DNA fragment EU350 (Fig. 1) was used. A protected region was seen extending from about base 108 to base 155 from the end of the transposon, and two bases within this region showed an increased susceptibility to cleavage by DNase I (Fig. 3). The region protected by Xis was further from the end of the transposon than the DR-2 repeats (see Fig. 5).

To determine whether the binding by Xis is specific, competition DNase I protection assays were done with a 1,000-fold excess of two specific competitors, the double-stranded region from the right end found to be protected (oligonucleotide 56; see Materials and Methods) and the double-stranded region from the left end found to be protected (oligonucleotide 53; see Materials and Methods), and a nonspecific competitor whose sequence includes the DR-2 repeat region bound by Int. Figure 4 shows the region of the Xis footprint in the presence

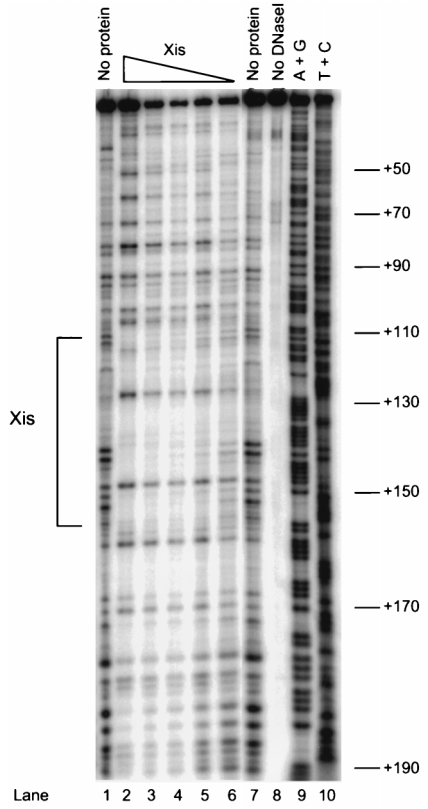

FIG. 3. DNase I protection of radiolabeled fragment EU350. Lanes 1 and 7, labeled DNA with no protein; lanes 2 to 6, labeled DNA with 4, 2, 1, 0.5, or 0.25 μg of Xis, respectively; lane 8, labeled DNA with no DNase I added; lanes 9 and 10, Maxam-Gilbert sequences. The bracket indicates the sequence protected by Xis (from +108 to +155).

of different concentrations of Xis and different competitors. A comparison of lanes 3 and 7 with lane 11 shows that the specific competitors were more effective than the nonspecific competitor. Thus, the binding of Xis to the right end of Tn*916* appears to be specific.

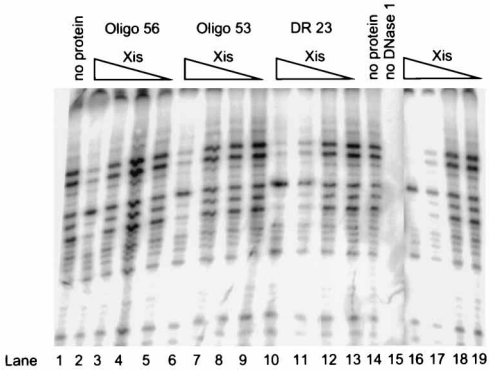

FIG. 4. Competition for DNase I protection of radiolabeled fragment EU350. Lanes: 1 and 14, labeled DNA with no protein; 16 to 19, labeled DNA incubated with 4, 2, 1, or 0.5 μg of Xis, respectively; 2 to 5, the same decreasing concentrations of Xis as in lanes 16 to 19 incubated with oligonucleotide 56 (a specific competitor); 6 to 9, the same decreasing concentrations of Xis as in lanes 16 to 19 incubated with oligonucleotide 53 (a specific competitor); 10 to 13, the same decreasing concentrations of Xis as in lanes 16 to 19 incubated with DR23 (a nonspecific competitor); lane 15, labeled DNA with no DNase I. Only the area of the gel with the Xis footprint is shown (from +120 to +160).

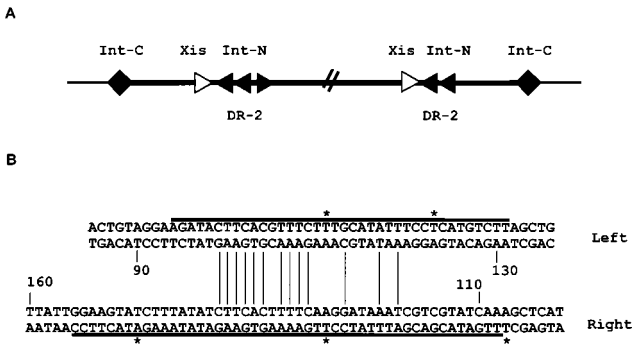

FIG. 5. (A) Position of Xis binding sites in Tn*916*. The thick line represents Tn*916* DNA, and the thin line represents flanking bacterial DNA. The open arrowhead labeled Xis represents the regions protected by Xis described in this paper. The solid arrowheads labeled Int-N represent DR-2 repeats which are bound by the N-terminal domain of Int. The solid diamonds labeled Int-C represent binding sites for the C-terminal domain of Int. (B) The Xis-protected sequences from the left and right ends of Tn*916*. The protected regions are indicated by the horizontal lines; identical bases within the protected regions are connected by vertical lines. Stars indicate bases with an increased susceptibility to cleavage. The bases are numbered from the ends of the transposon.

