Resolution of Mismatched Overlap Holliday Junction Intermediates by the Tyrosine Recombinase IntDOT

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ABSTRACT CTnDOT is an integrated conjugative element found in *Bacteroides* species. CTnDOT contains and transfers antibiotic resistance genes. The element integrates into and excises from the host chromosome via a Holliday junction (HJ) intermediate as part of a site-specific recombination mechanism. The CTnDOT integrase, IntDOT, is a tyrosine recombinase with core-binding, catalytic, and amino-terminal (N) domains. Unlike well-studied tyrosine recombinases, such as lambda integrase (Int), IntDOT is able to resolve Holliday junctions containing heterology (mismatched bases) between the sites of strand exchange. All known natural isolates of CTnDOT contain mismatches in the overlap region between the sites of strand exchange. Previous work showed that IntDOT was unable to resolve synthetic Holliday junctions containing mismatched bases to products in the absence of the arm-type sites and a DNA-bending protein. We constructed synthetic HJs with the arm-type sites and tested them with the *Bacteroides* host factor (BHFa). We found that the addition of BHFa stimulated resolution of HJ intermediates with mismatched overlap regions to products. In addition, the L1 site is required for directionality of the reaction, particularly when the HJ contains mismatches. BHFa is required for product formation when the overlap region contains mismatches, and it stimulates resolution to products when the overlap region is identical. Without this DNA bending, the N domain of IntDOT is likely unable to bind the L1 arm-type site. These findings suggest that BHFa bends DNA into the necessary conformation for the higher-order complexes, including the L1 site, that are required for product formation.

IMPORTANCE CTnDOT is a mobile element that carries antibiotic resistance genes and moves by site-selective recombination and subsequent conjugation. The recombination reaction is catalyzed by an integrase IntDOT that is a member of the tyrosine recombinase family. The reaction proceeds through ordered strand exchanges that generate a Holliday junction (HJ) intermediate. Unlike other tyrosine recombinases, IntDOT can resolve HJs containing mismatched bases in the overlap region *in vivo*, as is the case under natural conditions. However, HJ intermediates including only IntDOT core-type sites cannot be resolved to products if the HJ intermediate contains mismatched bases. We added arm-type sites in *cis* and in *trans* to the HJ intermediates and the protein BHFa to study the requirements for higher-order nucleoprotein complexes.

KEYWORDS conjugative transposons, DNA-protein interactions, Holliday junctions, integrative conjugative elements, integrase, recombination, tyrosine recombinases

CTnDOT is an integrated conjugative element (ICE, formerly known as conjugative transposons) found within *Bacteroides* species (1, 2). CTnDOT excises from a *Bacteroides* donor chromosome, transfers by conjugation, and integrates into the recipient host chromosome. The element is induced to transfer via conjugation by tetracycline

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and carries genes for resistance to both tetracycline and erythromycin (3). *Bacteroides* spp. are major components (20 to 30%) of the gut microbiota and can act as opportunistic pathogens, so these species can cause abscesses or anaerobic bacteremia if the gut is punctured (4–6). CTnDOT and similar elements have contributed to the increase in antibiotic resistance among *Bacteroides* spp. in the last 40 years (7).

CTnDOT integrates into and excises from the host chromosome by a site-selective recombination mechanism. The recombination reaction of CTnDOT is catalyzed by the integrase (Int) IntDOT, a member of the tyrosine recombinase family. Integration into the chromosome requires the joined ends of the element (*attDOT*), IntDOT, a host-encoded DNA-bending protein, and a suitable *attB* site within the chromosome (2, 8, 9). Excision from the chromosome is tightly regulated and involves the additional excisionase proteins Xis2c and Xis2d. A topoisomerase, Exc, stimulates the excision reaction (3, 10–13).

In both integration and excision, the necessary proteins bind to specific DNA sites to form higher-order nucleoprotein complexes called intasomes. While the integrative and excisive intasomes are composed of different participants and DNA sites, both proceed through a series of ordered strand exchanges by IntDOT monomers that generate a Holliday junction (HJ) intermediate. Two IntDOT monomers make the initial cleavages at the core-type sites, D and D', located 7 bp apart (Fig. 1A) (9, 10). The region between the core-type sites is called the overlap region.

