CTnDOT Integrase Interactions with Attachment Site DNA and Control of Directionality of the Recombination Reaction^{∇†}

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IntDOT is a tyrosine recombinase encoded by the conjugative transposon CTnDOT. The core binding (CB) and catalytic (CAT) domains of IntDOT interact with core-type sites adjacent to the regions of strand exchange, while the N-terminal arm binding (N) domain interacts with arm-type sites distal to the core. Previous footprinting experiments identified five arm-type sites, but how the arm-type sites participate in the integration and excision of CTnDOT was not known. *In vitro* integration assays with substrates containing arm-type site mutants demonstrated that *attDOT* sequences containing mutations in the L1 arm-type site or in the R1 and R2 or R1 and R2' arm-type sites were dramatically defective in integration. Substrates containing mutations in the L1 and R1 arm-type sites showed a 10- to 20-fold decrease in detectable *in vitro* excision, but introduction of multiple arm-type site mutations in *attR* did not have an effect on the excision frequency. A sixth arm-type site, the R1' site, was also identified and shown to be required for integration and important for efficient excision. These results suggest that intramolecular IntDOT interactions are required for integration, while the actions of accessory factors are more important for excision. Gel shift assays performed in the presence of core- and arm-type site DNAs showed that IntDOT affinity for the *attDOT* core was enhanced when IntDOT was simultaneously bound to arm-type site DNA.

Conjugative transposons (CTns), also known as integrative and conjugative elements (ICEs), are mobile genetic elements that are widespread in *Bacteroides* spp. and are implicated in the spread of antibiotic resistance. These elements are normally integrated into the host chromosome but can excise, replicate, and transfer to a recipient cell by conjugation (34). Since CTns commonly carry antibiotic resistance genes, it is likely that the increase in antibiotic-resistant *Bacteroides* strains has been mediated through the lateral transfer of these elements (36). One of the best-studied ICEs in *Bacteroides* is the conjugative transposon CTnDOT. CTnDOT is 65 kb in size and carries genes encoding resistance to tetracycline and erythromycin. Over the past 30 years, the incidence of tetracycline resistance has increased to 80% of *Bacteroides* isolates due to the presence of CTnDOT-type elements (36).

Integration and excision of CTnDOT results from site-specific recombination between regions of DNA known as attachment (*att*) sites. During integration, the joined ends of the closed circular intermediate (*attDOT*) recombine with the bacterial target sequence (*attB*) to form the recombinant sites (*attL* and *attR*). The integration reaction requires IntDOT, a CTnDOT-encoded protein that has been identified as a member of the tyrosine recombinase family, as well as a host factor encoded by *Bacteroides* (8, 21). Site-specific recombination between the *attL* and *attR* attachment sites results in excision of CTnDOT from the host chromosome. IntDOT is also required

for excision, as are three element-encoded proteins: Orf2c, Orf2d, and Exc, as well as a *Bacteroides* host factor (8, 38). The roles of these accessory proteins are not well understood, although Orf2c and Orf2d have been shown to bind DNA (unpublished results).

One of the best-studied tyrosine recombinases is the integrase (Int) of the lambda system. The C terminus of Int includes the core binding (CB) and catalytic (CAT) domains that bind to core-type sites, which flank the sites of cleavage and strand exchange (2, 24). The N-terminal arm-binding (N) domain binds to arm-type sites that are distal to the core-type sites. In the presence of the appropriate host and accessory factors, Int binding to arm-type sites is required for the formation of higher-order protein/DNA complexes known as intasomes, which are required for integration and excision (15, 18, 22). Int is capable of making intramolecular interactions (interactions between Int monomers on the same attachment site) and intermolecular interactions (interactions between Int monomers on different attachment sites) during recombination (15, 16). In the lambda system, the directionality of the reaction is regulated by Int interactions with arm-type sites in conjunction with the integration host factor (IHF) during the formation of an integrative intasome, or IHF, Xis, and FIS during the formation of the two excisive intasomes (1, 4, 42).

Presumably, IntDOT occupancy of specific arm-type sites in conjunction with interactions of accessory factors with *att* sites leads to the assembly of integrative or excisive intasomes and thus contributes to the directionality of IntDOT-mediated recombination. Previous DNase I footprinting experiments identified five arm-type binding sites on *attDOT* (11). In this study, mutations were constructed in the five sites to determine their roles in the integration and excision of CTnDOT. In addition, a sixth arm-type site was discovered that is important for both

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integrative and excisive recombination. The results of gel shift assays also show that the interaction of IntDOT with core-type sites and arm-type sites involves cooperative interactions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All oligonucleotides and bacterial strains are shown in Table S1 of the supplemental material. All *Escherichia coli* strains were grown in Luria-Bertani (LB) broth. Antibiotics were supplied by Sigma, and restriction enzymes were supplied by New England Biolabs. T4 polynucleotide kinase was obtained from Fermentas. The QuikChange II mutagenesis kit was supplied by Stratagene. IntDOT preparations used for this study were partially purified through heparin-agarose chromatography as previously described (11). The concentrations of antibiotics used in this study were as follows: ampicillin (Amp), 100 μ g/ml; kanamycin (Kan), 50 μ g/ml; chloramphenicol (Cam), 20 μ g/ml.