Comparison of Xis binding sites at the left and right ends of Tn*916*. Figure 5A shows the relative positions of the Xis binding sites and the DR-2 repeats on both the left and right ends of Tn*916*. The region protected by Xis is 5′ to the DR-2 repeats on both ends, with 10 intervening bases on the left end and 12 intervening bases on the right end. The protected regions are similar in size, and they are in the same orientation relative to the DR-2 repeats.

In Fig. 5B, the protected regions from the left and right ends of Tn*916* have been aligned to show their similarities. Ten of 11 bases were identical in the largest region.

Xis causes an extended pattern of increased susceptibility to cleavage by DNase I. An examination of the patterns of DNase I digestion in the experiments presented in Fig. 2 and 3 shows that at high concentrations of Xis a regular pattern of enhanced cleavages extends outward in both directions from the region protected by Xis (Fig. 2, lane 4, and Fig. 3, lane 2). This pattern is more obvious in the experiment, shown in Fig. 6 (lanes 2 to 4), using labeled DNA fragment BO11 containing sequences from the right end of the transposon. The sites with increased susceptibility to cleavage were 10 to 11 bases apart. In Fig. 6, reduced DNase I cleavage at some positions is evident, especially at bases −35 and −10 and between bases +20 and +30.

To determine if the extensive pattern of enhanced cleavages required specific Xis-DNA interactions, we carried out competition assays with competitor double-stranded nucleotides specific for the Xis binding site at the left end of Tn*916* (oligonucleotide 53; see Materials and Methods) and a nonspecific competitor (oligonucleotide DR23; see Materials and Methods). As shown in Fig. 6, at high concentrations of Xis, neither of the competitors had an effect on the pattern of enhanced cleavage. At the lowest concentration of Xis, diminution in the enhancement of cleavage and loss of protection at bases −35 and −10 and between bases +20 and +30 were evident in the presence of the nonspecific competitor as well as the two specific competitors. It therefore appears that the regular pattern of enhanced cleavage produced by Xis is due to nonspecific binding of the protein.

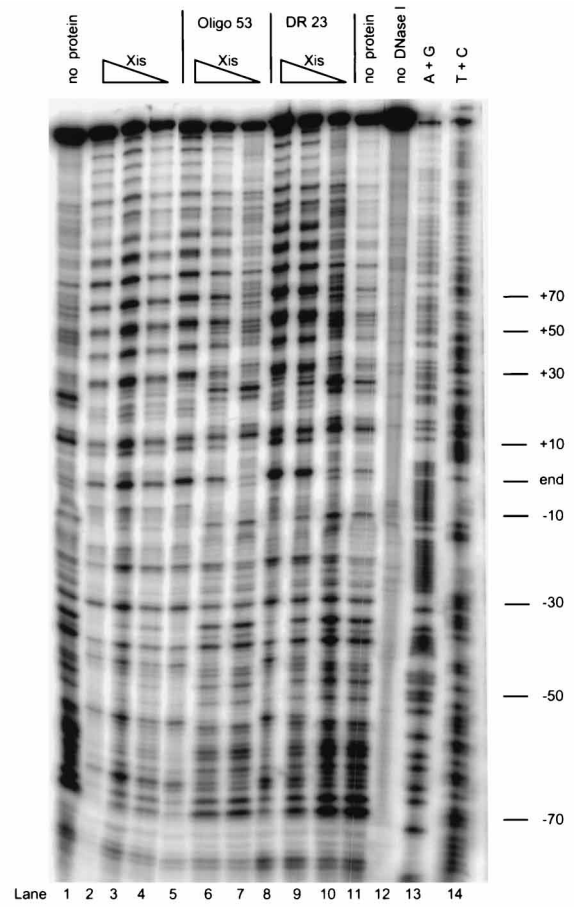

FIG. 6. DNase I protection of radiolabeled fragment BO11 and competition by specific and nonspecific competitor DNA. Lanes: 1 and 11, labeled DNA with no protein; 2 to 4, labeled DNA incubated with 4, 2, or 1 μg of Xis, respectively; 5 to 7, the same decreasing concentrations of Xis as in lanes 2 to 4 incubated with oligonucleotide 53 (a specific competitor); 8 to 10, the same decreasing concentrations of Xis as in lanes 2 to 4 incubated with DR23 (a nonspecific competitor); 12, labeled DNA with no DNase I; 13 and 14, Maxam-Gilbert sequences.