During the first strand exchanges, two IntDOT monomers each create a covalent 3'-phosphotyrosyl intermediate, leaving a 5' hydroxyl to attack the partner strand and form a phosphodiester bond (Fig. 1B). Once formed, the HJ intermediate isomerizes by a strand-swapping mechanism through the overlap region until a second set of strand exchanges carried out by an additional two IntDOT monomers resolve the junction (Fig. 1C) (14). The products of integrative HJ resolution are *attL* and *attR* (Fig. 1D). Unlike other tyrosine recombinases, IntDOT can resolve HJ intermediates that contain mismatched bases in the overlap region *in vivo*. In contrast, lambda integrase (Int) is unable to recombine sites that contain a single mismatch (15, 16).

Like lambda Int, IntDOT is a heterobivalent protein with three domains (10, 17). The core-binding (CB) domain of IntDOT interacts with the core-type sites, which are D and D' on *attDOT* and B and B' on *attB*. The catalytic (CAT) domain is responsible for the cleavage and ligation steps necessary for strand exchange between the DNA sites. The amino-terminal (N) domain of IntDOT interacts with the arm-type sites, which are positioned 40 bp (or more) away from the core-type sites (Fig. 1A). The N domain of lambda Int determines the order of strand exchanges and the directionality (integration versus excision) of the recombination reaction based on interactions with the arm-type sites (18–20). In contrast, the order of strand exchanges by IntDOT is determined by a conserved GC dinucleotide at the site of the first strand exchange within the D and B core-type sites rather than by interactions with the arm-type sites (9). These 2 bp of homology are essential, but the remaining 5 bp of the overlap region may be mismatched, particularly in recombination between natural sites (Fig. 2). All known *attB* sequences would result in mismatches during integrative recombination (2, 8). After resolution, these mismatched bases would remain as heteroduplexes within *attL* and *attR*. These heteroduplexes may be resolved by the host cell machinery during subsequent replication or DNA repair.

Synthetic HJ intermediates containing the IntDOT core-type sites but not arm-type sites can be processed *in vitro* to both products and substrates if the overlaps are identical (Fig. 2A) (21). However, if the overlaps contain mismatches, the HJs are only resolved back to substrates by exchanges at the GC dinucleotide (Fig. 2B). It is possible that nucleoprotein complexes containing both *Bacteroides* host factor (BHFa) and IntDOT are required for IntDOT to resolve core-only mismatched HJ intermediates toward products.

We hypothesized that both a DNA-bending protein and the arm-type sites are necessary to form intasomes and enable IntDOT to catalyze recombination through the mismatched bases. One example of a DNA-bending protein that can assist with

FIG 1 The integrative recombination reaction of CTnDOT. (A) Overview of the *attDOT* region of DNA. The IntDOT core type sites (D and D') are represented by circles and flank the overlap region. Arm-type sites are filled boxes. Sites that are required for integration are filled black boxes. Sites that act cooperatively in integration are gray boxes. BHFa binding sites are marked with white boxes. The central base of the overlap region is numbered zero. Bases toward the *attR* arm-type sites are negative; bases toward the L1 site are positive. (B) The first set of strand exchanges by IntDOT. Both the *attDOT* (red) and *attB* (blue) DNA

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FIG 2 Core-only Holliday junction intermediates. (A) The left portion shows the two double-stranded substrates for CTnDOT integration that will lead to an identical overlap region. The right portion shows the two double-stranded substrates for CTnDOT integration that will lead to a mismatched overlap region. The essential dinucleotide is capitalized, and lowercase letters represent the 5 bases that may be mismatched. The bases that will be mismatched in the right panel are capitalized and in red. (B) The left portion shows the core-only identical Holliday junction intermediate. The right portion shows the core-only mismatched Holliday junction intermediate. In both panels, black arrows indicate the sites of the first strand exchange. The open arrows are the sites for the second strand exchange. The circle at the 5' end of *attB* represents the ^{32}P label. Only *attB* (substrate) and *attR* (product) will be labeled. In the right HJ intermediate, mismatched bases are capitalized and in red.

CTnDOT integration is *Bacteroides* host factor A (BHFa), a host-encoded nucleoid-associated protein that enables integration in the CTnDOT *in vitro* integration reaction. BHFa has four binding sites (H1 to H4) within the *attDOT* region (Fig. 1A). Of the sites, BHFa has the highest affinity for the H2 site, less affinity for the paired H3-H4 sites, and the lowest affinity for the H1 site (22).