Site-directed mutagenesis of IntDOT arm-type binding sites. HindIII restriction sites were inserted in each of the putative IntDOT arm-type binding sites by using a Stratagene QuikChange II mutagenesis kit. The plasmid pGEM-T+*attDOT*, which contains the wild-type *attDOT* sequence, was used as a template for the mutagenesis reactions. Primers containing each of the desired mutations are listed in Table S2 of the supplemental material. Digestions with HindIII were used to identify mutants, and the *attDOT* sites of mutated plasmids were sequenced to ensure that there were no additional mutations in the *attDOT* sequence. Identical mutations were also constructed in plasmids containing *attL* (pYS55-1.1) and *attR* (pYS56-1) for analysis in *in vitro* excision assays.

***In vitro* integration competition assay with arm-type site mutants.** The *in vitro* integration competition assay we performed is a modified version of a gel-based assay described previously (11, 17). This assay utilizes two supercoiled plasmids: the wild-type *attDOT* sequence in a large (5.4-kb) pBAD18 vector and mutant *attDOT* sequences that were created in a smaller (3.5-kb) pGEM-T vector. The *attB* substrate was prepared as described previously (17). The *in vitro* integration competition assays were performed in 20- μ l reaction volumes containing 0.1 pmol of pBAD18+*attDOT* (containing the wild-type *attDOT* sequence) and pGEM-T+*attDOT* with the mutated arm-type site(s) along with three units of IntDOT, 3 pmol of *E. coli* IHF (which substitutes for an unidentified *Bacteroides* host factor) (9), and 260 mix (final concentrations, 20 mM Tris-HCl [pH 7.4], 5 mM dithiothreitol, 0.05 mg/ml bovine serum albumin, 1% glycerol, and 50 mM KCl). One unit of IntDOT is defined as the minimum amount of IntDOT required for maximum recombination between *attDOT* and *attB* (17, 25). Reaction mixtures were incubated at 37°C overnight, and reactions were stopped with a solution containing 30% glycerol, 10% SDS, 0.5% xylene cyanol, and 0.5% bromophenol blue. Aliquots of each reaction mixture were electrophoresed on a 1% agarose gel at 120 V for 2 h, and the gel was then dried and exposed to imaging plates (IPs) and visualized using a FujiFilm FLA-3000 PhosphorImager and FujiFilm Image Gauge software (version 3.4 for Macintosh). Levels of recombination with wild-type *attDOT* and *attB* sites were approximately 10 to 15% of that with the initial *attB* substrate.

***In vitro* excision competition assays with arm-type site mutants.** *In vitro* excision competition assays were performed as described previously (12, 38), with minor modifications. HindIII mutations in one or more arm-type sites were constructed in pGEM-T carrying either *attL*(pYS55-1.1) or *attR*(pYS56-1). Three different plasmids were incubated together in the *in vitro* excision competition assay: pGEM-T carrying a mutated *attL* or *attR* site and encoding ampicillin resistance; one pBAD18 plasmid containing the wild-type *attL* or *attR* sequence and encoding kanamycin resistance; a *pir*-dependent pEP185.2 plasmid carrying the wild-type partner site (*attR* or *attL*, respectively) and encoding chloramphenicol resistance. When recombination occurs between either the wild-type or mutant *att* site and the partner site, cointegrate plasmids containing recombinant attachment sites form. The cointegrates that form after recombination *in vitro* can be detected on selective media following transformation into a *pir* background. The excision frequencies for both the wild type and the mutants were calculated by dividing the number of cointegrate colonies by the number of ampicillin- or kanamycin-resistant colonies. The excision frequencies of the wild type and mutants can then be compared as a ratio where higher excision ratios indicate that the mutant excision frequency is reduced relative to the wild type. Wild-type control assays conducted with wild-type *att* sites in both pGEM-T and pBAD18 were used to ensure that the electroporation frequencies of the cointegrates were similar regardless of which plasmid underwent recombination. To ensure that mutant cointegrate colonies resulted from recombination events, at least five colonies from each assay were restreaked on selective media and plasmids were isolated and screened using XhoI restriction digests.

There is a single XhoI restriction site present on the mutant cointegrate plasmids, so linearized plasmids could be visualized by agarose gel electrophoresis to confirm that they were the correct size. Colony PCR was also utilized on 10 cointegrate colonies from each *in vitro* excision assay to confirm that recombination had occurred between *attL* and *attR* as described previously (12).

Gel shift analyses with a multiple arm-type site substrate. A 120-bp DNA substrate containing the R1', R1, R2, and R2' arm-type sites was PCR amplified from pGEM-T+*attDOT* plasmid using primers MM176F and MM25R and purified using the Qiagen PCR purification kit. The PCR products were 5'-radio-labeled using [γ -³²P]ATP and T4 polynucleotide kinase. The labeled DNA was run on a 5% polyacrylamide gel for 1.5 h, exposed to X-ray film for 2 min, and gel extracted. The DNA then was eluted from the polyacrylamide in low-salt buffer (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA) overnight at room temperature. After filtering away excess polyacrylamide, the DNA was cleaned up using DE-52 resin and eluted from the DE-52 columns using high-salt buffer (10 mM Tris-HCl [pH 8], 1 M NaCl, 1 mM EDTA). The substrates were then ethanol precipitated and resuspended in Tris-EDTA buffer.