DISCUSSION

We have shown that Xis binds specifically to the ends of Tn*916*. A possible role for Xis in excision of Tn*916* is suggested by comparison with the activities of lambda Xis. During excision of phage lambda, complex nucleoprotein structures that include lambda Int are formed at each end of the integrated prophage and allow these ends to interact (Fig. 7) (17, 18). Formation of these structures requires DNA bending induced by accessory proteins (17, 18) as well as direct protein-protein interactions (23). Lambda Xis binds only at the right end of the prophage (4, 38) (*att*R; Fig. 7). Two molecules of Xis bind cooperatively to two closely spaced DNA sequences with several consequences. First, Xis bends the DNA approximately 120° (37). Second, Xis stimulates binding of the N-terminal domain of lambda Int to an adjacent arm type site that is required for excision (11). Third, Xis inhibits binding of Int to a more distant arm type site that is required for integration (11). Consequently, Xis plays a critical role in determining that recombination leads to prophage excision (14).

In contrast to phage lambda Xis, Tn*916* Xis binds to a specific sequence at each end of the transposon (Fig. 7). The sizes of the regions protected from nuclease digestion by Tn*916* Xis are similar to the sizes of those protected by lambda

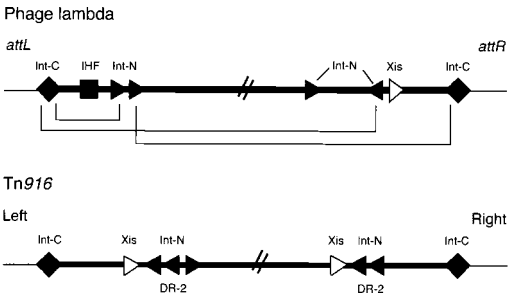

FIG. 7. Comparison of protein binding sites at the ends of phage lambda and Tn*916*. The upper part of the figure shows the ends of phage lambda and the binding sites important for excision. The thick line represents lambda DNA and the thin line represents flanking bacterial DNA. The open arrowhead labeled Xis represents two binding sites for Xis. The solid square labeled IHF represents a binding site for IHF. The solid arrowheads labeled Int-N represent arm type binding sites for the N-terminal domain of Int. The solid diamonds labeled Int-C represent core type binding sites for the C-terminal domain of Int. The thin lines joining core and arm type Int binding sites show the interactions of single Int molecules. The lower part of the figure shows the ends of Tn*916*. The thick line represents Tn*916* DNA, and the thin line represents flanking bacterial DNA. The open arrowheads labeled Xis represent the regions protected by Xis described in this paper. The solid arrowheads labeled Int-N represent DR-2 repeats which are bound by the N-terminal domain of Int. The solid diamonds labeled Int-C represent binding sites for the C-terminal domain of Int.

Xis in *att*R. Since lambda and Tn*916* Xis proteins are similar in size, it is possible that, like lambda Xis, Tn*916* Xis binds as a dimer.

The enhanced nuclease cleavage we observed at nucleotides within the regions protected by Tn*916* Xis suggests that binding of Xis to a specific site may bend the DNA. The specific Xis binding sequence at the left end of Tn*916* is located between binding sites for the N- and C-terminal domains of Int. Therefore, it is possible that Xis-induced DNA bending helps a molecule of Int bind simultaneously to a DR-2 repeat sequence and to the end of the transposon to form a DNA loop. A similar DNA loop is formed in *att*L of phage lambda, where bending of the DNA by IHF allows a single lambda Int molecule to bind simultaneously to core and arm type sequences (17, 18). It seems unlikely that bending of the DNA by Xis would facilitate Int binding at the right end of the transposon because the Xis binding sequence does not lie between the N- and C-terminal Int binding sites. If DNA bending by Xis does facilitate Int binding at the left end of Tn*916*, such binding may be the first step in the assembly of a nucleoprotein complex required for excision of the element.

The specific Xis binding sequences at each end of the transposon are in the same orientation relative to the DR-2 repeats, and they are sufficiently close to these repeats for protein-protein interactions to occur. Therefore, Xis may facilitate the binding of the N-terminal domain of Tn*916* Int to the DR-2 repeats by direct protein-protein interaction rather than by DNA bending. At both ends of Tn*916*, if Xis stimulated binding of the N-terminal domain of Int to a DR-2 repeat, it could help position an Int molecule so that its C-terminal domain can interact with the other end of the transposon.

At high concentrations, Xis binding also produces a pattern of enhanced cleavage by DNase I at 10- to 11-bp intervals that extends at least 40 bp in both directions from the Xis binding site at each end of the transposon. The DNA-protein interactions involved in the formation of this extended structure appear to be nonspecific, although they depend on the presence of the specific binding site. Since this pattern extends well beyond the regions specifically protected from nuclease digestion by Xis and into adjacent DNA, it appears that a structure may be formed in which the DNA is wrapped around the Xis protein. The importance in transposition of the DNA-protein interactions that cause this pattern of enhanced cleavage remains to be established.

We have shown that Tn*916* Xis binds specifically to both ends of the transposon. This interaction may facilitate binding of Tn*916* Int to its specific binding sites by DNA bending, by direct protein-protein interaction, or by a combination of both mechanisms. Further studies of the interactions between Tn*916* Int and Xis and of the role of Xis in excision and integration will be needed to test these proposals.

ACKNOWLEDGMENT

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