To test this hypothesis, we constructed larger synthetic HJs containing the arm-type sites. As with core-only identical HJs, these arm-type HJ intermediates were resolved into either products or substrates when the overlap region contained identical bases. We also constructed arm-type HJ intermediates with a mismatched overlap region. When arm-type mismatched HJ intermediates were incubated with IntDOT, they were resolved to substrates. However, in the presence of both IntDOT and BHFa, they were resolved to both substrates and products.

We also tested HJ intermediates with mutations in the arm-type sites to determine which arm-type sites are necessary for product formation when the overlap region is mismatched. The L1 arm-type site was required for product formation, but mutations in the R1' and R2-R2' arm-type sites did not affect overall resolution or product

FIG 1 Legend (Continued)

regions are shown. The *attB* core-type sites (B and B') are represented by gray circles. In the top portion, the four rounded rectangles indicate IntDOT monomers bound to core-type sites. The active pair of monomers is shaded yellow and generates 5'-hydroxyl groups (triangles). In the bottom portion, the first set of strand exchanges has generated the HJ intermediate. (C) Left, the HJ intermediate from panel B, redrawn to show the arms of the HJ intermediate. This HJ intermediate may isomerize by strand swapping. Right, after isomerization, the second pair of IntDOT monomers activated (shaded yellow) can complete another set of strand exchanges after generating 5'-hydroxyl groups (triangles). (D) Left, the HJ intermediate may be resolved back to the substrates (*attB* and *attDOT*). Right, after the second set of strand exchanges, the HJ intermediate can be resolved to recombinant products (*attL* and *attR*).

formation. In fact, DNA bending by BHFa appears more important than the *attR* arm-type sites tested for resolution of HJ intermediates. We conclude that when the HJ intermediate contains mismatches, the L1 arm-type site and the IntDOT- and BHFa-mediated complexes are all necessary for resolution to products.

RESULTS

Arm-type site DNA provided in *trans* to core-only mismatched HJ intermediates increases product formation. It was previously shown that HJ intermediates with only the core-type sites cannot be resolved to products if the overlap region is mismatched (21). The core-only HJ intermediates lack all of the five arm-type sites which would be present *in vivo*. Since IntDOT can bind and resolve HJ intermediates with only the core-type sites (D, D', B, and B'), it is expected that the CB domain of up to four IntDOT monomers will bind to the HJ intermediate (Fig. 1C). However, the IntDOT N domains will be unoccupied and able to bind arm-type DNA added in *trans*.

In CTnDOT recombination, the role of the arm-type sites is different than in the lambda system, where the arm-type sites determine directionality and the order of strand exchanges. Two of the CTnDOT arm-type sites (the L1 and R1' sites) are required for both integration and excision. Three additional sites act cooperatively in integration (R1, R2, and R2') but appear to be dispensable in excision (23, 24). While lambda Int has a higher affinity for the arm-type sites than the core-type sites, IntDOT has a higher affinity for the core-type sites (24, 25). In the absence of core-type DNA, IntDOT binds the L1 arm-type site but does not bind DNA fragments containing the R1', R1, R2, and R2' arm-type sites. Adding core-type DNA in *trans* improves the affinity of IntDOT for the *attR* arm-type sites. The inclusion of the DNA-bending protein IHF also facilitated IntDOT binding to a DNA fragment containing R1', R1, R2, and R2' sites (24). This suggests the importance of higher-order structures to promote IntDOT binding and subsequent recombination.

To test the importance of the arm-type sites for HJ resolution, we incubated the core-only HJ intermediates with the following DNA fragments in *trans*: one containing the *attL* arm-type site (L1 [L-ATS]), another containing the four *attR* arm-type sites (R1', R1, R2, and R2' [R-ATS]), and a 156-bp PCR product from the pUC19 plasmid that contains none of the arm-type site sequences (nonspecific DNA [NS]) (Fig. 3A to C).

When added to core-only identical HJ intermediates, IntDOT resolved the HJ into both products (25%) and substrates (57%) (Fig. 3D), as observed previously (21). The addition of arm-type DNA in *trans* did not further stimulate product formation (Fig. 3D, lanes 3 to 5). Higher concentrations of these DNA substrates than the ones shown (200 nM) did not increase overall resolution or alter the directionality (data not shown).