Gel shift assays were performed in GSB475 consisting of 50 mM Tris-HCl (pH 8), 1 mM EDTA, 50 mM NaCl, 10% glycerol, and 0.075 μ g/ μ l herring sperm DNA. Labeled substrate at 10 nM was incubated with 2 or 20 nM IHF and various dilutions of IntDOT for 15 min at room temperature and then loaded onto a prerun 8% polyacrylamide gel at 300 V, at which point the voltage was reduced to 200 V. The samples were allowed to electrophorese for approximately 2 h and 20 min. The gel was vacuum dried and exposed to IP as described above.

Gel shift assays with *attDOT* core- and arm-type site substrates. Oligonucleotides containing the top and bottom strands of *attDOT* core- and arm-type site DNA were 5'-radiolabeled as described above, separated from unreacted [γ -³²P]ATP, and annealed together. Gel shift assays were performed in gel shift binding buffer (GSBA75) as described above. Approximately 4 nM labeled substrate was incubated with various dilutions of IntDOT. To examine IntDOT complex formation in the presence of both the core- and arm-type site DNA, the same concentrations of unlabeled arm-type site or core DNA were added to the binding reaction mixtures prior to the addition of IntDOT. After samples were allowed to incubate at room temperature for 15 min, reaction mixtures were loaded on a prerun 8% polyacrylamide gel and electrophoresed at 20 mA for 1.5 to 2.5 h. The gel was vacuum dried, exposed to an IP, and visualized using a phosphorimager as described above.

RESULTS

The role of IntDOT arm-type binding sites in integration. In a previous study, DNase I footprinting was used to detect binding of IntDOT to the CTnDOT arm-type binding sites. Using the regions of protection observed in the footprinting assays, an arm-type site consensus sequence was derived, and five putative arm-type binding sites were proposed (11). Deriving a consensus sequence was difficult because of the high number of A/T base pairs in the *attDOT* sequence. Thus, we wanted to identify the arm-type site sequences by an independent approach which would also experimentally confirm the validity of the consensus sequence. Accordingly, we chose to determine which arm-type sites are required for integration and excision by using site-directed mutagenesis to mutate the *attDOT* arm-type sites. The arm-type site mutants were then analyzed using *in vitro* assays in an attempt to test the involvement of the arm-type sites in integration and excision.

An earlier mutational analysis of the lambda Int arm-type binding sites showed that single base pair mutations were not necessarily sufficient to cause dramatic defects in integration and excision. This result was due to cooperative interactions between Int monomers binding to *att* site DNA (3, 27, 33, 40). Taking into account the possibility that cooperative interactions may also play a role in IntDOT interactions with the *attDOT* sequence, HindIII restriction sites were constructed in each of the arm-type binding sites. The HindIII sites changed at least four of the bases in the consensus sequence for each of

FIG. 1. (A) The CTnDOT core- and arm-type sites. The black boxes indicate positions of arm-type sites. The gray ovals indicate core-type sites. The vertical arrows indicate sites of cleavage. (B) Alignment of CTnDOT arm-type sites. The boldface bases are the regions in each arm-type site that were mutated to a HindIII sequence (5'-AAGCTT-3'), while the underlined region denotes bases that were actually changed as a result of the mutagenesis. Six base pairs were mutated for all of the sites except for the R2 arm-type site, where the sixth-position T was not changed, and the R2' arm-type site, where the third-position G and fifth-position T were not changed. W represents A or T; D represents A, T, or G. The R1' arm-type site was a new site identified in this study.

the arm-type sites and were centered in the region where conserved bases were located (Fig. 1).

An *in vitro* integration competition assay was used to analyze the effects of the arm-type site mutations on the integration reaction. This assay is a modified version of a previously described gel-based *in vitro* integration assay (11, 17) that allows for comparison of recombination frequencies between two different *attDOT* sites in the same reaction mixture. Analyzing arm-type site mutants in this assay should allow for the comparison of integration of a wild-type *attDOT* with sites containing one or more arm-type site mutations. Initially, individual HindIII mutations were made in each of the five IntDOT arm-type binding sites, and the effects of the mutations were analyzed in the *in vitro* integration competition assay (Fig. 2A). The *attDOT* sites with mutations in the R1, R2, R2', and L2 arm-type sites showed levels of recombination between 10 and 15%, which is similar to that of the wild-type *attDOT* site. However, the mutation in the L1 arm-type site abolished detectable *in vitro* integration (Fig. 2A, lane 6), demonstrating that an intact L1 arm-type site is required for integration to occur.