When arm-type sites were provided in *trans*, core-only mismatched HJ intermediates were resolved to both products and substrates. Without arm-type DNA, we saw extremely low levels of product formation (4%) when the overlap was mismatched (Fig. 3D, lane 7). The *attL* arm DNA increased product formation to 10% (Fig. 3D, lane 8). The four *attR* arm-type sites also increased product formation to 12% (Fig. 3D, lane 9). Adding both the *attL* and *attR* arm-type sites simultaneously did not increase resolution compared to adding them separately (data not shown). The lack of improvement may be because the higher affinity of IntDOT for the L1 site prevented any further stimulation due to the *attR* arm-type sites.

As described above, when core-only HJ intermediates are incubated with arm-type DNA in *trans*, mismatched overlap HJ intermediates can be resolved to products by IntDOT, although product formation remains lower (12% at most, compared to 25% for identical overlap HJ intermediates) (Fig. 3E, compare columns B and I). It is unlikely that adding the arm-type DNA in *trans* fully mimics the coordination among IntDOT monomers that would be possible when the arm-type sites are present in *cis* with the core-type sites. This lack of coordination may account for the lower resolution levels, especially when the overlap region is mismatched. Overall, arm-type site DNA provided in *trans* stimulated resolution of core-only mismatched HJ intermediates to products. While the increased product formation was modest (10 to 12% compared to 4%

FIG 3 Resolution of core-only HJ intermediates with arm-type DNA in *trans*. (A) DNA sequence of the L-ATS fragment, which is the *attL* arm DNA fragment containing the L1 site. Numbers are in reference to the center base of the overlap region, which is 0. (B) DNA sequence of the R-ATS fragment, which is the *attR* arm DNA fragment

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without), it was statistically significant when the *attL* and *attR* arm-type containing fragments were added.

Addition of BHFa stimulates product formation in arm-type HJ intermediates.

We constructed and tested larger HJ intermediates containing the R1', R1, R2, and R2' arm-type sites and the L1 arm-type site. These arm-type HJ intermediates contained either an identical or mismatched overlap region (Fig. 4A). Of the four BHFa binding sites within the full *attDOT* sequence (Fig. 1A), only the H2 site is entirely present within the core-only HJ intermediate, since it is located between the D' core-type site and the L1 arm-type site (Fig. 4A). In the arm-type site HJ intermediates, there are additional BHFa binding sites. The H3 site and a small portion of the H4 site are present in the north arm. The H3 site overlaps with the L1 site. The H1 site is present in the east arm and overlaps with the R2-R2' arm-type sites (Fig. 4A).

We tested the core-only and arm-type HJ intermediates for resolution in the presence and absence of the DNA-bending protein BHFa. The addition of BHFa without IntDOT to HJ intermediates does not result in any resolution (data not shown).

The addition of BHFa to all core-only HJ intermediates reduced overall resolution (compare Fig. 4B, columns B and C, and E and F). For core-only identical HJ intermediates, adding BHFa reduced total resolution from 67% to 48% (Fig. 4B). For core-only mismatched HJ intermediates, adding BHFa decreased resolution from 64% to 33% (Fig. 4B). Possibly, BHFa binding to core-only HJs competes with IntDOT binding to the core-type sites, although previous IntDOT footprints do not overlap the H2 site (10). It is also possible that binding by BHFa bends the smaller core-only HJs into nonproductive conformations.

Similarly to the core-only HJ intermediates, the arm-type identical HJ intermediate was resolved to both substrates (56%) or products (16%) with IntDOT alone (Fig. 4B, column H). Adding BHFa increased product formation from 16% to 31% (Fig. 4B, column I), presumably by bringing the arm-type sites into a favorable conformation for resolution to products.

When the arm-type mismatched HJ intermediate was incubated with IntDOT alone, almost no product formation (3%) was seen. Resolution was primarily (61%) back to substrates (Fig. 4B, column K). The addition of BHFa increased product formation from 3% to 15% (Fig. 4B, column L). As with the arm-type identical HJ intermediate, total levels of resolution were similar with or without BHFa present (Fig. 4B). However, the addition of BHFa to arm-type HJ intermediates increased product formation.

L1 mutant arm-type identical HJ intermediates are defective in resolution to products. In order to evaluate the importance of the individual arm-type sites in resolution of HJ intermediates, we constructed additional versions of the arm-type HJ intermediates. The L1, R1', and R2-R2' arm-type sites were changed based on previous studies indicating their importance in integration and excision of CTnDOT (23, 24). The L1 and R1' sites are each required for integration and excision. Either the R2 site or R2' site is required in combination with the R1 site for integration (Fig. 1A). Mutating both the R2 and R2' sites did not previously eliminate integration or excision, but they were selected to test the cooperativity of the *attR* arm-type sites in HJ resolution.

As in the previous work, we changed each of the selected sites to a HindIII sequence (Fig. 5A). Mutated arm-type HJ intermediates containing either an identical or mis-

FIG 3 Legend (Continued)

containing the R1', R1, R2, and R2' sites. Numbers are as in panel A. (C) DNA sequence of the NS fragment, which is the nonspecific control. Since this is not *attDOT* DNA, numbers begin at zero at the 5' end of the top strand. (D) Resolution of core-only HJ intermediates with arm-type DNA added in *trans*. The *attR* product is 59 bp in size. The *attB* substrate is 50 bp in size. Lanes 1 to 5 contain core-only identical HJ intermediates. Lanes 6 to 10 contain core-only mismatched HJ intermediates. All DNA substrates in *trans* are present at 200 nM. IntDOT is present at 110 nM. HJ intermediates are present at 0.34 nM. (E) Quantification of the results of HJ intermediate resolution with arm-type site DNA in *trans*. Product indicates *attR*. Substrate indicates *attB*. Percent signal is calculated using the intensity of the bands visualized using the phosphorimager. The total signal is determined by adding together the substrate band (*attB*), the product band (*attR*), and any unresolved HJ intermediate. Total resolution indicates product added to substrate. Quantifications are based on three separate trials averaged together. Error bars are based on the standard error from the three trials.

FIG 4 Resolution of HJ intermediates containing arm-type and H sites. (A) The arm-type HJ intermediate. For reference, the core-only HJ intermediate is marked in red. The additional bases are in black. The arm-type sites are marked with gray boxes. The circle at the 5' end of *attB* indicates the ^{32}P label. Only *attB* (substrate) or *attR* (product) will be labeled. Solid lines indicate top strands, and dashed lines indicate bottom strands. Blue boxes indicate BHFa binding sites within the HJ intermediate. Numbers indicate bases protected by BHFa in footprinting trials. Positive numbers indicate the *attL* (north) arm. Sizes of the resulting substrates (*attB* and *attDOT*) and products (*attL* and *attR*) are indicated in the box. Shaded boxes indicate the products visible on the gel due to the 5' label. (B) Quantification of the results of HJ resolution with core-only or arm-type HJ intermediates. Product indicates *attR*. Substrate indicates *attB*. Total resolution indicates product added to substrate. Quantifications are based on three separate trials averaged together. Error bars are based on the standard errors from the three trials. Percent signal is calculated using the intensity of the bands visualized using the phosphorimager. The total signal is determined by adding together the substrate band (*attB*), the product band (*attR*), and any unresolved HJ intermediate.

FIG 5 Role of the arm-type sites in *cis* in resolution of HJ intermediates. (A) Four arm-type sites were mutated to include HindIII sites to test IntDOT interactions with the site in the context of HJ resolution. Original sequences are listed on the left. The mutated sequences are listed on the right. The changed bases (4 to 6 bases) are underlined, bolded, and in blue. Due to their adjacent locations and cooperativity, the R2-R2' sites were both changed. (B) Quantification of the results of HJ resolution with core-only or core plus arm-type site HJ intermediates. Product indicates *attR*. Substrate indicates *attB*. Total resolution indicates product added to substrate. Quantifications are based on three separate trials averaged together. Error bars are based on the standard errors from the three trials. Percent signal is calculated using the intensity of the bands visualized using the phosphorimager. The total signal is determined by adding together the substrate band (*attB*), the product band (*attR*), and any unresolved HJ.

matched overlap region were tested. Some of the altered arm-type site bases are within BHFa binding sites (H1 for the R2-R2' mutant and H3 for the L1 mutant). However, the BHFa binding site appears to be degenerate and involve contacts with numerous bases, much like IHF. The highest affinity H2 site was unaffected by any arm-type site mutations, and it is unlikely that all H sites are occupied by BHFa during integration or excision (26). The H1 site overlaps the R2-R2' arm-type sites, so 10 bases of the total 28 bases previously protected by BHFa were changed (Fig. 5A). The H3 site overlaps with the entire L1 arm-type site, but only 6 of the previously BHFa protected 37 bases were changed in the L1 mutant (L1^{Mut}) arm-type HJ intermediate (22).