It was possible that IntDOT binds to one or more arm-type sites through protein-protein interactions among IntDOT monomers, and this might compensate for mutated arm-type sites. In attempts to further disrupt IntDOT interactions with the arm-type binding sites as well as to uncover any intramolecular interactions, mutations were constructed in multiple arm-type sites in the same *attDOT* DNA and tested in the *in vitro* integration competition assay. Since the R2 and R2' arm-type sites are immediately adjacent to one another, it was possible that IntDOT may bind cooperatively to the two sites in a manner similar to Int interactions with the contiguous lambda P' arm-type sites, and this cooperativity may have overcome the defects of single arm-type site mutations (3, 18, 31). However, the *attDOT* site with both the R2 and R2'

FIG. 2. (A) Results of an *in vitro* integration competition assay using *attDOT* sequences containing mutated arm-type site sequences. Radiolabeled *attB* DNA was incubated with IntDOT, IHF, and two supercoiled plasmids, one containing the wild-type *attDOT* sequence and one containing a mutation in one of the five arm-type sites. The slower-migrating recombinant in each lane is the larger product resulting from the wild-type internal control, while the faster-migrating recombinant is the product resulting from recombination between the mutant *attDOT* site and the *attB* sequence. Lane 1 is a 1-kb ladder. Lane 2 is a control where both the smaller and larger *attDOT* plasmids contain the wild-type sequence. (B) Results of an *in vitro* integration competition assay using *attDOT* sequences containing multiple mutated arm-type sites. Lane 1 is a 1-kb ladder.

arm-type sites mutated showed levels of integration similar to the wild-type *attDOT* site (Fig. 2B, lane 2), suggesting that the integration reaction proceeds normally with mutations in these two sites. Substrates with mutations in both the R1 and R2 arm-type sites or the R1 and R2' arm-type sites (Fig. 2B, lanes 4 and 5) showed no detectable levels of recombination. This result demonstrated that *attDOT* sites with mutated R2 or R2' arm-type sites are defective when combined with a mutated R1 arm-type site and suggests that intramolecular interactions between IntDOT monomers are involved in the binding of IntDOT to the R1, R2, and R2' arm-type sites. Additionally, an *attDOT* site with three arm-type site mutations (R2, R2', and L2) showed no defect in integration frequency (data not shown), demonstrating that integration can proceed even with three of five arm-type sites mutated. This result suggests that there are no cooperative interactions between the left and right sides of the crossover region during integration. A summary of the integration results is shown in Table 1.

TABLE 1. Results of *in vitro* integration competition assays with the arm-type site mutations^a

Arm-type site mutation	Effect on integration ^b
Wild type	+
R1'	−
R1	+
R2	+
R2'	+
L1	−
L2	+
R1/R2	−
R1/R2'	−
R2/R2'	+
R1/R2/R2'	−
R2/R2'/L2	+

^a Data shown are the result of at least four independent assays.
^b Plus signs indicate wild-type levels of integration of 10 to 15%, and minus signs indicate no detectable integration.

Identification of an additional arm-type binding site. The results of the *in vitro* integration competition assays demonstrated that the L1 arm-type site was the only individual arm-type site absolutely required for integration to proceed. This was surprising, since multiple arm-type sites are required for integration reactions of other site-specific recombination systems (13, 27, 28). It was possible that there might be additional arm-type sites that were not detected by DNase I footprinting. Since the results above showed that the L1 arm-type site plays an important role in the integration reaction, we hypothesized that if the same sequence as the L1 site appeared elsewhere on the *attDOT* attachment site then it might also serve as an IntDOT arm-type site. Examination of the sequence around the R1 arm-type site showed that there is nearly an exact match to the L1 site immediately upstream of the R1 arm-type site. This sequence was partially protected by DNase I footprinting experiments done previously (11) (Fig. 3). A HindIII site was inserted into this sequence (Fig. 1B), and the effect of the mutation was examined in the *in vitro* integration competition assay. A mutation in this sequence abolished detectable *in vitro* integration (Table 1). This result demonstrated that this sequence was likely required for integration, and the sequence was named the R1' site. The identification of the R1' site indicated that both the R1' and L1 arm-type sites are required for integration.

Effects of arm-type binding site mutations on the *in vitro* excision reaction. To examine the roles of the arm-type sites in excision of CTnDOT, analogous HindIII mutations were made in the arm-type binding sites on the *attL* and *attR* sites and tested in an *in vitro* excision competition assay. The assay is a modified version of a previously developed intermolecular excision assay that uses three plasmids (12). Recombination results in the formation of cointegrate plasmids containing two drug resistance markers and can be detected on the appropriate selective media. Ratios of wild-type versus mutant excision frequencies were determined for each assay. The internal standard in the *in vitro* excision competition assay provides a consistent means of comparing wild-type and mutant excision frequencies.

The results of the *in vitro* excision competition assays are shown in Fig. 4. Substrates with HindIII sites in the R1, R1',

FIG. 3. The R1 region of protection. Bases shaded in gray represent regions protected by IntDOT in DNase I footprinting experiments performed previously, and the boldface type denotes sequence portions previously assigned to R1 (11). The boxes represent bases that were changed in site-directed mutagenesis of the R1' and R1 arm-type sites.

and L1 arm-type sites showed the greatest decrease in excision frequency, between 10- and 20-fold. Mutations in the R2 and R2' arm-type sites had less of an effect on excision. As was seen with integration, mutations in the L2 arm-type site caused no change in excision frequency. These results show that R1' and L1 are the only individual arm-type sites which affect both integration and excision.

attR sites containing combinations of two mutated arm-type sites were also constructed to determine if IntDOT protein-protein interactions were required for efficient excision, similar to what was seen in the integration reaction. The greatest decrease in excision frequency was seen with an *attR* site with mutated R1 and R2 arm-type sites, which showed a 15- to 20-fold decrease in excision. This is, at best, only a slightly greater defect than was seen with *attR* sites containing individual mutations in either the R1 or R2 arm-type sites. *attR* sites with mutated R1 and R2' arm-type sites or R2 and R2' arm-type sites showed approximately the same defect in excision as was seen with either of the individual arm-type site mutants. Interestingly, none of the *attR* sites with two mutated arm-type sites affected the excision reaction as dramatically as the effects observed in the *in vitro* integration competition assays.