It may be that nonspecific binding of BHFa to the HJ intermediates is sufficient for resolution. For example, the *Escherichia coli* integration host factor (IHF) stimulates integration in the *in vitro* CTnDOT integration assay even though *attDOT* does not contain any consensus IHF binding sites (8, 27). Therefore, it is possible that the

arm-type site mutations primarily affected interactions between the N domain of IntDOT and the arm-type site DNA rather than BHFa binding.

In L1^{Mut} arm-type identical HJ intermediates, product formation dropped from 16% (wild type [WT]) to 12% (L1^{Mut}) with IntDOT alone (Fig. 5B, columns B and E). Overall resolution increased by 12% but was biased toward substrates (Fig. 5B, column E). Adding BHFa to L1^{Mut} arm-type identical HJ intermediates improved product formation (from 12% up to 18%), but it remained lower than that for wild-type arm-type identical HJ intermediates incubated with BHFa as well (31% product) (Fig. 5B, columns C and F). Since the inclusion of BHFa improved product formation, we propose that BHFa still binds to L1^{Mut} HJ intermediates. However, binding by BHFa is not sufficient to restore the necessary contacts between the N domain of IntDOT and the *attL* arm when the L1 site is mutated. The loss of these interactions reduced product formation.

Mutations in either the R1' site or the R2-R2' sites together did not substantially affect overall resolution or product formation (data not shown). The results for the R1' mutant (R1'^{Mut}) and R2-R2' mutant (R2-R2'^{Mut}) identical overlap HJ intermediates are summarized in Table S4 in the supplemental material.

L1 mutant arm-type mismatched HJ intermediates cannot be resolved to products. We also tested the arm-type site mutants with mismatched overlap HJ intermediates. The L1^{Mut} arm-type mismatched HJ intermediates with IntDOT alone returned to background (2%) levels of product formation (Fig. 5B, column K). The addition of BHFa resulted in very low (4%) levels of product formation (Fig. 5B, columns K and L). Unlike the L1^{Mut} arm-type identical HJ intermediates, the addition of BHFa did not improve product formation. The addition of BHFa decreased overall resolution from 62% (without BHFa) to 51% (Fig. 5B, columns K and L). It appears that the contacts between the L1 site and the N domain of IntDOT are most important when the overlap region is mismatched (Fig. 5B, compare columns E and F to columns K and L). In the absence of the L1 site, overall resolution remains similar, but product formation drops sharply. This suggests that when the overlap region is mismatched, the L1 site is required for product formation.

As in the arm-type identical HJ intermediates, both the R1'^{Mut} and R2-R2'^{Mut} changes did not significantly affect overall resolution or product formation compared to the wild-type arm-type sites (data not shown). None of the *attR* sites tested were required for resolution or affected product formation (Table S4).

In summary, the L1 site was required for product formation in mismatched arm-type HJ intermediates. Without the L1 site, product formation returned to background levels. Whether the overlap was identical or mismatched, the L1^{Mut} arm-type HJ intermediates resolved similarly to the corresponding core-only HJ intermediates, suggesting that the L1 site is the most important arm-type site tested for product formation.

DISCUSSION

Tyrosine recombinases perform site-specific recombination reactions in a diverse array of systems (28). A common feature of this family of enzymes is the strict requirement for small regions of homology in the overlap regions between the sites of the strand exchanges involved in the reactions (29). IntDOT is unusual in that it does not have a stringent requirement for homology in the overlap region between the sites of the strand exchanges. The first strand exchange requires that the partner sites contain at least 2 bp of homology for the first strand ligation to occur (9, 30). However, the second strand exchanges are not homology dependent and result in short heteroduplexes in the recombinant sites. These heteroduplexes are eventually resolved by DNA replication or repair in the host cell.

Previous work showed that IntDOT cannot resolve core-only mismatched HJ intermediates to products. The core-only mismatched HJ intermediates are processed back to substrates by exchanges involving the complementary base pairs instead (21). The goal of this research was to determine if the ability of IntDOT to exchange HJ intermediates with mismatches in the overlap region is an intrinsic property of the

enzyme or if the mechanism is more complex and requires the assembly of nucleoprotein complexes with additional participants.

In this study, we tested whether interactions between IntDOT and arm-type sites are involved in processing HJ intermediates to products and whether BHFa facilitates those interactions. For lambda Int, it is known that adding arm-type DNA in *trans* can stimulate the activity of the C domain (CB and CAT) due to reducing inhibition by the N domain (31). In order to test whether the arm-type sites alone were sufficient to stimulate resolution to products, we used DNA fragments containing either the *attL* or *attR* arm-type sites.