The *in vitro* excision competition assays demonstrated that arm-type sites that are required for integration (R1', L1, and R1) are also required for efficient excision. Interestingly, none of the mutations or combinations of mutations tested completely abolished excision. It is possible that the action of accessory factors during excision may help compensate for

FIG. 4. Effects of arm-type site mutations on excision. Excision frequencies were calculated as described in Materials and Methods, and excision ratios were calculated by dividing the wild-type (WT) excision frequency by the mutant excision frequency. Wild-type excision frequencies averaged between 1 and 5%. The values displayed in the figure are the results of at least four independent assays.

FIG. 5. Gel shift analysis of IntDOT complex formation with the L1 arm-type site. Experiments were carried out with labeled wild-type L1 DNA alone (lanes 1 to 4) or labeled wild-type L1 DNA in the presence of unlabeled complementary oligonucleotides containing the *attDOT* core (lanes 5 and 6). IntDOT was added to the DNA substrates in increasing dilutions, as shown above lanes 2 to 6. An additional band labeled X is visible in all lanes, including the DNA-only lane 1. We believe this band is due to aberrant annealing of the top and bottom oligonucleotides used to make the gel shift substrate. The migration shift of this band in lanes 2 and 5 may be due to interactions of a contaminating DNA binding protein in the partially purified IntDOT preparation. The cartoons diagrammed on the left of the figure indicate possible stoichiometries of the protein-DNA complexes.

mutated arm-type sites, and protein-protein interactions between IntDOT monomers may not be required for excision.

Interactions of IntDOT with *attDOT* core- and arm-type sites. As demonstrated above, mutations in certain IntDOT arm-type binding sites disrupted the integration and excision of CTnDOT. However, the experiments did not reveal the affinity of IntDOT for the various arm-type binding sites or how that affinity may be affected by the presence of *attDOT* core DNA. Gel shift assays were performed with labeled arm-type site DNA to determine if IntDOT has a high enough affinity for any of the arm-type sites to detect binding in gel shift assays. Initially, gel shift assays were performed using labeled oligonucleotides containing the wild-type arm-type site sequences. Since the R2 and R2' arm-type sites are located immediately adjacent to one another, oligonucleotides containing both the R2 and R2' sites were utilized in the gel shift assays. There were no detectible gel shifts when IntDOT was incubated with DNA containing the R1, R1', or L2 arm-type sites or a substrate containing both the R2 and R2' arm-type sites (data not shown). IntDOT was able to shift the L1 arm-type site only weakly (Fig. 5, lanes 2 to 4), indicating that IntDOT affinity for the site is low. Since none of the other arm-type sites was shifted by IntDOT, IntDOT appears to have a stronger affinity

for the L1 arm-type site than for the other sites. The mutated L1 site containing a HindIII site was not shifted (data not shown), which indicated that IntDOT interactions with the wild-type L1 site are specific for that particular DNA sequence. Binding reactions were also performed with the L1 arm-type site in the presence of unlabeled oligonucleotides containing the *attDOT* core. As shown in Fig. 5, lanes 5 and 6, a supershifted complex was visible, demonstrating that IntDOT was able to form a complex with both the L1 arm-type site and the *attDOT* core.

We were also interested in examining interactions of IntDOT with the *attDOT* core. To gain insight into these interactions, gel shift assays were carried out with an 80-bp radiolabeled *attDOT* core containing the D and D' core-type sites. This substrate lacks any of the identified IntDOT arm-type binding sites. As shown in Fig. 6, lane 2, IntDOT was able to form a complex with the *attDOT* core. In addition to this complex, there was also aggregation of protein and DNA in the wells (Fig. 6, lane 2), which may be due to interactions between the unoccupied N domains of IntDOT monomers or nonspecific interactions between IntDOT and the *attDOT* core.