The addition of arm-type site DNA in *trans* (either the *attL* or *attR* arm) to core-only identical HJ intermediates did not affect resolution or product formation. For the core-only mismatched HJ intermediates, the addition of either the *attL* or *attR* arm-type sites slightly but significantly increased product formation. This result suggests that resolution of mismatched HJ intermediates is more complex and requires the assembly of higher-order nucleoprotein structures, including DNA-bending proteins in addition to IntDOT.

While the structures of the IntDOT intasomes (either integrative or excisive) are not known, it is likely that resolution is based on both protein-protein interactions between the IntDOT monomers and regulation by interactions from the N domain of each monomer. For example, a crystal structure of lambda Int tetramers associated with a Holliday junction indicated extensive interactions between the monomers (32). In the experiments described above with core-only HJ intermediates and arm-type site DNA in *trans*, the arm-type site DNA was presumably bound by the N domain of IntDOT, but any higher-order coordination was absent. Additionally, any supercoiling that would be present in the DNA is absent in the *in vitro* HJ resolution reaction. In the absence of higher-order coordination and DNA supercoiling, it is not surprising that levels of resolution to product were relatively low for core-only HJ intermediates.

In the core-only HJ intermediates, the inclusion of BHFa did not stimulate resolution to products and inhibited overall resolution. This suggests that there is no inherent property of BHFa binding that biases resolution toward products. Thus, we conclude that the role of BHFa is to bend HJ intermediate DNA into suitable conformations for IntDOT monomers to contact both core and arm-type sites.

Without the inclusion of BHFa, the arm-type sites were effectively absent in the arm-type HJ intermediates despite being present in *cis*. In arm-type identical HJ intermediates, BHFa stimulated product formation. In arm-type mismatched HJ intermediates, BHFa was required for product formation. Therefore, we suggest that BHFa was required for IntDOT to interact with the L1 site in *cis* to resolve the HJ intermediate to products. This suggests that the higher-order dynamics of the intasome are most important when the overlap region contains mismatches.

It is possible that the arm-type site mutants disrupted BHFa binding sites. However, we do not think it is likely that the reduced product formation was predominantly due to decreased BHFa binding. Both the R1^{Mut} and R2-R2'^{Mut} HJ intermediates followed the same trends as wild-type arm-type HJ intermediates when BHFa was included (see Table S4 in the supplemental material). This was the case for both identical and mismatched overlap regions. For L1^{Mut} arm-type identical HJ intermediates, the addition of BHFa slightly improved resolution to products (Fig. 5B, columns E and F). This suggests that BHFa was still able to bind to the L1^{Mut} arm-type HJ intermediate.

Product formation was reduced in L1^{Mut} arm-type identical HJ intermediates. This finding suggests that contacts between the N domain of IntDOT and the L1 site drive product formation. Both BHFa (to bend the DNA into the correct conformation) and the L1 site are required for product formation. Contact with the L1 site was essential rather than stimulatory when the overlap region was mismatched, so that the L1^{Mut} arm-type mismatched HJ intermediates were resolved back to substrates only when BHFa was absent.

IntDOT may require the N domain to bind to the L1 site to correctly position one monomer with any others participating in protein-protein interactions. Alternatively, N

domain binding to the L1 site may influence the catalytic domain of IntDOT. This would be similar to how the interaction of arm-type sites with the N domain of lambda Int regulates the activity of the core-binding and catalytic domains (31–34). Previous work had shown that the inclusion of the L1 site in *trans* stimulated the ligation activity of IntDOT (35). For other recombinases that are unable to resolve HJ intermediates containing mismatches in the overlap region, it is believed that the ligation step cannot proceed (15, 16, 36, 37). Therefore, the interactions between the L1 site and the N domain of IntDOT may allow ligation to proceed despite mismatches. Either or both of these possibilities could explain the requirement for the L1 site for product formation.

IntDOT resolution of HJ intermediates containing mismatched bases represents an unusual ability among tyrosine recombinases. Well-studied tyrosine recombinases, such as lambda Int, Cre, and Flp, are unable to tolerate mismatched bases within the overlap region. Other examples of recombinases that can resolve mismatched bases in the overlap region are rare: the integrase of Tn916 (another ICE) may be able to resolve mismatched bases in an HJ intermediate (38–41), and Tn916 has sequences that are functionally similar to arm-type sites, but the role of individual sites in that system has not yet been established. It is clear that the arm-type sites of IntDOT do not regulate directionality in the same manner as the arm-type sites of the lambda system. This study is the first to demonstrate that interactions between a host factor, tyrosine recombinase, and arm-type site are necessary to resolve a HJ intermediate with mismatched bases in the overlap region.

MATERIALS AND METHODS

Enzymes, reagents, and PCR primers. All oligonucleotides were ordered from IDT. Oligonucleotides were suspended in Tris-EDTA (TE) buffer (pH 8.0) and stored at -80°C . The sequences for all oligonucleotides can be found in Table S1 in the supplemental material. The *attR* (185 bp) and *attL* (114 bp) oligonucleotides were IDT Ultramers. The *attDOT* oligonucleotides (249 bp) were specially ordered from IDT.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was ordered from PerkinElmer. T4 polynucleotide kinase was obtained from Fermentas.

KOD PCR master mix was purchased from Novagen. Gel extractions were performed using Qiagen kits, according to the manufacturer's instructions. The PCR primer sequences can be found in Table S2.

Generating Holliday junction intermediates. Holliday junction intermediates were prepared similarly to the method of Kim and Gardner (21). Only the *attB* oligonucleotides (SK1 or SK7) were labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using polynucleotide kinase (Fermentas).

After labeling, equal amounts (24 pmol) of the other oligonucleotides (*attL*, *attR*, and *attDOT*) were added to the labeled oligonucleotide in annealing buffer (10 mM Tris-HCl [pH 8], 100 mM KCl, 5 mM EDTA). The mixture was heated to 95°C in a PCR machine and cooled by 1°C per 30 s until it reached 50°C . It was then cooled by 1°C per minute until reaching room temperature. The combinations of oligonucleotides used in the different HJ intermediates can be found in Table S3.

HJ intermediates were gel purified by loading on a prerun 8% polyacrylamide gel. The HJ intermediates were electrophoresed for about 2 h at 150 V. After that time, they were exposed to X-ray film and excised from the gel. The HJ intermediates were ethanol precipitated, resuspended in 0.5 M KCl, and stored at -20°C .

Recovered HJ intermediates were quantified using a Thermo Fisher Qubit 2.0 and single-stranded DNA (ssDNA) quantification kit, according to the manufacturer's instructions.

Resolving and visualizing Holliday junction intermediates and resolution results. The HJ resolution assays were performed in 50- μl reaction volumes containing 50 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM EDTA, 0.1 mg/ml bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), and 6 ng/ μl herring sperm DNA. Herring sperm DNA was added to reduce nonspecific DNA binding activity of IntDOT and BHF α . Herring sperm DNA was also found to increase resolution rates (data not shown).

Amounts of each HJ intermediate were standardized based on the Qubit 2.0 quantifications. Purified HJ intermediates were present at 0.34 nM. When present, IntDOT was present at 110 nM and BHF α was present at 90 nM.

The reactions were allowed to proceed for 2 h at 37°C . They were stopped by the addition of SDS to a final concentration of 0.4% and loaded onto a prerun 8% polyacrylamide gel. The gels were run for 6 h at 100 V. Gels were exposed to phosphorimager screens overnight and scanned, and the results were analyzed and quantified using the Fujifilm Image Gauge software (Macintosh version 3.4).

Percent product was calculated by dividing the number of counts in the product band (*attR*) by the total counts of the product band, the substrate band (*attB*), and the unresolved HJ band combined. Percent substrate was calculated by dividing the number of counts in the substrate band by the total counts of the product band, the substrate band, and the unresolved HJ band combined. In a comparison of product formation, we determined statistical significance using a *t* test (paired 2 sample for means) with an alpha level of 0.05 using the Data Analysis ToolPak in Microsoft Excel 2013.

Proteins. IntDOT was purified as described in reference 10, except that an *ihfA* overexpression mutant strain was used. BHF α was purified as described in reference 22 and diluted in IHF dilution buffer

(50 mM Tris HCl [pH 8], 10% glycerol, 2 mg/ml BSA, and 200 mM KCl). Both proteins were stored at -80°C . Proteins were quantified using a Qubit 2.0 and Qubit protein assay kit, according to the manufacturer's instructions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00873-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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