Unlabeled oligonucleotides containing either the wild-type or mutant L1 arm-type sites were also added to gel shift reaction mixtures containing the *attDOT* core to determine if the presence of an arm-type site affected IntDOT interactions with the core. In a reaction mixture containing IntDOT and labeled *attDOT* core, the addition of DNA containing the wild-type L1 arm-type site caused a detectable supershift in the migration of the IntDOT-core complex (Fig. 6, lane 3). This slower-migrating complex may correspond to two IntDOT monomers bound to the *attDOT* core with at least one wild-type L1 arm-type site bound to one of the monomers. No supershift was seen when complementary oligonucleotides containing the mutant L1 arm-type site were added (Fig. 6, lane 4). Gel shifts performed with unlabeled longer and shorter complementary oligonucleotides containing the wild-type L1 arm-type site and the labeled *attDOT* core showed that the DNA containing the L1 arm-type site was part of the supershifted complex (data not shown). The complex caused by aggregation in the wells was less pronounced in binding reactions in which complementary oligonucleotides containing the wild-type L1 site were present, suggesting that occupancy of the N domains of IntDOT monomers may prevent protein aggregation (Fig. 6, lane 2 or 4 compared to lane 3). Similar supershifted complexes were also seen when DNA containing the R1 arm-type site or both the R2 and R2' arm-type sites were added to binding reaction mixtures containing labeled *attDOT* core DNA (Fig. 6, lanes 5 and 7). It appears that there are two supershifted complexes visible in these lanes; we hypothesize that the faster-migrating band is a complex containing two IntDOT monomers bound to *attDOT* core with an arm-type site DNA bound to one of the monomers and that the slower-migrating band corresponds to a complex with arm-type site DNA bound to both IntDOT monomers interacting with the *attDOT* core. In contrast, no slower-migrating complexes were seen in binding reactions with the R1 and R2 and R2' arm-type sites containing HindIII mutations (Fig. 6, lanes 6 and 8). The formation of the supershifted complexes was not as efficient with the R1 and R2/R2' arm-type sites as with the L1 arm-type site. These results also showed that the formation of the IntDOT/core/arm-type site

FIG. 6. Gel shift analysis of IntDOT interactions with the *attDOT* core. Each indicated arm-type site (at approximately 250 nM) was incubated with 40 nM labeled *attDOT* core and a 1:2 dilution of IntDOT, and the mixtures were subjected to electrophoresis. The cartoons diagrammed on the right of the figure indicate possible stoichiometries of the protein-DNA complexes.

complex is specific for the wild-type arm-type sequences, and mutations in the arm-type sites disrupted IntDOT interactions with the arm-type sites.

To examine IntDOT interactions with the R1 and R1' arm-type sites in the presence and absence of *attDOT* core DNA,

two different substrates containing arm-type sites were utilized: one containing the R1' arm-type site alone and another containing both the R1 and R1' arm-type sites (Fig. 7A and B). IntDOT does not shift either substrate in the absence of *attDOT* core DNA. However, supershifts were visible when

FIG. 7. (A) DNA sequences containing the R1' arm-type site or the R1 and R1' arm-type sites. (B) Gel shift showing IntDOT binding to DNA containing the R1' or the R1 and R1' arm-type sites. Approximately 40 nM arm-type site DNA was incubated with IntDOT alone or with IntDOT and unlabeled *attDOT* core.

FIG. 8. IntDOT binding to a gel shift substrate containing four arm-type sites. A 120-bp radiolabeled fragment of *attDOT* was incubated with either 20 or 2 nM IHF and decreasing IntDOT concentrations for 15 min and then electrophoresed on an 8% polyacrylamide gel.

IntDOT was incubated with the labeled arm-type site DNA and unlabeled *attDOT* core (Fig. 7, lanes 3 and 6). The supershift seen with the substrate containing both the R1 and R1' arm-type sites was stronger than the one seen with the R1' arm-type site alone. This result indicates that IntDOT has a higher affinity for DNA that contains both arm-type binding sites in the presence of the *attDOT* core, suggesting that cooperative interactions may be involved in IntDOT binding to the arm-type sites when *attDOT* core DNA is also present.

IntDOT interactions with DNA containing the *attR* arm. As described above, IntDOT was only able to shift the L1 arm-type site. It was possible that IntDOT needed specific intramolecular contacts with other monomers to interact with the arm-type sites on the *attR* arm (R1', R1, R2, and R2'). We hypothesized that a DNA-bending protein may bend the *attR* arm DNA in such a way as to bring arm-type sites into closer proximity, thus facilitating protein-protein interactions. To investigate IntDOT interactions with the *attR* arm, gel shift assays were performed with a DNA substrate containing the R1', R1, R2, and R2' arm-type sites. IntDOT alone was unable to shift the DNA (Fig. 8, lanes 2 and 3). Binding reactions were also performed with IntDOT and the labeled substrate in the presence of *E. coli* IHF, because IHF has been shown to facilitate IntDOT binding to the full-length *attDOT* in gel shift assays (12). IHF shifted the DNA at a concentration of 20 nM (Fig. 8, lane 4) but did not shift it at a 10-fold-lower concentration (2 nM) (Fig. 8, lane 5). When IntDOT was incubated with DNA and IHF, two or three faint supershifts were visible (Fig. 8, lane 6). If IHF was diluted 10-fold such that it no longer shifted the DNA substrate, the supershifted complex disappeared (Fig. 8, lane 7). This further demonstrated that IntDOT binding was dependent on IHF also being bound to the substrate. Similar results were seen with slightly more diluted IntDOT (Fig. 8, lanes 8 and 9). It appears that at least two IntDOT-dependent complexes formed in the presence of IHF (Fig. 8, lanes 6 and 8). We do not know the exact composition of the complexes, but we hypothesize that the faster-migrating complex contained one IntDOT monomer and the slower-migrating complex contained at least two IntDOT monomers. These results suggest that intramolecular IntDOT

interactions may be required for IntDOT binding to *attR* arm DNA, since complex formation occurred only in the presence of IHF. IHF might bind nonspecifically between the R1 and R2 arm-type sites and bend the DNA, thus bringing the two pairs of arm-type sites closer to each other. When gel shifts were performed with a substrate containing the R1, R2, and R2' arm-type sites but lacking the R1' arm-type site, no supershifts were visible (data not shown), demonstrating that the R1' arm-type site is required for IntDOT complex formation.

DISCUSSION

Tyrosine recombinases that display directionality, such as the integrases of lambda, mycobacteriophage L5, HP1 of *Haemophilus influenzae*, and CTnDOT, interact with two different classes of binding sites: core-type sites and arm-type sites. These proteins contain three domains: the CB and CAT domains bind to the core-type sites during catalysis, and the N domain binds to arm-type sites that are distal to core-type sites (24, 31–33). In the lambda system, the binding of Int monomers, along with the action of the appropriate accessory factors, is responsible for the formation of productive integrative and excisive intasomes (15, 18, 22). The accessory factors bend the DNA in such a way that bivalent Int monomers can form inter- and intramolecular bridges between higher-affinity arm-type and lower-affinity core-type sites (2, 15, 20). Recent work suggests that N domain interactions with arm-type site DNA play a role in regulating DNA binding, cleavage, and Holliday junction resolution (5, 6, 30, 35, 41). Interactions with specific arm-type sites also influence the directionality of recombination. During integration, lambda Int is bound to the P1, P'1, P'2, and P'3 arm-type sites, while during excisive recombination the P2, P'1, and P'2 arm-type sites are occupied (3, 14, 27). Multiple arm-type sites on both sides of the crossover region are also required for the integration of phages L5 and HP1 (13, 28). We hypothesized that the IntDOT arm-type sites play a similar role in determining the directionality of CTnDOT recombination.

In this report we used a mutational analysis of the IntDOT arm-type binding sites to identify which arm-type sites are

FIG. 9. (A) Roles of the arm-type sites in integration of CTnDOT. The core-type sites D and D' are shown as gray ovals. Arm-type sites shown as black boxes are required for integration. Arm-type sites represented by gray boxes were found to be important for intramolecular interactions between IntDOT monomers during integration. Arm-type sites shown in white are not required for integration. (B) Roles of the arm-type sites in excision of CTnDOT. The core-type sites D and D' are shown as light gray ovals and the core-type sites B and B' are shown as dark gray ovals. Arm-type sites, represented by gray boxes, stimulate excision, and arm-type sites, shown as white boxes, are not required for excision. (The illustrations not drawn to scale.)

involved in the integration and excision of CTnDOT (Fig. 9). Five arm-type binding sites were identified in previous DNase I footprinting experiments (11), but exactly how the arm-type sites participated in the directionality of recombination was not known. We demonstrated that an intact L1 arm-type site is required for integration to occur. This result also led to the discovery of the R1' arm-type site, since a portion of its sequence is identical to that of L1. A mutation in the R1' arm-type site also abolished *in vitro* integration (Table 1), and this result demonstrated that the L1 arm-type site was not the only arm-type site required for *in vitro* integration.

Since the R1 and R1' sites are separated by only 5 bp, it is possible that this region actually contains only one arm-type site that overlaps with the mutated sequences. We believe that the R1 and R1' sites are separate arm-type sites for three reasons. First, a DNA substrate containing both the R1 and R1' arm-type sites appeared to increase the affinity of IntDOT for the *attDOT* core more than what was seen with DNA containing the R1' arm-type site alone (Fig. 7, lane 6 versus lane 3). Second, the observation that mutations in the R1 and R1' arm-type sites have different effects on *in vitro* integration while both mutations decrease *in vitro* excision suggests that they are two independent arm-type sites. Third, the length of the arm-type site sequences for phages lambda, P22, and L5 is 10 bases (28, 31, 37), so if the lengths of the CTnDOT arm-type sites are similar then there is enough space for two arm-type sites. In other site-specific recombination systems, such as in P22, HP1, L5, and Tn916, arm-type sites are often arranged in pairs (13, 19, 28, 37). It is likely that the R1 and R1' arm-type sites are also arranged in a pair but function as two independent arm-type sites.

In addition to the effects seen with *attDOT* sites with mutations in the R1' and L1 arm-type sites, integration was also abolished when the R1 and R2 or the R1 and R2' arm-type sites were both mutated on the same *attDOT* site (Table 1). This result indicates that interactions between IntDOT monomers may be required for IntDOT binding to the R1, R2, and R2' arm-type sites during the integration reaction. For example, an IntDOT monomer bound to one arm-type site may interact with a second IntDOT monomer bound to another arm-type site through an intramolecular bridging interaction.

It is possible that mutating one arm-type site does not sufficiently disrupt interactions to inhibit integration. Cooperativity has also been observed in lambda Int interactions with the P'123 arm-type sites (3, 18).

Further evidence for cooperative interactions between IntDOT monomers when binding the arm-type sites was seen in gel shift assays with a substrate containing four arm-type sites (R1', R1, R2, and R2') (Fig. 8, lanes 6 and 8). Presumably, four IntDOT monomers bind to *attDOT* during the integration reaction, with one monomer to cleave each strand of the DNA required for recombination. Since the R1' and L1 arm-type sites are also required for integration, it is possible that five IntDOT monomers may be bound to *attDOT* during integration, and one of the monomers may play a structural role in the formation of the integrative intasome. Since the only other protein required for integration is an unidentified *Bacteroides* host factor, it is likely that the interactions between IntDOT monomers play an important role in formation of a productive integrative intasome.

Mutational analysis of the arm-type sites revealed that the L1, R1', and R1 arm-type sites are also important for efficient excision, although mutations in multiple arm-type sites (e.g., R1 and R2, R1 and R2', or R2 and R2') still retained some *in vitro* excision activity. Since mutations in multiple arm-type sites did not abolish excision, it appears that intramolecular interactions between IntDOT monomers on *attR* are not as important for excision as they are for integration. This difference may be due to the action of accessory factors. Xis, an accessory protein in the lambda excision reaction, bends the DNA and interacts cooperatively with Int binding at the P2 arm-type site (7, 10, 26, 39, 40). Lambda Xis also helps drive recombination in the direction of excision by inhibiting Int binding to the P1 arm-type site, which is an interaction required for integration (23). The excision reaction of CTnDOT is complex and involves three element-encoded excision proteins as well as IntDOT and a *Bacteroides* host factor (8, 38). One or more of the excision proteins may help drive recombination toward excision by facilitating IntDOT interactions with one or more arm-type sites, even if the sites are mutated. Thus, binding of accessory factors may play an important role in regulating IntDOT interactions with its arm-type sites.

Lambda Int has been shown to form both intramolecular bridges on the same attachment site and intermolecular bridges between *attL* and *attR* during excision (15, 16), so it is possible that IntDOT utilizes similar interactions. In addition to effects that accessory factors may have on IntDOT binding, long-range intramolecular interactions between IntDOT monomers are likely to be important during excision. However, bridging intermolecular IntDOT interactions are also likely to play an important role in intasome formation during excision. For example, the N domain of one IntDOT monomer may bind to one of the arm-type sites on one attachment site (e.g., *attR*), and its CB and CAT domains may be interacting with a core-type site on the partner attachment site (e.g., *attL*). Previous work on the CTnDOT *attR* demonstrated that phasing mutations that affected the distances between arm-type sites and core-type sites had only a modest effect on *in vitro* excision frequency. This was hypothesized to be due to the intermolecular bridging that may occur during excision (11, 12). Since the mutagenesis of multiple arm-type sites on *attR* did not abolish *in vitro* excision, it is possible that IntDOT interactions with the R1, R2, or R2' arm-type sites during excision are more important for intermolecular bridging interactions.

Gel shift assays performed with DNA containing the *attDOT* core revealed that IntDOT was able to form stable complexes with core DNA but not with arm-type site DNA, which suggests that IntDOT has a higher affinity for the D and D' core-type sites. Lambda Int has a lower affinity for the core-type sites than the arm-type sites and is unable to gel shift the *attP* core unless arm-type site DNA is provided in *trans* (29, 31, 35). Studies with lambda Int have also demonstrated that the presence of P'1,2 arm-type site DNA in *trans* stimulates Int cleavage activity (35). Similar experiments performed with IntDOT in the presence of CTnDOT arm-type site DNA failed to show any detectable effect on cleavage or ligation activity (M. McCurdy, unpublished results). It is possible that IntDOT interactions with arm-type site DNA are less important for regulating catalytic activity.

Although IntDOT was able to shift DNA containing the *attDOT* core in gel shift assays, addition of arm-type site DNA in *trans* led to the formation of a supershifted complex containing both core and arm-type site DNA (Fig. 6, lane 3). We do not know the stoichiometry of IntDOT binding to the *attDOT* core substrate. Because the supershifted complex has a similar mobility to that of a complex seen occasionally in gel shifts performed only in the presence of IntDOT and the labeled *attDOT* core, we believe this complex contains two IntDOT monomers bound to one *attDOT* core sequence and one or two arm-type sites. The shift seen when IntDOT is incubated with the *attDOT* substrate alone (Fig. 6, lane 2) appears to correspond to only one IntDOT monomer bound to the DNA, so it is possible that the presence of arm-type site DNA in *trans* stimulates the cooperative binding of the second IntDOT monomer to the *attDOT* core. Our results also suggest that IntDOT forms the most stable complexes when both core-type and arm-type site DNAs are present.

The results of gel shift assays also demonstrated that IntDOT binding to arm-type site DNA was specific for wild-type sequences. When IntDOT was incubated with the labeled *attDOT* core and arm-type sites containing HindIII mutations, supershifted complexes were no longer detectable (Fig. 6,

lanes 4, 6, and 8). This demonstrates that the sequences that we mutated and tested in *in vitro* integration and excision assays are specifically bound by IntDOT and that we have identified regions of the arm-type sites that are important for IntDOT recognition and binding.

The interactions of IntDOT with its arm-type sites appear similar to what has been seen with lambda Int and other well-characterized tyrosine recombinases. The experiments detailed in this report suggest that IntDOT interactions with arm-type sites are enhanced when IntDOT simultaneously binds the *attDOT* core and that different types of interactions are required for integration versus excision. There is still much that is unknown about CTnDOT recombination, especially the complex excision reaction. The identity of the *Bacteroides* host factor(s) required for integration and excision is not known, and the binding sites of other accessory factors have yet to be identified. Experiments are currently under way to characterize the CTnDOT accessory factors, and the data obtained from these studies may help elucidate the structures of the integrative and excisive intasomes.

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